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ACCELERATED COMMUNICATION

Hormone-Induced Biosynthesis of Endothelium-Derived Relaxing Factor/Nitric Oxide-like Material in N1E-115 Neuroblastoma Cells Requires Calcium and Calmodulin

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SUMMARY

Stimulation of soluble guanylyl cyclase in rat fetal lung fibroblasts (RFL-6 cells) was used as a sensitive assay for endothelium-derived relaxing factor/nitric oxide (EDRF/NO) formation. Intact N1E-115 cells released an EDRF/NO-like material that enhanced cyclic GMP levels in RFL-6 cells. The synthesis of this substance could be stimulated with the receptor agonist neurotensin (10 μM) or by addition of the EDRF/NO substrate L-arginine (100 μM). In Ca^{2+}-free Locke's solution, stimulation of EDRF/NO production by both neurotensin and L-arginine was abolished. The EDRF/NO-synthesizing activity was localized in the cytosol of N1E-115 cells. The activity was lost after boiling and it was highly sensitive to Ca^{2+} with the major increase in activity occurring between 100 and 500 nM Ca^{2+}. L-Arginine and NADPH were required for maximal synthesis of EDRF/NO by the enzyme(s). The synthesis of EDRF/NO was inhibited by the following antagonists of calmodulin-regulated functions (with the approximate IC_{50} values given in parentheses): calmidazolium (7 μM), trifluoperazine (10 μM), fendiline (80 μM), W-7 (*N*-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide) (120 μM), and compound 48/80 (3 μg/ml). The EDRF/NO-synthesizing activity was partially purified from N1E-115 cytosol by DE 52 anion exchange chromatography. The activity was eluted with 0.1 M KCl. The enzyme(s) showed very little activity in the presence of L-arginine (100 μM) and NADPH (100 μM), but the activity could be fully restored by addition of exogenous calmodulin (EC_{50}, ~2 units/ml). At 0.3 M KCl, a fraction eluted from the DE 52 column that was also able to fully restore the EDRF/NO-synthesizing activity. Thus, this fraction is likely to contain the endogenous Ca^{2+}-binding protein. It is concluded that the activity of the EDRF/NO-synthesizing enzyme(s) in N1E-115 neuroblastoma cells is regulated by Ca^{2+} and calmodulin.

EDRF (1) is a labile substance that, by stimulating soluble guanylyl cyclase, increases cyclic GMP and relaxes vascular smooth muscle (1–5). NO is likely to account for the biological activity attributed to EDRF (6–8). EDRF/NO derives from a novel biosynthetic pathway that involves oxidation of a guanidino nitrogen of L-arginine or a related material (9, 10). During the past 2 years, it became apparent that other cell types can also produce EDRF/NO or a similar substance. These cells include activated murine macrophages (11), human neutrophils and HL-60 leukemia cells (12, 13), porcine kidney epithelial cells (14), murine adenocarcinoma cells (15), and murine neuroblastoma cells (14, 16, 17). Also in these cells, L-arginine or a related material is the substrate for EDRF/NO synthesis and NADPH is required as a cofactor (17–19). However, the mechanism regulating the activity of the EDRF/NO-producing enzyme(s) is still largely unclear. In endothelial cells, transmembrane influx of Ca^{2+} is essential for the production and/or release of EDRF/NO in response to hormonal stimulation (20–22). Recent evidence suggests that the activity of endothelial EDRF/NO-synthesizing enzyme(s) is Ca^{2+} dependent (23, 24).

We have reported previously that N1E-115 neuroblastoma cells have a high capacity to produce EDRF/NO (14, 16, 17). We now show that in these cells both hormonal and nonhormonal stimulation of EDRF/NO production (with L-arginine) depend critically on the presence of extracellular Ca^{2+}. Similar

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ABBREVIATIONS: EDRF, endothelium-derived relaxing factor; CaM, calmodulin; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; IBMX, 3-isobutyl-1-methyl xanthine; NO, nitric oxide; SOD, superoxide dismutase; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethane sulfonic acid.

to the endothelial cells (23, 24), the activity of the cytosolic enzyme(s) synthesizing EDRF/NO in N1E-115 cells was found to be Ca^{2+} sensitive. In addition, the enzyme activity is likely to be regulated by a Ca^{2+}-binding protein, presumably by CaM.

Materials and Methods

Assay of EDRF/NO using cultured RFL-6 cells. Rat fetal lung fibroblasts (RFL-6; American Type Culture Collection, Rockville, MD) were cultured as previously described (14, 17, 25). These cells contain considerable amounts of soluble guanylyl cyclase (26). Therefore, the increase in cGMP in RFL-6 cells was used as a measure of EDRF/NO activity (14, 17, 25). In brief, near-confluent RFL-6 cells in six-well plates were washed twice with Dulbecco's phosphate-buffered saline (Sigma, St. Louis, MO). Then they were incubated for 20–30 min either in 0.5 ml of Locke's solution containing 0.6 mM IBMX (Sigma) or in 0.5 ml of isotonic (280 mM) Tris·HCl buffer (pH 7.4) containing the same concentration of IBMX. The Locke's solution had the following composition: 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, and 10.0 mM HEPES (pH 7.4). After the preincubation, 0.5 ml of EDRF/NO-containing or -generating medium was added and the cells were incubated for another 2 or 3 min. Following the exposure to EDRF/NO, the medium was rapidly removed from the RFL-6 cells and 1.0 ml of ice-cold 50 mM sodium acetate (pH 4.0) was added to each well. The RFL-6 cells were immediately frozen in liquid nitrogen and stored at −70° until assayed for intracellular cGMP by radioimmunoassay (27).

EDRF/NO production by intact N1E-115 neuroblastoma cells. N1E-115 cells were cultured as described (14, 17). Cell suspensions (10^6 cells/ml) were prepared in Locke's solution containing SOD (20 units/ml; Boehringer Mannheim, FRG). In some experiments, cells were equilibrated for 20 min with L-arginine (100 μM) or for 1 min with neurotensin (10 μM). After the preequilibration, 0.5-ml aliquots of the N1E-115-conditioned medium, a filtrate of the cell suspensions obtained through Nalgene syringe filters (pore size, 0.2 μm), were added to cultured RFL-6 cells preincubated for 20 min in 0.5 ml of Locke's solution containing 0.6 mM IBMX. The RFL-6 cells were exposed to the N1E-115-conditioned medium for 2 min, after which their cGMP content was assayed as described above. The same experiments were repeated with N1E-115 cells suspended in Ca^{2+}-free Locke's solution containing 0.2 mM EGTA. The RFL-6 cells used in these experiments were preincubated in standard Locke's solution (2 mM Ca^{2+}) so that the final Ca^{2+} concentration on the RFL-6 cells was about 0.9 mM (versus 2.0 mM in the control experiments).

EDRF/NO synthesis by the cytosolic fraction of N1E-115 cells. N1E-115 cells were homogenized in ice-cold Locke's solution using a glass tissue grinder with a Teflon pestle. To obtain the cytosolic fraction, the homogenate was centrifuged at 100,000 × *g* for 1 hr. The supernatant fraction was collected, protein was determined using the Bradford reagent (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard, and protein concentration was adjusted to 200 μg/ml with Locke's solution. The RFL-6 cells were preincubated for 20 min in 0.5 ml of Locke's solution containing 0.6 mM IBMX and 20 units/ml SOD. Aliquots of diluted N1E-115 cytosol (0.5 ml) were added to the RFL-6 cells, together with L-arginine and NADPH. The N1E-115 protein was always added last to start the reaction and the cells were incubated at 37° for 3 min with gentle shaking. The different components and their final concentrations were: N1E-115 protein, 100 μg; IBMX, 0.3 mM; L-arginine, 100 μM; NADPH, 100 μM; and SOD, 10 units/ml. In some experiments, one of the following inhibitors was added: N^G-nitro-L-arginine (1–10 μM; Sigma), N^G-methyl-L-arginine (10–100 μM; CalBiochem, La Jolla, CA), hemoglobin (10 μM; Sigma), or methylene blue (10 μM; Sigma). After 3 min, the reaction was stopped and cGMP was determined in the RFL-6 cells as described above. For experiments with defined Ca^{2+} concentrations, N1E-115 cells were homogenized in ice-cold hypotonic (28 mM) Tris·HCl buffer (pH 7.4). After centrifugation at 100,000 × *g* for 1 hr, the supernatant fraction was made isotonic by adding 2.8 M Tris·HCl buffer (pH 7.4). The RFL-6 cells were preincubated for 20 min in 0.5 ml of 280 mM Tris·HCl containing 0.6 mM IBMX and 20 units/ml SOD. All reaction components and their final concentrations were the same as described above. Experiments with defined Ca^{2+} concentrations were performed in the presence of 1 mM EGTA; different amounts of $CaCl_2$ were added and free Ca^{2+} concentrations were calculated according to the method of Segal (28). A Tris·HCl solution with 1 mM EGTA and no added Ca^{2+} was considered Ca^{2+}-free; in experiments with 1 mM free Ca^{2+} no EGTA was added. In other experiments, the following inhibitors of CaM-mediated functions (29–33) were added to the reaction mixture before the N1E-115 protein: camidazolium (compound R24571, 3–30 μM final concentration; Sigma), trifluoperazine (3–30 μM; Sigma), fendiline (3–300 μM; Sigma), W-7 (3–300 μM; Sigma), and compound 48/80 (1–100 μg/ml; Sigma). The concentration of free Ca^{2+} was 500 nM (buffered with 1 mM EGTA) in experiments with CaM antagonists. In order to monitor possible nonspecific effects of the CaM antagonists on soluble guanylyl cyclase or overall viability of the RFL-6 detector cells, the inhibitors were also added to RFL-6 cells stimulated with sodium nitroprusside (10 μM) in the presence of IBMX (0.3 mM).

DE 52 anion exchange chromatography of the N1E-115 cytosolic fraction. N1E-115 neuroblastoma cells were suspended in 20 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol, 1

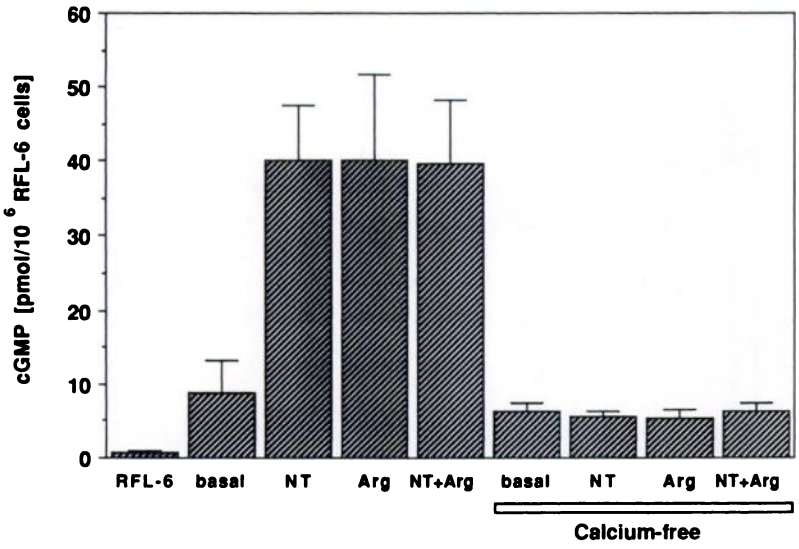


Fig. 1. Transfer experiment showing the effect of N1E-115 neuroblastoma cell-conditioned medium on RFL-6 cells. *First column*, cGMP levels in RFL-6 cells alone. N1E-115 cells released an EDRF/NO-like material that increased cGMP levels in RFL-6 cells (*basal*). The formation of this material was stimulated by neurotensin (*NT*, 10 μM), L-arginine (*Arg*, 100 μM), or a combination of both (*NT +Arg*). When the N1E-115 cells were incubated in Ca^{2+}-free Locke's solution containing 0.2 mM EGTA, the stimulating effects of neurotensin and L-arginine were abolished. *Columns*, means ± standard errors of 12–16 experiments.

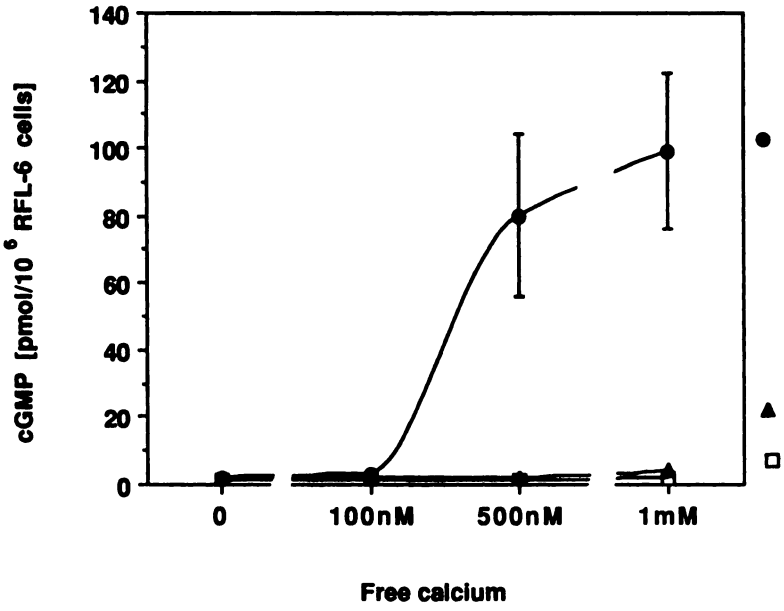


Fig. 2. Effect of free Ca^{2+} on the generation of cGMP-stimulating material (EDRF/NO) by the cytosolic fraction of N1E-115 neuroblastoma cells. N1E-115 cytosol (100 μg) was added to RFL-6 cells and incubations were performed at different Ca^{2+} concentrations (Ca^{2+} concentrations were buffered with 1 mM EGTA; 0 Ca^{2+} denotes the presence of 1 mM EGTA alone and at 1 mM Ca^{2+} no EGTA was added). In the presence of the substrate L-arginine (*Arg*, 100 μM) and the cofactor NADPH (100 μM), the enzyme(s) showed a clear Ca^{2+} dependency. Levels of cGMP in unstimulated RFL-6 cells alone were always below 1.0 pmol/10^6 cells. *Symbols*, means ± standard errors of six experiments.

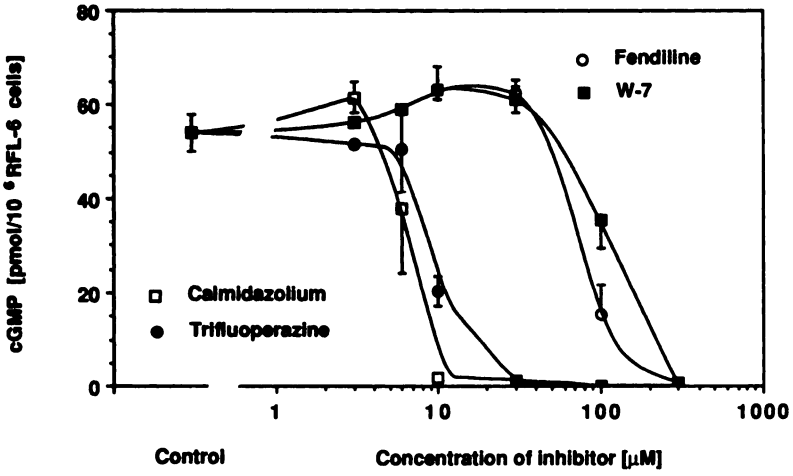
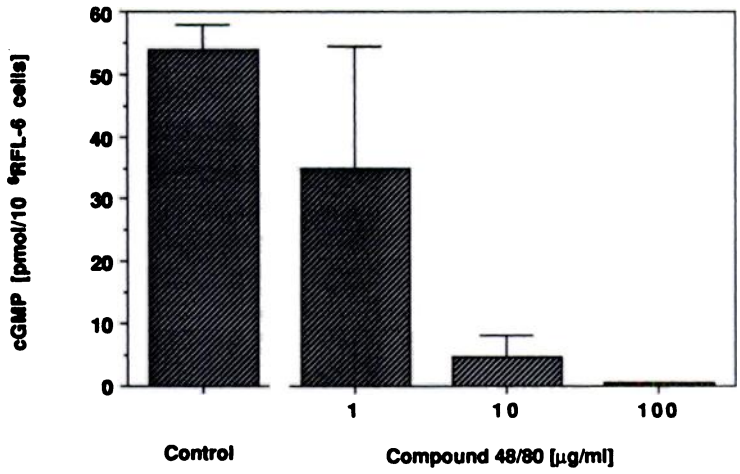


Fig. 3. Effect of various inhibitors of CaM-regulated functions on the production of cGMP-stimulating material (EDRF/NO) by the cytosolic fraction of N1E-115 cells. *Symbols* (*top*) or *columns* (*bottom*), means ± standard errors of four to six experiments.



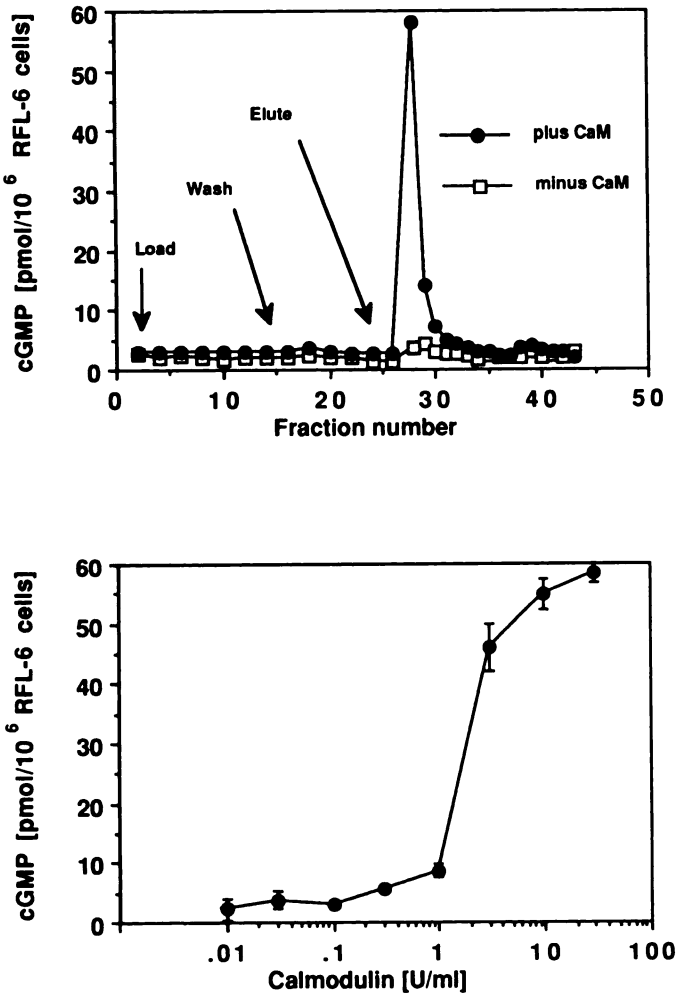


Fig. 4. DE 52 anion exchange chromatography of the N1E-115 cytosolic fraction (*top*). The column (1.5 × 6.0 cm) was equilibrated in potassium phosphate buffer, pH 7.6, containing 20% glycerol, 1 mM EDTA, and 12 mM 2-mercaptoethanol (running buffer). N1E-115 cytosol (25 mg of protein) was loaded onto the column at a flow rate of 0.4 ml/min (*Load*). Five-milliliter fractions were collected throughout the experiment and 100 μl thereof were assayed for cGMP-stimulating activity (EDRF) on RFL-6 cells. The assay was done in the absence or presence of CaM (10 units/ml). The column was washed with running buffer (*Wash*) and then eluted with running buffer containing 0.1 M KCl (*Elute*). The tracing is representative of four experiments. *Bottom*, the concentration-response relationship between added CaM and the formation of cGMP-stimulating activity (EDRF/NO). Twenty-microliter aliquots of fraction 28 (see *top*) were used for these experiments. *Symbols*, means ± standard errors of four experiments.

mM EDTA, 12 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride. The cytosolic fraction was obtained as described. DE 52 resin (Whatman) was equilibrated with 20 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol, 1 mM EDTA, and 12 mM 2-mercaptoethanol (running buffer), in a column measuring 1.5 × 6.0 cm. The N1E-115 cytosolic protein (25 mg) was loaded onto the column at a flow rate of 0.4 ml/min, and 5-ml fractions were collected. The column was washed with running buffer, and then the EDRF/NO-synthesizing protein fraction was eluted with running buffer containing 0.1 M KCl. The 0.1 M KCl wash contained a total of 1 mg of protein. In order to assay the EDRF/NO-forming activities of column fractions, RFL-6 cells preincubated for 30 min in Locke's solution with 0.6 mM IBMX were used. Locke's solution (0.4 ml) containing L-arginine, NADPH, and SOD was added to the RFL-6 cells. The reaction was initiated with 100-μl aliquots of the column fractions. The final concentrations of the components in 1 ml were: 0.3 mM IBMX, 100 μM L-arginine, 100 μM NADPH, and 10 units/ml SOD; incubation time was 3 min. In some experiments, CaM (from bovine brain, 10 units/ml, equivalent to 9.4 nM based on a molecular weight of 16,900; Sigma) was added to the mixture. In other experiments, troponin (from rabbit muscle, 1 and 10 μg/ml; Sigma) parvalbumin (from rabbit muscle, 1 and 10 μg/ml; Sigma), or phosphatidylserine (from bovine brain, 1 and 10 μg/ml; Sigma) were included. After the incubation, the protein suspension was removed from the cells, the reaction was stopped, and cGMP was determined in the RFL-6 cells as described. DE 52 column fraction 28, which contained about 80% of the EDRF/NO-synthesizing material, was used for CaM titration of the activity. CaM (0.01–30 units/ml) was added to the assay mixture and the reactions were started with 20-μl aliquots of column fraction 28. All other assay parameters were the same as described for the above column fractions.

Recombination experiments with different DE 52 column fractions. In these experiments, N1E-115 neuroblastoma cells were homogenized on ice in 50 mM Tris·HCl buffer, pH 7.6, containing 1 mM EDTA, 0.1 mM EGTA, 250 mM sucrose, and 12 mM 2-mercaptoethanol. The cytosolic fraction was obtained by centrifugation at 100,000 × *g* for 1 hr. DE 52 was equilibrated with the same Tris·HCl buffer and 25 mg of N1E-115 protein were loaded onto the column as described above. The column was washed with the Tris·HCl buffer and eluted stepwise with 0.1, 0.2, and 0.3 M KCl added to the Tris·HCl buffer. Each increase in KCl concentration resulted in the release of a protein peak from the column (0.1 M KCl, peak 1; 0.2 M KCl, peak 2; and 0.3 M KCl, peak 3). One hundred-microliter aliquots of each protein peak were assayed on RFL-6 cells. The material was assayed alone, in the presence of CaM, (10 units/ml), and in the presence of CaM and inhibitors of CaM-mediated functions (10 μM calmidazolium or 10 μg/ml compound 48/80). The assay conditions and the concentrations of all other components were as described above. In further experiments, 100-μl aliquots of the different protein peaks were assayed in combination (peaks 1 plus 2, peaks 1 plus 3, and peaks 2 plus 3). Again, the assay was done in the absence or presence of calmidazolium (10 μM) or compound 48/80 (10 μg/ml).

Results

Formation of EDRF/NO by intact N1E-115 cells. Under basal conditions, intact N1E-115 cells suspended in Locke's solution released an EDRF/NO-like material that could be transferred onto RFL-6 detector cells, where it increased cGMP (Fig. 1). Hormonal stimulation of the N1E-115 cells with neurotensin (10 μM) resulted in a five-fold increase in cGMP formation in the RFL-6 cells (Fig. 1). A similar stimulation of EDRF/NO formation was achieved with the EDRF/NO substrate L-arginine (100 μM). The effects of neurotensin and L-arginine were not additive (Fig. 1). When the same experiments were repeated in Ca^{2+}-free medium containing 0.2 mM EGTA, no stimulation of EDRF/NO production was seen with either agent (Fig. 1). Additions without N1E-115-conditioned medium had no effects on basal levels of cGMP in RFL-6 cells.

EDRF/NO synthesis by the N1E-115 cytosolic fraction. When the cytosolic and particulate fractions of N1E-115 cells were analyzed, the EDRF/NO-synthesizing activity was found exclusively in the cytosol. The activity was lost after boiling (100°, 3 min). In agreement with our previous findings (17), formation of EDRF/NO by the cytosolic enzyme(s) was inhibited in a concentration-dependent fashion by N^G-nitro-L-arginine (1–10 μM) and N^G-methyl-L-arginine (10–100 μM) (four experiments each; data not shown). The effect of the formed EDRF/NO on RFL-6 cells was abolished by hemoglobin (10 μM) or methylene blue (10 μM) (four experiments each; data not shown). The synthesis of EDRF/NO by the cytosolic fraction of N1E-115 cells was found to be completely Ca^{2+} depend-

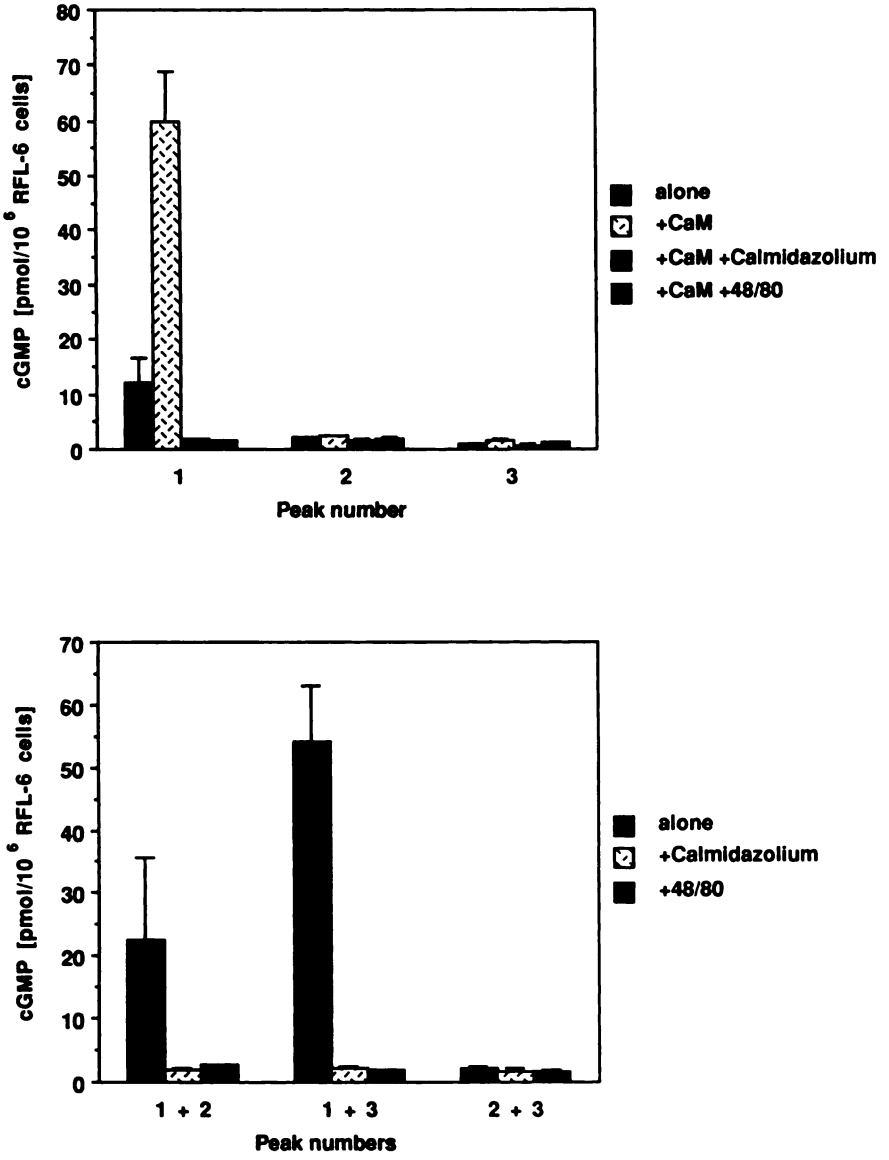


Fig. 5. DE 52 anion exchange chromatography of the N1E-115 cytosolic fraction. Loading of the column was similar to that in Fig. 4. After washing, the column was eluted stepwise with 0.1, 0.2, and 0.3 M KCl added to the running buffer. Each increase in KCl concentration resulted in the release of a protein peak from the column (0.1 M KCl, peak 1; 0.2 M KCl, peak 2; 0.3 M KCl, peak 3). *Top*, assay of 100-μl aliquots of these protein peaks on RFL-6 cells. The material was assayed alone, in the presence of CaM (10 units/ml), and in the presence of CaM and inhibitors of CaM-regulated functions (10 μM calmidazolium or 10 μg/ml compound 48/80). Only protein peak 1 contained EDRF-synthesizing activity, which was markedly stimulated by CaM and inhibited by the CaM antagonists. *Bottom*, recombination experiments performed with 100-μl aliquots of the three protein peaks. The cGMP-stimulating activity (EDRF) was assayed on RFL-6 cells in the absence (*alone*) or presence of calmidazolium (10 μM) or compound 48/80 (10 μg/ml). Protein peak 2 (eluted with 0.2 M KCl) and especially protein peak 3 (eluted with 0.3 M KCl) contained material that enhanced the activity of the EDRF-synthesizing enzyme(s) (protein peak 1). The effect of the stimulating material was completely inhibited by calmidazolium (10 μM) or compound 48/80 (10 μg/ml). *Columns*, means ± standard errors of four experiments.

ent. In Ca^{2+}-free solution containing 1 mM EGTA, the activity of the EDRF/NO-forming enzyme(s) was very low (1.9 ± 0.5 pmol of cGMP/10^6 RFL-6 cells in the presence of 100 μM L-arginine and 100 μM NADPH) (Fig. 2). When the free Ca^{2+} concentration was controlled by Ca^{2+}/EGTA buffers (28), there was a marked increase in enzyme activity between 100 and 500 nM free Ca^{2+} in the presence of L-arginine and NADPH (Fig. 2). The enzyme activity was near maximal at 500 nM Ca^{2+}; an increase of the Ca^{2+} concentration to 1 mM caused only a modest further increase in EDRF/NO synthesis (Fig. 2). A modest Ca^{2+} dependency was also observed for the low enzyme activity measured in the absence of L-arginine and NADPH. In Ca^{2+}-free solution (1 mM EGTA) it was 2.0 ± 0.4 pmol of cGMP/10^6 RFL-6 cells; at 1 mM Ca^{2+} it was 3.8 ± 0.3 pmol of cGMP/10^6 RFL-6 cells.

Inhibitors of CaM-regulated functions. In many systems CaM acts as a mediator of Ca^{2+} effects. Therefore, we tested different inhibitors of CaM-regulated functions for their effect on EDRF/NO synthesis by N1E-115 cytosol. Five CaM antagonists from different chemical groups inhibited EDRF/NO synthesis in a concentration-dependent fashion. As in other systems (31, 32), calmidazolium proved to be the most potent inhibitor (IC_{50} ~ 7 μM), followed by the phenothiazine derivative trifluoperazine (IC_{50} ~ 10 μM), the Ca^{2+} antagonist fendiline (IC_{50} ~ 80 μM), and the naphthalenesulfonamide W-7 (IC_{50} ~ 120 μM) (Fig. 3, *upper*). In addition, the histamine releaser compound 48/80, which has been reported to inhibit CaM-regulated functions (32), blocked EDRF/NO synthesis in N1E-115 cytosol (IC_{50} ~ 3 μg/ml) (Fig. 3, *lower*). When the same CaM antagonists were tested on sodium nitroprusside-stimulated increases in cGMP in RFL-6 cells, they were without effect (four experiments for each inhibitor). Only fendiline produced about 40% inhibition of cGMP formation at the highest concentration used (300 μM).

DE 52 anion exchange chromatography of N1E-115 cytosol. When N1E-115 cytosol was loaded onto DE 52 anion exchange columns, the protein that was eluted with 0.1 M KCl synthesized only very small amounts of EDRF/NO when assayed on RFL-6 cells under standard conditions in the absence of CaM (Fig. 4, *upper*). However, a significant peak of activity was seen when the activity of the partially purified enzyme was assayed in the presence of CaM (10 units/ml) (Fig. 4, *upper*).

The highest enzyme activity was found in column fraction 28 (compare Fig. 4, *upper*). Therefore, this fraction was used for CaM titration of the activity (Fig. 4, *lower*). The EC_{50} of CaM in restoring activity was found to be approximately 2 units/ml, and maximum EDRF/NO-synthesizing activity was achieved with 10 units/ml CaM (Fig. 4, *lower*). Other Ca^{2+}-binding proteins such as troponin (1 and 10 μg/ml) or parvalbumin (1 and 10 μg/ml) could not substitute for CaM in restoring EDRF/NO-synthesizing activity (two experiments each) and phosphatidylserine (1 and 10 μg/ml) also was without effect.

When DE 52 columns were eluted stepwise with 0.1, 0.2, and 0.3 M KCl, each increase in KCl concentration resulted in the elution of a peak of protein (0.1 M KCl, peak 1; 0.2 M KCl, peak 2; and 0.3 M KCl, peak 3). EDRF/NO-synthesizing activity eluted with 0.1 M KCl (peak 1). As before, this enzyme activity was markedly stimulated with CaM (10 units/ml) and was completely inhibited by calmidazolium (10 μM) or compound 48/80 (10 μg/ml) (Fig. 5, *upper*). In further experiments, the protein fractions that were eluted with different concentrations of KCl were recombined. Protein peak 3 (eluted with 0.3 M KCl) was able to fully restore the activity of the EDRF/NO-synthesizing enzyme(s) and, again, this activity was abolished by calmidazolium (10 μM) or compound 48/80 (10 μg/ml) (Fig. 5, *lower*). Protein peak 2 (eluted with 0.2 M KCl) also increased the enzyme activity, but to a lesser and more variable extent (Fig. 5, *lower*). Calmidazolium (10 μM) and compound 48/80 (10 μg/ml) inhibited this activity as well.

Discussion

EDRF/NO is produced by many different cell types (6–15) including N1E-115 neuroblastoma cells (14, 16, 17), but the intracellular mechanisms regulating the formation of this material are still poorly understood. It has been known for some time that hormone-induced production of EDRF/NO in endothelial cells depends critically on a transmembrane influx of extracellular Ca^{2+} (20–22). While the present work was in progress, two reports appeared showing that the cytosolic enzyme(s) responsible for EDRF/NO synthesis in endothelial cells is Ca^{2+} sensitive (23, 24), suggesting that hormone-induced increases in the intracellular Ca^{2+} concentration regulate EDRF/NO biosynthesis.

Data generated in the present study demonstrate that a similar mechanism is likely to be operative in a neuronally derived cell, the N1E-115 neuroblastoma cell. Both neurotensin-stimulated EDRF/NO formation in intact N1E-115 cells and the synthesis in response to the substrate L-arginine required extracellular Ca^{2+}. In addition, the cytosolic enzyme responsible for the synthesis was completely Ca^{2+} dependent. The Ca^{2+} sensitivity of the cytosolic enzyme is in accordance with a recent report showing Ca^{2+} dependency of an EDRF/NO-forming enzyme preparation from rat forebrain (34). Thus, there is evidence to suggest that free Ca^{2+} may serve as an intracellular signal regulating the activity of the EDRF/NO-synthesizing enzyme(s) in brain and N1E-115 neuroblastoma cells.

In various systems, transmembrane influx of Ca^{2+} is associated with the signal transduction initiated by the binding of a hormone to its membrane receptor. The increased intracellular concentration of Ca^{2+} results in the binding of Ca^{2+} to CaM, giving rise to a conformational change in CaM (for review see Refs. 35 and 36). This conformational change in CaM is believed to be an integral part of the signal transduction system leading to altered activities of the CaM-regulated target protein. Drugs from different chemical groups have been identified as inhibitors of CaM-mediated functions (29–33).

The first piece of evidence in this study for a possible involvement of CaM in the regulation of EDRF/NO formation came from the inhibitory effect of five chemically different CaM antagonists on EDRF/NO synthesis in N1E-115 cytosol. A nonspecific effect of the compounds on RFL-6 cell guanylyl cyclase or other cellular functions is unlikely, because the CaM antagonists did not inhibit sodium nitroprusside-induced stimulation of cGMP in RFL-6 cells (except for the highest concentration of fendiline, 300 μM, which produced about 40% inhibition). Further evidence for the requirement of CaM for EDRF/NO synthesis in N1E-115 cells was obtained using DE 52 anion exchange chromatography. This technique allowed the separation of the fraction that contained the EDRF/NO-synthesizing activity from other fractions that could be substituted for by CaM. These latter fractions are likely to contain the endogenous Ca^{2+}-binding protein. The binding of EDRF/NO synthase to CaM seems to be specific, because other Ca^{2+}-binding proteins such as troponin or parvalbumin could not replace CaM.

When we submitted our work we became aware of a paper by Bredt and Snyder (37), which reported the isolation of NO synthase from rat cerebellum. In agreement with our data, these authors found that the cerebellar enzyme was Ca^{2+} and CaM sensitive. However, they used citrulline formation as an index of enzyme activity and provided no direct evidence for the formation of EDRF/NO by the enzyme preparation.

Our data show that hormone-induced EDRF/NO synthesis in a neuronally derived cell line is regulated by free Ca^{2+} and requires CaM. The study suggests that hormonal regulation of cGMP by soluble guanylyl cyclase is mediated through Ca^{2+}-CaM regulation of EDRF/NO production. This signal transduction mechanism further adds to the unique mechanisms for hormonal regulation of the different isoforms of guanylyl cyclase (5, 25).

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