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DEPENDENCE OF ENDOTHELIN-1 SECRETION ON Ca^{2+}

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Abstract—The role of Ca^{2+} and protein kinase C (PKC) activity in the release of immunoreactive endothelin-1 (ET-1) from cultured porcine aortic endothelial cells of first or second passage has been studied. ET-1 accumulation within cells and secretion into cell-conditioned medium over 3 and/or 5 hr was measured. Confluent cells incubated in medium containing 1.8 mM Ca^{2+} (control condition) accumulated and released ET-1 in a time-dependent way. Reducing intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) by adding the Ca^{2+} entry blockers $NiCl_2$ (0.5 mM) and amiloride (1 mM) or the Ca^{2+} chelator EGTA (5 mM) to the incubation medium reduced ET-1 secretion to between 50 and 30% of controls, respectively ($P < 0.01$). To determine the effect of high $[Ca^{2+}]_i$ on ET-1 release, cells were incubated with thapsigargin (10–1000 nM) or Ca^{2+} ionophore A23187 (1 μM) which raised $[Ca^{2+}]_i$ progressively from 190 nM (control) to > 1 μM. Both agents reduced ET-1 secretion in a concentration-dependent manner to between 50 and 20% of controls ($P < 0.01$). Intracellular levels of ET-1 were also reduced at both low and high $[Ca^{2+}]_i$ ($P < 0.01$). In the presence of the PKC inhibitors chelerythrine (50 μM) and H-7 (60 μM), basal ET-1 secretion was reduced to below 20% of controls ($P < 0.01$). The PKC activator phorbol 12-myristate 13-acetate (0.4 μM) stimulated ET-1 release 1.4-fold ($P < 0.01$) and its effect was abolished by EGTA (5 mM). Increased $[Ca^{2+}]_i$ stimulated the production and release of cyclic guanosine-3′,5′-monophosphate, but basal ET-1 secretion rates correlated poorly with nucleotide levels. These data indicate that: (i) at resting $[Ca^{2+}]_i$ concentrations, ET-1 release is close to maximal and is reduced at lower and higher concentrations, resulting in a bell-shaped relationship between $[Ca^{2+}]_i$ and ET-1 release; and (ii) basal ET-1 release is largely determined by Ca^{2+}-dependent PKC activity.

Key words: basal endothelin-1 secretion; Ca^{2+} dependence; protein kinase C; Ca^{2+} ionophore; thapsigargin; spermine/NO

ET-1† is a recently discovered endogenous polypeptide with multiple biologic actions [1]. ET-1 potently contracts vascular and non-vascular smooth muscle, generally produces a biphasic systemic blood pressure response and modulates aldosterone secretion by the adrenal gland and the glomerular filtration rate of the kidney [2, 3]. Stationary cultures of endothelial cells or isolated organs show basal ET-1 secretion which is enhanced in response to a variety of stimuli, e.g. thrombin, angiotensin II, and ischaemia/reperfusion [4–7]. The second messengers involved in the regulation of basal and stimulated ED-1 secretion are not well understood. Basal ET-1 production was not affected by inhibiting PKC or reducing extracellular Ca^{2+} concentration with EGTA, whereas agonist-stimulated ET-1 release was diminished [5, 8]. The 8-bromo derivative of cGMP reduced basal ET-1 production in cultivated cells [9], but was without effect in native tissues [10, 11]. The role of $[Ca^{2+}]_i$ in ET secretion is also controversial since the Ca^{2+} ionophore A23187 was reported to stimulate [1] or inhibit ET-1 secretion [12].

One possible reason for these discrepancies is that different investigators used qualitatively different endothelial cells. For example, in the studies cited above, cells were subcultured between three [8] and 24 times [5]. Cells may progressively deteriorate, lack receptors or other cellular components necessary for signal transduction, or become deficient in regulatory functions. Another reason may be that the expression levels of distinct isoforms of PKC, e.g. the Ca^{2+}-dependent versus Ca^{2+}-independent forms [13], may differ in cells of different passages. Cell viability is also crucial. Increasing $[Ca^{2+}]_i$ with ionophores applied for extended periods of time [12] may irreversibly damage cells, which may in turn affect ET-1 secretion.

Therefore, the relative contribution of $[Ca^{2+}]_i$ and PKC activity to basal ET-1 secretion was reinvestigated, especially in the light of a recent report which showed ET-1 to be transported in vesicles in bovine aortic endothelial cells [14]. If vesicular transport does indeed occur, Ca^{2+} may play a crucial role. Evidence is presented that $[Ca^{2+}]_i$ determines basal and phorbol ester-stimulated ET-1 secretion and that low and high $[Ca^{2+}]_i$ reduce ET-1 synthesis.

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† Abbreviations: ET-1; endothelin-1; PKC, protein kinase C; c-GMP, guanosine cyclic 3′,5′-monophosphate; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PMA, phorbol 12-myristate 13-acetate; H-7, 1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine dihydrochloride; H-9, *N*-(2-aminoethyl)-5-isoquinoline-sulphonamide hydrochloride; IBMX, 3-isobutyl-1-methylxanthine; DMEM, Dulbecco's minimum essential medium; LDH, lactate dehydrogenase; RIA, radioimmunoassay; Sper/NO, spermine/nitric oxide; ECE, endothelin-converting enzyme.

Table 1. Time-dependence of ET-1 secretion into cell-conditioned medium and accumulation within cells*

Time (hr)	Baseline		PMA (0.4 μM)	
	Conditioned medium	Intracellular	Conditioned medium	Intracellular
	(pg/10^6 cells)		(pg/10^6 cells)	
1	164 ± 7	62 ± 7	253 ± 6	80 ± 6
3	295 ± 22	77 ± 7	504 ± 14	87 ± 3
5	499 ± 22	104 ± 7	676 ± 32	109 ± 4

* Extracellular Ca^{2+} concentration was 1.8 mM and $[Ca^{2+}]_i$ 190 nM. Values were determined directly in medium or in homogenates following freezing and thawing of cells (intracellular). Means ± SEM for 1, 3, and 5 hr (N = 3–6).

MATERIALS AND METHODS

Materials. [3-(^{125}I)Tyr]ET-1 (specific activity ~2000 Ci/mmol) was from ANAWA Trading (Wangen, Switzerland); PMA and chelerythrine chloride were from LC Services Corp. (Woburn, MA, U.S.A.); fura-2/AM (penta-acetoxymethyl ester of fura-2) was from Lambda Probes and Diagnostics (Graz, Austria); α-nicotinamide adenine dinucleotide (reduced form), sodium pyruvate, thrombin (from bovine plasma), Ca^{2+} ionophore A23187, H-7, H-9, collagenase type II, EGTA, EDTA, IBMX, trypsin, trypsin inhibitor and HEPES were from Sigma Chemicals Co. (Deisenhofen, Germany). The source of tissue culture media has been described previously [15]. Concentrations are expressed as final molar concentrations in the incubation buffer.

Cell culture. Porcine aortic endothelial cells were isolated and cultured as previously described [15]. Briefly, cells were generally grown for 6 days (primary culture), removed with trypsin (0.05% in PBS + 0.02% EDTA), centrifuged, reseeded in culture dishes (first passage) and subcultured once. Only confluent cultures of endothelial cells (~10^6 cells/well, or ~10^5 cells/cm^2) of first to second passage were used. Basal ET-1 release was similar in cultures of first and second passage, as was the increase of ET-1 release by PMA (0.4 μM). Purity of cells was > 99% as indicated by the typical cobblestone morphology and immunofluorescence detection of contaminating smooth muscle cells. For each experiment, only cells derived from one preparation (two to three aortas) were used. Approximately 20 hr before experiments, cells were transferred into DMEM without serum. Agents were dissolved in DMEM and cells incubated for 1, 3 or 5 hr as indicated ($CaCl_2$, 1.8 mM). Following incubation, the media were harvested, stored at −70° and ET-1 determined within 3 days.

Cell viability was determined morphologically by trypan blue exclusion and cell counting. In addition, LDH activity was measured in aliquots of cell-conditioned media. The incubations contained 0.1 M K^+-phosphate buffer (pH 7.4), 1.0 mM pyruvate, 0.1 mM NADH and 100 μL assay medium. Decreases in absorbance at 340 nm were determined over a 5-min period at 37°.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured by the fura-2 technique [16] using a dual wavelength temperature-controlled spectrofluorimeter (Shimadzu Rf 5000/PC, Shimadzu Corp., Vienna, Austria). Confluent endothelial cells were harvested by incubation with PBS containing 0.05% trypsin and 0.02% EDTA for 2 min; the suspension was then centrifuged, the supernatant aspirated and the cells incubated for 45 min with all agents used to adjust $[Ca^{2+}]_i$. Fura-2/AM (2 μM final concentration) was added and the cells incubated for another 45 min. Following this, cells were centrifuged, the medium discarded and the cells resuspended in HEPES buffer (~1 × 10^6 cells/mL), again containing all agents used to adjust intracellular Ca^{2+} concentration. Ca^{2+} determinations were carried out on separate plates of cells derived from the same aortas as those used for ET-1 determinations. Fura-2 fluorescence was monitored at 37° by the ratio technique (excitation at 340 and 380 nm, emission at 500 nm), and $[Ca^{2+}]_i$ calculated according to Grynkiewicz *et al.* [17].

Measurement of ET-1. ET-1 was measured by RIA using an antibody specific for ET-1 (RAS 6901, Peninsula Laboratories, Belmont, CA, U.S.A.). Cell-conditioned media and cell lysates obtained after freezing and thawing cells were diluted with assay buffer as appropriate, and 0.1 mL incubated with 0.1 mL assay buffer containing anti-ET-1 antibody for 24 hr at room temperature. ET-1 standards (0.25–32 pg/tube, 0.1 mL) were treated identically. One hundred microlitres of the radioactive tracer [3-(^{125}I)Tyr]ET-1 (spec. act. ~2000 Ci/mmol, 10,000 cpm/tube) were added at the same time as the antibody. To terminate incubations, 0.1 mL γ-globulin (11 mg/mL RIA buffer) and 0.75 mL polyethylene glycol 6000 (20% in water) were added to precipitate bound radioactivity, the mixture allowed to stand for 5 min at room temperature, and centrifuged for 20 min at 3000 × *g* to separate bound from free radioactivity. The supernatant was decanted and radioactivity contained in 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 four times in one run and in four different runs and were 5.0 and 5.8%,

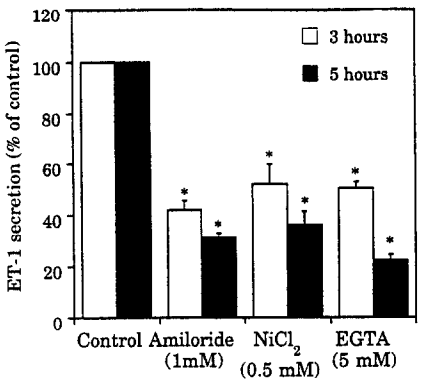

Fig. 1. Effect of agents reducing $[Ca^{2+}]_i$ on ET-1 secretion. Confluent endothelial cells were incubated without drug (Control) or with amiloride, $NiCl_2$ or EGTA for 3 or 5 hr and ET-1 determined in the cell-conditioned medium by RIA. The basal (control) ET-1 secretion rate is given in Table 1. EGTA reduced intracellular Ca^{2+} concentration to 50 nM. Means ± SEM, N = 9–12, *P < 0.01 versus control.

respectively. At the concentrations used in the experiments, none of the agents added (EGTA, thapsigargin, A23187, $CaCl_2$) affected measurement of ET-1.

Statistical analysis. Data are presented as arithmetic means ± SEM of N observations. Differences were tested for statistical significance by Student's unpaired *t*-test. P values of ≤ 0.05 were considered to be significant. Numbers of observations (N) refer to different cell cultures.

RESULTS

Confluent endothelial cells secreted ET-1 in a time-dependent way into the culture medium (Table 1). Only a small fraction was found within cells. ET-1 secretion was stimulated by PMA (0.4 μM) in a time-dependent way. Thrombin (5 U/mL) also stimulated ET-1 secretion time-dependently up to two-fold (data not shown).

The involvement of Ca^{2+} in basal and PMA-stimulated ET-1 secretion was studied using various agents affecting the levels of extracellular and intracellular Ca^{2+} (Fig. 1). When cells were incubated in medium containing 1.8 mM extracellular Ca^{2+}, amiloride (1 mM) and Ni^{2+} (0.5 mM), both of which reduce Ca^{2+} entry, caused a reduction in ET-1 secretion to approx. one-third of controls. When cells were incubated together with EGTA (5 mM) precipitating a drop in intracellular Ca^{2+} concentration to 50 nM, a similar reduction (to 50% and 22%, respectively) was observed ($P < 0.01$ in all cases). The effect of increased $[Ca^{2+}]_i$ on ET-1 secretion was tested using thapsigargin (10 nM–1 μM). This intracellular Ca^{2+} mobilizer increased $[Ca^{2+}]_i$ some fivefold over the basal level (190 nM, Fig. 2A) and reduced ET-1 secretion to 42% (3 hr) or 16% (5 hr) of controls (Fig. 2B). The Ca^{2+} ionophore A23187 (0.1–1 μM) also reduced ET-1 secretion in a concentration-dependent (IC_{50} ~0.2 μM) and time-dependent way (45% of control following 3 hr of incubation, $P < 0.01$, see point 7 in Fig. 3).

Since ET-1 secretion was apparently inhibited by EGTA and Ca^{2+} mobilizing agents, i.e. presumably in the presence of both low and high $[Ca^{2+}]_i$, we studied ET-1 secretion as a function of various cytosolic Ca^{2+} concentrations. $[Ca^{2+}]_i$ was determined by the fura-2 technique and was varied systematically between 50 nM and 1 μM by incubating the cells in solutions of varying Ca^{2+} content adjusted by EGTA or in the presence of thapsigargin. A more than 10-fold variation in $[Ca^{2+}]_i$ was thereby achieved (see abscissa in Fig. 3). ET-1 secretion was markedly inhibited at low $[Ca^{2+}]_i$ (50 and 110 nM)

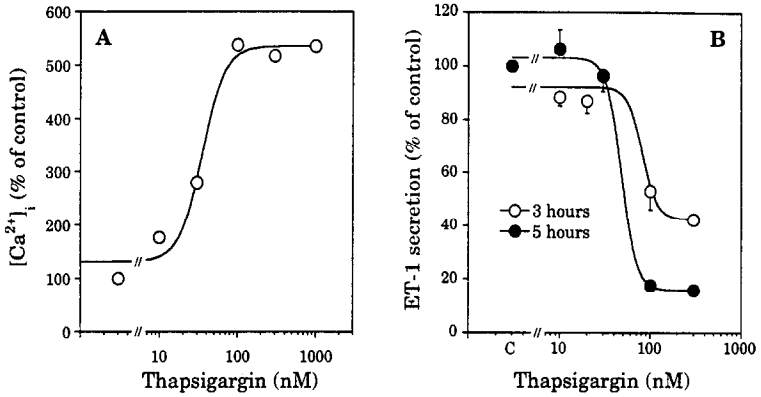

Fig. 2. Effect of thapsigargin on $[Ca^{2+}]_i$ and ET-1 secretion. (A) Confluent endothelial cells were incubated with thapsigargin (10–1000 nM) and $[Ca^{2+}]_i$ measured by the fura-2 technique. Control $[Ca^{2+}]_i$ (no thapsigargin) was 190 nM. Means of two determinations. (B) Confluent endothelial cells were incubated in the absence or together with thapsigargin (10–300 nM) for 3 or 5 hr and ET-1 secretion into cell-conditioned medium determined. Control secretion rate is given in Table 1. Means ± SEM, N = 3–6.

($P < 0.01$ versus control), reached a maximum between 190 and 420 nM $[Ca^{2+}]_i$, and was again reduced with further increases in $[Ca^{2+}]_i$. ET-1 secretion was similarly depressed following 3 or 5 hr incubation.

Intracellular ET-1 levels at the various Ca^{2+} concentrations were also determined. Compared to intracellular ET-1 accumulation at resting $[Ca^{2+}]_i$ (62 ± 7 pg/10^6 cells), accumulation of ET-1 over 3 hr was reduced to 46 ± 2.9 and $42 \pm 12.9\%$ at $[Ca^{2+}]_i$ of 50 nM and 1.2 μM, respectively ($N = 9$, $P < 0.01$ versus control). At 5 hr, the corresponding values were $81 \pm 6.3\%$, and $54 \pm 10.2\%$ of control ($N = 6$, $P < 0.01$ versus control) (data not shown). Thus, ET-1 accumulation within cells was similarly slowed at low and high Ca^{2+} levels as was secretion into conditioned medium.

To exclude the possibility that inhibition of ET-1 release was due to loss of normal cell functioning, cell viability was verified by optical inspection and cumulative LDH release. All cells appeared viable throughout incubation for 3 or 5 hr. Total LDH releasable by sonication of cells for 7 sec (repeated three times) was 350 ± 13 mU/mL (~10^6 cells, $N = 3$). Cells incubated in control Ca^{2+} (190 nM) released 3.32 ± 0.24 mU/mL LDH (1.0% of total); none of the agents used to adjust intracellular Ca^{2+} concentration for the time indicated affected LDH release ($N = 3$, $P > 0.05$) (data not shown).

The role of PKC in ET-1 secretion was studied using PKC inhibitors (Fig. 4). Chelerythrine (50 μM) and H-7 (60 μM) reduced basal ET-1 secretion to 12% and 16% of controls, respectively ($P < 0.01$). ET-1 secretion was increased to 138% by PMA (0.4 μM, $P < 0.01$), and this stimulation was antagonized by chelerythrine, H-7, and H-9 (0.3 mM), a mixed cyclic nucleotide dependent protein kinase/PKC inhibitor. Interestingly, the stimulatory effect of PMA on ET-1 secretion was also dependent on Ca^{2+}, since it was abolished in the presence of EGTA (5 mM).

The involvement of cGMP in the inhibitory effect of a low (EGTA, 5 mM) and high $[Ca^{2+}]_i$ (thapsigargin, 100 nM) on ET-1 secretion was studied (Fig. 5). ET-1 secretion was similarly reduced by both agents (50% of control), but cGMP levels in conditioned media differed more than 20-fold. On the other hand, the nitric oxide donor Sper/NO (1 μM) did not reduce ET-1 secretion, although it increased cGMP levels to the same extent as thapsigargin. Only following a very high concentration of Sper/NO (100 μM), which resulted in a massive (more than 300-fold) increase in cGMP, was ET-1 secretion reduced.

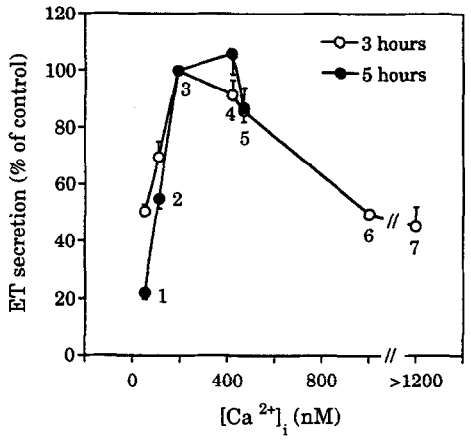

Fig. 3. Relationship between ET-1 secretion and $[Ca^{2+}]_i$. Ca^{2+} concentrations between 50 nM and 1 μM were obtained by incubating cells in culture medium together with the following agents: 1.8 mM $CaCl_2$ ('control Ca^{2+}') + 5 mM EGTA (Point 1); control Ca^{2+} + 1.7 mM EGTA + 10 nM thapsigargin (Point 2); control Ca^{2+} (Point 3 = reference); control Ca^{2+} + 20 nM thapsigargin (Point 4); 5 mM $CaCl_2$ + 10 nM thapsigargin (Point 5); control Ca^{2+} + 100 nM thapsigargin (Point 6), and control Ca^{2+} + 1 μM A23187 (Point 7). ET-1 was measured following 3 or 5 hr of incubation. Means ± SEM, N = 9–12 (ordinate) and N = 6 (abscissa). ET-1 secretion was reduced for points 1, 2, 6, and 7 versus reference; $P < 0.01$. The control secretion rate is given in Table 1.

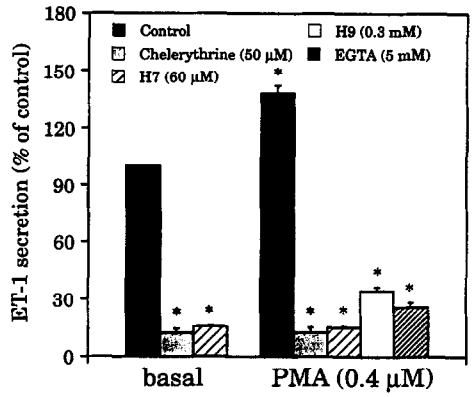

Fig. 4. Effect of inhibition of PKC and chelation of extracellular Ca^{2+} on basal and PMA-stimulated ET-1 secretion. Confluent endothelial cells were incubated without (Control) or with drug for 5 hr and ET-1 determined in cell-conditioned media. The control ET-1 secretion rate is given in Table 1. EGTA reduced intracellular Ca^{2+} concentration to 50 nM. Means ± SEM, N = 9. *$P < 0.01$ versus control.

DISCUSSION

The major finding of the present study was that ET-1 release rates are linked to $[Ca^{2+}]_i$. Depriving cells of extracellular Ca^{2+} reduced ET-1 secretion, as did manipulations designed to increase $[Ca^{2+}]_i$ beyond resting levels. The endothelial cells secreted immunoreactive ET-1 in a time-dependent manner and responded normally on challenge with thrombin, which is well known to stimulate ET-1 secretion [4]. Under control conditions (1.8 mM extracellular Ca^{2+}), the $[Ca^{2+}]_i$ of endothelial cells was 190 nM, similar to previous determinations [15, 18]. Since chelation of extracellular Ca^{2+} by EGTA reduced both intracellular levels and secretion of ET-1, Ca^{2+} is clearly obligatory in ET-1 synthesis. The lower

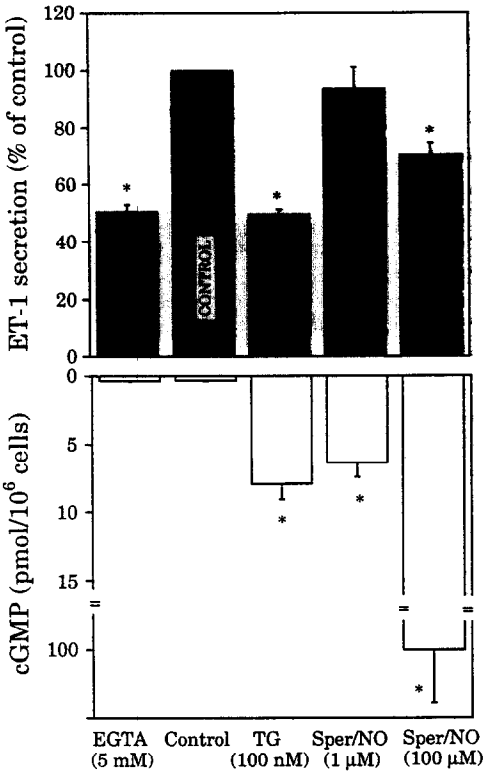

Fig. 5. Relationship between ET-1 release into cell-conditioned medium and cGMP levels following incubation of cells for 3 hr. Upper panel: ET-1 secretion at low (EGTA), control, and high (thapsigargin, TG) $[Ca^{2+}]_i$, as well as in the presence of spermine/NO complex (Sper/NO). Lower panel: cGMP released into culture medium under the same experimental conditions. Experiments were done in the presence of IBMX (1 mM). Means ± SEM, N = 6–9. *$P < 0.01$ versus control ($[Ca^{2+}]_i$ = 190 nM).

ET-1 levels after blockade of receptor-operated Ca^{2+} entry with Ni^{2+} or inhibition of Na^+/Ca^{2+}-exchange with amiloride are consistent with this conclusion. The present work was not designed to elucidate the mechanism of influx of extracellular Ca^{2+} which is still poorly understood with respect to its pathway and control mechanisms [19], since endothelial cells seem to lack Ca^{2+} channels [20]. Our results with Ni^{2+} and amiloride only suggest a participation of Ca^{2+} influx, possibly via a 'leak' channel, in maintaining basal ET-1 secretion. Alternatively, Ni^{2+} may prevent refilling of intracellular Ca^{2+} stores, depletion of which may cause reduced ET-1 synthesis and release.

Thapsigargin, known to elevate steady-state $[Ca^{2+}]_i$ to a higher level [21], was used to determine the effect of increased $[Ca^{2+}]_i$ on ET-1 secretion. As expected, this compound increased $[Ca^{2+}]_i$ in a concentration-dependent manner, ET-1 secretion being reduced in parallel (Fig. 2). This, together with the concentration-dependent reduction of ET-1 secretion in the presence of Ca^{2+} ionophore, is strong evidence for a dual regulation of ET-1 secretion governed by Ca^{2+} both in normoxic (this paper) and hypoxic atmosphere (unpublished). The suppressed ET-1 secretion was probably due to inhibition of ET-1 synthesis or processing of pro-forms, but not inhibition of outward transport, since intracellular ET-1 concentrations were similarly reduced, rather than increased, as extracellular levels. Hence, synthesis and/or processing involves a Ca^{2+} dependent step. The inhibition of ET-1 secretion following 3 hr of incubation at high $[Ca^{2+}]_i$ agrees well with a report of diminished mRNA transcription [22].

The physical condition and function of the cells following incubation with various agents affecting $[Ca^{2+}]_i$ was carefully controlled in the present study. A toxic effect is unlikely to explain the reduction in ET-1 secretion seen in Fig. 3 at either low or high Ca^{2+} concentrations, since incubation over 3 or 5 hr with various agents did not result in additional release of LDH compared to control release at resting Ca^{2+} level. Release of LDH is considered evidence for cell membrane defects and is frequently used to assess cell damage, e.g. following ischaemia and/or reperfusion [23].

Previously, inconsistent effects on ET-1 secretion were observed with certain Ca^{2+} mobilizing agents such as angiotensin II, which either stimulated ET-1 secretion [5] or was without effect [24]. The discrepancy may be explained by different $[Ca^{2+}]_i$ in the two cell populations. Ca^{2+} measurements recorded by Emori and co-workers [5] substantiate this conclusion. In their cells angiotensin II increased $[Ca^{2+}]_i$ up to ~200 nM, resulting in stimulated ET-1 release, which agrees well with the ascending leg of the present relationship (Fig. 3). No Ca^{2+} measurements were reported by Yoshida and Nakamura [24], but since their ET-1 secretion rate was ~5 times higher than that reported by Emori and co-workers [5], it may be assumed that the initial $[Ca^{2+}]_i$ was higher, thus precluding a further rise in ET-1 synthesis and secretion. The stimulation of ET-1 secretion by A23187 [1], the lack of effect on ET-1 secretion [25] and the inhibition of secretion [12, 26] may also, at least in part, be explained by the dual effects of different Ca^{2+} levels.

The mechanism of the inhibitory effect of low and high intracellular Ca^{2+} levels on ET-1 secretion are not understood at present. Raising $[Ca^{2+}]_i$ activates the nitric oxide synthase/cGMP pathway which may curtail ET-1 secretion [10]. Here, the NO donor Sper/NO [27] inhibited ET-1 secretion only at a very high concentration, and the correlation with cGMP levels was poor, suggesting that this is not the main mechanism. Nitric oxide synthase inhibition with N^G-nitro-L-arginine likewise had no effect on basal and stimulated ET-1 secretion [28]. One possible regulatory site of ET-1 production is the catalytic activation of the precursor peptide, big ET-1, via an ECE [29]. A metal chelator-inhibitable protease with ECE activity was recently cloned and expressed in heterologous ECE-deficient cells [30]. The reduced secretion of ET-1 observed in the present study at low $[Ca^{2+}]_i$ could result from inefficient conversion of big ET-1 to ET-1 due to lack of Ca^{2+}. This possibility and the question whether higher $[Ca^{2+}]_i$ than normal inhibit ECE activity will best be studied with the purified enzyme.

PKC has previously been implicated in regulation of ET-1 production [22]. In the cells studied here, PKC activity was largely responsible for basal ET-1 release, since the PKC inhibitors chelerythrine and H-7 greatly reduced secretion. As expected, ET-1 production stimulated by PMA was also antagonized by these agents. Thus, a regulatory role for this enzyme in ET-1 formation is suggested, although the effects of phorbol esters on prepro-ET-1 expression and peptide secretion are variable in magnitude and time-dependent [31]. As to the relative contribution of PKC activity versus $[Ca^{2+}]_i$ to ET-1 production, our data show that Ca^{2+} is the major determinant, since PMA-stimulated ET-1 secretion was also reduced in the presence of EGTA (Fig. 4).

In conclusion, our data suggest that basal release of ET-1 is regulated by $[Ca^{2+}]_i$. The relationship between ET-1 secretion and $[Ca^{2+}]_i$ is bell-shaped; i.e. at low Ca^{2+} concentrations ET-1 secretion is suppressed, possibly involving reduced ECE and/or Ca^{2+}-dependent PKC activity. At resting Ca^{2+} values, ET-1 secretion is close to maximal, and reduced again at high Ca^{2+} concentrations. If also operating *in vivo*, this dual effect of Ca^{2+} on ET-1 secretion may form a self-limiting signal to assure net vasodilation, rather than vasoconstriction.

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