The stimulation of phosphorylation of intracellular proteins in GH₃ rat pituitary tumour cells by phorbol esters of distinct biological activity

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Using a pituitary tumour cell line (GH₃), we have studied the phosphorylation of intracellular proteins induced by phorbol esters of diverse biological activity. All the active phorbol esters, including the weakly tumour-promoting but non-platelet aggregatory compound DOPPA, stimulated the phosphorylation of a cytosolic 80 kDa protein. A protein of this molecular mass has been suggested to be a marker of PKC activity. In contrast, only TPA and the non-tumour promoting but highly active phorbol ester SAP A stimulated the phosphorylation of a 130 kDa membrane protein. The results suggest that these phorbol esters activate PKC, but induce the differential phosphorylation of a variety of intracellular proteins.

Protein kinase C; Phorbol ester; Protein phosphorylation

1. INTRODUCTION

On binding to their receptor, a wide variety of hormones, neurotransmitters and growth factors stimulate the hydrolysis of the phosphoinositides by activation of phospholipase C. This enzyme hydrolyses phosphatidylinositol 4,5-bisphosphate to yield the two second messengers inositol 1,4,5-trisphosphate and diacylglycerol. IP₃ has been shown to be responsible for the rise in intracellular Ca^{2+} levels, whereas DAG provides the signal for the transmembrane control of protein

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Abbreviations: DOPPA, 12-deoxyphorbol-13-phenyl-acetate-20-acetate; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SAP A, sapintoxin A; IP₃, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; α-SAP Ac, α-sapinine acetate

phosphorylation by activation of the Ca^{2+}- and phospholipid-dependent protein kinase C. At low concentrations, DAG increases the affinity of PKC for Ca^{2+} and phospholipid, and renders the enzyme fully active in the absence of a further rise in Ca^{2+} levels. The enzyme then migrates to the plasma membrane where it exerts its full enzymatic activity [1].

Investigation into the role of PKC within the cell has been made considerably easier since the discovery that certain naturally occurring phorbol esters are able to activate PKC [2]. Subsequent studies have shown that the phorbol ester receptor co-purifies with PKC [3] and have provided further evidence that the phorbol esters directly activate PKC by substituting for DAG [4]. Phorbol esters are a group of toxic diterpenes isolated from the plant families Euphorbiaceae and Thymeleaceae. Biologically active phorbol esters have been shown to induce lymphocyte mitogenesis, stimulate platelet aggregation, increase the production of prostaglandin E_1, possess tumour-

promoting and pro-inflammatory activity, induce changes in phospholipid metabolism and protein synthesis, alter rates of DNA synthesis, affect polyamine biosynthesis and produce changes in cell morphology (reviews [5,6]). Our interest in this field lies in the use of the phorbol esters of diverse biological activity to determine substrate proteins for PKC which are involved in enzyme cascades leading to specific biological effects such as secretion and tumour promotion. Several substrate proteins have been identified in vitro, although only a few of these have been identified as in vivo substrates for the enzyme [7].

In the present study we have used the rat pituitary tumour cell line, GH_3. In these cells, phorbol esters have little effect on cell growth although they do stimulate growth hormone and prolactin secretion, as well as enhance the production of prolactin. Other effects include decreased thyrotropin releasing hormone (TRH), epidermal growth factor and somatostatin binding (review [8]). The pituitary peptide TRH has also been shown to induce prolactin and growth hormone secretion [9] by a mechanism which is believed to utilize the phosphoinositide hydrolysis pathway [10,11]. A small number of proteins have been shown to be phosphorylated in response to TPA treatment in both GH_3 and the related GH_4C_1 cells [12,13]. This is a potent phorbol ester, capable of inducing a broad spectrum of biological effects including platelet aggregation, tumour promotion and inflammation [5,6]. In order to identify substrates which may be involved in a particular biochemical response, we utilized three phorbol esters which have narrower spectra of activity, SAP A, DOPPA and α-SAP Ac. SAP A is an extremely potent inflammatory agent which also possesses platelet aggregatory and other properties similar to TPA. However, we have recently shown that SAP A is neither a first nor second stage tumour-promoting agent [14]. DOPPA has been shown to be weakly tumour-promoting [15] but non-platelet aggregatory [16], whereas α-SAP Ac possesses no biological activity [6] (see table 1). Consequently, it is of great interest to show whether these phorbol esters and TRH induce patterns of protein phosphorylation different to that of TPA.

2. MATERIALS AND METHODS

Ampholytes were obtained from LKB, KCl, NaCl, SDS, glucose, glycine, $MgCl_2$, $CaCl_2$ and glycerol were from BDH. $^{32}P_i$ was from Amersham International. Penicillin/streptomycin solution, donor horse serum, fetal calf serum and Ham's F10 culture medium were obtained from Gibco. The phorbol esters SAP A, α-SAP Ac and DOPPA were isolated as reported [17–19]. TRH and GH_3 cells were kindly donated by A.H. Drummond. All other reagents were obtained from Sigma.

2.1. Cell culture

GH_3 cells were grown in monolayer culture at 37°C under 95% air/5% CO_2 on 10 cm dishes in 9 ml of Ham's F10 culture medium, supplemented with 15% donor horse serum, 2.5% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, and 6.25 μg/ml neomycin. The cultures were refed at 3–4 day intervals for 10 days.

2.2. $^{32}P_i$ incorporation

Prior to labelling, cells were scraped off the dishes using a rubber policeman and were washed with balanced salt solution (135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM Hepes, pH 7.4, 0.1% bovine serum albumin, 0.01% sodium pyruvate). Cells were resuspended in this solution, incubated with 20 μCi/ml $^{32}P_i$ for 1 h at 37°C and stimulated with various agents for the indicated times.

2.3. Sample preparation for one-dimensional gel electrophoresis

Following phorbol ester stimulation, cells were rapidly washed with ice cold 0.3 M sodium phosphate and 0.15 M NaCl, and resuspended in a final volume of 50 μl of the same solution. The cells were rapidly frozen on dry ice then thawed in 50 μl of a protease/phosphatase inhibitor solution containing 0.2 M NaF, 20 mM EDTA, 50 mM Hepes, pH 7.5, and 1 mM 2-mercaptoethanol. All subsequent procedures were carried out at 4°C unless otherwise stated. The suspension was sonicated for 3 s using an MSE ultrasonic power unit – a procedure which has been reported to lyse cells but leave nuclei intact. The cell suspension was centrifuged at 15000 × g for 5 min using a

Heraeus Christ Biofuge to pellet cell debris and nuclei. The resultant supernatant, which contained the cytoplasm and membrane fragments, was then centrifuged at $100000 \times g$ for 20 min in a Beckman Airfuge to yield a supernatant (the 'cytoplasmic' fraction) and a pellet (the 'membrane' fraction). The membrane fraction was resuspended in 100 μl of 100 mM NaF, 10 mM EDTA, 0.5 mM 2-mercaptoethanol and 25 mM Hepes, pH 7.5. Both fractions were solubilized with 100 μl sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) at 100°C for 5 min. The suspension was centrifuged at $100000 \times g$ for 20 min using a Beckman Airfuge. The resultant supernatant was

counted for radioactivity by Cherenkov counting on an LKB rack-beta liquid scintillation counter. Equal counts were loaded onto one-dimensional SDS-polyacrylamide gels and run according to the method of Laemmli [20]. Proteins were resolved on 10% polyacrylamide slab gels at 45 mA constant current unless otherwise stated. Standard molecular mass markers used to calibrate the gels were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (98 kDa), bovine serum albumin (68 kDa) and carbonic anhydrase (29 kDa). After staining with Coomassie blue and drying, gels were autoradiographed using Kodak X-Omat S film with an intensifying screen at $-70°C$.
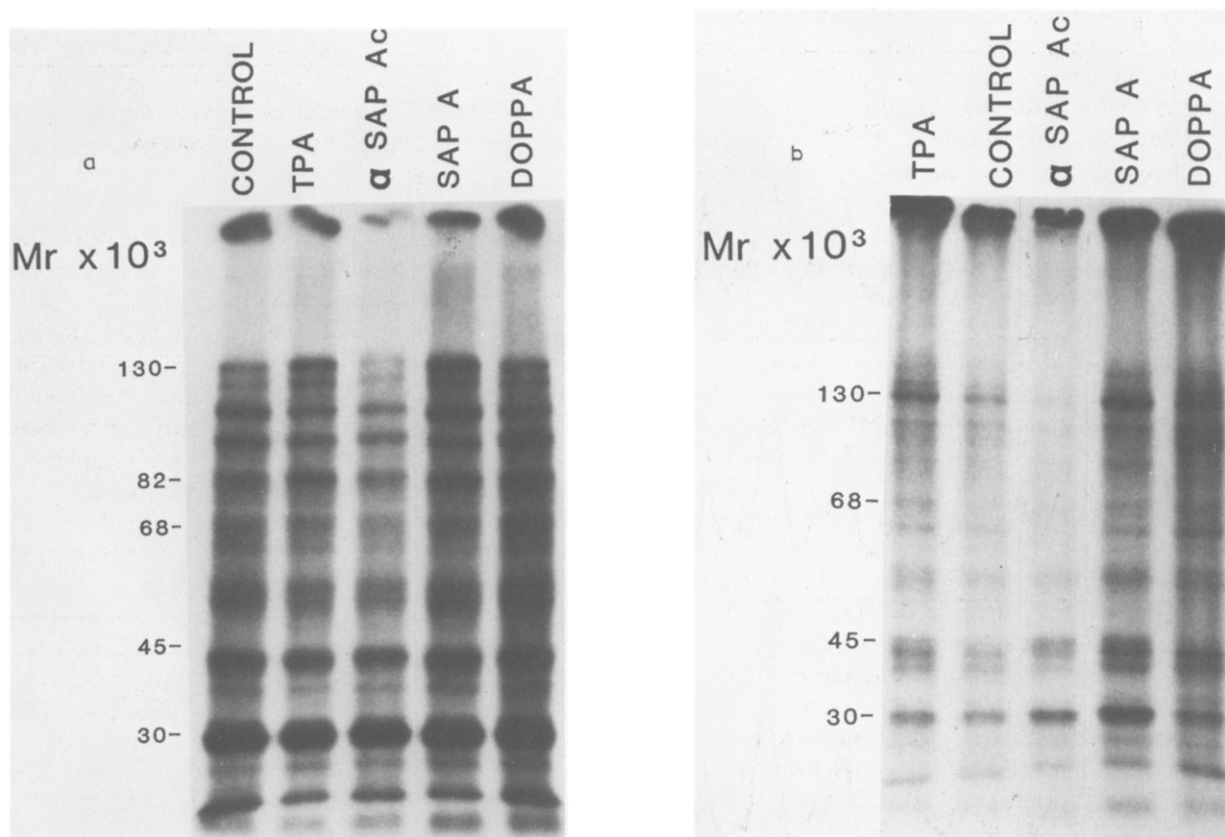

Fig.1. Autoradiographs of cytosolic (a) and membrane (b) proteins from GH$_3$ cells following treatment with various phorbol esters. Cells were labelled with ^{32}P$_i$ for 1 h, and incubated with no further additions (control), TPA (100 nM), SAP A (100 nM), DOPPA (300 nM) or α-SAP Ac (500 nM) for 10 min. The reaction was terminated by washing the cells in ice-cold PBS followed by lysis into a protease/phosphatase inhibitor solution. The lysate was centrifuged at $100000 \times g$ for 20 min to yield a supernatant cytosolic fraction and membrane pellet. These fractions were analysed by SDS-gel electrophoresis using a 10% acrylamide resolving gel.

2.4. Sample preparation for two-dimensional gel electrophoresis

Following phorbol ester stimulation cells were rapidly washed in phosphate buffered saline (PBS) containing 100 mM NaF and 10 mM EDTA, and resuspended in 50 μl of this solution with 20 μl of leupeptin solution (1 mg/ml in 20 mM Tris-HCl, pH 7.0). Cells were homogenized by rapid freez-

ing, thawing and sonicating as before. Subcellular fractions were prepared as for one-dimensional gels with the exception that the membrane fraction was resuspended in 50 μl of PBS containing 100 mM NaF, 10 mM EDTA and 20 μl of the leupeptin solution. To both fractions, 100 μl of a micrococcal nuclease solution (100 μg/ml in 20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$), 20 μl of
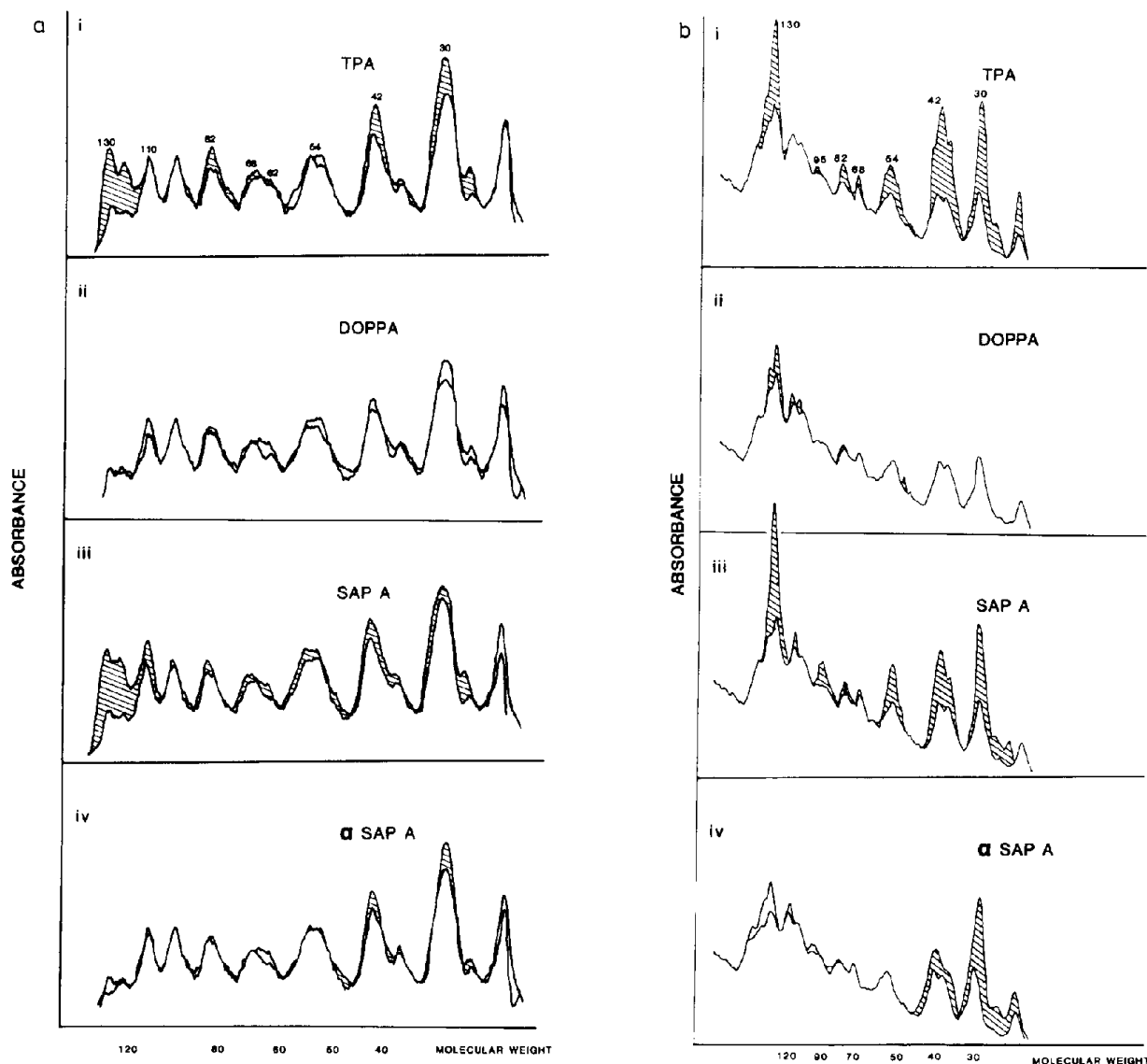

Fig.2. Densitometric scans of autoradiographs shown in fig.1. In each case, phorbol ester incubations are superimposed onto those of control scans, with increases in phosphate incorporation being represented by the shaded areas. Molecular masses of each protein peak are indicated by the figures in a(i) and b(i).

solubilizer (2% SDS, 0.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.0) and 10 μl of a DNase/RNase solution (2 mg/ml DNase, 1 mg/ml RNase, 0.5 M Tris-HCl, pH 7.0, 50 mM $MgCl_2$) were added, and incubated at 4°C for 10 min. The suspension was heated at 100°C for 15 min before lyophilizing. The residue was resuspended in 40 μl dilution buffer (9.5 M urea, 2% ampholytes, 5% 2-mercaptoethanol and 8% NP-40). Samples were then spun at $100000 \times g$ for 20 min using a Beckman Airfuge and the supernatant was counted for radioactivity. Equal counts were analysed by 2-dimensional gel electrophoresis [21] using 7.5 cm isoelectric focussing gels of the ampholyte composition pH 2.5–4:4–6:5–7:3.5–10, 2:2:2:1.

3. RESULTS

Several proteins have shown an increase in phosphorylation state in response to phorbol ester treatment of intact GH_3 cells. The autoradiographs of one-dimensional gels of cytosolic and membrane proteins phosphorylated in response to treatment with phorbol esters are shown in fig.1a and b. The corresponding densitometer scans are shown in fig.2a and b.

It would appear that the non-tumour promoting phorbol ester, SAP A, induces a very similar pattern of cytosolic and membrane protein phosphorylation to TPA although at the concentration used (100 nM), there is greater phosphorylation of some membrane proteins than that induced by the same dose of TPA. Both TPA and SAP A have similar K_a values for the activation of PKC (see table 1). In contrast, the phosphorylation pattern seen in response to DOPPA is markedly different. Using a dose of 300 nM, there is an obvious lack of phosphorylation of all membrane proteins above basal levels (fig.2b), although there is a detectable increase in the phosphorylation state of a 82 kDa cytosolic protein (fig.1a). The phosphorylation pattern induced by the biologically inactive phorbol ester, α-SAP Ac shows some similarities to that of DOPPA. However, the former compound does not induce the phosphorylation of the 82 kDa cytosolic protein (fig.1a), but does increase the phosphorylation state of a few lower molecular mass membrane proteins (figs 1b,2b). Unlike the phosphorylation patterns induced by the active phorbol esters TPA and SAP A, there is no evidence of phosphorylation of the 130 kDa membrane protein. Indeed, phosphate incorporation in this region appears to be reduced below basal levels. At the dose used in the present study (500 nM), α-SAP Ac has been reported to induce virtually no activation of PKC in vitro [22,23], although at doses higher than 1 μM some stimulation is seen.

To characterize further the proteins whose state of phosphorylation is differentially enhanced by phorbol esters of distinct biological activity we analysed subsequent subcellular fractions by two-dimensional gel electrophoresis. Both TPA and DOPPA stimulate the phosphorylation of proteins of molecular masses 23, 25 and 80 kDa, whereas

Table 1

Compound	Lymphocyte mitogenesis [6] ED_{40} (ng/ml)	Pro-inflammatory activity [6] ID_{50} (μg)	Human platelet aggregation [31] ED_{50} (μM)	Prostaglandin E_2 production [6] ED_4 (ng/ml)	Protein kinase C stimulation [30] K_a (nM)	Phorbol dibutyrate binding assay [23] IC_{50} (nM)
TPA	17	0.007	0.30	0.28	17	0.8
SAP A	34	0.02	1.76	0.26	36	22
DOPPA	564	0.04	– ve	46.0	100	240
α-SAP Ac	–	–	– ve	–	2600	10000

The compound TPA is a known tumour-promoting agent. Lymphocyte mitogenesis values are EC_{40} ng/ml. Pro-inflammatory activity is ID_{50} μg/ear, values more than 10 μg/ear are shown as negative (– ve). Platelet aggregation is ED_{50} μM. Values greater than 8 μM are shown as negative (– ve). ED_4 is the dose of phorbol ester required to raise the basal level of PGE_2 4-fold in human rheumatoid synovial cells. The phorbol dibutyrate binding values are shown as negative if more than 40 μM. (–) Figures not available

TPA additionally stimulates the phosphorylation of a 20 kDa protein(s) (fig.3). Incubation with TRH also resulted in increased phosphorylation of the 80 kDa protein although there was no detectable increase in phosphate incorporation into the 23 and 25 kDa proteins as seen with DOPPA and

TPA. Additionally, treatment with both TPA and TRH, but not DOPPA appears to induce a reduction in the phosphorylation of a 52 kDa protein(s) to below basal levels.

4. DISCUSSION

We have shown that a variety of phorbol esters differentially induce the phosphorylation of several cytosolic and membrane proteins in vivo. The reasons for the increase in phosphorylation in response to the supposedly inactive phorbol ester α-SAP Ac remains unclear. It is unlikely that this compound is degraded to an active metabolite as phorbol esters possessing the α-configuration are inherently inactive [6]. On the other hand, DOPPA shows poorer stimulation of protein phosphorylation, more in line with what would be expected from a phorbol ester with limited biological activity (see table 1). However, we have shown that this compound is capable of activating PKC in vitro, albeit at relatively high doses [22,23]. Consequently, at the dose used (300 nM), there is likely to be some activation of PKC with the resultant increase in substrate phosphorylation. As this compound is only weakly tumour-promoting and non-platelet aggregatory, it is improbable that the proteins phosphorylated in response to this phorbol ester (e.g. 82 kDa) are important regulatory enzymes for these biological effects.

A similar set of cytosolic proteins has been reported to be phosphorylated in response to TRH and TPA stimulation of GH_3 cells [12,24]. This peptide hormone stimulates phosphoinositide metabolism to yield the two intracellular second messengers IP_3 and DAG [10,11]. Consequently, it is likely that TRH acts by stimulating both Ca^{2+}-calmodulin dependent protein kinase and PKC. Indeed, it has been shown that PKC is responsible for some of the increases in protein phosphorylation seen on addition of TRH, as TPA treatment induces the phosphorylation of 5 of the 7 proteins induced by TRH [12]. These cytosolic proteins have molecular masses of 59 kDa (two bands), 65, 80 and 82 kDa, and there is also a reported decrease in the phosphorylation of a 55 kDa protein in response to treatment with TRH [24]. TPA treatment of cells in our system resulted in an increase in phosphate incorporation into
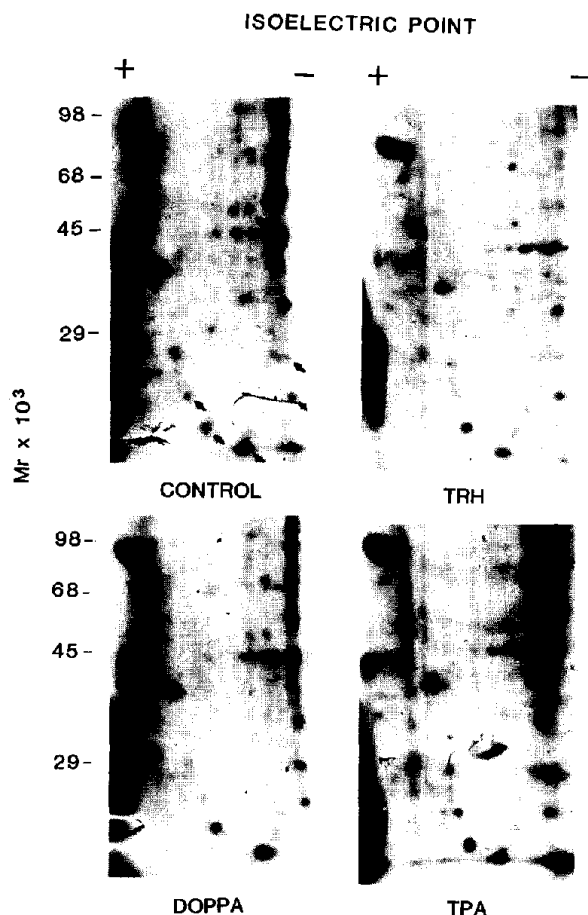
ISOELECTRIC POINT

Fig.3. Autoradiograph of cytosolic proteins of GH_3 cells analysed by 2D-SDS gel electrophoresis. Gels were labelled for 1 h with $^{32}P_i$ and incubated with no further addition (control), TPA (100 nM), DOPPA (300 nM) and TRH (10^{-7} M) for 10 min. The reaction was terminated by washing with ice-cold PBS and lysing the cells into a protease/phosphatase inhibitor solution. The lysate was centrifuged to yield the cytosolic supernatant. These fractions were lyophilized, solubilized and analysed by 2D-SDS gel electrophoresis using 10% acrylamide resolving gels. Proteins which show differences in phosphorylation following phorbol ester on TRH treatment are indicated by arrows on the control autoradiograph.

cytosolic proteins of molecular masses 23, 25 and 80 kDa, and a decrease of phosphorylation of two 52 kDa proteins. Additionally, a 135 kDa pellet protein has been shown to be phosphorylated in response to both TRH and TPA [24]. This could correspond to the 130 kDa membrane protein phosphorylated in response to TPA treatment in our system. Interestingly, this protein is also phosphorylated following treatment of the cells with the non-tumour-promoting phorbol ester SAP A, but not by DOPPA or α-SAP Ac. A protein of this molecular mass has been identified as vinculin, a cytoskeletal protein which is believed to be involved in the attachment of actin to membranes, and has also been shown to be a substrate for PKC both in vitro [25] and in vivo [26].

Other proteins of interest include the two 20 kDa proteins that appear to be exclusively phosphorylated in the presence of TPA, and the 52 kDa protein(s) whose phosphorylation state is to be altered in response to TPA and TRH treatment, but not by the phorbol ester of limited biological activity, DOPPA. It remains to be seen if the 52 kDa phosphoprotein corresponds to the 55 kDa protein previously reported to be dephosphorylated following incubation with TRH and TPA [24].

Explanations for the observed differences include the possibilities that the phorbol esters may alter the substrate specificity of the enzyme, or that the phorbol esters are activating isozymes of PKC [27] with different affinities. Heterogeneity of TPA and [^{3}H]phorbol dibutyrate binding in vivo and in vitro has previously been observed [28]. However, our results of the [^{3}H]phorbol dibutyrate binding assay indicate that all the phorbol esters bind to the same site on rat brain PKC preparations [23]. It appears that the majority of substrates for this protein kinase are cytosolic, which is somewhat surprising considering that the active form of PKC is attached to the plasma membrane [28]. It has been shown that in addition to inducing the relocalization of PKC to the membrane, TPA is also capable of inducing proteolysis of the enzyme to a cytoplasmic cofactor independent form [29].

In order to gain further insight into the role of these phosphoproteins in the induction of cellular events it is important to correlate phosphorylation of particular proteins with specific cellular

responses such as changes in morphology, prolactin synthesis and secretion. Ultimately, our aim is to fully characterize the proteins which mediate these specific biological events.

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