Biochemical Pharmacology, Vol. 55, pp. 1991–1999, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00069-0

Participation of Nitric Oxide and Cyclic GMP in the Supersensitivity of Acute Diabetic Rat Myocardium by Cholinergic Stimuli

*Miriam R. Wald, Enri S. Borda and Leonor Sterin-Borda**

Centro de Estudios Farmacologicos y Botanicos (CEFYBO), Consejo Nacional de Investigacion Cientificas y Tecnicas (CONICET), Catedra de Farmacologia de la Facultad de Medicina y Odontologia de la Universidad de Buenos Aires, 1414 Buenos Aires, Argentina

ABSTRACT. The purpose of this study was to explore the pharmacological and biochemical mechanisms involved in diabetic cardiomyopathy, with particular interest in the abnormal function of cholinergic neurotransmission at the onset of the pathology. The muscarinic acethylcholine agonist carbachol showed a negative inotropic response on both normal and diabetic isolated atria, but the latter showed a supersensitive response. No changes were found in muscarinic acethylcholine receptor (mAChR) expression. Measurements of mAChR-associated second messengers indicated no significant differences between normal and diabetic rat atria in the stimulatory effect of carbachol on protein kinase C activity and the production of inositol phosphates, or in the inhibitory effect induced by carbachol on cyclic AMP (cAMP) production. On the contrary, nitric oxide (NO) synthase activity and cyclic GMP production were higher in diabetic cardiac preparations than in normal ones. Moreover, in diabetic atria, nitric oxide synthase and guanylate cyclase inhibitors shifted the carbachol concentration–response curve on contractility to the right, reaching values similar to those of normal atria. These results suggest an early alteration in the mACh system during the diabetic state, associated with increased production of nitric oxide and cyclic GMP (cGMP). This, in turn, could increase the biological mechanical activity of the mAChR agonist, inducing in this way a higher pharmacological response, without changes in mAChR expression. BIOCHEM PHARMACOL **55**;12:1991–1999, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. nitric oxide; cyclic GMP; diabetic heart; cholinergic stimuli

Diabetes mellitus affects a large number of people from different social conditions throughout the world. Patients suffering from diabetes are particularly prone to cardiovascular disorders [1, 2], which have been associated with an early autonomic neuropathy [3]. Diabetic cardiomyopathy, as a distinct entity, was first recognized by Rubler *et al.*, as described in Ref. 4, in diabetic patients with congestive heart failure who had shown no evidence of coronary atherosclerosis. The increased incidence of congestive heart failure, mortality, and morbidity in diabetic patients—following myocardial infarction or coronary artery bypass graft—can be explained by the presence of diabetic cardiomyopathy [5].

STZ†-treated rats display many of the features observed in human subjects with uncontrolled diabetes mellitus [6]. Accordingly, STZ-treated rats show alterations in the intrinsic electrical and contractile properties of the heart, which may be related to alterations in adrenoceptor and cholinoceptor populations [7].

As regards the cholinergic muscarinic response to specific agonists, an alteration in the myocardium has been detected in the course of the diabetic syndrome. The alterations have been related to different phenomena, i.e. either supersensitivity or subsensitivity, with or without changes in the receptor expression [8–12]. However, these controversial conclusions could be due to differences in the experimental models and/or in the duration of the diabetic state in the experimental animals. Yet, little is known about the signal transduction mechanism involved in the altered response of the diabetic rat heart to an mAChR agonist.

The aim of this study was to establish the atrial contractile response to mAChR agonists in acutely diabetic rats, and the signal transduction mechanism involved in the response. Here we demonstrate an increased sensitivity to the negative inotropic response to carbachol acting on the M_2 mAChR without changes in the number and affinity of this receptor. Results show that the carbachol-induced supersensitivity response was accompanied by muscarinic hyperactivation of NO and cGMP production. Furthermore, the results demonstrated no changes in muscarinic

* Corresponding author: Leonor Sterin-Borda, M.D., CEFYBO-CONICET, Serrano 669, 1414 Buenos Aires, Argentina. FAX 54-1-856-2751.

† *Abbreviations:* cAMP, cyclic AMP; cGMP, cyclic GMP; IPs, inositol phosphates; KRB, Krebs-Ringer-bicarbonate; L-NMMA, N^G-monomethyl-L-arginine; mAChR, muscarinic acetylcholine receptor; MI, *myo*-inositol; NCDC, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate; NO, nitric oxide; NOS, NO synthase; PGE_2, prostaglandin E_2; PKC, protein kinase C; QNB, quinuclidinyl benzilate; and STZ, streptozotocin.

Received 4 August 1997; accepted 8 January 1998.

activation of PKC activity, production of IPs, or cAMP formation.

MATERIALS AND METHODS

Animals

Experimental short-term diabetes was induced, as reported previously [13], in Wistar male rats (200–250 g) by a single i.p. dose of STZ (85 mg/kg of body weight, s.c.). Animals with plasma glucose above 300 mg/dL and with glycosuria were considered diabetic. The rats were killed 72 hr after STZ injection.

Atrial Contractility

Diabetic and normal rats were decapitated. The entire heart was excised quickly and placed in Petri dishes filled with a modified KRB solution. This medium was kept at room temperature, gassed with a mixture of 95% O_2 and 5% CO_2. The atria were separated from the ventricles, carefully dissected, attached to a glass holder, and immersed in a tissue chamber filled with 20 mL of KRB solution. The tissue bath solution was gassed and maintained at a constant temperature of 30° and pH 7.4 throughout the experiments. One end of the preparation was anchored to a glass holder, and the other was connected to a force transducer coupled to an ink-writing oscillograph. A constant resting tension of 500 mg was applied to the atria by means of a micrometric device, and tissues were allowed to equilibrate for 1 hr. The preparations were paced with a bipolar electrode and an SK4 Grass Stimulator (Berger) [14]. Inotropic effects (dF/dt) were assessed by recording the maximum rate of isometric force development during electrical stimulation at a fixed rate (150 beats/min) with slightly suprathreshold (+10%) electrical square pulses of a 2-msec duration. Pulses were delivered by a conventional stimulator and conveyed to the tissue via two pointed platinum electrodes. 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 ± 0.7 g/sec in normal and 7.1 ± 0.5 g/sec in diabetic atria. Concentration–response curves to carbachol were obtained employing the method described by Van Rossum [15]. A maximal effect was achieved within 6 min after each concentration was added. Basal values of dF/dt were not affected by different blockers at the concentration used [16].

Radioligand Binding Assay

Membranes were prepared as described previously [17]. In brief, atria from normal and diabetic rats were mixed in 6 vol. of cold potassium phosphate buffer containing 0.25 M of sucrose, 1 mM of $MgCl_2$, pH 7.5, supplemented with 0.1 mM of phenylmethylsulfonyl fluoride (PMSF), 1 mM of EDTA, 5 μg/mL of leupeptin, and 1 μM of pepstatin A, and then were homogenized three times for 15 sec with an Ultraturrax T 25 (IKA-Labortechnik) at a setting of maximal speed. The homogenates were centrifuged twice at 1,000 *g* for 10 min, then at 12,000 and 40,000 *g* at 4° for 15 and 90 min, respectively. The pellet was resuspended in 50 mM phosphate buffer with the same protease inhibitors, pH 7.5 (buffer B). Receptor ligand binding was performed as described previously [17]. Aliquots of the membrane suspension (30–50 μg of protein) were incubated with different concentrations of 1-[*benzylic*-4,4′-^{3}H(N)]QNB ([^{3}H]QNB) (New England Nuclear, sp. act. 44.8 Ci/mmol) for 60 min at 25° in a total volume of 150 μL of buffer B. Binding was stopped by adding 2 mL of ice-cold buffer followed by rapid filtration (Whatman GF/c). Filters were rinsed with 12 mL of ice-cold buffer and transferred into vials containing 10 mL of scintillation fluid in a liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 5×10^{-8} M of atropine and never exceeded 10% of total binding. The radioactivity bound was lower than 10% of total counts. For competition binding experiments, atrial preparations were incubated with increasing concentrations of muscarinic antagonists (atropine, AFD-X 116, and pirenzepine) in the presence of 0.56 nM of [^{3}H]QNB. Binding data were analyzed with the computer-assisted curve-fitting program LIGAND [18] and the parameters calculated correspond to simultaneous fitting of N sets of binding data for each membrane.

Assay of PKC Activity

Atria from normal and diabetic rats were incubated for the indicated times alone or in the presence of carbachol, stimulant plus blockers, or blockers alone for a total incubation time of 30 min in KRB solution at 30° and were frozen immediately in liquid N_2. PKC was purified from subcellular fractions as described previously [19]. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1 [20]. Incubations were conducted for 30 min at 30° in a final volume of 85 μL. In the final concentrations, the assay mixture contained 25 μM of ATP (0.4 μCi), 10 mM of magnesium acetate, 5 mM of β-mercaptoethanol, 50 μg of histone H1, 20 mM of HEPES, pH 7.5, and, unless otherwise indicated, 0.2 mM of $CaCl_2$ and 10 μg/mL of phosphatidylserine vesicles. The incorporation of [^{32}P]phosphate into histone was linear for at least 30 min. The reaction was stopped by the addition of 2 mL of ice-cold 5% trichloroacetic acid, 10 mM of H_3PO_4. The radioactivity retained on the GF/c glass-fiber filters after filtration was determined by counting the oven-dried 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/mg of protein). Another known PKC substrate peptide, MBP (4–14) from Life Technologies, was also used for measuring PKC activity purified from subcellular cardiac fractions, following the instructions of the PKC assay

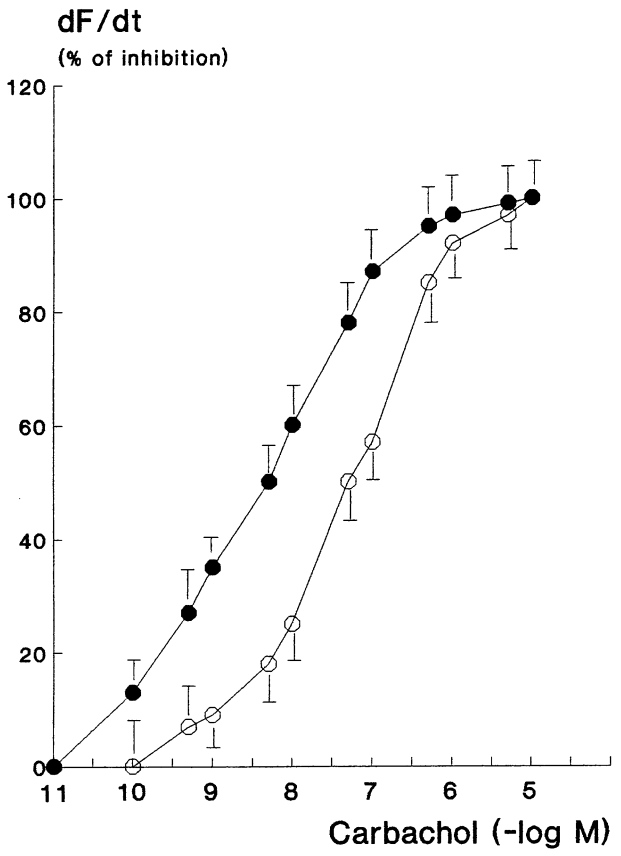


FIG. 1. Cumulative concentration–response curves of carbachol in rat isolated atria from normal (–○–) and diabetic (–●–) rats. dF/dt is expressed as a percent of decreased contractility calculated by comparison with the absolute values (g/sec: 7.4 ± 0.6 and 7.1 ± 0.5 for normal and diabetic atria, respectively) prior to the addition of carbachol. Values represent the means ± SEM of eight different determinations in each group.

system of Life Technologies. PKC specificity was confirmed by means of the PKC pseudosubstrate inhibitor peptide PKC (19–36) provided by Gibco.

Measurement of Total Labeled IPs

Rat atria were incubated for 120 min in 0.5 mL of KRB gassed with 1 μCi of *myo*-[1,2-^{3}H(N)] inositol ([^{3}H]MI) (sp. act. 45 Ci/mmol, DuPont/NEN), and 10 mM of LiCl was added for inositol monophosphate accumulation, according to the technique of Berridge *et al.* [21]. Carbachol was added 30 min before the end of the incubation period and the blockers 30 min before the addition of carbachol. After incubation, atria were quickly washed with KRB, and water-soluble IPs were extracted after a 120-min incubation. Briefly, atria were homogenized in 0.3 mL of KRB with 10 mmol/L of 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) was applied to a 0.7-mL column of Bio-Rad AG 1 × 8 anion exchange resin (100–200 mesh) suspended in 0.1 mol/L of formic acid, which had been washed previously with 10 mmol/L of Tris formic acid, pH 7.4. The resin was then washed with 20 vol. of 5 mmol/L of MI to get the first peak of [^{3}H]MI incorporated in the tissue, followed by 6 vol. of water, and finally IPs were eluted with 1 M of ammonium formate in 0.1 M of formic acid (second peak). One-milliliter 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 the absolute values of the area under the curve following the criteria of Simpson's equation. To determine the absence of [^{3}H]inositol in the eluted peaks of IPs, thin-layer chromatography on silica gel 60 F254 sheets (Merck) was performed using appropriate MI and IP standards as described before, following the procedure of Hokin-Neaverson and Sadeghian [22].

TABLE 1. EC_{50} of muscarinic antagonists in the carbachol inotropic effect on isolated normal and diabetic rat atria

Addition	EC_{50} ($\times 10^{-8}$ M)	
	Normal	Diabetic
Carbachol	4.8 ± 0.2	0.40 ± 0.02
+Atropine	79 ± 5*	8.2 ± 0.9*
+AFD-X 116	52 ± 7*	6.5 ± 0.5*
+Pirenzepine	6.9 ± 0.6	0.72 ± 0.08

Atria were preincubated for 30 min with different antagonists before concentration–response curves to carbachol were determined. The EC_{50} values were obtained from concentration–response curves in the presence or absence of 10^{-7} M AFD-X 116 or atropine or 10^{-6} M pirenzepine. Values are means ± SEM of eight different experiments.

*Significantly different from carbachol alone, $P < 0.001$.

Determination of NOS Activity

NOS activity was measured in atria by production of [U-^{14}C]citrulline from [^{14}C]arginine according to the procedure described by Bredt and Snyder [23] for brain slices. Briefly, after a 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 [^{14}C]arginine (0.5 μCi). Appropriate concentrations of drugs were added,

TABLE 2. Inhibition of [^{3}H]QNB binding to rat cardiac membranes

Cholinoceptor agent	K_i (nM)	
	Normal	Diabetic
Atropine	1.4	1.2
AFD-X 116	55	68
Pirenzepine	385	352

Equilibrium dissociation constant values (K_i) for the interaction of competing ligands were calculated from the equation (Cheng and Prusoff [26]) $X_i = IC_{50}/1 + (L)/K_d$, where IC_{50} is the competing ligand concentration that half-maximally inhibits the specific binding of the radioligand present at concentration (L). The IC_{50} values were obtained from seven competition experiments performed in duplicate at several concentrations of each agent.

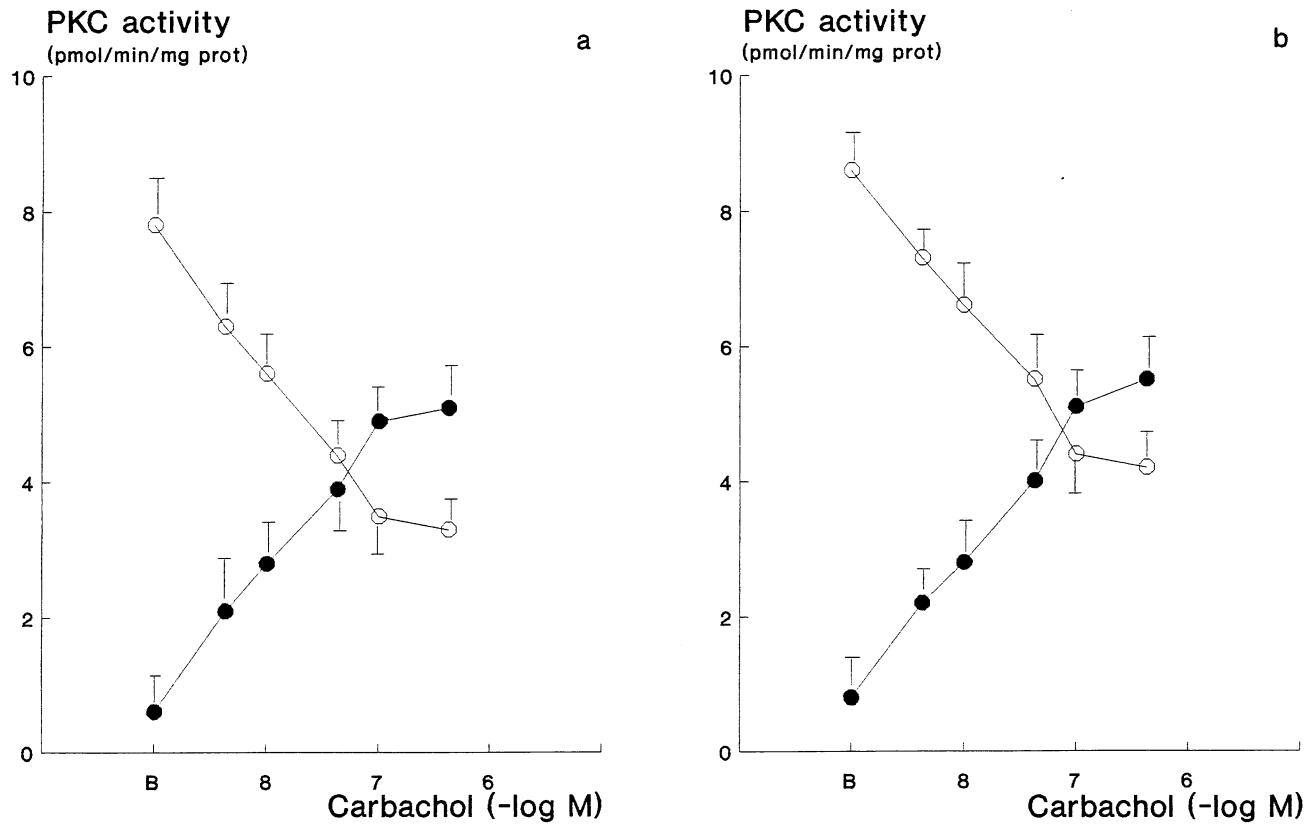


FIG. 2. Concentration–response curves of carbachol-induced activation of PKC activity. Atria from normal (panel a) and diabetic (panel b) rats were incubated with increasing concentrations of carbachol for 10 min, and PKC activity was determined on both cytosolic (–○–) and membrane (–●–) fractions as described. "B" represents basal values in (panel a) and (panel b). Enzymatic activity values shown are the means ± SEM of seven independent experiments performed in duplicate.

and the atria were incubated for 20 min at 37°. Then atria were homogenized with an Ultraturrax (IKA-Labortechnik) in 1 mL of medium containing 20 mM of HEPES, 0.5 mM of EGTA, 0.5 mM of EDTA, 1 mM of dithiothreitol, 1 μM of leupeptin, and 0.2 mM of PMSF, pH 7.4, at 4°. After centrifugation, supernatants were applied to columns of Dowex AG 50 WX-8 (sodium form), and [^{14}C]citrulline was eluted with 3 mL of water and quantified by liquid scintillation counting. Partial purification of NOS was done by a 2′,5′-ADP-Sepharose column, as previously described [16]. Measurements of basal NOS activity in whole atria by the above-mentioned procedures were inhibited 95% in the presence of 0.5 mM of L-NMMA. The results (pmol/g of tissue wet wt) obtained for whole atria were expressed as the difference between values in the absence (381 ± 21, N = 9) and in the presence (20 ± 5, N = 9) of L-NMMA.

cAMP and cGMP Assays

Tissues from normal and diabetic rats were incubated in 1 mL of KRB with 1 mM of 3-isobutyl-1-methylxanthine and were gassed, with shaking at 37° for 30 min; carbachol was added in the last 5 min. To evaluate the inhibitory effects on cAMP production, PGE_2 (1×10^{-6} M) was added in the last 3 min as an adenylate cyclase activator. 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. Then the pellets were rehomogenized in ethanol:water (2:1), and supernatants were collected and evaporated to dryness. Residues were resuspended in 5 mM of Tris–HCl, pH 7.4, containing 8 mM of theophylline, 0.45 mM of EDTA, and 6 mM mercaptoethanol and in 0.005 M of sodium acetate buffer, pH 6.2, for cAMP and cGMP assays, respectively. cAMP determination was developed by the competitive protein-binding assay described by Brown *et al.* [24], using [^{3}H]cAMP as the tracer. Determination of cGMP was done using a radioimmunoassay (RIA) procedure with a cGMP ^{125}I-RIA kit from DuPont/NEN.

Protein Determination

Protein was measured according to Lowry *et al.* [25] using bovine serum albumin as standard.

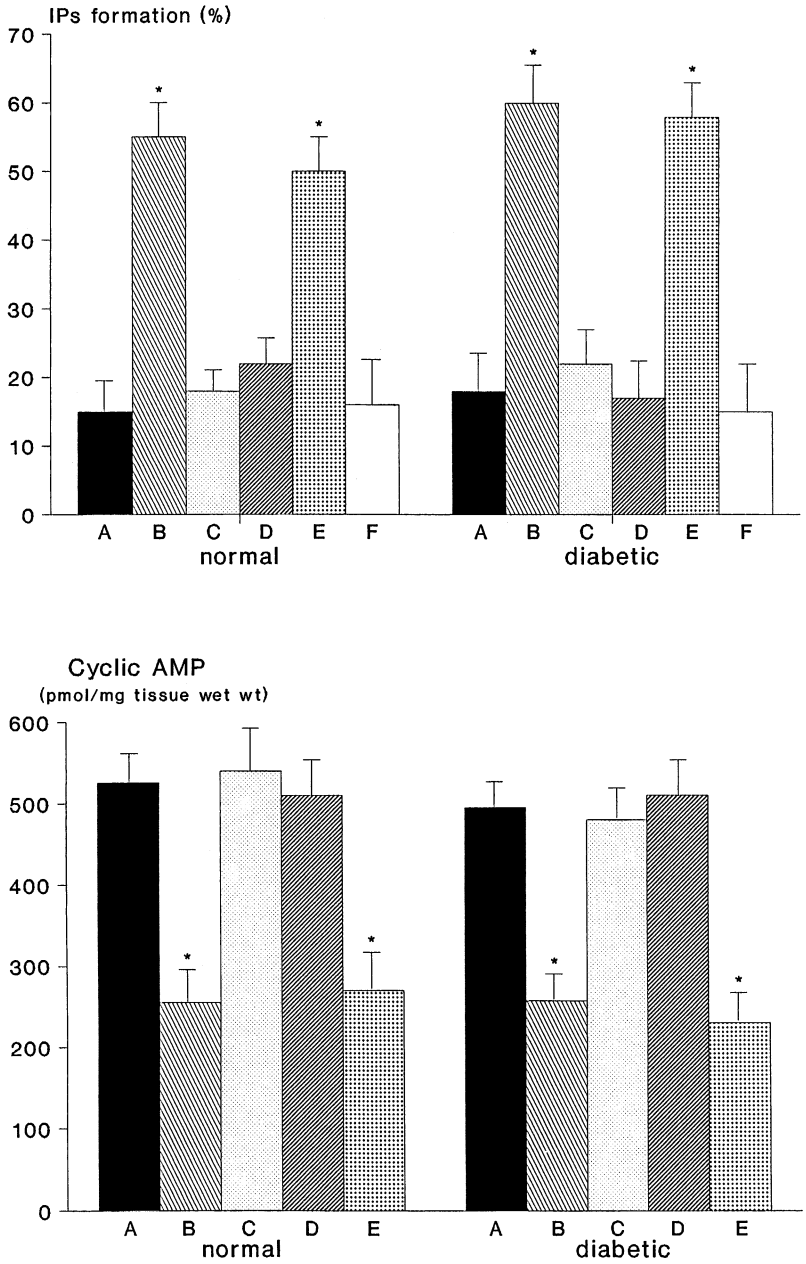


FIG. 3. Effect of carbachol upon phosphoinositide turnover (formation of IPs) (upper panel) and cAMP activity (lower panel). Atria from normal and diabetic rats were incubated for 30 min in the presence of 1×10^{-7} M of atropine (C), 1×10^{-7} M of AFD-X 116 (D), 1×10^{-6} M of pirenzepine (E), and 5×10^{-6} M of NCDC (F). Tissues were then left in the absence (A) or presence (B) of 1×10^{-7} M of carbachol. Absolute basal values of IPs expressed as area under the curve per milligram of wet weight tissue were: 135 ± 12 and 141 ± 15 for normal and diabetic atria, respectively. Atria were treated as described in Materials and Methods. Values are means ± SEM of six experiments performed in duplicate in each group. *Significantly different from basal values, $P < 0.001$.

Drugs

Freshly prepared solutions of the following drugs (purchased from the Sigma Chemical Co.) were used: carbachol, atropine, L-arginine, L-NMMA, pirenzepine, and NCDC. AFD-X 116 (11-[2-[(diethylamino)methyl]-1-piperdinyl-acetyl]-5,11-dihydro-6*H*-pyrido-[2,3-*b*][1,4]-benzodiazepine-6-one) was provided by Boehringer Ingelheim Pharmaceuticals, Inc. All concentrations quoted in the text represent the final ones in the solution.

Statistical Analysis

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

RESULTS

To study the muscarinic acetylcholine response in diabetic rat hearts, contractile assays were performed. Figure 1 shows the negative inotropism by increasing concentrations of carbachol on normal and diabetic rat atria. The potency (K_d) was greater on diabetic rat atria than on atria from normal controls, whereas the maximal effect (efficacy, E_{max}) was similar (Fig. 1). To assess which mAChR subtype was involved in the contractile action of carbachol, the effects of cholinoceptor antagonists were investigated. Atropine (10^{-7} M) and AFD-X 116 (10^{-7} M), but not

TABLE 3. Membrane PKC activity in normal and diabetic rat atria: Effects of different blockers

Additions	PKC activity (pmol/min/mg protein)	
	Normal	Diabetic
None (basal)	0.7 ± 0.1	0.9 ± 0.1
CCh	5.5 ± 0.3*	5.8 ± 0.2*
+Atropine	0.9 ± 0.1	0.8 ± 0.1
+AFD-X 116	0.5 ± 0.2	1.1 ± 0.2
+Pirenzepine	5.0 ± 0.2*	5.4 ± 0.3*
+NCDC	1.1 ± 0.1	0.8 ± 0.2

Rat atria were incubated for 30 min alone or with the muscarinic cholinergic blockers atropine, AFD-X 116 (1×10^{-7} M), pirenzepine (1×10^{-6} M), or the phospholipase C blocker NCDC (5×10^{-6} M). Incubations were continued for 10 min more in the presence of 1×10^{-7} M of carbachol. PKC activity was determined as indicated in membrane fractions. Results are the means ± SEM of four experiments performed in duplicate.

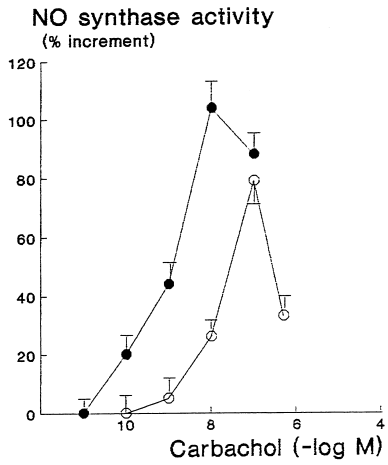
*$P < 0.05$, with respect to control basal values (none) obtained in the absence of drugs.

pirenzepine (10^{-6} M) significantly inhibited the carbachol-induced negative inotropic effect, indicating that the most important mediator in the negative inotropism induced by carbachol in both groups of atria is the M_2 mAChR subtype (Table 1). None of the drugs at the concentrations used were able to modify the basal dF/dt of normal and diabetic atria by themselves.

To determine if contractile supersensitivity of diabetic atria to carbachol is a consequence of changes in their receptors, radioligand binding assays were performed. The equilibrium parameters were similar in normal (K_d = 62 ± 3 pM, B_{max} = 425 ± 21 fmol/mg protein, N = 6) and in diabetic (K_d = 70 ± 5 pM, B_{max} = 521 ± 15 fmol/mg protein, N = 6) atria. Competitive binding assays with different antagonists confirmed that normal and diabetic atria preferentially express M_2 mAChR (Table 2).

To demonstrate the nature of the mechanism triggering the supersensitivity to carbachol in diabetic atria, we studied second messenger production in response to cholinergic stimulation of normal and diabetic atria. No significant differences between normal and diabetic rat atria were obtained in the stimulatory action of carbachol on PKC activity (Fig. 2) and the production of IPs (Fig. 3, upper panel), or in the inhibitory action of carbachol on PGE_2-stimulated cAMP production between normal and diabetic rat atria (Fig. 3, lower panel). It is noteworthy that the M_2 mAChR subtype mediated all these signal transduction events activated by carbachol (Fig. 3 and Table 3). Also, NCDC, the phospholipase C inhibitor (5×10^{-6} M), impaired the stimulatory action of carbachol on both production of IPs (Fig. 3) and PKC activity (Table 3), confirming a previous report [27].

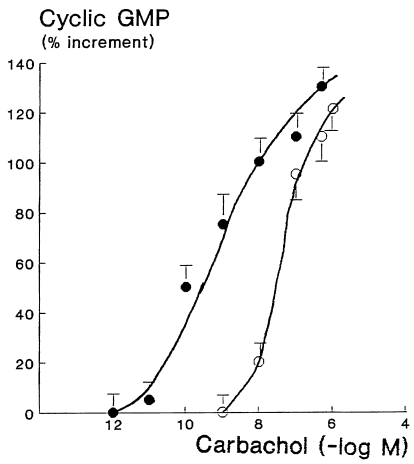
To determine if an endogenous NO signaling system participates in the cholinergic supersensitivity in diabetic atria, the actions of carbachol on NOS activity and cGMP production were assayed and compared in both normal and diabetic tissues. Figures 4 and 5 show the effect of increasing concentrations of carbachol on NOS activity (Fig. 4, left panel) and cGMP production (Fig. 5, left panel). It can be seen that carbachol induced a concentration-dependent increase in NOS activity and cGMP production, shifting to the left the agonist concentration–response curve in diabetic atria in a fashion similar to that observed in the contractile studies (Fig. 1). The inhibition of NOS by L-NMMA attenuated the muscarinic receptor-dependent activation of both second messengers, while the natural substrate L-arginine completely reversed the inhibitory



NO synthase activity
(pmol/g tissue wet wt)

Additions	Normal	Diabetic
None	345 ± 19	448 ± 21
Carbachol (CCh)	617 ± 25 *	914 ± 29 *
Atropine + CCh	358 ± 21	403 ± 23
AFD-X 116 + CCh	351 ± 19	466 ± 19
Pirenzepine + CCh	601 ± 29 *	875 ± 22 *
L-NMMA + CCh	024 ± 06 *	015 ± 09 *
L-NMMA+L-arginine+CCh	572 ± 24 *	891 ± 22 *

FIG. 4. Left panel: stimulation of NO synthase activity by increasing concentrations of carbachol on normal (–○–) and diabetic (–●–) atria. Values are given as the percentage of stimulation above basal values (see "none" in the right panel for absolute values). Right panel: NO synthase activity was measured after incubating atria with or without enzymatic inhibitors for 30 min and then for an additional 10 min with 5×10^{-7} M of carbachol under normal conditions or 5×10^{-8} M of carbachol under diabetic conditions. Results are means ± SEM 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, 1×10^{-6} M; L-NMMA, 5×10^{-5} M; and L-arginine, 5×10^{-4} M. *Significantly different from basal values, $P < 0.001$.



Cyclic GMP
(pmol/g tissue wet wt)

Additions	Normal	Diabetic
None	47.3 ± 2	42.6 ± 5
Carbachol (CCh)	95.6 ± 7 *	89.5 ± 7 *
Atropine + CCh	48.7 ± 5	46.4 ± 3
AFD-X 116 + CCh	45.0 ± 6	43.4 ± 6
Pirenzepine + CCh	94.6 ± 6 *	84.9 ± 8 *
L-NMMA + CCh	54.0 ± 7	55.4 ± 4
L-NMMA+L-arginine+CCh	94.4 ± 9 *	93.2 ± 9 *

FIG. 5. Left panel: stimulation of cGMP production by increasing concentrations of carbachol on normal (–○–) and diabetic (–●–) atria. Values are given as the percentage of stimulation above basal values (for absolute values see "none" in the right panel). Right panel: cGMP production was measured after incubating atria with or without enzymatic inhibitors for 30 min and then for an additional 10 min with 5×10^{-7} M of carbachol under normal conditions or 5×10^{-8} M of carbachol under diabetic conditions. Results are means ± SEM of five experiments performed in triplicate in each group. The final concentrations of the inhibitors were: atropine and AFD-X 116, 1×10^{-7} M; pirenzepine, 1×10^{-6} M; L-NMMA, 5×10^{-5} M; and L-arginine, 5×10^{-4} M. *Significantly different from basal values, $P < 0.001$.

action of L-NMMA on the effect of carbachol. Atropine and AFD-X 116, but not pirenzepine, inhibited the stimulatory action of carbachol on both cGMP production and NOS activity (Figs. 4 and 5, right panel).

To determine if endogenous NO and cGMP production participate in the supersensitivity to the negative inotropism triggered by carbachol in diabetic atria, inhibitors of the enzymatic pathways involved in the muscarinic receptor-dependent activation of NO and cGMP synthesis were studied. Table 4 shows that the inhibition of cGMP and NO production by methylene blue and L-NMMA shifted to the right the carbachol mechanical effect, reaching EC_{50} values that were similar in both normal and diabetic atria. Table 4 also shows that L-arginine reversed the inhibitory action of L-NMMA on the effect of carbachol.

TABLE 4. Influence of NO and cGMP inhibitors on the carbachol-induced negative inotropic effect on normal and diabetic rat atria

	EC_{50} ($\times 10^{-8}$ M)	
Addition	**Normal**	**Diabetic**
Carbachol	5.2 ± 0.2	0.45 ± 0.04
+L-NMMA	92 ± 5*	105 ± 9*
+L-NMMA + L-arginine	4.8 ± 0.2	0.70 ± 0.03
+Methylene blue	53 ± 3.1*	82.0 ± 6.2*

Atria were exposed to increasing concentrations of carbachol in the absence or presence, for 30 min, of NO and cGMP inhibitors. The EC_{50} values were obtained from those concentration–response curves. Results are means ± SEM of seven experiments in each group. The final concentrations of the drugs were: L-NMMA, 5×10^{-6} M; methylene blue, 1×10^{-5} M; and L-arginine, 5×10^{-5} M.

*Significantly different from carbachol alone, $P < 0.001$.

DISCUSSION

In this study, we examined the reactivity of short-term diabetic atria to the action of muscarinic cholinergic agonism. We used short-term STZ-induced diabetes to provide valuable information about the underlying pathophysiological changes that lead to chronic diabetic complications.

The present results demonstrated an increased ability of the acutely diabetic rat heart to respond to carbachol, pointing to a role for NO and cGMP in this response. The supersensitivity in the negative inotropic response to carbachol observed in diabetic atria contrasts with data from receptor binding studies, in which neither the affinity (K_d) nor the density (B_{max}) of the mAChR was modified.

A dissociation between the mechanical effect of the agonist and the number of receptor sites has been described previously. Carrier *et al.* [11] reported a post-junctional muscarinic supersensitivity to the negative chronotropic effect induced by cholinergic agonists, accompanied by a decrease in the atrial muscarinic receptors during diabetes. Moreover, hyperresponsiveness to the contractile effect of α- and β-adrenergic agonists was also found in isolated left atria from chronically and acutely diabetic rats [13, 28, 29], accompanied in the latter by a decrease in the number of α- and β-adrenoceptors.

The heart expresses M_2 mAChR preferentially [16]. Accordingly, in this paper, pharmacological analysis with cholinergic antagonists tends to support the hypothesis that carbachol effects on diabetic atria are also mediated by the M_2 mAChR subtype. The contrast between receptor binding data and the carbachol-induced contractile response suggests that downstream receptor levels could be involved

in the supersensitivity to the mAChR agonist in diabetic atria.

It is known that in normal rat atria the interaction between the agonist and the myocardial M_2 mAChR induces many different signal transduction pathways [16]: decreases contractility and cAMP production, and stimulates phosphoinositide-specific phospholipase C and cGMP production. The activation of NOS activity was also demonstrated. Furthermore, M_2 mAChR activation by carbachol exerts a negative inotropic effect, which is associated with an increased production of NO and cGMP [16].

Despite the supersensitivity to the negative inotropic response to the muscarinic agonist observed in diabetic atria, there was no difference in muscarinic activation of PKC activity, stimulation of IPs, or cAMP formation [30, 31]. The unique biochemical change in diabetic atria was the muscarinic activation of NOS and cGMP formation that were enhanced. However, the basal rate of NOS activity is higher in diabetic atria than in normal atria, although the basal production of cGMP is not different. This selective difference suggests that the biochemical alteration is at the NOS level rather than at the downstream guanylate cyclase level. An increment in endothelial NOS activity in diabetic rat heart has been observed [32]. Also, hyperreactivity to both acetylcholine and sodium nitroprusside in forearm vasculature in patients with insulin-dependent diabetes mellitus has been reported [33].

The fact that the supersensitivity to the negative inotropic response to carbachol in diabetic hearts was reversed by the inhibition of NOS and guanylate cyclase activities strongly suggests the participation of NO and cGMP overproduction in this phenomenon. NO- and cGMP-mediated supersensitivity is most relevant at lower than at higher concentrations of carbachol, because the maximal effect was similar in both normal and diabetic rat atria. This is consistent with reports suggesting that the NO-cGMP-mediated pathway predominates at low concentrations of carbachol, while more than one signaling cascade accounts for the maximal inotropic effect of muscarinic agonists in rat atria [16].

Although it has been reported that ventricular myocytes produce endogenous NO [34], we cannot rule out that a constitutive NOS from the endothelium contributes to the effect of carbachol in isolated rat atria. Moreover, NO production mediates the effects of muscarinic agonists on ventricular myocytes [34] and cholinergic inhibition of L-type calcium currents in mammalian cardiac pacemaker tissue [35]. It has been proposed that some of the physiological effects of NO on myocardial contractile behavior appear to be mediated by activation of guanylate cyclase [16, 36]. Also, cGMP is a mediator of the reduction of L-type calcium current induced by mAChR stimulation and by NO [37].

It is known that the mechanism by which mAChR increases cGMP production appears to occur secondarily to the stimulation of phosphoinositide turnover via phospholipase C activation. This, in turn, triggers a cascade of reactions involving calcium-calmodulin and PKC, leading to activation of NOS with activation of soluble guanylate cyclase by NO [16].

Our results indicate that even though the stimulatory action of carbachol increases the production of IPs and PKC activity to the same extent in normal and diabetic conditions, it is possible that in the diabetic heart an additional mechanism could be involved in the overactivation of NOS. A factor to take into account is the movement of calcium. Diabetes is associated with significant alterations in myocardial calcium homeostasis [14, 38–40]. These abnormalities with respect to calcium handling may lead to the occurrence of intracellular calcium overload. In rat atria, calcium-calmodulin and PKC stimulation are required for carbachol activation of the NO-cGMP-mediated pathway [16]. It is tempting to speculate that the increase in intracellular levels of calcium could activate the calcium-calmodulin-dependent NOS, which, in turn, triggers the signaling events that lead to the supersensitivity to cholinergic agonists observed in the acute diabetic state.

The authors gratefully acknowledge the technical help of Mrs. Elvita Vanucchi. This work was performed with the support of grants from CONICET-PID 3348PEI and UBACYT 0147/97 ME 056.

References

1. Garcia MJ, McNamara PM, Gordon T and Kannell WB, Morbidity and mortality in diabetics in the Framingham population: Sixteen year follow-up study. *Diabetes* **23:** 105–111, 1974.
2. Jarrett RJ, Cardiovascular disease and hypertension in diabetes mellitus. *Diabetes Metab Rev* **5:** 547–558, 1989.
3. Ewing DJ, Campbell IW and Clarke BF, Assessment of cardiovascular effects in diabetic autonomic neuropathy and prognostic implications. *Ann Intern Med* **92:** 308–311, 1980.
4. Fein FS and Sonnenblick EH, Diabetic cardiomyopathy. *Cardiovasc Drugs Ther* **8:** 65–73, 1994.
5. Bell DS, Diabetic cardiomyopathy. A unique entity or a complication of coronary artery disease? *Diabetes Care* **18:** 708–714, 1995.
6. Tomlinson KC, Gardiner SM and Bennett T, Diabetes mellitus in Brattleboro rats: Cardiovascular, fluid and electrolyte status. *Am J Physiol* **256:** R1279–R1285, 1989.
7. Tomlinson KC, Gardiner SM, Hebden RA and Bennett T, Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. *Pharmacol Rev* **44:** 103–150, 1992.
8. Vadlamudi RVSV and McNeill JH, Effect of alloxan- and streptozotocin-induced diabetes on isolated rat heart responsiveness to carbachol. *J Pharmacol Exp Ther* **225:** 410–415, 1983.
9. Li XS, Tanz RD and Chang KSK, Effect of age and methacholine on the rate and coronary flow of isolated hearts of diabetic rats. *Br J Pharmacol* **97:** 1209–1217, 1989.
10. Wong KK, Cardiac response to autonomic drugs in atria of diabetic mouse. *J Pharm Pharmacol* **46:** 513–514, 1994.
11. Carrier GO, Edwards AD and Aronstam RS, Cholinergic supersensitivity and decreased number of muscarinic receptors

in atria from short-term diabetic rats. *J Mol Cell Cardiol* **16:** 963–965, 1984.
12. Kofo-Abayomi A and Lucas PD, Muscarinic receptor density is reduced in diabetic rat atria, an effect prevented by the aldose reductase inhibitor, Statil. *J Pharm Pharmacol* **39:** 1019–1021, 1987.
13. Wald M, Pascual J and Sterin-Borda L, Role of thromboxanes in alterations of the diabetic β-adrenergic system. *Biochem Pharmacol* **38:** 3347–3355, 1989.
14. Borda E, Pascual J, Wald M and Sterin-Borda L, Hypersensitivity to calcium associated with an increased sarcolemmal Ca^{2+}-ATPase activity in diabetic rat heart. *Can J Cardiol* **4:** 97–101, 1988.
15. Van Rossum JM, Cumulative dose–response curves. II. Technique for the making of dose–response curves in isolated organs and the evaluation of drug parameters. *Arch Int Pharmacodyn Ther* **143:** 299–330, 1963.
16. Sterin-Borda L, Vila Echague A, Perez Leiros C, Genaro A and Borda E, Endogenous nitric oxide signalling system and the cardiac muscarinic acetylcholine receptor-inotropic response. *Br J Pharmacol* **115:** 1525–1531, 1995.
17. Goin JC, Borda ES, Perez-Leiros C, Storino R and Sterin-Borda L, Identification of antibodies with muscarinic cholinergic activity in human Chagas disease: Pathological implications. *J Auton Nerv Syst* **47:** 45–52, 1994.
18. Mundson PJ and Rodbard D, LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107:** 220–239, 1980.
19. Wald MR, Borda ES and Sterin-Borda L, Mitogenic effect of erythropoietin on neonatal rat cardiomyocytes: Signal transduction pathways. *J Cell Physiol* **167:** 461–468, 1996.
20. Genaro AM and Boscá L, Early signals in alloantigen induced B-cell proliferation. Comparison between B-cell triggering by intact allogeneic cells and solubilized alloantigen. *J Immunol* **151:** 1832–1843, 1993.
21. Berridge MJ, Downes CP and Hauley MR, Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem J* **206:** 587–595, 1982.
22. Hokin-Neaverson M and Sadeghian K, Separation of [^{3}H] inositol monophosphates and [^{3}H] inositol on silica gel glass-fiber sheets. *J Chromatogr* **120:** 502–505, 1976.
23. Bredt DS and Snyder SH, Nitric oxide mediates glutamate-linked enhancement of cyclic GMP levels in the cerebellum. *Proc Natl Acad Sci USA* **86:** 9030–9033, 1989.
24. Brown BL, Albano JDM, Elkins RP and Sherzi AM, A simple and sensitive saturation assay method for the measurement of adenosine 3′,5′-cyclic monophosphate. *Biochem J* **121:** 561–562, 1971.
25. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurements with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
26. Cheng Y and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:** 3099–3108, 1973.
27. Sterin-Borda L, Cremaschi G, Genaro AM, Vila Echague A, Goin JC and Borda E, Involvement of nitric oxide synthase and protein kinase C activation on chagasic antibodies action upon cardiac contractility. *Mol Cell Biochem* **161:** 75–82, 1996.
28. Jackson CV, McGrath GM and McNeill JH, Alterations in alpha 1-adrenoceptor stimulation of isolated atria from experimental diabetic rats. *Can J Physiol Pharmacol* **64:** 145–151, 1986.
29. Wald M, Borda ES and Sterin-Borda L, α-Adrenergic supersensitivity and decreased number of α-adrenoceptors in heart from acute diabetic rats. *Can J Physiol Pharmacol* **66:** 1154–1160, 1988.
30. Bergh CH, Hjalmarson A, Sjogren KG and Jacobsson B, The effect of diabetes on phosphatidylinositol turnover and calcium influx in myocardium. Horm Metab Res **20:** 381–386, 1988.
31. Wichelhaus A, Russ M, Petersen S and Eckel J, G protein expression and adenylate cyclase regulation in ventricular cardiomyocytes from streptozotocin-diabetic rats. *Am J Physiol* **267:** H548–H555, 1994.
32. Rosen P, Ballhausen T and Stockklauser K, Impairment of endothelium dependent relaxation in the diabetic rat heart: Mechanisms and implications. *Diabetes Res Clin Pract* **31** Suppl**:** S143–S155, 1996.
33. Makimattila S, Mantysaari M, Groop PH, Summanen P, Virkamaki A, Schlezka A, Fagerudd J and Yki-Jarvinen H, Hyperreactivity to nitrovasodilators in forearm vasculature is related to autonomic dysfunction in insulin-dependent diabetes mellitus. *Circulation* **95:** 618–625, 1997.
34. Balligand JL, Kelly RA, Marsden PA, Smith TW and Michel T, Control of cardiac muscle cell function by an endogenous nitric oxide signalling system. *Proc Natl Acad Sci USA* **90:** 347–351, 1993.
35. Han X, Shimoni Y and Giles WR, An obligatory role for nitric oxide in autonomic control of mammalian heart rate. *J Physiol* (*Lond*) **476:** 309–314, 1994.
36. Grocottmason R, Anning P, Evans H, Lewis MJ and Shah AM, Modulation of left ventricular relaxation in isolated ejecting heart by endogenous nitric oxide. *Am J Physiol* **36:** H1804–H1813, 1994.
37. Levi RC, Alloatti G, Penna C and Gallo MP, Guanylate-cyclase-mediated inhibition of cardiac ICa by carbachol and sodium nitroprusside. *Pflugers Arch* **426:** 419–426, 1994.
38. Golfman LS, Takeda N and Dhalla NS, Cardiac membrane calcium-transport in alloxan-induced diabetes in rats. *Diabetes Res Clin Pract* **31** Suppl**:** S73–S77, 1996.
39. Lagadic-Gossmann D, Buckler KJ, Le-Prigent K and Feuvray D, Altered calcium handling in ventricular myocytes isolated from diabetic rats. *Am J Physiol* **270:** H1529–H1537, 1996.
40. Yu JZ, Quamme GA and McNeill JH, Altered $[Ca^{2+}]_i$ mobilization in diabetic cardiomyocytes: Responses to caffeine, KCl, ouabain and ATP. *Diabetes Res Clin Pract* **30:** 9–20, 1995.