A comparative study of endothelin- and platelet-activating-factor-mediated signal transduction and prostaglandin synthesis in rat Kupffer cells

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Endothelin-3 (ET-3) stimulated phosphoinositide metabolism and synthesis of prostaglandins in cultured rat Kupffer cells. ET-3-induced hydrolysis of phosphoinositides was characterized by the production of various inositol phosphates and of glycerophosphoinositol. The mechanism of ET-3-stimulated metabolism of phosphoinositides and synthesis of prostaglandins appeared to be distinct from the effect of platelet-activating factor (PAF) on these processes described previously [Gandhi, Hanahan & Olson (1990) *J. Biol. Chem.* **265**, 18234–18241]. On a molar basis ET-3 was significantly more potent than PAF in stimulating phosphoinositide metabolism, e.g. ET-3-induced hydrolysis of phosphoinositides occurred at 1 pM, whereas PAF was ineffective at concentrations less than 1 nM. Upon challenging Kupffer cells with both ET-3 and PAF, an additive stimulation of phosphoinositide metabolism was observed, suggesting that the actions of these factors may be exerted on separate phosphoinositide pools. Treatment of Kupffer cells with pertussis toxin resulted in an inhibition of ET-3-induced phospholipase C activation; in contrast, cholera toxin treatment caused potentiation of ET-3-stimulated phospholipase C activity. Both toxins, however, inhibited PAF-stimulated phospholipase C activity. The present results suggest that the stimulatory effects of ET-3 and PAF on the phosphodiesteric metabolism of phosphoinositides in Kupffer cells require different guanine-nucleotide-binding proteins. Furthermore, the effects of bacterial toxins on ET-3- and PAF-induced phosphoinositide metabolism were not mediated by cyclic AMP. ET-3-induced metabolism of phosphoinositides was inhibited completely in Kupffer cells pretreated with ET-3, suggesting homologous ligand-induced desensitization of the ET-3 receptors. In contrast, similar experiments using PAF showed only a partial desensitization of subsequent PAF-induced phosphoinositide metabolism. In contrast to the increased production of prostaglandins E_2 and D_2 observed upon stimulation of Kupffer cells with PAF, ET-3 stimulated the biosynthesis of prostaglandin E_2 only. Consistent with their additive effects on phosphoinositide metabolism, PAF and ET-3 exhibited an additive stimulation of the synthesis of prostaglandin E_2 .

INTRODUCTION

Several mediators [e.g. eicosanoids and platelet-activating factor (PAF)] and various cytokines are synthesized by the liver in response to various types of stimulation. These mediators are synthesized primarily by endothelial and Kupffer cells, which reside in the hepatic sinusoids (Jones & Summerfield, 1988). Kupffer cells, the resident macrophages of the liver, detoxify endotoxin and phagocytose invading microbes, virus particles and senescent erythrocytes and platelets (Jones & Summerfield, 1988). Various hepatic metabolic events, including biosynthesis of mediators which accompany reticuloendothelial cell activation, are initiated following and hence are regulated by the phosphoinositide signalling cascade.

Endothelin (ET), a potent vasoactive peptide (Yanagisawa *et al.*, 1988; Yanagisawa & Masaki, 1989; Simonson & Dunn, 1990a) and PAF (Snyder, 1982; Vargaftig *et al.*, 1982), an ether-phospholipid autacoid, exhibit profound mediator effects in a variety of tissues. Both ET (Gandhi *et al.*, 1990a) and PAF (Buxton *et al.*, 1984) are potent agonists in the liver, stimulating glycogenolysis as well as causing alterations in oxygen consumption and vasoconstriction of the hepatic vasculature when infused into the perfused rat liver. A significant amount of ET-like immunoreactivity has been detected in the liver (Yoshimi *et al.*, 1989), and perfused rat liver challenged with bacterial endotoxin has been found to synthesize several lipid autacoid

mediators, including PAF (Duffy-Krywicki *et al.*, 1990). Also, endotoxin has been reported to cause increased release of ET into the circulation (Sugiura *et al.*, 1989). Furthermore, the endothelium releases contractile factors in response to anoxic conditions (De Mey & Vanhoutte, 1983; Vanhoutte & Katusis, 1988). Since liver receives nearly 80% of its blood supply from the portal vein, this organ is normally exposed to a somewhat hypoxic environment. Because the liver plays a crucial role in the detoxification of endotoxin, it might be conjectured that during conditions such as endotoxaemia, PAF and ET may play significant roles in intercellular signalling mechanisms in the hepatic responses to shock.

We have characterized PAF-mediated signalling events in cultured hepatic Kupffer cells (Gandhi *et al.*, 1990b; Gandhi & Olson, 1991). Bronchopulmonary effects of ET have been suggested to occur via PAF (Lagente *et al.*, 1989), and this lipid autacoid has also been implicated in ET-induced mortality of animals (Terashita *et al.*, 1989). Therefore it was of interest to characterize intra- and inter-cellular signalling systems in which these two autacoid mediators are involved.

EXPERIMENTAL

Materials

Collagenase (Type IV from *Clostridium histolyticum*), protease (Type XIV from *Streptomyces griseus*), BSA (fraction V), per-

Abbreviations used: ET-3, endothelin-3; GPIs, glycerophosphoinositol; IBMX, isobutylmethylxanthine; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycerophosphocholine); PGD₂, prostaglandin D₂; PGE₂, prostaglandin E₂.

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tussis toxin, cholera toxin, EGTA, phorbol 12-myristate 13-acetate and heat-inactivated fetal bovine serum were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Synthetic PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was obtained from Bachem, Bubendorf, Switzerland, and metrizamide [2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose] was from Nyegaard and Co., Oslo, Norway. *myo*-[2-³H]Inositol (20 Ci/mmol) and [5,6,8,9,11,12,14,15-³H]arachidonic acid (100 Ci/mmol) were purchased from Amersham, New York, NY, U.S.A., and DuPont, Boston, MA, U.S.A. respectively. ET-3 was purchased from Peninsula Laboratories, Belmont, CA, U.S.A. The stock solution of ET (10 μ M) was prepared in phosphate-buffered saline containing 0.2% BSA (fraction V) and stored in aliquots at -70 °C. All other chemicals and reagents were of the highest purity available.

Isolation and culture of Kupffer cells

Kupffer cells were isolated from livers of male Sprague-Dawley rats (200–250 g) using a modification of the centrifugal elutriation procedure of Knook & Sleyster (1976). Briefly, livers were perfused aseptically for 10 min at 37 °C via the portal vein and with perfusate collection via the inferior vena cava at a rate of 30 ml/min. The medium used for this perfusion was calcium- and magnesium-free Krebs-Henseleit buffer containing 0.2% glucose and 0.2% BSA, gassed with O₂/CO₂ (19:1). This initial perfusion interval was followed by a period of 5 min of recirculating perfusion with the same buffer containing 0.03% collagenase and 0.02% protease in the presence of 2 mM-Ca²⁺. Following digestion of the liver the perfusion was stopped, the liver capsule was opened and the cells were dispersed in Krebs-Henseleit buffer. The cell suspension was filtered through two layers of nylon mesh and the filtrate was centrifuged at 525 *g* for 45 s, followed by centrifugation of the supernatant at 830 *g* for 6 min. The pellet was suspended in Gey's balanced salt solution (GBSS) without NaCl containing 17.5% metrizamide. GBSS (1 ml) was layered over 6 ml of the above cell suspension and, after centrifugation for 20 min at 1400 *g*, non-parenchymal cells which collected at the interphase were aspirated and washed with Krebs-Henseleit buffer. Kupffer cells and endothelial cells were separated by centrifugal elutriation as described (Knook & Sleyster, 1976).

Primary culture of Kupffer cells

Kupffer cells were suspended in William's medium E supplemented with 2 mM-L-glutamine, 10% heat-inactivated fetal calf serum, 5000 units of penicillin/ml and 5000 μ g of streptomycin/ml. Portions of 2 ml (3×10^6 cells) of this cell suspension were plated on 35 mm-diam. tissue culture dishes and placed in an incubator at 37 °C in an atmosphere of O₂/CO₂ (19:1). Cells were allowed to attach for 2–3 h and were washed with incubation medium. After an overnight incubation, the attached cells were washed twice and placed in fresh medium containing 2% fetal calf serum and [³H]arachidonic acid (for determination of prostaglandins) or [³H]inositol (for determination of inositol phosphates). Following incubation for 24 h, the cells were washed twice with non-radioactive medium and stimulated with the various agonists. Reactions were terminated with the addition of ethanol for the determination of prostaglandins (Powell, 1982) or with ice-cold 5% trichloroacetic acid after rapidly aspirating the medium for the analysis of inositol phosphates.

Extraction of eicosanoids

Eicosanoids were extracted essentially as described by Powell (1982). Cells were scraped from the culture plates and both the medium and cells were transferred quantitatively to tubes. The

plates were washed with an additional 1 ml of ethanol and water was added to the combined extracts to bring the final concentration of ethanol to 15%. The extract was centrifuged and the pellet was washed with 3–4 ml of 15% ethanol. The combined supernatants were acidified to pH 3.5 with 1 M-HCl, applied to Sep-Pak columns (C₁₈ cartridges from Waters Associates) and washed with 15% ethanol, and finally eicosanoids were eluted with methyl formate.

Analysis of prostaglandins

Methyl formate was evaporated under N₂, and the residue was dissolved in 140 μ l of 36% acetic acid (0.1%, v/v) in acetonitrile and filtered (0.45 μ m Millipore Type HV filter). The filtrate (80–90 μ l) was used for the analysis of [³H]eicosanoids by h.p.l.c. The analysis of arachidonic acid metabolites was performed essentially as described by Peters *et al.* (1983) using a Varian h.p.l.c. with an Ultrasphere ODS column (4.6 mm $\times$ 25 cm). The solvent system used was 0.1% (v/v) aqueous acetic acid (pH 3.7) and acetonitrile, with a flow rate of 1.5 ml/min. Fractions (30 s) were collected and quantified by liquid scintillation spectroscopy. Various eicosanoids were identified by spiking the cell extracts with a mixture of [³H]eicosanoid standards.

Extraction and identification of [³H]inositol phosphates

Cells were scraped from culture plates and the plates were washed with an additional 0.5 ml of trichloroacetic acid. The combined extracts were centrifuged and the supernatant was washed with water-saturated ether, lyophilized and stored at -70 °C. [³H]Inositol phosphates were analysed by h.p.l.c. essentially as described by Dean & Moyer (1987). The identities of various inositol phosphate species were confirmed by spiking cell extracts with a mixture of [³H]inositol phosphate standards.

RESULTS

ET-3-induced metabolism of phosphoinositides: dose-response relationship and Ca²⁺ requirement

ET has been shown to stimulate the hydrolysis of phosphoinositides, intracellular Ca²⁺ mobilization and the influx of extracellular Ca²⁺ in various cells and tissues (Marsden *et al.*, 1989; Takuwa *et al.*, 1989a,b; Kuraja & Woodcock, 1990; Resink *et al.*, 1990; Lin *et al.*, 1990; Vigne *et al.*, 1990). Fig. 1 illustrates the effects of increasing concentrations of ET-3 on phosphoinositide metabolism via both phospholipase C and deacylation in primary cultures of rat Kupffer cells. Significant stimulation of phosphoinositide metabolism by ET-3 occurred at concentrations as low as 1 pM. It should be noted that several Ca²⁺-mobilizing glycogenolytic agents which stimulate phosphoinositide metabolism in isolated hepatocytes (Williamson *et al.*, 1985; Williamson, 1986) are not capable of stimulating phospholipase C action in Kupffer cells (Gandhi & Olson, 1991). Furthermore, PAF-induced metabolism of phosphoinositides did not occur at concentrations less than 1 nM. Thus ET appears to be a very potent agonist, capable of stimulating the phosphoinositide cascade in cultured Kupffer cells.

In Kupffer cells the production of InsP₃ and InsP₂, which occurred within seconds of stimulation by ET, was not inhibited in the presence of EGTA (Table 1); however, ET-stimulated InsP₁ and InsP₂ formation over longer intervals (i.e. 20 min) was inhibited significantly by EGTA or low Ca²⁺ concentrations in the extracellular medium (Table 1). This type of effect on the ET-3-mediated signalling response to manipulation of the extracellular Ca²⁺ concentration is similar to that observed for PAF-induced phosphoinositide metabolism in Kupffer cells (Gandhi *et al.*, 1990b; Gandhi & Olson, 1991) and is consistent with the

Fig. 1. Effect of ET-3 concentration on the metabolism of phosphoinositides in Kupffer cells

Kupffer cells in primary culture were washed and placed in 2 ml of William's medium E containing 2% fetal calf serum and 5 μ Ci of [3 H]inositol/ml. After labelling for 24 h, cells were washed and placed in a medium without radioactive inositol and containing 10 mM-LiCl. After 15 min, ET-3 at the indicated concentrations was added and the reaction was terminated at 20 min by rapidly aspirating the medium and adding trichloroacetic acid. Values represent averages $\pm$ S.E.M. of duplicate determinations from a representative of four separate experiments. For other details, see the Experimental section. * P < 0.05, ** P < 0.01 and *** P < 0.005 compared with controls.

existence of two separate phases of ET-3-induced phosphoinositide metabolism. It is known that various inositol phosphate(s) phosphatases when Li⁺ is added to the incubation medium (Storey *et al.*, 1984; Burges *et al.*, 1985; Hansen *et al.*, 1986; Turk *et al.*, 1986). In the present study, Li⁺ did not affect the formation of Ins(1,4,5) P_3 and Ins(1,4) P_2 upon hydrolysis of PtdIns(4,5) P_2 and PtdIns4 P_1 respectively in stimulated Kupffer cells at short time intervals, e.g. 15 s (results not shown). At later times, however, the levels of inositol phosphates returned rapidly to the basal values when the experiments were performed in the absence of Li⁺ (C. R. Gandhi, K. Stephenson & M. S. Olson, unpublished work; see also Fig. 7, in which, for the homologous ligand-induced down-regulation experiments, cells were incubated with ET-3 or PAF in the absence of Li⁺). Therefore Li⁺ was added to all assays described in this investigation.

Additive stimulation of phosphoinositide metabolism and prostaglandin (PG) synthesis by ET-3 and PAF

Experiments were designed to elucidate possible interactions between ET- and PAF-induced stimulation of phosphoinositide metabolism in Kupffer cells. As depicted in Fig. 2, ET-3 and PAF had an additive effect on the release of glycerophosphoinositol (GPIIns) as well as on the phosphodiesteric hydrolysis of phosphoinositides. These results infer that PAF and ET-3 stimulate the metabolism of separate pools of phosphoinositides in Kupffer cells.

Table 1. Effect of Ca²⁺ on ET-3-stimulated phosphoinositide metabolism in Kupffer cells at 15 s and 20 min

Kupffer cells in primary culture were labelled with 5 μ Ci of [3 H]inositol/ml for 24 h as described in the Experimental section. Cells were washed and placed in Hanks' balanced salt solution (HBSS, in mM: NaCl, 137; KCl, 5.4; KH₂PO₄, 0.44; Na₂HPO₄, 0.33; MgSO₄, 0.4; MgCl₂, 0.5; CaCl₂, 1.2; NaHCO₃, 4.0; buffered with 20 mM-Hepes, pH 7.4, containing 0.2% BSA) (control and ET-3), in HBSS in which Ca²⁺ was adjusted to 0.13 μ M [37.5 μ M-Ca²⁺ + 100 μ M-EGTA; Bartfai (1979); Gandhi & Ross (1988)] or in HBSS containing 5 mM-EGTA. Cells were then incubated with 25 nM-ET-3 for 15 s or 20 min in the presence of 10 mM-Li⁺. The reaction was terminated with trichloroacetic acid as described and [3 H]inositol phosphates were separated by h.p.l.c. The results described are averages of duplicate determinations $\pm$ S.E.M. from a representative of three to four identical experiments.

	Inositol phosphates (d.p.m./plate)			
	Control	ET-3-stimulated		
		1.2 mM-Ca ²⁺	130 nM-Ca ²⁺	+ EGTA
15 s				
Ins P_1	560 $\pm$ 49	620 $\pm$ 57	596 $\pm$ 38	539 $\pm$ 22
Ins(1,4) P_2	135 $\pm$ 13	655 $\pm$ 43	696 $\pm$ 52	623 $\pm$ 36
Ins(1,4,5) P_3	55 $\pm$ 4	201 $\pm$ 15	223 $\pm$ 23	198 $\pm$ 19
20 min				
Ins P_1	583 $\pm$ 37	5918 $\pm$ 163	1321 $\pm$ 135	1098 $\pm$ 41
Ins P_2	128 $\pm$ 9	2342 $\pm$ 59	1019 $\pm$ 125	782 $\pm$ 62
Ins P_3	48 $\pm$ 3	393 $\pm$ 23	318 $\pm$ 8	279 $\pm$ 70

Fig. 2. Additive effects of PAF and ET-3 on the metabolism of phosphoinositides in Kupffer cells

Kupffer cells in primary culture were labelled with 5 μ Ci of [3 H]inositol/ml for 24 h. After washing, the cells were challenged with 25 nM-ET-3, 25 nM-PAF or a mixture of PAF and ET-3 in the presence of 10 mM-LiCl, and the reaction was terminated at 20 min. For other details, see the Experimental section and the legend for Fig. 1. Values represent means $\pm$ S.E.M. of triplicate determinations from a representative of four separate experiments. * P < 0.01, ** P < 0.005 compared with control; † P < 0.02, †† P < 0.01 compared with ET-3 or PAF alone.

Kupffer cells influence the metabolism of other hepatic cell types by synthesizing and releasing several mediators, including PGs (Jones & Summerfield, 1988). ET has been shown to stimulate the synthesis of PGs in various cell types (De Nucci *et al.*, 1988; Reynolds *et al.*, 1989; Resink *et al.*, 1989; Simonson & Dunn, 1990b). Fig. 3 compares the effects of PAF and ET-3 on the synthesis of PGs in cultured Kupffer cells. The major difference in the action of these two agonists appears to be in their specificity towards the biosynthesis of individual prostanooids. PAF stimulated the biosynthesis of both PGD₂ and PGE₂, whereas ET-3 specifically stimulated the synthesis of PGE₂ only. Furthermore, consistent with the effects of these

Fig. 3. Effects of PAF and ET-3 on PG synthesis in Kupffer cells

Kupffer cells in primary culture were labelled with 0.5 μ Ci of [3 H]arachidonic acid/ml for 24 h, washed and placed in non-radioactive medium. After 15 min of equilibration in an incubator, cells were challenged with 25 nM-ET-3, 25 nM-PAF or a mixture of both. The reaction was terminated at 20 min with the addition of ethanol, and PGs were analysed by h.p.l.c. Each value is the mean $\pm$ S.E.M. of duplicate determinations from a representative of three experiments. * P < 0.01, ** P < 0.001 compared with control; † P < 0.02 or < 0.05 compared with ET-3 and PAF alone respectively.

agonists on the metabolism of phosphoinositides, an additive effect of PAF and ET-3 was observed on PGE₂ synthesis in Kupffer cells.

Differential effects of cholera toxin on the ET-3- and PAF-induced metabolism of phosphoinositides

Many receptor-mediated transmembrane signalling events, including the metabolism of phosphoinositides, occur via the activation of guanine-nucleotide-binding proteins (G-proteins) (Gilman, 1987), and these processes are frequently sensitive to cholera toxin and pertussis toxin (Gilman, 1987; Williamson, 1986). We have reported differential effects of cholera toxin on PAF-induced deacylation and phosphodiesteric hydrolysis of phosphoinositides in Kupffer cells, although pertussis toxin inhibited both of these processes (Gandhi *et al.*, 1990b). The results illustrated in Fig. 2 suggest that PAF- and ET-3-induced signal transduction may be coupled via different G-proteins. To evaluate the role of G-protein coupling in ET-induced phosphoinositide metabolism, Kupffer cells were first incubated with cholera toxin or pertussis toxin and then were challenged with ET-3. As illustrated in Fig. 4(a) and 4(b), pertussis toxin inhibited ET-3-induced phosphoinositide metabolism, both at an early time point (i.e. 15 s, extracellular-Ca²⁺-independent) and after 20 min (extracellular-Ca²⁺-dependent), consistent with the effect of pertussis toxin on these processes when PAF was employed as the agonist (Gandhi *et al.*, 1990b). In contrast, cholera toxin potentiated the ET-3-stimulated production of inositol phosphate(s) at both 15 s and 20 min (Fig. 4). In our previous study, it was demonstrated that PAF-mediated phosphodiesteric hydrolysis of phosphoinositides in Kupffer cells was inhibited by cholera toxin (Gandhi *et al.*, 1990b). Also, a cholera toxin-induced increase in the production of GPIIns in Kupffer cells was apparent (Gandhi *et al.*, 1990b). As shown in Fig. 4(b), addition of ET-3 to cholera toxin-treated cells caused an increase in GPIIns release greater than that observed with ET-3 alone, suggesting that this toxin had no effect on the deacylation process stimulated by ET-3.

Effect of cyclic AMP on the ET-3- and PAF-induced metabolism of phosphoinositides

Metabolic events in a cell in response to various agents are

Fig. 4. Effects of cholera toxin and pertussis toxin on ET-3-induced metabolism of phosphoinositides in Kupffer cells

Following a 24 h incubation of Kupffer cells in the presence of 5 μ Ci of [3 H]inositol/ml, the cells were washed and then incubated in the presence of 2.5 μ g of cholera toxin (CTX)/ml or 50 ng of pertussis toxin (PTX)/ml. After 3 h, the cells were challenged with 25 nM-ET-3 in the presence of 10 mM-LiCl. The reaction was terminated at 15 s (a) or 20 min (b) and the inositol phosphates were analysed as described. For other details see the Experimental section. Each value is the mean $\pm$ S.E.M. of duplicate determinations from a representative of three experiments. * P < 0.02, ** P < 0.001 compared with control; † P < 0.05, †† P < 0.02 and ††† P < 0.01 respectively compared with ET-3.

Table 2. Changes in cyclic AMP levels in Kupffer cells in response to bacterial toxins, PAF, ET-3 and forskolin

Kupffer cells in primary culture (2×10^6 cells/plate) were washed and placed in William's medium E containing 2% fetal calf serum. The cells were then challenged with various agents at the indicated concentrations, for 20 min except for cholera toxin and pertussis toxin (3 h incubation) in the presence or absence of 0.5 mM-IBMX. At the end of the incubation period the medium was aspirated and 0.5 ml of ice-cold 5% trichloroacetic acid was added to the plates. After scraping, the plates were washed with additional 0.5% trichloroacetic acid. The combined extracts were centrifuged and the supernatants were washed several times with water-saturated ether. The cyclic AMP content in the extracts was then determined by radioimmunoassay (Amersham). The values are the means $\pm$ S.E.M. of triplicate determinations from a representative experiment. * P < 0.005 compared with control.

Treatment	Cyclic AMP (pmol/plate)	
	+ IBMX	- IBMX
Control	2.75 $\pm$ 0.08	1.38 $\pm$ 0.19
PAF (25 nM)	2.72 $\pm$ 0.19	1.28 $\pm$ 0.06
ET-3 (25 nM)	2.73 $\pm$ 0.02	1.33 $\pm$ 0.32
Pertussis toxin (50 ng/ml)	2.67 $\pm$ 0.24	1.41 $\pm$ 0.33
Cholera toxin (2.5 μ g/ml)	7.84 $\pm$ 0.64*	2.13 $\pm$ 0.43
Forskolin (100 μ M)	24.8 $\pm$ 2.84*	9.22 $\pm$ 0.39*

Fig. 5. Effect of cyclic AMP on ET-3 and PAF-induced metabolism of phosphoinositides in Kupffer cells

Following a 24 h incubation of Kupffer cells in the presence of $5 \mu\text{Ci}$ of $[^3\text{H}]$ inositol/ml, the cells were washed and incubated in the presence or absence of 2 mM-dibutyl cyclic AMP (dbcAMP). To one set of plates $100 \mu\text{M}$ -forskolin was added 15 min prior to stimulation with 25 nM-ET-3 or 25 nM-PAF. The reaction was terminated at 20 min. Each value represents the mean $\pm$ S.E.M. of duplicate determinations from a representative of three or four experiments. Essentially similar results were obtained when the reaction was terminated at 15 s. * $P < 0.05$, ** $P < 0.01$ compared with control; † $P < 0.05$, †† $P < 0.02$ compared with ET-3 alone; ‡ $P < 0.01$ compared with PAF.

exerted via the production of intracellular second messengers; it is likely that such second messengers influence complex cellular metabolic events by interacting with different signal transduction mechanisms elicited by the individual agonists. Cholera toxin and pertussis toxin are known to interact with the G-proteins coupled to adenylate cyclase in many cells, elevating cellular cyclic AMP levels (Gilman, 1987). Therefore it was important to ascertain whether cholera toxin and pertussis toxin stimulate cyclic AMP synthesis in Kupffer cells and thus exert their effects on ET- and PAF-induced phosphoinositide metabolism via this second messenger. The results of these experiments are depicted in Table 2 and Fig. 5. In cells incubated with dibutyl cyclic AMP as well as with forskolin, an agent which stimulates cyclic AMP synthesis (Table 2), both ET-3- and PAF-induced phosphodiesteric hydrolysis of phosphoinositides was strongly attenuated. In contrast, production of GPIs stimulated by either of these agonists was not affected by cyclic AMP treatment or by forskolin (Fig. 5). Cholera toxin, which potentiated ET-3-induced phospholipase C activity, caused an increase in cyclic AMP levels in Kupffer cells (Table 2), whereas pertussis toxin did not alter cyclic AMP levels but strongly inhibited phospholipase C activity. Furthermore, cholera toxin caused significant increase in cyclic AMP levels in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Neither ET-3 nor PAF elicited any significant change in the cyclic AMP levels in Kupffer cells (Table 2).

Fig. 6. Effect of heterologous ligand-induced down-regulation on PAF- or ET-3-mediated metabolism of phosphoinositides in Kupffer cells

Kupffer cells were labelled with $2.5 \mu\text{Ci}$ of $[^3\text{H}]$ inositol/ml for 24 h, then washed and incubated in the presence of 25 nM-ET-3 (▨), 25 nM-PAF (■) or the carrier (□) for 3 h. The cells were then washed extensively and placed in a medium containing 10 mM-LiCl. After 15 min, the reaction was initiated with the addition of 25 nM-ET-3 or -PAF and terminated at 20 min. Values represent means $\pm$ S.E.M. of duplicate determinations from a representative experiment. * $P < 0.01$ and ** $P < 0.005$ compared with control.

Effects of PAF- and ET-3-mediated desensitization of the phosphoinositide signalling response

Experiments were designed to ascertain whether the actions of PAF and ET-3 on the hydrolysis of phosphoinositides in Kupffer cells were independent of each other. The data shown in Fig. 6 indicate that preincubation of Kupffer cells with either agonist had no effect on the metabolism of phosphoinositides induced by the other agonist. These results are consistent with the observations reported previously that PAF did not affect glycogenolysis, oxygen consumption or vasoconstriction of the hepatic vasculature induced by ET-3, or vice versa, in the isolated perfused rat liver (Gandhi *et al.*, 1990a).

Effects of preincubation with ET-3 or PAF on subsequent stimulation of phosphoinositide metabolism with homologous ligand

Repeated bolus infusion of ET-3 into the perfused rat liver resulted in a diminished glycogenolytic response but had little effect on the usual vasoconstrictive response to ET-3 (Gandhi *et al.*, 1990a). Also, it has been demonstrated that PAF-induced hepatic responses can be desensitized to subsequent PAF challenges (Buxton *et al.*, 1984). Therefore it was of interest to determine how Kupffer cells exposed to PAF or ET-3 respond to a subsequent challenge with the same agonist. Kupffer cells in primary culture labelled with $[^3\text{H}]$ inositol were washed and then incubated with ET-3 or PAF for 3 h. Following this treatment, the cells were washed extensively and challenged with the same agonist. It is evident from Fig. 7(a) that preincubation of Kupffer cells with ET-3 produced a strong attenuation of a subsequent ET-3-induced hydrolysis of phosphoinositides, suggesting some type of desensitization or regulation of ET-3 receptors. Preincubation of Kupffer cells with PAF partially desensitized the subsequent PAF-induced metabolism of phosphoinositides (Fig. 7b).

DISCUSSION

The present investigation describes similarities and differences between the effects of ET and PAF on phosphoinositide metabolism and PG synthesis in cultured Kupffer cells. Like PAF

Fig. 7. Homologous ligand-induced desensitization of PAF- and ET-3-mediated metabolism of phosphoinositides in Kupffer cells

Kupffer cells were labelled with 2.5 μ Ci of [3 H]inositol/ml for 24 h. After washing, the cells were incubated in the presence of carrier ($\square$) or 25 nM-ET-3 (a) or PAF (b) ($\blacksquare$) for 3 h. The cells were then washed extensively and placed in a medium containing 10 mM-LiCl; 15 min later the reaction was initiated with 25 nM-ET-3 (a) or PAF (b), and was terminated at 20 min with trichloroacetic acid. For other details, see the Experimental section. The values represent means $\pm$ S.E.M. of duplicate determinations from a representative experiment. * P < 0.05, ** P < 0.02, *** P < 0.01; † P < 0.005, †† P < 0.001 compared with control.

(Gandhi *et al.*, 1990b), ET-3 stimulated phosphodiesteric hydrolysis as well as deacylation of phosphoinositides in cultured Kupffer cells. On a molar basis ET-3 was more potent in eliciting these responses (Fig. 1) than PAF (Gandhi *et al.*, 1990b). Although both PAF (Buxton *et al.*, 1984) and ET-3 (Gandhi *et al.*, 1990a) exert glycogenolytic effects in the perfused liver, it should be noted that ET-3 (Serradeil-Le Gal *et al.*, 1991) but not PAF (Fisher *et al.*, 1984) stimulates conversion of glycogen phosphorylase from its inactive into its active form in isolated hepatocytes. Furthermore, ET-3 stimulates phospholipase C- and phospholipase A₂-mediated phosphoinositide metabolism in isolated hepatocytes (Gandhi *et al.*, 1990a), whereas PAF-induced metabolism of phosphoinositides occurs via phospholipase A₂ only (Shukla *et al.*, 1983; Gandhi & Olson, 1991). Hence, in certain respects, ET may be a more versatile agonist in the liver than PAF.

One of the functional responses that Kupffer cells exhibit to a variety of stimuli is the production of eicosanoids (Dieter *et al.*, 1986; Jones and Summerfield, 1988). In contrast to PAF, which stimulates the synthesis of both PGD₂ and PGE₂ in cultured Kupffer cells prelabelled with [3 H]arachidonic acid (Gandhi *et al.*, 1989; Gandhi & Olson, 1991), ET-3 specifically stimulated the biosynthesis of PGE₂ but not of PGD₂ (Fig. 3). Moreover, incubation of Kupffer cells with both ET-3 and PAF resulted in an additive stimulation of PGE₂ synthesis, whereas PGD₂ synthesis was not stimulated over that with PAF alone (Fig. 3).

These results are analogous to the additive effects of PAF and ET-3 on phosphoinositide metabolism in the Kupffer cell (Fig. 2). Thus the additive effects of these two agonists on the Kupffer cell infer that PAF and ET-3 act independently of each other in eliciting phosphoinositide metabolism and synthesis of eicosanoids. The effect of ET-3 on the selective stimulation of PGE₂ synthesis and an additive stimulation of PGE₂ synthesis by ET-3 and PAF in Kupffer cells is interesting. Although a definitive role for PGs in the liver has not been precisely defined, PGE₂ has been implicated as a mitogenic agent in cultured hepatocytes (Andreis *et al.*, 1981; Spolarics *et al.*, 1984) and as a vasodilator (Samuelsson *et al.*, 1978).

The results in Fig. 2 suggest that ET-3 and PAF stimulate phosphoinositide metabolism via different G-proteins. Consistent with the effect of pertussis toxin on PAF-mediated phosphoinositide metabolism (Gandhi *et al.*, 1990b), ET-3-induced phosphoinositide metabolism also was inhibited by this toxin (Fig. 4). Considering the inability of pertussis toxin to alter A23187-stimulated hydrolysis of phosphoinositides (Gandhi *et al.*, 1990b), it would appear that PAF- and ET-3-induced signal transduction occurs via a common pertussis toxin-sensitive G-protein. Interestingly, ET-induced metabolism of phosphoinositides in vascular smooth muscle cells has been reported to be pertussis toxin-insensitive (Takuwa *et al.*, 1990), indicating variability in the pertussis toxin-sensitive G-protein coupled to ET receptors in different cell types. In contrast to the effect of pertussis toxin, treatment of Kupffer cells with cholera toxin resulted in potentiation of ET-3-induced phosphodiesteric hydrolysis of phosphoinositides (Fig. 4). This observation differs from the inhibitory effect of this toxin on PAF-mediated phospholipase C activation (Gandhi *et al.*, 1990b). Thus a cholera toxin-sensitive G-protein coupled to the ET-3 receptor is complementary to the action of ET, but appears to be distinct from the PAF-receptor-coupled G-protein.

Cholera toxin and pertussis toxin are known to elevate cellular cyclic AMP levels by interacting with G-proteins coupled to adenylate cyclase (Gilman, 1987). Hence it was important to determine whether these toxins are capable of influencing PAF-induced (Gandhi *et al.*, 1990b; Gandhi & Olson, 1991) and ET-3-induced phosphoinositide metabolism via an elevation of cellular cyclic AMP levels. Cholera toxin treatment produced an increase in cyclic AMP in Kupffer cells, whereas pertussis toxin had no such effect (Table 2). Furthermore, dibutyryl cyclic AMP and forskolin, an activator of adenylate cyclase (Seamon & Daly, 1981) (Table 2), inhibited both ET-3 and PAF-induced phospholipase C activity (Fig. 5).

Interestingly, neither cholera toxin treatment nor preincubation of Kupffer cells with dibutyryl cyclic AMP or forskolin inhibited ET-3- or PAF-induced GPIs formation (Fig. 5; see also Gandhi *et al.*, 1990b). It should be noted that, in the absence of IBMX, a significant increase in the cyclic AMP levels following incubation of cells with cholera toxin did not occur (Table 2). These results indicate that the effects of cholera toxin on PAF- and ET-3-induced phosphoinositide metabolism in Kupffer cells were not mediated via cyclic AMP. Further, these experiments support our suggestion that different G-proteins are involved in the regulation of the signal transduction events induced by ET and PAF.

Desensitization of the ET-3-induced glycogenolytic response in the perfused rat liver occurred when this organ was subjected to successive ET-3 infusions; however, hepatic vasoconstriction induced by ET-3 was not affected under these conditions (Gandhi *et al.*, 1990a). In contrast, homologous desensitization of PAF-induced hepatic glycogenolysis as well as vasoconstriction has been described (Buxton *et al.*, 1984). Interestingly, neither PAF nor ET-3 altered vasoconstriction, glucose production or oxygen

consumption induced by the other agonist in the perfused rat liver (Gandhi *et al.*, 1990a). Consistent with this observation, preincubation of Kupffer cells with either agonist did not affect phosphoinositide metabolism induced subsequently by the other agonist (Fig. 6). Preincubation of Kupffer cells with ET-3 resulted in complete attenuation of the metabolism of phosphoinositides when the cells were challenged subsequently with the homologous agonist (Fig. 7a). In view of the decreased ^{125}I -endothelin binding to its receptors observed following exposure to ET in C-6 cells (Cozza *et al.*, 1990), cardiomyocytes (Hirata *et al.*, 1989) and hepatocytes (C. R. Gandhi, R. Behal, S. A. K. Harvey, T. A. Nouchi & M. S. Olson, unpublished work), the results described in Fig. 7(a) suggest that receptors for ET-3, coupled to transmembrane signalling via phosphoinositides in the Kupffer cell, were desensitized or possibly down-regulated, although definitive proof of the latter suggestion is not available at the present time. A similar experiment with PAF showed incomplete attenuation of subsequent PAF-induced phosphoinositide metabolism (Fig. 7a), suggesting partial desensitization or down-regulation of PAF receptors (Chao *et al.*, 1989). Alternatively, the possibility of PAF effects on phosphoinositide metabolism being mediated through some type of intracellular action (Marcheselli *et al.*, 1990) cannot be ruled out.

Administration of radiolabelled ET to rats followed by localization of the labelled peptide in various tissues indicated that a significant portion of the administered ET was found in the liver (Anggard *et al.*, 1989; Koseki *et al.*, 1989; Shiba *et al.*, 1989), suggesting that the liver may be a major site of ET action and/or metabolism. Under conditions such as hypoxia, and in response to endotoxin and thrombin, the endothelium synthesizes contractile factor(s), presumably ET (De Mey & Vanhoutte, 1982, 1983; Vanhoutte & Katusis, 1988; Sugiura *et al.*, 1989; Moon *et al.*, 1989). Such conditions are also known to enhance the synthesis of PAF in the liver (D. S. Lapointe & M. S. Olson, unpublished work; Duffy-Krywicki *et al.*, 1990). Physiologically, the liver is exposed to a somewhat hypoxic environment, since it receives nearly 80 % of its blood supply from the portal vein. The liver, and specifically the Kupffer cell, plays a significant role in the detoxification of endotoxin from circulating blood (Jones & Summerfield, 1988). It would follow that during various types of trauma, tissue injury, endotoxaemia and hypoxia, increased hepatic synthesis of PAF and/or ET occurs. Thus, under certain conditions, in view of the independent effects of PAF and ET on Kupffer cells, parenchymal cells and intact liver, these two autacoids may play significant roles in hepatic pathophysiology.

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