0022-1767/93/15010-4591$02.00/0
The Journal of Immunology

Vol 150, 4591–4599, No. 10, May 15, 1993
Printed in U.S.A.

Cyclosporin H Is a Potent and Selective Formyl Peptide Receptor Antagonist

Comparison with *N-t*-Butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L-leucyl-L-phenylalanine and Cyclosporins A, B, C, D, and E [1]

Katharina Wenzel-Seifert and Roland Seifert[2]

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69/73 D-1000 Berlin 45, Federal Republic of Germany

Abstract. The cyclic undecapeptide, cyclosporin (Cs) H, is a potent inhibitor of FMLP-induced superoxide anion (O_2^-) formation in human neutrophils. We studied the effects of CsH in comparison with those of *N-t*-butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L-leucyl-L-phenylalanine (BocPLPLP), a well known formyl peptide receptor antagonist, and of other Cs on activation of N^6,2′-*O*-dibutyryl adenosine 3:5′-monophosphate-differentiated HL-60 cells and human erythroleukemia cells (HEL cells). CsH inibited FMLP binding in HL-60 membranes with a K_i (inhibition constant) of 0.10 μM. CsH inhibited activation by FMLP of high affinity GTPase (the enzymatic activity of α-subunits of heterotrimeric regulatory guanine nucleotide-binding proteins) in HL-60 membranes with a K_i of 0.79 μM. CsH inhibited the stimulatory effects of FMLP on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), O_2^- formation, and β-glucuronidase release with K_i values of 0.08, 0.24, and 0.45 μM, respectively. BocPLPLP was 14-fold less potent than CsH in inhibiting FMLP binding and 4- to 6-fold less potent than CsH in inhibiting FMLP-induced GTP hydrolysis, rises in $[Ca^{2+}]_i$, O_2^- formation, and β-glucuronidase release. CsA reduced FMLP-induced O_2^- formation by 20%, but CsB, CsC, CsD, and CsE did not. CsA, CsB, CsC, CsD, and CsE did not affect FMLP-induced rises in $[Ca^{2+}]_i$. BocPLPLP inhibited leukotriene B_4-induced rises in $[Ca^{2+}]_i$ with a K_i of 0.33 μM, whereas CsH showed no inhibitory effect. CsH and BocPLPLP did not inhibit the rises in $[Ca^{2+}]_i$ induced by several other stimuli in HL-60 cells and HEL cells. Our results show that 1) CsH is a more potent formyl peptide receptor antagonist than BocPLPLP; 2) unlike BocPLPLP, CsH is selective; and 3) *N*-methyl-D-valine which is present at position 11 of the amino acid sequence of CsH but not of other Cs is crucial for FMLP antagonism. *Journal of Immunology,* 1993, 150: 4591.

The chemotactic peptide, FMLP, binds to formyl peptide receptors in human and rabbit neutrophils and differentiated HL-60 leukemic cells and activates pertussis toxin-sensitive G-proteins[3] which possess high affinity GTPase activity (for review, see Refs. 1–4). G-proteins mediate activation of phospholipase C with subsequent activation of protein kinase C and increase in $[Ca^{2+}]_i$ (1–4). In addition, FMLP stimulates NADPH oxidase-catalyzed O_2^- formation and β-glucuronidase release from azurophilic granules (1–4). Rises in $[Ca^{2+}]_i$ are evident with FMLP at much lower concentrations than activation of GTPase, O_2^- formation, and enzyme release (1–6). Tertiary butoxycarbonyl analogs of FMLP are well known competitive antagonists at formyl peptide receptors and inhibit chemotactic peptide-induced cell activation

Received for publication July 17, 1992. Accepted for publication February 4, 1993.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants of the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

[2] Address correspondence and reprint requests to Dr. Roland Seifert, Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, Federal Republic of Germany.

[3] Abbreviations used in this paper: G-protein, heterotrimeric regulatory guanine nucleotide-binding protein; B_{max}, maximal number of binding sites; Bt_2cAMP, N^6,2′-*O*-dibutyryl adenosine 3:5′-monophosphate; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; Cs, cyclosporin; BocPLPLP, *N-t*-butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L-leucyl-L-phenylalanine; HEL cells, human erythroleukemia cells; K_D, equilibrium dissociation constant; K_i, inhibition constant; LTB_4, leukotriene B_4; O_2^-, superoxide anion.

Table I
Amino acid sequence of various Cs[a]

Cs	1	2	3	4	5	6	7	8	9	10	11
CsA	C9-	Abu-	Sar-	MeLeu-	Val-	MeLeu-	Ala-	D-Ala-	MeLeu-	MeLeu-	MeVal
CsB	C9-	Ala-	Sar-	MeLeu-	Val-	MeLeu-	Ala-	D-Ala-	MeLeu-	MeLeu-	MeVal
CsC	C9-	Thr-	Sar-	MeLeu-	Val-	MeLeu-	Ala-	D-Ala-	MeLeu-	MeLeu-	MeVal
CsD	C9-	Val-	Sar-	MeLeu-	Val-	MeLeu-	Ala-	D-Ala-	MeLeu-	MeLeu-	MeVal
CsE	C9-	Abu-	Sar-	MeLeu-	Val-	MeLeu-	Ala-	D-Ala-	MeLeu-	MeLeu-	Val
CsH	C9-	Abu-	Sar-	MeLeu-	Val-	MeLeu-	Ala-	D-Ala-	MeLeu-	MeLeu-	D- MeVal

[a] The amino acid sequences of CsA, CsB, CsC, CsD, CsE, and CsH are shown (10). Cg, [2*S*,3*R*,4*R*,6*S*]-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid; Abu, L-aminobutyric acid; Sar, sarcosine; MeLeu, *N*-methyl-L-leucine; Val, L-valine; Meval, *N*-methyl-L-valine; D-MeVal, *N*-methyl-D-valine; Ala, L-alanine; D-alanine; Thr, L-threonine.

(7–9). BocPLPLP is one of the most potent formyl peptide receptor antagonists presently available (8). In rabbit neutrophils, the K_i values of BocPLPLP for inhibition of FMLP-stimulated azurophilic granule release and high affinity GTP hydrolysis are 74 and 230 nM, respectively (9).

The cyclic undecapeptide, CsH, is an analog of CsA (10). CsH possesses *N*-methyl-D-valine at position 11 of the amino acid chain, whereas CsA bears *N*-methyl-L-valine (Table I) (10). In contrast to CsA, which potently suppresses early events in the activation of T and B lymphocytes, CsH is without effect (10, 11). In addition to CsA and CsH, other structurally related Cs have been isolated from the fungus, *Tolypodadiam inflatum* (Table I) (10). We showed recently that CsH is a potent inhibitor of FMLP-induced O_2^- formation in human neutrophils (12). In addition, CsH inhibits FMLP binding in HL-60 membranes. Intriguingly, CsH does not inhibit neutrophil activation induced by the chemoattractants, C5a, platelet-activating factor, and LTB_4. Thus, CsH may be an FMLP antagonist (12). These findings prompted us to study in detail FMLP antagonism by CsH in Bt_2cAMP-differentiated HL-60 cells. For comparison, we also studied the effects of BocPLPLP and of CsA, CsB, CsC, CsD, and CsE. We show here that CsH is a potent and selective formyl peptide receptor antagonist.

Materials and Methods

Cs were kindly provided by Sandoz (Basel, Switzerland). BocPLPLP was from Sigma Chemie (Deisenhofen, Germany). Stock solutions of Cs (1 mM each) and BocPLPLP (10 mM) were prepared in 100% (v/v) DMSO and were stored in polypropylene tubes at −20°C under light protection for up to 4 wk. Dilutions of Cs and BocPLPLP were made immediately before experiments. As a control, experiments with DMSO (0.1–3.0%, (v/v)) were performed. FML[^{3}H]P (56 Ci/mmol) was purchased from Du Pont-New England Nuclear (Bad Nauheim, Germany). Sources of other materials have been described elsewhere (12–18).

Cell culture

HL-60 cells were cultured in suspension culture and were differentiated toward neutrophil-like cells upon incubation with Bt_2cAMP (0.2 mM) for 48 h (15, 18, 19). Human erythroleukemia cells (HEL cells) were cultured in suspension culture as previously described (17).

FMLP binding assay

FMLP binding was performed as described elsewhere (12) with modifications. In brief, reaction mixtures (100 µl) contained 30 µg of membrane protein of Bt_2cAMP-differentiated HL-60 cells, BSA (0.2%, w/v), 50 mM Tris/HCl, pH 7.3, 1 mM EDTA, and 5 mM $MgCl_2$ with or without Cs or BocPLPLP. Incubations were conducted for 30 min at 25°C and were initiated by the addition of FML-[^{3}H]P at various concentrations. Reactions were terminated by rapid filtration through glass fiber filters (Whatman, GF/C) followed by two washes with 5 ml of buffer (4°C) containing 50 mM Tris/HCl, pH 7.3, 1 mM EDTA, and 5 mM $MgCl_2$. Filters were dried, and radioactivity was determined in a liquid scintillation counter. Specific binding was calculated by subtracting the amount of FML[^{3}H]P bound in the presence of 10 µM FMLP from the total amount of FML[^{3}H]P bound. Unlabeled FMLP was added to reaction mixtures before FML[^{3}H]P. Nonspecific binding was less than 10% of total binding.

Measurement of GTPase activity

GTP hydrolysis was measured as previously described (16). Assay mixtures (100 µl) contained membranes from HL-60 cells (5.0–7.0 µg of protein/tube), 0.5 µM [γ-^{32}P]GTP (0.1 µCi/tube), 0.5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenosine 5′-(β,γ-imido)-triphosphate, 5 mM creatine phosphate, 40 µg of creatine kinase, 1 mM dithiothreitol, and 0.2% (w/v) BSA in 50 mM triethanolamine/HCl, pH 7.4. Reaction mixtures were preincubated for 5 min at 25°C in the presence of FMLP at various concentrations with or without CsH or BocPLPLP. Reactions were initiated by the addition of [γ-^{32}P]GTP and were conducted for 15 min. Low affinity GTP hydrolysis was determined in the presence of 50 µM GTP and amounted to <5% of total GTPase activity.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by using the fluorescent dye, Fura-2, according to the protocol described in detail by Seifert et al. (18). Briefly, HL-60 cells and HEL cells were suspended at 5×10^6 cells/ml in a buffer consisting of (in mM) 138 NaCl, 6 KCl, 1 $MgSO_4$, 1.1 $CaCl_2$, 0.1 EGTA, 1 Na_2HPO_4, 5 $NaHCO_3$, 5.5 glucose, 20 HEPES/NaOH, pH 7.4, supplemented with BSA (0.1%, w/v). Cells were incubated for 1 h at 37°C in the presence of Fura-2/acetoxymethylester (4 μM). Subsequently, cells were diluted with the above buffer to a final concentration of 0.5×10^6 cells/ml and were centrifuged at $250 \times g$ for 10 min at 20°C. Cells were suspended at 1.0×10^6 cells/ml in the above buffer and were kept at 20°C until measurement of $[Ca^{2+}]_i$. Fluorescence of cells (1.0×10^6 cells in 2 ml) was determined at 37°C under constant stirring at 10^3 rpm by using a Ratio II spectrofluorometer (Aminco, Silver Spring, MD). Cells were incubated for 3 min with or without Cs or BocPLPLP before the addition of stimuli. The excitation and emission wavelengths were 340 and 500 nm, respectively. Basal $[Ca^{2+}]_i$ values were subtracted from the corresponding peak $[Ca^{2+}]_i$ values stimulated by agonists. Basal $[Ca^{2+}]_i$ in freshly loaded HL-60 cells and HEL cells was 115 ± 15 nM and 145 ± 25 nM, respectively (mean ± SD, three to eight different preparations of cells). Experiments with HL-60 cells were performed within 4 h after loading with the dye; experiments with HEL cells were performed within 1 h. Within these times, basal $[Ca^{2+}]_i$ did not rise by more than 20 nM, and the responsiveness to none of the stimuli studied changed significantly.

Assay for O_2^- formation

O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase, with the use of an Uvikon 810 dual beam spectrophotometer (Kontron, Eching, FRG) (14). Reaction mixtures (0.5 ml) contained 100 μM ferricytochrome C and a buffer consisting of (in mM) 138 NaCl, 6 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5.5 glucose, and 20 HEPES/NaOH, pH 7.4. HL-60 cells (2.5×10^6 cells) were suspended in the solution described above and were incubated for 3 min in the absence or presence of Cs or BocPLPLP at 37°C. O_2^- formation was initiated by the addition of FMLP. Experiments were performed in the absence of cytochalasin B.

β-Glucuronidase release

Enzyme release was determined as previously described (15). Briefly, Bt_2cAMP-differentiated HL-60 cells (5.0×10^6 cells in 0.5 ml) were suspended in the buffer used for the determination of O_2^- formation. Cells were incubated for 5 min at 37°C in the presence of cytochalasin B (5 μg/ml) in the presence of CsH or BocPLPLP at various concentrations before the addition of FMLP (30 nM). Reactions were conducted for 10 min and were terminated by placing the tubes onto melting ice. Reaction mixtures were centrifuged at $1000 \times g$ for 10 min at 4°C. The determinations of the activities of lactate dehydrogenase and β-glucuronidase of supernatant fluids of reaction mixtures and of cell lysates were performed as described elsewhere (15). Lactate dehydrogenase release and basal β-glucuronidase release amounted to <5% of cellular content (data not shown).

Miscellaneous procedures

Protein was determined according to Lowry et al. (20). [γ-^{32}P]GTP was synthesized according to Johnson and Walseth (21). HL-60 membranes were prepared as previously described (13). K_d and B_{max} were calculated as described elsewhere (22). EC_{50} and IC_{50} values were obtained by graphically analyzing concentration-response curves. K_i values were calculated according to Cheng and Prusoff (23).

Results

First, FMLP binding to formyl peptide receptors in HL-60 membranes was studied. FML[^{3}H]P was employed at 0.1 to 30 nM. Analysis of the binding data in a Scatchard plot revealed a single high affinity binding site with a K_d of 0.70 ± 0.08 nM and a B_{max} of 956 ± 45 fmol/mg protein (means ± SD of three experiments, original binding data not shown). In agreement with these results, Boulay et al. (24) reported on a single class of high affinity binding sites (K_d = 1.5–2.0 nM) for a hydrophilic analog of FMLP in intact Bt_2cAMP-differentiated HL-60 cells.

The effects of CsH and BocPLPLP on binding of FMLP (3 nM) to HL-60 membranes are shown in Figure 1. CsH inhibited agonist binding with an IC_{50} of 0.7 μM. BocPLPLP was about 14-fold less potent than CsH. CsB, CsC, CsD, and CsE (1 μM each) did not inhibit FMLP binding (data not shown).

Modulation by CsH and BocPLPLP of high affinity GTPase was studied (Fig. 2). FMLP stimulated GTP hydrolysis with an EC_{50} of about 0.1 μM. CsH (1 μM) increased the EC_{50} for FMLP to 1 μM without reducing agonist efficacy. BocPLPLP (10 μM) increased the EC_{50} for FMLP by almost sevenfold. CsH and BocPLPLP did not inhibit basal GTP hydrolysis. CsH and BocPLPLP inhibited GTP hydrolysis stimulated by FMLP (100 nM) with IC_{50} values of 1.3 and 8.1 μM, respectively (Fig. 3).

The effects of Cs and BocPLPLP on $[Ca^{2+}]_i$ were studied. Cs (up to 10 μM) and BocPLPLP (up to 100 μM) did not cause damage of Bt_2cAMP-differentiated HL-60 cells as revealed by trypan blue dye exclusion and lactate dehydrogenase release and did not induce rises in $[Ca^{2+}]_i$ (data not shown). FMLP increased $[Ca^{2+}]_i$ with an EC_{50} of

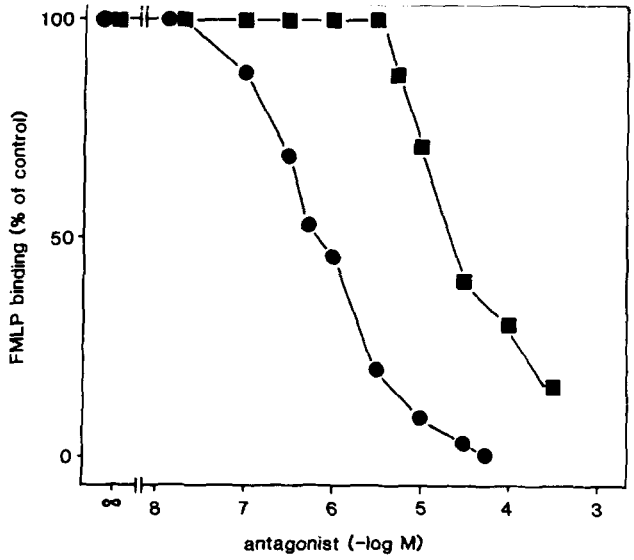

FIGURE 1. Inhibition by CsH and BocPLPLP of FMLP binding to formyl peptide receptors in membranes of Bt_2cAMP-differentiated HL-60 cells. CsH and BocPLPLP at the indicated concentrations were added to reaction mixtures 3 min before FML[^{3}H]P (3 nM) (17 nCi/tube). ●, CsH; ■, BocPLPLP. FMLP binding to membranes in the absence of CsH or BocPLPLP was 758 ± 45 fmol/mg protein (mean ± SD). Data shown are the means of assay triplicates; the SD values of the data were generally <10% of the means. Similar results were obtained in six experiments.

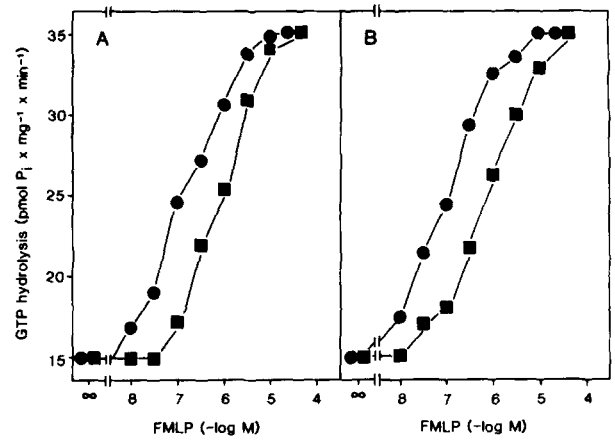

FIGURE 2. Effects of CsH and BocPLPLP on basal and FMLP-stimulated high affinity GTP hydrolysis in membranes of Bt_2cAMP-differentiated HL-60 cells. GTP hydrolysis in HL-60 membranes was determined in the presence of FMLP at various concentrations with CsH (1 μM), BocPLPLP (10 μM), or solvent (control). *A*, ●, Control; ■, CsH. *B*, ●, Control; ■, BocPLPLP. Data shown are the means of assay quadruplicates; the SD values of the data were generally <5% of the means. Similar results were obtained in four experiments.

1.6 nM and a maximum at 10 nM; CsH and BocPLPLP shifted the concentration-response curve for FMLP to the right without reducing agonist efficacy (data not shown). CsH and BocPLPLP inhibited the effect of FMLP (3 nM) on $[Ca^{2+}]_i$ with IC_{50} values of 0.23 and 1.25 μM, respectively (Fig. 4).

The effects of CsH and BocPLPLP on O_2^- formation in HL-60 cells were studied. FMLP activated O_2^- formation with an EC_{50} of 15–20 nM and a maximum at 300 nM (data not shown). CsH (1 μM) increased the EC_{50} for FMLP to 170 nM without changing its efficacy; BocPLPLP (10 μM) increased the EC_{50} for FMLP 14-fold (data not shown). CsH and BocPLPLP inhibited O_2^- formation induced by FMLP (30 nM) with IC_{50} values of 0.6 and 2.6 μM, respectively (Fig. 5).

The effects of CsH and BocPLPLP on FMLP-induced β-glucuronidase release were also studied. FMLP activated enzyme release with an EC_{50} of 13 nM and a plateau at 0.1 to 1 μM (data not shown). CsH and BocPLPLP inhibited β-glucuronidase release induced by FMLP (50 nM) with IC_{50} values of 2.2 and 8.8 μM, respectively (see Fig. 5).

To study the specificity of CsH, its effects on $[Ca^{2+}]_i$ and O_2^- formation were compared with those of CsA, CsB, CsC, CsD, and CsE (Table II). CsA, CsB, CsC, CsD, and CsE differ from CsH in the amino acid sequence at positions 2 or 11 (see Table I). Whereas CsH (1 μM) abolished the effect of FMLP (1 nM) on $[Ca^{2+}]_i$, none of the other Cs (1 μM each) was inhibitory. CsB, CsC, CsD, and CsE (1 μM each) did not inhibit O_2^- formation induced by FMLP (50 nM) (see Table II). CsH and CsA (1 μM each) inhibited FMLP-induced O_2^- formation by about 20% and 95%, respectively (see Table II).

Finally, the receptor selectivity of CsH and BocPLPLP was studied. HL-60 cells were stimulated with C5a (0.1 nM), platelet-activating factor (10 nM), ATP (30 nM), UTP (30 nM), and histamine (1 μM) (15, 16, 18, 25). Neither CsH (1 μM) nor BocPLPLP (10 μM) inhibited the stimulatory effects of these stimuli on $[Ca^{2+}]_i$ (data not shown). In addition, CsH (1 μM) and BocPLPLP (10 μM) did not inhibit the increases in $[Ca^{2+}]_i$ induced by NaF (10 mM), a direct activator of G-proteins (data not shown) (26). Unexpectedly, BocPLPLP shifted the concentration-response curve for LTB_4 to the right (Fig. 6). BocPLPLP inhibited the rise in $[Ca^{2+}]_i$ induced by LTB_4 (3 nM) with an IC_{50} of 0.73 μM (Fig. 6). By contrast, CsH did not inhibit LTB_4-induced rises in $[Ca^{2+}]_i$ (Fig. 6).

In HEL cells, thrombin, epinephrine (via α_2-adrenoceptors) and PGE_1 induce rises in $[Ca^{2+}]_i$ (17, 27). CsH (1 μM) and BocPLPLP (10 μM) did not inhibit the stimulatory effects of thrombin (0.1 and 0.3 U/ml), epinephrine (0.1 and 1 μM), and PGE_1 (30 and 100 nM) on $[Ca^{2+}]_i$ (data not shown).

Discussion

CsH is a competitive formyl peptide receptor antagonist. This view is supported by the following findings. CsH shifted the concentration-response curves for FMLP on high

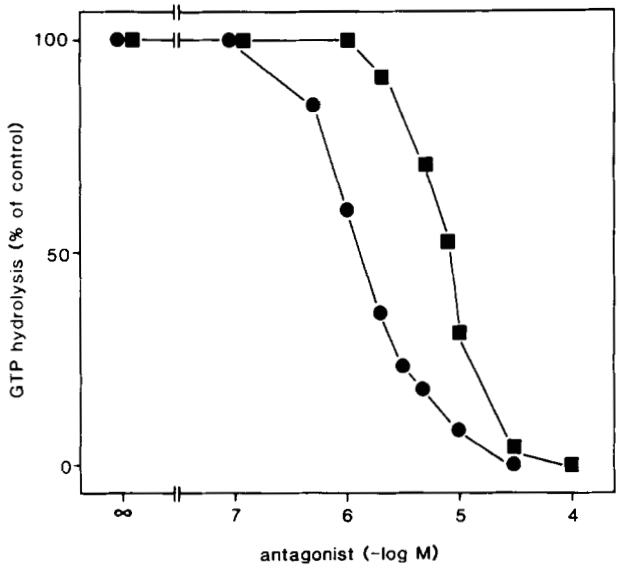

FIGURE 3. Concentration-response curves for the inhibitory effects of CsH and BocPLPLP on FMLP-stimulated high affinity GTP hydrolysis in membranes of Bt_2cAMP-differentiated HL-60 cells. GTP hydrolysis in HL-60 membranes was determined in the presence of FMLP (100 nM) with CsH and BocPLPLP at various concentrations. ●, CsH; ■, BocPLPLP. In the presence of solvent (control), FMLP (100 nM) increased GTP hydrolysis by 9.5 ± 1.2 pmol of P_i/mg/min above basal enzyme activity. Data shown are referred to the increase in GTP hydrolysis stimulated by FMLP and are the means of seven experiments performed in quadruplicate; the SD values of the data were generally <5% of the means.

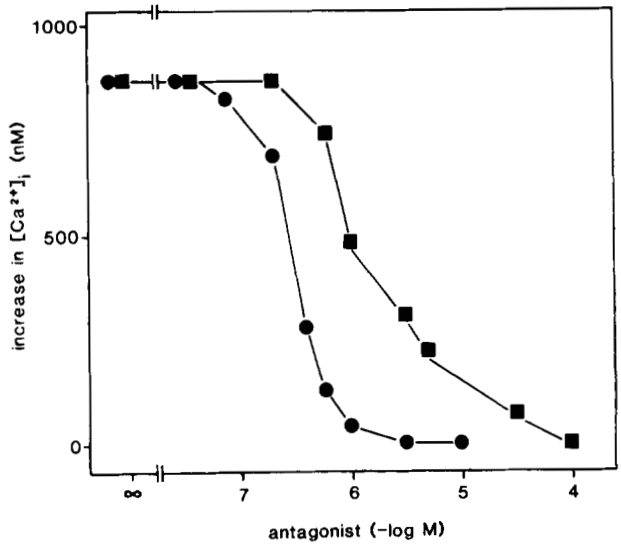

FIGURE 4. Concentration-response curves for the inhibitory effects of CsH and BocPLPLP on FMLP-induced rises in $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells. The increases in $[Ca^{2+}]_i$ induced by FMLP (3 nM) were determined in the presence of CsH and BocPLPLP at various concentrations. Antagonists or solvent (control) were added to cells 3 min before FMLP. ●, CsH; ■, BocPLPLP. In the presence of solvent, FMLP (3 nM) increased $[Ca^{2+}]_i$ by 734 ± 48 nM. Data shown are referred to this value and are the means of four experiments; the SD values of the data were generally <15% of the means.

affinity GTP hydrolysis, increase in $[Ca^{2+}]_i$, O_2^- formation, and β-glucuronidase release to the right (Fig. 2). The effects of CsH on these parameters were concentration-dependent (Figs. 3–5). In addition, CsH inhibited FMLP binding in a concentration-dependent manner (Fig. 1). Due to the pure competitive FMLP antagonism by CsH, K_i values were calculated (Table III).

Unlike in HL-60 cells, CsH is a mixed competitive/noncompetitive antagonist to inhibit FMLP-induced O_2^- formation in human neutrophils (12). Thus, the potency of CsH in neutrophils and HL-60 cells cannot be compared on the basis of K_i values. On the basis of IC_{50} values, CsH is about 15-fold more potent in neutrophils than in HL-60 cells in inhibiting O_2^- formation induced by FMLP (30 nM) (Fig. 5) (12). It remains to be determined whether the differences in the effects of CsH on O_2^- formation in these cell types are due to the expression of different formyl peptide receptor subtypes.

CsH did not inhibit the rises in $[Ca^{2+}]_i$ induced by various other stimuli, i.e., C5a, platelet-activating factor, LTB_4, ATP, UTP, histamine, thrombin, epinephrine, and PGE_1 (Fig. 6). Thus, CsH is a selective formyl peptide receptor antagonist. Additionally, CsH did not interfere with signal transduction processes at the level of G-proteins as the NaF-induced rise in $[Ca^{2+}]_i$ was not affected.

Regardless of whether FMLP binding, rises in $[Ca^{2+}]_i$, O_2^- formation, or β-glucuronidase release are considered, CsH is an at least fourfold more potent FMLP antagonist than BocPLPLP (Figs. 1–5, Table III). Unlike CsH, BocPLPLP is not a selective formyl peptide receptor antagonist but a mixed FMLP/LTB_4 antagonist (Fig. 6). The K_i of BocPLPLP for inhibition of LTB_4-induced rises in $[Ca^{2+}]_i$ was 0.33 μM. Thus, the potency of BocPLPLP at formyl peptide and LTB_4 receptors is similar (Figs. 4 and 6, Table III). 7-[3-(4-Acetyl-3-methoxy-2-propylphenoxy)propoxy]-3,4-dihydro-8-propyl- 2 *H* -1-benzopyran-2-carboxylic acid (SC-41930) is another mixed FMLP/LTB_4 antagonist, but its affinity to LTB_4 receptors is greater than to formyl peptide receptors (28).

Both CsH and BocPLPLP are very hydrophobic peptides (8, 10). This biophysical property may be important for FMLP antagonism. Interestingly, hydrophobic transmembrane domains are involved in the recognition of ligands at G-protein-coupled receptors (29, 30). However, other Cs are hydrophobic, too, but devoid of antagonistic properties at formyl peptide receptors (Table II). Apart from being hydrophobic peptides, CsH and BocPLPLP do not share amino acid sequence similarity (Table I) (8, 10). It cannot be excluded, however, that CsH and BocPLPLP possess similarities in the tertiary structure.

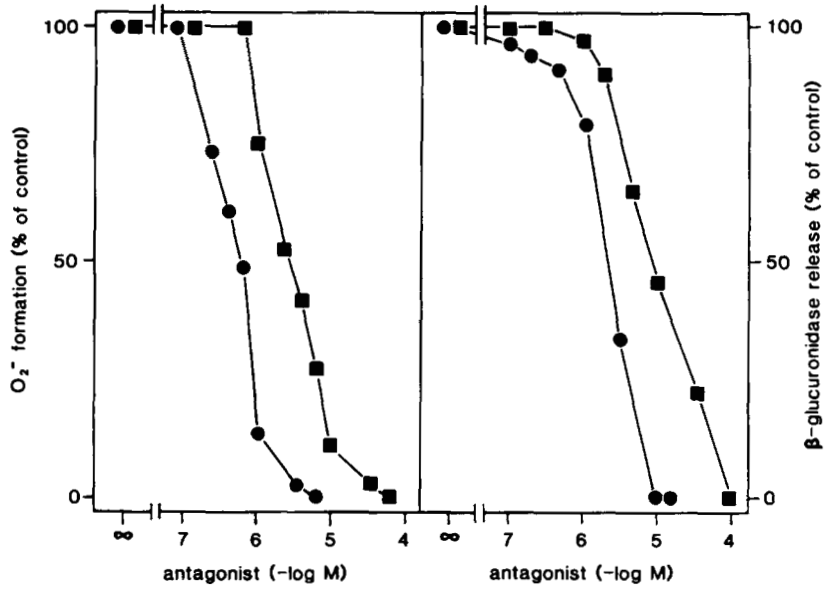

FIGURE 5. Concentration-response curves for the inhibitory effects of CsH and BocPLPLP on FMLP-induced O_2^- formation and β-glucuronidase release in Bt_2cAMP-differentiated HL-60 cells. CsH and BocPLPLP at various concentrations or solvent (control) were added to reaction mixtures before FMLP (30 nM). ●, CsH; ■ BocPLPLP. In the presence of solvent, FMLP (30 nM) induced the formation of 3.8 ± 0.3 nmol of O_2^- per 10^6 cells and the release of 12.6 ± 0.2% of the cellular content of β-glucuronidase. Data shown are referred to these values and are the means of four experiments; the SD values of the data were generally <10% of the means.

Table II
Effects of Cs on FMLP-induced rises in $[Ca^{2+}]_i$ and O_2^- formation in Bt_2cAMP-differentiated HL-60 cells[a]

Addition	Increase in $[Ca^{2+}]_i$ (nM)	O_2^- formation (nmol/10^6 cells)
Solvent	519 ± 68	4.3 ± 0.5
CsH	0[b]	0.3 ± 0.2[b]
CsA	536 ± 51[c]	3.4 ± 0.3[d]
CsB	494 ± 60[c]	4.0 ± 0.7[c]
CsC	485 ± 62[c]	4.6 ± 0.4[c]
CsD	553 ± 64[c]	3.8 ± 0.5[c]
CsE	522 ± 47[c]	4.0 ± 0.6[c]

[a] The effects of Cs (1 μM each) on rises in $[Ca^{2+}]_i$ induced by FMLP (1 nM) and on O_2^- formation induced by FMLP (50 nM) were studied. Cs or solvent (control) were added to reaction mixtures 3 min before FMLP. Data shown are the means of assays in triplicate or quadruplicate. Similar results were obtained in three experiments carried out with different preparations of HL-60 cells. The effects of Cs were assessed statistically using the Wilcoxon test.
[b] $p < 0.01$.
[c] NS.
[d] $p < 0.05$.

In differentiated HL-60 cells, formyl peptide receptors couple to G-proteins not only in the presence of FMLP, but also in its absence. This is suggested by the finding that pertussis toxin, which uncouples receptors from G-proteins by ADP-ribosylation of their α-subunits, substantially reduces basal high affinity GTP hydrolysis (data not shown) (6). Intriguingly, certain antagonists at δ-opioid peptide receptors inhibit high affinity GTP hydrolysis in the absence of agonists, i.e., they possess negative intrinsic activity (31). In view of these data it was of considerable interest to study the effects of CsH and BocPLPLP on basal GTP hydrolysis in HL-60 membranes. Neither CsH nor BocPLPLP reduced basal GTPase activity, indicating that they are neutral antagonists, i.e., devoid of negative intrinsic activity (Fig. 2). Similar to HL-60 membranes, BocPLPLP is a neutral antagonist in rabbit neutrophil membranes (9).

Bt_2cAMP-differentiated HL-60 cells express at least two very closely related formyl peptide receptor subtypes (24). The fact that FMLP is much more potent to increase $[Ca^{2+}]_i$ than to activate GTPase, O_2^- formation, and β-glucuronidase release may point to the involvement of different receptor subtypes in the activation of the above functions. Therefore, we compared the potency ratio CsH/BocPLPLP for the various parameters (Table III). With regard to all of the above-mentioned parameters CsH was four to six times more potent than BocPLPLP. The differences in potency ratio between the various functions are too small to support the involvement of different formyl peptide receptor subtypes (32). However, as formyl peptide receptor subtypes in Bt_2cAMP-differentiated HL-60 cells differ only in few amino acids, their relative affinities for CsH and BocPLPLP may be very similar (24).

Formyl peptide receptors in Bt_2cAMP-differentiated HL-60 cells and rabbit neutrophils possess the same functions, e.g., activation of high affinity GTPase and of azurophilic granule release (Figs. 2, 3, and 5) (9). Intriguingly, formyl peptide receptors in Bt_2cAMP-differentiated HL-60 cells and rabbit neutrophils show only less than 30% amino acid identity (33, 34). Thus, one may expect substantial pharmacological differences between formyl peptide receptors in both cell types. One important difference is that in Bt_2cAMP-differentiated HL-60 cells, the potency of FMLP and BocPLPLP to activate or inhibit GTPase, and azurophilic granule release is about 20- to 25-fold lower than in rabbit neutrophils (Fig. 2, Table III) (9, 15). For further analysis of the pharmacologic heterogeneity of formyl peptide receptors it would be desirable to compare the effects of CsH and BocPLPLP on separately expressed receptor subtypes.

With regard to FMLP binding, the potency ratio CsH/BocPLPLP was greater than for the other parameters measured (Table III). Binding experiments were conducted for

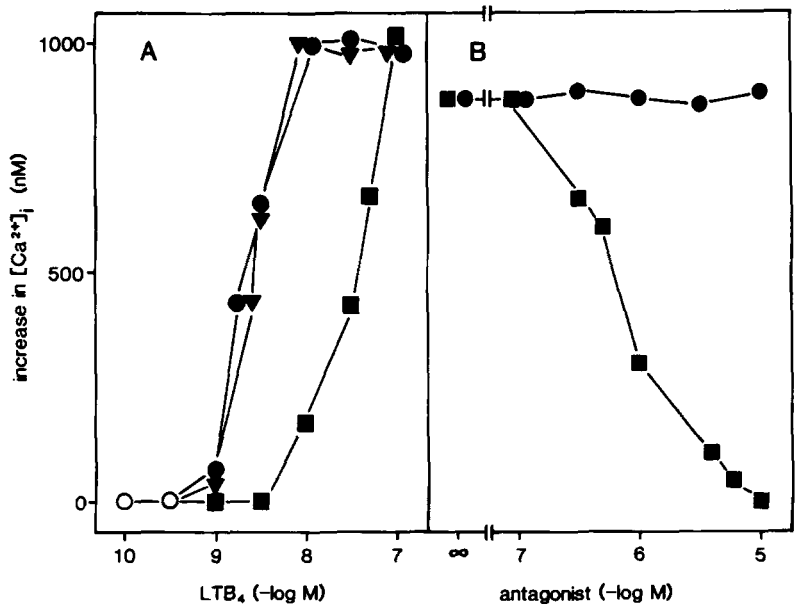

FIGURE 6. Effects of CsH and BocPLPLP on LTB_4-induced rises in $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells. Antagonists were added to cells 3 min before LTB_4. *A*, The increases in $[Ca^{2+}]_i$ induced by LTB_4 at various concentrations were assessed in the presence of solvent (control), CsH (1 μM) or BocPLPLP (10 μM). ▼, control; ●, CsH; ■, BocPLPLP. *Open circles* designate that LTB_4 at the indicated concentrations did not induce a rise in $[Ca^{2+}]_i$. Data shown are the means of assay triplicates; the SD values generally varied by less than 15% of the means. Similar results were obtained in three experiments carried out with different preparations of HL-60 cells. *B*, The increases in $[Ca^{2+}]_i$ induced by LTB_4 (3 nM) were determined in the presence of CsH and BocPLPLP at various concentrations. Antagonists or solvent (control) were added to cells 3 min before LTB_4. ●, CsH; ■, BocPLPLP. In the presence of solvent, LTB_4 (3 nM) increased $[Ca^{2+}]_i$ by 623 ± 39 nM. Data shown are referred to these values and are the means of four experiments; the SD values of the data were generally <15% of the means.

Table III
K_i values of CsH and BocPLPLP for inhibition of FMLP binding and functional effects of FMLP in Bt_2cAMP-differentiated HL-60 cells[a]

Parameter	K_i Values (μM)	
	CsH	BocPLPLP
FMLP binding	0.10	1.46 (14.6)
High affinity GTP hydrolysis	0.79	4.77 (6.0)
Increase in $[Ca^{2+}]_i$	0.08	0.43 (5.4)
O_2^- formation	0.24	1.04 (4.3)
β-Glucuronidase release	0.45	1.82 (4.0)

[a] K_i values were derived from the IC_{50} values of CsH and BocPLPLP for inhibition of FMLP binding and functional effects of FMLP (Figs. 1–5) and agonist affinity/potencies (see *Results*) according to Cheng and Prusoff (23). Numbers in parentheses designate the potency ratio CsH/BocPLPLP.

30 min, whereas all other experiments were conducted for times not longer than 15 min. The pH of buffers was 7.3–7.4 (see *Materials and Methods*). Interestingly, BocPLPLP is not stable, i.e., substantial decomposition of BocPLPLP occurs after prolonged incubation in buffer, pH 7.4 (7). Thus, partial degradation of BocPLPLP could explain its relatively low potency in binding studies in comparison to other parameters.

Among several Cs examined, CsH is the only one which shows antagonism at formyl peptide receptors as assessed by receptor binding studies and rises in $[Ca^{2+}]_i$ (Figs. 1 and 4, Table II). Thus, the question arises which structural properties of CsH are important for FMLP antagonism. All Cs are hydrophobic cyclic undecapeptides which differ in the amino acid sequence at positions 2 and 11 (Table I). Interestingly, CsH is the only Cs which bears *N*-methyl-D-valine at position 11. By comparison, CsA, CsB, CsC, and CsD possess *N*-methyl-L-valine, and CsE bears L-valine at this position (Table I). Exchange of *N*-methyl-D-valine by *N*-methyl-L-valine or L-valine resulted in complete loss of FMLP antagonism (Table II). Substitution of the D-amino acid by a L-amino acid is associated with a large conformational change of the peptide (10). Exchange of L-aminobutyric acid at position 2 in CsA by L-alanine (CsB), L-threonine (CsC), or L-valine (CsD) did not result in recovery of FMLP antagonism (Tables I and II). Thus, there is a high degree of structural specificity for FMLP antagonism of Cs, i.e., the presence of *N*-methyl-D-valine in position 11 is a conditio sine qua non. Synthesis of CsH derivatives may provide a more detailed insight into the structure/activity relationship for FMLP antagonism of Cs and may result in the identification of more potent antagonists than CsH. Eventually, highly potent CsH derivatives may be used as radioligands for formyl peptide receptors. Presently, these important tools for the characterization of formyl peptide receptors are not available as the affinity of both CsH and BocPLPLP is too low (Fig. 1, Table III). In this context it may be interesting to note that CsH is not the

only naturally occurring formyl peptide receptor antagonist, i.e., a retrovirus-derived hexapeptide is an FMLP antagonist as well (35). We anticipate that additional natural FMLP antagonists will be identified in plants, fungi, viruses, or even bacteria.

With regard to O_2^- formation, the situation concerning the specificity of the effects of Cs is more complicated than with regard to FMLP binding and rises in $[Ca^{2+}]_i$. In particular, not only CsH but also CsA inhibited, at least to some extent, FMLP-induced O_2^- formation (Table II). Inhibition by CsA of FMLP-induced O_2^- formation was also reported for human neutrophils (12, 36). This effect of CsA may be independent of FMLP antagonism as CsA inhibits also O_2^- formation induced by NaF, γ-hexachlorocyclohexane, and PMA, i.e., stimuli which act at post-receptor sites (12). However, a lack of effect of CsA on PMA-induced O_2^- formation was reported by Janco and English (37). Thus, further experimental work is required to understand the effects of CsA on O_2^- formation.

In addition to the pharmacologic characterization of formyl peptide receptors, could one envisage other potential applications of CsH and CsH derivatives? Although activation by formyl peptides of neutrophilic cells in vitro has been characterized very extensively, much less information is available concerning the role of bacterial and endogenous (e.g., mitochondrial) formyl peptides in the pathogenesis of inflammatory processes in vivo (1–4, 38, 39). One approach to learn more about the in vivo role of formyl peptides is to study the effects of formyl peptide receptor antagonists on the course of inflammatory processes in intact organisms. CsH may possess favorable properties as a drug for this purpose. Apart from its potency and selectivity for formyl peptide receptors, CsH is more stable than BocPLPLP (7, 40). The polarity of CsA and CsH is similar (40). Thus, one can anticipate that not only CsA but also CsH is absorbed after oral administration and shows substantial tissue penetration (41). By analogy to CsA, CsH would be expected to be resistant to degradation by proteases (10, 41). Finally, CsH does not affect activation of lymphocytes and basophils (10, 42).

In conclusion, CsH is a potent and selective formyl peptide receptor antagonist. CsH, CsH derivatives, and BocPLPLP may be important tools for further exploration of the functional heterogeneity of formyl peptide receptor subtypes. CsH may be a valuable drug to elucidate the still poorly defined role of endogenous and bacterial formyl peptides in the pathogenesis of inflammatory processes in vivo. Finally, CsH may be the starting point for the development of a new class of anti-inflammatory drugs, i.e., formyl peptide receptor antagonists.

Acknowledgments

The authors thank Mrs. E. Glaß for expert technical assistance.

References

1. Rossi, F. 1986. The O_2^--forming NADPH oxidase of phagocytes: nature, mechanisms of activation and function. *Biochim. Biophys. Acta 853:65.*
2. Sandborg, R. R., and J. E. Smolen. 1988. Biology of disease. Early biochemical events in leukocyte activation. *Lab. Invest. 59:300.*
3. Dillon, S. B., M. W. Verghese, and R. Snyderman. 1988. Signal transduction in cells following binding of chemoattractants to membrane receptors. *Virchows Arch. B Cell. Pathol. 55:65.*
4. Seifert, R., and G. Schultz. 1991. The superoxide-forming NADPH oxidase of phagocytes: an enzyme system regulated by multiple mechanisms. *Rev. Physiol. Biochem. Pharmacol. 117:1.*
5. Korchak, H. M., C. Wilkenfeld, A. M. Rich, A. R. Radin, K. Vienne, and L. E. Rutherford. 1984. Stimulus response coupling in the human neutrophil. Differential requirements for receptor occupancy in neutrophil responses to a chemoattractant. *J. Biol. Chem. 259:7439.*
6. Gierschik, P., D. Sidiropoulos, M. Steisslinger, and K. H. Jakobs. 1989 Na^+ regulation of of formyl peptide receptor-mediated signal transduction in HL 60 cells. Evidence that the cation prevents activation of the G-protein by unoccupied receptors. *Eur. J. Pharmacol. 172:615.*
7. Schiffmann, E., S. Aswanikumar, K. Venkatasubramaniam, B. A. Corcoran, C. B. Pert, J. Brown, E. Gross, A. R. Day, R. J. Freer, A. H. Showell, and E. L. Becker. 1980. Some characteristics of the neutrophil receptor for chemotactic peptides. *FEBS Lett. 117:1.*
8. Freer, R. J., A. R. Day, J. A. Radding, E. Schiffmann, S. Aswanikumar, H. J. Showell, and E. L. Becker. 1980. Further studies on the structural requirements for synthetic peptide chemoattractants. *Biochemistry 19:2404.*
9. Kanaho, Y., J. C. Kermode, and E. L. Becker. 1990. Comparison of stimulation by chemotactic formyl peptide analogs between GTPase activity in neutrophil plasma membranes and granule enzyme release from intact neutrophils. *J. Leukocyte Biol. 47:420.*
10. Von Wartburg, A., and R. Traber. 1986. Chemistry of the natural cyclosporin metabolites. *Prog. Allergy 38:28.*
11. Sigal, N. H., and J. Dumont. 1992. Cyclosporin A, FK-506, and rapamycin: pharmacologic probes of lymphocyte signal transduction. *Annu. Rev. Immunol. 10:519.*
12. Wenzel-Seifert, K., L. Grünbaum, and R. Seifert. 1991. Differential inhibition of human neutrophil activation by cyclosporins A, D, and H. Cyclosporin H is a potent and effective inhibitor of formyl peptide-induced superoxide formation. *J. Immunol. 147:1940.*
13. Seifert, R., and G. Schultz. 1987. Reversible activation of NADPH oxidase in membranes of HL-60 leukemic cells. *Biochem. Biophys. Res. Commun. 146:1296.*
14. Seifert, R., R. Burde, and G. Schultz. 1989. Lack of effect of opioid peptides, morphine and naloxone on superoxide formation in human neutrophils and HL-60 leukemic cells. *Naunyn-Schmiedeberg's Arch. Pharmacol. 340:101.*
15. Wenzel-Seifert, K., and R. Seifert. 1990. Nucleotide-, chemotactic peptide- and phorbol ester-induced exocytosis in HL-60 leukemic cells. *Immunobiology 181:298.*
16. Seifert, R., S. Serke, D. Huhn, W. G. Bessler, S. Hauschildt, J. Metzger, K.-H. Wiesmüller, and G. Jung. 1992. Incomplete

functional differentiation of HL-60-leukemic cells by synthetic lipopeptides. Partial inhibition by pertussis toxin of enhanced superoxide formation. *Eur. J. Biochem. 203:143.*

17. Schwaner, I., R. Seifert, and G. Schultz. 1992. Receptor-mediated increases in cytosolic Ca^{2+} in the human erythroleukemia cell line involve pertussis toxin-sensitive and -insensitive pathways. *Biochem. J. 281:301.*
18. Seifert, R., A. Höer, S. Offermanns, A. Buschauer, and W. Schunack. 1992. Histamine increases cytosolic Ca^{2+} in dibutyryl cyclic AMP-differentiated HL-60 cells *via* H_1-receptors and is an incomplete secretagogue. *Mol. Pharmacol. 42:227.*
19. Chaplinski, T. J., and J. E. Niedel. 1982. Cyclic nucleotide-induced maturation of human promyelocytic leukemia cells. *J. Clin. Invest. 70:953.*
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Rall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193:265.*
21. Johnson, R. A., and T. F. Walseth. 1979. The enzymatic preparation of [α-^{32}P]ATP, [α-^{32}P]GTP, [^{32}P]cAMP, and [^{32}P]-cGMP, and their use in the assay of adenylate and guanylate cyclases and cyclic nucleotide phosphodiesterases. *Adv. Cyclic Nucleotide Res. 10:135.*
22. Segel, I. H. 1976. The scatchard plot for equilibrium binding data. In *Enzyme Kinetics*, 2nd Ed. John Wiley, New York, p. 241.
23. Cheng, Y.-C., and W. H. Prusoff. 1973. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol. 22:3099.*
24. Boulay, F., M. Tardif, L. Brouchon, and P. Vignais. 1990. The human *N*-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry 29:11123.*
25. Dubyak, G. R., D. S. Cowen, and L. M. Meuller. 1988. Activation of inositol phospholipid breakdown in HL60 cells by P_2-purinergic receptors for extracellular ATP. Evidence for mediation by both pertussis toxin-sensitive and pertussis toxin-insensitive mechanisms. *J. Biol. Chem. 263:18108.*
26. English, D., M. T. Rizzo, G. Tricot, and R. Hoffman. 1989. Involvement of guanine nucleotides in superoxide release by fluoride-treated neutrophils. Implications for a role of a guanine nucleotide regulatory protein. *J. Immunol. 143:1685.*
27. Michel, M. C., L. F. Brass, A. Williams, G. M. Bokoch, V. J. LaMorte, and H. J. Motulsky. 1989. α_2-Adrenergic receptor stimulation mobilizes intracellular Ca^{2+} in human erythroleukemia cells. *J. Biol. Chem. 264:4986.*
28. Tsai, B. S., D. Villani-Price, R. H. Keith, J. M. Zemaitis, R. F. Bauer, R. Leonard, S. W. Djuric, and R. L. Shone. 1989. SC-41930: An inhibitor of leukotriene B_4-stimulated human neutrophil function. *Prostaglandins 38:655.*
29. Dohlman, H. G., J. Thorner, M. G. Caron, and R. Lefkowitz. 1991. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem. 60:653.*
30. Savarese, T. M., and C. M. Fraser. 1992. In vitro mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *Biochem. J. 283:1.*
31. Costa, T., and A. Herz. 1989. Antagonists with negative intrinsic activity at δ opiod receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. USA 86:7321.*
32. Kenakin, T. P., R. A. Bond, and T. I. Bonner. 1992. Definition of pharmacological receptors. *Pharmacol. Rev. 44:351.*
33. Murphy, P. M., and D. McDermott. 1991. Functional expression of the human formyl peptide receptor in *Xenopus* oocytes requires a complementary human factor. *J. Biol. Chem. 266:12560.*
34. Thomas, K. M., H. Y. Pyun, and J. Navarro. 1990. Molecular cloning of the fMet-Leu-Phe receptor from neutrophils. *J. Biol. Chem. 265:20061.*
35. Oostendorp, R. A. J., E. F. Knol, A. J. Verhoeven, and R. J. Scheper, R. J. 1992. An immunosuppressive retrovirus-derived hexapeptide interferes with intracellular signalling in monocytes and granulocytes through *N*-formylpeptide receptors. *J. Immunol. 149:1010.*
36. Kurokawa, T., T. Nonami, H. Kobayashi, W. Kishimoto, K. Uchida, H. Takagi, S. Sugiyama, and T. Ozawa. 1992. Inhibition by cyclosporine of the production of superoxide radicals. *N. Engl. J. Med. 326:840.*
37. Janco, R. L., and D. English. 1983. Cyclosporine and human neutrophil function. *Transplantation 35:501.*
38. Marasco, W. A., S. H. Phan, H. Krutzsch, H. J. Showell, D. E. Feltner, R. Nairn, E. L. Becker, and P. A. Ward. 1984. Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia coli. J. Biol. Chem. 259:5430.*
39. Carp, H. 1982. Mitochondrial *N*-formylmethionyl proteins as chemoattractants for neutrophils. *J. Exp. Med. 155:264.*
40. Traber, R., H.-R. Loosli, H. Hofmann, M. Kuhn, and A. von Wartburg. 1982. Isolierung und Strukturermittlung der neuen Cyclosporine E, F, G, H und I. *Helv. Chim. Acta 65:1655.*
41. Kahan, B. D. 1989. Cyclosporine. *N. Engl. J. Med. 321:1725.*
42. Cirillo, R., M. Triggiani, L. Siri, A. Ciccarelli, G. R. Pettit, M. Condorelli, and G. Marone. 1990. Cyclosporin A rapidly inhibits mediator release from human basophils presumably by interaction with cyclophilin. *J. Immunol. 144:3891.*