British Journal of Pharmacology (2009), 156, 575–586

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Activation of muscarinic receptors elicits inotropic responses in ventricular muscle from rats with heart failure through myosin light chain phosphorylation

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Background and purpose: Muscarinic stimulation increases myofilament Ca^{2+} sensitivity with no apparent inotropic response in normal rat myocardium. Increased myofilament Ca^{2+} sensitivity is a molecular mechanism promoting increased contractility in failing cardiac tissue. Thus, muscarinic receptor activation could elicit inotropic responses in ventricular myocardium from rats with heart failure, through increasing phosphorylation of myosin light chain (MLC).
Experimental approach: Contractile force was measured in left ventricular papillary muscles from male Wistar rats, 6 weeks after left coronary artery ligation or sham surgery. Muscles were also frozen, and MLC-2 phosphorylation level was quantified.
Key results: Carbachol (10 $\mu mol \cdot L^{-1}$) evoked a positive inotropic response only in muscles from rats with heart failure approximating 36% of that elicited by 1 $\mu mol \cdot L^{-1}$ isoproterenol (20 $\pm$ 1.5% and 56 $\pm$ 6.1% above basal respectively). Carbachol-evoked inotropic responses did not correlate with infarction size but did correlate with increased left ventricular end diastolic pressure, heart weight/body weight ratio and lung weight, primary indicators of the severity of heart failure. Only muscarinic receptor antagonists selective for M_2 receptors antagonized carbachol-mediated inotropic effects with the expected potency. Carbachol-evoked inotropic responses and increase in phosphorylated MLC-2 were attenuated by MLC kinase (ML-9) and Rho-kinase inhibition (Y-27632), and inotropic responses were abolished by *Pertussis* toxin pretreatment.
Conclusion and implications: In failing ventricular muscle, muscarinic receptor activation, most likely via M_2 receptors, provides inotropic support by increasing MLC phosphorylation and consequently, myofilament Ca^{2+} sensitivity. Enhancement of myofilament Ca^{2+} sensitivity, representing a less energy-demanding mechanism of inotropic support may be particularly advantageous in failing hearts.
British Journal of Pharmacology (2009) **156**, 575–586; doi:10.1111/j.1476-5381.2009.00016.x; published online 21 January 2009

Keywords: signal transduction; contractility; ventricular function; congestive heart failure; Rho-kinase; *Pertussis* toxin

Abbreviations: HF+, myocardial infarction with heart failure; HF–, myocardial infarction without heart failure; LVEDP, Left ventricular end diastolic pressure; MLC, Myosin light chain; RT, relaxation time; ROCK, RhoA/Rho-associated kinase; TPF, time to peak force; TR80, time to 80% relaxation

Introduction

According to the classical view, the parasympathetic nervous system regulates heart rate and contractility through cardiac muscarinic receptor activation (Brodde and Michel, 1999). In ventricular myocardium, the muscarinic M_2 receptor (receptor nomenclature conforms to Alexander *et al.*, 2008) inhibits receptor-mediated activation of adenylyl cyclase (Felder, 1995), accounting for the indirect negative inotropic response in the heart observed after pre-stimulation with a β-adrenoceptor agonist (Giessler *et al.*, 1999).

Cardiac muscarinic receptors can also elicit direct inotropic responses. Imai and Ohta (1988) and Eglen *et al.* (1988) reported a carbachol-evoked inotropic response in rat atria mediated by the breakdown of phosphatidylinositol. Inotropic responses after carbachol stimulation of M_2 receptors have also been reported in both guinea pig left ventricular papillary muscles (Korth and Kuhlkamp, 1987; Eglen *et al.*, 1988) and in developing chick ventricle (Nouchi *et al.*, 2007). Higher concentrations of carbachol (>10 $\mu mol \cdot L^{-1}$) are also reported to produce an inotropic response in normal human ventricular trabeculae (Du *et al.*, 1994).

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Received 26 June 2008; revised 20 August 2008; accepted 1 September 2008

Although the cAMP-independent mechanism mediating the muscarinic inotropic responses cited above remains unclear, a role for myosin light chain (MLC) phosphorylation seems likely. Contractility in smooth muscle is dependent upon the phosphorylation status of MLC, which alters the Ca^{2+} sensitivity of myofilaments (Hirano *et al.*, 2004). The MLC phosphorylation status is regulated primarily by the balance between the activity of MLC kinase and MLC phosphatase (Hirano *et al.*, 2003).

As in smooth muscle, also in normal cardiomyocytes, increasing Ca^{2+} sensitivity of the myofilaments through increased MLC-2 phosphorylation has been reported to be an important molecular mechanism of increasing contractility, following activation of α_1-adrenoceptors in rat ventricle (Andersen *et al.*, 2002) and human atrium (Grimm *et al.*, 2005), urotensin-II receptors in human atria (Russell and Molenaar, 2004) and endothelin receptors in rat ventricle (Rossmanith *et al.*, 1997). In normal rat myocardium, muscarinic stimulation by the agonist carbachol increases myofilament Ca^{2+} sensitivity with no apparent corresponding inotropic response (McIvor *et al.*, 1988; Puceat *et al.*, 1990).

Interestingly, stimulation of rat myocardial 5-HT_{2A} receptors elicited an inotropic response through increasing MLC-2 phosphorylation in left ventricle of failing hearts but not in normal hearts (Qvigstad *et al.*, 2005b; Birkeland *et al.*, 2007). Likewise, myofibrillar Ca^{2+} sensitivity is increased in failing canine heart through up-regulation of α_1-adrenoceptor signalling (Suematsu *et al.*, 2001). Possibly, enhancement of myofilament Ca^{2+} sensitivity through increased phosphorylation of MLC is a compensatory mechanism offering contractile support in failing myocardium. Thus, we hypothesized that the reported muscarinic receptor-mediated increase of myofilament Ca^{2+} sensitivity (McIvor *et al.*, 1988; Puceat *et al.*, 1990) may be enhanced in failing ventricle to such an extent that an inotropic response may be detected. Here, we report for the first time that activation of muscarinic receptors, most likely M_2 receptors, increases contractility in failing but not sham-operated left ventricle. The mechanism of the increased contractility involves increased MLC-2 phosphorylation, analogous to that observed for α_1-adrenoceptor signalling.

Methods

The experiments and animal care were in accordance with 'Regulations on Animal Experimentation' under The Norwegian Animal Welfare Act and were approved by the Norwegian Animal Research Authority. Animals were housed in a temperature-regulated room with a 12 h/12 h light/dark cycle (two animals per cage) and given access to food and water *ad libitum*.

Induction of myocardial infarction and congestive heart failure
Male Wistar rats (Møllegaard Breeding and Research Centre, Skensved, Denmark) weighing about 320 g, were intubated and ventilated with 68% N_2O, 29% O_2 and 2–3% isoflurane (Abbot Scandinavia, Solna, Sweden). An extensive myocardial infarction was induced by proximal ligation of the left coronary artery. Echocardiography was performed 5 weeks later. Six weeks after infarction, the rats were anaesthetized and ventilated with isoflurane, and the left ventricular pressures were measured as previously described (Sjaastad *et al.*, 2000). Rats were included in the myocardial infarction with heart failure group (HF+) if left ventricular end diastolic pressure (LVEDP) was $\geq$15 mm Hg, whereas rats with LVEDP < 15 mm Hg were included in the myocardial infarction without heart failure group (HF–). Sham-operated animals (Sham) were subjected to the same surgical procedures, without the ligation of the coronary artery. In a subset of rats, *Pertussis* toxin was administered at a dose of 30 $\mu g \cdot kg^{-1}$, i.p. 3 days prior to isolation of the muscles. Data from animals treated with *Pertussis* toxin were included only if carbachol inhibition of the β-adrenoceptor-mediated inotropic response was completely abolished.

Isolated papillary muscles
Posterior left ventricular papillary muscles and strips of left ventricles (diameter ~1.0 mm) were prepared, mounted in 31°C organ baths containing physiological salt solution with 1.8 $mmol \cdot L^{-1}$ Ca^{2+}, equilibrated and field-stimulated at 1 Hz (Skomedal *et al.*, 1982). Contraction–relaxation cycles were recorded and analysed as previously described (Sjaastad *et al.*, 2003; Qvigstad *et al.*, 2005b) with respect to time to peak force (TPF), time to 80% relaxation (TR80) and the relaxation time (RT) defined as TR80-TPF. Lusitropic responses were expressed as decrease in RT. Basal contractility was expressed as maximal developed force (F_{max}, mN). Inotropic responses to agonists were expressed as changes in the maximal development of force $[(dF/dt)_{max}]$. The values after agonist responses were expressed as a percentage of the control period (set to 100%) before the addition of kinase inhibitors. Blockers of α_1-adrenoceptors (prazosin 1 $\mu mol \cdot L^{-1}$) and β-adrenoceptors (timolol 1 $\mu mol \cdot L^{-1}$) were added 90 min before the muscarinic agonist, carbachol. Carbachol was added to the organ bath cumulatively (concentration–response curves) or as a bolus (10 $\mu mol \cdot L^{-1}$). The apparent inhibition constant K_b for the selective muscarinic receptor antagonists was calculated from ratios of carbachol EC_{50} values, with and without the antagonists. The muscarinic antagonists did not influence basal contraction–relaxation cycle characteristics or electrical stimulation threshold (not shown). Concentration–response curves were constructed by estimating centiles (EC_{10} to EC_{100}) and calculating the corresponding means, and the horizontal positioning is expressed as $-\log EC_{50}$ values (Sjaastad *et al.*, 2003).

Receptor binding assay
Papillary muscles snap frozen in liquid nitrogen were crushed to powder and placed into a microcentrifuge tube containing ~0.5 mL of ice cold 50 $mmol \cdot L^{-1}$ Tris-HCl (pH 7.5 at 20°C), 1 $mmol \cdot L^{-1}$ EDTA, 10 $nmol \cdot L^{-1}$ $MgCl_2$ with protease inhibitors (100 $\mu mol \cdot L^{-1}$ phenylmethylsulphonyl fluoride and 100 $\mu mol \cdot L^{-1}$ phenanthroline) and homogenized with an Ultra-Turrax homogenizer (5 × 10 s bursts). An equal volume of ice cold 1 M KCl was added to the homogenate, mixed and placed on ice for 10 min. The homogenate was centrifuged at 20 000× *g* for 12 min at 4°C. The membrane pellets were

resuspended in ice cold 50 mmol·L^{-1} Tris-HCl (pH 7.5 at 20°C), 1 mmol·L^{-1} EDTA buffer containing protease inhibitors and rehomogenized by an Ultra-Turrax at maximum speed (this procedure was repeated twice). The membrane preparation was then filtered through a nylon mesh (60 µmol·L^{-1} pore size) and used immediately for the binding assay. Affinity (pK_d) and receptor density (B_{max}) was determined from equilibrium binding analysis of the non-selective muscarinic antagonist L-quinuclidinyl[*phenyl*-4-^{3}H]benzilate ([^{3}H]QNB) (specific activity of 42 Ci·mmol^{-1}; GE Healthcare, Buckinghamshire, England) binding to each membrane preparation. Membranes were incubated with increasing concentrations of [^{3}H]QNB in the absence (total binding) or presence (non-specific binding) of 1 µmol·L^{-1} atropine for 2 h at 24°C (Myslivecek *et al*., 2003).

MLC-2 phosphorylation

MLC-2 phosphorylation at Ser-15 was determined in clamp frozen papillary muscles or ventricular strips as previously described by others (Hathaway and Haeberle, 1985). Briefly, non-phosphorylated and phosphorylated MLC-2 were separated by using glycerol polyacrylamide gel electrophoresis, and both forms of MLC-2 were identified with a mouse monoclonal antibody that recognizes rat and human ventricular MLC-2 (amino acid residues 45–59). To control for protein loading, MLC-2 phosphorylation is reported as a percentage of total MLC-2 within the same sample. The effect of the MLC kinase (ML-9) and Rho-kinase (Y-27632) inhibitors upon carbachol-stimulated MLC-2 phosphorylation was determined on ventricular strips taken from the same heart. The reported data were normalized to remove variability in differences of basal MLC-2 phosphorylation between hearts.

Statistics

Data are expressed as mean ± SEM. $P < 0.05$ was considered statistically significant (one way ANOVA with Bonferroni corrections made for multiple comparisons).

Drugs and solutions

We used anti-myosin mouse monoclonal antibody (1150-S; Biocytex, Marseille, France) and the secondary antibody ECL Mouse IgG, HRP-Linked Whole Ab (from sheep; NA931, GE Healthcare, Oslo, Norway). 1-(5-chloronaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-9) was from Sigma-Aldrich (St. Louis, Mo., USA). (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide dihydrochloride (Y-27632), 2-diethylaminoethyl 1-(4-nitrophenyl) cyclopentanecarboxylate hydrochloride (nitrocaramiphen), 5,11-dihydro-11-[(4-methyl-1-piperazinyl)acetyl]-6*H*-pyrido [2,3-b][1,4]benzodiazepin-6-one dihydrochloride (pirenzepine), 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b][1,4]benzodiazepin-6-one (AF-DX 116), *N*-[2-[2-[(dipropylamino)methyl]-1-piperidinyl] ethyl]-5,6-dihydro-6-oxo-11*H*-pyrido[2,3-b][1,4] benzodiazepine-11-carboxamide (AF-DX 384) and 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) were from Tocris (Bristol, UK). *Pertussis* toxin was from Merck Chemicals (Nottingham, UK).

Results

Animal characteristics

All rats in the HF+ group had large anterolateral infarctions and signs of congestion, including tachypnea, pleural effusion and increased lung weight. The rats in the HF– group had infarctions of variable sizes, no signs of congestion, and the lung weight was normal. Animal characteristics and haemodynamic data at 6 weeks after infarction are given in Table 1.

Inotropic responses of the muscarinic agonist carbachol

Administration of 10 µmol·L^{-1} carbachol elicited an initial, transient, negative inotropic response followed by a sustained positive inotropic response [$(dF/dt)_{max}$ 20 ± 1.5% above basal, $n = 14$] in the papillary muscles excised from HF+ rats (Figures 1B and 2A). The magnitude of the positive inotropic response elicited by carbachol was ~36% of the maximum inotropic response of 1 µmol·L^{-1} isoproterenol (56 ± 6.1% above basal, $n = 8$) in HF+ rats (Figure 2B). Administration of atropine (1 µmol·L^{-1}) completely reversed the carbachol-evoked positive inotropic response to basal values, indicating the response is mediated through muscarinic receptors (Figure 1B). The time from carbachol addition to 50% and to the maximal positive inotropic response was 122 ± 3 s and ~5 min respectively (Figure 1C). In papillary muscles from HF– rats, 10 µmol·L^{-1} carbachol also mediated a biphasic ino-

Table 1 Animal and papillary muscle characteristics

	Sham (n = *14*)	*HF–* (n = *22*)	*HF+ (n = 57)*
Body weight, g	395 ± 7	390 ± 6	379 ± 5
Heart weight, g	1.46 ± 0.04	1.83 ± 0.06*	2.36 ± 0.05**
Heart weight/body weight, g·kg^{-1}	3.7 ± 0.1	4.5 ± 0.1*	6.3 ± 0.1**
LVEDP, mm Hg	2.9 ± 0.5	7.2 ± 0.9	22.6 ± 1.0**
LVSP, mm Hg	102 ± 6	103 ± 5	94 ± 2
Lung weight, g	1.43 ± 0.03	1.57 ± 0.03	3.81 ± 0.12**
Basal F_{max}, mN·mm^{-2}	5.0 ± 0.5	4.8 ± 0.6	6.1 ± 0.6[a]

LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; F_{max}, maximal developed force.
[a]$n = 20$.
*HF– vs. Sham, $P < 0.05$.
**HF+ vs. HF– and Sham, $P < 0.05$ (one-way ANOVA with Bonferroni correction for multiple comparisons).

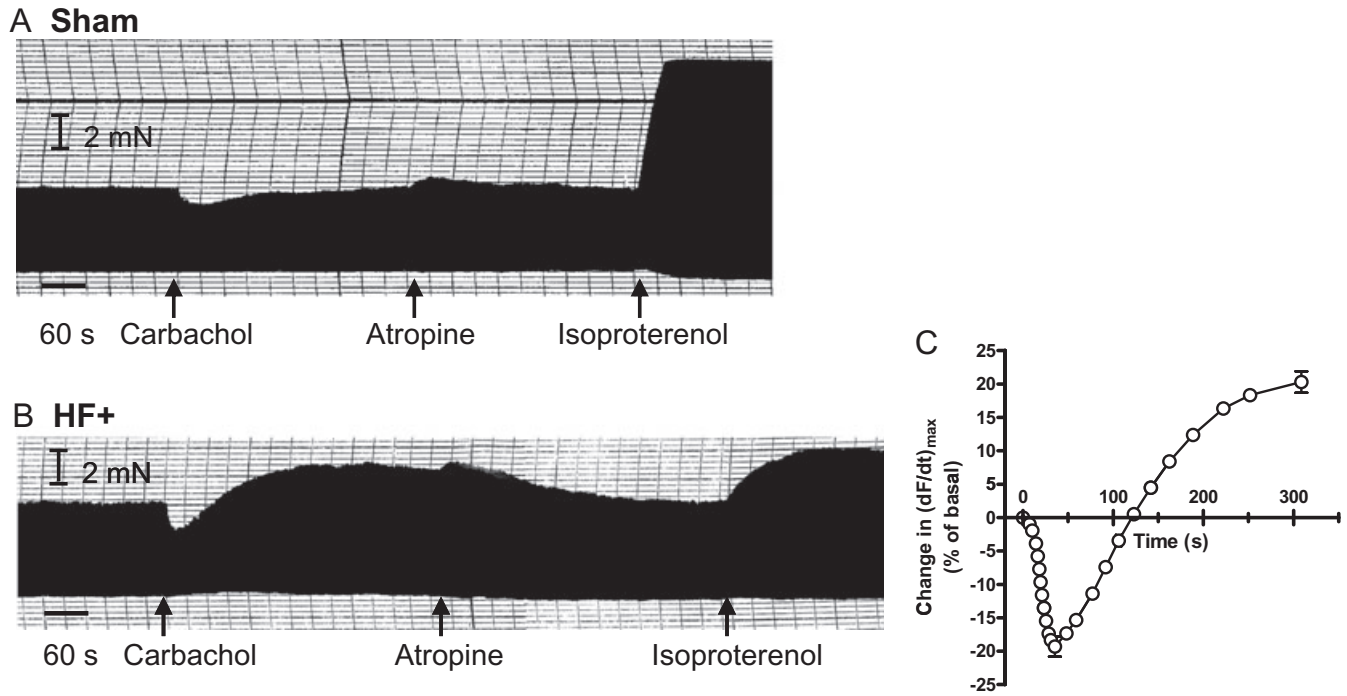

Figure 1 Extracts of representative original recordings of the inotropic effects of carbachol (10 μmol·L^{-1}) observed in Sham (A) or HF+ (B) papillary muscle. Atropine (1 μmol·L^{-1}) fully reversed the inotropic effect of carbachol. (C) Mean time course showing the development of the carbachol-induced inotropic response in HF+ papillary muscle ($n = 8$). Data are shown as the percentage change of $(dF/dt)_{max}$ relative to basal.

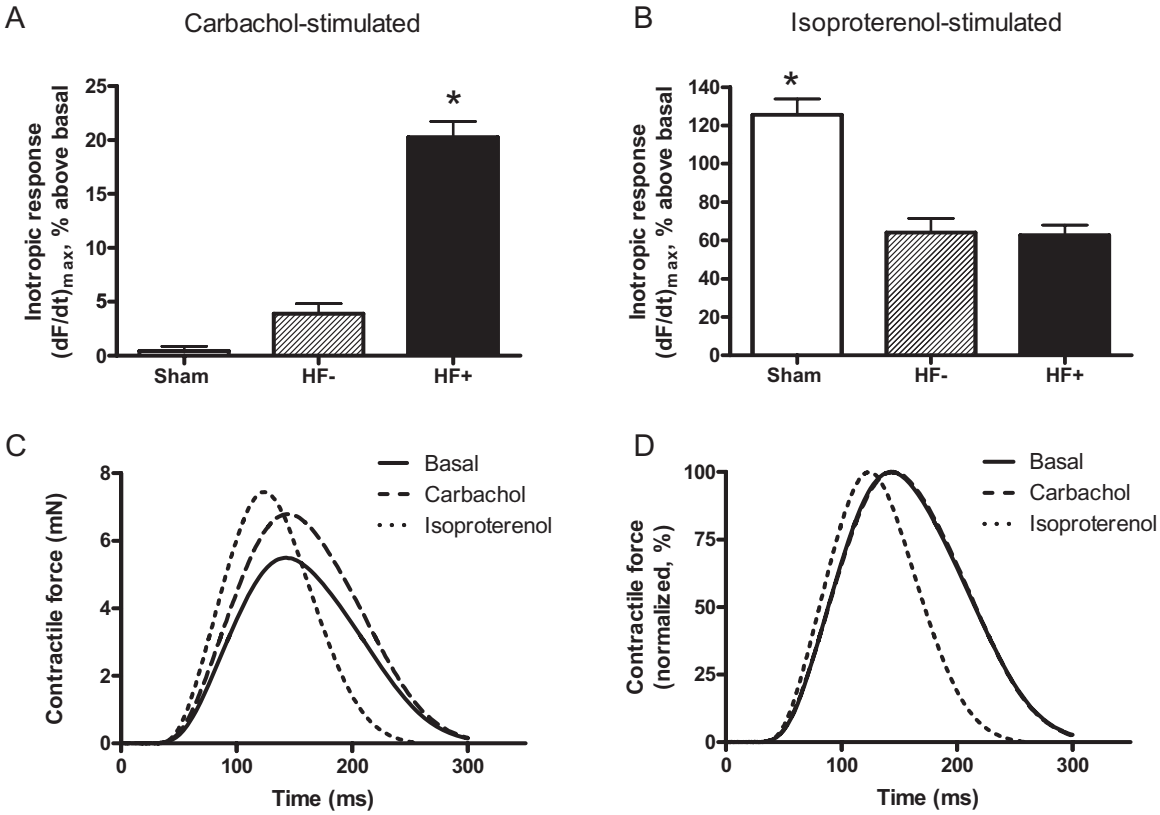

Figure 2 Inotropic response [increase in $(dF/dt)_{max}$] induced by (A) carbachol (10 μmol·L^{-1}) in Sham ($n = 7$), HF– ($n = 22$) and HF+ ($n = 14$), and (B) isoproterenol [10 μmol·L^{-1} in Sham ($n = 14$) and 1 μmol·L^{-1} in HF– ($n = 14$) and HF+ ($n = 10$)]. (C,D) Representative averaged contraction–relaxation cycles in papillary muscles taken from rat left ventricle with HF+ before and after administration of 10 μmol·L^{-1} carbachol and 1 μmol·L^{-1} isoproterenol expressed as developed force to illustrate the inotropic effect (C) and normalized to maximal force (100%) to illustrate the lusitropic effect (D). *$P < 0.05$.

Table 2 Characteristics of contraction–relaxation cycles in Sham and HF+ papillary muscles after addition of carbachol (10 μmol·L^{-1}) or isoproterenol (10 μmol·L^{-1} in Sham, 1 μmol·L^{-1} in HF+)

		Basal (ms)	*Carbachol (ms)*	*Carbachol (% of basal)*	*Basal (ms)*	*Isoproterenol (ms)*	*Isoproterenol (% of basal)*
Sham	TPF	102 ± 3	101 ± 3	99 ± 4	100 ± 2	92 ± 2*	86 ± 6
	TR80	191 ± 4	190 ± 6	100 ± 8	190 ± 2	150 ± 7*	79 ± 3
	RT	89 ± 3	89 ± 3	100 ± 4	89 ± 1	63 ± 1*	66 ± 4
HF+	TPF	142 ± 3	143 ± 3	100 ± 4	138 ± 2	113 ± 2*	84 ± 1
	TR80	246 ± 4	247 ± 4	100 ± 6	234 ± 7	183 ± 4*	78 ± 1
	RT	104 ± 2	104 ± 2	100 ± 2	100 ± 4	70 ± 2*	71 ± 1

Data were obtained from Sham ($n = 9$) or HF+ ($n = 14$) papillary muscles and represent the average results of 15–35 consecutive contraction–relaxation cycles. TPF, time to peak force; TR80, time to 80% relaxation; RT, relaxation time (TR80-TPF).
*$P < 0.05$ vs. basal.

tropic response, but the positive inotropic response was only ~20% (3.9 ± 0.9% above basal, $n = 22$) of those with HF+ (Figure 2A). In contrast, the magnitude of the isoproterenol-evoked positive inotropic response did not differ between HF– and HF+ animals but was reduced by 50% compared with Sham rats (Figure 2B). In papillary muscles from Sham rats, carbachol (10 μmol·L^{-1}) did not affect the baseline contraction after the initial transient negative inotropic response (Figures 1A and 2A).

Characteristics of the contraction–relaxation cycle

The time-course characteristics of the contraction–relaxation cycle were not significantly changed by carbachol in Sham, HF– or HF+ animals demonstrating a lack of a lusitropic effect (Table 2). As expected, β-adrenoceptor stimulation with isoproterenol produced a large lusitropic effect (reduced TPF & RT), a characteristic of effects mediated through activation of the cAMP/PKA pathway (Bers, 2002) (Table 2). The lusitropic effect was not attenuated in HF+. The lack of shortening of the contraction–relaxation cycle by carbachol is a characteristic shared with inotropic responses mediated by $G_{q/11}$-coupled receptors such as the α_1-adrenoceptor and 5-HT_{2A} receptors (Skomedal *et al.*, 1997; Qvigstad *et al.*, 2005b).

The carbachol-evoked inotropic response correlated well with parameters indicative of congestive heart failure, but not infarction size

The increased LVEDP, heart weight/body weight ratio, lung weight/body weight ratio and TPF, all primary indicators of congestive heart failure, correlated well with the magnitude of the carbachol-evoked inotropic response (Figure 3A,B,C). In the HF– group the carbachol-evoked inotropic response correlated poorly with infarction size (Figure 3D). These data suggest that the emergence of the carbachol-mediated inotropic response is associated with pathophysiological changes accompanying heart failure.

The inotropic response to carbachol appears to be mediated through the M_2 muscarinic receptor

To determine the muscarinic receptor subtype mediating the carbachol-evoked inotropic response, we determined the ability of several muscarinic antagonists to shift the concentration–response curve to carbachol. The following relatively selective antagonists were used: pirenzepine and nitrocaramiphen (M_1 receptor), AF-DX 116 and AF-DX 384 (M_2 receptor) and the $M_{3/5}$ receptor antagonist 4-DAMP. As shown in Figure 4, only the M_2 selective antagonists (AF-DX 116 and AF-DX 384) produced the expected shift (based on binding affinities) to higher values of the EC_{50} for carbachol ($-\log EC_{50}$ values of 5.68 ± 0.12, $n = 7$ with AF-DX 116; 5.05 ± 0.09, $n = 6$, with AF-DX 384 and 6.28 ± 0.10, $n = 9$ without antagonist). The estimated inhibition constant (pK_b) was 7.3 ± 0.1 for AF-DX 116 and 8.5 ± 0.1 for AF-DX 384, values consistent with those reported at M_2 receptors (7.1–7.2 and 8.2–9.0 respectively) (Caulfield and Birdsall, 1998). Whereas the antagonists nitrocaramiphen (M_1) and 4-DAMP ($M_{3/5}$) were without effect, the M_1 antagonist pirenzepine shifted the concentration–response curve to higher concentrations of carbachol ($-\log EC_{50} = 5.77 \pm 0.14$, $n = 6$). However, the estimated pK_b for pirenzepine was 7.5 ± 0.1, a value lower than that reported for the M_1 receptor (7.8–8.5) but too high if it were acting through the M_2 receptor (6.3–6.7) (Caulfield and Birdsall, 1998).

Pertussis *toxin inactivation of G_i abolished the carbachol inotropic effect*

The M_2 receptor is the dominant muscarinic receptor subtype in the heart, known to couple predominantly to G_i (Dhein *et al.*, 2001). To determine whether G_i signalling was required for the carbachol-evoked inotropic response, we pretreated HF+ rats with the G_i inactivating agent *Pertussis* toxin (Ramkumar and Stiles, 1990). The carbachol-evoked inotropic response was significantly attenuated in muscles from rats pretreated with *Pertussis* toxin (Figure 5). Basal contractility was not affected by *Pertussis* toxin pretreatment [$F_{max} = 5.6 \pm 0.7$ ($n = 8$), see Table 1 for comparison]. This finding suggests that activation of G_i is involved in the intracellular signal transduction cascade.

Binding characteristics of muscarinic receptors

The dissociation constant of [^{3}H]QNB for muscarinic receptors did not significantly differ among the groups (Table 3). The B_{max} was increased 26% in HF+ ventricular muscle compared with Sham, but the increase did not reach statistical significance ($P = 0.07$; Table 3). The competition curve for carbachol in Sham and HF+ was best fitted to a two-site binding

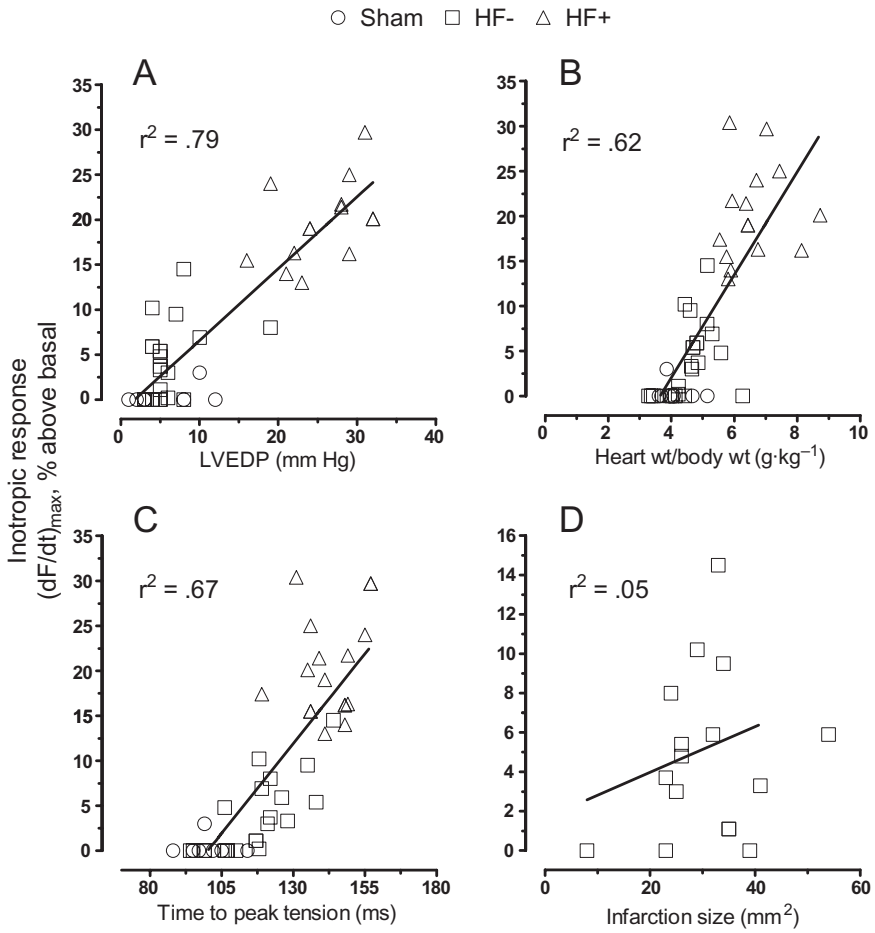

Figure 3 Scatter plots of the inotropic response (increase in $(dF/dt)_{max}$) to carbachol in rat left ventricular papillary muscles as a function of left ventricular end diastolic pressure (LVEDP) (A), heart weight/body weight (B), time to peak tension (C) or infarction size (D). An LVEDP > 15 mm Hg is a primary characteristic of congestive heart failure. Increased lung weight, an additional parameter of congestive heart failure also correlated with the inotropic response (data not shown).

model in the absence of GTP, indicating the presence of both a high and a low affinity binding site. No differences were observed in the fraction of the high and low affinity binding sites or their pK_i values between the groups (Table 4). Addition of 100 μmol·L^{-1} GTP significantly reduced the amount of high affinity binding, shifting the curve to better fit a model with a single binding site. There were no significant differences in the pK_i of the low binding site between groups in the presence of GTP. The pK_i was slightly higher in the presence of GTP than the pK_i of the low affinity binding site in the absence of GTP (Table 4).

The inotropic response to carbachol is accompanied by an increase of MLC-2 phosphorylation

The level of MLC-2 phosphorylation was determined in muscles that were clamp frozen after the carbachol-evoked inotropic response was fully developed (bolus dose of 10 μmol·L^{-1} for ~10 min). No significant change of basal MLC-2 phosphorylation was detected in Sham versus HF+ animals [19 ± 0.9% (n = 5) and 20 ± 0.7% (n = 21) MLC-2 phosphorylated respectively]. Carbachol did not modify phosphorylation levels of MLC-2 in Sham ventricle (20 ± 1.0%, n = 5). MLC-2 phosphorylation was increased by ~40% above basal levels in HF+ [26 ± 1.4% (n = 21) MLC-2 phosphorylated as percentage of total MLC-2 protein] (Figure 6A,B).

The MLC kinase inhibitor ML-9 (50 μmol·L^{-1}) reduced the carbachol-evoked inotropic response by ~70% [6 ± 0.9% above basal with ML-9 (n = 6) vs. 20 ± 1.5% above basal without (n = 8); P < 0.05] (Figure 7A). The increase of MLC-2 phosphorylation normally accompanying the inotropic response was nearly abolished by ML-9 pretreatment (Figure 7B). Basal contractility was reduced by 19 ± 6% (n = 6) after ML-9 administration (data not shown) with no effect upon basal MLC-2 phosphorylation levels. The selective Rho-associated kinase inhibitor Y-27632 (50 μmol·L^{-1}) reduced the carbachol-evoked inotropic response by ~75% [5 ± 0.7% above basal with Y-27632 (n = 6) vs. 20 ± 1.5% above basal without (n = 8); P < 0.05] (Figure 7A). As seen with ML-9 pretreatment, the increase in MLC-2 phosphorylation was also nearly abolished by Y-27632 pretreatment (Figure 7B). Basal contractility was reduced by 30 ± 3% (n = 6) after Y-27632 administration, accompanied by a modest (~20%) but non-significant reduction of MLC-2 phosphorylation (Figure 7B). These data indicate that carbachol is likely to increase activity

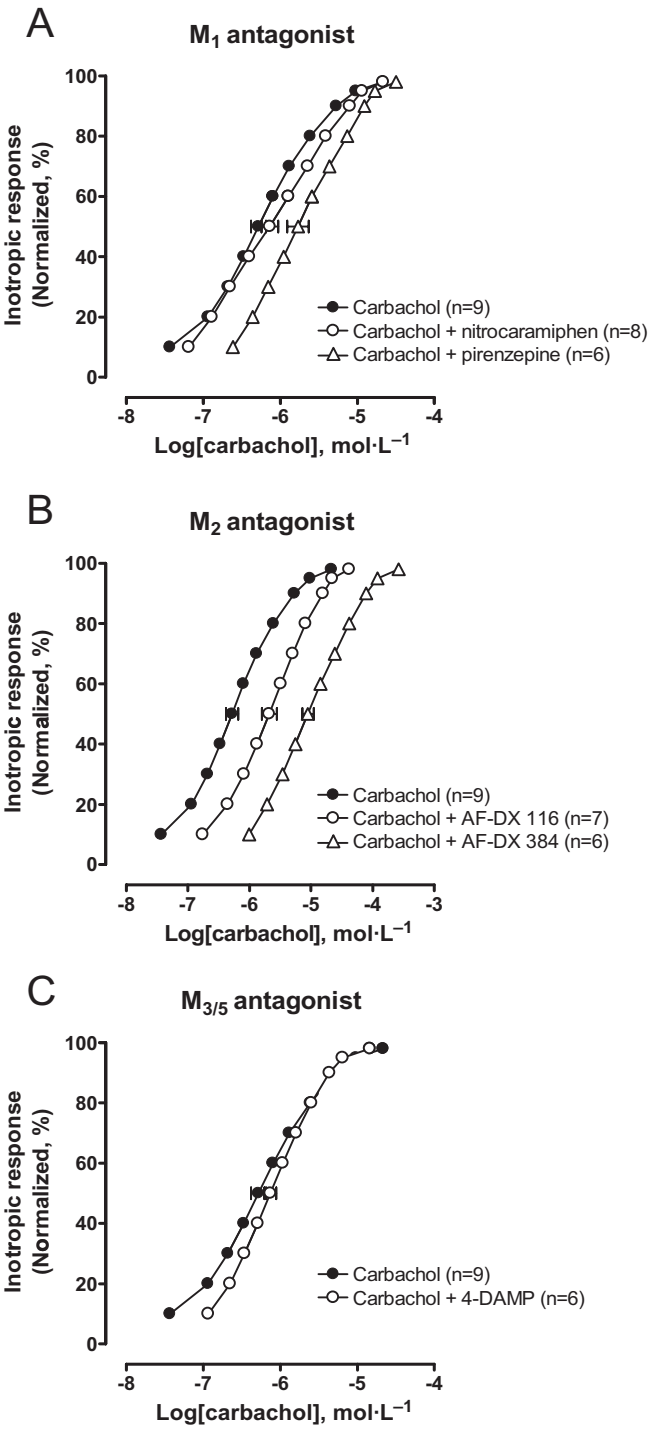

Figure 4 Concentration–response curves for carbachol in HF+ papillary muscles in the absence and presence of the M_1 antagonists nitrocaramiphen (11 nmol·L^{-1}) or pirenzepine (63 nmol·L^{-1}) (A), the M_2 antagonists AF-DX 116 (150 nmol·L^{-1}) or AF-DX 384 (54 nmol·L^{-1}) (B) or the M_3/M_5 antagonist 4-DAMP (1.7 nmol·L^{-1}) (C). Each antagonist was expected to shift the EC_{50} for carbachol by 0.5 (nitrocaramiphen, AF-DX 116, 4-DAMP) or 1 (AF-DX 384, pirenzepine) log unit if that receptor were mediating the inotropic effect. Inotropic response [increase in $(dF/dt)_{max}$] is expressed in percentage of maximum within each experiment.

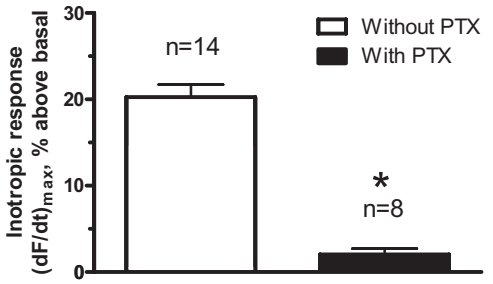

Figure 5 Effect of *Pertussis* toxin (PTX) treatment (30 μg·kg^{-1} 3 days prior to harvesting the muscle strips) on the inotropic effect of carbachol (10 μmol·L^{-1}) in HF+ rats. Data are mean ± SEM of one strip from n rats. *$P < 0.05$ compared with carbachol without PTX.

Table 3 Characteristics of muscarinic acetylcholine receptors in myocardium from Sham and HF+ rats measured by radioligand binding analysis

	Sham (n = *6*)	*HF+* (n = *6*)
K_d (nmol·L^{-1})	0.18 ± 0.06	0.15 ± 0.04
B_{max} (fmol·mg protein^{-1})	139 ± 11	175 ± 18

Table 4 Inhibition of [^{3}H]QNB binding with carbachol to myocardial muscarinic acetylcholine receptors in left ventricle of Sham or HF+ rats

	Sham (n = *8*)	*HF+* (n = *8*)
In the absence of GTP		
F_H	0.45 ± 0.07	0.34 ± 0.07
F_L	0.55 ± 0.07	0.66 ± 0.07
pK_{iH}	6.42 ± 0.23	6.79 ± 0.29
pK_{iL}	4.52 ± 0.14	4.62 ± 0.15
In the presence of 100 μmol·L^{-1} GTP		
F_H	0	0
F_L	1.0	1.0
pK_{iH}	–	–
pK_i	5.24 ± 0.11	5.20 ± 0.16

Values are given as mean ± SEM. Affinity for the high affinity site (K_{iH}) and affinity for the low affinity site (K_{iL}) are given as pK_i (–log K_i). Data in the absence of GTP were analysed by a two-site model. Data in the presence of GTP were analysed by a one-site model.
F_H, fraction high affinity; F_L, fraction low affinity.

of both MLC kinase and Rho-kinase in HF+ ventricle. Through this receptor-mediated regulation, MLC-2 phosphorylation is increased, presumably enhancing the Ca^{2+} sensitivity of the myofilaments. Neither ML-9 nor Y-27632 altered the β-adrenoceptor response to isoproterenol in the same papillary muscles [$(dF/dt)_{max}$ 48 ± 4% and 58 ± 6% above basal with ML-9 and Y-27632 respectively vs. 56 ± 6% above basal without inhibitors].

Discussion and conclusions

In this study, we report the emergence of an inotropic response to muscarinic receptor activation by carbachol in ventricular tissue from rats with heart failure, not observed in sham-operated controls. This finding demonstrates that the

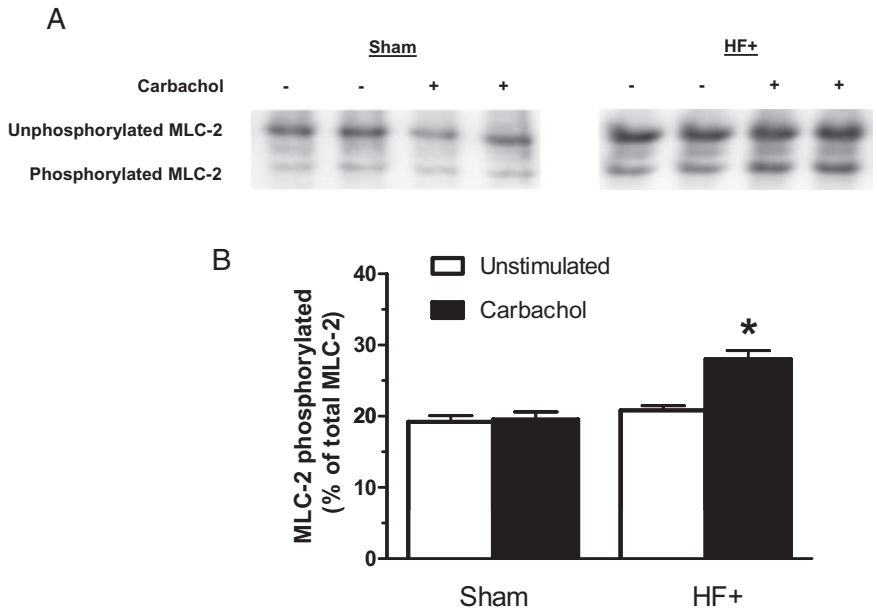

Figure 6 Representative Western blots (A) from glycerol polyacrylamide gels showing that carbachol stimulation (10 μmol·L^{-1} for 10 min) increased phosphorylation of myosin light chain-2 (MLC-2) in ventricular strips from HF+, but not Sham rats. Western blots were probed with an MLC2v antibody. The amount of phosphorylated MLC-2 was quantified by measuring the optical density of the bands. The data are reported as percentage phosphorylated MLC-2 of the total MLC-2 (B; Sham: $n = 5$, HF+: $n = 21$, $^*P < 0.05$).

functional role of muscarinic receptor activation is modified in ventricular myocardium of rats with heart failure. Therefore, the function of muscarinic receptors in failing myocardium extends beyond the classical role of 'indirect negative inotropic responses' revealed through attenuation of β-adrenoceptor-mediated inotropic responses by G_i-mediated inhibition of adenylyl cyclase (Caulfield, 1993). Secondly, we demonstrate that the carbachol-evoked inotropic response is dependent upon increasing phosphorylation of MLC-2, through regulation of both MLC kinase and Rho-kinase activity. This finding is, to our knowledge, also the first detailing a role for MLC-2 phosphorylation in mediating inotropic responses by cardiac muscarinic receptors. We propose that this results in an increase of myofilament sensitivity to Ca^{2+}, analogous to that proposed for both the α_1-adrenoceptor (Suematsu *et al.*, 2001; Andersen *et al.*, 2002; Grimm *et al.*, 2005), endothelin (Rossmanith *et al.*, 1997) and 5-HT_{2A} receptors (Qvigstad *et al.*, 2005b; Brattelid *et al.*, 2007a) in models of heart failure. Lastly, our data are largely consistent with the inotropic response being mediated by the predominant cardiac muscarinic receptor subtype, the M_2 receptor, with the subsequent downstream signal transduction dependent upon G_i activation.

It is well established that the muscarinic system antagonizes the inotropic responses mediated by β-adrenoceptors (Dhein *et al.*, 2001). This indirect negative inotropic response is mediated by the M_2 receptor, the predominant muscarinic receptor subtype in the mammalian ventricle (Dhein *et al.*, 2001), through activation of *Pertussis* toxin-sensitive G_i proteins and inhibition of adenylyl cyclase (Brodde *et al.*, 2001). The induction of a muscarinic receptor-mediated inotropic response in failing ventricle is not unprecedented, as muscarinic receptor activation can increase contractility in normal heart tissue. In fact, cardiac muscarinic receptors increase contractility in normal human ventricular trabeculae (Du *et al.*, 1994), guinea pig papillary muscles (Korth and Kuhlkamp, 1987) and embryonic chick ventricle (Protas, 1990; Nouchi *et al.*, 2007). However, until this report, inotropic responses to muscarinic agonists in rat heart were only observed in the atria, not the ventricle (Eglen *et al.*, 1988; Imai and Ohta, 1988).

The magnitude of the carbachol-evoked inotropic response correlated well with increased LVEDP, lung weight, heart weight/body weight ratio and an elongated TPF, all parameters indicative of established heart failure (Figure 3). In HF– rats, the size of the infarction did not correlate well with the carbachol-evoked inotropic response (Figure 3). This finding indicates that infarction alone is not sufficient for the induction of the muscarinic functional changes and that the functional shift develops as part of the decompensation into heart failure. In support of this, carbachol-, 5-HT_{2A}- and α_1-adrenoceptor-mediated inotropic responses were significantly larger in papillary muscles taken 3 days after infarction from rats with signs of heart failure than in those from rats 6 weeks after myocardial infarction and with no signs of heart failure, similar to those evaluated in the current study [(Qvigstad *et al.*, 2005b) and unpublished data]. In contrast, the magnitude of a 5-HT_4-mediated inotropic response increased with increasing infarction size in the same rat model of heart failure (Qvigstad *et al.*, 2005a). One possibility is that during decompensation, stretch receptors are triggered, activating genes (van Wamel *et al.*, 2000; Heineke and Molkentin, 2006; Brattelid *et al.*, 2007a) that support mechanisms known to increase calcium sensitivity of the myofilaments.

Given after carbachol, atropine rapidly and completely reversed the inotropic response to baseline levels (Figure 1B). To determine the muscarinic receptor subtype mediating the carbachol inotropic response, we used subtype selective mus-

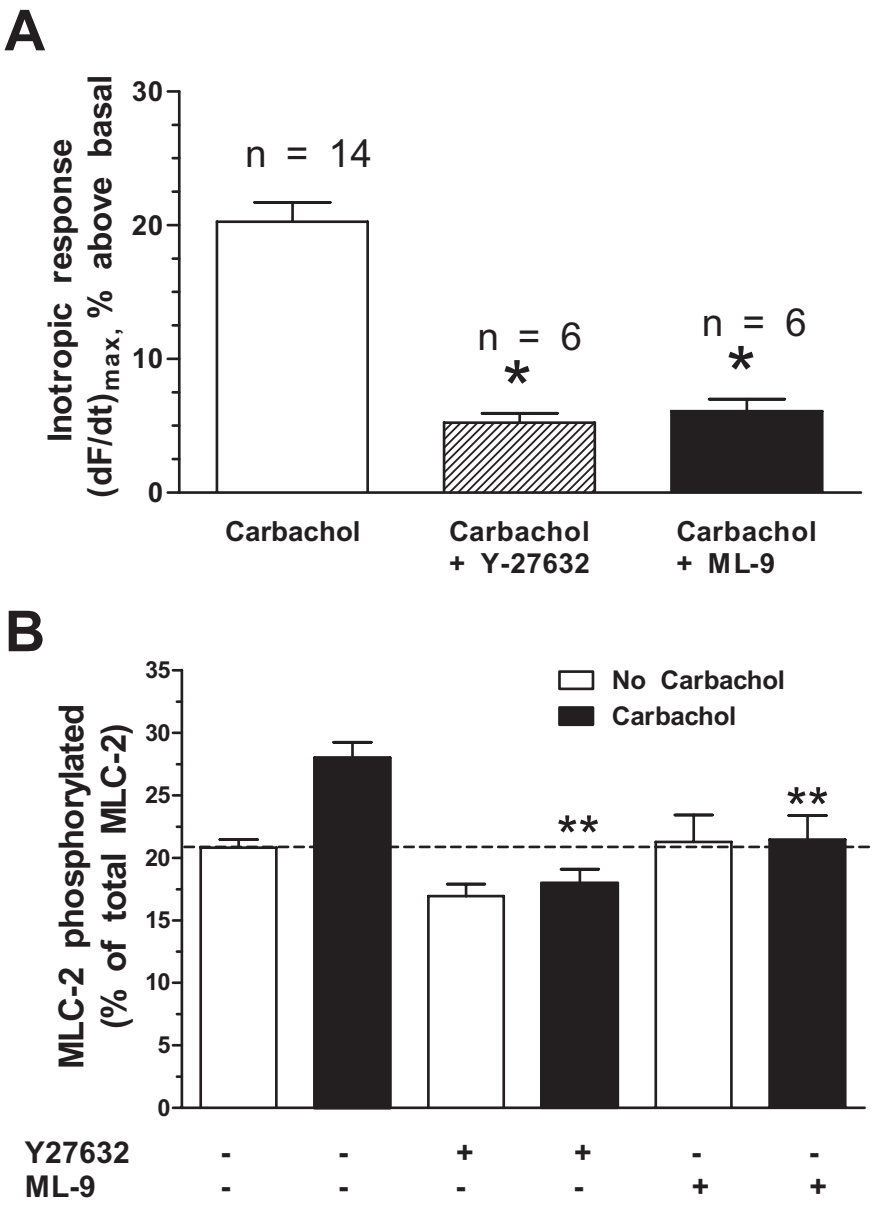

Figure 7 Effect of the MLC kinase inhibitor ML-9 (50 μmol·L^{-1}) and the Rho-kinase inhibitor Y-27632 (50 μmol·L^{-1}) on the 10 μmol·L^{-1} carbachol-induced inotropic effect (A) and MLC-2 phosphorylation (B; n = 5–20). The inhibitors were given ~45 min prior to carbachol. Both inhibitors significantly attenuated the carbachol-induced inotropic effect (*P < 0.05 vs. carbachol without inhibitor) and blocked the carbachol-induced enhancement of MLC-2 phosphorylation (**P < 0.05 vs. carbachol without inhibitor). Inotropic response was calculated as the increase in $(dF/dt)_{max}$ by carbachol in the presence of the inhibitor divided by the basal $(dF/dt)_{max}$ prior to the inhibitor. The amount of phosphorylated MLC-2 was quantified by measuring the optical density of the bands. The data are reported as percentage phosphorylated MLC-2 of the total MLC-2. The dashed line represents MLC-2 phosphorylation in the absence of any inhibitors or carbachol stimulation (control group).

carinic antagonists to shift the concentration–response curve of carbachol. Assuming that M_2 receptors mediated the inotropic response, the M_2 receptor antagonists AF-DX 116 and AF-DX 384 produced the expected shift of the carbachol concentration–response curve to higher concentrations. The affinity estimates (pK_b) calculated from our data for AF-DX 116 and AF-DX 384 fit well with those published previously for M_2 receptors (Caulfield, 1993). There is evidence that the inotropic response of isolated guinea pig ventricular myocytes is mediated via M_2 receptors (Matsumoto and Pappano, 1991). Although inotropic responses of muscarinic agonists in human atria appear mediated by a receptor subtype other than M_2 [see Brodde *et al.* (2001)], in ventricular myocardium the positive inotropic response of acetylcholine appears to be mediated through M_2 receptor activation (Du *et al.*, 1994).

Whereas both the M_1 antagonist nitrocaramiphen and the $M_{3/5}$ antagonist 4-DAMP did not, the M_1 antagonist pirenzepine did shift the carbachol concentration–response curve to higher concentrations (Figure 4). However, the pK_b calculated for pirenzepine was 7.5 ± 0.1, a value lower than that reported for the M_1 receptor (Caulfield, 1993). Possibly pirenzepine shifted the carbachol curve by binding at the M_2 recep-

tor. Ligand affinity estimates determined by functional studies in native tissue can differ substantially from those determined in transfected cell lines (Caulfield, 1993). As such, pirenzepine may have a higher affinity for M_2 receptors in heart tissue than that determined by radioligand binding in transfected cell lines over expressing M_2 receptors. Alternatively, it is possible that M_1 receptors contribute to the inotropic response as they are reportedly present in rat and guinea pig ventricular myocytes (Gallo *et al.*, 1993; Brattelid *et al.*, 2007b). Activation with high concentrations of carbachol increased L-type Ca^{2+} current and activated phospholipase C (Gallo *et al.*, 1993). Furthermore, the M_3 receptor is coupled to activation of the phospholipase C/IP_3 pathway in adult rat ventricular myocytes (Pönicke *et al.*, 2003). Even though mRNA levels for the M_3 receptor are significantly up-regulated in ventricular myocardium of rats with heart failure (Brattelid *et al.*, 2007b), the M_3 antagonist 4-DAMP did not shift the carbachol concentration–response curve in the present study. Therefore, it is unlikely that the inotropic response to carbachol was mediated by M_3 receptor activation. Although we can not rule out a role for the M_1 receptor, it seems unlikely based on the mismatch of estimated and expected affinity constants for pirenzepine at the M_1 and M_2 receptors in these studies as well as the lack of evidence for functional cardiac M_1 receptors. Furthermore, the robust inhibition mediated by *Pertussis* toxin pretreatment indicates that the carbachol-evoked inotropic response is mediated through activation of the G_i-coupled M_2 subtype, not the G_q-coupled M_1 subtype.

Carbachol evokes a biphasic inotropic response in HF+ left ventricle, which differs from that of G_s-coupled receptors. Composed of an initial, relatively fast, transient negative component followed by a more slowly developing sustained positive phase, it more closely resembles responses to α_1-adrenoceptor stimulation in rat (Sjaastad *et al.*, 2003). In contrast to cAMP-dependent effects on cardiac contractility (e.g. 5-HT_4, β-adrenoceptors) the M_2 response develops slowly (Figure 1B,C). It is also characterized by a symmetrical change in the contraction–relaxation cycle (Figure 2C,D) with unchanged or slightly prolonged time to peak tension and RT (Table 2). These characteristics are typical of effects elicited through cardiac G_q-coupled receptors (e.g. α_1-adrenoceptors, 5-HT_{2A} and endothelin receptors) (Rossmanith *et al.*, 1997; Skomedal *et al.*, 1997; Watanabe and Endoh, 1998; Andersen *et al.*, 2002; Grimm *et al.*, 2005). Regulation of cardiac contractility through G_q-coupled receptors, as with α_1-adrenoceptors or $ET_{A/B}$ receptors, is mediated through a Ca^{2+} sensitizing mechanism that may involve increased MLC-2 phosphorylation (Blinks and Endoh, 1986; Pieske *et al.*, 1999; Andersen *et al.*, 2002). Muscarinic receptor activation with carbachol has been reported to enhance the Ca^{2+} sensitivity of myofilaments (Puceat *et al.*, 1990). In addition, α_1-adrenoceptor-mediated inotropic responses are slightly increased (Sjaastad *et al.*, 2003), and a G_q-coupled 5-HT_{2A} receptor-mediated inotropic response emerges in the same rat model of heart failure (Qvigstad *et al.*, 2005b). On this background and given the similarity of the carbachol-evoked effect to both the 5-HT_{2A}- and α_1-adrenoceptor-mediated inotropic responses, it seemed likely that MLC-2 phosphorylation was involved in the carbachol-evoked inotropic response.

The MLC kinase inhibitor ML-9 (Reig *et al.*, 1993) inhibits cAMP-independent inotropic responses, for instance to phenylephrine or endothelin, in heart tissue (Rossmanith *et al.*, 1997; Andersen *et al.*, 2002), indicating that these effects are mediated via increased MLC-2 phosphorylation. Similarly, the carbachol-evoked inotropic response was nearly abolished by ML-9, as was the carbachol-evoked increased phosphorylation of MLC-2 at Ser-15. Accordingly, we suggest that the sustained positive inotropic response to carbachol, possibly mediated through M_2 receptors in chronic heart failure, depends on MLC kinase-mediated phosphorylation of MLC-2.

In smooth muscle, the RhoA/Rho-associated kinase (ROCK) pathway contributes to agonist-mediated contraction mainly through inhibition of myosin phosphatase, thus increasing phosphorylated MLC-2 (Uehata *et al.*, 1997). ROCK can be activated by G_q-coupled receptor signalling via the small G-protein RhoA (Shimokawa *et al.*, 1999). The pyridine derivative, Y-27632, is a selective ROCK inhibitor (Uehata *et al.*, 1997), and there is now increasing evidence that the RhoA/ROCK pathway is involved in agonist-mediated inotropic responses (Andersen *et al.*, 2002; Qvigstad *et al.*, 2005b). In the present study, the carbachol-evoked positive inotropic response was significantly attenuated by Y-27632, when added before carbachol. Furthermore, the carbachol-stimulated increase in MLC-2 phosphorylation was abolished by Y-27632 suggesting an involvement of the RhoA/ROCK pathway. Inhibition of RhoA/ROCK is likely to increase myosin phosphatase activity by preventing phosphorylation of the regulatory myosin binding subunit (Rajashree *et al.*, 2005). In contrast, neither ML-9 nor Y-27632 inhibited the cAMP-dependent β-adrenoceptor-mediated inotropic response, consistent with previous reports (Andersen *et al.*, 2002; Nishimaru *et al.*, 2003; Qvigstad *et al.*, 2005b).

In the current study, we have shown that the carbachol-evoked inotropic response was sensitive to *Pertussis* toxin inactivation of G_i (Figure 5). This finding lends support to the suggestion that the carbachol-mediated inotropic response is mediated by activation of the G_i-coupled M_2 receptor subtype. Studies indicate that G_i can mediate signal transduction of pathways known to activate either Rho-kinase or MLC kinase. In smooth muscle, activation of M_2 muscarinic receptors caused contraction due to phosphorylation of both CPI-17 and MLC-2 through activation of integrin-linked kinase only when p38 MAPK was inhibited (Huang *et al.*, 2006). Alternatively, in HF+ ventricle, M_2 receptors via $G_{i\beta\gamma}$ activation and PI3 kinase switch from Rac1 to RhoA activation, as reported in neonatal rat cardiomyocyte derived H10 cells (Vogt *et al.*, 2007). M_2 receptors might also couple to G_q or possibly $G_{12/13}$, G proteins purportedly involved in the signalling of other receptor systems known to mediate inotropic responses through increasing MLC-2 phosphorylation. Further investigation is needed to clarify the role of M_2 receptors and the signalling mechanisms mediating the carbachol-evoked inotropic response.

In conclusion, agonist stimulation of muscarinic cholinergic receptors, most likely of the M_2 subtype, elicited an inotropic response in ventricular muscle from rats with chronic heart failure, which was not observed in myocardium from Sham or normal rats, analogous to that observed for the

G_q-coupled 5-HT_{2A} receptor (Qvigstad *et al.*, 2005b). The mechanism of action of muscarinic receptor activation involves phosphorylation of MLC-2 similar to that following stimulation of 5-HT_{2A} and α_1-adrenoceptors. The α_1-adrenoceptor, 5-HT_{2A} and muscarinic systems share the feature of either up-regulation, induction or shift in function respectively, to mediate enhanced inotropic responses in failing myocardium, through the common mechanism of increasing Ca^{2+} sensitivity of myofilaments by increasing MLC-2 phosphorylation. This enhancement of Ca^{2+} sensitivity of myofilaments, representing a less energy-demanding mechanism of inotropic support may be advantageous in the failing heart, compared with cAMP-mediated mechanisms. However, the functional role of such changes in the whole pathophysiological background of heart failure cannot be decided by the present study. Thus further clarification is needed.

Acknowledgements

This study was supported by the Norwegian Council on Cardiovascular Diseases, the Research Council of Norway, Anders Jahre's Foundation for the Promotion of Science, the Novo Nordisk Foundation and the Family Blix foundation. Line Solberg provided technical assistance.

Conflicts of interest

The authors state no conflict of interest.

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