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PHORBOL ESTERS INDUCE INSULIN RECEPTOR PHOSPHORYLATION IN TRANSFECTED FIBROBLASTS WITHOUT AFFECTING TYROSINE KINASE ACTIVITY

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The effects of phorbol ester induced activation of protein kinase C on insulin receptor phosphorylation and tyrosine kinase activity have been investigated in transfected fibroblasts expressing high levels of the human insulin receptor. Receptor phosphorylation was stimulated more than two-fold over basal levels upon treating CHO.T cells with PMA. This phosphorylation was additive with, rather than antagonistic to, that induced by insulin. Furthermore, PMA treatment was completely without effect on insulin-stimulated receptor tyrosine kinase activity. Similar results were obtained in NIH3T3 HIR3.5 and Rat 1 HIRc-B cells. It is concluded that the previously reported inhibitory effect of PMA on receptor kinase activity is not of general regulatory significance in all cell types.

The binding of insulin to its receptor stimulates autophosphorylation and activation of the receptor's intrinsic tyrosine kinase, an event thought to be essential to the hormone's ability to evoke biologic responses within target cells [1]. The initial autophosphoryation of the insulin receptor occurs on tyrosine but is followed by phosphorylation of serine and threonine residues [2]. Treatment of cells with phorbol esters, which are presumed to activate protein kinase C, also elicits serine/threonine phosphorylation of the receptor β-subunit [3-7]. Analysis of phosphopeptide maps suggests that there are differences in the relative phosphorylation of individual serine/threonine residues in response to insulin and phorbol esters [4, 6, 7]. The only sites identified to date are threonine 1348 and serines 1305/1306, which are predominant sites of phosphorylation in response to phorbol esters and insulin respectively [8, 9].

Several studies have observed that increased receptor serine/threonine phosphorylation is associated with a reduced biologic response to insulin [3, 10-14]. In particular, treatment of cultured cells with phorbol esters has been reported to inhibit insulin-stimulated receptor autophosphorylation and tyrosine kinase activity [3, 10, 11], and this inhibition was attributed to protein kinase C-mediated receptor phosphorylation. This hypothesis was supported by the finding that protein kinase C directly phosphorylated the insulin receptor in vitro and reduced its tyrosine kinase activity [15]. Treatment of cells with catecholamines or cAMP analogues

Abbreviation : 4β-phorbol 12β-myristate 13α-acetate (PMA).

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has also been reported to inhibit insulin receptor tyrosine kinase activity [12-14]. However, there are conflicting data regarding the ability of cAMP-dependent protein kinase to phosphorylate and inhibit the receptor in vitro [16, 17].

Inhibitory effects of phorbol esters on insulin-stimulated receptor autophosphorylation have not been found universally, and in some cells the phosphorylation induced by phorbol ester and insulin appeared additive [5, 7, 18]. Differences between cell types might reflect phosphorylation of the receptor at distinct sites, perhaps resulting from the presence of different isoforms of protein kinase C. A number of transfected cell lines are available which stably express high levels of the human insulin receptor [19-21], including cells in which phorbol esters have [10] or have not [7] been reported to antagonize insulin receptor function. The aim of the present study was to compare the effects of phorbol esters on receptor phosphorylation and kinase activity in different cells, as a prelude to defining the regulatory significance of individual phosphorylation sites.

METHODS

Materials. The 11mer synthetic peptide used as the substrate for the tyrosine kinase assay (RDIFEADYFRK) was a generous gift from Dr D.D. Myles, Glaxo Group Research Ltd., Greenford, Middlesex, UK. P81 phosphocellulose paper was from Whatman International Ltd., Maidstone, Kent, UK. [32-P]orthophosphate was from Amersham International, Amersham, Bucks, UK. Molecular weight markers for SDS/PAGE were from Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, UK. Cell culture reagents were from ICN-Flow, High Wycombe, Bucks, UK, and other reagents were from Sigma Chemical Co. Ltd., Poole, Dorset, UK. 4β-phorbol 12β-myristate 13α-acetate (PMA) was dissolved in dimethyl-sulphoxide at a stock concentration of 1 mM.

Cell culture. Transfected Chinese Hamster Ovary cells (CHO.T) [19], mouse fibroblasts (NIH3T3.HIR3.5) [20], and rat 1 fibroblasts (HIRc-B) [21], which stably express approximately one million human insulin receptors per cell, were generously provided by Dr. L. Ellis, Dr. J. Whittaker and Dr J. Olefsky respectively. Rat Fao hepatoma cells were purchased from the European Collection of Animal Cell Cultures, Porton Down, Wilts, UK. Monolayer cultures were grown at 37°C in a humidified atmosphere of air:CO_2 (19:1) in Ham's F12 medium (CHO.T), Dulbecco's Modified Eagle's Medium (NIH3T3 HIR3.5) or a 50:50 mixture of these two media (HIRc-B and Fao). All media were supplemented with 10% (v/v) foetal calf serum (FCS), 50 IU/ml penicillin, 50 μg/ml streptomycin and 2mM glutamine. The media for the CHO.T and rat HIRc-B cells additionally included 200 μg/ml G418.

Metabolic labelling of receptors in intact cells was carried out as described [24], using 0.25mCi [32-P]orthophosphate per 10 cm^2 dish. For determination of tyrosine kinase activity, cells were grown to confluence in 28 cm^2 plastic dishes, and serum starved for four hours in medium in which FCS was replaced by 0.1% (w/v) bovine albumin, before making other additions.

Cells were incubated with PMA for 30 min followed by addition of insulin for 10 min as indicated. Cells were then extracted in 0.5ml lysis buffer, containing 50mM Hepes pH7.4, 150mM NaCl, 10mM EDTA, 1mM Na_3VO_4, 30mM NaF, 10mM $Na_2P_2O_7$, 2.5mM benzamidine, 1μg/ml of pepstatin, leupeptin and antipain, 0.5mM PMSF, and 1% (w/v) TritonX-100. Plates were shaken on ice for 5 minutes following which the cells were scraped and the lysates were centrifuged at 13,000g for 5 min at 4°C. Insulin receptor phosphorylation was determined following immunoprecipitation from cell lysates using the monoclonal antibody 83-14 [23] and analysis by SDS/PAGE and autoradiography [24, 25]. Samples of lysate were used directly for determination of tyrosine kinase activity.

Tyrosine kinase assay. Receptor tyrosine kinase activity was assayed essentially as described by Zhang et al [22]. A 96-well microtitre plate was coated with insulin receptor antibody 83-7 [23] (50 μl/well, 2.5 μg/ml in 20mM $NaHCO_3$, pH9.6). The plate was then washed with ice cold buffer A (50mM Hepes pH7.4, 150mM NaCl, 0.1% (w/v) TritonX-100, 0.1% (w/v) Tween20 and 0.1% (w/v) bovine albumin). Cell lysate (150 μl per well) was

added and incubated for 16 h at 4°C. The plate was again washed in ice-cold buffer A, before addition of tyrosine kinase reaction mixture (20 µl per well) containing 130mM Hepes pH7.4, 100 µg/ml bovine albumin, 1 mM EGTA, 12mM $MgCl_2$, 0.2mM peptide substrate and 2 µCi [γ-32P]ATP. After 10 min at 22°C, reactions were stopped by addition of 5 µl 2M HCl. Portions (10 µl) of the reaction mixture were spotted onto 2 cm^2 pieces of P81 phosphocellulose paper. Papers were washed in 5 x 1 l of 150mM H_3PO_4 and Cerenkov radiation counted in 10ml distilled water. Results are expressed as fmol ATP incorporated per minute per assay well.

RESULTS

We initially investigated effects of PMA on the cell line CHO.T [19]. Cells were incubated with PMA and/or insulin, before assay of receptor phosphorylation *in situ* and tyrosine kinase activity *in vitro*. Phosphorylation of the receptor β-subunit, as detected by metabolic labelling with [^{32}P]phosphate, was increased 2.9-fold by PMA and 7-fold by insulin, with an additive increase (8.7-fold) when both agents were used together (Figure 1). Tyrosine kinase activity was assayed in a microtitre plate antibody capture assay, using a peptide substrate related to the major receptor autophosphorylation domain. In control cells which had not been treated with PMA, basal kinase activity was almost unbdetectable, and maximal stimulation was obtained at 100 nM insulin, with half-maximal stimulation at 3-10 nM insulin (Figure 2). When CHO.T cells were pretreated with PMA (1 nM - 1 µM) there was no effect on tyrosine kinase activity stimulated by a maximally effective concentration of insulin (Figure 3). Therefore, in CHO.T cells, insulin receptor phosphorylation induced by PMA was not accompanied by any diminution in insulin-stimulated receptor autophosphorylation or tyrosine kinase activity.

We have previously shown that PMA and insulin were additive in their effects on receptor phosphorylation in NIH3T3.HIR3.5 cells [7, 26] In the present study we confirmed that pretreatment with 1 µM PMA also did not influence insulin-stimulated tyrosine kinase activity in these cells, under the same conditions as used for the experiment illustrated in Figure 3 (data not shown).

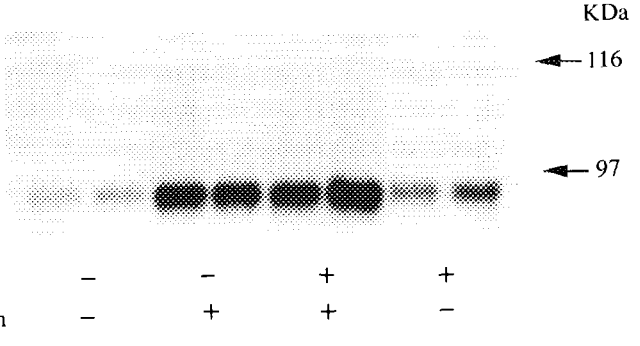

Figure 1. PMA and insulin induced phosphorylation of insulin receptor in intact CHO.T cells. Duplicate wells of CHO.T cells were labelled with ^{32}P and exposed to 1µM PMA (30 min) or DMSO vehicle followed by stimulation with or without 1µM insulin (10 min) as indicated. Insulin receptors were immunoprecipitated from solubilised cells and analysed by SDS/PAGE (7.5%) and autoradiography.

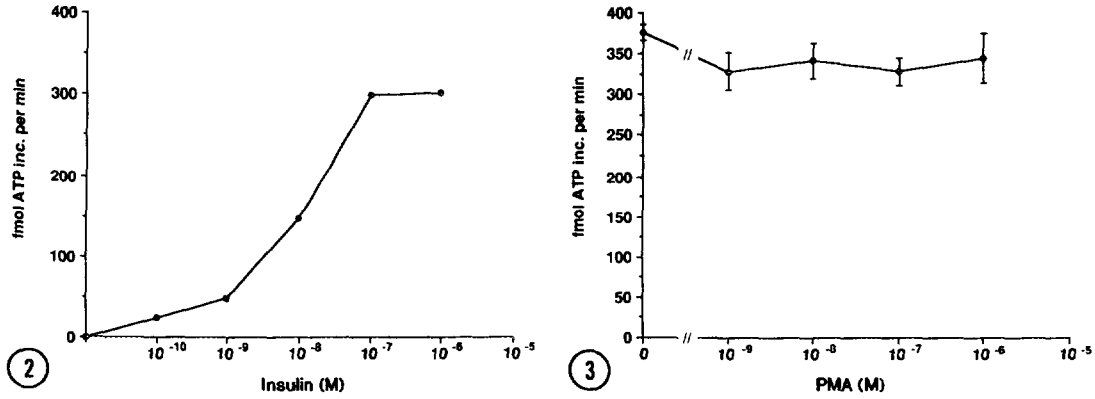

Figure 2. Insulin dose-reponse curve of receptor tyrosine kinase. CHO.T cells were stimulated with insulin at the concentration indicated for 10 mins. Cells were solubilised and the insulin receptor tyrosine kinase activity was assayed as described in the Methods section. Points represent the mean of two assay wells corrected for no-peptide control.

Figure 3. Effect of PMA on insulin receptor tyrosine kinase. CHO.T cells were pre-treated with PMA, or DMSO vehicle, at the indicated concentration for 30 mins before the addition of insulin (1μM) and assay of the receptor tyrosine kinase activity. Points represent the mean of three assay wells corrected for no-peptide control. Error bars denote standard deviation from the mean.

It has been reported that in transfected rat 1 fibroblasts (HIRc-B cells) pretreatment with phorbol esters resulted in inhibition of insulin-stimulated receptor tyrosine kinase activity [10]. However, we were unable to confirm this result either using conditions similar to those of Figure 3 (1 μM PMA followed by 1μM insulin; four independent experiments) or using the conditions of previous workers (162 nM PMA followed by 17 nM insulin, three separate experiments). Under all conditions pretreatment with PMA had no effect on tyrosine kinase activity measured either in the microtitre plate assay or the previously employed [10] immunoprecipitation assay (data not shown). However, we did confirm that PMA treatment increased the phosphorylation of insulin receptors in HIRc-B cells approximately 2-fold, under the same conditions as used for the experiment illustrated in Figure 1 (data not shown). Therefore in all three transfected cell lines we observed that PMA induced receptor serine/threonine phosphorylation without affecting receptor tyrosine kinase activity.

An inhibitory effect of phorbol ester on insulin receptor function was first reported in rat Fao hepatoma cells [3]. In a final attempt to demonstrate inhibition of receptor tyrosine kinase by PMA we investigated Fao cells, reproducing the conditions of the original authors as closely as possible. Cells were pretreated with 1.6 μM PMA, lysed, and receptors partially purified using wheat germ agglutinin agarose. Tyrosine kinase activity was assayed in the presence of 100 nM insulin using a peptide substrate (RFKRSYEEHIPYTHM) corresponding to the insulin receptor carboxyl-terminal autophosphorylation sites. In our hands the insulin-stimulated kinase activity was not influenced by PMA pretreatment (data not shown).

The cell types, assay formats and agonist concentrations used in our attempts to observe an inhibitory effect of PMA are listed in Table 1. In no case was PMA treatment found to inhibit the receptor kinase.

Table 1 : Summary of the cells, agonist concentrations and assay formats used in attempts to observe an inhibitory affect of PMA

Cell Line	Assay	Number of Experiments	[PMA] (M)	[Insulin]
NIH3T3Hir3.5	Plate	2	10^{-6}	10^{-6}
HIRc-B	Plate	4	10^{-6}	10^{-6} 10^{-7} 17.4×10^{-9}
		3	162×10^{-9}	17.4×10^{-9}
HIRc-B	Protein A (Ref 10)	1	10^{-6}	10^{-6}
			162×10^{-9}	17.4×10^{-9}
Fao	WGA (Ref 3)	1	1.62×10^{-6}	10^{-7}

DISCUSSION

Studies reporting an inhibitory effect of serine/threonine phosphorylation on insulin receptor tyrosine kinase activity have been widely quoted [27-32], on the assumption that these indicate a mechanism of physiological importance which might modulate insulin sensitivity. Impairment of insulin receptor tyrosine kinase activity has been described under pathophysiological conditions associated with insulin resistance [33-36], although in these cases the underlying regulatory mechanism remains to be elucidated and it has not been shown that phosphorylation is involved. Inhibitory effects on receptor function in cultured cells have usually been demonstrated by using pharmacological agents such as PMA or forskolin to induce receptor phosphorylation [3,10,12]. In the present work we sought to correlate PMA-mediated inhibition of the insulin receptor kinase with the extent and pattern of receptor phosphorylation, using transfected cell lines which reportedly differed in their sensitivity to PMA. In fact we were unable to demonstrate an inhibitory effect of PMA on the insulin receptor tyrosine kinase in any of the cell lines studied (CHO.T, NIH3T3.HIR3.5, Rat 1 HIRc-B and Fao), although PMA substantially ($\geq$ 2-fold) increased the level of receptor phosphorylation. In the case of HIRc-B and Fao cells our results conflict directly with previous reports [3, 10] for reasons which are not clear, every effort having been made to reproduce the original experimental conditions. However the present data are consistent with other studies which have demonstrated additivity, rather than antagonism, of PMA- and insulin-induced receptor phosphorylation in various cell types [5, 7, 18]. Furthermore, it has recently been reported that PMA treatment did not influence insulin-stimulated receptor tyrosine kinase activity either in freshly isolated rat hepatocytes or in Fao cells [37].

Our conclusion is that insulin receptor phosphorylation sites which are responsive to phorbol esters, particularly threonine 1348 [9], are not involved in a mechanism of general significance for the regulation of receptor tyrosine kinase activity. This is consistent with the previous demonstration that replacement of threonine 1348 or serines 1305/6 with alanine or aspartate residues did not affect the tyrosine kinase activity of receptors isolated from insulin treated cells [38]. These results do not exclude totally the involvement of serine/threonine phosphorylation in regulation of insulin receptor function, and sites phosphorylated by other kinases or by the receptor's intrinsic serine kinase activity [39] may play some role. It is also still possible that whilst PMA-induced receptor phosphorylation does not affect receptor kinase activity as assayed with artificial peptide substrates it may perturb receptor interactions with endogenous cellular substrates.

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