Effects of phorbol esters on α_1 -adrenergic-mediated and glucagon-mediated actions in isolated rat hepatocytes

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Phorbol 12-myristate 13-acetate (PMA) inhibited the stimulation of ureogenesis produced by adrenaline, but produced a minimal displacement to the right of the dose-response curve for glucagon. However, PMA diminished the accumulation of cyclic AMP induced by glucagon. Dissociation between the cyclic AMP concentrations and the metabolic effects induced by glucagon is evidenced in the presence of phorbol esters.

Phorbol esters are a series of mitogenic compounds which induce phenotypic changes in mammalian cells resembling those produced by oncogenic viruses. The mechanism of action of these tumour promoters is not completely known. However, some evidence indicates that phorbol esters bind and stimulate a Ca^{2+} -activated phospholipid-dependent kinase, known as protein kinase C (Castagna *et al.*, 1982; Kikkawa *et al.*, 1983). Activation of this kinase seems to be the major route whereby these tumour promoters exert their actions.

It has been shown that activation of protein kinase C by phorbol diesters can alter the cellular responsiveness to a variety of agents; this change in responsiveness seems to be associated with changes in the number, state of affinity or state of phosphorylation of the receptors (Shoyab *et al.*, 1979; Grumberger & Gordon, 1982; Cochet *et al.*, 1984; Corvera & García-Sáinz, 1984; Davies & Czech, 1984; Heyworth *et al.*, 1984; Iwasita & Fox, 1984; May *et al.*, 1984; Moon *et al.*, 1984). We have previously shown that in isolated hepatocytes PMA completely blocks α_1 -adrenergic stimulation of glycogenolysis (Corvera & García-Sáinz, 1984). This effect of phorbol esters seems to be quite specific for α_1 -adrenergic actions, since we observed that the stimulations of glycogenolysis by other agents such as vasopressin, angiotensin, ionophore A23187 and glucagon were not affected by these agents (Corvera & García-Sáinz, 1984). However, Heyworth *et al.* (1984) reported that

PMA inhibits glucagon-stimulated adenylate cyclase. In order to compare the findings of Heyworth *et al.* (1984) with ours (Corvera & García-Sáinz, 1984), the present study was performed. The effect of phorbol esters on the stimulations of ureogenesis by α_1 -adrenergic agents and glucagon and the effect of these tumour promoters on the concentration of cyclic AMP induced by glucagon were quantified. Our results confirmed the findings of Heyworth *et al.* (1984), but indicate that the inhibition of glucagon-mediated cyclic AMP accumulated by phorbol-esters has little, if any, metabolic significance. In contrast, phorbol esters markedly diminish α_1 -adrenergic effects.

Experimental

(-)-Adrenaline, ($\pm$)-propranolol, PMA, PDA, 4 β -phorbol and urease were obtained from Sigma Chemical Co. Glucagon was generously given by Eli Lilly. Collagenase and bovine serum albumin were obtained from Worthington and Reheis respectively. [^3H]Cyclic AMP was obtained from New England Nuclear. Forskolin (7 β -acetoxy-8,12-epoxy-12,6 β ,9 α -trihydroxylabd-14-en-11-one) was from Calbiochem. Other substances were of analytical grade.

Female Wistar rats (approx. 200 g) fed *ad libitum* with Purina rat chow were used. Hepatocytes were isolated by the method of Berry & Friend (1969). Isolation, washing and incubation of the cells were done in Krebs-Ringer bicarbonate buffer (120 mM-NaCl, 5 mM-KCl, 1.3 mM- CaCl_2 , 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 18 mM- NaHCO_3) saturated with

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PDA, phorbol 12,13-diacetate.

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O₂/CO₂ (19:1), pH 7.4, at 37°C, containing 1% bovine serum albumin. To determine the rate of ureogenesis, cells were incubated in 1 ml of Krebs-Ringer bicarbonate buffer supplemented with 10 mM-glutamine and 2 mM-ornithine. After 60 min, urea was determined enzymically in the cell supernatants (Gutman & Bergmeyer, 1974). In the experiments where cyclic AMP was quantified, the cells were preincubated for 15 min in the absence or presence of phorbol agents. After this preincubation, glucagon or vehicle was added and the cells were incubated for another 2 min. Cyclic AMP was determined in cells plus medium by the method of Gilman (1970). Free cyclic AMP was separated from bound cyclic AMP by charcoal adsorption as described by Brown *et al.* (1971).

Results

Adrenaline produced a dose-dependent stimulation of ureogenesis (Fig. 1). This effect is mediated through stimulation of α_1 -adrenoceptors (Corvera & García-Sáinz, 1981). Confirming our previous results on glycogenolysis (Corvera & García-Sáinz, 1984), it was observed that 0.1 μ M-PMA blocked this effect of adrenaline (Fig. 1). In contrast, the dose-response curve for glucagon was only minimally shifted to the right by 0.1–1 μ M-PMA (Fig. 2). PMA alone was without effect on this parameter. Identical results were obtained if the cells were preincubated with PMA for 15 min, washed and resuspended before the addition of adrenaline or glucagon (results not shown).

Fig. 1. Effect of PMA on the stimulation of ureogenesis by adrenaline

Hepatocytes were incubated in the absence (○) or presence (●) of 0.1 μ M-PMA and different concentrations of adrenaline. Basal urea production was 30 ± 1 nmol/mg wet wt. of cells. Results are means $\pm$ S.E.M. (bars) for five to eight experiments with different cell preparations.

Fig. 2. Effect of PMA on the stimulation of ureogenesis by glucagon

Hepatocytes were incubated in the absence (○) or presence of (●) 0.1 μ M- or (■) 1 μ M-PMA and different concentrations of glucagon. Results are means $\pm$ S.E.M. (bars) for five to eight experiments with different cell preparations.

Fig. 3. Effect of PMA on the stimulation of cyclic AMP accumulation by glucagon

Hepatocytes were incubated in the absence (○) or presence of (●) 0.1 μ M- or (■) 1 μ M-PMA for 15 min; glucagon or vehicle was then added, and 2 min later the incubation was ended. Basal cyclic AMP was 0.97 ± 0.09 pmol/mg wet wt. Results are means $\pm$ S.E.M. (bars) for five to eight experiments with different cell preparations.

Glucagon produced a dose-dependent increase in cyclic AMP accumulation (Fig. 3), which was significantly decreased by PMA (Fig. 3). This phorbol ester, at 0.1 and 1 μ M, clearly shifted the dose-response curve for glucagon to the right, decreased the maximal effect and diminished the steepness of the curve (Fig. 3), indicating clearly a non-competitive type of inhibition. PMA alone was without effect at the concentrations tested.

To characterize this effect further, a fixed concentration of glucagon was antagonized by different concentrations of phorbol derivatives (Fig. 4). PMA produced a dose-dependent inhibition of the effect of glucagon, reaching a maximal

Fig. 4. Effect of different phorbol derivatives on the accumulation of cyclic AMP produced by glucagon

Hepatocytes were incubated in the absence (Δ) or presence of different concentrations of PMA (●), PDA (○) or 4 β -phorbol (■) for 5 min; then glucagon (10 nM) was added, and 2 min later the incubation was ended. Basal cyclic AMP was 1.04 ± 0.10 pmol/mg wet wt. Results are means $\pm$ S.E.M. (bars) for five to eight experiments with different cell preparations.

inhibition of approx. 50% at 1 μ M (Fig. 4). PDA also produced a dose-dependent inhibition of similar magnitude, but it was about 1 order of magnitude less potent than PMA (Fig. 4). In contrast, 4 β -phorbol was completely inactive (Fig. 4).

Discussion

The ability of phorbol esters to inhibit glucagon-mediated cyclic AMP accumulation (Heyworth *et al.*, 1984) was confirmed in our studies. Interestingly, although phorbol esters produced a very

significant inhibition of the effect of glucagon on cyclic AMP concentrations (Fig. 3), a minimal shift in the dose-response curve for the effect of glucagon on ureogenesis was observed (Fig. 2). This is essentially consistent with our previous findings in which another metabolic parameter, glycogenolysis, was studied (Corvera & García-Sáinz, 1984). In other words, in the presence of phorbol esters, a clear dissociation between glucagon-mediated cyclic AMP accumulation and glucagon-mediated metabolic effects is observed. We (Cárdenas-Tanús *et al.*, 1982; Corvera *et al.*, 1984) and other authors (Okajima & Ui, 1976; Birnbaum & Fain, 1977; Fain & Shepherd, 1977; Khan *et al.*, 1980), using different experimental approaches, have been able to observe dissociation between cyclic AMP accumulation and the physiological responses produced by glucagon action. These data suggest that cyclic AMP might be the main, but not the sole, factor involved in the metabolic actions of glucagon on liver cells.

It is noteworthy that 0.1 μ M-PMA blocks the action of α_1 -adrenergic agents (Fig. 1), whereas the same concentration of PMA only partially decreases glucagon-mediated cyclic AMP accumulation (Figs. 3 and 4). It is difficult to interpret this difference at present. Both effects seem to be mediated through activation of protein kinase C. This is based on the potency order of the phorbol derivatives [PMA > PDA > > > phorbol (Fig. 4, and Corvera & García-Sáinz, 1984)], which is identical with that observed for binding and activation of protein kinase C (Castagna *et al.*, 1982; Kikkawa *et al.*, 1983). Furthermore, Heyworth *et al.* (1984) have shown that Ca^{2+} and phosphatidylserine, which are cofactors of protein kinase C (Castagna *et al.*, 1982), are required to reproduce the effect of PMA on adenylate cyclase in purified plasma membranes.

Heyworth *et al.* (1984) have suggested that the action of phorbol ester may be exerted at the point of regulation of adenylate cyclase by guanine nucleotides. On the other hand, we (Corvera & García-Sáinz, 1984) have suggested that the action of phorbol esters on α_1 -adrenergic-mediated effects probably occurs at the receptor level. Neither of these suggestions has been definitively demonstrated yet. However, the findings are consistent with the idea that activation of protein kinase C by phorbol esters alters the cell responsiveness by inducing significant changes in the metabolic dynamic of the plasma membrane.

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