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Control of cardiac muscle cell function by an endogenous nitric oxide signaling system

(guanylate cyclase/signal transduction/adrenergic receptors/cholinergic receptors/myocardial contractility)

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ABSTRACT **Nitric oxide (NO) synthesized from L-arginine is a ubiquitous intracellular chemical messenger and is involved in signal transduction in diverse mammalian cells, including vascular endothelium and neuronal tissues. The role of the NO-signaling pathway in the direct modulation of cardiac function is less well characterized. In this report, the effects of inhibitors of NO synthase (NOS) were examined in isolated neonatal and adult rat ventricular myocytes exposed to either muscarinic or adrenergic agonists. Carbachol (10 μM) caused a 91% inhibition of the spontaneous beating rate of cultured neonatal rat cardiac myocytes. N^{ω}-monomethyl-L-arginine, an L-arginine analog that inhibits NOS, and methylene blue, an inhibitor of NO, blocked the negative chronotropic effect of carbachol but had no effect on the basal beating rate of these cells. The inhibition by N^{ω}-monomethyl-L-arginine of the negative chronotropic effect of carbachol was reversed by adding excess L-arginine. The negative chronotropic effect of carbachol was also mimicked by analogs of cGMP, a second messenger implicated in mediating the action of NO in other cell types. Production of NO could be detected directly in carbachol-stimulated neonatal myocytes by using a reporter cell bioassay. The regulation of adrenergic responsiveness by the NO signaling system was also documented in studies of adult cardiac myocyte contractility. The NOS inhibitor N^{ω}-nitro-L-arginine significantly increased the inotropic effect of the β-adrenergic agonist isoproterenol on electrically stimulated adult rat ventricular myocytes, whereas this inhibitor had no effect on basal contractility. Inhibition of NO production by N^{ω}-monomethyl-L-arginine in these cells, as measured by reporter cell bioassay, was also reversible with excess L-arginine. Thus, the physiologic response of isolated neonatal and adult ventricular myocytes to both muscarinic cholinergic and β-adrenergic stimulation is mediated, at least in part, by products of an endogenous NOS.**

A major determinant of cardiac function is the activity of the autonomic nervous system. Although cAMP has been documented to mediate the positive inotropic response of the heart to β-adrenergic stimulation (1–3), the cyclic nucleotide-coupled signal-transduction mechanisms that mediate the action of muscarinic cholinergic agonists on heart rate (4) and on contractile function (5) are less clearly defined. Cholinergic agonists elevate intracellular levels of cGMP in heart muscle (6), and cGMP analogs produce a negative chronotropic effect in cultured rat heart cells (7) and can alter the contractile function of mammalian heart muscle (8, 9), raising the possibility that cGMP plays a role in the autonomic modulation of cardiac function. However, the mechanisms involved in the activation of guanylate cyclase in ventricular muscle cells are unknown.

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NO is now recognized to be present in many tissues, acting as both a paracrine autacoid and intracellular chemical messenger (for review, see ref. 10). In these tissues, the physiological effects of NO are mediated through activation of guanylate cyclase (11, 12) and, within the heart, guanylate cyclase is present within several cell types, including ventricular myocytes (13). Several isoforms of the enzyme NO synthase (NOS) responsible for the conversion of L-arginine to L-citrulline plus NO have been identified (10). To determine whether the L-arginine–NO pathway was constitutively present in neonatal and adult rat heart cell preparations and whether it played a role in the response of these preparations to autonomic stimuli, we studied the effects of NOS inhibitors on the contractile response of cardiac myocytes to β-adrenergic and cholinergic agonists.

METHODS

Isolation of Neonatal and Adult Ventricular Myocytes. Rat neonatal cardiac cells were isolated from 1- to 2-day-old newborn tissue by incubation with collagenase and trypsin, as described (14). All cell suspensions underwent two preplating steps to minimize contamination with nonmyocyte cells, lowered to 2–5% (15). Dissociated cells were placed in culture medium consisting of Dulbecco's modified essential medium, 7% fetal calf serum, and 0.1% penicillin–streptomycin. Cells were plated on glass coverslips and incubated at 37°C in 95% O_2/5% CO_2. The neonatal cardiac cells began to beat spontaneously after 2 days in culture. For the experiments using N^{ω}-monomethyl-L-arginine (*N*-MeArg), neonatal cells at day 3 in culture were incubated for 4 hr in L-arginine-depleted culture medium (MEM, Select Amine, GIBCO)/7% dialyzed fetal calf serum (GIBCO) containing either 1 mM *N*-MeArg or 1 mM L-arginine before the experiment. A third control group was incubated in L-arginine-depleted medium alone, for the same time.

Calcium-tolerant adult rat ventricular muscle cells were isolated by perfusion with a nominally calcium-free solution of collagenase and hyaluronidase, followed by dispersion in trypsin and deoxyribonuclease, as described (16). This technique included multiple washes and density-gradient sedimentation steps through a bovine serum albumin cushion, as well as differential cell attachment to laminin-coated coverslips, to minimize contamination with nonmyocyte cells [typically <1%, as described (16)]. Freshly isolated cells were plated on coverslips coated with collagen (Vitrogen, Palo Alto, CA) and kept in a nonserum-containing physio-

Abbreviations: *N*-NArg, N^{ω}-nitro-L-arginine; *N*-MeArg, N^{ω}-monomethyl-L-arginine; NOS, NO synthase.

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logical buffer (140 mM NaCl/4.0 mM KCl/0.5 mM $MgCl_2$/1.0 mM $CaCl_2$/10 mM Hepes, pH 7.4/12 mM glucose), with the addition of the following amino acid mixture (0.1 mM L-cysteine/0.22 mM L-histidine/0.4 mM L-leucine/0.07 mM L-lysine/0.1 mM L-methionine/0.2 mM L-phenylalanine/0.4 mM L-threonine/0.05 mM L-tryptophan/0.2 mM L-tyrosine/0.4 mM L-valine) for 2 hr before each experiment.

Measurement of Contractile Function. The frequency of spontaneous beating was measured in neonatal cardiac myocytes. In contrast to neonatal cells, myocytes freshly isolated from adult rat ventricles do not beat spontaneously, although their contractile function can be measured at baseline and in response to neurohumoral agents during electrical-field stimulation *in vitro*. The spontaneous beating rate of neonatal myocytes and the contractile amplitude of adult myocytes were determined on a temperature-controlled chamber on the stage of a microscope connected to a videomotion analyzer, as described (16), and superfused with the same buffer described above. Light–dark contrast at the cell edge provided the marker for measurement of the rate or amplitude of cell shortening. Cells were allowed to equilibrate for at least 10 min on the stage of the microscope before an experiment was begun.

cGMP Assay. This coincubation bioassay was done as described (17, 18). Briefly, a rat lung fibroblast cell line (RFL-6; American Type Culture Collection) or rat glomerular mesangial cells isolated and cultured from rat kidneys served as reporter cells for NO production by virtue of their NO-sensitive guanylate cyclase activity. Neonatal rat cardiac myocytes at day 3 in culture or adult rat cardiac myocytes that had been freshly isolated were plated on 6-well plates at 4.5×10^5 and 2×10^5 cells per well, respectively. The myocytes were gently washed with 2 ml of a physiological salt solution composed of 130 mM NaCl/5 mM KCl/10 mM D-glucose/1 mM $MgCl_2$/1.5 mM $CaCl_2$/25 mM Hepes, pH 7.4 with added L-arginine (5 mM) and/or *N*-MeArg (100 mM), where indicated. For the bioassay, glomerular mesangial cells grown on coverslips were directly coincubated for 1 hr with the donor cells (adult myocytes) (17), or an aliquot (800 μl) of neonatal myocyte-conditioned incubation buffer was directly added to RFL-6-containing wells, in the presence of superoxide dismutase (100 units/ml) and 3-isobutyl-1-methylxanthine (1 mM) (18). In both of these coincubation bioassays, measurement of reporter monolayer cell-associated cGMP levels allows the influence of NO released by a donor cell culture to be determined. The assay was terminated by adding either ice-cold 15% trichloroacetic acid (17) or 0.1 M hydrochloric acid (18). Glomerular mesangial cell- or RFL-6 cell-associated cGMP was determined by RIA, and results are expressed as pmol per well, as described (18). In the absence of coincubation with adult rat ventricular myocytes, the mean endogenous glomerular mesangial cell-associated cGMP was 0.10 ± 0.01 pmol per well, and this value was not affected by *N*-MeArg.

Materials. Carbachol, isoproterenol, N^{ω}-nitro-L-arginine (*N*-NArg), oxyhemoglobin, and methylene blue were purchased from Sigma. *N*-MeArg was purchased from Calbiochem. All other amino acids and reagents were purchased from Sigma.

Statistics. Data are expressed as means ± SEM. Comparisons were made with the unpaired two-tailed Student *t* test. The level of statistically significant difference was defined as $P < 0.05$.

RESULTS

Chronotropic Response of Neonatal Rat Myocytes to Carbachol: Effects of Methylene Blue and Oxyhemoglobin. Primary cultures of neonatal cardiac myocytes were studied 3 days after isolation and plating, at which time their spontaneous rate of contraction had stabilized. After at least 10 min of equilibration in superfusion buffer, the spontaneous beating rate was 37 ± 5 beats per min. Carbachol (10 μM) abruptly reduced the beating rate to 7 ± 2 beats per min ($n = 5$ from three different cultures; Fig. 1 *A* and *C*). The onset of the effect of carbachol was rapid, persisted through the duration of superfusion, and was completely reversible after drug washout. The cells were then reexposed to carbachol in the presence of methylene blue (10 μM), an agent that inhibits the activation of soluble guanylate cyclase by NO. Although the baseline spontaneous beating rate of these cells was unaffected by methylene blue (33 ± 5 beats per min; $n = 5$, in three separate cultures), coincubation of methylene blue with 10 μM carbachol abolished the negative chronotropic effect of the muscarinic agonist (28 ± 5 beats per min; $n = 5$, three cultures, Fig. 1*C*). After drug washout, repetitive applications of carbachol in control experiments caused similar marked declines in beating rate. Qualitatively similar results were also seen by superfusion with 1 μM oxyhemoglobin, an agent known to bind and inactivate NO, thus attenuating its physiologic and biochemical effects (Fig. 1*B*).

Effects of NOS Inhibitors on the Response of Neonatal Rat Cardiac Myocytes to Carbachol. The effects of graded increases in carbachol concentration (100 nM to 3 μM) were studied in rat neonatal cardiac cells exposed for 4 hr to three different conditions: L-arginine-depleted medium, L-arginine-depleted medium supplemented with L-arginine (1 mM), or L-arginine-depleted medium supplemented with *N*-MeArg (1 mM). Carbachol produced a concentration-dependent decrease in beating rate that was not different in L-arginine-depleted or L-arginine-containing medium (down to 8.8 ± 4.7% and 5.4 ± 3.5% of baseline beating rate with 3 μM carbachol, respectively; $n = 4$–12). As shown in Fig. 2, preincubation of the cells for up to 4 hr in L-arginine-depleted culture medium containing 1 mM *N*-MeArg markedly attenuated the ability of carbachol to reduce the beating rate of neonatal myocytes in primary culture ($P < 0.01$, $n = 4$–12, from three different cultures). Neither compound had any significant effect on baseline beating rate (data not shown). Addition of L-arginine (5 mM) to *N*-MeArg-containing superfusion media partially restored the negative chronotropic effect of carbachol in neonatal cardiocytes (down to 33.6 ± 8.27% of baseline with 3 μM carbachol; $P < 0.05$ compared with *N*-MeArg alone).

Action of cGMP Analogs and NO-Generating Drugs on the Spontaneous Beating Rate of Neonatal Rat Cardiac Myocytes. If carbachol were acting to generate NO and subsequently increase cGMP levels in neonatal rat myocytes, thereby decreasing their spontaneous beating rate, then cGMP analogs might mimic the effect of carbachol on these cells under the conditions used here. Superfusion of neonatal ventricular myocytes with 1 mM 8-bromo-cGMP markedly decreased myocyte beating rate, an effect completely reversible upon washout (Fig. 3). No change in the beating rate was seen in vehicle-treated cells studied under the same conditions. The effects of the vasodilator drugs sodium nitroprusside and molsidomine, which directly generate NO in solution (19), were also tested on the spontaneous beating rate of neonatal cardiocytes. Neither drug had any effect on the basal beating rate ($n = 3$). Sodium nitroprusside (10 μM) added in the superfusion buffer did not modify the chronotropic dose-dependent response to carbachol ($EC_{50} = 3.1 \pm 1.5 \times 10^{-7}$ M in control cells; $EC_{50} = 6.5 \pm 0.9 \times 10^{-7}$ M in the presence of sodium nitroprusside; $n = 3$; P not significant). Importantly, addition of 10 μM sodium nitroprusside did not restore the negative chronotropic response in cells preincubated with *N*-MeArg ($n = 3$).

Effect of Carbachol on the Production of NO by Neonatal Cardiac Myocytes. Neonatal rat cardiac myocytes produce detectable amounts of NO constitutively, as measured by the

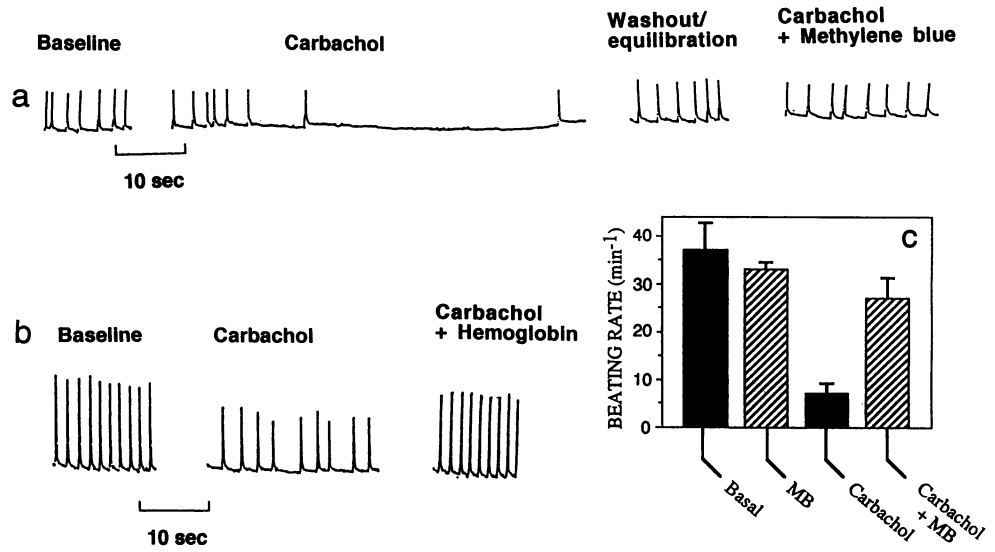

FIG. 1. Reversal by methylene blue and hemoglobin of the negative chronotropic action of carbachol in neonatal rat cardiac myocytes. (*a*) Representative tracings (of five cells from three preparations), illustrating the spontaneous rate of beating of neonatal rat cardiac myocytes at baseline after adding the cholinergic agonist carbachol and after adding nonspecific antagonists of NO action, methylene blue and hemoglobin. Cells were allowed to equilibrate for at least 10 min before determinations of basal beating rate and were then exposed to 10 μM carbachol, which markedly decreased beating rate. Myocytes were allowed to return to their baseline beating rate after carbachol washout before the cells were reexposed to carbachol and methylene blue (10 μM). Repetitive applications of carbachol, followed by washout, caused similar marked declines in beating rate. Methylene blue applied without carbachol had no effect on myocyte beating rate (see *c*, below). (*b*) In a protocol similar to that described for methylene blue, 10 μM hemoglobin also prevented any decline in beating rate with carbachol but had no effect on the basal rate of spontaneous beating. (*c*) Graphic representation of mean data from three different preparations (n = 5 cells for each condition), showing the basal beating rate and the beating rates with methylene blue (MB), carbachol alone, and carbachol with MB (mean ± SEM; $P < 0.001$ compared with carbachol alone; other differences are not significant).

RFL-6 reporter assay (Fig. 4). The cGMP content of the RFL-6 reporter cells incubated with the myocyte-conditioned buffer was significantly higher than the basal cGMP level of RFL-6 cells incubated with unconditioned buffer ($P < 0.05$). Furthermore, treatment of the myocytes with 10 μM carbachol for 3 min yielded a significant increase in cGMP levels in the reporter cell assay, compared with unstimulated cells ($P < 0.05$; n = 9 from four different cultures).

Effects of NOS Inhibitors on the Positive Inotropic Effect of Isoproterenol in Adult Ventricular Myocytes. We also examined whether an NO-mediated signaling system could regulate the contractile (inotropic) response of ventricular myocytes to the β-adrenergic agonist isoproterenol. After a 2-hr incubation in buffer containing either 0.6 mM L-arginine or 0.6 mM *N*-NArg, cells were allowed to equilibrate for 10 min, baseline contractile amplitude was recorded, and then 2 nM isoproterenol was added, a concentration determined to yield about a half-maximal increase in contractile amplitude. Contractility was recorded again when the increase in contractile amplitude had stabilized (≈5 min) (Fig. 5). *N*-NArg had no effect on baseline contractile function (n = 6 cells). With *N*-NArg, the inotropic response to isoproterenol significantly increased (130 ± 18% increase over baseline; n = 6 cells) compared with myocytes superfused with L-arginine-containing medium (77 ± 7% increase over baseline contractile amplitude; n = 8 cells; $P < 0.05$). Methylene blue, like *N*-NArg, significantly enhanced the positive inotropic effect of isoproterenol at a concentration that resulted in a half-

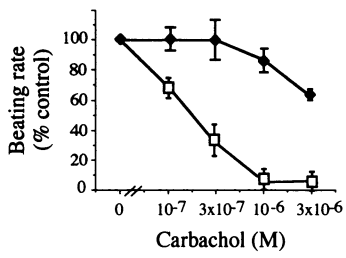

FIG. 2. Effect of *N*-MeArg on the negative chronotropic effects of carbachol in neonatal rat myocytes. After 3 days in primary culture, neonatal rat myocytes were preincubated for 4 hr in L-arginine-depleted culture medium containing either 1 mM *N*-MeArg (◆) or 1 mM L-arginine (□). Cultured cells (on coverslips) were then transferred to a superfusion chamber to determine beating rate and were superfused with physiological buffer with either L-arginine or *N*-MeArg added. After the initial equilibration period, cells were exposed to graded increases in carbachol (100 nM to 3 μM). In cells preincubated with *N*-MeArg, the concentration–effect curve of carbachol on beating rate was shifted to the right compared with cells preincubated with L-arginine ($P < 0.01$; n = 4–12). This experiment was repeated three times in three different cell preparations. A qualitatively similar effect was obtained with *N*-NArg (data not shown). Addition of 5 mM L-arginine to 1 mM *N*-MeArg resulted in a significant, although incomplete, restoration of the negative chronotropic effect of carbachol (n = 4).

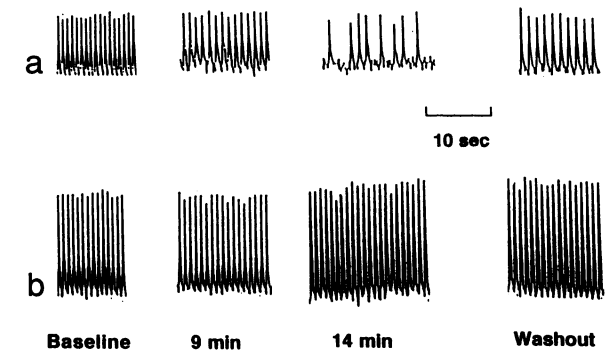

FIG. 3. Negative chronotropic effect of 8-bromo-cGMP in neonatal cardiocytes. Representative tracings are shown for beating rate of confluent neonatal rat myocytes during superfusion with physiological buffer. The spontaneous beating rate was allowed to equilibrate before adding 1 mM 8-bromo-cGMP. Tracings are shown at baseline, 9, and 14 min in an 8-bromo-cGMP-treated cell (*a*) and in a control cell (*b*) at the same time intervals. This experiment was repeated three times with similar results.

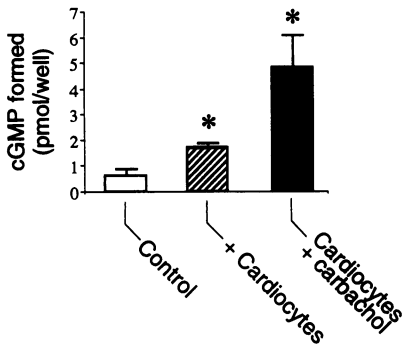

FIG. 4. NO production by carbachol-treated neonatal cardiocytes in the RFL-6 reporter cell bioassay. Conditioned buffer from neonatal rat cardiac myocytes cultured in 6-well plates for 3 days produced a significant increase in RFL-6-associated cGMP over baseline values with unconditioned medium (1.66 ± 0.18 compared with 0.62 ± 0.22 pmol per well, $n = 9$ from four different cultures, $P < 0.05$). After carbachol addition (10 μM) to the myocyte culture for 3 min, reporter cell-associated cGMP markedly increased (to 4.80 ± 1.27 pmol per well, $n = 9$ from four separate cultures, $P < 0.05$).

maximal contractile response. As with *N*-NArg, methylene blue did not have any effect on the baseline amplitude of contraction of single isolated adult rat ventricular myocytes (data not shown).

Effects of NOS Inhibitors on NO Production in Adult Cardiac Myocytes. Adult rat ventricular myocytes in culture produced detectable amounts of NO constitutively, as detected by measurement of intracellular cGMP levels in the reporter cell assay (0.30 ± 0.01 pmol per well; $n = 3$). cGMP levels were lower (0.10 ± 0.01 pmol per well) in reporter cells not incubated in the presence of myocytes. Basal rates of NO release were not further enhanced by adding 5 mM L-arginine to the myocyte incubation buffer (0.30 ± 0.01 pmol per well; $n = 3$). This constitutive rate of NO production by adult myocytes could be lowered significantly by 100 mM *N*-MeArg, resulting in a decrease in intracellular cGMP levels in reporter cells to 0.19 ± 0.01 pmol per well ($n = 3, P < 0.01$). This inhibition could be reversed by coincubation of the inhibitor with an excess of L-arginine (0.31 ± 0.01 pmol per well, $n = 3$; $P < 0.01$, compared with *N*-MeArg alone).

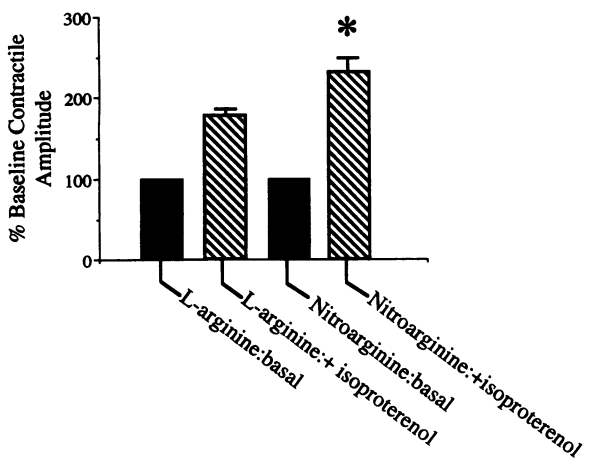

FIG. 5. Enhancement of positive inotropic action of isoproterenol by *N*-NArg in freshly isolated adult ventricular myocytes. Ventricular muscle cells from adult rat hearts were incubated for 2 hr in physiological buffer, with addition of the following amino acid mixture: 0.1 mM L-cysteine/0.22 mM L-histidine/0.4 mM L-leucine/0.07 mM L-lysine/0.1 mM L-methionine/0.2 mM L-phenylalanine/0.4 mM L-threonine/0.05 mM L-tryptophan/0.2 mM L-tyrosine/0.4 mM L-valine with either 0.6 mM L-arginine ($n = 6$) or 0.6 mM *N*-NArg ($n = 8$), and superfused with the same buffer throughout the experiment. Osmolality was maintained at 290 mOsm/l. Each cell was allowed to equilibrate for 10 min, baseline contractile amplitude was recorded, and then 2 nM isoproterenol was added, and contractility was recorded again when the increase in contractile amplitude had stabilized (≈5 min). Results are given as percentage of baseline contractile amplitude (mean ± SEM; *, $P < 0.05$ relative to isoproterenol augmentation of contractility in the presence of L-arginine; n = 6–8 cells from four different preparations).

DISCUSSION

These data indicate that cardiac myocytes respond functionally to products of an endogenous NOS. This conclusion is based upon several lines of evidence. The action of the cholinergic agonist carbachol on neonatal rat cardiac myocytes was inhibited both by nonspecific antagonists of NO (i.e., methylene blue and oxyhemoglobin) and by L-arginine analogs that inhibit NOS (i.e., *N*-NArg and *N*-MeArg). Additionally, the positive inotropic action of the β-adrenergic agonist isoproterenol on adult rat ventricular myocytes was significantly enhanced by *N*-NArg. Moreover, production of NO was demonstrated both from neonatal and freshly isolated adult rat ventricular myocytes, in which this production was also inhibited by *N*-MeArg.

The L-arginine analogs *N*-NArg and *N*-MeArg attenuate the production of NO in a variety of tissues by inhibiting the transformation of L-arginine to L-citrulline plus NO by members of the NOS protein family (20, 21). In a particular tissue, the potency of these inhibitors varies among the analogs, perhaps due to their relative affinities for different isoenzymes (22). Although the inhibition can be reversed with an excess of the natural substrate, L-arginine, in most tissues, some analogs, including *N*-MeArg, may irreversibly inactivate the enzyme (23). Thus, the incomplete restoration of the negative chronotropic effect of carbachol by L-arginine in the presence of *N*-MeArg may reflect the relatively greater affinity of the arginine analogs for NOS in neonatal cardiac cells, as well as possible irreversible inactivation of the enzyme. Less specific inhibitors of NO action, like methylene blue and oxyhemoglobin, produced qualitatively similar effects on the response to cholinergic and adrenergic agonists in neonatal and adult cardiac myocytes, consistent with the hypothesis that a NO signaling system is constitutively present in these cells and is involved in their response to autonomic stimulation. Although the inhibitory effect of hemoglobin might suggest that other nonmyocyte cells produce NO, acting in a paracrine fashion, other observations suggest that this compound acts as a "sink" for intracellular NO (24), thus arguing against this hypothesis. Although it seems plausible that cardiac muscle cells themselves are producing NO, this chemical signal may still originate from other cell types closely surrounding the myocytes in the ventricular wall. Future studies will be necessary to definitively assign the cellular source of the NO signal in this system.

In many vascular and nonvascular tissues, NO activates the soluble form of guanylate cyclase, with a resulting increase in cGMP (11, 12). Using a second independent approach, we also showed that neonatal and adult rat ventricular myocyte preparations constitutively produce NO by measuring reporter cell-associated cGMP, an observation also reported by others in adult myocytes (25). Addition of excess L-arginine did not increase cGMP production, suggesting that the availability of L-arginine is not rate-limiting in the synthesis of NO in this system, as has been shown with other preparations (26, 27). Although NO may not be the only mediator released by cardiac cells responsible for the increase in cGMP in reporter cells, *N*-MeArg significantly reduced cGMP levels, an effect that was also reversible by coincubation with L-arginine, indicating that at least part of the effect is NO-mediated. A constitutive NOS enzyme

activity is present in many tissues and cells, and the DNA sequences encoding different constitutive isoforms in rat brain and bovine or human endothelium have been described (28–30); the synthesis of biochemically distinct isoforms of the enzyme is induced in response to cytokines and inflammatory mediators (10), and the DNA sequence encoding the inducible isoform in a murine monocyte cell line has also been reported (31). These studies provide functional and biochemical evidence for generation of NOS products by an isoform of the enzyme that appears to be constitutively present in rat cardiocytes. Recent work has described the induction of NOS activity in rat vascular smooth muscle cells by interleukin 1 (24). Although induction of this isoenzyme of NOS could also occur in cardiac myocytes, it seems unlikely for several reasons that the NOS activity we observed in our contractility studies was cytokine-induced. (*i*) The NO-mediated response to carbachol was very rapid, as is characteristic of NO release by constitutive, Ca^{2+}/calmodulin-responsive isoforms of NOS. (*ii*) The studies on the adult ventricular myocyte contractility were done within 3–4 hr and were never done >6 hr after isolation, a time period probably insufficient for a significant induction of NOS.

Although cholinergic agonists can elevate intracellular levels of cGMP in heart muscle (6), other signal-transduction mechanisms have been well characterized as mediating at least part of the physiologic effect of muscarinic agonists. Our data document that the muscarinic agonist carbachol results in NO generation from cardiac myocytes. The role of cGMP in mediating or modifying the response to autonomic stimulation remains controversial (for review, see ref. 32). In our rat neonatal cardiac cell preparation, high concentrations of 8-bromo-cGMP mimicked the negative chronotropic action of carbachol, an observation also reported by others (7), suggesting that this action of carbachol is mediated, at least in part, by activation of guanylate cyclase, presumably a consequence of NO generation within these cells. On the other hand, two NO-generating drugs, sodium nitroprusside and molsidomine, failed to produce the same effect. This discordance between the actions of NO-generating drugs and the activation of an endogenous NO signaling system by a muscarinic agonist probably indicates that signal-transduction mechanisms other than activation of NOS are required for the negative chronotropic response to muscarinic agonists in these cells. Alternatively, this result could be due to the fact that products of an endogenous NOS may be distinct from NO as liberated by exogenous vasodilator drugs (33). Also, the efficiency of NO-generating drugs in activating soluble guanylate cyclase varies among different tissues, perhaps reflecting the capacity of these drugs to form biologically active *S*-nitrosothiols (34). If this explanation is correct, it would explain the well-known lack of effect of organic nitrates on heart function independent of direct effects on coronary arterial vasodilation.

The increase in the contractile response of adult rat ventricular myocytes to isoproterenol by NOS inhibitors suggests the existence of a countervailing, negatively inotropic, NO-mediated mechanism limiting the effect of β-adrenergic stimulation in adult myocytes. In this regard, the role of NO in cardiac muscle would be analogous to the control of the vascular tone by endothelium-derived NO (35, 36). In the adult myocardium, a rise in cGMP has been shown to attenuate calcium influx (37) and also to attenuate the positive inotropic effect of increases in cAMP after β-adrenergic stimulation (8). Future studies are needed to clarify whether these effects of cGMP mediate the negative inotropic action of NO on cardiac muscle.

In summary, these data document that the L-arginine–NO pathway is constitutively present in primary cultures of both adult and neonatal heart cells. Importantly, functional NO-mediated responses were detectable only in cells pretreated with a neurohumoral agonist, implying that the activity of the NO signaling system may be physiologically regulated in cardiac cells. Although NO synthesis may not account entirely for the physiological effects observed, this signaling pathway acts to modify myocardial responsiveness to both adrenergic and cholinergic stimuli. Inappropriate activation of this signaling system in disease states could result in regional or global depression of myocardial function.

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