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Short Communication

Enhancement of maximal activation of neuronal nitric oxide synthase at muscarinic M_1 receptors following prolonged agonist treatment

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Abstract

It was previously believed that the neuronal type of nitric oxide (NO) synthase was constitutive in nature, and that changes in the concentration of intracellular Ca^{2+} represent the sole input that regulates its activity. Recent reports, however, suggested that this enzyme could also be induced under certain conditions. We report here that prolonged stimulation of M_1 muscarinic acetylcholine receptors results in potentiation of maximal receptor-mediated activation of neuronal NO synthase in Chinese hamster ovary cells. This effect was dependent on the concentration of agonist during the treatment and was abolished by a muscarinic receptor antagonist. These findings are important for understanding the sequelae of prolonged administration of muscarinic agonists in vivo. © 1997 Elsevier Science B.V.

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1. Introduction

The effects of nitric oxide (NO) on blood vessels and cGMP formation have long been known before it was discovered that it can be produced endogenously by a group of enzymes called NO synthases (Ignarro et al., 1981; Palmer et al., 1987, 1988). NO also has diverse effects on neurons, and it has been shown to play an important role in learning (Dinerman et al., 1994), neurotoxicity (Nowicki et al., 1991; Dawson, 1994) and in regulation of neuronal excitability (Bagetta et al., 1992). Three isoforms of NO synthase have been cloned. Two isoforms are constitutive and their activity is Ca^{2+}-calmodulin dependent (Bredt and Snyder, 1990). These enzymes are expressed predominantly in neuronal and endothelial cells (Bredt and Snyder, 1992). The third is Ca^{2+} independent and its expression is induced by agents such as gamma-interferon and tumor necrosis factor and by foreign agents such as bacterially-derived lipopolysaccharides (Bredt and Snyder, 1994).

Recent evidence, however, has shown that the activity of neuronal NO synthase is not purely constitutive, since the level of expression of the enzyme is altered under certain conditions. These conditions include brain lesions (Yu, 1994) and ischemia (Kato et al., 1994). Since NO is not stored in neurons, its rate of formation is dependent on the level of activity of neuronal NO synthase, which in turn is dependent on the level of expression of the enzyme. In this report we used Chinese hamster ovary (CHO) cells which co-express M_1 muscarinic receptors and neuronal NO synthase to investigate whether the activity of this important enzyme is modulated by prolonged receptor activation. Our data demonstrate that maximal activation of neuronal NO synthase by M_1 muscarinic receptors is markedly potentiated following long-term exposure to the muscarinic agonist carbachol.

2. Materials and methods

2.1. Materials

[^{3}H] L-arginine was obtained from Amersham (Arlington Heights, IL). Carbachol and pirenzepine were purchased from Sigma Chemicals (St. Louis, MO).

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2.2. Cell culture

Chinese hamster ovary (CHO) cells that stably express human muscarinic M_1 receptors were donated by Dr. M. Brann (University of Vermont). These cells were stably transfected with the neuronal NO synthase gene obtained from S.H. Snyder (John Hopkins University). CHO transfects were grown in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) supplemented with 10% bovine calf serum (Hyclone, Logan, UT), in the presence of geneticin (50 μg/ml) and hygromycin 50 μg/ml. Cells were used when they reached confluency.

2.3. Assay of NO synthase activity

Intact cells were assayed for NO synthase activity as previously described (Wang et al., 1994). Briefly, cells were harvested, washed and suspended in a buffer containing (in mmol): NaCl, 109; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 1.0; HEPES, 20, glucose; 58 (pH, 7.4; osmolarity adjusted to 335–340 mOSM by addition of sucrose). Suspended cells were distributed in assay tubes (5×10^5 cells per tube) in a volume of 0.3 ml containing 0.6 μCi of [^{3}H] L-arginine, in the presence or absence of the indicated concentrations of carbachol for one hour at 37°C. The reaction was stopped with 0.75 ml of buffer containing 4 mM EDTA and 5 mM L-arginine. Afterwards, cells were centrifuged and the buffer containing excess radiolabeled substrate was aspirated. Cells were then lysed with 0.3 M $HClO_4$ and subsequently neutralized with 0.15 M K_2CO_3. [^{14}C] L-citrulline ($\cong$ 1500 d.p.m.) was added to each sample as an internal elution standard. Samples were transferred into Dowex AG50W-X8 (sodium form) columns and the flow through eluate was collected into scintillation vials, followed by the addition of 2 ml of H_2O that was also collected. Econolite scintillation fluid was added to the collected samples and radioactivity counted in a Beckman LS6000TA liquid scintillation counter.

2.4. Data analysis

Results of each experiment were expressed as percent of the respective control maximum [^{3}H] L-citrulline produced. Non-linear regression (variable slope) and one way analysis of variance (ANOVA) were generated by Graphpad Prism (Graphpad, San Diego, CA).

3. Results

CHO cells which co-express M_1 muscarinic receptors and neuronal NO synthase were preincubated in culture flasks with varying concentrations of carbachol for 48 h at 37°C. At the end of preincubation, cells were harvested and each group was incubated with [^{3}H] L-arginine with or without 100 μM carbachol for 1 h at 37°C. [^{3}H] L-citrulline formation was assayed as described in Section 2. Pretreatment with carbachol resulted in a concentration-dependent potentiation of subsequent agonist-induced activation of neuronal NO synthase. In contrast, there was no significant change in the basal level of neuronal NO synthase activity following agonist pretreatment (Fig. 1). Maximal potentiation averaged 210 $\pm$ 9% of the response

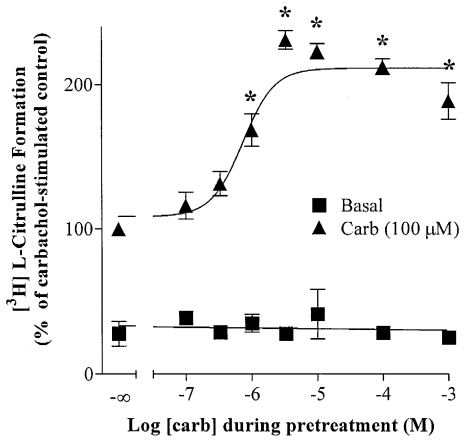

Fig. 1. Effects of prolonged carbachol pretreatment on M_1 muscarinic receptor-mediated activation of NO synthase. CHO cells which express the M_1 muscarinic receptor and neuronal NO synthase were incubated with or without increasing concentrations of carbachol for 48 h. This was followed by determination of synthesis of [^{3}H] L-citrulline in the presence or absence of 100 μM carbachol. Data are represented as percent of [^{3}H] L-citrulline formation in the presence of 100 μM carbachol in control cells for each experiment (mean $\pm$ S.E.M., $n = 4$). Carbachol stimulated [^{3}H] L-citrulline formation by 5.6 $\pm$ 0.7 fold in control cells. * $P < 0.01$, one way ANOVA. Carb = carbachol.

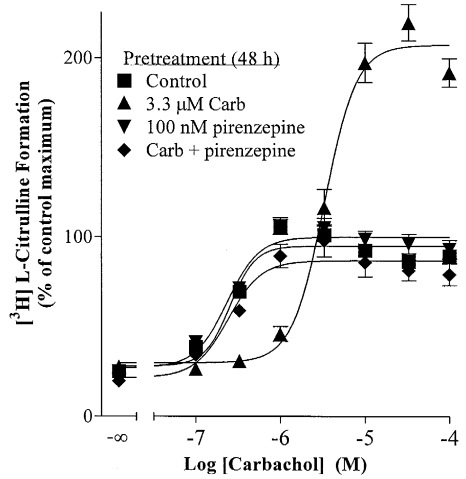

Fig. 2. Antagonism of carbachol-induced changes in M_1 muscarinic receptor coupling to activation of neuronal NO synthase by pirenzepine. Transfected CHO cells were preincubated with or without 3.3 μM carbachol for 48 h in the presence or absence of 100 nM pirenzepine. Washed cells were then stimulated in the presence or absence of increasing concentrations of carbachol. Data are presented as percent of the maximal response in control cells (mean $\pm$ S.E.M., $n = 5$). Maximal response averaged 4.0 $\pm$ 0.3 fold over basal in control cells. Carb = carbachol.

in control cells treated in parallel in the absence of carbachol during the preincubation phase, with a half maximal effective concentration (EC_{50}) of 543 ± 261 nM carbachol.

We studied the effects of pretreatment with 3.3 μM carbachol for 48 h on complete concentration–response curves of M_1 receptor-mediated activation of neuronal NO synthase. While there was an increase in the maximal response following this treatment (220 ± 17% of maximal stimulation of control, $p < 0.01$, one way ANOVA), there was a marked decrease in agonist potency noticed as an increase in EC_{50} from 0.23 ± 0.03 to 3.5 ± 0.5 μM for control and treated cells, respectively (Fig. 2). The effects of agonist pretreatment were abolished in the presence of 100 nM of the muscarinic M_1 receptor-selective antagonist, pirenzepine, during preincubation with carbachol (Fig. 2). Preincubation of cells with pirenzepine alone for 48 h followed by washing did not alter the profile of carbachol-induced activation of the enzyme when compared to control (Fig. 2). Together, these data suggest that the observed phenomenon is a specific consequence of muscarinic receptor activation.

4. Discussion

Preliminary studies show that pretreatment with 3.3 μM or 1 mM carbachol for 48 h both resulted in a marked increase in the expression of neuronal NO synthase. However, pretreatment of CHO cells with either concentration of carbachol was also accompanied by significant reduction in muscarinic receptor density, dampening of receptor-mediated generation of inositol phosphates and desensitization of the Ca^{2+} response to carbachol (Cuadra, unpublished data). These findings might explain the observed decrease in agonist potency in activation of NO synthase, due to the Ca^{2+} dependence of this enzyme (Bredt and Snyder, 1992).

Previous studies demonstrated an increase in both mRNA encoding neuronal NO synthase and the Ca^{2+}-stimulated activity of the enzyme in cytosolic preparations of rat brain after combined administration of the cholinesterase inhibitor, tacrine, and LiCl (Bagetta et al., 1993a,b). In addition, we have preliminary evidence obtained in N1E-115 mouse neuroblastoma cells that shows a considerable increase in the expression of endogenous neuronal NO synthase after 24 h pretreatment with 1 mM carbachol (Chell, unpublished observation). Together with our present findings, it is logical to speculate that prolonged activation of muscarinic receptors in vivo might result in an enhancement of maximal receptor-mediated activation of NO synthase. This possibility should be tested, since the proposed treatment of Alzheimer's dementia with M_1-selective muscarinic receptor agonists involves long-term administration of such agents (McKinney and Coyle, 1991). While an enhancement of maximal coupling of muscarinic receptors to generation of NO is expected to be beneficial for memory enhancement, it might also underlie certain adverse effects of chronic cholinergic treatment observed in animal studies, e.g. neurotoxicity (Veronesi et al., 1990) and induction of epileptic foci (Mollace et al., 1991; Bagetta et al., 1992). Future research should test the possible role of enhanced maximal muscarinic receptor-mediated generation of NO in the etiology of these serious untoward effects, since some studies have demonstrated neurotoxic and epileptogenic actions of NO (Nowicki et al., 1991; Dawson, 1994).

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