Phorbol-ester-induced alterations of free calcium ion transients in single rat hepatocytes

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The effect of the phorbol esters phorbol 12-myristate 13-acetate (TPA) and phorbol 12,13-dibutyrate (PDB) on changes in free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in single rat hepatocytes, microinjected with the photoprotein aequorin, were investigated. $[\text{Arg}^8]$ vasopressin and phenylphrine induced a series of repetitive $[\text{Ca}^{2+}]_i$ transients. Phorbol esters inhibited the α_1 -adrenoceptor-induced response; sub-nanomolar concentrations decreased the transient frequency, and higher concentrations abolished the transients. The inhibitory effect of PDB was readily reversible. Phorbol esters were less effective in decreasing the frequency of $[\text{Arg}^8]$ -vasopressin-induced transients, and the inhibition could be overcome by high $[\text{Arg}^8]$ vasopressin concentrations.

INTRODUCTION

We have previously shown that, in single enzymically dispersed rat hepatocytes, stimulation with phenylephrine or $[\text{Arg}]$ vasopressin will induce repetitive transients in free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Woods *et al.*, 1986, 1987). The frequency and shape of the transients are governed by the agonist concentration and species.

Ligand-stimulated increases in $[\text{Ca}^{2+}]_i$ arise as a result of membrane polyphosphoinositide hydrolysis, leading to the generation of inositol trisphosphate and diacylglycerol as hormone second messengers (Berridge, 1984; Williamson *et al.*, 1985). Inositol trisphosphate has been implicated in mobilizing intracellular Ca^{2+} in hepatocytes (Burgess *et al.*, 1984; Dawson & Irvine, 1984; Joseph *et al.*, 1984) and in other tissues (Suematsu *et al.*, 1984; Epstein *et al.*, 1985), whereas diacylglycerol, along with Ca^{2+} and phosphatidylserine, activates protein kinase C (PKC) (Nishizuka, 1984). One powerful tool in the study of the bifurcating signal-transduction pathway involves the use of tumour-promoting phorbol esters to activate PKC (Castagna *et al.*, 1982) by lowering the Ca^{2+} requirement of this enzyme (Takai *et al.*, 1979). In so doing, phorbol esters may mimic the action of endogenously produced diacylglycerol.

TPA has been shown to inhibit hepatocyte hormone-induced responses such as glycogenolysis (Corvera & Garcia-Sainz, 1984) and glucagon- (Staddon & Hansford, 1986) and α_1 -adrenoceptor- (Cooper *et al.*, 1985) mediated increases in $[\text{Ca}^{2+}]_i$. There is evidence, obtained in populations of cells (Corvera & Garcia-Sainz, 1984), of the differential sensitivity of α_1 - and V_1 -receptor responses to inhibition by TPA. We report here a similar differential sensitivity to inhibition by phorbol esters of repetitive $[\text{Ca}^{2+}]_i$ transients in single aequorin-microinjected hepatocytes. We show that low concentrations of phorbol esters can decrease the frequency of the transients, and higher concentrations can completely abolish the response.

MATERIALS AND METHODS

Hepatocyte preparation

Isolated viable hepatocytes were prepared from fed male Wistar-strain rats (180–250 g) by collagenase (BCL) perfusion, by using a modification of the procedure of Burgess *et al.* (1981) as described by Woods *et al.* (1987). Single hepatocytes of healthy appearance were microinjected with aequorin as described previously (Cobbold *et al.*, 1982, 1985; Woods *et al.*, 1986).

Chemicals

The phorbol esters TPA and PDB (Sigma) were dissolved in dimethyl sulphoxide and stored frozen at -70°C . Stocks ($10\ \mu\text{M}$) were prepared in Williams Medium E (Flow Laboratories) on the day of use. The sources of other chemicals have been described previously (Woods *et al.*, 1986, 1987).

Data collection and analysis

Photoelectron counts from single microinjected hepatocytes were collected, stored and analysed as described previously (Woods *et al.*, 1987).

Aequorin calibration

The relationship between the $-\log(\text{fraction of aequorin consumed/s})$ and pCa values between 8 and 6.5 at 1 mM free Mg^{2+} concentration was determined as described previously (Woods *et al.*, 1987).

RESULTS

Fig. 1 shows that 1 nM-TPA completely inhibits repetitive $[\text{Ca}^{2+}]_i$ transients in a single hepatocyte stimulated by $5\ \mu\text{M}$ -phenylephrine. In the absence of TPA and in the continued presence of agonist, single hepatocytes exhibit repetitive $[\text{Ca}^{2+}]_i$ transients for periods often in excess of 1 h (Woods *et al.*, 1987). TPA

* Abbreviations used: TPA, phorbol 12-myristate 13-acetate, PDB, phorbol 12,13-dibutyrate; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; PKC, protein kinase C.

Fig. 1. Inhibitory effect of 1 nM-TPA on 5 μ M-phenylephrine- (Phe)-induced $[Ca^{2+}]_i$ transients

A single rat hepatocyte was microinjected with aequorin and superfused with agonist and phorbol ester at the times indicated. The time constant for resting concentrations was 15 s, the time constant for transients 1 s. The results shown are typical of eight separate experiments.

at 1 nM and above reproducibly abolished $[Ca^{2+}]_i$ transients (eight cells). The inhibitory effects occurred rapidly, within 5 min, consistent with the time course of PKC activation (Rozengurt *et al.*, 1983). After 15 min exposure to TPA, a high concentration of phenylephrine (100 μ M) failed to elicit a $[Ca^{2+}]_i$ response. In similar experiments applications of very high phenylephrine concentrations (100 μ M–4 mM) failed to evoke the initial sustained rise and very fast $[Ca^{2+}]_i$ -transient behaviour previously observed (Woods *et al.*, 1987). In four cells high agonist concentrations resulted in single or paired transients, and in six cells no response to the supra-maximal stimuli occurred. The threshold for TPA-induced alteration of $[Ca^{2+}]_i$ transients was approx. 0.1 nM. However, the likelihood that TPA will partition into the cell from what is effectively an infinite volume of medium means that TPA effects are time-dependent, and therefore a firm threshold cannot be quoted. TPA at 0.01 nM had no effect on 2 μ M-phenylephrine-induced transients within 23 min. At concentrations around the apparent threshold, TPA (0.1–0.2 nM) induced a progressive decrease in transient frequency (results not shown).

In an attempt to reach equilibrium between phorbol esters in solution and in the single cell, we used the less hydrophobic phorbol ester PDB, which binds with a lower affinity to the cellular target, presumably PKC (Leach *et al.*, 1983). Fig. 2(a) demonstrates that 2 nM-PDB has a stable and prompt effect in decreasing the frequency of $[Ca^{2+}]_i$ transients induced by 2 μ M-phenylephrine. In this experiment the period rose from approx. 52 s to approx. 154 s. The effect was quickly reversible. These findings contrast with those for TPA, where a decrease of transient frequency to a stable value could

not be obtained. PDB had no effect on the inter-transient resting $[Ca^{2+}]_i$ (210 nM). In some experiments (three out of five cells) a decrease in peak $[Ca^{2+}]_i$, combined with an increase in transient length, was observed (Fig. 2b), but this was not always a consistent feature. A decrease in peak $[Ca^{2+}]_i$ was never seen without a decrease in transient frequency. PDB concentrations of approx. 10 nM were needed to abolish the $[Ca^{2+}]_i$ transients.

Concentrations of TPA (5 nM) and PDB (10 nM) that abolished α_1 -receptor-mediated increases in $[Ca^{2+}]_i$ resulted only in a decrease in transient frequency induced by $[Arg^8]$ vasopressin (three cells). Fig. 3 shows a representative result. In one cell 200 nM-TPA failed to abolish transients induced by 0.4 nM-vasopressin (results not shown). Increasing the concentration of vasopressin to 20 nM (Fig. 3) resulted in a sustained increase in $[Ca^{2+}]_i$ comparable with those observed previously (Woods *et al.*, 1987), and in complete contrast with the failure of high phenylephrine concentrations to overcome the effect of phorbol esters (see above). In control experiments, dimethyl sulphoxide had no effect on the $[Ca^{2+}]_i$ transients (results not shown).

DISCUSSION

Single rat hepatocytes when stimulated with glycolytic concentrations of agonist produce series of repetitive $[Ca^{2+}]_i$ transients (Woods *et al.*, 1986, 1987). Phorbol esters at nanomolar concentrations can inhibit the transients. The differential sensitivity between phenylephrine- and vasopressin-induced responses to blockage by phorbol esters, previously described for quin2 (Cooper *et al.*, 1985) and glycogenolysis (Corvera & Garcia-Sainz, 1984), is here shown to be reflected in the

Fig. 2. Inhibitory effect of PDB on phenylephrine- (Phe)-induced $[\text{Ca}^{2+}]_i$ transients

Conditions were as in Fig. 1 except that in (a) 2 μM -phenylephrine and 2 μM -phenylephrine with 2 nM-PDB were used, and in (b) 2 μM -phenylephrine and 2 μM -phenylephrine with 5 nM-PDB were superfused. The time constants for both (a) and (b) were as in Fig. 1. The results shown are typical of five separate experiments.

inhibition of repetitive $[\text{Ca}^{2+}]_i$ transients. Low concentrations of phorbol esters decrease the transient frequency, and higher concentrations can abolish them. The phorbol ester concentrations required to induce this inhibition are an order of magnitude higher for the vasopressin response than for the phenylephrine res-

ponse. The possibility that these changes are mediated via receptor or G-protein phosphorylation, dependent on PKC activation, is discussed.

The effects of the tumorigenic phorbol esters are usually interpreted in terms of their ability to activate phospholipid- Ca^{2+} -dependent PKC. One possible ex-

Fig. 3. Inhibitory effect of TPA on $[\text{Arg}^8]\text{vasopressin- (Vp)-induced } [\text{Ca}^{2+}]_i$ transients

Conditions were as in Fig. 1. A single hepatocyte was superfused with 0.4 nM- $[\text{Arg}^8]\text{vasopressin}$ and then in combination with 10 nM-TPA. The sustained increase in $[\text{Ca}^{2+}]_i$ was elicited with 20 nM- $[\text{Arg}^8]\text{vasopressin}$ in the presence of 200 nM-TPA. The time constants were as in Fig. 1. The results shown are typical of three separate experiments.

planation of our data is that PKC phosphorylates hormone receptors to decrease their affinity for ligands, as has been shown for the adrenergic receptor of hepatocytes (Schwartz *et al.*, 1985). A decrease in receptor affinity would act like a decrease in agonist concentration in decreasing the number of occupied receptors, and would therefore explain the decrease in frequency, since frequency depends on agonist concentration (Woods *et al.*, 1986, 1987). Since phosphatidylinositol bisphosphate hydrolysis produces diacylglycerol, the endogenous activator of PKC (Nishizuka, 1984), receptor phosphorylation should also occur as part of the normal response. This should act as negative-feedback control of the response, and could explain the slowing-down of transient frequency during prolonged phenylephrine stimulation (Woods *et al.*, 1987). There is evidence that exposure to phorbol esters results in a decrease in receptor affinity for ligands (Thomopoulos *et al.*, 1982) and has been shown to induce receptor phosphorylation (Leeb-Lundberg *et al.*, 1985). Phosphorylation of receptors may also mediate receptor down-regulation (Klausner *et al.*, 1984; Cooper *et al.*, 1985). A complicating factor in the interpretation of long experiments with phorbol esters may be down-regulation of PKC itself.

Another possible explanation of the effects of phorbol esters is PKC-induced phosphorylation of the GTP-binding G-proteins (Gp) thought to link ligand-receptor interaction to phospholipase C activation in hepatocytes (Cockcroft & Gomperts, 1985; Wallace & Fain, 1985; Litosch & Fain, 1986). We have suggested a model in which such Gp phosphorylation may curtail the Ca^{2+} increase at the peak of each transient (Woods *et al.*,

1987). Although phorbol-ester-induced phosphorylation of activated Gp during its build-up between transients could theoretically explain the inhibition of transient frequency, the known effects on receptors are a sufficient explanation. However, our model also predicts a decrease in peak $[\text{Ca}^{2+}]_i$ in the presence of phorbol esters. We did indeed see instances of a reversible decrease in peak $[\text{Ca}^{2+}]_i$ with PDB, but not consistently. Perhaps PKC is normally activated only briefly but nearly maximally during each transient by diacylglycerol and Ca^{2+} , so that the sustained application of phorbol esters can have little further effect during the transients. Transients might then be abolished by receptor phosphorylation before effects on peak $[\text{Ca}^{2+}]_i$ had become substantial. However, this explanation puts tight constraints on the model.

In conclusion, these data show that the inhibition of α_1 -receptor responses in hepatocytes by phorbol esters works by slowing down or abolishing the repetitive $[\text{Ca}^{2+}]_i$ transients. A sustained rise can still be induced by high vasopressin concentrations in the presence of phorbol esters, but can perhaps be affected via PKC-induced receptor down-regulation on a slower time scale. The data are consistent with receptor affinity modulation by PKC-mediated receptor phosphorylation, with α_1 -receptor both being more readily phosphorylated and undergoing a much more substantial change in affinity. Phosphorylation of G-protein remains a candidate for negative feedback in the oscillator that generates the transients, but this role is not strongly supported by the data. Finally, the data illustrate the need for single-cell measurements of the $[\text{Ca}^{2+}]_i$ response, since the primary effect of phorbol esters on this response is not directly visible in population studies.

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