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The regulation by phosphorylation of 'priming' of phospholipase A_2 activity in the neutrophil model system, differentiated HL60 cells

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1 Differentiated HL60 cells have been utilized as a model system to examine the 'priming' of neutrophil phospholipase A_2 activity. In control cells activation of phospholipase A_2 by a 5 min stimulation with the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (100 nM) was essentially undetectable. When cells were primed by preincubation with 5 μM cytochalasin B for 5 min arachidonate release, a measure of phospholipase A_2 activation, was observed within 20 s.

2 Priming by cytochalasin B did not involve or require a change in intracellular free calcium concentration.

3 Priming was associated with an increase in general protein tyrosine phosphorylation and could also be induced by the receptor tyrosine kinase agonist granulocyte macrophage colony-stimulating factor (GM-CSF, 20 ng ml^{-1}) and be mimicked by treatment with the phosphotyrosine phosphatase inhibitor perhydrovanadate (0.5 mM). However, an increase in MAP kinase activity was not involved in the priming process.

4 Western blot analysis demonstrated that phospholipase A_2 was phosphorylated in both control and primed cells, but that an increase in the amount of membrane associated enzyme was found in the primed cells.

5 Thus priming appears to be due to membrane association of the phospholipase and this may be regulated by tyrosine kinase activities.

Keywords: Phospholipase A_2; priming; phosphorylation; membrane localization; neutrophil

Introduction

The exposure of resting neutrophils to agents such as granulocyte/macrophage colony-stimulating factor (GM-CSF) is apparently without effect (Yuo *et al*., 1991). Nevertheless, incubation with such agonists results in enhanced responses to subsequent stimulation with, for example, formyl-methionyl-leucyl-phenylalanine (fMLP), an effect observed at both the level of superoxide-generating respiratory burst (Phillips & Hamilton, 1990) and of phospholipase activation (Cockcroft, 1992). This 'priming' effect is clearly apparent for the stimulation of phospholipase A_2 activity which catalyses the generation of arachidonate from phosphatidylcholine. The arachidonate can be utilized in the generation of eicosanoids, or it may play a distinct messenger role itself, e.g. in regulating rac function (see Dennis, 1994 for review).

The mechanism(s) involved in the priming response are unknown. However, a number of studies have suggested that changes in the cytoskeleton are critical, this is emphasized by the ability of cytochalasins to induce priming artificially (Honneycutt & Niedel, 1986). It has been further suggested that there is a role for protein kinase activation, though there are conflicting data as to whether this involves protein kinase C or a member of the tyrosine kinase family (Lloyds & Hallett, 1994). This is particularly relevant to the regulation of phospholipase A_2 activity since the enzyme can be phosphorylated and activated *in vitro* by mitogen-activated protein (MAP) kinase (Lin *et al*., 1993).

In order to investigate the regulation of priming of phospholipase A_2 activity, we have utilized the HL60 model cell system, these are differentiated into neutrophil-like cells by exposure to dimethyl sulphoxide. The experimental advantage provided by utilizing this cell line model, is that the cells can be labelled with radioactive precursors and reproducible populations of cells can be examined. Using this system we demonstrated that MAP kinase catalysed phosphorylation of phospholipase A_2 (PLA_2) is not sufficient to induce priming. Rather, we demonstrated that priming involves the activation of tyrosine kinases with consequent membrane localization of the phospholipse.

Methods

Cell culture

HL60 cells were seeded at 5×10^5 cells ml^{-1} in RPMI 1640 containing 15% (v/v) heat-inactivated foetal calf serum. For differentiation dimethyl sulphoxide (DMSO) was added to a final concentration of 1.3% (v/v) for 4 days. Rat-1 RafER4 fibroblasts were seeded at 1×10^4 cells ml^{-1} in DMEM containing 10% (v/v) NBCS and 400 μg ml^{-1} geneticin. When between 80–90% confluent, cells were quiesced overnight by replacing the medium with serum-free DMEM containing 400 μg ml^{-1} geneticin.

Arachidonate generation

Three day DMSO-differentiated HL60 cells were labelled with 0.25 μCi ml^{-1} [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid for 17 h. The cells were washed in RPMI 1640 (phenol red free) containing 20 mM HEPES pH 7.4, 10 mM glucose and 0.1% (w/v) bovine serum albumin (fraction V) (RBG) for 2 h, pelleted by centrifugation at 100 *g* for 3 min at 37°C and resuspended in fresh buffer at a density of 10^7 cells ml^{-1}.

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Incubations were performed as described and terminated by the addition of methanol:acetic acid (100:1.5) containing 4 μg arachidonic acid. The lipids were extracted into chloroform, dried under vacuum, resuspended in chloroform:methanol (2:1) and spotted onto heat-activated, plastic-backed silica G thin-layer chromatography plates which were developed in hexane:diethyl ether:acetic acid (70:30:2). The plates were air dried and the fatty acids identified by iodine staining. The areas corresponding to free fatty acids were excised and placed in a scintillation vial, solubilized by the addition of methanol and the radioactivity determined by liquid scintillation spectrophotometry.

Western blotting of cPLA$_2$ localization and phosphorylation state

Differentiated HL60 cells were washed twice in RBG; 2×10^7 cells were incubated with the stated additions at 37°C. Incubations were terminated by washing twice in ice-cold PBS. The washed pellet was resuspended in ice cold lysis buffer 1 (20 mM HEPES pH 7.4, 2.5 mM EGTA, 2.03 mM calcium chloride (a concentration which gave rise to a final free value of 150 nM), 20 mM PMSF, 2 mM orthovanadate, 40 μg ml^{-1} leupeptin and 40 μg ml^{-1} aprotinin) for 1 hour. The samples were homogenized, cell debris pelleted by centrifuging at $50 \times g$ for 10 min at 4°C and the membranes isolated by centrifugation at $150{,}000 \times g$ for 30 min at 4°C. An equal volume of ice-cold lysis buffer 2 (lysis buffer 1 containing 100 mM NaF, 10 mM $MgCl_2$, 2 mM EDTA, 80 mM sodium tetraphosphate and 2% (w/v) Triton X-100) was added to the cytosolic fractions, whilst the membrane pellet was solubilized in 0.5 ml lysis buffer 3 (lysis buffer 1 containing 50 mM NaF, 5 mM $MgCl_2$, 1 mM EDTA, 40 mM sodium tetraphosphate and 1% (w/v) Triton X-100). All samples were then sonicated briefly, insoluble material removed by centrifugation and protein content determined by use of the micro BCA protein assay kit.

Thirty micrograms of protein was precipitated with trichloroacetic acid (TCA), solubilized and separated on 10% polyacrylamide gels when the localization was examined, or 12.5% polyacrylamide gels when the phosphorylation state was analysed. Proteins were transferred onto nitrocellulose, blocked in NaTT (150 mM NaCl, 20 mM Tris/HCl (pH 7.2) and 0.4% (v/v) Tween 20) containing 5% (w/v) non-fat milk and 5% (v/v) goat serum, for 4 h at 4°C and probed with a 1:10,000 dilution of rabbit anti-peptide, anti-cPLA$_2$ polyclonal antibody (raised against amino acids 727–746) with detection by ECL. The efficiency and reproducibility of transfer was checked by staining the post-transfer gel with Coomassie Blue. Standard cPLA$_2$ was purified from baculovirus expressing sf9 cells.

Measurement of agonist-stimulated calcium release

Differentiated HL60 cells were washed twice in RBG and resuspended at a density of 10^7 cells per ml in RBG containing 2 μM Fura-2-AM before being incubated at 37°C for 45 min. The cells were then washed twice in RBG and resuspended to a density of 5×10^6 cells per ml.

1 ml of cell suspension was added to a cuvette containing a magnetic stirrer and incubated at 37°C either in the absence or presence of 5 μM cytochalasin B, 100 nM fMLP or 0.5 mM perhydrovanadate for the times stated. The cuvette was then transferred to the fluorimeter and the cells agitated by stirring. Stimulants in RBG were added to the cells through a porthole.

The fluorimeter detector was set at excitation wavelengths of 340 nm and 380 nm, which corresponded to Ca^{2+}-bound and -unbound Fura-2 and an emission wavelength of 510 nm. The ratio of bound:unbound Fura-2 fluorescence was monitored over the times stated. Quantitative values of Ca^{2+} concentration were determined by lysing the cells with Triton X-100, therefore giving a total Ca^{2+} concentration and then adding excess EDTA to give a background Ca^{2+} concentration.

Immunoblotting for MAP kinase localization and phosphorylation state

Differentiated HL 60 cells were washed twice in RBG and resuspended at a density of 2×10^6 cells per ml with the agonists described. Quiesced Rat-1 RafER 4 fibroblasts were washed twice with DBG and preincubated with 1 μM β-oestradiol to induce expression of the v-raf gene (Samuels *et al.*, 1993) and thus activate the MAP kinase or DBG for 45 min. Reactions were terminated by washing twice in ice-cold PBS. The cells were lysed in 1 mM potassium dihydrogen orthophosphate, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM EGTA, 40 μg ml^{-1} aprotinin, 40 μg ml^{-1} leupeptin and 0.5% (w/v) Triton X-100. Protein content was determined by the micro BCA protein assay kit and 30 μg protein separated on 12.5% polyacrylamide gels. Proteins were transferred onto nitrocellulose and blocked in NaTT containing 5% (w/v) bovine serum albumin (high purity fraction V) for 4 h at 4°C. A 1:50,000 dilution of mouse anti-ERK1/2 was added overnight in NaTT containing 1% (w/v) BSA at 4°C. Nitrocellulose was washed frequently over a 2 h period with NaTT before incubation for 1 h with an anti-mouse HRP-linked polyclonal antibody. The 2 h washing procedure was repeated and the HRP-highlighted proteins identified by ECL.

MAP kinase in vitro *activity measurements*

Cells were treated and lysed as described under MAP kinase immunoblotting. A volume of sample in MAP kinase lysis buffer, corresponding to 50 μg total protein, was diluted in

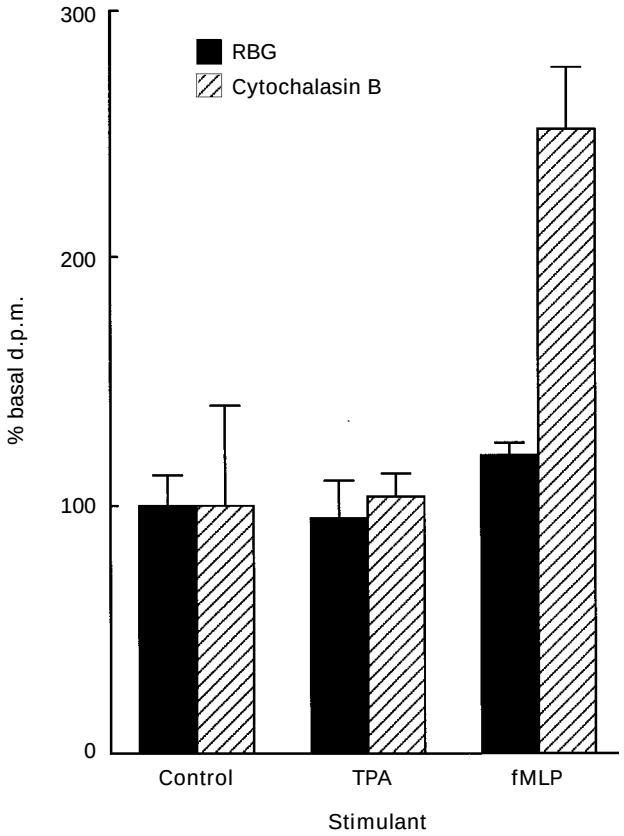

Figure 1 Comparison of agonist-stimulated [^{3}H]-arachidonate release in cytochalasin B primed and unprimed differentiated HL60 cells. [^{3}H]-arachidonate labelled cells were washed, preincubated for 5 min with either RBG or 5 μM cytochalasin B and then stimulated with either RBG, 100 nM TPA for 10 min or 100 nM fMLP for 5 min. Result is a representation of 3 individual experiments with values being presented as % basal d.p.m. where the basal was 400 ± 150 d.p.m.

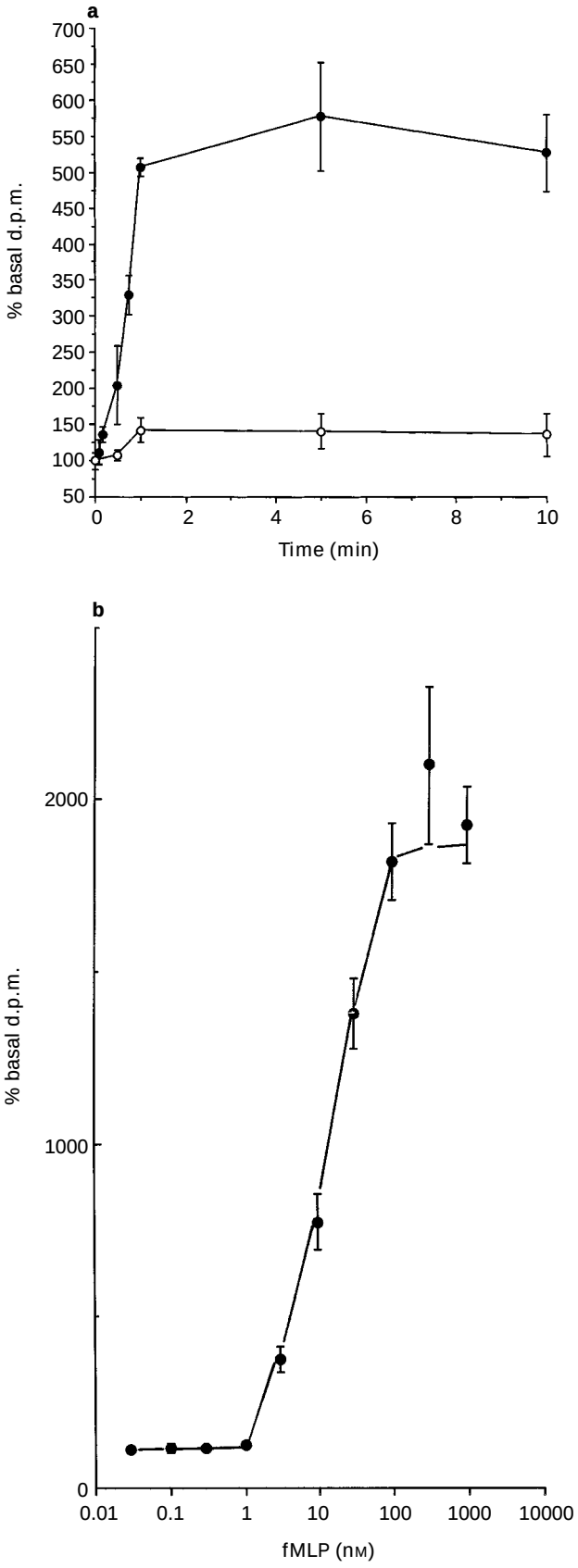

Figure 2 (a) fMLP-stimulated [^{3}H]-arachidonate release in cytochalasin B-primed differentiated HL60 cells. Cells, 10^6 per sample were incubated for 5 min with 5 μM cytochalasin B at 37°C. The cells were then stimulated with either RBG (○) or 100 nM fMLP (●) for the times indicated. Result shown is representative of 3 individual experiments with the values being presented as % basal d.p.m. where the basal value was 500±45 d.p.m. (b) Dose-dependency of fMLP-stimulated [^{3}H]-arachidonate release in cytochalasin B-primed differentiated HL60 cells. Cells, 10^6 per sample, had been incubated with 5 μM cytochalasin B for 5 min at 37°C before stimulation for 5 min with the various fMLP concentrations shown. Results are representative of 3 individual experiments with the values presented as % basal d.p.m. where the basal level, as determined by incubating with RBG in place of fMLP for 5 min had a mean value of 560±45 d.p.m. during the incubation time.

MAP kinase lysis buffer to a final volume of 0.4 ml. Four microlitres of rabbit anti-ERK1/2 polyclonal antibody was added and the samples mixed at 4°C for 2 h. The immunocomplexes were sedimented by mixing with 20 μl protein G for 1 h at 4°C and then centrifuging at 14,000 × *g* for 1 min at 4°C. The pellet was washed twice in 1 mM Tris/HCl pH 7.2, 0.5% (w/v) sodium deoxycholate, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate and 1% (w/v) Triton X-100, twice in 1 mM Tris/HCl pH 7.2, 1 M NaCl, 1 mM sodium orthovanadate and 0.1% (w/v) Triton X-100 and once in 5 mM Tris/HCl pH 7.2 and 150 mM NaCl. The washed pellet was resuspended in 20 μl assay buffer (50 mM MOPS, 3.75 mM EGTA, 25 mM $MgCl_2$, 0.25 mM sodium orthovanadate, 2 μM protein kinase A (PKA) inhibitor and 5 μM microcystin LR). Myelin basic protein, 0.5 mg was added and the reaction initiated by the addition of 5 μl 0.5 mM ATP containing 3 μCi [γ-^{32}P]-ATP per 5 μl and incubated at 30°C for 10 min. Reactions were terminated by the addition of 50 μl ice-cold 2 M HCl and centrifugation at 14,000 × *g* for 1 min at 4°C. Fifty microlitres of resuspended pellet was spotted onto P81 paper and transferred immediately to a bath of 150 mM orthophosphoric acid. The papers were then extensively washed, before a brief rinse in 100% ethanol and air dried. The radioactivity was quantified by liquid scintillation spectrometry.

Phosphotyrosine immunoblotting

Cells were treated and lysed as described under MAP kinase immunoblotting. A volume of sample corresponding to 15 μg total protein was added to an equal volume of 2 × Lamelli buffer, vortexed and boiled for 5 min, loaded onto 10% polyacrylamide gels and run at 16 mA, 30 V. The proteins were transferred onto nitrocellulose and blocked overnight at 4°C in NaTT containing 5% (w/v) BSA (electrophoresis grade). A 1:10,000 dilution of the antiphosphotyrosine PY54 antibody was then added overnight in NaTT containing 1% (w/v) BSA. The membranes were then washed stringently over 2 h with NaTT before a 1:10,000 dilution anti-mouse IgG HRP-linked antibody was added for 1 h at 4°C in NaTT containing 1% (w/v) BSA. The membranes were again washed in NaTT for 2 h and developed by ECl.

Perhydrovanadate stimulated very extensive tyrosine phosphorylation and ECL was found to be too sensitive enough to distinguish the bands of interest, therefore the ABC Detection system was utilized. The primary antibody was as used above, but a 1:5000 dilution of anti-mouse IgG Biotin-linked antibody was added for 1 h at room temperature in NaTT containing 1% (w/v) BSA. The membranes were washed in NaTT for 1 h and developed as instructed by the manufacturer.

Presentation of results

All experiments were performed at least three times. Where appropriate results are expressed as means ± s.d. Variation was sometimes observed in magnitude of response, this was possibly due to passage number and maybe degree of differentiation. However, the quantitative changes between experiments had no effect upon the qualitative responses which were extremely reproducible. Representative results are presented which reflect these changes. Statistical analysis was performed by Student's *t* test.

Materials

[5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid, [γ-^{32}P]-ATP, sheep anti-mouse IgG HRP-linked antibody and ECL detection kit

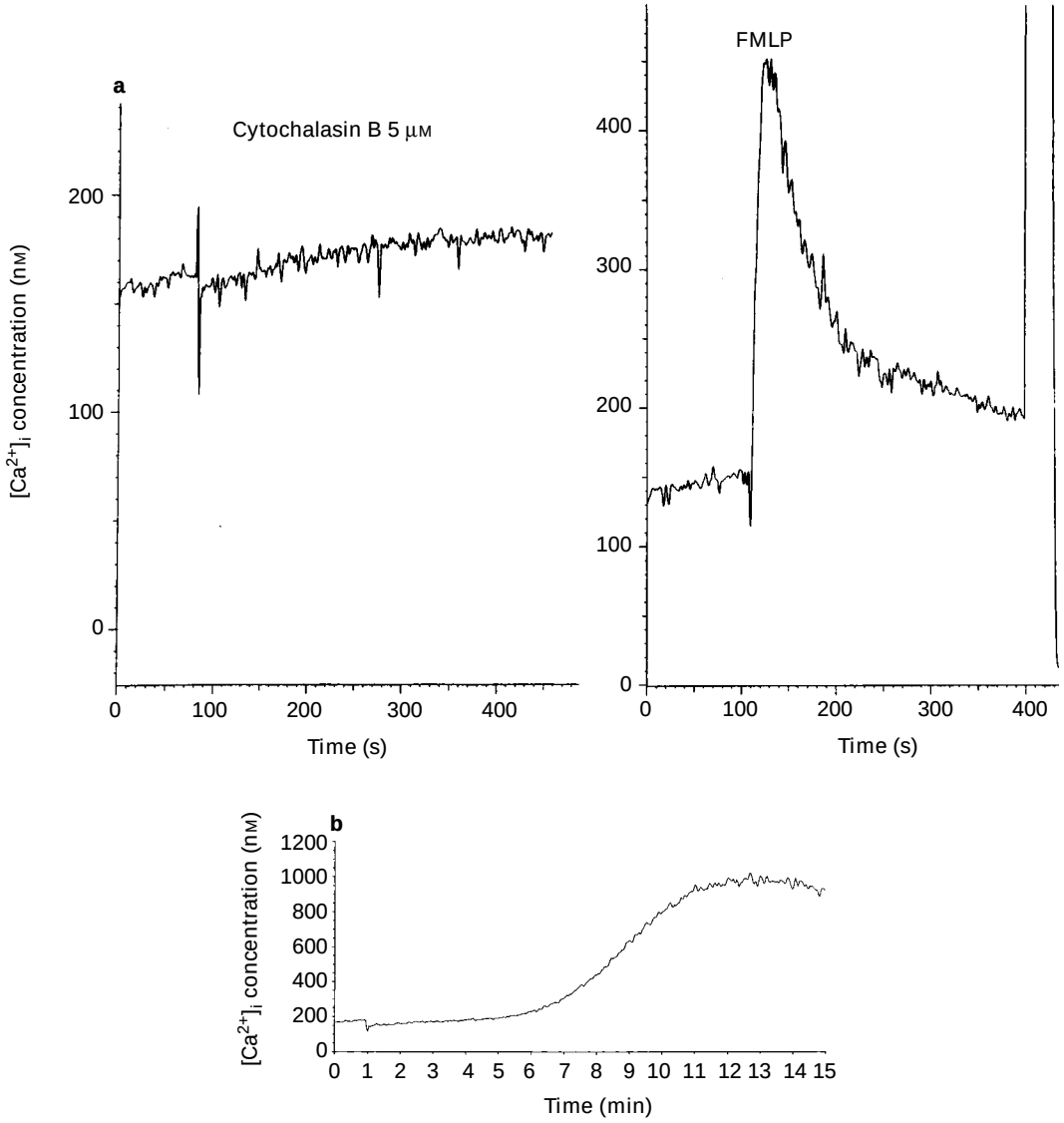

Figure 3 Cytochalasin B-, fMLP- and perhydrovanadate-stimulated changes in intracellular calcium concentration in differentiated HL60 cells. HL60 cells were washed in RBG (phenol red free) and then incubated at 37°C for 45 min in RBG (phenol red free) containing 1 μM Fura-2-am. (a) Fura-2 loaded HL60 cells (10^6) were treated with 5 μM cytochalasin B for the times indicated. Cells pretreated with 5 μM cytochalasin B for 5 min were then stimulated with 100 nM fMLP for the times indicated. (b) Fura-2 loaded cells were incubated with 0.5 mM perhydrovanadate for the times indicated. The ratio of calcium-bound and -unbound Fura-2 was measured as described in Methods. Results are representative of 3 individual experiments with fresh cell cultures utilized for each.

were purchased from Amersham International plc (Bucks., U.K.). Vector ABC Detection kit was purchased from Vector Laboratories Inc. (Burlingame, C.A., U.S.A.). PY 54 anti-phosphotyrosine antibody and monoclonal anti-ERK 1/2 were purchased from Affiniti Research Products Ltd. (Nottingham, U.K.), Microcystin LR from Boeringer Mannheim. Nitrocellulose from Costar (Cambridge, MA., U.S.A.). All other chemicals were purchased at the highest grade available from Sigma Chemical Co. (Poole, U.K.). Rabbit anti-MAP kinase antibody (ERK 16) was kindly donated by Dr J. Tavare (Department of Biochemistry, University of Bristol, Bristol).

Results

Stimulation of differentiated HL60 cells with platelet activating factor (PAF), lysophosphatidic acid (LPA), fMLP or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) elicited little or no arachidonate release (Figure 1). However, when the cells were preincubated for 5 min with cytochalasin B, stimulation of arachidonate generation was clearly observed in response to fMLP (Figure 1). In some, but not all experiments stimulation of arachidonate generation by LPA and PAF was also observed, the reason for this experimental variability is unclear. In the cytochalasin B-primed cells fMLP-stimulated arachidonate release was first detectable after 10 s and reached a maximum after 1 min (Figure 2a), the EC_{50} was 28 ± 6 nM ($n = 4$) and maximum stimulation was observed at concentrations greater than 100 nM (Figure 2b).

Cytochalasin B thus induces a priming effect upon stimulated arachidonate generation, i.e. activation of phospholipase A_2. However, the mechanism of priming is unclear and it has been proposed that cytochalasin B can stimulate an increase in intracellular free [Ca^{2+}] (Koenderman *et al.*, 1989). Since an elevation in [Ca^{2+}] has been associated with an increase in phospholipase A_2 activity, changes in the concentration of the metal ion in HL60 cells were examined by treating Fura-2 loaded cells with cytochalasin B alone or together with fMLP. The priming agent had no effect on intracellular [Ca^{2+}] even after a 60 min exposure, nevertheless fMLP stimulated a rapid increase in [Ca^{2+}] (Figure 3a).

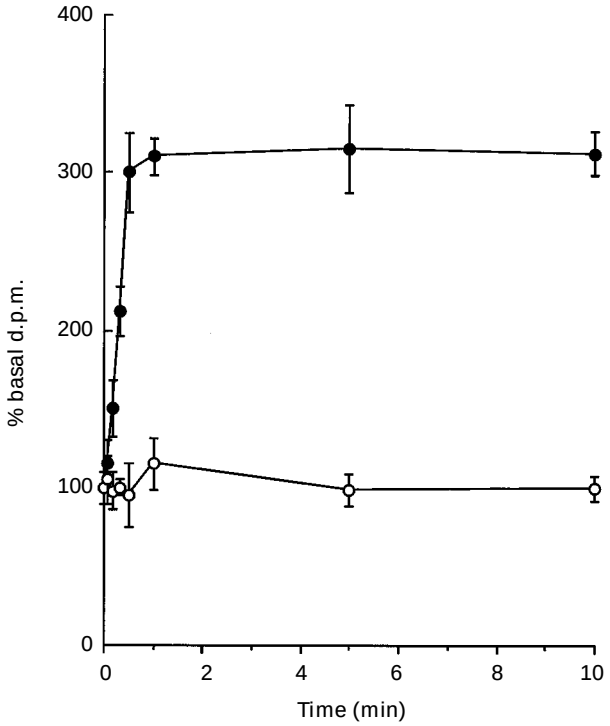

Figure 4 fMLP stimulated [^{3}H]-arachidonate release in GM-CSF-primed HL60 cells. Cells, 10^6 per sample, were incubated for 10 min with 20 ng ml^{-1} GM-CSF at 37°C. The cells were then stimulated with either RBG (○) or 100 nM fMLP (●) for the times indicated. Results shown are representative of 3 individual experiments with the values being presented as % basal d.p.m. where the basal value was 450±40 d.p.m.

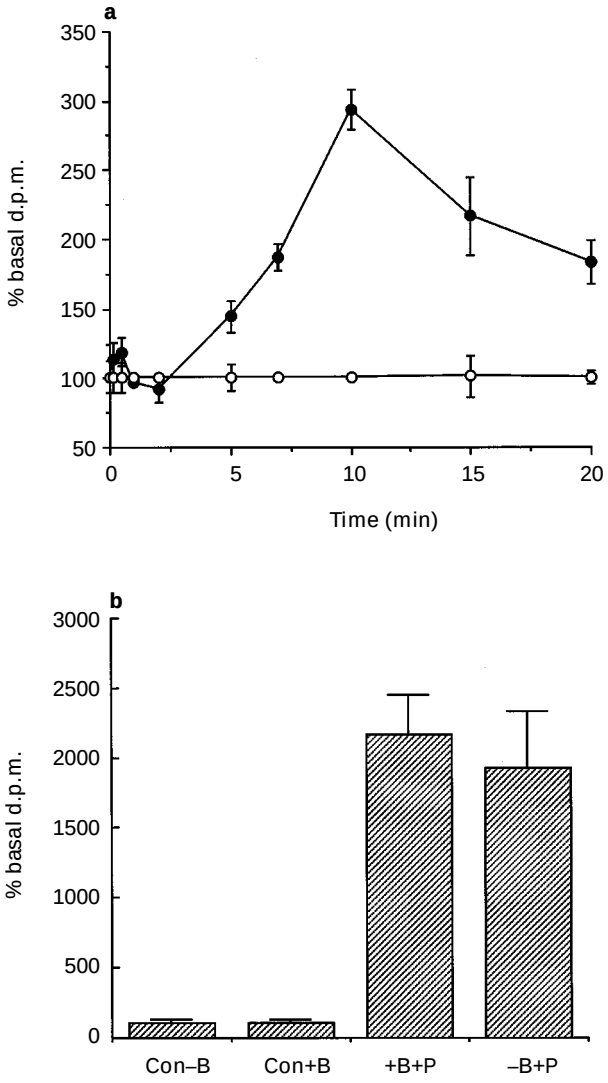

Figure 5 (a) Perhydrovanadate-stimulated [^{3}H]-arachidonate release in differentiated HL60 cells. Cells, 10^6, which had been preincubated for 5 min at 37°C with 5 μM cytochalasin B, were stimulated for the times shown with either 0.5 mM perhydrovanadate (●) in RBG or RBG alone (○). Result represents 3 individual experiments with values presented as % basal d.p.m. where the basal level was 500±100 d.p.m. (b) Perhydrovanadate-stimulated [^{3}H]-arachidonate release in cytochalasin B-primed and -unprimed differentiated HL60 cells. Cells, 10^6 per sample, were preincubated with either 5 μM cytochalasin B in RBG or RBG alone for 5 min at 37°C. Preincubated samples were then stimulated with either 0.5 mM perhydrovanadate in RBG or RBG alone for a further 5 min at 37°C. Results are representative of 3 individual experiments with values presented as % basal d.p.m. where the basal level was 800±150 d.p.m. with fresh cell cultures being used for each. Con-B: control cells no cytochalasin B preincubation; Con+B: control cells plus cytochalasin B preincubation; +B+P: pervanadate-stimulated, cytochalasin B preincubated cells; −B+P pervanadate-stimulated, non-cytochalasin B preincubated cells.

Priming in neutrophils can also be induced by, for example, GM-CSF an agonist which increases tyrosine kinase activity (Roberts *et al*., 1994. Figure 4 shows that a 10 min preincubation with 20 ng ml^{-1} GM-CSF also primes fMLP-stimulated arachidonate release in HL60 cells. Thus, the possibility that tyrosine phosphorylation is involved in priming was further examined by treating cells with the tyrosine phosphate phosphatase inhibitor perhydrovanadate. Addition of perhydrovanadate to unprimed cells stimulated arachidonate release. However, this was only apparent after a 5 min incubation with a maximum response being observed following a 10 min exposure (Figure 5a). Perhydrovanadate stimulated a slow, but sustained increase in intracellular [Ca^{2+}], the increase was first apparent after 5 min (Figure 3b). The stimulation of arachidonate generation by a 10 min exposure to perhydrovanadate was identical in control and cytochalasin B-primed cells (Figure 5b), at this time the [Ca^{2+}] was increased to 800 nM (Figure 3b). When cells were exposed to perhydrovanadate for 2 min (a period which did not induce an increase in [Ca^{2+}], Figure 3b) and then stimulated with fMLP there was rapid arachidonate release, i.e. perhydrovanadate had primed the cells (Figure 6).

Because these results suggested a role for tyrosine phosphorylation in the priming, cellular proteins were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed for phosphorylation on tyrosine residues with an anti-phosphotyrosine antibody. Treatment of cells with perhydrovanadate induced extensive tyrosine phosphorylation (Figure 7), as observed by other authors, GM-CSF also stimulated an increase in tyrosine phosphorylation (Figure 7). Cytochalasin B stimulated tyrosine phosphorylation in HL60 cells, whilst there appeared to be a general increase in protein tyrosine phosphorylation, an increase was particularly apparent for a number of proteins with molecular weights of approximately 60 and 120 kDa (Figure 7). However, it was not possible to identify particular bands that were phosphorylated.

An increase in protein tyrosine kinase activity can regulate the activity of many proteins, one of which, MAP kinase, has been implicated in the control of $cPLA_2$ (Lin *et al*., 1993). Changes in MAP kinase activity were examined both by electrophoretic band shift, where the phosphorylated protein migrates with a different mobility to the unphosphorylated molecule and by *in vitro* kinase assay of the immunoprecipitated enzyme. HL60 cells were found to express the 44 kDa (ERK1), but not the 42 kDa (ERK2) form of MAP kinase (Figure 8). The 44 kDa form was significantly phosphorylated in the control cells. Cytochalasin B had no extra effect upon

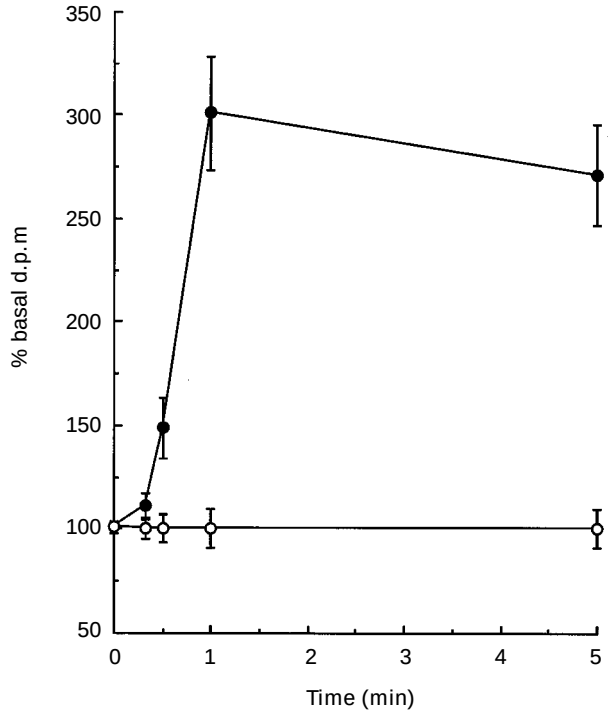

Figure 6 Priming of HL60 cells by perhydrovanadate. Cells, 10^6 per sample, were preincubated with 0.5 mM perhydrovanadate in RBG for 2 min at 37°C. Preincubated samples were then stimulated with either 100 nM fMLP (●) in RBG or RBG (○) alone for a further 5 min at 37°C. Results are representative of 3 individual experiments with values presented as % basal d.p.m. where the basal level was 700 ± 160 d.p.m. with fresh cell cultures being used for each.

the phosphorylation state, but the enzyme was hyper-phosphorylated in response to perhydrovanadate treatment. This increase in phosphorylation was reflected by an increase in the *in vitro* kinase activity (Table 1). As a control, Figure 8 also shows the MAP kinase phosphorylation state in Rat 1 RafER4 fibroblasts, since these contain both the 42 and 44 kDa forms.

The potential importance of MAP kinase activation in priming is that phospholipase A_2 is a substrate for the kinase, this enzyme also migrates with a different mobility when phosphorylated (Lin *et al*., 1993). The phosphorylation state of phospholipase A_2 was therefore examined in control and primed cells, but found to be high under both conditions (Figure 9a). However, treatment of the unprimed cells with either cytochalasin B or perhydrovanadate changed the intracellular loction of the phospholipase. In control cells the majority of the enzyme was found in the cytosolic fraction, but, following treatment with the priming agents, there was significant translocation of phospholipase A_2 to the membrane fraction (Figure 9b,c). Exposure to fMLP was able to increase further the apparent amount of phospholipase A_2 detectable at the membrane, whilst in longer incubations (greater than 1 min) the agonist itself caused a small increase in membrane-associated phospholipase in unprimed cells (Figure 9c).

Discussion

Neutrophil priming is a process whereby the cells are activated by an agent but do not appear to respond, nevertheless subsequent exposure of primed cells to an inflammatory stimulus generates an enhanced response. Primed cells are thus of importance in the enhanced reactivity of neutrophils in, for example, psoriasis and rheumatoid arthritis, and an understanding of the mechanisms involved in the priming response may point to sites of potential therapeutic intervention. The most significant observation presented in the work de-

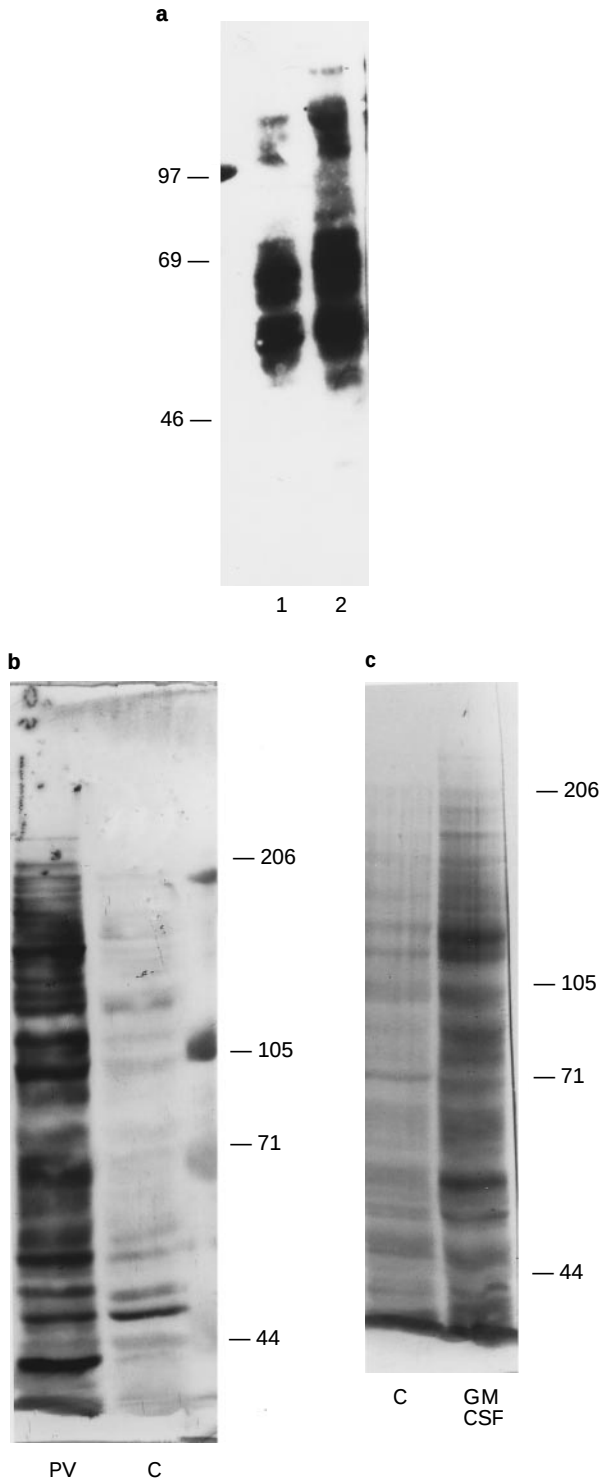

Figure 7 Western blot analysis of (a) 5 μM cytochalasin B-, (b) 0.5 mM perhydrovanadate- and (c) 20 ng ml^{-1} GM-CSF stimulated tyrosine phosphorylation in differentiated HL60 cells. (a) 10^7 washed cells were incubated for 5 min at 37°C with (lane 2) or without (lane 1) 5 μM cytochalasin B. Lysis was carried out as described in Methods and 10 μg total protein separated on 10% (w/v) polyacrylamide gels. Tyrosine phosphorylated proteins were visualized by ECL. Result is representative of 3 individual experiments. The position of the 97 kDa marker is shown. (b) 10^7 washed cells were incubated for 2 min at 37°C with (lane 1) or without (lane 2) 0.5 mM perhydrovanadate. (c) 10^7 washed cells were incubated for 10 min at 37°C without (lane 1) or with (lane 2) 20 ng ml^{-1} GM-CSF. Lysis was carried out as described in Methods and 10 μg total protein separated on 10% (w/v) polyacrylamide gels. Tyrosine phosphorylated proteins are visualized by the Vector ABC Detection kit. Result is representative of 3 individual experiments and the position of all markers is shown.

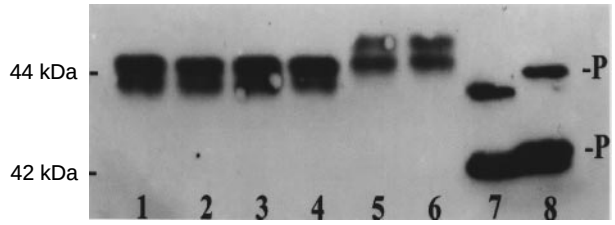

Figure 8 Western blot analysis of MAP kinase isoforms present in differentiated HL60 cells. Cells were washed in RBG before the stimulation of 10^7 cells with either RBG, 5 μM cytochalasin B, 100 nM fMLP or both cytochalasin B and fMLP. The cells were washed and lysed, 40 μg total protein was separated on 12.5% (w/v) polyacrylamide gels. MAP kinase isoforms were detected as described and visualized with ECL. Result is representative of 3 individual experiments. 1: control; 2: cytochalasin B; 3: fMLP; 4: cytochalasin B + fMLP; 5: perhydrovanadate; 6: perhydrovanadate + fMLP; 7: control Rat-1 RafER4 fibroblasts; 8: β-oestradiol-treated Rat-1 RafER4 fibroblasts. P denotes the phosphorylated bands.

Table 1 *In vitro* MAP kinase activity in HL60 cells

Control cells	7413.9 ± 208
Cytochalasin B treated	7022.8 ± 200
Perhydrovanadate treated	8992.4 ± 94.5*

Washed differentiated HL60 cells 10^6 were incubated with either RBG alone, RBG containing 5 μM cytochalasin B for 5 min at 37°C or RBG containing 0.5 mM perhydrovanadate for 2 min at 37°C. Incubations were terminated by washing with ice-cold PBS and lysates prepared and MAP kinase activity determined as described in Methods. Results are representative of 3 individual experiments and are presented as c.p.m. ^{32}P incorporated into myelin basic protein, ± s.d. *$P < 0.005$.

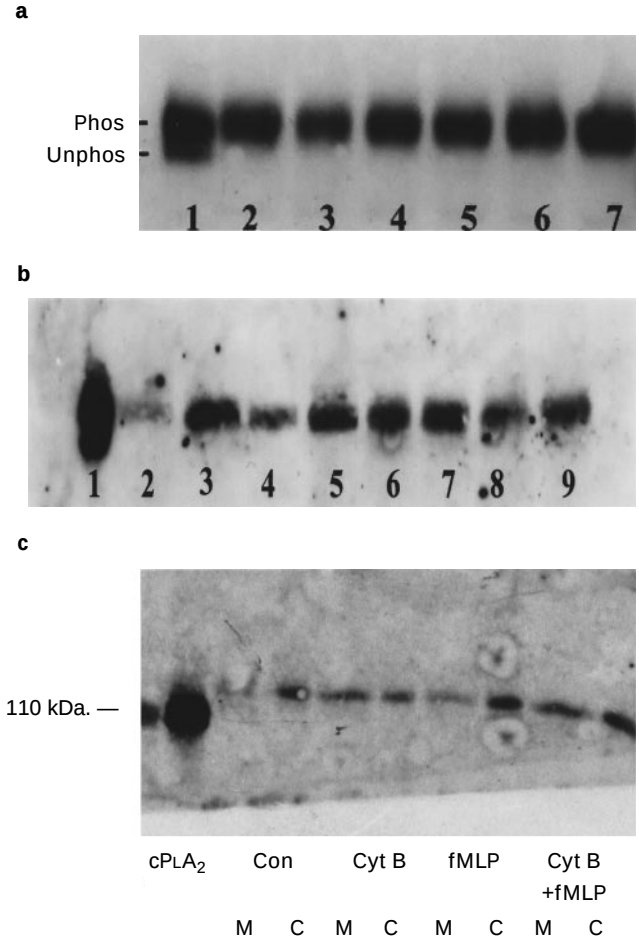

Figure 9 Localization and phosphorylation of phospholipase A_2 as determined by Western blotting in differentiated HL60 cells. Cells, 10^7 per sample, were preincubated at 37°C with either RBG, 5 μM cytochalasin B for 5 min, or 0.5 mM perhydrovanadate for 2 min and then stimulated with either RBG or 100 nM fMLP for 5 min at 37°C. (a) Cell lysate protein (10 μg) was separated on 12.5% polyacrylamide gels. (b,c) Cells were washed twice with ice-cold PBS and cytosolic and membrane fractions prepared. Total protein (10 μg) was separated on 12.5% (w/v) polyacrylamide gels. In each case Western blotting was performed and detected proteins were visualized with ECL. Results are representative of 3 individual experiments, utilizing freshly prepared lysates. (a) 1: $cPLA_2$ standard, 2: control cells, 3: + cytochalasin B, 4: + fMLP, 5: + cytochalasin B and fMLP, 6: + pervanadate, 7: + pervanadate and fMLP. (b) 1: $cPLA_2$ standard, 2: control membranes, 3: control cytosol, 4: membranes from cytochalasin B treated cells, 5: cytosol from cytochalasin B treated cells, 6: membranes from pervanadate treated cells, 7: cytosol from pervanadate treated cells, 8: membranes from cytochalasin B + pervanadate treated cells, 9: cytosol from cytochalasin B + pervanadate treated cells, 10: membrane from fMLP treated cells, 11: cytosol from fMLP treated cells. (c) Samples as shown.

scribed here is that priming is associated with an increase in the membrane localization of phospholipase A_2.

In this study we have utilized the neutrophil model system, DMSO-differentiated HL60 cells, in which priming can be achieved by treatment with cytochalasin B. Cytochalasin B is one of a family of cell permeable fungal metabolites which inhibit membrane ruffling and can stimulate adherent cells to round up (Cooper, 1987). These effects are probably due to binding of the cytochalasin to the barbed end and thus capping of actin fillaments. The capping results in the inhibition of actin fillament growth, the presence of shortened fillaments and thus a weakened cytoskeleton (Cooper, 1987). This cytoskeletal reorganisation can have a range of effects upon signalling, thus we have investigated the mechanism whereby priming was induced.

The results in this study suggest that stimulated protein tyrosine phosphorylation plays a key role in priming. Figure 7 shows that treatment of HL60 cells with cytochalasin B stimulated an increase in total protein tyrosine phosphorylation, with a number of proteins of approximate molecular weights of 60 kDa and 120 kDa being clearly phosphorylated. Phosphorylation of a range of cellular proteins was also observed in response to acute treatment with the protein tyrosine phosphate phosphatase inhibitor perhydrovanadate and by receptor tyrosine kinase agonist GM-CSF (Figure 7). Perhydrovanadate can both prime and stimulate phospholipase A_2 activity (Figure 5, 6). Priming was probably induced as a result of phosphorylation of similar proteins to those activated in response to cytochalasin B treatment. Perhydrovanadate treatment was also able to stimulate phospholipase A_2 activity, this is because incubation of cells with the non-specific phosphatase inhibitor activates a number of different proteins by tyrosine phosphorylation and thus additional signalling pathways to those activated by treatment with cytochalasin B. These additional pathways include the activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by phospholipase Cγ with a consequent stimulated increase in free $[Ca^{2+}]_i$. Figure 3b shows that perhydrovanadate only stimulated an increase in $[Ca^{2+}]_i$ after 5 min and it was only after this time that there was a stimulation of arachidonate release (Figure 5a). Thus the initial effect of perhydrovanadate was to induce priming whilst in the longer term it was able to activate phospholipase A_2 probably as a result of the increase in $[Ca^{2+}]$. Despite evidence to the contrary (Koenderman *et al*., 1989), we were unable to detect any change in $[Ca^{2+}]_i$ in response to cytochalasin B (Figure 3). This result would rule out a role for Ca^{2+} in the priming event because an increase in $[Ca^{2+}]_i$ is essential for maximum phospholipase A_2 stimulation (Dennis, 1994). This would also explain why cytochalasin B is only

capable of priming rather than also activating the phospholipase as observed in response to perhydrovanadate.

Despite the activation of tyrosine kinases which would be expected to stimulate MAP kinase activity, there did not appear to be a priming associated increase in the extent of phospholipase A_2 phosphorylation. It would thus appear that the high resting activity of MAP kinase in the HL60 cells (Table 1), which is presumably a consequence of the high serum concentration necessary to grow the cells, resulted in complete phospholipase A_2 phosphorylation (Figure 9). Phosphorylation of phospholipase A_2 *in vitro* has been shown to increase enzyme activity (Lin *et al*., 1993), nevertheless the data presented in this paper would strongly suggest that MAP kinase catalysed phosphorylation is insufficient to activate phospholipse A_2 in intact cells and clearly points to additional regulatory mechanisms.

The data in Figure 9 further demonstrate that phospholipase phosphorylation is not adequate for priming and also that membrane association cannot be regulated by MAP kinase catalyzed phosphorylation of phospholipase A_2. The results presented in Figure 9 show that the priming of the HL60 cells by both cytochalasin B and perhydrovanadate was associated with an increase in the amount of phospholipase A_2 detectable in the membrane rather than the cytosolic fraction. This translocation to a membrane fraction has also been shown for GM-CSF priming in human neutrophils (Durstin *et al*., 1994). Therefore, there must be an as yet unidentified tyrosine kinase regulated event, perhaps involving cytoskeletal reorganisation, which results in membrane localization and thus 'priming' of phospholipase A_2 activity. This proposal is supported by the observation that agonists such as fMLP which can prime following a sustained exposure (Figure 9c) also stimulate the activity of cytosolic tyrosine kinases in human neutrophils (Uings *et al*., 1992). A role for tyrosine phosphorylation in the 'priming' of neutrophil $cPLA_2$ by GM-CSF has been suggested (Nahas *et al*., 1996), thus this may represent a common mechanism. However, the kinase(s) responsible remains to be identified.

In conclusion, this work points to the importance of membrane localization of phospholipase A_2 potentially regulated by an unidentified tyrosine kinase regulated pathway as being critical for priming. Identification of the kinase responsible and its substrate(s) will thus be important in the understanding of priming and may point to a therapeutic target.

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