Inhibition of H₂ Histamine Receptor-Mediated Cation Channel Opening by Protein Kinase C in Human Promyelocytic Cells

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Histamine, through H2 receptors, triggers a prominent rise in intracellular free Ca2+ concentration ([Ca2+]i) in addition to an elevation of cAMP level in HL-60 promyelocytes. Here we show that the histamine-induced [Ca2+]i rise was due to influx of Ca2+ from the extracellular space, probably through nonselective cation channels, as incubation of the cells with SKF 96365 abolished the histamine-induced [Ca2+]i rise, Na+ influx, and membrane depolarization. The Ca2+ influx was specifically inhibited by pretreatment of the cells with PMA or extracellular ATP with 50% inhibitory concentrations of 0.12 ± 0.03 nM and 185 ± 17 μM, respectively. Western blot analysis of protein kinase C (PKC) isoforms revealed that PMA (≤1 nM) and ATP (300 μM) caused selective translocation of PKC-δ to the particulate/membrane fraction. Costimulation of the cells with histamine and SKF 96365 partially reduced histamine-induced granulocytic differentiation, which was evaluated by looking at the extent of fMet-Leu-Phe-charge, as immediate hypersensitivity reactions, alterations in vascular reactivity, activation of smooth muscle contraction, regulation of gastrointestinal mobility and gastric acid secretion, as well as in the modification of various leukocyte subset activities (1, 2). Histamine is also involved in the regulation of cell proliferation and differentiation, probably through stimulation of gene expression (3). These various effects of histamine are mediated by histamine receptors on the plasma membrane. Several lines of evidence involving the pharmacological analyses, second messenger responses, and differences in the predicted amino acid sequence of the histamine receptor proteins have led to the classification of histamine receptors into at least three subclasses, H1, H2, and H3 (4–6). Histamine H1 receptors are known to be coupled to the phosphatidylinositol hydrolysis pathway, where histamine binding results in the mobilization of intracellular Ca2+ ([Ca2+]i), whereas H2 receptors are coupled to adenylly cyclase, causing increases in intracellular cAMP. H3 receptors are negatively involved in the release of histamine and neurotransmitter via probably the inhibition of adenyl cyclase, activation of potassium channels, and inhibition of voltage-sensitive Ca2+ channels (7).

In human HL-60 promyelocytic leukemia cells, histamine treatment induces differentiation of the cells toward neutrophil-like cells (8, 9). The effect of histamine is known to be mediated by cAMP, which is produced upon activation of H2 receptors functionally coupled to adenyl cyclase through cholera toxin-sensitive Gs proteins (10). However, many recent studies have reported that histamine itself also elevates [Ca2+]i in an H2 receptor-dependent manner (11, 12); however, the mechanism and its physiological function are not yet completely understood for HL-60 cells. It seemed unlikely that histamine-mediated cAMP production was responsible for the rise in [Ca2+]i, inasmuch as forskolin or the cell-permeable cAMP analogs dibutyryl cAMP and 8-bromo-cAMP failed to increase [Ca2+]i, and had no effect on histamine-induced mobilization of Ca2+ (13). In the present study we found that the H2 receptor-mediated rises in [Ca2+]i, in HL-60 cells were mediated exclusively through nonselective cation channels, and that the channel opening was negatively regulated by treatment with ATP or PMA via specific activation of protein kinase C (PKC) isoform. This regulatory effect of PKC on histamine responses provides an example of the importance of cross-communication between receptors under physiological conditions. Finally, we provide evidence that the histamine-induced [Ca2+]i rise is involved in granulocytic differentiation.

\[ \text{Materials and Methods} \]

**Materials**

ATP, UTP, 3'-O-(4-benzoyl)benzoyl ATP (BzATP), dibutryl cAMP, 8-bromo-cAMP, iMLP, thapsigargin, sulfipyrazone, EGTA, EDTA, Trizma base, TCA, PMA, and inositol 1,4,5-trisphosphate (IP3) were purchased from Sigma (St. Louis, MO). [3H]IP3 and [3H]adenine were obtained from NEN Life Science Products (Boston, MA). [3H]adenine was obtained from NEN Life Science Products (Boston, MA). [3H]adenine was obtained from NEN Life Science Products (Boston, MA). [3H]adenine was obtained from NEN Life Science Products (Boston, MA). [3H]adenine was obtained from NEN Life Science Products (Boston, MA). 96365, and 6-[2-(4-imidazo-2(4-trifluoromethyl)phenyl)heptane-carboxamidemaleate (HTMT) were purchased from BIOMOL (Plymouth Meeting, PA). Histamine, dimaprit, ranitidine, triploridine, thioramide maleate, GF 109203X, and Ro 20–1724 were obtained from.
Research Biochemicals (Natick, MA). H89 was purchased from Seikagaku (Tokyo, Japan). Fura 2-penta-acetoxymethylene (fura 2-AM), sodium-binding benzofuran isophthalate tetra-acetoxymethyl ester (SBFI/AM), 2',7'-dichlorofluorescein diacetate (DCFH-DA), and bis-oxycon-DisBAC2(3) purchased from Molecular Probes (Eugene, OR).

Cell culture
Human promyelocytic leukemia HL-60 cells were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 20% heat-inactivated bovine calf serum (HyClone, Logan, UT) plus 1% (v/v) penicillin-streptomycin (Life Technologies) under a humidified atmosphere of 5% CO₂ at 37°C. Fresh medium was added to culture flasks every 2 days, and cells were subcultured once a week.

Measurement of [Ca²⁺]

The level of [Ca²⁺], was measured using fura 2-AM as previously described (14). Briefly, cells suspension were incubated in fresh serum-free RPMI 1640 medium with 3 μM fura 2-AM at 37°C for 40 min under continuous stirring. Thereafter, the cells were resuspended in Locke’s solution of the following composition: 154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, and 5 mM HEPES buffer adjusted to pH 7.4. In the Ca²⁺-free Locke’s solution, CaCl₂ was omitted, and 100 μM EGTA was included. Sulfonpyrazone (250 μM) was added to all solutions to prevent dye leakage (15). Changes in fluorescence ratios were measured at the dual excitation wavelengths of 340 and 380 nm, and the emission wavelength of 500 nm. [Ca²⁺] was calculated using the equation: 

\[ [Ca^{2+}]_{i} = K_{d}(R_{\text{max}} - R_{\text{min}})(R_{\text{min}} - R)/(S_{2} - S_{1}) \]

where \( R_{\text{min}} \) and \( R_{\text{max}} \) are the ratios obtained when fura 2 is saturated with Ca²⁺ and when EGTA is used to remove Ca²⁺, respectively. To obtain \( R_{\text{min}} \) and \( R_{\text{max}} \), the fluorescence ratios of the cell suspension were measured successively at final concentrations of 4 mM EGTA, 30 mM Trizma base, and 0.1% Triton X-100, and then at a final concentration of 4 mM CaCl₂, S₂, and S₁ are the proportionality coefficients of Ca²⁺-free fura 2 and saturated fura 2, respectively. Calibration of the fluorescence signal in term of [Ca²⁺] was performed according to the method described by Grynkiewicz et al. (16).

Measurement of intracellular Na⁺ level
The level of intracellular Na⁺ was determined by use of SBFI/AM, which is a fluorescence sodium indicator (17). Cells were harvested and incubated in serum-free RPMI 1640 medium with 15 μM SBFI/AM, 0.2% pluronic acid, and 250 μM sulfonpyrazone at 37°C for 90 min under continuous stirring. Then the cells were washed with serum-free RPMI 1640 solution with 250 μM sulfonpyrazone. Before measurement, a small aliquot of the cell suspension was withdrawn for fluorescence ratio measurement, 10% of the cell suspension was added for assay, centrifuged, and, after the supernatant was removed, resuspended in Locke’s solution. For these experiments, the increase in cytosolic Na⁺ was measured as an increase in the fluorescence ratio determined at the dual excitation wavelengths of 340 and 380 nm and the emission wavelength of 520 nm at 37°C. Because the calibrations of the obtained fluorescence ratios for Na⁺ concentrations were not absolute (18), we expressed our results as fluorescence ratios.

Measurement of [H³]cAMP
Intracellular cAMP generation was determined by [H³]cAMP competition assay in binding to cAMP binding protein as described previously by Park et al. (19) with some modification. To determine the cAMP production induced by histamine or ATP analogs, the HL-60 cells were stimulated with agonists for 20 min in the presence of the phosphodiesterease inhibitor Ro 2–1724 (5 μM), and the reaction was quickly terminated by three repeated cycles of freezing and thawing. The samples were then centrifuged at 2500 x g for 5 min at 4°C. The cAMP assay is based on the competition between [H³]labeled cAMP and unlabeled cAMP present in the sample for binding to a crude cAMP binding protein prepared from bovine adrenal cortex according to the method of Brown et al. (20).

Bound [H³]cAMP was adsorbed onto charcoal and removed by centrifugation. Bound [H³]cAMP in the supernatant was then determined by liquid scintillation counting. Each sample was incubated with 50 μl [H³]labeled cAMP (5 μCi) and 100 μl binding protein for 2 h at 4°C. Separation of protein-bound cAMP from unbound cAMP was achieved by adsorption of free cAMP onto charcoal (100 μl), followed by centrifugation at 12,000 x g at 4°C. Protein-bound cAMP in the sample was determined based on a standard curve and expressed as picomoles per number of cells.

Measurement of IP₃

The IP₃ concentration in the cells was determined by [H³]IP₃ competition assay in binding to IP₃ binding protein (21). To determine IP₃ production, the HL-60 cells were stimulated with agonists for specific periods of time, and the reaction was terminated by aspirating the medium off the cells followed by addition of 0.3 ml ice-cold 15% (v/v) TCA containing 10 mM EGTA. The samples were then centrifuged for 5 min at 4°C. The extraction was transferred to an Eppendorf tube, and TCA was removed by evaporation with diethyl ether four times. Finally, the extract was neutralized with 200 μl Trizma base, and its pH was adjusted to 7.4. Twenty microliters of the cell extract was added to 20 μl of the assay buffer (0.1 M tris(hydroxymethyl)-aminomethane buffer containing 4 mM EDTA and 0.01 mg/ml BSA) and 20 μl [H³]IP₃, (0.1 μCi/ml). Then 0.1 ml solution containing the binding protein was added, and the mixture was incubated for 15 min on ice and centrifuged at 2000 x g for 5 min. The pellet was resuspended in 100 μl water, and 1 ml scintillation cocktail was added to measure the radioactivity. The IP₃ concentration in the sample was determined based on a standard curve and expressed as picomoles per milligram protein. The IP₃ binding protein was prepared from bovine adrenal cortex according to the method of Challisi et al. (22).

Measurement of membrane potential with bisoxonol

Changes in membrane potential were monitored using a fluorescent potential-sensitive anionic dye, bisoxonol DisBAC2(3), as reported by Barry and Cheek (23) with minor modifications. Briefly, HL-60 cells, after preincubation for 1 h at 37°C in incubation buffer (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM Na₂HPO₄, 5.5 mM glucose, 5 mM NaHCO₃, 20 mM HEPES, and 200 μM EGTA, pH 7.4), were washed and resuspended with the above incubation buffer at a density of 1.5 x 10⁵ cells/ml. The cells were then incubated with 300 nM bisoxonol for 7 min at 37°C before the addition of stimuli. Fluorescence was measured at the excitation wavelength of 540 nm and the emission wavelength of 580 nm.

Measurement of intracellular reactive oxygen species production

The production of intracellular reactive oxygen species such as superoxide and hydrogen peroxide was determined by a method based on the changes in fluorescence of DCF-DA, an oxidation-sensitive fluorescence probe, following a previously published procedure (24, 25). Briefly, the cell suspension was incubated in fresh serum-free RPMI 1640 medium with 2 μM DCF-DA at 37°C for 40 min under continuous stirring. The loaded cells were then washed twice with Locke’s solution. Then 2 x 10⁵ cells were placed into a cuvette in a thermostatically controlled cell holder at 37°C and continuously stirred. Fluorescence was measured when excited at 488 nm, and emission was recorded at 530 nm. The change in fluorescence intensity was monitored.

Cell fractionation and Western blot analysis of PKC isoforms

To separate the cell material into soluble/cytosolic and particulate/membrane fractions, the HL-60 cells were suspended in buffer A (20 mM Tris-HCl, pH 7.5, containing 250 mM sucrose, 2 mM EGTA, 2 mM EDTA, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF, and 10 μg/ml apro- tin). The cells were sonicated for 5 s each time and centrifuged at 100,000 x g for 1 h. The supernatant was saved as the cytosolic fraction. The pellet was then extracted with buffer B (20 mM Tris-HCl, pH 7.5, containing 1% SDS, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and protease inhibitors as described above for buffer A). Following centrifugation the supernatant was saved as the particulate/membrane fraction (26).

Proteins (30 μg) from the cytosolic and particulate membrane fractions were separated by electrophoresis on an 8% polyacrylamide gel containing 0.1% SDS and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 3% nonfat dry milk in Tris-buffered saline. PKC isoforms were detected with isoform-specific anti-PKC mAbs against α, βI, ε, and ζ isoforms (Transduction Laboratories, Lexington, KY). The blots were developed using a peroxidase-conjugated secondary Ab, either goat anti-rabbit or anti-mouse IgG, using the ECL system (Amerham, Arlington Heights, IL).

Analysis of data

Quantitative data are expressed as the mean ± SEM. Comparison between groups was analyzed using Student’s unpaired t test, and differences were considered significant when the degree of confidence in the significance was 95% or better (p < 0.05). Calculation of the 50% effective concentration was performed with the ALLFIT program (27).
Results

Nonselective cation channels activated by the H₂ receptor

Exposure of HL-60 cells to histamine (100 μM) results in an increase in [Ca²⁺], in the presence of 2.2 mM extracellular CaCl₂ (Fig. 1A). The specific H₂ agonist dimaprit (100 μM) also increased [Ca²⁺], with an effectiveness comparable to that of histamine. However, removal of extracellular Ca²⁺ completely abolished the histamine- and dimaprit-induced rises (Fig. 1A, right panel), indicating that the histamine-induced increase in [Ca²⁺], is mediated by Ca²⁺ influx from the extracellular medium. As shown in Fig. 1B, histamine and dimaprit increased [Ca²⁺], in a concentration-dependent manner with 50% effective concentrations of 10 ± 3 and 17 ± 5 μM, respectively. Moreover, stimulation of the cells with the maximal concentration of dimaprit (100 μM) resulted in complete inhibition of the subsequent histamine-induced Ca²⁺ mobilization (data not shown), suggesting that the [Ca²⁺] increase induced by histamine or dimaprit was mediated through a common receptor. The H₁ agonist HTMT and the H₃ agonist R(-)-α-methylhistamine had little effect on [Ca²⁺], suggesting that the histamine-mediated Ca²⁺ influx occurred exclusively through H₂ receptors in these cells. This was further confirmed in the experiment using selective antagonists. Fig. 1C shows that addition of the H₂ antagonist triploridine and the H₃ antagonist thioperamide-maleate did not affect the histamine-mediated [Ca²⁺], rise up to a 100-μM concentration, whereas the H₂ receptor antagonist ranitidine concentration-dependently inhibited the response with a 50% inhibitory concentration of 0.6 ± 0.2 μM. Because Ca²⁺ mobilization from intracellular stores is mediated by IP₃, we examined whether histamine treatment produced IP₃ in HL-60 cells. Table I shows that histamine and selective agonists had no effect on the generation of IP₃, whereas treatment with ATP and UTP significantly increased IP₃ contents. Therefore, the data clearly suggest that the histamine-induced [Ca²⁺], rise must be due to Ca²⁺ influx from the extracellular medium following the stimulation of H₂ receptors.

SKF 96365 blocks nonselective cation channels and inhibits receptor agonist-activated Ca²⁺ influx in neutrophils and HL-60 cells (28, 29). In the presence of extracellular Ca²⁺ we found that treatment with 10 μM SKF 96365 inhibited the subsequent histamine-induced [Ca²⁺], rise by ~85–90% (Fig. 2A). SKF 96365 also inhibited the ATP- and thapsigargin-induced [Ca²⁺], rises by 20–30% (Fig. 2A). However, SKF 96365 did not affect the ATP- and thapsigargin-mediated Ca²⁺ mobilization from intracellular stores (Fig. 2B), indicating that the decreased responses were due to the inhibition of store-operated Ca²⁺ entry from extracellular space. As shown in Fig. 1A, histamine had no effect on Ca²⁺ mobilization and the histamine-mediated Ca²⁺ influx was almost completely blocked by SKF 96365 (Fig. 2B). Fig. 2C shows the concentration-dependent inhibitory effect of SKF 96365 on the histamine- and ATP-induced [Ca²⁺], rise. SKF 96365 at concentrations greater than 30 μM produced complete inhibition of the histamine response, whereas it had a maximal inhibitory effect on the ATP response of about 25%. The results demonstrate that the histamine-induced [Ca²⁺], rise in HL-60 cells is exclusively due to Ca²⁺ influx through SKF 96365-sensitive nonselective cation channels, whereas the ATP-induced [Ca²⁺], rise is caused by Ca²⁺ mobilization from intracellular stores and Ca²⁺ influx from extracellular space through the store-operated Ca²⁺ channels; the latter is also partially sensitive to SKF 96365 (Fig. 2B).

We tested the permeation of Na⁺ ions through nonselective cation channels upon histamine stimulation. Histamine triggered Na⁺ influx in SBIFI-loaded cells (Fig. 3A). However, the addition of ranitidine abolished the histamine-induced response, whereas it

Table I. Effects of histamine on IP₃ generation in HL-60 promyelocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IP₃ Generation (pmol/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>21.5 ± 1.6</td>
</tr>
<tr>
<td>Histamine</td>
<td>22.7 ± 1.2</td>
</tr>
<tr>
<td>HTMT</td>
<td>23.3 ± 2.6</td>
</tr>
<tr>
<td>Dimaprit</td>
<td>22.2 ± 2.8</td>
</tr>
<tr>
<td>R(-)-α-methylhistamine</td>
<td>20.3 ± 3.2</td>
</tr>
<tr>
<td>ATP</td>
<td>73.2 ± 6.5₈</td>
</tr>
<tr>
<td>UTP</td>
<td>65.6 ± 3.7₈</td>
</tr>
<tr>
<td>BzATP</td>
<td>25.3 ± 2.4</td>
</tr>
</tbody>
</table>

₈ p < 0.01, compared with control level.
Through the SKF 96365-sensitive cation channel.

and maximal inhibitions at

inhibited the histamine-mediated depolarization with half-maximal (data not shown). However, incubation of cells with SKF 96365 evoked membrane depolarization comparable to that of histamine.

C$_1$). However, Na$_1$ evoked (Fig. 3). The experiments were conducted more than five times, and typical Ca$_2^+$ transients are presented. B, Fura 2-AM-loaded HL-60 cells were stimulated with 100 µM histamine, 300 µM ATP, or 1 µM thapsigargin initially in the absence of external Ca$_2^+$, and then 3 mM CaCl$_2$ was added to the medium 1 min after histamine and ATP stimulation or 1.5 min after thapsigargin stimulation. C, Concentration-dependent inhibition of histamine- and ATP-mediated [Ca$^{2+}$], rise by SKF 96365. Cells pretreated with various concentrations of SKF 96365 were subsequently stimulated with histamine or ATP. The net increase in [Ca$^{2+}$], is expressed as a percentage of the control (histamine or ATP alone). Each point is the mean ± SEM of four independent experiments.

FIGURE 2. Effect of SKF 96365 on histamine-induced Ca$^{2+}$ mobilization. A, Fura 2-AM-loaded HL-60 cells treated with or without 10 µM SKF 96365 for 10 min were then stimulated with 100 µM histamine, 300 µM ATP, or 1 µM thapsigargin in the presence of extracellular 2.2 mM CaCl$_2$. The experiments were conducted more than five times, and typical Ca$_2^+$ transients are presented. B, Fura 2-AM-loaded HL-60 cells were stimulated with 100 µM histamine, 300 µM ATP, or 1 µM thapsigargin initially in the absence of external Ca$_2^+$, and then 3 mM CaCl$_2$ was added to the medium 1 min after histamine and ATP stimulation or 1.5 min after thapsigargin stimulation.

Negative regulation of histamine-activated nonselective cation channel upon PKC activation

Because it has been shown that histamine H$_2$ receptor-mediated signaling is regulated by various protein kinases (30–32), we examined the involvement of PKC in the modulation of the histamine-mediated [Ca$^{2+}$], in HL-60 cells. We found that treatment of the cells with 100 pM PMA significantly inhibited the histamine-induced cyslosic Ca$^{2+}$ increase (Fig. 5A). The inhibitory effect of PMA on the histamine-induced [Ca$^{2+}$], elevation was concentration dependent, with 50% inhibitory effect and maximum effect at 0.12 ± 0.03 and 1.0 ± 0.3 nM PMA, respectively (Fig. 5B). However, the cAMP generation induced by histamine was only barely influenced at the above concentrations and was maximally (60–65%) blocked at higher concentrations of PMA (≏100 nM). An inactive PMA analog, 4α-PMA, had no inhibitory effect at concentrations up to 100 nM (data not shown). The results thus indicate that the histamine-mediated cation channel activation was inhibited upon PKC activation without any link to the pathway of cAMP generation in HL-60 cells.

Because PKC is activated upon [Ca$^{2+}$], elevation and diaclylglycerol generation elicited by phospholipase C (PLC)-coupled receptor activation, we tested whether activation of the P2 purinoceptor also had an inhibiting effect like that caused by PKC in
HL-60 promyelocytes. Fig. 6A shows that ATP pretreatment inhibited the subsequent histamine-induced \([\text{Ca}^{2+}]_i\) rise. The histamine-induced response was affected by the extent of prior ATP stimulation, because the subsequent response was dependent on the concentration of ATP in the pretreatment. Fig. 6B shows that as the concentration of ATP increased, the subsequent response to histamine decreased, and pretreatment of the cells with a supra-maximal concentration of ATP (1 mM) almost completely inhibited the subsequent histamine-induced \([\text{Ca}^{2+}]_i\) rise. The effect of ATP was also dependent on the length of time elapsed between ATP and histamine stimulation. As the interval between the two stimulations increased, the inhibition decreased (Fig. 6C). To confirm the involvement of PLC-coupled P2 purinoceptors in HL-60 cells, we tested the effects of various nucleotides. Similar to the inhibitory effect caused by ATP were the effects obtained by treatments with ATPγS and UTP, both of which produce IP3 (Fig. 6D). However, BzATP, which elevates \([\text{Ca}^{2+}]_i\) without IP3 generation (Table I), and α,β-methylene ATP, a general P2X1 agonist, had no effect on the histamine response, indicating that the inhibitory effect of extracellular ATP is exclusively mediated through the activation of P2Y2 receptors. It has been known that ATP can trigger cAMP production. However, Table II shows that various stimulations that elevate cAMP did not affect the histamine-induced \([\text{Ca}^{2+}]_i\) rise. Treatment of the cells with the adenylyl cyclase activator forskolin, PGE2, or the cAMP analogs 8-bromo-cAMP and dibutyryl cAMP neither increased nor inhibited the subsequent histamine-mediated \([\text{Ca}^{2+}]_i\) rise.

To find out whether PKC activation was responsible for the inhibiting effect of the P2 receptor, a PKC inhibitor was administered before stimulation with ATP, and the subsequent histamine response was measured. Fig. 7 shows that the addition of 3 μM GF109203X for 10 min before ATP stimulation blocked the inhibitory effect of ATP (300 μM) on the subsequent histamine-induced \([\text{Ca}^{2+}]_i\) rise. In contrast, H89 (10 μM), which is a selective cAMP-dependent protein kinase inhibitor, had no impact on the ATP effect. These data clearly show that the signaling of the histamine receptor can be specifically inhibited by pretreatment with ATP via PKC activation.

**Differential regulation of PKC isoforms by PMA and extracellular ATP**

To investigate the PKC isoforms involved in the regulation of the histamine responses, we determined the isoforms of PKC translocated from cytosol to membranes upon treatment with PMA and ATP using Western blotting analysis with isoform-specific Abs. Each Ab recognized individual PKC isoforms in the lysate of rat brain, which was the positive control (33). The distribution of the expressed PKC isoforms between the soluble/cytosolic and the particulate/membrane fraction was determined by binding of Abs against the PKC-α, -βI, -δ, and -ε isoforms after stimulation of HL-60 cells (Fig. 8A). In untreated cells all PKC isoforms were detected predominantly in the cytosolic fraction. However, treatment of the cells with lower concentration (1 nM) of PMA selectively induced translocation of the cytosolic PKC-δ isoform to the particulate membrane fraction. Higher concentrations of PMA (>10 nM) selectively translocated PKC-ε in addition to PKC-δ, and at concentrations of >100 nM PMA all isoforms of PKC translocated to the membrane. The distribution of the atypical PKC-δ was not affected by treatment with PMA (data not shown). Treatment with ATP (300 μM) also specifically induced translocation of PKC-δ, whereas it had little effect on translocation of the other isoforms of PKC. However, histamine did not affect the translocation of any PKC isoform. Fig. 8B shows the time course of PKC translocation. The translocation of PKC-δ, but not other
types of PKC, was dramatically evoked within 5 min and sustained for up to 20 min after stimulation with ATP (300 μM) and PMA (1 nM). These data suggest that the specific translocation of novel PKC-δ may be involved in regulation of the nonselective channel-mediated Ca\(^{2+}\) influx induced by histamine.

Negative modulation of histamine-induced granulocytic differentiation of HL-60 cells

To assess the functional importance of the H\(_2\) receptor-mediated Ca\(^{2+}\) influx, we looked at its effect on cellular differentiation. Granulocytic differentiation of HL-60 promyelocytes results in increased expression of formyl peptide receptors that can be readily monitored by observing the increased effectiveness of fMLP in inducing a rise in [Ca\(^{2+}\)]\(_i\) (34). The responsiveness of HL-60 cells to fMLP was substantially increased when the cells were induced to differentiate by treatment with 1.25% DMSO, 100 μM histamine, or 100 μM dibutyryl cAMP (Fig. 9A). However, in cells simultaneously treated with histamine and SKF 96365, the fMLP

### Table II. Effect of cAMP pathway on histamine-induced [Ca\(^{2+}\)]\(_i\) rise\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Ca(^{2+})](_i), (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>54 ± 3(^b)</td>
</tr>
<tr>
<td>Dibutyl-cAMP</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>8-Bromo-cAMP</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>Forskolin</td>
<td>108 ± 9</td>
</tr>
<tr>
<td>Prostaglandin E(_2)</td>
<td>84 ± 8</td>
</tr>
</tbody>
</table>

\(^a\)Fura 2-AM-loaded cells preincubated with 300 μM ATP, 1 mM dibutyl-cAMP, 1 mM 8-bromo-cAMP, 100 μM prostaglandin E\(_2\), and 10 μM forskolin for 5 min were stimulated with 100 μM histamine. Net increase in [Ca\(^{2+}\)]\(_i\) is expressed as percent of the level obtained by treatment with histamine alone. The experiments were carried out three times in triplicate and data are the means ± SEM.

\(^b\)\(p < 0.01\), compared with control.
response in eliciting a \([\text{Ca}^{2+}]\) rise was significantly decreased, whereas inclusion of SKF 96365 had no effect on dibutyryl cAMP-mediated differentiation of HL-60 cells. Consistent with the \([\text{Ca}^{2+}]\) response, incubation of the cells with histamine together with SKF 96365 also resulted in a decrease in IMLP-stimulated IP\(_3\) generation (Fig. 9B). However, incubation with SKF 96365 had no effect on histamine-mediated cAMP generation (Fig. 9C), suggesting that H\(_2\) receptor-induced cAMP signaling to cellular differentiation was not affected by SKF 96365 treatment. The \([\text{Ca}^{2+}]\) rise on histamine-mediated differentiation of HL-60 cells was also detected by monitoring the production of reactive oxygen species. Fig. 9D shows that PMA evoked the generation of superoxide in DMSO-, histamine-, and dibutyryl cAMP-treated cells. However, simultaneous treatment of the cells with histamine and SKF 96365 resulted in a lesser elevation of fluorescence in cells loaded with DCFH-DA, whereas in cells treated with dibutyryl cAMP plus SKF 96365 PMA-mediated superoxide generation was not affected by SKF 96365 inclusion. Therefore, the results indicate that the H\(_2\) histamine receptor-mediated granulocytic differentiation could be functionally regulated by the \([\text{Ca}^{2+}]\) influx through H\(_2\) receptor-activated cation channels.

**Discussion**

The present study demonstrates that stimulation of HL-60 promyelocytes with histamine causes an increase in \([\text{Ca}^{2+}]\), through PLC-independent pathways and that the \([\text{Ca}^{2+}]\) rise is negatively regulated by PKC activation in a subtype-selective manner. Furthermore, we found that the H\(_2\) receptor-mediated \([\text{Ca}^{2+}]\), rise is involved in the histamine-mediated granulocytic differentiation of these cells. Thus, the aim of this work was to investigate the possible mechanism of PKC activation and the specific regulation of the H\(_2\) receptor-mediated signal transduction pathways and its contribution to differentiation.

The data presented in this study show that the effect of histamine on the \([\text{Ca}^{2+}]\) increase in HL-60 cells is mediated via H\(_2\) receptors, as had been suggested by several previous studies (11, 12, 35). The H\(_2\) receptor antagonist ranitidine completely inhibited the stimulatory effects of histamine on the \([\text{Ca}^{2+}]\), rise, whereas H\(_1\)- and H\(_2\)-selective antagonists did not have this effect (see Fig. 1). In addition, the H\(_2\) receptor agonist dimaprit increased \([\text{Ca}^{2+}]\), to an extent comparable to that of histamine, whereas the H\(_1\) agonist HTMT and the H\(_3\) agonist \(R(-)-\alpha\)-methylhistamine had little effect. The H\(_2\) receptor-mediated \([\text{Ca}^{2+}]\), increase resulted from \([\text{Ca}^{2+}]\) influx from the extracellular medium and not from \([\text{Ca}^{2+}]\) released from internal stores, because histamine did not induce a \([\text{Ca}^{2+}]\) rise in the absence of extracellular \([\text{Ca}^{2+}]\), and did not stimulate significant IP\(_3\) production (Fig. 1 and Table I). In addition, the experiment to test PKC activation subsequently occurred after activation of the PLC pathway shows that any translocation of PKC isoforms was not detected by the histamine treatment, suggesting that histamine does not apparently activate PLC. Our study also shows that the histamine-mediated increase in \([\text{Ca}^{2+}]\), resulted from the opening of nonselective cation channels, because SKF 96365 treatment could almost completely inhibit the histamine-stimulated \([\text{Ca}^{2+}]\) entry, Na\(^+\) influx, and membrane depolarization. Nevertheless, we cannot exclude the possibility that PLC activation may also be required to activate the nonselective cation channel. Previously, many studies have shown that nonselective cation channels are simultaneously activated upon stimulation of PLC-coupled receptors such as P2 purinoceptors or fMLP receptors in HL-60 cells, probably via a G-protein-dependent pathway (11, 36), but this mechanism has not yet been studied systematically. It seems likely that the activation of nonselective cation channel results from H\(_2\) receptor-coupled PLC activation, although in amounts below our detection limit. There still remains the possibility that a localized increase in IP\(_3\) close to a Ca\(^{2+}\) storage organelle might occur during the H\(_2\) histamine receptor activation and lead to the concomitant opening of nonselective cation channels. It has been known that activation of the H\(_2\) histamine receptor elicits both cAMP production and a rise in \([\text{Ca}^{2+}]\), in various cells. This was confirmed in experiments with a cloned H\(_2\) receptor in
which the direct linkage of a single receptor to both adenyllyl cyclase and PLC via separate GTP-dependent mechanisms was demonstrated (37, 38). Several other reports also showed that a single type of receptor may be associated with more than one G protein and thus lead to multiple intracellular signaling systems, although the mechanism by which different factors may be involved in regulation of the plurality of receptor-mediated signaling remains unknown.

The mechanism involved in the PKC-mediated regulation of the histamine receptor-histamine-mediated signaling is still poorly understood. Several preliminary studies have suggested that the inhibition of the histamine-mediated 
\[\text{Ca}^{2+}\] influx upon PKC activation may not be due to an alteration in the activity of the histamine receptor, because the binding affinity and total binding of \[^{[H]}\text{histamine}\] to the membrane receptor were not affected by short term treatment with PMA (30, 33). Previously, it has been observed that the activation of multiple subtypes of PKC by PMA resulted in phosphorylation of the terminal consensus sequences found in the third intracellular domains of the seven-transmembrane receptor (39). It has been suggested that phosphorylation of these consensus sequences causes a decrease in the receptor’s potency during acute PMA treatment (40). The histamine H2 receptors also contain in the corresponding region five potential consensus phosphorylation sites for PKC (41), although these sites have not yet been studied in a systemic manner to determine whether they actually are targets of that kinase. However, the structural requirements for H2 receptors for cAMP generation and \[\text{Ca}^{2+}\] elevation have not yet been fully elucidated. Recent studies have demonstrated that segments of the second and third intracellular loops containing the consensus sequence and the COOH-terminal tail couple in a differential manner to separate G proteins (2, 3). It has also been reported that in HL-60 cells the activation of nonselective cation channels occurs via G protein and that the intracellular application of a nonhydrolyzable GDP analog blocked the agonist stimulation of nonselective cation channels (36, 42). Therefore, the possibility exists that a selective uncoupling of the histamine receptor from specific G proteins might be induced by acute activation of PKC-\(\delta\) in a manner that does not affect receptor binding to adenyllyl cyclase-linked G proteins.

It has been generally accepted that multiple PKC isoforms are responsible for different specialized physiological processes and that many cell types express multiple PKC isoforms (43). Presently, 11 isoforms of PKC have been identified in mammalian tissue, and they have been divided into four groups based on their mechanism of activation (44). We showed here that from among the multiple PKC isoforms expressed in HL-60 cells, the novel type PKC-\(\delta\) was specifically activated by ATP or low concentrations of PMA (\(\leq 1\) nM), whereas another novel type PKC-\(\epsilon\) was activated by higher concentrations of PMA (\(> 10\) nM), as determined by translocation of cytosolic PKC to the membrane fraction. The concentration-response curve of PMA’s effect on the inhibition of the histamine-induced \[\text{Ca}^{2+}\] response matches the translocation of the novel PKC-\(\delta\). Therefore, the result suggests that PKC-\(\delta\) may be specifically involved in the inhibition of the histamine-mediated \[\text{Ca}^{2+}\] rise when the cells are treated with PMA (\(\leq 1\) nM) or ATP.

HL-60 cells are pluripotent and can differentiate into monocytes or neutrophils depending on the inducer of differentiation. Previous studies have shown that treatment with histamine resulted in differentiation toward neutrophil-like cells and expression of formyl peptide receptors in the cells, probably through the production of intracellular cAMP (8, 45, 46). However, our present study clearly shows that \[\text{Ca}^{2+}\] influx through nonselective cation channels is also involved in the histamine-induced differentiation, in as much as blockage of these channels by SKF 96365 treatment prevented differentiation of the cells. This observation is consistent with previous studies in which \[\text{Ca}^{2+}\] rise, induced by H2 receptor stimulation (11), P2 receptor stimulation (34), or \[\text{Ca}^{2+}\] ionophore (47) could cause differentiation of the cells. In the studies the cytosolic \[\text{Ca}^{2+}\] rise itself plays an important role in induction of granulocytic differentiation as well as sensitization of cells to the differentiating effect of other inducers such as retinoic acid, 1\(\alpha\),25-dihydroxyvitamin D3, and DMSO (48–50). Thus, histamine-mediated \[\text{Ca}^{2+}\] rise might act as an inducer of HL-60 differentiation and/or enhances the cAMP-mediated process of differentiation. At present, the action mechanism by which the \[\text{Ca}^{2+}\] rise induces differentiation of promyelocytes was not yet completely understood. Some studies reported that expression of the proto-oncogene c-myc is dramatically regulated by the transient elevation of \[\text{Ca}^{2+}\] in HL-60 cells (34, 47). In those studies the induction of membrane tyrosine kinase activity also accompanied with a significant reduction of c-myc expression. Therefore, there is a possibility that, as shown in our present studies (Fig. 9), the initial inhibition of histamine receptor-mediated cation channel activation and membrane depolarization might result in the induction of differentiation of HL-60 promyelocytes.

Although a physiological role for ATP in the immune system has not yet been firmly established, the high concentration of ATP stored in bone marrow-derived megakaryocytes and its release upon extracellular stimulation suggest a functional relevance for extracellular nucleotides in the physiology of hemopoietic cells (51). Moreover, it has been shown that P2 purinergic receptors are present on various immature bone marrow-derived cells and are involved in the regulation of the proliferation of hemopoietic stem cells by the release of histamine from mast cells (52, 53). Recently, Seifert et al. (11) also reported that the histamine-mediated \[\text{Ca}^{2+}\] rise plays a role in the cellular differentiation of HL-60 cells.

Therefore, we may yet discover an important physiological relevance in the cross-communication between PLC-coupled receptor and receptor-activated nonselective cation channel opening, displaying distinct biological characteristics. In conclusion, our results show that PMA and a physiological agonist, ATP, can inhibit \[\text{Ca}^{2+}\] influx induced by the natural stimulant histamine by selective activation of PKC-\(\delta\) in HL-60 cells.

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References


