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Endogenous nitric oxide signalling system and the cardiac muscarinic acetylcholine receptor-inotropic response

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1 In this paper we have determined the different signalling pathways involved in muscarinic acetylcholine receptor (AChR)-dependent inhibition of contractility in rat isolated atria.

2 Carbachol stimulation of M_2 muscarinic AChRs exerts a negative inotropic response, activation of phosphoinositide turnover, stimulation of nitric oxide synthase and increased production of cyclic GMP.

3 Inhibitors of phospholipase C, protein kinase C, calcium/calmodulin, nitric oxide synthase and guanylate cyclase, shifted the dose-response curve of carbachol on contractility to the right. These inhibitors also attenuated the muscarinic receptor-dependent increase in cyclic GMP and activation of nitric oxide synthase. In addition, sodium nitroprusside, isosorbide, or 8-bromo cyclic GMP, induced a negative inotropic effect, increased cyclic GMP and activated nitric oxide synthase.

4 These results suggest that carbachol activation of M_2 AChRs, exerts a negative inotropic effect associated with increased production of nitric oxide and cyclic GMP. The mechanism appears to occur secondarily to stimulation of phosphoinositides turnover via phospholipase C activation. This in turn, triggers cascade reactions involving calcium/calmodulin and protein kinase C, leading to activation of nitric oxide synthase and soluble guanylate cyclase.

Keywords: Heart contractility; cholinoceptors; nitric oxide synthase; cyclic GMP; phosphoinositide turnover; binding assay

Introduction

The activation of muscarinic acetylcholine receptors (AChR) by the agonist carbachol, triggers many different signal transduction pathways including: stimulation of guanosine 3':5'-cyclic-monophosphate (cyclic GMP) production, a decrease in adenosine 3':5'-cyclic monophosphate (cyclic AMP) through either attenuation of cyclic AMP synthesis or stimulation of cyclic AMP degradation, regulation of several ion channels and stimulation of phospholipase (PL) C, A2 and D (Feigenbaum & El-Fakahany, 1985; Liles *et al.*, 1986; Lai & El-Fakahany, 1987; Candell *et al.*, 1990; Entzeroth *et al.*, 1990; Hosey, 1992). Studies of signalling events on cloned muscarinic AChR subtypes have revealed differences in their abilities to modulate many different signal transduction pathways. The heart expresses only M_2 receptors (Peterson *et al.*, 1984) which are coupled to more than one G protein, regulating several signal transduction pathways (Brown *et al.*, 1985a; Ashkenzi *et al.*, 1989; Ikegaya *et al.*, 1990).

Decreased cardiac contractility is modulated by the activity of the cholinergic autonomic nerves. However, the signal transduction mechanisms that mediate the action of muscarinic cholinoceptor agonists on heart contractility (Trautwein *et al.*, 1982), have not been clearly defined.

Heart muscarinic AChR stimulation elevates intracellular levels of cyclic GMP (George *et al.*, 1970) and cyclic GMP analogues alter the contractility of mammalian heart (Shah *et al.*, 1991) but the involvement of cyclic GMP in cardiac contractility is still unclear. Among the mechanisms involved in the cholinoceptor dependent-activation of cyclic GMP synthesis, an increase in intracellular calcium and activation of calcium/calmodulin-dependent nitric oxide synthase (NOS) which in turn leads to activation of soluble guanylate cyclase has been described (Forstermann *et al.*, 1990).

It has been reported that ventricular myocytes produce endogenous nitric oxide (NO) and that some of the physiological effects of NO appear to be mediated by activation of guanylate cyclase. Moreover, the effect of muscarinic agonists on ventricular myocytes is mediated, at least in part, by production of endogenous NO (Balligand *et al.*, 1993).

In this paper we have determined the different signalling events involved in the M_2 muscarinic AChR-dependent inhibition of contractility in rat isolated atria. Results show that carbachol activation of M_2 AChRs exerts a negative inotropic effect which is associated with an increased production of NO and cyclic GMP. The mechanism by which muscarinic AChRs increase cyclic GMP production appears to occur secondarily to stimulation of phosphoinositides turnover (IPs) via phospholipase C activation (PLC). This in turn triggers cascade reactions involving calcium/calmodulin and protein kinase C (PKC), leading to activation of NOS and soluble guanylate cyclase activity. The increase in NO and cyclic GMP synthesis may contribute to the negative inotropic effect of low concentrations of carbachol in rat isolated atria. These and other signalling events modulated by cardiac M_2 muscarinic AChRs are shown.

Methods

Radioligand binding assay

Membranes were prepared as described previously (Goin *et al.*, 1994). In brief, atria were homogenized in an Ultraturrax at 4°C in 6 vol of potassium phosphate buffer, 1 mM $MgCl_2$, 0.25 M sucrose (buffer A) pH 7.5 supplemented with 0.1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM EDTA, 5 μg ml^{-1} leupeptin, 1 μM bacitracin and 1 μM pepstatin A. The homogenate was centrifuged twice for 10 min at 3000 *g*, then at 10000 *g* and 40000 *g* at 4°C for 15 and 90 min respectively. The resulting pellets were resuspended in 50 mM phosphate buffer with the same protease inhibitors pH 7.5 (buffer B). Receptor ligand binding was performed as described previously (Bacman *et al.*, 1990). Aliquots of the membrane suspension (30-50 μg protein) were incubated with different concentrations of 1-[benzilic-4,4'-^{3}H(N)]-quinuclidinyl benzilate ([^{3}H]-QNB) (New England Nuclear, Sp. Act. 44.8 Ci $mmol^{-1}$) for 60 min at 25°C in a total volume of 150 μl of buffer B. Binding was stopped by adding 2 ml ice-cold buffer followed by rapid filtration (Whatman GF/c). Filters were rinsed with 12 ml of ice-cold buffer, transferred into vials

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containing 10 ml of scintillation cocktail and counted in a liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 5×10^{-8} M atropine and never exceeded 10% of total binding. Radioactivity bound was lower than 10% of total counts.

Atrial preparation for contractility

Male Wistar rats were killed by decapitation. The atria were carefully dissected from the ventricles, attached to a glass holder and immersed in a tissue bath containing Krebs-Ringer Bicarbonate (KRB) solution gassed with 5% CO_2 in oxygen and maintained at pH 7.4 and 30°C. KRB solution was composed as described previously (Sterin-Borda *et al.*, 1986). A preload tension of 750 mg was applied to the atria and tissues were allowed to equilibrate for 1 h. The initial control values for contractile tension of the isolated atria were recorded by use of a force transducer coupled to an ink writing oscillograph (Borda *et al.*, 1984). 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 (*dF/dt*) were assessed by recording the maximum rate of isometric force development during electrical stimulation at a fixed frequency of 150 beats min^{-1}. 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 7.3 ± 0.6 g s^{-1}. Cumulative dose-response curves to carbachol were obtained according to the method of Van Rossum (1963). A maximal effect was achieved within 6 min after each dose.

Measurement of total labelled inositol phosphates (IPs)

Rat atria were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO_2 in O_2 with 1 μCi [myo-^{3}H]-inositol ([^{3}H]-MI) (Sp. Act. 15 Ci $mmol^{-1}$) from Dupont/New England Nuclear, LiCl (10 mM) was added for inositol monophosphate accumulation, according to the technique of Berridge *et al.* (1982). Carbachol was added 30 min before the end of the incubation period and the blockers 30 min before the addition of carbachol. Water-soluble IPs were extracted after 120 min incubation following the method of Berridge *et al.* (1982). Atria were quickly 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 2000 *g* for 15 min and the aqueous phase of the supernatants (1–2 ml) were applied to a 0.7 ml column of Bio-Rad AG 1 × 8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid which had 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 IPs were eluted with 1 M ammonium formate in 0.1 M formic acid. One ml fractions were recovered and radioactivity was determined by scintillation counting. Peak areas were determined by triangulation. Results corresponding to the second peak, were expressed as a percentage of the total radioactivity incorporated (1st plus 2nd peaks). In order to determine the absence of [^{3}H]-myo-inositol in the eluted peaks of IPs, chromatography in silica gel 60 F254 sheets (Merck) was performed using propan-2-ol/6, NH_4 (14:5) as the developing solvent (Hokin-Neaverson & Sadeghian, 1976). Spots were located by spraying with freshly prepared 0.1% ferric chloride in ethanol followed, after air-drying, with 1% sulphosalicylic acid in ethanol. To assay the radioactivity a histogram was constructed by cutting up the sheet gel, placing each sample in Triton-toluene based scintillation fluid and then counting.

Determination of nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in atria by production of [U-^{14}C]-citrulline from [U-^{14}C]-arginine according to the procedure described by Bredt & Snyder (1989) for brain slices. 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 the atria were incubated for 20 min under 5% CO_2 in O_2 at 37°C. Atria were then homogenized with a Ultraturrax 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 phenylmethanesulphonyl fluoride at 4°C. After centrifugation at 20000 *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. When partial purification of NOS was required, the atria were homogenized with an Ultraturrax as described above. After centrifugation at 20000 *g* for 10 min, the supernatant was partially purified by a 2′,5′-ADP-Sepharose column equilibrated with the homogenization buffer supplemented with 1 mM $MgCl_2$ and 100 mM NaCl. The column was washed with this medium until no more protein emerged after which NOS activity was eluted with homogenization buffer with 5 mM NADPH and 10% glycerol. Samples containing NOS activity were incubated in a buffer with 20 mM HEPES, 10 μM [U-^{14}C]-arginine (0.3 μCi), 0.5 mM NADPH and 10 μg ml^{-1} of calmodulin (200 μl of incubation volume). After 10 min incubation the reaction was stopped by addition of 1 ml ice-cold 10 mM EGTA, 1 mM citrulline and 100 mM PIPES pH 5.5. Fractions containing NOS activity were concentrated by ultrafiltration through cellulose citrate membrane with a cut-off of 30 kDa. All procedures were carried out at 4°C and enzyme activity was measured. Measurement of basal NOS activity in whole atria by the above mentioned procedure was inhibited 95% in the presence of 0.5 mM L-NMMA. The results (pmol g^{-1} tissue wet wt.) obtained for whole atria were expressed as the difference between values in the absence (287 ± 12, $n = 7$) and in the presence (13 ± 2, $n = 7$) of L-NMMA.

Cyclic GMP assay

Tissues were incubated in 1 ml KRB for 30 min and carbachol was added in the last 5 min. When blockers were used, they were added 25 min before the addition of carbachol. After incubation, atria were homogenized in 2 ml of absolute ethanol and centrifuged at 6000 *g* for 15 min at 4°C. Pellets were then rehomogenized in ethanol-water (2:1) and supernatants collected and evaporated to dryness as indicated above. Cyclic GMP in the residue was dissolved in 400 μl of 0.05 M sodium acetate buffer pH 6.2. Aliquots of 100 μl were taken for the nucleotide determination using RIA procedure with a cyclic GMP ^{125}I-RIA KIT from Dupont/New England Nuclear.

Drugs

Carbachol, atropine, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC), neomycin, methylene blue, oxyhaemoglobin, 8-bromo cyclic GMP, L-arginine, N^G-monomethyl-L-arginine (L-NMMA), trifluoperazine (TFP), sodium nitroprusside (NP), pirenzepine, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) and phorbol 12-myristate-13-acetate (PMA) were purchased from Sigma Chemical Company and AFD-X 116 (11-[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6-H-pyrido-[2,3-b][1,4]-benzodiazepine-6-one) was kindly provided by Boehringer Ingelheim Pharmaceuticals Inc. Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentrations stated in the text.

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 $P < 0.05$.

Results

To assess the muscarinic AChR subtypes involved in the biological effect of carbachol in rat atria, both radioligand binding and contractile assays were performed.

[^{3}H]-QNB binding to atrial membranes was a saturable process to a single class of binding sites, free of cooperative interactions. The equilibrium parameters calculated from Scatchard plots were K_d 70±3 pM and B_{max} 478±19 fmol mg^{-1} protein. K_i and pA_2 values, calculated from competitive binding assays and contractile studies showed that rat atria express preferentially M_2 muscarinic receptors (Table 1) and that the negative inotropism of carbachol is mediated by this receptor subtype (Table 2).

To determine if an endogenous nitric oxide (NO) signalling system participates in the contractile action of carbachol, rat isolated atria were incubated with different inhibitors of the enzymatic pathways involved in the muscarinic receptor-dependent activation of NO and cyclic GMP synthesis. As can be seen in Figure 1a the inhibition of nitric oxide synthase (NOS) activity by L-NMMA (2×10^{-6} M) or the inhibition of cyclic GMP production by oxyhaemoglobin (5×10^{-5} M) or methylene blue (1×10^{6} M); shifted to the right the dose-response curve of carbachol. The action of the inhibitors was more effective at low concentrations of the agonist. The inhibitory action of L-NMMA on the carbachol effect was reversed by L-arginine (Figure 1b).

In addition, the generation of NO by sodium nitroprusside (SNP) or isosorbide and 8-bromo cyclic GMP (cyclic GMP analogue), were able to induce a negative inotropic effect (Table 3). Moreover, oxyhaemoglobin and methylene blue blocked the inhibitory effect of SNP (Table 3).

To determine the participation of phospholipase C (PLC) in the negative inotropic effect of carbachol, the action of NCDC and neomycin were explored. As can be seen in Figure 2, NCDC (5×10^{-6} M) and neomycin (1×10^{-6} M) shifted to the right the dose-response curve of carbachol. To elucidate which pathways, gated by polyphosphoinositide turnover (PI), could be involved in this effect, atria were incubated in the presence of an inhibitor of protein kinase C (PKC) (H-7, 5×10^{-6} M) and an inhibitor of calcium-calmodulin (TFP, 5×10^{-6} M). Figure 2 shows that in the presence of either H-7 or TFP the dose-response curve of carbachol was also shifted to the right.

To assess which muscarinic AChR subtype was coupled to PI turnover in rat atria, the effect of carbachol in the presence or absence of cholinoceptor antagonists was investigated. As

Table 1 Inhibition of [^{3}H]-QNB binding to rat cardiac membranes

Cholinoceptor agent	K_i (nM)
Atropine	1.4
AFD-X 116	57
Pirenzepine	360

The equilibrium dissociation constant (K_i) for the interaction of competing ligands were calculated from the equation (Cheng & Prussoff) $Xi = IC_{50}/1+(L)/K_d$ where IC_{50} is the competing ligand concentration which half-maximally inhibits the specific binding of the radioligand present at a concentration (L). IC_{50}s were obtained from competition experiments performed in duplicate at several concentrations of each agent. Competition curves of carbachol were best fitted to a two state model (K_{iH}: 1.1±0.3 μM and K_{iL}: 20±4 μM).

Table 2 pA_2 (–log K_i) and K_i values (in parentheses) of muscarinic antagonists in the carbachol inotropic effect on rat isolated atria

Antagonists	*pA_2*
Pirenzepine	6.3 (500 nM)
AFD-X 116	7.9 (10 nM)
Atropine	8.2 (6 nM)

Atria were preincubated for 30 min with different antagonists before concentration-response curves to carbachol were performed. Values of EC_{50} were obtained from those concentration-response curves in the presence or absence of 1×10^{-7} M pirenzepine, AFD-X 116 or atropine and then K_i and pA_2 were calculated. Values are mean±s.e.mean.

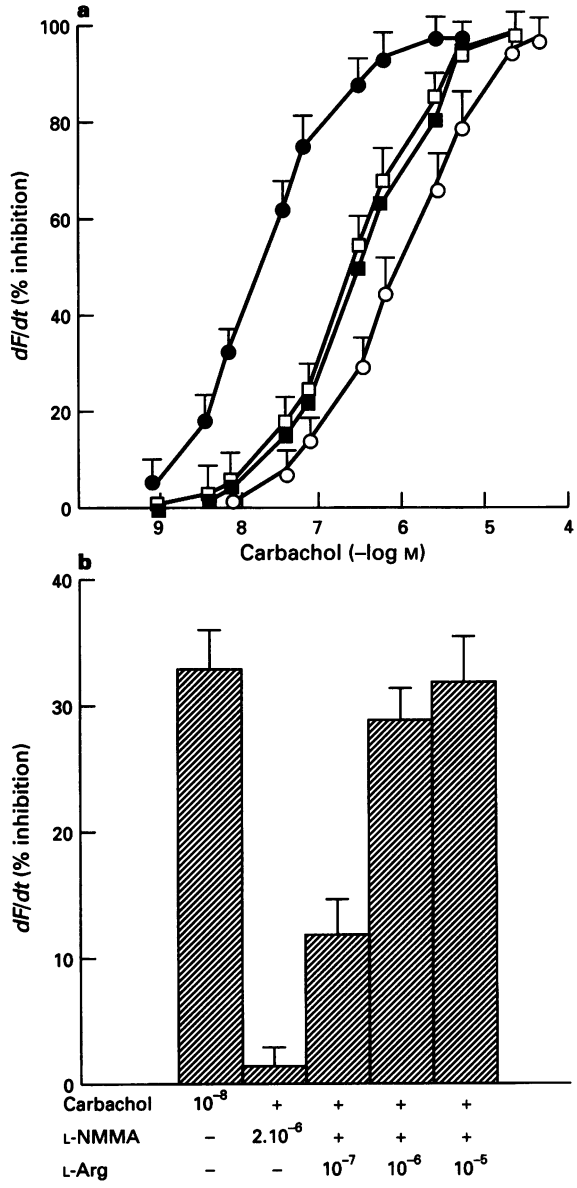

Figure 1 (a) Decrease in contractility (dF/dt) of rat isolated atria by increasing concentrations of carbachol (●). The inhibitory action of 5×10^{-5} M oxyhaemoglobin (□), 1×10^{-6} M methylene blue (■) and 2×10^{-6} M N^G-monomethyl-L-arginine (L-NMMA, ○) is also shown. (b) Inhibition of negative inotropic effect of 1×10^{-8} M carbachol by treatment of atria with 2×10^{-6} M L-NMMA and reversal of inhibition by increasing concentration of L-arginine (L-Arg). No inotropic effect of L-arginine was observed at concentrations used (data not shown). Values represent the mean±s.e.mean of eight different determinations in each group. Tissues were incubated for 30 min in presence or absence of different enzymatic inhibitors and then carbachol was added. Values are expressed as percentage changes calculated by comparison with the absolute values prior to the addition of carbachol.

Table 3 Comparative inotropic negative effect of different drugs on *dF/dt* of rat isolated atria

Drugs	dF/dt *(% of inhibition)*	n
Carbachol	97±5.1	7
8-Br cyclic GMP	69±3.2	6
Isosorbide	40±2.3	6
Sodium nitroprusside	43±2.2	7
Sodium nitroprusside + oxyhaemoglobin	3±0.2	6
Sodium nitroprusside + methylene blue	8±1.1	6

Values are mean±s.e.mean and represent the effect of maximum concentrations of carbachol (5×10^{-6} M), 8-bromo cyclic GMP (8-Br cyclic GMP), isosorbide and sodium nitroprusside (both at 5×10^{-5} M) of *n* indicated experiments. Oxyhaemoglobin (5×10^{-5} M) and methylene blue (1×10^{-6} M) were added 30 min before sodium nitroprusside (5×10^{-5} M).

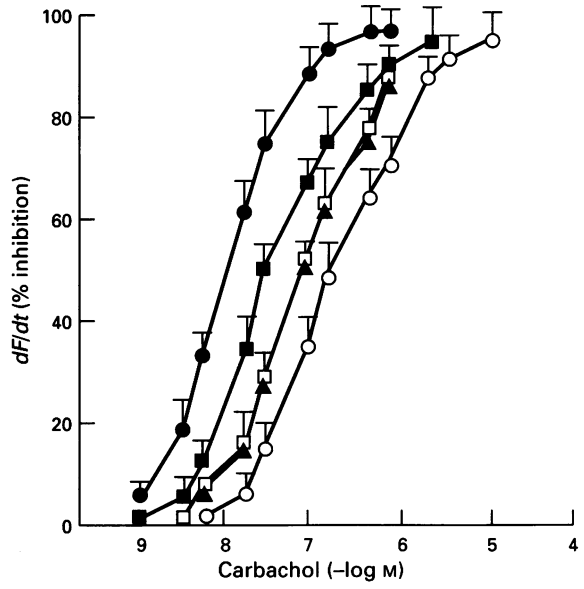

Figure 2 Effect of 5×10^{-5} M trifluoperazine (TFP, ■), 5×10^{-6} M 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC, ▲), 1×10^{-6} M neomycin (□) and 5×10^{-6} M 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7, ○) on the dose-response curve of carbachol (●) upon atrial *dF/dt*. Tissues were incubated for 30 min in presence or absence of different inhibitors and then the dose-response curves to carbachol were obtained. Values represent the means±s.e.mean of eight experiments in each group. For more details see Figure 1.

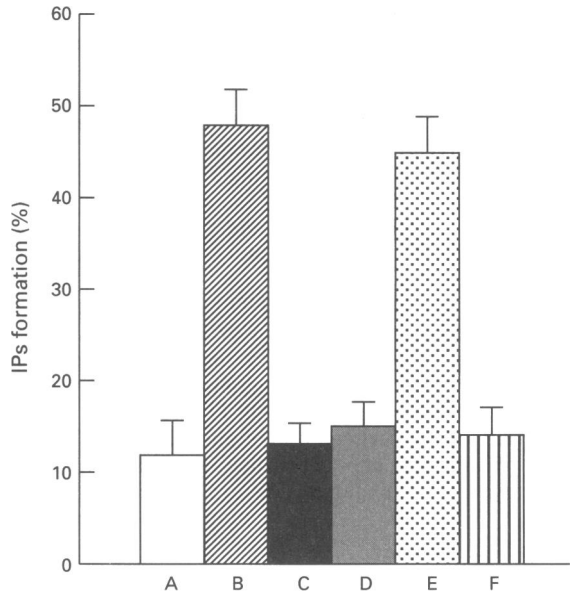

Figure 3 Effect of carbachol upon phosphoinositide turnover. Atria were incubated for 60 min with *myo*-[2-^{3}H]-inositol and for an additional 30 min in the presence of 1×10^{-7} M atropine (C), 1×10^{-7} M AFD-X 116 (D), 1×10^{-6} M pirenzepine (E) and 5×10^{-6} M 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC) (F). Tissues were then left for a further 40 min in the absence (A) or presence of 5×10^{-7} M carbachol (B). Results correspond to the percentage of the second peak related to the total radioactivity incorporated (1st+2nd peaks). Values are means±s.e.mean of six experiments performed in duplicate in each group.

can be seen in Figure 3, carbachol (5×10^{-7} M) increased PI formation. Atropine (1×10^{-7} M) and AFD-X 116 (1×10^{-7} M) but not pirenzepine (1×10^{-6} M) significantly inhibited the stimulatory action of carbachol, pointing to the participation of M_2 muscarinic AChR in the action of carbachol upon PI hydrolysis. As a control NCDC also abrogated this effect, indicating that PLC activation would be implicated in this phenomenon.

Carbachol also, increased cyclic GMP production and nitric oxide synthase activity (NOS) in rat isolated atria (Table 4).

To verify the nature of the mechanism by which the activation of muscarinic AChR increased cyclic GMP synthesis and NOS activity; rat atria were incubated with several inhibitors. Table 4 shows that atropine and AFD-X 116 but not pirenzepine inhibited the stimulatory action of carbachol on both cyclic GMP production and on NOS activity. Also the inhibition of PLC (by NCDC and neomycin), calcium/calmodulin (by TFP), PKC (by H-7) and NOS (by L-NMMA), attenuated the muscarinic receptor-dependent activation of cyclic GMP levels and NOS activity. Moreover, PMA (10 μg ml^{-1}) mimicked the effect of the muscarinic agonist on NOS activity (850±30 pmol g^{-1} tissue wet wt.). This effect was blocked by H-7 (274±10 pmol g^{-1} tissue wet wt.). Additionally, SNP increased cyclic GMP production (75±5 pmol g^{-1} tissue wet wt.).

None of the inhibitors had any effect *per se* on basal values of nitric oxide synthase activity, cyclic GMP levels, PI hydrolysis or cardiac contractility (data not shown). As expected, L-NMMA inhibited by 95% basal NOS activity (see Experimental Procedure). This explains the low values of the enzyme activity when carbachol was tested in the presence of this inhibitor of NOS activity (Table 4). It is important to note that this effect of L-NMMA was not dose-related, but occurred over a wide range of concentrations (2×10^{-6} M to 5×10^{-5} M), each exerting the same inhibitory action. The maximal effect of carbachol on NOS activity was at 5×10^{-7} M; at this concentration of carbachol, L-NMMA (2×10^{-6} M) inhibited the negative inotropic action of carbachol by 66%. Higher concentrations of L-NMMA (5×10^{-4} M) inhibited NOS activity by 95%.

Discussion

The present data indicate that both NO and cyclic GMP production are involved in the M_2 muscarinic AChR-mediated inotropic effects of carbachol in rat atria. The results point to a

Table 4 Effect of carbachol (CCh) upon cyclic GMP production and NO synthase activity of rat isolated atria: influence of enzymatic inhibitors

Additions	*Cyclic GMP* (pmol g^{-1} tissue wet wt)	*NO synthase activity* (pmol g^{-1} tissue wet wt)
None	45±2	274±12
Carbachol (CCh)	98±4*	798±20*
Atropine + CCh	40±2	192±9
AFD-X 116 + CCh	40±3	302±15
Pirenzepine + CCh	92±5*	742±19*
NCDC + CCh	50±5	330±14
Neomycin + CCh	58±4	302±13
TFP + CCh	52±3	590±17
H-7 + CCh	50±4	265±10
L-NMMA + CCh	58±5	18±2
L-NMMA + L-arginine + CCh	100±7*	850±20*
Methylene blue + CCh	48±4	nd

Cyclic GMP and nitric oxide (NO) synthase activity were measured incubating atria with or without enzymatic inhibitors for 20 min and then for an additional 10 min with 5×10^{-7} M carbachol (CCh). Results are means±s.e.mean of seven experiments performed in duplicate in each group. The final concentrations of the inhibitors were: atropine and AFD-X 116 1×10^{-7} M; pirenzepine, and H-7 1×10^{-6} M; L-NMMA 5×10^{-4} M; NCDC, neomycin and TFP 5×10^{-6} M; methylene blue 1×10^{-5} M and L-arginine 5×10^{-5} M. *Significantly different from basal values (none) with $P<0.001$.

role for PKC and calcium mobilization in the rapid activation of NOS. These conclusions are based on three main facts: first, the concentration-response curves of the muscarinic agonist carbachol, acting on M_2 muscarinic AChRs were shifted to the right when either NOS or soluble guanylate cyclase activities were inhibited. The mechanism seems to involve an increase in PI hydrolysis whose intermediates would turn on a calcium/calmodulin-dependent constitutive NOS, since various agents known to interfere with routes of calcium mobilization also caused a right-ward shift of the rapid effect of carbachol in rat atria. Surprisingly the PKC inhibitor (H-7), was the most efficient inhibitor of this effect. Second, the inhibitory effect of L-NMMA on carbachol-induced NOS activation could be reversed by L-arginine. Both SNP, as a NO source, and 8-bromo cyclic GMP, a lipid soluble cyclic GMP analogue, mimicked the inotropic effect of low doses of carbachol. Third, carbachol activation of M_2 muscarinic AChRs led to increased hydrolysis of PI and stimulated NOS activity and cyclic GMP levels. Both NOS activation and cyclic GMP stimulation, were decreased by NOS inhibitors as well by scavengers of released NO. As seen with the negative inotropic effect, PI turnover-gated calcium mobilization and PKC activity appear to play a role in the low carbachol concentration stimulation increase in NOS activity and cyclic GMP levels.

Reports pointing to the involvement of NO in the inotropic effect induced by high concentrations of carbachol in heart cells have been controversial (Schulz *et al.*, 1992; Balligand *et al.*, 1993; Stein *et al.*, 1993) and the possibility of species differences has been discussed. However, little is known about the relative importance of different signal transduction pathways including increases in NO and cyclic GMP in the negative inotropism induced by lower concentrations of carbachol.

Two results support the assumption that carbachol-promoted increases in NO and cyclic GMP are mediated by M_2 muscarinic AChRs: Firstly, because this subtype is preferentially expressed in rat atria (Tables 1 and 2) and secondly, because AFD-X 116 was able to inhibit both cyclic GMP and NO production (Table 4).

To evaluate further the role of constitutive NOS in the low concentration-range, we tested the ability of the stereospecific NOS inhibitor, L-NMMA (Mulsch & Busse, 1990) to alter the carbachol-induced contractile response, the NOS activity, and the cyclic GMP levels in rat atria (Figure 1a, Tables 3 and 4). The fact that, in the presence of L-NMMA, methylene blue and oxyhaemoglobin, the concentration-response curve was shifted to the right together with lower levels of NOS activity and cyclic GMP production strongly suggests that a constitutive NOS is active in this system, a result in agreement with previous reports using crude heart cell preparations (Moncada *et al.*, 1991).

The nature of the inhibition also suggests that the NO-mediated pathway would be more relevant at lower than at higher concentrations of carbachol. Consistent with this, are the results obtained from experiments with sodium nitroprusside and 8-bromo cyclic GMP where, even if they induced negative inotropism in rat atria, these agents were not as efficient as carbachol, since their maximal effects are comparable to that of carbachol at its EC_{50} (Table 3). However, the possibility cannot be ruled out that their lower effectiveness is due to an impaired access to their effectors when added exogenously or to different concentrations reached from those of the intermediates made *in situ*. Thus, it seems likely that the NO–cyclic GMP-mediated pathway predominates at low doses of carbachol while more than one signalling cascade accounts for the maximal inotropic effect of muscarinic agonists in rat atria. Our results pointing to a role for cyclic GMP in the inotropic effect induced by carbachol are in agreement with previous studies reporting the ability of a cyclic GMP analogue to decrease heart contractility (Shah *et al.*, 1991) as well as with the proposed role of this cyclic nucleotide on calcium channel activity (Mery *et al.*, 1991).

The role of NOS in the negative inotropic effect of carbachol was further confirmed by the reversibility of the inhibitory action of L-NMMA on both contractility and NOS activity by the natural substrate L-arginine (Figure 1b, Table 4). This restoration was total and is in contrast to results obtained in neonatal cardiac myocytes where L-arginine reversed only partially the effect of L-NMMA on high carbachol concentrations (Balligand *et al.*, 1993). The ability of L-arginine to reverse the effect of L-NMMA on the carbachol-induced inotropic effect seems not to be due to the activation of a different pool of NOS since L-arginine in a wide range did not show any effect when added alone. This confirms that the levels of substrate are not rate-limiting for cardiac constitutive NOS activity (Gold *et al.*, 1989; Wood *et al.*, 1990). The effect of L-arginine also rules out the possibility of a non-specific, direct, interaction of L-NMMA at mucarinic AChRs. This is in accordance with Buxton *et al.* (1993), who demonstrated that L-NMMA did not have any affinity for M_2 mAChRs.

In order to investigate the calcium/calmodulin-dependent nature of the NOS activity induced by low concentrations of carbachol, we pretreated atria with various calcium-mobilization related drugs (Figure 2). It is well known that carbachol stimulates hydrolysis of phosphoinositides in cardiac cells

(Brown & Brown, 1984; Brown *et al.*, 1985b) with the consequent calcium mobilization and activation of PKC (Berridge, 1984). The inclusion of PLC inhibitors (NCDC and neomycin) caused a shift to the right of the concentration-response curve for carbachol and the same was observed with a calcium/calmodulin inhibitor, trifluoperazine (TFP), though it was less effective than the PLC inhibitors. Moreover, the presence of the above inhibitors in experiments where low doses of carbachol were used to stimulate NOS activity and cyclic GMP levels, was sufficient to reduce those increments in a similar way to that seen in contractility studies (Table 4). The fact that TFP (5×10^{-5} M) was less effective at inhibiting NOS activity than at inhibiting cyclic GMP formation, even though its inhibition constant at NOS is around the concentration tested, suggests that cyclic GMP formation could depend on the activation of another pathway involving calcium mobilization but independent of phosphoinositide turnover. Moreover, the possibility that TFP was acting directly on muscarinic AChRs rather than by inhibition of calcium/calmodulin, seems not to be the case, since there were no differences between the effect of TFP on carbachol stimulated-cyclic GMP and NOS activity.

While the existence of calcium/calmodulin-dependent constitutive NOS involved in the stimulation of cyclic GMP levels has been proposed in vascular endothelial and smooth muscle cells (Mülsch & Busse, 1990), reports on the action of this enzyme in cultured cardiac cells are in apparent conflict (Schulz *et al.*, 1992; Stein *et al.*, 1993). We cannot rule out that a constitutive NOS from the endothelium contributes to the effect of low doses of carbachol in the rat isolated atria. A striking result was obtained with H-7 which, by inhibiting PKC activity showed the greatest right-ward shift of the concentration-response curve for carbachol and greatly inhibited NOS activity and cyclic GMP stimulated levels. Also, PMA, a known PKC activator, stimulated the activity of NOS. Although it is well known that H-7 is only relatively selective in inhibiting PKC activity (Hidaka & Hagiwara, 1987), the fact that PMA stimulated NOS activity strongly suggests that this kinase is the most important in the effect.

The requirement of calcium/calmodulin and PKC activation for the carbachol-induced stimulation of the NO-cyclic GMP mediated pathway in rat atrium shown in this paper is consistent with recent reports showing calcium/calmodulin kinase and PKC-mediated phosphorylation and regulation of NOS (Nakane *et al.*, 1991; Bredt *et al.*, 1992). Similarly, the inducible form of the enzyme has been reported to be regulated by PKC in hepatocytes and cultured peritoneal macrophages (Hortelano *et al.*, 1992; 1993). Although the levels at which calcium/calmodulin or PKC activity are involved cannot be calculated from our results as yet, it is tempting to speculate that the phosphorylation by PKC is more important in the above effects, since its inhibition promoted the most drastic shift of the inotropic effect as well as impairment of NOS activation by carbachol. It would be of interest to address the possibility that a particular isoform of NOS, sensitive to PKC phosphorylation and regulation could be present in cardiac tissue.

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