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

ROLAND SEIFERT, ASTRID HAGELÜKEN, ARIANE HÖER, DIETER HÖER, LORE GRÜNBAUM, STEFAN OFFERMANNS, INGO SCHWANER, VOLKMAR ZINGEL, WALTER SCHUNACK, and GÜNTER SCHULTZ

Institut für Pharmakologie (R.S., A.Ha., A.Hö., D.H., L.G., S.O., I.S., G.S.) and Institut für Pharmazie (V.Z., W.S.), Freie Universität Berlin, D-14195 Berlin, Germany

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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>]) via pertussis toxin-sensitive G proteins of the G 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.

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).
Among these substances, CPH is one of the most potent and selective H1 receptor agonists presently available (12). In the guinea pig ileum, CPH activates H1 receptors with potency and effectiveness comparable to those of HA (12). In the hamster ductus deferens smooth muscle cell line DDTMF-2, CPH is a potent partial H1 receptor agonist (13). Fig. 1 shows the structural formulae of HA and CPH. CPH activates H1 receptors with potency and effectiveness comparable to those of HA (12). In the hamster ductus deferens smooth muscle cell line DDTMF-2, CPH is a potent partial H1 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 H1 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 H1 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 G1 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°C. 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 [35S]GTPγS (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, 16–20).

**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 μg/ml) or its carrier (control) was added to cell cultures 24 hr before experiments or membrane preparation. Under these conditions, >98% of G1 protein α subunits were ADP-riboylated (4).

**Measurement of [Ca2+](i) and Mn2+ influx.** [Ca2+]i was determined using the fluorescent dye fura-2, as described (2). Fluorescence of HL-60 cells (1.0 × 106 cells in 2 ml) was determined at 37°C, with constant stirring at 1000 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 CaCl2. Desensitization of rises in [Ca2+]i, was determined according to the protocol described by Schwaner et al. (17). Mn2+ 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 μl) contained 30 μg 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°C and stopped by rapid filtration. Non-specific binding was determined in the presence of 10 μM fMLP and was <10% of total binding.

**Assay for phosphoinositide degradation.** Phosphoinositides in HL-60 cells were labeled by incubation with 2–5 μCi/ml myo-[3H]inositol for 48 hr (2). HL-60 cells (1 × 106 cells in 200 μl) were suspended in buffer and were exposed to CPH (100 μM) or solvent (control) for 30 sec at 37°C (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 × 106 cells/ml in culture medium and were incubated with 25–50 μCi/ml [9,10-3H]oleic acid for 16–20 hr. Labeled cells were centrifuged at 250 × g for 10 min at 20°C and were resuspended at 0.5 × 106 cells/ml in a buffer consisting of 125 mM NaCl, 0.7 mM MgCl2, 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 × 106 cells/ml. Reaction mixtures (50 μl) contained 0.5–1.0 × 106 cells in the aforementioned buffer supplemented with CaCl2 (free Ca2+ concentration, 1 mM) and were preincubated for 15 min at 37°C. Reactions were initiated by the simultaneous addition of CB (1 μg/ml), 0.5% (v/v) ethanol, and stimuli or solvent (control). Reactions were terminated by the addition of 150 μl of CHCl3/CH3OH/concentrated acetic acid (100:200:4, v/v/v). Phase separation was achieved by addition of 50 μl of CHCl3 and 50 μl of water. Samples were centrifuged at 3000 × g for 5 min and 1 ml of supernatant was transferred to a scintillation vial. For labeling of phosphoinositides, HL-60 cells were labeled with 25–50 μCi/ml [3H]fMLP (3 nM) and CPH at various concentrations or solvent (control). Reactions were conducted for 30 min at 25°C and stopped by rapid filtration. Non-specific binding was determined in the presence of 10 μM fMLP and was <10% of total binding.

**Protein Activation by CPH**

CPH and HA at various concentrations or solvent (control) were added to HL-60 cells. PTX had no effect on basal [Ca2+]i in HL-60 cells. CPH- and HA-induced rises in [Ca2+]i were assessed as described (18). Thereafter, cells were harvested and loaded with fura-2/ace-toxymethyl ester, and the effects of CPH and HA at various concentrations on [Ca2+]i were assessed as described in Experimental Procedures.

**Fig. 1.** Concentration-response curves for CPH- and HA-induced rises in [Ca2+]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/ace-toxymethyl ester, and the effects of CPH and HA at various concentrations on [Ca2+]i were assessed as described in Experimental Procedures. Control cells; PTX-treated cells. PTX had no effect on basal [Ca2+]i in HL-60 cells.
30 min at 4°C. The upper phase was removed, and 50 μl of the organic phase were spotted onto Whatman LKGD 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 CH3OH/1 M HCl (20:1, v/v), and radioactivity was determined in a liquid scintillation counter.

**Assay for tyrosine phosphorylation.** HL-60 cells (1 × 10⁶ cells in 40 μl) were suspended in buffer at 37°C and were incubated for 3 min in the absence or presence of CB (1 μg/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 O₂⁻ formation in HL-60 cells.** O₂⁻ formation was monitored at 550 nm by continuous measurement of ferricytochrome c reduction inhibited by superoxide dismutase, using an UV-810 dual-beam spectrophotometer (Kontron, Eching, Germany) (11). HL-60 cells (2.5 × 10⁶ cells in 500 μl) were incubated for 3 min at 37°C in the absence or presence of CB (1 μg/ml) before the addition of stimuli.

**Assays for the release of β-glucuronidase and lactate dehydrogenase.** Enzyme release was determined as described (4). HL-60 cells (5 × 10⁶ cells in 500 μl) were suspended in buffer at 37°C and were incubated for 5 min in the presence of CB (1 μg/ml) before exposure to stimuli for 10 min. Stopping of reactions and determination of the activities of β-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 μl) contained HL-60 membranes (3–7.0 μg of protein/tube), 0.5 μM [γ-³²P]GTP (0.1 μCi/tube), 0.5 mM MgCl₂, 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% (v/v) bovine serum albumin in 50 mM triethanolamine-HCl, pH 7.4, with substances at various concentrations. Reactions were conducted for 15 min at 37°C. Low affinity GTPase activity was determined in the presence of GTP (50 μM) and amounted to <5% of total GTPase activity. The determination of GTP hydrolysis in N-ethylmaleimide-treated HL-60 membranes was performed as described (9).

**GTP·S binding assay.** [³²P]GTP·S binding was assayed according to the method of Gierschik et al. (22), with modifications. In brief, reaction mixtures (100 μl) contained HL-60 membranes (3.0–5.0 μg of protein/tube), 0.4 mM [³²P]GTP·S, 5 mM MgCl₂, 1 mM EDTA, 150 mM NaCl, 0.5 μM GDP, 1 mM dithiothreitol, and 0.2% (v/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°C 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 MgCl₂, 1 mM EDTA, and 50 mM triethanolamine-HCl, pH 7.4. Non-specific binding was determined in the presence of 10 μM GTP·S and was <1% of total binding.

**Assay for photolabeling of membrane proteins.** HL-60 membranes (50 μg of protein in 60 μl) were incubated at 30°C in a buffer consisting of 0.1 mM EDTA, 5 mM MgCl₂, 1 mM benzamidine, 10 μM 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 [α-³²P]GTP azidoanilide (1 μCi/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 μl) contained membranes from about 1 × 10⁶ HL-60 cells, 3 μM [³²P]NAD (1 μCi/tube), 2.5 mM MgCl₂, 1 mM ATP, 0.2% (v/v) bovine serum albumin, 2 μg of activated CTX, and 0.1 M potassium phosphate, pH 7.4, with various substances. Reactions were conducted for 60 min at 30°C (9).

**Miscellaneous methods.** Cell viability was assayed by trypan blue dye exclusion (21). Protein was determined according to the method of Lowry et al. (23). [γ-³²P]GTP was prepared as described (24). [α-³²P]GTP azidoanilide was synthesized according to the method of Offermanns et al. (19). [³²P]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). Actinoluminographs 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 ± standard deviations of assay quadruplicates. Similar results were obtained in at least three independent experiments. Data shown in Table 3 are the means ± 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 [Ca²⁺], in HL-60 cells are shown in Fig. 1. HA increased [Ca²⁺], with an EC₅₀ of 5 μM and a maximum at 30–100 μM. In PTX-treated cells, the stimulatory effects of HA on [Ca²⁺], were partially inhibited. CPH increased [Ca²⁺], with an EC₅₀ of about 50 μM and a maximum at 75–100 μM. At a maximally effective concentration, CPH was by approximately 45% less effective than HA in increasing [Ca²⁺]. Treatment of HL-60 cells with PTX abolished the rises in [Ca²⁺], caused by CPH.

The effects of the H₁ receptor antagonists clemastine, chlorpheniramine, and diphenhydramine (10, 11), of the H₂ receptor antagonists cimetidine and famotidine (10, 26), and of the dual H₁/H₂ receptor antagonist impromidine (2, 10) on CPH- and HA-induced rises in [Ca²⁺], in HL-60 cells are summarized in Table 1. H₁ antagonist clemastine, chlorpheniramine, diphenhydramine, cimetidine, and famotidine was 10 μM, and that of impromidine was 100 μM. HA receptor antagonists by themselves had no effect on [Ca²⁺],.

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

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>Effects of HA receptor antagonists on HA- and CPH-induced rises in Ca²⁺, in HL-60 cells</strong></td>
</tr>
<tr>
<td><strong>Addition</strong></td>
</tr>
<tr>
<td><strong>HA</strong></td>
</tr>
<tr>
<td>Solvent (control)</td>
</tr>
<tr>
<td>Clemastine</td>
</tr>
<tr>
<td>Chlorpheniramine</td>
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<tr>
<td>Diphenhydramine</td>
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<tr>
<td>Cimetidine</td>
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<tr>
<td>Famotidine</td>
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<tr>
<td>Impromidine</td>
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HL-60 membranes. fMLP binding in HL-60 membranes amounted to 610 ± 21 fmol/mg of protein (mean ± standard deviation, four experiments). CPH (10 μM to 1 mM) had no inhibitory effect on fMLP binding (data not shown).

Time courses of the stimulatory effects of CPH on [Ca2+]i are depicted in Fig. 2. In the presence of extracellular Ca2+, CPH rapidly increased [Ca2+]i, to a peak that declined to a sustained plateau above basal values within 2 min. In the absence of extracellular Ca2+, the magnitude of the CPH-induced rise in [Ca2+]i was greatly diminished. The nonselective cation channel blocker SK&F 96365 [1-(α-[3-(4-methoxy-phenyl)]propoxy)-4-methoxyphenethyl]-1H-imidazole hydrochloride] (30 μM) (3) reduced the stimulatory effect of CPH on [Ca2+]i in the presence of extracellular Ca2+ (see Fig. 2) but not in its absence (data not shown). Fig. 2 also shows the effect of CPH on Mn2+ influx. At excitation wavelengths of 340 nm and 360 nm, CPH substantially increased the rate of basal Mn2+ influx. At the Ca2+-sensitive excitation wavelength of 340 nm, an additional transient increase in fluorescence was evident. All of these findings indicate that CPH activated both Ca2+ mobilization from intracellular stores and Ca2+ influx through nonselective cation channels (1, 3).

Because a part of the CPH-induced rise in [Ca2+]i was attributable to Ca2+ mobilization from intracellular stores (see Fig. 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 ± 38 dpm, 695 ± 37 dpm, and 168 ± 37 dpm, respectively (means ± standard deviations, three experiments). CPH (100 μM) increased these values to 924 ± 58 dpm (p < 0.05), 1056 ± 24 dpm (p < 0.05), and 234 ± 24 dpm (p < 0.05), respectively (means ± standard deviations, three experiments).

Desensitization of CPH- and HA-induced increases in [Ca2+]i, was studied (Fig. 3). HL-60 cells that had been exposed to HA (100 μM) did not undergo a second rise in [Ca2+]i when rechallenged with HA (100 μM or 1 mM; data shown only for 100 μM), i.e., the effect of HA was desensitized in a homologous manner. In contrast, the CPH-induced rises in [Ca2+]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 μM) 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 mM) was about 40% less effective than fMLP (10 μM) 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 phosphorylation were less effective than those of fMLP.
phosphorylation were already declining. CB by itself did not significantly stimulate tyrosine phosphorylation.

The effects of CPH and fMLP on O$_2^-$ formation were compared. fMLP (1 μM) rapidly activated O$_2^-$ formation, which ceased after about 5 min (Fig. 5A). CB potentiated the stimulatory effect of fMLP. CPH (1 mM) did not activate O$_2^-$ formation in the absence of CB. However, in its presence CPH (1 mM) activated O$_2^-$ 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$_2^-$ formation in the presence of CB. CPH activated O$_2^-$ formation with an EC$_{50}$ 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$_2^-$ formation (data not shown). In PTX-treated cells, CPH did not activate O$_2^-$ 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$_{50}$ of 15 nM and a maximum at 0.1–1.0 μM. CPH activated β-glucuronidase release with an EC$_{50}$ 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$_m$ of basal high affinity GTP hydrolysis 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$_{max}$ of GTP hydrolysis without affecting the K$_m$ (data not shown), indicating that the substance increased the catalytic rate of GTP turnover. CPH activated high affinity 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 GTPase and GTPγS binding.

N-Ethylmaleimide alkylates G 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-ethylemaleimide 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-ethylemaleimide-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 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).

**TABLE 2**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Solvent (basal)</th>
<th>CPH</th>
<th>fMLP</th>
</tr>
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<tr>
<td></td>
<td>dpm</td>
<td>dpm</td>
<td>dpm</td>
</tr>
<tr>
<td>Solvent (basal)</td>
<td>1429 ± 98</td>
<td>1428 ± 106</td>
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</tr>
<tr>
<td>CPH</td>
<td>1716 ± 101*</td>
<td>1354 ± 156*</td>
<td></td>
</tr>
<tr>
<td>fMLP</td>
<td>3546 ± 74*</td>
<td>1481 ± 68*</td>
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*p < 0.05.

*Not significant.

**TABLE 3**

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<tr>
<th>Experimental conditions</th>
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<tr>
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<tr>
<td>Solvent (control), 1 min</td>
<td>1.05 ± 0.07</td>
</tr>
<tr>
<td>fMLP, 1 min</td>
<td>1.85 ± 0.15</td>
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<tr>
<td>fMLP + CB, 1 min</td>
<td>3.20 ± 0.31</td>
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<tr>
<td>CB, 1 min</td>
<td>1.13 ± 0.08</td>
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<tr>
<td>CPH, 1 min</td>
<td>1.50 ± 0.13</td>
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<tr>
<td>CPH + CB, 1 min</td>
<td>3.07 ± 0.34</td>
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<tr>
<td>Solvent (control), 3 min</td>
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<td>CPH, 3 min</td>
<td>1.27 ± 0.06</td>
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<tr>
<td>CPH + CB, 3 min</td>
<td>2.03 ± 0.19</td>
</tr>
<tr>
<td>CB, 3 min</td>
<td>1.08 ± 0.09</td>
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10 9 8 7 6
fMLP (-log N)

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 μg/ml). □, β-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 protein α subunits in HL-60 membranes (see Fig. 8B).

Discussion

To clarify the functional relevance of H1 receptors in dibutyryl-cAMP-differentiated HL-60 cells (2), we studied the effects of CPH, one of the most potent and selective H1 receptor agonists presently available (12, 13), in these cells. We found that CPH, similarly to HA, activates phospholipase C, Ca2+ mobilization, and nonselective cation channels (see Figs. 1–3) (2). Additionally, HA and CPH are less effective than fMLP in inducing increases in [Ca2+], (see Fig. 1) (2). In view of our original assumption that CPH is an H1 receptor agonist not only in the guinea pig ileum and in DDT1MF-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 [Ca2+]i were abolished by PTX, whereas those of HA were only partially PTX sensitive (see Fig. 1) (2). These data indicate that only G proteins are involved in the signal transduction pathway activated by CPH. Additionally, in the guinea pig ileum and in DDT1MF-2 cells CPH is a similarly potent H1 receptor agonist, compared with HA, whereas in HL-60 cells CPH is approximately 10-fold less potent than HA in increasing [Ca2+]i (see Fig. 1) (2, 12, 13). Moreover, there are differences in the kinetics of HA- and CPH-induced increases in [Ca2+]i, (see Figs. 2 and 3) (2). Other differences between HA and CPH were observed with respect to activation of tyrosine phosphorylation, O2− 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 [Ca2+]i, O2− formation, and GTPase were completely resistant to inhibition by various HA receptor antagonists (see Table 1). Finally, the CPH-induced rises in [Ca2+]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 H1 receptor agonist in HL-60 cells although these cells express functional H1 receptors (see Table 1) (2) and although CPH has been shown to be a potent H1 receptor agonist in at least two systems (12, 13)? An explanation for these discrepancies may be that there are different H1 receptor reserves in the systems studied. Specifically, the H1 receptor reserve in DDT1MF-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 H1 receptor agonist (13). Possibly, the H1 receptor reserve in HL-60 cells is
TABLE 4
Effect of PTX on stimulation of GTP hydrolysis and GTP-S binding caused by CPH and fMLP in HL-60 membranes

<table>
<thead>
<tr>
<th>Addition</th>
<th>GTP hydrolysis (pmol/mg/min)</th>
<th>GTP-S binding (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PTX</td>
</tr>
<tr>
<td></td>
<td>17.5 ± 0.7</td>
<td>10.3 ± 0.2</td>
</tr>
<tr>
<td>Solvent (basal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPH</td>
<td>30.1 ± 1.3</td>
<td>9.0 ± 0.5</td>
</tr>
<tr>
<td>fMLP</td>
<td>43.1 ± 0.6</td>
<td>10.8 ± 0.3</td>
</tr>
</tbody>
</table>

Even lower than that in DDT;MP-2 cells, so that the expected agonistic effect of CPH at H1 receptors in HL-60 cells is too small to be detected. Thus, instead of activating H1 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 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).

Specifically, CPH also 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 proteins are higher than those needed for stimulation of H1 receptors (see Figs. 1 and 5–7) (12, 13).

fMLP is about 5-fold more potent in increasing [Ca2+]i, than in activating O2 formation and azurophilic granule release (1, 2, 4, 5). Two formyl peptide receptor subtypes have been identified at the cDNA level in dibutylcAMP-differentiated HL-60 cells (33), but it is still unknown, inasmuch as different formyl peptide receptor subtypes are responsible for mediating rises in [Ca2+]i, on one hand and activation of O2 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, proteins, rather than being due to activation of different receptor subtypes. If stimulation of increases in [Ca²⁺], required a smaller number of activated G, proteins than did stimulation of O₂⁻ 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 O₂⁻ formation caused by fMLP (8, 34). We found that CPH substantially activated tyrosine phosphorylation in the absence of CB but failed to activate O₂⁻ 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 O₂⁻ 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 O₂⁻ formation and that activation of additional effector systems, e.g., phospholipase D, is required (35).

In conclusion, we have shown that CPH activates G, 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, proteins directly. In addition, the structure-activity relationships of 2-substituted HA derivatives with respect to G, protein transduction processes.

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Send reprint requests to: Roland Seifert, Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69-73, D-14195 Berlin, Germany.