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Ca^{2+}-dependent formation of an L-arginine-derived activator of soluble guanylyl cyclase in bovine lung

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In a fraction of cytosolic proteins from bovine lung, soluble guanylyl cyclase was concentration-dependently stimulated by L-arginine but not by D-arginine. Stimulation was up to 20-fold with an EC_{50} of about 3×10^{-5} M. Activation of guanylyl cyclase by L-arginine was dependent on NADPH (EC_{50} about 5×10^{-7} M) and Ca^{2+} (EC_{50} about 1.4×10^{-6} M). The activation by L-arginine was inhibited by N^G-monomethyl-L-arginine and hemoglobin. The effect of L-arginine was dependent on the protein concentration and was not observed in preparations of purified gyanylyl cyclase. These results suggest that bovine lung contains a Ca^{2+}-regulated enzyme or enzyme system which converts L-arginine into an activator of soluble guanylyl cyclase.

Arginine; Ca^{2+}; Guanylyl cyclase; NADPH

1. INTRODUCTION

The endothelium-derived relaxing factor (EDRF) that directly activates soluble guanylyl cyclase [1,2] has been characterized as nitric oxide [3]. Biosynthesis of nitric oxide was recently described for various mammalian tissues [4–7] with L-arginine as a precursor [4,8,9]. Marletta et al. [10] have described a cytosolic enzyme in macrophages which converts L-arginine into citrulline and nitric oxide in the presence of NADPH. Palmer et al. [11] showed that endothelial cell homogenates convert L-arginine into citrulline, but the authors failed to detect a concomitant NO chemiluminescence signal. There is no study available until now which directly demonstrates enzymatic formation of a bioactive moiety from L-arginine.

In the present paper we describe a Ca^{2+}-regulated enzymatic conversion of L-arginine into a bioactive metabolite which activates soluble guanylyl cyclase and exhibits features similar to EDRF (NO). The enzymatic formation of the bioactive metabolite occurred in a fraction of cytosolic proteins from bovine lung containing soluble guanylyl cyclase, which was used as a detection system.

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2. MATERIALS AND METHODS

2.1. *Enzyme preparation*

Bovine lungs (5 kg) were mixed with 1000 ml of a 300 mM triethanolamine/HCl buffer, pH 7.0, containing 60 mM EDTA, 6 mM reduced glutathione, 6 mM benzamidine, 60 kE/l penicillin G and 60 mg/l streptomycin, and were homogenized by means of a Microcut (MC 10, 0.9 mm cutting rings). The homogenate was centrifuged for 60 min at 10000 × g_{av}, and phenylmethylsulfonyl fluoride and pepstatin A (final concentrations 0.5 mM and 0.5 μM, respectively) were added. The obtained cytosol was passed over a column of DEAE-Sepharose Fast Flow (5 × 13 cm) which had been equilibrated with a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 75 mM NaCl, 1 mM EDTA, 2 mM reduced glutathione and 0.2 mM benzamidine. The column was washed with two bed volumes of this buffer; then a linear gradient from 75 to 400 mM NaCl was applied, and 25-ml fractions were collected. Fractions containing guanylyl cyclase activity were pooled, mixed 1 : 1 with glycerol, and β-mercaptoethanol (50 mM final concentration) was added to this 'DEAE fraction'. The protein content was 4.0 mg/ml as estimated by the method of Lowry et al. modified according to Peterson [12]. The enzyme was stored at −80°C for at least two months without loss of enzyme activi-

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ty. Homogenous guanylyl cyclase was obtained by immunoaffinity chromatography ([13] and manuscript in preparation).

2.2. *Guanylyl cyclase assay*

Formation of ^{32}P-cGMP from [α-^{32}P]GTP was measured as previously described [14] in the presence of 0.1 mM [α-^{32}P]GTP (specific activity 3–5 TBq/mmol), 1.1 mM $MgCl_2$, 2 mM reduced glutathione, 1 mM isobutylmethylxanthine, 1 mM cGMP, 0.5 mg of bovine serum albumin per ml, 50 mM triethanolamine/HCl, pH 7.4). Superoxide dismutase, NADPH and Ca^{2+} buffers (see below) were included in the reaction mixture as indicated. Reactions were carried out in a total volume of 0.1 ml at 37°C for 10 min in the presence of 10 μl of enzyme (40 μg of protein) unless otherwise indicated.

2.3. *Calcium buffers*

Free Ca^{2+} was adjusted by including in the reaction mixture 1.5×10^{-5} M EGTA, 8.5×10^{-5} M EDTA and $CaCl_2$ as calculated from the respective stability constants given by Holloway and Reilley [15].

2.4. *Data evaluation*

Each experiment was performed three times, and each point was estimated in triplicate in the absence and presence of L-arginine. Guanylyl cyclase activity in the presence of L-arginine is given relative to the respective basal activity (fold-stimulation; v_{arg}/v_o), and geometric mean values and SEM were calculated.

2.5. *Materials*

Buffer salts of analytical grade were obtained from E. Merck, Darmstadt, FRG. [α-^{32}P]GTP was prepared from $^{32}PO_4^{3-}$ [16] obtained from DuPont de Nemours, Dreieich, FRG. DEAE-Sepharose Fast Flow was from Pharmacia, Freiburg, FRG, NAD, NADP and NADH were from Boehringer Mannheim, Mannheim, FRG, hemoglobin from Serva, Heidelberg, FRG, and N^G-monomethyl-L-arginine[di(*p*-hydroxyazobenzene-*p*'-sulfonate] from Calbiochem, Frankfurt, FRG. L- and D-arginine, sodium nitroprusside, NADPH, bovine serum albumin, manganese-containing superoxide dismutase, cyclic GMP, isobutylmethylxanthine, GTP, reduced glutathione, phenylmethylsulfonyl fluoride, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonimid (W-7), trifluoperazine, benzamidine hydrochloride, penicillin G and streptomycin were obtained from Sigma, Deisenhofen, FRG.

3. RESULTS AND DISCUSSION

L-Arginine concentration-dependently increased guanylyl cyclase activity in the DEAE fraction of cytosolic proteins from bovine lung when determined in the presence of 10^{-5} M NADPH and 10^{-5} M Ca^{2+}. As shown in fig.1, L-arginine caused an up to 20-fold increase in guanylyl cyclase activity, and the estimated EC_{50} was about 3×10^{-5} M. This value is within the range of the intracellular L-arginine concentration as determined by Palmer and Moncada [11] in endothelial cell homogenates.

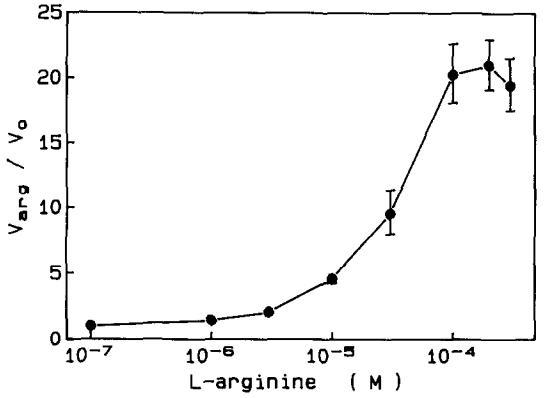

Fig.1. Effects of L-arginine on guanylyl cyclase activity. An aliquot of the DEAE fraction (40 μg of protein) was incubated for 10 min at 37°C in the presence of 50 U/ml superoxide dismutase, 10^{-5} M Ca^{2+} and 10^{-5} M NADPH with increasing concentrations of L-arginine.

Therefore, regulation of enzyme activity via substrate availability seems possible but rather unlikely.

L-Arginine did not activate guanylyl cyclase purified by immunoaffinity chromatography from the same lung preparation. Deguchi and Yoshioka [17] have identified L-arginine as an endogenous activator of guanylyl cyclase in neuroblastoma cells, but the authors did not test the possibility that an enzyme might be involved in guanylyl cyclase activation by L-arginine.

Further evidence for the assumption that activation of soluble guanylyl cyclase requires enzymatic conversion of L-arginine was obtained from experiments in which we compared the effect of sodium nitroprusside with the effect of L-arginine in the presence of increasing amounts of added protein. Fig.2A shows that the effect of sodium nitroprusside on guanylyl cyclase activity was independent of the protein concentration, whereas the effect of L-arginine increased linearly with increasing protein concentrations, suggesting that an activator of soluble guanylyl cyclase was formed from L-arginine.

L-Arginine was utilized stereoselectively by the L-arginine-converting enzyme, as D-arginine had no effect on soluble guanylyl cyclase activity (not shown). N-Benzoyl-L-arginine methylester and L-arginine-containing bioactive peptides such as bradykinin and tuftsin were only poor substrates. Up to a concentration of 10^{-4} M, these com-

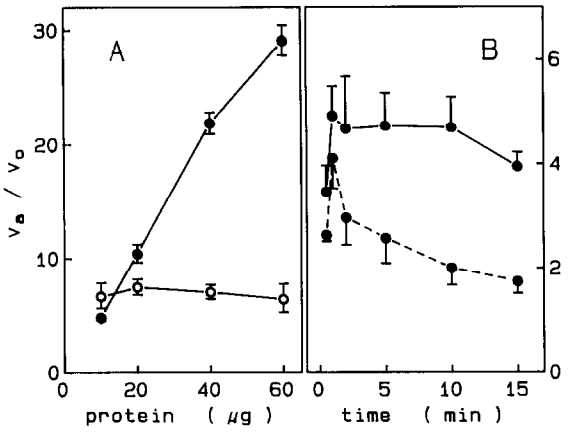

Fig.2. Effects of protein concentration and of incubation time on the activation of guanylyl cyclase by L-arginine and sodium nitroprusside. (A) Aliquots of the DEAE fraction with protein as indicated on the abscissa were diluted with a 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.5 mM EDTA, 0.5 mg of bovine serum albumin per ml, and 2 mM reduced glutathione; 20 μl of diluted enzyme were incubated for 10 min at 37°C in the presence of 50 U/ml superoxide dismutase, 10^{-5} M Ca^{2+} and 10^{-5} M NADPH in the presence or absence of 10^{-4} M L-arginine (filled symbols) or 10^{-7} M sodium nitroprusside (unfilled symbols) in a total volume of 0.1 ml. Guanylyl cyclase activity in the presence of activator is given relative to the basal activity (v_a/v_o). (B) An aliquot of the DEAE fraction (40 μg of protein) was preincubated for 2 min at 37°C with the reaction mixture described in section 2, containing 10^{-5} M NADPH and 10^{-5} M Ca^{2+} in the presence (solid line) and absence (dashed line) of 50 U/ml superoxide dismutase. Incubations were carried out for the indicated periods of time at 37°C without and with 10^{-4} M L-arginine.

pounds caused between 10 and 20% guanylyl cyclase stimulation when compared to L-arginine, which finding confirms the suggestion of Palmer et al. [11] that peptidyl-arginine deiminase may not be involved in the biosynthesis of EDRF.

N^G-Monomethyl-arginine (10^{-4} M), an inhibitor of nitric oxide biosynthesis and endothelium-dependent relaxation [11,18,19], and hemoglobin (10^{-6} M), an inhibitor of activation of guanylyl cyclase by NO-containing compounds and of endothelium-dependent relaxation [20], markedly decreased the effect of L-arginine to about 14% and 8%, respectively (not shown).

Superoxide dismutase has been described to stabilize nitric oxide [3]. Fig.2B shows the results obtained when DEAE fraction was incubated for increasing periods of time with L-arginine in the absence and presence of superoxide dismutase. The degree of guanylyl cyclase activation was considerably decreased as compared to other experiments described, probably due to an inactivation of the L-arginine-converting enzyme or an essential cofactor in the course of the required preincubation without added L-arginine and GTP (2 min, 37°C). A rapid increase in guanylyl cyclase activation occurred within the first 60 s of incubation time. Subsequently, the effect of L-arginine remained constant over a period of 15 min in the presence of superoxide dismutase, whereas it decreased continuously in the absence of superoxide dismutase. The observed enhancement of the effect of L-arginine by superoxide dismutase and the development of a steady state are consistent with the formation of a labile metabolite from L-arginine which is stabilized by superoxide dismutase.

The results obtained with N^G-monomethyl-arginine, hemoglobin and superoxide dismutase suggest that an activator of soluble guanylyl cyclase is formed which has features comparable with EDRF, and we, therefore, conclude that activation of guanylyl cyclase by L-arginine reflects biosynthesis of nitric oxide or a closely related compound.

Fig.3A shows that the guanylyl cyclase-stimulating activity of L-arginine was dependent on NADPH; this finding confirms results obtained previously on nitric oxide biosynthesis in macrophages [10]. The EC_{50} for NADPH was very low (about 5×10^{-7} M), and the enzyme appears to selectively depend on this nucleotide, since only about 16% of the effect were observed in the presence of 10^{-5} M $NADP^+$. NAD^+ as well as NADH (each up to 10^{-5} M) completely failed to act as cofactors for the L-arginine-converting enzyme.

The release of EDRF from endothelial cells was shown to be associated with increases of intracellular free Ca^{2+} [21]. Fig.3B shows the concentration–response curve which was obtained when the DEAE fraction was incubated in the presence and absence of 10^{-4} M L-arginine with increasing concentrations of free Ca^{2+}. Up to 10^{-4} M, Ca^{2+} did not alter basal guanylyl cyclase activity. No effect of L-arginine was observed when EDTA (1.35×10^{-4} M) and EGTA (1.5×10^{-5} M) were present in the incubation mixture without exogenously added Ca^{2+}. The concentration–response curve showed a maximum

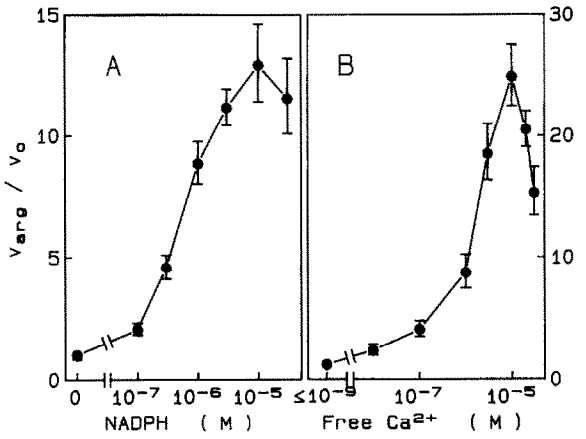

Fig.3. Effects of NADPH and of Ca^{2+} on the activation of guanylyl cyclase by L-arginine. (A) An aliquot of the DEAE fraction (20 μg of protein) was incubated for 10 min at 37°C in the presence of 10^{-5} M Ca^{2+} and 50 U/ml superoxide dismutase with increasing concentrations of NADPH. Stock solutions of NADPH were freshly prepared in 50 mM triethanolamine/HCl, pH 7.5. Guanylyl cyclase activity was estimated in the absence and presence of 10^{-4} M L-arginine. (B) An aliquot of the DEAE fraction (40 μg of protein) was incubated in the presence of 10^{-5} M NADPH and 50 U/ml superoxide dismutase for 10 min at 37°C with increasing concentrations of free Ca^{2+} (for adjusting the free Ca^{2+} concentrations see section 2). Guanylyl cyclase activity was estimated in the absence and presence of 10^{-4} M L-arginine.

of guanylyl cyclase activation at 10^{-5} M free Ca^{2+} with an EC_{50} of about 1.4×10^{-6} M. The L-arginine-converting enzyme, therefore, appears to be regulated directly or indirectly by physiologically occurring concentrations of free Ca^{2+}, which, therefore, may mediate hormone-induced EDRF release. The calmodulin antagonists, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and trifluoperazine [22], at concentrations up to 10^{-5} M did not alter activation of guanylyl cyclase by L-arginine, suggesting that the effect of Ca^{2+} was not mediated via calmodulin.

Summarizing the data presented in this paper, we conclude that in bovine lung, which is rich in vascular tissue, exists an enzyme which Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. This labile L-arginine metabolite appears to be identical with EDRF and may represent nitric oxide or a closely related compound. The bioassay applied in this study represents a powerful tool for the investigation of EDRF biosynthesis, especially in the purification of the enzyme involved.

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