Activation of membrane protein kinase C by glucagon and Ca^{2+} -mobilizing hormones in cultured rat hepatocytes

Role of phosphatidylinositol and phosphatidylcholine hydrolysis

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We found that glucagon stimulated membrane protein kinase C (PKC) activity and phosphatidylcholine hydrolysis in 24 h-cultured rat hepatocytes. Phorbol myristate acetate, 8-bromo cyclic AMP, vasopressin, noradrenaline and the Ca^{2+} ionophore A23187 also stimulated membrane PKC activity. However, only vasopressin and noradrenaline stimulated inositol phosphate accumulation, whereas all agonists stimulated the rate of release of water-soluble choline metabolites into the medium. Choline, and to a much lesser extent phosphocholine, were released, suggesting predominantly phospholipase D activation. This was supported by the finding that the accumulation of phosphatidate and diacylglycerol was enhanced by the agents in [^3H]myristate-labelled hepatocytes, as was [^{32}P]phosphatidylethanol formation. Since the time courses for the release of choline into the medium and the accumulation of phosphatidate and diacylglycerol caused by vasopressin and glucagon were similar, the more rapid activation of PKC by vasopressin probably reflects diacylglycerol formation from phosphoinositide breakdown. The inability of glucagon to stimulate inositol phosphate production was not due to the prolonged culture, since similar results were obtained in 4 h cultures. We conclude that the stimulation of membrane PKC activity by glucagon correlates with accumulation of diacylglycerol and phosphatidate derived from the hydrolysis of phosphatidylcholine.

INTRODUCTION

Protein kinase C (PKC) activity is found in both cytosolic and membrane-associated cell compartments. Activation of the enzyme in some cells is accompanied by its apparent translocation from the soluble to the membrane compartment, where catalytic activity is dependent on diacylglycerol, phosphatidylserine and Ca^{2+} (for reviews see [1,2]). Redistribution of PKC activity in response to hormonal agents can occur through the generation of diacylglycerol following receptor-mediated stimulation of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis by phospholipase C. Inositol trisphosphate (IP_3), which is also formed, acts as a signal for the release of Ca^{2+} sequestered in the endoplasmic reticulum [3].

Several studies have shown that Ca^{2+} -mobilizing hormones, such as vasopressin, angiotensin II [4], noradrenaline [4,5], thyrotropin-releasing hormone [6] and carbachol [7], which stimulate PIP_2 hydrolysis, also stimulate the translocation of PKC. However, PKC activation can also occur independently of inositol phosphate turnover, as has been shown with interleukin-3 in FDCP-Mix 1 cells [8]. It is also becoming apparent that PIP_2 , a minor component of the phospholipid family, cannot maintain elevated levels of diacylglycerol, and that phosphatidylcholine (PC) hydrolysis in liver [9] and other tissues [10–12] represents a much larger source of diacylglycerol.

In this report, we show that glucagon and 8-bromo cyclic AMP, as well as vasopressin, noradrenaline, phorbol 12-myristate 13-acetate (PMA) and the Ca^{2+} ionophore A23187, all transiently activate PKC and stimulate PC breakdown in cultured hepatocytes. However, only vasopressin and noradrenaline significantly stimulate phosphoinositide breakdown.

MATERIALS AND METHODS

Preparation and incubation of hepatocytes

Hepatocytes were prepared and attached to 100 mm-diam. Falcon tissue culture plates at a density of $(8.5\text{--}10) \times 10^6$ cells/plate for 1 h in 10 ml of a modified Liebowitz L15 medium containing 10% newborn calf serum [13,14]. They were subsequently maintained in fresh medium for 18 h before use. For labelling experiments, hepatocytes were attached to 35 mm-diam. Falcon multiwell tissue culture plates at a density of 1.25×10^6 cells/well in 1.5 ml of medium. Hepatocytes were maintained for 24 h before use. When comparing fresh and 24 h old cultures, hepatocytes were either labelled for 4 h before use or maintained for 20 h followed by 4 h of labelling before use.

PKC assay

Soluble and membrane PKC assays were based on published procedures [6,15]. Hepatocytes from two 100 mm culture plates (approx. 10 mg of protein) were combined for each determination. Cells were scraped into 3 ml of buffer containing 20 mM-Tris/HCl, pH 7.5, 5 mM-EGTA, 2 mM-EDTA, 5 mM-dithiothreitol (DTT), 2 mM-phenylmethanesulphonyl fluoride and 2 μg of leupeptin/ml, and homogenized with 6×1 s strokes using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 100 000 *g* for 30 min to obtain soluble and crude membrane fractions. Membrane pellets were resuspended by sonication in 3 ml of the same buffer containing 1% Triton X-100 and all fractions were stored at -80°C . Membrane fractions were found to contain less than 5% of total lactate dehydrogenase activity, indicating minimal contamination of membrane fractions with cytosol.

Abbreviations used: DTT, dithiothreitol; IP_1 , inositol monophosphate; IP_2 , inositol bisphosphate; IP_3 , inositol trisphosphate; IP_4 , inositol tetrakisphosphate; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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Samples were partially purified before assay by DEAE-Sephacel chromatography because of the low specific activity of PKC in crude hepatocyte homogenates and to remove possible inhibitors. Samples were applied to 0.4 ml DEAE columns equilibrated with 20 mM-Tris/HCl, pH 7.5, containing 5 mM-DDT, 1 mM-EGTA and 0.5 mM-EDTA. The columns were additionally washed with 5 ml of the same buffer. PKC was eluted with 1.2 ml of the same buffer containing 300 mM-NaCl.

PKC activity was measured by incubating, for up to 10 min at 30 °C, 50 μ l (approx. 150–300 μ g of protein) of eluent in a final volume of 200 μ l of buffer containing 25 mM-Tris/HCl, pH 7.5, 100 μ g of histone H1S/ml, 10 mM-MgCl₂, 25 μ M-ATP [γ -³²P]ATP, (3–5) $\times 10^5$ c.p.m.}, 1 mM-CaCl₂, 16 μ g of phosphatidylserine and 1.6 μ g of diolein. Incubations were terminated by the addition of 0.3 ml of 25 % trichloroacetic acid, and samples were kept at 4 °C for at least 30 min. Acid-insoluble material was collected on 25 mm nitrocellulose filters (0.45 μ m pore size) under vacuum and each filter was washed 3 times with 10 % trichloroacetic acid containing 10 mM-NaH₂PO₄ and 1 mM-EDTA. Filters were dissolved in Filter Count for scintillation counting.

PKC activity was determined as the difference in activity between samples incubated with phosphatidylserine, diolein and Ca²⁺ compared with incubations in the presence of 1 mM-EGTA and the absence of lipid or Ca²⁺. PKC activity in the absence of lipid and Ca²⁺ accounted for approx. 50 % of the total activity seen in the presence of lipid, and was unaltered at 5 min by any of the ligands shown in Table 1 (results not shown). PKC activity is expressed as pmol of ATP converted/min per mg of protein. Protein was determined by the method of Bradford [16], and in five experiments it was 182 $\pm$ 57 μ g/assay tube (mean $\pm$ S.E.M.) for the membrane fraction and 305 $\pm$ 52 μ g for the soluble fraction.

Measurement of inositol phosphate production

Hepatocytes were labelled for 4 h or 18 h with 5 μ Ci of [³H]inositol. At 5 min before the end of the appropriate incubations, a small amount of LiCl was added to the medium to give a final concentration of 20 mM. This medium was then replaced with fresh unlabelled serum-free medium containing 0.2 % (w/v) BSA and 20 mM-LiCl with or without hormones, agonists or solvents as indicated, and hepatocytes were further incubated for 5 min at 37 °C. [³H]inositol phosphates were extracted [14] and separated on Dowex-formate columns [17]. Results are expressed as the total amounts of inositol bisphosphate (IP₂), inositol tris- plus tetrakis-phosphate (IP₃₊₄) produced/plate. The levels of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and PIP₂ under basal conditions in the chloroform phase of hepatocyte extracts were 167,450 $\pm$ 17790, 3070 $\pm$ 1480 and 1037 $\pm$ 110 d.p.m. respectively (means $\pm$ S.E.M. of four independent experiments) after 24 h of labelling.

Measurement of choline release

For most experiments, hepatocytes were incubated for 18 h with 1–2 μ Ci of [³H]choline/well in 1.5 ml of Leibowitz L15 medium containing 10 % serum, and were further incubated for 4 h with unlabelled serum-free medium containing 0.2 % BSA before experiments. The plates were washed once with fresh medium and hepatocytes were incubated with 1 ml of medium for 5 min in the presence or absence of hormones, agonists or solvent. The medium was collected, and 1 ml of methanol and 1 ml of chloroform were added. The phases were separated by centrifugation, and radioactivity in the top phase was determined by scintillation spectroscopy. Radioactivity in the top phase is expressed as total water-soluble choline metabolites in the

medium produced/plate. This methodology has been used as an indicator of PC hydrolysis in various cell types [5,18–21]. The total ³H-labelled lipid in the chloroform phase of hepatocyte extracts under basal conditions was 138 560 $\pm$ 13 180 d.p.m. (from five independent experiments). For studies comparing fresh and 24-h-old cultures, hepatocytes were labelled for 4 h and the plates were then washed once with fresh unlabelled serum-free medium before use.

Separation of water-soluble choline metabolites

The method used for separation of choline, phosphocholine and glycerolphosphocholine was a modification of that described by Cook & Wakelam [22] using ion-exchange chromatography. A 1 ml portion of the medium to be analysed was extracted with 1 ml of methanol and 1 ml of chloroform, then 1 ml of the top phase was diluted to 3 ml with methanol/water (1:1, v/v) and was passed over a 1 ml column of Dowex 50X8-H⁺ (200–400 mesh). The separation of phosphocholine and glycerolphosphocholine was improved by eluting the latter with a mixture of methanol/water (1:1, v/v) instead of water alone. Routinely, glycerolphosphocholine was eluted with 10 ml of methanol/water (1:1, v/v), phosphocholine with 10 ml of water, and choline with 10 ml of 1 M-HCl, which was subsequently neutralized with NaOH. Radioactivity associated with each fraction was determined by scintillation spectroscopy.

Measurement of phosphatidic acid and diacylglycerol levels

Hepatocytes were incubated for 24 h with 1 μ Ci of [³H]myristate in 1.5 ml of Leibowitz medium containing 10 % serum. [³H]Myristate was used, as a large proportion of the incorporated label is found in PC and relatively little is found in the phosphoinositide lipids, as described by Martinson *et al.* [23] (results not shown). Changes in the levels of phosphatidate and diacylglycerol were much larger using myristate than with glycerol or oleate (results not shown). After the appropriate incubations, hepatocytes were scraped from the plates and extracted with chloroform/methanol/water (1:1:0.9, by vol.). The chloroform phase was removed, dried down under vacuum and resuspended in 100 μ l of chloroform. This was then subjected to t.l.c. analysis on silica gel G plates. For phosphatidate, the plates were developed in chloroform/methanol/water/acetic acid/acetone (10:1:2:4, by vol.). For diacylglycerol, the plates were developed in hexane/diethyl ether/acetic acid (60:40:1, by vol.), as described [14]. Spots corresponding to standards were visualized with iodine, and radioactivity was measured by scintillation spectroscopy.

Measurement of phosphatidylethanol formation

Hepatocytes were labelled for 24 h with 5–10 μ Ci of [³²P]P_i phosphate. The plates were washed with ³²P-free medium. Hepatocytes were then incubated for 20 min with the various hormones and agonists in the presence or absence of 2 % ethanol. Incubations were terminated by the addition of 1 ml of methanol. Hepatocytes were scraped from the plates and were extracted with 1 ml of chloroform and 1 ml of water. The chloroform phase was taken, dried under vacuum and resuspended in 100 μ l of chloroform and spotted on silica gel G plates. The plates were developed in chloroform/methanol/acetic acid (65:15:2, by vol.) as described [24]. Autoradiographs were then made of the plates to reveal the phosphatidylethanol formed. In this solvent system phosphatidylethanol (R_f 0.3–0.4) ran above phosphatidate (R_f 0.1–0.2) and other phospholipids (R_f 0–0.1). The area of the plate corresponding to ethanol-specific lipid formation was scraped off, and radioactivity was determined by scintillation spectroscopy.

Materials

[γ -³²P]ATP, [γ -³²P]P_i, *myo*-[2-³H]inositol and [methyl-³H]choline chloride were obtained from NEN Research Products; tissue culture plates were from Falcon; BSA was from Armour Pharmaceuticals; AG 1-X8 Dowex resin (100–200 mesh, formate form), 50X8-H⁺ (200–400 mesh) and Triton X-100 were from Bio-Rad; nitrocellulose filters were from Schleicher & Schuell; DEAE-Sephacel was Pharmacia; and collagenase, tissue culture medium and other reagents and hormones were from Sigma Chemical Co.

Fig. 1. Effects of vasopressin on the distribution of PKC activity

Hepatocytes were incubated with vasopressin (100 nM) for the times indicated and PKC activity was measured in the soluble (●) and membrane (○) fractions. Results are expressed as the percentage change in soluble and membrane PKC activity from basal values, which averaged 10 and 22 pmol of ATP converted/min per mg of protein respectively, in five independent experiments. The protein content of the soluble fraction was 1.7 times that of the membrane fraction, so the total PKC activity in the membrane fractions was only 7% greater under basal conditions than that in the soluble fraction. The significance of differences was calculated using a paired *t* test: * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, † $P < 0.005$ and †† $P < 0.001$. The inset shows the dose response to a 2 min exposure to vasopressin of membrane-associated PKC activity. Results are expressed as the percentage change of activity compared with basal values, and are means $\pm$ S.E.M. from four to five independent experiments.

Fig. 2. Effects of noradrenaline on the distribution of PKC activity

Hepatocytes were incubated with 20 μ M-adrenaline as described in the legend to Fig. 1, and the values are the means $\pm$ S.E.M. from four or five independent experiments. ○, Membrane fraction; ●, soluble fraction. Significance of differences is indicated as in Fig. 1.

Fig. 3. Effects of glucagon on the distribution of PKC activity

Hepatocytes were incubated with 10 nM-glucagon as described in the legend to Fig. 1, and the values are the means $\pm$ S.E.M. from four or five independent experiments. Significance of differences is indicated as in Fig. 1. ○, Membrane fraction; ●, soluble fraction.

RESULTS AND DISCUSSION

Glucagon stimulates PKC activity

Vasopressin (Fig. 1) and noradrenaline (Fig. 2) rapidly increased membrane-associated PKC activity by 60% within 20 s. PKC activity remained elevated for 5 min, before returning to basal values after 15–60 min. The increases in membrane-associated PKC activity were dose-dependent, requiring 1–10 nM-vasopressin (Fig. 1), or 2–20 μ M-noradrenaline (Fig. 2). Soluble PKC activity decreased by approx. 20–30% within 20 s, before returning to basal values within 60 min. There was some apparent translocation of PKC activity from the cytosol to the membrane-associated compartment, in agreement with the result of Hernandez-Sottomayor & Garcia-Sainz [4] in hepatocytes and Slivka *et al.* [5] in MDCK-D1 cells. The former authors found that the effects of noradrenaline were inhibited by prazosin and unaffected by propranolol. We have also seen that noradrenaline-induced inositol phosphate accumulation is not affected by propranolol, but is inhibited by prazosin, showing that in hepatocytes the effects of noradrenaline are mediated primarily through α_1 -adrenergic receptors, independently of cyclic AMP (results not shown).

The increases in membrane-associated activity were greater than the decreases in cytosolic activity, since the basal specific activity of membrane-associated PKC activity was 2.2-fold greater than that of the cytosol, whereas the relative amount of protein in the cytosol fraction was 1.7 times that in the membrane fraction. The total PKC activity of the membrane fraction was only 7% greater than that of the soluble fraction. It should be noted that all of our PKC assays utilized extracts partially purified by DEAE-Sephacel chromatography to remove soluble factors. Pelech *et al.* [25] have emphasized that caution must be exercised in interpreting PKC translocation studies based on unfractionated extracts. In NP-40 cells, platelet-activating factor decreased cytosolic activity and increased membrane-bound activity in unfractionated extracts, but increased the activity in both fractions if assayed after anion-exchange chromatography as was done in our experiments. Whether this net increase in PKC activity in membrane extracts reflects a covalent modification such as phosphorylation in the presence of ligands remains to be established.

Glucagon (10 nM) increased membrane-associated PKC activity by up to 50% (Fig. 3), which to our knowledge has not been reported before in hepatocytes. However, unlike with vasopressin and noradrenaline, the stimulation by glucagon was slower in

Fig. 4. Effects of A23187 on the distribution of PKC activity

Hepatocytes were incubated with 5 μ M-A23187 as described in the legend to Fig. 1, and the values are the means $\pm$ S.E.M. from three to four independent experiments. Significance of differences is indicated as in Fig. 1. $\circ$, Membrane fraction; $\bullet$, soluble fraction.

onset. No effect of glucagon was seen at 20 s, and maximal effects required a 2 min exposure. Membrane-associated PKC activity returned to basal values within 60 min, and the effects of glucagon required 1–10 nM (Fig. 3, inset). There appeared to be no significant effect of glucagon on soluble PKC activity. Either the assay used in our experiments was not sensitive enough to detect any small change, or glucagon specifically increased membrane-associated PKC activity without any apparent translocation. Activation without apparent translocation of PKC has also been reported with adrenocorticotrophic hormone in Y1 cells [26] and platelet-activating factor in platelets [25].

The effects of glucagon could be secondary to the elevation of cyclic AMP, since 8-bromo cyclic AMP (100 μ M) mimicked the effects of glucagon. A 50–60% increase in membrane-associated PKC activity was observed between 1 and 5 min with the cyclic AMP analogue before returning to basal values between 15 and 60 min (results not shown).

An activation of membrane-associated PKC activity was also seen with the Ca^{2+} ionophore A23187, which required 5 μ M-A23187 (Fig. 4 and inset). Again, membrane-associated PKC

Fig. 5. Effects of insulin and 8-bromo cyclic GMP on the distribution of PKC activity

Hepatocytes were incubated with either 50 nM-insulin (a) or 100 μ M-8-bromo cyclic GMP (b) as described in the legend to Fig. 1, and the values are the means $\pm$ S.E.M. from three to four independent experiments in (a) and means $\pm$ ranges from two independent experiments in (b). $\circ$, Membrane fraction; $\bullet$, soluble fraction.

activity was not increased at 20 s, but was increased at 1 min and returned to basal values within 15–60 min. Soluble PKC activity was decreased by less than 10%, which was statistically significant only at 1 min (Fig. 4). A23187, like glucagon, appeared to activate membrane PKC activity with little apparent translocation and a similar lag period.

Insulin (50 nM) and 8-bromo cyclic GMP were without effect on PKC activity at any of the time points tested (Fig. 5). However, insulin has been reported to increase both membrane and soluble PKC activity in myocyte extracts after anion-exchange chromatography [27].

Glucagon stimulates PC but not PI breakdown

Ca^{2+} -mobilizing hormones are thought to activate PKC secondary to stimulation of the hydrolysis of phosphoinositide lipids [1–3]. Table 1 shows the ability of ligands to affect inositol phosphate accumulation. Of the agents tested, only vasopressin and noradrenaline significantly stimulated inositol phosphate accumulation. The inositol phosphate response to noradrenaline was 3-fold less than that seen with vasopressin, even though the magnitude of PKC redistribution was similar for both hormones.

Table 1. Comparison of PKC redistribution with inositol phosphate, diacylglycerol, phosphatidic acid and phosphatidylethanol accumulation, as well as the release of choline metabolites

Hepatocytes were incubated with agonists for 5 min, except for measurement of phosphatidylethanol formation, where the incubation period was 20 min. Lipid- and Ca^{2+} -dependent PKC activity was determined in membrane fractions as described. Accumulation of $\text{IP}_2 + \text{IP}_{3+4}$, release of water-soluble choline metabolites, and accumulation of labelled myristate in phosphatidic acid and diacylglycerol were also determined after a 5 min incubation under the same conditions. Phosphatidylethanol formation was measured in the presence of 2% ethanol, and the incubation period was 20 min. Results are expressed as means $\pm$ S.E.M. for the numbers of experiments denoted in parentheses. Where only two experiments were performed, means $\pm$ ranges are presented. The significance of percentage changes from basal were calculated using a paired *t* test: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; † $P < 0.005$; and †† $P < 0.001$. Basal values were: membrane PKC activity, 22 pmol/min per mg; $\text{IP}_2 + \text{IP}_{3+4}$ accumulation, 305 d.p.m.; water-soluble choline metabolite release, 1330 d.p.m.; accumulation of labelled myristate in diacylglycerol and phosphatidic acid, 2910 and 5205 d.p.m. respectively; formation of phosphatidylethanol, 90 c.p.m.

Addition	Increase above basal (%)					
	Membrane PKC activity in the presence of lipid and Ca^{2+}	$\text{IP}_2 + \text{IP}_{3+4}$ accumulation	Water-soluble choline metabolite release	Accumulation of labelled myristate in:		Formation of phosphatidylethanol
				Diacylglycerol	Phosphatidic acid	
Glucagon (10 nM)	49 $\pm$ 15 (4)*	–2 $\pm$ 4 (4)	102 $\pm$ 11 (9)††	34 $\pm$ 4 (9)††	34 $\pm$ 10 (7)††	–2 $\pm$ 14 (6)
A23187 (5 μ M)	50 $\pm$ 13 (4)**	–1 $\pm$ 2 (4)	129 $\pm$ 37 (9)††	29 $\pm$ 4 (9)††	40 $\pm$ 14 (7)††	364 $\pm$ 103 (6)***
PMA (1 μ M)	82 $\pm$ 13 (2)	1 $\pm$ 4 (4)	89 $\pm$ 25 (9)†	27 $\pm$ 6 (9)†	43 $\pm$ 19 (7)†	220 $\pm$ 55 (6)***
Vasopressin (100 nM)	76 $\pm$ 14 (5)***	126 $\pm$ 14 (6)††	93 $\pm$ 20 (9)†	31 $\pm$ 6 (9)††	55 $\pm$ 24 (7)††	70 $\pm$ 7 (6)††
Noradrenaline (20 μ M)	58 $\pm$ 15 (5)**	42 $\pm$ 8 (6)†	133 $\pm$ 32 (9)†	33 $\pm$ 7 (9)†	66 $\pm$ 32 (7)†	59 $\pm$ 14 (6)†

This suggests that factors other than phosphoinositide breakdown may be involved in noradrenaline activation of PKC. We measured phosphoinositide breakdown based on the accumulation of the inositol bis- and tris-phosphates, since this is a more sensitive measurement of phosphoinositide breakdown in cultured hepatocytes than is total inositol phosphates accumulation [28].

PMA, glucagon, 8-bromo cyclic AMP and A23187 all increased membrane-associated PKC activity, but did not significantly change the level of inositol phosphates (Table 1). These results agree with our previous findings that glucagon and 8-bromo cyclic AMP do not stimulate inositol phosphate accumulation in hepatocytes [13,29]. A23187 at higher doses (10–20 μ M) does increase inositol phosphate accumulation by approx. 20–30% (results not shown), but at 5 μ M no significant effect was seen.

Phosphoinositide hydrolysis is not the only source of receptor-mediated diacylglycerol production. Vasopressin, noradrenaline and ATP have also been shown to stimulate PC hydrolysis in a variety of cells, including liver [5,9,19,30]. Phorbol esters have been shown to activate a phospholipase specific for PC [18–19,31]. PC breakdown is accompanied by a release of water-soluble choline metabolites into the medium [5,18–21].

Fig. 6 and Table 1 show the effects of ligands on the release of water-soluble choline metabolites into the medium by cultured hepatocytes. A23187, vasopressin, noradrenaline and PMA all increased the release of water-soluble choline metabolites by approx. 2-fold, as expected [5,9–21,30]. However, glucagon and 8-bromo cyclic AMP also increased the rate of choline release (Fig. 6 and Table 1). The effects of glucagon plus vasopressin on choline release were partially additive (102%, 70% and 145% increase over basal for glucagon, vasopressin and glucagon plus vasopressin respectively; means of four independent experiments), suggesting independent mechanisms of action. The release of choline metabolites into the medium in the presence of insulin was less than 10% of that seen with the other agonists (results not shown).

To determine whether the lack of effect of glucagon on inositol phosphate formation was due to the culture conditions, a comparison was made between fresh hepatocytes labelled in culture for 4 h and 24-h-old cultured hepatocytes labelled for the last 4 h of culture. Bouscarel *et al.* [32] reported that after the culture of rat hepatocytes for 24 h, angiotensin II could no

Fig. 6. Time course of the release of water-soluble choline metabolites

Hepatocytes were labelled with [³H]choline as described in the Materials and methods section and incubated for the times indicated in the absence (●) or presence of 5 μ M-A23187 (○), 100 nM-vasopressin (□), 20 μ M-noradrenaline (◇) or 10 nM-glucagon (△). Results are expressed as the increase in water-soluble choline metabolite release (d.p.m.) into the medium over that present at $t = 0$ (4150 $\pm$ 590 d.p.m.). Results are means of four independent experiments.

longer increase intracellular Ca²⁺ and the response to vasopressin was decreased by 66%.

In our studies, incorporation of label into lipid was decreased by approx. 10–15% over 20 h in culture compared with 4 h (Table 2). Vasopressin and angiotensin II stimulated inositol phosphate accumulation by 229% and 145% respectively in hepatocytes labelled during the first 4 h. This was decreased to 131% and 53% respectively in hepatocytes labelled during 20–24 h of culture. In contrast, there was no decrease in PC breakdown due to vasopressin in hepatocytes labelled during 20–24 h of culture (Table 2). However, the angiotensin II response was lowered from a 59% increase to a 23% increase over basal. Stimulation by noradrenaline of inositol phosphate accumulation

Table 2. Comparison of phosphoinositide and PC breakdown in 4-h- and 24-h-cultured hepatocytes

Hepatocytes were labelled during 0–4 or 20–24 h in culture with either 5 μ Ci of [³H]inositol or 1 μ Ci of [³H]choline. Intracellular inositol phosphate accumulation or release of water-soluble choline metabolites was determined after a 5 min incubation with agonists, as described. Results are expressed as the percentage change in inositol phosphate accumulation or choline metabolite release, and are means $\pm$ s.e.m. from five paired independent experiments, except for angiotensin II, where the values are from four experiments. Values for radioactivity in lipid (d.p.m.) were: inositol phosphate accumulation, 51 500 $\pm$ 4340 (0–4 h) and 45 100 $\pm$ 6260 (20–24 h); water-soluble choline metabolites, 304 800 $\pm$ 28 270 (0–4 h) and 262 000 $\pm$ 23 200 (20–24 h). Basal breakdown values (d.p.m.) were: inositol phosphate accumulation: 146 $\pm$ 12 (0–4 h) and 139 $\pm$ 15 (20–24 h); water-soluble choline metabolite accumulation, 6075 $\pm$ 775 (0–4 h) and 5050 $\pm$ 540 (20–24 h).

	Increase (%)			
	IP ₂ + IP ₃₊₄ accumulation		Water-soluble choline metabolite accumulation	
	0–4 h	20–24 h	0–4 h	20–24 h
Vasopressin (100 nM)	229 $\pm$ 66	131 $\pm$ 51	38 $\pm$ 11	39 $\pm$ 10
Noradrenaline (20 μ M)	72 $\pm$ 7	47 $\pm$ 12	83 $\pm$ 12	95 $\pm$ 25
Glucagon (10 nM)	–5 $\pm$ 6	–2 $\pm$ 8	137 $\pm$ 30	93 $\pm$ 18
Angiotensin II (100 nM)	145 $\pm$ 45	53 $\pm$ 17	59 $\pm$ 12	23 $\pm$ 10

Fig. 7. Time course of the release of glycerolphosphocholine (a), phosphocholine (b) and choline (c)

Hepatocytes were labelled with [${}^3\text{H}$]choline as described in the Materials and methods section and incubated in the absence (●) or presence of 10 nM-glucagon (△), 20 μM -noradrenaline (◇), 100 nM-vasopressin (□) or 5 μM -A23187 (○) for the times indicated. The medium was removed and water-soluble choline metabolites were separated as described. Results are expressed as the increase in glycerolphosphocholine, phosphocholine and choline (d.p.m.) over that present at $t = 0$ (2585 ± 545 , 535 ± 35 and 1080 ± 135 d.p.m. respectively). Results are the means of four independent experiments.

stimulate inositol phosphate production was not due to the prolonged culture conditions, since similar results were obtained in 4 h cultures.

Time course of stimulation of phosphatidylcholine breakdown by glucagon

A time course for the release of water-soluble choline metabolites into the medium is shown in Fig. 6. Glucagon, vasopressin, noradrenaline and ionophore A23187 all stimulated the release of water-soluble choline metabolites into the medium. The time of onset of release was similar for all agents (Fig. 6).

The principal choline metabolites, glycerolphosphocholine, phosphocholine and choline, were separated by ion-exchange chromatography. Choline was the primary metabolite released, suggesting phospholipase D activation (Fig. 7). However, small amounts of phosphocholine and glycerolphosphocholine were also released, which suggests that phospholipases C and A_2 may also be involved to a lesser extent. Addition of up to 10 mM-choline or -phosphocholine to the medium did not affect the release or relative distribution of choline metabolites into the medium (results not shown). This suggests that minimal interconversion of choline and phosphocholine takes place in pools that equilibrate with externally added choline or phosphocholine. Glucagon, vasopressin and noradrenaline did not significantly elevate intracellular phosphocholine or choline accumulation (results not shown). The relatively small release of phosphocholine compared with choline suggests that this is not due to the inability of phosphocholine to cross the plasma membrane.

Stimulation of phosphatidate and diacylglycerol accumulation by glucagon

Time courses for the increases in phosphatidate and diacylglycerol are shown in Fig. 8 for glucagon, vasopressin, noradrenaline and A23187. These agents and PMA (results not shown) all increased the accumulation of labelled phosphatidate and diacylglycerol in [${}^3\text{H}$]myristate-labelled hepatocytes at 2 and 5 min. Phosphatidate accumulated to a greater extent than

Fig. 8. Time course of accumulation of phosphatidate (□) and diacylglycerol (△)

Hepatocytes were labelled for 24 h with 1 μCi of [${}^3\text{H}$]myristate/ml and incubated for the indicated times. Radioactivity in phosphatidate and diacylglycerol is expressed as the increase at each time point over basal values (4340 ± 550 and 1400 ± 195 d.p.m. respectively). The data are the means $\pm$ S.E.M. from three independent experiments. Additions: (a) vasopressin; (b) glucagon, (c) noradrenaline and (d) A23187 at the concentrations given in the legend to Fig. 1.

Table 3. Effects of glucagon on phosphatidylethanol formation

Hepatocytes were incubated for 20 min with the agents described in the presence or absence of 2% ethanol. Results are expressed as a percentage of [³²P]phosphatidylethanol formation in basal conditions (91 ± 18 c.p.m.), and are means ± S.E.M. from four paired independent experiments. The significance of the stimulation of phosphatidylethanol formation by glucagon over that by vasopressin or A23187 was calculated using a paired *t* test: ** *P* < 0.02; †† *P* < 0.001.

Addition	Phosphatidylethanol accumulation (% of basal)	
	– Glucagon	+ Glucagon (10 nM)
Basal	100	118 ± 18
Vasopressin (100 nM)	162 ± 7	217 ± 8††
A23187 (5 μM)	338 ± 29	476 ± 16**

diacylglycerol, although on a percentage basis the increases were similar, as basal values for phosphatidate were higher at 5 min (Table 1). Phosphatidate and diacylglycerol were found to increase simultaneously (Fig. 8), in agreement with the results of Martin and co-workers [33–35]. Phosphatidate did not precede diacylglycerol, in contrast with the findings of Bocckino *et al.* [36] in freshly isolated rat hepatocytes. Increases in phosphatidate that precede diacylglycerol suggest activation of phospholipase D, whereas concurrent increases in diacylglycerol and phosphatidate do not differentiate between phospholipase C versus D activation, since the lipids are rapidly interconverted. The use of [³H]myristate favours the labelling of phosphatidylcholine, as described in the Materials and methods section. The changes in phosphatidate and diacylglycerol were correlated with the changes seen in choline release. The more rapid accumulation of diacylglycerol from hydrolysis of inositol-containing lipids caused by vasopressin and noradrenaline may not be detected due to the relatively poor labelling of these lipids using myristate. Since we have shown that glucagon and A23187 do not significantly increase inositol phosphate production, the changes in phosphatidate and diacylglycerol probably reflect hydrolysis of PC.

Effect of glucagon on phosphatidylethanol formation

The formation of phosphatidylethanol has been used as an index of phospholipase D activation [24,37]. Ethanol substitutes for water during the cleavage of PC to yield phosphatidylethanol instead of phosphatidic acid. Table 1 shows the effects of various agents on the levels of phosphatidylethanol at 20 min, since a 5 min incubation was not sufficient to demonstrate phosphatidylethanol formation. All of the agents tested, except glucagon, stimulated phosphatidylethanol formation at 20 min. A23187 and PMA produced a much larger response than did the other agents. That glucagon did not have an effect was a surprise, considering the similarity of the effects of glucagon with those of the other agents on choline release as well as on phosphatidate and diacylglycerol formation. Ethanol (2%) did not impair the ability of glucagon or cyclic AMP to stimulate choline release (results not shown). The failure of glucagon to stimulate PC release may be related to the 20 min incubation period, as it could be a weak stimulator over the period of 5–20 min.

This was supported by the finding that it was possible to see an effect of glucagon on stimulation of phosphatidylethanol formation at 20 min in the presence of vasopressin and A23187 (Table 3). Glucagon, while having no significant effect alone, significantly enhanced the ability of vasopressin and A23187 to

stimulate phosphatidylethanol formation. The small stimulations by vasopressin, noradrenaline and glucagon of phosphatidylethanol formation relative to that seen with PMA and A23187 suggest that phospholipase D activation accounts for a greater fraction of choline release with PMA and A23187 over a 20 min incubation as compared with the hormones (Table 1).

Conclusions

The present data suggest that stimulation of PKC by glucagon is correlated with a stimulation of PC breakdown, but do not prove that there is a causal relationship. The data could also be due to a cyclic AMP-dependent activation of both PKC and the phospholipases involved in PC breakdown. The stimulation of membrane-bound PKC activity by glucagon did not appear to involve a translocation, so it may not necessarily be secondary to elevation of diacylglycerol by glucagon. Most studies on translocation have involved crude extracts, whereas in extracts prepared by anion-exchange chromatography an increase in activity could represent a covalent modification resulting in an increase in maximal activity when the enzyme is subsequently assayed in the presence of saturating concentrations of Ca²⁺, diacylglycerol and phosphatidylserine. The effects of glucagon appear to be secondary to the elevation in cyclic AMP, since they are mimicked by 8-bromo cyclic AMP. Glucagon [38], as well as the other agonists tested, all increase cytosolic Ca²⁺. It is not known whether a concomitant rise in intracellular Ca²⁺ is an absolute requirement for the activation of PKC. Studies performed *in vitro* suggest that diacylglycerol decreases the Ca²⁺ requirement for PKC activity to levels found in the cytosol under basal conditions [2]. However, phorbol esters and synthetic diacylglycerols can activate PKC in intact cells without any associated increase in cytosolic Ca²⁺ levels [2].

Stimulation of PC breakdown by glucagon appears to involve phospholipase D activation, since the accumulation of labelled phosphatidate and diacylglycerol was concurrently increased by glucagon. This assumption was supported by the data on phosphatidylethanol formation, which was stimulated by glucagon over a 20 min incubation in the presence of either vasopressin or the Ca²⁺ ionophore A23187. The regulation of a phospholipase D for PC is more complex than that of phosphoinositidase C, since PMA, A23187 and glucagon, as well as cyclic AMP, all activate the former but not the latter enzyme. Some investigators have attributed all effects of ligands on phospholipase D to PKC activation, but this is clearly not the entire story, as indicated by the present results and the demonstration by Bocckino *et al.* [36,37] of a stimulation of this enzymic activity by guanosine 5'-[γ-thio]triphosphate in isolated rat liver membranes.

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