Diacylglycerols and PMA induce actin polymerization and distinct shape changes in lymphocytes: relation to fluid pinocytosis and locomotion

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Summary

Shape changes have been determined in human blood lymphocytes stimulated with OAG, diC8, PMA, colchicine or the hexapeptide fNLPNTL in short-term assays (30 min). Distinct types of shape-change responses were observed. Colchicine was active in generating a relatively small proportion of polarized lymphocytes (front–tail polarity). OAG, diC8 and PMA produced different types of shape change (non-polar cells with surface projections), and these were closely associated with an increase in actin polymerization and a shift of F-actin into the projections at the cell periphery. The diacylglycerols OAG and diC8 produced biphasic dose-response curves leading to rounding up of cells at very high stimulant concentrations. PMA produced no comparable biphasic response when tested over a much wider concentration range. Though the non-polar cells with surface projections generated by OAG, diC8 or PMA showed vigorous shape changes, they lacked significant locomotor activity. α-Phorbol, 4α-PDD, lumicolchicine or fNLPNTL were inactive. Small blood lymphocytes stimulated by OAG, diC8 or PMA showed a very small increase in the net uptake of FITC-dextran by fluid pinocytosis. Unlike neutrophils, which show a high net uptake, lymphocytes did not concentrate FITC-dextran in large granules, indicating that they do not develop a 'storage' compartment in the form of large vesicles. However, small fluorescent spots were consistently found in at least a fraction of blood lymphocytes. The results indicate that stimulated surface movement may be instrumental in fluid pinocytosis. Diacylglycerols may act as second messengers to induce pinocytosis, shape changes and altered actin polymerization in lymphocytes.

Key words: diacylglycerols, PMA, lymphocyte locomotion, fluid pinocytosis, actin polymerization.

Introduction

Normal lymphocytes from peripheral human blood are in general spherical and non-motile, but various stimuli can induce shape changes following short-term or long-term stimulation (for review, see Wilkinson, 1987). Previous studies with neutrophils indicated that PMA or diacylglycerols (DAGs) can elicit a type of movement and a shape that differs from the characteristic polarized shape (front–tail polarity) produced following stimulation with chemotactic peptides (Keller et al. 1987; Roos et al. 1987; Zimmermann et al. 1988; Robinson et al. 1987). Neutrophils treated with phorbol myristate acetate (PMA) or diacylglycerols showed vigorous movements with numerous surface projections and intracellular vacuoles in the absence of front–tail polarity. We observed that the lymphocytes contaminating the neutrophil preparations showed similar types of shape changes as neutrophils. This agrees with independent experiments with PMA done by Wilkinson et al. (1988), except that we also observe similar effects with DAGs.

A correlation between cell shape and function has been postulated. Front–tail polarity has been shown to be associated with locomotor activity (Lewis, 1931; Keller, 1983; Wilkinson, 1987), whereas cells characterized by multiple projections in the absence of front–tail polarity show no or little locomotor activity (Keller, 1983; Roos et al. 1987). However, uptake of FITC-dextran by fluid pinocytosis in non-polar cells with surface projections was much more pronounced than in polarized cells (Keller & Zimmermann, 1987). It was the purpose of the present study to describe the morphologically distinct response of lymphocytes to diacylglycerols or PMA as opposed to colchicine. A further aim was to test the correlation between PMA or diacylglycerol-induced shape changes and actin polymerization on the one hand, and locomotion or fluid pinocytosis in small human blood lymphocytes on the other hand.

Materials and methods

Materials

Reagents and suppliers were: N-formyl-L-norleucyl-L-leucyl-
l-phenylalanyl-l-norleucyl-l-tyrosyl-l-lysine (fNLPNTL), Bachem, Bubendorf, Switzerland; human serum albumin (HSA), Behringwerke, Marburg, FRG; 1-oleoyl-2-acetylglycerol (OAG), 1,2-dioctanoyl-sn-glycerol (diC8), NBD-phallacidin, Molecular Probes Inc., Junction City, OR, USA, 4α-phorbol, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (4α-PDD), FITC-dextran FD-70, lysolecithin (l-α-lysophosphatidylcholine, palmityl), paraformaldehyde, EGTA, Sigma Chemical Co., St Louis, MO, USA; colchicine, lumicolchicine, glutaraldehyde, N,N,N,N′-tetraethylenepiperazine-N′-2-ethanesulfonic acid (Hepes), Serva Feinbiochemica, Heidelberg, FRG; neutrophil isolation medium (NIM), Packard Instrument International SA; Percoll, Pharmacia AG, Uppsala, Sweden. Water-insoluble compounds were dissolved in dimethylsulfoxide (DMSO; Fluka AG, Buchs, Switzerland). Triton X-100 was obtained as a 10% solution in water from Pierce (Oud-Beijerland, Netherlands) and stored under nitrogen. Actin was prepared from rabbit skeletal muscle water from Pierce and stored as described by Pardee & Spudich (1982). The medium was usually prepared as follows: 138 mm-NaCl, 6 mm-KCl, 1·1 mm-EGTA, 1 mm-Na2HPO4, 5 mm-NaHCO3, 1 mm-MgSO4, 5·5 mm-glucose, 20 mm-Hepes and 2% (w/v) human serum albumin (HSA). Exceptions are stated below (actin polymerization experiments). The medium (pH 7·4) was prepared without added Ca2+ and Mg2+ in order to prevent cell aggregation.

Cell preparation

Neutrophils were isolated from heparinized (10 units ml−1) human blood withdrawn from apparently healthy volunteers. The lymphocytes were then separated using either NIM (Ferrante & Thong, 1980) or a two-step procedure, the first step involving separation of white cells from red cells using Isopaque–Methocel (Böyum, 1968) followed by separation of lymphocytes by means of Percoll (Phillips et al. 1983). Both procedures yielded about 80–95% small lymphocytes. Similar results have been obtained with cells from the two types of isolation procedures. Most experiments shown have been performed with Percoll-separated cells. Lymphocytes prepared by these procedures showed hardly any signs of morphological activation, whereas contaminating phagocytes were usually activated in Percoll-separated cells as indicated by numerous surface projections and vacuoles.

A more sophisticated separation procedure was used for actin polymerization assays. This was necessary to exclude the possibility that monocytes or platelets can make major contributions to the actin assay. Blood was first separated by NIM (Ferrante & Thong, 1980) and in the second step the fraction containing the mononuclear cells was passed over a discontinuous Ficoll gradient (Böyum, 1968). The fraction with mononuclear cells was then passed over a Percoll gradient. The mononuclear cells were collected and passed through a column of loosely packed nylon wool (Mirnepore L Travenol AG, Dietikon, Switzerland). Centrifuge tubes (50 ml) were packed with nylon wool up to 30 ml, washed with 50 ml plain medium and warmed at 37°C. Afterwards the cells were applied to the column, incubated at 37°C for 30 min and eluted with 100 ml of medium through an injection needle placed at the bottom of the tube without applying pressure. The cells were centrifuged at 300 g for 10 min and pooled. The procedure yields preparations consisting of 90–97% lymphocytes and 3–10% monocytes. There were 0–2–5 platelets per lymphocyte.

Determination of cell shape

The analysis was restricted to small lymphocytes, which could be distinguished on the basis of their size. Cells (106 in 1 ml) were preincubated in a recirculating water bath at 37°C for 10 min and then exposed to uniform concentrations of stimuli for the time indicated, i.e. usually 30 min. The cells were fixed in 1% glutaraldehyde at 37°C for 30 min, washed and resuspended in 0·9% NaCl containing 1 ml of NaN3 per ml. The shape of small lymphocytes was determined in Sykes-Moore chambers using differential interference contrast (DIC; Nomarski) optics (Zeiss IM 35 microscope, ×100; NA 1·25 objective). Continuous shape changes in living cells were observed on the microscope stage heated to approximately 37°C with an Air Stream Stage Incubator (Nicholson Precision Instruments Inc., Bethesda, MD).

Small lymphocytes were classified as follows (Fig. 1): (1) spherical cells: they exhibit an uninterrupted circular contour (Fig. 1A1 and A2). The spherical cells of unstimulated control lymphocytes appear to have a fairly smooth ‘surface’ structure (Fig. 1A1). Spherical cells in lymphocytes stimulated with high (10−2 M) diacylglycerol concentrations (Fig. 1A2) tend to have a more irregular surface (top), may not be ideally spherical (middle) or show a hyaline periphery (bottom). The variants shown on A1 and A2 have not been quantified separately because there are too many intermediate forms. (2) Spherical cells with unifocal projections: basically spherical cells with a circular outline except for unifocal projection(s) on one side (Fig. 1B). (3) Non-polar lymphocytes with surface projections: cells without morphological front–tail polarity but...
with numerous major surface projections that may be distributed all over the surface giving a complex irregular outline (Fig. 1C). Projections are extended and retracted along multiple axes. (4) Polarized lymphocytes: these are elongated cells showing front-tail polarity, i.e. protrusions at the front and a contracted area at the opposite (rear) end (Fig. 1D). One hundred lymphocytes per sample for each experiment were classified.

**Random locomotion in vitro**

Cells (2×10^6 lymphocytes/sample) were preincubated (10 min at 37°C) in plain medium, then incubated with or without stimulus (30 min at 37°C). Samples of 1 ml were spun (5 min at 1200 revs min^{-1}) and the pellet was resuspended in 20 μl of medium with or without stimulus. A sample of between 3 and 4 μl (10^5 cells μl^{-1}) was placed on a cleaned slide, covered with a circular coverslip (25 mm in diameter) and the preparation was sealed with paraffin. Cells are slightly squeezed between slide and coverslip. Thus, these narrow chambers are suitable for inducing cell—substratum contacts and assessing locomotion of cells such as Walker carcinosarcoma cells (Keller et al. 1985) or lymphocytes, which otherwise (in deeper chambers such as Sykes-Moore chambers) had little or no contact with the substratum. The preparation was placed on the heated stage (37°C) of a Leitz Durovert microscope in a controlled-environment chamber. One microscopic field of each sample was recorded for 10 min using a ×40 objective, a Newvicon video camera (Robert Bosch GmbH, Germany) and a time-lapse video recorder (NV8050, Panasonic Video Systems). Preparations with floating cells were discarded. The positions of cells within the recording time of 10 min were traced. Only cells that had moved outside the circumference of their initial position were recorded as migrating cells. The proportion of migrating cells and the average speed of all cells was determined by means of morphometry (IBAS, Zeiss, FRG).

**Fluid pinocytosis of FITC—dextran**

Fluid pinocytosis was determined by measuring the net uptake of fluorescinated dextran (Davis et al. 1982). Lymphocyte suspensions (10^6 cells ml^{-1}) were preincubated in plain medium at 37°C for 10 min and then incubated in the presence of FITC—dextran (5 or 10 mg ml^{-1} final concentration) with or without chemical stimuli at the concentrations indicated for 30 min. The reaction was stopped by fixation in 1% glutaraldehyde at 37°C for 30 min. The cells were then washed five times with phosphate-buffered saline, pH 7.4. Fluorescence was determined immediately by flow cytometry (Ortho cytofluorograph H-50, Ortho Instruments, Raritan, NJ, USA) using a 5 W argon laser, an excitation wavelength of 488 nm and a long pass filter giving an emission wavelength between 515 and 555 nm. The median channel number was determined, the non-specific fluorescence, which was mainly due to glutaraldehyde fixation, being relatively high. The value for the unstimulated control with added FITC—dextran was taken as 100% and the preparations with floating cells were discarded. The positions of cells were also examined by means of fluorescence microscopy (Zeiss IM 35) using a ×100 objective. Photographs were taken using a Kodak T-Max P3200 film exposed at 400 ASA.

**F-actin assays (NBD-phallacidin staining)**

Highly purified lymphocytes (see above) were used. The topography of F-actin distribution and the relative amount was determined after paraformaldehyde fixation and NBD—phallacidin staining as described (Roos et al. 1987; Keller et al. 1985). One series of experiments was performed with the usual medium containing 2% HSA (Fig. 2) and another series with an incubation scheme similar to that used for the cytoskeletal actin assay, i.e. with 0.1% instead of 2% HSA (Table 1).
Table 1. Effects of PMA and OAG on actin polymerization in lymphocytes as determined by two different methods

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Method A</th>
<th>Method B</th>
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<tr>
<td></td>
<td>% of total cellular actin associated with cytoskeleton (relative amount)</td>
<td>Relative amount of NBD-phallacidin bound</td>
</tr>
<tr>
<td>None</td>
<td>20 ± 3-1</td>
<td>100</td>
</tr>
<tr>
<td>DMSO (0-143 %)</td>
<td>19 ± 2-1</td>
<td>110 ± 1</td>
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<tr>
<td>PMA (10^-4 M)</td>
<td>31 ± 2-9</td>
<td>159 ± 13</td>
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<tr>
<td>OAG (10^-4 M)</td>
<td>32 ± 9-7</td>
<td>124 ± 10</td>
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The extent of actin polymerization was determined either by quantification of the % of total cellular actin associated with the Triton X-100-insoluble cytoskeleton (method A), or by measurement of the amount of NBD-phallacidin bound to fixed and permeabilized cells (method B), as described in Materials and methods. The data represent mean ± S.D.M. of three (OAG) or four (all other data) experiments. Cells were preincubated for 10 min at 37°C, followed by the addition of stimuli, as indicated, and a further incubation at 37°C for 30 min. The amount of DMSO added to the cells was maximally 0-143 % (v/v) in the case of OAG.

Distribution of cellular actin between the cytosol and cytoskeleton

Highly purified lymphocytes were suspended in 0·1 % HSA before stimulation. Cytoskeletal structures were subsequently isolated as described by White et al. (1982) with small modifications. Briefly, cells (450 μl, 8 x 10^6 cells ml^-1) were preincubated in Eppendorf tubes for 10 min. Medium (PMA or OAG, in 50 μl) was then added to the cells, followed by a 30-min incubation at 37°C. The reaction was stopped by the addition of 500 μl of an ice-cold Triton X-100 stock solution containing 2 % Triton X-100, 160 mM-KCl, 20 mM-Tris-HCl, pH 7·5, 20 mM-EGTA, 0·02 % NaN₃. The tubes were incubated on ice for 120 min, followed by centrifugation in a Heraeus Biofuge for 10 min at 4°C (10 000 g). After decanting the supernatant, 1 ml of the Triton stock solution, diluted 1:1 with medium (without HSA), was added and the tubes were centrifuged as before. The resulting pellets were solubilized in 100 μl of 10 % SDS, 50 mM-dithiothreitol (DTT), 15 % (v/v) glycerol, 0·25 % Triton-HCl, pH 6·8, 0·001 % Bromphenol Blue, at 95°C, with vortexing (10–15 min). To samples (70 μl) of the supernatants resulting from the first centrifugation, 2 % SDS, 0·25 % Triton-HCl, pH 6·8, 15 % glycerol, 50 mM-DTT and 0·001 % Bromphenol Blue (final concentrations) were added, followed by incubation for 5 min at 95°C. Pellets and supernatants, together with known amounts of purified rabbit skeletal muscle actin, were subsequently electrophoresed through a 7·5 % to 15 % gradient polyacrylamide slab gel (Laemmli, 1970) and the proteins were stained with Coomassie Brilliant Blue R. Actin bands were then scanned at 590 nm with a Camag TLC scanner. From comparison with known amounts of purified actin, the amount of actin in the cytoskeleton and the cytosol could be determined.

Results

Shape changes induced by PMA and diacylglycerols (OAG and diC8)

PMA produced an increase in the proportion of non-polar lymphocytes with surface projections and spherical cells with unifocal projection. Most of these morphologically altered cells were non-polar with surface projections (Fig. 2). There was a reciprocal reduction in the proportion of spherical small lymphocytes with an ED₅₀ of about 10⁻⁹ M-PMA. The response reached a plateau at concentrations between 10⁻⁸ and 10⁻⁶ M-PMA, with only about 10 % spherical cells left. α-Phorbol (10⁻⁸ to 10⁻⁶ M) and DMSO alone, at the solvent concentrations used with PMA, OAG or diC8, were inactive under these conditions (data not shown).

As with PMA, treatment with OAG or diC8 produced a substantial proportion of motile, non-polar cells with surface projections (Fig. 3). But the response to diacylglycerols was nevertheless different in some respects: (1) higher concentrations of diacylglycerols as compared to PMA were required to elicit responses. (2) A smaller proportion of lymphocytes (about 70 %) appeared to respond to diacylglycerols as compared to PMA (about 90 %). (3) Responses to OAG and diC8 were clearly biphasic. There was a relatively narrow peak of morphologically activated cells and at higher concentrations the proportion of spherical cells returned to 80–90 % (Fig. 2). In contrast, PMA showed no clear-cut biphasic response within the limits of the experiment even though PMA was tested over a much wider concentration range. The aspect of spherical cells at high diacylglycerol concentrations appeared to be slightly different from that of unstimulated spherical cells (Fig. 1). In contrast to unstimulated spherical lymphocytes, non-polar cells with surface projections elicited by OAG, diC8 or PMA also showed fairly vigorous and continuous shape changes (Fig. 3). The solvent used for diacylglycerols had no effect at concentrations corresponding to 3 x 10⁻⁴ M of OAG or diC8, whereas at DMSO concentrations corresponding to 10⁻⁴ M-diacylglycerol the response to PMA was found to be inhibited by 25–40 %, indicating that the biphasic response reflects the activity of diacylglycerols (up to 3 x 10⁻⁴ M) and not, or only to a limited extent (at 10⁻³ M), solvent effects.

Effect of colchicine or the chemotactic peptide fNLPNTL on the shape of blood lymphocytes

In contrast to neutrophils (Keller et al. 1984), small peripheral blood lymphocytes showed only a very small immediate shape response to stimulation with colchicine. The proportion of lymphocytes with front–tail polarity (see Fig. 1D) observed in the presence of 10⁻⁸ M to 3 x 10⁻⁷ M–colchicine was no different from unstimulated controls (1·3 ± 0·7 %) or 10⁻⁶ M-lumicolchicine-treated controls (1·7 ± 0·3 %). A relatively small increase was observed with 10⁻⁶ M (5·7 ± 1·3 %) or 10⁻⁵ M–colchicine (14·5 ± 0·3 %). This proportion (mean of three experiments ± S.D.M.) was not further increased following exposure to 10⁻⁵ M–colchicine for 60 min (12 %) instead of 30 min (14·5 %). Colchicine had no substantial effect on the proportion of non-polar cells with surface projections. The hexapeptide fNLPNTL had no significant effect at all. The proportion of spherical cells in the presence at 10⁻¹² to 10⁻⁶ M–fNLPNTL did not differ significantly from control values (95·6 ± 1·9 %; mean of three experiments ± S.D.M.).
Fig. 4. Distribution pictures (top) and fluorescence pictures (below) of unstimulated lymphocytes (C), cells stimulated with $10^{-9}$M-PMA (PMA), $10^{-4}$M-OAG (OAG) or $3 \times 10^{-7}$M-diC8 (diC8) for 30 min, fixed and stained with NBD-phallacidin. Bar, 10 $\mu$m.

contact and to prevent them from floating. Therefore, we interpret the findings to mean that the non-polar type of movement induced by PMA is not instrumental in locomotion.

Basically similar results were obtained when diC8 was tested. Only two out of the 114 cells in unstimulated controls and none out of 215 cells stimulated with diC8 ($10^{-6}$ to $10^{-3}$M) migrated. Thus, diC8 at concentrations between $10^{-3}$ and $10^{-6}$M (2 experiments) induced shape changes but not locomotor activity.

Pinocytosis of FITC-dextran in the presence of PMA, OAG or diC8

In initial experiments using medium with added Ca$^{2+}$ and Mg$^{2+}$, the small lymphocyte fraction gave a clearly positive result in flow cytometry, but microscopic evaluations showed that platelets containing fluorescent label were attached to small lymphocytes. In the first series of experiments in the absence of Ca$^{2+}$ and Mg$^{2+}$, one out of three experiments showed an increase in the fluorescence intensity of small lymphocytes stimulated with PMA. In another series of experiments with another lot of FITC-dextran, PMA produced a small dose-dependent increase in fluorescence at PMA concentrations $>10^{-9}$ (Fig. 5) in all three experiments. This was confirmed in numerous later experiments including experiments using 20 mg ml$^{-1}$ FITC-dextran. The inactive phorbol ester 4$\alpha$-PDD had no effect. Direct examination of lymphocytes showed that fluorescent spots were detectable by fluorescence microscopy in 32$\pm$2% of cells stimulated with $10^{-3}$M-PMA as compared to 3$\pm$1% in unstimulated controls (mean of four experiments $\pm$ S.D.M.), whereas contaminating phagocytes showed usually multiple large fluorescent dots (Fig. 6).

In two out of three experiments, OAG or diC8 produced a small dose-dependent increase in the uptake of fluorescent dextran. Mean values for three experiments ($\pm$ S.D.M.) were 100$\pm$2% for unstimulated FITC-stained controls as compared to 108$\pm$2, 119$\pm$4 and 133$\pm$21% for $10^{-7}$M, $3 \times 10^{-4}$M and $10^{-3}$M-OAG, respectively. The values for $10^{-4}$M, $3 \times 10^{-4}$M and $10^{-3}$M-diC8 were 113$\pm$1, 116$\pm$1 and 124$\pm$15%.

Discussion

Both PMA and diacylglycerols are capable of activating protein kinase C, which is, therefore, the likely target of
Effects on actin polymerization

PMA produced a dose-dependent increase in NBD-phallacidin binding over unstimulated controls, indicating a rise in F-actin, which closely parallels the morphological changes (Fig. 2). The correlation is even better than the value suggests because F-actin experiments and shape-change experiments were carried out with different cell populations. At \(10^{-10}\) M-PMA the particular F-actin experiments showed a weak increase in F-actin (112 and 128% as compared to untreated controls (100%)) and morphological activation (32 and 42% non-polar cells with surface projections) in two out of three experiments, whereas the third experiment showed no change in F-actin and morphology. A relative increase in NBD-phallacidin binding as compared to untreated controls (100%) was elicited by \(10^{-4}\) M-OAG (119 ± 2.9%) and by \(3 \times 10^{-4}\) M-diC8 (119 ± 4.9%) (mean of three experiments ± s.d.m.). Dose–response curves showed that the OAG or diC8 concentrations that were optimal in shape change assays were also optimal for actin polymerization (data not shown). DMSO alone had no stimulating effect (97.3 ± 3.7%). Similar results were obtained if the window of the flow cytometer was set for leucocytes or for lymphocytes only. Unstimulated spherical cells show diffuse staining of the narrow cytoplasm as shown by comparison of fluorescence and DIC pictures (Fig. 4c). In PMA- or DAG-stimulated lymphocytes the label was located preferentially in the surface projections (Fig. 4). No such labelled surface projections were found in response to α-phorbol or 4 α-PDD (data not shown).

Parallel measurements of NBD-phallacidin binding and of cytoskeleton-associated actin (on samples of the same cell population) were performed (Table 1). The results obtained with the two different methods correlated well. They show a marked increase in actin polymerization with \(10^{-8}\) M-PMA or with \(10^{-4}\) M-OAG and little if any effect with DMSO.

Effect of PMA and diacylglycerols on locomotion of blood lymphocytes in vitro

We tested whether shape changes and actin polymerization are related to locomotor activity. Migrating lymphocytes were extremely rare. In four experiments (2 experiments each with Percoll- or NIM-separated lymphocytes) with a total of 438 cells, only one lymphocyte in one control sample (=4.5%) and one cell in a corresponding sample with \(10^{-11}\) M-PMA (=3.8%) of one experiment had migrated. No migrating lymphocytes were found at PMA concentrations between \(10^{-10}\) and \(10^{-7}\) M, even though the cells at \(10^{-8}\) and \(10^{-7}\) M-PMA constantly changed shape. Absence of migration of PMA-stimulated cells could not be attributed to excessive cell–substratum adhesion. For the locomotion assay the cells were squeezed between slide and coverslip to induce

**Fig. 3.** Time course of shape changes induced by PMA, OAG or diC8. A. Plain medium; B, medium + PMA \((10^{-8}\) M); C, medium + OAG \((10^{-4}\) M); D, medium + diC8 \((3 \times 10^{-4}\) M). Lymphocytes were preincubated in suspension at \(37^\circ\)C for 10 min in plain medium, then incubated with or without PMA, OAG or diC8 for 30 min, transferred into Sykes-Moore chambers at \(37^\circ\)C and photographed sequentially at 20-s intervals (shown in row D) using DIC optics. Bar, 10 μm.

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with $10^{-8}$ M-PMA. Small lymphocytes (arrows) show no or little granular fluorescence, whereas large cells, i.e., phagocytes (arrowheads), show strong granular accumulation of FITC–dextran ($10 \text{ mg ml}^{-1}$) as previously demonstrated in neutrophil granulocytes (Keller & Zimmermann, 1987). Top: Nomarski optics; bottom: fluorescence picture. Bar, 10 μm.

**Fig. 6.** Uptake of FITC–dextran by leucocytes stimulated with $10^{-8}$ M-PMA. Small lymphocytes (arrows) show no or little granular fluorescence, whereas large cells, i.e., phagocytes (arrowheads), show strong granular accumulation of FITC–dextran ($10 \text{ mg ml}^{-1}$) as previously demonstrated in neutrophil granulocytes (Keller & Zimmermann, 1987). Top: Nomarski optics; bottom: fluorescence picture. Bar, 10 μm.

these stimuli (Niedel et al. 1983). There is evidence that diacylglycerols and protein kinase C are important for signal transduction and that they mediate or modulate a great variety of lymphocyte responses including growth control (Yap et al. 1986; Isakov & Altman, 1987), lytic function (Russel et al. 1986), and immunoglobulin secretion (Shearer et al. 1988). However, diacylglycerols and related compounds may also act through other pathways (Goppelt-Strübe et al. 1987). Our experiments suggest a novel role for diacylglycerols in lymphocytes. They may act as second messengers for signals eliciting pinocytosis and changes in shape and actin polymerization. Diacylglycerols or PMA can induce continuous shape changes associated with the formation of non-polar cells with surface projections and altered actin polymerization. In contrast to PMA, diacylglycerols produce a biphasic dose–response curve within a relatively narrow concentration range. This biphasic dose–response curve of lymphocytes to diacylglycerols resembles the biphasic dose–response curve of neutrophils to PMA (Roos et al. 1987) or diacylglycerols (Zimmermann et al. 1988) and should, therefore, not be interpreted as evidence that diacylglycerols act through a basically different pathway from PMA. PMA was found to induce actin polymerization in neutrophils (Sha’ah et al. 1983) and mononuclear cells (Rao, 1985). The finding that diacylglycerols and PMA elicit increased actin polymerization in highly purified lymphocyte preparations, as measured by gel electrophoresis of isolated cytoskeletal proteins and as determined by flow cytometry using a window selecting lymphocytes, suggests that this is indeed a lymphocytic response and not due to contaminating platelets, monocytes or neutrophils. This is substantiated by the finding that F-actin is redistributed in diacylglycerol- or PMA-treated lymphocytes (Fig. 4).

The role of movements and altered actin polymerization induced by diacylglycerols and PMA has been explored in respect of two functions, locomotor activity and fluid pinocytosis. It has been shown previously that front–tail polarity in lymphocytes is associated with locomotor activity (Lewis, 1931) and that the stimulating effect of PMA on locomotion is small within the first two hours and substantial after overnight culture (Wilkinson, 1987). In the present short-term experiments ($\leq 1$ h), cells with front–tail polarity and non-polar cells with surface projections have been distinguished and quantified. The results show that the formation of non-polar cells with surface projections is not associated with the development of substantial locomotor activity. This is consistent with observations in neutrophils indicating that formation of non-polar cells with surface projections by PMA or diacylglycerols favors pinocytosis rather than efficient locomotion, whereas chemotactic peptides preferentially stimulate locomotion rather than pinocytosis (Roos et al. 1987; Keller & Zimmermann, 1987). PMA or diacylglycerols may in fact suppress front–tail polarity and locomotion of neutrophils (Keller et al. 1987; Roos et al. 1987; Zimmermann et al. 1988). Only cells with a one-sided pseudopod formation and a contracted opposite end were listed as polarized, whereas cells that were elongated with a pseudopod on one end, but without contracted opposite side, were not. The sequential photographs show that elongated cells with one-sided pseudopods but without a contraction area on the other side (e.g. see Fig. 3, OAG at 0 min and 1 min or diC8 at 1 min) show no persistent behavioral polarity compatible with the finding that PMA or DAG do not induce locomotor activity under the experimental conditions used. This indicates that a distinction between elongated cells and cells with clear-cut antero–posterior polarity is reasonable. The leading pseudopod may have to develop a certain length (i.e. about 3–5 μm in neutrophils) before behavioral polarity persists (Zigmond et al. 1981) and, in addition to the contraction ring (Wilkinson et al. 1988), it is possible that the formation of a contracted tail is another important requirement. Lymphocyte responses appear to be different after long-term exposure of lymphocytes to PMA, which leads to the development of front–tail polarity and locomotor activity (Wilkinson et al. 1988). The finding that synthetic chemotactic peptides are inactive is consistent with earlier results (Wilkinson, 1987) and it is probably due to lack of appropriate surface receptors.

Two pathways of pinocytosis have been demonstrated in phagocytes: one pathway is associated with rapid return of pinocytosed material to the medium (reversible pinocytosis), whereas another pathway is associated with fusion of pinosomes with electron-dense granules leading to the formation of storage granules (Daukas et al. 1983;
The pinocytic responses of lymphocytes are certainly different from those of neutrophils. Using the technique that was effective with neutrophils (Keller & Zimmermann, 1987), there was only a small increase in net uptake of FITC-dextran by PMA- or DAG-stimulated cells. Direct morphological analysis showed tiny fluorescent spots in a fraction of lymphocytes but not the large vacuoles containing FITC-dextran, which may be characteristic of the storage (low turnover) compartment. Specific granules are involved in the formation of these vesicles in granulocytes (Robinson et al. 1987; White & Estensen, 1974). Lympocytes contain fewer granules and they appear to be of a different type (Timonen & Pakkanen, 1987; Tschopp & Nabholz, 1988), and may, therefore, not be fit for large vesicle formation or there may be too few. Our experiments do not exclude the possibility that substantial reversible pinocytosis takes place that is not fully detected by the technique used. The results suggest that there is an increase in net uptake of FITC-dextran that is, however, quite small and close to the limits of detection by the technique used. The pinocytic response (Fig. 5) parallels changes in shape and F-actin (Fig. 2) quite closely. Experiments with more sensitive techniques for measuring pinocytosis are required to extend these findings and to perform kinetic measurements of the turnover of the rapidly reversible pinocytosis pool.

Furthermore, the diacylglycerol- and PMA-induced changes in shape and actin polymerization may play a role in other functions. One interesting possibility is the stimulation of cell–cell contacts. Phorbol esters and diacylglycerols were found to induce cell–cell contacts in human mononuclear cells (Patarroyo & Jondal, 1985). The present experiments were performed without addition of Ca++ and Mg++ to the medium in order to prevent aggregation. Experiments with cells from lymphocyte leukaemias (Pollack et al. 1986) and our preliminary experiments with human blood lymphocytes in the presence of divalent cations have shown aggregates consisting of lymphocytes and/or neutrophils as well as platelet–lymphocyte interaction. The projections of the stimulated cells may stabilize cell–cell interaction by interdigitation and facilitate exchange of signals. Furthermore, DAG and PMA have been shown to inhibit chemotactic migration of neutrophils (Roos et al. 1987; Zimmermann et al. 1988). Also lymphocytes show chemotactic responses (Potter & van Epps, 1987) and these may also be inhibited by these activators of protein kinase C. After completion of this work a paper appeared that reported similar changes in actin to those reported here (Phatak et al. 1988).

We thank Miss Ch. Schmidhalter, Miss M. Kilchenmann and Mr D. Meier for technical assistance. The work was supported by the Swiss National Science Foundation and the 'Jubiläumsstiftung der Schweizerischen Lebensversicherungs- und Rentenanstalt für Volks gesundheit und medizinische Forschung'.

References


(Received 22 February 1989 – Accepted 4 April 1989)