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Calcium mobilization and Na^+/H^+ antiport activation by endothelin in human skin fibroblasts

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Endothelin (ET-1) has been shown to exert vasoconstrictor activity in vivo and mobilize Ca^{2+} in vascular smooth muscle cells in culture. In this paper we show that the human skin fibroblast exhibits specific receptors to ET-1 and that activation of these receptors results in increased intracellular Ca^{2+} (Ca_i^{2+}) and accelerated Na^+/H^+ antiport activity. ET-1 raised Ca_i^{2+} in a dose–response manner; the peak Ca_i^{2+} rise was from basal levels of 112.2 ± 21.9 to 299.2 ± 49.7 nM at 300 nM ET-1. This rise was attenuated by removal of extracellular $Ca_i^{2+}0$. Although ET-1 did not alter basal intracellular pH, it enhanced Na^+/H^+ antiport activity of acidified cells. Fibroblasts demonstrated 156 ± 18 (mean ± SE) ET-1 receptors per unit cell and an equilibrium dissociation constant of 203.4 ± 35.6 pM. Inasmuch as ET-1 plays a role in the metabolism of cells such as the undifferentiated fibroblast, an important action of this peptide may be to act as a growth factor.

Endothelin; Growth factor; (Human skin fibroblast)

1. INTRODUCTION

Endothelin-1 (ET-1), a peptide derived from endothelium [1], affects different cells including vascular smooth muscle cells (VSMC) [1–5], mesangial cells [6], atrial myocytes [7] and juxtaglomerular cells [8]. ET-1 increases intracellular Ca^{2+} (Ca_i^{2+}) in cultured VSMC and has vasoconstrictor effects in vascular beds and isolated arterial segments [1–5,9–11]. ET-1 mediated increases in Ca_i^{2+} may result from activation of voltage-sensitive Ca^{2+} channels, as many of the observed responses are dihydropyridine-sensitive and dependent on extracellular Ca^{2+} [1,3,10,11]. However, in VSMC ET-1 also increases inositol phosphates in concert with a rise in Ca_i^{2+} [2,5,12,13], indicating Ca^{2+} mobilization from intracellular stores.

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Only recently has it been appreciated that several vasoconstrictors can act as growth factors for VSMC [14,15]. These include ET-1, which stimulates VSMC proliferation, DNA synthesis, and the induction of c-*fos* and c-*myc* mRNAs [3,4]. Since ET-1 elicits growth-related responses in VSMCs, we investigated its action in an undifferentiated cell, the human skin fibroblast. We first examined whether fibroblasts could respond to ET-1 by mobilizing Ca^{2+} and activating Na^+/H^+ antiport activity. These two intracellular responses are associated with increased growth [16,17]. Second, we explored whether these cells contained receptors specific for the ET-1 peptide.

2. MATERIALS AND METHODS

2.1. *Materials*

Synthetic ET-1 was from Cambridge Biochemicals (Valley Stream, NY), and ^{125}I-ET-1 from Peninsula Laboratories (Belmont, CA). Fura 2-AM and BCECF-AM were from Molecular Probes (Eugene, OR). All other chemicals and ionophores were from Sigma (St. Louis, MO).

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2.2. *Cell culture*

Human fibroblasts, derived from arm biopsies (2 × 3 mm) of 13 volunteers, were processed as previously described [18]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; 95% air, 5% CO_2 with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin). Cells (passages 6–11) were inoculated 3–4 days prior to experiments on 13.8 × 30 mm glass coverslips in Nunc 6-well plates for subsequent Ca_i^{2+} and pH_i measurements. Binding experiments were performed on cells grown directly in the Nunc 6-well plates. Cells were grown as above (minus antibiotics) until confluent, and were made quiescent by a 24 h FBS depletion. No effects of passage number, or gender or age of donor were observed on the various cell parameters.

2.3. *Measurement of Ca_i^{2+}*

Coverslips (containing 3–7 × 10^5 cells) were incubated with 5 μM fura 2-AM in 2 ml (37°C) of Hepes-buffered solution (HBS) containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 glucose and 0.1% BSA (fraction V), pH 7.35. Cells were exposed to the dye for 60 min, after which they were washed 3 times with HBS and secured in a quartz cuvette in a SPEX CM3 fluorescence spectrometer (equipped with a thermostatically controlled (37°C) cell holder, stirrer, and suction device for removing solutions). Excitation wavelengths were set at 340 and 380 nm and emission wavelength at 505 nm. Cells were preincubated 1–3 min until signal stabilized; at the end of the experiment autofluorescence was determined for each coverslip (by subjecting cells to 2 mM Mn^{2+} and 10 μM ionomycin for 5 min) and subtracted from the fluorescent signal. Calculations of R_{max}, R_{min} and S_{f2}/S_{b2} for Ca^{2+} calibration [19] were performed on similarly prepared coverslips by exposing cells to 3 mM EGTA, 10 μM ionomycin (without and with 0.1% BSA), and 5 mM Ca^{2+}, 10 μM ionomycin (without and with 0.1% BSA).

2.4. *Measurement of cytosolic pH_i*

Coverslips were incubated with 5 μM BCECF-AM at 37°C in HBS for 40 min. After 3 washes in HBS, they were placed in the SPEX (excitation wavelengths 440 and 503 nm and emission wavelength 530 nm) and monitored for 5–7 min until pH_i stabilized. Calibration of pH_i was performed by subjecting cells to 5 μg/ml nigericin in HBS (minus BSA) at pH values ranging between 6.30 and 7.50 and a K^+ of 140 mM (KCl substituted isosmotically for NaCl) [20]. Na^+/H^+ antiport activity was assessed after acidification of cells with an NH_4Cl prepulse, by measuring the Na^+-dependent recovery in pH_i during the initial 10–30 s of pH_i change. This pH_i recovery is inhibited by the amiloride analog 5-(*N*-methyl-*N*-isobutyl)amiloride, indicating that the Na^+/H^+ antiport is activated by cellular acidification in nominally bicarbonate free medium (data not shown). HBS solutions in which the $[Na^+]$ was less than 140 mM contained *N*-methyl-D-glucamine substituted isosmotically for Na^+.

2.5. *^{125}I-ET-1 binding to fibroblasts*

^{125}I-ET-1 binding was performed by incubating fibroblasts for 90 min at 22°C in 1 ml of phosphate-buffered saline (PBS). Binding medium contained 44.4 pM ^{125}I-ET-1 (spec. act. = 1200 Ci/mmol), 0.2% BSA, 100 KU/ml aprotinin and varying concentrations (0–1.2 nM) of unlabeled ET-1. In preliminary experiments, binding of ET-1 reached a plateau phase within 60 min and remained stable for at least 120 min. Thereafter, the medium was aspirated and cells were washed 5 times with 2 ml ice-cold PBS. Cells were extracted with 5% trichloroacetic acid and ^{125}I-radioactivity was measured in a gamma counter. Total binding averaged 0.21 ± 0.03% of total activity added to each well. Nonspecific binding (60.0 ± 4.5% of total binding) was determined in the presence of excess (400 nM) unlabeled ET-1. Cell number was determined by a Coulter counter and averaged 1147970 ± 8470 cells/well.

2.6. *Data analysis*

Data are presented as means ± SE, with *n* equal to the number of individuals studied. Where noted, statistical comparisons were made using the paired Student's *t*-test.

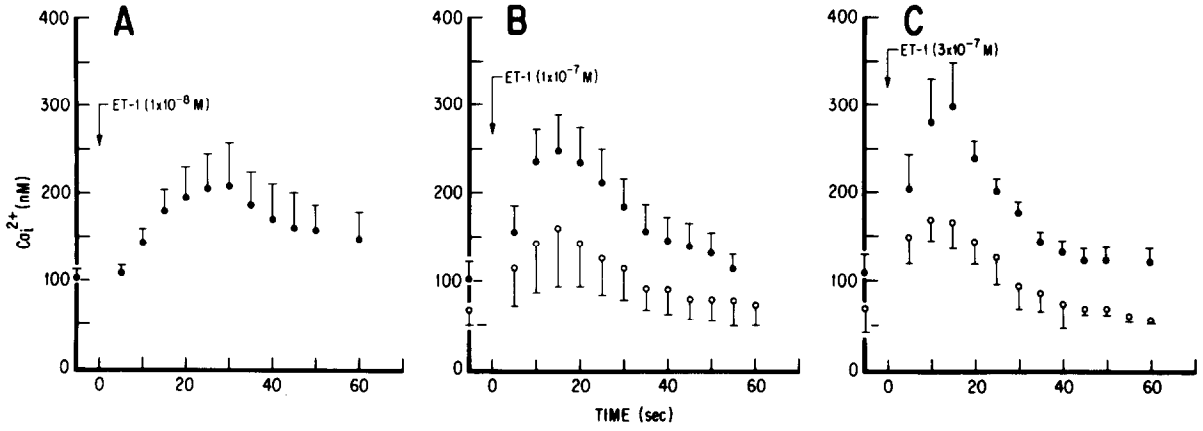

Fig.1. (A–C) Ca_i^{2+} profiles elicited by increasing doses of ET-1 (arrows). Closed symbols ($[Ca^{2+}]_o$ = 1.8 mM) and open symbols ($[Ca^{2+}]_o$ < 1 nM) represent the $[Ca^{2+}]_i$ of 5 individuals responding to ET-1. ET-1 (10 nM) failed to increase $[Ca^{2+}]_i$ in cells exposed to Ca^{2+} free medium.

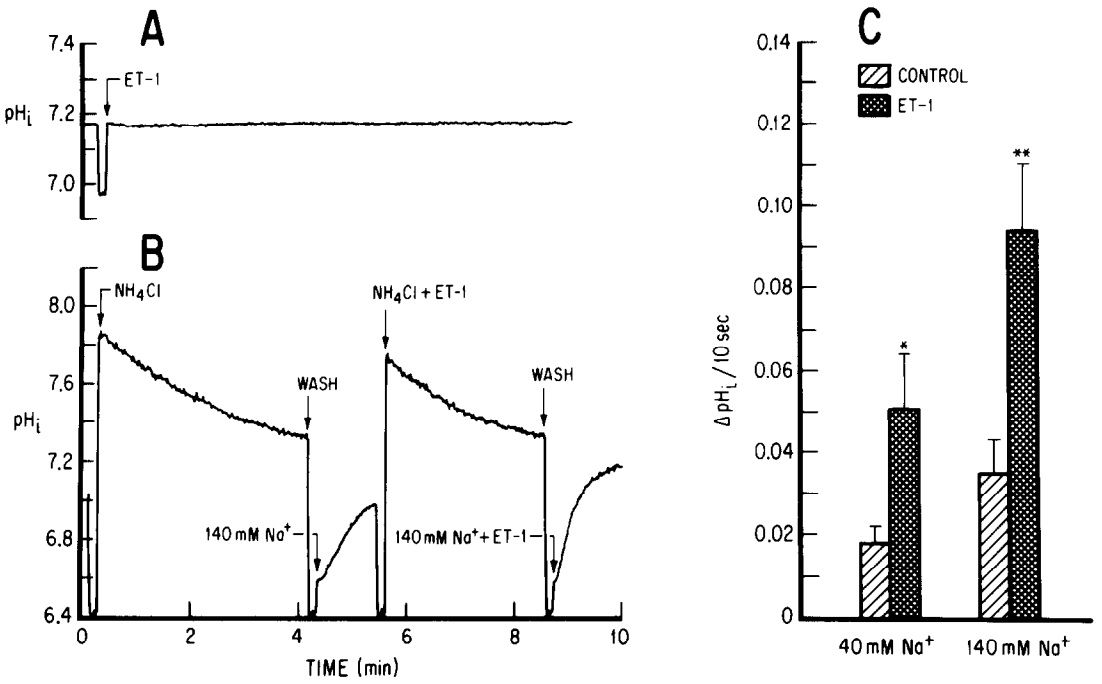

Fig.2. (A–C) Effects of ET-1 on pH_i (A) and Na^+-dependent pH_i recovery in acidified fibroblasts (B and C). 100 nM ET-1 (arrow) was added to cells at rest or after acidification by NH_4Cl prepulse. (B) The NH_4Cl prepulse procedure is illustrated and the effect of ET-1 on the alkalinization response is shown. Recovery solutions contained 140 mM Na^+. In control experiments (not shown), the pH_i values for Na^+-dependent recoveries were not different after two consecutive acidifications by the NH_4Cl prepulse method (0.027 ± 0.004 and 0.026 ± 0.007 pH_i units/10 s, respectively, $n = 5$). (C) Cells were acidified as illustrated in B, and exposed to 40 mM or 140 mM Na^+-containing HBS, without and with 100 nM ET-1 ($n = 7$, * $p = 0.020$, ** $p = 0.003$).

3. RESULTS

Fig.1 shows averaged Ca_i^{2+} transients in fibroblasts from 5 individuals responding to ET-1. In Ca_i^{2+} containing media, the threshold for increases in Ca_i^{2+} occurred at 1–3 nM and maximum Ca_i^{2+} responses were observed at 300 nM ET-1. Peak $[Ca^{2+}]_i$ at 15 s after exposure to ET-1 were significantly greater when measured in the presence of extracellular Ca^{2+} than in Ca^{2+} free medium (containing 1 mM EGTA) (282.0 ± 34.6 vs 161.3 ± 67.5 nM at 100 nM ET-1 and 299.2 ± 49.7 vs 170 ± 23.7 nM at 300 nM ET-1, respectively, $n = 5$, $p < 0.05$). In fibroblasts from 7 other individuals, ET-1 elicited no measurable response in Ca^{2+} free or Ca^{2+}-containing media. The reason for this lack of response was not apparent.

Agonist-mediated Ca_i^{2+} signalling is frequently coupled with activation of the Na^+/H^+ antiport. Moreover, mitogenesis and the action of growth factors are commonly associated with stimulation of the Na^+/H^+ antiport in several cell types, including fibroblasts [14–16]. We measured the effect of ET-1 on pH_i and Na^+/H^+ antiport activity in human fibroblasts (fig.2). ET-1 added to cells under basal conditions ($pH_i = 7.23 \pm 0.05$) exerted no change in pH_i over a 10 min interval (pH_i at 10 min = 7.23 ± 0.05, $n = 5$, fig.2A). However, in cells acidified by the NH_4Cl prepulse method, 100 nM ET-1 significantly increased the rate of Na^+-dependent alkalinization by 1.7-fold (fig.2B,C). These effects were demonstrated in 6 of 7 individuals studied.

^{125}I-ET-1 binding to fibroblasts showed saturable binding. The displacement of labeled ET-1 by the unlabeled peptide (fig.3) indicated a B_{max} value of 156 ± 18 binding sites/cell and a K_d value of 203.4 ± 35.6 pM. The Hill coefficient was 0.99, indicating no evidence of cooperativity in ET-1 binding to fibroblasts.

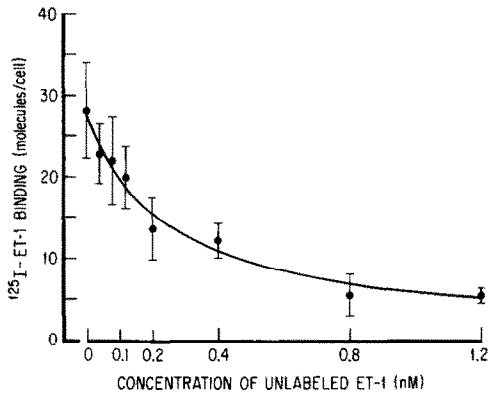

Fig.3. Displacement of ^{125}I-ET-1 from specific receptors by unlabeled ET-1. The curve depicts the fit of the model, described by eqn. 1, to the data,

$$B = B_{max}\{L/K_d \times [1 + (i/K_d)^N] + L\} \quad (1)$$

where B is the specific ET-1 binding, B_{max} is the maximum specific binding, K_d is the equilibrium dissociation constant, L is the concentration of ^{125}I-ET-1, i is the concentration of unlabeled ET-1, and N is the Hill coefficient. Nonlinear regression analysis of ET-1 binding was performed on an IBM compatible personal computer using an NLIN regression procedure of SAS [22].

4. DISCUSSION

Specific receptors for ET-1 have been demonstrated in cultured human VSMCs [5,12] and cultured myometrial cells (Maher, E. and Aviv, A., unpublished data). We report that human skin fibroblasts which respond to ET-1 do so in a dose-response manner with a partial dependence on extracellular Ca_i^{2+}. These cells also exhibit ET-1-dependent changes in pH_i when subjected to an acid load. Finally, human fibroblasts exhibit high-affinity receptors specific to ET-1. These findings suggest ET-1 related Ca_i^{2+} transients and activation of the Na^+/H^+ antiport are receptor mediated, possibly occurring through the phosphoinositide and diacylglycerol signalling systems as shown in other cells [2,6].

The Ca_i^{2+} transients following ET-1 addition to monolayers were similar to VSMCs and other cells [2,6,9,10], with respect to their dose-response curves and extracellular Ca^{2+} dependency. Although a number of preparations failed to show an increase in Ca^{2+} in response to ET-1, specific ^{125}I-binding to cells was readily demonstrated in all preparations studied. These observations suggest that the lack of the Ca^{2+} response in these cells is at a level distal to the receptor-ligand interaction.

There are few reports on ET-1 activation of the Na^+/H^+ antiport. In work with cultured mesangial cells, Badr et al. [6] reported that endothelin increased pH_i from basal values (pH_i = 6.79) to a pH_i of 7.27. We observed no change in resting pH_i with 100 nM ET-1. This difference may be due to the lower basal pH_i in mesangial cells. That is, ET-1 may have shifted the pH_i dependence of the antiporter in mesangial cells to an alkaline pH_i value that resulted in stimulation of Na^+/H^+ exchange and cellular alkalinization. It has previously been demonstrated that serum and specific growth factors activate the Na^+/H^+ antiport by increasing the H^+ affinity of this transport process [22–24]. In cultured fibroblasts, the resting pH_i exhibited by these cells may have been too alkaline, such that activation of the antiport could only be demonstrated following intracellular acidification. Thus, the mechanism of ET-1 stimulation of the Na^+/H^+ antiport may be similar to that suggested for phorbol esters, hormones and growth factors that activate phosphoinositide hydrolysis [25].

The presence of specific ET-1 receptors in human skin fibroblasts and their coupling with Ca_i^{2+} signalling and the Na^+/H^+ antiport suggest a metabolic role for ET-1. Recently, Takuwa et al. [26] reported that endothelin transiently increased c-*fos* and c-*myc* protooncogenes in Swiss 3T3 cells via activation of protein kinase C-dependent mechanisms. We propose that ET-1 acts as a growth factor for fibroblasts and other, more specialized cells possessing ET-1 receptors.

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