嘉南藥理學院專題研究計畫成果報告

The Study of NO on Neuronal System

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ABSTRACT

The modulatory role of nitric oxide (NO) has been implicated in various physiological functions. Due to the death of the rat after the surgery, the study model was switched to the other nervous system: the chromaffin cell. We found NO indeed regulate catecholamine (CA) release of chromaffin cells. In order to clarify the possible role of NO in altering CA release caused by different stimulants, we used the specific NO pathway blocker, N^G-nitro-L-arginine methyl ester (L-NAME), to study its effect on neuronal cell secretion. It was found that CA release induced by nicotinic receptor agonist, DMPP, was inhibited by pretreating the cultured bovine chromaffin cells with 200 μM L-NAME. But the inibitory effect of L-NAME was not found in high K^+ induced secretion. Therefore, these results suggested that in bovine chromaffin cells the NO might play some role in regulating DMPP-induced CA release. The further mechanism remained to be investigated.

INTRODUCTION

Nitric oxide (NO), which accounts for the biological activity of endothelium derived relaxing factor (EDRF) (Palmer et al., 1987), plays tremendous roles in all kinds of physiological responses. NO was found to be involved in blood coagulation system and immune system (Geiger et al., 1992; Salvemini et al., 1989; Morgan and Newby, 1989). NO also acts as a neuromodulator in both central nervous system and peripheral nervous system, involving in synaptic plasticity and long term potentiation (Garthwaite, 1991; Vincent and Hope, 1992), and had an inhibitory effect on the adrenergic neurotransmission (Greenberg et al., 1989). NO was synthesized by NO synthase (NOS) which deamined the substrate, L-arginine, to give citrulline and NO. The isoforms of NOS were classified to be calcium/calmodulin (Ca/CaM)-dependent, which found in the neural and endothelial tissue, and Ca/CaM-independent, which was presented in macrophages. The effects of NO is conducted by its binding with iron in a heme moiety of guanylate cyclase, causing the activation of this enzyme and thus rising the intracellular concentration of cyclic GMP (cGMP) (Moncada et al., 1991; Snyder and Bredt, 1991).

NOS has been shown to be present in bovine adrenal medulla by either immunostaining (Palmer and Moncada, 1989) or some indirect evidences (O'Sullivan and Burgoyne, 1990; Oset-Gasque et al., 1994). Since increase of cGMP had long been found during the activation of chromaffin cells (Schneider et al, 1979; Yanagihara et.al., 1979; Derome et al., 1981), and NO generators also increased catecholamine (CA) release (Dohi et al., 1983;

O'Sullivan and Burgoyne, 1990), the involvement of NO in modulating the CA release had been speculated. However, the role of NO on regulating CA release of chromaffin cells seemed to be controversial (O'Sullivan and Burgoyne, 1990; Oset-Gasque et al., 1994). There was an enhancement in secretion by NO (Oset-Gasque et al., 1994), while an inhibitory effect was also found (Marley et al., 1995; Rodriguez-Pascual et al., 1996). Recent study found NO donor and db-cGMP may modulate calcium mobilization in bovine chromaffin cells（Shono et al., 1997）and NO could serve as both an intracellular and intercellular messenger（Oset-Gasque et al., 1998）. The role of NO in regulating the CA release in chromaffin cells still remains to be elucidated. In this study, we used cultured bovine chromaffin cells to investigate the role of NO in modulating CA secretion. We found the specific NO pathway blocker, N^G-nitro-L-arginine methyl ester (L-NAME), inhibited the CA release induced by agonist of nicotinic receptor, 1,1-dimethyl-4-phenylpiperazinium (DMPP). The IC_{50} of L-NAME was 200μM, which is correlated with other report (Rodriguez-Pascual et al., 1995). However, L-NAME was ineffective on high-K^+ induced secretion. These results suggested that NO pathway might play a positive role in potentiating CA release of bovine chromaffin cells.

MATERIALS AND METHODS

Preparation of chromaffin cells

Bovine adrenal medullary chromaffin cells were isolated from adrenal glands obtained from a local slaughter house. The glands were removed immediately after the animals were killed, and were kept on ice until cells were isolated, which usually took 5 hr. The chromaffin cells were isolated as described. In brief, adrenal glands were first perfused via the adrenal vein for 3 to 5 times using a syringe containing perfusion solution (145 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 10 mM glucose, and 15 mM HEPES, pH 7.4) over a period of 30 min at 4°C. The glands were then perfused 2 times with a collagenase solution (0.2% collagenase and .002% deoxyribonuclease I in perfusion buffer). After perfusion, the medulla was separated from the cortex and cut into small pieces. Minced medulla was then further digested for 30 min with collagenase solution (0.05% collagenase and 0.0004% deoxyribonuclease I in perfusion solution) at 37°C. The isolated cells were filtered through 250 mm nylon mesh and collected by centrifugation (2,300 × g) at 4°C. The isolated chromaffin cells were either used fresh or cultured in 96-well culture plates (2×10^5 cells/well) for the measurement of secretion. Cultured cells were used 3 to 7 days after isolation.

Measurement of catecholamine secretion.

The chromaffin cells cultured in 96-well culture plates were washed three times every 10 min with loading buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4). 40 ml loading buffer with D,L-(7-^{3}H)norepinephrine (1μCi/ml), 0.5% bovine serum albumin (BSA) and 250 mM ascorbic acid was added into each well, incubated for 2 to 3 hr. at 37°C.. Each well was then washed three time with loading buffer (0.5% BSA) every 10 min. 50 ml loading buffer with stimulant was added into each well. At designated times the supernatant was removed and a solution of 0.1% Triton X-100 was added to the pellet. Radioactivities of the supernatant and pellet were counted and used to calculated the percentage of the total radioactivity of the cells which was secreted. Results are expressed as mean ±SEM of at least three determinations in each of three experiments using different batches of cells.

RESULTS AND DISCUSSION

In bovine chromaffin cells stimulation of 3 μM DMPP for 10 min induced catecholamine secretion upto 10.3 ± 2.4% (n =18) as compared with the basal secretion （4.6 ±0.5%, n =3）. If the cells were challenged with 55 mM K^+, the CA release was 7.9 ±2.7% (n =6). When chromaffin cells were stimulated with 3 μM DMPP in the presence with 200 μM L-NAME, a NO pathway blocker, the secretion decreased and the inhibitory effect was 48.4 ±14.9% (n=9） (Fig.1). This inhibitory effect was statistically significant and was not found in high K^+-induced secretion (Table 1). The effective inhibitory concentration of L-NAME was close to the IC_{50} (232μM) reported in the literature (Rodriguez-Pascual et al., 1995).

In accordance with an increase of cGMP after stimulating bovine chromaffin cells with muscarinic receptor agonist, methacholine, found in the early nineties, a modulatory role of cGMP on CA release was speculated, but the mechanism of cGMP regulation was not clear (Schneider et al, 1979; Yanagihara et.al., 1979; Derome et. al., 1981). After EDRF was proved to be NO (Palmer et al, 1987), the NO pathway was studied thoroughly, and a rise of cGMP in response to activation of guanylyl cyclase by NO seemed to partially link the gap found in cGMP regulation on CA release. The immunoreactivity studies also showed that NOS was present in pre- and post-synaptic elements of the sympathetic ganglia（Dun et al., 1993）and in bovine adrenal medulla. These findings suggested NO might play some roles in regulating the secretion of chromaffin cells. In our experiment we found that L-NAME, the specific NO blocker, could inhibit the DMPP-induced exocytosis. This inhibition of L-NAME could be partially due to its blockage on calcium influx induced by stimulating with the secretagogue in chromaffin cells. The specific action site of NO on CA release still remained to be identified. But the possible mechanism might be that NO provided an alteration via S-nitrosylation of the affinities of proteins, which were associated to form the secretory vesicle（Meffert et al., 1996）.

In conclusion, by using specific NO pathway blocker, we found the CA release in bovine chromaffin cells was modulated by L-NAME. This regulation was only found in DMPP-induced CA release. In the future study, it needs to further confirm that the action site of L-NAME inhibitory effect on CA release in chromaffin cells.

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Table 1. |The inhibition effect of L-NAME on DMPP- and high K^+-induced secretion.

Treatment	% of secretion	P

DMPP	100(10.3 ± 2.4, n=18)	
DMPP + L-NAME		
200μM	48.4 ±14.9 (n=9)	<0.0002
1mM	56.4 ± 2.0 (n=6)	<0.00005
K^+	100 (7.9 ± 2.7, n=6)	
K^+ + L-NAME		
200μM	96.6 ± 6.1(n=6)	
1mM	97.4 ± 2.5(n=6)	

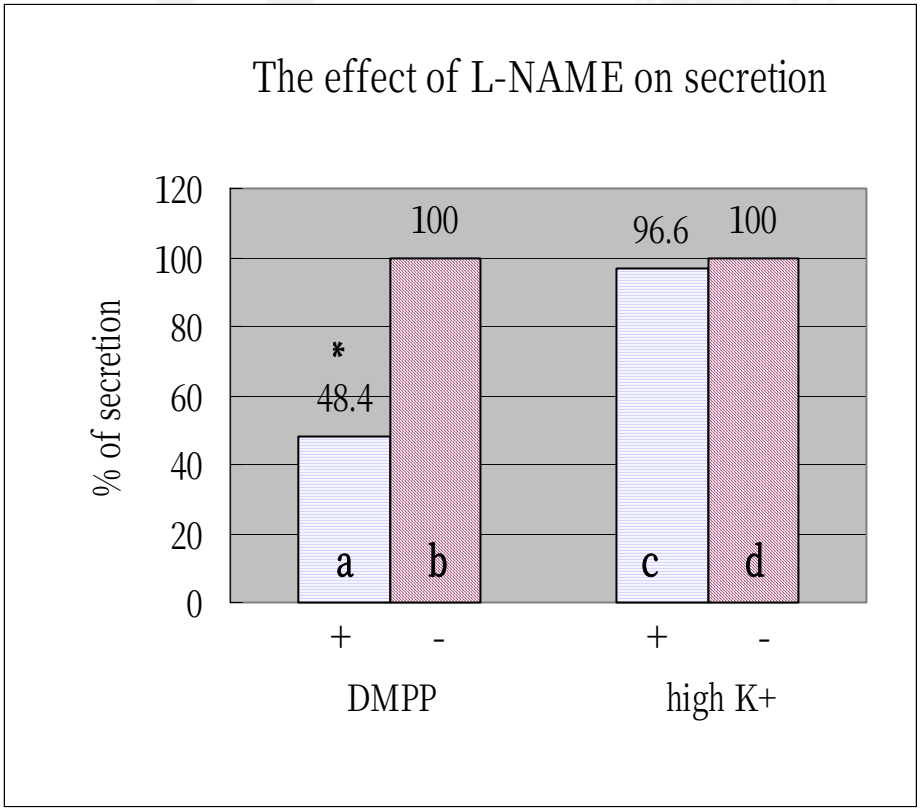

Figure 1. The effect of L-NAME on the secretion. The percentage of secreation was measured in the presence with (+, bar a and c) or without(-, bar b and d) 200 μM L-NAME. The SEMs were 23.3, 14.9, 34.2 and 6.1 referred to bar a, b, c and d, respectively. * P<0.0002