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Indirect CB_2 Receptor and Mediator-Dependent Stimulation of Human Whole-Blood Neutrophils by Exogenous and Endogenous Cannabinoids

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ABSTRACT

Immunomodulatory effects of endogenous and exogenous cannabinoids have been investigated in numerous studies, mostly performed with isolated cells or transformed cell lines, but only sparse data exist on human polymorphonuclear neutrophils (PMNs). We therefore investigated the respiratory burst reaction of human whole-blood PMNs under the influence of cannabinoids using flow cytometry. In their natural whole-blood milieu, a CB_2 receptor-dependent stimulation of the PMN respiratory burst was found at nanomolar concentrations of CP55 940 [(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol] and methanandamide after a 3-h incubation period, whereas the short-living and rapidly hydrolyzed endogenous ligand anandamide did not alter the burst reaction of whole-blood PMNs under the same experimental conditions. The stimulatory cannabinoid effect was totally absent in isolated PMNs but could be transferred onto isolated PMNs by adding the cell-free low-molecular mass plasma fraction (<5000 Da) of cannabinoid-incubated blood, indicating an indirect mechanism depending on humoral products or mediators. Results of our further experiments suggest that products of the arachidonic acid metabolism are mediators of the cannabinoid-induced enhancement of the respiratory burst reaction of whole-blood PMNs.

After detection of the "peripheral" cannabinoid receptor (CB_2R) on leukocytes, numerous in vitro studies evaluated the potential effects of natural exogenous (Klein et al., 2003) and endogenous cannabinoid ligands on various immune functions, mainly in lymphocytes and macrophages.

Suppression of natural killer cell cytotoxicity (Massi et al., 2000), B-lymphocyte activity (Klein et al., 1985), and impairment of macrophage functions (Lopez-Cepero et al., 1986; Baldwin et al., 1997) have been described as well as an enhancement of oxygen radical production in alveolar macrophages (Sarafian et al., 1999) and an increased B-cell proliferation response (Derocq et al., 1995). Some authors also found an altered cytokine production with a shift from T-helper cell 1 to T-helper cell 2 cytokines and an impairment of macrophage/T-cell cooperation induced by certain cannabinoids (Klein et al., 1991, 1998).

To date, cannabinoids are considered to act mainly as immunosuppressive agents in animals and humans because they are potent inhibitors of the adenylate cyclase activity and thus may alter leukocyte functions by reduction of intracellular cAMP levels (Slipetz et al., 1995; Schatz et al., 1997). Reduced intracellular cAMP levels in polymorphonuclear neutrophils have been shown to increase chemotaxis, lysosomal enzyme release, and respiratory burst reaction (Wright et al., 1990). Therefore G_i-coupled receptors such as CB_2 would be expected to enhance rather than inhibit neutrophil function. However, not all effects elicited by cannabinoid receptor activation can be explained by cAMP-dependent mechanisms, and most in vitro studies were performed with either isolated cells from animals or transformed cell lines expressing the CB_2 receptor. The data reported from these in vitro studies are often unequivocal and difficult to interpret depending on cell type, animal species, cannabinoid compound, concentration, and cellular environment.

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ABBREVIATIONS: CB_2R, cannabinoid receptor; PMN, polymorphonuclear neutrophil; THC, Δ9-tetrahydrocannabinol; CP55 940, (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; AEA, anandamide; MethAEA, methanandamide; SR144 528, *N*[1*S*)-*endo*-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; COX, cyclooxygenase; LOX, lipoxygenase; MK886, 3-[1-(*p*-chlorobenzyl)-5(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid,Na; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; ANOVA, analysis of variance; 2-AG, 2-arachidonoylglycerol.

Even from in vitro and animal studies, only sparse data exist on the influence of cannabinoids on the major leukocyte population of polymorphonuclear neutrophils (PMNs), although these phagocytes are the first line defense against bacterial and fungal infections. Two independent previous reports showed a suppression of the oxygen radical production of isolated human PMNs at high micromolar concentrations of the natural cannabinoid Δ^9-tetrahydrocannabinol (THC) and the synthetic THC analog CP55 940 in vitro (Djeu et al., 1991; Kraft et al., 2004). In both studies, the concentrations necessary for this suppression were far above the range that can be reached in vivo, and no data are available on cannabinoid effects under more physiological conditions, i.e., in whole blood. Since dibenzopyrane cannabinoids such as THC (Marinol) or nabilone (Cesamet) are therapeutically used in immunocompromised HIV and cancer patients as antiemetics and to improve appetite, a potential impairment of the phagocytic and oxidative microbicidal activity of human PMNs would be clinically relevant and the conditions of its appearance should be known in more detail.

Therefore, the objective of the present study was to investigate the effects of relevant concentrations of the synthetic THC-analog CP55 940, the endogenous cannabinoid anandamide, and its more stable derivative methanandamide on the respiratory burst of human PMNs in the whole-blood milieu.

Materials and Methods

Cannabinoid Compounds

Three different ligands of the two cannabinoid receptors, CB_1R and CB_2R, were tested: the synthetic dibenzopyrane cannabinoid CP55 940 as an analog of the marijuana cannabinoid Δ^9-THC, the endogenous eicosanoid compound *N*-arachidonylethanolamide (anandamide; AEA), and its nonhydrolyzable and thus more stable derivative methanandamide (MethAEA) (Devane et al., 1992; Martin et al., 1999). CP55 940 (Tocris Cookson Inc., Ellisville, MO) and the specific CB_2-antagonist SR144 528 (kindly provided by SANOFI Research Center, Montpellier, France) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and further diluted with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) to the final working concentrations. AEA (Cayman Chemical, Ann Arbor, MI) and MethAEA (Cayman Chemical) were dissolved in ethanol and also diluted with PBS. The sterile solutions were always freshly prepared for each experiment, and the final DMSO or ethanol concentration was 0.001% (v/v) in each test.

Inhibitors of the Cyclooxygenase and Lipoxygenase Enzymes

To characterize the cannabinoid-induced mechanisms and the potential humoral mediators involved, three different inhibitors of cyclooxygenase (COX) or lipoxygenase (LOX) enzymes were used: meclofenamic acid (Sigma-Aldrich), an inhibitor of COX with some LOX-inhibitory effects at higher concentrations (IC_{50} = 47 μM) (Conroy et al., 1991; Streefkerk et al., 2003); flurbiprofen (Aldrich Chemical Co., Milwaukee, WI), a COX inhibitor without strong suppressive effects on the neutrophil respiratory burst (Parij et al., 1998); and MK886 (kindly provided by Merck Frosst, Canada), an inhibitor of the 5-lipoxygenase activator protein (Daniels et al., 1998). The three inhibitors were dissolved in DMSO and diluted with PBS to the respective working concentrations. The final concentrations in the tests were 50 and 100 μM for meclofenamic acid (Ramos et al., 1994), 10 and 25 μM for flurbiprofen, and 4 and 40 μM for MK886, as previously described by Daniels et al. (1998).

In Vitro Assessment of the Respiratory Burst Reaction in Human Whole-Blood PMNs

After approval by our institutional ethics committee, heparinized venous blood obtained from informed and consenting healthy volunteers (six males, two females, median age 36 years) was incubated with CP55 940 at logarithmic concentration steps from 10^{-11} to 10^{-4} M. After incubation, the respiratory burst reaction of the whole-blood PMNs was determined using the commercially available Bursttest (Orpegen Inc., Heidelberg, Germany) as described by Rothe et al. (1998). The synthetic PMN stimulant *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; final concentration 10^{-7} M) or plain buffer were added to 100-μl triplicate aliquots of the whole-blood samples to detect potential stimulatory effects, and the two strong and almost maximum stimulants, the phorbolester PMA (final concentration, 8.1×10^{-7} M) and a suspension of *Escherichia coli* (10^9 bacteria/ml), were used for detection of a potential suppressive effect. The burst reaction was determined by the conversion of 123-dihydrorhodamine to the fluorescent dye rhodamine in the cytoplasm of activated PMNs. Rhodamine fluorescence was measured using a FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer with a 488-nm argon laser as previously described (Kraft et al., 2004). Other blood cells were excluded from analysis by a live gate on the PMN cluster in the SSC/FSC dot plot. For each sample, 10,000 events were acquired and aggregation artifacts or cell detritus were detected by the addition of 200 μl of propidium iodide solution (125 mg/ml) after lysing the erythrocytes and washing (live gate on the Fl2 histogram). Data were analyzed with CellQuest software (BD Biosciences), and the mean cellular fluorescence (Fl1), which is proportional to the amount of produced oxygen radicals, as well as the percentage of stimulated rhodamine positive cells were determined to assess the activation of the oxidative burst reaction in whole-blood PMNs (Kraft et al., 2004).

Discrimination of Direct Cellular from Humorally Mediated Cannabinoid Effects

Direct Cellular Effects on PMNs. To further characterize the role of direct cannabinoid effects on PMNs, heparinized venous blood from each healthy donor was divided into two aliquots: one for the whole-blood incubation with the respective cannabinoids as already described above and the other aliquot for the separation of PMNs as described below (Deusch et al., 2003; Kraft et al., 2004). In brief, from the plasma supernatant obtained by Ficoll-Hypaque (Pfizer, Inc., New York, NY) sedimentation, PMNs were separated by centrifugation (20°C, 25 min, 250*g*) through a two-step Percoll (Pfizer, Inc.) density gradient (62 and 73% v/v). Separated PMNs (2.5×10^6 cells/ml) were resuspended in RPMI 1640 medium supplemented with 5% fetal calf serum and maintained at 37°C. Cell viability was monitored by trypan blue exclusion (>95%), and PMN enrichment was verified by differential count (>95%).

Aliquots of the remaining whole-blood sample from the same donor were incubated with vehicle as controls or the respective cannabinoid concentrations. After centrifugation (20°C, 10 min, 250*g*), the supernatants were removed and separated into high-molecular mass (>5000 Da) and a low-molecular mass (<5000 Da) fractions by centrifugation through a molecular pore filter system (Centrisart I; Sartorius Inc., Göttingen, Germany) with a cut-off threshold of 5000 Da. Aliquots of the isolated PMNs were incubated (30 min, 37°C) either with the cannabinoid dilution alone or with the respective low- and high- molecular plasma fractions derived from the whole-blood incubation experiments; thereafter, the PMN respiratory burst reaction was examined as described before.

COX- or LOX-Dependent Mediators. The unseparated whole-blood samples were preincubated with meclofenamic acid, flurbiprofen, or MK886 before adding CP55 940 (0.1 and 1.0 nM). Thereafter, the plasma supernatants were obtained by centrifugation (20°C, 10 min, 250*g*) as described above, and the freshly isolated PMNs from the same donor were incubated with the supernatants (30 min, 37°C) followed by the Bursttest, as described in detail before.

Statistical Analysis. Data were analyzed by means of Jandel Sigma Stat 2.0 software for Windows (SPSS Inc., Chicago, IL). Unless otherwise indicated, results are expressed as a percentage of the control measurements with vehicle alone. For multiple comparisons, one-way ANOVA followed by Bonferroni's post hoc test was used for normally ANOVA-on ranks followed by Dunn's post hoc test for non-normally, distributed values, respectively. Where appropriate, paired Student's t test was applied. $P < 0.05$ was considered significant.

Results

Pilot experiments were performed to determine the optimum incubation period for whole-blood PMNs using two high (10^{-6} and 10^{-5} M) and two low (10^{-10} and 10^{-9} M) concentrations of CP55 940. The micromolar concentrations as well as the long incubation periods of up to 180 min were chosen based on our previous report (Kraft et al., 2004). Because of the CB_2R dissociation constants for the cannabinoids studied, the nanomolar concentrations were tested because they are considered more relevant to determine CB receptor-mediated effects. The oxygen radical production of fMLP-stimulated whole-blood PMNs was activated at 10^{-10} and 10^{-9} M CP55 940, starting after a 90-min incubation period and reaching a maximum after a 120- to 180-min duration of cannabinoid exposure (Fig. 1).

Therefore, a 180-min exposure period to CP55 940 was used in all further experiments. Resting (Fig. 2A) as well as fMLP-stimulated (Fig. 2B) PMNs showed a statistically significant stimulation of the respiratory burst reaction at subnanomolar and nanomolar concentrations of CP55 940 in both the percentage of activated rhodamine-positive PMNs as well as the oxygen radical generation expressed as mean fluorescence per cell (data not shown). The observed stimulation by CP55 940 was completely abolished after preincubation of the whole-blood aliquots with the specific CB_2R antagonist SR144 528 (100 nM) (Fig. 2, A and B).

In contrast to the experiments with the weak stimulus

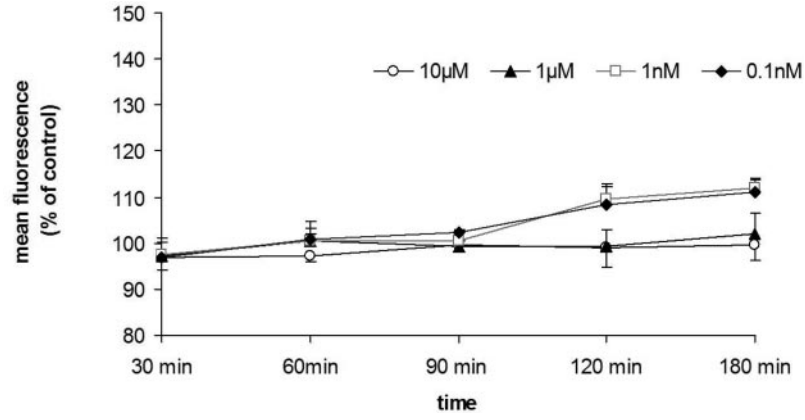

Fig. 1. Time-dependent oxygen radical generation in fMLP-stimulated whole-blood PMNs under CP55 940 exposure. After more than 90 min of incubation, only exposure to 10^{-10} and 10^{-9} M CP55 940 showed a significant increase. Values are means of the mean log fluorescence channel number (Fl1) (percentage of control) ± S.E.M. obtained from five independent triplicate tests.

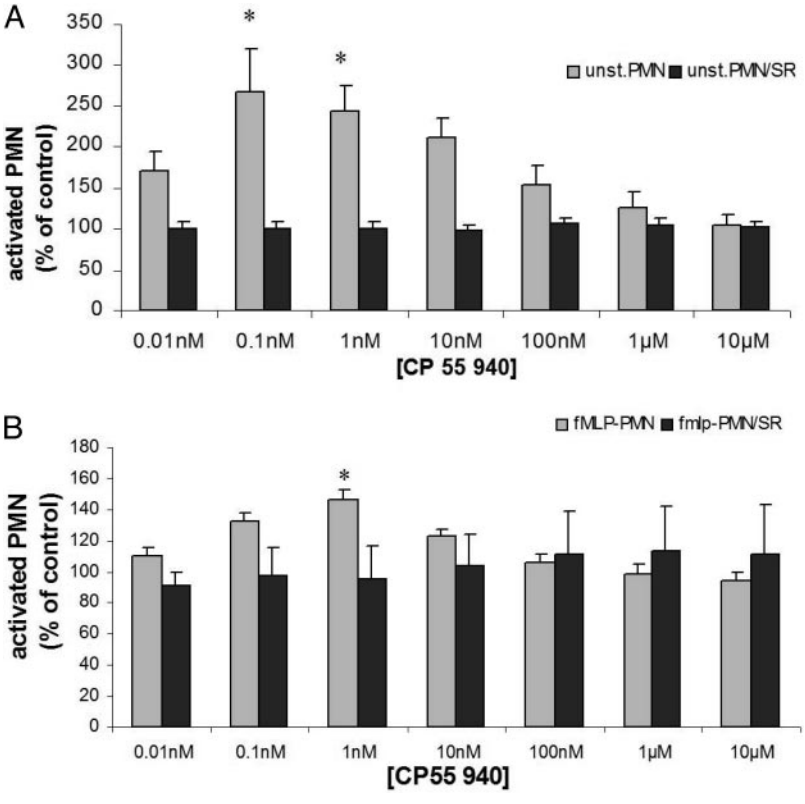

Fig. 2. Stimulation of the respiratory burst reaction of human whole-blood PMNs by CP55 940 and antagonism by preincubation with 100 nM SR144 528 (PMN/SR). A, resting PMNs (3.61 ± 0.61% rhodamine-positive cells), significant activation of PMNs at 0.1 and 1.0 nM CP55 940. B, fMLP-stimulated whole-blood PMNs (fMLP/PMN, 13.70 ± 1.11% rhodamine-positive cells), significant stimulation at 1.0 nM CP55 940. Data are expressed as mean values (percentage of control) ± S.E.M. of eight independent triplicate tests. *, $P < 0.05$, ANOVA.

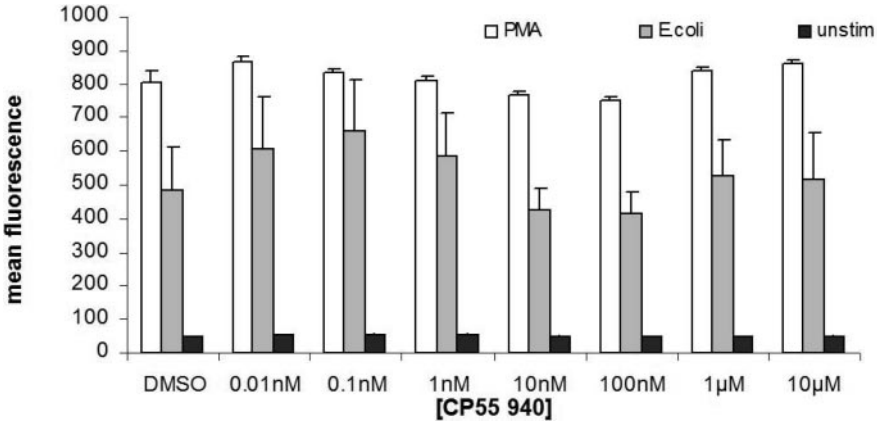

Fig. 3. Oxygen radical production of whole-blood PMNs under exposure to CP55 940 and stimulated with PMA or *E. coli*. Data are shown as mean log fluorescence channel number (Fl1) ± S.E.M. obtained from eight independent triplicate tests.

fMLP, the burst enhancement with *E. coli* was so strong that an additional stimulation with CP55 940 between 0.1 and 1 nM did not reach statistical significance. As expected, PMA produced a maximum stimulation that could not be enhanced by CP55 940 (Fig. 3).

To rule out an exclusive CP55 940-specific effect, the endocannabinoid AEA and its stable derivative MethAEA were also investigated in our experimental setting. Although anandamide, which is rapidly degraded by fatty acid amido hydrolase in cell-containing media, had neither a stimulatory nor a suppressive effect (data not shown), its nonhydrolyzable derivative MethAEA showed an activation of resting (Fig. 4) and fMLP-stimulated whole-blood PMNs similar to CP55 940 and this effect was also CB_2R-dependent as shown by its antagonism with SR144 528 (Fig. 4).

To further characterize the mechanisms underlying this cannabinoid-induced burst stimulation in whole-blood PMNs, isolated human PMNs were exposed under identical incubation conditions to concentrations of CP55 940 that produced stimulation in whole blood (Fig. 5). The cannabinoid did not exert any detectable effect on isolated neutrophils, in contrast to the significant enhancement of the respiratory burst in whole-blood PMNs.

To evaluate the involvement of humoral factors, aliquots of whole blood were incubated for 180 min with stimulatory concentrations of CP55 940 at 10^{-10} and 10^{-9} M as described before. The plasma supernatants of the aliquots were then added to isolated PMNs of the same donor, and after a further 30-min incubation, the respiratory burst was measured by flow cytometry. The isolated PMNs were stimulated by the plasma supernatants of the CP55 940-containing samples but not by the CP55 940-free plasma controls, clearly indicating that inducible humoral factors were responsible for this effect (Table 1).

When the supernatants were separated into a high-molecular mass fraction expected to contain cytokines and a low-molecular mass fraction containing the smaller mediator molecules, such as prostaglandins and leukotrienes, isolated PMNs showed a significant stimulation only after incubation (30 min) with the low-molecular mass fraction but not with the high-molecular mass fraction of the cannabinoid-primed plasma supernatant (Fig. 6).

To clarify the potential involvement of other mediators in the cannabinoid-induced PMN stimulation, whole-blood samples were incubated (37°C, 30 min) with three different inhibitors of the COX and/or LOX enzymes prior to the incubation with CP55 940 at 10^{-10} (Tables 2 and 3) and 10^{-9} M (data not shown), respectively. After preincubation with meclofenamic acid (50 and 100 μM), the stimulatory effect of CP55 940-primed plasma supernatant was dose dependently abolished. This was true for the percentage of rhodamine-positive PMNs as well as the mean cellular fluorescence (Tables 2 and 3). In these experiments, meclofenamic acid alone did not significantly alter the burst activity of resting PMNs, whereas the fMLP-stimulated PMN activity was insignificantly reduced by 10 to 20% (Table 3).

The results with the COX inhibitor flurbiprofen (10 and 25 μM, data not shown) were similar to those obtained with

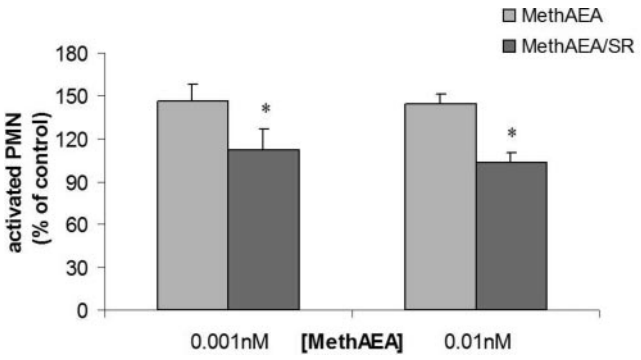

Fig. 4. Activation of resting whole-blood PMNs after incubation with MethAEA and complete antagonization by preincubation with 100 nM SR144 528 (SR/MethAEA). Bars represent the means ± S.D. (percentage of control) of eight independent triplicate tests. *, $P < 0.05$, ANOVA.

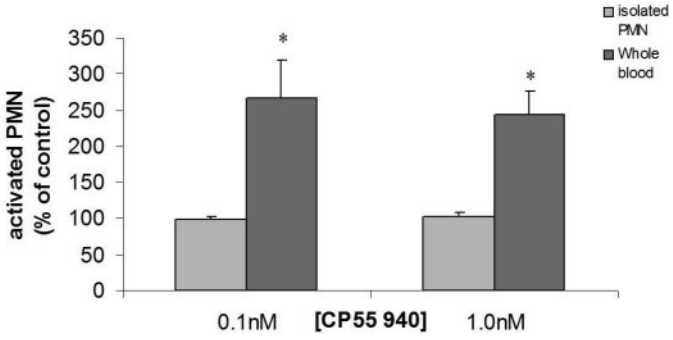

Fig. 5. Differential effects of CP55 940 on isolated and whole-blood PMNs from the same blood sample. Data represent activated PMNs after 180-min incubation with CP55 940 expressed as a percentage of unstimulated controls. Mean values ± S.E.M. of eight independent experiments. *, $P < 0.05$, ANOVA.

TABLE 1

Activation of isolated PMNs by CP55 940-primed plasma supernatants

Relative number of activated rhodamine-positive (Rhod. pos.) PMNs and mean cellular fluorescence (FL1) with or without fMLP stimulation. Data are presented as a percentage of the effects of CP55 940-free control plasma. Mean ± S.D. of three independent triplicate experiments.

CP55 940	0.1 nM Plasma	1.0 nM Plasma
	% Control	
Rhod. pos. PMNs (without fMLP)	272.66 ± 156.18	244.96 ± 123.51
Rhod. pos. PMNs (with fMLP)	204.44 ± 141.55	170.58 ± 85.78
FL1 (without fMLP)	116.76 ± 21.42	117.52 ± 3.75
FL1 (with fMLP)	128.91 ± 34.60	121.32 ± 21.11

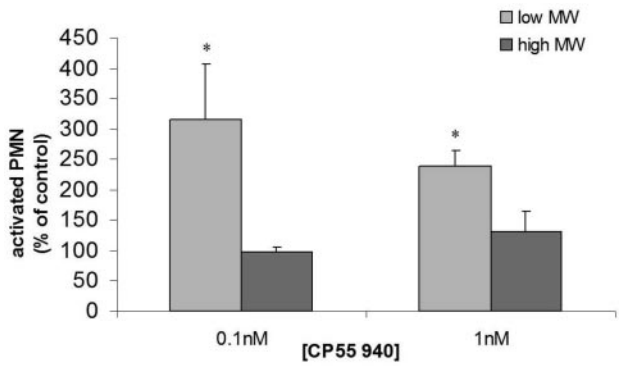

Fig. 6. Increase of the relative number of activated rhodamine positive PMNs after incubation with the low-molecular mass plasma fraction. Mean values ± S.E.M. of six independent triplicate tests. *, $P < 0.05$, ANOVA.

meclofenamic acid. The preincubation with flurbiprofen also produced a dose-dependent and significant inhibition of the PMN burst activation mediated by the CP55 940-primed plasma. This effect could be observed in both the resting and fMLP-stimulated PMNs for the percentage of rhodamine-positive cells, as well as for the oxygen radical generation. The preincubation with MK886 (4 and 40 μM), an inhibitor of the 5-lipoxygenase activator protein, did not significantly inhibit the CP55 940-induced stimulation at 10^{-10} (Tables 2 and 3) and 10^{-9} M (data not shown).

TABLE 2

Inhibition of the CP55 940 supernatant-induced stimulation of resting PMN by preincubation with the COX inhibitor meclofenamic acid and the FLAP inhibitor MK886

Data are presented as a percentage of the cannabinoid-free control without COX and LOX inhibitors (resting PMN, 3.61% ± 0.61 rhodamine-positive cells; fMLP-stimulated PMN, 13.70% ± 1.11 rhodamine-positive cells). Mean ± S.E.M. of six independent triplicate experiments. Concentrations are given as final concentrations in the tests.

	Without CP	0.1 nM CP
Rhod. pos. PMNs (% control)		
No inhibitor	100	155.69 ± 23.79
50 μM meclo	83.91 ± 7.12	112.47 ± 9.35
100 μM meclo	78.75 ± 6.93	80.46 ± 6.04*
4 μM MK886	104.84 ± 5.14	135.34 ± 6.57
40 μM MK886	109.31 ± 6.07	140.80 ± 7.96
Mean fluorescence		
No inhibitor	100	149.72 ± 8.47
50 μM meclo	93.70 ± 6.52	105.01 ± 6.92*
100 μM meclo	91.77 ± 3.11	92.69 ± 6.49*
4 μM MK886	113.19 ± 3.26	131.64 ± 9.05
40 μM MK886	118.44 ± 3.96	137.61 ± 7.51

Rhod. pos., rhodamine-positive PMN.
* $P < 0.05$, ANOVA.

TABLE 3

Inhibition of the CP55 940 supernatant-induced stimulation of fMLP-treated PMNs by preincubation with the COX inhibitor meclofenamic acid and the FLAP inhibitor MK886

Data are presented as a percentage of the cannabinoid-free control without COX and LOX inhibitors (resting PMN, 3.61% ± 0.61 rhodamine-positive cells; fMLP-stimulated PMN, 13.70% ± 1.11 rhodamine-positive cells). Mean ± S.E.M. of six independent triplicate experiments. Concentrations are given as final concentrations in the tests.

	Without CP	0.1 nM CP
Rhod. pos. PMNs (% control)		
No inhibitor	100	241.85 ± 29.41
50 μM meclo	93.84 ± 6.41	139.40 ± 4.84*
100 μM meclo	97.96 ± 3.64	109.92 ± 6.41*
4 μM MK886	101.62 ± 18.34	149.99 ± 20.19
40 μM MK886	104.39 ± 6.49	168.47 ± 26.63
Mean fluorescence		
No inhibitor	100	194.20 ± 63.71
50 μM meclo	103.31 ± 2.46	121.80 ± 7.64*
100 μM meclo	100.66 ± 3.98	111.43 ± 8.47*
4 μM MK886	106.74 ± 6.42	136.91 ± 11.68
40 μM MK886	109.21 ± 5.35	142.25 ± 11.48

Rhod. pos., rhodamine-positive PMN.
* $P < 0.05$, ANOVA.

Discussion

Our study demonstrated for the first time a significant stimulatory effect of the cannabinoids MethAEA and the THC analog CP55 940 on human PMNs when incubated in their natural whole-blood milieu. In clear contrast to the previously described receptor-independent suppressive action of CP55 940 (Kraft et al., 2004) and Δ^9-THC (Djeu et al., 1991) on isolated human PMNs, this stimulation occurred at much lower and pharmacologically more relevant concentrations of the cannabinoid agents and showed a bell-shaped dose-response relationship with a maximum stimulation between 0.1 and 1.0 nM. Although the 10^6-fold concentration range of CP55 940 covered more than only the pharmacologically relevant concentrations, those lower than 0.01 nM were not tested; thus, the dose-response relationship was not determined below half-maximum stimulation. However, at high CP55 940 concentrations, the decline in stimulatory activity probably reflects the counteracting receptor-independent, direct inhibitory effect of CP55 940 on PMNs that has been shown to occur in isolated PMNs at similar concentrations. The slight statistically not significant suppression of the respiratory burst reaction of fMLP-treated PMNs at concentrations above 100 nM also supports this interpretation and confirms data from the literature, reporting a concentration-dependent appearance of receptor-mediated versus receptor-independent cannabinoid actions on immune cells. Although the suppressive effect on isolated PMNs was neither CB_1R nor CB_2R dependent, the stimulatory activity in whole-blood PMNs could be completely blocked by coincubation with the specific CB_2R-antagonist SR144 528. In addition, the subnanomolar and low nanomolar concentrations of CP55 940 and the even lower concentrations of MethAEA sufficient to enhance the respiratory burst of resting as well as fMLP-stimulated whole-blood PMNs are consistent with their respective K_i values for CB_2 receptors (Rinaldi-Carmona et al., 1998; Howlett et al., 2002).

Together with the SR144 528 antagonism, these findings indicate an involvement of CB_2R-mediated mechanisms. Since human PMNs isolated from the blood of healthy individuals lack functional CB_2R (Deusch et al., 2003), the failure to detect any direct stimulatory effect of the two cannabi-

noids on isolated PMNs in the present study confirms our previous results and fits the impression that, although mRNA for CB_2R was found by reverse transcription-polymerase chain reaction in PMNs (Bouaboula et al., 1993; Galiegue et al., 1995), human circulating PMNs are not a direct target for cannabinoid actions. Instead, they may be indirectly influenced by the interactions of the cannabinoid agents with other blood cells. The present results with unfractioned and fractioned cell-free plasma supernatants from whole blood exposed to cannabinoids argue for a humoral mechanism that is clearly dependent on a CB_2R activation of cannabinoid-sensitive blood cells, presumably macrophages or other peripheral mononuclear leukocytes. The fact that the cannabinoid-induced burst stimulation in whole-blood PMNs did not show a rapid onset but started slowly after an at least 90-min incubation gives further evidence for such an indirect, mediator-dependent mechanism.

Previous reports demonstrated the release of arachidonic acid (Diaz et al., 1994) and the modulation of cytokine production of mononuclear leukocytes (Zhu et al., 1994; Klein et al., 2003) by the marijuana cannabinoid Δ^9-THC. Both mediator pathways are known to be physiological and pathophysiological activators of PMNs, but the fact that in the present study the stimulating activity was exclusively found in the low-molecular mass fraction of the plasma incubation supernatants strongly argues in favor of an the involvement of arachidonic acid or its metabolites. Interactions between prostanoid metabolism and cannabinoids have already been described by various investigators (Burstein et al., 1988; Perez-Reyes et al., 1991) and illustrate the close relationship between arachidonic acid metabolism and endogenous cannabinoid ligands (Edgemond et al., 1998; Pestonjamasp and Burstein, 1998). The activity of COX enzymes and the release of prostaglandin E_2 and arachidonic acid were stimulated by cannabinoids in astrocytes (Shivachar et al., 1996), cortical slices (Reichmann et al., 1987), and also lymphocytes (Audette and Burstein 1990). Diaz et al. (1994) demonstrated that THC increased the production of leukotriene B_4 and 12-hydroxyeicosatetraenoic acid from mononuclear blood cells. Arachidonic acid and leukotriene B_4 are known to be potent chemoattractants, activating PMN migration and oxygen radical generation (Liu et al., 2003). Thus, arachidonic acid and its metabolites are promising candidates for a potential involvement in the observed indirect, low-molecular mass mediator-dependent stimulatory effect of cannabinoids on whole-blood PMNs.

The results with the COX and LOX inhibitors, respectively, suggest mainly the involvement of COX-dependent pathways induced by a CB_2R-evoked interaction of the cannabinoids with blood cells others than PMNs. These findings further support the idea of a stimulation of eicosanoid synthesis by cannabinoids as suggested in former investigations by Hunter and Burstein (1997).

Although the short-living, rapidly hydrolyzed endogenous cannabinoid AEA (Di Marzo et al., 1999) had no stimulatory effect on the burst reaction of whole-blood PMNs, its stable, nonhydrolyzable derivative MethAEA enhanced the respiratory burst of PMNs in whole blood, similar to the synthetic Δ^9-THC analog CP55 940. Our observations confirm the CP55 940 data by using a second completely different CB_2R ligand and suggest a potential physiological role of endocannabinoids as indirect regulators of PMN activity in humans by means of the COX- or LOX-dependent arachidonic acid pathways in other blood cells. Macrophages (Diaz et al., 1994) or mast cells (Samson et al., 2003) are possible candidates for the release of arachidonic acid into whole blood after cannabinoid incubation. Interestingly enough, the exposure to marijuana smoke was recently reported to increase the oxygen radical production from alveolar macrophages in humans, resulting in oxidative stress and inflammation (Baldwin et al., 1997).

Although the endogenous cannabinoid, 2-arachidonylglycerol (2-AG), also acts on CB_2R and is supposed to play a role in immunomodulation (Sugiura and Waku, 2000), experiments with 2-AG would have been hampered by its significant instability in cell culture media (Rouzer et al., 2002). Even under cell-free conditions, 2-AG rapidly rearranges to 1- or 3-arachidonylglycerol in a first order process with a half-life of only 2.3 min in RPMI 1640 medium containing 10% fetal calf serum. Because AEA resembles 2-AG in its affinity to the CB_2R (Gonsiorek et al., 2000) and, what is more important from a practical point of view, has the stable derivative MethAEA available, only AEA and MethAEA were used as endocannabinoid substances in our experiments.

In conclusion, our results suggest that human circulating PMNs are not a direct cellular target of endogenous or exogenous cannabinoids but are nevertheless strongly activated by a CB_2R-evoked COX-dependent mediator pathway induced by cannabinoid interactions with other blood cells. Thus, there is no evidence for a potential cannabinoid-induced suppression of PMN functions in healthy human individuals, but in contrast, even an enhancement of oxidative burst activity is to be expected.

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References

Audette CA and Burstein SH (1990) Inhibition of leukocyte adhesion by the in vivo and in vitro administration of cannabinoids. *Life Sci* **47:**753–759.

Baldwin CG, Tashkin DP, Buckley DM, Park AN, Dubinett SM, and Roth MD (1997) Marijuana and cocaine impair alveolar macrophage function and cytokine production. *Am J Resp Crit Care Med* **156:**1606–1613.

Bouaboula M, Rinaldi M, Carayon P, Carillon C, Delpech B, Shire D, Le Fur G, and Casellas P (1993) Cannabinoid-receptor expression in human leukocytes. *Eur J Biochem* **214:**173–180.

Burstein SH, Hull K, Hunter SA, and Letham V (1988) Cannabinoids and pain responses: a possible role for prostaglandins. *FASEB J* **2:**3022–3026.

Conroy MC, Randinitis EJ, and Turner JL (1991) Pharmacology, pharmacokinetics and therapeutic use of meclofenamate sodium. *Clin J Pain* **7 (Suppl 1):**44–48.

Daniels I, Lindsay MA, Keany CIC, Burden RP, Fletcher J, and Haynes AP (1998) Role of arachidonic acid and its metabolites in the priming of NADPH oxidase in human polymorphonuclear leukocytes by peritoneal dialysis effluent. *Clin Diagn Lab Immunol* **5:**683–689.

Derocq JM, Segui M, Marchand J, Le Fur G, and Casellas P (1995) Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett* **369:** 177–182.

Deusch E, Kraft B, Nahlik G, Weigl L, Hohenegger M, and Kress HG (2003) No evidence for direct modulatory effects of delta 9-tetrahydrocannabinol on human polymorphonuclear leukocytes. *J Neuroimmunol* **141:**99–103.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Wash DC)* **258:**1946–1949.

Diaz S, Specter S, Vanderhoek JY, and Coffey RG (1994) The effect of delta-9-tetrahydrocannabinol on arachidonic acid metabolism in human peripheral blood mononuclear cells. *J Pharmacol Exp Ther* **268:**1289–1296.

Di Marzo V, De Petrocellis L, Bisogno T, and Melck D (1999) Metabolism of anandamide and 2-arachidonoylglycerol: an historical overview and some recent developments. *Lipids* **34:**319–325.

Djeu JY, Wand M, and Friedman H (1991) Adverse effect of Δ^9-tetrahydrocannabinol on human neutrophil function. *Drug Abuse Immunomodul Immunodefic* **288:**57–61.

Edgemond WS, Hillard CJ, Falck JR, Kearn CS, and Campbell WB (1998) Human platelets and polymorphonuclear leukocytes synthesize oxygenated derivatives of

arachidonylethanolamide (anandamide): their affinities for cannabinoid receptors and pathways of inactivation. *Mol Pharmacol* **54:**180–188.
Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G, and Casellas P (1995) Expression of central and peripheral cannabinoid receptors in human tissues and leukocyte subpopulations. *Eur J Biochem* **232:**54–61.
Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, and Hipkin RW (2000) Endocannabinoid 2-arachidonylglycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. *Mol Pharmacol* **57:**1045–1050.
Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, et al. (2002) International Union of Pharmacology: XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54**: 161–202.
Hunter SA and Burstein SH (1997) Receptor mediation in cannabinoid stimulated arachidonic acid mobilization and anandamide synthesis. *Life Sci* **60:**1563–1573.
Klein TW, Kawakami Y, Newton C, and Friedman H (1991) Marijuana components suppress induction and cytolytic function of murine cytotoxic T cells in vitro and in vivo. *J Toxicol Environ Health* **32:**465–477.
Klein TW, Newton C, Larsen K, Lu L, Perkins I, Nong L, and Friedman H (2003) The cannabinoid system and immune modulation. *J Leukoc Biol* **74:**486–496.
Klein TW, Newton C, and Specter S (1998) Marijuana, immunity and infection. *J Neuroimmunol* **83:**102–115.
Klein TW, Newton CA, Widen R, and Friedman H (1985) The effect of delta-9-tetrahydrocannabinol and 11-hydroxy-delta-9-tetrahydrocannabinol on T-lymphocyte and B-lymphocyte mitogen responses. *J Immunopharmacol* **7:**451–466.
Kraft B, Wintersberger W, and Kress HG (2004) Cannabinoid receptor-independent suppression of the superoxide generation of human neutrophils (PMN) by CP55 940, but not by anandamide. *Life Sci* **75:**969–977.
Liu J, Liu Z, Chuai S, and Shen X (2003) Phospholipase C and phosphatidylinositol 3-kinase signalling are involved in the exogenous arachidonic acid-stimulated respiratory burst in human neutrophils. *J Leukoc Biol* **74:**428–437.
Lopez-Cepero M, Friedman M, Klein TW, and Friedman H (1986) Tetrahydrocannabinol-induced suppression of macrophage spreading and phagocytic activity in vitro. *J Leukoc Biol* **39:**679–686.
Martin BR, Mechoulam R, and Razdan RK (1999) Discovery and characterization of endogenous cannabinoids. *Life Sci* **65:**637–644.
Massi P, Fuzio D, Vigano D, Sacerdote P, and Parolaro D (2000) Relative involvement of cannabinoid CB(1) and CB(2) receptors in the Delta(9)-tetrahydrocannabinol-induced inhibition of natural killer activity. *Eur J Pharmacol* **387:**343–347.
Parij N, Nagy AM, Fondu P, and Neve J (1998) Effects of non-steroidal anti-inflammatory drugs on the luminol and lucigenin amplified chemiluminescence of human neutrophils. *Eur J Pharmacol* **352:**299–305.
Perez-Reyes M, Burstein SH, White WR, McDonald SA, and Hicks RE (1991) Antagonism of marihuana effects by indomethacin in humans. *Life Sci* **48:**507–515.
Pestonjamasp VK and Burstein SH (1998) Anandamide synthesis is induced by arachidonate mobilizing agonists in cells of the immune system. *Biochem Biophys Acta* **1394:**249–260.
Ramos CL, Pou S, and Rosen GM (1994) Effect of antiinflammatory drugs on myeloperoxidase-dependent hydroxyl radical generation by human neutrophils. *Biochem Pharmacol* **49:**1079–1084.
Reichmann M, Nen W, and Hokin LE (1987) Effects of delta 9-tetrahydrocannabinol on prostaglandin formation in brain. *Mol Pharmacol* **32:**686–690.
Rinaldi-Carmona M, Barth F, Millan J, Derocq JM, Casellas P, Congy C, Oustric D, Sarran M, Bouaboula M, Calandra M, et al. (1998) SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* **284:**644–650.
Rothe G, Oser A, and Valet G (1998) Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. *Naturwissenschaften* **75:**354–355.
Rouzer CA, Ghebreselasie K, and Marnett LJ (2002) Chem stability of 2-arachidonylglycerol under biological conditions. *Chem Phys Lipids* **119:**69–82.
Samson MT, Small-Howard A, Shimoda LM, Koblan-Huberson M, Stokes AJ, and Turner H (2003) Differential roles of CB1 and CB2 cannabinoid receptors in mast cells. *J Immunol* **170:**4953–4962.
Sarafian TA, Magallanes JA, Shau H, Tashkin DP, and Roth MD (1999) Oxidative stress produced by marijuana smoke: an adverse effect enhanced by cannabinoids. *Am J Resp Cell Mol Biol* **20:**1286–1293.
Schatz AR, Lee M, Condie RB, Pulaski JT, and Kaminski NE (1997) Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. *Toxicol Appl Pharmacol* **142:**278–287.
Shivachar AC, Martin BR, and Ellis EF (1996) Anandamide- and delta9-tetrahydrocannabinol-evoked arachidonic acid mobilization and blockade by SR141716A [*N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride]. *Biochem Pharmacol* **51:**669–676.
Slipetz DM, O'Neill GP, Favreau L, Dufresne C, Gallant M, Gareau Y, Guay D, Labelle M, and Metters KM (1995) Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl cyclase. *Mol Pharmacol* **48:**352–361.
Streefkerk JO, Pfaffendorf M, and van Zwieten PA (2003) Endothelium-dependent, vasopressin-induced contractions in rabbit renal arteries. *J Cardiovasc Pharmacol* **42:**703–709.
Sugiura T and Waku K (2000) 2-Arachidonylglycerol and the cannabinoid receptors. *Chem Phys Lipids* **108:**89–106.
Wright CD, Kuipers PJ, Kobylarz-Singer D, Devall LR, Klinkefus BA, and Weishaar RE (1990) Differential inhibition of human neutrophil functions: role of cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase. *Biochem Pharmacol* **40:**699–707.
Zhu W, Newton C, Daaka Y, Friedman H, and Klein TW (1994) Δ^9-THC enhances the secretion of interleukin 1 from endotoxin stimulated macrophages. *J Pharmacol Exp Ther* **270:**1334–1339.

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