Phorbol 12-myristate 13-acetate-mediated signalling in murine bone marrow cells

Dora VIŠNJIĆ,* Drago BATINIĆ,† Zoran LASIĆ,* Mladen KNOTEK,* Matko MARUŠIĆ* and Hrvoje BANFIĆ*‡

*Department of Physiology, School of Medicine, University of Zagreb, 41000 Zagreb, and †Department of Clinical Laboratory Diagnosis, Clinical Hospital Center, 41000 Zagreb, Croatia

Phorbol 12-myristate 13-acetate (PMA)-mediated signalling was investigated in relation to the ability of murine (CBA) bone marrow cells to form colonies *in vitro*. Treatment of marrow cells with PMA did not influence the 1,2-diacylglycerol or cyclic AMP concentrations, the intracellular Ca^{2+} concentration or phospholipase D activity. PMA increased particulate phospholipase A_2 (PLA_2) activity, lysophosphatidylcholine formation and arachidonic acid release from bone marrow cells; these effects were abolished when cells were pretreated with the putative PLA_2 inhibitors heparin and mepacrine. While indomethacin and nordihydroguaiaretic acid inhibited either the cyclo-oxygenase or lipoxygenase pathway of arachidonic acid metabolism, as measured by their products prostaglandin E_2 and leukotriene B_4, they did not influence PMA-mediated PLA_2 activation or translocation of protein kinase C (PKC) from the soluble to the particulate fraction. Treatment of cells with PMA increased the amounts of membrane-bound α, β, δ, ϵ and ζ isoforms of PKC in bone marrow cells. Pretreatment of cells with PLA_2 inhibitors reduced the amount of membrane-bound PKC-ζ in unstimulated cells and diminished PMA-induced translocation of PKC-ζ to membranes without affecting other PKC isoforms. This effect could be overcome by exogenous addition of arachidonic acid, suggesting that PKC-ζ may operate downstream of the activated PLA_2. On the other hand, wortmannin, an inhibitor of phosphatidylinositol 3-kinase, did not influence the amount of PKC-ζ associated with particulate fractions in control cells and could not abolish the PMA-mediated translocation of this isoform. Short-term exposure (45 min) of bone marrow cells to PMA, phorbol 12,13-dibutyrate or arachidonic acid increased the number of colonies formed over 7 days in a methylcellulose-based culture *in vitro*. The effects of PMA, but not those of arachidonic acid, could be prevented by putative PLA_2 inhibitors. This suggests that PMA-mediated activation of conventional PKCs and novel PKCs leads to PLA_2 activation which, by releasing arachidonic acid from phospholipids, activates PKC-ζ. This signalling pathway appears to be mitogenic for bone marrow cells.

INTRODUCTION

The protein kinases C (PKCs) constitute a family of phospholipid-dependent and diacylglycerol (DAG)-activated protein kinases which play key roles in signal transduction and cell regulation [1]. PKC isoenzymes exhibit distinct tissue distribution patterns, with most cell types expressing more than one PKC isotype. These isoenzymes have been grouped into three functional classes depending on their requirements for phosphatidylserine, calcium and DAG. The first group, the conventional PKCs (cPKCs; isoforms α, $\beta1$, $\beta2$ and γ), require Ca^{2+} and DAG to be activated, whereas the second group, the novel PKCs (nPKCs; isoforms δ, ϵ, θ and η), are Ca^{2+}-independent [1,2]. Common features of the C_1 domain of cPKCs and nPKCs are a conserved pseudosubstrate site and two adjacent N-terminal cysteine clusters which are responsible for phorbol ester binding. The stimulation of PKCs by phorbol 12-myristate 13-acetate (PMA) has been widely used in studies of PKC-mediated cell signalling [1,2]. More recently, the third group, termed the atypical PKCs (aPKCs; isoforms ζ, λ, ι and μ) has been identified. aPKCs are dependent on phosphatidylserine, but are not affected by DAG, phorbol esters or Ca^{2+} [1,3].

All mature blood cells in the body are derived from pluripotent stem cells which generate lineage-restricted progenitor cells through the process of differentiation. Haematopoiesis is regulated by a series of soluble and membrane-bound molecules which act on responding cells via specific membrane receptors [4,5]. Although many haemopoietic growth factors have been purified to homogeneity and their genes cloned, post-receptor signalling events elicited by the occupation of their respective receptors remain only partially described [4–6]. The role of PKC in haematopoietic cell function has been widely examined and it has been shown that proliferation, differentiation and colony formation of both normal and leukaemic haematopoietic cells can be stimulated by treatment of the cells with PMA [2,4,7,8]. Therefore the aim of the present study was to investigate PMA-mediated signalling in murine bone marrow cells and to compare it with the ability of the cells to form colonies *in vitro*.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: Iscove's modified Dulbecco's medium, CHAPS, EGTA, EDTA, Hepes, nordihydroguaiaretic acid (NDGA), indomethacin, PMA, phorbol 12,13-dibutyrate (PDBu), cytochalasin B, fura 2-AM, ionomycin, A23187, dithiothreitol (DTT), wortmannin, lipid standards, Triton X-100, histone IIIS, ATP, mepacrine and heparin from Sigma, St. Louis, MO, U.S.A.; [γ-^{32}P]ATP, [^{3}H]alkyl-lysophosphatidylcholine ([^{3}H]alkyl-lysoPtdCho), [*ara*-

Abbreviations used: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; DAG, 1,2-diacylglycerol; PLD, phospholipase D; (c)PLA_2, (cytosolic) phospholipase A_2; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; PtdEt, phosphatidylethanol; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PGE_2, prostaglandin E_2; LTB_4, leukotriene B_4; cAMP, cyclic AMP; MAP kinase, mitogen-activated protein kinase; DTT, dithiothreitol; NDGA, nordihydroguaiaretic acid; GM-CSF, granulocyte/macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor.

‡ To whom correspondence should be addressed: Zavod za fiziologiju, Medicinski fakultet, Šalata 3, POB 978, 41001 Zagreb, Croatia.

chidonyl-^{14}C]PtdCho, [*choline*-^{14}C]PtdCho, [^{3}H]arachidonic acid, [^{3}H]choline chloride, kits for measurement of cyclic AMP (cAMP), prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) and enhanced chemiluminescence kit from Amersham International, Amersham, Bucks., U.K.; anti-PKC anti-peptide antibodies (against isoforms α, β, γ, δ, ϵ and ζ) from Gibco-BRL, Gaithersburg, MD, U.S.A.; MethoCult M3430 from Stemcell Technologies, Vancouver, BC, Canada. All other chemicals were of analytical grade. All studies were performed on CBA mice of both sexes bred at the Department of Physiology, School of Medicine, University of Zagreb, Croatia.

Isolation of bone marrow cells

Bone marrow cells from 8–12-week-old CBA mice were obtained by flushing the femoral and tibial shafts with ice-cold Iscove's modified Dulbecco's medium and the concentration of the cells was adjusted to 10×10^6 per ml. If not stated otherwise, all incubations were performed with the above-mentioned concentration of cells and medium.

Mass assay of DAG

DAG from the cells was extracted using 1.5 ml of chloroform/methanol (1:2, v/v). Further extraction was performed as described by Folch et al. [9]. The lipid extract was dissolved in 0.5 ml of chloroform and loaded on a silicic acid column (0.5 ml; made in a Pasteur pipette), eluted with 1 ml of chloroform, dried again and the mass measurement for DAG was performed using DAG kinase whose purification was achieved in a single step from rat brain using DEAE-Sepharose column as described by Divecha and Irvine [10].

The mass measurement for DAG was performed in the following manner. The dried lipid was dissolved by adding 20 μl of CHAPS (9.2 mg/ml) and sonicated at room temperature for 15 s. After addition of 80 μl of buffer (50 mM Tris/acetate, 80 mM KCl, 10 mM magnesium acetate, 2 mM EGTA, pH 7.4), the reaction was started by adding 20 μl of DAG kinase followed by 80 μl of buffer containing 20 μM ATP and 1 μCi of [^{32}P]ATP. After 1 h of incubation at room temperature the reaction was stopped by adding 750 μl of chloroform/methanol/HCl (80:160:1, by vol.). Phosphatidic acid (PtdOH) was extracted as described by Folch et al. [9] and chromatographed on oxalate (1%)-sprayed TLC plates using the solvent system chloroform/methanol/conc. ammonia/water (45:35:2:8, by vol.). After autoradiography, the spots corresponding to PtdOH were scraped off and their ^{32}P content determined by scintillation counting. The DAG mass content in each sample was adjusted to between 5 and 50 pmol, since the sensitivity of the mass assay is highest within this range [10].

Determination of phospholipase D (PLD) activity

Cells were labelled with 5 μCi/ml [^{3}H]alkyl-lysoPtdCho for 30 min at 37 °C in a buffer comprising 20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose and 1 mg/ml fatty-acid-free BSA (pH 7.2) as described by Stutchfield and Cockcroft [11]. The labelled cells were washed twice, resuspended in the same buffer and equilibrated for 30 min at 37 °C. Cytochalasin B was added to the cells (5 μM final concentration) and 100 μl samples were transferred to tubes containing PMA with ethanol (2%, v/v, final concentration). After incubation, the cells were quenched with chloroform/methanol (1:2, v/v). At this stage, a mixture of unlabelled PtdOH and phosphatidylethanol (PtdEt) was added for localization of the reaction products by TLC. Phase separation was achieved by sequential addition of 2 M KCl and chloroform. Lipids were extracted as described by Folch et al. [9] and chromatographed on oxalate (1%)-sprayed TLC plates using the solvent system chloroform/methanol/acetic acid/water (75:45:3:1, by vol.). Lipids were visualized using iodine vapour and the spots corresponding to PtdOH and PtdEt standards were scraped off and their ^{3}H content determined by scintillation counting.

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Isolated bone marrow cells were incubated in Hanks' physiological salt solution with 2 mM fura 2-AM at 37 °C for 30 min, washed and resuspended in fresh solution. Cells (13×10^6) were transferred to a 3 ml cuvette for measurement of $[Ca^{2+}]_i$. The fluorescence was measured in a Perkin Elmer LS50 spectrofluorimeter with excitation at 340 and 380 nm and emission at 510 nm. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio, as described by Grynkiewicz et al. [12]. Maximum fluorescence was determined by adding 10 μM ionomycin, and the minimum fluorescence was determined by adding 50 mM EGTA.

Determination of phospholipase A_2 (PLA_2) activity

After incubation, the cells were washed once with PBS and put into 0.5 ml of homogenization buffer: 50 mM Hepes (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 μM NaF, 10 μM leupeptin, 0.15 mM PMSF and $CaCl_2$ to give free Ca^{2+} concentration of 150 nM. The cells were homogenized with 10 strokes using a Potter–Elvehjem motor-driven Teflon pestle at 600 rev./min. The homogenate obtained was then centrifuged at 104000 g for 60 min in a Beckman TL-100 ultracentrifuge. The resulting supernatant was designated the soluble fraction and the pellet was designated the particulate fraction.

Determination of PLA_2 enzymic activity was carried out at 37 °C for 60 min in 200 μl of buffer (0.1 M Tris/HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 10 μM leupeptin, 0.15 mM PMSF, $CaCl_2$ to give a free Ca^{2+} concentration of 150 nM and 5 mM DTT to inactivate secretory PLA_2) containing 50 μg of protein and 3 nmol of [*arachidonyl*-^{14}C]PtdCho or [*choline*-^{14}C]PtdCho (the specific radioactivity was adjusted to 10000 d.p.m./nmol by adding unlabelled PtdCho). The reaction was terminated by adding 4 μl of acetic acid and 800 μl of chloroform/methanol (2:1, v/v) containing 20 μg of arachidonic acid. After centrifugation, the lower phase was concentrated and chromatographed on TLC plates using chloroform/methanol/water (65:25:3, by vol.) or light petroleum (b.p. 35–80 °C)/diethyl ether/acetic acid (60:40:1, by vol.) as solvent systems. When [*arachidonyl*-^{14}C]PtdCho was used as substrate, two radioactive spots corresponding to fatty acid and PtdCho were detected by autoradiography. When [*choline*-^{14}C]PtdCho was used as a substrate, radioactive spots corresponding to lysoPtdCho and PtdCho were detected [13]. The spots were scraped off and their ^{14}C content was determined by scintillation counting.

Measurement of arachidonic acid release and formation of lysoPtdCho

For the study of arachidonic acid release, bone marrow cells were prelabelled at 37 °C for 60 min with 5 μCi/ml [^{3}H]arachidonic acid, washed twice and resuspended in the medium; 100 μl samples were transferred to tubes containing PMA and/or different inhibitors. After incubation, cells were spun down and 50 μl of supernatant was counted for ^{3}H radioactivity.

For the study of lysoPtdCho formation, bone marrow cells were prelabelled at 37 °C for 60 min with 30 μCi/ml [^{3}H]choline

chloride, washed and incubated as described above. After incubation, cells were quenched with chloroform/methanol (1:2, v/v). A mixture of unlabelled lysoPtdCho and PtdCho was added at this stage for localization of the products by TLC. Lipids were extracted as described by Folch et al. [9] and chromatographed on TLC plates using the solvent system chloroform/methanol/7 M NH_3 (12:7:1, by vol.). Lipids were visualized using iodine vapour and the spots corresponding to lysoPtdCho and PtdCho standards were scraped off and their ^3H content determined by scintillation counting.

Determination of PKC

Particulate and soluble fractions from bone marrow cells were prepared as described above (for the determination of PLA_2 activity), particulate PKC was extracted from the membranes using 0.1% Triton X-100 and PKC activity was determined by a modification of the method used by Dawson and Cook [14]. The sample (50 μl; protein concentration 2 mg/ml) was added into 150 μl of buffer for determination of PKC activity (20 mM Tris, 40 mM magnesium acetate, 25 μg of histone IIIS, 0.75 mM $CaCl_2$, pH 7.5) also containing 100 μM ATP and 1 μCi of [^{32}P]ATP. The mixture was incubated at 30 °C for 5 min with or without 24 μg of phosphatidylserine and 0.8 μg of diolein. The reaction was stopped by adding 1 ml of ice-cold 5% (w/v) trichloroacetic acid. Radioactivity incorporated into the histone was trapped on filters (0.45 μm pore size) by washing twice with 3 ml of ice-cold 5% trichloroacetic acid. The filters with the remaining radioactivity were then placed into scintillation vials and radioactivity was measured by liquid scintillation counting. PKC activity was taken as the difference in counts of ^{32}P between samples with activators (phosphatidylserine and diolein) and without them (control).

SDS/PAGE and immunoblotting

Proteins for electrophoresis were prepared so that the concentration of each sample was 50 μg/25 μl of sample loading buffer [15]. Electrophoresis was carried out using a Bio-Rad Minigel apparatus at an acrylamide concentration of 10% (w/v). After electrophoresis, the proteins were transferred to nitrocellulose using a Bio-Rad wet-blotting system. After blocking the nitrocellulose in 20 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween 20 and 4% (w/v) dried milk (Marvel), the blots were incubated with primary antibody (1:1000) for 2 h, washed in the above buffer and incubated with the secondary antibody conjugated to horseradish peroxidase. Visualization was carried out using the ECL kit (Amersham).

Colony-forming assay

Isolated cells were incubated at (2–3) $\times 10^6$ per ml at 37 °C with PMA and/or different inhibitors. Cells were washed, resuspended and the concentration was adjusted to 1×10^6 per ml, and 0.1 ml of this suspension was mixed with 1 ml of MethoCult M3430. Methylcellulose cell suspensions were equally divided into two 24-well plates. After 7 days of incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, discrete cellular aggregates of > 50 cells were scored as colonies using an inverted microscope at 25× magnification.

Measurements of cAMP, PGE_2, LTB_4 and proteins

cAMP, PGE_2 and LTB_4 were determined as described using commercial kits (Amersham). Proteins were determined by the Bradford method [16].

Statistical evaluation

The data are shown as means ± S.E.M. For statistical analyses, Student's t test for unpaired samples at the level of significance of 0.05 was used.

RESULTS

As shown in Figure 1, the treatment of bone marrow cells with PMA (500 nM) did not produce any increase in the measured DAG concentration. In order to measure PLD activity, the cells were prelabelled with [^{3}H]alkyl-lysoPtdCho. Over 85% of the

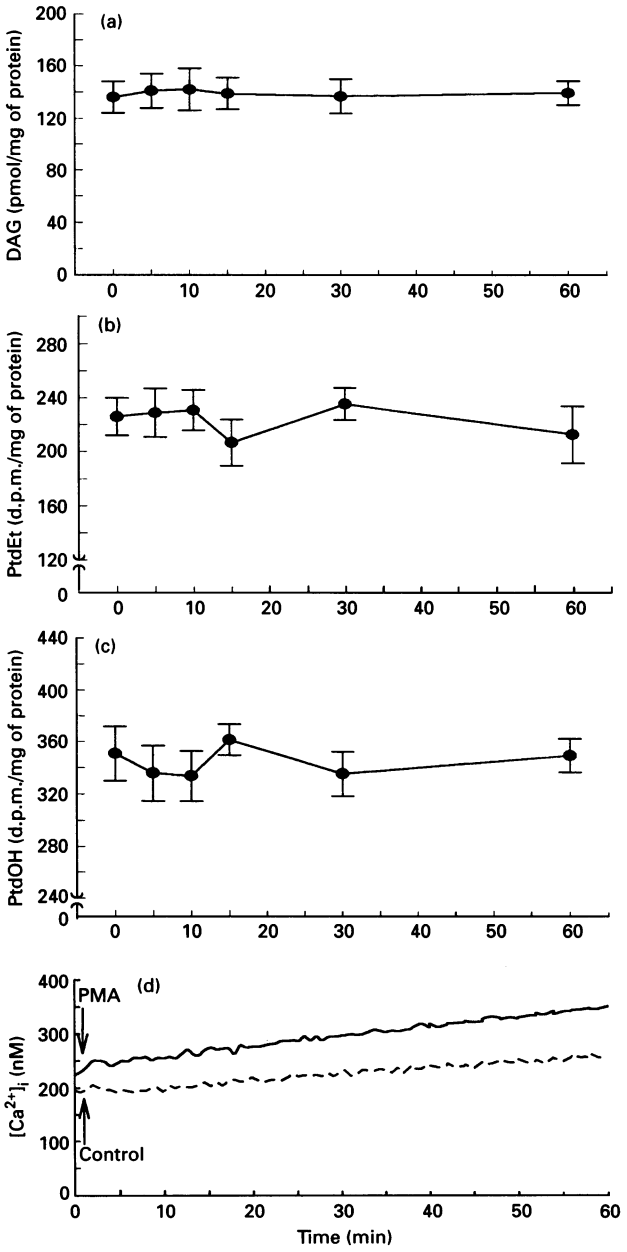

Figure 1 Time courses of changes in (a) DAG concentration, (b) PtdEt and (c) PtdOH formation, and (d) representative $[Ca^{2+}]_i$ response in a suspension of bone marrow cells stimulated with PMA (500 nM)

Each point represents the mean ± S.E.M. from four different experiments performed in duplicate. All other details are as described in the Materials and methods section.

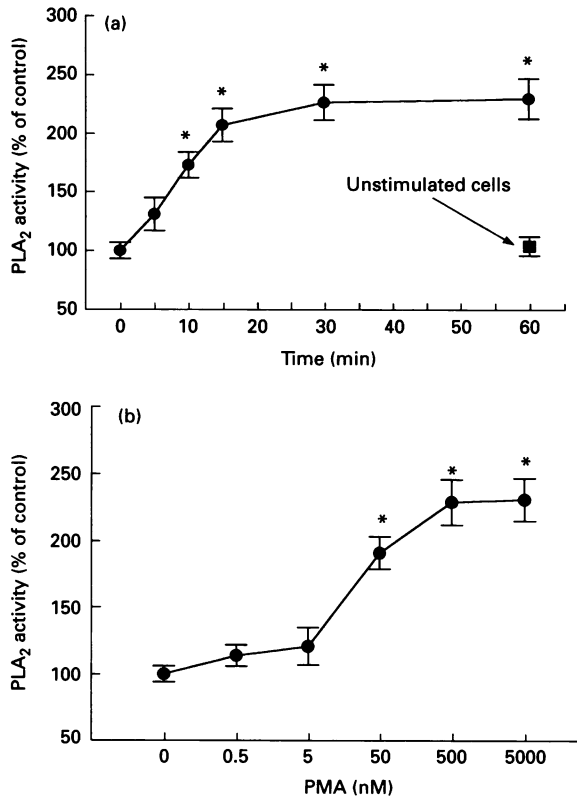

Figure 2 (a) Time course of changes in membrane-bound PLA_2 activity in bone marrow cells stimulated with PMA (500 nM), and (b) dose–response curve for PMA-stimulated increase in membrane-bound PLA_2 activity in bone marrow cells after a 30 min incubation

The activity of membrane-bound PLA_2 in the control bone marrow cells was 35.7 ± 4.2 pmol/min per mg of protein ($n = 16$). *Significantly different ($P < 0.05$; Student's t test) from controls. All other details are as described in the Materials and methods section and the legend to Figure 1.

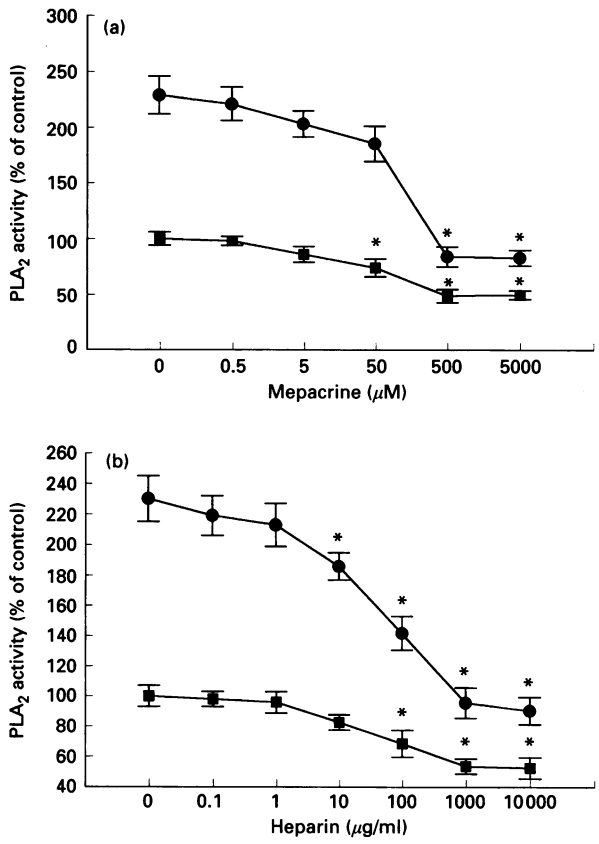

Figure 3 Dose-dependent inhibition by (a) mepacrine and (b) heparin of membrane-bound PLA_2 activity in control (■) and PMA-stimulated (●) bone marrow cells

To investigate the effects of mepacrine and heparin, bone marrow cells were pretreated with inhibitors for 15 min and the inhibitors were also present during treatment of the cells with or without 500 nm PMA for 45 min. *Significantly different ($P < 0.05$; Student's t test) from controls. All other details are as described in the Materials and methods section and the legend to Figure 2.

lipid-soluble radioactivity was associated with PtdCho (results not shown). Activation of PLD generates PtdOH which can be converted into DAG in a time-dependent manner by the enzyme PtdOH phosphohydrolase. When the cells are stimulated in the presence of 2% (v/v) ethanol, PtdEt is formed at the expense of PtdOH, and as the former is a more stable product than PtdOH, the sensitivity of the assay will be increased. Despite using 2% ethanol in the assays, we could not observe any increase in radioactivity either in PtdOH or in PtdEt upon stimulation of bone marrow cells with PMA (500 nM) (Figure 1). Moreover, when the cAMP concentration was measured in bone marrow cells over 60 min of incubation in the presence of 5 mM 3-isobutyl-1-methylxanthine, no significant difference between control and PMA (500 nM)-treated cells could be observed (untreated cells, 2.28 ± 0.12 pmol/mg of protein; PMA-treated cells, 2.41 ± 0.22 pmol/mg of protein; number of cell preparations = 8].

Figure 1 also shows a representative $[Ca^{2+}]_i$ response in a suspension of murine bone marrow cells stimulated with PMA. Resting $[Ca^{2+}]_i$ varied from 38 to 232 nM (mean value 161 ± 25 nM; number of cell preparations = 8), which is similar to the typical $[Ca^{2+}]_i$ measured in unstimulated haematopoietic cells [17]. During a 45 min incubation a slow increase in $[Ca^{2+}]_i$ was observed, but there was no statistical difference in $\Delta[Ca^{2+}]_i$ between control and PMA-stimulated cells at the end of incubation period ($\Delta[Ca^{2+}]_i$ in control cells was 111 ± 32 nM, while in PMA-treated cells it was 108 ± 25 nM). Moreover, the shapes of the $[Ca^{2+}]_i$ curves were similar in the two groups of cells. Because an increase in $[Ca^{2+}]_i$ could be detected, experiments in which cells were stimulated with calcium ionophore were designed to investigate how an acute increase in $[Ca^{2+}]_i$ caused by ionophore will influence PLC and PLD activity. Short-term exposure (10 min) of cells to calcium ionophore A23187 (1 μM) significantly increased the DAG concentration, as well as the amount of radioactivity found in PtdOH and PtdEt. On the other hand, simultaneous 10 min treatment of cells with both PMA (500 nM) and A23187 (1 μM) did not produce any further increase in the concentration and the radioactivity of the compounds mentioned above when compared with the cells stimulated by ionophore alone (results not shown).

Figure 2 shows the time- and dose-dependent activation of membrane-bound PLA_2. At 10 min after the stimulation of the cells with 500 nM PMA, a significant increase in the particulate PLA_2 activity could already be observed. In unstimulated cells, no change in the particulate PLA_2 activity could be observed during the incubation period. A small increase in enzyme activity

Table 1 Effects of PMA and different inhibitors on arachidonic acid release and on the formation of lysoPtdCho and PtdCho in bone marrow cells

Bone marrow cells were treated for 45 min with PMA (500 nM), for 60 min with mepacrine (500 μM) plus heparin (1 mg/ml), or pretreated for 15 min with mepacrine (500 μM) plus heparin (1 mg/ml), indomethacin (50 μM) or NDGA (50 μM) and then stimulated with PMA (500 nM) for 45 min. Results are given in d.p.m./mg of protein. All other details are described in the Materials and methods section. All data are from four different experiments and each measurement was performed in duplicate. *Significantly different ($P < 0.05$; Student's t test) from controls.

Treatment	Arachidonic acid (d.p.m./mg)	LysoPtdCho (d.p.m./mg)	PtdCho (d.p.m./mg)
None	1412 ± 147	911 ± 62	35812 ± 911
PMA	4578 ± 371*	2271 ± 179*	36217 ± 1321
Mepacrine + heparin	207 ± 33*	206 ± 29*	35458 ± 1972
Mepacrine + heparin + PMA	231 ± 40*	207 ± 26*	36371 ± 2178
Indomethacin + PMA	4271 ± 305*	2091 ± 159*	35378 ± 2221
NDGA + PMA	4351 ± 309*	2171 ± 206*	34358 ± 2198

could be detected with 5 nM PMA, while the increase in activity became statistically significant at a concentration of 50 nM. Since it is known that the free concentration of Ca^{2+} in the homogenization buffer can influence the cellular distribution of PLA_2 [18], the concentration of free Ca^{2+} in both the homogenization and PLA_2 assay buffers was adjusted to 150 nM, which is similar to the measured $[Ca^{2+}]_i$ (Figure 1). The assays were performed in the presence of 5 mM DTT to inactivate the secretory PLA_2 [19]. It is important to note that under the experimental conditions described we were not able to observe any difference in soluble PLA_2 activity between control and PMA-treated (500 nM over 30 min) groups (results not shown).

Figure 3 shows that the inhibitors of PLA_2, mepacrine and heparin [20,21], can diminish particulate PLA_2 activity in both control and PMA-treated bone marrow cells. In the present study, heparin of low molecular mass (approx. 3000 Da) was used, and although it was introduced extracellularly, its intracellular effect on PLA_2 activity might be explained by its endocytosis, which is known to occur in leukaemic haematopoietic cells [22], and its partial release from endocytotic vesicles within the cell. When the inhibitors were applied together (mepacrine 500 μM + heparin 1 mg/ml), more than 80% of initially measured particulate PLA_2 activity in untreated cells could be inhibited (control, 17 ± 4%; PMA-treated cells, 16 ± 3%; all other details are as described in the legend to Figure 3).

Additional experiments in which phospholipids were prelabelled with arachidonic acid were performed and arachidonic acid release from bone marrow cells was monitored. Table 1 shows that the treatment of cells with PMA significantly increased arachidonic acid release and formation of lysoPtdCho. Mepacrine and heparin diminished the release of arachidonic acid into the medium, as well as the radioactivity found in lysoPtdCho, in both control and PMA-treated groups, which is a similar result to that obtained when PLA_2 activity was assayed under *in vitro* conditions (Figure 3). On the other hand, pretreatment of cells with indomethacin, an inhibitor of cyclo-oxygenase [20], or NDGA, an inhibitor of lipoxygenase [23], had no effect on PMA-mediated arachidonic acid release. Moreover, when metabolites of the cyclo-oxygenase pathway (PGE_2) or the lipoxygenase pathway (LTB_4) were measured (Table 2), it could be observed that mepacrine and heparin not only abolished PMA-mediated increases in their production, but also decreased metabolite concentrations below control levels, probably by reducing the amount of available arachidonic acid (Table 1). Indomethacin could prevent the PMA-mediated increase in PGE_2, while NDGA could prevent the PMA-mediated increase in LTB_4.

The simplest explanation for all the above results is that mepacrine and heparin effectively inhibit PLA_2 activity, perhaps by inhibition of translocation, while indomethacin and NDGA are capable of inhibiting either the cyclo-oxygenase or the lipoxygenase pathway of arachidonic acid metabolism. As inhibitors might also influence the PMA-mediated translocation of PKC from soluble to particulate fractions, the influence of these compounds on PKC activity was examined in a classical PKC histone kinase assay. As shown in Table 3, none of the inhibitors

Table 2 Effects of PMA and different inhibitors on PGE_2 and LTB_4 production from bone marrow cells

Bone marrow cells were treated for 45 min with PMA (500 nM), or pretreated for 15 min with mepacrine (500 μM) plus heparin (1 mg/ml), indomethacin (50 μM) or NDGA (50 μM) and then stimulated with PMA (500 nM) for 45 min. All other details are described in the Materials and methods section and the legend to Table 1. *Significantly different ($P < 0.05$; Student's t test) from controls.

Treatment	PGE_2 (ng/ml)	LTB_4 (ng/ml)
None	2.72 ± 0.34	0.86 ± 0.11
PMA	13.58 ± 1.26*	2.25 ± 0.21*
Mepacrine + heparin + PMA	0.52 ± 0.11*	0.12 ± 0.04*
Indomethacin + PMA	2.68 ± 0.29	2.31 ± 0.30*
NDGA + PMA	14.02 ± 1.03*	0.68 ± 0.12

Table 3 Effects of PMA and different inhibitors on PKC activity in bone marrow cells

Bone marrow cells were treated for 45 min with PMA (500 nM), for 60 min with mepacrine (500 μM) plus heparin (1 mg/ml), indomethacin (50 μM) or NDGA (50 μM), or pretreated for 15 min with mepacrine (500 μM) plus heparin (1 mg/ml), indomethacin (50 μM) or NDGA (50 μM) and then stimulated with PMA (500 nM) for 45 min. All other details are described in the Materials and methods section and the legend to Table 1. *Significantly different ($P < 0.05$; Student's t test) from controls.

	PKC activity (pmol/min per mg of protein)	
Treatment	Soluble	Particulate
None	1356 ± 107	306 ± 27
PMA	731 ± 65*	651 ± 60*
Mepacrine + heparin	1305 ± 77	341 ± 33
Mepacrine + heparin + PMA	678 ± 53*	605 ± 41*
Indomethacin	1291 ± 121	327 ± 36
Indomethacin + PMA	711 ± 61*	632 ± 51*
NDGA	1334 ± 101	341 ± 28
NDGA + PMA	721 ± 61*	622 ± 36*

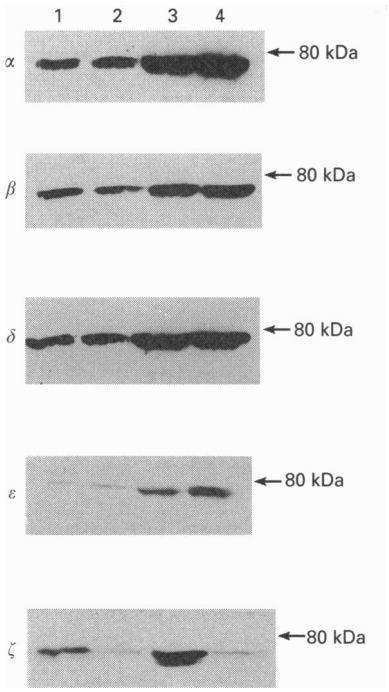

Figure 4 Translocation of different PKC isoforms to particulate fractions

Particulate fractions were isolated as described in the Materials and methods section (determination of PLA_2 activity). Protein (50 μg) was subjected to SDS/PAGE, transferred to nitrocellulose and probed with different anti-PKC antibodies. Lane 1, particulate fractions isolated from unstimulated bone marrow cells; lane 2, particulate fractions isolated from bone marrow cells treated for 60 min with mepacrine (500 μM) and heparin (1 mg/ml); lane 3, particulate fractions isolated from bone marrow cells stimulated for 45 min with PMA (500 nM); lane 4, particulate fractions isolated from bone marrow cells pretreated with inhibitors (mepacrine 500 μM + heparin 1 mg/ml) for 15 min and then stimulated with PMA (500 nM) for 45 min. All other details are as described in the Materials and methods section.

influenced PMA-mediated translocation of PKC from the soluble to the particulate fraction in bone marrow cells. It is important to note that PKC-δ, PKC-ϵ and PKC-ζ could not be detected by the typical PKC histone kinase assay [24,25], and therefore Western blot analysis of PKC isoforms was performed using commercially available antibodies against the α, β, γ, ϵ and ζ isoforms. As expected, PKC-γ was not found in bone marrow cells, since it is known that this isoform is expressed only in the brain [1]. As shown in Figure 4, all other isoforms were found associated with the particulate fraction of bone marrow cells (lane 1), as well as in the soluble fraction (results not shown), and their translocation to a membrane fraction could be stimulated by treatment of the cells with PMA (lane 3). PLA_2 inhibitors did not influence the amounts of cPKCs and nPKCs associated with the particulate fraction in either control or PMA-treated bone marrow cells (Figure 4, lanes 2 and 4). On the other hand, the pretreatment of cells with mepacrine and heparin lowered the amount of atypical PKC-ζ associated with the particulate fraction of control cells (Figure 4, lane 2) and diminished PMA-induced translocation of PKC-ζ to the membrane fraction (lane 4), suggesting that PKC-ζ may operate downstream of PLA_2 and is activated by arachidonic acid. Treatment of bone marrow cells with arachidonic acid induced translocation of PKC-ζ to the membrane fraction, which was not influenced by PLA_2 inhibitors (results not shown). It is important to note that in the unstimulated bone marrow cells only about 5% of the total PKC-ζ is associated with the particulate fraction, while 95% is found

Table 4 Effects of PMA, arachidonic acid and different inhibitors on colony formation by murine bone marrow cells

Bone marrow cells were treated for 45 min with PMA (500 nM) or arachidonic acid (15 μM), for 60 min with mepacrine (500 μM) plus heparin (1 mg/ml), indomethacin (50 μM) or NDGA (50 μM), or pretreated for 15 min with mepacrine (500 μM) plus heparin (1 mg/ml), indomethacin (50 μM) or NDGA (50 μM) and then stimulated with PMA (500 nM) or arachidonic acid (15 μM) for 45 min. All other details are described in the Materials and methods section. After a 7 day incubation, the number of colonies scored in control wells varied from 18 to 151 (mean ± S.E.M. 58 ± 6; $n = 17$). All data are from at least three different experiments performed in duplicate. ND, not determined. *Statistically significant difference ($P < 0.05$; Student's t test) from control; †statistically significant difference from PMA-treated in the absence of inhibitors.

	Colony formation (% of control)		
Treatment	Control	PMA	Arachidonic acid
None	100.0 ± 2.9	241.2 ± 30.8*	219.3 ± 23.6*
Mepacrine + heparin	67.5 ± 20.5	74.5 ± 20.3†	209.5 ± 19.6*
Indomethacin	101.2 ± 6.7	228.3 ± 28.6*	ND
NDGA	92.3 ± 6.6	194.8 ± 17.5*	ND

to be in the soluble fraction (assessed by densitometric analysis of the Hyperfilm after enhanced chemiluminescent detection of Western blots). Because PMA induced an approx. 1.5-fold increase in the membrane-bound PKC-ζ, no clear decrease in the cytosolic PKC-ζ could be detected on the Western blots (results not shown). Since it is known that under *in vitro* conditions PtdInsP_3 may also be a PKC-ζ activator [26], we used wortmannin, an inhibitor of PtdIns 3-kinase [27], as a tool to prevent PtdInsP_3 formation in the bone marrow cells. A 15 min pretreatment of cells with wortmannin (100 nM), which is known to be sufficient to inhibit PtdIns 3-kinase and its physiological effects [27], did not influence the amount of PKC-ζ associated with the particulate fraction in control cells and could not abolish its PMA-mediated translocation (results not shown).

When grown in semi-solid medium containing an adequate supply of growth factors, bone marrow cells form colonies, which is used as a proliferation and differentiation test [7,8]. Therefore the effects of short-term exposure of cells to PMA, arachidonic acid and different inhibitors were investigated on the ability of murine bone marrow cells to form colonies in methylcellulose supplemented with an optimal level of cytokines. As shown in Table 4, treatment of cells for 45 min with PMA or arachidonic acid significantly increased the number of colonies counted after 7 days. As it is known that PMA is very poorly released/washed out of cells and that PMA treatment might not be limited to only 45 min for the colony formation assay, bone marrow cells were stimulated with the more hydrophilic PDBu (500 nM) for 45 min, and a significant increase in the number of colonies was counted after 7 days when compared with the control group (207.5 ± 26.2; result is from three different experiments performed in duplicate and all other details are as shown in Table 4). When cells were treated simultaneously with PMA and arachidonic acid, no further increase in the number of colonies could be observed when compared with the groups treated with either PMA or arachidonic acid alone (240.2 ± 18.9; result is from three different experiments performed in duplicate and all other details are as shown in Table 4). While exposure of cells to PLA_2 inhibitors reduced the number of colonies formed in the control group and abolished the increase in the PMA-stimulated group, the treatment of cells with arachidonic acid overcame the effect of inhibitors on colony formation. Moreover, the treatment of cells with either cyclo-oxygenase or lipoxygenase

inhibitors, which effectively blocked production of arachidonic acid metabolites (Table 2), did not influence the PMA-mediated increase in colony formation, further suggesting that arachidonic acid, but not its metabolites, is essential in the signalling pathway that is responsible for PMA-mediated effects in bone marrow cells.

DISCUSSION

PKC activation is caused by increased amounts of DAG in membranes, which is generated primarily by stimulation of the inositol lipid/Ca^{2+} signalling pathway. In recent years it has become clear that the products of hydrolysis of other membrane phospholipids, particularly PtdCho, by PLC, PLD and/or PLA_2 may enhance and prolong the activation of PKC, which may be essential for long-term cellular responses such as cell proliferation and differentiation [1,17]. Tumour-promoting phorbol esters have been widely used to by-pass initial signalling processes in studies of the downstream effects of PKC [1], especially in complex experimental systems such as haematopoiesis, where signal transduction pathways that are activated after ligand–receptor interactions are poorly understood but where PKC plays an important role in the control and regulation of cell growth [4,6]. In the present investigation, PMA-mediated activation of PKC in bone marrow cells did not influence $[Ca^{2+}]_i$ homeostasis, PLC or PLD activity (which could be increased only when cells were stimulated by calcium ionophore) or the concentration of cAMP, even in leukaemic and haematopoietic cells where 'cross-talk' between these signalling pathways has been shown to be modulated by PKC [1,2]. Nevertheless, it was observed that PMA-mediated PKC activation in bone marrow cells leads to increased PLA_2 activity.

In cells, two major groups of PLA_2s are recognized: a low-molecular-mass secretory PLA_2 and a high-molecular-mass cytosolic PLA_2 ($cPLA_2$) [19]. The latter is known to be selective for arachidonic acid, to be activated by nanomolar concentrations of Ca^{2+} and to be insensitive to DTT [18,19,28]. Upon stimulation of various cell types with activators of PKC or protein tyrosine kinases (e.g. epidermal growth factor), $cPLA_2$ is phosphorylated and translocated to cell membranes [28,29]. Present data show that treatment of bone marrow cells with PMA increased particulate PLA_2 activity that is insensitive to DTT. This was accompanied by an increase in arachidonic acid release from cells, so it seems that $cPLA_2$ is activated by PKC-mediated phosphorylation and perhaps in part by translocation, although other possible mechanisms of PLA_2 stimulation, such as activation by phospholipase-activating protein, lipocortin or G-proteins, could not be completely ruled out.

Recently it has been demonstrated that $cPLA_2$ activation depends on phosphorylation of serine-505 by mitogen-activated protein (MAP) kinase, which can be achieved through both PKC-dependent and PKC-independent mechanisms [30]. Moreover, it is known that, in leukaemic and haematopoietic cell lines, activation of the MAP kinase pathway by either granulocyte/macrophage colony-stimulating factor (GM-CSF) or PKC will stimulate cell proliferation and differentiation [31–33]. This, together with evidence that macrophage colony-stimulating factor (M-CSF) and GM-CSF activate $cPLA_2$ [34,35] and the present observation of PMA-mediated activation of PLA_2 which correlates with the increased ability of bone marrow cells to form colonies *in vitro* (a well known assay for both proliferation and differentiation), not only suggests a possible involvement of PKC-mediated activation of the MAP kinase pathway in the activation of PLA_2 but also gives PLA_2 an important role in the control of mitogenesis in bone marrow cells. This was further supported by using the putative PLA_2 inhibitors heparin and mepacrine [20,21]. Heparin has anti-proliferative effects on many cell types including endothelial cells [36], Schwann cells [37] and muscle cells [38] where it inhibits the PKC-dependent pathway for proto-oncogene induction [39], as well as MAP kinase activation [40], while mepacrine blocks growth factor- and serum-stimulated proliferation of endothelial and smooth muscle cells [41]. We observed that exposure of cells to these compounds inhibited more than 80% of the initial PLA_2 activity, diminished the release of arachidonic acid from the cells and abolished the increase in the number of colonies formed after treatment of the cells with PMA. Therefore current results corroborate the hypothesis formulated by Reilly et al. [42] that heparin regulates cell proliferation and that one possible mechanism is to inhibit, together with mepacrine, PLA_2 activity.

PMA-sensitive PKCs are involved in mitogenic signal transduction in many cell systems [1], although this does not appear to be a general phenomenon. Recently it has been observed that the PKC-ζ isoform, which is insensitive to PMA, is critical for mitogenic signal transduction and is associated *in vitro* with the MAP kinase pathway [43,44]. Although the mechanisms of PKC-ζ activation are only partially understood, it is known that PtdInsP_3 and unsaturated fatty acids can enhance PKC-ζ activity under *in vitro* conditions [25,45,46]. Since the pretreatment of cells with wortmannin did not influence the association of PKC-ζ with particulate fractions in control or PMA-stimulated bone marrow cells, it seems that in the experimental model used in this study and under *in vivo* conditions PtdInsP_3 does not comprise a part of the signalling pathway leading to the activation of PKC-ζ. On the other hand, pretreatment of cells with mepacrine and heparin, which effectively inhibit PLA_2 activity, reduced the amount of PKC-ζ associated with the particulate fraction in control cells and diminished the PMA-induced translocation of PKC-ζ. These observations correlate with the effects of these inhibitors on the ability of bone marrow cells to form colonies *in vitro*. Therefore it seems probable that in bone marrow cells PKC-ζ is important for mitogenic signal transduction, as has been shown in oocytes and fibroblasts [44]. In addition, in this experimental model the activation of PKC-ζ lies downstream to PLA_2 activation, since exogenous addition of arachidonic acid caused translocation of PKC-ζ to the particulate fraction and increased the number of colonies formed, and neither effect was influenced by PLA_2 inhibitors. It is also important to note that the pattern of translocation of PKC-ζ, as measured by Western blot analysis, should be interpreted with great caution, since anti-peptide antibodies directed against the C-terminus of PKC-ζ may also recognize a newly described PKC-ι, as well as a member of the cPKC subfamily which is thought to be distinct from either PKC-α or -β [3,25,47].

In conclusion, the present data suggest that PMA-mediated activation of cPKCs and nPKCs in bone marrow cells leads to PLA_2 activation which, by releasing arachidonic acid from phospholipids, activates PKC-ζ; this signalling pathway appears to be mitogenic. As it is well known that PKC has an apparent role not only in the development but also in the lineage commitment of myeloid progenitor cells [48], particularly in M-CSF-stimulated macrophage development from granulocyte/macrophage colony-forming cells [49], it will be of major importance to investigate the PMA-mediated signalling pathway in purified myeloid progenitor cells, as well as in leukaemia cell lines. The results obtained from signalling pathway studies should be correlated with the ability of cells to proliferate and differentiate, since PMA (present paper), M-CSF and GM-CSF [34,35] share at least a part of a common signalling process which, by affecting $cPLA_2$ and PKC-ζ, might not only control mitogenesis

as has been suggested [43,44] but also have a pivotal role in the development and lineage commitment of haematopoietic cells.

We thank Robin F. Irvine, Leonard R. Stephens, Nullin Divecha, David Lander and Andrew Letcher for valuable help and advice, and Rene Lui for editing the manuscript before submission. This work was supported by the Ministry for Science of the Republic of Croatia and by the Wellcome Trust.

REFERENCES

1 Nishizuka, Y. (1992) Science **258**, 607–614
2 Hug, H. and Sarre, T. F. (1993) Biochem. J. **291**, 329–343
3 Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y. and Biden, T. (1993) J. Biol. Chem. **268**, 24296–24302
4 Dexter, T. M. and Spooncer, E. (1987) Annu. Rev. Cell Biol. **3**, 423–441
5 Ogawa, M. (1993) Blood **81**, 2844–2853
6 Kishimoto, T., Taga, T. and Akira, S. (1994) Cell **76**, 253–262
7 Stuart, R. K. and Hamilton, J. A. (1980) Science **208**, 402–404
8 Sieber, F., Stuart, R. K. and Spivak, J. L. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 4402–4406
9 Folch, J., Lees, M. M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. **226**, 497–509
10 Divecha, N. and Irvine, R. F. (1990) in Methods in Inositide Research: Mass Measurement of Phosphatidylinositol 4-Phosphate and *sn*-1,2-Diacylglycerols (Irvine, R. F., ed.), pp. 179–185, Raven Press, New York
11 Stutchfield, J. and Cockcroft, S. (1993) Biochem. J. **293**, 649–655
12 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
13 Tamiya-Koizumi, K., Umekawa, H., Yoshida, S., Ishihara, H. and Kojima, K. (1989) Biochim. Biophys. Acta **1002**, 182–188
14 Dawson, D. W. and Cook, J. J. (1987) J. Cell. Physiol. **132**, 104–110
15 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
16 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
17 Berridge, M. J. (1993) Nature (London) **361**, 315–325
18 Channon, J. Y. and Leslie, C. C. (1990) J. Biol. Chem. **265**, 5409–5413
19 Dennis, E. A. (1994) J. Biol. Chem. **269**, 13057–13060
20 Blackwell, G. J., Duncombe, W. G., Flower, R. J., Parsons, M. F. and Vane, J. R. (1977) Br. J. Pharmacol. **59**, 353–366
21 Diccianni, M. B., Mistry, M. J., Hug, K. and Harmony, J. A. K. (1990) Biochim. Biophys. Acta **1046**, 242–248
22 Favre, C. J., Lew, D. P. and Krause, K. H. (1994) Biochem. J. **302**, 155–162
23 Capdevila, J., Gill, G., Orellana, M., Marnett, L. J., Mason, I., Yadagiri, P. and Flack, J. R. (1988) Arch. Biochem. Biophys. **261**, 257–263
24 Olivier, A. R. and Parker, P. J. (1991) Eur. J. Biochem. **200**, 805–810
25 Zhou, G., Wooten, M. W. and Coleman, E. S. (1994) Exp. Cell Res. **214**, 1–11
26 Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993) J. Biol. Chem. **268**, 13–16
27 Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) J. Biol. Chem. **269**, 3568–3573
28 Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, M. A., Lin, A. Y., Millona, N. and Knopf, J. L. (1991) Cell **65**, 1043–1049
29 Lin, L. L., Lin, A. Y. and Knopf, J. L. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6147–6151
30 Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A. and Davis, R. J. (1993) Cell **72**, 269–278
31 Bashey, A., Healy, L. and Marshall, C. J. (1994) Blood **83**, 949–957
32 Carroll, M. P. and May, W. S. (1994) J. Biol. Chem. **269**, 1249–1256
33 Kharbanda, S., Saleem, A., Emoto, Y., Stone, R., Rapp, U. and Kufe, D. (1994) J. Biol. Chem. **269**, 872–878
34 Nakamura, T., Lih-Ling, L., Kharbanda, S., Knopf, J. and Kufe, D. (1992) EMBO J. **11**, 4917–4922
35 Durstin, M., Durstin S., Molski, T. F. P., Becker, E. L. and Sha'afi, R. I. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3142–3146
36 Thornton, S. C., Mueller, S. N. and Levine, E. M. (1983) Science **222**, 623–625
37 Ratner, N., Bunge, R. P. and Glaser, L. (1985) J. Cell Biol. **101**, 744–754
38 Clowers, A. W. and Karnovsky, J. S. (1977) Nature (London) **265**, 625–626
39 Pukac, L. A., Castellot, J. J., Wright, T. C., Caleb, B. L. and Karnovsky, M. J. (1990) Cell Regul. **1**, 435–443
40 Ottlinger, M. E., Pukac, L. A. and Karnovsky, M. J. (1993) J. Biol. Chem. **268**, 19173–19176
41 Dethlefsen, S. M., Shepro, D. and D'Amore, P. A. (1994) Exp. Cell Res. **212**, 262–273
42 Reilly, C. F., Fritze, L. M. S. and Rosenberg, R. D. (1987) J. Cell. Physiol. **131**, 149–157
43 Berra, E., Diaz-Meco, M., Dominguez, I., Municio, M. M., Sanz, L., Lozano, J., Chapkin, R. S. and Moscat, J. (1993) Cell **74**, 555–563
44 Diaz-Meco, M. T., Dominguez, I., Sanz, L., Dent, P., Lozano, J., Municio, M. M., Berra, E., Hay, R. T., Sturgill, T. W. and Moscat, J. (1993) EMBO J. **13**, 2842–2848
45 Nakanishi, H. and Exton, J. H. (1992) J. Biol. Chem. **267**, 16347–16354
46 Kochs, G., Hummel, R., Meyer, D., Hug, H., Marme, D. and Sarre, T. F. (1993) Eur. J. Biochem. **216**, 597–606
47 Battle, E., Fabre, M. and Garcia de Herros, A. (1994) FEBS Lett. **344**, 161–165
48 Liebermann, D., Hoffmann-Liebermann, B. and Sachs, L. (1981) Int. J. Cancer **28**, 285–291
49 Whetton, A. D., Heyworth, C. M., Nicholls, S. E., Evans, C. A., Lord, J. M., Dexter, T. M. and Owen-Lynch, P. J. (1994) J. Cell Biol. **125**, 651–659

Received 9 November 1994/29 March 1995; accepted 10 April 1995