

# The H<sub>1</sub> Receptor Agonist 2-(3-Chlorophenyl)histamine Activates G<sub>i</sub> Proteins in HL-60 Cells through a Mechanism that Is Independent of Known Histamine Receptor Subtypes

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## SUMMARY

In dibutyryl-cAMP-differentiated HL-60 cells, histamine H<sub>1</sub> and formyl peptide receptors mediate increases in the cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) via pertussis toxin-sensitive G proteins of the G<sub>i</sub> family. We compared the effects of 2-(3-chlorophenyl)histamine (CPH) [2-[2-(3-chlorophenyl)-1H-imidazol-4-yl]ethanamine], one of the most potent and selective H<sub>1</sub> receptor agonists presently available, with those of histamine and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) in these cells. CPH increased [Ca<sup>2+</sup>]<sub>i</sub> through Ca<sup>2+</sup> mobilization and Ca<sup>2+</sup> influx. Unlike histamine-induced rises in [Ca<sup>2+</sup>]<sub>i</sub>, those induced by CPH were not desensitized in a homologous manner, and there was no cross-desensitization between CPH and histamine. Like fMLP, CPH activated phospholipases C and D, tyrosine phosphorylation, superoxide anion formation, and azurophilic granule release. The effects of CPH on [Ca<sup>2+</sup>]<sub>i</sub>, phospholipase D, and superoxide anion formation were inhibited by pertussis toxin.

CPH and fMLP stimulated high affinity GTP hydrolysis by G<sub>i</sub> proteins in HL-60 membranes. They also enhanced binding of guanosine-5'-O-(3-thio)triphosphate and GTP azidoanilide to, and cholera toxin-catalyzed ADP-ribosylation of, G<sub>i</sub> protein  $\alpha$  subunits. Histamine receptor antagonists did not inhibit the stimulatory effects of CPH, and CPH did not reduce fMLP binding in HL-60 membranes. Our data suggest that CPH activates G<sub>i</sub> proteins in HL-60 cells through a receptor agonist-like mechanism that is, however, independent of known histamine receptor subtypes and formyl peptide receptors. CPH may be an agonist at an as yet unknown histamine receptor subtype or, by analogy with other cationic-amphiphilic substances, may activate G proteins directly. Future studies will have to take into consideration the fact that CPH, in addition to activating H<sub>1</sub> receptors, may show other, most unexpected, stimulatory effects on G protein-mediated signal transduction processes.

Dibutyryl-cAMP-differentiated HL-60 leukemic cells possess receptors for the chemotactic peptide fMLP (1-5). Agonist-occupied formyl peptide receptors activate PTX-sensitive G proteins of the G<sub>i</sub> family, leading to activation of phospholipase C (2, 6, 7). This enzyme catalyzes the formation of inositol trisphosphate, which mobilizes Ca<sup>2+</sup> from intracellular stores (2). Additional events in the cellular activation process caused by fMLP are the stimulation of Ca<sup>2+</sup> influx via nonselective cation channels, stimulation of phospholipase D, and tyrosine phosphorylation of 100/110-kDa proteins (2, 3, 8, 9). Ultimately, stimulation of HL-60 cells by fMLP results in the

activation of the O<sub>2</sub><sup>-</sup>-forming NADPH oxidase and azurophilic granule release (1-5).

In addition to formyl peptide receptors, dibutyryl-cAMP-differentiated HL-60 cells possess H<sub>1</sub> receptors coupled to G<sub>i</sub> proteins and, possibly, to PTX-insensitive G proteins (2). H<sub>1</sub> receptors mediate activation of phospholipase C and of nonselective cation channels (2). However, unlike fMLP, HA does not activate tyrosine phosphorylation and shows no stimulatory effects on O<sub>2</sub><sup>-</sup> formation and azurophilic granule release, i.e., HA is an incomplete secretagogue (2). Thus, the functional relevance of H<sub>1</sub> receptors in dibutyryl-cAMP-differentiated HL-60 cells remains obscure.

In the past, the functional characterization of H<sub>1</sub> receptors was hampered by the lack of availability of potent and selective agonists for these receptors (10, 11). Recently, we reported on the synthesis of a series of substituted 2-phenylhistamines (12).

This paper is dedicated to our colleague, the late Eycke Böhme, who made important contributions to the field of signal transduction.

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**ABBREVIATIONS:** fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; [Ca<sup>2+</sup>]<sub>i</sub>, cytosolic Ca<sup>2+</sup> concentration; CB, cytochalasin B; CPH, 2-(3-chlorophenyl)histamine; CTX, cholera toxin; GTP $\gamma$ S, guanosine-5'-O-(3-thio)triphosphate; HA, histamine; PTX, pertussis toxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

Among these substances, CPH is one of the most potent and selective  $H_1$  receptor agonists presently available (12). In the guinea pig ileum, CPH activates  $H_1$  receptors with potency and effectiveness comparable to those of HA (12). In the hamster ductus deferens smooth muscle cell line DDT<sub>1</sub>MF-2, CPH is a potent partial  $H_1$  receptor agonist (13). Fig. 1 shows the structural formulae of HA and CPH.

The original aim of our present study was to clarify the functional relevance of  $H_1$  receptors in dibutyryl-cAMP-differentiated HL-60 cells, using CPH as a tool. Most unexpectedly, however, we noticed several differences in the effects of CPH and HA, suggesting that CPH does not act as a  $H_1$  receptor agonist in HL-60 cells. We discuss the possibilities that CPH either is an agonist at an as yet unknown HA receptor subtype or, by analogy with other cationic-amphiphilic substances (14, 15), activates  $G_i$  proteins directly.

### Experimental Procedures

**Materials.** CPH was synthesized as described (12). Stock solutions of CPH (30 mM) were prepared in distilled water and were stored at  $-20^\circ$ . Lipids were from Sigma Chemie (Deisenhofen, Germany). PTX was from List Biological Laboratories (Campbell, CA). [9,10- $^3H$ ]Oleic acid (2-10 Ci/mmol) and [ $^{35}S$ ]GTP $\gamma$ S (1200-1500 Ci/mmol) were obtained from New England Nuclear (Bad Homburg, Germany). Sources of other materials have been described elsewhere (1-5, 7-9, 16-19).

**Cell culture and membrane preparation.** HL-60 cells were grown in suspension culture and were differentiated towards neutrophil-like cells with dibutyryl-cAMP (0.2 mM) for 48 hr (1). For determination of the activity of high affinity GTPase, for fMLP binding, and for photolabeling, HL-60 membranes were prepared as described (18). PTX (1  $\mu$ g/ml) or its carrier (control) was added to cell cultures 24 hr before experiments or membrane preparation. Under these conditions, >98% of  $G_i$  protein  $\alpha$  subunits were ADP-ribosylated (4).

**Measurement of  $[Ca^{2+}]_i$  and  $Mn^{2+}$  influx.**  $[Ca^{2+}]_i$  was determined using the fluorescent dye fura-2, as described (2). Fluorescence of HL-

60 cells ( $1.0 \times 10^6$  cells in 2 ml) was determined at  $37^\circ$ , with constant stirring at  $10^3$  rpm, using a Ratio II spectrofluorometer (Aminco, Silver Spring, MD). Cells were incubated for 3 min in the presence of various substances before the addition of stimuli. The excitation and emission wavelengths were 340 and 500 nm, respectively. Unless stated otherwise, experiments were performed in the presence of 1 mM  $CaCl_2$ . Desensitization of rises in  $[Ca^{2+}]_i$  was determined according to the protocol described by Schwaner *et al.* (17).  $Mn^{2+}$  influx was assessed according to the method of Seifert *et al.* (2).

**fMLP binding assay.** [ $^3H$ ]fMLP binding was determined as described (5). Reaction mixtures (100  $\mu$ l) contained 30  $\mu$ g of membrane protein from HL-60 cells, [ $^3H$ ]fMLP (3 nM), and CPH at various concentrations or solvent (control). Reactions were conducted for 30 min at  $25^\circ$  and stopped by rapid filtration. Nonspecific binding was determined in the presence of 10  $\mu$ M fMLP and was <10% of total binding.

**Assay for phosphoinositide degradation.** Phosphoinositides in HL-60 cells were labeled by incubation with 2-5  $\mu$ Ci/ml *myo*-[2- $^3H$ ] inositol for 48 hr (2). HL-60 cells ( $1 \times 10^6$  cells in 200  $\mu$ l) were suspended in buffer and were exposed to CPH (100  $\mu$ M) or solvent (control) for 30 sec at  $37^\circ$  (2). Experiments were carried out in the absence of LiCl. Stopping of reactions, extraction of lipids, and detection of radioactivity by scintillation counting were performed as described (2).

**Assay for phosphatidylethanol formation.** For labeling of phospholipids, HL-60 cells were seeded at  $0.5 \times 10^6$  cells/ml in culture medium and were incubated with 25-50  $\mu$ Ci/ml [9,10- $^3H$ ]oleic acid for 16-20 hr. Labeled cells were centrifuged at  $250 \times g$  for 10 min at  $20^\circ$  and were resuspended at  $0.5 \times 10^6$  cells/ml in a buffer consisting of 125 mM NaCl, 0.7 mM  $MgCl_2$ , 0.5 mM EGTA, 10 mM glucose, 0.1% (w/v) fatty acid-free bovine serum albumin, and 25 mM HEPES/NaOH, pH 7.2. Centrifugation was repeated. Cells were resuspended at  $2.5-5.0 \times 10^6$  cells/ml. Reaction mixtures (50  $\mu$ l) contained  $0.5-1.0 \times 10^6$  cells in the aforementioned buffer supplemented with  $CaCl_2$  (free  $Ca^{2+}$  concentration, 1 mM) and were preincubated for 15 min at  $37^\circ$ . Reactions were initiated by the simultaneous addition of CB (1  $\mu$ g/ml), 0.5% (v/v) ethanol, and stimuli or solvent (control). Reactions were terminated by the addition of 150  $\mu$ l of  $CHCl_3/CH_3OH$ /concentrated acetic acid (100:200:4, v/v/v). Phase separation was achieved by addition of 50  $\mu$ l of  $CHCl_3$  and 50  $\mu$ l of water. Samples were centrifuged at  $3000 \times g$  for

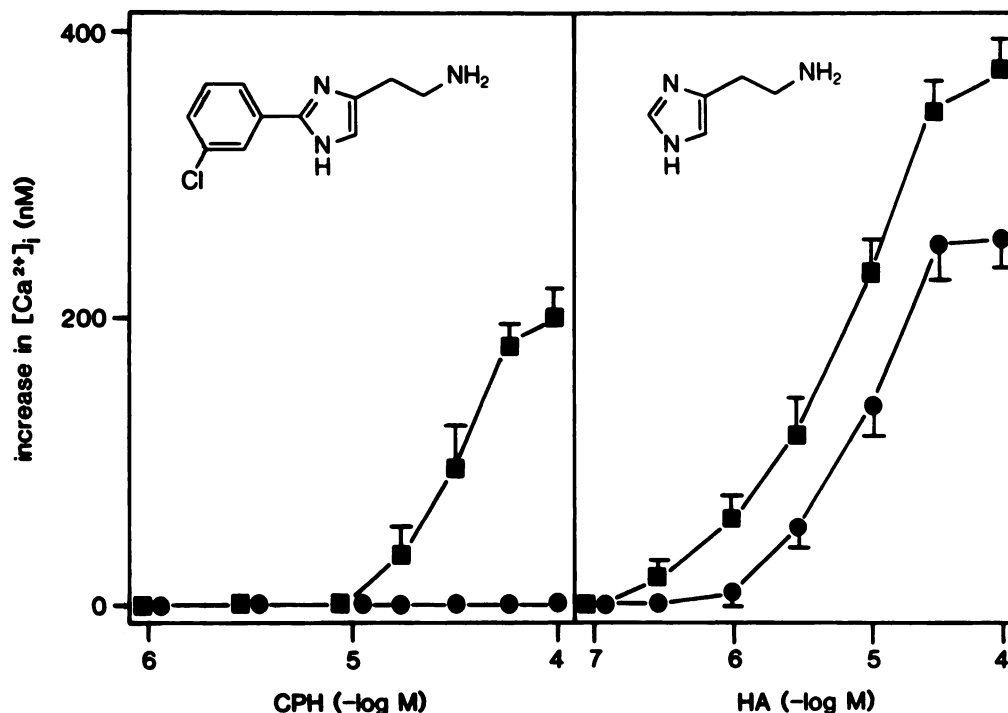


Fig. 1. Concentration-response curves for CPH- and HA-induced rises in  $[Ca^{2+}]_i$  in HL-60 cells and effects of PTX. HL-60 cells were treated with PTX or carrier (control) as described in Experimental Procedures. Thereafter, cells were harvested and loaded with fura-2/acetoxymethyl ester, and the effects of CPH and HA at various concentrations on  $[Ca^{2+}]_i$  were assessed as described in Experimental Procedures. ■, Control cells; ●, PTX-treated cells. PTX had no effect on basal  $[Ca^{2+}]_i$  in HL-60 cells.

30 min at 4°. The upper phase was removed, and 50  $\mu$ l of the organic phase was spotted onto Whatman LK6D thin layer chromatography plates. Nonradioactive phosphatidylethanol was synthesized as described (20) and was added to thin layer chromatography plates. Plates were developed in a system consisting of the upper phase of ethyl acetate/isooctane/concentrated acetic acid/water (65:10:15:50, v/v/v). Phosphatidylethanol was detected by exposure to iodine vapor, and the areas corresponding to phosphatidylethanol were scraped off after sublimation of iodine. Lipids were eluted from the silica gel with 0.5 ml of CH<sub>3</sub>OH/1 M HCl (20:1, v/v), and radioactivity was determined in a liquid scintillation counter.

**Assay for tyrosine phosphorylation.** HL-60 cells ( $1 \times 10^6$  cells in 40  $\mu$ l) were suspended in buffer at 37° and were incubated for 3 min in the absence or presence of CB (1  $\mu$ g/ml) before exposure to stimuli for 1 or 3 min (8). Stopping of reactions and immunological detection of tyrosine-phosphorylated proteins were performed as described by Offermanns *et al.* (8).

**Assay for O<sub>2</sub><sup>-</sup> formation in HL-60 cells.** O<sub>2</sub><sup>-</sup> formation was monitored at 550 nm by continuous measurement of ferricytochrome c reduction inhibitable by superoxide dismutase, using an Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, Germany) (1). HL-60 cells ( $2.5 \times 10^6$  cells in 500  $\mu$ l) were incubated for 3 min at 37° in the absence or presence of CB (1  $\mu$ g/ml) before the addition of stimuli.

**Assays for the release of  $\beta$ -glucuronidase and lactate dehydrogenase.** Enzyme release was determined as described (4). HL-60 cells ( $5 \times 10^6$  cells in 500  $\mu$ l) were suspended in buffer at 37° and were incubated for 5 min in the presence of CB (1  $\mu$ g/ml) before exposure to stimuli for 10 min. Stopping of reactions and determination of the activities of  $\beta$ -glucuronidase and lactate dehydrogenase in supernatant fluids of reaction mixtures and in cell lysates were performed as described (21).

**GTPase assay.** GTP hydrolysis was determined as described (5). Reaction mixtures (100  $\mu$ l) contained HL-60 membranes (3.0–7.0  $\mu$ g of protein/tube), 0.5  $\mu$ M [ $\gamma$ -<sup>32</sup>P]GTP (0.1  $\mu$ Ci/tube), 0.5 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenosine-5'-( $\beta$ , $\gamma$ -imido)triphosphate, 5 mM creatine phosphate, 40  $\mu$ g of creatine kinase, 1 mM dithiothreitol, and 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine-HCl, pH 7.4, with substances at various concentrations. Reactions were conducted for 15 min at 25°. Low affinity GTPase activity was determined in the presence of GTP (50  $\mu$ M) and amounted to <5% of total GTPase activity. The determination of GTP hydrolysis in *N*-ethylmaleinimide-treated HL-60 membranes was performed as described (9).

**GTP $\gamma$ S binding assay.** [<sup>35</sup>S]GTP $\gamma$ S binding was assessed according to the method of Gierschik *et al.* (22), with modifications. In brief, reaction mixtures (100  $\mu$ l) contained HL-60 membranes (3.0–5.0  $\mu$ g of protein/tube), 0.4 nM [<sup>35</sup>S]GTP $\gamma$ S, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 150 mM NaCl, 0.5  $\mu$ M GDP, 1 mM dithiothreitol, and 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine-HCl, pH 7.4. Reaction mixtures additionally contained various substances. Reactions were conducted for 30 min at 25° and were terminated by rapid filtration through cellulose nitrate BA 85 filters (Schleicher & Schuell, Dassel, Germany), followed by two washes with 5 ml of buffer (4°) consisting of 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 50 mM triethanolamine-HCl, pH 7.4. Non-specific binding was determined in the presence of 10  $\mu$ M GTP $\gamma$ S and was <1% of total binding.

**Assay for photolabeling of membrane proteins.** HL-60 membranes (50  $\mu$ g of protein in 60  $\mu$ l) were incubated at 30° in a buffer consisting of 0.1 mM EDTA, 5 mM MgCl<sub>2</sub>, 1 mM benzamide, 10  $\mu$ M GDP, and 30 mM HEPES/NaOH, pH 7.4. After exposure to various substances for 3 min, samples were incubated for another 3 min with 10 nM [ $\alpha$ -<sup>32</sup>P]GTP azidoanilide (1  $\mu$ Ci/tube). Stopping of reactions and irradiation of samples were performed as described (7).

**Assay for CTX-catalyzed ADP-ribosylation of membrane proteins.** For these experiments, membranes were prepared as described by Klinker *et al.* (9). Reaction mixtures (50  $\mu$ l) contained membranes from about  $1 \times 10^7$  HL-60 cells, 3  $\mu$ M [<sup>32</sup>P]NAD (1  $\mu$ Ci/

tube), 2.5 mM MgCl<sub>2</sub>, 1 mM ATP, 0.2% (w/v) bovine serum albumin, 2  $\mu$ g of activated CTX, and 0.1 M potassium phosphate, pH 7.4, with various substances. Reactions were conducted for 60 min at 30° (9).

**Miscellaneous methods.** Cell viability was assessed by trypan blue dye exclusion (21). Protein was determined according to the method of Lowry *et al.* (23). [ $\gamma$ -<sup>32</sup>P]GTP was prepared as described (24). [ $\alpha$ -<sup>32</sup>P]GTP azidoanilide was synthesized according to the method of Offermanns *et al.* (19). [<sup>32</sup>P]NAD was synthesized according to the method of Cassel and Pfeuffer (25). SDS-polyacrylamide gel electrophoresis and autoradiography were performed as described by Rosenthal *et al.* (16). Autoluminographs of SDS gels from tyrosine phosphorylation experiments and autoradiographs of SDS gels from photolabeling experiments were subjected to densitometric analysis using a LKB Ultrascan densitometer. The statistical significance of the effects of stimuli on inositol phosphate and phosphatidylethanol formation was assessed using the Wilcoxon test.

**Data reproducibility.** Data shown in Figs. 1, 5B, 6, and 7 and Tables 1, 2, and 4 are the means  $\pm$  standard deviations of assay quadruplicates. Similar results were obtained in at least three independent experiments. Data shown in Table 3 are the means  $\pm$  standard deviations of three independent experiments. Data shown in Figs. 2–4, 5A, and 8 are representative of at least three independent experiments.

## Results

Concentration-response curves for the stimulatory effects of CPH and HA on [Ca<sup>2+</sup>]<sub>i</sub> in HL-60 cells are shown in Fig. 1. HA increased [Ca<sup>2+</sup>]<sub>i</sub> with an EC<sub>50</sub> of 5  $\mu$ M and a maximum at 30–100  $\mu$ M. In PTX-treated cells, the stimulatory effects of HA on [Ca<sup>2+</sup>]<sub>i</sub> were partially inhibited. CPH increased [Ca<sup>2+</sup>]<sub>i</sub> with an EC<sub>50</sub> of about 50  $\mu$ M and a maximum at 75–100  $\mu$ M. At a maximally effective concentration, CPH was by approximately 45% less effective than HA in increasing [Ca<sup>2+</sup>]<sub>i</sub>. Treatment of HL-60 cells with PTX abolished the rises in [Ca<sup>2+</sup>]<sub>i</sub> caused by CPH.

The effects of the H<sub>1</sub> receptor antagonists clemastine, chlorpheniramine, and diphenhydramine (10, 11), of the H<sub>2</sub> receptor antagonists cimetidine and famotidine (10, 26), and of the dual H<sub>1</sub>/H<sub>3</sub> receptor antagonist impromidine (2, 10) on CPH- and HA-induced rises in [Ca<sup>2+</sup>]<sub>i</sub> in HL-60 cells are summarized in Table 1. H<sub>1</sub> but not H<sub>2</sub> receptor antagonists inhibited HA-induced rises in [Ca<sup>2+</sup>]<sub>i</sub>. In contrast, none of the HA receptor antagonists studied had an inhibitory effect on CPH-induced rises in [Ca<sup>2+</sup>]<sub>i</sub>.

We studied the effect of CPH on binding of FMLP (3 nM) in

TABLE 1  
Effects of HA receptor antagonists on HA- and CPH-induced rises in [Ca<sup>2+</sup>]<sub>i</sub> in HL-60 cells

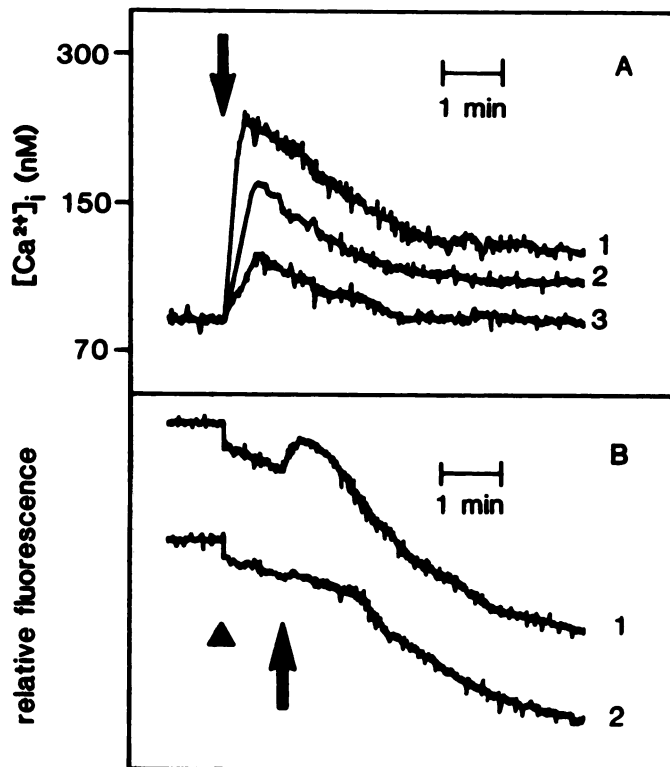
HL-60 cells were harvested and loaded with fura-2/acetoxymethyl ester, and the effects of HA and CPH (100  $\mu$ M each) on [Ca<sup>2+</sup>]<sub>i</sub> were assessed in the absence or presence of various HA receptor antagonists, as described in Experimental Procedures. HA receptor antagonists or solvent (control) was added to cells 3 min before HA or CPH. The concentration of clemastine, chlorpheniramine, diphenhydramine, cimetidine, and famotidine was 10  $\mu$ M, and that of impromidine was 100  $\mu$ M. HA receptor antagonists by themselves had no effect on [Ca<sup>2+</sup>]<sub>i</sub>.

Addition	Increase in [Ca <sup>2+</sup> ] <sub>i</sub>	
	HA	CPH
	<i>nm</i>	
Solvent (control)	385 $\pm$ 34	205 $\pm$ 23
Clemastine	0	198 $\pm$ 22
Chlorpheniramine	0	210 $\pm$ 10
Diphenhydramine	12 $\pm$ 8	199 $\pm$ 19
Cimetidine	365 $\pm$ 23	211 $\pm$ 25
Famotidine	392 $\pm$ 28	201 $\pm$ 9
Impromidine	178 $\pm$ 22	199 $\pm$ 23

HL-60 membranes. fMLP binding in HL-60 membranes amounted to  $610 \pm 21$  fmol/mg of protein (mean  $\pm$  standard deviation, four experiments). CPH ( $10 \mu\text{M}$  to  $1 \text{mM}$ ) had no inhibitory effect on fMLP binding (data not shown).

Time courses of the stimulatory effects of CPH on  $[\text{Ca}^{2+}]_i$  are depicted in Fig. 2. In the presence of extracellular  $\text{Ca}^{2+}$ , CPH rapidly increased  $[\text{Ca}^{2+}]_i$  to a peak that declined to a sustained plateau above basal values within 2 min. In the absence of extracellular  $\text{Ca}^{2+}$ , the magnitude of the CPH-induced rise in  $[\text{Ca}^{2+}]_i$  was greatly diminished. The nonselective cation channel blocker SK&F 96365 [1-( $\beta$ -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride] ( $30 \mu\text{M}$ ) (3) reduced the stimulatory effect of CPH on  $[\text{Ca}^{2+}]_i$  in the presence of extracellular  $\text{Ca}^{2+}$  (see Fig. 2) but not in its absence (data not shown). Fig. 2 also shows the effect of CPH on  $\text{Mn}^{2+}$  influx. At excitation wavelengths of 340 nm and 360 nm, CPH substantially increased the rate of basal  $\text{Mn}^{2+}$  influx. At the  $\text{Ca}^{2+}$ -sensitive excitation wavelength of 340 nm, an additional transient increase in fluorescence was evident. All of these findings indicate that CPH activated both  $\text{Ca}^{2+}$  mobilization from intracellular stores and  $\text{Ca}^{2+}$  influx through nonselective cation channels (1, 3).

Because a part of the CPH-induced rise in  $[\text{Ca}^{2+}]_i$  was attributable to  $\text{Ca}^{2+}$  mobilization from intracellular stores (see Fig.



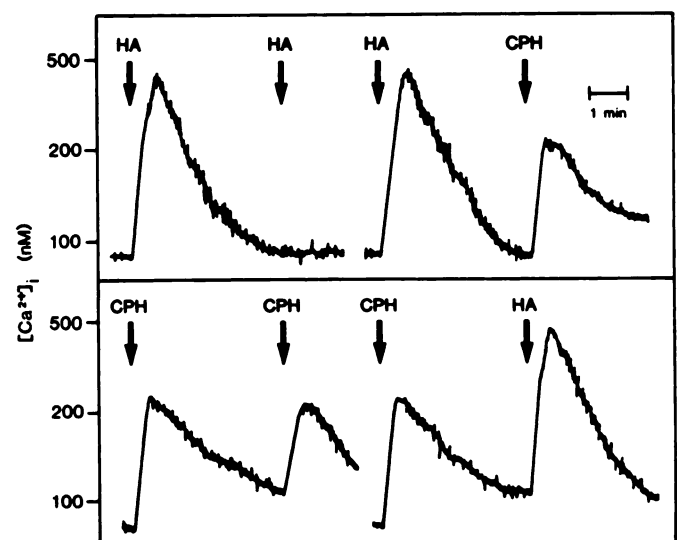
**Fig. 2.** Time courses of the stimulatory effects of CPH on  $[\text{Ca}^{2+}]_i$  and  $\text{Mn}^{2+}$  influx in HL-60 cells. HL-60 cells were harvested and loaded with fura-2/acetoxymethyl ester, and the effects of CPH ( $100 \mu\text{M}$ ) on  $[\text{Ca}^{2+}]_i$  (A) and  $\text{Mn}^{2+}$  influx (B) were assessed as described in Experimental Procedures. A, Arrow, addition of CPH. Trace 1, presence of  $\text{CaCl}_2$  ( $1 \text{mM}$ ) plus solvent (control); trace 2, presence of  $\text{CaCl}_2$  ( $1 \text{mM}$ ) plus SK&F 96365 ( $30 \mu\text{M}$ ); trace 3, presence of EGTA ( $1 \text{mM}$ ) without added  $\text{CaCl}_2$ . B, Experiments were performed without added  $\text{CaCl}_2$ . The emission wavelength was 500 nm. Arrowhead, addition of  $\text{MnCl}_2$  ( $100 \mu\text{M}$ ); arrow, addition of CPH. Trace 1, excitation wavelength of 340 nm; trace 2, excitation wavelength of 360 nm. Superimposed original fluorescence tracings are shown.

2), we studied the effects of CPH on phospholipase C-catalyzed inositol phosphate formation. Basal formation of inositol phosphate, inositol bisphosphate, and inositol trisphosphate amounted to  $767 \pm 38$  dpm,  $695 \pm 37$  dpm, and  $168 \pm 37$  dpm, respectively (means  $\pm$  standard deviations, three experiments). CPH ( $100 \mu\text{M}$ ) increased these values to  $924 \pm 58$  dpm ( $p < 0.05$ ),  $1056 \pm 24$  dpm ( $p < 0.05$ ), and  $234 \pm 24$  dpm ( $p < 0.05$ ), respectively (means  $\pm$  standard deviations, three experiments).

Desensitization of CPH- and HA-induced increases in  $[\text{Ca}^{2+}]_i$  was studied (Fig. 3). HL-60 cells that had been exposed to HA ( $100 \mu\text{M}$ ) did not undergo a second rise in  $[\text{Ca}^{2+}]_i$  when rechallenged with HA ( $100 \mu\text{M}$  or  $1 \text{mM}$ ; data shown only for  $100 \mu\text{M}$ ), i.e., the effect of HA was desensitized in a homologous manner. In contrast, the CPH-induced rises in  $[\text{Ca}^{2+}]_i$  were not desensitized in a homologous manner. Additionally, there was no cross-desensitization between HA and CPH.

We also studied the effects of CPH on the formation of phosphatidylethanol, i.e., a transphosphatidylation product specifically formed by phospholipase D (21, 27, 28), in HL-60 cells. CPH increased phosphatidylethanol formation by 20%, and in PTX-treated cells the stimulatory effect of CPH was abolished (Table 2). fMLP ( $1 \mu\text{M}$ ) stimulated phosphatidylethanol formation by about 150%, and this effect was also sensitive to PTX.

Fig. 4 shows the effects of fMLP and CPH on tyrosine phosphorylation of 100/110-kDa proteins in HL-60 cells. Table 3 summarizes the densitometric evaluation of the effects of fMLP and CPH on tyrosine phosphorylation. Experiments were performed in the absence or presence of CB, which inhibits actin polymerization and is known to potentiate several responses to fMLP (1, 4, 29). At an incubation time of 1 min, CPH ( $1 \text{mM}$ ) was about 40% less effective than fMLP ( $10 \mu\text{M}$ ) in stimulating tyrosine phosphorylation. CB potentiated the stimulatory effects of fMLP and CPH on tyrosine phosphorylation. In the presence of CB, fMLP and CPH were similarly effective in activating tyrosine phosphorylation. At an incubation time of 3 min, the stimulatory effects of CPH on tyrosine



**Fig. 3.** Desensitization of CPH- and HA-induced rises in  $[\text{Ca}^{2+}]_i$  in HL-60 cells. HL-60 cells were harvested and loaded with fura-2/acetoxymethyl ester, and the effects of CPH and HA on  $[\text{Ca}^{2+}]_i$  were assessed as described in Experimental Procedures. Arrows, addition of CPH or HA ( $100 \mu\text{M}$  each). Original fluorescence tracings are shown.

TABLE 2

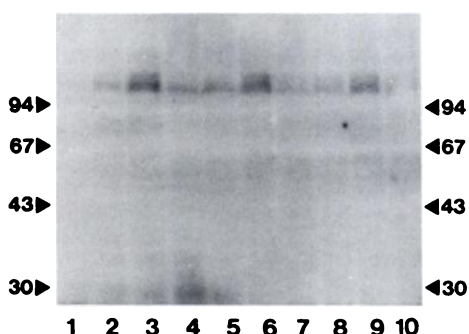
**Stimulation by CPH and fMLP of phosphatidylethanol formation in HL-60 cells and effects of PTX**

Pretreatment of HL-60 cells with PTX or carrier (control) was performed as described in Experimental Procedures. HL-60 cells were harvested and labeled with [<sup>3</sup>H]oleate as described in Experimental Procedures. HL-60 cells were exposed to CPH (1 mM), fMLP (10 μM), or solvent (basal) in the presence of CB (1 μg/ml) and 0.5% (v/v) ethanol. Extraction of lipids and detection of radioactive phosphatidylethanol were performed as described in Experimental Procedures. The statistical significance of the effects of CPH and fMLP versus solvent on phosphatidylethanol formation was assessed as described in Experimental Procedures.

Addition	Phosphatidylethanol formation	
	Control	PTX
	dpm	
Solvent (basal)	1429 ± 98	1428 ± 106
CPH	1716 ± 101 <sup>a</sup>	1354 ± 156 <sup>b</sup>
fMLP	3546 ± 74 <sup>a</sup>	1481 ± 68 <sup>b</sup>

<sup>a</sup>  $p < 0.05$ .

<sup>b</sup> Not significant.



**Fig. 4.** Effects of CPH and fMLP on tyrosine phosphorylation in HL-60 cells. HL-60 cells were exposed to fMLP (10 μM), CPH (1 mM), or solvent (control), in the absence or presence of CB (1 μg/ml), for 1 min (lanes 1-6) or 3 min (lanes 7-10). Proteins were analyzed as described in Experimental Procedures. The autoluminogram of an SDS gel containing 9% (w/v) acrylamide is shown. Lane 1, solvent (control); lane 2, fMLP; lane 3, fMLP plus CB; lane 4, CB; lane 5, CPH; lane 6, CPH plus CB; lane 7, solvent (control); lane 8, CPH; lane 9, CPH plus CB; lane 10, CB. Numbers on the left and right, molecular masses of marker proteins (in kDa).

TABLE 3

**Densitometric analysis of the stimulatory effects of CPH and fMLP on tyrosine phosphorylation of 100/110-kDa proteins in HL-60 cells**

HL-60 cells were exposed to fMLP (10 μM), CPH (1 mM), or solvent (control), in the absence or presence of CB (1 μg/ml), for the indicated periods of time. Proteins were analyzed as described in Experimental Procedures. The 100/110-kDa regions of autoluminographs were subjected to densitometric analysis.

Experimental conditions	Absorbance of 100/ 110-kDa regions
	arbitrary units
Solvent (control), 1 min	1.05 ± 0.07
fMLP, 1 min	1.85 ± 0.15
fMLP + CB, 1 min	3.20 ± 0.31
CB, 1 min	1.13 ± 0.08
CPH, 1 min	1.50 ± 0.13
CPH + CB, 1 min	3.07 ± 0.34
Solvent (control), 3 min	1.06 ± 0.03
CPH, 3 min	1.27 ± 0.06
CPH + CB, 3 min	2.03 ± 0.19
CB, 3 min	1.08 ± 0.09

phosphorylation were already declining. CB by itself did not significantly stimulate tyrosine phosphorylation.

The effects of CPH and fMLP on O<sub>2</sub><sup>-</sup> formation were compared. fMLP (1 μM) rapidly activated O<sub>2</sub><sup>-</sup> formation, which

ceased after about 5 min (Fig. 5A). CB potentiated the stimulatory effect of fMLP. CPH (1 mM) did not activate O<sub>2</sub><sup>-</sup> formation in the absence of CB. However, in its presence CPH (1 mM) activated O<sub>2</sub><sup>-</sup> formation, although with much less effectiveness than fMLP (1 μM). Fig. 5B shows the concentration-response curve for the stimulatory effects of CPH on O<sub>2</sub><sup>-</sup> formation in the presence of CB. CPH activated O<sub>2</sub><sup>-</sup> formation with an EC<sub>50</sub> of 0.65 mM and a plateau at 1-2 mM. Clemastine and famotidine (10 μM each) had no inhibitory effect on CPH-induced O<sub>2</sub><sup>-</sup> formation (data not shown). In PTX-treated cells, CPH did not activate O<sub>2</sub><sup>-</sup> formation.

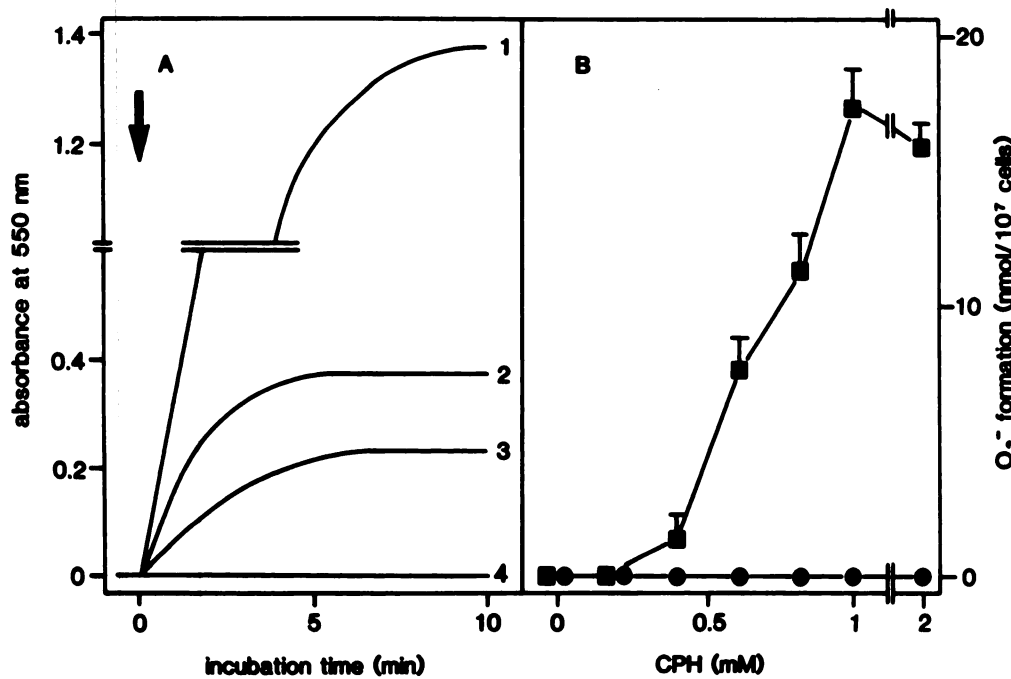
Fig. 6 shows the effects of fMLP and CPH on enzyme release in HL-60 cells. fMLP stimulated azurophilic granule release with an EC<sub>50</sub> of 15 nM and a maximum at 0.1-1.0 μM. CPH activated β-glucuronidase release with an EC<sub>50</sub> of 0.15 mM and a maximum at 0.3-1.0 mM. CPH was less effective than fMLP in stimulating azurophilic granule release. fMLP and CPH had no stimulatory effect on the release of the cytosolic enzyme lactate dehydrogenase. In addition, CPH and fMLP did not stimulate uptake of the dye trypan blue into HL-60 cells (data not shown). The latter two findings indicate that CPH was not cytotoxic at the concentrations studied.

Next, we studied the effects of CPH and fMLP on high affinity GTP hydrolysis, i.e., the enzymatic activity of G protein α subunits, and on GTPγS binding in HL-60 membranes. The K<sub>m</sub> of basal high affinity GTPase in HL-60 membranes was 0.43 ± 0.08 μM (mean ± standard deviation, three experiments). As is the case for fMLP (9), CPH increased the V<sub>max</sub> of GTP hydrolysis without affecting the K<sub>m</sub> (data not shown), indicating that the substance increased the catalytic rate of GTP turnover. CPH activated high affinity GTP hydrolysis and GTPγS binding with an EC<sub>50</sub> of about 0.3 mM and a maximum at 1-3 mM (Fig. 7). Clemastine and famotidine (10 μM each) had no effect on CPH-stimulated GTP hydrolysis in HL-60 membranes (data not shown).

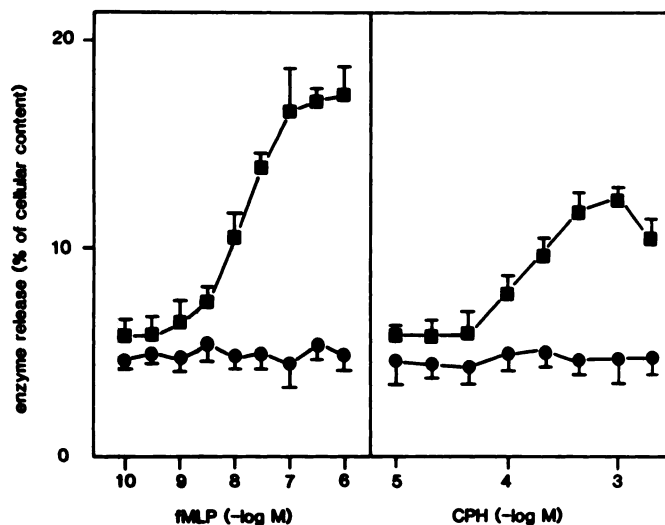
Table 4 compares the stimulatory effects of fMLP (10 μM) and CPH (1 mM) on high affinity GTP hydrolysis in HL-60 membranes. In control membranes, fMLP increased GTP hydrolysis and GTPγS binding by about 150%. CPH was about 50% less effective than fMLP in stimulating GTPase and GTPγS binding. Pretreatment with PTX resulted in almost complete or complete inhibition of the stimulatory effects of CPH and fMLP on GTP hydrolysis and GTPγS binding.

N-Ethylmaleinimide alkylates G<sub>i</sub> protein α subunits and thereby uncouples receptors from G proteins in a manner similar to that of PTX-catalyzed ADP-ribosylation (30). Pretreatment of HL-60 membranes with N-ethylmaleinimide reduces the stimulatory effect of fMLP on GTPase by about 40% (9). The stimulatory effect of CPH (1 mM) on GTP hydrolysis was reduced from 70% in control membranes to 15% in N-ethylmaleinimide-treated membranes (data not shown).

fMLP not only stimulated GTP hydrolysis and GTPγS binding in HL-60 membranes in a PTX-sensitive manner but also stimulated the incorporation of GTP azidoanilide into, and CTX-catalyzed ADP-ribosylation of, 40/41-kDa proteins (representing G<sub>i</sub> protein α subunits) in HL-60 membranes (Fig. 8) (9). Densitometric analysis revealed that fMLP increased photolabeling by 53 ± 18% (mean ± standard deviation, three experiments). Compared with fMLP, CPH was about 50% less effective in stimulating photolabeling (26 ± 15% stimulation, mean ± standard deviation, three experiments) (see Fig. 8A).



**Fig. 5.** Effects of CPH and fMLP on  $O_2^-$  formation in HL-60 cells. HL-60 cells were harvested and  $O_2^-$  formation was assessed under various experimental conditions, as described in Experimental Procedures. **A,** Time course of  $O_2^-$  formation. Arrow, addition of stimuli. Experiments were performed in the absence or presence of CB (1  $\mu$ g/ml). Trace 1, fMLP (1  $\mu$ M) plus CB; trace 2, fMLP (1  $\mu$ M); trace 3, CPH (1 mM) plus CB; trace 4, CPH (1 mM). Superimposed original spectrophotometric recordings are shown. **B,** Concentration-response curves for CPH-induced  $O_2^-$  formation. Cells were treated with PTX or carrier (control) as described in Experimental Procedures. ■, Control cells; ●, PTX-treated cells.



**Fig. 6.** Effects of CPH and fMLP on enzyme release in HL-60 cells. HL-60 cells were harvested and enzyme release was determined in the presence of CPH or fMLP at the indicated concentrations, as described in Experimental Procedures. Reaction mixtures contained CB (1  $\mu$ g/ml). ■,  $\beta$ -Glucuronidase release; ●, lactate dehydrogenase release.

As was the case for photolabeling, CPH (1 mM) was less effective than fMLP in enhancing CTX-catalyzed ADP-ribosylation of  $G_i$  protein  $\alpha$  subunits in HL-60 membranes (see Fig. 8B).

## Discussion

To clarify the functional relevance of  $H_1$  receptors in dibutyl- $\alpha$ -cAMP-differentiated HL-60 cells (2), we studied the effects of CPH, one of the most potent and selective  $H_1$  receptor agonists presently available (12, 13), in these cells. We found that CPH, similarly to HA, activates phospholipase C,  $Ca^{2+}$  mobilization, and nonselective cation channels (see Figs. 1–3) (2). Additionally, HA and CPH are less effective than fMLP in inducing increases in  $[Ca^{2+}]_i$  (see Fig. 1) (2). In view of our

original assumption that CPH is an  $H_1$  receptor agonist not only in the guinea pig ileum and in DDT<sub>1</sub>MF-2 cells but also in HL-60 cells, these findings were not unexpected.

Most unexpectedly, however, we noticed several differences in the effects of HA and CPH in HL-60 cells. The effects of CPH on  $[Ca^{2+}]_i$  were abolished by PTX, whereas those of HA were only partially PTX sensitive (see Fig. 1) (2). These data indicate that only  $G_i$  proteins are involved in the signal transduction pathway activated by CPH. Additionally, in the guinea pig ileum and in DDT<sub>1</sub>MF-2 cells CPH is a similarly potent  $H_1$  receptor agonist, compared with HA, whereas in HL-60 cells CPH is approximately 10-fold less potent than HA in increasing  $[Ca^{2+}]_i$  (see Fig. 1) (2, 12, 13). Moreover, there are differences in the kinetics of HA- and CPH-induced increases in  $[Ca^{2+}]_i$  (see Figs. 2 and 3) (2). Other differences between HA and CPH were observed with respect to activation of tyrosine phosphorylation,  $O_2^-$  formation, and azurophilic granule release, i.e., unlike HA, CPH was stimulatory (see Figs. 4–6) (2). Most importantly, the stimulatory effects of CPH on  $[Ca^{2+}]_i$ ,  $O_2^-$  formation, and GTPase were completely resistant to inhibition by various HA receptor antagonists (see Table 1). Finally, the CPH-induced rises in  $[Ca^{2+}]_i$ , unlike the HA-induced increases, were not subject to homologous desensitization, and there was no cross-desensitization between HA and CPH (see Fig. 3). From all of these data we conclude that CPH activates HL-60 cells through a mechanism that is independent of known HA receptor subtypes.

Why does CPH not act as an  $H_1$  receptor agonist in HL-60 cells although these cells express functional  $H_1$  receptors (see Table 1) (2) and although CPH has been shown to be a potent  $H_1$  receptor agonist in at least two systems (12, 13)? An explanation for these discrepancies may be that there are different  $H_1$  receptor reserves in the systems studied. Specifically, the  $H_1$  receptor reserve in DDT<sub>1</sub>MF-2 cells is apparently lower than that in the guinea pig ileum (13). In the former system, unlike in the latter, CPH is only a partial  $H_1$  receptor agonist (13). Possibly, the  $H_1$  receptor reserve in HL-60 cells is

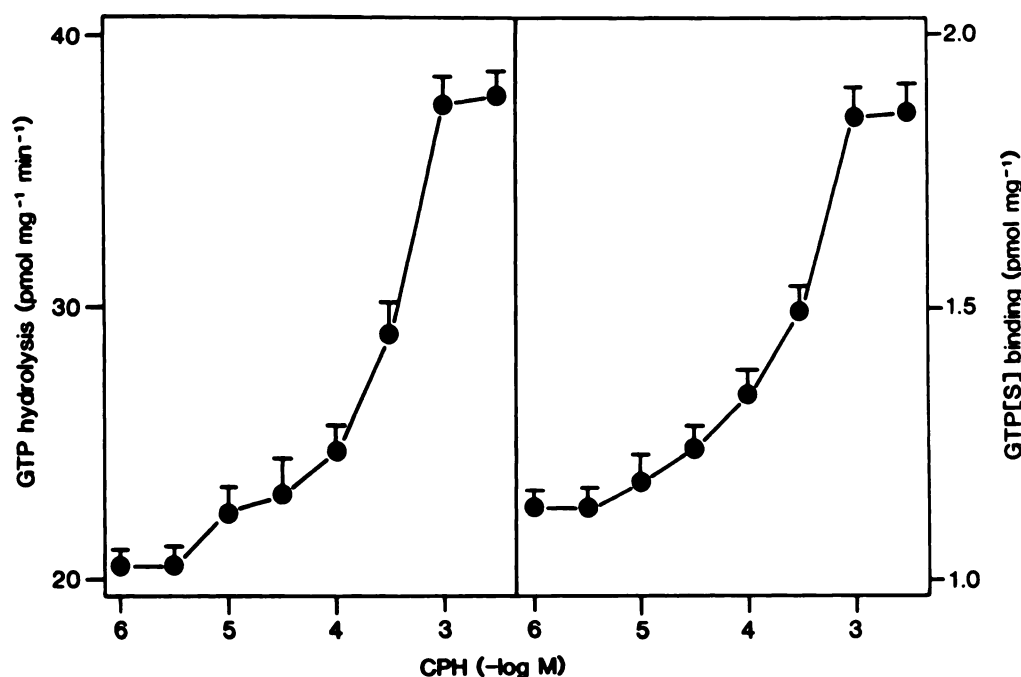


Fig. 7. Concentration-response curves for the stimulatory effects of CPH on GTP hydrolysis and GTP $\gamma$ S binding in HL-60 membranes. GTP hydrolysis and GTP $\gamma$ S (GTP[S]) binding in HL-60 membranes were determined in the presence of CPH at the indicated concentrations, as described in Experimental Procedures.

TABLE 4

**Effect of PTX on stimulation of GTP hydrolysis and GTP $\gamma$ S binding caused by CPH and fMLP in HL-60 membranes**

Pretreatment of HL-60 cells with PTX or carrier (control) was performed as described in Experimental Procedures. GTP hydrolysis and GTP $\gamma$ S binding in HL-60 membranes were determined in the presence of CPH (1 mM), fMLP (10  $\mu$ M), or solvent (basal), as described in Experimental Procedures.

Addition	GTP hydrolysis		GTP $\gamma$ S binding	
	Control	PTX	Control	PTX
	pmol/mg/min		pmol/mg	
Solvent (basal)	17.5 $\pm$ 0.7	10.3 $\pm$ 0.2	1.27 $\pm$ 0.10	0.99 $\pm$ 0.05
CPH	30.1 $\pm$ 1.3	9.0 $\pm$ 0.5	2.08 $\pm$ 0.09	0.93 $\pm$ 0.06
fMLP	43.1 $\pm$ 0.6	10.6 $\pm$ 0.3	3.16 $\pm$ 0.17	1.02 $\pm$ 0.03

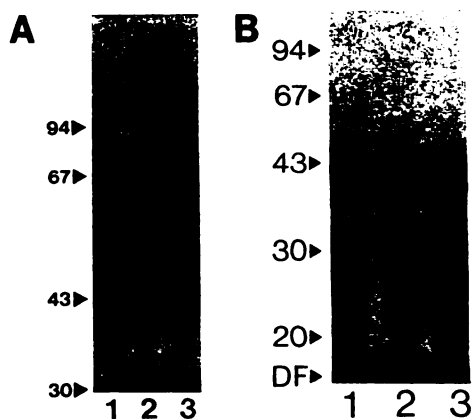


Fig. 8. Effects of CPH and fMLP on incorporation of GTP azidoanilide into, and CTX-catalyzed ADP-ribosylation of, G $_i$  protein  $\alpha$  subunits in HL-60 membranes. A, Photolabeling was performed as described in Experimental Procedures. The autoradiogram of an SDS gel containing 4 M urea and 9% (w/v) acrylamide is shown. Lane 1, solvent (control); lane 2, fMLP (10  $\mu$ M); lane 3, CPH (1 mM). B, ADP-ribosylation was performed as described in Experimental Procedures. The autoradiogram of an SDS gel containing 10% (w/v) acrylamide is shown. Lane 1, solvent (control); lane 2, CPH (1 mM); lane 3, fMLP (10  $\mu$ M). Numbers on the left, molecular masses of marker proteins (in kDa). DF, dye front.

even lower than that in DDT $_1$ MF-2 cells, so that the expected agonistic effect of CPH at H $_1$  receptors in HL-60 cells is too small to be detected. Thus, instead of activating H $_1$  receptors in HL-60 cells, CPH apparently interacts with a different site, which may be an as yet unknown HA receptor subtype. Although CPH mimicked many of the effects of fMLP in intact HL-60 cells and in HL-60 membranes (see Figs. 1 and 4–8 and Tables 2–4) (1–9), it is very unlikely that the substance acts as a formyl peptide receptor agonist, because it did not compete with fMLP for binding.

Another possible mechanism of action of CPH could be that this substance activates G $_i$  proteins directly, i.e., in a receptor-independent manner. It is well known that various cationic-amphiphilic substances, such as substance P, compound 48/80, and the wasp venom mastoparan, stimulate G proteins in such a way (14, 15). In fact, CPH also is a cationic-amphiphilic substance. Specifically, CPH possesses a hydrophobic halogen-substituted phenyl residue and a basic domain (see Fig. 1) (12). At pH 7.2–7.4 (the pH values of buffers used in the various assays; see Experimental Procedures), the amino group of CPH is, for the most part, positively charged (12, 31, 32). In accordance with this suggested mode of action of CPH may be our finding that the concentrations of the substance required to activate G $_i$  proteins are higher than those needed for stimulation of H $_1$  receptors (see Figs. 1 and 5–7) (12, 13).

fMLP is about 5-fold more potent in increasing [Ca $^{2+}$ ] $_i$  than in activating O $_2^-$  formation and azurophilic granule release (1, 2, 4, 5). Two formyl peptide receptor subtypes have been identified at the cDNA level in dibutylryl-cAMP-differentiated HL-60 cells (33), but it is still unknown, inasmuch as different formyl peptide receptor subtypes are responsible for mediating rises in [Ca $^{2+}$ ] $_i$  on one hand and activation of O $_2^-$  formation and enzyme release on the other (5). Because the differences in amino acid sequences of formyl peptide receptors in HL-60 cells are only small (33), one could also envisage that the differences in potency of fMLP with respect to the aforementioned parameters are attributable to differential requirements

for the number of activated  $G_i$  proteins, rather than being due to activation of different receptor subtypes. If stimulation of increases in  $[Ca^{2+}]_i$  required a smaller number of activated  $G_i$  proteins than did stimulation of  $O_2^-$  formation and azurophilic granule release, CPH would be expected to be more potent with respect to the former parameter than with respect to the latter two. This is, in fact, the case (see Figs. 1, 5, and 6).

Stimulation of tyrosine phosphorylation is thought to play a role in the activation of  $O_2^-$  formation caused by fMLP (8, 34). We found that CPH substantially activated tyrosine phosphorylation in the absence of CB but failed to activate  $O_2^-$  formation in its absence (see Figs. 4 and 5 and Table 3). In addition, fMLP and CPH are similarly effective activators of tyrosine phosphorylation in the presence of CB, but CPH is a considerably less effective activator of  $O_2^-$  formation than is fMLP under these conditions (see Figs. 4–6 and Table 3). These findings may indicate that tyrosine phosphorylation is not sufficient for activation of  $O_2^-$  formation and that activation of additional effector systems, e.g., phospholipase D, is required (35).

In conclusion, we have shown that CPH activates  $G_i$  proteins in dibutyryl-cAMP-differentiated HL-60 cells through a receptor agonist-like mechanism that is independent of known HA receptor subtypes and formyl peptide receptors. Future studies will have to answer the question of whether CPH is an agonist at an as yet unknown HA receptor subtype or activates  $G_i$  proteins directly. In addition, the structure-activity relationships of 2-substituted HA derivatives with respect to  $G_i$  protein activation in HL-60 cells have to be determined. Future studies will have to take into consideration the fact that CPH, in addition to activating  $H_1$  receptors, may show other, most unexpected, stimulatory effects on G protein-mediated signal transduction processes.

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#### References

- Seifert, R., R. Burde, and G. Schultz. Activation of NADPH oxidase by purine and pyrimidine nucleotides involves G proteins and is potentiated by chemotactic peptides. *Biochem. J.* **259**:813–819 (1989).
- Seifert, R., A. Höer, S. Offermanns, A. Buschauer, and W. Schunack. Histamine increases cytosolic  $Ca^{2+}$  in dibutyryl-cAMP-differentiated HL-60 cells via  $H_1$  receptors and is an incomplete secretagogue. *Mol. Pharmacol.* **42**:227–234 (1992).
- Krautwurst, D., R. Seifert, J. Hescheler, and G. Schultz. Formyl peptides and ATP stimulate  $Ca^{2+}$  and  $Na^+$  inward currents through nonselective cation channels via G proteins in dibutyryl cyclic AMP-differentiated HL-60 cells: involvement of  $Ca^{2+}$  and  $Na^+$  in the activation of  $\beta$ -glucuronidase release and superoxide production. *Biochem. J.* **288**:1025–1035 (1992).
- Wenzel-Seifert, K., and R. Seifert. Nucleotide-, chemotactic peptide- and phorbol ester-induced exocytosis in HL-60 leukemic cells. *Immunobiology* **181**:298–316 (1990).
- Wenzel-Seifert, K., and R. Seifert. 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. *J. Immunol.* **150**:4591–4599 (1993).
- Gierschik, P., D. Sidiropoulos, and K. H. Jakobs. Two distinct  $G_i$ -proteins mediate formyl peptide receptor signal transduction in human leukemia (HL-60) cells. *J. Biol. Chem.* **264**:21470–21473 (1989).
- Offermanns, S., R. Schäfer, B. Hoffmann, E. Bombien, K. Spicher, K.-D. Hinsch, G. Schultz, and W. Rosenthal. Agonist-sensitive binding of a photoreactive GTP analog to a G protein  $\alpha$ -subunit in membranes of HL-60 cells. *FEBS Lett.* **260**:14–18 (1990).
- Offermanns, S., R. Seifert, J. W. Metzger, G. Jung, A. Lieberknecht, U.

- Schmidt, and G. Schultz. Lipopeptides are effective stimulators of tyrosine phosphorylation in human myeloid cells. *Biochem. J.* **282**:551–557 (1992).
- Klinker, J., A. Höer, I. Schwaner, S. Offermanns, K. Wenzel-Seifert, and R. Seifert. Lipopeptides activate  $G_i$ -proteins in dibutyryl cyclic AMP-differentiated HL-60 cells. *Biochem. J.* **296**:245–251 (1993).
- Buschauer, A., W. Schunack, J.-M. Arrang, M. Garbarg, J.-C. Schwartz, and J. M. Young. Histamine receptors, in *Clinical Pharmacology, Vol. 13, Receptor Pharmacology and Function* (M. Williams, R. A. Glennon, and P. B. M. W. M. Timmermans, eds.). Marcel Dekker, New York, 293–348 (1989).
- Hill, S. J. Distribution, properties, and functional characteristics of three classes of histamine receptors. *Pharmacol. Rev.* **42**:45–83 (1990).
- Zingel, V., S. Elz, and W. Schunack. Histamine analogues. 33. 2-Phenylhistamines with high histamine  $H_1$ -agonistic activity. *Eur. J. Med. Chem.* **25**:673–680 (1990).
- White, T. E., J. M. Dickenson, and S. J. Hill. Histamine  $H_1$  receptor-mediated inositol phospholipid hydrolysis in DDT<sub>1</sub>MF-2 cells: agonist and antagonist properties. *Br. J. Pharmacol.* **108**:196–203 (1993).
- Higashijima, T., S. Uzu, T. Nakajima, and E. M. Ross. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding proteins (G proteins). *J. Biol. Chem.* **263**:6491–6494 (1988).
- Mousli, M., C. Bronner, J. Bockaert, B. Rouot, and Y. Landry. Interaction of substance P, compound 48/80 and mastoparan with the  $\alpha$ -subunit C-terminus of G protein. *Immunol. Lett.* **25**:355–358 (1990).
- Rosenthal, W., D. Koesling, U. Rudolph, C. Kleuss, M. Pallast, M. Yajima, and G. Schultz. Identification and characterization of the 35-kDa  $\beta$  subunit of guanine-nucleotide-binding proteins by an antiserum raised against transducin. *Eur. J. Biochem.* **158**:255–263 (1986).
- Schwane, I., R. Seifert, and G. Schultz. Receptor-mediated increases in cytosolic  $Ca^{2+}$  in the human erythroleukemia cell line involve pertussis toxin-sensitive and -insensitive pathways. *Biochem. J.* **281**:301–307 (1992).
- Seifert, R., and G. Schultz. Reversible activation of NADPH oxidase in membranes of HL-60 leukemic cells. *Biochem. Biophys. Res. Commun.* **146**:1296–1302 (1987).
- Offermanns, S., G. Schultz, and W. Rosenthal. Identification of receptor-activated G proteins with photoreactive GTP analog, [ $\alpha$ - $^{32}P$ ]GTP azidoanilide. *Methods Enzymol.* **195**:286–301 (1991).
- Liscovitch, M. Phosphatidylethanol biosynthesis in ethanol-exposed NG108–15 neuroblastoma  $\times$  glioma hybrid cells: evidence for activation of a phospholipase D phosphatidyl transferase activity by protein kinase C. *J. Biol. Chem.* **264**:1450–1456 (1989).
- Absolom, D. R. Basic methods for the study of phagocytosis. *Methods Enzymol.* **132**:95–180 (1986).
- Gierschik, P., R. Moghtader, C. Straub, K. Dietrich, and K. H. Jakobs. Signal amplification in HL-60 granulocytes: evidence that the chemotactic peptide receptor catalytically activates guanine-nucleotide-binding regulatory proteins in native plasma membranes. *Eur. J. Biochem.* **197**:725–732 (1991).
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
- Walseth, T. F., P. S. T. Yuen, and M. C. Moos, Jr. Preparation of  $\alpha$ - $^{32}P$ -labeled nucleoside triphosphates, nicotinamide adenine dinucleotide, and cyclic nucleotides for use in determining adenyllyl and guanylyl cyclases and cyclic nucleotide phosphodiesterase. *Methods Enzymol.* **195**:29–44 (1991).
- Cassel, D., and T. Pfeuffer. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **75**:2669–2673 (1978).
- Schunack, W. What are the differences between the  $H_2$  receptor antagonists? *Aliment. Pharmacol. Ther.* **1**:493S–503S (1987).
- Kanaho, Y., K. Takahashi, U. Tomita, T. Iiri, T. Katada, M. Ui, and Y. Nozawa. A protein kinase C inhibitor, staurosporine, activates phospholipase D via a pertussis toxin-sensitive GTP-binding protein in rabbit peritoneal neutrophils. *J. Biol. Chem.* **267**:23554–23559 (1992).
- Stutchfield, J., and S. Cockcroft. Correlation between secretion and phospholipase D activation in differentiated HL60 cells. *Biochem. J.* **293**:649–655 (1993).
- Jesaitis, A. J., J. O. Tolley, and R. A. Allen. Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. *J. Biol. Chem.* **261**:13662–13669 (1986).
- Jakobs, K. H., P. Lasch, M. Minuth, K. Aktories, and G. Schultz. Uncoupling of  $\alpha$ -adrenoceptor-mediated inhibition of human platelet adenylate cyclase by *N*-ethylmaleimide. *J. Biol. Chem.* **257**:2829–2833 (1982).
- Dziuron, P., and W. Schunack. Struktur-Wirkungs-Beziehungen bei Histaminanaloga. VIII. Synthese und Wirkung 2-substituierter Histamine. *Eur. J. Med. Chem.* **10**:129–133 (1975).
- Ganellin, C. R. Chemistry and structure-activity relationships of drugs acting



- at histamine receptors, in *Pharmacology of Histamine Receptors* (C. R. Ganellin and M. E. Parsons, eds.). Wright, Bristol, UK, 10–102 (1982).
33. Boulay, F., M. Tardif, L. Bouchon, and P. Vignais. The human *N*-formylpeptide receptor: characterization of two cDNA isolates and evidence for a new subfamily of G protein-coupled receptors. *Biochemistry* **29**:11123–11133 (1990).
34. Berkow, R. L., R. W. Dodson, and A. S. Kraft. Human neutrophils contain distinct cytosolic and particulate tyrosine kinase activities: possible role in neutrophil activation. *Biochim. Biophys. Acta* **997**:292–301 (1989).
35. Rossi, F., M. Grzeskowiak, V. Della Bianca, F. Calzetti, and G. Gandini. Phosphatidic acid and not diacylglycerol generated by phospholipase D is functionally linked to the activation of the NADPH oxidase by FMLP in human neutrophils. *Biochem. Biophys. Res. Commun.* **168**:320–327 (1990).

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