Possible Involvement of Nitric Oxide in Acetylcholine-Induced Increase of Intracellular Ca^{2+} Concentration and Catecholamine Release in Bovine Adrenal Chromaffin Cells

Yoshiko Uchiyama, Katsuya Morita, Shigeo Kitayama, Tetsuo Suemitsu, Naoki Minami#, Takanori Miyasako and Toshihiro Dohi*

Department of Pharmacology, Hiroshima University School of Dentistry, Kasumi 1-2-3, Minami-ku Hiroshima 734, Japan

Received December 2, 1993 Accepted March 14, 1994

ABSTRACT—The role of nitric oxide (NO) in neurotransmitter release was studied using bovine adrenal medullary chromaffin cells. L-Arginine and sodium nitroprusside (SNP) slightly increased the intracellular free calcium concentration ($[Ca^{2+}]_i$), and the effects of the agents were dependent on the presence of the extracellular Ca^{2+} ($[Ca^{2+}]_o$), but were not blocked by verapamil (30 μM) or diltiazem (30 μM). SNP enhanced the acetylcholine (ACh)-induced rise in $[Ca^{2+}]_i$ in the presence but not in the absence of $[Ca^{2+}]_o$. The effects of L-arginine but not those of SNP were inhibited by N^{ω}-nitro-L-arginine (L-NNA). L-NNA significantly reduced the ACh-induced rise in $[Ca^{2+}]_i$ and catecholamine (CA) release, and the reduction was restored by L-arginine but not by D-arginine. These results suggest a possible involvement of NO in ACh-induced $[Ca^{2+}]_i$ rise and CA release in bovine adrenal chromaffin cells.

Keywords: Nitric oxide, Catecholamine, Chromaffin cell

Endothelium-derived relaxing factor: nitric oxide (NO) has recently been found to mediate diverse physiologic functions in many organs. The synthesis of NO from the terminal guanidino nitrogen atom(s) of L-arginine was originally demonstrated in vascular endothelial cells. In the peripheral nervous system, recent experiments have provided compelling evidence that the previously unidentified neurotransmitter released by non-adrenergic, noncholinergic nerves, which supply a variety of tissues, is in fact NO (for review, see ref. 1). In the central nervous system, NO may link activation of postsynaptic NMDA receptors to functional modifications in neighboring presynaptic terminals and glial cells (for review, see ref. 2). In both cases, a major action of NO is to activate soluble guanylate cyclase and thereby raise cGMP levels in the target cells. We have previously shown that NO and other nitric compounds could stimulate catecholamine (CA) release from perfused adrenal glands and suggested the involvement of cGMP in the process (3). In this study, the involvements of NO in acetylcholine (ACh)-induced CA release and in the movement of intracellular calcium concentration ($[Ca^{2+}]_i$) were examined in cultured bovine adrenal chromaffin cells.

Chromaffin cells of bovine adrenal glands were isolated enzymatically according to the procedure described by Fenwick et al. (4) with some modifications (5). Chromaffin cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.1 mM ascorbate and 5 mM HEPES in suspension culture for 24-72 hr or in monolayer culture for 5-7 days at 37℃ under 5% CO_2/95% air as described previously (5). Cells were washed and suspended before use in a medium containing 150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.3 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES-Tris buffer and 0.5% BSA, pH 7.4. When Ca^{2+}-deficient medium was used, $CaCl_2$ was omitted and 10 μM EGTA was added in the medium. $[Ca^{2+}]_i$ was estimated by the use of the calcium-sensitive dye fura-2 as described previously (5), except that fluorescence was measured with a two-wavelength fluorescence spectrophotometer (Hitachi F2000, Tokyo) with excitation at 340 nm and 380 nm and emission at 510 nm. The procedures for the measurement of CA release from a monolayer culture have been described

Present address: Department of Pharmacology, Nara Medical University, Kashihara 634, Japan

* To whom correspondence should be addressed.

previously (5). Statistical analyses were performed by Student's *t*-test.

L-Arginine, but not D-arginine, caused a small increase in $[Ca^{2+}]_i$, and this effect of L-arginine was inhibited by Ca^{2+} deprivation in the medium or by pretreatment with N^{ω}-nitro-L-arginine (L-NNA) (Fig. 1A). L-Arginine, but not D-arginine, slightly increased CA release, and this effect of L-arginine disappeared by pretreatment with L-NNA (CA released in $\mu g/10^6$ cells during the 10-min incubation were 0.17 ± 0.002, 0.23 ± 0.007 and 0.15 ± 0.019 for the basal level, with 1 mM L-arginine, and with 100 μM L-NNA and 1 mM L-arginine, respectively). Sodium nitroprusside (SNP) caused a gradual increase in basal $[Ca^{2+}]_i$, and the effect of SNP was dependent on the presence of extracellular Ca ($[Ca^{2+}]_o$), but was not blocked by pretreatments of Ca-antagonists, verapamil and diltiazem (Fig. 1B-1). ACh caused a sharp and transient increase and a subsequent sustained increase in $[Ca^{2+}]_i$ (Fig. 1B-2). The former increase markedly decreased but still remained in the absence of $[Ca^{2+}]_o$, and the latter in-

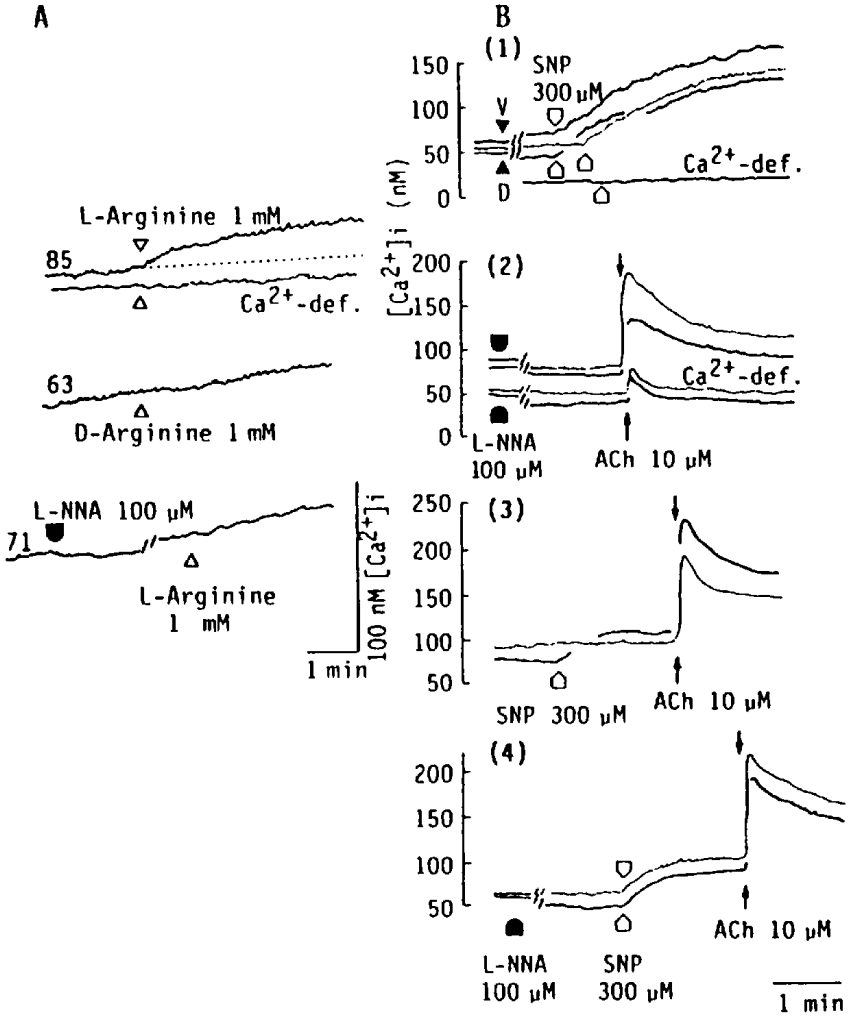

Fig. 1. Effects of arginine and sodium nitroprusside (SNP) on basal and acetylcholine (ACh)-induced $[Ca^{2+}]_i$ rise in isolated bovine adrenal chromaffin cells. Cells were washed and suspended in normal medium (containing 1.3 mM $CaCl_2$, except as indicated) or Ca^{2+}-deficient medium (Ca^{2+}-def.). A 1-ml aliquot of the cell suspension was preincubated in a cuvette at 37°C for 5 min and then challenged with various agents dissolved in the corresponding medium in a volume of 10 μl at the time point indicated. These and the following experiments are representative examples of experiments performed with very similar results in at least three different batches of cells. (A) L-arginine-induced $[Ca^{2+}]_i$ rise and the effect of N^{ω}-nitro-L-arginine (L-NNA). L-NNA was added 5 min before the addition of L-arginine. Each basal value of $[Ca^{2+}]_i$ was represented by Arabic numerals. (B) SNP- and ACh-induced $[Ca^{2+}]_i$ rise and the effects of various treatments. Thin curves are the controls of the various treatments (thick curves). Treatment with verapamil (V, 30 μM) or diltiazem (D, 30 μM) was performed 5 min before the addition of SNP (1). Treatment with L-NNA was performed 5 min before the addition of ACh (2). SNP and ACh were added at the time point indicated in (3) and (4), and L-NNA was added 5 min before the addition of ACh (4).

crease disappeared under this condition. L-NNA (100 μM) reduced the ACh-induced $[Ca^{2+}]_i$ transient in the presence but not in the absence of $[Ca^{2+}]_o$. SNP potentiated the ACh-induced $[Ca^{2+}]_i$ rise (Fig. 1B-3) and CA release: the increases of CA release induced by 5 μM of ACh without and with 100 μM of SNP were 0.41 ± 0.05 and 0.54 ± 0.06*, and the increases of CA release induced by 10 μM of ACh without and with SNP were 0.93 ± 0.08 and 1.21 ± 0.09* $\mu g/10^6$ cells/3 min, respectively (*significantly different at $P < 0.05$, $n = 5$). The effects of L-arginine and SNP were small but constantly observed. Pretreatment with L-NNA had no effect on the SNP-induced $[Ca^{2+}]_i$ rise and ACh-induced rise in the presence of SNP (Fig. 1B-4). Other nitric compounds such as sodium azide, hydroxylamine and sodium nitrite also induced the rise of $[Ca^{2+}]_i$ (data not shown). The effects of L-NNA on the transient rise in $[Ca^{2+}]_i$ and CA release induced by ACh are shown in Fig. 2. L-NNA significantly decreased the ACh-induced $[Ca^{2+}]_i$ rise at concentrations of more than 50 μM (Fig. 2A). The effects of L-NNA were restored with L-arginine, but not with D-arginine (Fig. 2A). CA release evoked by ACh was also decreased by L-NNA (Fig. 2B). The reduction of ACh (30 μM)-induced CA release by L-NNA reached maximum at 10–20 μM, and no further reduction was obtained by increasing the concentrations of L-NNA up to 100 μM. Similar results, although the effects were less potent, were obtained with another inhibitor of NO synthase (NOS), N^G-methyl-L-arginine (data not shown).

NO has been recognized recently as a prominent neuronal messenger. The presence of NOS in neuronal tissues including the adrenal medulla has been demonstrated an immunohistochemical study (6). A constitutive form of NOS (cNOS) has been purified, cloned and sequenced from rat brain (7). We have previously shown that various nitro-compounds including NO stimulate CA release from perfused dog adrenal glands accompanied by the increased output of cGMP (3). ACh also stimulated both CA release and cGMP output (8). Results of the present study show that L-arginine increased $[Ca^{2+}]_i$ and CA release, and the effects of L-arginine may be mediated by NO, because both responses were blocked by L-NNA. Although the CA release and $[CA^{2+}]_i$ rise in response to L-arginine in chromaffin cells were marginal compared to the L-arginine-induced dopamine release reported in superfused rat striatal slices (9), it is noticeable that ACh-induced $[Ca^{2+}]_i$ rise and CA release were significantly reduced by L-NNA. Thus, it seems likely that the L-arginine: NO pathway may play a significant role in these responses to ACh. The counter effects of L-arginine but not D-arginine against the L-NNA-induced reduction of ACh-induced $[Ca^{2+}]_i$ rise support this idea.

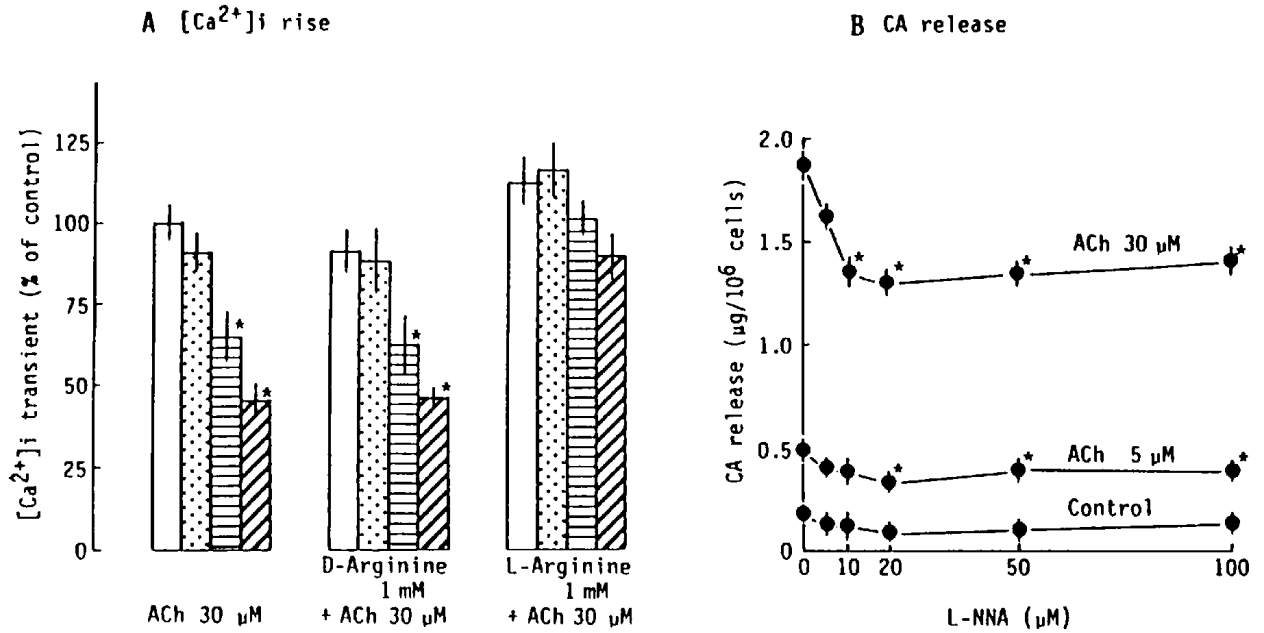

Fig. 2. Effects of N^{ω}-nitro-L-arginine (L-NNA) on acetylcholine (ACh)-induced rise of $[Ca^{2+}]_i$ and catecholamine (CA) release in isolated bovine adrenal chromaffin cells. (A) Cells were preincubated with L-NNA in combinations with or without L- and D-arginine for 10 min and then challenged with ACh. Values are the mean ± S.E.M. of the peak rise in $[Ca^{2+}]_i$ induced by ACh. *Significantly different from the control at $P < 0.05$ ($n = 5$) by Student's t-test. □ Control; ⊡ L-NNA, 20 μM; ▤ L-NNA, 50 μM; ▨ L-NNA, 100 μM. (B) Cells were preincubated with various concentrations of L-NNA for 30 min, and then the medium was replaced with fresh medium containing corresponding concentrations of L-NNA and ACh, and incubated for an additional 3 min. Values are the mean ± S.E.M. of the CA released during the last 3-min incubations. *Significantly different from the control at $P < 0.05$ ($n = 5$).

The evidence that SNP enhanced the ACh-induced rise of $[Ca^{2+}]_i$ and that L-NNA specifically antagonized the effects of L-arginine but not those of SNP on the $[Ca^{2+}]_i$ transient also suggest that NO possibly produced within the cells from L-arginine or arginine-containing peptide by the stimulation with ACh might act to enhance the ACh responses.

In the light of the concept of stimulus-secretion coupling in the adrenal medulla, it is considered that stimulation of the nicotinic ACh receptor (nAChR) initiates depolarization by stimulating Na^+ and Ca^{2+} entry through the receptor channels, followed by activation of voltage-sensitive Na^+ and Ca^{2+} channels (VOC), consequently resulting in Ca^{2+} entry. These are the initial steps in CA secretion in response to nAChR stimulation. Although, stimulation of muscarinic ACh receptors (mAChR) causes various biochemical responses involving increases of $[Ca^{2+}]_i$ (5), cGMP formation (10), Ca^{2-} fluxes (11) and phosphatidylinositol turnover (5), the role of mAChR in CA release is insignificant because mAChR agonists have little or no effect on CA release in bovine adrenal chromaffin cells. However, ACh-induced $[Ca^{2+}]_i$ rise and CA release were partly reduced by the M_1-antagonist pirenzepine in the present study (% reduction of ACh-induced $[Ca^{2+}]_i$ rise and CA release by 0.1 μM pirenzepine were 30.1 ± 2.9 and 21.2 ± 1.8, respectively). Pirenzepine (0.1 μM) did not affect 12–25 mM KCl-evoked $[Ca^{2+}]_i$ rise and CA release (data not shown). Therefore it may be assumed that mAChR activation does not stimulate CA release directly by itself but is involved in the ACh-induced $[Ca^{2+}]_i$ rise and CA release in bovine adrenal chromaffin cells.

It has been recognized that the activity of cNOS is regulated by Ca/calmodulin (1). It may be assumed that stimulation of nAChR and mAChR on chromaffin cells by ACh allows the $[Ca^{2+}]_i$ to increase, thereby activating cNOS.

A major action of NO is to activate soluble guanylate cyclase. Actually, the guanylate cyclase of adrenal medulla homogenate was found to be activated by NO (3). Thus to test the possibility that the effect of NO on $[Ca^{2+}]_i$ transient is mediated by cGMP, the effect of cGMP on the nicotine-induced $[Ca^{2-}]_i$ rise was examined. Increases in $[Ca^{2+}]_i$ in bovine adrenal chromaffin cells induced by a 30-sec incubation with nicotine in combination with 8Br-cGMP were as follows: the 3-μM nicotine-induced $[Ca^{2+}]_i$ rise without or with 0.1 mM of 8Br-cGMP was 74.9 ± 6.2 and $122.9 \pm 11.4^*$ nM/3×10^6 cells (*significantly different at $P < 0.05$, $n = 5$), and the 10-μM nicotine-induced rise without or with 8Br-cGMP was 277.0 ± 12.5 and $424.9 \pm 18.3^*$ nM/3×10^6 cells (*significantly different at $P < 0.05$, $n = 5$), respectively. 8Br-cGMP by itself, had no effect on $[Ca^{2+}]_i$. These results are in agreement with the observation of O'Sullivan and Burgoyne (12) that cGMP potentiated the nicotine-induced CA release from bovine adrenal chromaffin cells, although cGMP failed to alter the nicotine-induced $[Ca^{2+}]_i$ rise. We have recently demonstrated that cAMP, formed via Ca/calmodulin in chromaffin cells upon stimulation with ACh, acts to potentiate the effects of ACh on $[Ca^{2+}]_i$ rise and CA release (13). For the mechanism of cAMP-potentiation, it is proposed that cAMP primarily inhibits the Na^+,K^+-ATPase of the plasma membrane of chromaffin cells and thus enhances stimulation-induced accumulation of Na^+, resulting in further increases in membrane depolarization, opening of VOC, Na^+-Ca^{2+} exchange through the plasma membrane and $[Ca^{2+}]_i$ rise (14). The effects of L-arginine and SNP on the $[Ca^{2+}]_i$ transient are probably due to facilitation of Ca^{2+} influx through the plasma membrane, because their effects were dependent on the presence of the extracellular Ca^{2+}, and L-NNA decreased the ACh-induced $[Ca^{2+}]_i$ transient in the presence of $[Ca^{2+}]_o$, but not in Ca^{2+}-deficient medium. Verapamil and diltiazem, at concentrations that effectively inhibited the elevation of the KCl-evoked CA release (13), did not affect the SNP-induced $[Ca^{2+}]_i$ rise. Thus, the facilitation of Ca^{2+} influx does not seem to be mediated via activation of VOC. Although the mechanism for NO/cGMP-induced enhancement of the $[Ca^{2+}]_i$ transient is not known at present, we assume that the L-arginine: NO-cGMP pathway permits the enhancement of stimulation-evoked $[Ca^{2+}]_i$ rise, resulting in the potentiation of the secretory response. Findings from the study by Palacios et al. (15) indicating the formation of NO by L-arginine: NOS and its stimulation of the soluble guanylate cyclase in adrenal glands are in good agreement with this interpretation.

In summary, the demonstrations of the modification by a substrate or inhibitors of NOS and nitric compounds of $[Ca^{2+}]_i$ transient and CA release in bovine adrenal chromaffin cells support the idea that NO participates in ACh-induced neurotransmitter release in these cells.

REFERENCES

1 Snyder SH: Nitric oxide: first in a new class of neurotransmitters? Science **257**, 494–496 (1992)
2 Garthwaite J: Gultamate, nitric oxide and cell-cell signalling in the nervous sytem. Trends Neurosci **14**, 60–67 (1991)
3 Dohi T, Morita K and Tsujimoto A: Effect of sodium azide on catecholamine release from isolated adrenal gland and on guanylate cyclase. Eur J Pharmacol **94**, 331–335 (1983)
4 Fenwick EM, Fajdiga PB, Home NBS and Livett BG: Functional and morphological characterization of isolated bovine adrenal medullary cells. J Cell Biol **76**, 12–30 (1978)
5 Ohtsuki H, Morita K, Minami N, Suemitsu T, Tsujimoto A and Dohi T: Involvement of Ca^{2+} entry and inositol trisphosphate-induced internal Ca^{2+} mobilization in muscarinic receptor-

mediated catecholamine release in dog adrenal chromaffin cells. Neurochem Int **21**, 259–267 (1992)

6 Bredt DS, Hwang PM and Snyder SH: Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature **347**, 768–770 (1990)

7 Bredt DS, Hwang PM, Gltt CE, Lowenstein C, Reed RR and Snyder SH: Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature **351**, 714–718 (1991)

8 Tsujimoto A, Morita K, Nishikawa T and Yamada S: Cyclic nucleotide elevation preceding catecholamine release in isolated dog adrenals. Arch Int Pharmacodyn Ther **245**, 262–270 (1980)

9 Zhu X-Z and Luo L-G: Effect of nitroprusside (nitric oxide) on endogenous dopamine release from rat striatal slices. J Neurochem **59**, 932–935 (1992)

10 Yanagihara N, Isosaki M, Ohuchi T and Oka M: Muscarinic receptor-mediated increase in cyclic GMP level in isolated bovine adrenal medullary cells. FEBS Lett **105**, 296–298 (1979)

11 Oka M, Isosaki M and Watanabe J: Calcium flux and catecholamine release in isolated bovine adrenal medullary cells: effects of nicotinic and muscarinic stimulation. Adv Biosci **36** (Synthesis, Storage and Secretion of Adrenal Catecholamine), 29–36 (1982)

12 O'Sullivan AJ and Burgoyne RD: Cyclic GMP regulates nicotine-induced secretion from cultured bovine adrenal chromaffin cells: effects of 8-bromo-cyclic GMP, atrial natriuretic peptide, and nitroprusside (nitric oxide). J Neurochem **54**, 1805–1808 (1990)

13 Morita K, Dohi T, Kitayama S and Tsujimoto A: Mechanism of cyclic AMP facilitation of stimulation-evoked catecholamine release in adrenal chromaffin cells—1. Evidence for enhancement of evoked increase in cytosolic free Na^+ concentration by cyclic AMP elevation. Neurochem Int **19**, 71–79 (1991)

14 Morita K, Dohi T, Minami N, Kitayama S and Tsujimoto A: Mechanism of cyclic AMP facilitation of stimulation-evoked catecholamine release in adrenal chromaffin cells—II. Inhibition of Na^+,K^+-ATPase by cyclic AMP. Neurochem Int **19**, 81–85 (1991)

15 Palacios M, Knowles RG, Palmer RMJ and Moncade S: Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. Biochem Biophys Res Commun **165**, 802–809 (1989)