Biochem. J. (1992) 286, 945–950 (Printed in Great Britain)

Thrombin promotes actin polymerization in U937 human monocyte-macrophage cells

Analysis of the signalling mechanisms mediating actin polymerization

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The U937 human monocyte-macrophage cell line was used to examine the effect of thrombin, an ill-defined chemoattractant, on the polymerization of actin, a process essential for cell motility. In differentiated macrophage-like U937 cells, thrombin (0.5–50 units/ml) caused a rapid dose-dependent increase in the formation of filamentous (F-) actin, detected by the staining of F-actin with the fluorescent toxin, 7-nitrobenz-2-oxa-1,3-diazole-phallacidin. In contrast with other chemoattractants such as *N*-formylmethionyl-leucylphenylalanine or C5a, actin polymerization in response to thrombin occurred via a pertussis-toxin-insensitive G_i-(inhibitory G-protein) independent signalling pathway. Further, this response was not affected by the Ca^{2+} chelator EGTA or by the specific protein kinase C (PKC) inhibitor RO-31-8220. The response to thrombin was not mimicked by the Ca^{2+} ionophore ionomycin or by the direct PKC activator phorbol 12-myristate 13-acetate. The thrombin response was, however, inhibited by the non-specific protein kinase inhibitor staurosporine. The present results suggest that in U937 cells thrombin stimulates the formation of F-actin via a signalling pathway independent of (i) the activation of PKC, (ii) the mobilization of intracellular Ca^{2+} and (iii) the activation of Ca^{2+}-dependent protein kinases, but dependent on the activation of an undefined staurosporine-sensitive protein kinase.

INTRODUCTION

The accumulation of leucocytes at sites of inflammation and the subsequent phagocytosis and destruction of invading organisms or cells is dependent on the integrity of the peripheral cytoskeleton of these cells [1,2]. The major component of the cytoskeleton is actin, which in response to chemotactic stimuli rapidly and transiently is converted from a monomeric, globular form, G-actin, to a needle-like, filamentous form, F-actin [1,2].

The signal-transduction mechanisms that link chemoattractant-receptor occupancy to actin polymerization in leucocytes is not well understood, but has been best documented for the formylated peptide *N*-formylmethionyl-leucylphenylalanine (FMLP) in neutrophils [3–7] and the complement fragment C5a in monocytic cells [8,9]. Leucocyte receptors for FMLP or C5a are in each case coupled via a pertussis-toxin-sensitive G-protein to the activation of the enzyme phospholipase C (PLC), with the resultant formation of inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol [7,9,10]. $InsP_3$ and diacylglycerol respectively mobilize intracellular Ca^{2+} and activate the enzyme protein kinase C (PKC) [11]. Both mobilization of intracellular Ca^{2+} and PKC activation occur with time courses that precede or accompany the initiation of actin polymerization, suggesting a cause–effect relationship between the second messengers and actin polymerization [6]. However, the requirement for mobilization of intracellular Ca^{2+} or PKC activation as initiating signals for actin polymerization is ambiguous, with studies either supporting a role [6,12] or no role [8,13–15] for the second messengers in this response. The increase in cellular F-actin content in response to FMLP or C5a does, however, involve a G-protein, as this response is sensitive to pertussis toxin [4,8,15]. Although the primary role of G-proteins in actin polymerization is most likely at the level of PLC activation, a more direct role of G-proteins downstream of PLC has also been suggested [15]. This is based on the observation that the introduction of guanine nucleotides into the cytosol of electropermeabilized neutrophils triggers actin polymerization independently of PLC activation [15].

The serine proteinase thrombin is chemotactic for human peripheral-blood monocytes [16] and various monocyte-macrophage cell lines [17], in addition to its well-known stimulatory effects on blood platelets, vascular smooth muscle or endothelium [18–21]. However, in contrast with the chemoattractants FMLP or C5a, little if anything is known about the effect of thrombin on actin polymerization and the signalling pathways initiating this response in monocyte-macrophage cells. In the present study we have employed the U937 human monocytic cell line [22,23], a cell line known to respond chemotactically to thrombin [17], and the selective fluorescent F-actin stain, 7-nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin [3], to characterize the effect of thrombin on actin polymerization in monocytic cells.

MATERIALS AND METHODS

Materials

NBD-phallacidin was purchased from Molecular Probes (Eugene, OR, U.S.A.). Pertussis toxin was purchased from Porton Products (Salisbury, U.K.). Indo-1/AM and ionomycin were obtained from NovaBiochem, Nottingham, U.K. FMLP, EGTA, bovine thrombin (100 units/mg of protein) and phorbol 12-myristate 13-acetate (PMA) were bought from Sigma, Poole, Dorset, U.K. RPMI-1640 medium and foetal-calf serum were obtained respectively from Flow, Irvine, Scotland, U.K., and Gibco, Paisley, Scotland, U.K. The PKC inhibitors staurosporine and RO-31-8220 were generously provided by Ciba–Geigy, Basle, Switzerland, and Roche Products, Welwyn Garden City, Herts., U.K., respectively.

Methods

Cell culture conditions. U937 cells were grown in RPMI-1640

Abbreviations used: FMLP, *N*-formylmethionyl-leucylphenylalanine; PLC, phospholipase C; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular Ca^{2+} concn.; NBD-, 7-nitrobenz-2-oxa-1,3-diazole-; PMA, phorbol 12-myristate 13-acetate; RFI, Relative Fluorescence Index.

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medium (plus 10 % foetal-calf serum and 1.5 mM-glutamine) at 37 °C in an air/CO_2 (19:1) humidified atmosphere. The primary human bladder carcinoma cell line HTB 5637 (American Type Culture Collection, Bethesda, MD, U.S.A.) was used to prepare conditioned medium [24]. U937 cells (1×10^5/ml) were exposed to 20 % (v/v) conditioned medium for 48 h. Several studies have reported that treatment with conditioned medium results in differentiated, functionally more mature, U937 cells [22–24]. Our own studies have shown that after 48 h conditioned-medium-differentiated U937 cells, as compared with undifferentiated cells, display significantly increased expression of the cell-surface antigen CD23 [24] and increased agonist-stimulated mobilization of intracellular Ca^{2+} and thromboxane B_2 synthesis [23,24]. Consequently, in the present study, after 48 h the differentiated cells were harvested, washed and resuspended to a cell density of $(4–6) \times 10^6$/ml in a buffer, pH 7.4, containing 10 mM-Hepes, 140 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 10 mM-glucose and 1 mg of BSA/ml. Cell viability was greater than 95 %, as assessed by Trypan Blue dye exclusion.

Analysis of cellular F-actin content. The cellular content of polymerized actin (F-actin) was determined by NBD-phallacidin staining of fixed U937 cells, by a modification of the method of Howard & Meyer [3]. Briefly, 100 µl portions of cell suspensions incubated at 37 °C were treated with the appropriate vehicle, drug or agonist, and the reaction was terminated at designated time points by addition of 100 µl of a lysis staining/phosphate-buffered saline (PBS; 137 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4), pH 7.0, solution containing 8 % (w/v) paraformaldehyde, 0.30 µM-NBD-phallacidin and 0.20 mg of lysophosphatidylcholine/ml. The cells were left at room temperature for 30 min, sedimented at 13000 *g* (MSE Microcentaur) for 90 s, resuspended in 500 µl of PBS, washed again and finally resuspended in 500 µl of PBS.

The stained cells were analysed on an Epics Profile fluorescence activated cell sorter (Coulter Electronics), with excitation at 488 nm by an argon laser and emission recorded at 525 nm. A minimum of 5000 cells per sample were counted, with cell debris and cell aggregates gated out. The mean fluorescence intensity per cell was used as a measure of the F-actin content per cell. The results are expressed as the Relative Fluorescence Index (RFI), which represents the ratio of mean fluorescence intensity of drug or agonist-treated cells to the mean fluorescence intensity of vehicle-treated cells.

Measurement of intracellular Ca^{2+}. Differentiated U937 cells (3×10^6/ml) resuspended in the Hepes buffer, pH 7.4, were incubated at 37 °C for 45 min with 3 µM of the fluorescent Ca^{2+}-sensitive dye Indo-1/AM [25]. The cells were washed and resuspended in the Hepes buffer, pH 7.4, to a cell count of $(1–1.5) \times 10^6$/ml. Cell samples (500 µl) were incubated at 37 °C with stirring and the fluorescence levels measured using a Shimadzu RF-5000 spectrofluorophotometer. Fluorescence was monitored at an excitation wavelength of 355 nm and emission wavelengths of 400 nm and 490 nm. Intracellular calcium levels ($[Ca^{2+}]_i$) were determined by the method of Grynkiewicz *et al.* [25]. The results are expressed as the maximum change in $[Ca^{2+}]_i$ {$\Delta[Ca^{2+}]_i$ (nM): peak stimulated $[Ca^{2+}]_i$ minus resting $[Ca^{2+}]_i$}.

Measurement of superoxide generation. The release of superoxide was determined by its ability to reduce ferricytochrome *c* [26]. Differentiated U937 cells [$(3.5–4.0) \times 10^6$/ml] in Hepes buffer, pH 7.4, were plated out as 0.25 ml samples into 24-well 1 ml tissue-culture plates. Each cell sample was diluted to 0.5 ml with a solution of ferricytochrome *c* (Sigma, horse heart type III) in Hepes buffer, pH 7.4, to give a final ferricytochrome *c* concentration of 80 µM. The cells were preincubated at 37 °C for 15 min before addition of vehicle or drug for 2–10 min before being stimulated with 160 nM-PMA for 60 min. In some experiments, cells were stimulated in the presence of 30 µg of superoxide dismutase/ml. Reactions were stopped by transferring the reaction mixtures into micro-centrifuge tubes left on ice, followed by centrifugation at 13000 *g* for 90 s. The supernatants (300 µl) were removed, diluted 5-fold with the Hepes buffer, pH 7.4, and the A_{550} was determined spectrophotometrically. Reaction mixtures with no cells were used as blanks. The results were expressed as nmol of superoxide formed/60 min per 10^6 cells, and were calculated from the absorption coefficient $\Delta A_{550} = 21 \times 10^3\ M^{-1} \cdot cm^{-1}$ [27]. In experiments with superoxide dismutase, the reduction of ferricytochrome *c* in response to PMA was inhibited by superoxide dismutase by > 95 %, indicating that the generation of superoxide was responsible for the reduction of ferricytochrome *c* (results not shown).

Statistical analysis. Analysis of the data for statistical significance was carried out by Student's *t* test. Means were considered significantly different when $P < 0.05$.

RESULTS

Effect of thrombin on the cellular F-actin content

We have previously reported, using conditioned-medium-differentiated U937 cells, that thrombin stimulated the formation of InsP_3 and mobilized intracellular Ca^{2+}, with maximal effects occurring at approx. 50 units of thrombin/ml [23]. Fig. 1 demonstrates that thrombin also causes a dose- and time-dependent increase in F-actin content in conditioned-medium-differentiated U937 cells, as assessed by the increase in fluores-

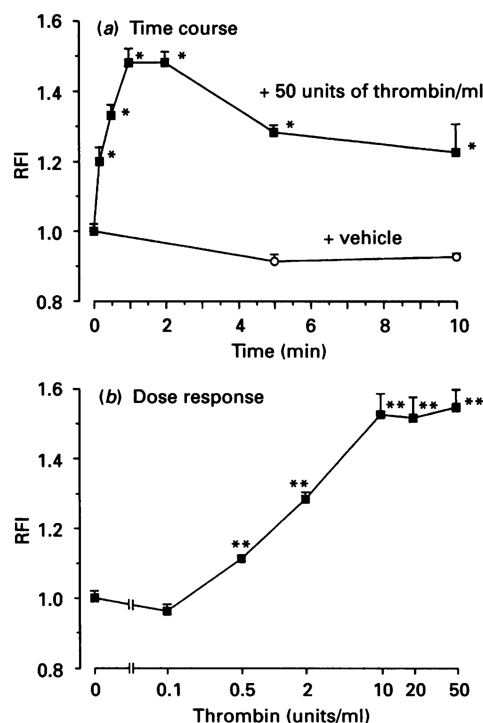

Fig. 1. Time course (*a*) and dose–response (*b*) effect of thrombin on the F-actin content in differentiated U937 cells

Macrophage-like U937 cells were (*a*) challenged with saline vehicle or 50 units of thrombin/ml for selected times or (*b*) challenged for 2 min with various concentrations of thrombin before being fixed and fluorescently stained with NBD-phallacidin, as described under 'Methods'. The stained cells were analysed by flow cytometry, and the results expressed as RFI values, as described under 'Methods'. The results are means ± S.E.M. ($n = 4$) from two separate experiments: *$P < 0.05$ compared with the 0 min time point; **$P < 0.05$ compared with the 2 min vehicle control.

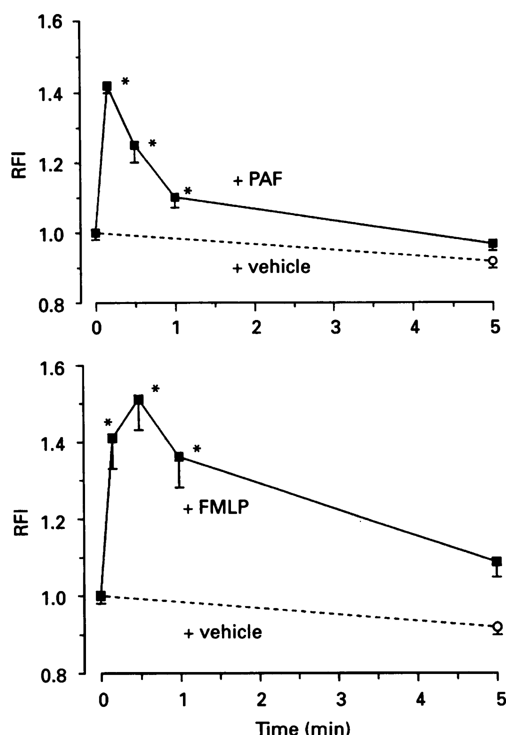

Fig. 2. Effect of platelet-activating factor (PAF) or FMLP on F-actin formation

Cells were stimulated with saline vehicle, 1 μM-PAF or FMLP for various periods of time. All other conditions are as described in the legends to Fig. 1: $*P < 0.05$ compared with the 0 min time point.

cence of NBD-phallacidin-stained cells. In response to 50 units of thrombin/ml a significant increase in F-actin content was detected 10 s after cell stimulation, with the response reaching a peak 1–2 min later and remaining significantly above resting levels 10 min after stimulation (Fig. 1*a*). A similar time course was also seen in undifferentiated monocytic U937 cells (result not shown). By using a fixed time point of 2 min to examine the dose–response effect of thrombin, a significant increase in F-actin content was seen in response to 0.5 unit of thrombin/ml, with $\geqslant$ 10 units/ml being maximally effective (Fig. 1*b*). In all subsequent experiments U937 cells were stimulated with 10 units of thrombin/ml for 2 min.

The time course of thrombin-stimulated F-actin formation was distinct from that seen in response to two other chemotactic agents, 1 μM-FMLP or 1 μM-platelet-activating factor. Despite these agonist concentrations evoking similar maximum increases in F-actin content as 50 units of thrombin/ml (Figs. 1 and 2), the increase in F-actin reached a peak 10–30 s after addition of FMLP or platelet-activity factor, and diminished to negligible levels 5 min later.

Effect of pertussis toxin on chemoattractant-stimulated F-actin formation

Earlier studies, including our own, using differentiated U937 cells, have reported that pertussis-toxin pretreatment results in the ADP-ribosylation and inactivation of the 40–41 kDa inhibitory G-protein, G_i, with concomitant inhibition of PLC activation and intracellular Ca^{2+} mobilization in response to FMLP [10,23]. Table 1 shows that pertussis-toxin pretreatment (500 ng/ml for 3 h at 37 °C) also significantly inhibited the increase in F-actin content in response to 1 μM-FMLP. In contrast, in the same pertussis-toxin-pretreated cells the formation of F-actin in response to thrombin was not significantly

Table 1. Effect of pertussis toxin on thrombin- or FMLP-stimulated F-actin formation

Cells were pretreated with 0.42 % (v/v) glycerol vehicle or 500 ng of pertussis toxin/ml for 3 h at 37 °C before being challenged with saline vehicle or 10 units of thrombin/ml for 2 min, or 1 μM-FMLP for 10 s. Pertussis toxin alone had no significant effect on the content of F-actin. The results are means $\pm$ S.E.M. ($n = 6$) from three separate experiments, each experiment performed in duplicate: $*P < 0.05$ compared with glycerol vehicle + FMLP.

	RFI	
	+ glycerol vehicle	+ pertussis toxin
+ saline vehicle	1.00 $\pm$ 0.07	0.96 $\pm$ 0.06
+ thrombin	1.42 $\pm$ 0.09	1.40 $\pm$ 0.11
+ FMLP	1.36 $\pm$ 0.05	1.08 $\pm$ 0.08*

altered ($P > 0.05$ compared with vehicle + thrombin). These results suggest that in U937 cells thrombin, unlike other chemoattractants such as FMLP (Table 1) or C5a [8], stimulates actin polymerization via a G_i-independent pertussis-toxin-insensitive signalling pathway.

Effect of rises in $[Ca^{2+}]_i$ on cellular F-actin content

In Indo-1-loaded macrophage-like U937 cells, thrombin (10 units/ml) caused a rapid 111 $\pm$ 6 nM increase in $[Ca^{2+}]_i$ above resting levels (102 $\pm$ 4 nM) (mean $\pm$ S.E.M., $n = 6$ from two separate experiments). This increase was predominantly due to the influx of extracellular Ca^{2+} into the cell cytosol, as a 2 min preincubation with the extracellular-Ca^{2+} chelator EGTA (5 mM) significantly decreased the rise in $[Ca^{2+}]_i$ in response to thrombin to only 30 $\pm$ 9 nM above resting levels (80 nM). However in parallel experiments a 2 min pretreatment with EGTA had no significant effect on thrombin-stimulated F-actin formation, i.e. RFI values were: vehicle alone 1.00 $\pm$ 0.06, vehicle + thrombin 1.60 $\pm$ 0.14, EGTA + thrombin 1.52 $\pm$ 0.08 ($P > 0.05$ compared with vehicle + thrombin). EGTA alone had no significant effect on the F-actin content (RFI value 1.12 $\pm$ 0.08; $P > 0.05$ compared with vehicle). These results suggest that the influx of extracellular Ca^{2+} is not a prerequisite for F-actin formation. It is also unlikely that the intracellular Ca^{2+} released from the internal Ca^{2+} stores by thrombin would be sufficient to trigger actin polymerization. In support of this, differentiated U937 cells were challenged with the Ca^{2+} ionophore ionomycin (200 nM) and the level of either $[Ca^{2+}]_i$ or F-actin was determined. Despite ionomycin causing an increase of ~ 300 nM in $[Ca^{2+}]_i$ from resting levels, it failed to increase the cellular content of F-actin over a 5 min time course (Fig. 3). Taken together, these results suggest that a rise in $[Ca^{2+}]_i$ is an insufficient signal for thrombin-stimulated actin polymerization.

Role of PKC in actin polymerization

To assess whether PKC activation is a signal for thrombin-stimulated actin polymerization, use was made of two PKC inhibitors, staurosporine and its analogue RO-31-8220 [28]. Although both compounds are potent PKC inhibitors with similar IC_{50} values *in vitro* of 10 nM, and IC_{50} values *in vivo* in cells such as blood platelets or T-cells of less than 1 μM [28,29], staurosporine is relatively non-specific, inhibiting tyrosine-, cAMP- and Ca^{2+}/calmodulin-dependent protein kinases with potencies similar to that required for the inhibition of PKC [28,30]. In contrast, RO-31-8220 is highly specific, being 100–1000 times more potent for PKC relative to the other protein kinases [28]. Fig. 4 demonstrates that a 2 min preincubation with

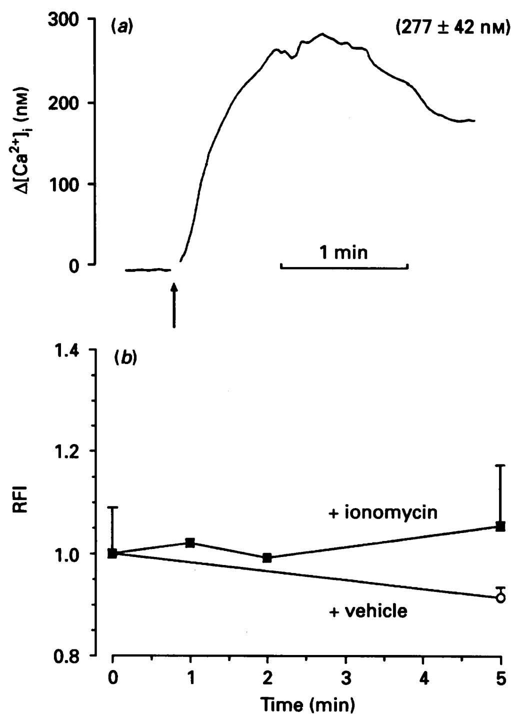

Fig. 3. Effect of ionomycin on $[Ca^{2+}]_i$ and F-actin content

Indo-1-loaded cells were challenged with 200 nM-ionomycin (as indicated by the arrow), and either (*a*) $\Delta[Ca^{2+}]_i$ or (*b*) the content of F-actin was measured. The values beside the $[Ca^{2+}]_i$ trace, which is a representative one, denotes the maximum change in $[Ca^{2+}]_i$ from resting levels. The results are means ± S.E.M. ($n = 6$) from two separate experiments, each performed in triplicate. All other conditions are as described in the legend to Table 1.

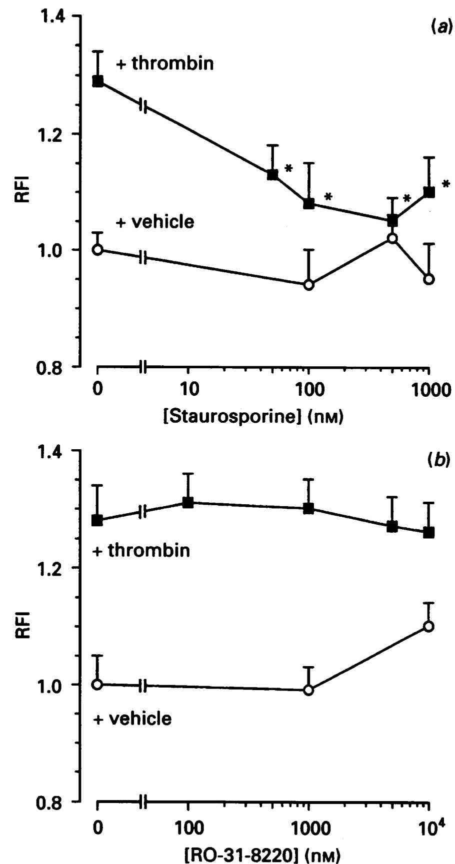

Fig. 4. Effect of staurosporine or RO-31-8220 on thrombin stimulated F-actin formation

Cells were preincubated with (*a*) 0.01% dimethyl sulphoxide (DMSO) vehicle or staurosporine for 2 min or (*b*) 0.1% DMSO vehicle or RO-31-8220 for 10 min before addition of saline vehicle or 10 units of thrombin/ml for a further 2 min. The results are means ± S.E.M. ($n = 6$–9) from three separate experiments: *$P < 0.05$ compared with the DMSO vehicle + thrombin.

staurosporine (50 nM–1 μM) led to a concentration-dependent inhibition of thrombin-stimulated F-actin formation. Significant inhibition occurred with 50 nM-staurosporine, with maximal inhibition occurring at ⩾ 0.5 μM staurosporine. In the absence of thrombin, staurosporine alone had no significant effect on the F-actin content ($P > 0.05$ compared with vehicle). It seems unlikely, however, that the inhibition by staurosporine of F-actin formation in response to thrombin could be due to PKC inhibition, since a 10 min preincubation with RO-31-8220 (0.1–10 μM) had no significant effect on the thrombin-stimulated response, even at 10 μM-RO-31-8220 (Fig. 4). RO-31-8220 alone had no effect on the cellular F-actin content, although a small, though not significant ($P > 0.05$), increase was seen with 10 μM-RO-31-8220. Neither staurosporine (1 μM) nor RO-31-8220 (10 μM) was cytotoxic, as cell viability was > 95% in thrombin-stimulated cells pretreated with either inhibitor.

To confirm that both staurosporine and RO-31-8220 were inhibiting PKC in differentiated U937 cells, the effect of the two inhibitors on superoxide generation in response to the direct PKC inhibitor PMA [31] was assessed. In cells pretreated with 1 μM-staurosporine for 2 min or with 10 μM-RO-31-8220 for 10 min, superoxide generation in response to 160 nM-PMA was significantly inhibited ($P < 0.05$ compared with PMA alone) by 92% and 99% respectively, i.e. nmol of superoxide/60 min per 10^6 cells: vehicle (2 min) + vehicle (60 min) 4.57 ± 0.45, vehicle (2 min) + PMA (60 min) 19.83 ± 0.69, staurosporine + PMA 5.74 ± 0.49, vehicle (10 min) + vehicle (60 min) 2.90 ± 0.33, vehicle (10 min) + PMA (60 min) 12.0 ± 1.49, RO-31-8220 (10 min) + PMA (60 min) 2.91 ± 0.03 (mean ± S.E.M., $n = 6$–8 from two or three separate experiments). Neither inhibitor alone had any

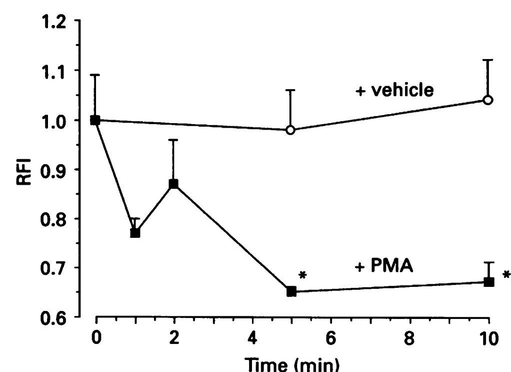

Fig. 5. Effect of PMA on F-actin content

PMA (160 nM) was added to differentiated U937 cells, and the content of F-actin was measured over a 10 min period. The results are means ± S.E.M. ($n = 6$) from two separate experiments, each performed in triplicate: *$P < 0.05$ compared to the 0 min time point.

significant effect on superoxide generation in unstimulated cells (results not shown). Further, in preliminary experiments (result not shown) the effect of RO-31-8220 (10 μM) and staurosporine (0.5 μM) on the protein [^{32}P]phosphorylation of a 45 kDa protein, the major PKC substrate in U937 cells [32], in response to PMA

was assessed. Both RO-31-8220 and staurosporine inhibited agonist-stimulated 45 kDa-protein phosphorylation. These results indicate that in U937 cells both agents were effective PKC inhibitors.

On the basis of the increased inhibitory selectivity of RO-31-8220 relative to staurosporine for PKC over other protein kinases [28], the present results suggest that the activation of PKC is unlikely to be a signal for thrombin-stimulated actin polymerization. The lack of PKC involvement in this response is also supported by the observation that PMA (160 nM) failed to increase the content of F-actin, and in fact caused significant actin depolymerization 5 min after cell stimulation (Fig. 5).

DISCUSSION

In the present study we have used the U937 human monocyte-macrophage cell line to characterize the effect of the chemotactic agent thrombin [16,17] on the polymerization of actin, a process essential for cell motility [1,2]. Our results demonstrate that thrombin behaves like other chemotactic agents, e.g. FMLP, PAF and C5a (Fig. 2) [8], in causing a rapid increase in F-actin formation. In terms of the signalling pathway initiating actin polymerization, the finding that pertussis toxin failed to affect thrombin-stimulated F-actin formation indicates that this response is mediated via a G_i-independent signalling pathway. As such, the thrombin signalling pathway is clearly distinct from the pertussis-toxin-sensitive G_i-dependent signalling pathway initiating actin polymerization in response to other chemoattractants (Table 1) [4,7,8,15].

We have also addressed the nature of the G_i-independent signalling pathway for thrombin-dependent actin polymerization. For example, this signalling pathway could be derived from the activation of a pertussis-toxin-insensitive PLC. In fact, in a previous study in macrophage-like U937 cells [23], we reported that pertussis toxin only partially inhibited intracellular Ca^{2+} mobilization in response to thrombin, whereas it totally inhibited the $[Ca^{2+}]_i$ response to FMLP. This suggested that U937-cell receptors for thrombin, though not for FMLP, were coupled via pertussis-toxin-sensitive and -insensitive G-proteins to PLC. Relating this earlier observation to the present study, it is possible that actin polymerization in response to thrombin is initiated by signals derived from the activation of the pertussis-toxin-insensitive G-protein-coupled PLC. (A corollary to this is that, at present, there is no information as to whether pertussis-toxin-insensitive G-proteins such as Gq or Gz [33,34] exist in U937 cells.) The results of this study suggest, however, that the potential end products of PLC activation, $[Ca^{2+}]_i$ and PKC activation, are unlikely to be the initiating signals for actin polymerizatiion.

The requirement for a rise in $[Ca^{2+}]_i$ for actin polymerization in monocytic cells and other leucocyte cell populations such as neutrophils remains unclear. Studies in neutrophils have reported that Ca^{2+} ionophores can stimulate actin polymerization [6,13,14], though in most cases their effects seem to be indirect [13,14]. For example, ionomycin-stimulated actin polymerization was shown to be inhibited by a leukotriene B_4 (LTB_4) antagonist [14], implying that ionomycin caused the formation of LTB_4, with LTB_4 in turn triggering actin polymerization. Further, although a rise in $[Ca^{2+}]_i$ may not be an initiating signal, it may modulate the rate of actin polymerization/depolymerization [5,14]. In the present study using macrophage-like U937 cells, the finding that the thrombin-stimulated rise in $[Ca^{2+}]_i$ is not an initiating signal is suggested by the fact that the Ca^{2+} chelator EGTA had no effect on thrombin-stimulated F-actin formation, whereas ionomycin failed to mimic the effect of thrombin on F-actin formation.

The requirement for PKC activation in actin polymerization in leucocytes is equally ambiguous. Earlier studies in neutrophils with direct PKC activators such as PMA have shown that PKC activation can result in actin polymerization [6,12]. In contrast, in the present study PMA caused actin depolymerization, whereas the highly specific PKC inhibitor RO-31-8220, at concentrations that substantially inhibit PKC (the present study) [28,29], had no effect on thrombin-stimulated F-actin formation. This suggests that PKC activation is not involved in actin polymerization, at least in U937 cells, although it may be involved in actin depolymerization.

Based on our results with RO-31-8220 and with PMA, the inhibition by staurosporine of thrombin-stimulated F-actin formation cannot be due to the inhibition of PKC activation. As staurosporine potently inhibits other protein kinases in addition to PKC, e.g. tyrosine-, cyclic AMP- and Ca^{2+}/calmodulin-dependent protein kinases [28,30], our results suggest that thrombin-stimulated F-actin formation may be regulated by a staurosporine-sensitive PKC-independent protein kinase. However, the inhibition by staurosporine of cyclic-AMP- or Ca^{2+}/calmodulin-dependent protein kinases cannot explain the inhibitory effect of staurosporine on F-actin formation, since (i) thrombin has no effect on adenylate cyclase in U937 cells [23], so cannot activate cyclic-AMP-dependent protein kinase, and (ii) a rise in $[Ca^{2+}]_i$ is not a signal for actin polymerization, so concomitantly Ca^{2+}/calmodulin-dependent protein kinases cannot be involved. The possibility therefore exists that in U937 cells thrombin activates a staurosporine-sensitive tyrosine kinase signalling pathway linked to actin polymerization. Although this aspect remains to be examined, recent reports have suggested a role for tyrosine kinases in neutrophil chemotaxis and actin polymerization [35,36], and thrombin is known to activate tyrosine kinases in some cell systems [37,38].

In conclusion, we have shown that thrombin stimulates actin polymerization in U937 cells. The signalling pathway initiating this response is independent of intracellular Ca^{2+} mobilization or PKC activation. Further, the thrombin signalling pathway does not involve G_i, which contrasts with other monocyte chemoattractants. However, actin polymerization is regulated by a staurosporine-sensitive protein kinase, the identity of which remains to be established.

We thank Gillian Angus, Department of Immunology, Royal Postgraduate Medical School, for her help with the flow-cytometry studies. This work was supported by a grant from the National Asthma Campaign.

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Received 15 January 1992/25 March 1992; accepted 7 April 1992