# The phorbol ester TPA prevents the expression of both glucagon desensitisation and the glucagon-mediated block of insulin stimulation of the peripheral plasma membrane cyclic AMP phosphodiesterase in rat hepatocytes

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The phorbol ester TPA (12-O-tetradecanoyl phorbol-13-acetate) causes a dose-dependent inhibition of the glucagon-stimulated adenylate cyclase activity expressed in plasma membranes isolated from TPA-treated hypatocytes. However, no observable inhibitory effect of TPA on adenylate cyclase activity was observed in cells which had been exposed to glucagon for 5 min, prior to isolation, to desensitise adenylate cyclase. The degree of inhibition of adenylate cyclase elicited by both glucagon desensitisation and TPA treatment of hepatocytes was identical. Pre-treatment of hepatocytes with TPA was also found to prevent glucagon from blocking insulin's activation of the peripheral plasma membrane cyclic AMP phosphodiesterase in intact hepatocytes. TPA treatment also inhibited the ability of cholera toxin to activate the peripheral cyclic AMP phosphodiesterase in intact hepatocytes. It is suggested that in these particular instances TPA and glucagon elicit mutually exclusive processes rather than TPA mimicking glucagon desensitisation per se.

*Glucagon* *Tumor promotor* *Adenylate cyclase* *Phorbol ester* *Desensitisation* *Cyclic AMP phosphodiesterase*

## 1. INTRODUCTION

TPA (12-*O*-tetradecanoyl phorbol-13-acetate) is a potent tumour agent [1]. It expresses the ability to activate the ubiquitous enzyme protein kinase C, a route whereby it might exert at least certain of its actions on target cells [2].

Glucagon binds to high-affinity receptors on the liver cell surface. These interact with the stimulatory guanine nucleotide regulatory protein $N_s$, which in turn interacts with, and activates, the catalytic unit of adenylate cyclase [3,4]. However, in intact hepatocytes, glucagon also causes the rapid desensitisation of adenylate cyclase [5–8]. This desensitisation is thought to result from either a modification of $N_s$ [5,6] or the indirect activation [5,7] of $N_i$, a distinct guanine nucleotide regulatory protein which mediates the action of inhibitory hormones on adenylate cyclase (see [4]).

Treatment of hepatocytes with glucagon also induces an 'insulin-resistant' state which is selective to certain actions of insulin [9,10]. In particular, it prevents insulin from activating a distinct, high-affinity, peripheral plasma membrane AMP phosphodiesterase [9]. The process by which glucagon elicits such an action is cyclic AMP independent [9] and has the same time and dose dependence as that process by which glucagon elicits the desensitisation of adenylate cyclase [5,8].

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Furthermore, both of these actions of glucagon are obliterated by the presence of adenosine and $N^6$-phenylisopropyladenosine (PIA) functioning through high-affinity 'R'-type receptors [11].

We have demonstrated previously that TPA inhibits glucagon-stimulated adenylate cyclase activity in hepatocytes through a mechanism thought to involve the action of protein kinase C [12]. This action of TPA was located functionally at the point of regulation of adenylate cyclase by guanine nucleotide. Here we show that like adenosine and PIA [11], TPA can prevent the expression of both glucagon desensitisation and glucagon's block of the stimulatory action of insulin on the peripheral plasma membrane cyclic AMP phosphodiesterase.

## 2. MATERIALS AND METHODS

Hepatocytes from male Sprague-Dawley rats (200–300 g) were prepared and incubated as described by us [5]. The Krebs-Henseleit incubation medium contained 2.5% bovine serum albumin and $CaCl_2$ at a final concentration of 2.5 mM.

A membrane fraction was obtained from these hepatocytes as in [10,13] and used for the assay of adenylate cyclase activity. This membrane preparation was assayed for adenylate cyclase activity basically as described in [10]. 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 utilised a muscle binding protein assay performed as described by us in [10].

To assess intracellular cyclic AMP accumulation, cells (4–5 mg dry wt/ml) were pre-incubated for 20 min at 37°C either with isobutylmethylxanthine (IBMX) (10 mm), to inhibit cyclic AMP phosphodiesterase (see [5]) or not. After an appropriate period of incubation, aliquots were taken for the determination of intracellular cyclic AMP content as in [5].

Assessment of activation of the plasma membrane cyclic AMP phosphodiesterase activity by insulin was made as before [9,14] using the rapid Percoll fractionation procedure to separate out the various subcellular components of the hepatocytes. As before it was crucial to ensure a good separation of the plasma membranes from the endoplasmic reticulum fractions otherwise the significant activity in those fractions obscured the activation of the plasma membrane enzyme. This was ensured by 'calibrating' each batch of Percoll to give marker enzyme separation profiles as detailed previously [9]. Such calibration involved adjusting the speed of centrifugation in order to obtain the desired separation profile [9].

Cyclic AMP phosphodiesterase activity was determined at a final substrate concentration of 100 nM cyclic AMP using a 2-step assay procedure [15] as modified and described in some detail by us [16]. Initial rates were taken from linear time courses.

TPA, bovine serum albumin, theophylline and IBMX were from Sigma, Poole, England. Creatine kinase, phosphocreatine, triethanolamine HCl, cyclic AMP, collagenase guanine nucleotides, A23187 and all other biochemicals were from Boehringer, Lewes, England. Glucagon was a kind gift from Dr W.W. Bromer, Eli Lilly & Co., Indianapolis, IN. All general biochemicals were from BDH Chemicals, Poole, England. All radiochemicals were from Amersham International, Amersham, England.

## 3. RESULTS

Fig.1 demonstrates that the treatment of hepatocytes with TPA causes a dose-dependent decrease in the glucagon-stimulated adenylate cyclase activity expressed in plasma membranes isolated from such cells. The degree of inhibition elicited by TPA is similar to that observed when assessing its interaction by following glucagon-stimulated cyclic AMP production in intact hepatocytes [12]. We also observed here (fig.1) as before [5], that, if hepatocytes were treated with glucagon ($10^{-8}$ M) for 5 min, prior to isolating plasma membranes, then a loss/desensitisation of the glucagon-stimulated adenylate cyclase activity occurred in these membranes. However, we show here that the absolute degree of inhibition of glucagon-stimulated adenylate cyclase activity observed in such glucagon-pretreated cells was

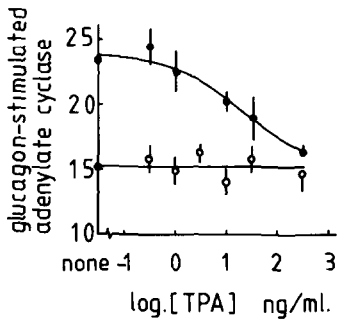


Fig.1. Changes in glucagon stimulated adenylate cyclase activity expressed in membranes from hepatocytes treated with TPA. Adenylate cyclase activity is expressed in pmol/min per mg protein. Hepatocytes were incubated at 37°C as described in section 2 with increasing concentrations of TPA for a 15 min period (●). They were then isolated and a washed membrane fraction prepared for the assay of glucagon-stimulated adenylate cyclase activity. In some instances (○) hepatocytes were subsequently incubated with glucagon (10 nM) for 5 min prior to harvesting. No difference in the results for the action of TPA in control (glucagon absent) experiments was observed if incubations were performed for 20 min rather than 15 min. Data are shown with SD for 3 separate experiments using different cell preparations ($n$ = 3). In each experiment adenylate cyclase assays were performed in triplicate.

constant regardless of the presence of a variety of inhibitory concentrations of TPA (fig.1).

As before [9], incubation of intact hepatocytes with insulin (10 nM) for 5 min led to the activation of the peripheral plasma membrane cyclic AMP phosphodiesterase. This effect blocked by pre-exposure of the cells to glucagon (10 nM) for 5 min prior to the addition of insulin (table 1). We see here, however, that pre-incubation of the cells with TPA, whilst having no effect on the plasma membrane phosphodiesterase activity observed in control and in insulin- and glucagon-treated hepatocytes, actually allowed insulin to activate this enzyme in hepatocytes which had been pre-exposed to glucagon (table 1). TPA pre-treatment also decreased the ability of cholera toxin to activate the plasma membrane cyclic AMP phosphodiesterase (table 1).

TPA pre-treatment had no effect (8% difference) on the activation of the 'dense-vesicle' phosphodiesterase that was elicited by insulin, glucagon, insulin plus glucagon and cholera toxin.

Table 1

The effect of the tumour promoting agent TPA on the ability of hormones and cholera toxin to regulate the peripheral plasma membrane cyclic AMP phosphodiesterase in intact hepatocytes

| Ligand | Phosphodiesterase activity with respect to control (100%) | |
|---|---|---|
| | Control cells | TPA-pre-treated cells |
| Insulin (10 nM), 5 min | 153 ± 9 | 165 ± 10 |
| Glucagon (10 nM), 5 min | 104 ± 2 | 105 ± 2 |
| Glucagon for 5 min then insulin for 5 min | 102 ± 5 | 145 ± 10 |
| Cholera toxin (1 g/ml), 30 min | 157 ± 9 | 127 ± 8 |

Hepatocyte suspensions (4–5 mg dry wt/ml) were incubated for 20 min at 37°C in either the presence or absence of TPA (10 ng/ml). They were then exposed either to hormones as defined above or to cholera toxin prior to harvesting, disruption and assessment of changes in activity in various subcellular fractions [9]. Incubation with TPA for this period of time did not affect the specific activity of the plasma membrane phosphodiesterase. This remained at 97 ± 12% of that observed in control studies. All data are shown ± SD taken for 3 separate experiments ($n$ = 3) using different cell preparations. As before triplicate cyclic AMP phosphodiesterase assays were performed on the gradients

This particular enzyme is associated with an intracellular membrane fraction [9] and is activated by both glucagon and insulin. It has been shown to be activated by insulin through a route which is distinct from that used to activate the plasma membrane enzyme [14]. These results are consistent with such a conclusion.

## 4. DISCUSSION

We have previously demonstrated that treatment of hepatocytes with TPA leads to an inhibition of the glucagon-stimulated cyclic AMP accumulation that occurs in these cells in the presence of a phosphodiesterase inhibitor [12]. Here, we confirm our conclusion that TPA inhibits adenylate cyclase by demonstrating that the glucagon-

stimulated adenylate cyclase activity in membranes isolated from such TPA-treated cells is indeed inhibited.

Normally, exposure of hepatocytes to glucagon for 5 min, prior to membrane isolation, leads to a dose-dependent desensitization of glucagon-stimulated adenylate cyclase activity in isolated membranes [5]. Our present intriguing observation is, however, that this cyclic AMP-independent [5,8] action of glucagon is completely obliterated in TPA pre-treated hepatocytes (fig.1). Indeed the degree of inhibition of glucagon-stimulated adenylate cyclase activity by both desensitisation and TPA-treatment is comparable (fig.1). This suggests that TPA might either mimic glucagon desensitisation or elicit a mutually exclusive inhibitory action.

Now, whatever mechanism elicits glucagon desensitisation bears very close relationships as regards time and dose dependence to that by which glucagon blocks insulin's stimulation of the peripheral plasma membrane cyclic AMP phosphodiesterase in intact hepatocytes [5,9]. They also both appear to be cyclic AMP-independent actions of glucagon [5,9]. Furthermore adenosine and its analogues [11] and also islet activating protein (pertussis toxin) [17] both prevent glucagon desensitisation from occurring and prevent glucagon's blockade on insulin's activation of the plasma membrane phosphodiesterase. This again suggests that these 2 actions of glucagon have a common origin. We see here (table 1) that TPA acts identically to these agents in preventing the glucagon-mediated block of insulin's stimulation of the plasma membrane phosphodiesterase. This observation might suggest that TPA, in inhibiting adenylate cyclase, does not trigger the same process as glucagon desensitisation but rather elicits a mutually exclusive action. The net effect of this is for TPA to not only mimic the degree of inhibition of adenylate cyclase elicited by glucagon desensitisation but also to prevent glucagon's blockade on insulin's stimulation of the plasma membrane phosphodiesterase.

Insulin's stimulation of the plasma membrane cyclic AMP phosphodiesterase has been suggested to be mediated by a distinct guanine nucleotide regulatory protein [9,10,18]. This has been tentatively identified as a 25 kDa species, called $N_{ins}$, which can be ADP-ribosylated and activated by cholera toxin [19]. Whilst elevated cyclic AMP concentrations do not activate the plasma membrane phosphodiesterase, treatment with cholera toxin does [9]. This may well be mediated via the ribosylation and activation of the 25 kDa protein called $N_{ins}$. We see here that TPA inhibits the cholera toxin-mediated activation of the plasma membrane phosphodiesterase (table 1). This is consistent with our observations that in TPA-treated hepatocytes, the ability of cholera toxin to ribosylate specifically $N_{ins}$ was markedly reduced [19], so providing further support to our contention [9,10,18] that insulin activates the plasma membrane phosphodiesterase through a distinct guanine nucleotide regulatory protein called $N_{ins}$.

TPA is believed to exert its physiological effects through activation of protein kinase C [2]. Whilst we [12] have presented some evidence which is consistent with TPA inhibiting adenylate cyclase through such a route, the putative target for phosphorylation has yet to be identified, although it is suggested that the perturbation lies at the level of regulation of this enzyme by guanine nucleotides. These studies pose the possibility that the putative $N_{ins}$ may be a substrate for phosphorylation. In this latter aspect, it is of interest that phorbol esters can stimulate the phosphorylation of the insulin receptor itself [20] and have also been shown to modify other actions of insulin such as lipogenesis [21].

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