Activation of mitogen-activated protein kinase by protein kinase C isotypes α, β_I and γ, but not ε

Stephen W. Young, Martin Dickens, Jeremy M. Tavaré*

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK

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Abstract Treatment of CHO.T cells with either PMA or insulin led to the activation of MAP kinase by ~3-fold, and $p90^{rsk}$ by ~4-fold. Over-expression of the α, β_I or γ isoforms of protein kinase C caused a substantial enhancement of the effect of PMA on the activation of MAP kinase and $p90^{rsk}$, however, the effect of insulin was unchanged. Over-expression of the ε isoform of protein kinase C did not alter the effect of either PMA or insulin on the activation of MAP kinase and $p90^{rsk}$. The results suggest that protein kinase C isotypes α, β_I and γ, but not ε, can mediate MAP kinase activation by PMA, and strongly support the hypothesis that protein kinase C isoforms can initiate distinct signalling pathways.

Key words: MAP kinase; Phorbol ester; Insulin; Protein kinase C

1. Introduction

The mitogen-activated protein (MAP) kinases are central to the effects of a number of extracellular stimuli on cell growth and development [1,2]. These enzymes (which include the Erk1 and Erk2 isoforms) are stimulated by growth factors, neurotrophins and insulin (reviewed in [1,2]) and it has been known for some time that they are also activated by phorbol esters [3]. The pathway by which Erk1 and Erk2 are activated by growth factors is relatively well characterized. Erk1 and Erk2 are phosphorylated and activated by the dual-specificity MAP kinase kinase, which is in turn activated through phosphorylation by Raf-1 (reviewed in [4,5]). Raf-1 appears to be activated by a rather complex mechanism which involves, in part, its association with the GTP-bound form of Ras [6,7] as well as other proteins such as 14-3-3 [8,9] and v-Src [10]. It has also been suggested that protein kinase C can phosphorylate and activate Raf-1 [11].

Protein kinase C comprises a group of related serine/threonine-specific protein kinases of which there are at least ten distinct members. These isozymes can be classified into conventional (e.g., α, β and γ), novel (e.g., δ and ε) and atypical (e.g., ζ) isoforms. The conventional and novel isoforms, but not the ζ isoform, bind phorbol esters (reviewed in [12]).

The fact that multiple isoforms of protein kinase C exist, and that they exhibit distinct tissue distribution profiles and are activated by different external stimuli (reviewed in [13]), suggests their functional divergence. For example, distinct isoforms may phosphorylate different substrates and thus activate distinct cellular signalling pathways. A limited number of studies have provided evidence that this may be the case. For example, Goode et al. [14] found that glycogen synthase kinase-3β was a good substrate in vitro for the α, β_I and γ, but not ε, isoforms of protein kinase C. Hata et al. [15] found that the α, β_{II} and ε isoforms could mediate transcriptional activation of promoters possessing either a TPA-response element (TRE) or a serum-response element (SRE). On the other hand, the γ isoform mediated activation of the SRE, but not TRE-dependent transcription. Spence et al. [16] found that the α and ε isoforms of PKC, but not β_I or γ, exhibited an induction of expression of phosphodiesterase activity, largely through a transcription-dependent mechanism. The data suggest that the protein kinase C isoforms subserve distinct intracellular signalling roles. In the current study this possibility was tested by measuring the ability of the α, β_I, γ and ε isoforms of protein kinase C to activate mitogen-activated protein kinase.

2. Materials and methods

2.1. Materials

All reagents were as previously described [17] except tissue culture media and serum which were from Gibco BRL (Paisley, UK). An antiserum (pan-Erk1/2) reactive towards both Erk1 and Erk2 was raised towards the C-terminal 15 amino acids of Erk1 and has been previously described [18]. CHO.T cells and CHO.T cells overexpressing the α, β_I, γ or ε isoforms of protein kinase C have been previously described [18] and were maintained in Ham's F12 medium supplemented with 5% foetal calf serum and 200 μg/ml G418.

2.2. MAP kinase and $p90^{rsk}$ assays

Approx. 2×10^6 cells in 60 mm dishes were serum starved by incubation at 37°C for 16 h in Ham's F12 medium containing 1% FCS and then for 2 h in serum free Ham's F12. After incubation with ligands for the times and at the concentrations indicated in the figure legends, the cells were washed with 2 ml of ice-cold phosphate buffered saline and extracted rapidly by scraping into 0.5 ml of ice-cold extraction buffer (50 mM β-glycerophosphate (pH 7.4)/1.5 mM EGTA/1 mM benzamidine/1 mM dithiothreitol/0.5 mM Na_3VO_4/0.1 mM PMSF/1 μM microcystin/1 μg/ml each of pepstatin, antipain and leupeptin). MAP kinases were assayed either in the crude cell lysate [19] or after immunopurification with a pan-Erk antiserum that is specific for Erk1 and Erk2 as described [17], using 0.5 mg/ml-myelin basic protein as substrate. $p90_{rsk}$ was assayed in the crude cell lysates using the peptide RRLSSLRA as substrate [19].

2.3. Two-dimensional phosphopeptide mapping of the human insulin receptor

Cells were pre-equilibrated with 1 mCi of $^{32}P_i$ in 2 ml phosphate-free and serum-free DMEM for 3 h and then incubated in the absence or presence of 1.6 μM PMA for 30 min at 37°C. Cells were extracted and the insulin receptor isolated, digested with trypsin and the resultant phosphopeptides were separated by two-dimensional thin-layer

*Corresponding author. Fax: (44) (117) 928 8274.
E-mail: tavare@bsa.bristol.ac.uk

Abbreviations: MAP kinase, mitogen-activated protein kinase; Erk, extracellular signal-regulated protein kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; MBP, myelin basic protein.

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chromatography as previously described [20]. The thin-layer plate was subjected to autoradiography for 7 days.

3. Results and discussion

CHO.T cells express approx. 10^6 human insulin receptors per cell and have also been shown to express low levels of endogenous PKCα, PKCγ and PKCζ [18]. We have previously reported the generation of CHO.T cell lines that stably over-express PKCα, PKCβI, PKCγ or PKCε [18]. We utilised these cell types to examine the ability of the various protein kinase C isotypes to activate MAP kinase.

CHO.T cells (controls) or CHO.T cells over-expressing the various PKC isotypes were incubated in the absence or presence of insulin or phorbol ester. As shown in Fig. 1a both insulin and phorbol ester stimulated the activity of MAP kinase in control CHO.T cells by ~3-fold. Upon over-expression of any of the protein kinase C isotypes the effect of insulin remained about the same as that observed in control CHO.T cells (Fig. 1a). However, the effect of phorbol ester was enhanced approx. 5-fold upon over-expression of PKCα and PKCβI, and by approx. 2-fold in cells over-expressing PKCγ. The effect of phorbol ester was unchanged in cells over-expressing PKCε (Fig. 1a). Similar data were obtained by assaying MAP kinase in immunoprecipitates using anti-MAP kinase specific antisera (Fig. 1b), where over-expression of PKCα and PKCβI caused a marked enhancement of the ability of phorbol esters to activate MAP kinase. This was also observed, but to a lesser extent, in cells over-expressing PKCγ. Again, cells over-expressing PKCε showed no difference from the control CHO.T cells (Fig. 1b).

The results strongly suggest that the α, βI and γ, but not ε, isotypes of protein kinase C can mediate the activation of MAP kinase by PMA in these cells. Furthermore, they also suggest that protein kinase C does not mediate the ability of insulin to activate MAP kinase. Consistent with the latter proposal, insulin still promoted a quite normal activation of MAP kinase in cells in which the phorbol ester-sensitive PKC isotypes were down-regulated by long-term treatment with PMA (M.D., unpublished observations). One of the target substrates for MAP kinase is the serine/threonine kinase $p90^{rsk}$ [21]. Phosphorylation of the enzyme by MAP kinase is accompanied by its activation. The activity of $p90^{rsk}$ should, therefore, reflect changes in the activity of its upstream regulator, MAP kinase. We thus examined the activation of this enzyme in cells over-expressing the various PKC isoforms using a specific substrate for $p90^{rsk}$ based on the sites phosphorylated in the ribosomal subunit S6; we have previously shown this to be a specific assay for the activity of this enzyme in crude cell lysates [19]. As shown in Fig. 2, both PMA and insulin had approx. 4-fold effects on the activation of $p90^{rsk}$.

Over-expression of PKCα, PKCβI and PKCγ all led to approx. 3-fold enhancement of the effect of phorbol ester on $p90^{rsk}$ activation. By contrast, and consistent with what is observed with MAP kinase in Fig. 1, over-expression of PKCε did not change the response of $p90^{rsk}$ to phorbol ester stimulation. The effect of insulin on $p90^{rsk}$ activation was unchanged upon over-expression of any of the PKC isoforms (Fig. 2).

It is interesting that the enhanced activation of $p90^{rsk}$ upon over-expression of PKCγ reaches the same level as that observed when over-expressing PKCα or βI (Fig. 2). This con-

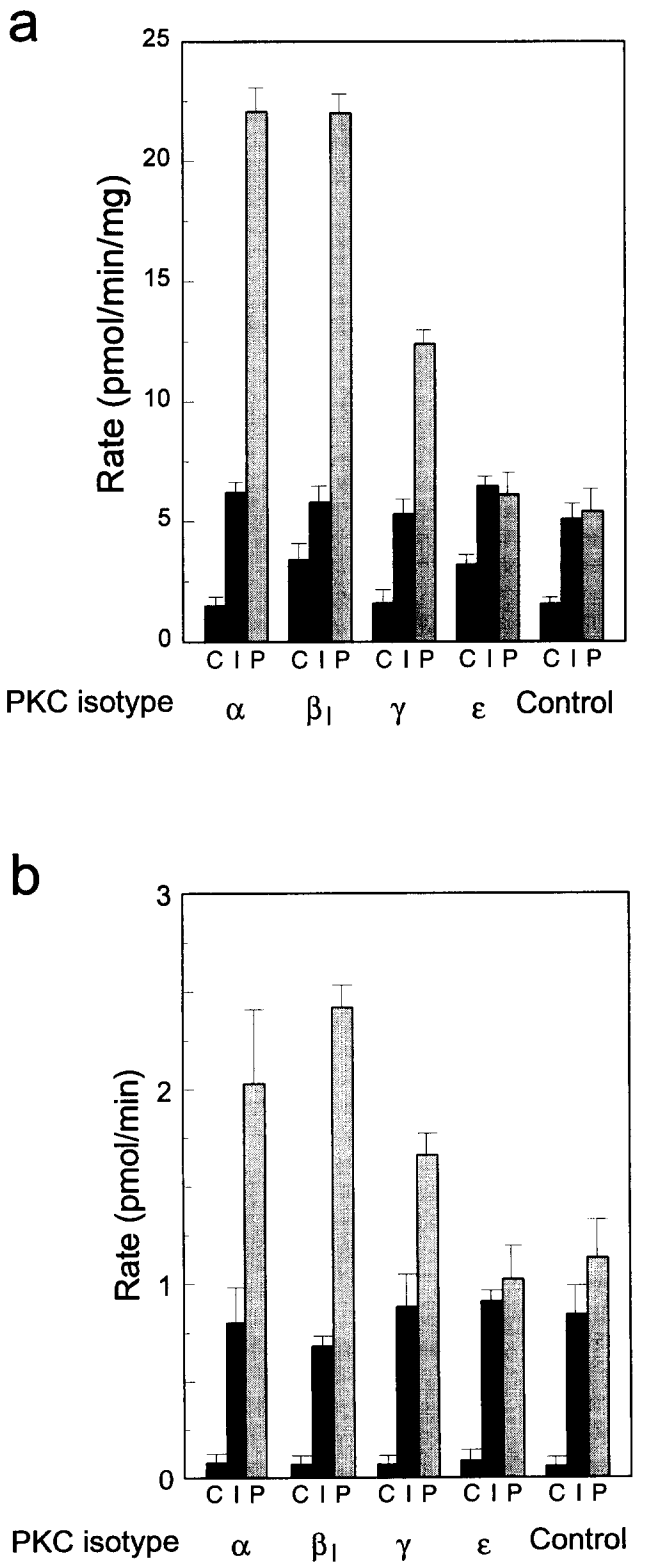

Fig. 1. Activation of MAP kinase by phorbol esters and insulin. CHO.T cells (control) or cells over-expressing the various PKC isoforms (as indicated) were serum starved and incubated without (C) or with either 100 nM insulin for 5 min (I) or 1.6 μM PMA for 30 min (P). The cells were extracted and MAP kinase activity assayed in the crude cell lysates (a) or after specific immunoprecipitation with anti-MAP kinase antibodies (b) as described in section 2. The data shows the results pooled from four (a) or three (b) independent experiments (means ± S.E.M.).

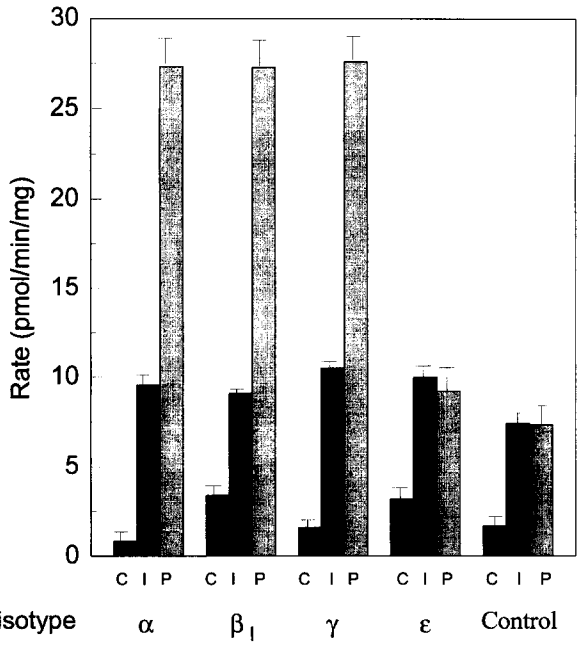

Fig. 2. Activation of p90^{rsk} by phorbol esters and insulin. CHO.T cells (control) or cells over-expressing the various PKC isoforms (as indicated) were incubated as described for Fig. 1. The cells were extracted and p90^{rsk} activity assayed in the crude cell lysate as described in section 2. The data shows the results pooled from four independent experiments (mean ± S.E.M.).

trasts with the ability of PKCγ over-expression to induce a lower enhancement of MAP kinase activity when compared to that induced by PKCα and β_I over-expression (Fig. 1). This suggests a degree of signal amplification between MAP kinase and p90^{rsk}, such that only a submaximal level of MAP kinase activation is required to produce maximal activation of p90^{rsk}.

We next wanted to confirm that PKCε was active in the CHO.T-PKCε cell line. We have previously demonstrated that over-expression of PKCα enhances the effect of phorbol esters on the phosphorylation of the human insulin receptor on serine and threonine residues [18]. We thus performed tryptic phosphopeptide mapping experiments on insulin receptors isolated from CHO.T cells over-expressing PKCβ_I or PKCε which were metabolically labelled with $^{32}P_i$. The maps obtained were compared with peptide maps derived from insulin receptors isolated from similarly treated parental CHO.T cells. In CHO.T cells, PMA promoted an increase in the phosphorylation of an insulin receptor phosphothreonine-containing peptide (peptide 'T'). In the experiment shown in Fig. 3, and using scanning densitometry, this increase in T-peptide phosphorylation was shown to be 1.9-fold. We have previously shown this peptide to correspond to Thr-1348 [22]. In CHO.T cells over-expressing PKCε, the effect of PMA was enhanced to 4.5-fold (Fig. 3). A quantitatively similar enhancement in Thr-1348 phosphorylation was observed in cells over-expressing PKCβ_I (4.3-fold; Fig. 3) or PKCγ (see [18]). In two additional independent experiments, the effect of PMA on Thr-1348 phosphorylation in CHO.T and CHO.T-PKCε cells was 1.9-versus 3.0-fold, and 0.45-versus 2.2-fold, respectively. This demonstrates that PKCε is not only active in these cells, but confirms that it can mediate enhanced phosphorylation of insulin receptor Thr-1348, but not enhanced MAP kinase activation by PMA.

Secondly, in the same clone of CHO.T cells as used in our studies, Spence et al. [16] have shown that over-expression of PKCε enhances the basal expression of the PDE1 isoform of phosphodiesterase when compared to the parental CHO.T cell line.

The data, therefore, strongly suggest that at least in CHO cells PKCα, PKCβ_I and PKCγ, but not PKCε, can mediate phorbol ester-stimulation of MAP kinase activity. The results are consistent with studies by Yamaguchi et al. [23] who found that PKCα and PKCδ enhanced the ability of phorbol esters to activate MAP kinase. The point at which the PKCα, β_I, δ and γ isoforms feed into the MAP kinase pathway has not been addressed, nor has the molecular basis for the inability of PKCε to couple to this pathway. However, it may be due to Raf-1 being an efficient substrate for PKCα, PKCβ_I, PKCδ and PKCγ, but not for PKCε. This intriguing possibility requires further testing. It would also be consistent with the fact that PKCε exhibits a quite distinct substrate specificity in vitro to PKCα, PKCβ and PKCγ [14,24].

Interestingly, in CHO cells the subset of isoforms of protein kinase C that lead to MAP kinase activation (i.e., α, β_I and γ, but not ε) differs from that leading to elevated basal expression of the phosphodiesterase PD1 (i.e., PKCα and ε, but not β_I or γ; [16]). These observations greatly increase the apparent complexity of signalling initiated by protein kinase C isoforms, and suggests that even within a subgroup (i.e., conventional isoforms) there is a further level of signalling specificity.

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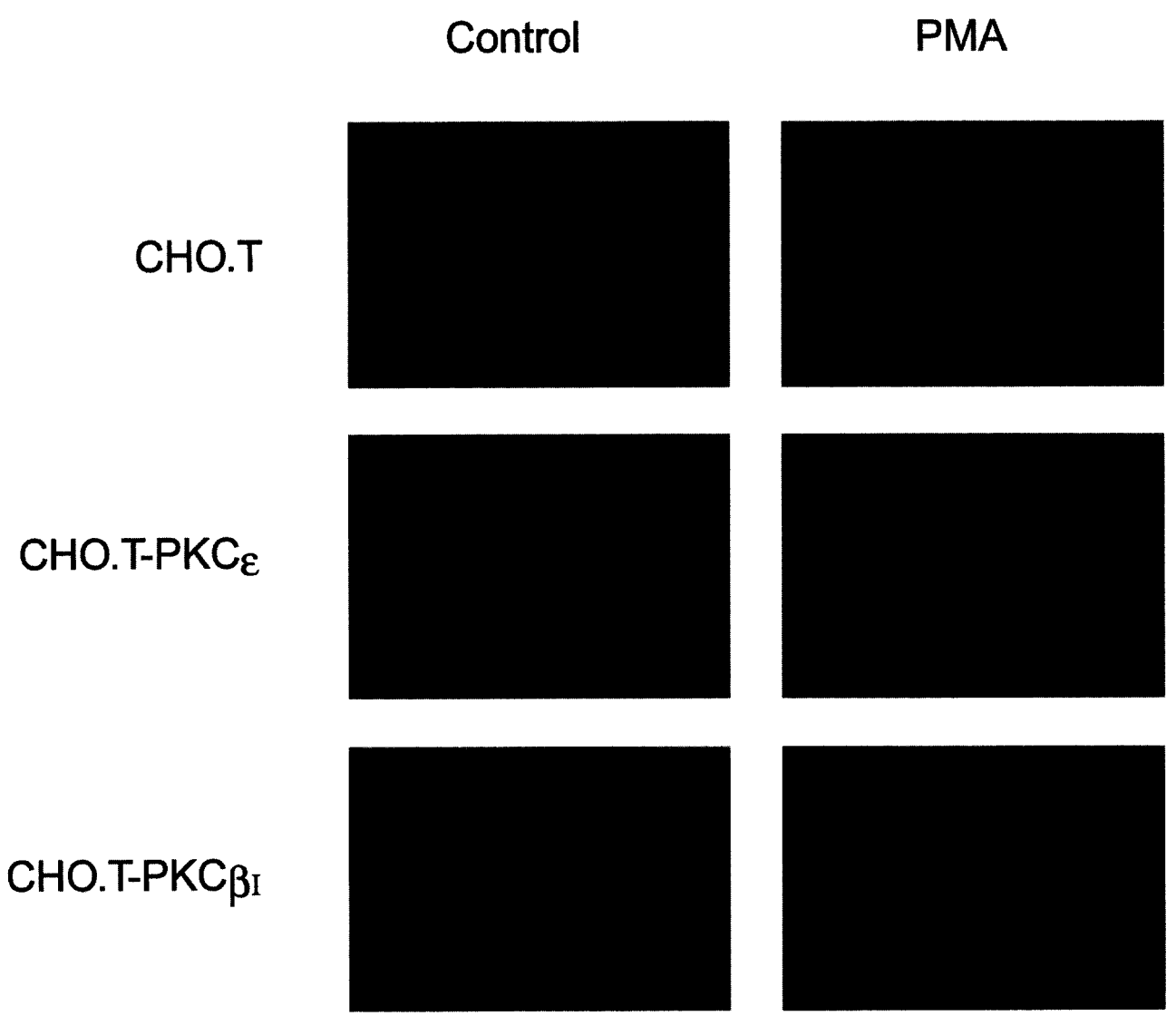

Fig. 3. Over-expression of PKCε enhances phorbol ester-stimulated phosphorylation of insulin receptor Thr-1348. CHO.T cells, or cells over-expressing $PKC\beta_I$ or PKCε, were pre-equilibrated with $^{32}P_i$ and incubated in the absence (control) or presence of 1.6 μM PMA, as indicated, for 30 min at 37°C. Cells were extracted and the insulin receptor isolated, digested with trypsin and the resultant phosphopeptides separated by two-dimensional thin-layer chromatography. The figure shows an autoradiograph of the resultant plate. The origin of sample application is indicated by 'O' and the Thr-1348 containing peptide by 'T'.

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