Glucagon, vasopressin and angiotensin all elicit a rapid, transient increase in hepatocyte protein kinase C activity

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Challenge of intact hepatocytes with one of the hormones vasopressin, angiotensin and glucagon or with the phorbol ester phorbol 12-myristate 13-acetate (PMA) led to a rapid increase in the activity of protein kinase C found in both cytosol and membrane fractions. Maximal activation by hormones occurred within 1–6 min of challenge of cells, after which activity declined. In membrane fractions protein kinase C activity return to basal levels some 15 min after exposure of cells to either angiotensin or glucagon. In cytosol fractions of cells challenged with hormones a second phase of activation ensued after about 10 min, with levels of protein kinase C activity remaining elevated above basal level 15 min afterwards. Activity changes elicited by PMA were rather different; it took about 15 min to achieve maximal activation of cytosolic protein kinase C activity. In membranes of cells challenged with PMA, an initial rapid and transient activation was followed by a sustained increase in activity occurring about 10 min after exposure of cells to this ligand. Only when hepatocytes were challenged with PMA was the translocation of protein kinase C from the cytosol to membrane fraction observed. The kinetics of PMA-induced translocation suggested that it accounted for the second phase of the increase in membrane protein kinase C activity which was unique to this ligand.

INTRODUCTION

Many hormones and neurotransmitters exert actions on cells by eliciting the receptor-coupled stimulation of inositol phospholipid metabolism [1–3]. This produces two second messengers, namely $\text{Ins}(1,4,5)\text{P}_3$, which elevates intracellular Ca^{2+} , and 1,2-diacylglycerol (DAG), which activates protein kinase C [1–3]. Protein kinase C is a Ca^{2+} -activated and phospholipid-dependent protein kinase which is believed to play an important role in signal propagation by causing the phosphorylation of a set of cellular target proteins [3–5]. Protein kinase C serves as the putative intracellular receptor for tumour promoters, which in a number of cells have been shown to cause the redistribution of this enzyme from the cytosol to the plasma membrane [6–9]. It has also been suggested [10,11] that activation of protein kinase C plays a pivotal role in modulating the functioning of a variety of signal transduction pathways.

In hepatocytes, challenge with glucagon elicits a rapid rise in intracellular cyclic AMP concentrations (see, e.g. [12,13]). The transient nature of this process is due in part to the activation of a specific high-affinity cyclic AMP-specific phosphodiesterase [12,14–16] and also to the rapid desensitization of adenylate cyclase itself [14,17]. This desensitization has been shown by us and others to be mediated through a cyclic AMP-independent process [18–21] which we have suggested involves the activation of protein kinase C [17–19]. Certainly, such desensitization can be mimicked by treating hepatocytes with phorbol esters capable of activating protein kinase C [18,22] and by hormones able to stimulate inositol phospholipid metabolism [18]. Indeed, we and others have shown that glucagon itself can elicit a weak stimulation of inositol phospholipid metabolism [18,21,23–25] and is capable of generating DAG in hepatocytes [26].

This study was aimed at determining whether treatment of intact hepatocytes with glucagon alters the subcellular distribution and functioning of protein kinase C activity.

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. Leupeptin was from Peptide Synthesis Inc., via Scientific Marketing Associates, London, U.K. Phosphatidylserine (PtdSer) was from either Sigma, Poole, Dorset, U.K., or Lipid Products, London, U.K. Collagenase was from Worthington, Cambridge Bioscience, Cambridge, U.K. Angiotensin II was from Calbiochem, Nottingham, U.K. 1,2-Dihexanoylglycerol, 1,2-dioleoylglycerol, histone H1 (type IIIS), glucagon, pertussis toxin and aprotinin were from Sigma. Bradford reagent was from Bio-Rad. All other reagents were at least AnalaR grade, from either Sigma or BDH.

Preparation of hepatocytes

Hepatocytes were prepared from fed male Sprague–Dawley rats (200–250 g) essentially as previously described by us [17] using an adaptation of the method of Berry & Friend [27] involving a Krebs–Henseleit buffer (gassed with O_2/CO_2 , 19:1) supplemented with 5 mM-glucose. The liver was first perfused with 100 ml of perfusion buffer containing 1 mM-EDTA followed by 100 ml of perfusion buffer. Collagenase digestion was carried out by perfusing with the collagenase-containing perfusion buffer (0.4 mg of collagenase/ml, 0.25 mg of soya bean trypsin inhibition/ml) for 20–30 min. Hepatocytes were harvested and washed once with perfusion buffer (10 vol.). The cells were further washed twice in Hanks balanced salt solution supplemented with 2.5% (w/v) dialysed BSA (2×10 vol.). Hepatocytes were pre-incubated in 50 vol. of the same medium at 37 °C for around 30 min before any experimental manipulation. Over 95% of the hepatocytes prepared by this method excluded Trypan Blue.

Hepatocyte incubation and preparation of cell extracts

Hepatocyte suspensions (15 ml), containing approx.

Abbreviations used: DAG, 1,2-diacylglycerol; PtdSer, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate; TEMED, $\text{NNN}'\text{N}'$ -tetramethylethylenediamine.

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2 mg dry wt. of cells/ml, were incubated at 37 °C in a 100 ml siliconized glass conical flask. Ligands (in 150 μ l) were added to the desired concentration and incubations were performed for the specified time. At the end of the incubation the cell suspensions were decanted into 30 ml of ice-cold cell washing buffer (20 mM-Tris/HCl, 150 mM-NaCl, 5 mM-glucose, 10 μ g of leupeptin/ml and 2 μ g of aprotinin/ml, pH 7.4) and the cells were immediately sedimented at 1000 *g* for 2 min. A washing procedure was then carried out with 30 ml of cold washing buffer and the final cell pellets were resuspended in 2 ml of ice-cold homogenization buffer (20 mM-Tris/HCl, 2 mM-EDTA, 2 mM-EGTA, 6 mM- β -mercaptoethanol, 20 μ g of leupeptin/ml and 4 μ g of aprotinin/ml, pH 7.4). All procedures were carried out at 4 °C from this point unless otherwise stated. The suspensions were sonicated on ice with a micro-probe attachment for 3 $\times$ 10 s bursts, with 10 s between each burst, on a Dawes sonicator at 80 W (setting 1.5). The resulting homogenates (2 ml) were centrifuged at 1000 *g* at 4 °C for 10 min. The pellets, which contained unbroken cells, nuclei and mitochondria, were discarded. An aliquot of the resulted supernatants (1 ml) was centrifuged at 430 000 *g* at 4 °C for 30 min on a Beckman TL-100 ultracentrifuge. The resulting supernatants were collected and made to 15% (v/v) glycerol, and are referred to as cytosolic fractions. The pellets were extracted by being resuspended in 0.8 ml of homogenization buffer containing 1% (v/v) Nonidet P-40. The extraction was performed for 30 min, after which the mixture was centrifuged at 430 000 *g* for 30 min. The supernatant from each was supplemented with 15% (v/v) glycerol and is referred to here as the membrane extract. Both cytosolic and membrane extracts were used immediately for the next step. The protein concentration was determined by the Bradford method [28] using dialysed BSA as standard.

Quantitative analysis and separation of protein kinase C

Quantitative analysis of protein kinase C was carried out using non-denaturing PAGE as described by Fabbro *et al.* [29]. Tube gels (internal diameter 5 mm) composed of 1.6 ml of resolving gel (10% acrylamide, 2% cross-linker, 375 mM-Tris/HCl, 0.15 mg of potassium persulphate/ml, 5 μ g of riboflavin/ml, 0.2% Triton X-100 and 0.1% TEMED) and 0.7 ml of stacking gel (4% acrylamide, 20% cross-linker, 60 mM-Tris/phosphate, 0.15 mg of potassium persulphate/ml, 5 μ g of riboflavin/ml, 0.2% Triton X-100 and 0.1% TEMED) were subjected to photopolymerization at 4 °C. Cytosolic or membrane extracts (between 200 and 600 μ g of protein), further supplemented with 0.2% Triton X-100, 12.5 μ g of Amaranth dye/ml and 10% cathode buffer (see below), were loaded on to the gel surface. Both anode buffer (60 mM-Tris/HCl, pH 7.51) and cathode buffer [42.6 mM-Tris/HCl/46.1 mM-glycine/0.2% (v/v) Triton X-100, pH 8.9] were pre-cooled at 4 °C. Electrophoresis was carried out at constant voltage, i.e. at 110 V while the dye boundary moved through the stacking gel, and at 160 V while the dye progressed through the resolving gel until it had reached 5 mm from the bottom of the gel.

At the end of the separation, the stacking gels were discarded and the resolving gels were removed from the tubes and frozen immediately using solid CO₂. Slices of 1 mm were taken from the resolving gel to yield some 80–85 slices in total. Slices 11–40 (containing the lipid-dependent protein kinase C activity) were retained, and the others (which express lipid-independent kinase activity [29]) were discarded. The gel slices were placed separately in 0.4 ml of gel elution buffer (20 mM-Tris/HCl, 0.1 mM-EGTA, 50 mM-NaCl, 50 mM- β -mercaptoethanol, 20 μ g of leupeptin/ml and 2 μ g of aprotinin/ml, pH 7.4) and left to elute overnight with shaking.

Protein kinase C microassay

Protein kinase C was assayed in 96-well flexible assay plates (Falcon) at 30 °C for 10 min. Briefly, prior to assay, 150 μ l of the gel eluent was placed into each well and allowed to equilibrate to 30 °C (15 min). The reaction was then started by adding 50 μ l of reaction mixture [20 mM-Tris/HCl, 160 μ M-ATP, 0.5 μ Ci of [γ -³²P]ATP, 40 mM-Mg(NO₃)₂, 10 mM-CaCl₂, 17.5 μ g of PtdSer, 50 μ g of histone (IIIS; type 1) and 80 mM-dihexanoylglycerol; final pH 7.4] with an 8-channel pipette. At the end of incubation a 150 μ l sample was withdrawn from each cell and spotted on to Whatman 17 Chr chromatography paper (6 mm $\times$ 4 mm). This was immediately immersed into ice-cold 10% trichloroacetic acid. The papers were then washed for 4 $\times$ 15 min, each with fresh ice-cold trichloroacetic acid. The papers were then rinsed finally with methanol, and radioactivity was measured by scintillation counting. Protein kinase C activity was calculated by determining total activity in the presence of PtdSer and DAG, and subtracting from this the basal protein kinase activity assessed in the absence of these ligands by the integrative analysis of activities obtained by analysis of gel slices as shown in Fig. 1.

Immunoblotting of protein kinase C

The monoclonal antibody MC5 was used to detect protein kinase C in the extracts. This antibody is able to detect all major isoforms of protein kinase C [30]. Cytosolic extracts (600 μ g of protein) and membrane extracts (200 μ g of protein) were separated by SDS/PAGE (7.5% gels). Western blotting on to nitrocellulose was carried out in blotting buffer (0.003% SDS, 5 g of Tris/litre and 14.4 g of glycine/litre) for 2 h. The blots were blocked with 3% skimmed milk in TBS (20 mM-Tris/HCl, 500 mM-NaCl, final pH 7.4) overnight, followed by incubating with the monoclonal antibody MC5 at 1 μ g/ml in 3% skimmed milk/TBS for 4 h. The blots were rinsed four times for 15 min each with TBS/0.2% (v/v) Nonidet P-40 and then probed with ¹²⁵I-labelled anti-(mouse antibody) (0.5 μ Ci/ml) for 2 h and rinsed as above. The blots were dried and immunoreactive bands were visualized by autoradiography and subsequently analysed by densitometric scanning as described by us [31]. We ensured that a linear relationship was present between the absorbance of the scanned gels and the applied radioactivity. In some instances labelled bands were excised from the gel and analysed on a γ -radiation counter, with similar results being obtained.

RESULTS

Assay of protein kinase activity in crude hepatocyte cytosolic and membrane extracts, using histone type IIIS as substrate, showed that as much as 60–85% (range of three separate experiments using different cell preparations) of the protein kinase activity was not PtdSer + DAG- Ca²⁺-dependent. Thus, in order to assess protein kinase C activity accurately, a method of resolving this enzyme from other kinases and endogenous modulators must be used. A number of methods have been described which employ DE52-cellulose chromatography to resolve kinase activities [32–35]. However, by using one-step salt procedures [28–30,32] we found that the single peak of protein kinase C activity was contaminated with varying amounts (20–35%; range of four experiments using different membrane preparations) of PtdSer + DAG- and Ca²⁺-independent kinase activity. A NaCl gradient procedure [29] gave much better separation, yielding a sample showing 90–95% PtdSer + DAG/ Ca²⁺-dependent protein kinase activity (results not shown), but imposed limitations on the number of samples we could evaluate. This was circumvented by using the quantitative preparative PAGE method developed by Fabbro and co-

Fig. 1. PAGE of protein kinase C

Samples from control (untreated) cells (*a, c*) and samples from cells treated with 100 nM-PMA for 3 min (*b, d*) were electrophoretically separated, and slices were assayed for protein kinase C activity in the presence of PtdSer plus DAG (■) or in the presence of PtdSer and EGTA (○). Samples were from either cytosol (*a, b*) or membrane (*c, d*) preparations, with loading of 600 µg of protein on gels.

Table 1. Activity of protein kinase C in cytosol and membrane fractions of hepatocytes

Cells were treated with the stated ligands for the periods specified. Harvesting and preparation of protein kinase C for assay was done as described in the Materials and methods section using a 600 µg protein sample. Values in parentheses are the percentage increases in activity over that in the basal state (no ligand challenge). Results are means ± S.D. for experiments done with three separate incubations of cells.

Treatment	Protein kinase C activity (pmol/min per sample)	
	Cytosol	Membrane
None (basal)	468 ± 12	38 ± 4
PMA (100 nM, 3 min)	697 ± 29 (49)	114 ± 22 (200)
Glucagon (10 nM, 3 min)	749 ± 12 (60)	79 ± 13 (110)
Angiotensin (10 nM, 3 min)	838 ± 48 (79)	84 ± 19 (121)
8-Bromo-cyclic AMP (20 µM, 5 min)	389 ± 30	37 ± 14

workers [29]. Such a methodology allowed us to resolve, from both cytosol and membrane extracts of hepatocytes, a single

peak of PtdSer + DAG/Ca²⁺-dependent protein kinase activity (Fig. 1). In contrast with other methods we evaluated, and when using crude extracts, the protein kinase activity of this resolved peak was completely (over 98 %) abolished when DAG/Ca²⁺ were removed from the assay (Fig. 1). This method was thus employed for the remainder of our studies analysing both membrane-bound and soluble protein kinase C activity in hepatocytes challenged with various agents.

When hepatocytes were incubated with glucagon (10 nM) for 3 min, we observed an increase in both cytosolic and membrane-associated protein kinase C activity (Table 1). This enhanced activity was seen in the presence of maximally activating concentrations of PtdSer + DAG and Ca²⁺. In contrast with studies done with glucagon, incubation of hepatocytes with 8-bromo-cyclic AMP (20 µM) failed to stimulate either cytosolic or membrane-associated protein kinase C activity (Table 1). Analogous to results with glucagon, a similar challenge of hepatocytes with angiotensin II (10 nM) also increased protein kinase C activity in both cytosol and membrane fractions. In each instance the increases in the cytosol- and membrane-associated protein kinase activities achieved by challenge of cells with those hormones were comparable (Table 1). Challenge of cells for 3 min with the phorbol ester PMA also yielded an increase in protein kinase C activity in both cytosol and membrane fractions. However, with PMA the degree of activation present in the membrane fraction was considerably greater than that found after challenge of cells with either glucagon or angiotensin (Table 1).

The time courses of activation of protein kinase C by both glucagon and angiotensin were investigated (Fig. 2). In the case of glucagon, cytosolic protein kinase C activity increased very rapidly during the first 1 min, reaching a maximum level within 6 min. After this time, the degree of activation fell rapidly over the next 2 min, before the occurrence of a small second phase of activation. This reached a maximum some 10 min after the cells had been challenged with either glucagon or angiotensin, and cytosolic protein kinase C activity still remained above control levels some 15 min after hormonal challenge (Figs. 2*a* and 2*c*). When cells were challenged with either glucagon or angiotensin a very rapid increase in membrane-associated protein kinase C activity was observed (Figs. 2*b* and 2*d*), which was nearly maximal within 1 min of exposure of cells to these hormones. However, after some 5–6 min of exposure the degree of activation decreased, and activity had decayed back to basal levels by 15 min after the time of the original challenge of cells with hormones (Figs. 2*b* and 2*d*).

Challenge of hepatocytes with the phorbol ester PMA (100 nM) also led to a time-dependent increase in the activity of both cytosolic and membrane protein kinase C (Figs. 2*e* and 2*f*). Cytosolic activity (Fig. 2*e*) showed a steady increase, attaining a maximum value some 15 min after challenge of cells with PMA. Activity then declined over the next 15 min, but still remained above basal levels even some 30 min after the original challenge of cells with PMA. Membrane-associated protein kinase C activity showed a dramatic increase within 1 min of challenge of cells with PMA (Fig. 2*f*). However, activity then declined very rapidly, reaching a minimum value some 10 min after first challenging cells with PMA. After this time, however, a sustained increase in membrane protein kinase C activity was noted which continued even some 30 min after first challenging cells with PMA (Fig. 2*f*).

We also determined whether translocation of protein kinase C from the cytosol to the membrane fraction occurred during ligand challenge of the hepatocytes. This was done by immunoblotting samples that had been resolved by SDS/PAGE using the monoclonal antibody MC5, which is able to detect all major

Fig. 2. Time courses of activation of protein kinase C by glucagon, angiotensin and PMA

Hepatocytes were treated with 10 nM-glucagon (*a, b*), 10 nM-angiotensin (*c, d*) or 100 nM-PMA (*e, f*) for the indicated periods of time. Cells were harvested and protein kinase C was isolated and assayed as described in the Materials and methods section. Data are shown for both cytosolic (*a, c, e*) and membrane (*b, d, f*) protein kinase C activities. Data are from experiments done three times on different cell preparations and are means $\pm$ S.D.

forms of protein kinase C [27]. Analysis of cytosol and membrane fractions identified an 80 kDa band which co-migrated with a sample of authentic brain protein kinase C (results not shown). Consistent with our finding that the majority of protein kinase C activity was present in the cytosolic fraction (Table 1), we found a similar distribution of protein kinase C upon immunoblot analysis (cytosol/membrane ratio 16 ± 2 ; $n = 4$) as we did when assessing activity (~ 12 ; Table 1). In cells challenged with the phorbol ester PMA, no increase in membrane-associated protein kinase C was evident over the first 5 min of exposure (Fig. 3). Indeed, no increase in the amount of membrane protein kinase C or decrease in the levels of cytosolic enzyme was evident in cells challenged with either glucagon or angiotensin over periods of up to 5 min (results not shown), during which marked changes in activity had been noted (Fig. 2). Nevertheless, in cells challenged with PMA, we observed at later time points a translocation of

Fig. 3. PMA-mediated changes in protein kinase C distribution

Cells were challenged with the phorbol ester PMA for the indicated times, prior to harvesting and the preparation of membrane (□) and cytosol (■) fractions. These were subjected to SDS/PAGE and Western blotting analysis using an antiserum to protein kinase C (see the Materials and methods section). Visualization was achieved using an ^{125}I -labelled second antibody and autoradiography. Quantification was done either by densitometric scanning or by excision of labelled bands and analysis by γ -radiation counting. The antiserum detected a single 80 kDa band which migrated like authentic protein kinase C. Data are expressed as arbitrary units for analyses done on equal amounts of protein from cytosol and membrane fractions. A typical experiment is shown of four carried out on different cell preparations.

protein kinase C from the cytosol to the membrane fraction; this was most obvious some 15–20 min after exposure of cells to PMA (Fig. 3).

It has been shown that if cells are homogenized in the presence of high concentrations of Ca^{2+} then the amount of protein kinase C bound to the membrane fraction can be increased [5]. When we homogenized hepatocytes in the presence of 1 mM- Ca^{2+} there was an approx. 4.5–5.6-fold increase (range in four separate experiments using different cell preparations) in the amount of protein kinase C found associated with the membrane fraction upon immunoblotting analysis, and a 5–5.8-fold increase (range of four separate experiments using different cell preparations) detected by assay of protein kinase C activity in membrane fractions.

DISCUSSION

It is well established that glucagon can activate adenylate cyclase and raise intracellular cyclic AMP concentrations in hepatocytes (see [10–12] for a discussion). However, there is considerable evidence to suggest that glucagon can also exert actions on hepatocytes that appear to occur independently of any increase in cyclic AMP and may be due to activation of lipid signalling pathways [14,18–24,36–39]. In this study we have attempted to ascertain whether we could identify any glucagon-mediated changes in protein kinase C functioning or distribution that might correlate with the ability of the hormone to cause the desensitization of adenylate cyclase. A number of investigators have shown that treatment of cells with agents capable of activating protein kinase C can lead to the translocation of the enzyme from the cytosol to a membrane location [4–7]. However, although we were able to observe this upon prolonged treatment

of hepatocytes with PMA (Fig. 3) and could show that translocation could be triggered artificially by homogenizing cells in a high- Ca^{2+} medium, we found no indication that challenge of intact hepatocytes with either angiotensin or glucagon could elicit the translocation of protein kinase C. Indeed, during the course of this study we noted that other investigators [40] were unable to observe the translocation of protein kinase C in hepatocytes treated with vasopressin, a hormone capable of stimulating hepatocyte inositol phospholipid metabolism. It may be that there is a difference in the action of PMA in modulating hepatocyte protein kinase C redistribution compared with hormones which activate this enzyme through the elevation of intracellular DAG levels. Perhaps phorbol esters stimulate the translocation of specific isoforms of protein kinase C which are unaffected by the DAG produced by hormone action [5,41]. Indeed, several investigators [41–46] have questioned the reliability of using translocation as an index for monitoring receptor-mediated events which lead to DAG generation and, certainly, the basis of translocation has yet to be ascertained. Nevertheless, as PMA, angiotensin, vasopressin and glucagon can all elicit the desensitization of adenylate cyclase, it would appear that the translocation of protein kinase C from cytosol to membrane fractions is not a pre-requisite for such action.

We demonstrated, however, that treatment of intact hepatocytes with either angiotensin or glucagon led to a rapid increase in protein kinase C activity associated with both the membrane and cytosolic fractions. Indeed, agonist-mediated increases in protein kinase C activity have been reported in membrane fractions from BC3H-1 myocytes [45] and in adipocytes [47–49]. Some investigators have suggested that protein kinase C activation seen in cell extracts may be due to DAG contamination occurring in either the crude enzyme extracts or the DEAE-cellulose column fractions employed, or the consequence of proteolysis leading to the constitutive activation of the regulatory subunit of protein kinase C and loss of the dependence of activity on DAG addition [50]. However, in the present study, protein kinase C was separated by non-denaturing PAGE in the presence of 0.2% Triton X-100, which will remove endogenous DAG. Consistent with this, the protein kinase C we resolved was dependent upon the addition of exogenous DAG for activity to be displayed. This could be presented in the form of either 1,2-dioleoylglycerol or 1,2-dihexanoylglycerol, which gave essentially similar results. Furthermore, all protein kinase activity in the protein kinase C fractions isolated could be abolished by using 1 mM-EGTA instead of Ca^{2+} /PtdSer in the assay. Thus it appears that treatment of hepatocytes with these ligands can increase the total DAG-stimulated protein kinase C activity. As this increased activity was evident in assays where maximally effective concentrations of exogenous DAG were present, this suggests either that latent activity has been exposed by hormonal challenge or that some modification of cellular protein kinase C activity has resulted, yielding a heightened (activated state).

Activation of both membrane and cytosolic protein kinase C activity was also elicited upon challenge of cells with the phorbol ester PMA (Figs. 2e and 2f). The initial rapid, transient, increase in membrane-bound activity presumably occurred via a mechanism akin to that employed by angiotensin or glucagon, as it showed similar kinetics and no significant translocation of protein kinase C occurred over this period (Fig. 3). Unlike DAG, PMA is not metabolized by cells, and thus the transience of the activation of protein kinase C might be due to a distinct deactivation process. In contrast to challenge of cells with either glucagon or angiotensin, PMA elicited a second phase of increased membrane protein kinase C activity which occurred some 10 min or so after challenge of cells with the ligand (Fig. 2). We believe that this second phase of increased membrane protein

kinase C activity was due to the translocation of the enzyme from the cytosol to the membrane compartment, as the kinetics for the two processes were similar (Figs. 2 and 3) and neither of the hormones effect either translocation or such a second phase activation. PMA, as with the hormones investigated, caused an elevation in cytosolic protein kinase C activity. The rate of activation was, however, slower and no second phase of activation occurred. One can estimate from immunoblotting studies (Fig. 3) that approx. 22% of the cytosolic protein kinase C was translocated to the membrane fraction some 30 min after challenge of cells with PMA (Fig. 3). From the maximal cytosolic protein kinase C activity seen at 15 min it can be calculated that cytosolic protein kinase C activity had fallen by 33% at 30 min after PMA challenge (Fig. 2). These changes are comparable in magnitude, and imply that the fall in cytosolic protein kinase C activity seen at later time points may be largely due to translocation. This implies that PMA may achieve a sustained, or only very slowly decaying, activation of cytosol protein kinase C in hepatocytes. The amount of cytosolic protein kinase C translocated after 30 min can be calculated as being capable of increasing the membrane-associated activity by around 600%. However, the degree of apparent activation recorded was around 300% (Fig. 2f). This might imply that the translocated membrane-associated protein kinase C has a slightly lower activity than when it is in a soluble state in the cytosol.

The mechanism underlying the protein kinase C activation observed in this study still remains unclear. A possible candidate is the regulation of protein kinase C by phosphorylation. This is unlikely to be due to any action of protein kinase A, as angiotensin, which neither activates protein kinase A nor elevates cyclic AMP levels in hepatocytes, can elicit the increase in protein kinase C activity (Figs. 2c and 2d). Furthermore, treatment of hepatocytes with 8-bromo-cyclic AMP for up to 15 min had no effect on the activity of protein kinase C (Table 1). It has, however, been reported that protein kinase C can become phosphorylated in intact cells [51,52]. This can occur as a result of receptor stimulation of inositol phospholipid metabolism [52], and has also been observed using purified enzyme preparations, resulting in an alteration in the kinetic properties of the enzyme [53–55]. However, it has yet to be established as to whether phosphorylation in intact cells results from the action of either protein kinase C itself or another species of protein kinase. Thus the molecular basis of the activation of protein kinase C identified here remains to be established.

The fact that the time course for the hormone-stimulated increase in membrane protein kinase C activity, and for the first phase of activation of the cytosolic activity, parallels that for the desensitization of adenylate cyclase ([17,18] Fig. 2) suggests that these may well be interlinked regulatory events occurring in intact hepatocytes. Indeed, the observation that an increase in DAG-stimulated protein kinase C activity can be triggered by glucagon action adds further support to the hypothesis that glucagon may exert certain of its actions in hepatocytes through this enzyme.

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