all-trans-Retinal stimulates superoxide release and phospholipase C activity in neutrophils without significantly blocking protein kinase C

(cell-stimulation/phospholipids/inositol phosphates)

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Communicated by Eugene P. Kennedy, July 3, 1986

ABSTRACT all-trans-Retinal was previously shown to stimulate high levels of superoxide release by guinea pig neutrophils. When the cells, previously labeled with [3H]inositol, are treated with all-trans-retinal, they exhibit a decrease in the levels of [3H]inositol phospholipids and an increase in the accumulation of [3H]inositol phosphates. The maximal accumulation of inositol phosphates and the optimal rate of superoxide release occurred together at ≈7 min after stimulation. The levels of [3H]inositol phosphates accumulated were comparable to those observed when the cells were stimulated with a chemotactic peptide. In direct measurements, using concentrations that stimulate intact cells maximally, all-trans-retinal was found not to inhibit protein kinase C from the cytosol of neutrophils significantly. This contrasts with the situation with this kinase obtained from other sources. These observations represent additional effects of vitamin A on cells.

Retinoids have recently attracted considerable attention due to their ability to affect cellular differentiation and inhibit carcinogenesis (for review, see ref. 1). These substances may exert their effects by influencing the expression of certain genes (e.g., see ref. 2), by functioning as glycolipid intermediates in glycosylation reactions (e.g., see ref. 3), by increasing the liquid crystalline fraction of membranes (4, 5), and by acting as inhibitors of protein kinase C (e.g., see refs. 6–8). The last-mentioned enzyme is itself the cellular receptor for the tumor-promoting phorbol esters (9, 10). Diglyceride, formed by a phospholipase C-catalyzed hydrolysis of inositol phospholipids, is the physiological activator of protein kinase C (e.g., see ref. 10), and phorbol esters can substitute for diglyceride in this role (10).

Certain phorbol esters (e.g., phorbol 12-myristate 13-acetate (PMA)) have been widely used as inducers of high levels of superoxide (O2−) release by neutrophils (e.g., see refs. 11 and 12). Superoxide is a key component of the oxygen-dependent antimicrobial mechanisms of phagocytic leukocytes (for review, see ref. 13). Activation of phospholipase C and of protein kinase C are considered to be critical initial events in the stimulation of this release (for review, see ref. 14).

Retinoids are known to block the effects of phorbol esters on a wide variety of cells (6, 15, 16). However, we have recently reported that they stimulate high levels of O2− release by neutrophils (17). The mechanism of the stimulation by retinoids is of interest since this is a situation in which retinoids mimic, rather than inhibit, a cellular effect of phorbol esters. In this paper, we report effects of all-trans-retinal on phospholipase C and protein kinase C of neutrophils.

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MATERIALS AND METHODS

myo-[2-3H]inositol (10–20 Ci/mmol; 1 Ci = 37 GBq) and [γ-32P]ATP (2–10 Ci/mmol) were purchased from Du Pont-NEN Products. all-trans-Retinal (Type XVI), phosphatidylserine, 1,2-dioleoyl-rac-glycerol, histone (type III-S), neomycin sulfate, and inositol phospholipid standards were obtained from Sigma. Anion exchange resin AG1-X8 (formate form) (200–400 mesh) was a product of Bio-Rad. Glycophase G glass beads (CPG/200) were obtained from Pierce. Sources of all other materials used have been described elsewhere (12, 17).

Preparation of Neutrophils. Guinea pig peritoneal neutrophils were prepared as described (18).

Superoxide Release. Superoxide release by neutrophils was measured as outlined earlier (12). Solutions of all-trans-retinal (10 mM) were prepared in dimethyl sulfoxide immediately prior to use (17). Stock solutions of fMet-Leu-Phe and PMA were prepared in dimethyl sulfoxide and stored at −20°C until needed. These compounds were diluted with dimethyl sulfoxide so that the final concentration of solvent in the assays was 0.25% (vol/vol) in all cases. This concentration of solvent did not itself cause the effects noted.

Labeling of Cells with [3H]inositol. Neutrophil inositol phospholipids were labeled by incubating the cells (108 cells per ml) for 2 hr at 37°C in an isotonic Hepes buffer (124 mM NaCl/5 mM KCl/10 mM Hepes, pH 7.35) containing [3H]inositol (125 μCi/ml). The cells were washed in a modified Hanks’ Hepes buffer (124 mM NaCl/5 mM KCl/0.5 mM CaCl2/0.2 mM MgCl2/10 mM Hepes, pH 7.35/5 mM glucose), resuspended in 50 ml of this buffer at a concentration of 2 × 108 cells per ml, and equilibrated to 37°C. They were stimulated with all-trans-retinal (25 μM), with fMet-Leu-Phe (1 μM), or with PMA (0.05 μM) for various times, and the reactions were terminated by rapidly pouring the cell suspensions into 500-ml flasks containing 100 ml of the modified Hanks’ Hepes buffer chilled to 4°C. The cells were transferred to 50-ml centrifuge tubes and collected by centrifugation for 10 min at 4°C. Cell pellets were lysed by the addition of 0.67 ml of 4.5% perchloric acid, and the water extracts were combined and neutralized by the procedure of Bradford and Rubin (19). The water-insoluble lipid residue was saved for analysis of inositol phospholipid labeling.

Separation and Measurement of [3H]inositol Phosphates. The combined neutralized extracts from 108 cells were applied to 1.0-ml columns of AG1-X8 resin (formate form), and myo-inositol and glycerophosphoinositol were eluted as outlined in ref. 20. The inositol phosphates—inositol mono-

Abbreviations: PMA, phorbol 12-myristate 13-acetate; InsP1, inositol triphosphate; InsP2, inositol bisphosphate; InsP3, inositol monophosphate.

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phosphate, inositol bisphosphate, and inositol trisphosphate (InsP$_2$, InsP$_3$, and InsP$_4$) were then sequentially eluted with a stepwise gradient of ammonium formate (ref. 20; see legend to Fig. 2). Fractions (1.0 ml each) were collected and 0.5 ml was used to determine the $^3$H content by scintillation counting.

Measurement of [${}^{3}$H]inositol Phospholipid. Lipids were extracted from the insoluble residue that remained from the perchlorate extraction procedure and were washed by the method of Schacht (21). The organic phase was washed twice more with 0.50 M KCl. The extracts were applied to immobilized neomycin glycolipase columns, and the individual inositol phospholipids were eluted with increasing concentrations of ammonium formate (22). It was necessary to raise the final formate concentration to 2.0 M in order to elute all of the phosphatidylinositol 4,5-bisphosphate, as noted previously (23). Phospholipids were measured as total lipid phosphorous after washing (24).

Protein Kinase C. This enzyme was assayed by measuring the incorporation of $^{32}$P from $[\gamma-^{32}P]ATP$ into histone type III-S. The assay conditions were those described by Hirota et al. (6). The neutrophil cytosol fraction was prepared by disrupting $10^7$ cells in 4.0 ml of buffer A (6) by sonication or freeze-thawing and centrifuging the resulting homogenate at 100,000 x g for 1 hr at 4°C. Cytosol fractions prepared from cells disrupted by either method exhibited identical results.

RESULTS

Stimulation of O$_2^-$ Release by Neutrophils. Upon exposure to all-trans-retinal neutrophils release O$_2^-$, detected by the reduction of ferricytochrome c. A lag period of several minutes was observed between the addition of this stimulus and the attainment of the linear steady-state rate. In contrast, a latency period was not observed when the chemotactic peptide fMet-Leu-Phe served as the stimulus, and only a brief lag ($\approx$30 sec) occurred when the phorbol ester PMA was used (Fig. 1). The maximal rates of O$_2^-$ release, derived from the slopes of the steepest linear regions of the progress curves, were virtually identical for all of these stimuli at optimal concentrations (i.e., $\approx$50 nmol of O$_2^-$ per min per $10^7$ cells) (12, 17).

When the combined effects of retinal and PMA were checked, the progress curve for O$_2^-$ release was very similar to that observed for retinal alone—i.e., a single sustained rate was seen; the maximal rate was that for either stimulus alone—i.e., the rates were not additive (cf. Fig. 1).

Neutrophils labeled with [${}^{3}$H]inositol exhibited lag times identical to those observed for freshly harvested unlabeled cells. The maximal rates of O$_2^-$ release exhibited by these labeled cells stimulated with all-trans-retinal (25 µM), PMA (0.05 µM), or fMet-Leu-Phe (1 µM) were 91.0% ± 5.0% (SD; n = 3), 71.0% ± 16.0% (SD; n = 3), and 67% (n = 2; range, 50–84), respectively, of the values for unlabeled cells; i.e., cells not subjected to a previous incubation step. Thus, the labeling procedure only modestly affected O$_2^-$ release when the cells were stimulated later.

Accumulation of Inositol Phosphates in Neutrophils. Unstimulated neutrophils contain measurable quantities of [${}^{3}$H]labeled inositol phosphates, particularly InsP$_3$ (Fig. 2). The large amount of this entity is probably a reflection of the higher concentration and greater specific activity of [${}^{3}$H]phosphatidylinositol in these cells compared to the other inositol phospholipids (Table 1).

Stimulation of neutrophils with all-trans-retinal resulted in increases of the radioactivity in InsP$_2$ and InsP$_3$ of $\approx$4.5- and $\approx$2.7-fold, respectively, whereas that in InsP$_1$ was increased by a factor of only 1.5 (Fig. 3). Maximal accumulation of radioactive inositol phosphates occurred at $\approx$7 min after stimulation with all-trans-retinal, the same time at which optimal O$_2^-$ release was observed with this stimulus (Fig. 3). These increases in inositol phosphates were comparable to those observed upon stimulation of the cells with fMet-Leu-Phe (Table 2). In contrast, stimulation with the tumor promoter PMA, which bypasses the phospholipase C step and activates protein kinase C directly (e.g., see ref. 10),
produced only marginal effects on the levels of these substances. The time periods chosen in Table 2 to compare the levels of inositol phosphates in cells stimulated with various agents were those at which the cells responded maximally in terms of O$_2$ release (Fig. 1).

The distribution of [H]inositol in the membrane lipids (inositol phospholipids) was examined in cells stimulated for 7 min with all-trans-retinal (25 μM), and compared to the values observed in unstimulated cells. The percentages of radioactivity in phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate were 93 ± 12, 51 ± 8, and 75 ± 6 (SEM; n = 3), respectively.

Table 1. Labeling of the inositol phospholipids of guinea pig neutrophils with [H]inositol

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Mass, nmol</th>
<th>Radioactivity, cpm</th>
<th>Specific activity, cpm/nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdIns</td>
<td>30.0 ± 3.72</td>
<td>187,945 ± 9770</td>
<td>6378 ± 444</td>
</tr>
<tr>
<td>PtdInsP$_2$</td>
<td>2.3 ± 0.24</td>
<td>6,167 ± 421</td>
<td>2846 ± 443</td>
</tr>
<tr>
<td>PtdInsP$_3$</td>
<td>1.4 ± 0.1</td>
<td>2,236 ± 357</td>
<td>1556 ± 229</td>
</tr>
</tbody>
</table>

Table 2. Effects of various stimulators on inositol phosphate accumulation in neutrophils

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Effect of stimulation, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>InsP</td>
<td></td>
</tr>
<tr>
<td>InsP$_2$</td>
<td></td>
</tr>
<tr>
<td>InsP$_3$</td>
<td></td>
</tr>
<tr>
<td>all-trans-Retinal (25 μM)</td>
<td>149 ± 440 440 ± 48 277 ± 49</td>
</tr>
<tr>
<td>fMet-Leu-Phe (1 μM)</td>
<td>141 ± 457 457 ± 164 192 ± 50</td>
</tr>
<tr>
<td>PMA (0.05 μM)</td>
<td>115 ± 15 121 ± 2 92 ± 10</td>
</tr>
</tbody>
</table>

Guinea pig neutrophils were labeled with [H]inositol, and the inositol phospholipids were subsequently extracted and quantified. Data are expressed as mean ± SEM for three different preparations of cells. Values are for 10^6 cells. PtdIns, phosphatidylinositol; PtdInsP$_2$, phosphatidylinositol 4-phosphate; PtdInsP$_3$, phosphatidylinositol 4,5-bisphosphate.

Neutrophils labeled with [H]inositol were incubated with the stimuli listed for a period of time during which each agent maximally superoxide release. These data are expressed as the percentages of the values for these substances in unstimulated cells. The data for all-trans-retinal are the means ± SEM of three separate experiments. The data for fMet-Leu-Phe and PMA represent mean values ± ranges for two separate experiments. Each separate experiment used a different preparation of cells.

The increase in superfolding of protein kinase C in cells stimulated with all-trans-retinal (25 μM) for 10 min had no effect on the total amount of protein kinase C activity that could be recovered—for example, by lysis of the cells in the presence of 5 mM EDTA. The values for recovered protein kinase C were as follows: control cells, 6389 ± 873 (n = 6); retinal-treated cells, 5814 ± 839 (n = 6) (pmol of P$_i$ per 4 min per 10^6 cells ± SEM).

The physiologic substrates of protein kinase C in neutrophils are not presently known. We therefore evaluated the effects of all-trans-retinal on this kinase in vitro by using an exogenous substrate. Neutrophil protein kinase C activity is in the cytosol (25 μM): it is stimulated severalfold by calcium in the presence of phosphatidyserine and diolein (protein kinase C) (Fig. 4). Calcium alone produced some stimulation (≈50%), whereas diolein alone was without effect on the enzyme activity (unpublished data). all-trans-Retinal at 10 and 100 μM inhibited this protein kinase C activity by almost 20% and 47%, respectively, and did not significantly affect the values of the enzyme observed for unstimulated cells. (The last two values are significantly lower than 100%, P < 0.01.)

Effect of all-trans-Retinal on Protein Kinase C Activity. Treatment of intact cells with retinal (25 μM) for 10 min had no effect on the total amount of protein kinase C activity that could be recovered—for example, by lysis of the cells in the presence of 5 mM EDTA. The values for recovered protein kinase C were as follows: control cells, 6389 ± 873 (n = 6); retinal-treated cells, 5814 ± 839 (n = 6) (pmol of P$_i$ per 4 min per 10^6 cells ± SEM).

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the basal activity (i.e., activity measured in the absence of Ca²⁺, diolein, and phosphatidylserine) (Fig. 4). Under identical conditions, all-trans-retinal at 10 μM completely inhibits protein kinase C from cultured pituitary cells and increases the basal kinase activity sevenfold (6). The assay conditions used in Fig. 4 were chosen for comparative purposes but are not optimal. No inhibition of protein kinase C activity from neutrophils by all-trans-retinal (10 or 100 μM) was observed when optimal conditions (i.e., those used in ref. 26) were employed (W.H., unpublished data).

**DISCUSSION**

We have recently reported that compounds known to increase the liquid crystalline fraction of membranes (e.g., cis-unsaturated fatty acids (27, 28), retinoids (4, 5)) induce high levels of O₂⁻ release by neutrophils (17, 29). Cis-unsaturated fatty acids stimulate purified phosphatidylinositol-specific phospholipase C activity in vitro (30, 31), and this effect has been attributed to alteration of the physical properties of the lipid substrate (30). The loss of cellular [³H]inositol phospholipids (see text) and the accumulation of [³H]inositol phosphates (Fig. 3; Table 2) upon treatment of neutrophils with all-trans-retinal are consistent with activation of phospholipase C in intact neutrophils by this substance. Similar studies on inositol phospholipid metabolism of neutrophils incubated with cis-unsaturated fatty acids were not undertaken since these stimuli have damaging effects on cells shortly (<3 min) after stimulation. Phosphatidylinositol-specific phospholipase C catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce diglyceride and InsP₃. Both products have important “second messenger” roles in stimulating certain cellular responses (for review, see ref. 32). As noted earlier, diglyceride is the physiological activator of protein kinase C. InsP₃ stimulates the release of Ca²⁺ from intracellular storage sites. These messengers may interact synergistically to stimulate neutrophils to produce O₂⁻ (for review, see ref. 14). Neutrophils stimulated with all-trans-retinal may similarly utilize these “second messengers,” since all-trans-retinal does not significantly inhibit protein kinase C from these cells at concentrations at which it elicits O₂⁻ production (Fig. 4). This contrasts with the situation regarding the kinase obtained from other sources (6).

Previous studies have shown that neutrophils treated with fMet-Leu-Phe degrade their inositol phospholipids and accumulate inositol phosphates, with the maximal effects occurring at 20–30 sec after stimulation (19, 33–35). This rapid response is compatible with the absence of a lag period in the progress curve for O₂⁻ release with this stimulus (Fig. 1). In contrast, neutrophils stimulated with all-trans-retinal exhibit a pronounced lag period in O₂⁻ release (Fig. 1) and do not exhibit maximal accumulation of labeled InsP₂ and InsP₃ until several minutes after stimulation (Fig. 3). The lag period of O₂⁻ release may therefore correlate with attainment of a critical level of a particular component. In our experiments, fMet-Leu-Phe and all-trans-retinal induced similar levels of inositol phosphates, with InsP₂ predominating in the case of both stimuli (Fig. 2). Reasons for the apparently high levels of labeled InsP₂ (measured as [³H]) may include the larger quantity and greater specific activity of the substrate phosphatidylinositol 4-phosphate compared to phosphatidylinositol 4,5-bisphosphate (Table 1), the possibility that phosphatidylinositol 4-phosphate is the preferred substrate (36), and/or the existence of phosphatases of high activity in guinea pig neutrophils that degrade InsP₃ (37).

Retinoids at concentrations similar to those used here affect differentiation and growth of normal and malignant cells, even in cells that lack specific cytosolic-binding proteins for these substances (ref. 38 and refs. cited therein). In this paper, we report an effect of all-trans-retinol on a particular cell type (i.e., activation of phospholipase C). In addition, we show that the neutrophil protein kinase C is largely insensitive to all-trans-retinal when examined in cell extracts (Fig. 4). Studies to determine whether retinoids activate phospholipase C in cells other than neutrophils would be of interest.

This work was supported by U.S. Public Health Service Grant 1 ROI GM35307-01. J.A.B. is the recipient of a Research Career Development Award (1 K04 AI 00672-01) from the National Institute of Allergy and Infectious Diseases. W.H. was supported by the Deutsche Forschungsgemeinschaft.