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Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells

(RFL-6 cells/cyclic GMP/calcium/L-arginine/detergents)

ULRICH FÖRSTERMANN*†‡, JENNIFER S. POLLOCK*, HARALD H. H. W. SCHMIDT*†, MICHAEL HELLER*†, AND FERID MURAD*†

*Abbott Laboratories, Abbott Park, IL 60064; and †Department of Pharmacology, Northwestern University Medical School, Chicago, IL 60611

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ABSTRACT **Endothelium-derived relaxing factor/nitric oxide (EDRF/NO) synthesized by bovine aortic endothelial cells and subcellular fractions thereof was assayed by its stimulating effect on soluble guanylyl cyclase of rat fetal lung fibroblasts (RFL-6 cells). The release of EDRF/NO by intact endothelial cells could be stimulated with bradykinin, thrombin, or ADP and was abolished in Ca^{2+}-free medium. When subcellular fractions were analyzed, some EDRF/NO-synthesizing activity was found in the cytosolic fraction, but most of the activity was associated with the particulate fraction. Both enzyme activities required L-arginine and NADPH for EDRF/NO synthesis, both were inhibited by N^G-nitro-L-arginine and N^G-methyl-L-arginine, and hemoglobin or methylene blue abolished the effect of the EDRF/NO produced by both enzymes. Both enzymes were highly sensitive to Ca^{2+}; the major increase in activity occurred between 100 and 500 nM free Ca^{2+}. Exposure of the particulate enzyme activity to 1 M KCl removed 39% of the protein and reduced total activity by 46%, but the activity was restored when exogenous calmodulin (CaM) was added. Further KCl washes caused little further loss of protein or EDRF/NO synthase activity. The KCl-washed particulate enzyme could be solubilized with the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The CaM antagonists calmidazolium and trifluoperazine as well as the CaM-binding protein calcineurin inhibited the EDRF/NO synthesis by both the cytosolic and the particulate enzyme. These effects were partially reversed with exogenous CaM. Partial purification of the cytosolic and solubilized particulate enzymes by affinity chromatography on adenosine 2′,5′-bisphosphate-Sepharose resulted in EDRF/NO synthase activities dependent on exogenous CaM. We conclude that endothelial cells contain both cytosolic and particulate enzymes that synthesize EDRF/NO. Both enzymes are regulated by free Ca^{2+} and, at least in part, by CaM.**

Endothelial cells synthesize and release endothelium-derived relaxing factor (EDRF) (1). This compound stimulates soluble guanylyl cyclase (sGC) and increases cGMP, thereby relaxing vascular smooth muscle and inhibiting platelet aggregation (1–6). Nitric oxide (NO) is likely to account for the biological activity of EDRF (7–9), and L-arginine or a related material seems to be the precursor for its biosynthesis (10, 11). Besides endothelial cells, EDRF/NO formation has been detected in macrophages (12), neutrophils and HL-60 leukemia cells (13, 14), kidney epithelial cells and adenocarcinoma cells (15, 16), as well as neuroblastoma cells and brain tissue (14, 17–21). In macrophages (22, 23), neuroblastoma cells (18, 20), and brain tissue (19, 21, 24), the EDRF/NO-forming enzymes have been characterized as exclusively cytosolic.

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Less is known, however, about the subcellular localization and regulation of EDRF/NO-synthesizing enzymes in endothelial cells. We have found recently that homogenates of bovine aortic endothelial cells (BAE cells) synthesize EDRF/NO from L-arginine (15). Another report described Ca^{2+}-dependent as well as Ca^{2+}-independent EDRF/NO-forming activity in the cytosolic fraction of BAE cells (25). Other workers found NO generation by a presumably membrane-associated endothelial enzyme that was not dependent on exogenous Ca^{2+} (26). This enzyme activity, however, was observed only at 0°C and not under physiological conditions (i.e., 37°C) (26).

We therefore investigated this issue in more detail and now report that EDRF/NO is mainly generated by the particulate fraction of BAE cells; less activity is present in the cytosolic fraction. Both EDRF/NO-forming enzymes depend on Ca^{2+} in the physiological intracellular range and both seem to be regulated by calmodulin (CaM). Some of these observations have been reported in abstract form (27).

METHODS

Intact BAE Cells. The increase in cGMP in rat fetal lung fibroblasts (RFL-6 cells; ref. 28) was used as a measure of EDRF/NO activity. The assay was performed as described (15, 18, 20, 21). Briefly, RFL-6 cells cultured in six-well plates as described (15, 18, 20) ($\approx 10^6$ cells per well) were washed with phosphate-buffered saline (PBS) and equilibrated for ≥20 min in 1 ml of Locke's solution (154 mM NaCl/5.6 mM KCl/2 mM $CaCl_2$/1.0 mM $MgCl_2$/3.6 mM $NaHCO_3$/5.6 mM glucose/10.0 mM Hepes, pH 7.4) containing 3-isobutyl-1-methylxanthine (IBMX, 0.6 mM). BAE cells (Stanford University) were cultured in six-well plates as described (15) ($\approx 8 \times 10^5$ cells per well). After washing with PBS, they were equilibrated for 20 min in 1 ml of Locke's solution containing superoxide dismutase (SOD, 40 units/ml). EDRF/NO stimulators (bradykinin, thrombin, ADP) were added during the last 2 min of this incubation. Then the BAE cell-conditioned medium (1 ml) was transferred within 5 sec onto the RFL-6 cells (final volume, 2 ml). The incubation was continued for another 2 min. The reaction was then stopped by aspirating the medium, adding 1 ml of ice-cold sodium acetate buffer (20 mM, pH 4.0) to the RFL-6 cells, and rapidly freezing the samples with liquid nitrogen. The

Abbreviations: 2′,5′-ADP, adenosine 2′,5′-bisphosphate; BAE cells, bovine aortic endothelial cells; CaM, calmodulin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDRF, endothelium-derived relaxing factor; IBMX, 3-isobutyl-1-methylxanthine; NMA, N^G-methyl-L-arginine; NNA, N^G-nitro-L-arginine; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase.

‡To whom reprint requests should be addressed at: Abbott Laboratories, Department 47S, Abbott Park, IL 60064.

content of cGMP in each sample was determined by radioimmunoassay (29).

Subcellular Fractions of BAE Cells. BAE cells were cultured in plastic roller bottles (15). The cells were gently scraped from the roller bottles, washed in PBS, and suspended in either ice-cold Tris/HCl buffer (50 mM, pH 7.4) containing EDTA (0.1 mM), EGTA (0.1 mM), and 2-mercaptoethanol (12 mM) (buffer 1) or potassium phosphate buffer (20 mM, pH 7.4) containing EDTA (0.1 mM), EGTA (0.1 mM), and 2-mercaptoethanol (12 mM) (buffer 2). Phenylmethylsulfonyl fluoride (1 mM), leupeptin (3 μM), and pepstatin A (1 μM) were added and the cells were homogenized on ice by using a glass tissue grinder with a Teflon pestle. The homogenates were centrifuged at 100,000 × *g* for 1 hr. The supernatant and pellet fractions were collected, and the pellet was washed once with buffer 1 or 2, recentrifuged, and then resuspended by sonication. Glycerol (10%, vol/vol) was added to both fractions and protein was determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as the standard. The particulate enzyme was preserved only in a buffer containing reduced thiol, glycerol, and protease inhibitors. Under less conserving conditions, we found less activity in the particulate fraction and this material showed little Ca^{2+} sensitivity (27). RFL-6 cells were preincubated for 20 min in 1 ml of Locke's solution containing IBMX (0.3 mM) and SOD (20 units/ml). Aliquots of BAE cytosolic or particulate fractions were added in small volumes to the RFL-6 cells together with L-arginine and NADPH (final concentrations, 1 mM) and the detector cells were incubated at 37°C for 3 min. The reaction was stopped and cGMP was determined in the RFL-6 cells as described above. Experiments with defined Ca^{2+} concentrations (Figs. 2 and 4) were performed in 230 mM Tris/HCl (pH 7.4) containing IBMX (0.3 mM), SOD (20 units/ml), and EGTA (1 mM). Various amounts of $CaCl_2$ were added and free Ca^{2+} concentrations were calculated (30).

In many experiments, the particulate fraction was washed with 1 M KCl (in buffer 1 or 2 containing 10% glycerol) for 5 min at 4°C. After centrifugation (100,000 × *g*, 30 min), the pellet was resuspended in buffer 1 or 2, and both the pellet and the KCl wash were assayed for EDRF/NO-synthesizing activity in the absence and presence of CaM (from bovine brain, Sigma). In some experiments, the KCl wash was repeated three times.

The cytosolic marker enzyme lactate dehydrogenase (EC 1.1.1.27) was measured in parallel with EDRF/NO synthase to assess the amount of cytosolic protein peripherally associated with the particulate fraction and to monitor the successful removal of that protein after the KCl wash. Lactate dehydrogenase activity was measured spectrophotometrically (at 30°C) as the amount of pyruvate consumed, by continuously monitoring the decrease in absorbance due to oxidation of NADH at 339 nm (31).

In further experiments, the particulate fraction prepared in buffer 3 (buffer 1 containing 10% glycerol) was treated with detergent for 20 min at 4°C with rotation of the samples. The detergent was 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, 20 mM), Triton X-100 (1%, vol/vol), Lubrol PX (0.2%, vol/vol), or deoxycholate (5 mM). After centrifugation (100,000 × *g*, 30 min), the supernatants were collected, the pellets were resuspended in buffer 3, and both the detergent-containing supernatants and the pellets were assayed on RFL-6 cells for EDRF/NO-synthesizing activity.

In another series of experiments, one of the following inhibitors of CaM-mediated functions (32) was added to the reaction mixture prior to the BAE protein: calmidazolium (compound R24571, Sigma), trifluoperazine (Sigma), or the CaM-binding phosphoprotein phosphatase calcineurin (Sigma). Calcineurin was dialyzed twice against 100 volumes of PBS (at 4°C) prior to use. Protein was determined and the calcineurin concentration was calculated based on a molecular mass of 85 kDa. In several experiments, CaM was added to the reaction mixture.

Partial Purification of EDRF/NO Synthases. The crude cytosolic fraction in buffer 3 and the KCl-washed and CHAPS-solubilized particulate fraction in buffer 4 (buffer 3 plus 20 mM CHAPS) were loaded onto 100 μl of preswollen adenosine 2',5'-bisphosphate (2',5'-ADP)-Sepharose in a fritted chromatography column. The crude enzyme preparations were recirculated through the columns for 3 hr. Then the columns were washed with 1 ml of buffer 3 or 4, respectively, 400 μl of buffer 3 or 4 containing 0.5 M NaCl, and 1 ml of buffer 3 or 4 again. EDRF/NO synthase was eluted with 400 μl of buffer 3 or 4 containing 10 mM NADPH. Aliquots of column eluates were assayed on RFL-6 cells for EDRF/NO-synthesizing activity in the presence or absence of CaM (1 μM).

Statistics. Statistical differences between mean values were determined by analysis of variance followed by the Fisher protected least-significant-difference test for comparison of different means.

RESULTS

EDRF/NO Production by Intact BAE Cells. When conditioned medium from unstimulated BAE cells (Locke's solution containing SOD at 20 units/ml) was transferred within 5 sec to RFL-6 cells, it produced a modest increase in cGMP in these cells (Fig. 1). When the BAE cells were stimulated with bradykinin (0.1 μM), thrombin (1 unit/ml), or ADP (10 μM), the increase in cGMP was markedly enhanced (Fig. 1). Pretreatment of the BAE cells with N^G-methyl-L-arginine (NMA, 100 μM, 20 min), N^G-nitro-L-arginine (NNA, 10 μM, 20 min) or addition of hemoglobin (10 μM) to the BAE cells or methylene blue (10 μM) to the RFL-6 cells prevented the cGMP-stimulating effect of the BAE cell-conditioned medium ($n \geq 4$, data not shown). In the absence of Ca^{2+}, the EDRF/NO release from BAE cells was abolished (Fig. 1).

EDRF/NO Synthesis by Subcellular Fractions of BAE Cells. When BAE cells were homogenized in buffer 1 or 2 (see *Methods*), some EDRF/NO-synthesizing activity was found in the cytosolic fraction, but most of the activity was asso-

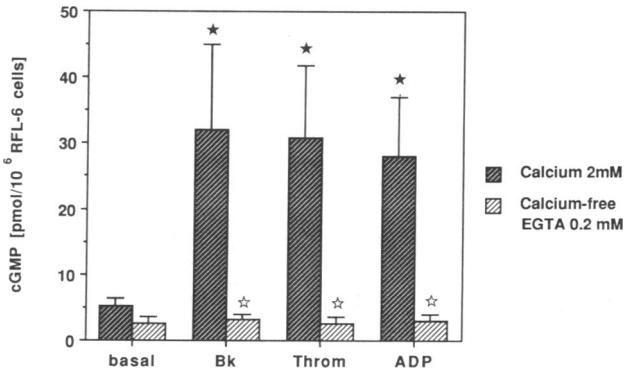

FIG. 1. Production of EDRF/NO by BAE cells. BAE cells released EDRF/NO that increased cGMP levels in RFL-6 cells (basal). The formation of this material was markedly stimulated by bradykinin (Bk, 0.1 μM), thrombin (Throm, 1 unit/ml), or ADP (10 μM). When the BAE cells were incubated in Ca^{2+}-free Locke's solution containing 0.2 mM EGTA, EDRF/NO production was abolished. Unstimulated RFL-6 cells contained 2.5 ± 1.0 pmol of cGMP per 10^6 cells. Nonconditioned media containing the agonists did not increase this value. Bars represent means ± SEM, $n = 6$–8. Filled stars indicate significant stimulation by agonists ($P < 0.05$); open stars indicate significant inhibition in Ca^{2+}-free medium ($P < 0.05$).

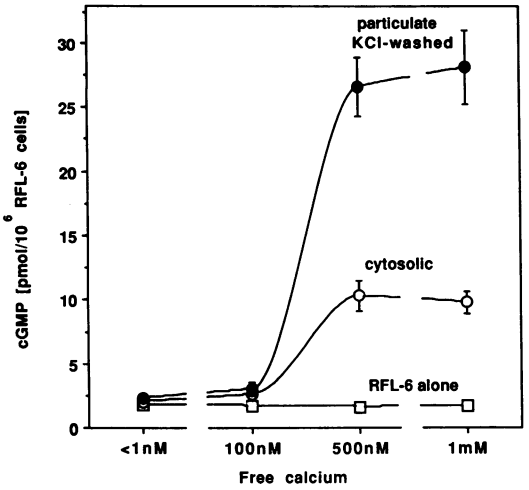

FIG. 2. Effect of the concentration of free Ca^{2+} on the generation of EDRF/NO by the cytosolic and particulate fractions of BAE cells. The particulate material was washed once with 1 M KCl to remove contaminating soluble proteins. Cytosolic or particulate fractions of BAE cells (100 μg of protein) were added to RFL-6 cells and incubations were performed at different Ca^{2+} concentrations buffered with EGTA (1 mM). L-Arginine (1 mM) and NADPH (1 mM) were present in the medium. Symbols represent means ± SEM, $n = 6$.

ciated with the particulate fraction (Fig. 2 and Table 1). There was no difference in total activity or activity distribution between buffers 1 and 2. The soluble and particulate enzyme preparations showed a significant Ca^{2+}-sensitivity with the major increase in activity occurring between 100 nM and 500 nM Ca^{2+}. Mg^{2+} (1 mM) could not substitute for Ca^{2+} (1 mM) in stimulating EDRF/NO synthesis in either the cytosolic or the particulate fraction ($n = 2$ for each).

Characterization of the sGC-Stimulating Material. NNA and NMA inhibited the formation of sGC-stimulating material by both the cytosolic and particulate fractions. Half-maximal inhibition was achieved with about 50 μM NNA and 500 μM NMA, respectively (measured in the presence of 1 mM L-arginine and 1 mM NADPH; $n = 6$ for each). Hemoglobin (10 μM) and methylene blue (10 μM) abolished the cGMP increase in RFL-6 cells in response to the stimulating material formed by either enzyme ($n = 6$ for each).

Further Characterization of the Particulate EDRF/NO-Synthesizing Activity. When the enzyme activity in the particulate fraction was exposed to 1 M KCl for 5 min, this removed 39% of the protein and reduced total EDRF/NO-synthesizing activity by 46% (Table 1). Specific enzyme activity was only slightly decreased. Total activity of the KCl-washed material could be restored if CaM (1 μM) was added to the assay mixture (Table 1). When measured in the presence of CaM, specific activity actually increased in the KCl-washed material, indicating that the KCl wash removed little EDRF/NO synthase but probably removed endogenous CaM. This is confirmed by the finding that only minimal EDRF/NO-synthesizing activity was present in the KCl wash (<10 pmol of cGMP per mg of protein per 3 min in 10^6 RFL-6 cells; $n = 6$). Subsequent KCl washes removed little additional protein and decreased EDRF/NO-synthesizing activity only slightly (Table 1). In BAE homogenates, 27% of the total activity of the cytosolic marker enzyme lactate dehydrogenase was found to be associated with the particulate fraction, but >90% of this contamination was removed with one KCl wash (Table 1).

When the KCl-washed particulate enzyme was treated with the detergent CHAPS (20 mM for 20 min), >80% of the EDRF/NO-synthesizing activity was removed (Fig. 3*a*). At the same time, significant EDRF/NO-synthesizing activity was detected in the detergent wash, indicating solubilization of the enzymatic activity (Fig. 3*a*). At the final concentration occurring on the RFL-6 detector cells (≤1 mM), CHAPS did not interfere with the RFL-6 assay (Fig. 3*b*). Other detergents (1% Triton X-100, 0.2% Lubrol PX, or 5 mM deoxycholate) also removed the enzymatic activity from the particulate fraction ($n \geq 2$ for each), but at the resulting final concentrations they markedly inhibited the cGMP response of RFL-6 cells to sodium nitroprusside (10 mM) or 3-morpholinosydnonimine (1 μM), thus precluding the quantification of EDRF/NO-synthesizing activity in the detergent wash.

CaM Dependence of EDRF/NO-Synthesizing Activities. When CaM (100 nM or 1 μM) was added to the crude cytosolic or particulate fractions, there was no significant increase in EDRF/NO-synthesizing activity (Table 1 and Fig. 4). However, the activity of the KCl-washed particulate material showed a partial dependence on exogenous CaM (Table 1). When soluble and solubilized particulate EDRF/NO synthases were partially purified by 2′,5′-ADP-Sepharose affinity chromatography, the activities in the NADPH eluate (measured in the RFL-6 cell assay) were also dependent on exogenous CaM. The specific activity of the partially purified cytosolic EDRF/NO synthase in the absence of CaM was 3.1 ± 0.2 nmol of cGMP per mg of protein per 3 min in 10^6 RFL-6 cells and increased to 7.4 ± 0.4 nmol in the presence of CaM (1 μM) (means ± SEM, $n = 3$). The specific activity of the partially purified particulate EDRF/

Table 1. EDRF/NO synthase activity in various fractions obtained from a homogenate of 3×10^9 cultured BAE cells (74.3 mg of total protein)

Fraction*	Protein, mg	EDRF/NO synthase activity†			Lactate dehydrogenase activity‡		
		pmol per mg per 3 min	nmol per 3 min	% of total§	units/mg	units	% of total§
Supernatant	14.9	60.5 ± 1.7 (72.0 ± 2.8)	0.9 ± 0.3 (1.1 ± 0.4)	4.6 (5.7)	4.59 ± 0.2	68.4 ± 2.9	72.7
Pellet							
Not washed	57.0	330.6 ± 27.5 (310.9 ± 16.9)	18.9 ± 1.6 (17.7 ± 0.9)	95.4 (94.3)	0.45 ± 0.1	25.7 ± 5.7	27.3
1× KCl-washed	34.9	289.8 ± 13.3 (483.2 ± 5.6)	10.1 ± 0.5 (16.9 ± 0.2)	51.2 (89.7)	0.07 ± 0.0	2.4	2.6
2× KCl-washed	33.1	255.8 ± 14.8	8.5 ± 0.5	42.9	0.05 ± 0.0	1.7	1.8
3× KCl-washed	31.7	249.2 ± 9.4	7.9 ± 0.3	40.0	0.05 ± 0.0	1.6	1.7

The homogenate was prepared in buffer 1 (see *Methods*). The activity of the cytosolic enzyme lactate dehydrogenase is given for comparison. Values are means ± SEM ($n = 3$–4). The preparation is representative of $n = 3$.

*From centrifugation at 100,000 × *g*. Pellet was washed as indicated with 1 M KCl for 5 min at 4°C.

†Measured as cGMP produced by 10^6 RFL-6 detector cells. Values in parentheses indicate activity measured in the presence of 1 μM CaM.

‡One unit = 1 μmol of NADH oxidized per min.

§Total activity defined as the sum of the activities associated with the soluble and particulate fractions.

NO synthase in the absence of CaM was 22.3 ± 3.1 nmol of cGMP per mg of protein per 3 min in 10^6 RFL-6 cells and increased to 94.8 ± 6.7 nmol in the presence of CaM (1 μM) (n = 3). The CaM antagonists calmidazolium (10 μM) and trifluoperazine (30 μM) produced partial inhibition of the crude cytosolic and particulate enzymes (Fig. 4). A similar degree of inhibition was achieved with the CaM-binding phosphoprotein phosphatase calcineurin (1 μM). Addition of exogenous CaM partially antagonized the effects of these inhibitors (Fig. 4).

DISCUSSION

The major finding is that most of the EDRF/NO-synthesizing activity in BAE cells is associated with the particulate fraction. We found a similar distribution of EDRF/NO-synthesizing activity in *porcine* aortic endothelial cells (unpublished observation). In contrast, the NO-synthesizing activity in brain, N1E-115 neuroblastoma cells, and macrophages was detected exclusively in the cytosolic fraction (18–23). The particulate EDRF/NO-synthesizing enzyme in endothelial cells seems to be an integral constituent of a membrane, as it could not be dissociated from the particulate fraction with multiple KCl washes, whereas contamination of the particulate fraction with the cytosolic marker enzyme lactate dehydrogenase was removed in one KCl wash (Table 1). The particulate EDRF/NO-synthase activity could, however, be solubilized with detergent. These observations as well as the amount of activity found in the particulate fraction argues against this activity being trapped cytosolic material. Therefore, the particulate enzyme must be an integral part of a membrane or anchored in the membrane.

The synthesis of EDRF produced by the cytosolic or particulate fraction was inhibited by NNA and NMA and its effect on the sGC of RFL-6 cells was blocked by hemoglobin and methylene blue. This is consistent with NO being the active principle of the EDRF produced by both enzymes. Both the cytosolic and particulate endothelial enzymes were sensitive to Ca^{2+} in the physiological intracellular range. This suggests that both enzymes could be involved in Ca^{2+}-dependent synthesis of EDRF/NO in intact endothelial cells. Both enzymes were partially inhibited by CaM antagonists, including the CaM-binding protein calcineurin. This inhibition could be partially overcome with exogenous CaM. Under physiological conditions both enzymes seem to be saturated with CaM, since addition of exogenous CaM to the crude enzyme preparations did not increase their activity. After a salt wash, however, the particulate enzyme showed a partial dependence on exogenous CaM (Table 1) and partial purification of the enzymes by 2′,5′-ADP-Sepharose affinity chromatography revealed a CaM dependence of both the cytosolic and particulate activities. The CaM dependence, however, was not complete as has been found for the rat brain

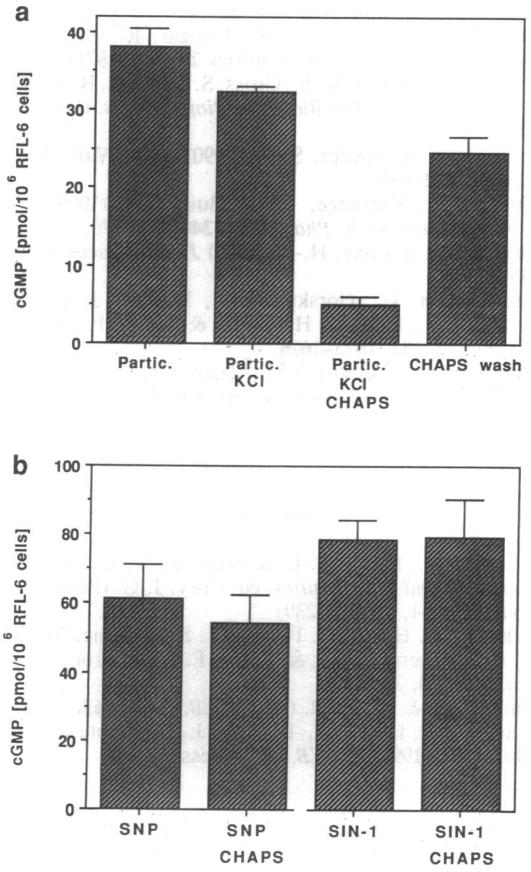

FIG. 3. (*a*) Effect of a wash with KCl (1 M, 4°C, 5 min) and a subsequent exposure to the detergent CHAPS (20 mM, 4°C, 20 min) on the particulate (Partic.) EDRF/NO synthase activity of BAE cells. After exposure of the particulate fraction to CHAPS, the EDRF/NO-synthesizing activity (measured as cGMP increase in RFL-6 detector cells) was reduced by >80%. Significant amounts of EDRF/NO-synthesizing activity were detected in the detergent wash, indicating solubilization. (*b*) At the final concentrations resulting on the RFL-6 detector cells, CHAPS (1 mM) had no significant effect on the cGMP response of these cells to sodium nitroprusside (SNP, 10 mM) or 3-morpholinosydnonimine (SIN-1, 1 μM). The cGMP level in unstimulated RFL-6 cells was 1.72 ± 0.2 for *a* and 1.69 ± 0.3 for *b*. Bars represent means ± SEM, n = 4–6.

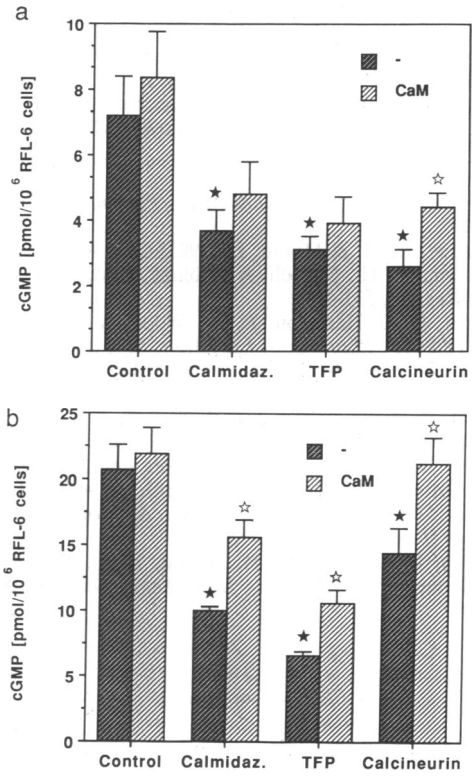

FIG. 4. Effect of CaM inhibitors on EDRF/NO synthesis by the cytosolic fraction (100 μg of protein) (*a*) and the particulate fraction (100 μg of protein) (*b*) of BAE cells. The inhibitors were calmidazolium (Calmidaz., 10 μM), trifluoperazine (TFP, 30 μM), and the CaM-binding phosphoprotein phosphatase calcineurin (1 μM). CaM (0.1 μM for control, calmidazolium, and TFP; 1 μM for calcineurin) partially antagonized the effect of the inhibitors. Filled stars indicate significant inhibition of EDRF/NO synthesis ($P < 0.05$); open stars indicate significant antagonism of this effect by CaM ($P < 0.05$). Bars represent means ± SEM, n = 6–8. The cGMP level in unstimulated RFL-6 cells was 1.84 ± 0.3 for *a* and 2.17 ± 0.1 for *b*.

enzyme after 2',5'-ADP-Sepharose affinity chromatography (33). Thus, there may be some differences in the CaM requirement or CaM-binding between the endothelial enzymes and the NO synthase from rat brain. Nevertheless, the activity of both endothelial enzymes is likely to be regulated, at least in part, by CaM. While this manuscript was under review, a report appeared showing CaM dependence of the cytosolic EDRF/NO synthase from BAE cells (34).

The L-arginine-converting EDRF/NO synthase seems to exist in at least three isoforms: A constitutive cytosolic Ca^{2+}/CaM-regulated, tetrahydrobiopterin-dependent isoform that has been characterized and purified from brain cells (type I; refs. 19–21, 24, 33, and 35); a cytosolic tetrahydrobiopterin/FAD-dependent, Ca^{2+}-independent isoform that can be induced in macrophages (type II; refs. 22 and 23); and the particulate Ca^{2+}/CaM-dependent isoform found in endothelial cells (type III). While we suspect that the cytosolic and particulate activities in endothelial cells may also represent two different isoenzymes (probably types I and III), further purification and analyses are required to investigate this possibility.

In conclusion, our study characterizes a particulate isoenzyme of EDRF/NO synthase in BAE cells. BAE cells also possess cytosolic enzyme activity that synthesizes EDRF/NO. Both activities are Ca^{2+}-dependent and seem to be regulated by CaM. These enzymes may have distinct functions in endothelial EDRF/NO synthesis in response to various stimuli.

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