Restrained Adenyl Cyclase in Human Neutrophils: Stimulation of Cyclic Adenosine 3′:5′-Monophosphate Formation and Adenyl Cyclase Activity by Phagocytosis and Prostaglandins

By Viktor Stoic

We observed that prostaglandins (PG) E1, E2, A1, A2, B1, and F2 alpha increase cAMP concentration in resting neutrophils. In phagocytosing cells, an additional enhancement was seen with PG E1 and PG A2. Such a stimulatory effect of PG E1 was noted any time during phagocytosis of latex microparticles (3–18 min). Adrenergic inhibitors, phentolamine and propranolol, did not interfere with the stimulatory action of PG E1 on cAMP level in resting and phagocytosing leukocytes. Both aforementioned inhibitors enhanced cAMP level in resting and phagocytosing neutrophils at 1 mM concentration, and an additional increase was seen in PG E1-stimulated leukocytes. Although phosphodiesterase activity was unchanged by phagocytosis and PG E1 action, adenylate cyclase (AC) activity was significantly higher in neutrophil homogenates after phagocytosis of latex particles. In contrary, contact of human lymphocytes with latex particles revealed a decrease in AC activity. These data indicate that AC-cAMP system is partly in a restrained state in resting neutrophils and can be relieved by phagocytosis.

Recently, the activity of adenyl cyclase (AC) and the formation of cyclic 3′,5′-monophosphate (cAMP) have been extensively studied in human neutrophils. In spite of this, little is known about cAMP production and AC activity in phagocytosing neutrophils. It has been shown that phagocytosis does not induce cAMP formation in a human neutrophil-enriched population; however, an earlier report to the contrary was positive, probably because of contamination of the neutrophil population by other mononuclear leukocytes. On the other hand, it seems that cAMP in leukocytes inhibits random and directed neutrophil motility, iodide accumulation and organification, release of granule enzymes into the phagocytic vacuole, antigenic release of histamine, and phagocytosis of paraffin oil emulsions. It has also been reported that cAMP regulates selective extrusion of lysosomal enzymes in phagocytosing neutrophils and impairs the neutrophil’s ability to kill Candida albicans, although a recent paper by the same authors now casts doubt on the previous conclusions that cAMP regulates these two functions of neutrophils.

Materials and Methods

Human polymorphonuclear leukocytes were obtained from healthy blood donors. The blood was drawn into plastic bags containing acid citrate-dextrose anticoagulant and centrifuged at
3000 rpm for 3.5 min. Plasma was separated from blood cells and the buffy coat obtained after the second centrifugation (4100 rpm for 5 min) was mixed with two volumes of solution containing 0.9% sodium chloride, 3% dextran (General Biochemicals, mol wt 200,000-300,000) and heparin, 3 USP units/ml (Organon, Liquaemin sodium 10, 1000 USP units/ml). The erythrocytes were allowed to sediment for 45 min at room temperature, and the leukocytes were harvested from the supernatant fluid by centrifugation at 600 g for 2 min, and the lysis of erythrocytes was repeated once more. The cells were then washed and suspended in Krebs-Ringer-Tris (KRT), pH 7.4, containing 1.31 mM CaCl$_2$. The polymorphonuclear neutrophils (PMN) were separated from other leukocytes using a modification of Boyum’s method.$^{11}$ The leukocyte suspension in 4 ml of KRT was layered onto 15 ml of water solution of 9% Ficoll (Sigma) and 36% Hypaque (Winthrop), mixed at a ratio of 24:10. Centrifugation was carried out at 15°C for 20 min at 650 g. After the centrifugation, the white blood cell pellet was washed twice and finally resuspended in KRT. The neutrophil specimen was 94%-98% pure.

*Cyclic AMP Estimation*

Approximately 2 x 10$^7$ PMN cells were incubated in triplicate in 3-ml portions of KRT. The incubations were carried out at 37°C in 25-ml Erlenmeyer flasks with gentle shaking (35 strokes per minute). Phagocytosis was induced by latex microparticles (Dow, 0.79 µ in diameter) at a cell:bead ratio of 1:200. Latex was extensively dialyzed against water and KRT before it was used. The phagocytosis was monitored by microscopic examination of leukocyte smears. After the incubation, flasks with cell suspensions were inserted into ice water and the contents transferred into precooled tubes and centrifuged at 5°C for 2 min at 600 g. Supernate was discarded, and the cells were disrupted by sonication in 0.5 ml of 6% trichloroacetic acid (TCA). Cyclic AMP was determined by Gilman’s method$^{12}$ in an aliquot of the ether-extracted TCA supernate.

*Adenyl Cyclase Assay*

The neutrophils for AC assay were incubated with or without particles at 37°C for 15 min and then centrifuged for 2 min at 600 g at 4°C. Phagocytosis was induced by latex microparticles (Dow, 0.79 µ in diameter) or zymosan at cell:bead ratios of 1:200 or 1:5, respectively. The cell homogenates were prepared in an all-glass homogenizator or by sonication. Both types of particles or procedures of cell disintegration revealed the same decreased AC activity in phagocytosing neutrophils.

AC assay was carried out according to the method of Krishna et al.$^{13}$ The incubation mixture in triplicate contained, in a volume of 50 µl, Tris (hydroxymethyl) aminomethane, 21 mM; theophylline, 10 mM; adenosine triphosphate 8-32P or 14C, 1 mM and 1 µCi; MgCl$_2$, 2 mM; phosphoenol pyruvate, 16 mM; albumin 0.04%; pyruvate kinase, 2.5 µg; and approximately 400 µg of enzyme or 6 x 10$^6$ neutrophils. The pH of Tris-theophylline solution was adjusted by HCl to 7.4. The reaction was started by adding the enzyme. Tubes were incubated at 37°C for 10 min, and the reaction was terminated by addition of 50 µl of solution containing ATP, 5 mM, and $^3$H-cAMP, 5 mM, 0.02 µCi. The tubes were immediately immersed into boiling water for 3 min. Radioactive cAMP was separated from ATP and other nucleotides by DOWEX-50-H$^+$ column chromatography and Ba(OH)$_2$-Zn(SO$_4$)$_2$ precipitation. Counting of radioactivity was performed in a Packard liquid scintillation spectrometer, and counts were corrected for recovery of cAMP. Recovery average was 60% ± 10%.

AC assays were performed in triplicate, and the cell blanks in which the enzyme was inactivated by boiling were included in each experiment. Enzyme activity is expressed in pmoles of cAMP formed per milligram of protein per 1 min. Enzyme activity was linear with time (1-10 min) and proportional to the amount of protein (0.1-2.0 mg).

*Phosphodiesterase Assay*

Phosphodiesterase assay was performed in a volume of 50 µl. The incubation solution contained $^3$H-cAMP, 0.1 µCi, 1 mM; MgCl$_2$, 2 mM; albumin, 0.04%; Tris (hydroxymethyl) amino-methane-HCl, 21 mM, pH 7.4. Aminophylline solution, when used, was mixed with Tris buffer, and pH was adjusted by HCl to 7.4. The reaction was started by addition of the leukocyte homogenate, approximately 130 µg of enzyme, or 2 x 10$^6$ neutrophils. The incubation
tion was performed for 5 min at 37°C and was terminated by addition of 50 µl solution containing 5 mM concentrations of ATP, AMP, and cAMP. The cell suspension was boiled for 3 min, and radioactive cAMP was separated from other compounds by the same procedure as described in AC assay.

Prostaglandins E1, E2, A1, A2, F1 alpha, F2 alpha, and B1 were a generous gift from Dr. John Pike (Upjohn). The prostaglandin compounds were dissolved in 0.1 ml of ethyl alcohol, and 0.9 ml Na₂CO₃ (0.2 mg/ml) solution was added to produce prostaglandin concentration of 1 mg/ml. These solutions were stored in a freezer at -20°C. A solution containing the same volumes of ethyl alcohol and Na₂CO₃ served as a control and was added to all control samples. The final concentration of ethyl alcohol in the incubation medium was 0.01% (v/v).

RESULTS

As shown in Fig. 1, PG E1 significantly increased cAMP formation in resting and phagocytosing neutrophils any time during phagocytosis. Surprisingly, cAMP level was always higher in phagocytosing neutrophils than in the resting ones.

In the subsequent experiments, we studied the effect of various prostaglandins on cAMP formation in neutrophils. As shown in Table 1, all prostaglandins under investigation, except PG F1 alpha, significantly increased cAMP concentration in resting neutrophils. An additional rise was found in phagocytosing cells. Part of this increase was due to the stimulatory effect of ethyl alcohol, which was used to dissolve the prostaglandin specimens. The level of cAMP in the phagocytosing neutrophils which were incubated without addition of ethyl alcohol was not significantly different from the cAMP concentration in the resting neutrophils (data are not shown). The net increase of cAMP concentration in phagocytosing neutrophils stimulated by prostaglandins was significantly higher in the cells exposed to PG E1 and PG A2 than in those exposed to the other prostaglandins.

The adrenergic inhibitors used at 0.1- and 1.0 mM levels had no inhibitory effect on cAMP concentrations in resting and phagocytosing neutrophils stimulated by PG E1 (Table 2). Both propranolol and phentolamine at 1.0 mM concentration stimulated cAMP production in the resting and phagocytosing neutrophils. Both compounds also additionally increased cAMP level in the PG E1-stimulated neutrophils.

The phosphodiesterase assay did not show any difference in PDE activity between resting and phagocytosing neutrophils. PG E1 had no effect on PDE
Table 1. Effect of Various Prostaglandins on cAMP Formation in Resting and Phagocytosing Neutrophils

<table>
<thead>
<tr>
<th>Prostaglandin (ng/ml)</th>
<th>pmoles cAMP per 10^7 Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting</td>
</tr>
<tr>
<td>Control</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>A1</td>
<td>34.3 ± 3.1</td>
</tr>
<tr>
<td>A2</td>
<td>16.7 ± 2.5</td>
</tr>
<tr>
<td>B1</td>
<td>20.9 ± 3.2</td>
</tr>
<tr>
<td>E1</td>
<td>57.0 ± 4.9</td>
</tr>
<tr>
<td>E2</td>
<td>44.2 ± 6.0</td>
</tr>
<tr>
<td>F1 alpha</td>
<td>9.7 ± 1.9</td>
</tr>
<tr>
<td>F2 alpha</td>
<td>15.4 ± 2.8</td>
</tr>
</tbody>
</table>

The neutrophils were preincubated with 1 mM aminophylline and with or without latex microparticles for 15 min at 37°C, and then prostaglandins were added for an additional 3 min. Mean ± SE.

On the other hand, adenylyl cyclase activity was significantly increased in phagocytosing neutrophils (p < 0.001), in comparison to the resting cells (Table 3). Prostaglandin E1 added to the cell homogenates prepared from resting or phagocytosing neutrophils stimulated more AC activity in the latter than in the former ones. In contrast, AC activity in lymphocytes that were incubated with latex particles was significantly lower than in the cells not exposed to latex.

DISCUSSION

According to current thinking, cAMP mediates the hormonal effects and serves as a second messenger in the tissue cells. In leukocytes, the problem of cAMP as a second messenger has not been solved, because the biochemical nature of the hormone (first messenger) that regulates the leukocyte function or development has not been discovered. Although the stimulatory effect of prostaglandins on cAMP production in neutrophils is a significant one, similar actions of prostaglandins on many other body cells suggest that prostaglandins do not play a role as a specific leukocyte-related first messenger. In spite of that,
Table 3. Effects of Latex Microparticles on Adenylate Cyclase in Human Neutrophils and Lymphocytes

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Latex</th>
<th>PG E1 (3 μg/ml)</th>
<th>pmoles cAMP per mg Protein per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>-</td>
<td>-</td>
<td>1.71 ± 0.42</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>+</td>
<td>-</td>
<td>2.18 ± 0.45</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>-</td>
<td>+</td>
<td>2.38 ± 0.30</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>+</td>
<td>+</td>
<td>2.80 ± 0.41</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>-</td>
<td>-</td>
<td>2.66 ± 0.68</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>+</td>
<td>-</td>
<td>1.76 ± 0.49</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>-</td>
<td>+</td>
<td>3.57 ± 0.86</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>+</td>
<td>+</td>
<td>2.52 ± 0.70</td>
</tr>
</tbody>
</table>

The leukocytes were preincubated with or without latex microparticles for 15 min and then separated from medium by centrifugation. The harvested cells were homogenized by sonication, and part of the crude homogenate was used for AC assay. Statistical pair analysis test shows that AC activity is significantly (p < 0.001) higher in phagocytic neutrophils and significantly lower in lymphocytes exposed to latex particles. Mean ± SE for nine assays.

It seems that prostaglandins play an important role in the inflammatory process, and the high concentration of prostaglandins in the loci of inflammation could contribute to the regulation of the metabolic activity of neutrophils via the AC-cAMP system. It is possible that the first messenger-hormone stimulates prostaglandin synthesis and that the activation of the AC-cAMP system is only secondary to this. A feedback mechanism of the AC-cAMP system with prostaglandin formation is not excluded, and just recently such a possibility was advanced for the thyroid gland.

We demonstrated that stimulation of cAMP formation by PG E1 is not related to adrenergic-type receptors, because neither phentolamine nor propranolol had any inhibitory effect on cAMP formation in PG E1-treated neutrophils. Our data confirm the observation of Bourne and Melmon, who found that the effect of PG E1 on AC activity was not blocked by the aforementioned inhibitors. However, it seems that both alpha and beta adrenergic inhibitor stimulates cAMP formation in addition to the effect of high doses of PG E1. This would suggest that at least two types of receptors control adenyl cyclase activity in leukocytes, one being related to prostaglandin stimulation and the other to the action of adrenergic compounds. Although we did not study the effect of isoproterenol, epinephrine, or norepinephrine on cAMP formation in leukocytes, such an action has been shown by others.

It seems that prostaglandin E1 and A2 especially stimulate cAMP formation in phagocytosing neutrophils. This is probably due to the AC activation by phagocytosis, because we show that AC is significantly increased in phagocytosing neutrophils. These data suggest that AC is held in cell membranes in a restrained state which is relieved by the changes in membrane structure. Similar observations were reported recently about the AC of rat parotid gland and cerebral cortex.

REFERENCES

2. Park BH, Good RA, Beck NP, Davis BB:


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