Endothelin Action in Rat Liver

Receptors, Free Ca^{2+} Oscillations, and Activation of Glycogenolysis

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Abstract

High affinity binding sites for endothelin (ET) were identified on rat liver plasma membranes. Binding of ^{125}I-ET-1 with its site was specific, saturable, and time dependent (k_{obs} = 0.019±0.001 min^{-1}), but dissociation of receptor-bound ligand was minimal. A single class of high affinity binding sites for ^{125}I-ET-1 was identified with an apparent K_d of 32.4±9.8 pM and a B_{max} of 1084±118 fmol/mg protein. ET-3 and big-ET-1 (1–38) (human) inhibited ^{125}I-ET-1 binding with IC_{50} values of 1.85±1.03 nM and 43±6 nM, respectively. Aequorin measurements of cytosolic free Ca^{2+} in single, isolated rat hepatocytes showed that ET-1 at subnanomolar concentrations induced a series of repetitive, sustained Ca^{2+} transients. ET-1 had no effect on cAMP production. Finally, ET-1 caused a rapid and sustained stimulation of glycogenolysis in rat hepatocytes. A 1.8-fold maximal increase in glycogen phosphorylase *a* was observed at 1 pM ET-1, with an EC_{50} of 0.03 pM. Stimulation of the enzyme was specific for ET-1 since the order of potency of related peptides was similar to that in binding experiments (ET-1 > ET-3 > big ET-1). These data constitute the first demonstration of the presence of ET-1 binding sites in liver which is associated with a rise in cytosolic free Ca^{2+} and a potent glycogenolytic effect. We conclude that ET-1 behaves as a typical Ca^{2+} mobilizing hormone in liver. (*J. Clin. Invest.* 1991. 87:133–138.) Key words: endothelin • hepatocyte • Ca^{2+} mobilizing hormone • receptor binding • glycogenolysis • single cell

Introduction

Endothelin-1 (ET-1)[1] is a potent vasoconstrictor peptide of 21 amino acid residues purified from the culture supernatant of porcine aortic endothelial cells (1). ET-1 is synthesized as a 203 amino acid prepropeptide which is proteolytically cleaved to produce big ET-1 (1–38) (human) or (1–39) (porcine), and then processed to mature ET-1 (1–21) by a putative endothelin-converting enzyme (2, 3). Two other types of endothelin genes are found in human genomic DNA, demonstrating the existence of three isoforms of human endothelin, called ET-1, ET-2, and ET-3, with distinct structure and pharmacological activity (4). Recently, the presence of a new peptide of the endothelin family has been also demonstrated in the mouse intestine (5). In addition to its potent, long lasting vasoconstrictor and pressor actions, ET-1 has now been shown to exert a wide variety of biological activities in nonvascular tissues, including positive inotropic and chronotropic actions and stimulation of atrial natriuretic factor release in heart, inhibition of Na^+/K^+ ATPase and renin release in kidney, stimulation of aldosterone production in zona glomerulosa cells, mitogenic actions in fibroblasts, vascular smooth muscle cells and mesangial cells, and actions on the central nervous system and on neuronal excitability (for a review see reference 6). In agreement with these observations, autoradiographic experiments and immunoreactive studies have shown that specific high affinity binding sites for ET-1 and presence of ET-1-like immunoreactivity are localized not only in blood vessels but also in the peripheral tissues and central nervous system (7, 8). We report here that ET-1 binds with a high affinity to rat liver plasma membranes, and demonstrate that ET-1 binding to its sites induces sustained Ca^{2+} mobilization and activation of glycogenolysis in rat hepatocytes. This is the first demonstration that ET-1 behaves as a Ca^{2+} mobilizing hormone in liver, where it exerts a major metabolic effect.

Methods

ET-1 and ET-3 were purchased from Novabiochem Ltd. (UK). Big ET-1 (1–38) (human) was obtained from Peninsula Laboratories Ltd. (Europe). Glucagon was from Novo (France), epinephrine from Sigma Chemical Co. (L'isle d'Abeau Chesnes, France), and collagenase from Worthington (Freehold, NJ). ^{125}I-ET-1 (2,000 Ci/mmol) and [^{14}C] glucose-1 phosphate (150 mCi/mmol) were purchased from Amersham Corp. (Les Ulis, France). Male Sprague-Dawley rats (250 g body weight) were obtained from Charles River Breeding Laboratories, Inc. (Saint Aubin les Elbeuf, France). Medium E was from Flow laboratories Inc. (McLean, VA). Radioimmunoassay kits for cyclic AMP were obtained from Institut Pasteur Production (Marnes La Coquette, France).

Preparation of liver plasma membranes. Rat liver plasma membranes were prepared by the method of Prpic et al. (9). The resulting pellet was suspended in 50 mM Tris/HCl, pH 8.0, at a final protein concentration of ~ 10 mg/ml, and stored as aliquots in liquid nitrogen until used.

Preparation of rat hepatocytes. Hepatocytes were isolated according to the procedure of Seglen (10). For measurements of cytosolic free Ca^{2+}, hepatocytes were prepared for microinjection with aequorin as described previously (11). For measurements of glycogen phosphorylase *a* activity and cAMP determinations, damaged and nonparenchymal cells were removed after dispersion by three centrifugations at 90 *g* for 50 s. in a Hanks' medium containing 20 mM Hepes, pH 7.4, and hepatocytes were finally resuspended in the same medium. After isolation, the cells (8.10^6 cells/ml) were preincubated for 30 min at 37°C under continuous gassing with O_2/CO_2 (95, 5%) atmosphere in Hanks' medium containing 20 mM Hepes, pH 7.4, and 4 g/liter glucose.

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1. *Abbreviations used in this paper:* ET, endothelin.

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Binding of ^{125}I-ET-1 to liver plasma membranes. Rat liver plasma membranes (10–20 μg/ml) were incubated for 90 min at 22°C in a 200-μl Krebs-Ringer medium (pH 7.4) containing 20 mM Hepes, 1% (wt/vol) bovine serum albumin, 300 μg/ml bacitracin, and 60 pM ^{125}I-ET-1. The incubation was stopped by adding 3 ml of ice cold Krebs-Ringer (pH 7.4), 20 mM Hepes buffer containing 1% (wt/vol) bovine serum albumin.

The contents of the assay tubes were filtered rapidly through Whatman GF/C filters presoaked in 1% polyethyleneimine then rinsed with 9 ml of ice cold wash buffer. The radioactivity of the filter was counted in an LKB multiwell gamma counter. Nonspecific binding was determined by incubating with 0.1 μM unlabeled ET-1. Saturation experiments were performed with increasing concentrations of ^{125}I-ET-1 (4.5–750 pM) to study the affinity of the iodinated ligand for the receptors. Competition studies were done with 60 pM of ^{125}I-ET-1 in the presence of increasing concentrations of unlabeled ET-1, ET-3, big ET-1 (1–38) (human), or 10^{-5} M neuropeptide Y, vasoactive intestinal peptide, vasopressin, nifedipine, verapamil, GTP-γ-S, or Gpp-NHp. The association constant was determined by measuring the binding of 60 pM ^{125}I-ET-1 to the liver plasma membranes at various times and dissociation was initiated after adding 0.2 μM unlabeled ET-1. Data from saturation, competition, and association experiments were analyzed using a nonlinear regression program (12).

To assess the stability of ^{125}I-ET-1, the assay medium containing 60 pM ^{125}I-ET-1 and 10–20 μg/ml of liver plasma membranes was incubated under standard conditions at various times (0–90 min) at 22°C and filtered through 0.8 μ Millex AA (Millipore Continental Water Systems, Bedford, MA). The ^{125}I-ET-1 in the resulting supernatants was analyzed by reverse-phase HPLC on a Synchropak RP-4 column (SynChrom, Inc., Linden, IN) using an 18–60% aqueous gradient of acetonitrile in 0.1% trifluoroacetic acid over 20 min at a 1 ml/min rate.

Aequorin measurements of cytosolic free Ca^{2+} in single rat hepatocytes. Measurements of cytosolic free Ca^{2+} were performed in single, aequorin-injected hepatocytes, as described before (13). The experimental medium was Williams medium E (13) to which ET-1 was added.

Glycogen phosphorylase a assay and cAMP determination. Dilution of peptides were made in Hanks' medium containing 20 mM Hepes, pH 7.4, and 0.1% BSA, and used immediately. Incubation of rat hepatocytes (7–9.10^6 cells/ml) with peptides was performed in a final volume of 100 μl for 90 s (except otherwise indicated) at 37°C, and stopped by quick freezing of the tubes in liquid nitrogen. The frozen tubes were kept at −80°C until use.

For cAMP determinations, each 100-μl aliquot of cells was diluted with an equal volume of 6% (vol/vol) trichloracetic acid. The resulting suspension was allowed to stand overnight at 4°C, then transferred to glass tubes and centrifuged at 1,500 *g* for 30 min at 4°C. cAMP determinations were performed in each supernatant by means of radioimmunoassays. For the measurement of glycogen phosphorylase *a* activity the frozen hepatocytes were homogenized according to Hue et al. (14; and as previously described in reference 15). Glycogen phosphorylase *a* was assayed according to Gilboe et al. (16; as described in reference 15), using 50 mM [^{14}C]glucose-1-phosphate (20,000 cpm/assay). In some experiments, phosphorylase *a* was assayed according to Hue et al. (14) and P_i content was determined according to Kallner (17).

Proteins. Membrane proteins were determined by the method of Bradford (18) with bovine serum albumin as standard.

Results

Binding of ET-1 to rat liver plasma membranes. Binding of ^{125}I-ET-1 at 22°C to rat liver membranes was time dependent ($t_{1/2}$ = 37±2 min; n = 3) and reached an apparent equilibrium at ~90 min (k_{obs} = 0.019±0.001 min^{-1}) (Fig. 1). Dissociation of receptor-bound ligand was minimal: only 5% of dissociation was observed 5 h after the addition of 0.2 μM unlabeled ET-1 (Fig. 1).

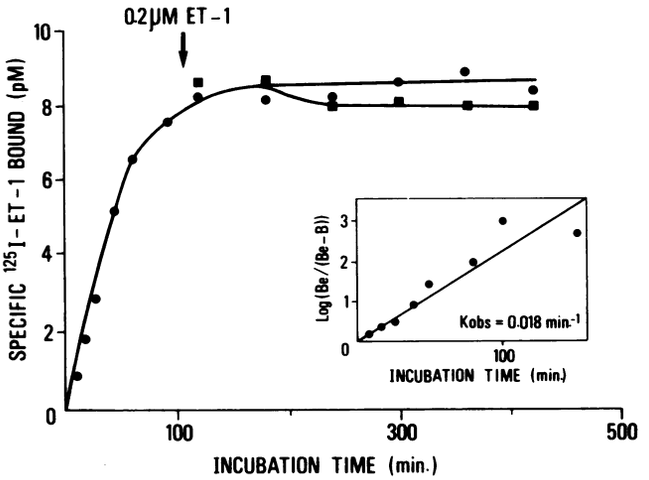

Figure 1. Time course of the ^{125}I-ET-1 association (●) with rat liver plasma membranes and dissociation (■) after addition of 0.2 μM unlabeled ET-1. Inset represents data from a typical evaluation of the bimolecular rate constant (k_{obs}) for the ET-1 receptor association: the equation of the curve is Log (Be/Be-B) = f(t) where Be is the radioactivity specifically bound at equilibrium and B is the radioactivity specifically bound at different times.

Nonspecific binding, determined by incubating 60 pM of ^{125}I-ET-1 and 10–20 μg/ml liver membranes in the presence of 0.1 μM unlabeled ET-1, represented 7% of bound radioactivity. During the incubation period, stability of the ^{125}I-ET-1 was assessed by HPLC analysis of the supernatant obtained after filtration of the incubation medium containing liver plasma membranes. The degradation of ^{125}I-ET-1 represented ~10% under standard assay conditions (data not shown). Binding of ^{125}I-ET-1 to rat liver membranes was saturable and showed high affinity (Fig. 2). Scatchard analysis revealed the presence of a single class of binding sites with an apparent K_d of 32.4±9.8 pM (mean±SEM, n = 3) and a maximal binding site capacity (B_{max}) of 1084±118 fmol/mg protein. As shown in Fig. 3, ^{125}I-ET-1 binding was inhibited in a dose-dependent manner by ET-3 and big ET-1 with IC_{50} values of 1.85±1.03 nM and 43±6 nM, respectively (n = 4). The Hill coefficient of displacement by ET-3 (nH = 0.50±0.07) suggested the presence of two sites of high and low affinities in our membrane preparation.

Binding was highly specific for ET-1 since peptides such as

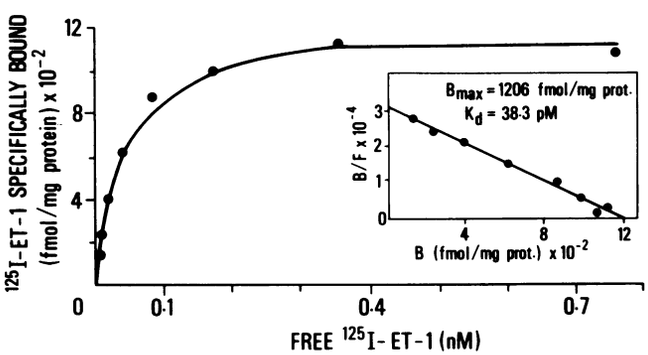

Figure 2. Specific binding of ^{125}I-ET-1 to rat liver plasma membranes at varying concentrations of ^{125}I-ET-1. (*Inset*) Scatchard plot. Results represent data of a typical experiment which was repeated three times.

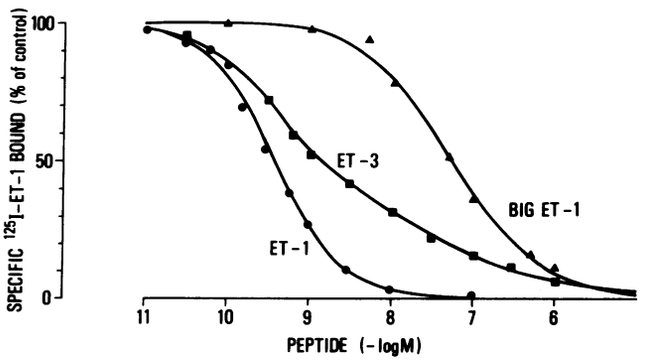

Figure 3. Inhibition of ^{125}I-ET-1 binding by unlabeled ET-1 (●), ET-3 (■), and big ET-1 (▲) in rat liver plasma membranes. Results represent data of a typical experiment which was repeated four times.

neuropeptide Y, vasoactive intestinal peptide, vasopressin, and Ca^{2+} channel antagonists, nifedipine or verapamil, when used at 10 μM concentration, were unable to inhibit the binding of ^{125}I-ET-1 to rat liver receptors. Nor did GTP-γ-S or Gpp-NHp (10 μM) modify this binding (not shown).

Effects of ET-1 on cytosolic free Ca^{2+} in single, isolated, aequorin-injected hepatocytes. In single, isolated, aequorin-injected hepatocytes, ET-1 at 0.2 nM induced a series of repetitive, free Ca^{2+} transients (Fig. 4) that occurred at 90-s intervals after an initial lag of 3–4 min, which could be reduced to 1–2 min depending on the cell. A similar variable delay has been reported for vasopressin-induced Ca^{2+} oscillations in hepatocytes (11, 19). Of 12 cells that were sensitive to phenylephrine, 8 cells responded to 0.1–1 nM ET-1, while 4 cells failed to respond. In each transient, free Ca^{2+} rose from a resting level of 250 nM to above 800 nM. However, in contrast to other agonists such as vasopressin or phenylephrine (not shown; see reference 19), it was difficult to wash off ET-1 to terminate the free Ca^{2+} response. In fact, ET-1 caused a sustained response that lasted for at least 10 min (Fig. 4).

Table I. Effects of ET-1 and Glucagon on cAMP Levels in Hepatocytes

Addition	cAMP
	% of control
None	100
Glucagon 100 nM	306±30
ET-1 5 pM	107±8
ET-1 100 pM	101±4
ET-1 5 nM	90±6

Hepatocytes were incubated for 90 s with ET-1 or glucagon and cAMP levels were determined as described under Methods. The same results were obtained after 300 s incubation with the peptides. In each experiment, data points were paired with their control cAMP levels measured in the absence of peptides and normalized to 100%. Data expressed as a percentage of control cAMP levels are the mean±SEM of three experiments. Mean basal cAMP levels were 4.7±1.1 pmol/mg protein.

Effects of ET-1 on cyclic AMP production. Cyclic AMP was assayed after 2- and 5-min exposures to varying concentrations of ET-1 from 5 pM to 5 nM and to 100 nM glucagon as control. As shown in Table I, while glucagon caused a threefold increase in cAMP levels, ET-1 had no significant effect.

Effects of ET-1 on glycogen phosphorylase a activity in hepatocytes. Incubation of hepatocytes with ET-1 resulted in a rapid and sustained activation of glycogen phosphorylase *a* activity (Fig. 5). A 1.8-fold maximal activation was observed at 1

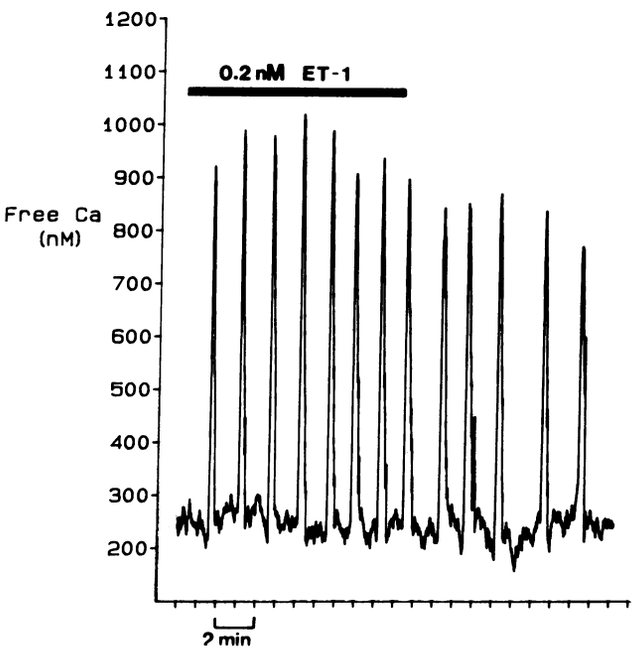

Figure 4. Repetitive free Ca^{2+} transients monitored with aequorin in a single rat hepatocyte exposed to 0.2 nM ET-1. Time constants were 1 s for transients and 10 s for resting levels. Duration of exposure to ET-1 is indicated by the horizontal bars.

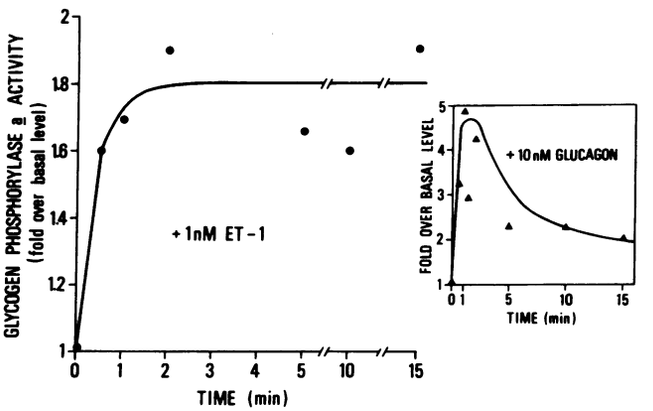

Figure 5. Time course of glycogen phosphorylase *a* activation by ET-1 and glucagon. Hepatocytes (8.10^6 cells/ml) were incubated at 37°C in a final volume of 2 ml containing 1 nM ET-1 or 10 nM glucagon (*inset*). The reaction was stopped by freezing 100-μl aliquots in liquid nitrogen and phosphorylase *a* activity was assayed as described under Methods. Data are expressed as fold increase over basal glycogen phosphorylase *a* activity, which was measured in the presence of Hanks' medium containing 0.1% BSA and 20 mM Hepes, pH 7.4. Basal activity was 30±8 nmol glucose-1-phosphate transformed/min per mg during the first 5-min incubation and decreased to 19±2 nmol glucose-1-phosphate transformed/min per mg between 10 and 15 min. Results are the mean of four experiments.

min and persisted at least for 15 min. In contrast, the maximal increase in the enzyme activity induced by 10 nM glucagon was reached after 1 min and declined after 2 min. The effect of ET-1 on glycogen-phosphorylase *a* was dose dependent, and a maximal 1.8-fold was attained at 1 pM, half-maximal stimulation being observed for 0.03 pM ET-1 (Fig. 6). This degree of activation was comparable to the maximal twofold increase in the enzyme activity obtained with 100 μM epinephrine. (Fig. 6, inset). The specificity of the action of ET-1 was assessed on glycogen phosphorylase *a* using ET-3 and big ET-1. Both peptides were less active than ET-1, causing a maximal activation of 1.5- and 1.2-fold of the enzyme activity, at a final concentration of 100 pM. They were less potent than ET-1 since half-maximal activation was observed for 30 pM ET-3 and for 70 pM big ET-1 (Fig. 6).

Discussion

We report here that, in rat liver, ET-1 has specific receptors and is a typical Ca^{2+} mobilizing hormone that induces activation of glycogenolysis. ET-1 receptors are a single class of high affinity and high capacity binding sites in rat liver plasma membranes, with an apparent K_d of 32.4 pM±9.8 pM, i.e., one order of magnitude higher than that reported in other tissues (20–26), and a B_{max} of 1084±118 fmol/mg protein, comparable to the number of sites reported in aorta, lung, kidney, and brain (20, 27, 28). Moreover, ET-3 discriminates between two ET-receptor subtypes on rat liver plasma membranes as recently reported in chick heart (29), rat lung (30), and on rat vascular smooth muscle cells (31). The binding for ET-1 is specific and saturable within 90 min. The exceeding high affinity of the peptide for its receptor is also reflected by the low reversibility of ET-1 binding (5% after 5 h; Fig. 1), even in the presence of guanine nucleotides (data not shown). This may not be attributed to ET-1 degradation which was minimal during the incubation period. ^{125}I-ET-1 binding kinetics with slow or minimal dissociation of endothelin from its binding sites has been reported in various tissues including aorta and lung (20), brain (28), placenta (32), kidney (27), vascular smooth muscle cells (33, 23), and adrenal glomerulosa cells (22), and could explain the potent long lasting effect of this peptide (1, 6). Elevation of intracellular free Ca^{2+} and not of cAMP has been invoked to mediate the action of ET-1; only in mesangial cells (34) and Swiss 3T3 fibroblasts (35) has a small increase (34) or decrease (35) in cAMP levels been reported in response to ET-1. Our results clearly show that ET-1 does not affect cAMP levels in rat hepatocytes (Table I), but provokes oscillations in cytosolic free Ca^{2+} in single aequorin-injected hepatocytes, similar to that produced by other Ca^{2+} mobilizing hormones (11, 19). We have previously shown that the time course of the transients generated by an individual hepatocyte are characteristic of the stimulating agonist. Oscillations of free Ca^{2+} induced by ET-1 are reminiscent of those caused by vasopressin or angiotensin II, which evoke longer spikes than those observed with phenylephrine (11, 19). However a particularity of ET-1-induced Ca^{2+} transients is that the spikes lasted even after removing the peptide from the perfusion (Fig. 4). The sustained elevation of intracellular free Ca^{2+} in response to ET-1 has been observed in various tissues (36–40), and is associated in hepatocytes with a long lasting activation of glycogenolysis (Fig. 5). The effect of ET-1 on glycogen phosphorylase *a* is specific and the order of potencies of related peptides is similar in binding experiments (Fig. 3) and in glycogen phosphorylase *a* assays (Fig. 6) (ET-1 > ET-3 > big ET), suggesting that the ET-1 binding site defined in liver plasma membranes is correlated to activation of glycogen phosphorylase *a* in isolated rat liver cells. There is up to two orders of magnitude difference between the apparent affinity of ET-1 for its binding sites and for activation of glycogen phosphorylase *a*. This apparent discrepancy has also been reported in liver for vasopressin and angiotensin II, which are 20- to 100-fold weaker for binding to their sites than for activating glycogen phosphorylase *a* (41, 42). In other biological systems such a discrepancy has been already observed, for example, for vasopressin binding potency and antidiuretic response (43).

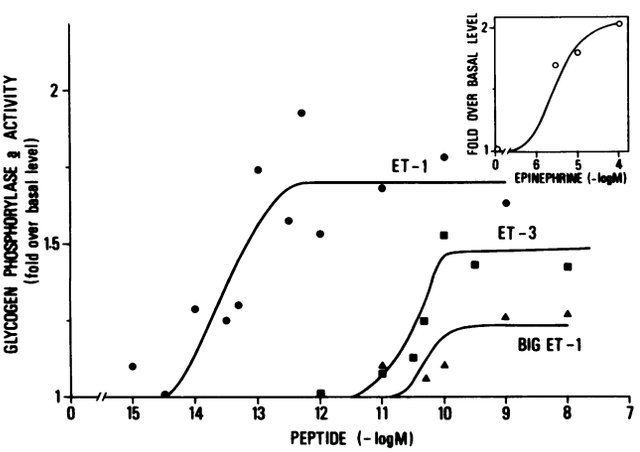

Figure 6. Effects of ET-1, ET-3, big ET-1, and epinephrine on glycogen phosphorylase *a* activity. Hepatocytes (8.10^6 cells/ml) were incubated in a final volume of 100 μl with varying concentrations of either ET-1 (●), ET-3 (■), big ET-1 (▲), or epinephrine (*inset*) for 90 s at 37°C. Glycogen phosphorylase *a* activity was assayed as described under Methods. Data are expressed as fold increase over basal glycogen phosphorylase *a* activity, which was measured in the presence of Hanks' medium containing BSA 0.1% and 20 mM Hepes pH 7.4 (25±5 nmol glucose-1-phosphate transformed/mg per min). Results are the mean of six experiments for ET-1 and two experiments for ET-3 and big ET-1.

This is usually explained by the possible existence of a "receptor reserve". According to this hypothesis, there would be more ET-1 receptors in liver plasma membranes than are necessary to provoke a maximal, functional activation of glycogenolysis. Therefore the maximal glycogenolytic response would be elicited only when a small fraction of ET-1 receptors are occupied. Moreover, the disparity between the potency of ET-1 toward binding and glycogen phosphorylase *a* activation may also be explained by the poor reversibility of the ET-1 binding to its sites, which precludes the exact determination of binding parameters based on complete reversibility such as K_d values.

The presence of high affinity binding sites for ET-1 on hepatocytes raises the question as to the origin of ET-1 which binds to liver plasma membranes. In vascular tissues, it has been shown that ET-1 is elaborated locally by endothelial cells adjacent to its target tissue, while it can also function as a circulating hormone (6). Although most liver functions are performed in parenchymal cells, other cell types are also present in this organ, in particular endothelial and Kupffer cells. Recent studies have stressed the importance of interaction between

liver parenchymal and nonparenchymal cells and suggested that activation of the liver vascular endothelial system results, through vasoconstriction, in localized anoxia and release of various mediators. In fact, PGD_2, or other mediators produced by liver reticuloendothelial cells (44), reportedly mediate stimulation of glycogenolysis by phorbol esters (45), platelet-activating factor (46), and endotoxin (47). Since a recent study indicates that endotoxin stimulates endothelin release in rat circulating blood (48), it is tempting to speculate that endothelin may be locally released by liver reticuloendothelial cells, and would then contribute to the stimulation of glycogenolysis by endotoxin.

The presence of high affinity and high capacity ET-1 receptors in liver plasma membranes, associated with Ca^{2+} mobilization and potent glycogenolytic effect in hepatocytes, in addition to significant amounts of ET-1-like immunoreactivity detected in liver (8), are in favor of a local production of endothelin by liver reticuloendothelial cells. This possibility is now being explored in our laboratory.

Acknowledgments

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