Okadaic acid activates p42 mitogen-activated protein kinase (MAP kinase; ERK-2) in B-lymphocytes but inhibits rather than augments cellular proliferation: contrast with phorbol 12-myristate 13-acetate

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Ligation of the membrane immunoglobulin M receptor as well as stimulation with the protein kinase C agonist phorbol 12-myristate 13-acetate leads to a B-lymphocyte proliferation and differentiation. Both stimuli activate p42 mitogen-activated protein (MAP) kinase in human B-lymphocytes [Casillas, Hanekom, Williams, Katz and Nel (1991) J. Biol. Chem. **266**, 19088–19094]. MAP kinase activation is dependent on tyrosine as well as threonine phosphorylation of the kinase and its activity is inhibited by tyrosine as well as threonine/serine phosphatases. Okadaic acid, a specific inhibitor of type 1 and 2A serine/threonine phosphatases, induced MAP kinase activity in a potent and dose-dependent fashion, but failed to induce [^{3}H]thymidine incorporation into normal human tonsil B-cells. Moreover, in combination with membrane immunoglobulin M ligation, okadaic acid decreased rather than increased [^{3}H]thymidine incorporation. The kinetics of MAP kinase activation by okadaic acid differed from phorbol 12-myristate 13-acetate and anti-membrane immunoglobulin M stimulation. Okadaic acid induced tyrosine phosphorylation of 42 kDa and 44 kDa proteins which co-electrophoresed and co-chromatographed with ERK-2 and ERK-1 respectively. Ramos cells also contained a constitutively active 46 kDa MAP kinase which appeared as a separate peak in chromatography and could be immunoprecipitated by an antiserum against a rat ERK-1 fusion protein.

INTRODUCTION

The induction of B-lymphocyte growth and differentiation by surface receptors such as membrane IgM (mIgM) and the protein kinase C (PKC) agonist, phorbol 12-myristate 13-acetate (PMA), is dependent on reversible phosphorylation of proteins [1]. Although the roles of protein kinases have been extensively explored in B-cells, not much is known about the role of phosphatases in signalling. Protein phosphatases are clearly important, since lymphocytes contain all four major serine/threonine protein phosphatase (PP) activities: PP1, PP2A, PP2B and PP2C [2–4]. It has become possible to study the role of PP1 and PP2A in mammalian cells more directly due to the availability of okadaic acid (OA), a potent and specific inhibitor of these phosphatases *in vitro* as well as in intact cells [2–4]. Moreover, OA is a tumour promoter in mouse skin, which suggests that OA affects the phosphorylation of substrates involved in cellular growth [2,5].

As one may expect from a serine/threonine phosphatase inhibitor, the result of OA treatment is complex and can affect the phosphorylation of a large number of intracellular proteins [2]. We were particularly interested in the effect of OA on p42 microtubule-associated protein-2 kinase (MAP-2K), also known as mitogen-activated protein kinase (MAP kinase), which is controlled by a process of reversible phosphorylation [1,6,7]. MAP kinase is a serine kinase which is activated during mIgM ligation or PMA treatment of B-lymphocytes [1] and is identical to p42^{MAPK}, which is induced by a variety of growth factor receptors in fibroblasts, adipocytes and neuronal cells [8–11]. p42^{MAPK} belongs to a kinase family some of the members of which have been cloned and are included in the extracellular signal-regulated kinase (ERK) family [12]. According to the latter nomenclature, ERK-1 represents p44 MAP-2K or p44^{MAPK} and ERK-2 is p42 MAP-2K or p42^{MAPK} [12,13]. p42^{MAPK} phosphorylates several substrates *in vitro*, including microtubule-associated protein-2 (MAP-2) and a peptide, APRTPGGRR, which constitutes a consensus site for MAP-kinase phosphorylation in bovine myelin basic protein (MBP) [13]. The kinase itself is a threonine and tyrosine phosphoprotein and can be inactivated by CD45, a tyrosine phosphatase, and PP2A, a serine/threonine phosphatase [14]. There is much debate currently as to whether the addition of the threonine and tyrosine phosphate groups to p42^{MAPK} reflects the effect of afferent kinases or autophosphorylation; however, in T-lymphocytes we and others have shown that the tyrosine protein kinase (TPK) p56^{lck} may be involved [15–17].

It has been shown that OA can induce p42 MAP kinase activity in fibroblasts and PC12 cells [6,7,18]. We were interested to see whether PP1/PP2A exert the same effect in B-lymphocytes and if this biomedical response, as for PMA, is associated with cellular growth. In the Ramos B-lymphoblastoid cell line, we were able to demonstrate an OA-activated MAP kinase activity which co-chromatographed with ERK-2 (p42^{MAPK}) and ERK-1 (p44^{MAPK}). PMA, but not OA, stimulated the proliferation of normal human B-lymphocytes.

MATERIALS AND METHODS

Reagents

OA was purchased from the LC Service Corporation (Woburn, MA, U.S.A.). PMA was from Sigma (St. Louis, MO, U.S.A.). [γ-^{32}P]ATP was from ICN Radiochemicals (Costa Mesa, CA,

Abbreviations used: mIgM, membrane immunoglobulin M; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase; MAP-2K, microtubule-associated protein-2 kinase; OA, okadaic acid; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; TPK, tyrosine protein kinase; APT, anti-phosphotyrosine; PP1/PP2A, protein phosphatase type 1 or 2A; MBP, myelin basic protein; SAC, *Staphylococcus aureus*, Cowan strain I; [^{3}H]TdR, [^{3}H]thymidine.

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U.S.A.) and ^{125}I-Protein A was from Amersham (Arlington Heights, IL, U.S.A.). Fetal calf serum and tissue culture reagents were from Gibco (Grand Island, NY, U.S.A.). [^{3}H]Thymidine ([^{3}H]TdR) was obtained from NEN Products (Boston, MA, U.S.A.). *Staphylococcus aureus*, Cowan strain I (SAC), was from Calbiochem (La Jolla, CA, U.S.A.).

Antibodies

A biotinylated goat anti-human IgM antibody was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL, U.S.A.). Affinity-purified rabbit anti-phosphotyrosine (APT) was donated by Dr J. Ledbetter (Oncogen Corp., Seattle, WA, U.S.A.). This antiserum is specific for tyrosine phosphoproteins [19]. Rabbit antiserum 691, which was raised against a synthetic peptide encompassing residues 305–327 (subdomain XI) or ERK-1 and -2 proteins [20], was generously provided by Dr M. Cobb (University of Texas, Southwestern Graduate School of Biomedical Sciences, Dallas, TX, U.S.A.). We also produced a rabbit antiserum against a denatured and incomplete rat ERK-1 fusion protein. A cDNA copy of rat ERK-1 (nucleotides 481–986) was obtained by PCR and was inserted into the pGEX-3X vector (Pharmacia) as a fusion with the glutathione S-transferase (GST) [12,21] gene. The vector was introduced into *Escherichia coli*. Because the fusion protein was insoluble in 1% Triton X-100, the pellet was boiled in SDS-sample buffer and the proteins resolved by preparative-scale SDS/PAGE. The fusion protein was electroeluted from gel slices and used to immunize rabbits. The IgG fraction was purified across a Protein A–Sepharose column (Bio-Rad) and shown by Western blotting to react with the fusion protein and the ERK-1 fragment, but not GST.

Cell culture and stimulation

The human Burkitt's lymphoma cell line, Ramos (mIgM+), was grown in RPMI 1640 with 10% fetal calf serum and antibiotics (complete medium) at 37 °C in a 5% CO_2 atmosphere [22]. Duplicate cell aliquots of 5×10^6 cells in 300 μl of complete medium were stimulated with the indicated concentrations of anti-IgM, PMA or OA. Control cultures received dimethyl sulphoxide (DMSO; carrier for OA and PMA) to a final concentration of 0.1%. Tonsillar lymphocytes were obtained from surgical specimens and enriched for B-cells (> 97% pure) by depleting T-cells (E-rosetting) and adherent cells. [^{3}H]TdR uptake was determined using 10^4 cells/well in triplicate on microtitre plates together with appropriate stimuli (PMA, OA and SAC) for 24–72 h at 37 °C in a 5% CO_2 atmosphere; 2 μCi of [^{3}H]TdR/well was added for the last 18 h before cells were harvested and counted for radioactivity in a scintillation counter.

Preparation of cellular lysates and rapid purification of MAP kinase

Where MAP kinase activity was simultaneously measured in a large number of samples, duplicate cellular aliquots of 5×10^6 cells were briefly centrifuged in an Eppendorf Microfuge and lysed in 20 mM Tris (pH 7.4), 1 mM EGTA, 1 mM phenylmethylsuphonyl fluoride, 100 μM Na_3VO_4, 50 mM NaFl and protease inhibitors (buffer A) [1,16,23–25]. The supernatants were added to 100 μl of a 50% DEAE-Sephacel suspension, following which the kinase was eluted over the salt range 100–200 mM NaCl [1,16,23–25].

Mono-Q chromatography

Large-scale purification of MAP kinase was carried out by sonication lysis of 200×10^6 stimulated or unstimulated cells in 3 ml of buffer B, containing 50 mM β-glycerophosphate, 1.5 mM EGTA, 100 μM Na_3VO_4, 1 mM dithiothreitol, 1 mM benzamidine, leupeptin (10 μg/ml) and aprotinin (100 μg/ml) (pH 7.3) [9]. Subsequently, the lysate was centrifuged at 100000 *g* at 4 °C for 20 min. Equal amounts of supernatant protein (12 mg) were loaded onto a 1.5 cm × 2 cm Mono-Q column, followed by washing with 40 ml of buffer B. The column was eluted in 0.5 ml fractions by 45 ml of a linear 0–400 mM NaCl gradient. MAP kinase activity was determined in even-numbered fractions.

Phosphorylation protocols

MAP kinase activity was measured *in vitro* by adding 5 μg of DEAE-eluate protein or 45 μl of Mono-Q fractions into reaction vials containing 2 mM $MnCl_2$, 5 μg of MAP-2 substrate (prepared as described in [1]), and 10 μM [γ-^{32}P]ATP (2–8 μCi/nmol) [1,16,23–25]. The reaction was carried out for 20 min at 4 °C and stopped by adding SDS sample buffer and boiling. The phosphorylated substrate was separated by 4–11% SDS/PAGE and ^{32}P incorporation determined by doing Čerenkov counts on gel slices [1,16,23–25]. We have previously shown that this assay selects for MAP kinase [1,16]. Confirmation of the specificity of the kinase was also sought by *in vitro* phosphorylation of a specific MAP kinase peptide substrate, APRTPGGRR (results not shown) [13]. The phosphorylated peptide was absorbed onto 2 cm × 2 cm phosphocellulose squares which were washed in 10 mM H_3PO_4 and counted for radioactivity [13].

Immunoblotting

The soluble fraction from 3×10^6 resting or stimulated (anti-IgM, PMA, OA) Ramos cells were boiled with 3 × SDS sample buffer and resolved by SDS/PAGE (10% gels). After electrophoretic transfer to Immobilin-P membranes, these were blocked for 2 h at 20 °C in 6% bovine serum albumin (fraction V), 0.1 M NaCl and 50 mM Tris (pH 7.4). The membranes were incubated with APT (0.25 μg/ml), 691 (1:2000) or the IgG fraction of the rabbit anti-ERK-1 antibody (1:500) for 2 h. Membranes were washed and overlaid with ^{125}I-Protein A (0.5 μCi/ml) for 1 h, washed and autoradiographed [1].

Immune affinity chromatography

Peak I MAP kinase activity (1 ml) obtained by Mono-Q chromatography (fractions 26 and 27 from control cells) was passed through a Hydrazide Avidchrom (Bioprobe International, Inc.) column (1.0 ml bed vol.) onto which 500 μg of the IgG fractions of the anti-ERK-1 fusion protein antibody had been immobilized according to the manufacturer's instructions [1]. The column was washed in buffer B and eluted with 50 mM glycine/HCl (pH 2.0). Fractions of 0.5 ml were collected and immediately neutralized to pH 7.4 by adding them to 15 μl of 2 M Tris-base in collection tubes. MAP kinase activity was measured in the starting material, the wash fractions and the eluate.

RESULTS

OA induces MAP kinase activity in B-cells

Treatment of the Ramos B-cell line with 0.001–1.0 μM OA induced MAP kinase activity in a dose-dependent fashion (Table 1). The results were similar whether MAP-2 protein (Table 1) or

Table 1 Dose–response for OA stimulation

Duplicate aliquots of 5×10^6 Ramos cells were stimulated with the indicated final concentrations of OA for 60 min, after which cells were lysed and MAP kinase activity determined by *in vitro* phosphorylation of MAP-2 protein, as described in Materials and methods. Similar results were obtained with the peptide substrate, APRTPGGRR. Results are means ± S.E.M.

Cell stimulator	MAP kinase activity as ^{32}P incorporation (c.p.m.)
Unstimulated	1874 ± 300
100 nM OA	2753 ± 107
250 nM OA	4771 ± 145
500 nM OA	15463 ± 53
1 μM OA	15324 ± 188

Table 2 Time course of stimulation by OA

Duplicate aliquots of 5×10^6 Ramos cells were stimulated with 1 μM OA for the time periods indicated. After lysis, MAP kinase activity was determined *in vitro* using the peptide substrate, APRTPGGRR, as described in Materials and methods. (Compare with OA stimulation shown in Figure 1a). Results are means ± S.E.M.

Time after stimulation	MAP kinase activity as ^{32}P incorporation (c.p.m.)
Unstimulated	5976 ± 1923
30 min	8827 ± 177
60 min	10464 ± 480
90 min	17332 ± 3672
120 min	22155 ± 2344

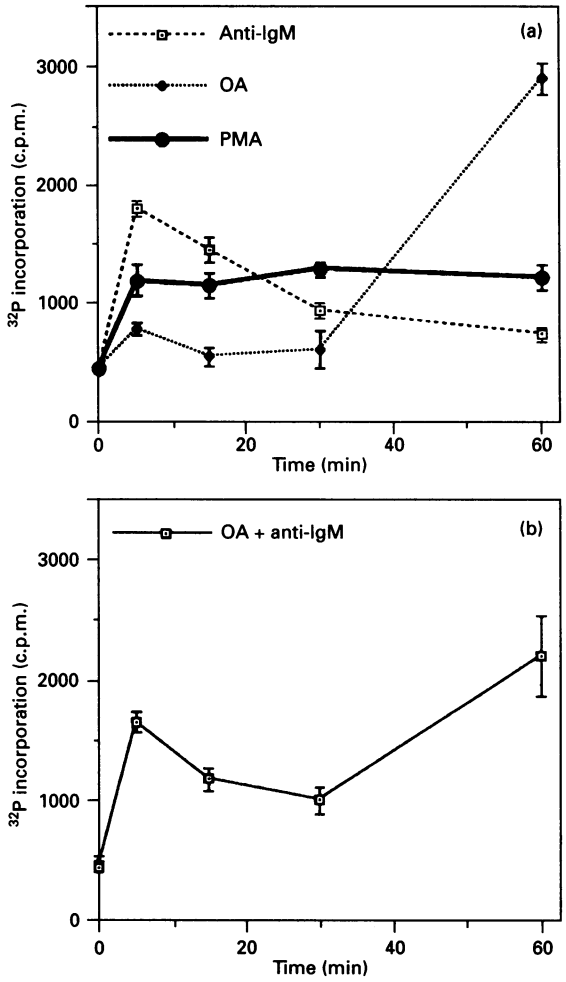

Figure 1 OA, PMA and α-IgM activate MAP kinase with unique kinetics

(**a**) Ramos cells were stimulated with 1 μg/ml biotinylated anti-IgM (secondarily crosslinked with 50 μg/ml avidin), 50 nM PMA and 1 μM OA for the time periods indicated. MAP kinase activity was purified by DEAE batch absorption and *in vitro* activity measured by the ability to phosphorylate MAP-2 protein as described [23–25]. (**b**) The combination of 1 μM OA and 1 μg/ml anti-IgM plus 50 μg/ml avidin was added at time zero.

the peptide, APRTPGGRR, was used as a substrate (results not shown, but see Table 2). The optimal stimulatory dose was 0.5–1 μM, which is in agreement with previous findings and the estimated intracellular concentration of these phosphatases [2,4,6]. Similar results were obtained in normal human tonsillar B-lymphocytes (not shown). MAP kinase activity was also induced by calyculin-A, another and even more potent inhibitor of PP1/PP2A. In a representative experiment, ^{32}P-incorporation into MAP-2 increased from 500 ± 30 c.p.m. in control cells to 2170 ± 300 c.p.m. after exposure to 2 nM calyculin-A for 60 min.

The kinetics of MAP kinase activation by OA differ from those of PMA or anti-IgM activation

OA and PMA are potent tumour promoters in mouse skin [2,5]. Both induced MAP kinase activity in B lymphocytes (Figure 1). Whereas the kinetics of MAP kinase activation by PMA consisted of rapid activation (peak < 5 min) with a slow return to baseline, OA induced a more gradual response with a discernible increase in activity from 30 to 60 min (Figure 1a). When MAP kinase activity was tested using the peptide substrate APRTPGGRR, similar results were seen and the kinase was still active 2 h later (results not shown), and no effect on cell viability was noted by Trypan Blue exclusion. Membrane IgM ligation induced an evanescent MAP kinase response (Figure 1a). Furthermore, co-stimulation of Ramos cells with anti-IgM and OA yielded dual activation, with a peak at 5 min and then beginning to rise again after 30 min (Figure 1b). These activities coincide with anti-mIgM and OA stimulation respectively, which suggests that OA does not affect the physiological signalling pathway.

PMA and OA differ in their mitogenic effects on human tonsillar B cells

PMA and IgM ligation, through their ability to activate PKC, act as mitogens for human tonsillar B-cells (Table 3). Contrary to predictions from the MAP kinase data, 72 h of OA treatment failed to induce [^{3}H]TdR incorporation and suppressed DNA synthesis by SAC (Table 3). This was not due to toxicity, because OA did not significantly change the viability compared with the control or the PMA-stimulated cells (> 83% viability in all cases). To exclude the possibility that OA may have induced an S phase which terminated before [^{3}H]TdR was added, we also studied [^{3}H]TdR uptake after 24 and 48 h (1.4×10^3 and 2.0×10^3 c.p.m., respectively) but did not see a discernible increase compared to control (1.3×10^3 c.p.m.). An absence of mitogenesis under MAP kinase activation probably reflects the dual nature of PP1/PP2A effects on the cell cycle.

Table 3 Differences in the mitogenic effects of PMA and OA on human tonsillar B-cells

Human tonsillar B-cells (5×10^4) were stimulated in triplicate microtitre wells with the indicated doses of PMA, OA, SAC or SAC + OA (200 μl) for 72 h. Each well was pulsed with 2 μCi of [^{3}H]TdR over the last 18 h. Cells were harvested onto glass-fibre filters and radioactivity was counted in a β-counter in a scintillation cocktail. Cell viability by Trypan Blue exclusion was > 83% in all samples.

Cell stimulation	$10^{-3} \times$ [^{3}H]TdR incorporation in normal human B-cells (c.p.m.)
Unstimulated	0.8 ± 0.02
PMA (50 nM)	16.7 ± 0.06
OA (10 nM)	0.8 ± 0.02
OA (100 nM)	2.1 ± 0.02
OA (1 μM)	1.8 ± 0.05
SAC (0.0005%)	17.0 ± 0.25
SAC + OA (1 nM)	13.9 ± 3.40
SAC + OA (10 nM)	10.2 ± 0.16

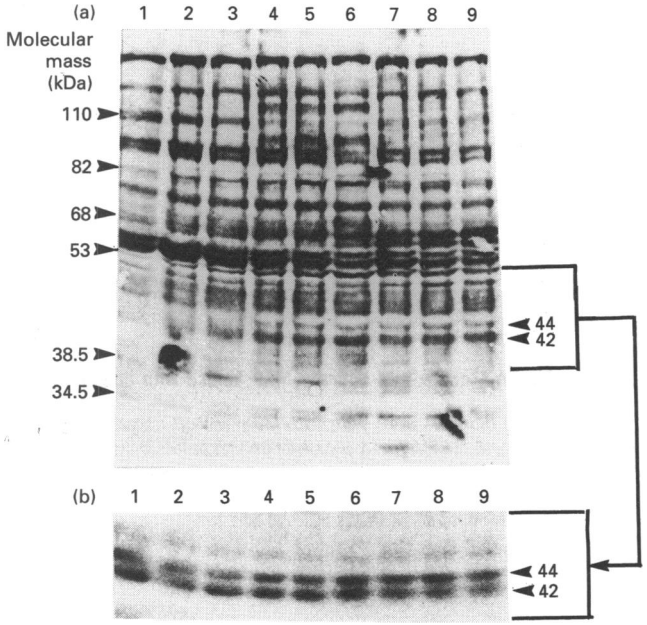

Figure 2 Induction of tyrosine phosphoproteins which co-electrophorese with ERK-1 and ERK-2

Ramos cells were stimulated in the presence of 1 μM OA (lanes 2–6) or 50 nM PMA (lanes 7–9). The soluble fraction from 3×10^6 cells was boiled with 3 × SDS sample buffer, resolved by SDS/PAGE (10% gels) and transferred to Immobilin-P membranes as described. Immunoblotting was first performed with APT antibodies (**a**), then the membrane was stripped and reprobed with Ab 691 (**b**), which recognizes ERK-1 and ERK-2. Lane 1, control; lanes 2–5, OA for 5, 15, 30 and 60 min, respectively; lanes 6–9, PMA for 5, 15, 30 and 60 min respectively. The experiment was reproduced once.

OA induces tyrosine phosphorylation of p42 MAP kinase

We have previously demonstrated that a 42 kDa tyrosine phosphoprotein induced by PMA and anti-IgM in Ramos cells can be purified as MAP kinase [1]. OA also induced tyrosine phosphorylation of a 42 kDa and, to a lesser extent, a 44 kDa protein (Figure 2a). This response followed the more gradual kinetics of MAP kinase activation by OA (compare Figures 1 and 2a). In contrast, more rapid induction of p42 and p44 by

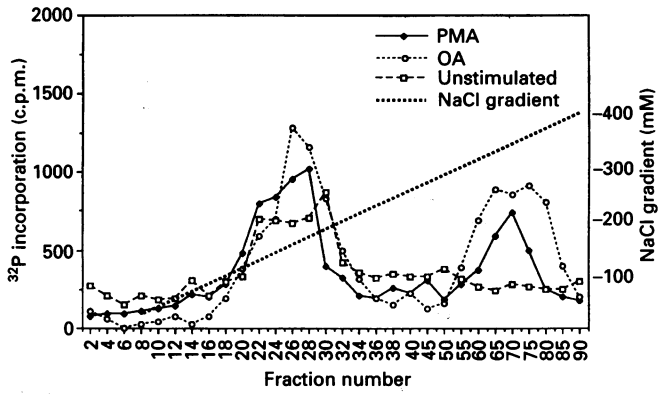

Figure 3 OA and PMA activate the same peak of MAP kinase activity as determined by Mono-Q chromatography

Ramos cells (200×10^6) were stimulated with 1 μM OA, 100 nM PMA or 0.1% dimethyl sulphoxide alone (control) and processed as described in Materials and methods for Mono-Q chromatography. Fractions of 0.5 ml were collected and assayed for MAP-2 phosphorylation as described. The results of stimulation were compared with unstimulated cells. PMA and OA both induced peak II, which eluted at 300–380 mM NaCl. A peak I MAP kinase activity was seen to elute at 100–200 mM NaCl and was present in control as well as stimulated cell populations. The findings from control, PMA- and OA-treated cells were reproduced once. Using PMA-treated cell lysates and the MBP peptide, APRTPGGRR, the same profile was obtained (results not shown).

PMA accompanied more rapid MAP kinase activation by the same agent (Figure 2a). When the APT immunoblot shown in Figure 2(a) was reprobed with antiserum 691, a 42/44 kDa doublet representing ERK-2 and ERK-1, respectively, was seen to co-electrophorese with p42 and p44 (Figure 2b) [20]. Although post-translational modification of MAP kinases results in electrophoretic retardation, this SDS/PAGE analysis did not show a mobility shift (Figure 2b), but such an effect could be seen in other experiments (data not shown).

OA and PMA both activate the same peak of MAP kinase activity during Mono-Q chromatography

Lysates obtained from PMA- and OA-treated cells after fractionation on Mono-Q columns yielded two peaks of activity which phosphorylate MAP-2 protein. Peak I eluted at 100–200 mM NaCl, while peak II eluted at 300–380 mM NaCl (Figure 3). The same profile was obtained when the MBP peptide was used as substrate (data not shown). Unstimulated cells contained a single peak of activity, peak I (Figure 3). The relative abundance of peak I activity was only slightly less than in stimulated cells, which implies that this is a constitutively active kinase (Figure 3). Peak II, in contrast, is clearly an inducible activity.

In order to determine how these peaks relate to the proteins detected in Figure 2(a), we immunoblotted Mono-Q fractions (Figure 3) from control, OA-, and PMA-treated samples with antiserum 691 [20]. Immunoblotting revealed a 42/44 kDa doublet which was present in peak II of OA- (Figure 4b) as well as PMA- (Figure 4c) treated populations, but was absent from peak I or the trough between these peaks (Figures 4a, 4b and 4c). In contrast, untreated Ramos cells yielded only small amounts of the 42/44 kDa doublet at the start of peak II (Figure 4a). We therefore looked for ERK-1 and ERK-2 in Mono-Q flow-through and trough fractions. No immunoreactive bands were detected in the Mono-Q flow-through or wash fractions (results not shown), but a 42/44 kDa doublet can be seen when the trough between peaks I and II was more extensively investigated (Figure 4d). Corresponding fractions from PMA- and OA-

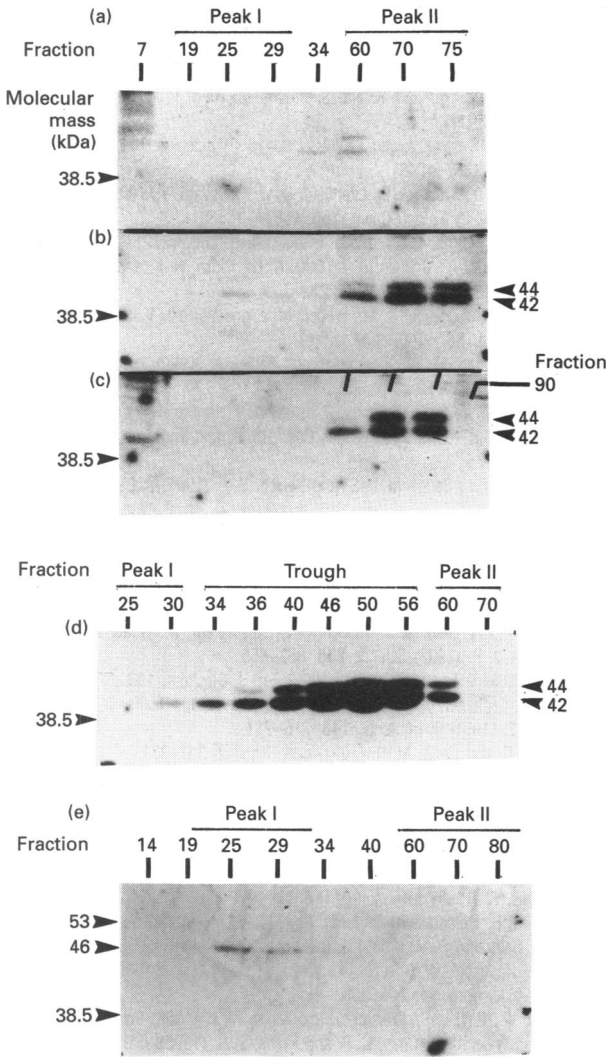

Figure 4 Immunoblotting with antiserum 691 and an antibody to an ERK-1 fusion protein

Mono-Q fractions obtained from control (**a**), OA- (**b**) and PMA- (**c**) treated cells (Figure 3) were immunoblotted with antiserum 691 (1:1000 dilution). The 38.5–53 kDa region of the autoradiogram shows a 42/44 kDa doublet in peak II from OA- and PMA-stimulated Ramos cells. In contrast, unstimulated cells showed a small amount of the 42/44 kDa doublet in trough fractions and at the beginning of peak II. In a separate blot (**d**) on control cells, fractions intermediate between peaks I and II were immunoblotted with Ab 691 (1:1000 dilution). The autoradiogram shows that most of the 42/44 kDa doublet eluted before the onset of peak II and suggests that after cellular activation ERK-1/ERK-2 elute at higher salt from Mono-Q resin. Corresponding fractions from PMA- or OA-stimulated cells did not reveal 691 immunoreactivity (data not shown). Fractions shown from the experiment in (**b**) were also immunoblotted and antiserum raised against a denatured ERK-1 fusion protein (see Materials and methods) (**e**). A single 46 kDa protein could be seen to elute in fractions corresponding to peak I and notably was absent in trough and peak II fractions. Similar results were obtained in PMA-treated samples.

stimulated cells did not reveal ERK-1 or ERK-2 proteins (results not shown). This implies that ERK-1 and ERK-2 elute at higher salt concentration from Mono-Q columns following cellular activation with OA and PMA.

Although peak I had no immunoreactivity with antiserum 691, we discerned a 46 kDa protein in this peak with our own antiserum against an ERK-1 fusion protein (Figure 4e). It is possible that the denatured state of the fusion protein antigen (purified by SDS/PAGE) may have exposed an epitope which is more abundantly expressed in the 46 kDa protein than in ERK-1. In order to prove that the 46 kDa protein is a MAP kinase, we immobilized the IgG fraction of our antiserum to a support resin and passed an aliquot of peak I (from control cells) through the column. More than 80% of initial MAP kinase activity bound to the column (Table 4). About half of the initial activity could be recovered by elution with an acidic buffer (Table 4). This suggests that a major portion of peak I kinase activity is contained in an unidentified but constitutively active 46 kDa MAP kinase.

Table 4 Binding of peak I to anti-ERK fusion protein antiserum

The IgG fraction of the anti-ERK fusion antiserum was immobilized on a column (Hydrazide Avidchrom) and a 1.0 ml aliquot of peak I material (control cell fractions 26 and 27) was allowed to bind. The starting material (peak I) and non-bound fractions were assayed for MAP kinase activity using MAP-2 protein as the substrate. The column was washed with 10 ml of buffer B and eluted with 50 mM glycine/HCl (pH 2.0). Fractions of 0.5 ml were collected and neutralized with 15 μl of 1 mM Tris-base. MAP kinase activity was recovered in fraction 8 (0.5 ml). Results are expressed as a percentage of total activity in starting material (peak I).

Fraction	MAP-2 phosphorylation activity immunoprecipitated by anti-ERK fusion antisera (% of total)
Peak I	100
Non-bound fraction	16
Eluate	43

DISCUSSION

In this paper, we provide evidence for the diverse effects of OA on B-lymphocyte mitogenesis. Although OA activated an early biochemical event associated with cellular growth, it failed to promote [^{3}H]TdR uptake at the intact cell level. This implies that PP1/PP2A may exert both stimulatory and inhibitory effects at different stages of the cell cycle.

The progression of B-lymphocytes through the cell cycle is dependent on reversible phosphorylation of signalling intermediates. Although some knowledge exists about the role of protein kinases in these events, not much is understood about the role of phosphatases. With the introduction of OA, it was possible to study the effect of PP1/PP2A at the intact cell level and relate this to a signalling response, namely the activation of MAP kinase. We show that OA induced MAP kinase activity in a dose- and time-dependent fashion in normal human B-lymphocytes as well as the B-lymphoblastoid cell line, Ramos (Tables 1 and 2, Figure 1). MAP kinase has the potential to regulate transcriptional activity through phosphorylation of c-*jun* and c-*myc*, and also activates ribosomal S6 kinase II (p90) [26–28]. It is probably relevant, therefore, that OA has been found to induce AP-1 activity in human lymphocytes by transcriptional activation of *jun* and *fos* [29]. In spite of activating a signalling pathway associated with cellular growth, OA failed to stimulate growth of normal human B-lymphocytes (Table 3). This contrasts with the effect of another tumour promoter and MAP kinase inducer, PMA [1]. PMA is a PKC agonist and a potent B-cell mitogen (Table 3). The difference in the biological response to PMA and to OA probably derives from the ability of the latter agent to exert more diverse effects than PMA. The range of OA substrates may include phosphoproteins that act downstream of MAP kinase but in an opposing fashion. In this paper we have not attempted to define the substrates or points in the cycle at which OA inhibits cycle events, but it is interesting that Metcalfe and Milner have previously shown that delayed addition of OA to concavalin A (Con A)-stimulated T-lymphocytes prevented these cells from entering S phase [30].

As defined by Mono-Q chromatography, OA induced the same peak of MAP kinase activity as did PMA (Figure 3). This peak co-chromatographed with ERK-1 and ERK-2, as defined by immunoblotting with antiserum 691 (Figure 4). In crude cell lysates, OA and PMA induced tyrosine phosphorylation of 42 kDa and 44 kDa proteins which co-electrophoresed with ERK-2 and ERK-1 respectively (Figure 2). The kinetics of tyrosine phosphorylation of these proteins differed for OA and PMA in accordance with their kinetics of MAP kinase activation (Figures 1 and 2). This indicates possible differences in the way in which these tumour promoters trigger the MAP kinase cascade, an idea which was further supported by dual MAP kinase responses when Ramos cells were simultaneously stimulated with anti-IgM and OA (Figure 2b). MAP kinase activation by mIgM is partially dependent on PKC [1]. Although the mechanisms of activation by PMA and OA remain to be elucidated, it would appear that tyrosine phosphorylation of ERK-1 and ERK-2 is common to both pathways (Figure 2a). Afferent signalling components which could theoretically be affected by serine/threonine phosphorylation include TPKs [17], a MAP kinase activator [31], and a MAP kinase kinase [32]. Experimental evidence for each of these afferent events exists, which implies that several afferent pathways may converge on MAP kinase [31–33].

The presence of a constitutively active MAP kinase was an interesting finding during fractionation of crude cell lysates on Mono-Q columns (Figure 3). We found that peak I MAP kinase co-eluted with a 46 kDa protein, which binds to an antibody to an ERK-1 fusion protein and so is ERK-1 related (Figure 4e). Using an immunoaffinity approach (Table 4), we demonstrated that this protein is a MAP kinase. Peak I also phosphorylated the MBP peptide, APRTPGGRR (results not shown). The inability of Ab 691 to bind to peak I material means that this protein differs from conventional ERK-1. This kinase requires further study and may have a different role from that of p42 MAP kinase.

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