Carbachol-induced suppression of contraction rhythm in spontaneously beating cultured cardiac myocytes from neonatal rats.

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Abstract

Nitric oxide (NO) and reactive oxygen species (ROS) are known to play various functional and pathophysiological roles as an intracellular messenger in the heart. In this study, we investigated whether the increased production of NO and/or ROS was involved in the cholinergic regulation of rhythmic contraction in spontaneously beating cultured cardiac myocytes from neonatal rats. Exposure of cultures to carbachol, an agonist of muscarinic acetylcholine receptors (mAchR), produced a dose-dependent decrease in the beat rate of cultured cardiac myocytes, and such a effect was significantly attenuated by pre-treatment with an NOS inhibitor, as well as an NO scavenger. In addition, exposure to an NO donor (SNAP) also decreased the beat rate dose-dependently. Carbachol - or SNAP-induced suppression of the contraction rhythm was significantly attenuated by co-treatment with 5-hydroxydecanoate (5-HD). In contrast, treatment with diazoxide decreased the beat rate dose-dependently. Carbachol treatment increased the intensity of 2',7'-dichlorodihydrofluorescein fluorescence, suggesting that the production of ROS was enhanced by the treatment. In addition, the carbachol- or diazoxide-induced suppression of contraction rhythm was attenuated by co-treatment with 2-mercaptopropionyl glycine, a scavenger of ROS. The present study has suggested that the mAchR-NO-mitoK_{ATP}-ROS pathway is a factor responsible for

carbachol-induced suppression of contraction rhythm in cultured cardiac myocytes.

Key words: carbachol, contraction rhythm, NO, ROS, mitoK_{ATP} channels

1. Introduction

Nitric oxide (NO) is a ubiquitous signaling molecule produced from L-arginine by nitric oxide synthase (NOS) (Nathan, 1992), and is known to play a variety of functional and/or pathophysiological roles in living tissues. Recently, we have demonstrated that the activity of NOS varies with cell differentiation, suggesting that NO functions as an important signaling molecule in differentiated cells (Kawahara et al., 2002b). We have also demonstrated in cultured astrocytes that propagating Ca^{2+} waves can be evoked by the local photolysis of caged calcium ionophore (Iwabuchi et al., 2002), and wave propagation is modulated by endogenously produced NO (Kawahara et al., 2003a). In the heart, NO plays a physiological role in mediating the effect of vagal stimulation in the autonomic regulation of heart functions (Brady et al., 1992). Han et al. (1994) investigated cholinergic modulation of heart rate in isolated sino-atrial node cells, and demonstrated that NO is responsible for the muscarinic acetylcholine receptor (mAchR)-mediated control of mammalian heart rate. Previous studies have shown that cultured cardiac myocytes from neonatal rats have both mAchR (Sterin-Borda et al., 1995; Sun et al., 1996) and constitutive NOS (cNOS; i.e., eNOS and nNOS) (Kanai et al., 2001; Xu et al., 1999; Yamamoto et al., 1999). In fact, treatment of cultured cardiac myocytes with NOS inhibitors attenuates the negative chronotropic effect of mAchR activation (Ballingand et al.,

1993), indicating that NO produced by NOS is possibly involved in mediating the effect of parasympathetic stimulation of the heart rate. In addition, isolated and cultured neonatal cardiac myocytes contract spontaneously and cyclically (Harary & Farley, 1963). Thus, cultured cardiac myocytes from neonatal rats seem to be an appropriate experimental model for analyzing the mAchR-NOS-NO signaling pathway responsible for the cholinergic regulation of contraction rhythm of cardiac cells.

In isolated dog hearts, treatment with glibenclamide, a blocker of ATP-sensitive potassium (K_{ATP}) channels, antagonized the Ach-induced negative chronotropic and inotropic effects (Murakami et al., 1992). In addition, increase in the heart rate caused by cardiac sympathetic nerve stimulation is antagonized by treatment with diazoxide, an opener of mitochondrial K_{ATP} (mitoK_{ATP}) channels, in the guinea pig double atrial/right stellate ganglion preparation (Mohan & Paterson, 2000). Recent studies have revealed that there is an intracellular signaling pathway linking the activation of AchR with the opening of mitoK_{ATP} channels in smooth muscle cell line (Olenberg et al., 2002), as well as in intact hearts (Cohen et al., 2001). A recent finding by us using Langendorff-perfused rat hearts also demonstrated that the NO-mitoK_{ATP} pathway is possibly involved in increased vulnerability to ischemia/reperfusion-induced ventricular tachyarrhythmias (Kawahara et al., 2003b). All these findings have led to a suggestion that opening of mitoK_{ATP} channels is one of the

downstream targets of AchR activation. However, functional relationships between the activation of AchR, the resultant increase in the production of NO, the opening of mitoK_{ATP} channels, and the AchR-induced suppressive effect on contraction rhythm remain largely unclarified.

In this study, we have tried to elucidate what mechanisms are involved in the carbachol-induced suppression of contraction rhythm in spontaneously beating cultured cardiac myocytes from neonatal rats. Results of the present study suggest that the mAchR-NO-mitoK_{ATP}-ROS pathway is a factor responsible for the carbachol-induced suppression of contraction rhythm in cultured cardiac myocytes.

2. Materials and methods

The animal experiments conformed to the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1996), as well as the "guide for the care and use of laboratory animals", Hokkaido University School of Medicine.

2.1 Culture of cardiac myocytes

The method of cardiac myocyte culture was described elsewhere in detail (Kawahara et al., 2002a; Yamauchi et al., 2002). In short, cardiac myocytes were prepared from 1-3 day old neonatal Wistar rat ventricles removed after decapitation. The ventricles were rinsed in a 25 mM HEPES buffered minimum salt solution (MSS) to remove contaminating blood cell components and then minced with scissors into fragments to be digested with 0.1 % collagenase (Wako Chemical, Tokyo, Japan) in MSS at 37 ℃ for 60 min. The digested fragments were centrifuged at 1000 rpm for 2 min (LC-100, TOMY, Japan) and precipitated cell components were washed twice with MSS to terminate the effects of collagenase. The cell components were suspended in MCDB 107 (Research Institute for the Functional Peptides, Yamagata, Japan) containing 5 % FCS (MBL, Nagoya, Japan), and then passed through a wire mesh screen (90 μm porosity) to remove large aggregates of cells; the filtered suspension contains cardiac myocytes and fibroblasts. To separate cardiac myocytes from fibroblasts based on the

selective adhesion technique, the cell suspension was poured into petri dishes (ϕ 60 mm, Falcon), and incubated for 60 min at 37 ° C, in 5 % CO2 and 95 % air. By virtue of the procedure, most of the fibroblasts adhere to the dish. After the incubation, the suspension, mostly containing cardiac myocytes, was collected. The suspension was centrifuged at 700 rpm for 5 min to separate the remaining blood cell components in the supernatant. The precipitated cells were resuspended in MCDB 107 containing 5 % FCS, transferrin (10 μ g/mL, Sigma, St. Louis, MO), and insulin (10 μ g/mL, Yamanouchi, Tokyo, Japan). The cell suspension was passed through a fine wire mesh screen (25 μ m porosity) to remove remaining small aggregates of myocytes, and finally the isolated myocytes remaining were cultured at a density of about 3.0×10^5 cells/ml in a petri dish (ϕ 30 mm, Falcon) coated beforehand with fibronectine (10 μ g/mL, Sigma). Cardiac myocytes cultured for 5-7 days were used in this study.

2.2 Image analysis

In this study, the spontaneous contraction rhythm of cultured myocytes was evaluated by a video image recording method. This procedure is described elsewhere in detail (Kawahara et al., 2002a; Yamauchi et al., 2002). In short, images of beating myocytes were recorded with a CCD camera (WV-BD400, Panasonic, Japan) through a phase-contrast microscope (IX70,

OLYMPUS, Japan). A spontaneously beating myocyte was arbitrarily selected from myocytes in the video image. A small area (a square of about 20 pixels) of the myocyte where brightness changed considerably with contraction was selected, and the video signals were digitized to an 8-bit number every video frame (30 frames/s) by a video capture board in a personal computer (Power Macintosh 7500/100, Apple) for 5 min.

A reference frame was arbitrarily selected and cross-correlograms were calculated between pixels of the reference frame and those of other frames, to represent temporal variation of brightness in the selected area corresponding to the contraction rhythm of the cardiac myocyte. Beat-to-beat intervals were calculated from the intervals between upward or downward peaks of the cross-correlogram in each series of data, and were averaged.

2.3 Fluorescence measurements

ROS generation in cardiac myocytes was assessed using the probe 2',7'-dichlorofluorescin diacetate (H_2DCFDA), a membrane-permeable diacetate form of 2',7'-dichlorofluorescin (DCFH). H_2DCFDA was added to the myocyte cultures at a final concentration of 5 μmol/L for 20 min. ROS in the cells oxidizes DCFH, yielding the fluorescent product 2',7'-dichlorofluorescein (DCF) (Rothe & Valet, 1990). The probe DCFH in cardiac myocytes is readily oxidized by hydrogen peroxide or hydroxyradical but is relatively

insensitive to superoxide (Vanden Hoek et al., 1998). Fluorescent intensity was measured using an excitation wavelength of 480 nm, and an emission of 535 nm. Fluorescent images were acquired at 2 sec intervals with a cooled CCD camera (C4880-80; Hamamatsu Photonics, Hamamatsu, Japan). Fluorescent intensity was normalized with the initial value (relative intensity).

2.4 Experimental protocol

The serum-containing MCDB107 was replaced by serum-free DMEM (Gibco BRL, Invitrogen Corp., Carlsbad, CA) 3 h before the measurement, and the cells were incubated at 37℃ in a humidified atmosphere of 95% room air and 5% CO_2. We then measured the spontaneous rate of beating in cultured cardiac myocytes for 3 min. An agonist of mAchR (carbachol) was added to DMEM, and the final concentration in DMEM was adjusted to 1, 10 or 100 μ mol/L. The change in beat rate was measured for 20 sec at 2 min, 5 min and 10 min after the exposure to carbachol. We analyzed the effects of pre-treatment with either 1 μ M atropine, an antagonist of mAchR, for 10 min, 100 μ mol/L L-NMMA, an NOS inhibitor for 2 h, or 10 μ mol/L carboxy-PTIO, a scavenger of NO for 30 min on carbachol-induced change in the beat rate of cultured myocytes. We also analyzed the change in the beat rate at 30 min and 1 h after the start of treatment with 10, 100, and 500 μ mol/L SNAP, an NO donor.

To investigate whether the activation of mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels was involved in the negative chronotropy in cardiac myocytes, cultures were pre-treated with 1 mmol/L 5-hydroxydecanoate (5-HD), a selective inhibitor of mitoK_{ATP} channels, for 15 min. The change in beat rate was measured at 30 min and 1 h after the start of treatment with diazoxide, a selective mitoK_{ATP} activator. To investigate whether the increased production of ROS was involved in negative chronotropy, cultures were pre-treated with 1 mmol/L 2-mercaptopropionyl glycine (2-MPG), an ROS scavenger, for 20 min.

2.5 Chemicals

Carbachol, atropine, N^G-monomethyl-L-arginine (L-NMMA), 2-mercaptopropionyl glycine (2-MPG), and diazoxide were obtained from Sigma. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl3-oxide (Carboxy-PTIO), S-nitroso-N-acetyl-DL-penicillamine (SNAP), and (±)-N-[(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR 4) were obtained from Dojindo Lab., Inc. (Kumamoto, Japan). 5-hydroxydecanoate (5-HD) was obtained from BIOMOL Res. Lab., Inc. (Plymouth, PA). 2',7'-dichlorofluorescin diacetate (H_2DCFDA) was obtained from Calbiochem (La Jolla, CA). The other chemicals were from Wako Chem.

2.6 Statistical analysis

The data are expressed as mean ± S.D. Comparisons were performed using the one-way analysis of variance (ANOVA) followed by a paired t-Test. A *P* value of less than 0.05 was considered significant.

3. Results

After 5-7 days in culture, the contraction rhythm of each of the cultured cardiac myocytes in an aggregate became stable and synchronized. The average rate of beating was 141.6 ± 30.2 beats/min. The data from cells beating at less than 50 beats/min were excluded, since such low rates were considered to reflect a reduced viability of cultured myocytes (Kawahara et al., 2002a).

3.1 Carbachol-induced suppression of contraction rhythm in cultured cardiac myocytes

To investigate the changes in the spontaneous rate of beating caused by the activation of muscarinic acetylcholine receptors (mAchR) in cultured cardiac myocytes, we treated myocytes with 1, 10 or 100 μ mol/L carbachol, an agonist of mAchR. Carbachol treatment resulted in the dose-dependent prolongation of beat-to-beat intervals; that is, decrease in the rate of beating (Fig. 1A). Treatment of myocytes with carbachol at all concentrations used produced a significant decrease in the rate of beating (Fig. 1B). Such carbachol-induced suppression of contraction rhythm was rapid, and could be observed as early as 2 min after the treatment (Fig. 1A2 and B). To ascertain whether carbachol-induced suppressive effect was actually caused by the activation of mAchR, cardiac myocytes were pre-treated with 1 μ mol/L atropine, an antagonist of mAchR, for 10 min before the start of carbachol treatment and during treatment.

Atropine treatment significantly antagonized the suppressive effect on the rate of beating induced by 100 μ mol/L carbachol (Fig. 1C). Treatment of myocytes with 1 μ mol/L atropine itself did not have any appreciable effect on the spontaneous beating rate of cardiac myocytes (data not shown). These results suggest that treatment of cultures with carbachol resulted in the decrease of beating rate via activation of mAchR in cultured cardiac myocytes.

3.2 Involvement of nitric oxide (NO) in carbachol-induced suppression of contraction rhythm

Previous studies have demonstrated that the activation of mAchR results in the activation of NOS and increases the production of NO in cardiac cells (Han et al., 1994; Sterin-Borda et al., 1995). We then investigated whether the activation of NOS and the resultant increase in NO production was involved in carbachol-induced suppression of the rate of beating in our cultured cardiac myocytes. To do this, cardiac myocytes were treated with either L-NMMA, an inhibitor of NOS, or carboxy-PTIO, a scavenger of NO. Pre-treatment with either 100 μ mol/L L-NMMA for 2 h or 10 μ mol/L carboxy-PTIO for 30 min significantly attenuated the 100 μ mol/L carbachol-induced negative chronotropy in cardiac myocytes (Fig. 2A). Treatment of myocytes with either 100 μ mol/L L-NMMA or 10 μ mol/L carboxy-PTIO without carbachol treatment did not show any appreciable effects on the spontaneous beating rate of cardiac myocytes (data not shown).

We then investigated whether exogenously applied NO caused a decrease in the rate of beating in cultured cardiac myocytes. To do this, myocytes were treated with 10, 100, or 500 μ mol/L SNAP, an NO donor (Fig. 2B). Treatment with 100 or 500 μ mol/L SNAP resulted in a significant decrease in the rate of beating in a dose-dependent manner at 30 min and 1 h after the start of SNAP-treatment. In contrast, treatment either with 1 % DMSO or 10 μ mol/L SNAP did not have significant effects on the spontaneous beating of myocytes (Fig. 2B). Treatment of cultures with a different kind of NO donor, NOR 4 (10 μ mol/L) , resulted in a decrease in the beat rate (Fig. 2C). All these results support the assumption that the activation of mAchR by carbachol resulted in the activation of NOS, and the resultant increase in the production of NO contributed to carbachol-induced suppression of the rate of beating in spontaneously beating cardiac myocytes in culture.

3.3 Involvement of mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$) in carbachol-induced suppression of contraction rhythm

Previous studies have suggested that NO directly activates mitoK$_{ATP}$ channels in cardiac myocytes (Ockali et al., 1999; Sasaki et al., 2000). Therefore, we investigated whether the activation of mitoK$_{ATP}$ channels was involved in carbachol-induced suppression of contraction rhythm in spontaneously beating cultured cardiac myocytes. To do this, myocytes were

pre-treated with 5-hydroxydecanoate (5-HD), a specific blocker of mitoK$_{ATP}$ channels (Sato et al., 2000), and the change in the beat rate caused by carbachol exposure was analyzed. Pre-treatment with 1 mmol/L 5-HD for 15 min significantly attenuated the decrease in the beat rate induced by 100 μ mol/L carbachol at 2 min after treatment (Fig. 3A). To confirm that the increased production of NO was necessary for activation of mitoK$_{ATP}$ channels, we investigated whether 5-HD treatment also affected SNAP-induced suppression of the rate of beating in cultured myocytes. Pre-treatment with 1 mmol/L 5-HD for 15 min significantly attenuated a decrease in the beat rate caused by 500 μ mol/L SNAP exposure at 30 min after treatment (Fig. 3A). These results suggest that the mAchR-NO-mitoK$_{ATP}$ pathway was responsible for the carbachol-induced decrease in the spontaneous beat rate in cultured cardiac myocytes.

We further investigated whether activation of mitoK$_{ATP}$ channels was actually involved in the decrease in the beat rate of cardiac myocytes. Treatment of myocytes with diazoxide, a selective opener of mitoK$_{ATP}$ channels (Sato et al., 2000), resulted in a significant decrease in the beat rate dose-dependently (Fig. 3B). Diazoxide (100 μ mol/L)-induced decrease in the beat rate was significantly attenuated by pre-treatment with 1 mmol/L 5-HD for 15 min (data not shown).

3.4 Reactive oxygen species (ROS) are involved in carbachol-induced suppression of

contraction rhythm

A previous study has demonstrated that the activation of AchR results in the activation of mitoK_{ATP} channels, which then increases the production of ROS, and contributes to the establishment of ischemic tolerance in cultured cardiac myocytes (Wang et al., 2001). Therefore, we investigated whether the increased production of ROS caused by the activation of mitoK_{ATP} channels was responsible for carbachol-induced suppression of the rate of beating in cultured cardiac myocytes. At first, we investigated whether the production of ROS actually increased by the treatment of cultures with carbachol (Fig. 4). Carbachol treatment increased fluorescent intensity of 2',7'-dichlorofluorescein (DCF), a fluorescent probe for ROS (Rothe & Valet, 1990; Vanden Hoek et al., 1998), suggesting that the treatment actually increased production of ROS (Fig. 4B & C). In contrast, sham treatment did not result in any appreciable change in DCF fluorescence (Fig. 4A & C).

We then investigated whether increased production of ROS was responsible for the carbachol-induced suppressive effect on the rate of beating in cultured cardiac myocytes. Pre-treatment of spontaneously beating cultured myocytes with 1 mmol/L 2-mercaptopropionyl glycine (2-MPG), a scavenger of ROS, for 20 min significantly attenuated the 100 μ mol/L carbachol-induced decrease in the beat rate of cardiac myocytes (Fig. 5). In addition, pre-treatment with 1 mmol/L 2-MPG for 20 min significantly attenuated the 500 μ mol/L

diazoxide-induced decrease in the beat rate (Fig. 5). These results suggest that activation of mAchR by carbachol resulted in activation of mitoK_{ATP} channels, which then increased production of ROS, and contributed to the suppression of contraction rhythm in spontaneously beating cultured cardiac myocytes.

Discussion

The present study has suggested that mAchR-NO-mitoK_{ATP}-ROS signaling was one pathway responsible for carbachol-induced suppression of contraction rhythm in cultured cardiac myocytes. Treatment of cultures with carbachol, an agonist of muscarinic acetylcholine receptors (mAchR), resulted in a dose-dependent decrease in the beat rate of spontaneously beating cultured cardiac myocytes (Fig. 1). The carbachol-induced suppressive effect on the rate of beating was attenuated by pre-treatment with either atropine, L-NMMA, or carboxy-PTIO, suggesting that activation of mAchR and nitric oxide synthase (NOS), and the resultant increase in production of nitric oxide (NO) were involved in the suppressive effect. In addition, treatment with either SNAP or NOR, NO donors, resulted in a dose-dependent decrease in the spontaneous beat rate (Fig. 2).

Carbachol-induced suppression of contraction rhythm was significantly attenuated by pre-treatment of cultures with 5-hydroxydecanoate (5-HD), a selective blocker of mitoK_{ATP} channels (Sato et al., 2000). In addition, treatment of cardiac myocytes with diazoxide, an opener of mitoK_{ATP} channels (Sato et al., 2000), suppressed the spontaneous beat rate dose-dependently (Fig. 3B). These results suggest that the activation of mitoK_{ATP} channels was responsible for carbachol-induced suppression of contraction rhythm in cultured cardiac myocytes. SNAP-induced suppression of the rate of beating was also attenuated by treatment

of cultures with 5-HD, suggesting the possibility that the activation of mitoK_{ATP} channels were downstream of the carbachol-induced increase in production of NO. Previous studies have demonstrated that NO either directly or indirectly activates mitoK_{ATP} channels in cardiac myocytes (Ockaili et al., 1999; Sasaki et al., 2000; Wang et al., 2001), although the underlying mechanisms still remain unknown. The present study suggested that NO-mitoK_{ATP} signaling was one possible pathway responsible for carbachol-induced suppression of contraction rhythm in cultured cardiac myocytes.

A recent report by Yao et al. in cultured chick embryonic cardiac myocytes (Yao et al., 1999) has revealed that prior Ach administration reduces ischemic cell death and results in the increased production of ROS attenuated by either antioxidant 2-mercaptopropionyl glycine (2-MPG) or 5-HD, suggesting that AchR- mitoK_{ATP}-ROS signaling is crucially involved in Ach-induced ischemic preconditioning of cardiac myocytes. In the present study, treatment of spontaneously beating cultured cardiac myocytes with carbachol also increased production of ROS (Fig. 4). Carbachol-induced suppression of contraction rhythm was attenuated by treatment with the antioxidant 2-MPG, suggesting that increased production of ROS by carbachol was involved in decrease in the rate of beating in cultured myocytes (Fig. 5). In addition, diazoxide-induced suppression of contraction rhythm was also attenuated by co-treatment with 2-MPG (Fig. 5), suggesting that ROS signaling for the suppression of beat

rate was downstream of the activation of mitoK$_{ATP}$ channels.

The question as to what mechanisms are involved in NO-mitoK$_{ATP}$-ROS signaling–induced negative chronotropy observed in this study remains unanswered. The mitoK$_{ATP}$-ROS signaling has been known to activate some protein kinases, such as extracellular signal-regulated kinases (ERKs) (Samavati et al., 2002). The activation of such kinases then modulates the activity of a variety of sarcolemmal ion channels such as L-type calcium channels (Murata et al., 1999). The increased production of ROS is also known to cause enhancement of Na^+/Ca^{2+} exchanger, promoting intracellular calcium overload during ischemia/reperfusion (Goldhaber, 1996). Calcium overload itself possibly affects the activity of sarcolemmal ion channels. In addition, increased ROS during reperfusion possibly activates sarcolemmal ATP-sensitive potassium channels, increasing vulnerability to reperfusion-induced tachyarrhythmias (Tokube et al., 1996; Dhein & Pejman, 2000). Recently, we demonstrated that the NO-mitoK$_{ATP}$ signaling is one pathway responsible for increased vulnerability to reperfusion-induced tachyarrhythmias such as ventricular tachycardia and fibrillation (Kawahara et al., 2003b). However, the mechanisms responsible for the NO-mitoK$_{ATP}$-ROS signaling–induced suppression of the spontaneous rate of beating in cardiac myocytes remain largely unknown at present. The detailed signaling pathway responsible for carbachol-induced suppression of contraction rhythm in spontaneously beating cultured cardiac myocytes is being

under investigation.

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Figure legends

Fig. 1

Carbachol-induced negative chronotropy in cultured cardiac myocytes. Cultures were treated with carbachol and the beating rhythm of cardiac myocytes was measured. Figures A1-A4 show the contraction rhythm of myocytes before carbachol (100 μmol/L) treatment (A1), 2 min (A2), 5 min (A3), and 10 min (A4) after the treatment. Figure B represents the dose-dependent effect of carbachol-induced negative chronotropy at 2, 5, and 10 min after the onset of carbachol treatment. The beat rate was normalized with the mean rate 5 min before carbachol treatment. Such a decrease in beat rate caused by carbachol treatment resulted from activation of muscarinic acetylcholine receptors (C). Cultures were pre-treated with atropine (1.0 μmol/L) for 10 min before and during carbachol. Each bar indicates the mean+SD (n=4 different cultures). * $P < 0.05$ vs carbachol treatment.

Fig. 2

Involvement of nitric oxide (NO) in carbachol-induced negative chronotropy. Cultured cardiac myocytes were pre-treated with either L-NMMA (100 μmol/L) or carboxy-PTIO (10 μmol/L) before the start of carbachol treatment and during the treatment. Either L-NMMA or

carboxy-PTIO treatment significantly attenuated carbachol-induced negative chronotropy in spontaneously beating cultured cardiac myocytes (A). The beat rate of cardiac myocytes was measured 30 min after the beginning of carbachol treatment. Figure B shows the dose-dependent decrease in the beat rate caused by treatment of cultures with various concentrations of SNAP, a donor of NO measured at 30 and 60 min after the start of SNAP treatment. Figure C shows the decreace in the beat rate by treatment with 10 μ mol/L NOR 4. * $P < 0.05$ vs CCh (A) or 1 % DMSO (B) treatment. Each bar in A and B shows the mean+SD ($n \geqq 4$ different cultures). Abbreviation: DMSO, dimethylsulphoxide (a vehicle of SNAP).

Fig. 3

Involvement of mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels in carbachol-induced negative chronotropy. Pre-treatment of cultured cardiac myocytes with 1.0 mmol/L 5-hydroxydecanoate (5-HD), a selective blocker of mitoK_{ATP} channels, attenuated the 100 μ mol/L carbachol-induced decrease in the beat rate of cardiac myocytes (A). 5-HD treatment also attenuated the 500 μ mol/L SNAP-induced negative chronotropy (A). Figure B shows the dose-dependent suppressive effect on the beat rate caused by treatment with various concentrations of diazoxide, an opener of mitoK_{ATP} channels. * $P < 0.05$ vs carbachol, SNAP (A) or 1 % DMSO (B), a vehicle of diazoxide, treatment. Each bar in A and B shows the

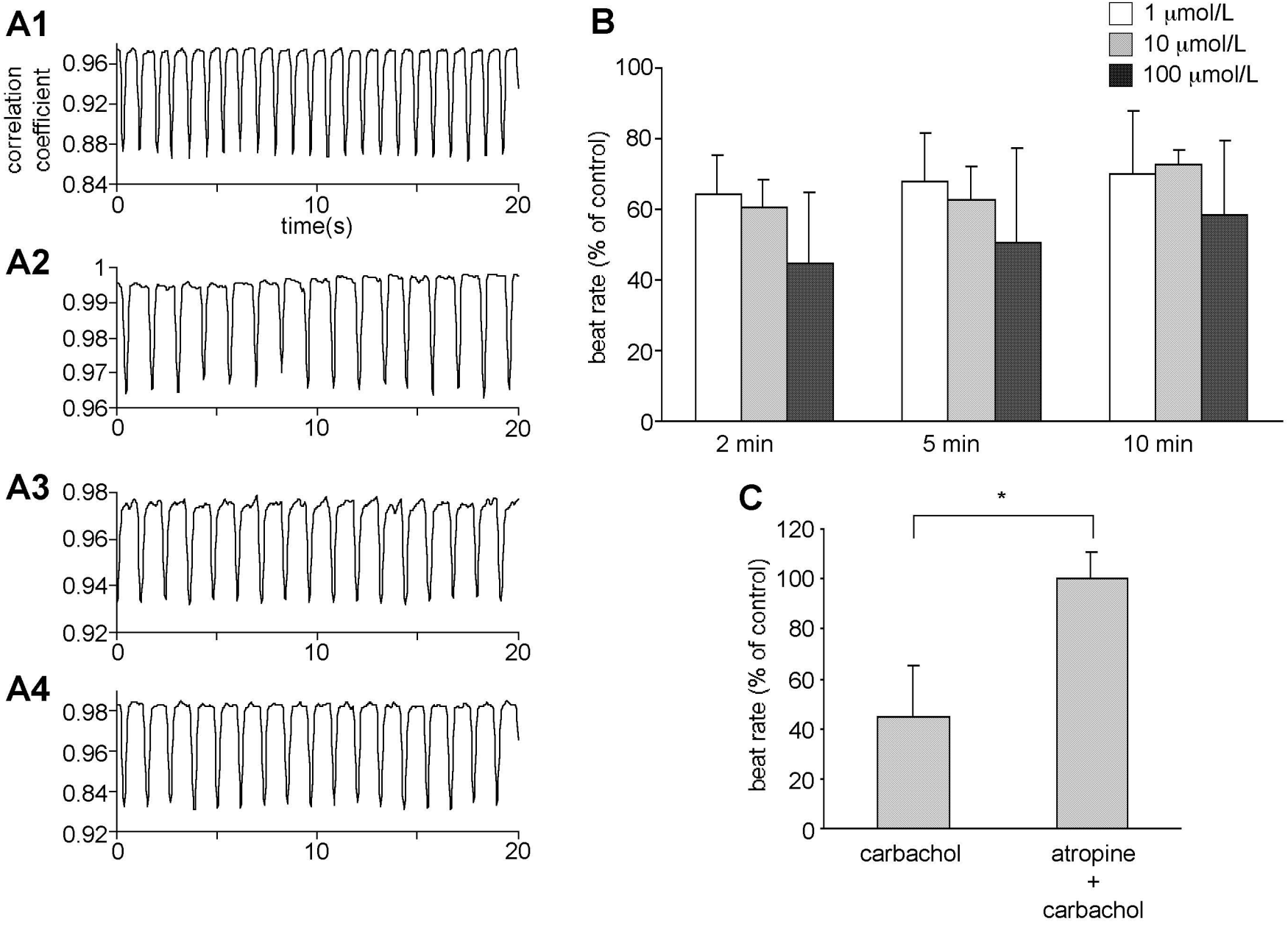
mean+SD (n≧4 different cultures).

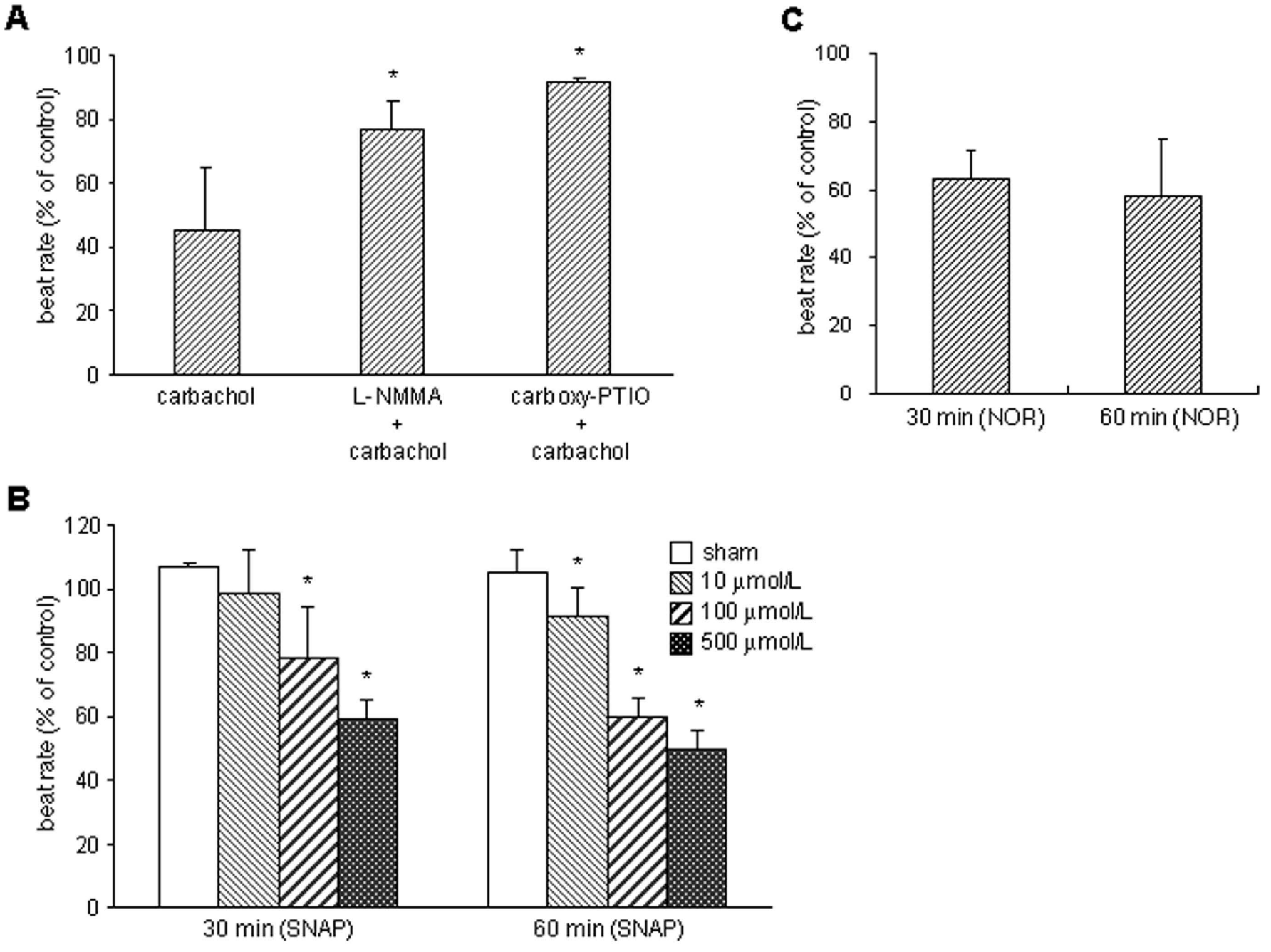
Fig. 4

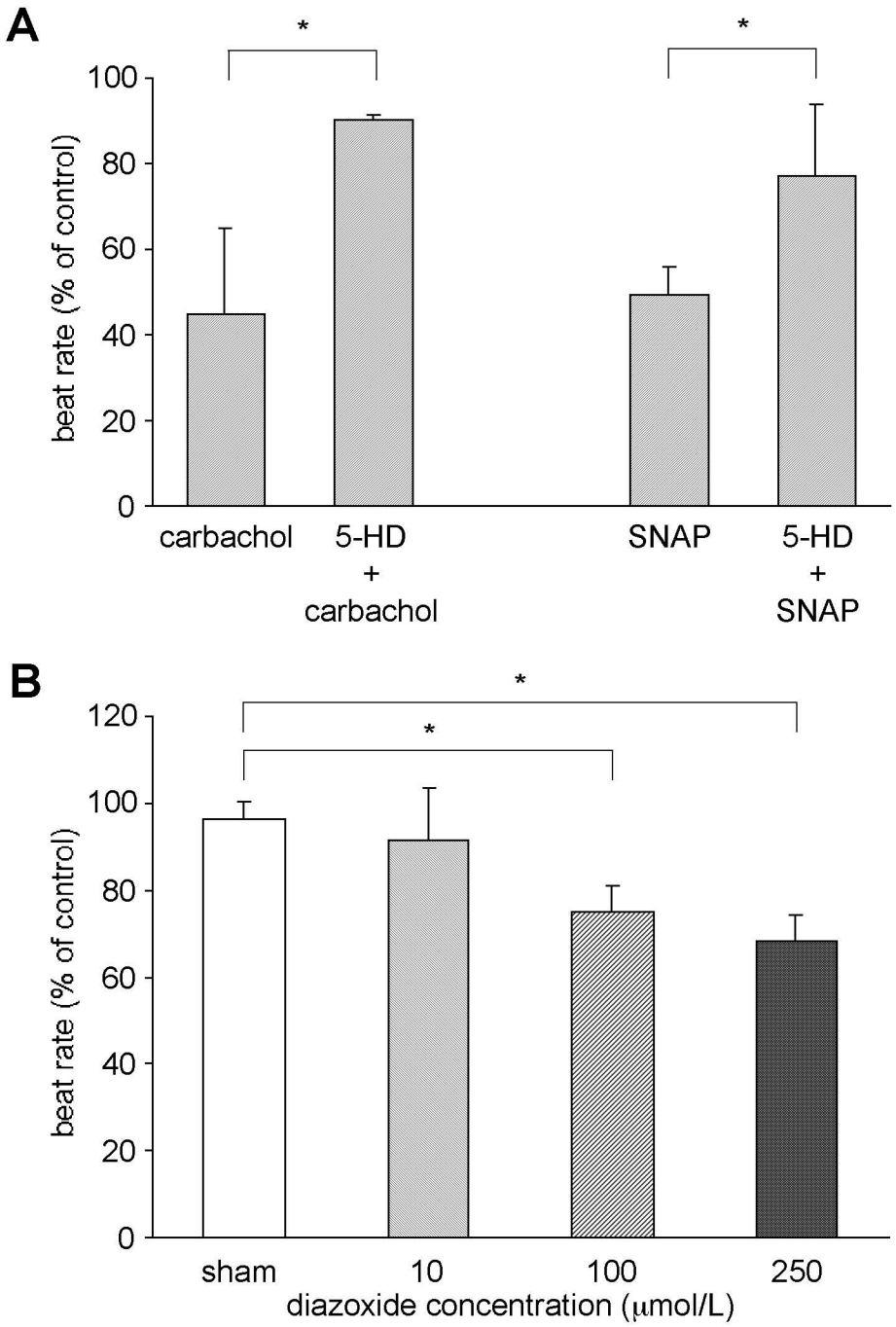
Carbachol increased the production of reactive oxygen species (ROS). Treatment of cultures with carbachol increased the intensity of 2',7'-dichlorofluorescein (DCF) fluorescence (B & C), but sham treatment did not (A & C), suggesting that production of intracellular ROS was enhanced by treatment with carbachol. Figures a1 and a2 show the DCF fluorescence of cardiac myocytes at 0 min and 30 min after the onset of the fluorescence measurement, respectively. Figures b1 and b2 show the DCF fluorescence at 0 min and 30 min (i.e., 20 min after the exposure to carbachol) after the start of fluorescence measurement, respectively. Pink circles in these images show the region of interest (ROI) in the cardiac myocyte. A scale bar in a1 indicates 100 μm. Figure C shows the mean DCF fluorescence at 25 - 30 min after the onset of measurement of DCF fluorescent intensity (t=0 min). The fluorescent intensity was normalized with the mean intensity at 0 - 5 min. * $P < 0.05$ vs sham treatment. Each bar in C shows the mean+SD (n=20 cells from 4 different cultures). Abbreviation: PBS, phosphate-buffered saline.

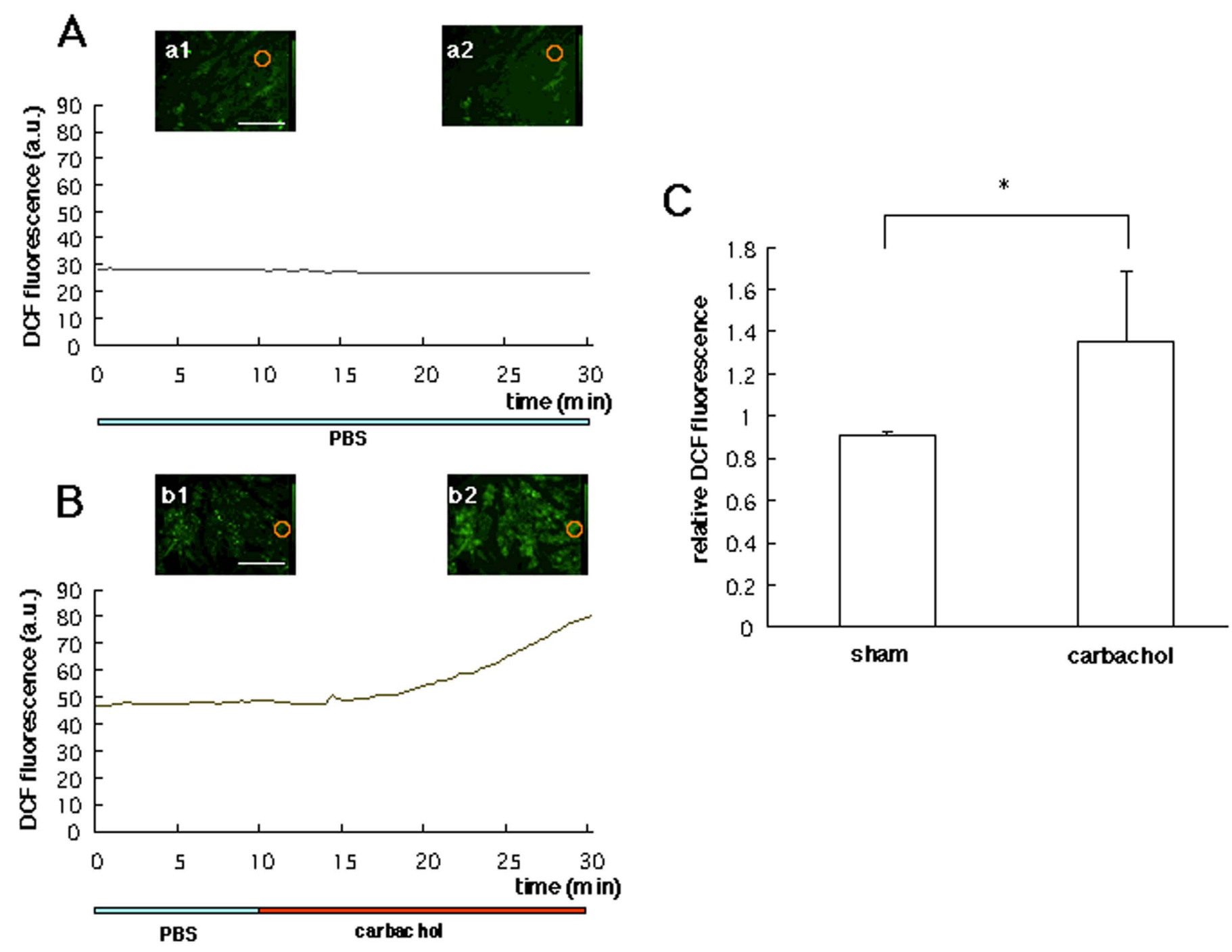
Fig. 5

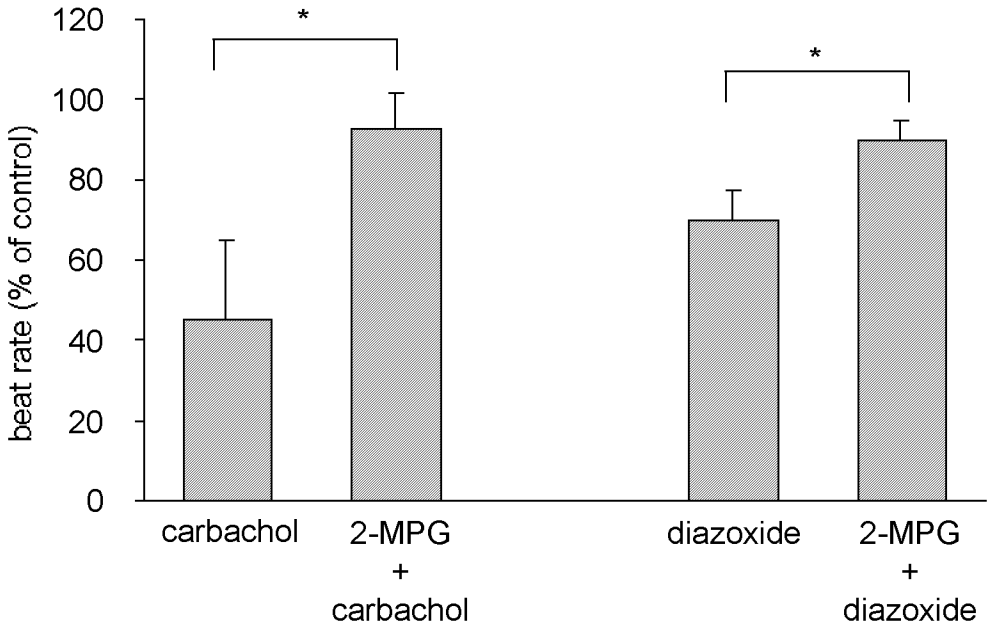
Involvement of reactive oxygen species (ROS) in carbachol-induced negative chronotropy. Carbachol-induced negative chronotropic effect on the spontaneous beating in cardiac myocytes was significantly attenuated by pre-treatment with 2-MPG. Diazoxide-induced negative chronotropy was also significantly attenuated by pre-treatment with 2-MPG . * $P < 0.05$ vs CCh or diazoxide treatment. Each bar in E shows the mean+SD (n=4 different cultures). Abbreviations are the same as those in Fig. 1 and Fig. 2.


A1
correlation coefficient
0.96
0.92
0.88
0.84
0
10
20
time(s)
A2
1
0.99
0.98
0.97
0.96
0
10
20
A3
0.98
0.96
0.94
0.92
0
10
20
A4
0.98
0.96
0.84
0.92
0
10
20
B
1 μmol/L
10 μmol/L
100 μmol/L
beat rate (% of control)
100
80
60
40
20
0
2 min
5 min
10 min
C
*
beat rate (% of control)
120
100
80
60
40
20
0
carbachol
atropine
+
carbachol


A
beat rate (% of control)
100
80
60
40
20
0
*
*
carbachol
L-NMMA
+
carbachol
carboxy-PTIO
+
carbachol
C
beat rate (% of control)
100
80
60
40
20
0
30 min (NOR)
60 min (NOR)
B
beat rate (% of control)
120
100
80
60
40
20
0
sham
10 µmol/L
100 µmol/L
500 µmol/L
*
*
*
*
30 min (SNAP)
60 min (SNAP)


A
*
*
100
80
60
40
20
0
beat rate (% of control)
carbachol
5-HD + carbachol
SNAP
5-HD + SNAP
B
*
*
120
100
80
60
40
20
0
beat rate (% of control)
sham
10
100
250
diazoxide concentration (μmol/L)


A
a1
a2
DCF fluorescence (a.u.)
0
10
20
30
40
50
60
70
80
90
0
5
10
15
20
25
30
time (min)
PBS
B
b1
b2
DCF fluorescence (a.u.)
0
10
20
30
40
50
60
70
80
90
0
5
10
15
20
25
30
time (min)
PBS
carbachol
C
*
relative DCF fluorescence
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
sham
carbachol


120
100
80
60
40
20
0
beat rate (% of control)
*
*
carbachol
2-MPG
+
carbachol
diazoxide
2-MPG
+
diazoxide