L-Arginine Identified as an Endogenous Activator for Soluble Guanylate Cyclase from Neuroblastoma Cells*

(Received for publication, March 30, 1982)

Takeo Deguchi‡
From the Department of Medical Chemistry, Tokyo Metropolitan Institute for Neurosciences, 2-6, Musashidai, Fuchu-city, Tokyo 183 Japan

Masanori Yoshioka§
From the Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113 Japan

Guanylate cyclase in neuroblastoma N1E 115 cells was readily solubilized upon homogenization of the cells with hypotonic buffer. When the supernatant was passed through cation exchangers such as a Chelex 100 Na+ column, the guanylate cyclase activity in the effluent fraction decreased to 4-6% of the original supernatant. The addition of the acid extract of neuroblastoma cells or rat tissues to the effluent restored guanylate cyclase activity, indicating that the supernatant of neuroblastoma cells contained an acid-soluble endogenous activator for guanylate cyclase which was adsorbed on cation exchangers. The activator was purified from rat brain and identified as L-arginine by 13C- and 1H-NMR spectroscopy and paper partition chromatography. L-Arginine, at a concentration of 1-2 × 10^{-5} M, stimulated guanylate cyclase activity in the effluent fraction 15-25-fold, whereas D-arginine and other basic L-amino acids were ineffective. Peptides that contained L-arginine at the NH2- or COOH-terminus also resulted in an activation of guanylate cyclase to the extent similar to that of L-arginine, while peptides that contained L-arginine inside the peptide chain failed to stimulate the activity. The activation by L-arginine seemed to operate by a mechanism similar to that induced by nitroso compounds.

It has been established well that cholinergic and α-adrenergic agonists and other transmitters elevate the intracellular cyclic GMP content in a variety of tissues in the presence of extracellular calcium (1-6). Although the increase of cyclic GMP levels by these agents has been thought to be due to an increased synthesis of cyclic GMP, the mechanism by which they stimulate the activity of guanylate cyclase (EC 4.6.1.2), the enzyme responsible for the synthesis of cyclic GMP (7-10), has remained to be elucidated. Acetylcholine and other transmitters failed to activate guanylate cyclase in broken tissues and in purified enzyme preparations (9-12).

Recently, a number of agents have been reported to activate guanylate cyclase in cell-free systems: nonionic detergents (10, 13-16), nitroso compounds (17-29), lipids (24-28), and others (29-31). However, some of these agents are not present in normal tissues, and there is no evidence that such agents regulate the synthesis of cyclic GMP in intact tissues.

We have previously reported the evidence that the synaptosomal soluble fraction of rat brain contains an endogenous activator for soluble guanylate cyclase (12) and that the activator is presumably involved in the synthesis of cyclic GMP in intact tissues in response to transmitters (32). The presence of such an activator in the synaptosomal soluble fraction of rat brain has lately been confirmed by another laboratory (33). We further demonstrated that the supernatant fraction of neuroblastoma N1E 115 cells, whose cyclic GMP content markedly responded to acetylcholine, prosta
glandins, and histamine (6, 34), also contains an endogenous activator of small molecules (35). However, because of difficulty in obtaining a large quantity of the synaptosomal soluble fraction and the supernatant of neuroblastoma cells, we succeeded in only a partial purification of the activator. Recently, we detected the same activator in the acid extract of the supernatant of rat brain and succeeded in a purification and identification of the endogenous activator as L-arginine. The detailed account of the study is described in this report.

MATERIALS AND METHODS

Chemicals-[8-3H]GTP (11 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, United Kingdom). Creatine kinase from rabbit muscle, creatine phosphate, and GTP were the products of Boehringer Mannheim (West Germany). L-Arginine hydrochloride, L-arginine methyl ester, L-arginyl-L-aspartic acid, and cyclic GMP were obtained from Sigma. L-Arginine hydrochloride and L-homoarginine were purchased from Nakarai Chemical Co. (Kyoto, Japan), and peptides from Protein Research Foundation (Osaka, Japan). Chelex 100 was obtained from Bio-Rad, and Sephadex gels and ion exchangers from Pharmacia Fine Chemicals (Uppsala, Sweden). Other chemicals were obtained from commercial sources.

Cell Culture—Neuroblastoma N1E 115 cells were donated by Dr. Takehiko Amano, Mitsubishi-Kasei Institute of Life Sciences, and were grown in 60-mm Nunc plastic dishes in 4 ml of Dulbecco-Vogt modified Eagle's minimal essential medium supplemented with 5% fetal calf serum (Gibco Laboratories, Grand Island, NY) in a humidified atmosphere of 10% CO2, 90% air at 37 °C as described (36). The medium was changed every day and cells were usually grown for 5-7 days to confluence. After the medium was removed, cells were collected in phosphate-buffered saline (0.14 M NaCl, 0.903 M KCl, and 0.01 M phosphate buffer, pH 7.4) by use of a rubber policeman and centrifuged at 1,000 × g for 10 min. The cells were homogenized with 5 mm Tris/HCl (pH 7.6, 100-150 μl/dish) and centrifuged at 105,000 × g for 30 min.

Preparation and Assay of Guanylate Cyclase—The supernatant (5-7 ml) of neuroblastoma cells was placed on a Chelex 100 Na+ column (1 × 25 cm) and eluted with distilled water at 4 °C. Guanylate cyclase activity was detected in the effluent fraction. Guanylate

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‡ Supported by Grant-in-Aid 56120005 for Special Project Research on "Metabolic Responses and Their Disorders."

§ Supported by Grant-in-Aid 5575095 from the Ministry of Education, Science, and Culture of Japan.
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cyclase activity was assayed as described previously (15). Briefly, the reaction mixture contained 7.5 µmol of Tris/HCl, pH 7.6, 0.75 µmol of creatine phosphate, 0.5 µmol of cyclic GMP, 0.45 µmol of MgCl₂, 1.2 µmol of theophylline, 0.6 µmol of creatine kinase, and 37.5 nmol of [8-³²P]GTP (10-12 µCi/µmol) in a total reaction volume of 150 µl. When indicated in Fig. 4, 3 mM MnCl₂ was used as a metal cofactor in place of 3 mM MgCl₂. The incubations were carried out at 37°C for 10 min, and the radioactive cyclic GMP produced was isolated as described (15). Protein was determined by the method of Lowry et al. (37).

Purification of Endogenous Activator from Rat Brain—Whole rat brains were homogenized in 9 volumes of distilled water and centrifuged at 15,000 × g for 30 min. All subsequent procedures were carried out at 0-4°C. To the supernatant, perchloric acid (60%) was slowly added to a final concentration of 2%, and denatured protein was removed by centrifugation at 15,000 × g for 20 min. The supernatant was neutralized to pH 7.0-7.3 by the addition of 4 mM KOH, and the resulting precipitate was removed by centrifugation. The supernatant was lyophilized to 7% volume and mixed with an equal volume of acetone. After the precipitate was discarded by centrifugation, the supernatant was mixed with an equal volume of chloroform and centrifuged at 2,000 × g for 10 min. The aqueous phase was lyophilized to dryness. The residue was dissolved in 70 µl of distilled water and placed on a Dowex 1-X8 (100-200 mesh) formate column (1.5 × 10 cm), followed by washing with 7 bed-volumes of distilled water. The effluent and washings were combined, lyophilized to 10 ml, and placed on a Sephadex G-10 column (2.5 × 100 cm). The activator-containing fractions were combined and placed on a DEAE-Sephadex A-25 Cl⁻ column (1.5 × 10 cm), followed by washing with 3 bed-volumes of distilled water. The efficient and washings were combined, lyophilized to 5-10 ml, and placed on a Sephadex G-10 column (1.9 × 100 cm). The activator was eluted out between 150 and 180 ml of distilled water. The fractions that contained the activity were lyophilized to dryness. The residue was dissolved in 100 µl of distilled water and placed on Whatman No. 3MM paper, which was developed in 1-butanol/ethanol/acetic acid/H₂O (3:1:1:2) at 4°C. The strip that contained the activity (Rf = 0.38) was eluted with distilled water. The fractions that contained the activity were placed on a sulphopropyl-Sephadex C-25 Na⁺ column (1.5 × 20 cm). After the column was washed with 30 ml of distilled water, the activator was eluted with 25 ml of 0.25 M NaCl. The eluate was lyophilized to 5-10 ml and placed on a Sephadex G-10 column (1.9 × 100 cm). The activator-containing fractions were combined and placed on a DEAE-Sephadex A-25 Cl⁻ column (1.5 × 10 cm), followed by washing with 3 bed-volumes of distilled water. The efficient and washings were combined, lyophilized to 5-10 ml, and placed on a Sephadex G-10 column (1.9 × 100 cm). The activator was eluted out between 150 and 180 ml of distilled water. The fractions that contained the activity were lyophilized to dryness. The residue was dissolved in 100 µl of distilled water and placed on Whatman No. 3MM paper, which was developed in 1-butanol/ethanol/acetic acid/H₂O (3:1:1:2) at 4°C. The strip that contained the activity (Rf = 0.38) was eluted with distilled water. The fractions that contained the activity were placed on a sulphopropyl-Sephadex C-25 Na⁺ column (1.5 × 20 cm).

Identification of the Purified Activator—¹H- and ³¹P-NMR spectroscopy were done with a pulsed Fourier transform spectrometer JNM-FX-400. The purified activator (6-8 mg) was dissolved in 0.3 ml of 5% D₂O. 1D and 2D NMR spectra were measured at room temperature. Dioxane was used as an external reference to correct chemical shifts from tetramethylsilane.

Polarimetry was done at room temperature with a digital polarimeter JASCO DIP-140. The activator (3-4 mg) was dissolved in about 1 ml of water in a cell 5 cm long. l-Arginine/HCl was used as a standard.

Paper partition chromatography of the purified activator was done under the same conditions for the purification step. Spray reagents of ninhydrin reaction for amine and of Sakaguchi reaction for guanidine were made according to the regular method described (46).

RESULTS

Presence of an Endogenous Activator for Guanylate Cyclase—Guanylate cyclase in neuroblastoma N1E 115 cells was easily solubilized, and all of the activity was recovered in the supernatant when the cells were homogenized in a hypotonic buffer (35). MgCl₂, as well as MnCl₂, served as a metal cofactor for the guanylate cyclase from the cells, and the maximum enzyme activity obtained with MgCl₂ was comparable to that with MnCl₂ (35). The specific enzyme activity in the supernatant of neuroblastoma cells was 2 nmol/mg of protein/min (Table I), which was the highest activity so far reported for the soluble enzyme from other tissues. However, when the supernatant was passed through a Chelex 100 Na⁺ column, a cation exchanger, the guanylate cyclase activity in the effluent decreased to 4-6% of the original supernatant (Table I) although almost all of the protein was recovered in the effluent fraction (data not shown). The addition of the supernatant, heated at 65°C for 3 min, to the effluent restored guanylate cyclase activity to the level similar to that in the original supernatant. The acid extract of the supernatant also stimulated the enzyme activity 3-13-fold. A comparable result was obtained when the supernatant of neuroblastoma cells was passed through a SP-Sephadex C-25 Na⁺ or carboxymethyl-Sephadex C-25 Na⁺ column and the effluents were used as enzyme preparations (data not shown). This observation indicates that the supernatant of neuroblastoma cells contains an acid-soluble endogenous activator for guanylate cyclase which was adsorbed by cation exchangers. The acid extracts of the synaptosomal soluble fraction, the supernatant of rat brain, lung, and liver, also stimulated guanylate cyclase activity although the maximum extent of the activation was variable depending on the tissues from which acid extracts were prepared. Nitroprusside, the agent that has been shown to activate soluble guanylate cyclase from a variety of tissues (22), activated the enzyme in the Chelex 100 effluent fraction to the level similar to that induced by acid extract of tissues. The addition of both tissue extract and nitroprusside did not show an additive effect (data not shown). In contrast, neither tissue extract nor nitroprusside stimulated guanylate cyclase activity in the original supernatant, indicating that the enzyme in the supernatant was in an activated state (Table I).

Purification and Identification of the Endogenous Activator from Rat Brain—The activator was purified from the supernatant of rat brain as described under "Materials and Methods." Approximately 3-4 mg of material was obtained from 200 g of rat brain. The elution pattern on Sephadex G-10 and G-15 columns indicated that the molecular weight of the activator was between 200 and 400 (data not shown), in accordance with our previous observation of the endogenous activator in the synaptosomal soluble fraction of rat brain (12). The activator was not soluble in organic solvents such as chloroform, ether, or 98.5% ethanol and showed essentially no

TABLE I

<table>
<thead>
<tr>
<th>Addition</th>
<th>Guanylate cyclase activity (nmol/min)</th>
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<tbody>
<tr>
<td>None</td>
<td>4.35</td>
</tr>
<tr>
<td>Heated neuroblastoma supernatant</td>
<td>4.76</td>
</tr>
<tr>
<td>Acid extract of neuroblastoma supernatant</td>
<td>4.16</td>
</tr>
<tr>
<td>Acid extract of synaptosomal soluble fraction</td>
<td>4.27</td>
</tr>
<tr>
<td>Acid extract of brain supernatant</td>
<td>4.31</td>
</tr>
<tr>
<td>Acid extract of lung supernatant</td>
<td>3.97</td>
</tr>
<tr>
<td>Acid extract of liver supernatant</td>
<td>3.92</td>
</tr>
<tr>
<td>Nitroprusside (0.1 mM)</td>
<td>4.53</td>
</tr>
</tbody>
</table>

Effect of acid extracts of various tissues on guanylate cyclase activity from neuroblastoma cells

The supernatant of neuroblastoma cells and the effluent fraction through a Chelex 100 Na⁺ column were prepared as described under "Materials and Methods." The supernatant (192 µg of protein) or the Chelex 100 effluent (190 µg of protein) was used for each assay. A part of the neuroblastoma supernatant was heated at 65°C for 3 min in a water bath. To prepare acid extracts, rat tissues were homogenized with 7 volumes of distilled water and centrifuged at 15,000 × g for 30 min. To the supernatant, perchloric acid (60%) was added to a final concentration of 2%, and denatured protein was removed by centrifugation. The supernatant was neutralized to pH 7.0-7.3 by the addition of 4 mM KOH, and the resulting precipitate was discarded. Acid extracts (25-50 µl) was added to the assay of guanylate cyclase activity. The values are the means of duplicate determinations.
absorption in the visible and ultraviolet regions (data not shown).

Although the amount of the activator purified was small, we succeeded in taking beautiful $^{13}$C- and $^1$H-NMR spectra. Six carbons were distinctly present in the structure (Fig. 1). It was easily determined that carbon a at 24.72 ppm, b at 28.33 ppm, and c at 41.36 ppm were fragments $-\text{CH}_2-$; d at 55.08 ppm was fragment $-\text{CH}-$; and e at 158.0 ppm and f at 174.9 ppm were fragments $-\text{C}-$ and $-\text{C}-$, respectively. On the other hand, a 400.5-MHz $^1$H-NMR spectrum showed resonances at 1.75 ppm (multiplet, 2H), 1.98 ppm (multiplet, 2H), 3.90 ppm (triplet, 2H), and 3.85 ppm (triplet, 1H) (data not shown). From the assignment of both spectra, a basic structure was assumed as $-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}-$, $-\text{C}-$ and $-\text{C}-$. Furthermore, the activator was positive for ninhydrin reaction and seemed amphoteric from the behaviors observed during the purification steps. The data of amino acids containing six carbons were easily covered (38). Arginine hydrochloride had completely the same spectrum as the activator. The chemical shift of each carbon of arginine hydrochloride was 24.8, 28.4, 41.5, 55.3, 157.7, and 175.0 ppm (38); thus, the activator should be arginine.

The chemical shift of each carbon of arginine hydrochloride was completely the same spectrum as the activator. The chemical shift of each carbon of arginine hydrochloride was 24.8, 28.4, 41.5, 55.3, 157.7, and 175.0 ppm (38); thus, the activator should be arginine.

To confirm this estimation, paper partition chromatography was done. As shown in Fig. 2, the stimulatory effect of the activator on guanylate cyclase activity (A) correspond to the spots positive for ninhydrin reaction for amine (B) and for Sakaguchi reaction for guanidine (C). Thin layer chromatography of the activator with or without dansylation also showed a single spot which coincided with L-arginine, but not with L-homoarginine nor with L-arginine methyl ester (Fig. 3, data with dansylation were not shown). The elution pattern of the activator from a Sephadex G-10 column (1.9 x 100 cm) was the same as that of L-arginine (data not shown). The activator gave positive optical rotation as expected, indicating the L-form of arginine (data not shown). The amount of the activator, however, was too small to calculate the accurate rotation. Comparing the stimulatory effect of L- and D-arginine on guanylate cyclase activity as described later, it was concluded that the activator was L-arginine.

**Specificity of the Activation of Guanylate Cyclase by L-Arginine**—The dose responses of guanylate cyclase activation to the activator and L-arginine are shown in Fig. 4. There was a 5-fold activation of guanylate cyclase activity at 0.06 μg/150 μl and a maximum activation at 0.6-1.2 μg/150 μl of the activator. The maximum activation in this enzyme preparation was 16-fold over the basal activity. L-Arginine resulted in a 15-fold activation at 0.1 mM, whereas D-arginine had no effect at any concentration examined. L-Arginine methyl ester and L-homoarginine also stimulated the enzyme activity 16-fold when assayed with 3 mM MgCl$_2$, which was comparable to that induced by the activator. When assayed with 3 mM MnCl$_2$, the basal activity was 2-fold higher and the maximum activation was lower than that with MgCl$_2$. There was a 5-6-fold activation by L-arginine with MnCl$_2$. In both cases, a significant activation was observed at $1 \times 10^{-6}$ M and a maximum activation at $1-2 \times 10^{-5}$ M of L-arginine. The time course of guanylate cyclase activity was followed in the absence and in the presence of 0.06 mM L-arginine. There was no lag phase of the reaction in both cases (data not shown), indicating that L-arginine itself, rather than its metabolites, stimulated guanylate cyclase activity.

The specificity of the activation of L-arginine and its related compounds was examined (Table II). L-Arginine resulted in a 15-fold activation at 0.1 mM, whereas D-arginine had no effect at any concentration examined. L-Arginine methyl ester and L-homoarginine also stimulated the enzyme activity to a similar extent as L-arginine, while guanidine, its derivatives, and other basic L-amino acids failed to stimulate the activity, indicating a specificity of the activation to L-arginine and its analogues.

The effect of L-arginine-containing peptides was examined for guanylate cyclase activation (Table III). L-Arginyl-L-aspartic acid, tuftsin, bradykinin and its derivatives that con-
tained L-arginine at either the NH2- or COOH-terminal or both terminals also stimulated guanylate cyclase activity 15-25-fold, whereas luteinizing hormone-releasing hormone, neurotensin, and angiotensin I (human) that contained L-arginine inside the peptide chain showed no or a slight stimulation of the activity. Substance P, which contained L-arginine at the NH2-terminal, failed to stimulate the activity, the reason of which is not elucidated at present. Thyrotropin releasing hormone or melanocyte stimulating hormone-release inhibiting factor, peptides without L-arginine, were without effect on the activity. There was no lag phase in the activation reaction by bradykinin (data not shown). The dose response of the activation by bradykinin was examined. A significant activa-

![Graph](image)

**FIG. 4**. Dose responses of the stimulation of guanylate cyclase activity to the activator (A) and L-arginine (B). The effluent fraction (166 µg of protein) of the neuroblastoma supernatant through a Chelex 100 Na+ column was used for each assay. The activator purified to the final step (A) or L-arginine (B) was added to the assay in the amount indicated. Guanylate cyclase activity was measured with either 3 mM MgCl₂ (C) or 3 mM MnCl₂ (X). The values are the means of duplicate determinations.

**TABLE II**

Effect of L-arginine and related compounds on guanylate cyclase activity from neuroblastoma cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>Amount (µM)</th>
<th>Guanylate cyclase (nmol/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>(0.1)</td>
<td>4.59</td>
</tr>
<tr>
<td>D-Arginine</td>
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<td>0.29</td>
</tr>
<tr>
<td>L-Arginine methyl ester</td>
<td>(0.1)</td>
<td>4.53</td>
</tr>
<tr>
<td>L-Homoarginine</td>
<td>(0.1)</td>
<td>3.67</td>
</tr>
<tr>
<td>Guanidine</td>
<td>(1.0)</td>
<td>0.28</td>
</tr>
<tr>
<td>Guanidoicetic acid</td>
<td>(1.0)</td>
<td>0.30</td>
</tr>
<tr>
<td>β-Guanidinopropionic acid</td>
<td>(1.0)</td>
<td>0.32</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>(0.1)</td>
<td>0.31</td>
</tr>
<tr>
<td>L-Citrulline</td>
<td>(1.0)</td>
<td>0.26</td>
</tr>
<tr>
<td>L-Ornithine</td>
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<td>0.27</td>
</tr>
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</table>


**TABLE III**

Effect of L-arginine and peptides on guanylate cyclase activity from neuroblastoma cells

The Chelex 100 effluent (262 µg of protein) of the neuroblastoma supernatant was used for each assay. The values are the means of duplicate determinations. LHRH, luteinizing hormone-releasing hormone; TRH, thyrotropin releasing hormone; MIF, melanocyte stimulating hormone-releasing inhibiting factor.

<table>
<thead>
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<th>Addition</th>
<th>Amount (µM)</th>
<th>Guanylate cyclase (nmol/10 min)</th>
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<tr>
<td>None</td>
<td></td>
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<tr>
<td>L-Arginine</td>
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<tr>
<td>L-Arynyl-L-aspartic acid</td>
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<td>Tuftsin</td>
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<td>Bradykinin</td>
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<td>Lysylbradykinin</td>
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</tr>
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<td>Desargetylbradykinin</td>
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<tr>
<td>LHRH</td>
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<td>TRH</td>
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</tr>
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<td>MIF</td>
<td>(0.3)</td>
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</table>
| N-methyl-N'-nitro-N-nitrosoguanidine, N-methylnitroso-urea, nitroprusside, or nitric oxide activate the soluble guanylate cyclase from a variety of tissues (17-23). The observations that L-arginine and nitroso compounds showed no ad-

**DISCUSSION**

This study has confirmed our previous findings that the synaptosomal soluble fraction of rat brain and the supernatant of neuroblastoma N1E 115 cells contain an endogenous activator for soluble guanylate cyclase (12, 35). We now purified the activator from the supernatant of rat brain and identified it as L-arginine. L-Arginine activated guanylate cyclase after the supernatant of neuroblastoma cells or the synaptosomal soluble fraction was passed through cation exchangers such as Chelex 100, SP-Sephadex C-25, or CM-Sephadex C-25 columns. The activation was observed at a low concentration of L-arginine with a maximum activation of 15-25-fold when assayed with MgCl₂. The activation was specific to L-arginine; DG-arginine and other basic L-amino acids and related compounds were ineffective on the enzyme activity. Peptides that contained L-arginine at the NH₂- or COOH-terminal also stimulated guanylate cyclase activity to the same extent as L-arginine. It is, therefore, undetermined which of the L-arginine or L-arginine-containing peptides are responsible for the activation of guanylate cyclase in tissues.
Activation of Guanylate Cyclase by L-Arginine

Vanant advice concerning the NMR analysis. We examined whether or not L-arginine can activate a highly purified guanylate cyclase from neuroblastoma cells. When the effluent fraction of neuroblastoma supernatant through a Chelex 100 Na' column was adjusted to pH 5.2, all of guanylate cyclase activity was precipitated. L-Arginine failed to activate the guanylate cyclase in the pH 5.2 precipitate fraction. However, recombination of the pH 5.2 precipitate and the pH 5.2 supernatant together with L-arginine resulted in a marked stimulation of guanylate cyclase activity, indicating that another factor which remained in the supernatant at pH 5.2 was required for the activation of the enzyme by L-arginine. It has been shown that guanylate cyclase contained a heme-Cu**, which was dissociated from the enzyme at pH 5 (40, 41). We are presently studying whether or not another factor such as heme is required for the activation of guanylate cyclase by L-arginine after a purification of guanylate cyclase from neuroblastoma cells.

Little has been known as to the biological action of L-arginine in vivo and in vitro systems. Several laboratories have reported that L-arginine inhibited the growth of experimental tumors in vivo and the growth of human breast cancer cells in culture (42-45). Some of the effect was ascribable to the inhibition of ADP ribosylation and to the enhancement of cyclic AMP synthesis in the cells (45). Although the concentration of L-arginine required for the growth arrest of tumor cells is much higher than that needed for the activation of guanylate cyclase and there is no correlation at present between these two observations, L-arginine may have unknown effects on the metabolisms in tissues. This study raises a possibility that L-arginine might be an intracellular mediator of neural transmission and hormone action via the stimulation of cyclic GMP synthesis in cells. The elucidation of the activation mechanism of guanylate cyclase reported here gives us a clue to a new role of L-arginine.

Acknowledgment—We are deeply grateful to Dr. Shiguo Iwasaki, Institute of Applied Microbiology, University of Tokyo, for his relevant advice concerning the NMR analysis.

REFERENCES