0026-895X/08/7302-441–450$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 73:441–450, 2008

Vol. 73, No. 2
41863/3293422
Printed in U.S.A.

Inhibition of Human Neutrophil Chemotaxis by Endogenous Cannabinoids and Phytocannabinoids: Evidence for a Site Distinct from CB_1 and CB_2

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Received September 18, 2007; accepted October 26, 2007

ABSTRACT

Here, we show a novel pharmacology for inhibition of human neutrophil migration by endocannabinoids, phytocannabinoids, and related compounds. The endocannabinoids virodhamine and *N*-arachidonoyl dopamine are potent inhibitors of *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine-induced migration of human neutrophils, with IC_{50} values of 0.2 and 8.80 nM, respectively. The endocannabinoid anandamide inhibits human neutrophil migration at nanomolar concentrations in a biphasic manner. The phytocannabinoid (−)-cannabidiol is a partial agonist, being ~40 fold more potent than (+)-cannabidiol; abnormal-cannabidiol is a full agonist. Furthermore, the abnormal-cannabidiol (CBD) analog *trans*-4-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-methyl-1,3-benzenediol (O-1602) inhibits migration, with an IC_{50} value of 33 nM. This reported profile of agonist efficacy and potency parallels with the pharmacology of the novel "abnormal-cannabidiol" receptor or a related orphan G protein-coupled receptor, which are already known to modulate cell migration. Although having no effect alone, *N*-arachidonoyl L-serine attenuated inhibition of human neutrophil migration induced by anandamide, virodhamine, and abnormal-CBD. Our data also suggest that there is cross-talk/negative co-operativity between the cannabinoid CB_2 receptor and this novel target: CB_2 receptor antagonists significantly enhance the inhibition observed with anandamide and virodhamine. This study reveals that certain endogenous lipids, phytocannabinoids, and related ligands are potent inhibitors of human neutrophil migration, and it implicates a novel pharmacological target distinct from cannabinoid CB_1 and CB_2 receptors; this target is antagonized by the endogenous compound *N*-arachidonoyl L-serine. Furthermore, our findings have implications for the potential pharmacological manipulation of elements of the endocannabinoid system for the treatment of various inflammatory conditions.

The endocannabinoid system comprises two known receptors (CB_1 and CB_2); a family of endogenous ligands (endocannabinoids); and specific molecular machinery for the synthesis, transport, and inactivation of these ligands (Pertwee and Ross, 2002). The most studied endocannabinoids are arachidonoylethanolamide (AEA), also known as anandamide, and 2-arachidonoyl glycerol (2-AG), both of which are synthesized on demand, and they are rapidly hydrolyzed by the enzymes fatty acid amide hydrolase and monoacyl glycerol lipase, respectively (Fowler et al., 2005). There are a number of additional endocannabinoids; these include *N*-arachidonoyl

This study was funded by Allergan Inc., GW Pharma, and National Institutes of Health/National Institute on Drug Abuse Grant DA018244.

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Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.041863.

ABBREVIATIONS: CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; AEA, anandamide, *N*-arachidonoyl ethanolamide; 2-AG, 2-arachidonoyl glycerol; NADA, *N*-arachidonoyl dopamine; Δ^9-THC, Δ^9-tetrahydrocannabinol; CBD, cannabidiol; Abn-CBD, abnormal-cannabidiol, *trans*-4-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol; O-1602, *trans*-4-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-methyl-1,3-benzenediol; LPI, lysophosphatidylinositol; PMN, polymorphonuclear neutrophil; PBS, phosphate-buffered saline; fMLP, *N*-formyl-methionine-leucine-phenylalanine; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance; CP55940, 5-(1,1-dimethylheptyl)-2-(5-hydroxy-2-(3-hydroxypropyl)cyclohexyl)phenol; JWH-133, 1,1-dimethylbutyl-1-deoxy-Δ^9-tetrahydrocannabinol; LTB_4, Leukotriene B_4; PEA, palmitoylethanolamide; ARA-S, *N*-arachidonoyl-L-serine; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; CPZ, capsazepine, *N*-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothioamide; AM630, [6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone; JWH-015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; SR144528, 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-*N*-[(1*S*,4*R*,6*S*)-1,5,5-trimethyl-6-bicyclo-[2.2.1]heptanyl]pyrazole-3-carboxamide.

dopamine (NADA) and virodhamine (De Petrocellis et al., 2004). The major constituents of cannabis include Δ^9-tetrahydrocannabinol (Δ^9-THC), which is psychoactive and (−)-cannabidiol (CBD), which is nonpsychoactive (Di Marzo and Petrocellis, 2006).

The pharmacology of both the endocannabinoids and the phytocannabinoids seems to be increasingly complex, their actions being mediated by cannabinoid CB_1 and CB_2 receptors and by putative non-CB_1, non-CB_2 receptors (Pertwee, 2004; Begg et al., 2005; Mackie and Stella, 2006). One such novel target, the "abnormal-cannabidiol" (abn-CBD) receptor, has been implicated in modulation of endothelial, microglial, and glioma cell migration (Franklin and Stella, 2003; Walter et al., 2003; Mo et al., 2004; Vaccani et al., 2005). Furthermore, *N*-arachidonoyl-L-serine, an endocannabinoid-like brain constituent, has been reported to be an agonist at this receptor (Milman et al., 2006). It has recently emerged that various cannabinoids, including abnormal-CBD, its analog O-1602, virodhamine, and anandamide bind to and activate the orphan G protein-coupled receptor GPR55, which is reported to be coupled to $G\alpha_{13}$ and to activate rhoA, cdc42, and rac1 (for commentaries, see Hiley and Kaup, 2007; Johns et al., 2007; Pertwee, 2007; Ryberg et al., 2007). In contrast, others find that lysophosphatidylinositol (LPI), but not cannabinoid ligands, induce extracellular signal-regulated kinase phosphorylation in GPR55-expressing cells (Oka et al., 2007).

There is a growing body of evidence suggesting that neutrophils make a crucial contribution to a number of autoimmune, autoinflammatory, and neoplastic disorders (Nathan, 2006). This study was directed at investigation of the modulation of human neutrophil migration. Cannabinoid CB_2 receptors are primarily expressed in immune cells. In neutrophils, the CB_2 receptor plays a key role in differentiation (Derocq et al., 2000; Alberich Jordà et al., 2004), and it has been implicated in the development of leukemia. 2-AG is reported to exert CB_2-mediated stimulation of hematopoietic cell migration (Jordà et al., 2002; Kishimoto et al., 2003; Walter et al., 2003; Oka et al., 2004; Kishimoto et al., 2005; Sacerdote et al., 2005). Conversely, inhibition of migration by 2-AG, AEA, synthetic cannabinoids, and the phytocannabinoid Δ^9-THC has also been reported previously (Sacredote et al., 2000; Steffens et al., 2005; Ghosh et al., 2006; Kurihara et al., 2006).

In this milieu, the primary aim of our study was to investigate the modulation of human neutrophil migration by cannabinoids, focusing on the underlying receptor pharmacology and the contribution, if any, of non-CB_1 and non-CB_2 receptors. Included in the study are endocannabinoids and related endogenous lipids and phytocannabinoids and structurally related ligands; specifically those that have been demonstrated to interact with either the abnormal-CBD receptor or GPR55.

Materials and Methods

Isolation of Neutrophils

Polymorphprep is a ready-made, sterile, and endotoxin-tested solution designed to isolate polymorphonuclear granulocytes. Whole blood separates into distinct bands of plasma, mononuclear leukocytes, polymorphonuclear neutrophils (PMNs), and red blood cells when centrifuged over the solution. Five milliliters of normal whole blood was carefully layered over 5 ml of Polymorphprep in 12-ml centrifuge tubes. The filled tubes were centrifuged at 550*g* for 35 min at 20°C, and the PMN layer was removed with a fine-tipped Pasteur pipette. To remove the residual Polymorphprep, the cells were suspended in a universal container with 20 ml of sterile RPMI 1640 medium at 37°C and centrifuged (Mistral 1000 centrifuge; MSE, Ltd., London, UK) at 450*g* for 10 min at 20°C. The supernatant fluid was discarded, and the pellet was resuspended with 10 ml of PBS (containing $CaCl_2$ and $MgCl_2$) and 10 ml of 4°C distilled H_2O for further washing. The fluid was centrifuged again at 450*g* for 10 min at 20°C. The supernatant fluid was discarded, and the pellet resuspended with 210 μl of PBS (containing $CaCl_2$ and $MgCl_2$). An estimation of the cell concentration was determined using 0.4% trypan blue solution and a hemocytometer. An appropriate amount of PBS (containing $CaCl_2$ and $MgCl_2$) was used to resuspend the PMNs at a concentration of 1×10^6 cell ml^{-1}.

Boyden Chamber Assay

In vitro cell migration assays were performed using a modified 48-well Boyden chamber. The lower chamber wells were loaded with 27 μl of chemoattractant fluid such that a slight positive meniscus formed to prevent air bubbles. Polyvinylpyrrolidone-free polycarbonate filters with pores 3 μm in diameter were used. The upper wells were filled with 45 μl of cell suspension at a concentration of 1×10^6 cells ml^{-1} in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$. The Boyden chamber was then placed in an incubator with a 5% CO_2 atmosphere at 37°C for 30 min. After incubation, the filter was removed and placed, with the "nonmigrated cell side" facing downward, in a Petri dish containing 70% ethanol for 7 min and then in another dish containing distilled H_2O for 3 min, to reduce the degree of adhesion between the nonmigrated cells and the filter. Nonmigrated cells were then removed by carefully drawing the filter over a wiper blade. The filter was allowed to air dry before fixation and staining with Diff-Quik stain set. Finally, the filter was mounted onto a microscope slide using xylene and *p*-xylene-bis-pyridinium bromide. The migrated cells were counted in 10 nonoverlapping fields (40× magnification) with a light microscope and the order in which wells were counted was randomized. The migrated cells were counted by one scorer. The order in which wells were counted was randomized before each experiment.

The setup of the Boyden chamber and the incubation time of the cells varied depending on whether stimulation or inhibition of migration was being investigated according to the following protocols.

Protocol I. Stimulation of Neutrophil Migration. Neutrophils, at a concentration of 1×10^6 cells/ml were loaded into the top wells of the Boyden chamber, whereas the bottom wells contained test compound. fMLP (1 μM) acted as positive control.

Protocol II. Inhibition of Neutrophil Migration. Neutrophils were preincubated with test compound(s) for 30 min at 37°C in a water bath before loading into the top wells. The bottom wells contained the corresponding concentration of test compound and 1 μM fMLP. This arrangement ensures that the only concentration gradient present is that generated by fMLP as it diffuses through the pores in the filter.

Analysis of Data

The mean number of neutrophils that migrated in response to 1 μM fMLP in the presence of test compounds was normalized against the mean number of migrated neutrophils elicited by 1 μM fMLP with test compound vehicle (0.01% DMSO); the number of migrated cells in the presence of basal (fMLP vehicle only) was subtracted, as illustrated by the equation Migration (percentage of fMLP-induced migration) = {number of cells migrated [(test compound + fMLP) − (basal)]/number of cells migrated [(fMLP + vehicle) − (basal)]} × 100.

All data are expressed as means ± S.E.M. IC_{50} and E_{max} values, together with the S.E.M. or 95% confidence intervals were calculated

by nonlinear regression analysis using the equation for sigmoidal concentration-response curve (GraphPad Prism 4; GraphPad Software Inc., San Diego, CA). Statistical analyses were performed with GraphPad Prism 4. A one-sample t test was used to compare the percentage of neutrophil migration in the presence of test compounds with 100%, which is the level of migration in the presence of vehicle alone. Analysis of variance (ANOVA) followed by Dunnett's test or Students' paired t test was used to compares the percentage of migration in the presence of vehicle with the presence of antagonists or enzyme inhibitors. A P value <0.05 was considered to be significant. In all cases, the n number given in the text represents individual measurements from separate Boyden chamber experiments performed using neutrophils derived from blood donated by at least three different donors.

Results

Stimulation of Migration

The ability of various cannabinoids to induce human neutrophil migration was investigated using protocol I (see *Materials and Methods*). The endogenous cannabinoid AEA (Fig. 1A) and 2-AG (Fig. 1B), induced a modest stimulation of migration of neutrophils that was not concentration related. Neither the synthetic nonselective CB_1/CB_2 receptor agonist CP55940 nor the CB_2 receptor selective agonist JWH-133 produced a significant migration of human neutrophils (data not shown). In Fig. 1, the data are expressed as the number of neutrophils from the count of 10 nonoverlapping fields (40× magnification) in each well. This highlights the variability inherent in using freshly isolated human neutrophils. Over a large number of experiments ($n = 103$), the neutrophils in each well over 10 nonoverlapping fields was 200 ± 25 and 1983 ± 145 in the presence of vehicle (basal) and 1 μM fMLP, respectively ($P < 0.001$; Student's paired t test). Because of the interdonor variability in migration, in subsequent experiments the number of migrated neutrophils was normalized to a 1 μM fMLP control for each donor, and the data are expressed as a percentage of this donor-specific control.

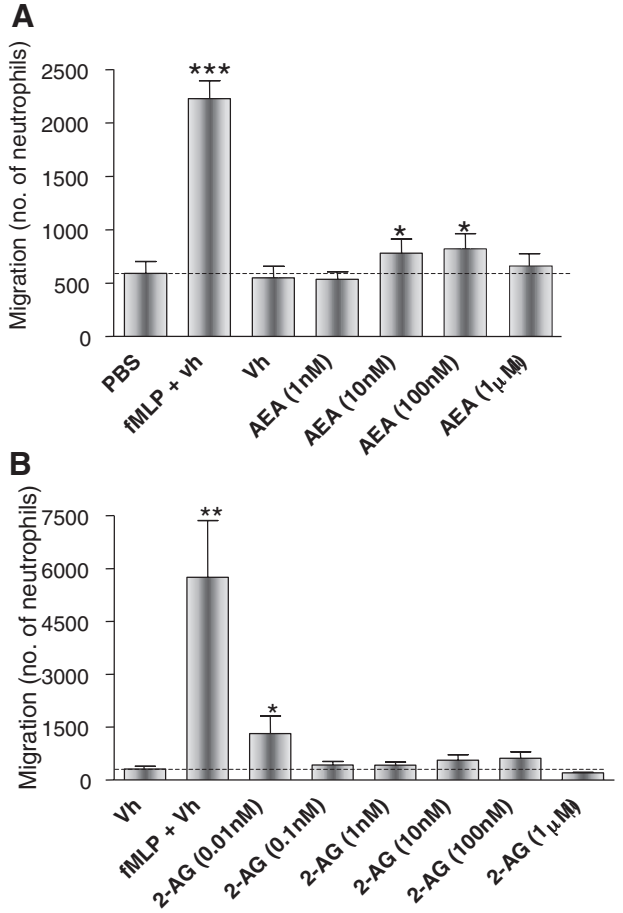

Fig. 1. Histograms showing the number of neutrophils induced to migrate by AEA (A) and 2-AG (B). The data represent the mean number of neutrophils migrated ± S.E.M. (n = 3–9). *, $P < 0.05$; **, $P < 0.01$, one-way ANOVA.

AEA Inhibited Human Neutrophil Migration

After preincubation with AEA (protocol II; see *Materials and Methods*), neutrophil migration induced by 1 μM fMLP was significantly attenuated (Fig. 2A). AEA also significantly attenuated human neutrophil migration induced by 100 nM LTB_4 (one-sample t test; $n = 9$) (Fig. 2B). In both cases, significant inhibition was observed at concentrations as low as 0.1 nM.

AEA Did Not Affect Cell Viability

Using the trypan blue exclusion method, the estimated percentage viability of neutrophils incubated for 30 min in vehicle (0.01% DMSO) or 100 nM AEA was 97.2 ± 0.52 and 97.5 ± 0.35%, respectively. These values were not significantly different from one another ($P > 0.05$; Student's unpaired t test; $n = 3$).

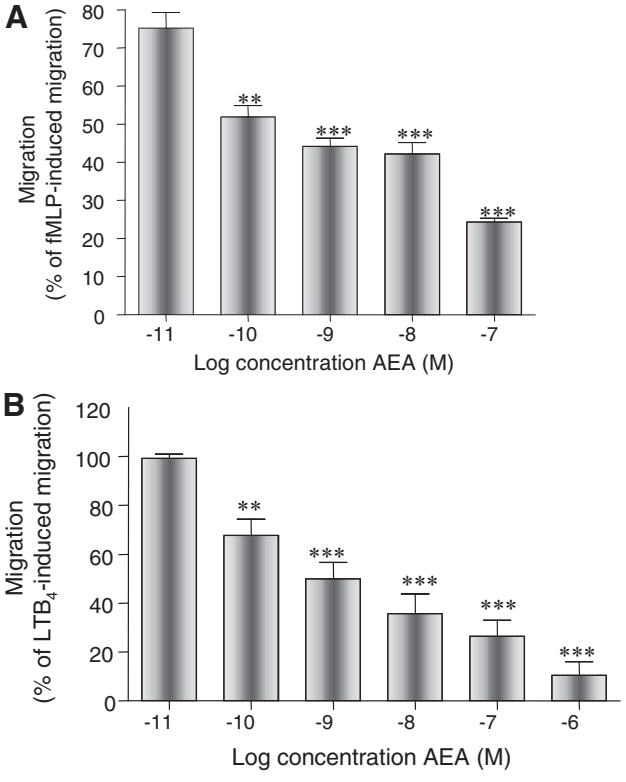

Fig. 2. Histograms showing neutrophil migration (A) induced by 1 μM fMLP after preincubation with AEA (B) induced by 100 nM LTB_4 after preincubation with increasing concentrations of AEA. The data represent the mean number of neutrophils migrated ± S.E.M. ($n = 9$) as a percentage of the migration induced by fMLP or LTB_4 in the presence of vehicle (0.01% DMSO). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, one-sample t test.

Certain Endogenous Lipids, Phytocannabinoids, and Related Ligands Inhibit Human Neutrophil Migration

Endocannabinoids and Endogenous Lipids. Next, we investigated the effect of various endocannabinoids and other endogenous lipids that have been reported to interact with either the abnormal-CBD receptor (Begg et al., 2005) or GPR55 (Ryberg et al., 2007) (Table 2).

The endocannabinods virodhamine and NADA significantly inhibited neutrophil migration induced by fMLP (n = 9; Fig. 3A). The IC_{50} and E_{max} values for these compounds are shown Table 1.

2-AG and palmitoylethanolamide (PEA) had no significant effect on the neutrophil migration induced by fMLP (n = 9–12; Fig. 3A) using protocol II (see *Materials and Methods*).

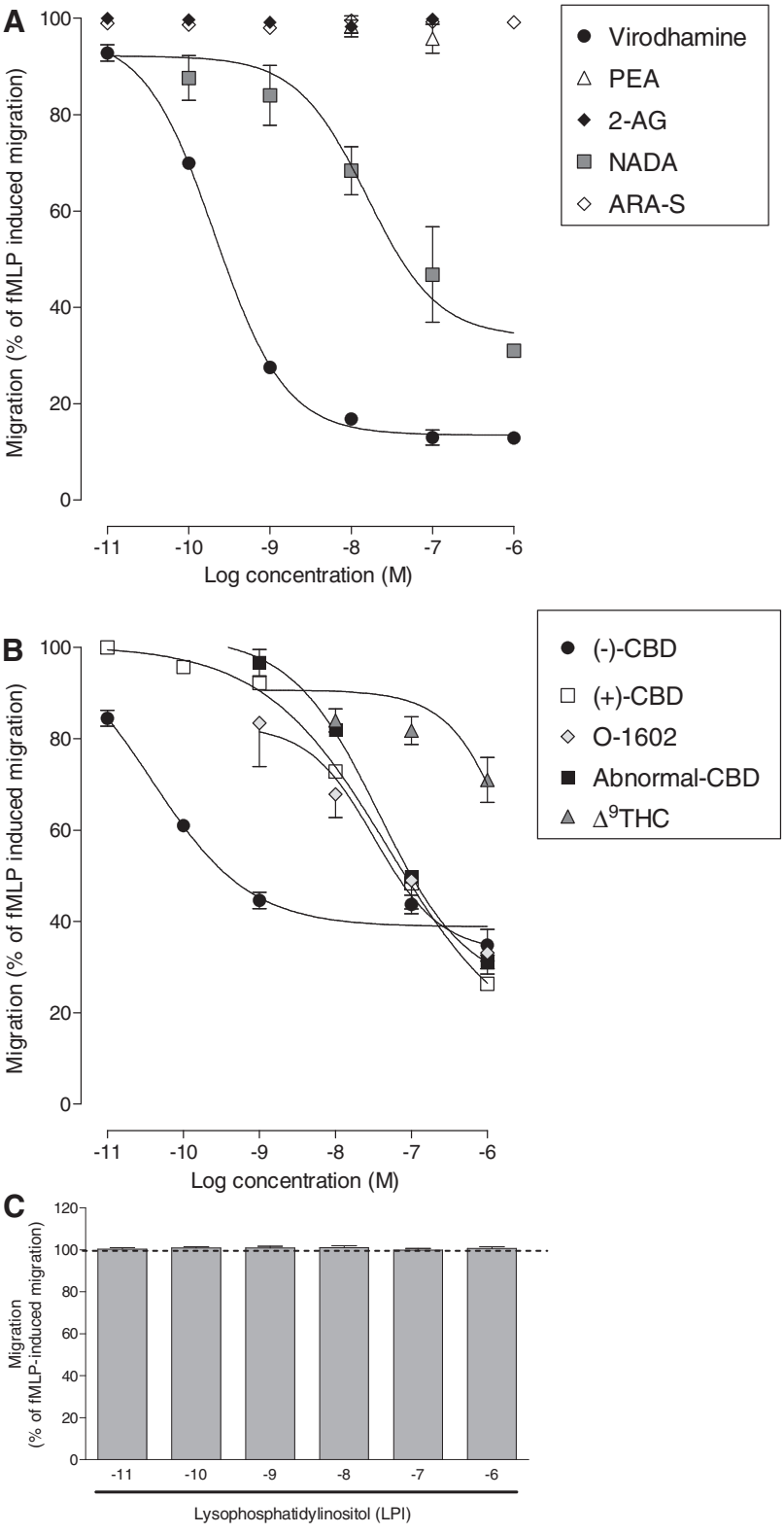

Fig. 3. Log concentration-response curves for neutrophil migration induced by 1 μM fMLP in the presence of various ligands (response calculated using the equation under *Materials and Methods*). A, endocannabinoids and related endogenous lipids; AEA 2-AG, PEA, NADA, and ARA-S. B, phytocannabinoids and their analogs: CBD, Abn-CBD, O-1602, and Δ^9-THC. C, histogram showing the effect of LPI. Data are the mean ± S.E.M. (n = 3–12).

At concentrations up to 1 μM, *N*-arachidonoyl-L-serine (ARA-S) did not inhibit human neutrophil migration (Fig. 3A). However, this compound behaved as an antagonist (see later). The putative GPR55 agonist (Oka et al., 2007) LPI did not inhibit human neutrophil migration (Fig. 3C).

Phytocannabinoids and Related Ligands. We also investigated the effects of a range of compounds that are found in *Cannabis sativa* and structurally related ligands that have been reported to interact with either the abnormal-CBD receptor or GPR55.

(−)-CBD did not stimulate neutrophil migration ($P > 0.05$; one-sample *t* test; $n = 9$), with the extent of migration being 0.09 ± 0.26, −0.02 ± 0.18, −0.03 ± 0.07, −0.02 ± 0.24, −0.20 ± 0.13, and 0.25 ± 0.25% of the 1 μM fMLP control stimulated by 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, and 1 μM (−)-CBD, respectively (Protocol I; see *Materials and Methods*). In contrast, (−)-CBD significantly inhibited neutrophil migration induced by 1 μM fMLP (one-sample *t* test; $n = 9$–15; Fig. 3B). Whereas (−)-CBD was highly potent (IC_{50} of 0.45 nM), it had a significantly lower efficacy (E_{max}) than virodhamine (Table 1). Using the trypan blue exclusion method, the estimated percentage of viability of neutrophils incubated for 30 min in vehicle (0.01% DMSO) or 1 μM (−)-CBD was 96.6 ± 0.5 and 96.8 ± 0.3%, respectively, which was not significantly different from one another ($P > 0.05$; Student's unpaired *t* test; $n = 3$).

(+)-CBD also significantly inhibited neutrophil migration induced by 1 μM fMLP (one-sample *t* test; $n = 9$; Fig. 3B), but it was ~40-fold less potent than its stereoisomer (−)-CBD (Table 1). The close structural analog of CBD, abn-CBD significantly inhibited neutrophil migration induced by 1 μM fMLP (one-sample *t* test; $n = 9$; Fig. 3B). Abn-CBD was ~70-fold less potent than (−)-CBD, but it seemed to have a higher efficacy (E_{max}) (Table 1).

The abnormal CBD analog, O-1602, which is an agonist of the abn-CBD receptor (Járai et al., 1999) and GPR55 (Johns et al., 2007; Ryberg et al., 2007), inhibited human neutrophil migration with a similar potency to abnormal-CBD (Tables 1 and 2; Fig. 3B). The major psychoactive constituent of cannabis, Δ^9-THC, had no significant effect on the neutrophil migration induced by 1 μM fMLP (one-sample *t* test; $n = 9$; Fig. 3B).

Effect of Receptor Antagonists on Cannabinoid-Mediated Inhibition of Human Neutrophil Migration

In the next series of experiments, we investigated the receptors underlying the inhibition of human neutrophil migration by cannabinoids and related ligands. The effect of the CB_1 receptor antagonist SR141716A (SR141), the CB_2 receptor antagonists SR144528 and AM630, and the vanilloid receptor subtype 1 receptor antagonist capsazepine (CPZ) on the ability of AEA to inhibit fMLP-induced neutrophil migration was tested using protocol II (see *Materials and Methods*).

The inhibition of fMLP-induced neutrophil migration by 100 nM AEA was significantly attenuated by 1 μM SR141 ($P < 0.001$; one-way ANOVA; $n = 9$) (Fig. 4A). AEA-mediated inhibition of fMLP-induced migration was significantly enhanced in the presence of the CB_2 antagonist SR144 (100 nM) ($P < 0.001$; one-way ANOVA; $n = 9$). CPZ had no significant effect on the ability of 100 nM AEA to inhibit fMLP-induced neutrophil migration ($P > 0.05$; one-way ANOVA; $n = 9$) (Fig. 4A). Because 100 nM SR144 enhanced the inhibition of fMLP-induced neutrophil migration by 100 nM AEA, the effect of a structurally distinct CB_2 receptor antagonist AM630 was also investigated. AM630 also significantly enhanced the ability of 100 nM AEA to inhibit fMLP-induced neutrophil migration ($P < 0.001$; Student's unpaired *t* test; $n = 9$) (Fig. 4B). Neither 1 μM SR141, 100 nM SR144, 1 μM CPZ, nor 100 nM AM630 alone had any significant effect on fMLP-induced migration (one-sample *t* test; $n = 9$) (Fig. 4D).

The ability of AEA to inhibit fMLP-induced neutrophil migration in the presence of the CB_1 receptor antagonist SR141 (1 μM) was further investigated. Again, 1 μM SR141 alone had no significant effect on fMLP-induced migration (one-sample *t* test; $n = 3$), the migration being 100.6 ± 2.03% ($P > 0.05$) of fMLP with vehicle. The log concentration-response curve for AEA appeared to be biphasic (Fig. 4C); in the presence of 1 μM SR141, there was a rightward shift in the first phase of the log concentration-response curve for AEA (Fig. 4C).

Inhibition of fMLP-induced migration by virodhamine (10 nM) was significantly attenuated in the presence of the CB_1 antagonist SR141 (1 μM) ($P < 0.05$; one-way ANOVA; $n = 12$) (Fig. 5A). In contrast, 10 nM virodhamine-mediated inhibi-

TABLE 1

Inhibition of fMLP-induced migration of human neutrophils by cannabinoids

IC_{50} and E_{max} values (percentage of inhibition of fMLP-induced migration) were calculated from sigmoidal concentration-response curves constructed in GraphPad Prism 4. The data represent the mean with 95% confidence limits, $n = 3$ to 9.

Compound	IC_{50} (95% Confidence Limits)	E_{max} (95% Confidence Limits)
	nM	%
Endogenous lipid mediators		
Virodhamine	0.18 (0.17–0.19)	86.3 (85.7–86.8)
NADA	8.80 (4.7–16.2)	64.0 (56.6–71.5)
AEA	0.14 (0.12–0.17)[a]	54.0 (53.0–55.2)[a]
2-AG	>1000	
ARA-S	>1000	
PEA	>1000	
LPI	>1000	
Phytocannabinoids and analogs		
(−)-CBD	0.45 (0.39–0.53)	59.5 (58.0–61.0)
(+)-CBD	18.8 (15.4–23.0)	70.0 (67.1–72.8)
Abnormal-CBD	33.1 (30.0–36.4)	69.9 (68.6–71.3)
O-1602	32.6 (24.1–44.2)	66.6 (64.1–69.1)
Δ^9-THC	>1000	

[a] The concentration-response curve to anandamide is biphasic. These values represent the first phase of inhibition.

tion of fMLP-induced migration was enhanced in the presence of the CB_2 antagonists SR144 (100 nM) and AM630 (1 μM) ($P < 0.01$; one-way ANOVA; $n = 12$) (Fig. 5A). The vanilloid receptor subtype 1 receptor antagonist CPZ had no effect on virodhamine-mediated inhibition of migration ($P > 0.05$; one-way ANOVA; $n = 12$) (Fig. 5A).

In the presence of the CB_2 receptor antagonist SR144, the synthetic CB_1/CB_2 receptor antagonist CP55940 significantly inhibited fMLP-induced inhibition of human neutrophil migration, an effect that was not seen in the absence of the antagonist (Fig. 5B).

ARA-S Antagonized the Inhibition of Human Neutrophil Migration Induced by Virodhamine and Abnormal-CBD

As shown in Fig. 3A, at concentrations up to 1 μM, *N*-arachidonoyl-L-serine did not inhibit human neutrophil migration. However, at 1 μM this compound significantly attenuated the inhibition of fMLP-stimulated human neutrophil migration induced by anandamide ($P < 0.001$; Student's unpaired *t* test) (Fig. 6A). Furthermore, this compound abolished the inhibition of human neutrophil migration induced by abnormal-CBD (Fig. 6B), and it significantly reduced the E_{max} for inhibition of migration by virodhamine to 45% (95% confidence limits, 42–47) (Fig. 6C).

Discussion

Our investigation revealed an intriguing pharmacology underlying the inhibition of human neutrophil chemotaxis indicating that certain endocannabinoids and phytocannabinoids are potent inhibitors of human neutrophil migration.

CB_1 receptor expression in human neutrophils is low- (Galiègue et al., 1995), and the weight of evidence indicates that cannabinoid CB_1 receptors do not play a role in the cannabinoid-mediated inhibition of induced human neutrophil migration observed in this study. First, a number of compounds display potent inhibitory behavior that is

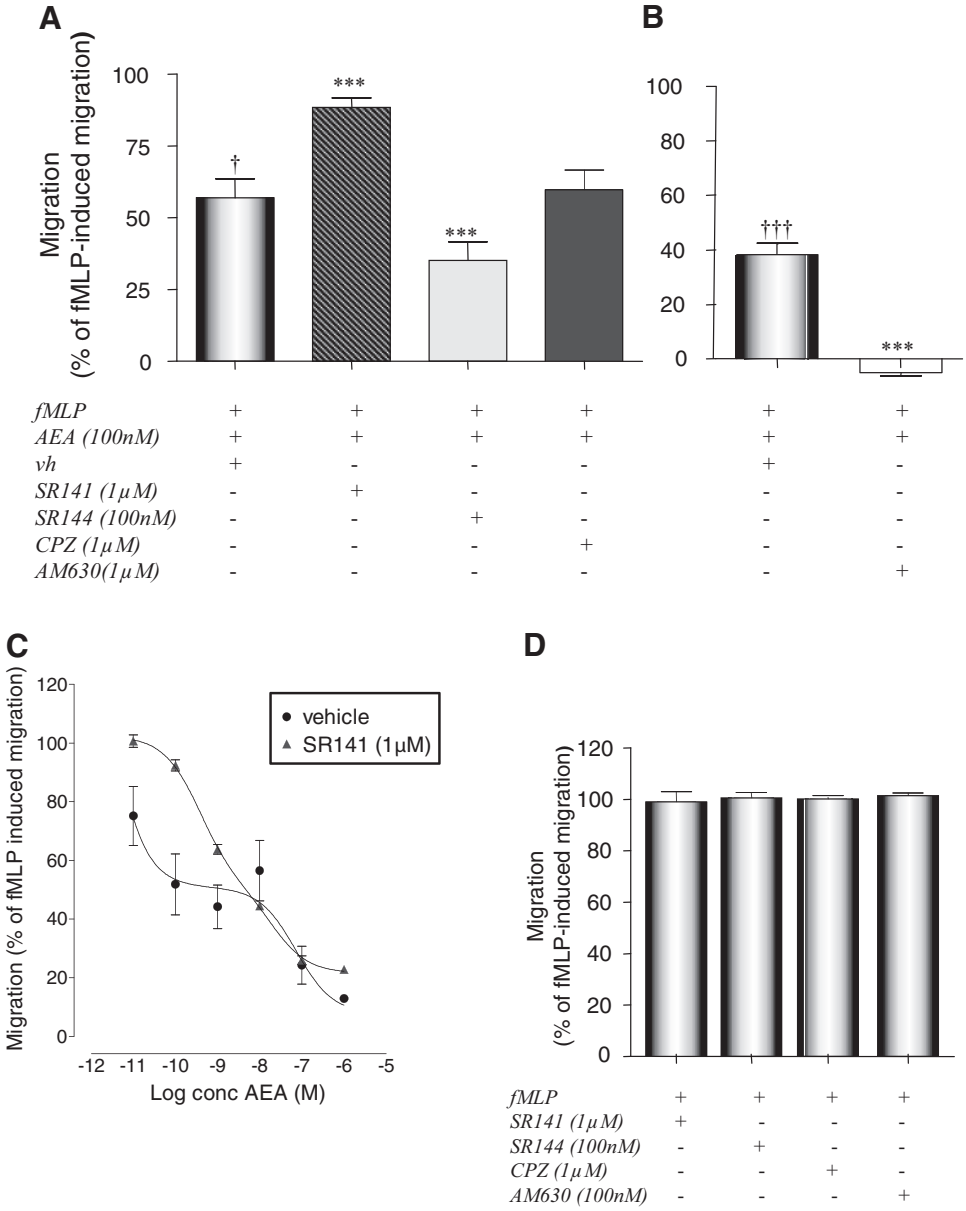

Fig. 4. A, histogram showing neutrophil migration induced by 1 μM fMLP after preincubation with AEA (100 nM) + vehicle, AEA (100 nM) + SR141 (1 μM), AEA (100 nM) + SR144 (100 nM), and AEA (100 nM) + CPZ (1 μM). AEA (100 nM) significantly inhibits (†, $P < 0.05$; one-sample *t* test) migration induced by fMLP (1 μM). In the presence of SR141 (1 μM), the inhibition is significantly attenuated (***, $P < 0.001$; one-way ANOVA). In the presence of SR144 (100 nM), the inhibition is significantly enhanced (***, $P < 0.001$; one-way ANOVA). B, histogram showing neutrophil migration induced by 1 μM fMLP after preincubation with AEA (100 nM) + vehicle or AEA (100 nM) + AM630 (100 nM). AEA (100 nM) significantly inhibits (†††, $P < 0.001$; one-sample *t* test) neutrophil migration induced by fMLP (1 μM). In the presence of AM630 (100 nM), the inhibition is significantly enhanced (***, $P < 0.001$; Student's unpaired *t* test). C, log concentration-response curves showing the percentage of fMLP (1 μM)-induced migration in the presence of increasing concentrations of AEA with either vehicle or 1 μM SR141. D, preincubation with SR141 (1 μM), SR144 (100 nM), CPZ (1 μM), or AM630 (100 nM). None of these antagonists have any significant ($P > 0.05$; one-sample *t* test) effect on neutrophil migration induced by 1 μM fMLP. The data represent the mean number of neutrophils migrated ± S.E.M. ($n = 9$) as a percentage of the migration induced by 1 μM fMLP in the presence of vehicle (0.01% DMSO).

inconsistent with their pharmacology at CB_1 receptors; virodhamine is a CB_1 receptor antagonist (Porter et al., 2002) and (−)-CBD has low affinity for the CB_1 receptor (Thomas et al., 1998; Bisogno et al., 2001); 2-AG and Δ^9-THC, which are both agonists of the CB_1 receptor (Pertwee and Ross, 2002), do not inhibit neutrophil migration. It has been demonstrated that the CB_1 receptor antagonist SR141 can antagonize non-CB_1 receptors at concentrations in the micromolar range (Drmota et al., 2004; Begg et al., 2005); therefore, we made a conscious decision to use a concentration of 1 μM. The inhibition of neutrophil migration by virodhamine was attenuated by 1 μM SR141. The inhibition of neutrophil migration by anandamide seems to be biphasic in nature, and the first phase of this inhibition is by the CB_1 receptor antagonist 1 μM SR141. The nature of the log concentration-response curve for AEA suggests that more than one target may be involved in the inhibition of human neutrophil migration by this endocannabinoid. Taken together, these results are consistent with a role for a non-CB_1 receptor in mediating the effect of these endocannabinoids (MacLennan et al., 1998; Pertwee, 1999; Begg et al., 2005).

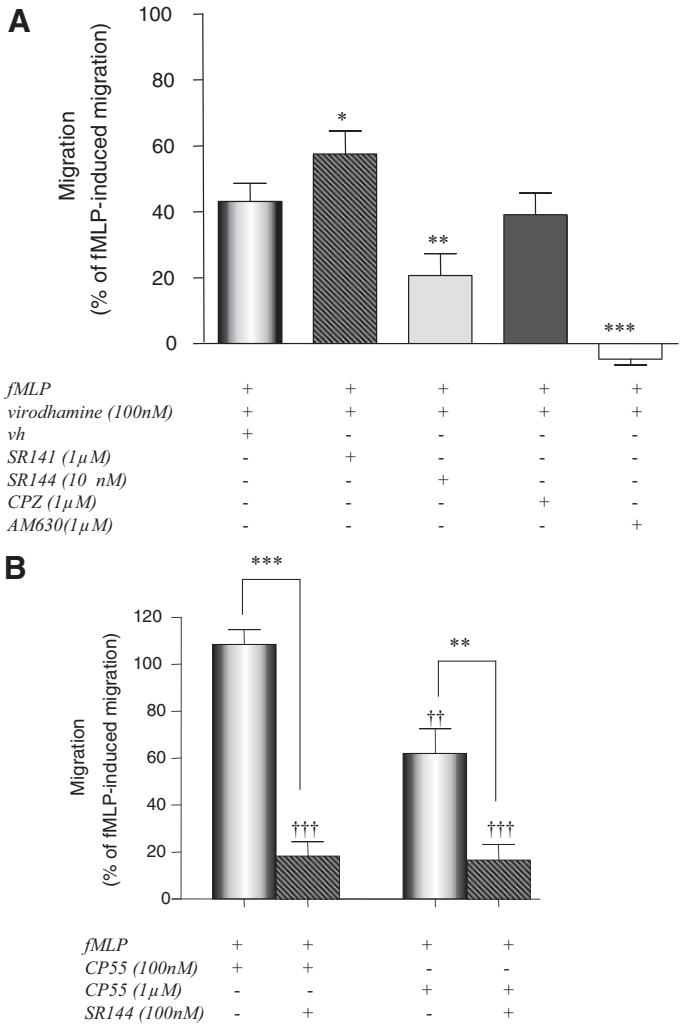

Fig. 5. A, histogram showing neutrophil migration induced by fMLP (1 μM) after preincubation with virodhamine (10 nM), virodhamine + SR141 (1 μM), virodhamine (10 nM) + SR144 (100 nM), virodhamine (10 nM) + CPZ (1 μM), and virodhamine (10 nM) + AM630 (100 nM). Virodhamine (10 nM) significantly inhibits (†, $P < 0.05$; one-sample t test) migration induced by fMLP (1 μM). Asterisks represent P values calculated from one-way ANOVA compared with virodhamine alone. The data represent the mean number of neutrophils migrated ± S.E.M. ($n = 9$) as a percentage of the migration induced by 1 μM fMLP in the presence of vehicle (see the equation under *Materials and Methods*). B, histogram showing neutrophil migration induced by fMLP (1 μM) followed by preincubation with CP55950 at 100 nM and 1 μM, in the absence and presence of SR144 (100 nM). CP55940 (1 μM) significantly inhibits (††, $P < 0.01$; one-sample t test) neutrophil migration induced by fMLP (1 μM). In the presence of SR144 (100 nM), both 100 nM and 1 μM inhibit fMLP induced migration (†††, $P < 0.001$; one-sample t test). The inhibitory effect of both concentrations of CP55940 is significantly different in the presence of the antagonist (**, $P < 0.01$; ***, $P < 0.001$; Student's unpaired t test).

Kurihara et al. (2006) recently found surface expression of CB_2 receptors on neutrophil-like HL60 cells and human neutrophils. In our investigation, a role for CB_2 receptors in the modulation of neutrophil migration was implied by the fact that two, structurally distinct, CB_2-selective antagonists significantly enhanced the cannabinoid-mediated inhibition. There is considerable evidence that cannabinoid CB_1 and CB_2 receptors exist in a conformation that is precoupled to the G protein (Pertwee, 2005). Both SR144 and AM630 are CB_2 receptor inverse agonists (Bouaboula et al., 1999; Ross et al., 1999), and, as such, they bind with a high affinity to the precoupled CB_2 receptors. CB_2 receptors on human neutrophils may be autoactivated, exerting high basal levels of CB_2 receptor-mediated signaling; thereby precluding inhibition mediated by certain other receptors. Furthermore, autoactivation may underlie the lack of significant stimulation of neutrophil migration by the CB_2 receptor agonists 2-AG, CP55940, JWH-133, and JWH015 (Kurihara et al., 2006; this study). In line with our findings, Lunn et al. (2006) have demonstrated that CB_2 receptor inverse agonists inhibit leukocyte migration both in vivo and in vitro, and the level of effect is proportional to the degree of inverse efficacy.

Particularly pertinent to our neutrophil study is the non-CB_1, non-CB_2 pharmacological target named the abn-CBD receptor, which is antagonized by SR141, but only at concentrations considerably higher than those predicted from its CB_1 receptor affinity (Begg et al., 2005). Evidence for the existence of abn-CBD receptors initially emerged from studies in certain blood vessels, which relax in response to AEA and abn-CBD; an effect that is maintained in vessels from $CB_1^{-/-}$ mice (Járai et al., 1999). It is noteworthy that neither 2-AG nor Δ^9-THC relaxed these vessels (Ho and Hiley, 2003). Although CBD acts as an antagonist of the abn-CBD receptor in certain vessels, in other vessels CBD seems to act as an agonist, sharing the ability of abn-CBD to relax vessels. In addition, virodhamine and NADA induce a relaxation of mesenteric arteries, being more potent than either AEA or abn-CBD (Ho and Hiley, 2004; O'Sullivan et al., 2004; Begg et al., 2005). Probably of the greatest relevance to our data are studies in microglial cells, which provide robust evidence for a role of Abn-CBD receptors in cell migration (Walter et al., 2003). Thus, 2-AG triggers microglial cell migration by acting through CB_2 and abn-CBD receptors. CBD acts as a partial agonist, thereby inducing migration alone but also attenuating migration induced by a full agonist. Stimulation of microglial cell migration by cannabinoids is antagonized by high but not low concentrations of SR141 (Franklin and Stella, 2003). It is apparent that there are certain parallels between the pharmacology that we observed in human neu-

trophils and pharmacology of the abn-CBD receptor, which is summarized in Table 2. First, the effects of both AEA and virodhamine are sensitive to antagonism by 1 μM SR141, which is in line with the proposed affinity of the CB_1 receptor antagonist for the abn-CBD receptor (Begg et al., 2005). Second, agonist efficacy and potency closely parallel that previously obtained for the abn-CBD receptor in blood vessels and microglial cells (Table 2); of the phytocannabinoids and structurally related ligands, (−)-CBD has low efficacy, but it is more potent than both (+)-CBD and abn-CBD; O-1602, an analog of abn-CBD in which the pentyl side chain was shortened to a methyl group, is an agonist and Δ^9-THC is inactive; of the endocannabinoids and related lipids, AEA, NADA, and virodhamine are active, the later having the highest potency; palmitoylethanolamide is inactive. Perhaps the more intriguing finding in the current study is the antagonism of various cannabinoids by ARA-S, a brain constituent previously shown to be an agonist at the abn-CBD receptor (Milman et al., 2006). Here, we find that this endogenous compound attenuates the inhibition of human neutrophil migration by AEA, virodhamine, and abn-CBD. It is also notable that although 1 μM ARA-S abolishes the effect of abn-CBD, it reduces the E_{max} value of virodhamine. Thus, the effect of virodhamine may involve more than one receptor, only one of which is blocked by ARA-S. Alternatively, ARA-S may be acting as an allosteric inhibitor of the target receptor; a reduction in E_{max} is characteristic of an allosteric inhibitor. In line with our findings, in a recent abstract Zhang et al. (2006) report that abn-CBD inhibits angiogenesis, an effect that is antagonized by ARA-S. This raises two possibilities: either this endogenous lipid mediator is a partial agonist at the abn-CBD receptor and thereby also acts as antagonist in certain conditions; or the effects observed in this study are due, at least in part, to activation of another, perhaps related target.

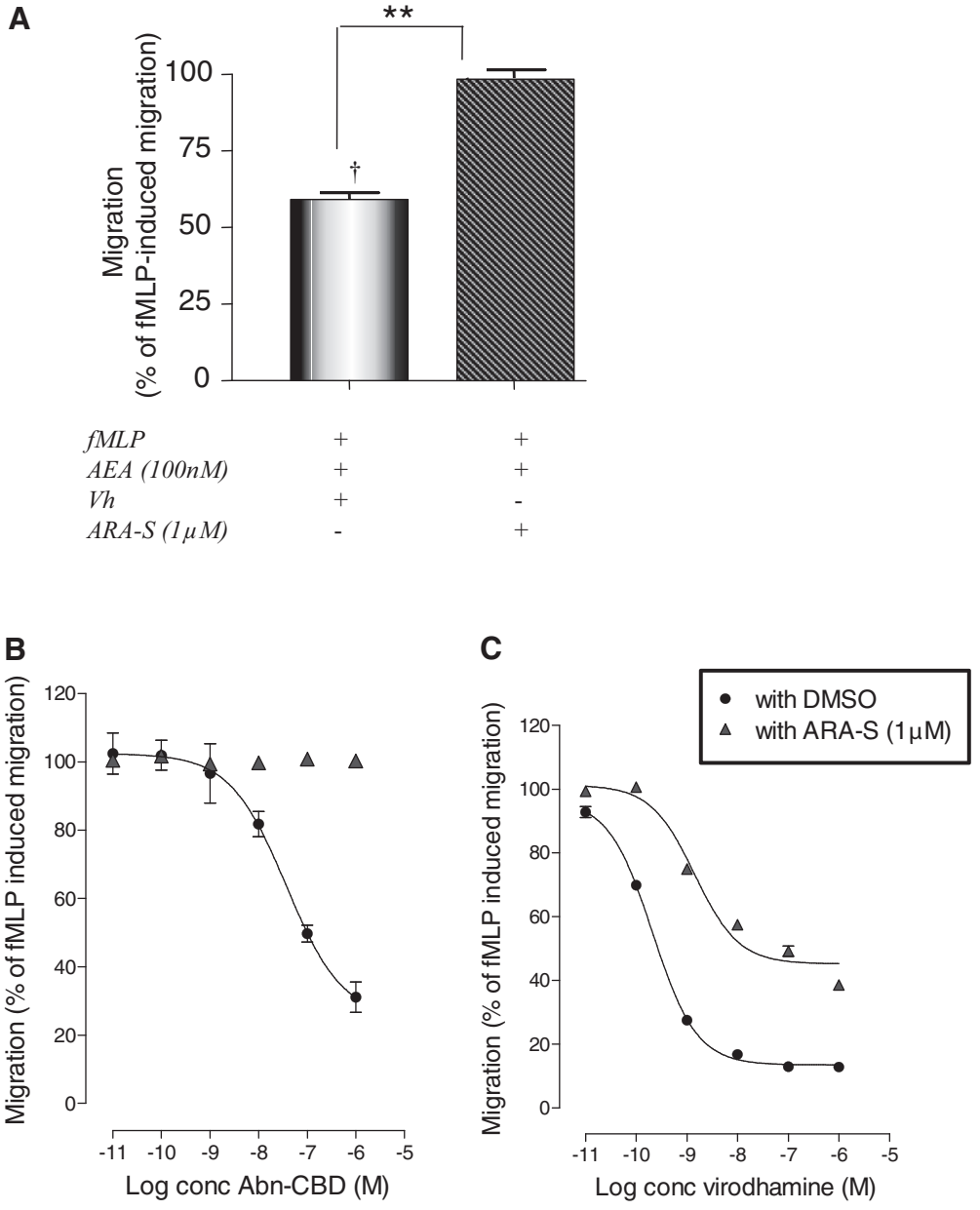

Fig. 6. A, histogram showing neutrophil migration induced by fMLP (1 μM) after preincubation with either vehicle (Vh) (0.01% DMSO) or ARA-S (1 μM) before subsequent preincubation with AEA. AEA significantly inhibits (†, $P < 0.05$; one sample t test) migration induced by fMLP (1 μM). The migration induced by 1 μM fMLP in the presence of AEA + Vh (0.01% DMSO) is significantly lower than compared with AEA + ARA-S (**, $P < 0.001$, Student's unpaired t test). B, histogram showing neutrophil migration induced by fMLP (1 μM) after preincubation with either Vh or ARA-S (1 μM) before subsequent preincubation with Abn-CBD. C, histogram showing neutrophil migration induced by fMLP (1 μM) after preincubation with either Vh or ARA-S (1 μM) before subsequent preincubation with virodhamine. The data represent the mean number of neutrophils migrated ± S.E.M. (n = 9–12) calculated using the equation under *Materials and Methods*.

Recent publications (Johns et al., 2007; Ryberg et al., 2007) have emerged suggesting certain cannabinoid ligands interact with an orphan receptor GPR55 and that this, and possibly other orphan receptors, may account for the pharmacological and functional evidence for some novel CB receptors (Baker at al, 2006; Pertwee, 2007). Although it has been suggested that the abn-CBD receptor is, in fact, GPR55, there are notable differences in the reported pharmacology of these two targets, as summarized in Table 2 (Baker et al., 2006; Mackie and Stella, 2006). It is particularly notable that although abn-CBD has high affinity for GPR55, it retains the vasodilator effects in mice lacking GPR55, the implication being that this atypical cannabinoid activates more additional novel receptors (Hiley and Kaup, 2007; Johns et al., 2007). With regard to human neutrophils, it is noteworthy that GPR55 is expressed in splenic tissue and that virodamine, which was the highest efficacy and potency compound in our study, is also reported to be a high-efficacy agonist of GPR55 (Ryberg et al., 2007). Reports suggest that GPR55 and related orphan receptors are G_{13}-coupled and that they can activate the Rho pathway (Ryberg et al., 2007), which plays an important role in the regulation of myosin light chain phosphorylation and subsequent cytoskeletal-dependent locomotion (Buhl et al., 1995; Kozasa et al., 1998). Therefore, as a consequence of its G_{13}-coupling, it seems that inverse agonism of GPR55 would be necessary to deliver an antimigratory signal to the cellular locomotory machinery. However, there is considerable evidence for cross-desensitization of G protein-coupled receptors, leading to inhibition of fMLP-induced migration (Ali et al., 1999), thereby affording speculation that agonists of this receptor may be inhibitory. Kurihara et al. (2006) have recently demonstrated that the CB_2 receptor plays a role in human neutrophil migration by modulating RhoA activation; one might speculate that cross-talk between the CB_2 receptor and the novel target located in human neutrophils occurs at the level of Rho signaling. Some controversy surrounds the pharmacology of GPR55; Oka et al., (2007) report that LPI, but not cannabinoid ligands, induce extracellular signal-regulated kinase phosphorylation in GPR55-expressing cells. Here, we report that LPI does not inhibit human neutrophil migration.

Our data suggest that in human neutrophils, there is cross-talk/negative co-operativity between the cannabinoid CB_2 receptor and a novel receptor such that inhibition of precoupled CB_2 receptors enhances the inhibition observed in response to activators of this receptor. This is in line with the positive co-operatively observed between the CB_2 and abn-CBD receptor in microglial cells (Walter et al., 2003). A realistic explanation for the apparent bidirectional co-operativity exhibited between these two receptors is a difference in underlying signal transduction depending on cell type. Taken together, our data provide the first evidence that human neutrophil migration can be modulated by a novel receptor and that, in these cells, the CB_2 receptor exerts negative co-operativity upon this receptor.

This study reveals that certain endogenous lipids, phytocannabinoids and related ligands are potent inhibitors of human neutrophil migration, and it implicates a novel pharmacological target distinct from cannabinoid CB_1 and CB_2 receptors; this target is antagonized by the endogenous compound *N*-arachidoloyl L-serine. These findings corroborate the emerging clinical and animal model data

TABLE 2
Comparison of the profile of various compounds as inhibitors of human neutrophil migration (this study) with the reported pharmacology of these ligands at abnormal-CBD receptors and the orphan receptor GPR55

Compound	Inhibition of Neutrophil Migration	Abnormal-CBD Receptor	GPR55
Endogenous lipid mediators			
Virodhamine	+++	++[a]	++[b]
NADA	++	++[a]	N.D.
AEA	+++	+[a]	++[b]
2-AG	0	0[a]	++[b]
		++[c]	
ARA-S	Antagonist	Agonist[d]	N.D.
		Antagonist[e]	
PEA	0	0[c]	++[b]
LPI	0	N.D.	++[f,g]
Phytocannabinoids and related ligands			
(−)-CBD	+++	+[a]/++[c]	++[b]
	Partial agonist	Partial agonist	Antagonist
(+)-CBD	++	N.D.	N.D.
Abnormal-CBD	++	++[c]	++[h]
		+[a]	+[b]
O-1602	++	+[i]	++[h]
			++[b]
Δ^9-THC	0	0[a,c]	++[b]
Other			
CP55940	++	0[a]	++[b]
SR141716A	Antagonist	Antagonist[a,c]	Partial agonist[j]

The symbols represent the potency or affinity of compounds being in the picomolar range (+++), nanomolar range (++), micromolar range (+), or not determined (N.D.).
[a] Begg et al. (2005).
[b] Ryeberg et al. (2007).
[c] Walter et al. (2003).
[d] Millman et al. (2005).
[e] Zhang et al. (2006).
[f] Oka et al. (2007).
[g] Oka et al. (2007) report that cannabinoid ligands do not activate GPR55.
[h] Johns et al. (2007).
[i] Járai et al. (1999).
[j] Drmota et al. (2004).

demonstrating that the nonpsychoactive phytocannabinoid, CBD and its structural analogs are effective in alleviating arthritis (Malfait et al., 2000; Blake et al., 2006). Furthermore, our findings have implications for the potential pharmacological manipulation of elements of the endocannabinoid system for the treatment of various inflammatory conditions.

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