The phorbol ester, TPA inhibits glucagon-stimulated adenylate cyclase activity

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The ability of glucagon (10 nM) to increase hepatocyte intracellular cyclic AMP concentrations was reduced markedly by the tumour-promoting phorbol ester TPA (12-*O*-tetradecanoyl phorbol-13-acetate). The half-maximal inhibitory effect occurred at 0.14 ng/ml TPA. This action occurred in the presence of the cyclic AMP phosphodiesterase inhibitor isobutylmethylxanthine (1 mM) indicating that TPA inhibited glucagon-stimulated adenylate cyclase activity. TPA did not affect either the binding of glucagon to its receptor or ATP concentrations within the cell. TPA did inhibit the increase in intracellular cyclic AMP initiated by the action of cholera toxin (1 μg/ml) under conditions where phosphodiesterase activity was blocked. TPA did not inhibit glucagon-stimulated adenylate cyclase activity in a broken plasma membrane preparation unless Ca^{2+}, phosphatidylserine and ATP were also present. It is suggested that TPA exerts its inhibitory effect on adenylate cyclase through the action of protein kinase C. This action is presumed to be exerted at the point of regulation of adenylate cyclase by guanine nucleotides.

Protein kinase C *Guanine nucleotide regulation* *Tumor promoter* *Adenylate cyclase* *Phorbol ester* *Calcium*

1. INTRODUCTION

TPA (12-*O*-tetradecanoyl phorbol-13-acetate) is one of the most potent tumour promoters that is known to date [1,2]. It binds specifically with high affinity to cell surface receptors [3,4], inducing pleiotropic and reversible effects on cell structure and function (see [1,4]). Recently, the exciting observation was made that the so-called phorbol ester receptors are associated with the activation of protein kinase C [7,8]. This enzyme appears to occur universally in eukaryotes [7] and may be the route whereby phorbol esters exert at least some of their effects on target cells [6,8].

Hormones, such as glucagon, activate adenylate cyclase through a well-elucidated mechanism. Thus, glucagon binds with high affinity to receptors on the hepatocyte surface, whereupon adenylate cyclase becomes activated, giving rise to a rapid and dramatic increase in intracellular cyclic AMP concentrations (see [9]). This involves the occupied hormone receptor activating a distinct guanine nucleotide regulatory protein, called N_s, which subsequently interacts with, and activates, the catalytic unit of adenylate cyclase (see [10]). Alterations in intracellular cyclic AMP concentrations have been observed in transformed cells, and proposed to exert effects on cell growth and development [9,10]. Here we use the glucagon-stimulated adenylate cyclase activity from hepatocytes, as a model system, to show that the phorbol ester, TPA, can inhibit adenylate cyclase activity.

2. MATERIALS AND METHODS

Hepatocytes, from male Sprague-Dawley rats

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(200–300 g) were prepared and incubated as in [9].

A membrane fraction was obtained from these hepatocytes as in [13] and used for the assay of adenylate cyclase activity. This membrane preparation was assayed for adenylate cyclase activity basically as described by us in [14]. Briefly the incubation mixture contained final concentrations of 1.5 mM ATP, 5 mM $MgSO_4$, 10 mM theophylline, 7.4 mg/ml phosphocreatine, 1 mg/ml creatine kinase and 25 mM triethanolamine·HCl at a final pH of 7.4. Membranes were present at 1 mg/ml and rates were determined from linear time courses obtained over 10 min incubation at 30°C. Measurement of cyclic AMP produced was performed as described by us in [14].

To assess intracellular cyclic AMP accumulation, cells (4–5 mg dry wt/ml) were pre-incubated for 20 min at 37°C with isobutylmethylxanthine (10 mM), to inhibit cyclic AMP phosphodiesterases (see [9]). After an appropriate period of incubation, aliquots were taken for the determination of intracellular cyclic AMP content as described by us in [15].

The specific binding of ^{125}I-labelled glucagon to the intact hepatocytes was measured essentially as in [16] except that the cells were separated from the incubation medium by centrifugation through bromododecane for 1 min at 400 × *g*. Non-specific binding was assessed by the inclusion of unlabelled glucagon (10 μM) in the incubation medium.

Rat liver plasma membranes from male, Sprague Dawley rats were isolated and purified as before [14,17]. Adenylate cyclase activity in these purified membranes was assessed essentially as in [14]. Here, incubation mixtures contained glucagon (10 nM), GTP (10 μM), $CaCl_2$ (50 μM), a sonicated dispersion of phosphatidylserine (20 μg/ml), ATP (1.5 mM), $MgSO_4$ (1 mM), theophylline (10 mM), phosphocreatine (7.4 mg/ml), creatine kinase (1 mg/ml) and triethanolamine·HCl (25 mM) at a final pH of 7.4 plus membranes (500 μg/ml). Linear time courses over 10 min at 30°C were obtained under all assay conditions. TPA was freshly prepared dissolved in DMSO at 1 mg/ml so that the DMSO concentration in the assay was always $<0.001\%$. Incubation mixtures were always freshly prepared on the day of use. Controls also had the appropriate DMSO content.

3. RESULTS AND DISCUSSION

When intact hepatocytes were challenged with glucagon (10 nM), intracellular cyclic AMP concentrations rose from around 2 ± 0.5 to 84.0 ± 4.5 pmol/mg dry wt cells after 5 min (SE, $n = 3$). Such experiments were performed in the presence of the cyclic AMP phosphodiesterase inhibitor, isobutylmethylxanthine at concentrations (1 mM) which blocked the breakdown of cyclic AMP in hepatocytes [9]. If the cells were pre-treated with the phorbol ester TPA, prior to challenge with glucagon (10 nM), this increase in intracellular cyclic AMP was reduced markedly (fig.1a). The half-maximal inhibitory effect (ID_{50}) occurred at 0.14 ± 0.02 ng TPA/ml (SE, $n = 3$), which is within the range where TPA is believed to elicit its biological effects, including its promotional activity [1,4,6]. We noted that the non-promoting analogue of TPA, 4-*O*-methyl-TPA [18] exerted no such inhibitory effect on cyclic AMP accumulation. The effect of TPA must therefore be to inhibit cyclic AMP production as under these conditions cellular cyclic AMP phosphodiesterase activity was blocked [9] and no TPA-induced increase in extracellular cyclic AMP was observed (unpublished). We also observed (fig.1) that ^{125}I-labelled glucagon specific binding was unaffected, indicating that TPA either inhibited the catalytic unit of adenylate cyclase per se or the functioning of the guanine nucleotide regulatory protein (N_s) which effects the cholera toxin and the hormone-receptor mediated stimulation of adenylate cyclase (see [10]). The functioning of adenylate cyclase was not altered by any change in substrate concentration as intraeellular ATP concentrations were 9.54 ± 0.21 nmol/mg dry wt cells in control experiments and 9.38 ± 0.21 nmol/mg dry wt in cells treated for 20 min with 10 ng/mg TPA ($n = 4$, SE).

Cholera toxin can increase intracellular cyclic AMP concentrations by causing the ADP-ribosylation of N_s, which subsequently activates adenylate cyclase [10]. After 30 min incubation of hepatocytes at 37°C with cholera toxin (1 μg/ml) in the presence of isobutylmethylxanthine (1 mM), to inhibit cyclic AMP phosphodiesterase (see [9]), intracellular cyclic AMP concentrations had risen to 82 ± 4 pmol cyclic AMP/mg dry wt cells. If, however, the cells were treated with 1 ng TPA/ml,

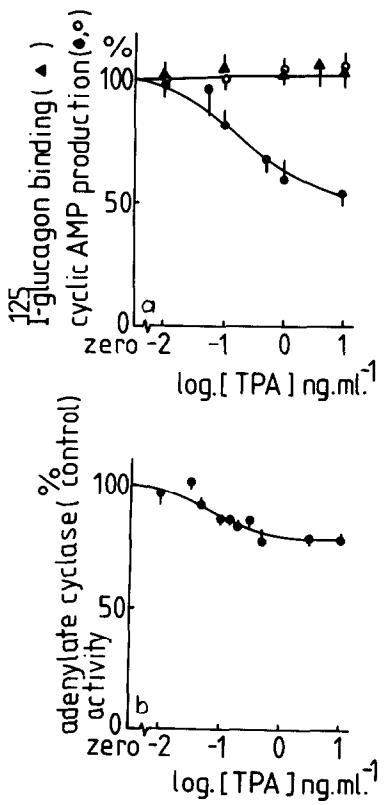

Fig.1. The effect of TPA on glucagon binding to hepatocytes and on the glucagon-stimulated adenylate cyclase activity in intact hepatocytes and purified plasma membranes. (a) Action of TPA on the specific ^{125}I-labelled glucagon binding to intact hepatocytes (○); change in intracellular concentrations of cyclic AMP in hepatocytes treated with either TPA (●) or 4-*O*-methyl-TPA (▲). These ligands were added to the intact hepatocytes, as per the diagram, and incubation continued for a further 10 min. The cells were then challenged with glucagon (10 nM) and after 10 min harvested and their intracellular cyclic AMP content determined. The mean of 4 experiments (n = 4) using different hepatocyte preparations is shown (errors are SE) with triplicate determinations of cyclic AMP. (b) Action of TPA on the glucagon-stimulated adenylate cyclase activity of isolated, purified rat liver plasma membranes. The specific activity of the purified membranes was 58.6 ± 11.6 pmol/min per mg protein for 3 different membrane preparations (errors are SD). We found it crucial that assay mixtures were freshly prepared each day and plasma membranes were stored at −80°C.

then cyclic AMP concentrations rose to only some 45 ± 4.5 pmol cyclic AMP/mg dry wt after exposure to cholera toxin (n = 4, SE). As TPA clearly cuased inhibition of cyclic AMP accumulation, under conditions where phosphodiesterase activity was blocked, then its site of action does not appear to be at the level of receptor-N_s coupling but would seem to occur either at the catalytic unit of adenylate cyclase itself or at the level of N_s-adenylate cyclase coupling. This is supported by our observation that not only is the activity of glucagon-stimulated adenylate cyclase inhibited in membranes prepared from TPA-treated cells, but so also is the stimulation achieved by the non-hydrolysable GTP analogue p[NH]ppG, which acts to stimulate adenylate cyclase through N_s. Indeed, the action of TPA would appear to be exerted at the level of regulation by guanine nucleotides as basal adenylate cyclase activity, presumably a measure of the functioning of the catalytic unit itself, was unchanged in membranes from TPA-treated cells (table 1).

In a number of instances [19], inhibitory effects exerted on adenylate cyclase through receptor-coupled systems have been shown to be exerted through a distinct type of inhibitory guanine nucleotide regulatory protein (N_i). We, however,

Table 1

The adenylate cyclase activity of membranes from TPA-treated hepatocytes

Activated state	Specific activity in control membranes	% Inhibition of adenylate cyclase in membranes from TPA-treated cells
Basal	0.79 ± 0.21	7 ± 8
p[NH]ppG	10.9 ± 2.8	22 ± 1.5
Glucagon + GTP	25.0 ± 2.6	31 ± 5.0

Hepatocytes were incubated as in [9] for 15 min at 37°C with TPA (10 ng/ml) after which a membrane fraction was prepared as in [13]. The appropriate stimulatory ligand was also present as per the table. In this instance GTP was added at 100 μM, glucagon at 10 nM and guanosine 5′-[β,γ-imido]triphosphate (p[NH]ppG) at 100 μM. Errors are SE from 3 separate experiments (n = 3) using different hepatocyte preparations. Adenylate cyclase assays were performed in triplicate in each instance

consider it extremely unlikely that TPA acts through N_i. Firstly, the effects of TPA are not blocked by islet activating protein from *Bordatella pertussis* (unpublished) which prevents the functioning of N_i [20]. Secondly, to observe inhibition of hormone-stimulated activity in a broken membrane system much higher GTP concentrations are required [19] than were used here (table 1) and thirdly, inhibitory effects exerted through N_i are not observed in broken membranes under conditions of high p[NH]ppG concentrations [19] such as have been employed in this study (table 1). It is possible, therefore, that TPA exerts its effects at the level of the stimulatory guanine nucleotide regulatory protein, N_s.

Stimulatory and inhibitory hormones acting upon adenylate cyclase will exert their effects in broken membrane systems [10,19]. In contrast, the addition of TPA to purified rat liver plasma membranes failed to exert any effect on adenylate cyclase activity (fig.2). This suggests that TPA is affecting adenylate cyclase through a novel mechanism.

Recently it was demonstrated that TPA can stimulate the phospholipid and Ca^{2+}-dependent protein kinase C (see [8]) which may be an integral part of the phorbol ester receptor itself [21]. Here we see that using purified liver plasma membranes, which have been shown to exhibit protein kinase C activity [22], then TPA will indeed inhibit glucagon-stimulated adenylate cyclase provided that both Ca^{2+} and phosphatidylserine are present in the incubation mixture (fig.2). The inhibition of adenylate cyclase caused by TPA is dose dependent (fig.1b) with an ID_{50} of 0.21 ± 0.09 ng TPA/ml (n = 3 with different membrane preparations, SE). This paralleled that observed in the intact cell (fig.1a), although the magnitude of the inhibition appeared to be somewhat less. As protein kinase C is found in both soluble and particulate fractions and TPA is believed to cause the recruitment of additional enzyme to the plasma membrane fraction [23], then this may have enhanced the response to TPA in the intact cell. Such an inhibitory effect was not observed with 4-*O*-methyl-TPA, the non-promoter analogue of TPA (unpublished) yet was mimicked, to a somewhat lesser degree, with diolein which also activates protein kinase C (fig.2). The effect on adenylate cyclase would appear to involve phosphorylation as no inhibition

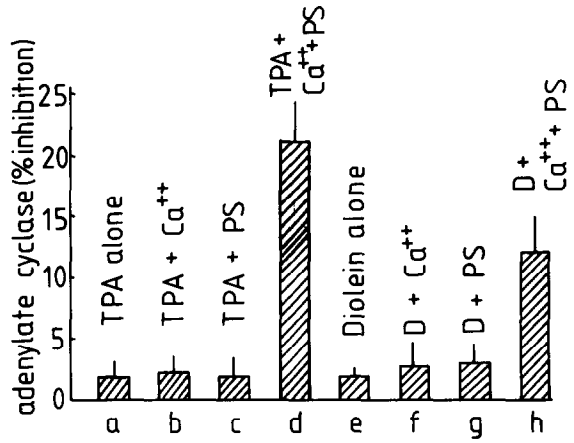

Fig.2. The inhibitory action of TPA and diolein on the glucagon-stimulated adenylate cyclase activity of purified plasma membranes is dependent upon Ca^{2+} and phosphatidylserine. The (glucagon + GTP)-stimulated adenylate cyclase activity of purified rat liver plasma was measured as described in section 2 except that additions of $CaCl_2$ (50 μM), phosphatidylserine (PS, 20 μg/ml), TPA (10 ng/ml) and diolein (0.5 μg/ml) were only made as and when indicated. Rates were obtained from linear time courses over 10 min at 30°C. If the concentration of Mg^{2+} was elevated to 5 mM, rather than being at 1 mM, then the inhibitory effects were reduced by about 50%. Errors are SD for 3 experiments (n = 3) using different membrane preparations where adenylate cyclase activity was assessed in triplicate. In each instance activities were expressed as a percentage of their appropriate control activity. The specific activity of these membranes is as in the legend to fig.1b. Data are expressed as percentage inhibition to enable comparison. This is particularly important as, under these conditions, Ca^{2+} itself causes a substantial inhibition of adenylate cyclase activity in the assay (see [24]). All solutions were prepared fresh each day.

(<4%, n = 3) occurred if ATP[γ-S], which can be used as a substrate by adenylate cyclase but not by protein kinases, replaced ATP in the incubation mixture of TPA + Ca^{2+} + phosphatidylserine (as in the legend to fig.2).

We propose that TPA exerts its effect on adenylate cyclase through activation of protein kinase C. The site at which protein kinase C exerts its effect is possibly the guanine nucleotide regulatory protein (N_s) which is involved in cyclase activation. This is a dimeric molecule consisting of an M_r 35000 β-subunit and an M_r 42000 α-subunit which both could presumably form targets for the

action of protein kinase C. In this respect one of the subunits of the GTP-binding protein, eukaryotic initiation factor 2, has been shown to be phosphorylated by protein kinase C [25]. Also, TPA has been demonstrated to phosphorylate an M_r 42000 protein in a number of cell types [26,27].

This action of TPA may lend insight into certain of the molecular mechanisms involved in tumour promotion, and may also identify a novel mode of regulation of adenylate cyclase.

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