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Effect of intracellular Ca^{2+} concentration on endothelin-1 secretion

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Abstract

The role of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cellular regulation of endothelin-1 (ET-1) secretion was investigated in cultured porcine aortic endothelial cells of first passage. Intracellular Ca^{2+} concentrations were adjusted between 50 nM and 1 μM using EGTA and thapsigargin, respectively. ET-1 secretion was maximal at $[Ca^{2+}]_i$ of 190–470 nM, and reduced at low (50 and 110 nM) and high (>470 nM) $[Ca^{2+}]_i$. The Ca^{2+} ionophores A23187 and ionomycin (each 1 μM), both of which raise $[Ca^{2+}]_i$ above 1 μM, also potently inhibited ET-1 secretion under basal and stimulated conditions. The A23187-induced reduction in ET-1 secretion was not affected by N^G-nitro-L-arginine (0.1 mM). Our results provide evidence that basal ET-1 secretion is regulated by Ca^{2+} and that Ca^{2+} ionophores reduce ET-1 secretion due to the inhibitory effect of high $[Ca^{2+}]_i$.

Key words: Endothelin-1 secretion; Cytosolic free Ca^{2+}; Cultured endothelial cell; Ca^{2+} ionophore; Nitric oxide synthase inhibitor; Porcine aorta

1. Introduction

The endothelins (ET) are recently discovered endogenous polypeptides with multiple biologic actions [1]. Endothelins are produced by cultured and native endothelial cells [2], isolated tissues and whole organs [3–5] under basal conditions and in response to a variety of chemical stimuli, including thrombin [6,7] and pressor hormones [8]. The second messengers governing ET secretion are not well understood. Since several of these stimuli elevate the intracellular free Ca^{2+} concentration, Ca^{2+} may mediate the action of these agents. This is supported by the finding that Ca^{2+} ionophores induce a time-dependent increase in ET-1 secretion [8,9] and endothelial prepro-ET-1 mRNA expression after short incubation periods [10]. Recently, however, the Ca^{2+} ionophore A23187 was shown to inhibit rather than stimulate ET-1 secretion [11], and the Ca^{2+} channel blocker nisoldipine reduced basal ET-1 secretion from cultured endothelial cells in a concentration-dependent manner [12]. The goal of the present study was to determine the dependence of ET-1 release on $[Ca^{2+}]_i$ in cultured endothelial cells, whose Ca^{2+} concentration was adjusted to different levels. Since high Ca^{2+} levels maintained for extended periods of time are toxic, particular attention was paid to cell viability. We present evidence that basal ET-1 secretion is close to maximal at physiological $[Ca^{2+}]_i$ but reduced at low and high concentrations due to inhibition of synthesis.

2. Materials and methods

2.1. Materials

[3-(^{125}I)Tyr]-ET-1 (specific activity ~2,000 Ci/mmol) was from ANAWA Trading, Wangen, Switzerland. Fura-2/AM was from Lambda Probes and Diagnostics, Graz, Austria. The source of all other chemicals and tissue culture media was described previously [13]. Concentrations are expressed as final molar concentrations in the buffered incubation medium.

2.2. Cell culture

Porcine aortic endothelial cells were isolated according to the method of Sturek et al. [14] and cultured as previously described [13]. Only confluent cultures of endothelial cells (~10^6 cells per well, or ~10^5 cells per cm^2) of first passage (~10 days after isolation) were used. For each experiment, a separate preparation derived from the pooled cells of 1–3 aortae was used. Confluent cells were incubated in fresh Dulbecco's minimum essential medium (DMEM) without serum for 3 h together with all agents. Following incubation the media were harvested, the cells lysed, and the ET-1 concentration determined by radioimmunoassay (RIA). Cell viability was judged: (i) by optical inspection; (ii) in terms of lactate dehydrogenase (LDH) release into cell-conditioned medium; and (iii) using the colorimetric MTT (tetrazolium) assay developed by Mosmann [15] as modified in the EZ4U-test (Biomedica, Vienna, Austria).

2.3. Ca^{2+} measurement

$[Ca^{2+}]_i$ was measured with the fura-2 technique using a dual wavelength temperature-controlled spectrofluorimeter (Shimadzu Rf 5000/PC, Shimadzu Corp., Vienna, Austria). Confluent endothelial cells were harvested by incubation with protein buffered saline (PBS) containing 0.05% trypsin and 0.02% EDTA for 2 min, the suspension centrifuged, the supernatant aspirated, and the cells resuspended for 45 min in DMEM containing all agents used to adjust $[Ca^{2+}]_i$. Following this, 2 μM fura-2/AM were added for 40 min. Cells were centrifuged, the medium discarded, and the cells resuspended in HEPES buffer (~1×10^6 cells per ml), again containing all agents used to adjust the intracellular Ca^{2+} concentration. Ca^{2+} determinations were carried out on separate plates of cells derived from the same aorta(e) as those which were used for ET-1 determinations. Fura-2 fluorescence was monitored at 37°C by the ratio fluorescence spectroscopy at least 1.5 h after arranging the various Ca^{2+} concentrations (excitation at 340

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Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Fura-2/AM, penta-acetoxymethyl ester of fura-2; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[ethanesulfonic acid]; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration(s); L-NNA, N^G-nitro-L-arginine; PMA, phorbol 12-myristate 13-acetate.

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and 380 nm, emission at 500 nm). Autofluorescence was corrected and $[Ca^{2+}]_i$ calculated according to Grynkiewicz et al [16].

2.4. Measurement of ET-1

Endothelin-1 was measured by RIA using an antibody specific for ET-1 (RAS 6901, Peninsula Laboratories, Belmont, CA, USA). Samples and standards (0.1 ml) were incubated with 0.1 ml assay buffer containing anti-ET-1 antibody for 24 h at room temperature. The radioactive tracer, [3-(^{125}I)Tyr]-ET-1 (specific activity ~2,000 Ci/mmol, 10,000 cpm/tube), was added at the same time as the antibody. To terminate incubation, 0.1 ml γ-globulin (11 mg/ml RIA buffer) and 0.75 ml polyethylene glycol 6,000 (20% in water) was added, the mixture allowed to stand for 5 min at room temperature, and centrifuged for 20 min at 3,000 × g to separate bound from free radioactivity. The supernatant was decanted and the pellet counted in a gamma counter (Packard-Canberra, Vienna, Austria). The intra- and inter-assay coefficients of variation were determined with 3.0 pg ET-1 assayed 4 times in one run and in 4 different runs and were 5.0 and 5.8%, respectively.

2.5. Statistical analysis

Data are presented as arithmetic means ± S.E.M. Differences were tested for statistical significance using Student's unpaired t-test. A P-value ≤ 0.05 was considered to be significant. P-values ≤ 0.01 or lower were not indicated separately. Numbers of experiments (n) refer to different cell cultures.

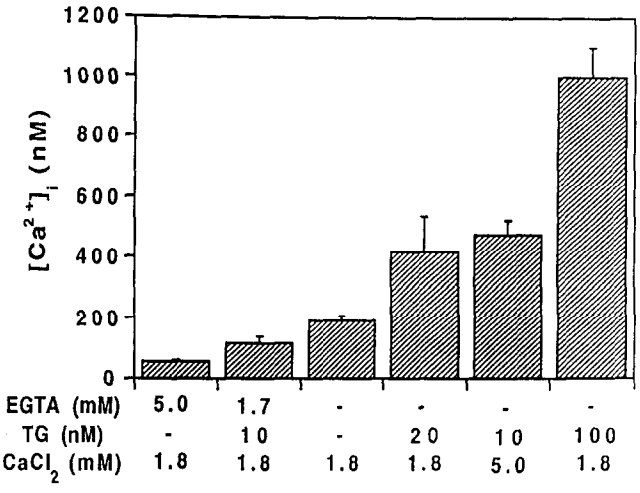

Fig. 2. Adjustment of intracellular free Ca^{2+} concentration using EGTA, thapsigargin (TG) and $CaCl_2$ at the concentrations indicated. $[Ca^{2+}]_i$ was measured by the ratio technique 1.5 h after addition of agents. Data are means ± S.E.M. of 7 different determinations. All values were different from control (1.8 mM extracellular Ca^{2+}).

3. Results

Fig. 1 illustrates the cumulative basal ET-1 release from confluent endothelial cells and ET-1 levels within cells as a function of time under control conditions (1.8 mM extracellular Ca^{2+}). Only a small fraction of ET-1 accumulated within cells (17%), the greater portion (83%) was released into the culture medium. The concentration in conditioned medium after 3 h of incubation was 295 ± 22 pg ET-1 per 10^6 cells. In the presence of phorbol 12-myristate 13-acetate (PMA; 0.4 μM), ET-1 levels in the culture medium increased by 36% (3 h, $P < 0.05$), whereas intracellular levels were not affected. To adjust $[Ca^{2+}]_i$ of cells to different levels, plates of endothelial cells were incubated at different Ca^{2+} concentrations as shown in Fig. 2. An extracellular Ca^{2+} concentration of 1.8 mM (control) resulted in a mean resting $[Ca^{2+}]_i$ of 190 nM. Addition of EGTA (5 mM) or EGTA (1.7 mM) plus thapsigargin (10 nM) [17] to control incubation medium reduced intracellular Ca^{2+} to 50 and 110 nM, respectively, whereas higher extracellular Ca^{2+} (1.8 mM or 5.0 mM) and/or thapsigargin concentrations (10–100 nM) increased $[Ca^{2+}]_i$ up to ~1 μM. The Ca^{2+} ionophores A23187 and ionomycin (1 μM) increase $[Ca^{2+}]_i$ above 1 μM [18]. ET-1 secretion at these different intracellular Ca^{2+} concentrations is shown in Fig. 3. At 190 nM (control), ET-1 secretion was maximal, but reduced to ~50% of control at both the lowest and highest Ca^{2+} concentrations, thus resulting in a bell-shaped relationship between ET-1 secretion and $[Ca^{2+}]_i$. In the presence of A23187 (1 μM), inhibition of ET-1 secretion was indistinguishable (~50%) from that at a 10 times lower Ca^{2+} concentration. A second Ca^{2+} ionophor, ionomycin (1 μM) applied over 5 h, reduced ET-1 secretion to 11.0 ± 1.0% at control extracellular Ca^{2+} (1.8 mM) and to 16.0 ± 1.2% in the additional presence of 5 mM EGTA (n = 3, data not shown). To ascertain that reduced secretion rates were not due to endothelial cell death, viability was judged by visual inspection and verified by two different quantitative assays. No change in appearance of cells incubated with the different agents was noticed. As a measure of cell membrane integrity, LDH release was measured in conditioned media. Control LDH release was less than 5% of total releasable LDH, and enzyme release was not increased in the presence of the various agents, including the Ca^{2+} ionophores. Identical results were obtained using the modified MTT conversion assay, which quantifies release of intracellular dehydrogenase activities (data not shown).

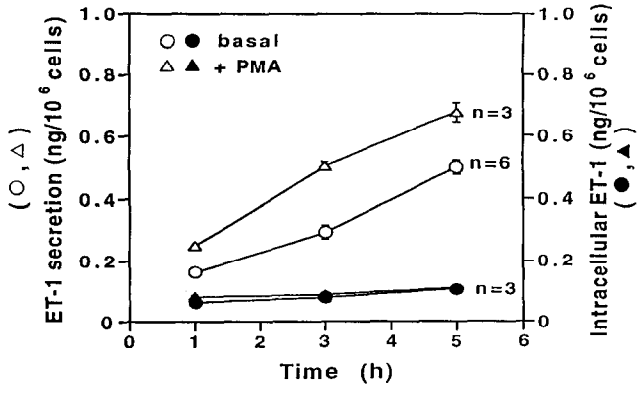

Fig. 1. Time-dependence of cumulative ET-1 secretion into culture medium and accumulation in endothelial cells under basal conditions and in the presence of phorbol 12-myristate 13-acetate (PMA; 0.4 μM). Extracellular Ca^{2+} concentration was 1.8 mM. Data are means ± S.E.M. of 3 or 6 experiments. The effect of PMA on ET-1-secretion from cells was significant.

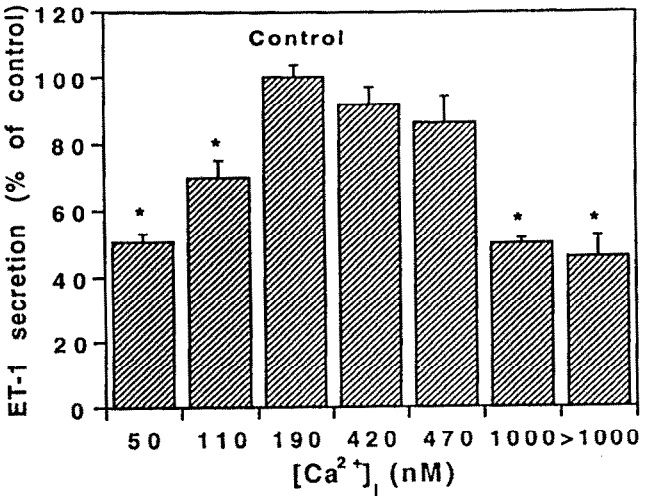

Fig. 3. Relationship between cumulative ET-1 secretion over 3 h and intracellular free Ca^{2+} concentration. ET-1 levels were determined on separate plates of cells derived from the same aorta as those in which Ca^{2+} was measured (see ordinate of Fig. 2). Data are means ± S.E.M. of 7 different determinations. $^*P < 0.05$ vs. control.

The involvement of nitric oxide in the inhibitory effect of A23187 on ET-1 secretion was analyzed at basal and stimulated conditions. Neither inhibition of basal secretion nor secretion stimulated by PMA (0.4 μM) was affected by N^G-nitro-L-arginine (L-NNA, 0.1 mM; Fig. 4).

4. Discussion

Our study shows that basal ET-1 secretion is governed by the intracellular free Ca^{2+} concentration. Depriving cells of extracellular Ca^{2+} decreased ET-1 secretion, as did manipulations designed to increase $[Ca^{2+}]_i$ beyond resting levels. The Ca^{2+} ionophores A23187 and ionomycin reduced basal and PMA-stimulated ET-1 production, and this inhibition was time-dependent. Since both ionophores are known to stimulate Ca^{2+} mobilization and entry, the simplest interpretation of these data is that the Ca^{2+} ionophores inhibited ET-1 secretion subsequent to raised intracellular Ca^{2+} levels. The endothelial cells used were of first passage to assure a homogeneous population with highly reproducible peptide secretion rates; they released ET-1 in a time-dependent manner and at rates similar to published reports. On challenge with PMA, a well-known secretagogue [8], they responded with increased secretion rates as previously reported [7].

Under control conditions (1.8 mM extracellular Ca^{2+}), $[Ca^{2+}]_i$ was 190 nM, similar to our previous determinations [13,19]. When intracellular Ca^{2+} levels were reduced by incubating cells with EGTA, ET-1 secretion was suppressed, probably due to inhibition of ET-1 synthesis or processing of proforms. The availability of extracellular Ca^{2+} together with a graded intracellular mobilization by thapsigargin [17,20] reproducibly raised $[Ca^{2+}]_i$ up to ~1 μM, whereas ionomycin (1 μM) increases levels of Ca^{2+} in endothelial cells substantially above 1 μM [18]. The initial increase in ET-1 secretion is consistent with Ca^{2+}-dependent ET-1 synthesis and/or processing. This is also supported by the pronounced inhibition of ET-1 secretion in the presence of ionomycin and EGTA, a situation where the chelator deprives the cells of extracellular and, in turn, intracellular Ca^{2+}. The concentration- and time-dependent inhibition of ET-1 secretion observed here following 3 h of incubation at high $[Ca^{2+}]_i$ agrees well with a report of diminished ET-1 mRNA transcription after 2 h of incubation with Ca^{2+} ionophore [10].

The physical condition of the cells following incubation with various agents affecting $[Ca^{2+}]_i$ is of critical importance in the present study. A toxic effect, however, cannot explain the reduction in ET-1 secretion seen in Fig. 3 at either low or high Ca^{2+} concentrations, since incubation over 3 h did not result in toxic intracellular Ca^{2+} levels as evident from several stringent criteria of cell viability, which all showed that treated cells were as viable as non-treated control cells. Ca^{2+} ionophores applied for as long as 13 h [11] or 24 h [21] may, however, damage cells and compromize ET production.

Our findings that ET-1 secretion is critically dependent on $[Ca^{2+}]_i$ may explain the inconsistent effects on ET-1 secretion obtained with Ca^{2+} mobilizing agents. Thus, angiotensin II was reported to stimulate ET-1 secretion [8] or was without effect [22]. This may be resolved if, given the bell-shaped relationship, the $[Ca^{2+}]_i$ were different in the two populations of cells, allowing an increase in ET-1 secretion [8] or precluding it because the maximal rate had already been attained. Similarly,

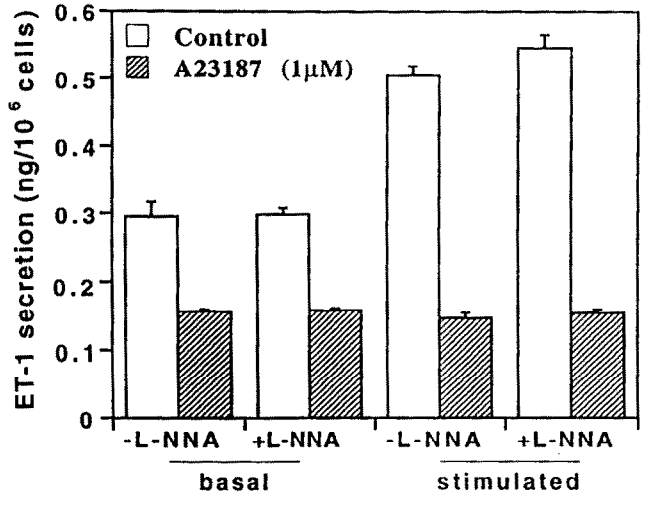

Fig. 4. Effect of L-NNA (0.1 mM) on ET-1 secretion under basal, stimulated (PMA, 0.4 μM), and A23187-inhibited conditions. Endothelial cells were incubated for 3 h, and ET-1 was measured in cell conditioned media. Extracellular Ca^{2+} concentration was 1.8 mM. The data are means ± S.E.M. of 3 different determinations. L-NNA had no effect on either condition.

the stimulatory effects of A23187 [8,9], the lack of effect [12], or the inhibition of secretion (present report) may be due to different resting Ca^{2+} levels.

It is presently unclear how low and pathophysiologically high intracellular Ca^{2+} levels inhibit ET-1 secretion. Nitric oxide does not seem to be involved since both basal and stimulated ET-1 secretion was unaffected by L-NNA which inhibits nitric oxide synthase [23]. At low intracellular Ca^{2+} levels, reduced Ca^{2+}-dependent protein kinase C activity may be limiting ET synthesis (F. Brunner, unpublished observation), whereas high levels of Ca^{2+} might suppress ET synthesis to avoid deleterious effects on blood vessel tone.

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