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Inhibition by the protein kinase C activator 4β-phorbol 12-myristate 13-acetate of the prostaglandin $F_{2\alpha}$-mediated and noradrenaline-mediated but not glucagon-mediated activation of glycogenolysis in rat liver

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In perfused rat livers, infusion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or noradrenaline increased glucose and lactate output and reduced flow. Glucagon increased glucose output and decreased lactate output without influence on flow. Infusion of phorbol 13-myristate 14-acetate (PMA) for 20 min prior to these stimuli strongly inhibited the metabolic and hemodynamic effects of noradrenaline, reduced the metabolic actions of $PGF_{2\alpha}$ but did not alter the effects of glucagon.

In isolated rat hepatocytes $PGF_{2\alpha}$, noradrenaline and glucagon activated glycogen phosphorylase but only $PGF_{2\alpha}$ and noradrenaline increased intracellular inositol 1,4,5-trisphosphate ($InsP_3$). The noradrenaline- or $PGF_{2\alpha}$-elicited activation of glycogen phosphorylase and increase in $InsP_3$ were largely reduced after preincubation of the cells for 10 min with PMA, whereas the glucagon-mediated enzyme activation was not affected. In contrast to PMA, the phorbol ester 4α-phorbol 13,14-didecanoate, which does not activate protein kinase C, did not attenuate the $PGF_{2\alpha}$- and noradrenaline-elicited stimulation of glucose output, glycogen phosphorylase and $InsP_3$ formation. Stimulation of $InsP_3$ formation by AlF_4^-, which activates phospholipase C independently of the receptor, was not attenuated by prior incubation with PMA.

Plasma membranes purified from isolated hepatocytes had both a high-capacity, low-affinity and a low-capacity, high-affinity binding site for $PGF_{2\alpha}$. The K_d of the high-capacity, low-affinity binding site was close to the concentration of $PGF_{2\alpha}$ that increased glycogen phosphorylase activity half-maximally. Binding to the high-capacity, low-affinity binding site was enhanced by guanosine 5′-*O*-(3-thio)triphosphate (GTP[S]). This high-capacity, low-affinity site might thus represent the receptor. The B_{max} and K_d of the high-capacity site, as well as the enhancement by GTP[S] of $PGF_{2\alpha}$ binding to this site, remained unaffected by PMA pretreatment.

It is concluded that, in hepatocytes, activation of protein kinase C by PMA interrupted the $InsP_3$-mediated signal pathway from $PGF_{2\alpha}$ via a $PGF_{2\alpha}$ receptor and phospholipase C to glycogen phosphorylase at a point distal of the receptor prior to phospholipase C.

Prostaglandins are involved in the regulation of liver metabolism and hemodynamics by humoral factors [1–9] and hepatic nerves [10–12]. In perfused rat liver, glycogenolysis was increased most potently by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), followed by prostaglandin D_2 (PGD_2) and E_2 (PGE_2) [12–14]. In isolated hepatocytes glycogenolysis was activated by $PGF_{2\alpha}$ and PGE_2 via a putative prostaglandin receptor that elicited an increase in intracellular inositol trisphosphate ($InsP_3$) [15, 16]. The activation of glycogenolysis by noradrenaline or vasopressin via $InsP_3$ formation was inhibited by activation of protein kinase C with phorbol esters [17]. It was postulated that activation of protein kinase C by diacylglycerol, which is released concomitantly with $InsP_3$, might constitute a feedback inhibition loop for signal pathways involving activation of phospholipase C. In the current investigation evidence is provided that, in line with this proposal, activation of protein kinase C attenuated also the $PGF_{2\alpha}$-mediated activation of glycogenolysis and the increase in $InsP_3$ formation in perfused liver and isolated hepatocytes.

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Abbreviations. $InsP_3$, inositol 1,4,5-trisphosphate; PGD_2, PGE_2 and $PGF_{2\alpha}$, prostaglandins D_2, E_2 and $F_{2\alpha}$; PMA, phorbol 12-myristate 13-acetate; 4αPDD, 4α-phorbol 12,13-didecanoate; GTP[S], guanosine 5′-*O*-(3-thio)triphosphate; G-protein, trimeric GTP-binding protein.

MATERIALS AND METHODS

Chemicals

All chemicals were reagent grade and from commercial sources. The Merck glucose system was purchased from Merck. Lactate dehydrogenase and glutamic–pyruvic transaminase were from Bochringer Mannheim, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from Paesel and [^{3}H]prostaglandin $F_{2\alpha}$ from New England Nuclear. The inositol 1,4,5-trisphosphate ($InsP_3$) assay kit was obtained from Amersham Buchler, glass fiber filters GF52 from Schleicher & Schüll and percoll from Pharmacia.

Animals

Male Wistar rats (160–200 g, Winkelmann) were kept on a 12-h day/night rhythm (light from 7.00 to 19.00) with free access to water and food (standard rat diet, Ssniff). All experiments were begun between 9.00 and 10.00. Rats were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg body mass).

Liver perfusion

The liver was perfused *in situ* via the portal vein at constant pressure without recirculation as described previously [18] using an erythrocyte-free Krebs-Henseleit bicarbonate buffer, which contained 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate and was equilibrated with 95% O_2/5% CO_2 (by vol.). At the times indicated, $PGF_{2\alpha}$ (5 μM), noradrenaline (1 μM) or glucagon (1 nM) were infused for 2 min. The flow was determined by quantitating the perfusate fractions collected at 1-min intervals.

Hepatocyte suspensions

Hepatocytes were isolated according to Meredith without the use of collagenase [19]. The method was slightly modified. The liver was perfused without recirculation with a Ca^{2+}-free Krebs-Henseleit buffer containing 15 mM glucose, 2 mM lactate, 0.2 mM pyruvate and 2 mM EDTA for 30–40 min. Viable hepatocytes were purified by differential centrifugation and then centrifuged through a gradient with 58% Percoll to remove residual detritus and possible minor contaminations with non-parenchymal liver cells, which were floating on top of the gradient. Hepatocytes were free of contamination by non-parenchymal liver cells as judged by light microscopy.

Hepatocytes were preincubated for 1 h in a Hepes-buffered saline (20 mM Hepes, 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$) pH 7.4 with 20 mM glucose. They were then sedimented and resuspended in Hepes-buffered saline pH 7.4. The incubation mixture contained a final concentration of 4 mM glucose and 2 mM lactate. Cells were incubated for 10 min with or without phorbol myristate acetate (PMA) (100 nM); then $PGF_{2\alpha}$ (10 μM), noradrenaline (0.1 μM) or glucagon (1 nM) was added. The incubation was stopped with trichloroacetic acid at −1 min and 30 min for glucose and at 30 s for $InsP_3$ determination or by freezing in liquid nitrogen after 2 min for the determination of glycogen phosphorylase activity. Glucose was assayed in the perfusate and the hepatocyte supernatants using a commercial test kit (Merck glucose system) based on glucose dehydrogenase [20]. Lactate was measured in a combined optical test using lactate dehydrogenase and glutamic–pyruvic transaminase [21]. Glycogen phosphorylase activity was determined by a standard assay [22], protein by the method of Lowry [23] and $InsP_3$ by using the Amersham radioligand assay.

Hepatocyte plasma membranes

A plasma-membrane-enriched fraction was obtained from purified control or PMA-treated rat hepatocytes, which were free of contamination by non-parenchymal liver cells as judged by light microscopy, according to Pietras and Szego [24] by separation of membrane subfractions on sucrose gradients. The homogenization buffer contained 10 mM Tris/HCl pH 7.4 and 250 mM sucrose, or in the majority of the experiments, 200 mM sucrose and in addition 50 mM NaF to inhibit phosphatases. Ligand binding was measured in saturation binding assays. Plasma membranes (3 mg membrane protein/ml) were incubated for 1 h at room temperature in a final volume of 0.1 ml Hepes-buffered saline with 0.5 nM–25 μM $PGF_{2\alpha}$ containing either 7.66, 0.15 or 0.015 TBq/mmol [^{3}H]$PGF_{2\alpha}$. An aliquot at each concentration was taken for measuring total radioactivity. Bound label was separated from free ligand by vacuum filtration using GF52 glass fiber filters. The binding constants were calculated using the computer program LIGAND [25] provided by the National Institutes of Health.

RESULTS

Inhibition by PMA of the $PGF_{2\alpha}$- and noradrenaline-mediated increase in glucose and lactate output in perfused liver

In perfused rat liver, infusion of $PGF_{2\alpha}$ (5 μM) or noradrenaline (1 μM) increased glucose and lactate output and reduced flow. Glucagon (1 nM) in a subsequent stimulation period increased glucose release and reduced lactate output but left the flow unaffected (Fig. 1, Table 1).

Infusion of PMA (100 nM) for 20 min, which suffices to activate protein kinase [17], initially caused a slow 10–20% reduction of flow, which reached a new steady state after 15 min. It did not increase glucose output (not shown) in contrast to its action in glucose-free perfused liver [26]. Prior treatment with PMA clearly diminished the $PGF_{2\alpha}$-mediated increases in glucose and lactate output but not the reduction in flow. Likewise, it almost completely abolished the noradrenaline-mediated increases in glucose and lactate output and, in contrast to $PGF_{2\alpha}$, also the reduction in flow. However, it did not significantly affect the glucagon-mediated increase in glucose output and decrease in lactate output (Fig. 1, Table 1). The peak value of glucose output was slightly lower and was delayed in PMA-treated livers, probably due to the somewhat lower flow rate; the area under the curve was, however, not significantly different from the control. Thus, in perfused liver, activation of protein kinase C reduced the metabolic effects of the two agonists that act via an increase in $InsP_3$ and intracellular calcium whereas it did not interfere with the action of the agonist that operates via cAMP.

Inhibition by PMA of the $PGF_{2\alpha}$- and noradrenaline-mediated activation of glycogenolysis and $InsP_3$ formation in hepatocytes

In order to examine the mechanism by which PMA interrupted the $PGF_{2\alpha}$ and noradrenaline signal chains, the studies were continued with isolated hepatocytes instead of the more complex system of the perfused liver. Hepatocytes, that were essentially free of contamination with non-parenchymal liver cells, were incubated at 37 °C in Hepes-buffered saline pH 7.4. The glucose concentration in the cell suspension at the beginning of the experiment was about 4 mM. The cells released glucose at a rate of 0.5 $\mu mol \times min^{-1} \times g$ wet $mass^{-1}$. The basal glycogen phosphorylase activity was 4.2 U/g and the $InsP_3$ level was 0.2 $nmol \times g^{-1}$. $PGF_{2\alpha}$ (10 μM) increased glucose output by about 25%, glycogen phosphorylase by about 60% over basal (Fig. 2, left). Noradrenaline (0.1 μM) elevated glucose release by about 50%

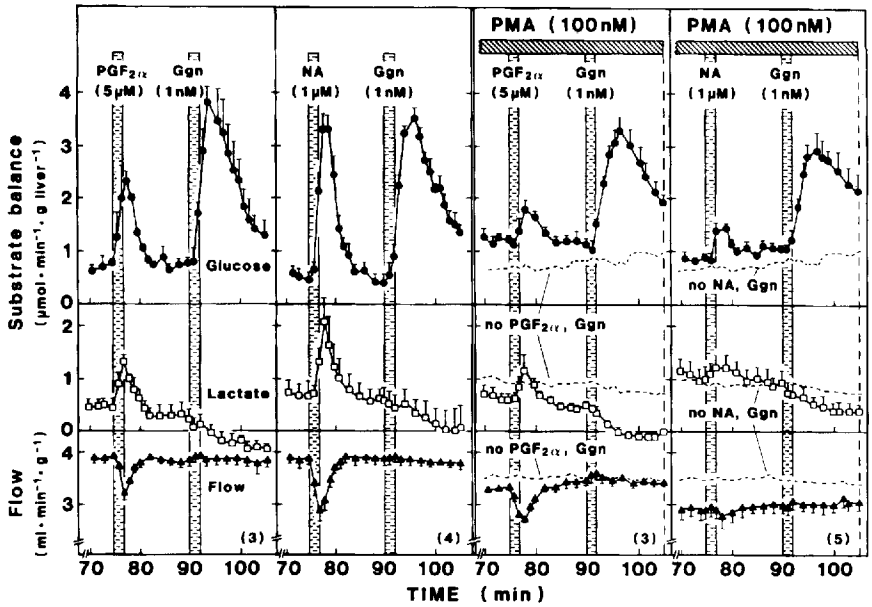

Fig. 1. Inhibition by PMA of $PGF_{2\alpha}$- and noradrenaline-mediated increases in glucose and lactate output and noradrenaline-mediated reduction in flow in perfused rat livers. Rat livers were perfused as described in Materials and methods. At the times indicated, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 5 μM), noradrenaline (NA, 1 μM) or glucagon (Ggn, 1 nM) were infused to the final concentrations indicated. Where indicated PMA was added 20 min prior to the first stimulus. Substrate balance is given as (posthepatic concentration − prehepatic concentration) × $flow^{-1}$ × $liver\ mass^{-1}$. The calculated liver mass was assumed to be 4% of the total body mass. Values are means ± SEM of the number of experiments given in parentheses.

Table 1. Changes in glucose and lactate output and flow after $PGF_{2\alpha}$, noradrenaline or glucagon treatment in the presence or absence of PMA in perfused rat liver. Rat livers were perfused as described in the legend to Fig. 1. $PGF_{2\alpha}$, noradrenaline or glucagon were infused to final concentrations of 5 μM, 1 μM and 1 nM, respectively, for 2 min. Where indicated, PMA was infused to a final concentration of 100 nM for 20 min prior to these stimuli. Values represent the means ± SEM of n experiments of the areas under the curve of Fig. 1. The baseline was determined by linear regression through all non-stimulation values; the area under the curve was calculated during the time period of stimulation onset until it reached the baseline (noradrenaline, $PGF_{2\alpha}$) or end of the experiment (glucagon). Statistical analysis was performed with the Student's t-test for unpaired samples. $^{*}P < 0.025$, $^{**}P < 0.01$, n.d. = not detectable.

Stimulus	PMA	Output of		Flow reduction	n
		glucose	lactate		
		μmol · g^{-1}		ml · g^{-1}	
$PGF_{2\alpha}$	−	6.2 ± 0.6	3.1 ± 0.1	−1.6 ± 0.2	3
	+	2.3 ± 0.3 **	1.7 ± 0.4 *	−1.9 ± 0.2	3
Noradrenaline	−	11.5 ± 0.5	4.8 ± 0.9	−2.7 ± 0.6	4
	+	2.7 ± 0.0 **	1.4 ± 0.1 **	−0.5 ± 0.3 **	5
Glucagon	−	24.8 ± 2.8	−5.4 ± 0.8	n. d.	7
	+	20.4 ± 2.5	−5.5 ± 0.5	n. d.	8

and glycogen phosphorylase by about 100% over basal. The augmentation in glucose output and glycogen phosphorylase was most pronounced after glucagon (1 nM) which increased glucose output by 260% and glycogen phosphorylase by 180% over basal. Lower concentrations (0.1 nM and 0.01 nM) increased glycogen phosphorylase activity by 128 ± 16% and 39 ± 10%, respectively. $PGF_{2\alpha}$ and noradrenaline but not glucagon (1 nM) increased $InsP_3$ twofold and threefold, respectively (Fig. 2, left) reaching a maximum about 30 s after administration (time course not shown).

If cells were treated with 100 nM PMA 10 min prior to the administration of $PGF_{2\alpha}$, noradrenaline or glucagon, the $PGF_{2\alpha}$- and noradrenaline-mediated increases in glucose output and glycogen phosphorylase were largely reduced or abolished, whereas the increase in glucose output and glycogen phosphorylase activity elicited by 1 nM glucagon remained unaltered (Fig. 2, middle). However, PMA treatment reduced the increase in glycogen phosphorylase activity caused by 0.1 nM and 0.01 nM glucagon to 73 ± 13% and 27 ± 10% of the increase in activity in control cells. PMA treatment also abolished the increases in $InsP_3$ levels after $PGF_{2\alpha}$ or noradrenaline. Apparently, activation of protein kinase C with PMA attenuated the $PGF_{2\alpha}$- and noradrenaline-mediated increase in glucose output by interrupting the signal chain prior to the generation of the second messenger $InsP_3$ (cf. Fig. 4).

Incubation of the hepatocytes with the phorbol ester 4αPDD, which does not activate protein kinase C, inhibited neither the $PGF_{2\alpha}$-, noradrenaline- nor glucagon-mediated increases in glucose output and glycogen phosphorylase activity nor the $PGF_{2\alpha}$- and noradrenaline-mediated increase in $InsP_3$ formation; the minor changes in $InsP_3$ following gluca-

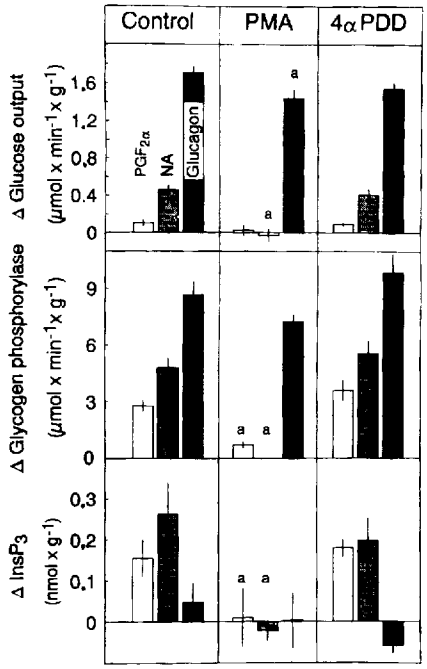

Fig. 2. Inhibition by PMA of $PGF_{2\alpha}$- and noradrenaline-mediated increases in glucose release, glycogen phosphorylase activity and $InsP_3$ concentration in rat hepatocyte suspensions. Purified hepatocytes (30 mg/ml) were suspended in a Hepes-buffered saline containing 4 mM glucose, 2 mM lactate and 0.2 mM pyruvate. After a 10-min incubation with or without 100 nM PMA or 4αPDD, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM), noradrenaline (NA, 100 nM) or glucagon (1 nM) were added. The incubation was stopped with trichloroacetic acid at −1, and 30 min for glucose and at 30 s for $InsP_3$ determination or by freezing in liquid nitrogen after 2 min for the determination of glycogen phosphorylase. The increases in glucose output, glycogen phosphorylase and $InsP_3$ are shown. Basal values were: glucose output 0.5 μmol × min^{-1} × g wet $mass^{-1}$; glycogen phosphorylase, 4.2 U × g^{-1}; $InsP_3$ 0.2 nmol × g^{-1}. For PMA-treated cells these values were 0.64 μmol × min^{-1} × g^{-1}, 3.1 U × g^{-1} and 0.2 nmol × g^{-1}, respectively. The values shown are means ± SEM of six controls and three experiments each with PMA or 4αPDD. Statistics: Student's *t*-test for unpaired samples compared to control: a = $P < 0.05$.

gon were not significant either in the presence or the absence of PMA or of 4αPDD (Fig. 2). Thus, the interruption of the signal chain by PMA apparently was not an unspecific side effect of the phorbol ester but depended on its capability to activate protein kinase C.

$InsP_3$ formation could also be stimulated unspecifically by AlF_4^-, which activates phospholipase C by a receptor-independent G-protein mediated mechanism [27]. The AlF_4^--elicited increase in $InsP_3$ formation was not attenuated in PMA-treated hepatocytes (Table 2). This is in contrast to previous findings, that the AlF_4^--mediated increase in $InsP_3$ formation and internal Ca^{2+} concentration was inhibited by PMA pretreatment [27] in hepatocytes. There are, however, other reports [28] that in chromaffin cells the AlF_4^--elicited increase in$InsP_3$ formation was not attenuated by PMA treatment. In the current investigation, the increase in $InsP_3$ formation by AlF_4^- in PMA-treated hepatocytes can only be taken as an indication that the activation of protein kinase C did not inactivate phospholipase C (cf. Fig. 4).

Table 2. Increase in $InsP_3$ formation by AlF_4^- in control and PMA-treated hepatocytes. Purified hepatocytes (30 mg/ml) were suspended in a Hepes-buffered saline containing 4 mM glucose, 2 mM lactate and 0.2 mM pyruvate. After a 10-min incubation with or without 100 nM PMA, NaF and $KAl(SO_4)_2$ were added to final concentrations of 10 mM and 100 μM, respectively. The incubation was stopped with trichloroacetic acid at 30 s for inositol 1,4,5-trisphosphate ($InsP_3$) determination. The increase of $InsP_3$ was calculated as the mean of the differences between AlF_4^--stimulated and control value in every experiment. Values are means ± SEM of six experiments. Statistical analysis with Student's *t*-test for unpaired samples. + = not significantly different from control, $P > 0.05$; * = significantly higher than basal, $P < 0.01$.

Addition	PMA	$InsP_3$ level	
		basal	increase over basal
		nmol · g^{-1}	
None	−	0.76 ± 0.12	−
	+	0.49 ± 0.08 +	−
AlF_4^-	−	1.75 ± 0.12 *	0.99 ± 0.19
	+	1.42 ± 0.08 *	0.93 ± 0.12 +

$PGF_{2\alpha}$ binding to plasma membranes in PMA-treated hepatocytes

In order to examine whether the binding constants of the $PGF_{2\alpha}$ receptor in hepatocytes might be altered by PMA, saturation binding assays were performed with plasma membranes obtained from control and PMA-treated hepatocytes. Binding studies could not be performed with intact cells, since considerable trapping of the ligand within the cells occurred even at 4 °C, this being tenfold higher than the specific binding of $PGF_{2\alpha}$ to surface receptors (not shown).

Saturation binding studies with purified hepatocyte plasma membranes yielded non-linear Scatchard plots. Non-linear regression analysis [25] of pooled data of seven experiments of independent plasma membrane preparations revealed the existence of a high-capacity, low-affinity and a low-capacity, high-affinity binding site for $PGF_{2\alpha}$ in control plasma membranes. The B_{max} of the high-capacity binding site was 570 fmol/mg membrane protein with a K_d of 620 nM. The B_{max} of the low-capacity binding site was 3 fmol/mg membrane protein, with a K_d of about 1 nM (Fig. 3).

Treatment of the hepatocytes with PMA prior to the purification of the plasma membranes left the B_{max} and the K_d of the high-capacity, low-affinity binding site unaffected; for the low-capacity, high-affinity site, the increase in values was not significant (Fig. 3). PMA as such did not interfere directly with the binding of $PGF_{2\alpha}$ (not shown). There was no difference whether plasma membranes were purified in presence or absence of NaF to inhibit phosphatases (not shown). Thus, activation of protein kinase C by PMA did not affect $PGF_{2\alpha}$ binding to the high-capacity, low-affinity binding site, the putative receptor (cf. Fig. 4).

GTP[S] enhanced $PGF_{2\alpha}$ binding, if the $PGF_{2\alpha}$ concentration was close to the K_d of the high-capacity, low-affinity site, i.e. 200 nM (Table 3), but not when the $PGF_{2\alpha}$ concentration was close to the K_d of the low-capacity, high-affinity site [29]. $PGF_{2\alpha}$ binding was enhanced by GTP[S] in plasma membranes of control and PMA-treated hepatocytes to a similar extent (Table 3). This indicated that the connection be-

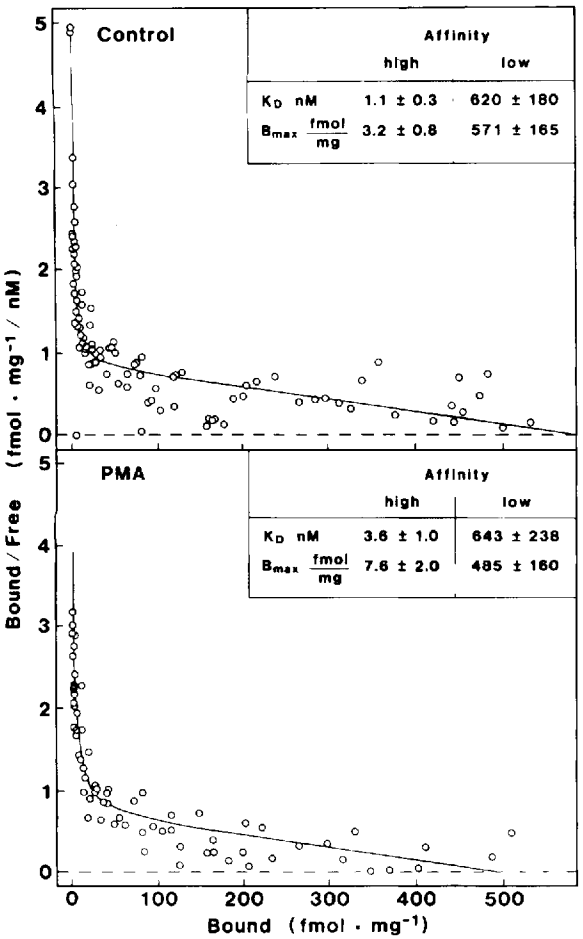

Fig. 3. Scatchard analysis of the binding of $PGF_{2\alpha}$ to hepatocyte plasma membranes from PMA-treated hepatocytes. Control or PMA-treated hepatocytes were homogenized in the presence of 50 mM NaF to inhibit phosphatases. Plasma membranes were purified from the homogenates by centrifugation on sucrose gradients. Binding data were determined in saturation binding assays. The binding data were analysed using the program LIGAND [25], K_d and B_{max} are given as means ± SEM. The Scatchard plots shown are pooled data fits of seven separate plasma membrane preparations of controls and five separate plasma membrane preparations of PMA-treated hepatocytes.

Table 3. Increase in $PGF_{2\alpha}$ binding by GTP[S] in hepatocyte plasma membranes of control and PMA-treated cells. Rat hepatocyte plasma membranes were prepared as described in the legend to Fig. 3. They were incubated with 200 nM [^{3}H]$PGF_{2\alpha}$ (0.10 TBq/mmol) with or without 100 μM GTP[S]. Unspecific binding was determined by including a 5000-fold excess of unlabelled $PGF_{2\alpha}$ to the reaction mixture. The difference between total and unspecific binding was defined as specific binding. Values are means ± SEM of five experiments. Statistical analysis with Student's *t*-test for unpaired samples. + = not significantly different from control, $P > 0.05$; * = significantly higher than without GTP[S], $P < 0.01$.

Additions	Specific $PGF_{2\alpha}$ binding	
	Control	+PMA
	dpm	
None	316 ± 102	328 ± 53 +
GTP[S]	668 ± 32 *	761 ± 125 + *

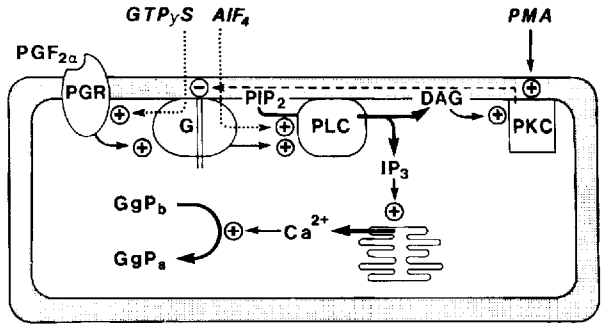

Fig. 4. Hypothetical signal chain from $PGF_{2\alpha}$ to glycogen phosphorylase and the site of its interruption by PMA. DAG, diacylglycerol; G, G-protein linked to the $PGF_{2\alpha}$-receptor and phospholipase C; GgPa, glycogen phosphorylase a; GgPb, glycogen phosphorylase b; $GTP_\gamma S$, guanosine 5′-*O*-(3-thio)triphosphate; IP_3, 1,4,5-inositol trisphosphate; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGR, prostaglandin $F_{2\alpha}$ receptor; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PIP_2, phosphatidyl inositolbisphosphate.

tween the putative receptor and the G-protein was not interrupted by the activation of protein kinase C (cf. Fig. 4).

DISCUSSION

Physiological significance of a protein-kinase-C-mediated interruption of the $PGF_{2\alpha}$ signal chain

Metabolism

Activation of protein kinase C by short-term incubation with the phorbol ester PMA interrupted the $PGF_{2\alpha}$ and noradrenaline signal chains, which increased InsP_3 formation, glycogen phosphorylase activity and glucose output in perfused rat liver and isolated hepatocytes. Yet, PMA incubation did not interfere with the cAMP-mediated metabolic effects of glucagon. A protein-kinase-C-elicited interruption by phosphorylation of one or more proteins of the signal chain leading to an increase in InsP_3 might constitute a physiological feedback loop [17], since the natural protein kinase C activator, diacylglycerol, is released concomitantly with InsP_3. Such a putative feedback inhibition mechanism has also been observed with two other hormones that mediate their signal via an increase in InsP_3: vasopressin [17] in hepatocytes and bradykinin in the smooth muscle cell line MF-2 [30]. In perfused liver the α_1-receptor-mediated increase in glucose and lactate output elicited by nerve stimulation and noradrenaline was inhibited by incubation with noradrenaline or adrenaline [31]. The inhibition could be partially reversed if, during the preincubation period, the protein kinase C inhibitor H7 was added to the perfusion medium. The noradrenaline-mediated increases in glucose and lactate output in perfused liver were inhibited by prior application of vasopressin or angiotensin, whereas the glucagon-mediated increase in glucose output remained unaffected (H. Miura, A. Gardemann, A. Hunger and K. Jungermann, unpublished). This feedback loop might thus be operative not only in homologous (noradrenaline vs noradrenaline) but also in heterologous (vasopressin or angiotensin vs noradrenaline) desensitization between different InsP_3-linked signal chains.

Hemodynamics

In perfused liver, flow was reduced by $PGF_{2\alpha}$ and noradrenaline. As has been shown previously [32], the increases

in glucose and lactate output were not secondary to the flow reduction: nifedipine or sodium nitroprusside abolished $PGF_{2\alpha}$- and noradrenaline-mediated flow reduction without affecting the increase in metabolite output. The flow reduction after noradrenaline was abolished by PMA treatment, whereas the $PGF_{2\alpha}$-mediated flow reduction was not significantly affected. Apparently, the mechanisms by which noradrenaline and $PGF_{2\alpha}$ reduced flow were different. This view is supported by the observation that the $PGF_{2\alpha}$-mediated but not the noradrenaline-mediated flow reduction was inhibited by leukotriene and thromboxane receptor antagonists [11, 33]. Possibly the paracrine and autocrine production of other eicosanoids such as thromboxane and leukotrienes, which have previously been shown to reduce flow or increase pressure in perfused liver [12, 13, 34], was involved in flow reduction by $PGF_{2\alpha}$. Eicosanoid receptors on the cells that regulate hepatic blood flow, i.e. vascular smooth muscle cells and/or perisinusoidal cell, the latter of which have recently been demonstrated to respond to thromboxane A_2, and $PGF_{2\alpha}$ with slow contractions [35], may not be inhibited by activation of protein kinase C, so that PMA would not be expected to affect flow reduction by $PGF_{2\alpha}$.

Interruption of prostaglandin signal chains by protein kinase C in other cell types

Interruption by protein kinase C action of a prostaglandin-elicited increase in $InsP_3$ has also been shown in other cell types. In chromaffin cells the PGE_2-induced $InsP_3$ formation was inhibited by treatment of the cells with PMA; as in this study, the maximum specific binding of [^{3}H]PGE_2 was not altered [28]. Likewise, in NIH-3T3 cell lines that had been transfected with oncogenes of the *ras* family and showed an amplification of the protein kinase C activity, the $PGF_{2\alpha}$-mediated increase in $InsP_3$ was attenuated without a reduction of the receptor number or the K_d [36]. The K_d for the $PGF_{2\alpha}$ binding site was 430 nM and thus close to the K_d of the low-affinity binding site reported here.

Site of the protein-kinase-C-mediated interruption of the $PGF_{2\alpha}$ signal chain

Receptor

Binding studies revealed the existence of two $PGF_{2\alpha}$ binding sites, one with high-capacity and low-affinity and the other with low-capacity and high-affinity. The high-capacity, low-affinity binding site had an apparent K_d close to the concentration of $PGF_{2\alpha}$ which caused a half-maximal stimulation of glycogen phosphorylase in hepatocytes [37] and of glucose output in perfused liver [12]. GTP[S] enhanced $PGF_{2\alpha}$ binding to this site but it did not influence $PGF_{2\alpha}$ binding to the other site. The high-capacity, low-affinity site might thus represent the prostaglandin receptor that mediated the $InsP_3$-dependent metabolic alterations in hepatocytes. The low-capacity, high-affinity binding site is most unlikely to represent the functional receptor, since concentrations of $PGF_{2\alpha}$ that already saturate this binding site (100 nM) did not increase glucose output or glycogen phosphorylase activity in hepatocyte suspensions [37]. This is reminiscent of the glucagon receptor system where the low-affinity rather than the high-affinity binding site is linked to the physiologically relevant second messenger cAMP [38, 39]. The portal concentrations of $PGF_{2\alpha}$ are in the range of about 0.5–1 nM [44] and thus below the K_d of the low-affinity site of about 600 nM and the $PGF_{2\alpha}$ concentrations of 2–10 μM [11] that increase glycogen phosphorylase activity. However, locally in the small space of Disse micromolar concentrations may be found: if a non-parenchymal liver cell with a diameter of 8 μm releases 2 amol $PGF_{2\alpha} \times min^{-1}$ (corresponding to 2 pmol × $min^{-1} \times (10^6$ Kupffer cells$)^{-1}$ [40] into the surrounding space of 1 μm width for 1 min, the local concentration can be expected to be about 4 μM [28]. If the prostaglandin were released vectorially only into the space of Disse, the local concentration would be even higher.

Neither the B_{max} nor the K_d of the high-capacity, low-affinity $PGF_{2\alpha}$ binding site were altered by PMA pretreatment. This could indicate that the signal chain was interrupted at a site distal to the $PGF_{2\alpha}$ receptor (Fig. 4). These data are in line with experiments showing that in hepatocytes the vasopressin-mediated increase in $InsP_3$ formation was attenuated by PMA without changing the affinity or density of the receptor [17]. Similarly the stimulation of the glycogen phosphorylase activity by noradrenaline in hepatocytes was inhibited by PMA with no influence of PMA on the binding characteristics of the α_1-receptor [41]. Yet, in other studies, PMA-treatment of hepatocytes has been reported to reduce the affinity of the α_1-receptor [42] as well as the number of binding sites [17, 43]. The same group later showed that both heterologous and homologous desensitization of the α_1-receptor in MT-2 cells were accompanied by a receptor phosphorylation; however, receptor sequestration and thus reduction of the binding sites in plasma membranes occurred only if the receptor was occupied by its ligand in homologous desensitization [30].

There is currently no direct evidence for a function of the high-affinity binding site. Yet, the affinity of this site (1 nM) is in the range of the $PGF_{2\alpha}$ concentrations normally found in the portal vein (0.5 nM) [44]. Prostaglandins are very efficiently removed from the hepatic circulation [44, 45] and the hepatovenous $PGF_{2\alpha}$ concentration is only about 25% of the portal concentration [44]. The high-affinity $PGF_{2\alpha}$-binding site might thus be part of a scavenger system located in the distal perivenous zone [46, 47] that extracts prostaglandins from the hepatic circulation.

Plasma membranes were isolated from purified hepatocytes. Therefore, most likely, both sites were located on hepatocyte plasma membranes. However, a minor contamination with non-parenchymal liver cell plasma membranes cannot be excluded with absolute certainty. Thus, the possibility must be envisaged that the high-affinity binding site, which represents only 1% of the total binding, may be located on non-parenchymal liver cell plasma membranes.

Receptor–G-protein interaction

The enhancement of $PGF_{2\alpha}$ binding to the high-capacity, low-affinity site by GTP[S] indicated that this putative receptor was linked to a G-protein. This increase in binding was found only when the concentration of $PGF_{2\alpha}$ was in the range of the K_d of the low-affinity site but not when it was in the range of the high-affinity site [29]. Frequently, a decrease of binding of ligands by GTP[S] has been reported that is due to a shift from a high-affinity state of a receptor to a low-affinity state of a receptor. Such a shift cannot be the cause for the twofold increase in low-affinity binding of $PGF_{2\alpha}$ by GTP[S], since there were only 3.2 fmol/mg high-affinity site but 629 fmol/mg low-affinity site (Table 3). The data can only be taken as an indication that the influence of GTP[S] on $PGF_{2\alpha}$ binding, and thus the interaction between a putative

G-protein and the putative receptor was not affected by PMA treatment. This finding indicates that the signal chain was not interrupted between the receptor and the G-protein but probably at a more distal site (Fig. 4). Similar findings were reported for the muscarinic receptor in astrocyte membranes [48] and the PGE_2 receptor in chromaffin cells [28].

Glycogen phosphorylase and phospholipase C

The regulation of the glycogen phosphorylase activity was not impaired in PMA-treated cells, since its activity could still be increased by 1 nM glucagon to the same extent as in control cells. However, PMA treatment partially inhibited the increase in glycogen phosphorylase activity by 0.1 nM and 0.01 nM glucagon, concentrations at which glucagon has been proposed to act at least partially via a cAMP-independent Ca^{2+}, $InsP_3$-dependent signal chain [38]. These results are in line with previous reports [28, 49] that the glucagon-mediated increase in Ca^{2+} and $InsP_3$ were inhibited by PMA treatment of hepatocytes without affecting the glycogen phosphorylase activity or cAMP formation at glucagon concentrations ≥ 1 nM [17, 28]. The $PGF_{2\alpha}$-mediated stimulation of $InsP_3$ formation was abolished by PMA treatment of the hepatocytes, whereas the unspecific receptor-independent stimulation of $InsP_3$ formation by high concentrations of AlF_4^- was not affected. Therefore, activation of protein kinase C must have interrupted the $PGF_{2\alpha}$ signal chain at a point proximal to phospholipase C (Fig. 4).

Conclusion

It appears that activation of protein kinase C interrupted the $PGF_{2\alpha}$ signal chain at a site distal to the interaction between the receptor and the G-protein and proximal of the phospholipase C. Such a site might be the receptor-dependent G-protein-mediated activation of phospholipase C.

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