The phorbol ester TPA inhibits cyclic AMP phosphodiesterase activity in intact hepatocytes

Fiona Irvine, Nigel J. Pyne and Miles D. Houslay

Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

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Treatment of intact hepatocytes with the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) potentiated the ability of glucagon to increase intracellular cyclic AMP concentrations. This effect was dose-dependent upon TPA, exhibiting an EC_{50} of 0.39 ng/ml and such activation was observed at both saturating and sub-saturating concentrations of glucagon. However, this stimulatory effect of TPA was completely abolished by the presence of the cyclic AMP phosphodiesterase inhibitor 1-isobutyl-3-methylxanthine, when TPA now inhibited the glucagon-stimulated increase in intracellular cyclic AMP concentrations. It is suggested that, as well as inhibiting glucagon-stimulated adenylate cyclase activity, TPA also inhibits cyclic AMP phosphodiesterase activity in intact hepatocytes. Treatment of either hepatocyte homogenates or purified cyclic AMP phosphodiesterase with TPA failed to show any direct inhibitory effect of TPA on activity showing that TPA did not exert any direct inhibitory action on phosphodiesterase activity. However, homogenates made from hepatocytes that had been pre-treated with TPA did show a reduced cyclic AMP phosphodiesterase activity. It is suggested that TPA might inhibit cyclic AMP phosphodiesterase activity through phosphorylation by C-kinase.

Adenylate cyclase *Glucagon* *Phosphorylation* *Tumor promotor* *Cyclic-AMP phosphodiesterase* *Hepatocyte*

1. INTRODUCTION

Tumour-promoting phorbol esters such as TPA (12-*O*-tetradecanoyl phorbol-13-acetate) have been shown to exert a variety of effects upon cells [1,2]. It is believed that they achieve such effects by stimulating the activity of protein kinase C which, in turn, modulates the activity of target proteins by eliciting their phosphorylation [3,4]. Recently, there has been considerable interest in the possibility that C-kinase activation can regulate signal transmission through the adenylate cyclase pathway [5–15]. Indeed, phorbol esters have been shown to inhibit the functioning of the inhibitory guanine nucleotide regulatory protein G_i, by causing its phosphorylation [9,10]. This elimination of an inhibitory input is believed to account for the TPA-mediated potentiation of adenylate cyclase activity that has been observed in S49 mouse lymphoma cells [11]. However, in a number of other systems, phorbol esters have been shown to inhibit hormone-stimulated adenylate cyclase activity [5–8,12,13]. This inhibitory effect appeared to result as a consequence of the loss of productive coupling between receptors and the stimulatory guanine nucleotide regulatory protein, G_s [5,6,12,13]. In this way phorbol esters can be seen to mimic the rapid uncoupling/desensitization of hormone-stimulated adenylate cyclase systems [6,12,13].

We show here that signal transduction by the cyclic AMP pathway can be perturbed by another route, namely the inhibition of cellular cyclic AMP phosphodiesterase activity.

2. MATERIALS AND METHODS

Hepatocytes from male Sprague-Dawley rats

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(200–300 g) were prepared and incubated as described before by us [16]. The Krebs-Henseleit incubation medium contained 2.5% bovine serum albumin and 1.5 mM $CaCl_2$. Intracellular cyclic AMP determinations were performed as described before [17] by us, as was the assay of adenylate cyclase [16,17]. Cyclic AMP phosphodiesterase activity was assessed at a final substrate concentration of 100 nM cyclic AMP using a two-step assay procedure [18] as described in some detail by us [19]. Initial rates were taken from linear time courses. Purification of the 'dense-vesicle', peripheral plasma membrane [20] and cyclic GMP-stimulated cyclic AMP phosphodiesterases [21] was performed as described previously by us. Hepatocyte homogenates were made as described before by us [22].

TPA, bovine serum albumin and IBMX were from Sigma (Poole, England). Cyclic AMP, triethanolamine HCl, collagenase and all other biochemicals were from Boehringer (UK) Ltd. Glucagon was a kind gift from Dr W.W. Bramer, Eli Lilly & Co. (IN, USA). All radiochemicals were from Amersham International (Amersham, England).

3. RESULTS AND DISCUSSION

Challenge of intact hepatocytes with glucagon (10 nM) has been shown by us and many others previously (see e.g. [16]) to cause a transient rise in the intracellular concentration of cyclic AMP (fig.1a). This action is markedly potentiated (fig.1b) by the presence of the non-selective cyclic AMP phosphodiesterase (PDE) inhibitor 1-isobutyl-3-methylxanthine (IBMX) which inhibits over 98% of hepatocyte PDE activity [16]. If hepatocytes were pre-incubated with TPA (10 ng/ml, 15 min), in the presence of IBMX (1 mM), then intracellular cyclic AMP accumulation triggered by the addition of glucagon (10 nM) was markedly reduced. This observation is in accord with our observations [5,6] that TPA treatment of intact hepatocytes inhibited the glucagon-stimulated adenylate cyclase activity expressed by membranes isolated from them. However, if such experiments were repeated in the absence of the cyclic AMP phosphodiesterase inhibitor IBMX then, in contrast to the above results, the presence of TPA actually enhanced the

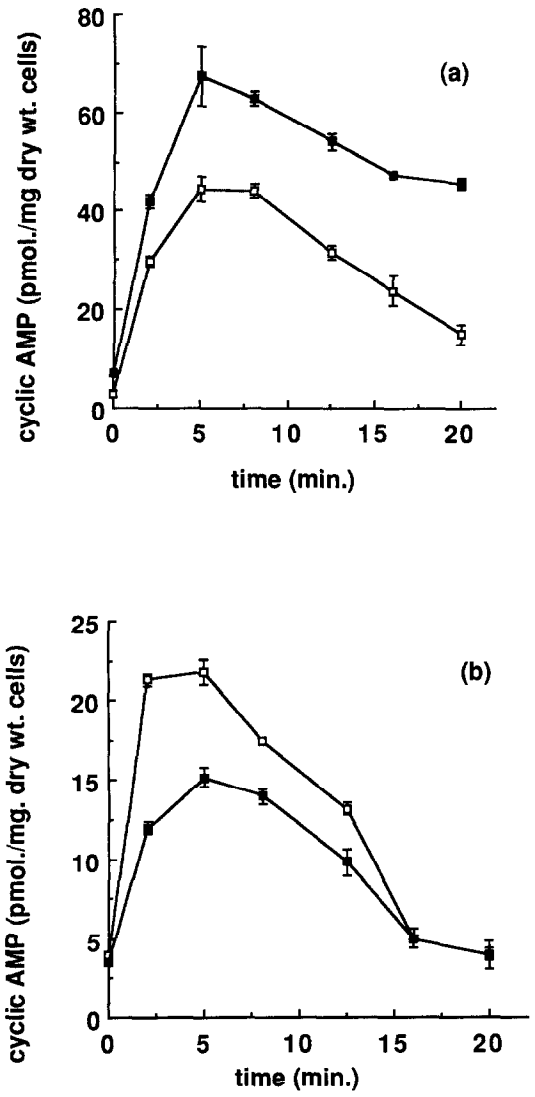

Fig.1. Time course of glucagon-stimulated cyclic AMP accumulation in intact hepatocytes. (a) Hepatocytes were pre-incubated with the cyclic AMP phosphodiesterase inhibitor IBMX (1 mM) for 20 min prior to challenge with glucagon (10 nM) (■). In one instance (□), TPA (10 ng/ml) was added 15 min prior to the addition of glucagon. Cells were harvested at the indicated times for determination of their intracellular cyclic AMP content. (b) As in (a) except that IBMX was not added to the cells. Experiments were done in the presence (□) or absence (■) of TPA (1 ng/ml) as detailed above. Data are given with SD for $n = 3$ experiments using different cell preparations each with triplicate samples and triplicate cyclic AMP determinations.

increase in intracellular cyclic AMP concentrations elicited by glucagon markedly (fig.1). These effects were seen clearly over the entire dose range for which glucagon stimulated the intracellular accumulation of cyclic AMP (fig.2).

Furthermore, the ability of TPA to potentiate the glucagon-stimulated increase in cyclic AMP, seen in the absence of IBMX, was shown to be dose dependent upon TPA (fig.3) with an EC_{50} of 0.39 ± 0.05 ng/ml TPA. This value is very similar to that observed for the action of TPA in inhibiting adenylate cyclase activity in intact hepatocytes [5].

In the absence of IBMX, then TPA (10 ng/ml for 15 min) even increased ($p \leqslant 0.001$) basal cyclic AMP concentrations from 2.8 ± 0.1 to 4.0 ± 0.2 pmol/mg dry wt cells (errors are SD, $n = 3$ separate experiments, Student's t-test). This contrasted with a decreased ($p \leqslant 0.001$) basal accumulation from 14.4 ± 1.0 pmol/mg dry wt cells to, with TPA (10 ng/ml) present, 5.1 ± 0.3 pmol/mg dry wt cells over a 20 min period at 37°C with IBMX (1 mM) present (errors are SD, $n = 3$ separate experiments, Student's t-test).

These results imply that the TPA-mediated potentiation of glucagon-stimulated cyclic AMP accumulation, which was only seen in the absence of the PDE inhibitor IBMX, was due to the ability of TPA to inhibit hepatocyte PDE activity. Indeed, the relative effectiveness of TPA to inhibit PDE activity can be gauged from the observation (fig.1) that IBMX caused a 4.5-fold potentiation of the elevated cyclic AMP accumulation seen after 5 min exposure to glucagon whereas, in the absence of IBMX, TPA caused an approx. 1.3-fold potentiation of the stimulatory effect of glucagon. If one takes account of the fact that TPA exerts an inhibitory effect on glucagon-stimulated adenylate cyclase (~30–40% inhibition; observed here and see [5,6]) the maximum effect of TPA at potentiating the glucagon-stimulated cyclic AMP accumulation through PDE inhibition can be estimated as being around 2-fold. This approximates to TPA exerting an inhibitory effect on cyclic AMP phosphodiesterase activity which was around 25% of the potency of the effect elicited by IBMX, which inhibits >98% of hepatocyte PDE activity [16].

The addition of a range of concentrations of TPA (10–100 ng/ml) to assays of total hepatocyte

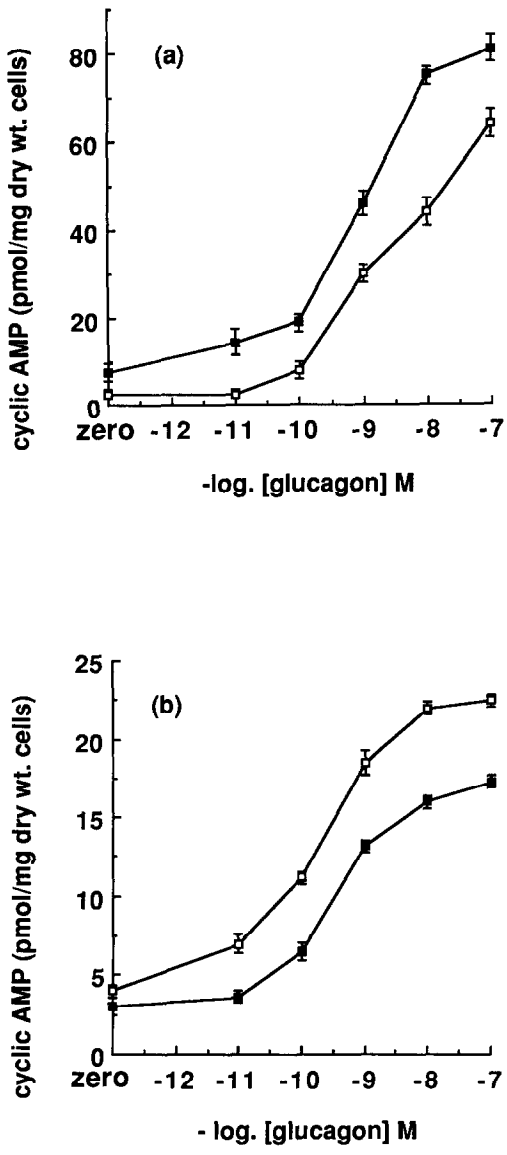

Fig.2. Dose-response curve to glucagon-stimulated cyclic AMP accumulation in intact hepatocytes. (a) Hepatocytes were pre-incubated with IBMX (1 mM) for 20 min prior to challenge with glucagon at the indicated concentrations. After 5 min the cells were harvested for determination of their intracellular cyclic AMP content (■). In one instance (□), TPA (10 ng/ml) was added 15 min prior to challenge with glucagon. (b) As in (a) except that IBMX was absent. Cells either were (□) or were not (■) pre-treated with TPA (10 ng/ml) as above. Data are given with SD for $n = 3$ experiments using different cell preparations each with triplicate samples and triplicate cyclic AMP determinations.

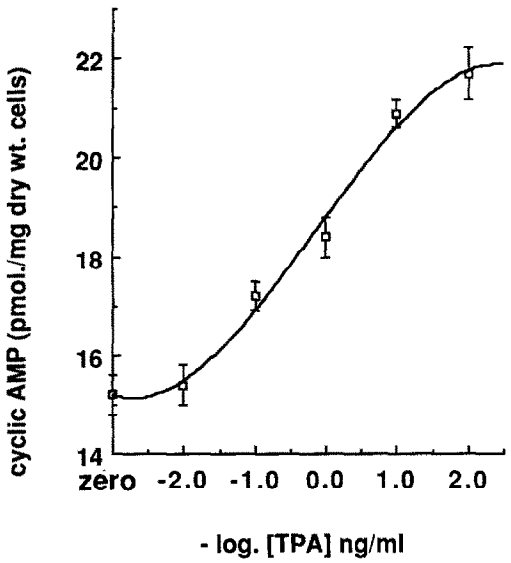

Fig.3. In intact hepatocytes TPA produces a dose-dependent enhancement of the glucagon-stimulated increase in cyclic AMP when cyclic AMP phosphodiesterase inhibitors are absent. Hepatocytes were incubated at 37°C with increasing concentrations of the phorbol ester TPA for a 10 min period. After this, they were challenged with glucagon (10 nM) for a 5 min period prior to harvesting for analysis of their intracellular cyclic AMP content. Data are given with SD for $n = 3$ experiments using different cell preparations each with triplicate samples and triplicate cyclic AMP determinations.

homogenate PDE activity or to purified preparations of rat liver dense-vesicle PDE, peripheral plasma membrane PDE and cyclic GMP-stimulated PDE failed to exert any effect on activity (less than 5% change). However, we did observe inhibition of the PDE activity exhibited by hepatocyte homogenates made from intact cells which had been pre-treated with TPA. In such preparations, hepatocytes were pre-incubated with TPA (100 ng/ml) for 15 min prior to homogenisation in the presence of 50 mM β-glycerophosphate, which has been shown to inhibit the dephosphorylation of proteins phosphorylated by the action of C-kinase [23]. These studies showed that TPA elicited a 20 ± 3% inhibition of total homogenate PDE activity (errors are SD, $n = 6$ separate experiments). Such a degree of PDE inhibition is similar to that estimated above to account for TPA's ability to potentiate the glucagon-stimulated increase in intracellular cyclic AMP accumulation observed in the absence of IBMX.

These experiments demonstrate that, as well as modulating adenylate cyclase functioning, TPA can also inhibit hepatocyte PDE activity. This observation may have important physiological consequences as well as demanding a re-investigation of the actions of TPA on intracellular cyclic AMP accumulation in studies performed in the absence of PDE inhibitors (e.g. see [15]). Our investigations also suggest that ligands which initiate diacylglycerol production in cells, by stimulating inositol phospholipid metabolism, may also regulate cyclic AMP phosphodiesterase activity through a route similar to that employed by TPA.

Rat hepatocytes contain a family of distinct PDE isoenzymes whose relative importance in controlling intracellular cyclic AMP concentrations is only partially understood [24–26]. It remains to be determined as to which species are regulated by TPA and whether phosphorylation by C-kinase is involved.

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