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Nitric Oxide-dependent Parasympathetic Signaling Is Due to Activation of Constitutive Endothelial (Type III) Nitric Oxide Synthase in Cardiac Myocytes*

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Nitric oxide synthase (NOS) isoforms are discovered in an increasing variety of cell types with different roles in signaling. The inducible NOS (*i.e.* iNOS or NOS II) is expressed in cardiac myocytes in response to specific cytokines. Independent of iNOS induction, however, receptor-dependent signaling is modulated by a constitutive nitric oxide (NO) synthase isoform in these cells (Balligand, J. L., Kelly, R. A., Marsden, P. A., Smith, T. W., and Michel, T. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 347–351). We now show that cardiac myocytes constitutively express the endothelial isoform of NO synthase (ecNOS or NOS III). Transcripts for NOS III were detected by Northern blot in myocyte extracts using as a probe a polymerase chain reaction-generated cDNA amplified with isoform and species-specific primers. In subcellular fractionation experiments, a calcium-sensitive NO synthase activity was present primarily in the particulate fraction, coinciding with the distribution of NOS III analyzed by protein immunoblotting. The localization of NOS III within cardiac myocytes was further demonstrated by immunohistochemistry. The functional role of NOS III was explored by analyzing the effects of NOS inhibitors on single myocyte L-type calcium current and contractility. Inhibition of NOS blocked the attenuation by carbamylcholine of the increases in both parameters induced by β-adrenergic stimulation. We conclude that NO-dependent parasympathetic signaling is mediated by NOS III in cardiac myocytes.

Three NO[1] synthase isoforms have been identified to date, and although originally classified according to the tissue of origin, all three have been subsequently found in disparate cell types. The constitutive isoform first identified in brain (ncNOS) (1) has recently been found to be expressed in skeletal muscle cells (2, 3). The inducible isoform, or iNOS, was first characterized in a macrophage cell line (4, 5) and is now known to be expressed in a wide variety of cells after stimulation with inflammatory mediators (6), including cardiac myocytes (7–9). The constitutive isoform originally characterized in endothelial cells (ecNOS) (10) has now been identified in rat hippocampal pyramidal cells (11). A numerical nomenclature recently proposed by Nathan and Xie (6) identifies ncNOS, iNOS, and ecNOS as NOS I, NOS II, and NOS III, respectively, and will be used here.

Nitric oxide regulates cardiac muscle function in cardiac myocytes *in vitro* (12–15), in isolated cardiac muscle preparations (16), and in hearts examined *in situ* in experimental animals (17). Even in the absence of NOS II induction, inhibition of NO synthase modifies the contractile responsiveness of cardiac myocytes to autonomic nervous system agonists, implying the presence of a constitutively active NO synthase isoform in heart muscle (12, 17). However, the specific cell type(s) producing NO to alter cardiac myocyte function *in vivo* or *in vitro* has not been definitively identified, nor has the identity of the isoform of NOS constitutively expressed in cardiac myocytes previously been determined.

In this study, we characterize the constitutive isoform of NO synthase in cardiac myocytes as that originally identified in endothelial cells (10) (*i.e.* NOS III) and provide evidence for its role in mediating muscarinic cholinergic and β-adrenergic regulation of voltage-dependent calcium current and contraction in isolated ventricular myocytes.

EXPERIMENTAL PROCEDURES

Cell Culture—Purified adult rat ventricular myocytes were prepared as previously described (18), plated on laminin, and cultured for 24 h, unless specified otherwise.

Measurement of NO Synthase Activity—Myocyte NOS activity was quantified by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, as previously described (7).

Total cellular homogenates from approximately 2×10^6 myocytes were prepared by sonication in a buffer containing 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1 mM tetrahydrobiopterin, 1 mM leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, and 20 mM Tris-HCl (pH 7.4, 37 °C). After centrifugation of the homogenates (1,000 × *g*, 5 min, 4 °C) and determination of the protein content (Bradford assay), aliquots (approximately 80 μg) were added to 200 μl of buffer containing 50 mM Tris-HCl (pH 7.4), tetrahydrobiopterin (10 μM), dithiothreitol (1 mM), calmodulin (10 μg/ml), FAD (4 μM), FMN (4 μM), NADPH (1 mM), L-arginine (2 μM), purified L-[^{3}H]arginine (2×10^5 cpm, Amersham Corp.) and incubated for 60 min at 37 °C in the presence (1 mM $CaCl_2$) or absence of calcium (*i.e.* nominally calcium free, with 2 mM EGTA). Parallel reactions were analyzed in the presence of 1 mM L-*N*-monomethylarginine, an inhibitor of NO synthase. The reaction was stopped by addition of ice-cold sodium acetate (20 mM, pH 5.5) containing 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA, and the L-[^{3}H]citrulline content was determined by anion-exchange chromatography. The cal-

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[1] The abbreviations used are: NO, nitric oxide; NOS, nitric oxide synthase; bp, base pair(s); ecNOS, endothelial constitutive NO synthase; ncNOS, neuronal constitutive NO synthase; iNOS, inducible NO synthase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PCR, polymerase chain reaction; CCh, carbamylcholine; ISO, isoproterenol.

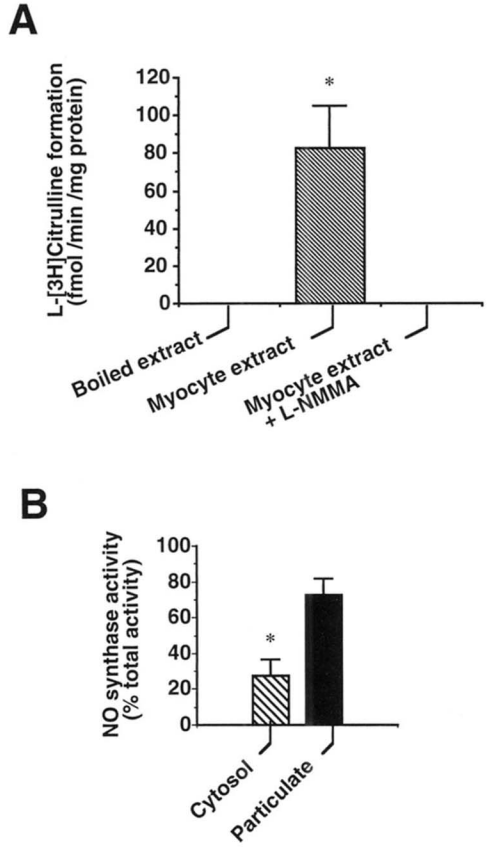

FIG. 1. **NO synthase activity and subcellular distribution in adult rat ventricular myocytes.** *A*, whole cell extracts from purified cardiac myocytes were assayed for the enzymatic conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in the presence and absence of calcium (see "Experimental Procedures"), and the calculated calcium-sensitive activity is compared with background levels obtained from boiled extracts (*left*) or extracts incubated in the presence of the NO synthase inhibitor L-*N*-monomethylarginine (L-NMMA) (*right*). *B*, the same extracts were fractionated by ultracentrifugation (see "Experimental Procedures"), and the activity, assayed as described above, is compared in the cytosolic and particulate fractions.

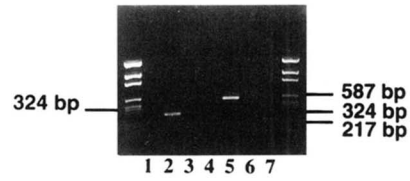

FIG. 2. **Identification of transcripts for NO synthase isoforms in adult rat ventricular myocytes by reverse transcription-PCR.** Poly(A)$^+$ RNA from purified adult rat ventricular myocytes or total RNA from rat brain was reverse transcribed with random hexamers and amplified by PCR with rat-specific oligonucleotide primers for NOS I (ncNOS), NOS II (iNOS), or NOS III (ecNOS) and analyzed by agarose gel electrophoresis and ethidium bromide staining. *lanes 1–4*, samples from freshly dissociated, untreated ventricular myocytes; no PCR product was obtained from the control sample in the absence of reverse transcription (*lane 1*); a 324-bp single band was amplified from cDNA with NOS III (ecNOS) primers (*lane 2*); with the same cDNA, NOS I (*lane 3*)- or NOS II (*lane 4*)-specific amplimers gave no product. *Lane 5*, cDNA from rat brain; NOS I primers amplified the expected 587-bp product (positive control). *lanes 6–7*, cDNA from purified myocytes treated for 18 h with recombinant human interleukin-1β (2 ng/ml) and recombinant mouse interferon γ (500 units/ml); a 324-bp band was obtained with NOS III (ecNOS) primers (*lane 6*) and a 217-bp band with the iNOS primers (*lane 7*, Ref. 7).

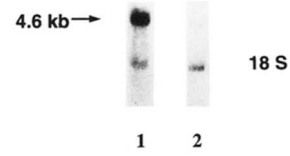

FIG. 3. **Identification of NOS III transcripts in adult rat ventricular myocytes by Northern analysis.** Total RNA from purified ventricular myocyte primary isolates was analyzed by Northern blot using the 324-bp NOS III (ecNOS) cDNA fragment obtained by PCR (see Fig. 2) as a probe. *Lane 1*, RNA from untreated ventricular myocytes. *lane 2*, RNA from ventricular myocytes treated for 24 h with recombinant human interleukin-1β (2 ng/ml) and recombinant mouse interferon γ (500 units/ml).

cium-sensitive activity was calculated by subtraction of the activities measured in the presence and absence of calcium after normalization for protein content from at least three separate experiments, each in duplicate.

For cell fractionation experiments, homogenates were prepared as described above. Aliquots were ultracentrifuged for 1 h at 100,000 $\times$ *g*. The supernatant (cytosolic fraction) was kept on ice for subsequent assay, and the pellet was resuspended in a buffer containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 2 mM β-mercaptoethanol, protease inhibitors (as above), 10% glycerol, and 1 M KCl. After a second ultracentrifugation (100,000 $\times$ *g*, 30 min), the second pellet was resuspended in the same buffer containing 20 mM CHAPS, but without KCl, and sonicated; aliquots were assayed as above. About 70% of the total activity was recovered in both fractions.

PCR Cloning and Sequencing of NOS III mRNA from Cardiac Myocytes—The reverse transcription reaction was primed with random hexamers and done as previously described (7). For PCR amplification (35 cycles), the following oligonucleotide primers were used: 5′-GGGCCAGGGTGATGAGCTCTG-3′ (sense) and 5′-CCCTCCTGGCTTCCAGTGTCC-3′ (antisense) for NOS III; 5′-GAGGCACCCCAACTCTGTG-3′ (sense) and 5′-TCCTGATTCCCGTTGGTGTGG-3′ (antisense) for NOS I; for NOS II, the primers were as described in Ref. 7. An annealing temperature of 60 °C was used.

The NOS III PCR product was purified by agarose gel electrophoresis, cloned into pBluescript (Stratagene), and used to transfect DH5a *Escherichia coli*. The nucleotide sequence was determined for cDNA inserts from three positive clones on both strands by the dideoxy chain termination technique using a Sequenase kit (U. S. Biochemical Corp.).

Northern and Western Blotting—Northern blots were performed as previously described (7). The 324-bp NOS III cDNA insert was ^{32}P radiolabeled using random hexanucleotide primers according to the manufacturer's protocol (Boehringer Mannheim). Blots were washed in 0.2 $\times$ SSC and 0.1% SDS up to 65 °C and then autoradiographed at −70 °C with intensifier screens for 12–14 h.

For Western blotting, aliquots of whole homogenates or subcellular fractions of ventricular myocytes were run on a 12% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. Equal transfer among lanes was verified by reversible staining with Ponceau red. Western analysis was performed as previously described (7) using a mouse monoclonal antibody raised against a polypeptide (residues 1030–1209) of the human NOS III protein (Transduction Laboratories).

Immunohistochemical Analysis—Cryostat sections of heart tissue or cytospin preparations of suspensions of freshly isolated myocytes and cultured myocytes that had been cultured in defined medium for 24 h were fixed in buffered 2% paraformaldehyde for 5 min, followed by 10 min in 100% methanol. After rinsing, immunostaining was performed by sequential application of primary antibody (mouse anti-human NOS III, 5 μg/ml (see above)), goat anti-mouse IgG (1/50, Steinberger Monoclonals, Inc.), mouse peroxidase-anti-peroxidase complex (1/100, Steinberger Monoclonals, Inc.), followed by labeling with the chromogen diaminobenzidine (Sigma) and H_2O_2. The slides were washed with water, counterstained with hematoxylin, dehydrated, and mounted for light microscopy. For negative controls, mouse myeloma IgG (Sigma) was used as the primary antibody, and the samples were processed identically.

Measurements of Calcium Current and Contraction—Adult rat ventricular myocytes were isolated as described above. All cells selected for electrophysiological study had a resting potential of −75 mV or more negative in buffer containing 5.4 mM $[K^+]_o$. Unloaded cell shortening was recorded with a video edge detector (12). The nystatin-perforated (19) patch-clamp technique in the whole cell configuration (20) was used to allow simultaneous recordings of single L-type Ca^{2+} current ($I_{Ca\text{-}L}$) along with cell shortening. The membrane-ruptured patch-clamp technique was used to internally dialyze the cell with methylene blue. Transient outward current (I_{TO}), which interferes with the measurement of $I_{Ca\text{-}L}$, was blocked by 4-aminopyridine (3 mM, in nystatin-perforated experiments) or by using Cs^+ to substitute for K^+ during internal dialysis.

RESULTS AND DISCUSSION

All three NOS isoforms characterized to date depend upon calmodulin activation, but NOS I and NOS III are distinguished from NOS II by their calcium sensitivity for the binding of activator calmodulin and enzymatic activity within the physiological range for intracellular calcium (6). A constitutive NO synthase activity was detected and quantified in whole extracts of freshly isolated adult rat ventricular myocytes, and this activity was dependent upon the presence of calcium (Fig. 1*A*). Duplicate extracts were assayed both in the presence of a maximally activating concentration of calcium (1 mM) and in a nominally calcium-free buffer containing EGTA (2 mM). This calcium-dependent NO synthase activity was about one-tenth of the calcium-independent NO synthase activity we measured in isolated adult ventricular myocytes after induction of NOS II activity with interleukin-1β and interferon γ (7).

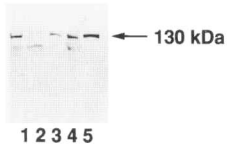

FIG. 4. **Detection of NOS III protein by Western analysis in extracts of adult rat ventricular myocytes.** Equal amounts of protein from whole extracts or subcellular fractions of purified myocytes were analyzed by Western blotting using a monoclonal antibody specific for NOS III (ecNOS) (see "Experimental Procedures"). The abundance of the specific 130-kDa protein is shown for *lanes 1–5*. *Lanes 1* and *4*, whole extract of ventricular myocytes; *lanes 2* and *3*, cytosolic and particulate fractions of ventricular myocytes, respectively; *lane 5*, human endothelial cell extract (positive control).

The NO synthase isoforms are also distinguished on the basis of their subcellular distribution. NOS III is found primarily in the particulate subcellular fraction of endothelial cells (21, 22), but in most tissues, NOS I and NOS II are cytosolic (6), although a recent report has described association of NOS I with skeletal muscle membranes (3). To examine the pattern of distribution of constitutive NOS activity in cardiac myocytes, whole cell extracts were resolved into cytosolic and particulate fractions by ultracentrifugation and assayed for NOS activity (Fig. 1*B*). Most of the calcium-sensitive NO synthase activity segregated with the particulate fraction. Therefore, both the calcium dependence and subcellular localization of the cardiac myocyte constitutive NOS activity suggested that the responsible NOS isoform was either NOS I or NOS III. We pursued molecular cloning and immunohistochemical analyses to definitively identify the isoforms expressed by these cells.

The three isoforms of NOS are encoded by different genes (1, 4, 5, 10). We have shown previously that NOS II is expressed after cytokine stimulation in purified primary isolates of adult ventricular myocytes but cannot be detected in untreated cells (7). We developed isoform- and species-specific oligonucleotide probes to amplify cDNA for the constitutive isoforms of NOS in cardiac myocytes by reverse transcription-PCR. Amplimers specific for the rat NOS I or NOS III were designed according to the previously published rat brain cDNA sequence (1) and a partial rat endothelial cDNA sequence deposited in GenBank

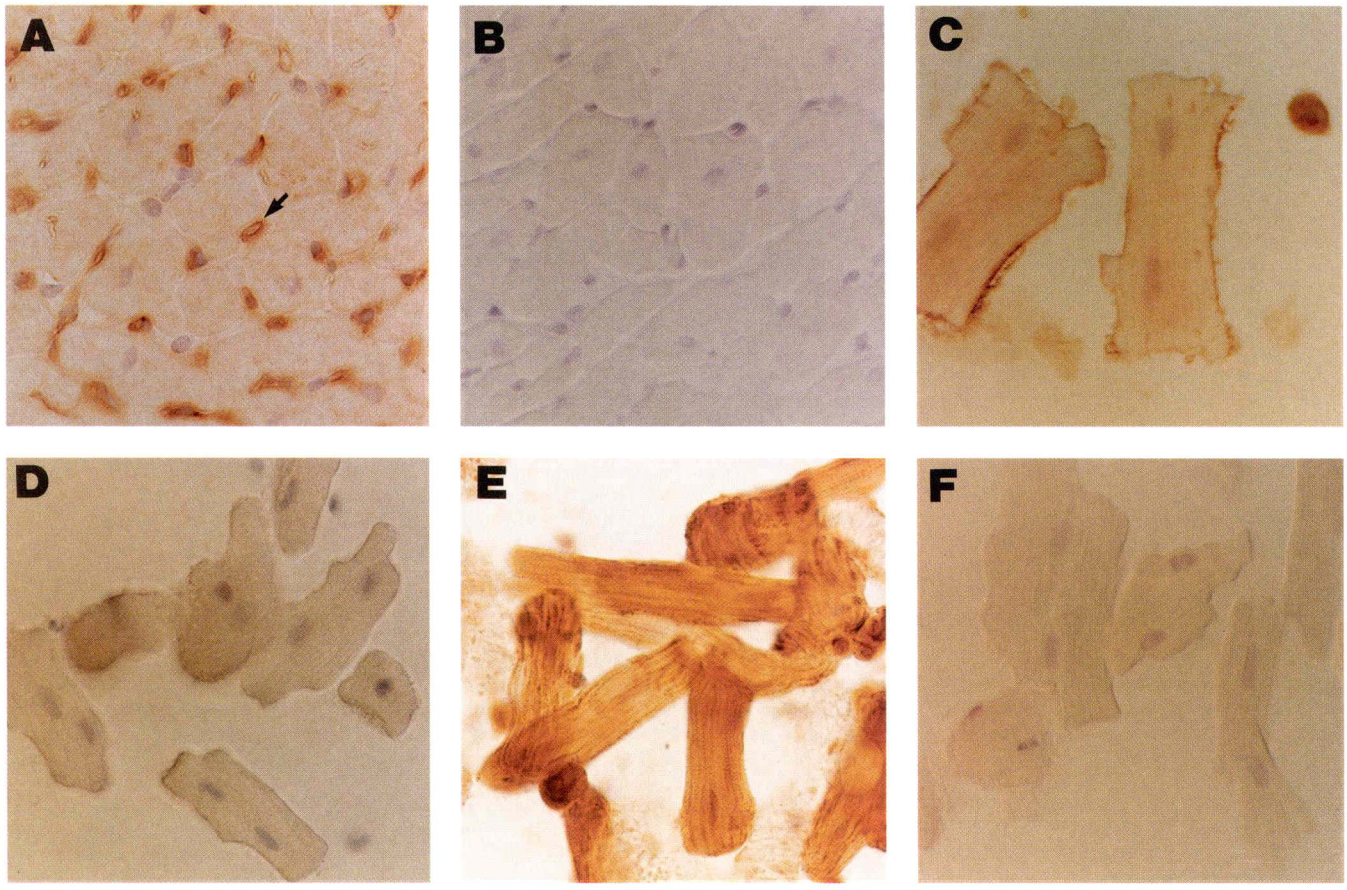

FIG. 5. **Immunohistochemical detection of NOS III protein in freshly isolated primary isolates of adult rat ventricular myocytes and sections of adult rat ventricular myocardium.** Immunostaining of sections of rat heart tissue (*A* and *B*) and freshly isolated (*C* and *D*) and cultured (*E* and *F*) rat ventricular myocytes. *A*, rat heart myocytes *in situ* show distinct immunostaining with anti-NOS III (ecNOS); note strongly positive adjacent capillaries (*arrow*). *C*, cytospin preparations of unpurified fresh rat heart cell suspensions show prominent immunostaining of both myocytes and endothelial cells. *E*, purified rat ventricular myocytes prepared, as previously described (18) and plated on laminin and cultured for 24 h, show prominent immunostaining with anti-NOS III (ecNOS). *B*, *D*, and *F*, no labeling is seen with control IgG either in rat heart tissue or in freshly isolated or cultured myocytes (respectively). Original magnification (200×), except for *C* (400×) is shown.

(accession no. RNU02534), respectively. The results of the amplification reactions using RNA from purified (typically 95–98% homogenous) ventricular myocytes are shown in Fig. 2. No PCR product was obtained from freshly isolated ventricular myocytes when amplified with primers specific for either rat brain (NOS I) or inducible NOS (NOS II). Products amplified with these primers were present in the appropriate positive controls, namely rat brain cDNA (for NOS I) and cDNA from rat cardiac myocytes treated with cytokines (for NOS II), as previously reported (7). Using amplimers specific for NOS III, a single 324-bp product was amplified from reverse-transcribed RNA from purified ventricular myocytes but not from negative controls consisting of samples processed in parallel containing either no cDNA or RNA samples processed in the absence of reverse transcriptase. Cloning and nucleotide sequence analysis of the PCR product (three independent clones, sequenced on both strands) revealed a sequence 84 and 91% identical to the originally described bovine endothelial NOS III isoform at the nucleotide and amino acid levels, respectively. Of note, the 324-bp-specific product was amplified from cytokine-treated cardiac myocytes in which a NOS II product was also identified, as previously reported (7).

To quantitate the abundance of the corresponding transcripts, the cloned NOS III cDNA was used as a probe in Northern analyses of total RNA from purified primary isolates of adult cardiac myocytes under high stringency hybridization conditions. A transcript of a size (4.6 kilobase pairs) consistent with that of NOS III was detected (Fig. 3). Notably, the abundance of the transcript for NOS III was markedly decreased after 24 h of treatment of adult myocytes with cytokines, similar to the effect described in other cell types (Refs. 10 and 23 and Fig. 3).

Using a monoclonal antibody directed against human NOS III, we detected a protein that was consistent in size (130 kDa) with NOS III in whole extracts of purified myocytes by Western analysis (Fig. 4). Quantitative comparison of the abundance of NOS III protein in subcellular fractions of cardiac myocytes revealed that most of the protein was present in the particulate fraction, consistent with the distribution of NOS enzymatic activity as described above.

The same antibody was used for the immunohistochemical localization of NOS III in single ventricular myocytes in culture and in sections of rat ventricular myocardium (Fig. 5). No staining was detectable above background when the primary antibody was omitted or nonspecific mouse myeloma IgG was used. With the NOS III-specific antibody, a strong signal was detected in endothelial cells from the microvasculature in myocardial sections. In addition, a distinct signal was observed in cardiac myocytes both in primary isolates and *in situ*.[2] No other cell type stained positively.

We have shown previously that NO antagonists and inhibitors of NO synthase attenuate the effect of muscarinic cholinergic agonists on the spontaneous beating rate of neonatal rat cardiocytes (12). The regulation by NOS III of both L-type calcium current and contractility in response to cholinergic and adrenergic agonists was further studied in single adult rat ventricular myocytes using NO antagonists and a NO synthase inhibitor (Fig. 6). Carbamylcholine, a muscarinic cholinergic agonist that is resistant to degradation, attenuated the isoproterenol-stimulated increase in $I_{Ca\text{-}L}$ and amplitude of unloaded cell shortening in paced ventricular myocytes (Fig. 6*A*). After extracellular perfusion (Fig. 6*B*) or internal dialysis (Fig. 6*C*) of the cell with methylene blue, the antagonistic effect of carbamylcholine on β-adrenergic stimulation of both $I_{Ca\text{-}L}$ and the amplitude of unloaded cell shortening was totally abolished. Internal dialysis with the NO synthase inhibitor L-*N*-monomethylarginine also completely abrogated carbamylcholine's effect on $I_{Ca\text{-}L}$ (Fig. 6*D*). A similar reversal of carbamylcholine's effect on contractility was also observed after NO blockade with

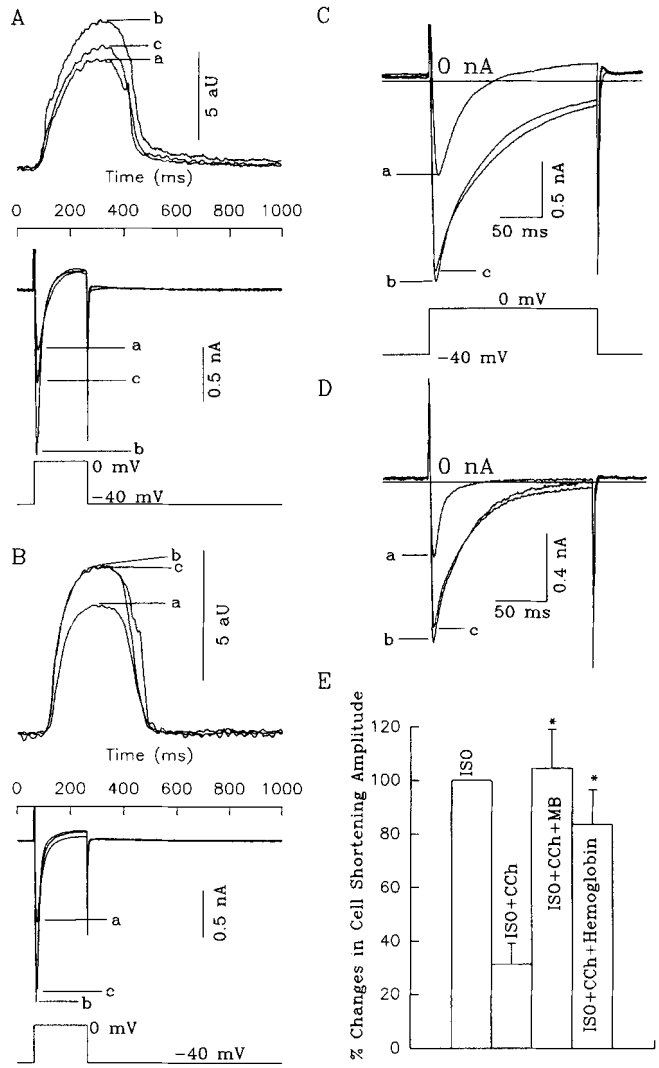

FIG. 6. **NO antagonists block the carbamylcholine-induced attenuation of L-type calcium current ($I_{Ca\text{-}L}$) and cell shortening in adult rat ventricular myocytes stimulated with isoproterenol.** Simultaneous recordings of $I_{Ca\text{-}L}$ and amplitude of shortening obtained in single rat ventricular myocytes are shown. *Panels A* and *B*, superimposed traces showing changes in cell shortening (in arbitrary units, *aU*, *top*) and $I_{Ca\text{-}L}$ (*bottom*) under control (*a*), isoproterenol alone (ISO, 1 μM) (*b*), and isoproterenol and carbamylcholine (CCh, 1 μM) (*c*). Results illustrated in *panels A* and *B* were obtained in the absence and presence of 10 μM methylene blue in the extracellular buffer, respectively. *Panel C*, superimposed current traces recorded under control (*a*), ISO (1 μM) (*b*), and CCh (1 μM) (*c*) in the presence of ISO in a cell internally dialyzed with methylene blue (20 μM). *Panel D*, the same experiment as in *C*, in a cell internally dialyzed with L-*N*-monomethylarginine (1 mM). *Panel E*, graphic representation of mean changes in cell shortening amplitude, relative to the maximum response to isoproterenol (1 μM, 100%), in the presence of ISO alone, isoproterenol with carbachol (1 μM, *ISO + CCh*), isoproterenol with carbachol in the presence of methylene blue (10 μM, *ISO + CCh + MB*), or hemoglobin (10 μM, *ISO + CCh + HB*). *, $p < 0.05$ compared with ISO + CCh. Note that ISO significantly increased $I_{Ca\text{-}L}$ and contraction and that both actions were attenuated by CCh (*panel A*, $n = 11$). Methylene blue and hemoglobin block the attenuating effects of CCh on force of contraction (*panels B* and *E*) and $I_{Ca\text{-}L}$ (*panels B* and *C*, $n = 4$ and 3 cells, respectively), as does L-*N*-monomethylarginine (*panel D*, $n = 7$ cells).

[2] Similar immunohistochemical detection of NOS III in cardiac myocytes was observed in sections of human heart ventricle (L. Kobzik and J.-L. Balligand, unpublished observations).

hemoglobin (Fig. 6*E*).

The regulation of voltage-dependent calcium channels by sympathetic agonists has been clearly demonstrated as a control mechanism of the rate and force of contraction in the mammalian ventricle (24, 25). The mechanism by which acetylcholine modulates the adrenergic effect on cardiac action potential differs according to the region of the heart, with attenuation of β-adrenergic agonist increases in $I_{Ca\text{-}L}$ being predominant in the mammalian ventricle (26, 27). In this study, we provide evidence that the cholinergic attenuation of β-adrenergic increases in $I_{Ca\text{-}L}$ in ventricular myocytes involves nitric oxide, as shown by the abolition of carbamylcholine's effect with methylene blue and hemoglobin, both blockers of NO, and L-*N*-monomethylarginine, a NO synthase inhibitor. That this has important functional consequences is shown by a quantitatively similar effect of methylene blue and hemoglobin on the cholinergic attenuation of the contraction of the same cells after isoproterenol stimulation.

Previous studies have demonstrated a calcium-sensitive NO synthase activity in human cardiac muscle (28) and in isolated cardiac myocyte preparations from different species (12, 29). In the present study, we identify NOS III (ecNOS) as the isoform that is constitutively expressed in adult ventricular myocytes at the transcript and protein levels, including immunohistochemical localization in single cells *in vitro* and *in vivo*. Endothelial cells from the heart microvasculature strongly expressed NOS III *in situ*, as detected by immunohistochemistry.[3] However, given the low percentage of non-myocytes (typically 2–5%, including endothelial cells) in the preparations of purified cardiac myocytes used in this study, it is unlikely that any substantial component of the NOS signal detected by Northern and Western analysis came from endothelial contaminants. The expression of type III NOS in cardiac myocytes themselves is clearly demonstrated by immunohistochemical detection in single cells.

Since its original cloning from endothelial cells, NOS III has also been identified in hippocampal pyramidal neurons (11). Among other cell types (for review, see Ref. 6), NOS I has also recently been localized in fast twitch skeletal muscle cells (3). We excluded the presence of NOS I in ventricular myocytes by Northern analysis and reverse transcription-PCR using species-specific probes. NOS II is expressed in response to several inflammatory stimuli in a variety of cell types, including neonatal and adult rat cardiac myocytes (7, 9). Together with our previous data (7), this study shows that ventricular myocytes can express both NOS II and III (Fig. 2) and that specific cytokines down-regulate the abundance of NOS III transcript in ventricular myocytes. Determination of whether this is mediated through changes in the half-life of the NOS III transcript, as previously shown in aortic endothelial cells (23), will await further experiments.

Overall, our data further support the concept of a more widespread tissue distribution and diverse signaling roles for NOS III beyond the paradigm originally described in the vascular wall.

[3] This is in contrast to cardiac microvascular endothelial cells maintained in serum-containing culture medium for 2 weeks, in which no detectable constitutive NOS activity can be measured (30).

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