Hepatic effects of endothelin

Receptor characterization and endothelin-induced signal transduction in hepatocytes

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Endothelin, a potent vasoactive peptide originally isolated from the vascular endothelial cells, exerts glycogenolytic and vasoconstrictive actions in the perfused rat liver. In this paper we demonstrate high-affinity binding sites for endothelin-1 (ET-1) on rat hepatocytes. Upon incubation at 37 °C, association of ET-1 with hepatocytes occurred in a time-dependent manner, was maximal between 3 and 6 h, and subsequently declined; at this temperature ET-1 was rapidly internalized with the internalized ligand exceeding the surface-bound ligand at all time points. The rate of association of ¹²⁵I-ET-1 with hepatocytes was much slower when the binding assay was performed at 4 °C; sequestration of ET-1 in hepatocytes was also substantially reduced at this temperature. ET-1 was extremely potent in stimulating phosphoinositide metabolism in hepatocytes, with significant activation of this signal transduction process occurring at ET-1 concentrations as low as 0.1 pM, with an EC₅₀ of 1 pM. The effect of ET-1 was coupled via a pertussis toxin-sensitive G-protein. Cholera toxin did not affect ET-1-mediated phosphoinositide metabolism and neither toxin influenced the association of ¹²⁵I-ET-1 with hepatocytes. PAGE of hepatocyte membranes following exposure of the cells to ¹²⁵I-ET-1 and cross-linking revealed labelling of three major proteins with apparent molecular masses of 32, 49 and 72 kDa. ¹²⁵I-ET-1 labelling of each of these proteins was inhibited by unlabelled ET-1, whereas unlabelled ET-3 inhibited the labelling of only the 32 and 49 kDa proteins. ¹²⁵I-ET-3 labelled the 49 kDa protein and this labelling was inhibited by both unlabelled ET-1 and ET-3. Each of these receptors appears to be functional, since both ET-1 and ET-3 stimulated phosphoinositide metabolism in hepatocytes. Down-regulation of ET-1 association and desensitization of ET-1-induced phosphoinositide metabolism occurred upon incubation of hepatocytes with the homologous ligand. Following down-regulation, the ET-1 receptor was restored to the surface of the hepatocyte by prolonged incubation, although the ET-1-stimulated phosphoinositide response remained inhibited even after complete recovery of the ET-1 association capability. These results demonstrate the presence of multiple high-affinity receptors for ET-1 on hepatocytes and the direct action of this peptide on hepatic parenchymal cells via the phosphoinositide signal transduction pathway.

INTRODUCTION

Endothelin (ET), a potent vasoactive 21-amino-acid peptide originally isolated from porcine endothelial cells (Yanagisawa *et al.*, 1988), exerts a multitude of effects in a variety of tissues and cells (for reviews, see Yanagisawa & Masaki, 1989; Simonson & Dunn, 1990; Rubanyi & Parker-Botelho, 1991). Apart from its vasoactive properties, ET modulates the release of hormones from the kidney (Goetz *et al.*, 1988; Miller *et al.*, 1989), the adrenal gland (Cozza *et al.*, 1989; Miller *et al.*, 1989) and the atrium of the heart (Goetz *et al.*, 1988; Fukuda *et al.*, 1988), stimulates phosphoinositide metabolism and an increase in intracellular Ca²⁺ (Badr *et al.*, 1989; Muldoon *et al.*, 1989; Simonson *et al.*, 1989; Takuwa *et al.*, 1989; Vigne *et al.*, 1989; Danthuluri & Brock, 1990; Serradeil-Le Gal *et al.*, 1991; Gandhi *et al.*, 1992), stimulates phospholipase A₂ and the synthesis of eicosanoids (De Nucci *et al.*, 1988; Reynolds *et al.*, 1989; Resink *et al.*, 1989; Gandhi *et al.*, 1992), and exerts mitogenic effects in Swiss 3T3 fibroblasts (Takuwa *et al.*, 1989), vascular smooth muscle cells (Komura *et al.*, 1988; Dubin *et al.*, 1989) and glomerular mesangial cells (Simonson *et al.*, 1989). Receptors for ET have been identified on several types of cells and ET immunoreactivity has been demonstrated in vascular as well as peripheral and neuronal tissues (Saida *et al.*, 1989; Giaid *et al.*, 1989; Haegerstrand *et al.*, 1989; Kosaka *et al.*, 1989; Yoshizawa

et al., 1990). Increased ET immunoreactivity has been detected in blood in conditions such as hypertension (Saito *et al.*, 1989), renal failure (Tomita *et al.*, 1989; Warrens *et al.*, 1990), diabetes (Takahashi *et al.*, 1990), liver cirrhosis (Schrader *et al.*, 1990) and following orthotopic liver transplantation (Lerman *et al.*, 1991), as well as upon administration of endotoxin (Morel *et al.*, 1989; Sugiura *et al.*, 1989b) and thrombin (Moon *et al.*, 1989). These findings suggest that ET may exhibit both humoral and autocrine hormonal or agonist activities.

Upon administration of ¹²⁵I-ET to rats, a significant portion of the radioactivity was found to localize in the liver (Anggard *et al.*, 1989; Koseki *et al.*, 1989; Shiba *et al.*, 1989), essentially in the hepatic parenchyma (Shiba *et al.*, 1989). These observations indicate that hepatic parenchymal cells may be a major site of action and/or metabolism of ET. Recently we have characterized glycogenolytic and haemodynamic actions of ET in perfused rat liver (Gandhi *et al.*, 1990a) and ET-induced signalling events in cultured Kupffer cells (Gandhi *et al.*, 1992). The current investigation was initiated to characterize mechanisms involved in the interaction of ET with isolated rat hepatocytes.

MATERIALS AND METHODS

Materials

Collagenase (type IV from *Clostridium histolyticum*) and

Abbreviations used: DAG, diacylglycerol; ET-1, endothelin-1; ET-3, endothelin-3; GPI, glycerophosphoinositol; HBSS, Hanks' buffered saline solution.

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William's medium E were obtained from Gibco. ET-1, ET-3 and sarafotoxin S6c were purchased from Peninsula Laboratories Inc., Belmont, CA, U.S.A. ^{125}I -ET-1, ^{125}I -ET-3 (2200 Ci/mmol) and $[^3\text{P}]\text{NAD}^+$ (1000 Ci/mmol) were obtained from DuPont-New England Nuclear, Boston, MA, U.S.A., and *myo*-[2- ^3H]-inositol (20 Ci/mmol) was from Amersham, New York, NY, U.S.A. Heat-inactivated fetal bovine serum, ethylene glycol-bis(succinic acid *N*-hydroxysuccinimide ester) and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., unless stated otherwise.

Preparation and primary cultures of hepatocytes

Hepatocytes were isolated from livers of male Sprague-Dawley rats (200–250 g) as described previously (Fisher *et al.*, 1984). The cells were washed three or four times and then suspended in William's medium E supplemented with 10% fetal bovine serum, 7 mg of insulin/litre, 5000 units of penicillin/ml and 5000 μg of streptomycin/ml and 2 mM-L-glutamine (medium A) at 5×10^5 cells/ml. The viability of hepatocytes obtained using this procedure was 80–90% as indicated by Trypan Blue exclusion. Portions of 2 ml of the hepatocyte suspension were placed in 35 mm tissue culture dishes (Falcon) and were maintained in an incubator at 37 °C in a humidified atmosphere of O_2/CO_2 (19:1). The medium was replaced after 18 h and subsequently every other day. Experiments were performed on day 4 or 5 of the culture interval; hepatocytes showed normal responses to glucagon (cyclic AMP generation) and to phenylephrine (inositol phosphate formation).

^{125}I -ET binding assay

Hepatocytes were washed three times with Hanks' balanced salt solution (HBSS) containing 0.1% BSA and were placed in 1 ml of this buffer containing 10 pM- ^{125}I -ET-1. Following incubation at 37 °C or 4 °C for the times indicated in the Figure legends, cells were washed four times with ice-cold HBSS/0.1% BSA and digested with 0.5 M-NaOH, and radioactivity in the NaOH fractions was determined. Non-specific binding was assessed in the presence of excess (25 nM) unlabelled ET-1.

Measurement of phosphoinositide metabolism

Cells were washed and then placed in medium A containing 10 μCi of $[^3\text{H}]\text{inositol}$ /ml. Following incubation at 37 °C for 24 h, the cells were washed and placed in prewarmed medium A without serum and containing 10 mM-LiCl. Cells were challenged with ET-1 and the reaction was terminated at 20 min by rapidly aspirating the medium and adding 0.5 ml of ice-cold 5% trichloroacetic acid. After scraping, the culture plates were washed with an additional 0.5 ml of trichloroacetic acid. The combined extracts were centrifuged, and supernatants were washed extensively with water-saturated ether and then lyophilized. $[^3\text{H}]\text{inositol}$ phosphates were measured using Dowex column chromatography (Downes & Michell, 1981). The identity of individual $[^3\text{H}]\text{inositol}$ phosphates was confirmed by using a modification of the h.p.l.c. procedure of Dean & Moyer (1987), as described previously (Gandhi *et al.*, 1990b).

Determination of surface-bound and internalized ET-1

In order to measure cell-surface-bound and sequestered ET, hepatocytes which had been exposed to ^{125}I -ET-1 were washed four times with ice-cold HBSS containing 0.1% BSA and then were placed in 1 ml of ice-cold 50 mM-glycine/100 mM-NaCl, pH 3.0 (Ascoli, 1982) or in 0.2 M-acetic acid/0.5 M-NaCl, pH 2.5 (Haigler *et al.*, 1980). The plates were placed on ice for 10 min and the medium was aspirated. This treatment was repeated once and the combined medium fractions and the cell digests were analysed for radioactivity.

ADP-ribosylation

An ADP-ribosylation assay was performed as described by Burch *et al.* (1988). Briefly, cells were scraped from the culture plates; following centrifugation, the cell pellet was stored at –80 °C until use. The cells were suspended in 10 mM-Tris buffer (pH 7.5) containing 5 mM-MgSO₄ and homogenized in an all-glass homogenizer. Following centrifugations at 500 g (10 min) and 50000 g (20 min), the resultant pellet was resuspended in Tris/MgSO₄ buffer at 1 mg of protein/ml. ADP-ribosylation was performed in a reaction volume of 75 μl containing 66 mM-potassium phosphate (pH 7.4), 10 mM-thymidine, 1 mM-ATP, 25 μM - $[^3\text{P}]\text{NAD}^+$ (5.3×10^3 mCi/mmol), 1 mM-GTP and a 25 μl aliquot of membrane protein. Pre-activated cholera toxin and pertussis toxin (incubated for 30 min at room temperature in 0.1 M-potassium phosphate buffer containing 25 mM-dithiothreitol) were added at 1 μg /tube. Following incubation at 37 °C for 20 min, membranes were pelleted by centrifugation, solubilized and subjected to SDS/PAGE on 12% acrylamide gels (Laemmli, 1970).

Covalent labelling of ET receptors

Covalent labelling and cross-linking of the putative ET receptor was performed as described by Martin *et al.* (1990). Hepatocytes in primary culture were incubated as described in the ET binding/uptake assay procedure in the presence of 100 pM- ^{125}I -ET-1 or ^{125}I -ET-3 and in the presence or absence of 100 nM unlabelled ET-1 or ET-3 for 4 h at 4 °C. Following this incubation, cells were washed three times and were incubated in the presence of 2.5 mM-ethylene glycol-bis(succinic acid *N*-hydroxysuccinimide ester) in HBSS for 1 h. The reaction was terminated by the addition of an equal volume of 400 mM-EDTA/1 M-Tris/HCl, pH 6.8. The cells were scraped from the plate, washed three times with ice-cold HBSS minus BSA, solubilized (Laemmli, 1970) and subjected to SDS/PAGE on 12% acrylamide gels.

RESULTS

Characteristics of the association of ET-1 with hepatocytes

In whole cells, methods for examining the relationship between the binding of a ligand and its sequestration include (1) performing the assay at a reduced temperature to minimize or attenuate ligand uptake and (2) exposing the cells to washing conditions which are known to disrupt receptor–ligand association without affecting the cells' integrity. Upon application of these approaches to examine the ET–hepatocyte interaction, cell-associated ET at 4 °C was observed to be about one-third of that at 37 °C (e.g. 6 h time point in Fig. 1). Furthermore, using acid-washing procedures at 37 °C, 93% of the cell-associated ET appeared to be internalized. When incubations were performed at 4 °C, the acid-resistant ligand decreased greatly (about 44% of the total cell-associated ET being acid-wash-resistant), suggesting that the reduced temperature minimized internalization, which is probably energy-dependent.

As illustrated in Fig. 1, the association of ET-1 with hepatocytes at 37 °C increased with time, was maximal between 3 and 6 h and subsequently declined. In contrast, association of ET-1 with hepatocytes at 4 °C was slow in onset and tended to plateau following 6 h of incubation. At 37 °C the amount of internalized (acid-wash-resistant) ligand exceeded the membrane-bound (acid-releasable) pool at all time points (Fig. 1). Essentially similar results were obtained whether the acid-wash procedure was performed using glycine/NaCl buffer, pH 3.0, or using acetic acid/NaCl, pH 2.5. Furthermore, subcellular fractionation of ^{125}I -ET-1-labelled hepatocytes revealed that only 8–10% of the

Fig. 1. Time course of the association and internalization of ^{125}I -ET-1 by hepatocytes

Hepatocytes in primary culture were incubated at 37 °C with 10 pM ^{125}I -ET-1 in the presence or absence of 25 mM unlabelled ET-1 in HBSS/0.1% BSA for the times indicated. The cells were washed three times and placed in 50 mM-glycine buffer, pH 3.0, containing 100 mM-NaCl. Following incubation for 10 min on ice, medium was aspirated and the cells were again treated with glycine buffer. The combined acid washes (●) and the cell digests (○) were analysed for radioactivity. The values presented are calculated from the difference between ^{125}I -ET-1 bound in the presence and absence of unlabelled ET-1 and are means $\pm$ S.D. from triplicate determinations from a representative of three or four repeats. Hepatocytes were incubated with ^{125}I -ET-1 with or without unlabelled ET-1, as described above, at 4 °C for the times indicated, following which cells were digested in 0.5 M-NaOH and cell-associated radioactivity was determined (□). Each value represents the mean $\pm$ S.D. of triplicate determinations of specific association (i.e. the difference between the ^{125}I -ET-1 associated in the presence and absence of 25 nM unlabelled ET-1) from an experiment representative of three or four repeats.

Fig. 2. Competitive binding of ^{125}I -ET-1 to cultured hepatocytes

Hepatocytes were incubated with 10 pM ^{125}I -ET-1 and the indicated concentrations of unlabelled ET-1 (●, 5 h at 4 °C, ○, 4 h at 37 °C) or ET-3 (□; 4 h at 37 °C). The cells were washed four times with ice-cold HBSS/0.1% BSA and the cell-associated radioactivity was determined following digestion with 0.5 mM-NaOH. Other details of this experiment are presented in the legend for Fig. 1. Values presented [percentages of ^{125}I -ET-1 associated in the absence of unlabelled ET-1, i.e. control (100%), 4.4 ± 0.8 fmol/ 10^6 cells at 37 °C and 1.3 ± 0.4 fmol/ 10^6 cells at 4 °C] are means $\pm$ S.D. from triplicate determinations in a representative of four experiments.

cell-associated ET-1 was associated with plasma membranes (results not shown), which was comparable with the acid-sensitive fraction apparent in Fig. 1.

Competition binding experiments were performed at 4 °C in order to minimize the effect of ligand internalization (Fig. 2). Under these conditions, half-maximal competition of ^{125}I -ET-1

association with hepatocytes occurred at approx. 50 pM unlabelled ET-1. In the competition experiments performed at 37 °C for comparison with signal transduction experiments performed at the physiological temperature (Fig. 3), half-maximal competition of ^{125}I -ET-1 association with hepatocytes occurred between 100 and 200 pM unlabelled ET-1 (Fig. 2). Similar concentrations of ET-3 (Fig. 2) and sarafotoxin S6c (results not shown), close structural analogues of ET-1, were found to be poor competitors of ^{125}I -ET-1 association with hepatocytes, emphasizing the specificity of the ET-1 association process. At concentrations several fold higher, however, ET-3 produced significant inhibition of ET-1 association with hepatocytes (results not shown).

The characteristics of the binding of ET to rat liver plasma membranes have been described, with a K_d of 33 pM and a B_{max} of ~ 1 pmol/mg of protein (Serradeil-Le Gal *et al.*, 1991). It is known that internalization of peptide ligands by intact cells undermines the validity of Scatchard analysis of cell-ligand interactions (Wileman *et al.*, 1985). The dynamics of the endothelin-cell interaction in the present study were such that the analytical method formulated by Wiley & Cunningham (1981) (i.e. assumption of a new steady state) was of limited use. We reasoned that the 4 °C data would most closely resemble the membrane data, since internalization is minimized (see above and Fig. 1). Thus we found that a Scatchard-format graph of our data yielded a curvilinear plot (results not shown), suggesting a family of apparent K_d values. The minimum limit value from this plot (given by the tangent to the curve where it intersected the y-axis) yielded a K_d of 31 ± 4 pM, which is similar to that reported by Serradeil-Le Gal *et al.* (1991), and a B_{max} of 1200 ± 230 sites/cell.

ET-1-induced phosphoinositide metabolism

Several agents exert glycogenolytic effects in the intact liver and in isolated hepatocytes by stimulating phosphoinositide metabolism and subsequent Ca^{2+} mobilization or by elevating cellular cyclic AMP, leading to the conversion of glycogen phosphorylase to its active form. We have demonstrated previously that ET stimulates glucose production in the perfused rat liver (Gandhi *et al.*, 1990a). In order to ascertain whether ET exerts a direct action on hepatocytes we challenged [^3H]inositol-labelled hepatocytes with ET and measured the levels of [^3H] inositol phosphates. Both ET-1 (Fig. 3) and ET-3 (results not shown) stimulated the phosphoinositide signalling system in hepatocytes with the release of inositol phosphates (phospholipase C activation) and glycerophosphoinositol (GPI) (deacylation). ET exhibited a very high potency in stimulating the phosphoinositide signal transduction pathway, with measurable effects at concentrations as low as 0.1 pM and an EC_{50} of ~ 1 pM. However, neither ET-1 nor ET-3 affected cyclic AMP levels in hepatocytes (results not shown).

Modulation of ET-1 association and desensitization of ET-1-induced phosphoinositide metabolism in hepatocytes: effects of homologous and heterologous ligands

Fig. 4 illustrates a time course for the homologous-ligand-induced inhibition of ET-1 association with hepatocytes. Incubation of hepatocytes with unlabelled ET-1 for various time periods produced a progressive decrease in the subsequent association of ^{125}I -ET-1 with hepatocytes. Interestingly, after 6 h of incubation with ET-1, recovery of the ET-1 association capacity of the hepatocytes occurred, and this apparent recovery of receptor function was significant at 24 h ($P < 0.005$ versus ET-1 association at 6 h). When cells were incubated with additional unlabelled ET-1 at this time point (24 h), the association of ^{125}I -ET-1 with the hepatocytes was maxi-

Fig. 3. Effect of ET-1 concentration on hepatocyte phosphoinositide metabolism

Hepatocytes were labelled with 10 μ Ci of [3 H]inositol/ml in William's medium E for 24 h as described in the Materials and methods section. After washing, the cells were placed in pre-warmed serum-free William's medium E containing 10 mM-LiCl and were challenged with the indicated concentrations of ET-1 for 20 min. The reaction was determined with 5% ice-cold trichloroacetic acid, and after washing the supernatants with water-saturated ether, inositol phosphates were analysed by Dowex column chromatography. The values represent means of duplicate determinations from a representative of three experiments. For other details, see the Materials and methods section. O, GPI, ●, inositol phosphates.

Fig. 4. Time course of homologous-ligand-induced down-regulation and recovery of ET-1 association with hepatocytes

Hepatocytes were incubated for the indicated times in the presence of 5 nM unlabelled ET-1 (O), washed five times and subjected to an 125 I-ET-1 association assay. At 24 h (*), where 125 I-ET-1 associated with hepatocytes was significantly higher than at 6 h ($P < 0.005$) (arrow), an additional 5 nM-ET-1 was added, and after incubation for 3 h the cells were washed prior to the 125 I-ET-1 association assay. Hepatocytes were treated with 5 nM-ET-1 for 4 h. The cells were washed, placed in medium A and the 125 I-ET-1 association assay was performed at the indicated times as described above (●). Each point [percentage of control, i.e. 125 I-ET-1 associated with untreated hepatocytes (3.8 ± 0.9 fmol/ 10^6 cells)] represents the mean $\pm$ S.D. of specific 125 I-ET-1 associated from four experiments, each done in triplicate.

mally inhibited. ET-1-induced phosphodiesteric metabolism of phosphoinositides was inhibited strongly after 3 h of pre-treatment with the homologous ligand and remained inhibited at 24 h (Table 1). Partial recovery of the capacity of hepatocytes to interact with ET-1 occurred at this time (Fig. 4). However, treatment of hepatocytes with ET-1 as described above did not deplete the [3 H]inositol phospholipid pools, as suggested by the quantification of [3 H]inositol-labelled phosphoinositides (results not shown).

Table 1. ET-1-induced phosphoinositide metabolism in hepatocytes following homologous ligand-induced down-regulation and recovery of the 125 I-ET-1 association capacity of hepatocytes

Hepatocytes were labelled with 10 μ Ci of [3 H]inositol/ml for 24 h in the absence (A,B) and the presence (D) of 5 nM ET-1. To one set of cells (C), 5 nM-ET-1 was added at 21 h of incubation with [3 H]inositol and the incubation was continued for an additional 3 h. Another set of cells (E) was incubated in the presence of 5 nM-ET-1 for 3 h, then extensively washed and incubated in the presence of 10 μ Ci of [3 H]inositol/ml for 24 h. Following these treatments, cells were washed five times and challenged with 20 nM-ET-1 in a medium containing 10 mM-LiCl for 20 min. Inositol phosphates were determined by Dowex chromatography. The [3 H]inositol content of the cellular lipids was found not to be altered by such incubations with ET-1. Values are presented as percentage of control values (non-stimulated) cells and are the means $\pm$ S.D. of duplicate determinations from four experiments.

	Total [3 H]inositol phosphates (% of control)
A Non-stimulated	100 $\pm$ 12
B ET-1-stimulated	573 $\pm$ 83
C ET-1-down-regulated (3 h)	169 $\pm$ 45
D ET-1-down-regulated (24 h)	181 $\pm$ 35
E Up-regulated (24 h)	193 $\pm$ 33

To ascertain whether the association of ET-1 with hepatocytes could be influenced by heterologous ligands such as the Ca^{2+} -mobilizing agents (e.g. phenylephrine, angiotensin II and vasopressin), and activators of adenylate cyclase (e.g. glucagon and isoprenaline), all of which stimulate hepatic glycogenolysis, the association of ET-1 with hepatocytes was assessed in cells incubated in the presence of these agents for 18 h or by adding these agents to incubations during the 125 I-ET-1 association assay. No appreciable change in the association of ET-1 with hepatocytes was observed with any of these agents under either treatment condition (results not shown).

Time course of the recovery of ET-1 association following homologous down-regulation

Fig. 4 illustrates the time course of the recovery of the association of ET-1 with hepatocytes following treatment with homologous ligand. Hepatocytes were incubated for 4 h with ET-1 to down-regulate the receptors and then were washed thoroughly to remove excess ET-1. When the cells were placed in the normal medium following this treatment, rapid recovery of the ET-1 association with hepatocytes occurred for up to 10 h. Thereafter, the recovery of ET-1 association with hepatocytes was gradual and nearly complete by 48 h. Interestingly, ET-1-induced phosphoinositide metabolism remained inhibited at both 24 h (Table 1) and 48 h (results not shown) of the recovery period, despite the fact that the capacity of hepatocytes to bind ET-1 was restored completely (Fig. 4). It should be noted that phenylephrine-induced phosphoinositide metabolism was not affected following treatment of hepatocytes with ET-1 (results not shown).

Considering the inability of ET-3 to effectively compete with the association of ET-1 with hepatocytes (Fig. 2), experiments were conducted to ascertain whether ET-3 could down-regulate ET-1 association and inhibit ET-1-induced phosphoinositide metabolism. As illustrated in Figs. 5(a) and 5(b), incubation of hepatocytes with ET-3 had a comparatively smaller effect on the association of ET-1 with hepatocytes and ET-1-induced phosphoinositide metabolism. Even at a concentration at which ET-1 produced maximal inhibition of these processes (5–10 nM),

Fig. 5. Effect of ET-3-induced down-regulation on ¹²⁵I-ET-1 association and ET-1-induced phosphoinositide metabolism in hepatocytes

(a) Hepatocytes were incubated in the presence of the indicated concentrations of ET-1 or ET-3 for 3 h. After washing five times, the ¹²⁵I-ET-1 association assay was performed at 37 °C for 4 h as described in the Materials and methods section. Values presented [percentage of control, i.e. ¹²⁵I-ET-1 associated with untreated hepatocytes (4.1 ± 0.75 fmol/10⁶ cells)] are the means ± S.D. of triplicate determinations from three experiments. (b) Following labelling of hepatocytes with 10 μCi of [³H]inositol/ml of William's medium E containing 10 % fetal calf serum for 24 h, cells were incubated with the indicated concentrations of ET-1 or ET-3 for 3 h. Cells were washed extensively, placed in serum-free medium containing 10 mM-LiCl and challenged with 20 nM-ET-1 for 20 min. Inositol phosphates were determined by Dowex chromatography. Values presented (percentage of control, i.e. unstimulated cells) are mean ± S.D. of duplicate determinations from three experiments. ○, ET-1-treated; ●, ET-3-treated.

hepatocytes incubated with ET-3 still exhibited significant ¹²⁵I-ET-1 association and ET-1-induced phosphoinositide metabolism (Fig. 5).

Heterogeneity of ET receptors in hepatocytes

As demonstrated in Figs. 2 and 5, ET-3 had significantly smaller effects than ET-1 on the association of ¹²⁵I-ET-1 with hepatocytes and ET-1-induced phosphoinositide metabolism. Hence it was of interest to determine whether there are separate receptors for ET-1 and ET-3 in hepatocytes. Upon affinity labelling and cross-linking followed by SDS/PAGE, three major protein bands with molecular masses of approx. 35, 52 and 75 kDa were labelled by ¹²⁵I-ET-1 (Fig. 6). Considering the molecular masses of ET and the cross-linking reagent, the apparent molecular masses of these proteins would reduce to 32, 49 and 72 kDa respectively. While the labelling of each of these proteins was inhibited in the presence of unlabelled ET-1, ET-3 inhibited ¹²⁵I-ET-1 labelling of only the 32 and 49 kDa bands. In contrast to ¹²⁵I-ET-1, ¹²⁵I-ET-3 labelled the 49 kDa band but not the 32 and 72 kDa bands, and the labelling by ¹²⁵I-ET-3 was inhibited by both unlabelled ET-1 and ET-3 (Fig. 6).

Fig. 6. Autoradiogram of PAGE of ¹²⁵I-ET-1 and ¹²⁵I-ET-3 covalently cross-linked to the receptors

Hepatocytes were incubated with 100 pM-¹²⁵I-ET-1 or ¹²⁵I-ET-3 for 4 h at 4 °C. Following cross-linking, PAGE was performed under non-reducing (lanes A–F) and reducing (lanes G–L) conditions. Lanes A, G, 100 pM-¹²⁵I-ET-1; B, H, ¹²⁵I-ET-1 + 100 nM unlabelled ET-1; C, I, ¹²⁵I-ET-1 + 100 nM unlabelled ET-3; D, J, 100 pM-¹²⁵I-ET-3; E, K, ¹²⁵I-ET-3 + 100 nM unlabelled ET-1; F, L, ¹²⁵I-ET-3 + 100 nM unlabelled ET-3. For other details, see the Materials and methods section.

Table 2. Effects of cholera toxin and pertussis toxin on ET-1-induced phosphoinositide metabolism in hepatocytes

Hepatocytes were incubated in William's medium E containing 10 % fetal calf serum and 10 μCi of [³H]inositol/ml. At 12 h, 50 ng of pertussis toxin/ml or 2.5 μg of cholera toxin/ml was added. After an additional incubation for 12 h, cells were washed and challenged with 20 nM-ET-1 for 20 min in the presence of 10 mM-LiCl. Inositol phosphates were determined by Dowex chromatography. Results are expressed as percentages of control (i.e. cells not challenged with ET-1) and are the means ± S.D. of duplicate determinations from four experiments.

	Total [³ H]inositol phosphates (% of control)
Control	100 ± 16
ET-1-stimulated	491 ± 74
Cholera toxin-treated/ET-1	501 ± 59
Pertussis toxin-treated/ET-1	183 ± 33

G-protein involvement

We reported previously differential effects of cholera toxin and pertussis toxin on platelet-activating-factor and ET-induced phosphoinositide metabolism and synthesis of prostaglandins in hepatic Kupffer cells (Gandhi *et al.*, 1990b, 1992; Gandhi & Olson, 1991). These toxins modulate several receptor-mediated signal transduction events by interacting with G-proteins (Gilman, 1987). Treatment of hepatocytes with pertussis toxin, but not cholera toxin, prior to stimulation with ET-1 resulted in an inhibition of ET-1-induced phosphoinositide metabolism (Table 2). Signal transduction events occur within seconds following the binding of an agonist to its receptor, with subsequent activation of G-protein(s). Since GTP has been shown to decrease the affinity of several receptors for their respective ligands by modulating the G-proteins, it was important to ascertain whether pertussis toxin produced inhibition of ET-1-induced phosphoinositide metabolism by affecting the binding of the peptide to its receptor. Preincubation of hepatocytes with either toxin had no effect on the association of ET-1 with hepatocytes (results not shown).

In order to assess the nature of the G-protein involvement in

Fig. 7. ADP-ribosylation of hepatocyte membrane proteins in response to bacterial toxins and ET-1

(a) Hepatocyte membranes were incubated with 50 nM-ET-1, 1 μ g of dithiothreitol-treated cholera toxin (CTX) or pertussis toxin (PTX)/ml, ET-1 + CTX or ET-1 + PTX in the presence of [32 P]NAD $^{+}$ for 20 min; CT, control. (b) Hepatocytes were incubated in the presence of 5 nM ET-1, 2.5 μ g of CTX/ml and 50 ng of pertussis toxin (PTX)/ml for 12 h and were washed extensively before the preparation of membranes. Membranes were incubated in the presence of 50 nM-ET-1 (lanes 2) and 1 μ g of dithiothreitol-treated cholera toxin (lanes 3) or pertussis toxin (lanes 4) in an incubation buffer containing [32 P]NAD $^{+}$ for 20 min at 37 $^{\circ}$ C; lanes 1, control. SDS/PAGE was performed using the solubilized membrane proteins. The gel was stained in Coomassie Brilliant Blue and exposed to Kodak XAR-5 film. For other details, see the Materials and methods section.

ET-1-induced phosphoinositide metabolism in hepatocytes, the extent of ADP-ribosylation using [32 P]NAD $^{+}$ in hepatocyte membranes was determined in the presence and absence of ET-1, cholera toxin and pertussis toxin. As illustrated in Fig. 7(a), upon incubation of hepatocyte membranes with [32 P]NAD $^{+}$, a 63 kDa protein was labelled in the presence and absence of ET-1 and the toxins and the labelling of this protein was not affected in membranes from cells treated with ET-1 or the toxins (Fig. 7b). Cholera toxin caused the labelling of three proteins (29, 45 and 52 kDa). Pretreatment of whole cells with cholera toxin abrogated subsequent labelling of 45 and 52 kDa proteins, indicating that the principal effect of cholera toxin in whole cells was to ADP-ribosylate only these proteins. Similarly, pertussis toxin labelled 31 and 43 kDa proteins (Fig. 7a), but pretreatment of whole cells with this toxin abrogated subsequent labelling of only the 43 kDa protein (Fig. 7b), indicating that this 43 kDa protein was the principal target of pertussis toxin in whole cells. In conjunction with the inhibition of the ET-1-induced accumulation of inositol

phosphates by pertussis toxin, these data demonstrate that in hepatocytes a 43 kDa pertussis toxin-sensitive G-protein may be responsible for ET-1-mediated signal transduction.

DISCUSSION

In this investigation we have demonstrated the presence of multiple receptors for ET and ET-induced signal transduction in cultured rat hepatocytes. Hepatocytes internalized ET-1 rapidly as demonstrated by two different acid-wash treatments (e.g. glycine buffer, pH 3.0, and acetic acid, pH 2.5) of the cultured cells. ET-1 was internalized in a time-dependent fashion (Fig. 1), and even at 4 $^{\circ}$ C a significant portion of the added ET-1 was internalized by hepatocytes. In vascular smooth muscle cells, internalization of ET was shown to be temperature-dependent with no internalization at 4 $^{\circ}$ C (Resink *et al.*, 1990). These findings, along with the observation that hepatocyte-associated ET-1 was incorporated into intracellular compartments (results not shown), suggest that hepatic parenchymal cells possess an efficient mechanism for sequestering ET. This observation is consistent with reports that a significant portion of intravenously administered ET is localized in the hepatic parenchyma (Shiba *et al.*, 1989).

Reversible binding of ET-1 to A10 vascular smooth muscle cells and C6 glial cells (Martin *et al.*, 1990) and the irreversible nature of ET binding to vascular smooth muscle cells (Hirata *et al.*, 1988; Clozel *et al.*, 1989) and to adrenal glomerulosa cells (Cozza *et al.*, 1989) suggest variability in the interactions between this peptide and its receptor in different cell types. Irreversible binding of ET-1 to vascular smooth muscle cells may be due to the internalization of the peptide, as demonstrated by Resink *et al.* (1990). Irreversible binding of ET-1 to placental membranes (Fischli *et al.*, 1989), glomerular and papillary membranes (Martin *et al.*, 1989) and a very slow reversal of the ET-1 binding (Serradeil-Le Gal *et al.*, 1991) to hepatic membranes suggest a very stable interaction between the peptide and its receptor in plasma membrane preparations. However, binding studies using intact hepatocytes are complicated by an active process of internalization of the peptide even at low temperatures.

In intact liver, ET induces a sustained and persistent vasoconstrictive response even after a lengthy ligand-free perfusion interval (Gandhi *et al.*, 1990a). Based on its prolonged vasoactive effects, Yanagisawa *et al.* (1988) suggested that ET binds with very high affinity to its receptor and that the ligand-receptor association is resistant to sample washing protocols. In contrast to its vasoactive effect in the hepatic vasculature, ET-induced hepatic glycogenolysis is transient (Gandhi *et al.*, 1990a) and at least a portion of this glycogenolytic effect appears to be due to a direct action of the peptide on the hepatocytes. In this regard ET has been reported to stimulate phosphorylase activity in isolated hepatocytes (Serradeil-Le Gal *et al.*, 1991), which is consistent with the ET-induced phosphoinositide hydrolysis, a signal transduction mechanism used by several glycogenolytic agonists, e.g. phenylephrine, vasopressin, angiotensin etc.

One of the major post-receptor binding effects of ET is phosphodiesteric hydrolysis of phosphoinositides and the activation of subsequent Ca $^{2+}$ - and DAG-mediated processes (Badr *et al.*, 1989; Muldoon *et al.*, 1989; Simonson *et al.*, 1989; Takuwa *et al.*, 1989; Vigne *et al.*, 1989; Danthuluri & Brock, 1990). In hepatic Kupffer cells (Gandhi *et al.*, 1992) and hepatocytes (present investigation) ET clearly stimulates the metabolism of phosphoinositides, and in neither cell type did ET affect cyclic AMP levels. In Kupffer cells, a primary functional response following ET-induced signal transduction appears to be eicosanoid synthesis (Gandhi *et al.*, 1992), whereas in hepatocytes ET stimulates glycogenolysis (Gandhi *et al.*, 1990a; Serradeil-Le

Gal *et al.*, 1991). ET-induced glycogenolysis in hepatocytes appears to occur predominantly via stimulation of phosphoinositide metabolism, as ET does not stimulate cyclic AMP synthesis in these cells (results not shown; also see Gandhi *et al.*, 1990a; Serradeil-Le Gal *et al.*, 1991). Significant stimulation of the ET-1-induced phosphoinositide metabolism in hepatocytes occurred at ET-1 concentrations as low as 0.1 pM. Although ET exhibits an EC_{50} of 0.5–10 nM in stimulating the metabolism of phosphoinositides in other cells (Muldoon *et al.*, 1989; Dantluri & Brock, 1990; Gandhi *et al.*, 1992), an EC_{50} of 0.03 pM for the activation of phosphorylase (Serradeil-Le Gal *et al.*, 1991) and stimulation of ET-1-induced phosphoinositide metabolism with an EC_{50} of ~ 1 pM indicate that this peptide is much more potent in stimulating signalling events in hepatocytes compared with other cell types.

Upon binding of ligands to specific receptors, the resultant ligand–receptor complexes are internalized by the process of endocytosis. The fate of the receptor under these conditions varies; the receptor may be recycled, degraded or extruded from the cell (Pastan & Willingham, 1981; Wiley & Cunningham, 1981; Forgac, 1988). We observed that incubation of hepatocytes with ET-1 resulted in a rapid down-regulation of subsequent association of the homologous ligand (Fig. 4). Upon thorough washing of the cells, restoration of the capacity of ET-1 to associate with hepatocytes also was rapid for 10 h and had reached the original level by 48 h (Fig. 4). Consistent with the homologous ligand-induced down-regulation of the ET-1 interaction with hepatocytes, such treatments also led to an inhibition of the phosphoinositide response to subsequent ET-1 challenge (Table 1). Surprisingly, at a time point when the capacity of hepatocytes to bind ET-1 was nearly completely recovered, the phosphoinositide response remained inhibited in these cells (Table 1), although phenylephrine evoked a normal effect on phosphoinositide metabolism. This raises the possibility that ET-1 receptors coupled to phosphoinositide metabolism and receptors involved in the sequestration of ET-1 may be different. Another possible explanation for this observation is that, following receptor-induced phosphoinositide metabolism, [3H]inositol may be incorporated into ET-1-insensitive pools.

Both ET-1 and ET-3 were found to evoke hepatic vasoconstriction and glycogenolysis (Gandhi *et al.*, 1990a), suggesting the possibility of the presence of multiple receptors for ET in hepatic cells. The existence of distinct receptor subpopulations has been demonstrated in various cells and tissues (Masuda *et al.*, 1989; Sugiura *et al.*, 1989a; Nakajo *et al.*, 1989; Watanabe *et al.*, 1989; Martin *et al.*, 1990). We observed specific labelling with ^{125}I -ET-1 of proteins exhibiting approximate molecular masses by SDS/PAGE of 32, 49 and 72 kDa (Fig. 6). In rat A10 vascular smooth muscle cells and C6 glial cells, receptors with an apparent molecular mass of ~ 72 kDa that are specific for ET-1 have been reported (Martin *et al.*, 1990). The 72 kDa protein band demonstrated in the present investigation (Fig. 6) appears to be similar to that described by Martin *et al.* (1990), the labelling of which by ^{125}I -ET-1 was not inhibited in the presence of unlabelled ET-3. The presence of an ET-1-specific receptor (72 kDa) in hepatocytes is consistent with the observations that ET-3 did not compete effectively with ^{125}I -ET-1 association, exhibited a smaller effect on the down-regulation of ET-1 interactions with the cells and did not lead to the inhibition of ET-1-induced phosphoinositide metabolism (Figs. 2 and 5). It is possible that the 32 and 49 kDa receptors may have higher affinity for ET-1 than for ET-3, as only at very high concentrations did ET-3 produce substantial inhibition of the association of ^{125}I -ET-1 with hepatocytes. Recently an ET receptor from bovine lung (Arai *et al.*, 1991) and rat lung (Sakurai *et al.*, 1991), with a molecular mass of ~ 49 kDa, was cloned and

sequenced. The bovine lung receptor seems to have higher specificity for ET-1 than for ET-3 (Arai *et al.*, 1991), whereas the rat lung receptor has a similar specificity for ET-1 and ET-3 (Sakurai *et al.*, 1991). This receptor is expressed in rat liver (Sakurai *et al.*, 1991). Affinity labelling of the 49 kDa receptor by both radioactive ET-1 and ET-3 and the inhibition of this labelling in the presence of either unlabelled ligand in excess (Fig. 6) suggests that this receptor may be similar to that observed in rat lung. Depending upon the procedure employed for binding and cross-linking, some of these protein bands may be proteolytic degradation products. We observed that unlabelled ET-1 or ET-3 inhibits ^{125}I -ET-1 labelling of the 32 kDa protein. Although this species may be a degradation product of the higher-molecular-mass ET receptor(s), it is noteworthy that an ET receptor with an apparent molecular mass of 32 kDa has been observed in the placenta (Nakajo *et al.*, 1989).

ET-1-induced phosphoinositide metabolism was not affected by cholera toxin. A 72 kDa protein was labelled in the absence of ET-1 and the labelling of this protein was somewhat enhanced in the presence of ET-1 (Fig. 7a). The labelling of this protein was inhibited by simultaneous addition of pertussis toxin; pre-treatment of whole cells with ET-1 but not pertussis toxin abrogated subsequent labelling of the 72 kDa protein in the membrane assay. Although the precise function of this 72 kDa protein is unclear at the present time, the differential effects of pertussis toxin and ET-1 on the labelling of this protein are of obvious interest. In other cell types, including Kupffer cells (Gandhi *et al.*, 1992), ET-induced phosphoinositide metabolism has been shown to be coupled via a pertussis toxin-sensitive G-protein. The evidence presented in this paper (i.e., labelling of a 43 kDa protein using [^{32}P]NAD $^+$ in the presence of pertussis toxin and inhibition of ET-1-induced phosphoinositide metabolism by pertussis toxin) suggests that the pertussis toxin-sensitive 43 kDa G-protein may be involved in ET-1-mediated signalling in hepatocytes.

The liver is one of the major organs involved in the clearance of circulating ET, as is evident from the tissue distribution of administered radioactive peptide (Anggard *et al.*, 1989; Koseki *et al.*, 1989; Shiba *et al.*, 1989). In the liver, ET-associated radioactivity was found primarily in the hepatic parenchyma (Shiba *et al.*, 1989). The inability of circulating proteolytic enzymes to metabolize ET (Shiba *et al.*, 1989) suggests that lung, kidney and liver play important roles in the uptake and subsequent metabolism of this peptide. In this regard we have observed that hepatic cells (both Kupffer and parenchymal) efficiently metabolize ET (C. R. Gandhi, S. A. K. Harvey & M. S. Olson, unpublished work). Although circulating levels of ET are usually very low (i.e. less than 0.5 pM), endotoxin infusion (Morel *et al.*, 1989; Sugiura *et al.*, 1989b), thrombin administration (Moon *et al.*, 1989) and conditions such as hypertension (Saito *et al.*, 1989) elevate significantly the circulating levels of ET. Additionally, because hepatic endothelial cells have been reported to synthesize ET (Yoshimi *et al.*, 1989; Rieder *et al.*, 1991), pathophysiological conditions such as sepsis, anoxia and other types of systemic trauma may lead to an enhanced rate of hepatic synthesis of this potent vasoactive peptide. The ability of the liver to synthesize, respond to and degrade this peptide suggests that ET is an authentic autacoid in this organ.

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