Mechanism of hepatic glycogen synthase inactivation induced by Ca^{2+} -mobilizing hormones

Studies using phospholipase C and phorbol myristate acetate

Peter F. BLACKMORE,* W. Garrison STRICKLAND, Stephen B. BOCKKINO and John H. EXTON

Laboratories for the Studies of Metabolic Disorders, Howard Hughes Medical Institute, and Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.

Incubation of hepatocytes with the protein kinase C activator and tumour promoter 4 β -phorbol 12 β -myristate 13 α -acetate (PMA) produced a time- and concentration-dependent inactivation of glycogen synthase, but no change in phosphorylase. The same rate and extent of inactivation occurred in hepatocytes depleted of Ca^{2+} by treatment with the Ca^{2+} chelator EGTA. When hepatocytes were treated with the Ca^{2+} -mobilizing hormone vasopressin (10 nM), the rate of glycogen synthase inactivation was similar to that observed with PMA (1 μM). Depletion of intracellular Ca^{2+} stores with EGTA abolished the ability of vasopressin to mobilize Ca^{2+} and activate phosphorylase without abolishing its ability to inactivate glycogen synthase and increase 1,2-diacylglycerol (DAG), the endogenous activator of protein kinase C. Protein kinase C, either in membranes or after partial purification, was shown to be activated *in vitro* by PMA in the presence of very low concentrations of Ca^{2+} . Exogenous phospholipase C from *Clostridium perfringens*, at low concentrations, inactivated glycogen synthase and increased DAG without affecting cell Ca^{2+} or phosphorylase. It is proposed that the inactivation of glycogen synthase elicited by the Ca^{2+} -mobilizing hormones is due, at least in part, to generation of DAG and activation of protein kinase C.

INTRODUCTION

Vasopressin, angiotensin and α_1 -adrenergic agonists can inactivate hepatic glycogen synthase in a Ca^{2+} -dependent manner (e.g. Strickland *et al.*, 1980). The mechanism by which the Ca^{2+} -dependent hormones inactivate glycogen synthase *in vivo* is unknown (Imazu *et al.*, 1984). Several Ca^{2+} -dependent protein kinases have been proposed to be the target for the Ca^{2+} that is mobilized. These include phosphorylase kinase (Roach *et al.*, 1978), the Ca^{2+} -calmodulin-dependent protein kinase (Payne & Soderling, 1980) and the phospholipid- and Ca^{2+} -dependent protein kinase C (Takai *et al.*, 1979; Kishimoto *et al.*, 1978). Although evidence *in vitro* has been presented for the involvement of each of these protein kinases, no general consensus exists as to which enzyme(s) is involved *in vivo* (Imazu *et al.*, 1984).

Several studies have shown that *myo*-inositol 1,4,5-trisphosphate (IP_3), a product of phosphatidylinositol 4,5-bisphosphate hydrolysis, is probably the second messenger for intracellular Ca^{2+} mobilization in liver and other tissues (Burgess *et al.*, 1984; Joseph *et al.*, 1984; Berridge & Irvine, 1984). In addition, evidence is accumulating which shows that 1,2-diacylglycerol (DAG), another product of phosphatidylinositol 4,5-bisphosphate hydrolysis, also acts as a second messenger (Nishizuka, 1984). Protein kinase C from brain has been shown to phosphorylate purified hepatic glycogen synthase (Ahmad *et al.*, 1984; Imazu *et al.*, 1984). In one study, inactivation of muscle glycogen synthase was observed after phosphorylation (Ahmad *et al.*, 1984), whereas in the other no alteration in the activity of liver

glycogen synthase was observed (Imazu *et al.*, 1984). We decided to investigate the role of protein kinase C in this inactivation in isolated hepatocytes. The results of this study indicate that protein kinase C is responsible, in part at least, for the inactivation of glycogen synthase induced by the Ca^{2+} -dependent hormones (vasopressin, angiotensin and α_1 -adrenergic agonists) and by AlF_4^- (Blackmore *et al.*, 1985), since they all increase DAG (Bockkino *et al.*, 1985), the putative endogenous activator of protein kinase C.

The data presented herein also confirm and extend previous observations showing that PMA does not activate phosphorylase or increase $[\text{Ca}^{2+}]_i$ (Charest *et al.*, 1985; Lynch *et al.*, 1985), but causes inactivation of glycogen synthase (Roach & Goldman, 1983). In contrast, vasopressin both activates phosphorylase and inactivates glycogen synthase (Strickland *et al.*, 1980), since it increases both DAG (Bockkino *et al.*, 1985) and $[\text{Ca}^{2+}]_i$ (Charest *et al.*, 1983).

EXPERIMENTAL

Materials

[8-Arginine]vasopressin, phospholipase C (type X; *Clostridium perfringens*), EGTA and PMA were from Sigma. A23187 was a gift from Lilly. Collagenase type I was from Worthington Diagnostic Systems. $[\text{U}-^{14}\text{C}]$ Glucose 1-phosphate and $[\text{U}-^{14}\text{C}]$ UDP-glucose were from ICN. Phenyl-Sepharose CL-4B and DEAE-Sepharose CL-6B were from Pharmacia. The zwitterionic detergent CHAPS {3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate} was from Calbiochem.

Abbreviations used: PMA, 4 β -phorbol 12 β -myristate 13 α -acetate; IP_3 , *myo*-inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; $[\text{Ca}^{2+}]_i$, free cytosolic Ca^{2+} concentration; DTT, dithiothreitol.

* To whom correspondence should be addressed, at the Howard Hughes Medical Institute, 731 Light Hall, Vanderbilt University, Nashville, TN 37232, U.S.A.

Preparation of hepatocytes

Hepatocytes were prepared from fed male rats (200–250 g body wt.) as described previously (Blackmore & Exton, 1985). The animals were fed *ad libitum* on a high-carbohydrate diet (Purina Lab Chow). For glycogen synthase measurements, hepatocytes were preincubated for 15 min with 30 mM-glucose to activate the enzyme before hormones or other agents were added. Methods for the measurement of cell Ca^{2+} (Blackmore & Exton, 1985), phosphorylase *a* activity (Blackmore & Exton, 1985), glycogen synthase activity (Strickland *et al.*, 1980), cytosolic Ca^{2+} (Charest *et al.*, 1983) and DAG (Bocchino *et al.*, 1985) have been described previously. Ca -EGTA buffers were used in the protein kinase C assays, and the concentration of free Ca^{2+} was calculated by using the COMICS program (Perrin & Sayce, 1967). Association constants were from Sillen & Martell (1971).

Purification and measurement of protein kinase C

Protein kinase C activity was measured as previously described (Lynch *et al.*, 1985). Liver plasma membranes (Prpić *et al.*, 1984) were solubilized in 10 mM-Hepes/5 mM-DTT/0.75% CHAPS/2 mM-EDTA (pH 7.5), containing 100 μg of leupeptin/ml and 100 μg of antipain/ml

(1 ml/5 mg of protein) and centrifuged at 30000 *g* for 30 min. The proteinase inhibitors were present in all subsequent steps. The supernatant was applied to a 1 ml DEAE-Sepharose CL-4B column equilibrated with 10 mM-Hepes/5 mM-DTT, pH 7.5. The column was washed with 8 ml of 10 mM-Hepes/5 mM-DTT/50 mM-KCl/0.2% CHAPS, pH 7.5, and the protein kinase C activity was then eluted with 3 ml of 10 mM-Hepes/5 mM-DTT/200 mM-KCl/0.2% CHAPS, pH 7.5. The DEAE-Sepharose eluate was adjusted to 0.5 M-NaCl and applied to a 1.5 ml phenyl-Sepharose CL-6B column equilibrated with 10 mM-Hepes/5 mM-DTT/0.5 M-NaCl, pH 7.5. The column was washed with 15 ml of this buffer and the protein kinase C activity was eluted with 3 ml of 10 mM-Hepes/5 mM-DTT/1 mM-EDTA, pH 7.5. Enzyme purified by this method routinely had a specific activity greater than 1 μmol per min/mg, but was only about 20% pure as assessed by SDS/polyacrylamide-gel electrophoresis and Coomassie Blue staining.

RESULTS

Dose-response of PMA to inactivate glycogen synthase in the presence and absence of Ca^{2+}

The data in Fig. 1 show the effect of cellular Ca^{2+} depletion on the ability of PMA to inactivate glycogen synthase activity. In confirmation of the studies by Roach

Fig. 1. Concentration-dependence of PMA to inactivate glycogen synthase in normal- Ca^{2+} -containing hepatocytes and in Ca^{2+} -depleted hepatocytes

Hepatocytes were preincubated for 15–20 min with 30 mM-glucose to activate glycogen synthase. The cells were then further incubated for 5 min with the indicated concentration of PMA. The control was with 1% (v/v) dimethyl sulphoxide. The Ca^{2+} -depleted hepatocytes were prepared by incubating the cells (in 2.4 mM- Ca^{2+} -containing medium) with 3.5 mM-EGTA for 20 min. The glycogen synthase activity ratio is the activity of glycogen synthase measured in the absence of added glucose 6-phosphate divided by the activity measured in the presence of 10 mM-glucose 6-phosphate. Results are representative of three such experiments, and the values shown are means of triplicate incubations assayed in duplicate.

Fig. 2. Time course of vasopressin (10 nM), A23187 (10 μM) and PMA (1 μM) to inactivate glycogen synthase (a) and activate phosphorylase (b)

See legend of Fig. 1 for details. The control shown was 1% dimethyl sulphoxide, the solvent used to dissolve PMA and A23187. Results are from a representative of three experiments, and the values shown are means of duplicate incubations assayed in duplicate. Abbreviation: VASO, vasopressin.

Fig. 3. Effect of Ca^{2+} depletion on the ability of vasopressin to activate phosphorylase (a), mobilize intracellular Ca^{2+} (c), increase DAG (b) and inactivate glycogen synthase (d)

Hepatocytes were incubated for 20 min under the conditions shown before 10 nM-vasopressin was added. The cells were incubated in normal 2.4 mM- Ca^{2+} -containing medium, in medium in which no Ca^{2+} was added (which was approx. 100 μM total Ca^{2+}), or in medium to which no Ca^{2+} and 1.0 mM-EGTA were added. Values shown are mean $\pm$ S.E.M. from triplicate incubations. A representative experiment of three is shown. $\square$, Control; $\blacksquare$, + vasopressin (10 nM).

& Goldman (1983), PMA in the presence of Ca^{2+} was able to inactivate glycogen synthase activity in a dose-dependent manner, with maximal effects being observed with 0.1 μM -PMA. The ability of PMA to inactivate glycogen synthase activity was not impaired when extracellular Ca^{2+} was depleted by the inclusion of 4 mM-EGTA in the incubation medium. This amount of EGTA depletes intracellular Ca^{2+} by approx. 80% when the cells are treated for 20 min. This shows that PMA is able to activate protein kinase C at very low $[\text{Ca}^{2+}]_i$.

The time course of glycogen synthase inactivation by PMA is shown in Fig. 2(a). The rate of glycogen synthase inactivation induced by PMA was not modified when the hepatocytes were depleted of Ca^{2+} by treatment with EGTA (results not shown). In a series of six experiments, the rate of inactivation induced by a maximally effective concentration of PMA (1 μM) was slightly less than that elicited by a maximally effective dose of vasopressin (10 nM) (e.g. Fig. 2).

The slightly faster rate of inactivation with vasopressin observed after 1 min may be due to an additional effect of a Ca^{2+} -activated kinase such as phosphorylase kinase. This kinase is activated by vasopressin in these experiments, as indicated by an increase in phosphorylase

a (Fig. 2b). A23187 (10 μM) inactivated glycogen synthase at the same rate as did vasopressin. However, it should be noted that 0.1–10 μM -A23187 not only increases cytosolic Ca^{2+} but also increases DAG (Bocchino *et al.*, 1985). PMA (1 μM) does not raise cytosolic Ca^{2+} (Lynch *et al.*, 1985), and does not activate phosphorylase in liver (Fig. 2b).

Effects of vasopressin to inactivate glycogen synthase in the presence and absence of Ca^{2+}

To evaluate the role of Ca^{2+} in the inactivation of glycogen synthase by vasopressin, hepatocytes were incubated in media containing amounts of free Ca^{2+} ranging from 2 mM to 30 nM, and the effects on phosphorylase, total cell Ca^{2+} , glycogen synthase activity and DAG concentrations were measured. The data in Fig. 3(c) show that incubation of cells in low- Ca^{2+} medium (30 nM free Ca^{2+}) for 20 min decreased cell Ca^{2+} by approx. 75% and abolished the ability of vasopressin to mobilize intracellular Ca^{2+} . Under this condition, there was no phosphorylase activation (Fig. 3a). The inactivation of glycogen synthase by vasopressin was attenuated, but not abolished, by Ca^{2+} depletion to this extent (Fig. 3d). The ability of vasopressin to increase DAG was also

Fig. 4. Effect of gradual Ca^{2+} depletion on vasopressin actions

At zero time 4 mM-EGTA was added to hepatocytes in medium containing 2.4 mM- Ca^{2+} . At the indicated times of EGTA treatment, the effect of 10 nM-vasopressin (↑) on total cell Ca^{2+} , phosphorylase and glycogen synthase activity were measured. Each value is the mean from duplicate incubations assayed in duplicate.

decreased, but not eliminated, by this degree of Ca^{2+} depletion (Fig. 3b). More extensive depletion of cellular Ca^{2+} by washing hepatocytes four times with medium containing 1 mM-EGTA does result in an inhibition of the ability of the Ca^{2+} -mobilizing hormones to inactivate glycogen synthase (Strickland *et al.*, 1980).

This differential sensitivity to Ca^{2+} of phosphorylase activation and glycogen synthase inactivation by vasopressin is also illustrated by the experiment represented in Fig. 4. The addition of 4.0 mM-EGTA to hepatocytes incubated in 2.4 mM- Ca^{2+} decreased total cell Ca^{2+} such that by approx. 20 min vasopressin was no longer able to

Fig. 5. Dose-response of vasopressin to activate phosphorylase and inactivate glycogen synthase in normal- Ca^{2+} -containing cells and in Ca^{2+} -depleted cells

Hepatocytes were depleted of Ca^{2+} by adding 4 mM-EGTA to medium containing 2.4 mM- Ca^{2+} for 20 min before vasopressin was added. Each value is the mean $\pm$ S.E.M. from an experiment performed in triplicate and assayed in duplicate. A representative experiment of two is shown.

mobilize cellular Ca^{2+} and activate phosphorylase (Figs. 4a and 4b). In contrast, glycogen synthase was still inactivated to the same extent at 35 min as it was at zero time of EGTA addition (Fig. 4c).

The concentration-dependence of vasopressin to activate phosphorylase and inactivate glycogen synthase in the presence and absence of Ca^{2+} is shown in Fig. 5. In the presence of Ca^{2+} , the inactivation of glycogen synthase induced by vasopressin occurred with lower concentrations of agonist than those required to activate phosphorylase (Fig. 5b). This difference was also seen if the enzymes were assayed at 2 or 10 min (results not shown). In the absence of Ca^{2+} (Fig. 5a), vasopressin was unable to activate phosphorylase, but was still able to inactivate glycogen synthase, although to a lesser extent and with slightly less sensitivity compared with the 'plus Ca^{2+} ' condition (Fig. 5b).

Generation of DAG *in situ* by exogenous phospholipase C

The addition of phospholipase C (from *Clostridium perfringens*) to isolated hepatocytes resulted in a concentration-dependent increase in DAG (Fig. 6a). A 73% increase in DAG accumulation was observed with 0.005 unit of phospholipase C/ml. This is similar to the DAG accumulation seen with 0.5 nM-vasopressin. No

Fig. 6. Dose-response of phospholipase C on hepatocyte Ca^{2+} (b), DAG content (a), phosphorylase (c) and glycogen synthase (d) activities

Hepatocytes were incubated in medium containing 2.4 mM- Ca^{2+} . Each value is the mean $\pm$ S.E.M. of triplicate incubations assayed in duplicate from a representative of three experiments. In this experiment vasopressin (10 nM) increased DAG to 382 ± 18 ng/mg wet wt. * $P < 0.005$; ** $P < 0.05$.

change in total cell Ca^{2+} was observed until 0.1 unit of phospholipase C/ml was added (Fig. 6b). Consistent with this lack of a detectable cell Ca^{2+} change, no activation of phosphorylase or elevation of cytosolic Ca^{2+} as measured with Quin 2 (Charest *et al.*, 1983) (result not shown) was seen until 0.05 unit of phospholipase C/ml was added (Fig. 6c). However, inactivation of glycogen synthase was evident with the lowest dose of phospholipase C added (0.005 unit/ml) (Fig. 6d). These data are consistent with DAG, but not Ca^{2+} , mediating the inactivation of glycogen synthase after the treatment of

hepatocytes with low concentrations of phospholipase C. This finding implicates protein kinase C in the inactivation of glycogen synthase by this lipase. Treatment of *myo*-[^3H]inositol-prelabelled hepatocytes with phospholipase C (0.005–0.2 unit/ml) did not increase the [^3H]IP $_3$ (results not shown).

The synergistic action of PMA, which activates protein kinase C, and A23187, which increases [Ca^{2+}] $_i$, has been observed on several biological processes (Nishizuka, 1984). The data in Table 1 show that, when submaximally effective concentrations of PMA and A23187 are combined, there is no synergism on glycogen synthase inactivation.

Ca^{2+} -sensitivity and activation of protein kinase C by PMA *in vitro*

Protein kinase C is generally described as a phospholipid-dependent Ca^{2+} -requiring enzyme (Nishizuka, 1984). However, for this enzyme to be activated by PMA in Ca^{2+} -depleted hepatocytes (Fig. 1), it must have only a minimal requirement for Ca^{2+} . The data in Fig. 7 show that, in the virtual absence of Ca^{2+} (35 nM), plasma-membrane protein kinase C can be activated by PMA. The Ca^{2+} requirement for the enzyme in plasma membranes is thus very different from that of the partially purified solubilized enzyme. In the presence of PMA, the half-maximally effective concentrations of free Ca^{2+} for activating protein kinase C in the plasma membrane and after partial purification are approx. 0.1 μM and 10 μM respectively (Fig. 7). This difference could reflect alterations either in the environment of the enzyme or in the structure of the enzyme itself. The proteinase inhibitors leupeptin and antipain (100 $\mu\text{g}/\text{ml}$) were present throughout the purification to minimize proteolysis by the calpains. It is noteworthy that the solubilized enzyme shows a similar Ca^{2+} -dependence to the soluble enzyme from rat brain (Castagna *et al.*, 1982).

DISCUSSION

In this study, we investigated the role of protein kinase C in the inactivation of hepatic glycogen synthase. Use was made of the protein kinase C activator PMA together with a phospholipase C, which increases intracellular DAG, the putative modulator *in vivo* of protein kinase C activity. Comparisons were made with the effects of the Ca^{2+} -mobilizing hormone vasopressin, which increases intracellular DAG (Bocckino *et al.*, 1985) and [Ca^{2+}] $_i$ (Charest *et al.*, 1983, 1985).

The inactivation of rat liver glycogen synthase has previously been shown to be elicited by the Ca^{2+} -mobilizing hormones vasopressin, angiotensin and α_1 -adrenergic agonists (e.g. Strickland *et al.*, 1980). It was originally hypothesized that Ca^{2+} acted by allosterically activating a Ca^{2+} -sensitive enzyme such as phosphorylase kinase, which subsequently phosphorylated and inactivated glycogen synthase, and activated phosphorylase (Strickland *et al.*, 1980). With the discovery of a calmodulin-dependent protein kinase active on glycogen synthase (Payne & Soderling, 1980), it was proposed that this enzyme was involved (Payne & Soderling, 1980; Payne *et al.*, 1983). More recently another putative second messenger has been shown to be produced by Ca^{2+} -mobilizing hormones in liver, namely DAG (Bocckino *et al.*, 1985). One source of this agent is hydrolysis of the inositol-containing phospholipids.

Table 1. Effect of A23187, PMA and PMA plus A23187 on glycogen synthase inactivation

Each value is the mean of duplicate incubations assayed in duplicate.

Expt. no.	Agent and concn.	Change in glycogen synthase activity	
		(- glucose 6-phosphate/ + glucose 6-phosphate)	Theoretical change in glycogen synthase activity ratio, if additive
1	PMA (2 nM)	0.02	
	PMA (5 nM)	0.04	
	A23187 (0.1 μ M)	0.06	
	A23187 (0.2 μ M)	0.11	
	PMA (2 nM) + A23187 (0.1 μ M)	0.06	0.07
	PMA (2 nM) + A23187 (0.2 μ M)	0.10	0.13
	PMA (5 nM) + A23187 (0.1 μ M)	0.04	0.10
	PMA (5 nM) + A23187 (0.2 μ M)	0.10	0.15
2	PMA (5 nM)	0.06	
	PMA (10 nM)	0.11	
	A23187 (0.1 μ M)	0.11	
	A23187 (0.2 μ M)	0.28	
	PMA (5 nM) + A23187 (0.1 μ M)	0.11	0.17
	PMA (5 nM) + A23187 (0.2 μ M)	0.32	0.34
	PMA (10 nM) + A23187 (0.1 μ M)	0.17	0.22
	PMA (10 nM) + A23187 (0.2 μ M)	0.41	0.39

Fig. 7. Dose-response of Ca^{2+} in the presence and absence of PMA (100 nM) on the activity of protein kinase C in plasma membranes and after partial purification

For details see the Experimental section. Each value is the mean of duplicate incubations from a representative experiment of three.

DAG has been shown to activate the phospholipid-dependent protein kinase C by lowering its requirement for Ca^{2+} (Nishizuka, 1984).

There are two commonly used non-hormonal means of activating protein kinase C in cells; they involve adding either PMA or synthetic diacylglycerols such as 1-oleoyl-2-acetyl-glycerol. The studies by Roach & Goldman (1983) showed that PMA, when added to hepatocytes isolated from overnight-starved rats in the presence of 20 mM-glucose, inactivated glycogen synthase. In the present study we similarly showed that PMA inactivated glycogen synthase in hepatocytes from fed rats in the

presence of 30 mM-glucose. The inactivation occurred after a lag of approx. 1 min (Fig. 2) and at a slightly slower rate compared with that observed with a maximally effective dose of vasopressin. Vasopressin increases DAG as well as cytosolic free Ca^{2+} (Charest *et al.*, 1983; Fig. 3). PMA, on the other hand, does not increase cytosolic free Ca^{2+} (Lynch *et al.*, 1985) or increase phosphorylase *a* (Roach & Goldman, 1983; Garrison *et al.*, 1984; Lynch *et al.*, 1985; Fig. 2b; cf. Fain *et al.*, 1984). The slightly faster rate of glycogen synthase inactivation induced by vasopressin could reflect the fact that vasopressin increases two second messengers capable of inactivating glycogen synthase. The increase in Ca^{2+} could activate phosphorylase kinase (Roach *et al.*, 1978), and also the calmodulin-dependent protein kinase (Payne & Soderling, 1980) and protein kinase C (Nishizuka, 1984), whereas PMA would only activate protein kinase C (Nishizuka, 1984).

The ability of vasopressin to inactivate glycogen synthase is attenuated in Ca^{2+} -depleted hepatocytes (Figs. 3 and 5), suggesting that the involvement of a Ca^{2+} -activated kinase(s) is required to elicit a full inactivation of the enzyme. [This interpretation needs to be qualified, since Ca^{2+} depletion also decreases the ability of vasopressin to increase DAG (Bocckino *et al.*, 1985).] On the other hand, PMA was able to inactivate glycogen synthase in Ca^{2+} -depleted hepatocytes as effectively as in Ca^{2+} -containing cells (Fig. 1). This suggests that PMA was not activating a Ca^{2+} -modulated enzyme such as phosphorylase kinase or a calmodulin-dependent kinase, but was stimulating protein kinase C, which has a low requirement for Ca^{2+} (Fig. 7).

The maximal activation of phosphorylase by the Ca^{2+} -mobilizing hormones occurs within a few seconds (Blackmore *et al.*, 1983). However, glycogen synthase inactivation exhibits a lag and takes 5–10 min to reach completion (Strickland *et al.*, 1980). The increase in IP_3 , the second messenger for intracellular Ca^{2+} mobilization, occurs within a few seconds, and the subsequent increase

in free cytosolic Ca^{2+} and phosphorylase activation occur concurrently or slightly thereafter (Charest *et al.*, 1985). The increase in total DAG can be detected after 1 min of exposure to vasopressin (10 nM); however, it continues to increase until approx. 8 min (Bocckino *et al.*, 1985). This relatively slow increase in DAG (Bocckino *et al.*, 1985) corresponds to the slow inactivation of glycogen synthase (Fig. 2, and Strickland *et al.*, 1980).

There are several possible explanations for the relatively slow rate of glycogen synthase inactivation induced by PMA and the Ca^{2+} -dependent hormones. If protein kinase C is activated *in vivo* by PMA or by DAG after hormone treatment, then this most probably occurs in the plasma membrane, since several studies have shown a translocation of the enzyme from the soluble fraction of the cell into the plasma membrane (e.g. Hirota *et al.*, 1985). Alternatively, protein kinase C may become more tightly bound to the plasma membrane after the increase in DAG or the intercalation of PMA in cell membranes (Nishizuka, 1984). Thus protein kinase C probably becomes associated with the plasma membrane, whereas glycogen synthase is tightly associated with glycogen (Imazu *et al.*, 1984). This may present a physical problem of substrate and enzyme coming together. Another possibility is that protein kinase C does not directly inactivate liver glycogen synthase (Imazu *et al.*, 1984; cf. Ahmad *et al.*, 1984), but that there are intervening enzymes, either phosphatases or kinases, which are phosphorylated and regulated by protein kinase C and which modulate the activity of glycogen synthase by altering its phosphorylation state.

Attempts to mimic the effects of PMA by addition of synthetic diacylglycerols to hepatocytes *in vitro* repeatedly have failed in our hands (results not shown). The most likely reason(s) for this is that these synthetic diacylglycerols are either rapidly deacylated or converted into their phosphatidate derivatives, as observed in platelets (Kaibuchi *et al.*, 1983). We thus performed experiments whereby DAG was generated *in vivo*, independent of hormone addition. This was achieved by adding phospholipase C to hepatocytes. Addition of low concentrations of phospholipase C to hepatocytes generated DAG (Fig. 6a) and produced an inactivation of glycogen synthase without any Ca^{2+} mobilization or activation of phosphorylase (Fig. 6). Higher concentrations of phospholipase C caused Ca^{2+} entry into hepatocytes (Fig. 6b), eliciting activation of phosphorylase (Fig. 6c).

Exogenous phospholipase C does not increase IP_3 (results not shown). This is consistent with phosphatidylinositol 4,5-bisphosphate being confined to the inner leaflet of the plasma membrane and thus being inaccessible to exogenous phospholipase C (Houslay & Stanley, 1984). The increase in total cell Ca^{2+} most probably represents an increased permeability of the plasma membrane. This may reflect an increased conversion of DAG into phosphatidate, which can under certain conditions allow Ca^{2+} entry into cells (Putney *et al.*, 1980; Salmon & Honeyman, 1980). Alternatively, the plasma membrane may be rendered more permeable after phospholipase C hydrolysis of the outer phospholipid leaflet, which is composed mainly of phosphatidylcholine (Houslay & Stanley, 1984).

There are several examples of physiological processes regulated by both Ca^{2+} and DAG (Nishizuka, 1984). This phenomenon can be observed after addition of a low

concentration of the Ca^{2+} ionophore A23187 to cells in the presence of a low concentration of PMA. Low doses of each agent alone are ineffective, but when they are combined a full biological effect is seen (e.g. Kaibuchi *et al.*, 1982; Knight & Baker, 1983; Nishizuka *et al.*, 1984). In contrast with the above findings, we have previously observed no synergism between PMA and A23187 to activate phosphorylase in hepatocytes, and no effect of PMA alone (Lynch *et al.*, 1985). Similar results have been obtained with PMA and ionomycin by Cooper *et al.* (1985), and Garrison *et al.* (1984) have reported that PMA does not stimulate the phosphorylation of phosphorylase, and that PMA in combination with A23187 does not increase the phosphorylation state of phosphorylase above that observed with A23187 alone. Likewise, Corvera & Garcia-Sáinz (1984) found no effect of PMA alone to stimulate glycogenolysis in hepatocytes and no enhancement of the effect of A23187, and Roach & Goldman (1983) observed no effect of PMA on phosphorylase in hepatocytes. On the other hand, Fain *et al.* (1984) reported an activation of phosphorylase by PMA alone (1.6 μM), which was synergistically increased by A23187, whereas van de Werve *et al.* (1984) reported no effect of PMA alone, and an enhancement of the effect of A23187. However, high concentrations of PMA were required (8–16 μM), and the increase observed was less than that reported by Fain *et al.* (1984). van de Werve *et al.* (1985) attributed the failure of some previous workers to observe an interaction between PMA and A23187 to the low concentrations of phorbol ester employed (usually 1 μM). As shown in Table 1, we attempted to find synergistic effects of A23187 and PMA on the inactivation of glycogen synthase in hepatocytes. The effects of the ionophore and PMA were found to be additive or less than additive and not synergistic. The simplest explanation for this is that the agents have separate mechanisms of action. However, it should be noted that A23187 increases not only cytosolic Ca^{2+} but also DAG in hepatocytes (Bocckino *et al.*, 1985). Furthermore, PMA itself can increase DAG (Bocckino *et al.*, 1985). For these reasons, the interpretation of the effects of ionophores and phorbol esters in hepatocytes may be far from simple, especially in experiments in which these agents are combined. It is also possible that ionophores and phorbol esters may increase DAG in other cell types.

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