Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin

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We investigated whether calmodulin mediates the stimulating effect of Ca^{2+} on nitric oxide synthase in the cytosol of porcine aortic endothelial cells. Nitric oxide was quantified by activation of a purified soluble guanylate cyclase. The Ca^{2+}-sensitivity of nitric oxide synthase was lost after anion exchange chromatography of the endothelial cytosol and could only be reconstituted by addition of calmodulin or heat-denatured endothelial cytosol. The Ca^{2+}-dependent activation of nitric oxide synthase in the cytosol was inhibited by the calmodulin-binding peptides/proteins melittin, mastoparan, and calcineurin (IC_{50} 450, 350 and 60 nM, respectively), but not by the calmodulin antagonist, calmidazolium. In contrast, Ca^{2+}-calmodulin-reconstituted nitric oxide synthase was inhibited with similar potency by melittin and calmidazolium. The results suggest that the Ca^{2+}-dependent activation of nitric oxide synthase in endothelial cells is mediated by calmodulin.

Nitric oxide; Soluble guanylate cyclase; Endothelial cytosol; Calmodulin; Nitric oxide synthase

1. INTRODUCTION

Endothelial cells synthetize and release a labile factor that relaxes vascular smooth muscle (endothelium-derived relaxing factor; EDRF) [1] via stimulation of soluble guanylate cyclase [2]. This factor is identical or closely related to nitric oxide [3]. An enzyme activity present in the cytosol of endothelial cells catalyzes the formation of nitric oxide from L-arginine in a NADPH-dependent manner [4,5]. It has been shown that the cytosolic nitric oxide formation is activated by free Ca^{2+} [4,5] in concentrations which occur upon stimulation with EDRF/nitric oxide-releasing agonists [6]. However, the mechanism by which this Ca^{2+}-dependence is achieved in endothelial cells is still unknown. Therefore, the objective of the present study was to investigate whether calmodulin mediates the regulatory function of Ca^{2+} on endothelial nitric oxide synthase. We studied the effect of several natural and pharmacological calmodulin antagonists as well as exogenously added calmodulin on nitric oxide formation by crude and partially purified nitric oxide synthase from porcine aortic endothelial cells.

2. MATERIALS AND METHODS

2.1. Isolation of endothelial cells and preparatin of cytosol

Endothelial cells were isolated from porcine aortae as described recently [4,7]. Cells were washed 2 times in 15 mM Hepes, pH 7.5 ($1000 \times g$, 5 min, 4°C), disintegrated by sonication (3×10 s, 100 W), and centrifuged (1 h, $100\,000 \times g$) for preparation of the cytosol. For removal of the endogenous calmodulin, the cytosol was loaded on a Mono-Q column (Pharmacia, Freiburg, FRG) and nitric oxide synthase was eluted with a linear salt gradient (0–0.5 M NaCl). Active fractions were pooled and aliquots were stored at −30°C. Protein content was determined by the Biorad assay (Biorad, München, FRG).

2.2. *Detection of cytosolic oxide formation by activation of soluble guanylate cyclase*

Crude or partially purified nitric oxide synthase (0.1 mg protein/ml) was incubated (30 min) at 37°C (final volume 50 μl) in a buffer containing 1 μg/ml homogeneously purified guanylate cyclase from bovine lung [4] and (in mM) 0.3 L-arginine, 0.1 NADPH, 0.1 [α-^{32}P]GTP (0.2 μCi), 0.1 cGMP, 2 glutathione, 15 Hepes, pH 7.5, 4 $MgCl_2$, 1,3-isobutyl-1-methylxanthine, 3.5 creatine phosphate, 4.8 units creatine phosphokinase, 0.1 mg/ml bovine γ-globulin and 0.1 EGTA. Isolation of [^{32}P]cGMP and calculation of guanylate cyclase activity (nmol cGMP per min per mg purified guanylate cyclase) was performed as described [4]. Endothelial guanylate cyclase activity accounted for about 1% of the cGMP formed in the presence of purified guanylate cyclase and was therefore not considered for calculation of the results. The concentration of free Ca^{2+} was adjusted by addition of $CaCl_2$ and was quantified by fluorescence measurement with the fluorescent Ca^{2+} indicator, indo-1 [6].

2.3. *Determination of the calmodulin content of endothelial cytosol*

The content of endogenous calmodulin in the cytosolic preparations was determined by radioimmunoassay. Non-heated calmodulin standard was used for calibration, since endothelial cytosol was not heated during the preparation. Cytosolic preparations contained 1.9 ± 0.6 mg protein/ml ($n = 27$) and were diluted 10–20-fold.

2.4. *Materials*

The calmodulin-RIA kit was obtained from NEN DuPont (Bad Homburg, FRG). The second (precipitating) antibody (donkey anti-sheep IgG) directed against the primary calmodulin antibody,

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calcineurin (bovine brain, 3000 units/mg protein), melittin, mastoparan, calmidazolium (R 24571), and trifluoperazin were purchased from Sigma (München, FRG). Porcine brain calmodulin and insulin were supplied by Boehringer (Mannheim, FRG). L-arginine, and NADPH were from Serva (Heidelberg, FRG). Fendiline-HCl was kindly donated by Thiemann Arzneimittel (Waltrop, FRG). Calcineurin was dissolved in bidistilled water and passed through a Sephadex G25 column (2 ml) equilibrated with 15 mM Hepes, pH 7.5, to remove low molecular weight constituents of the lyophilisate. Proteins and peptides were diluted in 15 mM Hepes, pH 7.5, containing 0.1 mg/ml bovine γ-globulin. All other substances were supplied and solutions were prepared as described [4, 7].

2.5. *Data evaluation*

Data represent means ± SE of at least 3 independent determinations, each performed in triplicate, if not indicated otherwise. Significance of differences was tested by the Student's *t*-test, with the Bonferroni-correction for the comparison of multiple means [8]. $P<0.05$ was considered significant.

3. RESULTS

The activity of a purified soluble guanylate cyclase was stimulated from 23.0 ± 1.5 nmol · mg^{-1} · min^{-1} to 56.0 ± 2.8 nmol · mg^{-1} · min^{-1} ($n=20$) by endothelial cytosol (0.1 mg protein/ml) in nominally Ca^{2+}-free buffer (about 20 nM free Ca^{2+}). Guanylate cyclase activity was further increased to 129.0 ± 8.3 nmol · mg^{-1} ($n=20$) by 2 μM free Ca^{2+}, indicating a direct Ca^{2+}-dependency of endothelial nitric oxide synthase. The half-maximal effect of Ca^{2+} was observed with 0.3 μM free Ca^{2+}. Addition of bovine brain calmodulin (3 μM) increased guanylate cyclase activity from 51 ± 6 to 100 ± 9 nmol · mg^{-1} · min^{-1} in the presence of 10 nM free Ca^{2+} and from 174 ± 11.5 to 212 ± 15 nmol · mg^{-1} · min^{-1} in the presence of 2 μM free Ca^{2+}. The effects of calmodulin were not mimicked by other hydrophobic proteins like insulin or bovine serum albumin (data not shown). Heat-denatured endothelial cytosol (10 min, 95°C; 0.05 mg protein/ml) did not activate guanylate cyclase in the presence of L-arginine, but significantly increased the calcium-dependent activation of guanylate cyclase in the presence of native endothelial cytosol (0.05 mg protein/ml) by 35 ± 7% ($P<0.05$; $n=3$). The heat-stable activator of calcium-dependent nitric oxide synthesis was probably identical with calmodulin, since 3.0 ± 0.1 μM calmodulin/mg cytosolic protein was detected in endothelial cytosol ($n=12$) by a specific radioimmunoassay.

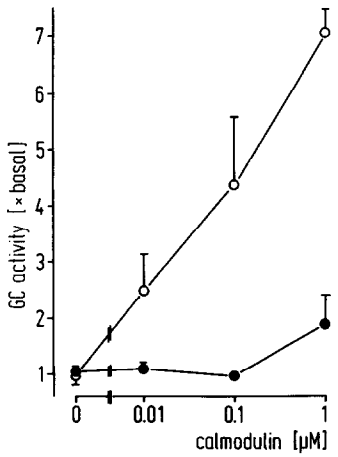

Fig. 1. Ca^{2+}-calmodulin-dependence of nitric oxide formation by partially purified nitric oxide synthase. Nitric oxide synthase was co-incubated with purified guanylate cyclase (GC) at two different Ca^{2+}-concentrations (20 nM (•) and 2 μM (○) free Ca^{2+}) with increasing concentrations of calmodulin in the presence of L-arginine (0.3 mM) and NADPH (0.1 mM) for 30 min at 37°C. Data are means ± SE of 3 independent experiments.

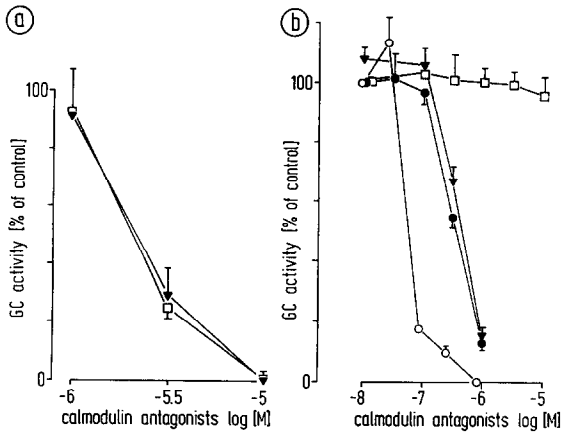

Fig. 2. Effects of calmodulin inhibitors (calcineurin (○), melittin (▼), mastoparan (•) and calmidazolium (□) on nitric oxide-formation by partially purified Ca^{2+}-calmodulin-reconstituted nitric oxide synthase (a) and by endothelial cytosol (b). Effects were quantified by inhibition of guanylate cyclase (GC) activity (% of control in the absense of inhibitors). Ca^{2+}-calmodulin (0.3 μM)-reconstituted nitric oxide synthase (a) or endothelial cytosol (b) was incubated with purified GC at different concentrations of calmodulin inhibitors in the presence of L-arginine (0.3 mM) and NADPH (0.1 mM) for 30 min at 37°C. For further details see text. Results from at least 3 independent experiments performed in triplicate.

The endogenous calmodulin was removed by partial purification of nitric oxide synthase utilizing anion exchange chromatography. Nitric oxide synthase activity in the effluent from a Mono-Q column was not detectable unless procine brain calmodulin *and* 2 μM free Ca^{2+} were added. As shown in Fig. 1, partially purified nitric oxide synthase was half-maximally activated by 100 nM calmodulin. Ca^{2+}-calmodulin (0.3 μM)-reconstituted partially purified nitric oxide synthase was inhibited with similar potency by the peptide calmodulin antagonist, melittin, and by calmidazolium (Fig. 2a). In contrast, the Ca^{2+}-dependent activation of nitric oxide synthase in the cytosol was inhibited by the Ca^{2+}-calmodulin-dependent protein phosphatase calcineurin and the peptides mastoparan and melittin with an IC_{50} of 60, 350 and 450 nM but not by calmidazolium (Fig. 2b). The inhibition of Ca^{2+}-dependent crude nitric oxide synthase in the cytosol by the peptide calmodulin inhibitors (1 μM) was

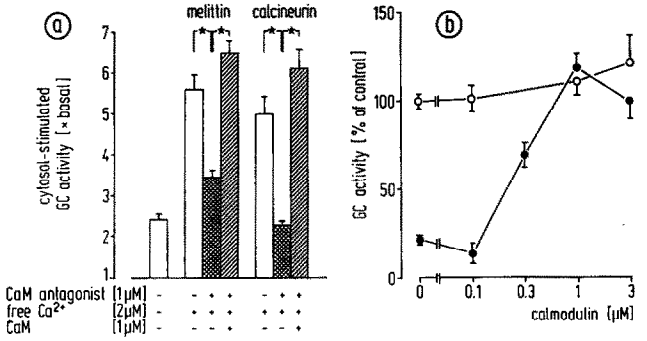

Fig. 3. (a) Free Ca^{2+} (2 μM) enhances the increase in guanylate cyclase (GC) activity elicited by endothelial cytosol (0.1 mg protein/ml) in the presence of L-arginine (0.3 mM) and NADPH (0.1 mM) (open columns). Calmodulin (CaM) antagonists (melittin, calcineurin, both 1 μM) significantly inhibit Ca^{2+}-cytosol-stimulated GC activity ($P<0.05$; $n=20$; cross-hatched columns). Porcine brain calmodulin (1 μM) significantly reverses this inhibition (hatched columns). (b) Porcine brain calmodulin reverses melittin-induced inhibition (1 μM) of Ca^{2+}-cytosol-stimulated guanylate cyclase (GC) activity (•); (○) control in the absence of melittin. Results from at least 4 independent experiments performed in triplicate. Incubation conditions as in Fig. 2.

concentration-dependently reversed by simultaneous addition of calmodulin (1 μM) (Fig. 3a and b).

4. DISCUSSION

The Ca^{2+}-dependent formation of an activator of soluble guanylate cyclase from L-arginine by cytosol from native and cultured aortic endothelial cells has recently been reported [4,5,7]. This activator was probably identical with nitric oxide, since nitric oxide was detected in cytosolic incubates by chemiluminescence [9]. According to these findings the Ca^{2+}-dependency of agonist-induced EDRF/nitric oxide release from intact endothelial cells [6,10] is caused by a direct Ca^{2+} sensitivity of the endothelial nitric oxide-forming enzyme.

In the present investigation we provide evidence that calmodulin or a calmodulin-like protein mediates the stimulatory effect of Ca^{2+} on endothelial nitric oxide synthase. This is substantiated by the findings that: (i) reconstitution of calmodulin to partially purified nitric oxide synthase was required for the stimulating effect of Ca^{2+}; (ii) the Ca^{2+}-calmodulin-reconstituted enzyme was inhibited by the calmodulin antagonists melittin and calmidazolium; (iii) the Ca^{2+}-dependent formation of nitric oxide in endothelial cytosol was inhibited by the calmodulin antagonists, melittin, mastoparan and calcineurin, which bind with high affinity to calmodulin (K_d around 1 nM) [11–13]; (iv) calmodulin and heat-denatured endothelial cytosol, which contained high amounts of calmodulin as detected by radioimmunoassay, competitively reversed the inhibition of Ca^{2+}-dependent nitric oxide synthesis by calmodulin inhibitors.

The lack of inhibition of nitric oxide synthesis in the cytosol by the pharmacological calmodulin inhibitors, calmidazolium and trifluoperazine, as observed in this study and by others [5,14] may be explained by the observation that pharmacological calmodulin inhibitors are by orders of magnitude less potent in crude tissue extracts than expected from their K_i values obtained with purified calmodulin-dependent target enzymes [15,16].

Previous studies have shown a stimulating effect of calmodulin inhibitors on EDFR release from intact cells [17,18]. At first glance, this stimulation is difficult to reconcile with the Ca^{2+}-calmodulin-dependence of nitric oxide synthesis. However, the endothelium-dependent relaxations elicited by melittin [17], and the potentiation of ATP-induced EDRF release from cultured endothelial cells by calmidazolium and fendiline [18] may be caused by the membrane-perturbing properties of these compounds, resulting in an increased Ca^{2+} influx into the endothelial cells [18,19]. Thus the effect of calmodulin inhibitors on intact endothelial cells may be the net result of a complex interference of these compounds with the membrane, the Ca^{2+} homeostasis, and the calmodulin-dependent nitric oxide synthesis.

During preparation of this manuscript, a similar Ca^{2+}-calmodulin-dependency of nitric oxide synthase isolated from rat cerebellum has been reported [20]. In contrast, activation of nitric oxide synthase from LPS-stimulated murine bone marrow macrophages was completely independent of the cytosolic Ca^{2+} level [21]. Therefore it appears that only those cells (e.g. endothelial and neuronal cells) which are supposed to respond quickly to humoral signals, possess a Ca^{2+}-calmodulin-dependent nitric oxide synthase. This enzyme allows the signal transduction of agoinst-induced Ca^{2+}-transients into nitric oxide formation.

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