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RESEARCH PAPER

Mechanisms involved in the regulation of mRNA for M_2 muscarinic acetylcholine receptors and endothelial and neuronal NO synthases in rat atria

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Background and purpose: Agonists of the M_2 muscarinic acetylcholine receptor (mAChR) increase mRNA for this receptor and mRNA for endothelial and neuronal isoforms of NO synthase (eNOS or nNOS). Here we examine the different signalling pathways involved in such events in rat cardiac atria.
Experimental approach: In isolated atria, the effects of carbachol on mRNA for M_2 receptors, eNOS and nNOS were measured along with changes in phosphoinositide (PI) turnover, translocation of protein kinase C (PKC), NOS activity and atrial contractility.
Key Results: Carbachol increased mRNA for M_2 receptors, activation of PI turnover, translocation of PKC and NOS activity and decreased atrial contractility. Inhibitors of phospholipase C (PLC), calcium/calmodulin (CaM), NOS and PKC prevented the carbachol-dependent increase in mRNA for M_2 receptors. These inhibitors also attenuated the carbachol induced increase in nNOS- and eNOS-mRNA levels. Inhibition of nNOS shifted the dose response curve of carbachol on contractility to the right, whereas inhibition of eNOS shifted it to the left.
Conclusions and implications: From our results, activation of M_2 receptors induced nNOS and eNOS expression and activation of NOS up-regulated M_2 receptor gene expression. The signalling pathways involved included stimulation of PI turnover via PLC activation, CaM and PKC. nNOS and eNOS mediated opposing effects on the negative inotropic effect in atria, induced by stimulation of M_2 receptors. These results may contribute to a better understanding of the effects and side effects of cholinomimetic treatment in patients with cardiac neuromyopathy.
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Keywords: M_2 mAChR; mRNA; nNOS–eNOS CaM; PKC; PLC; atrial contractility

Abbreviations: AF-DX 116, 11-((2-((diethylamino)methyl)-1-piperidinyl) acetyl)-5,11-dihydro-6*H*-pyrido (2,3-b)(1,4)benzodiazepin-6-one; CaM, calcium/calmodulin; eNOS, endothelial nitric oxide synthase; G_3PDH, glyceraldehyde-3-phosphate dehydrogenase; KRB, Krebs Ringer bicarbonate; L-NIO, N5-(1-iminoethyl)-L-ornithine dihydrochloride; L-NMMA, N^G-monomethyl-L-arginine; M_2 mAChR, M_2 muscarinic acetylcholine receptor; [^{3}H]MI, [myo-^{3}H]inositol; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NZ, *N*-propyl-L-arginine; PCR, polymerase chain reaction; PI, phosphoinositide; PKC, protein kinase C; PLC, phospholipase C; SNP, sodium nitroprusside; U-73122, 1-6-17β-3-methoxgestra-1,3,5 (10)-trien-17yl-aminohexyl-1-*H*-pyrrole-2,5-dione; WT, wild type

Introduction

A number of transcription factors that have the capacity to alter the rate of transcription of specific target genes in response to receptor-mediated signaling events at the cell membrane have been identified (Sheng and Greenberg, 1990). Many of these putative transcription factors are rapidly and transiently induced following receptor activation, and, therefore, can be classified as early genes.

A positive regulation of the mRNA for the M_1 muscarinic acetylcholine receptor (M_1 mAChR) in the early stages after treatment with carbachol has been described in the rat striatum (Chou *et al*., 1993) and in rat cerebral frontal cortex (Sterin-Borda *et al*., 2003). Within 1 h of M_1 receptor activation, the expression of immediate early genes is induced. These genes play important roles in coupling receptor stimulation to long-term neuronal responses

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(Albrecht *et al*., 2000). Three hours after M_1 receptor activation, a significantly higher number of genes, some expressing differential patterns, were observed. This other set of genes represents later effector or downstream target genes (von der Kammer *et al*., 1999). These target genes may be under the control of the immediate early genes. A feedback mechanism whereby upregulated acetylcholinesterase gene expression leads to an acceleration of acetylcholine breakdown at the cholinergic synapse, limiting cholinergic transmission has been demonstrated (von der Kammer *et al*., 2001).

Most studies conducted to identify early genes targeted by mAChR activation were performed in extracts of the CNS, which contain a high proportion of M_1 receptors. This raised the question whether other subtypes of mAChR, such as those expressed in cardiac tissue, also have the capacity to regulate the transcription of specific target genes in response to receptor stimulation.

The negative contractile effect in mammalian heart caused by the activation of mAChRs is mediated mainly by the M_2 subtype of receptors (Hosey, 1992; Sterin-Borda *et al*., 1995). Previously, we have determined the different signaling events involved in the M_2 mAChR-dependent inhibition of contractility in rat-isolated atria, which is associated with increased production of nitric oxide (NO). The mechanism appears to occur secondarily to stimulation of phosphoinositide (PI) turnover, via phospholipase C (PLC) activation. This in turn triggers a further cascade of reactions involving calcium/calmodulin (CaM) and protein kinase C (PKC), leading to activation of nitric oxide synthase (NOS) and soluble guanylate cyclase activity (Sterin-Borda *et al*., 1995).

Recent work has shown that NO is a powerful controller of gene expression, upregulating mRNAs encoding c-*fos* and microtubule-associated protein-2 and downregulating mRNAs encoding gonadotropin-releasing hormone and CAM-dependent protein kinase II α (Peunova and Enikolopov, 1993; Johnston and Morris, 1995; Belsham *et al*., 1996; Eigenthaler *et al*., 1998). It has also been reported that NO is able to alter dramatically the pattern of early gene expression in hippocampal granule cells (Morris, 1995). However, little is known about the reciprocal stimulation of immediate early gene expression between M_2 mAChRs and direct enzymic activation of NOS isoforms or an increase in their expression.

Therefore, the aim of these experiments was to determine whether the mAChR agonist carbachol acts as an early positive regulator of M_2 receptor, neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) gene expressions. Moreover, we were interested to see whether there is a reciprocity between the mRNAs for M_2 receptors and for isoforms of NOS and the common enzymic pathways involved. Our results showed that activation of M_2 receptors induced a complex pattern of gene expression of NOS isoforms that may be involved in the control of cardiac function as well as in the regulation of the mRNA for the M_2 receptor.

Methods

Animals

Male Wistar rats (obtained from the Pharmacology Unit, School of Dentistry, University of Buenos Aires, Argentina), and C57B1/6J wild type (WT) and homozygous for the $NOS3^{tm1Unc}$ targeted mutation (eNOS−/−) mice (3–5 months; Jackson Laboratories, Bar Harbor, ME, USA) were used. The animals housed in standard environmental conditions were fed a commercial pellet diet and water *ad libitum* and kept in automatically controlled lighting (lights on 0800–1900) and uniform temperature (25°C) conditions. Animals were cared for in accordance with the principles and guidelines of the NIH.

mRNA isolation and cDNA synthesis

Total RNA was extracted from rat atria by homogenization using the guanidinium isothiocyanate method (Chomozynski and Saachi, 1987). A 20 μl reaction mixture contained 2 ng of mRNA, 20 units of RNase inhibitor, 1 mM dNTPs and 50 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). First-strand cDNA was synthesized by incubating rat atria in Krebs Ringer bicarbonate (KRB) gassed with 5% CO_2 in O_2, (pH 7.4) at 37°C for 60 min. In a selected tube, the reverse transcriptase was omitted to control for amplification from contaminating cDNA or genomic DNA.

Quantitative PCR

Quantitation of NOS isoforms and M_2 mAChR-mRNA levels was performed by a method that involves simultaneous coamplification of both the target cDNA and a reference template (MIMIC) with a single set of primers (Sterin-Borda *et al*., 2003). MIMIC for nNOS, eNOS, M_2 mAChR and glyceraldehyde-3-phosphate dehydrogenase (G_3PDH) were constructed using a PCR MIMIC construction kit (Clontech Laboratories, Palo Alto, CA, USA). Each PCR MIMIC consists of a heterologous DNA fragment with 5′ and 3′-end sequences recognized by a pair of gene-specific primers. The sizes of PCR MIMIC were distinct from those of the native targets. The sequence of oligonucleotide primer pairs used for construction of MIMIC and amplification of mRNA for the NOS isoforms, M_2 mAChR and G_3PDH is listed in Table 1. Aliquots were taken from pooled first-strand cDNA from the same group and constituted one sample for PCR. A series of 10-fold dilutions of known concentrations of the MIMIC were added to PCR amplification reactions containing the first-strand cDNA. PCR MIMIC amplification was performed in 100 μl of a solution containing 1.5 mM $MgCl_2$, 0.4 μM primer, dNTPs, 2.5 U Taq DNA polymerase and 0.056 μM Taq Start antibody (Clontech Laboratories). After an initial denaturation at 94°C for 2 min, the cycle conditions were 30 s of denaturation at 94°C, 30 s of extension at 60°C and 45 s for enzymatic primer extension at 72°C for 45 cycles for NOS isoforms and 30 cycles for eNOS. For M_2 mAChR, each cycle consisted of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C for 30 cycles. The internal control was the mRNA of the housekeeping gene, G_3PDH. PCR amplification was performed with initial denaturation at 94°C for 2 min followed by 30 cycles of amplification. Each cycle consisted of 35 s at 94°C, 35 s at 58°C and 45 s at 72°C. Samples were incubated for an additional 8 min at 72°C before completion. PCR products were subjected to electrophoresis on ethidium

Table 1 Oligonucleotides of primers for PCR

Gene product	*Sense*	*Antisense*	*Predicted size (bp)*
nNOS	5′-GCGGA GCAGA GCGGC CTTAT-3′	5′-TTTGGT GGGAG GACCG AGGG-3′	240
eNOS	5′-CCGCA CTTCT GTGCC TTTGC TC-3′	5′-GCTCG GGTGG ATTTGC TGCTCT-3′	360
M_2 mAChR	5′-GATGA AAACAC AGTT TCCAC TTC-3′	5′-ATGAT GAAAGC CAACA GGATA GCC-3′	280
G_3PDH	5′-ACCAC AGTCCA TGCCAT CAC-3′	5′-TCCAC CACCC TGTTG CTGTA-3′	452

Abbreviations: eNOS, endothelial nitric oxide synthase; G_3PDH, glyceraldehyde-3-phosphate dehydrogenase; M_2 mAChR, M_2 muscarinic acetylcholine receptor; nNOS, neuronal nitric oxide synthase; PCR, polymerase chain reaction.
Neuronal (nNOS) and endothelial (eNOS) nitric oxide synthase (Qi *et al*., 2001); M_2 mAChR (Steel and Buckley, 1993); G_3PDH (Bishop-Bailey *et al*., 1997).

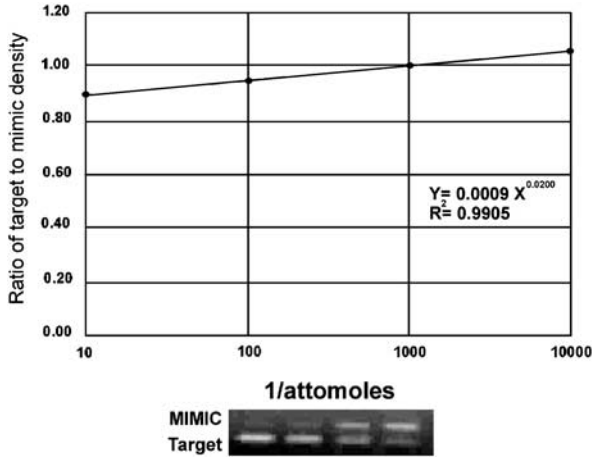

Figure 1 Analysis of target mRNA levels by competitive PCR. The upper panel shows a quantitative analysis of the competitive PCR experiment. Ordinate, ratio of the target to MIMIC products as determined by band densitometry. Abscissa, inverse amount of MIMIC added to the amplification. When the resultant target and MIMIC densitometric values are equal ($y=1$), extrapolation can be performed to yield the target amount.The lower panel shows an autoradiogram of neuronal nNOS in digoxigenin-labeled competitive PCR. The same applies for Figures 2, 5, 6 and 7.

bromide-stained gels. Band intensity was quantitated by densitometry using NIH Image software. Levels of mRNA were calculated from the point of equal density of the sample and MIMIC PCR products (Figure 1) (Sterin-Borda *et al*., 2003). NOS- and M_2 receptor mRNA levels were normalized against the levels of G_3PDH-mRNA present in each sample, which served as control for variations in RNA purification and cDNA synthesis. The relative mRNA expression of NOS and M_2 receptors in each group was compared with those from the respective normal group and reported as a percentage of normal.

Contractility in isolated atria

Rats and mice were killed by decapitation. The left atria were carefully dissected from the ventricles, attached to a glass holder and immersed in a tissue bath containing KRB solution gassed with 5% CO_2 in O_2 and maintained at pH 7.4 and 30°C. The composition of the KRB solution has been described previously (Pascual *et al*., 1986). A preload tension of 750 and 500 mg was applied to the rat and mice atria, respectively. Tissues were allowed to equilibrate for 1 h. The initial control values for contractile tension of the isolated atria were recorded by the use of a force transducer coupled to an ink-writing oscillograph. The preparations were paced with a bipolar electrode and an SK4 Grass Stimulator. The stimuli had a duration of 2 ms and the voltage was 10% above threshold. Inotropic effects (expressed as dF/dt) were assessed by recording the maximum rate of isometric force development during electrical stimulation at a fixed frequency of 150 and 250 beats min^{-1} to the rat and mice atria, respectively. Control values (=100%) refer to the dF/dt before the addition of drugs. The absolute value for dF/dt at the end of the equilibration period (60 min) was 7.8 ± 0.5 and 4.2 ± 0.4 g s^{-1} for rat and mice atria respectively. Cumulative dose–response curves were obtained according to the method of Van Rossum (1963). A maximal effect was achieved within 5 min after each dose was assayed. Basal values of dF/dt were not affected by the different antagonists or inhibitors at the concentrations used.

Measurement of total labeled PI

Rat-atria slices were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO_2 in O_2 with 1 mCi [myo-^{3}H]inositol ([^{3}H]MI) (special activiry, 15 Ci $mmol^{-1}$) from Dupont/New England Nuclear (Boston, MA, USA) and LiCl (10 mM) was added for determination of inositol monophosphate accumulation according to the technique described previously (Sterin-Borda *et al*., 1995). Carbachol and the blockers were added 60 min before the end of the incubation period. Water-soluble PI were extracted after 120-min incubation. Tissues were washed with KRB and homogenized in 0.3 ml of KRB with 10 mM LiCl and 2 ml chloroform/methanol (1:2, v/v) to stop the reaction. Then, chloroform (0.62 ml) and water (1 ml) were added. Samples were centrifuged at 3000 *g* for 10 min and the aqueous phase of the supernatant (1–2 ml) was applied to a 0.7-ml column of Bio-Rad AG (Formate form) 1×8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid that has been previously washed with 10 mM Tris-formic (pH 7.4). The resin was then washed with 20 volumes of 5 mM myo-inositol followed by 6 volumes of water and PI were eluted with 1 M ammonium formate in 0.1 M formic acid. One-milliliter fractions were recovered and radioactivity was determined by scintillation counting. Peak areas were determined by triangulation and results corresponding to the second peak and were expressed as area mg^{-1} following previous criteria (Sterin-Borda *et al*., 1995).

PKC activity assay

Atria were incubated alone or in the presence of each concentration of carbachol, stimulant plus blockers or blockers alone for a total incubation time of 60 min in KRB solution at 30°C and were frozen immediately in liquid nitrogen. PKC activity was purified from subcellular fractions as described previously (Wald *et al*., 1996); and was assayed on both cytosolic and membrane preparations from atria by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone H_1. Incubations were conducted for 30 min at 30°C in a final volume of 85 μl. In final concentrations, the assay mixture contained 25 μM ATP (0.4 μCi), 10 mM magnesium acetate, 5 mM β-mercaptoethanol, 50 μg of histone H_1, 20 mM HEPES, [pH 7.4] and unless otherwise indicated, 0.2 mM $CaCl_2$ and 10 μg ml^{-1} of phosphatidylserine vesicles. The incorporation of ^{32}P-phosphate into histone H_1 was linear for at least 30 min. The reaction was stopped by the addition of 2 ml of ice-cold 5% trichloroacetic acid and 10 mM H_3PO_4. The radioactivity retained on GF/C glass-fiber filters after filtration was determined by counting the filters in 2 ml of scintillation fluid. PKC activity was determined after subtracting the incorporation in the absence of Ca^{2+} and phospholipids. The data were expressed in picomoles of phosphate incorporated into the substrate per minute and per milligram of protein (pmol min^{-1} mg^{-1} protein). Another known PKC substrate peptide, myelin basic protein-4-14 from Life Technologies (Carlsbad, CA, USA), was also used for measuring PKC activity purified from subcellular cardiac fractions, following the instructions of the supplier. PKC specificity was confirmed by means of the PKC pseudosubstrate inhibitor peptide PKC (19–36) provided by Gibco (Wald *et al*., 1998). The data were expressed in picomoles of phosphate incorporated into the substrate per minute and per milligram of protein (pmol min^{-1} mg^{-1} protein).

Determination of NOS activity

NOS activity was measured in atria by production of [U-^{14}C]citrulline from [U-^{14}C]arginine according to the procedure described previously (Sterin-Borda *et al*., 1995). Briefly, after 20 min preincubation in KRB solution, atria were transferred to 500 μl of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of [U-^{14}C]arginine (0.5 μCi). Appropriate concentrations of drugs were added, and atria were incubated for 60 min under 5% CO_2 in O_2 at 37°C. Then tissues were homogenized with an Ultraturrax (IKA-Labortechnik, Staufen, Germany) in 1 ml of medium containing 20 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, 1 μM leupeptin and 0.2 mM phenylmethanesulfonyl fluoride at 4°C. After centrifugation at 20 000 *g* for 10 min at 4°C, supernatants were applied to 2 ml columns of Dowex AG 50 WX-8 (sodium form); [^{14}C]-citrulline was eluted with 3 ml of water and quantified by liquid scintillation counting. NOS partially purified using a 2′,5′-ADP-Sepharose column, as described previously (Sterin-Borda *et al*., 1995). Basal NOS activity in whole atria was inhibited by 95% by 0.5 mM N^G-monomethyl-L-arginine (L-NMMA). The results, expressed as picomoles per gram of tissue wet weight (pmol g^{-1} tissue) obtained for whole atria were expressed as the difference between values in the absence and in the presence of 0.5 mM L-NMMA.

Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P<0.05$.

Drugs

Carbachol, trifluoperazine, L-NMMA, calphostin C, sodium nitroprusside (SNP), isoprotererol and pirenzepine were purchased from Sigma Chemical Company (St Louis, MO, USA); 11-((2-((diethylamino)methyl)-1-piperidinyl)acetyl)-5,11-dihydro-6*H*-pyrido(2,3-b)(1,4)benzodiazepin-6-one (AF-DX 116), N5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO) and *N*-propyl-L-arginine (NZ) were purchased from Tocris Cookson Inc. (Baldwin, MO, USA); 1-6-17β-3-methoxgestra-1,3,5 (10)-trien-17yl-aminohexyl-1-*H*-pyrrole-2,5-dione (U-73122) from ICN Pharmaceuticals Inc. (Aurora, OH, USA). Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentration stated in the text.

Results

The products of reverse transcription-polymerase chain reaction (RT-PCR) using specific oligonucleotide primers for receptors showed single clear bands of the predicted size (Figure 2). Semi-quantitative RT-PCR analysis demonstrated that stimulation with carbachol for 2 h increased mRNA for M_2 receptors in a concentration-dependent manner, with a maximal response at 1×10^{-7} M (Figure 2a and b). Furthermore, a reduction in the carbachol-induced elevation of M_2 receptor mRNA levels was observed in the presence of AF-DX 116 (Figure 2a and c). Pirenzepine (1×10^{-7} M) and isoproterenol (from 10^{-9} to 10^{-6} M) had no effect (data not shown). This indicates that transcription of the gene for the M_2 receptor results specifically from the activation of M_2 receptors.

Carbachol stimulation resulted in activation of a number of enzymatic pathways commonly associated with receptor signaling. Figure 3 shows that stimulation of M_2 receptors triggered a pronounced increase of NOS activity (a), PI accumulation (b) and membrane PKC translocation (c). The degree of stimulation was directly proportional to agonist concentrations from 1×10^{-9} to 1×10^{-7} M, decreasing thereafter, to values still significantly higher than basal. All these effects of carbachol were inhibited by AF-DX 116 (Figure 3a–c). Figure 4a demonstrates that under identical experimental conditions, there was a significant correlation ($\alpha=0.05$) between carbachol-stimulated NOS activity and increase of PI accumulation and PKC translocation (Pearson *r*: PI, 0.9775; PKC, 0.9667). Figure 4b also shows a significant correlation ($\alpha=0.05$) between the increased mRNA for M_2

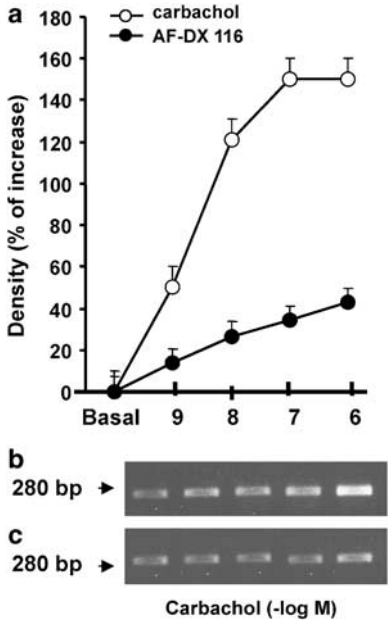

Figure 2 Effects of carbachol on semi-quantitative RT-PCR analysis of mRNA for M_2 receptors from rat atria incubated for 2 h with each concentration of carbachol. (**a**) Dose–response curve of carbachol in absence or in presence of 1×10^{-7} M AF-DX116. Basal values correspond to mRNA levels after 2-h incubation without carbachol. Values are mean ± s.e.m. of six experiments in each group. The RT-PCR products obtained from carbachol dose–response curve in absence (**b**) and in the presence (**c**) of AF-DX 116 are shown. $P < 0.001$ between carbachol and carbachol + AF-DX 116 at all concentrations used.

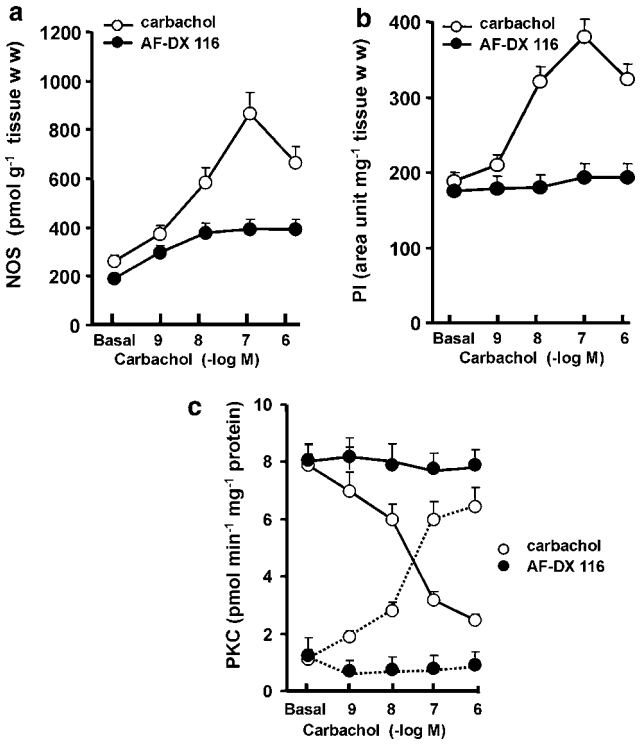

Figure 3 Concentration–response curve of carbachol-induced NOS activity (**a**) PI production (**b**) and membrane PKC translocation (**c**) in rat atria, with or without 1×10^{-7} M AF-DX 116. (**c**) PKC translocation from cytosol (full line) to membrane (dotted line) is shown. Basal values correspond to data before addition of carbachol. Values are mean ± s.e.m. of six experiments in each group.

receptors and NOS stimulation (Pearson *r*: 0.9850), both induced by carbachol.

To determine whether these enzyme activities were dependent on each other, atria were incubated with inhibitors of different enzymic pathways involved in mAChR activation. Table 2 shows that inhibition of PLC by U-73122 (5×10^{-6} M) (Smallridge *et al*, 1992) attenuated the stimulation of PI accumulation, membrane PKC translocation and NOS activity induced by 1×10^{-7} M carbachol. Trifluoperazine (5×10^{-6} M) and calphostin C (5×10^{-9} M) were used to inhibit CaM (Scharff and Foder, 1984) and PKC (Kobayashi *et al*., 1989), respectively. Trifluoperazine inhibited carbachol activation of NOS whereas calphostin C prevented the activation of NOS and PKC translocation from cytosol to membrane. As a control, L-NMMA (1×10^{-5} M) inhibited the carbachol-induced increase of NOS activity and L-arginine (1×10^{-4} M) reversed the L-NMMA effect (data not shown).

To find out if these enzymic pathways could be involved in the increased M_2 receptor gene expression induced by carbachol, atria were incubated with the inhibitory agents at the same concentrations as used above. Figure 5 shows that the inhibition of PLC, CaM, NOS and PKC decreased the effects of carbachol on M_2 receptor mRNA levels. Furthermore, SNP increased the levels of this mRNA. These results indicate that activation of M_2 receptors increased the mRNA for these receptors as a result of the stimulation of PI turnover, PKC translocation and NOS activation. Thus the endogenous NO signaling system participates in the early gene expression of M_2 receptors.

To define the role of different NOS isoforms in the regulation of M_2 receptors, isolated rat atria were incubated with selective inhibitors for the different NOS isoforms. As can be see in Figure 6a, eNOS inhibition by L-NIO (5×10^{-6} M) or nNOS inhibition by NZ (5×10^{-6} M) prevented the carbachol-induced stimulation of NOS activity. However, while the inhibition of eNOS was more effective at low agonist concentrations, whereas the inhibition of nNOS was significantly higher at the higher concentrations. The same profile was observed when specific oligonucleotide primers of eNOS and nNOS were used in RT-PCR amplification. Figure 6b shows that carbachol increased nNOS mRNA levels in a concentration-dependent manner. In the case of eNOS, its maximal action was observed at 1×10^{-9} M, decreasing thereafter.

Figure 7 shows that AF-DX 116 (5×10^{-7} M), U-73122 (5×10^{-6} M), trifluoperazine (5×10^{-6} M) and calphostin C (5×10^{-9} M) inhibited the stimulatory action of 1×10^{-7} M carbachol on eNOS- and nNOS-mRNA levels.

For further studies of the role of NOS isoforms in the effect of carbachol on contractility in atria, we used the selective NOS isoform inhibitors. Figure 8 shows that inhibition of nNOS by NZ (5×10^{-6} M) shifted to the right the dose–response curve of carbachol on atrial contractility (expressed as d*F*/d*t*). Contrasting sharply, the dose–response curve of

carbachol was shifted to the left by the eNOS inhibitor (L-NIO 5×10^{-6} M).

All the inhibitory agents at the concentrations used here did not have any effect upon basal values of mRNA for M_2 receptors, eNOS or nNOS (Table 3); nor on basal values of d*F*/d*t* (Sterin-Borda *et al*., 2006).

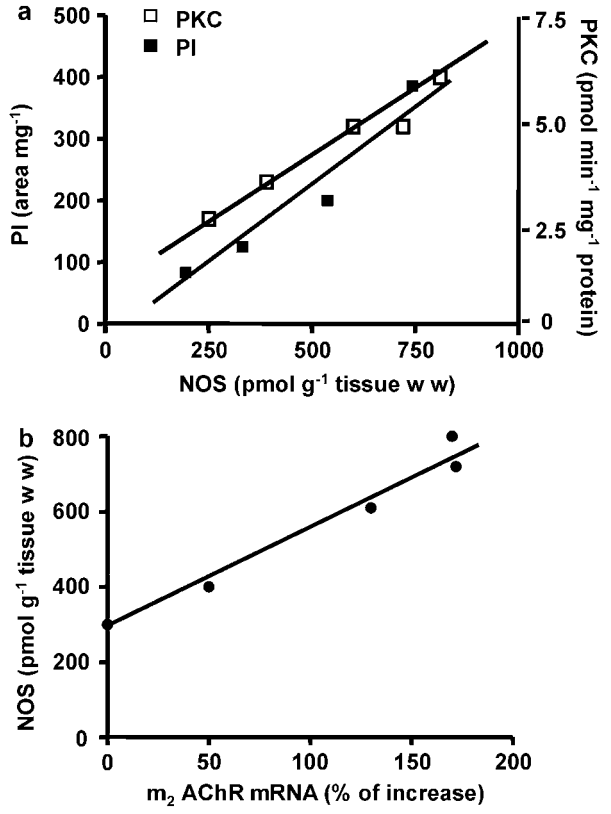

Figure 4 (**a**) Correlation between the stimulatory effects of carbachol (1×10^{-9} to 1×10^{-6} M) on NOS, membrane PKC activities and PI accumulation. NOS was plotted as a function of both PI ($r^2 = 0.9556$) and PKC ($r^2 = 0.9405$). Values are mean ± s.e.m. of five experiments on each group. (**b**) Correlation between the stimulatory effect of carbachol (1×10^{-9}–1×10^{-6} M) on M_2 receptor mRNA and NOS activity. M_2 receptor mRNA was plotted as a function of NOS activity ($r^2 = 0.9704$). Values are mean ± s.e.m. of six experiments in each group. For other details see legend of Figure 3.

To confirm the role of NOS isoforms in the regulation of atrial contractility by activation of M_2 receptors, genetically engineered mice deficient in eNOS (eNOS−/−) were used. Figure 9 shows that there was an almost 10-fold difference in the EC_{50} values of carbachol in atria from the eNOS−/− mice and in those from the corresponding WT strain. Thus, the EC_{50} was 1.34×10^{-7} M in WT mice, but in eNOS−/− mice, the EC_{50} was 1.86×10^{-8} M. The nNOS inhibitor, NZ (5×10^{-6} M) shifted the dose–response curve to the right in atria from both, WT mice (EC_{50} 6.8×10^{-7} M) and eNOS−/− mice (EC_{50} 1.68×10^{-6} M). Figure 9 also shows the effect of carbachol on NOS activity in atria of eNOS−/− and WT mice. Carbachol had a greater effect in WT atria than that in eNOS−/− atria. Further, NZ, but not L-NIO inhibited the stimulatory action of carbachol on NOS activity in atria from eNOS−/− mice, whereas both NZ and L-NIO had an inhibitory effect on NOS activity from WT atria.

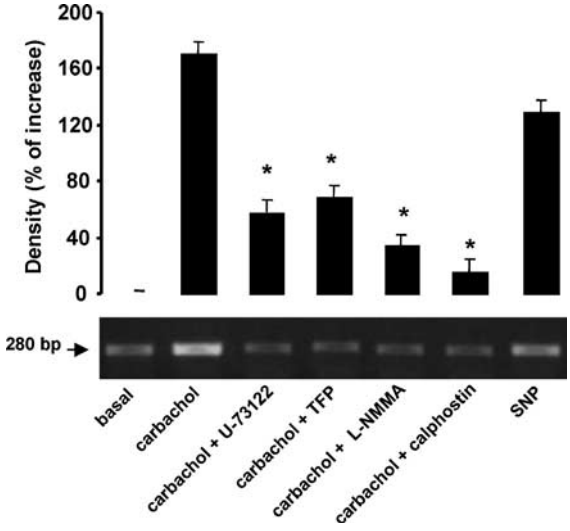

Figure 5 Carbachol-induced increase in mRNA for M_2 receptors in rat atria. Preparations were incubated for 2 h with 1×10^{-7} M carbachol in the absence or in the presence of 5×10^{-6} M U-73122 or 5×10^{-6} M trifluoperazine or 1×10^{-5} M L-NMMA or 5×10^{-9} M calphostin. The effect of 5×10^{-5} M SNP is also shown. Values are mean ± s.e.m. of six experiments in each group. *$P < 0.001$ versus carbachol alone. An example of the RT-PCR products obtained from this analysis is shown in the lower panel.

Table 2 Effect of carbachol upon enzymatic activities and PI turnover. Influence of enzymatic inhibitors

Additions	*Phosphoinositide (area/mg)*	*PKC (pmol/min/ mg protein)*	*NOS (pmol/g tissue wet wt)*
Basal	192 ± 12	1.2 ± 0.1	240 ± 18
Carbachol	383 ± 22*	5.8 ± 0.3*	848 ± 21*
Carbachol + U-73122	178 ± 15*	1.5 ± 0.1**	250 ± 15**
Carbachol + Trifluoperazine	356 ± 21	5.4 ± 0.2	485 ± 20**
Carbachol + Calphostin	422 ± 35	1.1 ± 0.1**	323 ± 20**
Carbachol + L-NMMA	356 ± 25	5.5 ± 0.2**	198 ± 15**

Abbreviations: L-NMMA, N^G-monomethyl-L-arginine; NOS, neuronal nitric oxide synthase; PKC, protein kinase C; PI, phosphoinositide; U-73122, 1-6-17β-3-methoxgestra-1,3,5 (10)-trien-17yl-aminohexyl-1-*H*-pyrrole-2,5-dione.

Values are mean ± s.e.m. of five experiments in each group performed in duplicate. Enzyme activities were measured after incubation rat atria after 2 h in the presence of carbachol with or without enzyme inhibitors. Carbachol was used at 1×10^{-7} M and U73122 5×10^{-6} M, TFP 5×10^{-6} M, calphostin 5×10^{-9} M and L-NMMA 5×10^{-5} M. *$P < 0.001$ comparing with basal values (no additions); **$P < 0.001$ comparing with carbachol alone.

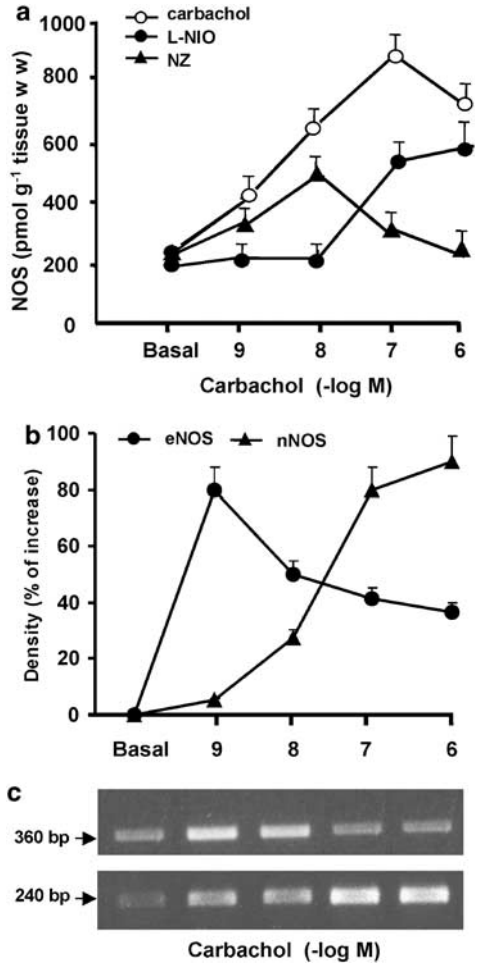

Figure 6 (**a**) Concentration–response curve of NOS activity induced by carbachol, in the absence or in the presence of 5×10^{-6} M L-NIO or 5×10^{-6} M NZ. Values are mean ± s.e.m. of six experiments in each group. (**b**) Carbachol effects on semi-quantitative RT-PCR analysis for nNOS and eNOS mRNA expression. Rat atria were incubated for 2 h in the absence (basal) or in the presence of different carbachol concentrations. Values are mean ± s.e.m. of seven experiments in each group. (**c**) RT-PCR products obtained from carbachol dose–response curves are illustrated for eNOS (360 bp) and nNOS (240 bp).

Discussion

It has become increasingly clear that agents classically thought to act as neurotransmitters can also alter gene expression. To understand the early events by which agonists at the mAChRs can affect gene expression, we have studied the transcription of several early genes that could be under the control of M_2 receptors. A positive feedback mechanism, where carbachol upregulated the mRNA for M_2 receptors, nNOS and eNOS was observed. Our present data indicated that the endogenous NO signaling system was involved in this positive feedback between activated M_2 receptors and the increase in mRNA for these M_2 receptors. The results also pointed to a role for PKC and Ca^{2+} mobilization in rapid activation of NOS and in the expression of early genes.

A positive regulation of mRNA for M_1 receptors soon after treatment with carbachol has been described in rat cerebral frontal cortex (Sterin-Borda *et al*., 2003). The present work shows that M_1 receptor subtype seems not to be involved in the induction of the mRNA by carbachol for M_2 receptors, as pirenzepine was not able to modify this effect. This is in agreement with the fact that mRNA for the M_1 receptors represents only 0.8% whereas the mRNA for the M_2 receptor represents 94% of total mAChR-mRNA in rat atria (Krejci and Tucek, 2002). Moreover, all the carbachol-mediated biological effects on adult rat atria are coupled to M_2 and not to M_1 receptors (Sterin-Borda *et al*., 1995; Borda *et al*., 1997).

The induction of immediate early genes could play an important role in coupling receptor stimulation to long-term responses (Albrecht *et al*., 2000). At the same time, long-term treatment of cardiac cell cultures with carbachol decreased the levels of mRNA for M_2 receptors (Mckinnon *et al*., 1997) as a result of the activation of a different set of genes that represents later effector or downstream target genes. These target genes may be under the control of the immediate-early genes (von der Kammer *et al*., 1999).

It has been suggested that events triggered within the first minute of agonist binding to mAChRs are sufficient for induction of gene expression (Trejo and Brown, 1991). However, different pathways for the regulation of mAChR responsiveness by short- and long-term agonist exposure have been described. The mobilization of intracellular Ca^{2+}, and activation of PKC were crucial in inducing immediate early gene expression, while inhibition of cAMP was associated with long-term responses (Trejo and Brown, 1991). It has been reported that NO also is able to alter dramatically the pattern of early gene expression (Morris, 1995). However, little is known about the reciprocal stimulation of immediate early gene expression between M_2 receptors and direct enzymatic activation of NOS isoforms or an increase in their expression.

In this paper, we propose a novel insight into the mechanism for the regulation of the positive feedback, where carbachol upregulates the mRNA for M_2 receptors, nNOS and eNOS. In rat-isolated atria, we observed that the activation of M_2 mAChR was able to promote nNOS and eNOS gene expression, and vice versa, that the gene for M_2 receptors was under the control of the endogenous NO signaling system. The mechanism by which carbachol increased mRNA for M_2 receptors in atria seemed to involve stimulation of PI hydrolysis by activation of PLC, which in turn, induced CaM, PKC and NOS activation. Two lines of evidence support this statement. On the one hand, we showed that agents known to interfere with these enzyme activities inhibited the stimulatory effect of carbachol on M_2 receptor mRNA levels. On the other, we found that, under identical experimental conditions, carbachol stimulated NOS activity, increased PI accumulation and PKC translocation, showing a significant correlation between activation of these enzymes by carbachol. Moreover, a correlation between the increase in mRNA for M_2 receptors and NOS activity was demonstrated. All these results indicate that activation of atrial M_2 receptors caused the increase in mRNA for M_2 receptors as a result of increased NOS activity. The fact that an NO donor (SNP) mimicked the carbachol effect provided more evidence that NO was the key player in the autoregulation of expression of the M_2 receptors.

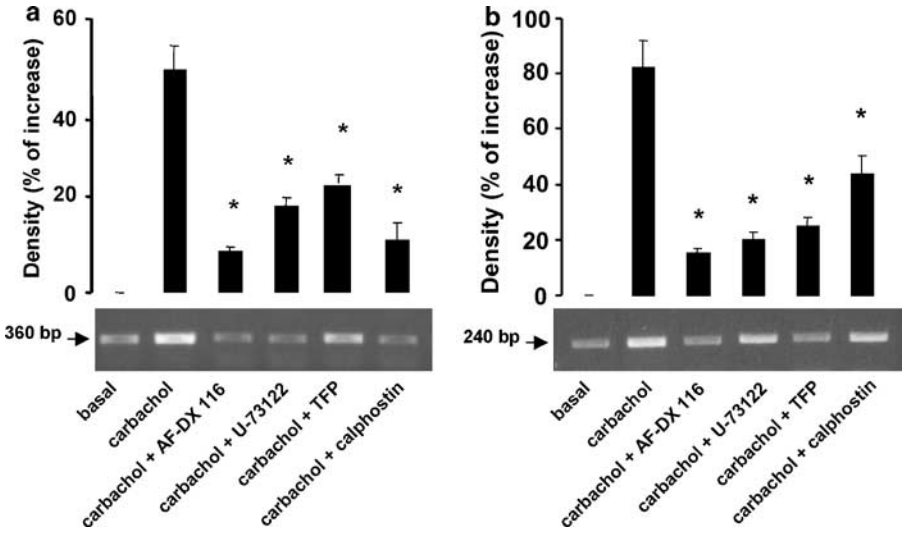

Figure 7 Effects of carbachol on semi-quantitative RT-PCR analysis for eNOS (**a**), nNOS (**b**) mRNA levels from rat atria incubated for 2 h with 1×10^{-7} M carbachol in the absence or in the presence of 1×10^{-7} M AF-DX116 or 5×10^{-6} M U-73122 or 5×10^{-6} M trifluoperazine or 5×10^{-9} M calphostin. Basal values correspond to mRNA levels after 2-h incubation without carbachol. Values are mean ± s.e.m. of six experiments in each group. The RT-PCR products obtained from carbachol alone or in presence of different blockers are shown in the lower panel. *$P < 0.001$ between carbachol and carbachol + inhibitors.

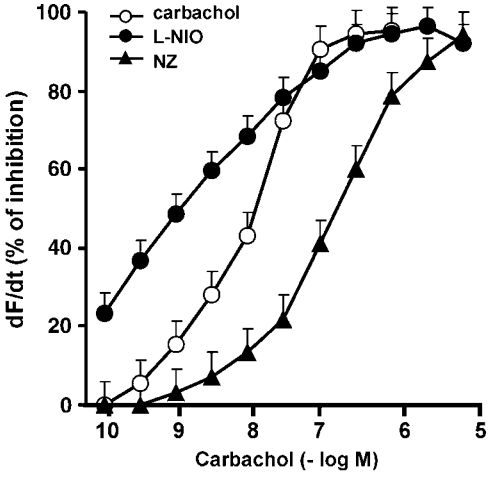

Figure 8 Carbachol-induced decrease of contractility (expressed as d*F*/d*t*) in rat-isolated atria. Preparations were incubated for 30 min with 5×10^{-6} M NZ, $1-10^{-6}$ M L-NIO or vehicle (shown as carbachol) the concentration–response curves of carbachol were plotted out. Values are mean ± s.e.m. of six experiments in each group.

Table 3 Influence of inhibitory agents upon basal mRNA levels of nNOS, eNOS, iNOS and M_2 mAChR

Inhibitors	*M_2 (%)*	*nNOS (%)*	*eNOS (%)*	N
None	100	100	100	12
AF-DX 116	98 ± 7	93 ± 8	93 ± 9	5
U-73122	110 ± 9	91 ± 7	108 ± 7	5
Trifluoperazine	95 ± 8	93 ± 9	109 ± 8	5
Calphostin	118 ± 7	101 ± 10	102 ± 7	5
L-NMMA	96 ± 8	104 ± 9	88 ± 6	5
L-NIO	97 ± 8	95 ± 6	102 ± 9	5
NZ	112 ± 9	97 ± 7	108 ± 7	5

Abbreviations: AF-DX 116, 11-((2-((diethylamino)methyl)-1-piperidinyl) acetyl)-5,11-dihydro-6*H*-pyrido (2,3-b)(1,4)benzodiazepin-6-one; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; L-NMMA, N^G-monomethyl-L-arginine; L-NIO, N5-(1-iminoethyl)-L-ornithine dihydrochloride; M_2 mAChR, M_2 muscarinic acetylcholine receptor; nNOS, neuronal nitric oxide synthase; U-73122, 1-6-17β-3-methoxgestra-1,3,5 (10)-trien-17yl-aminohexyl-1-*H*-pyrrole-2,5-dione; NZ, *N*-propyl-L-arginine.
Results are mean ± s.e.m. of *N* number of experiments and are expressed in percentage of variation from controls without drugs (none) taken as 100%.

All this raises the question whether other agonists, capable of raising intracellular Ca^{2+} concentration and NO, could similarly increase M_2 receptor expression. In this paper, we demonstrate that β-adrenergic stimulation did not modify M_2 receptor gene expression. Moreover, the stimulation of β-adrenoceptors selectively activated nNOS without affecting eNOS (Sterin-Borda *et al*., 2006). Thus, agonists at other receptors capable of raising intracellular Ca^{2+} and enhancing NOS gene expression and activation did not lead to increased mRNA for M_2 receptors.

The effect of the isoforms of NOS, induced at different agonist concentrations, appeared to be a pivotal factor in the regulation of gene expression. Our findings showed a positive correlation between agonist concentration and the mRNA and activity of nNOS. However, eNOS decreased with increasing concentrations of carbachol. Thus, a negative cross-talk between eNOS and nNOS was observed. A similar negative feedback regulation of eNOS activity and endothelial cell function by NO has been described (Buga *et al*., 1993; Schwatz *et al*., 1997).

The subcellular localization of NOS isoforms and the effector molecules is an important regulatory mechanism on their influence upon several biological parameters, including cardiac contraction (Fang *et al*., 2000, Massion *et al*., 2003). Stimulation of eNOS activity has been identified as a key inducer of plasmalemma caveolae budding and the internalization of the mAChR–eNOS complex, which terminates mAChR–eNOS coupling in cardiac myocytes (Dessy *et al*., 2000). The M_2 mAChR is translocated in calveolae after

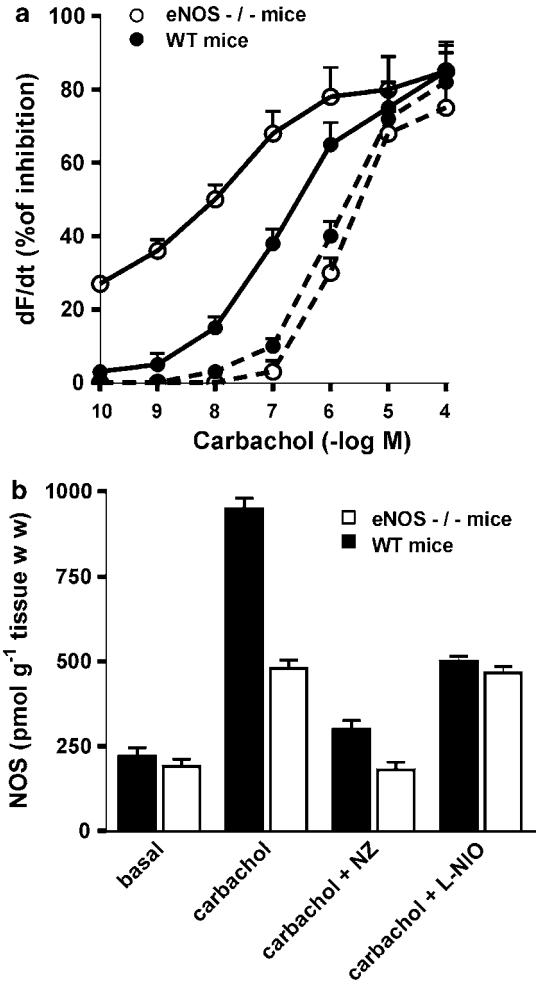

Figure 9 Effects of carbachol on atrial contractility (d*F*/d*t*) (**a**) and NOS activity (**b**) from WT mice or eNOS−/− mice, in absence (full line) or in presence of 5×10^{-6} M NZ (dotted line). Contractility (d*F*/d*t*) is expressed in % of basal values (before addition of the first concentration of the agonists) taken as 100%. Actual basal values were: 4.2±0.4, WT mice ($n=5$); and 3.9±0.4, eNOS−/− mice ($n=5$); in the presence of NZ, 4.6±0.5 WT mice ($n=5$) and 4.2±0.5 eNOS−/− mice ($n=5$). In (**b**), the NOS activity triggered by 1×10^{-7} M carbachol alone or in the presence of 5×10^{-6} M NZ and 5×10^{-6} M L-NIO in atria from WT mice or eNOS−/− mice is also shown. Data shown are the means±s.e.m. of five experiments in each group.

agonist stimulation (Feron *et al*., 1997). In cardiac myocytes transfected with acylation-deficient eNOS, which does not translocate to caveolae (Feron *et al*., 1998), carbachol was unable to inhibit the spontaneous beating rate of the mycocytes showing that caveolar location of eNOS was essential for the NO-dependent effect of the M_2 receptor agonist. Thus, the interaction between eNOS and caveolin may not only restrain basal NO production, but may also concentrate the enzyme in discrete locations, both promoting activation by agonist and the subsequent termination of signaling (Feron and Kelly, 2001).

On the basis of subcellular location of both NOS isoforms, our hypothesis is that the divergent effects on contractility in response to M_2 receptor activation observed in this paper could be because of the altered myocyte Ca^{2+} cycling (Petroff *et al*., 2001). Our striking current finding is that intracrine NO production, depending on which NOS isoform is its source, has opposite effects on the contractile response to M_2 agonists. Thus, inhibition of nNOS activity shifted the dose–response curve for carbachol to the right, whereas inhibition of eNOS activity enhanced the effect of low concentrations of carbachol. These results were corroborated by those from eNOS−/− mice, where an enhanced contractile response to carbachol was demonstrated. Moreover, inhibition of nNOS resulted in an inverse inotropic effect. In the heart, NO inhibits the L-type of Ca^{2+} channels (Mery *et al*., 1993), but stimulates sarcoplasmic reticulum Ca^{2+} release (Petroff *et al*., 2001), leading to variable effects on myocardial contractility. Our results suggest that nNOS may act as a negative cardiac regulator, whereas eNOS acts as a positive cardiac regulator. Accordingly, in rat atria, nNOS acts in a countervailing mechanism limiting the positive chronotropic and inotropic effect of β-adrenoceptor agonists (Sterin-Borda *et al*., 2006), because of the inhibition of Ca^{2+} influx (Vandecasteele *et al*., 1999; Sears *et al*., 2003). Although this study supports the view that eNOS attenuates mAChR-mediated control of the heart, this is challenged by opposite results (Han *et al*., 1996; Feron *et al*., 1998). These conflicting results might be the consequence of various factors, such as differences in mammalian species and the anatomical region within the myocardium itself (Massion *et al*., 2003). Thus, both eNOS and nNOS enzymes are constitutively expressed in rat atria, but in rat ventricle, the nNOS does not appear to be expressed constitutively (Sterin-Borda *et al*., 2006). The lack of nNOS in ventricle probably accounts for the discrepancies between the contributions of eNOS to mAChR signaling pathways in atria or ventricle.

Strikingly, calphostin C, known to inhibit PKC activity, was able to inhibit both eNOS and nNOS signaling pathways, triggered by mAChR activation. This suggests that PKC is an important activator of constitutive NOS in atria. The requirement of CaM and PKC activation observed for the mAChR agonist-induced stimulation of the NO-mediated pathway in rat atrium, revealed in this paper, is consistent with reports showing CaM kinase and PKC-mediated phosphorylation and regulation of NOS (Nakane *et al*., 1991; Bredt *et al*., 1992).

Myocardial production of NO is one element in a wide range of autonomic controls of normal cardiac contraction. It is also one of the pathogenic mediators of the loss of such controls in a failing heart (Massion *et al*., 2003). The discovery of the selective modulation of cardiac contractility by NOS isoforms has stimulated considerable interest in the possibility of reversing cardiac dysfunction with isoform-selective inhibitors of NOS.

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Conflict of interest

The authors state no conflict of interest.

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