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EFFECTS OF ENDOTHELIN-1 ON BOVINE PARATHYROID CELLS

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Endothelin-1 (ET-1) is synthesized and released by parathyroid epithelium. The effects of endothelin isopeptides were studied in clonal bovine parathyroid endothelial (BPE) cells. BPE cells did not produce ET-1, but showed ET_A receptors (Kd=0.1±0.02 nM, mean ± SE). ET-1 (10^{-8}-10^{-11} M) increased the intracellular calcium ion concentration ($[Ca^{2+}]i$) in BPE cells, while endothelin-3 (ET-3) was ineffective. The increase in $[Ca^{2+}]i$ was less sustained in the absence of extracellular Ca^{2+} ions. Moreover ET-1 induced phospholipase C (PLC) activation, as demonstrated by the increase in inositol trisphosphate. Cell growth was not affected by ET-1 in a wide range of concentrations. The present findings demonstrate: 1) BPE cells possess ET_A receptors; 2) the peptide activates PLC and increases cytosolic $[Ca^{2+}]i$ via both a release of Ca^{2+} ions from intracellular calcium pool(s) and an influx of the cation from the extracellular milieu. A possible role of ET-1 as a paracrine factor in parathyroid tissue can be hypothesized.

During the last decade it has been shown that vascular endothelial cells have a number of specific physiological functions, including regulation of vascular tone, transport of metabolic substrates, production and release of many biologically-active substances. One of these substances, endothelin-1 (ET-1), a vasoconstrictor peptide, was originally discovered in the conditioned medium of cultured porcine aortic endothelial cells (1). Later on, related peptides (ET-2, ET-3, VIC, SRTX) were also isolated (2-5). Endothelin isopeptides have been identified not only in vascular endothelial cells, but also in many other extravascular tissues (6-9). It is generally accepted that this family of peptides participates in the regulation of several physiological functions, including the endocrine system (9-13).

Specific transcripts for ET-1 and the related peptide have been demonstrated in parathyroid glands (8), but only the epithelial component of the parathyroid tissue expressed ET-1 mRNA and released the peptide, while cloned bovine parathyroid endothelial (BPE) cells were not able to synthesize ET-1 (8). In order to investigate whether BPE cells represent a target for ET-1 in parathyroid glands, we studied the presence of ET-1 receptor and ET-1 biological activity in this clonal cell line. Our study suggests a paracrine role of ET-1 in parathyroid tissue.

Abbreviations: ET-1, ET-2, ET-3: endothelin-1, endothelin-2, endothelin-3; PLC: phospholipase C; VIC: vasoactive intestinal contractor; SRTX: sarafotoxin B.

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MATERIALS AND METHODS

Materials. [^{125}I] Porcine endothelin-1 ([^{125}I]ET-1) (sp. act. 74 TBq/mmol, 2000 Ci/mmol) and myo-[^{3}H]inositol (118.5 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). ET-1, SRTX and polyclonal antibody to ET-1 RAS 6901 were purchased from Peninsula Laboratories Inc. (San Carlos, CA); ET-2 and ET-3 were obtained from NovaBiochem (Läufelfingen, CH), VIC from Sigma (St. Louis, MO).

Cell cultures. The BPE cells used in this study were a continuous line of endothelial cells selected and cloned from bovine parathyroid tissue as previously described (14). BPE cells were grown in Coon's modified Ham's F12 containing 1 mM $CaCl_2$, 10% Nu-Serum, 2% Ultroser-G, 200 µg/ml D-galactose, 100 U/ml penicillin, 100 µg/ml streptomycin.

[^{125}I]-ET-1 binding experiments. Binding studies were performed as previously described (10). Subconfluent (0.6-1.5x10^5 cells/well) BPE cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) containing 20 mM Hepes, 10 mM $MgSO_4$, 0.1% BSA, pH 7.4 and incubated in the same medium at 22°C for 90 min with a fixed concentrations (15 pM) of [^{125}I] ET-1 with or without increasing concentrations of unlabelled compounds (10^{-11}-10^{-5}M). After incubation, cells were extensively washed with ice cold PBS, 0.1% BSA, dissolved in 0.1 M NaOH and the cell bound radioactivity was determined. Experiments were carried out in triplicate.

Intracellular calcium measurements. BPE cells were directly grown on glass coverslips for 48 h, loaded with 10 µM Fura-2/AM at 37°C for 45 min (15) in Hepes buffer (137 mM NaCl, 5.6 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5mM glucose, 20 mM Hepes, pH 7.4). Coverslips were then washed and mounted in a perfusion chamber; 500 µl of prewarmed Hepes buffer, 0.1 mM $CaCl_2$ were added. Intracellular cytosolic calcium ion concentration ([Ca^{2+}]i) was measured in single cells using an image analysis system, "Magiscan" (double excitation wavelengths). $CaCl_2$ and endothelin isopeptides were added directly to the perfusion chamber just after recording the [Ca^{2+}]i basal value. Experiments were carried out at 32°C. [Ca^{2+}]i was calculated using the Joyce Loebl software "Tardis" by creating ratio images (340 nm/380 nm) after background subtraction. Calibration curve was performed according to Grynkiewicz et al. (16). Cells appeared uniformly loaded when observed both with CF 100x and CF 40x Nikon objectives. Although otherwise indicated, the 40x objective was used for the experiments.

Inositol phosphate assay . BPE cells, grown to confluence on 100 mm diameter plates, were prelabelled with myo-[^{3}H]inositol (10 µCi/ml) in Coon's modified Ham's F 12 for 48 h. Labelled monolayers were washed twice with an incubation buffer containing 115 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 10 mM $MgCl_2$, 5.6 mM glucose, 10 mM Hepes, 10 mM LiCl, 0.1% BSA, pH 7.4. After treatment with ET-1, inositolphosphates were separated using Dowex AG1-X8 formate resin and radioactivity was counted (17).

[^{3}H]Thymidine incorporation .The assay was performed as previously described (18). Confluent cells were incubated for 48 h in serum-free Coon's modified Ham's F 12. At the end of incubation, ET-1 was added and samples were incubated at 37°C for 48 h in the presence of protease inhibitors (20 U/ml aprotinin and 1 µM PMSF). [^{3}H]-Thymidine (0.5 µCi/ml) was added for the last 4 h. [^{3}H]-Thymidine incorporated into TCA-insoluble cellular fractions was counted .

Microfluorimetric determination of cell growth. Cell proliferation was measured by a semiautomatic microfluorimetric method using Hoechst 3334 DNA stain (19).

Statistical analysis . Statistical analysis was performed with Student's unpaired *t* test. $P<0.05$ was accepted as the level of significance. Binding and inositol trisphoshate data were evaluated quantitatively with a non-linear least- squares curve fitting using the computer programs LIGAND (20) and ALLFIT (21).

RESULTS

In preliminary experiments we found that binding reaction of [^{125}I] ET-1 to BPE cells at 22°C was time-dependent, reaching a maximum at 60-90 min . Moreover, the divalent cations Ca^{2+} and Mg^{2+} significantly ($p<0.005$) increased the specific binding of [^{125}I] ET-1 to BPE cells (data not shown). Incubation for all experiments was therefore performed at 22 °C for 90 min in the presence of 1 mM $CaCl_2$ and 10 mM $MgSO_4$. Scatchard analysis of equilibrium binding curves indicated the presence of an essentially linear relationship. The simultaneous computer modelling, using the program LIGAND (20), of 12 different experiments on BPE cells confirmed that a model involving a single class of binding sites was satisfactory. The estimated affinity constant (K_d) of [^{125}I] ET-1 binding was 0.1±0.02 nM (mean±SE) with a binding capacity of 52.9±9.4 fmol / 10^6 cells (mean±SE). To further characterise the [^{125}I] ET-1 binding characteristic in BPE cells, we performed several families of competition curves using [^{125}I] ET-1 displaced by the corresponding unlabelled peptide and structurally-related peptides ET-2, ET-3, SRTX and VIC (Fig. 1). In BPE cells, ET-1, ET-2 and VIC competed with high affinities (IC_{50}=0.1-0.9 nM) for [^{125}I] ET-1 binding sites, while SRTX and ET-3 showed from 500- to 3300-fold lower potencies than ET-1, respectively (Table 1). The analysis with a logistic model (21) of all the competition curves indicated that the slope factor B (pseudo-Hill coefficient) of the curves was not significantly different from unity (Table 1). This strongly suggested the homogeneity of sites for ET-1, in agreement with the one-site model estimated with the LIGAND program (20).

BPE cells preloaded with Fura-2 showed a basal $[Ca^{2+}]i$ of 101±6.4 nM (mean ±SE) (n= 164), measured with an external calcium ion concentration ($[Ca^{2+}]out$) of 0.1 mM. No change in $[Ca^{2+}]i$ was observed when $[Ca^{2+}]out$ was increased to 1.1 mM.

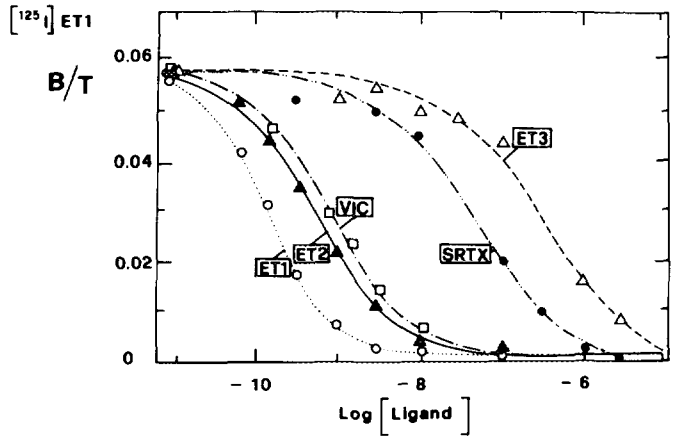

Fig.1. **Effect of ET-1 and related peptides on specific [^{125}I] ET-1 binding**. BPE cells ($0.8x10^5$ cells/well) were incubated for 90 min at 22°C with 56 pM [^{125}I] ET-1 in the presence or absence of incresing concentrations of ET-1 (open circles), ET-2 (closed triangles), ET-3 (open triangles), VIC (open boxes) and SRTX (closed circles).

Table 1. Analysis of competition curves of four - parameter logistic model

Ligand	IC50	Slope	n.
ET-1	0.11±0.03	0.99±0.09	(5)
ET-2	0.89±0.06	1.10±0.03	(2)
ET-3	376.00±22	0.95±0.07	(4)
VIC	0.60±0.15	0.97±0.11	(2)
SRTX	57.10±13	1.20±0.21	(2)

Values ± SE are derived from the simultaneous computer analysis of displacement curves using [^{125}I]ET-1 as labelled ligand. In brackets,the number of experiments.

Figure 2 depicts a typical experiment showing the effect of 10^{-8}M ET-1 on BPE cells ([Ca^{2+}]out 1.1 mM) . ET-1 induced a biphasic effect: a transient conspicuous increase in [Ca^{2+}]i followed by a more moderate and sustained plateau, that decreased in 64 out of 70 analysed cells. The early [Ca^{2+}]i increase ranged from 250 nM to 2130 nM. Figure 3 shows the effect of 10^{-8}M ET-1 on BPE cells (magnification: 100x). In this experiment, [Ca^{2+}]out was increased from 0.1 mM to 1.1 mM at time 33 s and 10^{-8}M ET-1 was administered at time 70 s. The A panel of the figure shows the basal [Ca^{2+}]i ([Ca^{2+}]out 1.1 mM). Panels B, C and D show, respectively,the cells after the administration of ET-1 (time=80.72 s), at time =98.76 s, and time =197.92 s. ET-1 (10^{-9} M) induced an increase in [Ca^{2+}]i in only 48% of the analyzed cells (n=23) and 10^{-10} M ET-1 in 42% of the 38 analyzed cells, while only 11% of cells responded to 10^{-11}M ET-1 (n=9, Table 2). ET-3 (10^{-8} M) induced an increase in [Ca^{2+}]i in 2 out of the 24 analyzed cells (Table 2).

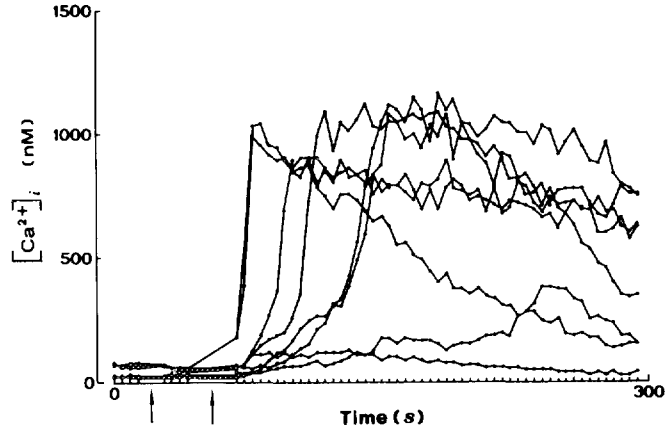

Fig.2. **Effect of 10^{-8} M ET-1 on intracellular free Ca^{2+} concentration of BPE cells**. Cells were incubated for 45 min at 37°C with 5 μM Fura 2-AM and [Ca2+]i was measured in each single cell using digital analysis system. Ratio elaborated images were obtained every 2 s for a total time of 300 s. Each trace represents the [Ca2+]i in a single cell (n=8). [Ca2+]out was increased from 0.1 mM to 1.1 mM at the first arrow and 10^{-8} M ET-1 was applied at the second arrow.

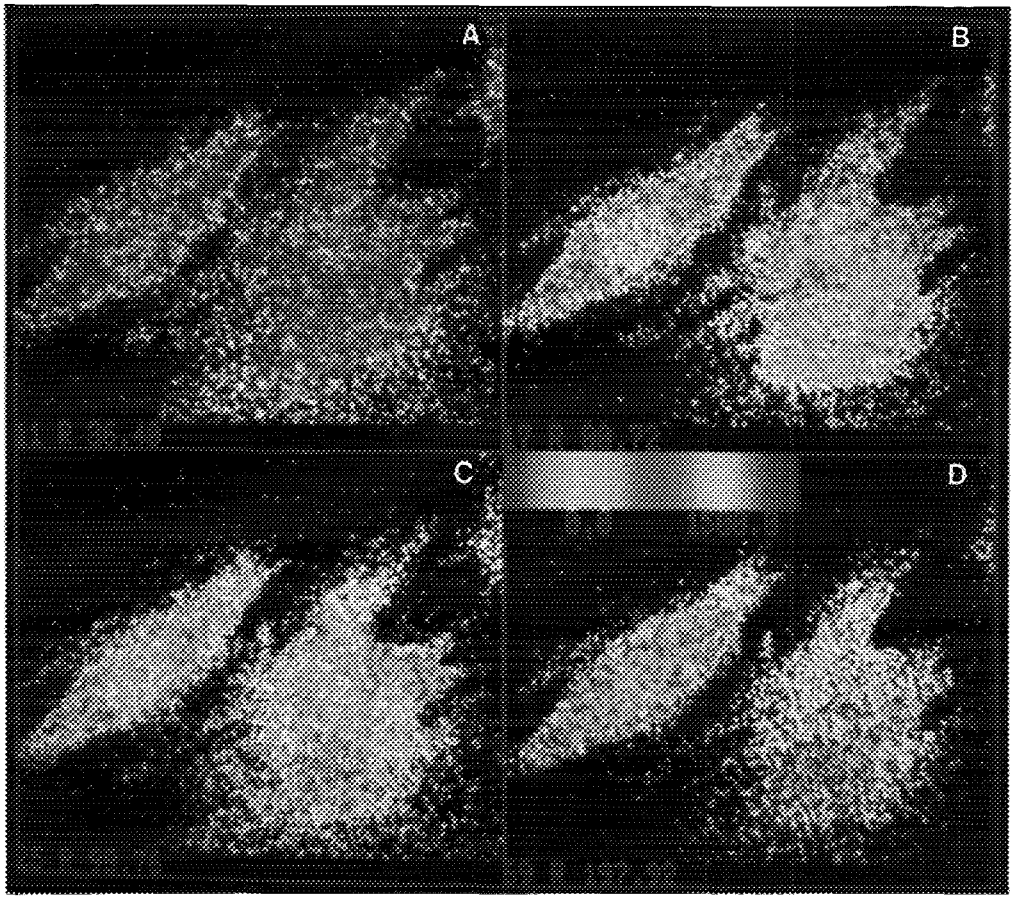

Fig.3. **Effect of 10^{-8} M ET-1 on intracellular free Ca^{2+} concentration of BPE cells**. Cells were incubated for 45 min at 37°C with 5 μM Fura 2-AM and $[Ca^{2+}]i$ was measured in each single cell using digital analysis system. The figure represents the digitalised images of 3 analysed cells (100x). The numbers on the panels indicate the number of the elaborated ratio frame and the time after the beginning of the experiment at which the frame was collected. The upper left panel shows the spatial distribution of $[Ca^{2+}]i$ in the presence of 1.1 mM $[Ca^{2+}]out$ before ET-1 administration (11 R, 36.40 s); the upper right, the lower left and the lower right panels show the spatial distribution of $[Ca^{2+}]i$ 2 s after (17 R, 80.72 s), 20.04 s (25 R, 98.76 s) and 119.20 s (69 R,197.92 s) after 10^{-8} M ET-1 administration, respectively. Spectral bars represent the [Ca2+]i scale (0-1500 nM).

Table 2. % "responder " BPE cells measured at mM external $CaCl_2$ after stimulation with ET-1 and ET-3

	n. responder cells	n. analysed cells	% of responders
10^{-8} M ET-1	64	70	91
10^{-9} M ET-1	11	23	48
10^{-10} M ET-1	16	38	42
10^{-11} M ET-1	1	9	11
10^{-8} M ET-3	2	24	8

The administration of 10^{-8}M ET-1 at $[Ca^{2+}]$out = 0 by addition of EGTA induced an increase in $[Ca^{2+}]$i in 23 out of 26 cells, but the effect was transient and rapidly $[Ca^{2+}]$i returned to the control value (data not shown).

As shown in Figure 4 (inset), 10^{-8}M ET-1 induced a significant increase in inositol trisphosphate (IP_3) production by 15 s, reaching a maximum of twice control value between 15 and 30 s. This elevated level of IP_3 was maintained for at least 10 min. ET-1-stimulated IP_3 formation was dose-dependent with an EC_{50} of 0.11 ± 0.24 nM (mean±SE) (Fig. 4).

The administration of ET-1 (10^{-8}-10^{-12} M) did not increase [^{3}H]-thymidine incorporation in BPE cells (data not shown). Furthermore, DNA content measured with a microfluorimetric assay was not modified after 96 h of incubation with the same doses of ET-1. The addition of 100 ng/ml bFGF to BPE cells increased [^{3}H]-thymidine incorporation by 720 ± 62 % (mean± SE) with respect to control values.

DISCUSSION

We have previously reported that parathyroid epithelial cells produce ET-1 mRNA and synthesise and secrete ET-1(8), while BPE cells do not. In this study we demonstrate the presence of an apparently homogeneous class of binding sites for ET-1 in BPE cells.The endothelin binding

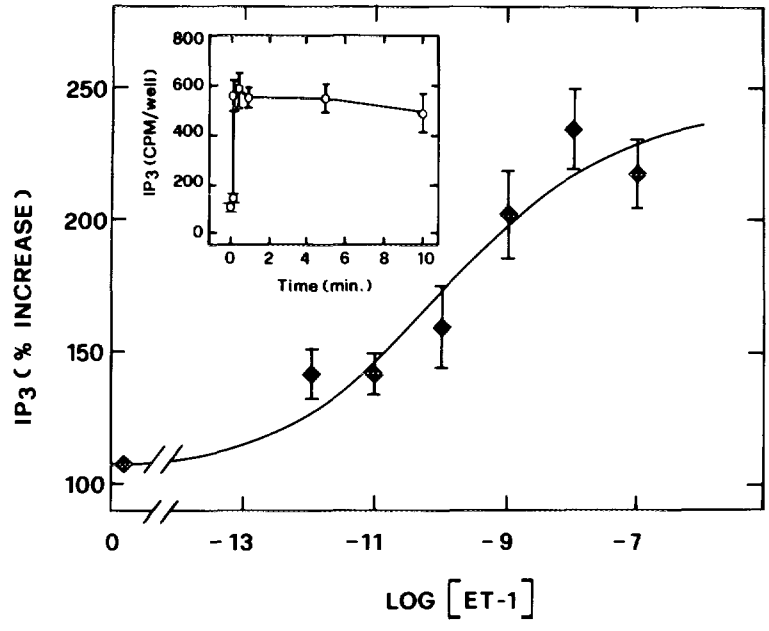

Fig.4. **Dose-response curve for ET-1 stimulation of [^{3}H]inositol trisphosphate formation in BPE cells**. Time of exposure to ET-1 was 10 min. Data were expressed as % increase with respect to control values. Each point represents the mean ± SE of 4 experiments in triplicate. (Inset) Time course of ET-1- induced IP_3 formation in BPE cells. Labelled monolayers were treated with or without 10^{-8} M Et-1 for the indicated times. The basal levels of IP_3 production were subtracted from the stimulated values. Each point represents the mean ± SE (n=3).

site described in this paper possesses high affinity for ET-1, while it binds SRTX and ET-3 with low affinity (ET-1< ET-2 = VIC<< SRTX<< ET-3), and is therefore virtually identical to the ET_A receptor, cloned by Arai et al. from a bovine lung cDNA library (22). The binding of [^{125}I]ET-1 on BPE cells does not depend on the presence of Ca^{2+} and Mg^{2+}, but both cations increase the specific binding as reported in other binding studies on a variety of tissues, including myocardium (23) and myometrium (10).

Functionally, the administration of ET-1 induces an increase in [Ca^{2+}]i in Fura-2 preloaded BPE cells, both in the presence of [Ca^{2+}]out and in its absence, while the administration of ET-3 is ineffective, confirming the results of binding studies. A number of features of the ET-1-induced [Ca^{2+}]i increase in BPE cells deserves to be discussed. Firstly, the dose-response curve in the range from 10^{-8}M to 10^{-11}M can be constructed by analysing the number of "responder" cells. Secondly, the starting time and the time of maximal response are not the same in all analysed cells. This phenomenon might be explained by the fact that BPE cells are not cell-cycle synchronised and therefore the response to exogenous stimuli is not homogeneous in time and potency. Thirdly, the increase in [Ca^{2+}]i in some cells seems to start from a region inside the cell. This confirms the finding that the increase in [Ca^{2+}]i in the single cell is still observed in the absence of [Ca^{2+}]out, suggesting a release from intracellular calcium-storing pool(s). In the absence of [Ca^{2+}]out the [Ca^{2+}]i increase is transient and [Ca^{2+}]i rapidly returns to the basal level.

Furthermore, ET-1 induces a rapid, dose-dependent increase in IP_3, confirming earlier reports (24-26). ET-1 has been indicated as a mitogenic factor in both endothelial (24) and non-endothelial (27-29) cells "in vitro". However, no mitogenic effects of ET-1 were observed on BPE cells.

In conclusion, BPE cells express ET-1 specific receptors; ET-1 activates PLC and increases the [Ca^{2+}]i in these cells. The presence of ET-1 specific receptors in parathyroid endothelial cells and the production of peptide by the epithelial component of parathyroid tissue (8) would suggest a functional paracrine communication between parathyroid epithelial and endothelial cells.

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