Redox regulation of the mitogen-activated protein kinase pathway during lymphocyte activation

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Abstract

We have previously demonstrated an obligatory requirement for intracellular reactive oxygen species generation during T lymphocyte activation, and have proposed that intracellular reactive oxygen species may act as signalling agents in the regulation of certain cellular processes, for example, during cell cycle entry. To test this hypothesis, we have been interested to determine which, if any, cell cycle entry events are affected by oxidative signalling. In earlier studies, we have identified the transcription factors NF-κB and AP-1 as molecular targets for oxidative signalling processes during cell cycle entry, and have shown that oxidative signalling is involved in the regulation of early changes in gene expression during the G_0 to G_1 phase transition. To extend these initial observations, we have examined the effect of antioxidant treatment on the activity of the mitogen-activated protein kinases erk1 and erk2, as members of a signal transduction pathway known to directly regulate transcription factor function. Using as a probe cysteamine, an aminothiol compound with both antioxidant and antiproliferative activity, we have identified erk2, a key element of the MAP kinase pathway, as being responsive to oxidative signalling during lymphocyte activation. These observations provide further evidence to suggest a role for intracellular oxidant generation as a regulatory mechanism during cell cycle entry, and establish a link between oxidative signalling and other aspects of the intracellular signalling network that is activated in response to mitogenic stimulation.

Keywords: Lymphocyte activation; Reactive oxygen species; Mitogen-activated protein kinase; Gene expression

1. Introduction

T lymphocyte proliferation is an integral part of the cellular immune response. Under physiological conditions, T lymphocyte proliferation is initiated following recognition of antigen, processed and presented by accessory cells in the context of self MHC, by the T cell antigen receptor complex. The resulting antigen-induced ligation of the TCR complex triggers an ordered series of dependent biochemical events which drive entry into the cell cycle and ultimately culminate in cell division [1]. As an immediate consequence of mitogenic stimulation, cellular signal transduction mechanisms are activated and the expression of a number of early genes is initiated. The initial stimulus provided by ligand binding to the TCR complex results in the rapid activation of membrane-associated tyrosine kinases such as p70^{ZAP}, p56^{lck} and p59^{fyn} [2,3]. Key substrates for early tyrosine phosphorylation include the CD3 subunits of the TCR complex [4], and 'adaptor' proteins such as *shc*, *vav* and GRB-2 [5–7]. These early tyrosine phospho-

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rylation events are crucial for the activation of downstream effectors such as protein kinase C and p21[ras] [1–3], required for the amplification and propagation of the initial signal provided by TCR ligation. In response to protein kinase C and p21[ras] activation, a number of intermediary serine/threonine kinases are activated, including p72[raf1] and the various members of the mitogen-activated protein kinase pathway, for example p42[erk2], which have been implicated in the regulation of transcription factor function during cell cycle entry [8]. There is considerable cross-talk and a degree of redundancy between these initiating pathways; protein kinase C is involved in the activation of both p72[raf1] and mitogen-activated kinases such as p42[erk2], while p21[ras] can also interact with and activate p72[raf1] [9–12]. Thus, the initial signal provided by TCR ligation is both amplified and prolonged, and ultimately leads to the initiation of changes in gene expression that are required for cell cycle entry, and the activation of the kinase system that drives the cell cycle itself.

In T lymphocytes, the intracellular redox balance is intimately linked to the cellular proliferative capacity [13,14]. We have previously demonstrated, by assessing intracellular oxidation of dichlorofluorescin to dichlorofluorescein, the occurrence of a burst of oxidative activity commencing 30–60 min after mitogenic stimulation of normal peripheral blood T lymphocytes [15]. In addition, we have shown that certain antioxidant compounds such as radical scavengers, iron chelators and aminothiols not only abrogate this oxidative response but also inhibit T cell proliferation, preventing transit through the G_1 phase of the cell cycle [16–18]. Given the apparent obligatory requirement for reactive oxygen species (ROS) formation during T cell activation, and that ROS are short-lived products whose generation and destruction are tightly controlled, we have proposed that ROS may act as signalling agents in the regulation of certain cellular processes, for example, during cell cycle entry [13,14].

Recent evidence has suggested an involvement of oxidative signalling in the activation of cellular signal transduction pathways; for example, ionizing radiation and H_2O_2, agents that induce oxidative stress, have been demonstrated to induce tyrosine phosphorylation events and activate downstream kinases such as PKC, p56[lck] and p72[raf1] [19–21]. Furthermore, it has very recently been shown that the major T lymphocyte costimulatory signal, provided by ligation of the CD28 receptor, results in the generation of intracellular ROS, and that this ROS generation is required for early tyrosine phosphorylation events and interleukin 2 expression [22]. Having established that oxidative signalling is required during cell cycle entry, in lymphocytes at least, we have been interested to identify which, if any, commitment events are affected by oxidative signalling during the G_0 to G_1 phase transition. In a previous study, we have identified transcription factors NF-κB and AP-1 as molecular targets for oxidative signalling processes during cell cycle entry [23]. Given both the requirement for altered gene expression and the involvement of the MAP kinase pathway in the regulation of transcription factor function during cell cycle entry [8], in this study we have examined the effect of antioxidant treatment on MAP kinase activity during lymphocyte activation in an effort to explore the nature of the relationship between oxidative signalling and other, better-described, signalling mechanisms that operate during the G_0 to G_1 phase transition.

2. Materials and methods

2.1. Cell culture

In this study, two cell types were used; normal, untransformed human peripheral blood mononuclear cell preparations, and the human leukaemic T cell line Jurkat, commonly used as a model system for the study of lymphocyte activation events. Mononuclear cells were isolated from human peripheral blood buffy coat preparations by centrifugation over Ficoll-Hypaque lymphocyte separation medium, followed by passage over Sephadex G10 columns to deplete adherent cells. The resultant cell preparations were plated at a density of 5×10^5 cells/cm^3 and cultured in RPMI 1640 medium + 10% v/v foetal calf serum in an atmosphere of 95% air/5% CO_2 at 37°C for up to 72 h post-isolation with little loss of viability, as assessed by trypan blue staining. Jurkat cells were cultured under the same conditions, and maintained at densities between 10^5 and 10^6 cells/cm^3. Cultures of human peripheral blood mononuclear cells were mitogenically activated by treatment with either phyto-

haemagglutinin (PHA) at a concentration of 10 mg/ml, or a combination of phorbol myristate acetate (PMA) at 20 ng/ml and ionomycin (IoM) at 100 ng/ml as required [13]. Jurkat cells were stimulated by treatment with 50 ng/ml PMA. The antioxidant used in this study was the aminothiol compound cysteamine, which we previously have shown to inhibit lymphocyte proliferation with essentially complete inhibition occurring at a dose of 400 μM [18,23]. Other inhibitors employed were cyclosporin A, which induces cell cycle arrest via a non-oxidative mechanism [2], and chelerythrine, a non-competitive inhibitor of protein kinase C [24]. These compounds were used at doses of 200 ng/ml and 1.3 μM respectively, the 90% inhibitory dose in each case, and lymphocyte proliferation was assessed by determining the incorporation of [^{3}H]-thymidine into DNA 48 h after stimulation [13].

2.2. Nuclear protein extraction and electrophoretic mobility shift assays

For nuclear protein extractions, 1×10^7 cells per sample were stimulated by treatment with either PMA/IoM or PHA, with or without inhibitors, added as required at the time of stimulation. These cultures were plated in 6 well tissue culture trays in a total volume of 4 ml medium. Nuclear proteins were harvested from each culture 4 h post-stimulation by hypotonic lysis followed by salt extraction, as follows: Culture medium was removed gently, and the cells were resuspended in 4 ml ice-cold PBS, and recovered by centrifugation. The cell pellets were washed once in 1 ml ice-cold PBS, transferred to microfuge tubes and resuspended in 100 μl lysis buffer, containing 20 mM HEPES, pH 7.9/25 mM KCl/1 mM EDTA/1 mM DTT/1 μg/ml each aprotinin, leupeptin and PMSF, and left to swell on ice for 10 min. Each tube was then vortexed briefly and nuclei were pelleted by centrifugation for 10 s in a microfuge. The nuclear pellets were then resuspended in 20 μl extraction buffer, containing 50 mM HEPES, pH 7.9/50 mM KCl/300 mM NaCl/1 mM DTT/20% glycerol and protease inhibitors as above, and nuclear proteins extracted by incubation on ice for 30 min with periodic gentle mixing. After the extraction period, the suspensions were cleared by centrifugation for 5 min and the supernatant solutions

retained as the nuclear protein fraction. Before use the protein concentration of each sample was determined using the Bradford assay.

Binding assays were performed with 2.5 μg protein per reaction in a total volume of 9 μl, and consisted of 12 mM HEPES, pH 7.9/5% glycerol/100 mM KCl/0.25 mM DTT/1 μg poly d[I-C] and 20,000 cpm [^{32}P] end-labelled AP-1 oligomer probe. Binding reactions were initiated by the addition of protein, and were continued for 15 min at room temperature. Reactions were terminated by the addition of 4% Ficoll 400 loading dye and electrophoresis over low ionic strength 6% polyacrylamide minigels. Gels were then dried and autoradiographed using Kodak X-OMAT K film. Specificity of binding was determined by cold competition assays in which a 100-fold molar excess of unlabelled relevant or unrelated oligomer was included in the binding assay as required.

2.3. Immune complex kinase assays

For each assay, 1×10^7 cells were plated at a density of $5 \times 10^6/cm^3$ and the appropriate inhibitors added as required, 90 min prior to stimulation. The cells were then stimulated, and culturing was continued for up to 90 min prior to harvesting. At the end of the culture time, the cultures were resuspended, diluted fivefold by the addition of ice-cold PBS, and the cells recovered by centrifugation in a bench-top centrifuge. The cell pellets were then washed in 1 ml ice-cold PBS, transferred to microfuge tubes, and pelleted by centrifugation for 10 s in a microfuge. The PBS was then removed, and the cells were resuspended in 1 ml RIPA buffer with freshly added protease and phosphatase inhibitors (PBS/1% NP40/0.5% sodium deoxycholate/0.1% SDS/1 mM PMSF/1 mM sodium orthovanadate/25 mM sodium β-glycerophosphate/20 mM sodium fluoride). The lysates were left on ice for 5 min, then the cells were further disrupted and the DNA sheared by forcing each lysate through a 23 g needle 4 times. The lysates were left another 15 min on ice, then cleared by centrifugation at 16 000 rpm for 20 min at 4°C, and the supernatants from each lysate were retained as the protein source for the kinase assays. The protein concentration of each lysate was determined using the Bradford assay, and equivalent

amounts of total protein per lysate were used in each kinase assay.

For immunoprecipitation of either erk1 or erk2, up to 1 μg of the appropriate antibody (Santa Cruz SC154) was added to each lysate as required, followed by a 1-h incubation on ice, with periodic mixing. After this initial incubation, 30 μl of protein A-Sepharose slurry was added to each lysate, and immune complex formation was continued for a further 30–60 min at 4°C, with constant mixing. The protein A-Sepharose bound complexes were then recovered by rapid centrifugation in a microfuge, and washed three times with RIPA buffer containing protease and phosphatase inhibitors. The complexes were then washed one more time in kinase buffer containing protease and phosphatase inhibitors (50 mM Tris-HCl, pH 7.4/150 mM NaCl/10 mM MgCl$_2$/1 mM DTT/100 μg/ml BSA/1 mM PMSF/1 mM sodium orthovanadate/25 mM sodium β-glycerophosphate/20 mM sodium fluoride) and the supernatant removed as completely as possible without disturbing the pellet. The complexes were then resuspended in 20 μl kinase buffer + inhibitors, and the kinase reactions initiated by the addition of 10–20 μg myelin basic protein as a substrate, together with 10 μCi [^{32}P]ATP (3000 Ci/mmol) per reaction. The reactions were run for 30–45 min at 30°C, then terminated by the addition of 30 μl SDS-PAGE loading dye and heating to 95°C prior to electrophoresis. 20 μl of each reaction was then subjected to SDS-PAGE, and the resulting gels were dried and autoradiographed, using Kodak X-OMAT K film. Exposure times were between 20 min to 2 h. Quantitation of the kinase reactions was achieved using a filter binding assay in which 5 μl of each reaction were spotted onto Whatman DE81 filter discs, which were then washed 4 times in 1% orthophosphoric acid, 5 min for each wash, followed by a 1-min wash in absolute ethanol. The discs were then air-dried, and the extent of binding of labelled proteins determined by liquid scintillation counting.

3. Results and discussion

MAP kinases play a significant role in the regulation of transcription factor activity [8]. Various members of the MAP kinase family have been shown to phosphorylate c-jun within its activation domain, and to be involved in the activation of AP-1 function [25,26]. Furthermore, MAP kinases target TCF/Elk1, potentiating their activity and thereby increasing c-fos transcription [27]. We have previously identified c-jun as a target for oxidative signalling in human PBMC [23], and therefore, in this study we have examined the effect of antioxidant treatment on MAP kinase activity in both normal human PBMC, and in a human leukaemic T-cell line, Jurkat.

Treatment of both normal human PBMC and Jurkat cells with either the aminothiol compound cysteamine, the protein kinase C inhibitor chelerythrine, or the macrolide immunosuppressant cyclosporin A, at doses of 400 μM, 1.3 μM, or 200 ng/ml, respectively, was found to inhibit proliferation by greater than 90% (Fig. 1). Electrophoretic mobility shift assays were used to examine the effect of cysteamine on AP-1 DNA binding activity (Fig. 2). In human PBMC cultures, cysteamine treatment was found to inhibit PMA + IoM-induced AP-1 activity. However, in Jurkat cells, cysteamine treatment was found to result in enhanced PMA-induced AP-1 DNA binding activity, as has been reported for other cell types [28]. In nuclear extracts from PBMC cultures two forms of AP-1 complex were detected, while in Jurkat cells only the more slowly-migrating form was seen. Both forms of the complex detected in PBMC cultures were inhibited by cysteamine treatment. Thus it would appear that the composition of AP-1 differs in PBMCs and Jurkat cells, and it is possible that the various isoforms of AP-1 are differentially affected, directly or indirectly, by oxidative signalling.

Interestingly, cysteamine treatment was found to result in a dose-dependent inhibition of both PMA + IoM and PHA-induced erk2 activity, as assessed by immune complex kinase assays, not only in normal human PBMC but also in Jurkat cells (Fig. 3). Erk1 activity was not significantly induced in response to either PMA/IoM or PHA stimulation (data not shown). As expected, treatment of both PBMC or Jurkat cells with chelerythrine was found to block both PMA/IoM and PHA-induced erk2 activation, indicating that protein kinase C is involved in the activation of MAP kinases by these agents in lymphocytes, in agreement with other published studies [29,30]. In order to determine whether the antioxidant-mediated inhibition of erk2 activity was due to

interference with oxidative signalling processes, or the result of inhibition of other early activation events leading to cell cycle arrest, we examined the effect of cyclosporin A treatment on erk2 activity in both normal human PBMCs and Jurkat cells. This agent inhibits lymphocyte proliferation by blocking cal-cineurin function, which is required for the activation of NF-AT and hence transcription from the IL-2 promoter. Significantly, treatment with cyclosporin A at a dose which resulted in essentially complete inhibition of proliferation had little effect on either PMA/IoM- or PHA-induced erk2 activity in either
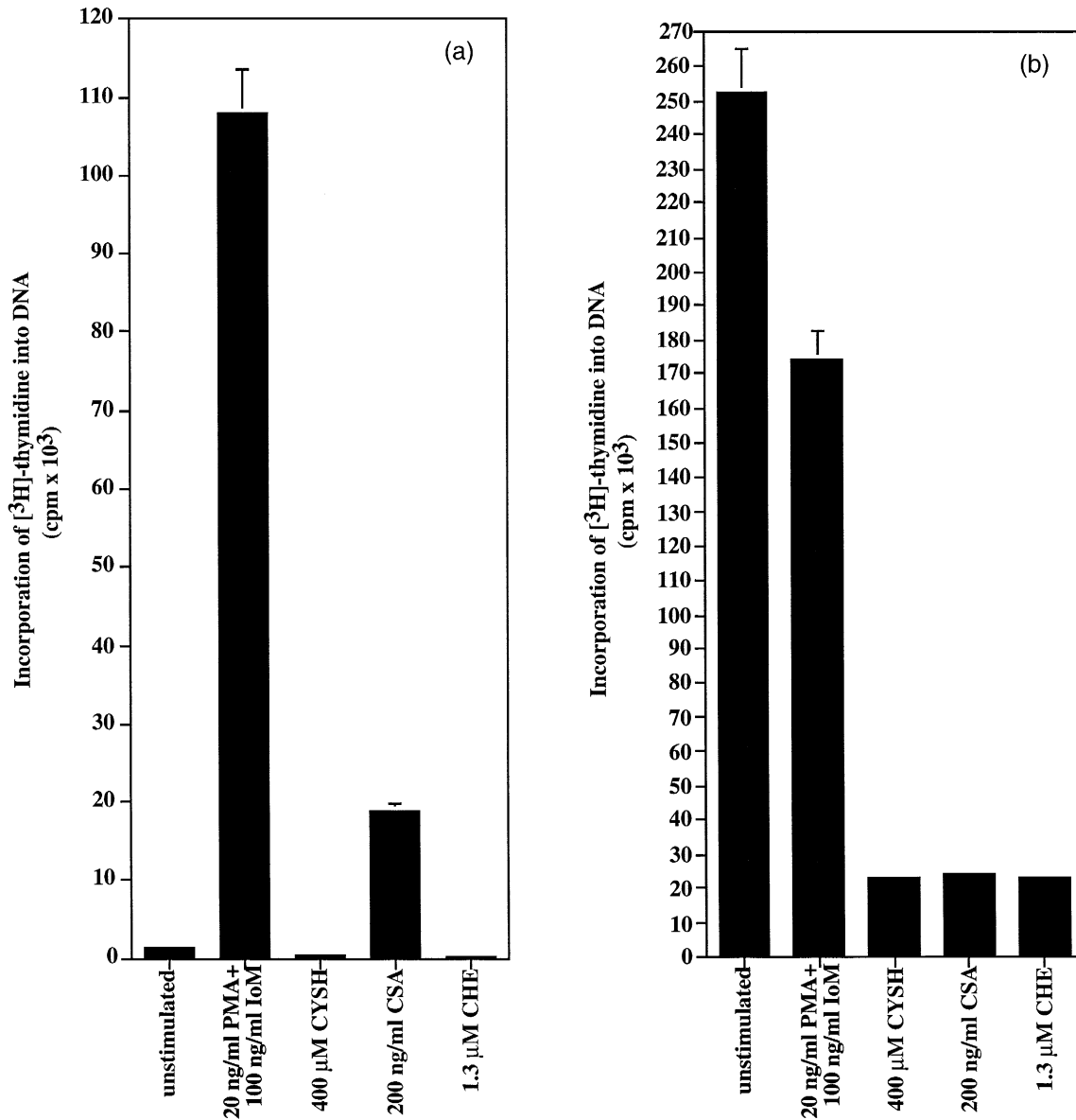
Fig. 1. The effect of cysteamine, cyclosporin A, and chelerythrine on the proliferation of both human peripheral blood mononuclear cell cultures (A) and Jurkat cells (B) was determined by assaying the incorporation of [^{3}H]-thymidine into DNA after a 48-h culture period. The inhibitors were added to the relevant cultures 90 min prior to the time of mitogenic stimulation, using a combination of 20 ng/ml phorbol myristate acetate (PMA) and 100 ng/ml ionomycin (IoM), with cysteamine (CYSH) being used at a final dose of 400 μM, and cyclosporin A (CSA) and chelerythrine at final doses of 200 ng/ml and 1.3 μM, respectively. The data shown are representative of four independent experiments, with the mean and SEM of the mean shown for each treatment group.
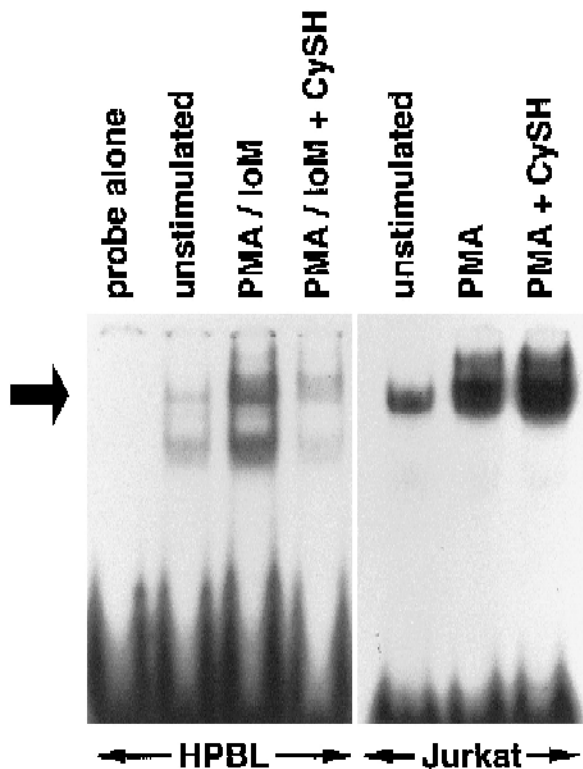
Fig. 2. The effect of cysteamine treatment on AP-1 DNA binding activity was determined in both human peripheral blood mononuclear cell cultures and in Jurkat cells using electrophoretic mobility shift assays. Nuclear proteins were prepared from cultures 4 hrs after stimulation with either 20 ng/ml PMA plus 100 ng IoM (HPBL cultures), or 50 ng/ml PMA (Jurkat cells), with and without the addition of 400 μM CYSH as required, and 2.5 μg of each nuclear extract was probed using 20,000 cpm of a ^{32}P-end-labelled oligonucleotide containing the AP-1 DNA binding site (top strand sequence AGCTTGTGAGTCAGCCG). Specificity of binding was demonstrated by the inclusion of a 100-fold excess of unlabelled relevant oligomer in the binding reaction (data not shown). The major AP-1 complex common to both cell types is indicated by an arrow. The faster migrating form seen in extracts from HBPL cultures is not found in Jurkat cells and presumably reflects differences in AP-1 isoforms present in the two different cell types.

tive signalling differs in continuously cycling cells and cells entering the cell cycle. For example, it is possible that intracellular signal transduction pathways may be affected differently by oxidative signalling in continuously cycling cells and those exiting quiescence. While it would appear that erk2 is equally sensitive to interference with oxidative signalling in continuously cycling cells (Jurkat) and those entering the cell cycle (human PBMC), other signal transduction pathways which target AP-1 may be differentially regulated according to cell cycle status, as exemplified by our data.

In summary, we have identified erk2, a key element of the MAP kinase pathway, as being responsive to oxidative signalling during lymphocyte activation. These observations provide further evidence to suggest a role for intracellular oxidant generation as a
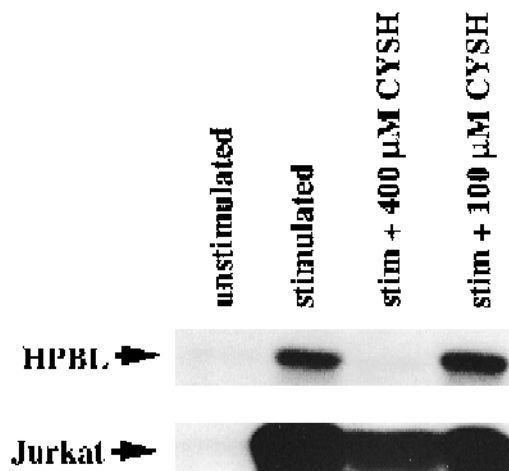

Fig. 3. The effect of cysteamine treatment on the induction of erk2 kinase activity was determined in both human peripheral blood mononuclear cell cultures and in Jurkat cells, using immune-complex kinase assays. For each assay, 10^7 cells were plated, and where required, cysteamine was added to a final concentration of either 100 or 400 μM, 90 min prior to mitogenic activation using either 20 ng/ml PMA plus 100 ng/ml IoM (HPBM cultures), 50 ng/ml PMA (Jurkat cultures), or 10 μg/ml PHA (both cell types). Cleared lysates were prepared from each culture 15 min after stimulation, and erk2 immunoprecipitated from each lysate using a polyclonal antibody (Santa Cruz SC 154). The kinase activity of the recovered immune complexes was determined using myelin basic protein as a substrate for the reaction and including [^{32}P]ATP as a phosphate donor source. The data shown are from PMA/IoM stimulated cultures, a similar effect was observed in PHA-stimulated cultures (data not shown).
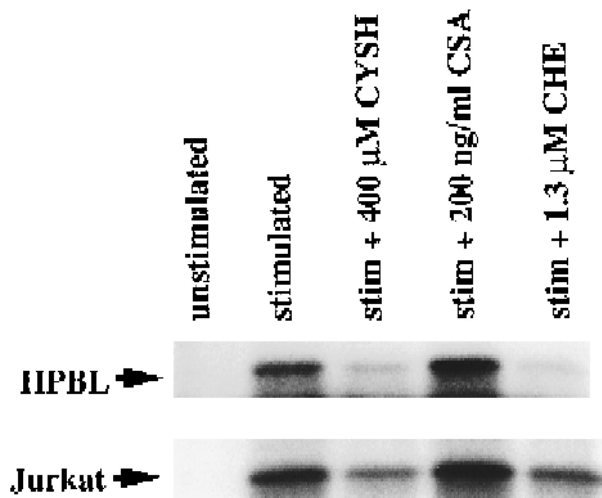
PBMC or Jurkat cells (Fig. 4), suggesting that antioxidant-induced inhibition of erk2 activity was not simply a consequence of cell cycle arrest and that oxidative signalling may be required for activation of the MAP kinase pathway, in lymphocytes at least.

Our observations of differences in the response of downstream effectors such as AP-1 to antioxidant treatment suggest that the cellular response to oxida-

Fig. 4. The effect of either cysteamine, cyclosporin A, or chelery-thrine on the induction of erk2 kinase activity was determined in both human peripheral blood mononuclear cell cultures and in Jurkat cells, using immune complex kinase assays as previously described. Each inhibitor was used at a dose previously determined to inhibit proliferation by at least 90%, and inhibitors were added 90 min prior to mitogenic stimulation, using either a combination of 20 ng/ml PMA and 100 ng/ml IoM, or 10 μg/ml PHA. The data shown are from PMA/IoM-stimulated cultures, and are similar to those obtained from PHA-stimulated cells (data not shown).

regulatory mechanism during cell cycle entry, and establish a link between oxidative signalling and other aspects of the intracellular signalling network that is activated in response to mitogenic stimulation. Further studies will be necessary to determine the mechanism by which oxidative signalling targets MAP kinases, for example by direct oxidation or by modulating the activity of upstream regulators of MAP kinase activity, and to establish which other sites within the mitogenic signalling network are sensitive to redox regulation.

Acknowledgements

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