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Role of nitric oxide production in carbachol-induced negative chronotropy in cultured rat ventricular myocytes

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Abstract

It has been reported that nitric oxide (NO) plays a physiological role in mediating the effect of vagal stimulation in the autonomic regulation of the heart. In this study, the changes in NO production induced by carbachol were investigated by measuring the NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), with a high-performance liquid chromatography-Griess reaction system, and the carbachol-induced chronotropic response was simultaneously investigated. Cultured rat ventricular myocytes exhibited a dose-dependent negative chronotropic response and NO metabolite production in response to carbachol. The negative chronotropy and the enhancement of NO metabolite production induced by 10^{-4} M carbachol were completely abolished by 10^{-6} M atropine. Both of these effects of carbachol were completely abolished by NO synthase inhibitors such as 3×10^{-4} M N^G-monomethyl-L-arginine acetate and 10^{-5} M methylene blue. Furthermore, the negative chronotropic effect induced by 10^{-4} M carbachol was also abolished by 10^{-6} M 1*H*-[1,2,4]oxadiazolo[4,3-α]quanoxalin-1-one, a selective guanylyl cyclase inhibitor. In addition, 10^{-4} M 8-bromoguanosine 3′:5′-cyclic monophosphate, a cell-permeable analogue of guanosine 3′:5′-cyclic monophosphate, caused a negative chronotropic effect. These results suggest that the NO-signaling pathway may play an important role in the muscarinic cholinergic regulation of myocardial function.

Keywords: Nitric oxide (NO); Nitric oxide (NO) metabolite; Nitric oxide (NO) synthase; Negative chronotropy; Muscarinic receptor; Myocyte, cultured

1. Introduction

Many observations have revealed that endogenous nitric oxide (NO) regulates physiologic functions of mammalian tissues, including blood vessels, neurons and immune systems, and sometimes acts as a toxic agent (Furchgott and Zawadzki, 1980; Garthwaite et al., 1988; Gillespie et al., 1989; Nathan and Hibbs, 1991). The human myocardium contains constitutive NO synthase and has the capacity to express inducible NO synthase (iNOS) (De Belder et al., 1993). In fact, it was found that the induction of iNOS and subsequent excessive production of NO within the heart contribute to the pathophysiology of several inflammatory conditions such as sepsis, transplant rejection, myocarditis and idiopathic dilated cardiomyopathy (Brady et al., 1992; Schulz et al., 1992; Balligand et al., 1993a; Winlaw et al., 1994).

Furthermore, some results indicated that NO also plays a physiological role in mediating the effect of vagal stimulation in the autonomic regulation of the heart (Brady et al., 1993). Using spontaneously beating neonatal rat cardiac myocytes, Balligand et al. (1993b) showed that muscarinic cholinergic stimulation induced by carbachol caused a negative chronotropic effect. They reported that analogs of guanosine 3′:5′-cyclic monophosphate (cGMP), a second messenger of the NO-signaling pathway, mimicked the negative chronotropic effect of carbachol. In adult rat ventricular myocytes, they also observed that NO antagonists inhibited the carbachol-induced attenuation of L-type Ca^{2+} current ($I_{Ca\ (L)}$) and cell shortening amplitude (Balligand et al., 1995).

However, there has been no direct evidence that this muscarinic cholinergic agonist induces NO production and

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simultaneous negative chronotropy in cultured myocytes. To unequivocally establish the relationships of these parameters to carbachol stimulation, simultaneous measurements were performed on the same individual cell preparations. In this study, we investigated the enhancement of NO production induced by carbachol by measuring the nitrite plus nitrate (NO_x) level and simultaneously investigated the carbachol-induced negative chronotropy in cultured rat ventricular myocytes. We observed that these effects of carbachol on the muscarinic cholinergic-NO signaling pathway were blocked by several drugs. In addition, we detected a negative chronotropic effect of 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br-cGMP), an analog of cGMP. Our results indicate an important role of NO in the negative chronotropic effect induced by muscarinic cholinergic stimulation in cultured rat ventricular myocytes.

2. Materials and methods

2.1. Preparation of cultured rat ventricular myocytes

All experiments were performed under the supervision of the Animal Care Committee of Sapporo Medical University. Primary cultured cardiac myocytes were prepared from ventricles of neonatal Wistar rats (1 to 3 days old) by collagenase digestion, as described previously (Kimura et al., 1993). Briefly, neonatal rat ventricles were removed and incubated with collagenase (200 unit/ml) in Ca^{2+}- and Mg^{2+}-free Hanks' salt balanced solution (pH 7.4) under 95% O_2 and 5% CO_2 gas bubbling at 37°C. The isolated ventricular myocytes were seeded at the density of 1.5×10^6 cells/35 mm dish and cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2. Serum-containing medium was replaced by fresh medium every 2 days. All experiments were carried out at 6 to 7 days of culture.

2.2. Measurement of the beating rate

Spontaneous contraction of cultured myocytes was measured with a Fotonic Sensor™, a fiber optic displacement measurement instrument (MTI 1000, MTI Co., USA) (Kawana et al., 1993, 1994; Kimura et al., 1994, 1998; Yamamoto et al., 1998). The principle of measurement is to detect changes in the distance between the probe and myocytes vertically extruded by contraction. The fiber optic probe consists of adjacent pairs of light-transmitting and light-receiving fibers. The ratio of reflected light to transmitted light changes in proportion to the distance between the probe and an object at a certain range shown by a calibration curve specific to each probe. We used a needle-type probe (MTI-3806R), 0.7-mm in diameter, which could cover about 100–150 myocytes. The serum-containing medium was replaced by 2000 μl of serum-free DMEM buffered with *N*-2-hydroxy-ethylpiperazine-*N*′-ethanesulfonic acid (HEPES) (SFD) for 2 h before measurement and the cells were stabilized in a multigas incubator (Sanyo, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The probe was set perpendicular to myocytes in the dish. They were analyzed after being agitated on a horizontally rotating shaker following 15 min in an incubator for stabilization. After collecting a 200 μl sample from SFD in the dish to measure the control level of NO metabolites, 200 μl of vehicle or a tenfold concentrated solution of carbachol was added to 1800 μl of SFD in the dish and then mixed with the shaker for 15 s at 60 rpm. The resultant concentrations of carbachol in SFD in the dish were from 10^{-6} to 10^{-3} M. We measured the changes in spontaneous beating rates of myocytes with the Fotonic Sensor™ for 10 min after vehicle or carbachol addition. We also measured the effects of pretreatment with 10^{-6} M atropine for 10 min, 3×10^{-4} M N^G-monomethyl-L-arginine acetate (L-NMMA) for 60 min, 10^{-5} M methylene blue for 10 min and 10^{-6} M 1*H*-[1,2,4]oxadiazolo[4,3-α]quanoxalin-1-one (ODQ) for 60 min on 10^{-4} M carbachol-induced chronotropy. Furthermore, we measured the changes of spontaneous beating rates after 10^{-4} M 8-Br cGMP stimulation.

2.3. Measurement of NO metabolite production

We did quantitative analysis of NO metabolite production in cultured myocytes by measuring the levels of nitrite and nitrate in a high-performance liquid chromatography (HPLC)-Griess reaction system (ENO-11, EICOM, Japan) according to the method of Yamada and Nabeshima (1997) and simultaneously measured the beating of myocytes. After collecting a 200 μl sample from SFD in the dish to measure the control NO_x level in SFD, we collected 200 μl samples at 5 min and 10 min after vehicle or carbachol addition. We also collected 200 μl samples from SFD in the dish at 5 min after 10^{-4} M carbachol stimulation following pretreatment of 10^{-6} M atropine for 10 min, 3×10^{-4} M L-NMMA for 60 min or 10^{-5} M methylene blue for 10 min. The NO_x production by 1.5×10^6 cells/dish in 5 min and 10 min was calculated from the change of the NO_x level, taking into account the NO_x in SFD used as a vehicle of drugs.

2.4. Western blot analysis of NO synthase isoforms

Gel electrophoresis and immunoblotting were performed using the procedures previously described (Miyamoto et al., 1992; Miyamoto et al., 1994). Culture dishes were rinsed twice with cold phosphate-buffered saline solution (136.9 mM NaCl, 2.7 mM KCl, 0.2 mM KH_2PO_4, 7.8 mM Na_2HPO_4) and 1 ml of ice-cold solution A (50 mM Tris–HCl, pH 7.4), containing 10 μg/ml soybean trypsin inhibitor, 5 μg/ml leupepsin, 200 μg/ml

bacitracin, 2 mM EDTA, and 100 μg/ml phenylmethylsulfonyl fluoride added to prevent proteolysis of protein. Myocytes were then scraped from culture dishes on ice with a rubber policeman. Collected myocytes were centrifuged at $1000 \times g$ for 15 min at 4°C. Sedimented myocytes were resuspended in solution A and homogenized by a Kinematica polytron (10 s at setting 6) on ice and stored at −80°C. On the day of the experiment, sample buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 0.025% bromophenol blue and 4% 2-mercaptoethanol was added to myocyte membrane suspensions and the mixture was boiled for 5 min. Aliquots (50 μg protein/lane) were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis with 4/20% polyacrylamide gel, and proteins were transferred electrophoretically (30 V, 90 min) to nitrocellulose membranes. Blots were washed with 0.01 M Tris buffer (pH 7.4) containing 0.9% NaCl, blocked by incubation (42°C) with 0.01 M Tris/0.9% NaCl buffer containing 3% bovine serum albumine. After subsequent incubations with the primary NO synthase monoclonal antibody, and anti-mouse IgG labeled with horseradish peroxidase, immunoreactivity was detected with the enhanced chemiluminescence (ECL) Western Blot Detection system followed by exposure to Hyperfilm-ECL. Immunolabeled NO synthase bands were identified by laser densitometry (PDSI, Molecular Dynamics, USA).

2.5. Measurement of protein

Protein was determined by the Coomassie Blue binding method (Bradford, 1976) with bovine serum albumine as a standard.

2.6. Materials

DMEM, collagenase and fetal bovine serum were obtained from Dainihon Seiyaku (Japan), Wako Chemical (Japan) and Flow Laboratories (UK), respectively. HEPES, carbachol, atropine sulphate, methylene blue and 8-Br-cGMP were obtained from Sigma (USA). ODQ was obtained from Tocris Cookson (USA). L-NMMA was obtained from RBI (USA). NO synthase monoclonal antibodies (neuronal NO synthase (nNOS), ecNOS and iNOS) were obtained from Transduction Laboratories (USA). All other reagents were either of reagent grade or molecular biology grade, and were purchase from commercial sources.

2.7. Statistical analysis

Mean ± S.E. was calculated and significant differences between mean values were estimated by means of one-factor analysis of variance. A P value smaller than 0.05 was considered to be significant.

3. Results

3.1. Changes in chronotropic responses to carbachol

Myocytes cultured for 6–7 days beat regularly at the rate of 139 ± 3 beats/min ($n = 217$). In the following experiments, the chronotropic effect of the drug was expressed as the percent of control under each set of conditions. Vehicle stimulation did not affect the beating rate and carbachol (10^{-6}–10^{-3} M) stimulation caused a negative chronotropic effect in a dose-dependent manner ($P < 0.05$) (Fig. 1). The pretreatment with 10^{-6} M atropine for 10 min completely abolished ($P < 0.05$) the negative chronotropic effect of 10^{-4} M carbachol at 5 min after stimulation (Fig. 2). Furthermore, to evaluate the implication of NO in the negative chronotropic effect caused by carbachol stimulation, we investigated whether L-NMMA or methylene blue affected it. The pretreatment with 3×10^{-4} M L-NMMA or 10^{-5} M methylene blue markedly attenuated ($P < 0.05$) the negative chronotropic effect of 10^{-4} M carbachol at 5 min after stimulation (Fig. 2). In this study, we used SFD as a vehicle of drugs. We found that 10^{-6} M atropine, 3×10^{-4} M L-NMMA, 10^{-5} M methylene blue and 10^{-6} M ODQ had no effect on the spontaneous beating rate of myocytes (data not shown).

3.2. Changes in NO_x production by carbachol

We investigated the change of NO metabolite production after carbachol stimulation in cultured myocytes. Because there was no change in nitrate level in our cell

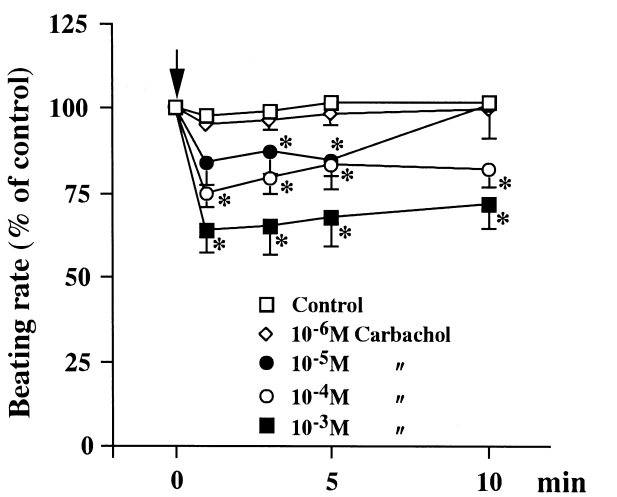

Fig. 1. The negative chronotropic effect induced by carbachol stimulation in cultured rat ventricular myocytes. The figure shows beating rates after vehicle stimulation ($n = 18$) and 10^{-3} M ($n = 14$), 10^{-4} M ($n = 17$), 10^{-5} M ($n = 13$) and 10^{-6} M ($n = 10$) carbachol stimulation. Data are expressed as percent of control (before stimulation) and show mean ± S.E. Carbachol stimulation decreased beating rate in a dose-dependent manner. Asterisks indicate $P < 0.05$ vs. vehicle.

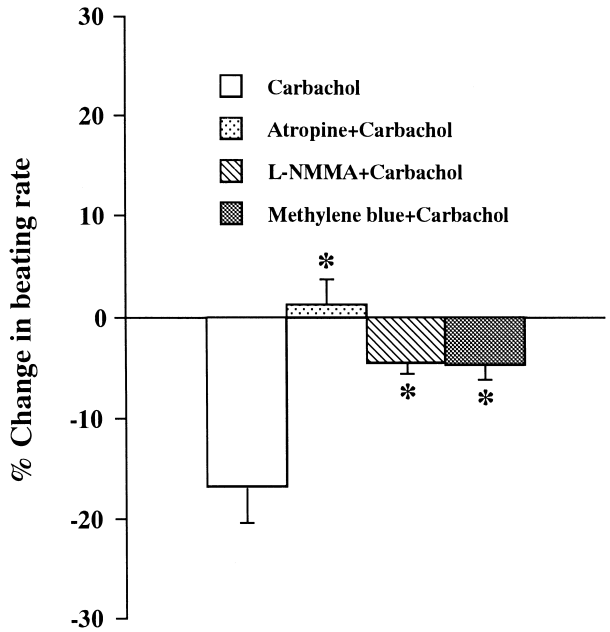

Fig. 2. Effects of atropine, L-NMMA and methylene blue on the negative chronotropic effect induced by carbachol stimulation in cultured rat ventricular myocytes. The figure shows the percent change in beating rate at 5 min after 10^{-4} M carbachol stimulation alone ($n=12$) and after 10^{-4} M carbachol with 10^{-6} M atropine ($n=11$), 3×10^{-4} M L-NMMA ($n=10$) or 10^{-5} M methylene blue ($n=10$). Data are expressed as percent of control (before stimulation) and show mean ± S.E. The percent changes in beating rate induced by 10^{-4} M carbachol were decreased by pretreatment with 10^{-6} M atropine for 10 min, 3×10^{-4} M L-NMMA for 60 min or 10^{-5} M methylene blue for 10 min. Asterisks indicate $P<0.05$ vs. carbachol alone.

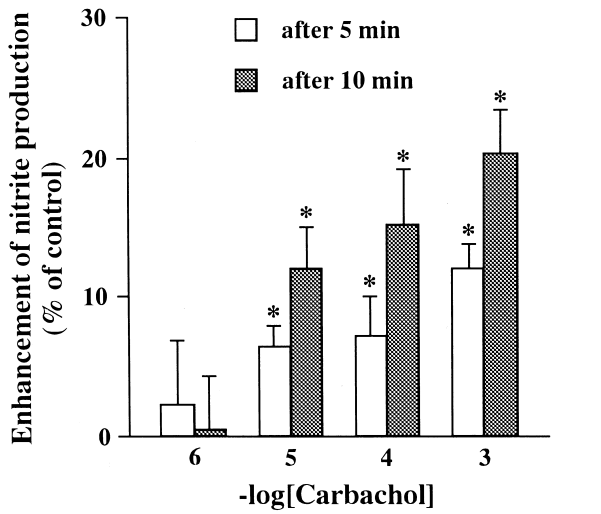

Fig. 3. The effect on nitrite production induced by carbachol stimulation on cultured rat ventricular myocytes. The figure shows the enhancement of nitrite production at 5 and 10 min induced by carbachol stimulation (10^{-6}–10^{-3} M, $n=6\sim12$). Enhancement was measured by subtracting the nitrite level produced spontaneously after vehicle stimulation (data not shown, $n=13$) from the nitrite level produced after carbachol stimulation. Data are expressed as percent of control (before stimulation) and show mean ± S.E. Enhancement of nitrite production after carbachol stimulation was increased in a dose-dependent manner. Asterisks indicate $P<0.05$ vs. vehicle.

preparations, we present only changes in nitrite production. Cultured myocytes spontaneously produced 307 ± 91 pmol/dish at 5 min and 593 ± 94 pmol/dish at 10 min above the control level after vehicle addition. In the following experiments, the nitrite production was expressed as a percent of control under each set of conditions. Cultured myocytes spontaneously produced nitrite (9 ± 3% at 5 min and 19 ± 4% at 10 min after vehicle addition) and carbachol (10^{-6}–10^{-3} M) stimulation caused enhancement of the nitrite production in a dose-dependent manner ($P<0.05$) (Fig. 3). We measured the enhancement of nitrite production by subtracting the level of nitrite produced spontaneously after vehicle addition from the level of nitrite produced after carbachol stimulation. Pretreatment with 10^{-6} M atropine for 10 min abolished the enhancement of nitrite production at 5 min after 10^{-4} M carbachol stimulation ($P<0.05$) (Fig. 4). Furthermore, we investigated the effects of L-NMMA and methylene blue on the enhancement of nitrite production induced by 10^{-4} M carbachol stimulation. Pretreatment with 3×10^{-4} M L-NMMA for 60 min and that with 10^{-5} M methylene blue for 10 min both decreased the enhancement of nitrite production at 5 min after 10^{-4} M carbachol stimulation (Fig. 4). However, 10^{-6} M atropine, 3×10^{-4} M L-NMMA and 10^{-5} M methylene blue had no effect on spontaneous nitrite production (data not shown).

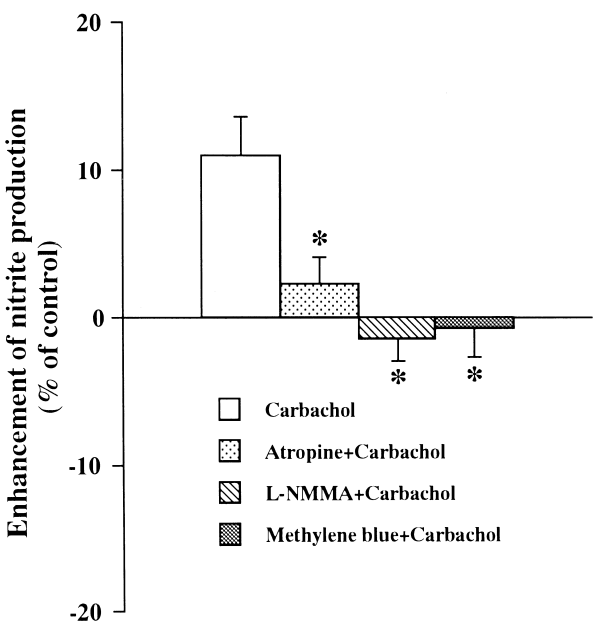

Fig. 4. Effects of atropine, L-NMMA and methylene blue on the nitrite production induced by carbachol stimulation in cultured rat ventricular myocytes. The figure shows the enhancement of nitrite production at 5 min after 10^{-4} M carbachol stimulation alone ($n=12$) and after 10^{-4} M carbachol with 10^{-6} M atropine ($n=11$), 3×10^{-4} M L-NMMA ($n=10$) or 10^{-5} M methylene blue ($n=10$). Data are expressed as percent of control (before stimulation) and show mean ± S.E. The enhancement of nitrite production induced by 10^{-4} M carbachol were abolished by pretreatment with 10^{-6} M atropine for 10 min, 3×10^{-4} M L-NMMA for 60 min or 10^{-5} M methylene blue for 10 min. Asterisks indicate $P<0.05$ vs. carbachol alone.

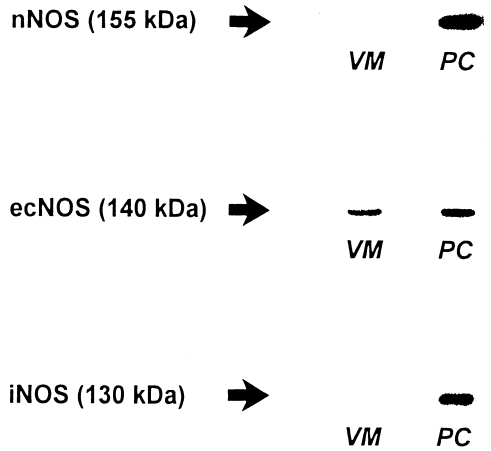

Fig. 5. Western blotting with monoclonal antibodies of NO synthase isoforms in cultured rat ventricular myocytes. We found that ecNOS was present but both nNOS and iNOS were absent in myocytes (VM); (PC = positive control).

3.3. Western blot analysis of NO synthase isoform

As shown in Fig. 5, we used nNOS (derived from rat pituitary), ecNOS (from Human Endothelial cells) and iNOS (from mouse macrophages) as positive controls in Western blotting experiments. We found that ecNOS was present but both nNOS and iNOS were absent in our cell preparations.

3.4. Evaluation of the involvement of cGMP in the carbachol-induced NO-signaling pathway

To evaluate the involvement of cGMP in the carbachol-induced NO-signaling pathway, we investigated the effect of ODQ, a potent and selective inhibitor of NO-stimulated guanylyl cyclase, on the negative chronotropic effect of carbachol stimulation. Pretreatment with 10^{-6} M ODQ for 60 min inhibited the negative chronotropic effect of 10^{-4} M carbachol at 5 min after stimulation ($P < 0.05$) (Fig. 6). This concentration of ODQ had no effect on the spontaneous beating rate of myocytes. Furthermore, we observed that 10^{-4} M 8-Br-cGMP caused a negative chronotropic effect in cultured rat ventricular myocytes ($P < 0.05$) (Fig. 7).

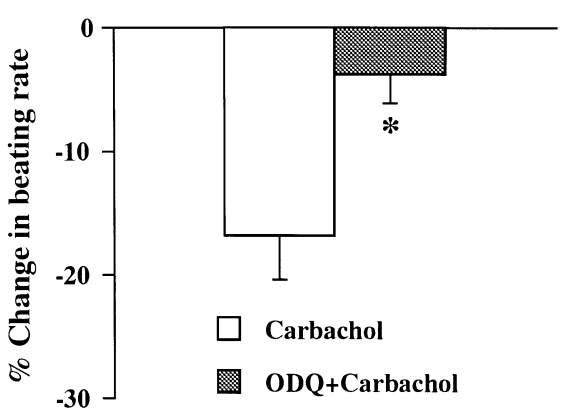

Fig. 6. The effect of ODQ on the negative chronotropic effect induced by carbachol stimulation in cultured rat ventricular myocytes. The figure shows the percent change in beating rate at 5 min after 10^{-4} M carbachol stimulation with ($n = 6$) or without ($n = 17$) pretreatment by 10^{-6} M ODQ for 60 min. Data are expressed as percent of control (before stimulation) and show mean ± S.E. The percent change in beating rate induced by 10^{-4} M carbachol stimulation was decreased by the pretreatment with 10^{-6} M ODQ. Asterisk indicates $P < 0.05$ vs. without ODQ.

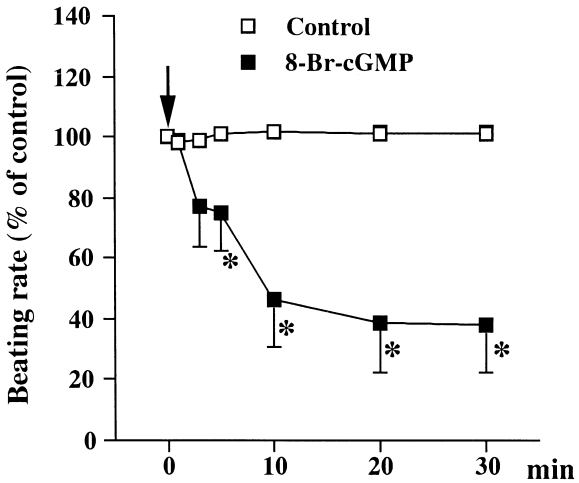

Fig. 7. The negative chronotropic effect of 8-Br-cGMP on cultured rat ventricular myocytes. The figure shows the beating rate after vehicle ($n = 18$) and 10^{-4} M 8-Br-cGMP stimulation ($n = 10$). Data are expressed as percent of control (before stimulation) and show mean ± S.E. 10^{-4} M 8-Br-cGMP stimulation decreased the beating rate. Asterisks indicate $P < 0.05$ vs. vehicle.

4. Discussion

There is accumulating evidence that rat cardiac myocytes express muscarinic acetylcholine receptors, which are responsible for the carbachol-induced chronotropic effect (Sterin-Borda et al., 1995; Sun et al., 1996). Han et al. (1994) investigated cholinergic modulation of heart rate in isolated rabbit sino-atrial node cells using the patch whole-cell voltage-clamp technique. They reported that carbachol inhibited $I_{Ca\ (L)}$ augmented by β-adrenoceptor stimulation, and this effect of carbachol was blocked by atropine, L-NMMA or N^G-nitro-L-arginine methylester. Using spontaneously beating cardiac myocytes from neonatal rat heart, Balligand et al. (1993b) showed that muscarinic cholinergic stimulation induced by carbachol caused a negative chronotropic effect, which was prevented by NO synthase inhibitors such as methylene blue and L-NMMA. These findings indicate that NO plays a physiological role in the autonomic regulation of myocar-

dial contractility mediated by muscarinic acetylcholine receptors in spontaneously beating myocytes. However, there has been no investigation that directly measured NO production coinciding with the negative chronotropic response induced by muscarinic acetylcholine receptor stimulation in cultured myocytes. In this study, we investigated the negative chronotropic effect (Fig. 1) and the enhancement of nitrite production (Fig. 3) induced by carbachol in a dose-dependent manner. We directly quantified the NO metabolite production by measuring the NO_x, i.e., nitrite and nitrate in an HPLC-Griess reaction system. There was no significant change in nitrate level induced by stimulation with drugs in our cell preparations. NO is generated by NO synthase from L-arginine and metabolized to nitrite and nitrate. There are few metabolic pathways of NO to nitrite and nitrate. It was reported that the principal spontaneous oxidation product of NO in aqueous solution in the absence of contaminating biological constituents such as hemoprotein is nitrite (Ignarro et al., 1993). No hemoprotein such as hemoglobin existed in our cell preparation, which could explain the different reactions of nitrate and nitrite.

The stimulation of muscarinic acetylcholine receptors by carbachol triggers many different signal transduction pathways. They include an increase in cGMP, a decrease in adenosine 3′:5′-cyclic monophosphate, regulation of ion channels and stimulation of phospholipase C (Liles et al., 1986; Lai and el-Fakahany, 1987; Candell et al., 1990; Entzeroth et al., 1990; Hosey, 1992). The activation of these pathways in the heart leads to inhibition of $I_{Ca\ (L)}$, inward current and myofilament Ca^{2+} sensitivity, and subsequent modulation of the myocardial contractile function (Giles and Shibata, 1981; Brown, 1982; Hartzell, 1988; Irisawa et al., 1993; DiFrancesco, 1993). In our study, the complete antagonism of the chronotropic effect by 10^{-6} M atropine indicated that the effect of carbachol was receptor-mediated even by the relatively high concentration of carbachol, 10^{-4} M, used in our experiment. In contrast, carbachol-induced negative chronotropy was inhibited 75% by L-NMMA and 71% by methylene blue. In the preliminary experiment, increases in the dose and pretreatment time did not reinforce this inhibition. Thus, NO-independent pathways of signal transduction were responsible for the incomplete antagonism by L-NMMA and methylene blue.

Methylene blue has been considered to inhibit the activation of soluble guanylyl cyclase by NO (Miki et al., 1977; Gruetter et al., 1979; Gruetter et al., 1981). Recently, some studies indicated that methylene blue was a potent inhibitor for endothelium-dependent relaxation rather than nitrovasodilator-induced vasodilation (Martin et al., 1985; Watanabe et al., 1988). Mayer et al. (1993) reported that methylene blue acts as a direct inhibitor of NO synthase and is a much less specific and potent of inhibitor of guanylyl cyclase. Therefore, we considered methylene blue to be a direct inhibitor of NO synthase. Brady et al. (1992) referred to the toxic effect of methylene blue on myocytes in their report.

They used 5×10^{-6} M methylene blue in their study and found that some myocardial cells showed signs of calcium overload with hypercontractility and fibrillation after 20 min superfusion with methylene blue. We observed that some myocytes pretreated with 10^{-4} M methylene blue for 10 min stopped their spontaneous beating (data not shown). The concentration of methylene blue used in our study was 10^{-5} M, which had no effect on the spontaneous beating rate of cultured myocytes.

There are at least three NO synthase isoforms in mammalian species, nNOS, ecNOS and iNOS. In this study, the existence of ecNOS was confirmed in cultured myocytes. This result is consistent with a previous study (Balligand et al., 1995), which concluded that NO-dependent parasympathetic signaling was mediated by ecNOS in rat ventricular myocytes. Nevertheless, there are various possible sources of NO production in the heart such as endothelial cells, vascular smooth muscle cells, neurons, myocytes and immune cells. Although, phosphotungstic acid hematoxylin dyeing showed that the ratio of myocytes to nonmyocardial cells was 85–90% in our preparation (Kawana et al., 1994), further study is necessary to clarify which cell is the predominant source of NO production in our system.

NO stimulates the soluble form of guanylyl cyclase and elicits an accumulation of cGMP in target cells. Balligand et al. (1993b) reported that 8-Br-cGMP, the cell-permeable analogue of cGMP, mimicked the negative chronotropic effect of carbachol. We also investigated the effect of ODQ on carbachol-induced negative chronotropy and the chronotropic effect of 8-Br-cGMP to determine the function of cGMP in our preparation. Garthwaite et al. (1995) reported that ODQ potently and selectively inhibits NO-sensitive guanylyl cyclase activity and does not affect NO synthase activity, so we used ODQ as a selective inhibitor of guanylyl cyclase. In our experiments, ODQ inhibited the negative chronotropic effect induced by carbachol, and 8-Br-cGMP caused a negative chronotropic effect.

These results demonstrated that cGMP was the second messenger of the NO-signaling pathway mediated by the muscarinic receptor in cultured rat ventricular myocytes.

In conclusion, we simultaneously investigated the negative chronotropic effect and directly measured the enhancement of NO metabolite production induced by carbachol stimulation in rat ventricular myocytes. These effects of carbachol were completely abolished by atropine. It has been reported that cultured neonatal rat cardiac myocytes possess both muscarinic acetylcholine receptor subtypes, i.e., M_2 and M_3 (Sun et al., 1996). The exact mechanisms of muscarinic receptor subtypes during carbachol stimulation remain to be clarified. In addition, we found that the negative chronotropic effect induced by carbachol was also attenuated by L-NMMA, methylene blue and ODQ, and that 8-Br-cGMP caused the negative chronotropic effect to

occur. These results suggest that the NO-signaling pathway may play an important role in the muscarinic cholinergic regulation of myocardial function.

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