β_2-Adrenergic and M_2-muscarinic receptors decrease basal t-tubular L-type Ca^{2+} channel activity and suppress ventricular contractility in heart failure

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Abstract

L-type Ca^{2+} channels (LTCC) play a crucial role in cardiac excitation-contraction coupling. We previously found that in failing ventricular myocytes of mice chronically treated with isoproterenol, basal t-tubular (TT) LTCC activity was halved by activation of protein phosphatase (PP)2A whereas basal surface sarcolemmal (SS) LTCC activity was doubled by inhibition of PP1. Interestingly, chronic treatment of these mice with pertussis toxin almost completely normalized TT and SS LTCC densities and cardiac contractility. In the present study, we therefore sought to identify the $G_{i/o}$ protein-coupled receptors in cardiac myocytes (i.e. β_2-adrenergic, M_2-muscarinic and A_1-adenosine receptors) that are responsible for these abnormalities in heart failure by chronically administrating mice a selective antagonist of each receptor (ICI118,551, atropine and 8-cyclopentyl-1,3-dipropilxanthine (DPCPX), respectively) with isoproterenol. Compared with mice treated with isoproterenol alone, mice treated with isoproterenol plus ICI118,551 or atropine, but not DPCPX showed significantly lower lung weight/tibial length, higher fractional shortening, lower left ventricular end-diastolic pressure and higher dP/dt_{max} and dP/dt_{min}. In addition, ventricular myocytes of mice treated with isoproterenol plus ICI118,551 or atropine, but not DPCPX exhibited significantly higher TT and lower SS LTCC current densities than those of mice treated with isoproterenol alone due to normalization of the PP activities. These results indicate that β_2-adrenergic, M_2-muscarinic, but not A_1-adenosine receptors contribute to reduced ventricular

contractility at least partially by decreasing basal TT LTCC activity in heart failure. Therefore, antagonists of β_2-adrenergic and/or M_2-muscarinic receptors can be good adjuncts to β_1-adrenergic receptor antagonists in the treatment of heart failure.

Keywords

Heart failure; L-type Ca^{2+} channels; Protein phosphatase; β_2-adrenergic receptor; M_2-muscarinic receptor; A_1-adenosine receptor

1. Introduction

Despite recently advanced medical therapies, heart failure is one of the leading risks of death in developed nations (Go et al., 2013). Thus, development of medical treatment based on further understanding of the pathophysiology of heart failure is required to improve the quality of life of patients with heart failure. Maladaptive remodeling of ventricular excitation-contraction (EC) coupling caused by such as chronic β-adrenergic receptor stimulation, and activation of the renin-angiotensin-aldosterone system plays a predominant role in an impaired cardiac function in heart failure (Mann, 2008; Osadchii, 2007; Rockman et al., 2002). The underlying primary defect is a decrease in sarcoplasmic reticulum (SR) Ca^{2+} content due to decreased activity and/or expression of SR Ca^{2+}-ATPase 2, increased expression of sarcolemmal Na^{+}/Ca^{2+} exchangers and diastolic Ca^{2+} leak from the SR via ryanodine receptors (Bers, 2008).

L-type Ca^{2+} channel (LTCC) currents play a crucial role in cardiac EC coupling (McDonald et al., 1994). In most cardiac hypertrophy and failure, whole-cell LTCC current density is reported not to change markedly (Bers, 2008; Mukherjee and Spinale, 1998). However, we previously found for the first time that basal t-tubular (TT) LTCC activity was halved by activation of protein phosphatase (PP)2A whereas basal surface sarcolemmal (SS) LTCC activity was doubled by inhibition of PP1 in failing ventricular myocytes of mice chronically treated with isoproterenol (Horiuchi-Hirose et al., 2011; Kashihara et al., 2012). Because $G_{i/o}$ proteins are known to activate

PP2A (Herzig et al., 1995; Ke et al., 2004; Liu and Hofmann, 2003) and suppress PP1 in cardiac myocytes (Zhu et al., 2008), we examined the effect on isoproterenol-treated mice of pertussis toxin, a selective inhibitor of the coupling between G protein-coupled receptors and $G_{i/o}$ proteins (Kashihara et al., 2012). Pertussis toxin significantly increased basal TT LTCC activity, decreased basal SS LTCC activity and increased cardiac contractility independently of protein kinase A in isoproterenol-treated mice.

In this study, we sought to identify the G protein-coupled receptors that lead to abnormal LTCC activity and cardiac dysfunction in heart failure. In the heart, M_2-muscarinic and A_1-adenosine receptors are the principal G protein-coupled receptors coupled with $G_{i/o}$ proteins. Moreover, the phosphorylation of β_2-adrenergic receptors by protein kinase A or G protein-coupled receptor kinase, which are expected to occur in heart failure, switches their coupling from G_s to G_i (Daaka et al., 1997; Liu et al., 2009); therefore, we examined whether any of these three types of receptors are involved in the abnormalities in isoproterenol-treated mice with a selective antagonist for each receptor.

In the present study, we found that chronic activation of β_2-adrenergic and M_2-muscarinic, but not A_1-adenosine receptors decreases cardiac contractile function at least partially by decreasing basal TT LTCC activity in heart failure. Therefore, antagonists of β_2-adrenergic and M_2-muscarinic receptors can be good adjuncts to β_1-adrenergic receptor antagonists in the treatment of heart failure.

A part of the preliminary results of this study has been published elsewhere in the form of an abstract (Kashihara et al., 2013).

2. Materials and Methods

2.1 *Chemicals*

Chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise indicated. Isoproterenol (Sigma-Aldrich, Inc., St. Louis, MO, USA) and atropine were dissolved in saline. ICI118,551 (Sigma-Aldrich) and 8-cyclopentyl-1,3-dipropilxanthine (DPCPX) (Sigma-Aldrich) were dissolved in 100% dimethyl sulfoxide (DMSO) at 50 and 6.6 mM, respectively. They were then diluted 3.3 and 20 times, respectively, with saline before administration to mice. Okadaic acid (Calbiochem, La Jolla, CA, USA) and fostriecin were dissolved at 1.0 mM in 100% DMSO. Final 0.1% DMSO did not significantly affect LTCC currents in isolated myocytes.

2.2 *Animal model*

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experiments were carried out in accordance with the Guidelines for Animal Experimentation of Shinshu University and approved by the Committee for Animal Experimentation. Male C57BL/6 adult mice (8-10 weeks) were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). Cardiac hypertrophy and failure were induced in the mice by subcutaneous injection of 6 mg/kg/day isoproterenol once daily from Day 0 to 21 as previously described (Horiuchi-Hirose et al., 2011). Age-matched normal control mice received the same volume of saline only. To examine the effect of

the inhibition of β_2-adrenergic, M_2-muscarinic and A_1-adenosine receptors on isoproterenol-induced cardiac hypertrophy and failure, mice were treated with 0.5 mg/kg/day ICI118,551, 3 mg/kg/day atropine or 1 mg/kg/day DPCPX along with isoproterenol. ICI118,551 and atropine were applied to mice through osmotic minipumps (model 1004; Alzet, Cupertino, CA, USA) whereas DPCPX was applied daily intraperitoneally because it is too hydrophobic to be used with osmotic minipumps. The dose of ICI118,551 (0.5 mg/kg/day) was the maximum applicable to mice with the pump. The blood concentration of ICI118,551 in mice applied with this dose of the agent was 0.54 ± 0.07 nM whereas the pA_2 value of this agent for β_1 and β_2-adrenergic receptors is 7.17 and 9.26, respectively. The dose of atropine was determined according to previous reports (Jumrussirikul et al., 1998; Uechi et al., 1998). In pilot studies, mice injected with 1, 3 or 10 mg/kg/day of atropine along with 6 mg/kg/day isoproterenol exhibited the fractional shortening of 32.7 ± 1.9, 36.4 ± 1.0, 35.0 ± 1.4%, respectively. Thus, we used the maximum effective dose of atropine (i.e. 3 mg/kg/day). The dose of DPCPX was determined according to a previous report (Koeppen et al., 2009). The half maximum inhibitory dose of intravenously applied N6-cyclopentyladenosine, a selective A_1 adenosine receptor agonist in causing negative chronotropic effect was 7.09 and 226 μg/kg in mice injected with saline or 1 mg/kg/day DPCPX. Pumps were implanted under pentobarbital sodium (30 mg/kg) anesthesia administered intraperitoneally on Day -1. These antagonists were applied to mice from Day -1 to 21, whereas isoproterenol was applied from Day 0 to 21.

All animals were used for experiments on Day 22. Animals were sacrificed by sodium pentobarbital (30 mg/kg) anesthesia administrated intraperitoneally. The hearts and lungs were excised from the animals, rinsed in ice-cold modified Tyrode solution containing (in mmol/l): NaCl, 136.5; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.53; HEPES (Dojindo Laboratories, Kumamoto, Japan), 5.5; and glucose, 5.5 (pH = 7.4 with NaOH) and weighed. Because the body weight of all groups of mice treated with drugs was significantly higher than that of saline-treated mice (Horiuchi-Hirose et al., 2011), the heart weight (HW; in mg) and lung weight (LW; in mg) were normalized to the tibial length (TL; in mm), which was not significantly different among the five groups of mice (i.e. HW/TL and LW/TL).

2.3 *Gross and histological evaluation of hearts*

The morphology of the heart was evaluated as previously described (Horiuchi-Hirose et al., 2011). Briefly, the hearts were fixed in 20% phosphate-buffered formalin at room temperature for 24 h and cut transversely to obtain ventricular cross sections. The tissues were then embedded in paraffin and cut into 4-μm thick slices. Histological sections were stained with hematoxylin-eosin and examined with a light microscope (BX-51; Olympus Corp., Tokyo, Japan). Wall thickness and the chamber diameter of the left ventricle were measured with an objective micrometer as the scale on digital microscopic images taken from histological sections.

2.4 *Measurement of heart rate (HR) and mean systemic blood pressure (MBP).*

HR and MBP in conscious, warmed and restrained mice were measured by the tail-cuff method with a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) once a week between Day -1 and 22. Mice were restrained for 3 min with a temperature-controlled warming holder (37°C) before these measurements. Each estimation was the average of three records taken at 1 min intervals. These measurements were performed 20 h after the isoproterenol injection.

2.5 *Ultrasound cardiography*

Cardiac function was assessed with ultrasound cardiography (GE Yokogawa Medical System K.K., Tokyo, Japan) in anesthetized mice as previously described (Horiuchi-Hirose et al., 2011). Briefly, hearts were viewed at the level of the papillary muscles in the short axis. On M-mode tracings, the average of three consecutive beats was used to measure the following parameters: left ventricular (LV) end-diastolic diameter (EDD); LV end-systolic diameter (ESD); and fractional shortening (FS), calculated as (EDD − ESD)/EDD × 100%.

2.6 *Cardiac catheterization.*

Under 2,2,2-tribromoethanol (250 mg/kg) anesthesia administrated intraperitoneally, a micro-tipped pressure catheter transducer (model 420 LP; Samba Sensor, Gothenburg, Sweden) was inserted into the right carotid artery and advanced into the LV cavity. After stabilization for 5 min, the signal was continuously recorded with a Samba 201 system (Samba Sensor) coupled with a PowerLab system (Chart4; ADInstruments, Dunedin, New Zealand), stored and displayed on a

computer. LV preload was assessed by end-diastolic pressure (LVEDP; in mmHg); contractility, by dP/dt_{max} (in mmHg/s); and relaxation, by dP/dt_{min} (in mmHg/s).

2.7 *Isolation of cardiac myocytes.*

Ventricular myocytes were enzymatically isolated as previously described (Horiuchi-Hirose et al., 2011). Briefly, the heart mounted on a Langendorff apparatus was digested with nominally Ca^{2+}-free Tyrode solution containing 0.80 mg/ml collagenase, 0.06 mg/ml protease (Sigma-Aldrich, Inc.), 1.20 mg/ml hyaluronidase (Sigma-Aldrich, Inc.), 0.03 mg/ml DNase I (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and 0.50 mg/ml bovine serum albumin at 37°C for 2 min. Isolated ventricular myocytes were suspended in Ca^{2+}-free Tyrode solution containing 1 mg/ml bovine serum albumin at room temperature, and the Ca^{2+} concentration was gradually increased to 1.8 mmol/l.

2.8 *Detubulation*

Detubulation of isolated ventricular myocytes was carried out with osmotic shock as previously described (Horiuchi-Hirose et al., 2011; Kawai et al., 1999). Briefly, myocytes were treated with modified Tyrode solution containing formamide (1.5 mol/l) for 15 min and returned to the modified Tyrode solution. A membrane-staining dye, di-8-ANEPPS clearly stained t-tubules in non-detubulated but barely in detubulated myocytes (Horiuchi-Hirose et al., 2011), suggesting that detubulation detached the t-tubular invaginations from the surface sarcolemma because hydrophobic

di-8-ANEPPS could stain the t-tubules that were occluded yet connected to the sarcolemma. In different experiments, detubulation significantly reduced cell membrane capacitance (Table 1), indicating that intra-t-tubular space was electrically disconnected from extracellular space. Taken together, it is likely that t-tubular LTCC currents were not measured in the whole-cell configuration in detubulated myocytes.

2.9 *Electrophysiology*

The current of isolated ventricular myocytes was studied in the whole-cell configuration of the patch-clamp technique at 35-37°C with a patch-clamp amplifier (EPC8; HEKA Instruments Inc., Bellmore, NY, USA) as previously described (Horiuchi-Hirose et al., 2011). Series resistance was always kept <7 MΩ and was routinely compensated using an amplifier by ~75%. This resulted in a voltage drop in series resistance of <3.5 mV in the presence of 2 nA membrane currents. LTCC currents were measured with pipette solution containing (in mmol/l): D-glutamate, 90; N-methyl-D(-)-glucamine, 10; $MgCl_2$, 5; tetraethylammonium chloride, 20; EGTA, 10; HEPES, 20; and ATP 3 (pH = 7.3 with CsOH). The bathing solution contained (in mmol/l): N-methyl-D(-)-glucamine, 150; CsCl, 5.4; $CaCl_2$, 2; $MgCl_2$, 1.2; 4-aminopyridine, 2; HEPES, 5; and glucose, 5.5 (pH = 7.4 with HCl). The membrane potential was stepped from -90 mV to -50 mV for 200 msec and then for 500 msec to potentials between -110 and +70 mV with a 10 mV increment every 5 sec. LTCC currents were isolated as the current inhibited by Cd^{2+} (100 μmol/l) plus nifedipine (10 μmol/l) (Yamada et

al., 2008). The peak amplitude of LTCC currents evoked by the 500 msec test pulse was plotted against the membrane potential.

To assess SS and TT LTCC current densities, the peak LTCC current amplitude at 0 mV and the membrane capacitance were measured in non-detubulated and detubulated myocytes (Table 1). SS and TT LTCC current densities were calculated from these values as previously described (Horiuchi-Hirose et al., 2011). Briefly, the LTCC current density in SS and TT (D_{SS} and D_{TT}, respectively) was calculated with the following equations:

$$D_{SS} = (I_D - \alpha * I_N) / (C_D - \alpha * C_N) \quad \text{Eq.1}$$

$$D_{TT} = (I_N - I_D) / (C_N - C_D) \quad \text{Eq. 2}$$

, where I_N and I_D are the peak LTCC current amplitude at 0 mV of non-detubulated and detubulated myocytes, respectively; C_N and C_D, the membrane capacitance of non-detubulated and detubulated myocytes, respectively; and α, fraction of TT membranes remaining in detubulated myocytes. α was estimated as 0.17 from binary image analysis of detubulated myocytes stained with di-8-ANEPPS. Because non-detubulated and detubulated myocytes were different groups of myocytes, the above calculation was carried out with the mean membrane capacitance and current amplitude of each

group.

The liquid junction potential of the pipette solution in relation to the modified Tyrode solution was -10 mV and taken into consideration throughout this study.

2.10 *Statistical analysis.*

Data are shown as the mean ± S.E.M. Statistical significance was evaluated with Student's unpaired *t*-test. For multiple comparisons of data, analysis of variance with Bonferroni's test was used. $P<0.05$ was considered significant.

3. Results

3.1 *Effect of inhibition of $G_{i/o}$ protein-coupled β_2-adrenergic, M_2-muscarinic and A_1-adenosine receptors on cardiac hypertrophy and failure in mice chronically treated with isoproterenol.*

We first examined whether inhibition of cardiac major $G_{i/o}$-coupled receptors (β_2-adrenergic, M_2-musucarinic, and A_1-adenosine receptors) with antagonists ameliorated the cardiac hypertrophy and failure of isoproterenol-treated mice. Namely, mice were treated with saline alone, isoproterenol alone, isoproterenol plus ICI118,551, isoproterenol plus atropine, or isoproterenol plus DPCPX. Mice treated with these drugs showed cardiac hypertrophy and dilation of ventricular chambers compared with those treated with saline (Fig. 1A). Although the left ventricular wall thickness (LVWT) was comparable in all groups, the left ventricular chamber diameter (LVCD) and HW/TL were significantly higher in mice treated with the drugs than those treated with saline (Fig. 1B), indicating that neither β_2-adrenergic, M_2-musucarinic, nor A_1-adenosine receptors contributed to isoproterenol-induced cardiac hypertrophy. LW/TL was also significantly higher in mice treated with the drugs than those treated with saline; however, LW/TL of mice treated with isoproterenol plus ICI118,551 or atropine was significantly lower than that of mice treated with isoproterenol alone (Fig. 1B), indicating that ICI118,551 and atropine ameliorated the congestive heart failure caused by isoproterenol.

Table 2 summarizes alterations in the HR and MBP during the treatment of mice with

saline or the drugs. HR of mice treated with isoproterenol alone progressively decreased as compared with those treated with saline, which was not attenuated by ICI118,551, atropine or DPCPX, most likely as a consequence of downregulation of β_1-adrenergic receptors. MBP also progressively decreased in mice treated with the drugs as compared with those treated with saline; however, MBP of mice treated with isoproterenol plus ICI118,551 or atropine was significantly higher than that of mice treated with isoproterenol alone, indicating that the effect of ICI118,551 and atropine on pulmonary edema was not due to their effect on the afterload of the heart.

M-mode ultrasound cardiogram showed that mice treated with isoproterenol alone exhibited increased end-diastolic and -systolic left ventricular diameters and decreased wall motion as compared with saline-treated mice as previously reported (Fig. 2A) (Horiuchi-Hirose et al., 2011). Mice treated with isoproterenol plus ICI118,551 or atropine exhibited smaller end-systolic left ventricular diameters and better wall motion than those treated with isoproterenol alone. These parameters of mice treated with isoproterenol plus DPCPX were comparable with those of mice treated with isoproterenol alone. Calculated fractional shortening (FS) of mice treated with isoproterenol plus ICI118,551 or atropine was significantly lower than that of mice treated with saline but significantly higher than that of mice treated with isoproterenol alone (Fig. 2B). FS was greatly decreased in mice treated with isoproterenol alone or isoproterenol plus DPCPX as compared with those treated with saline.

Hemodynamic assessment with catheterization showed that mice treated with the drugs exhibited significantly higher LVEDP and significantly smaller dP/dt_{max} and dP/dt_{min} than saline-treated mice (Fig. 3A, B and C) (Horiuchi-Hirose et al., 2011). However, mice treated with isoproterenol plus ICI118,551 or atropine exhibited significantly higher dP/dt_{max} and dP/dt_{min} and lower LVEDP than those treated with isoproterenol alone. These parameters of mice treated with isoproterenol plus DPCPX were comparable with those of mice treated with isoproterenol alone; therefore, β_2-adrenergic and M_2-muscarinic, but not A_1-adenosine receptors were involved in the cardiac dysfunction of isoproterenol-treated mice.

3.2 *Effect of inhibition of $G_{i/o}$ protein-coupled β_2-adrenergic, M_2-muscarinic and A_1-adenosin receptors on the L-type Ca^{2+} channel current density in mice chronically treated with isoproterenol.*

We previously demonstrated that chronic G protein-coupled receptor-mediated activation of $G_{i/o}$ decreased cardiac contractility at least partially by decreasing TT LTCC current density in isoproterenol-treated mice (Kashihara et al., 2012). We therefore assessed whether inhibition of β_2-adrenergic, M_2-muscarinic, and A_1-adenosine receptors normalized LTCC current density in myocytes of mice chronically treated with isoproterenol. Figure 4A (solid lines) shows representative whole-cell LTCC currents of myocytes of each group of mice in response to depolarization to 0 mV from the prepulse of -50 mV. To separately assess SS and TT LTCC current densities, we measured LTCC currents in ventricular myocytes with detubulation, a procedure to

acutely occlude TT in isolated ventricular myocytes (Horiuchi-Hirose et al., 2011; Kawai et al., 1999). As shown in Fig. 4A (broken lines), detubulation substantially reduced LTCC current amplitude in all groups of myocytes.

Figure 4B shows the pooled data of the peak current-voltage relationships. Detubulation reduced the LTCC current amplitude by 62% in myocytes of saline-treated mice but by only 30% in those of isoproterenol-treated mice (Fig. 4A and B), indicating that TT LTCC currents were decreased in isoproterenol vs. saline-treated mice. On the other hand, detubulation reduced the LTCC current amplitude by 60% in myocytes of mice treated with isoproterenol plus ICI118,551; by 57%, isoproterenol plus atropine; and by 35%, isoproterenol plus DPCPX. Thus, inhibition of β_2-adrenergic and M_2-muscarinic, but not A_1-adenosine receptors may have attenuated the decreased TT LTCC current density found in myocytes of mice treated with isoproterenol.

From the peak LTCC current amplitude at 0 mV and the membrane capacitance of non-detubulated and detubulated myocytes (Table 1), we could assess the LTCC current density in SS and TT membrane compartments (Horiuchi-Hirose et al., 2011). There were no significant differences in whole-cell (WC) LTCC current density in each group of myocytes (Fig. 4C). In myocytes of saline-treated mice, there was ~5 times higher LTCC current density in the TT membrane than in the SS membrane (Horiuchi-Hirose et al., 2011). In myocytes of isoproterenol-treated mice, there were significantly higher SS and lower TT LTCC current densities than in

myocytes of saline-treated mice. Notably, myocytes of mice treated with isoproterenol plus ICI118,551 or atropine but not DPCPX showed significantly lower SS and higher TT LTCC current densities than those of mice treated with isoproterenol alone. The LTCC current densities in myocytes of mice treated with isoproterenol plus ICI118,551 and atropine were similar to those of myocytes of saline-treated mice; therefore, β_2-adrenergic and M_2-muscarinic receptors may have induced cardiac dysfunction in isoproterenol-treated mice at least partially by decreasing TT LTCC activity.

3.3 Effect of inhibition of protein phosphatases on the L-type Ca^{2+} channel current density in each group of mice.

We previously reported that in isoproterenol-treated mice, SS LTCC current density is increased due to $G_{i/o}$-mediated suppression of PP1 whereas TT LTCC current density is decreased due to $G_{i/o}$-mediated activation of PP2A (Horiuchi-Hirose et al., 2011; Kashihara et al., 2012). Thus, we finally examined whether ICI118,551 and atropine normalized SS and TT LTCC current densities by ameliorating the abnormal PP activities. Figure 5A shows the WC, SS and TT LTCC current densities in each group of mice in the presence of okadaic acid (OA) (1 μM), a PP1 and PP2A inhibitor, where the difference in SS and TT LTCC densities among groups was abolished. This indicates that isoproterenol caused the abnormal LTCC densities whereas ICI118,551 and atropine, but not DPCPX normalized the LTCC densities by modifying PP1 and/or 2A. Figure 5B shows the

results in the presence of fostriecin (1 μM), a selective PP2A inhibitor. Under this condition, the difference in TT but not SS LTCC densities among groups was abolished, indicating that isoproterenol decreased the TT LTCC density by stimulating PP2A whereas ICI118,551 and atropine, but not DPCPX increased the TT LTCC density by inhibiting PP2A. Together with Fig. 5A, Fig. 5B also indicates that isoproterenol increased the SS LTCC density by inhibiting PP1 whereas ICI118,551 and atropine, but not DPCPX decreased the SS LTCC density by activating PP1.

Discussion

In heart failure, chronic and excessive β-adrenergic receptor stimulation causes remodeling of the myocardium and aggravates cardiac function (Osadchii, 2007; Rockman et al., 2002). This concept is the theoretical basis for the treatment of heart failure with β-adrenergic antagonists. In experiments, transgenic overexpression of β_1-adrenergic receptors in the murine heart causes dilated cardiomyopathy (Engelhardt et al., 1999). We also found that metoprolol, a selective β_1-adrenergic antagonist, almost completely prevented cardiac hypertrophy and failure of isoproterenol-treated mice (data not shown), indicating that excessive stimulation of β_1-adrenergic receptors is the primary cause of myocardial remodeling in isoproterenol-treated mice (Osadchii, 2007; Xiao et al., 2006). However, we also previously found that chronic administration of pertussis toxin significantly improved the cardiac function of isoproterenol-treated mice, indicating that $G_{i/o}$ chronically activated by some G protein-coupled receptors participate in the cardiac dysfunction of isoproterenol-treated mice (Kashihara et al., 2012). Consistent with this observation, it is reported that the increased activity of $G_{i/o}$ suppresses cardiac function in heart failure (El-Armouche et al., 2003). In the present study, we sought to identify $G_{i/o}$ protein-coupled receptors contributing to the cardiac dysfunction of isoproterenol-treated mice. Compared with mice treated with isoproterenol alone, mice treated with isoproterenol plus ICI118,551 or atropine, but not DPCPX showed significantly lower LW/TL (Fig. 1B) and better hemodynamic parameters (i.e. higher FS (Fig. 2B), lower LVEDP, and higher dP/dt_{max}

and dP/dt_{min} (Fig. 3B and C)). In addition, myocytes of mice treated with isoproterenol plus ICI118,551 or atropine, but not DPCPX exhibited significantly higher TT and lower SS LTCC current densities than those of mice treated with isoproterenol alone (Fig. 4C) due to normalization of PP activities (Fig. 5). Thus, chronic activation of β_2-adrenergic and M_2-muscarinic, but not A_1-adenosine receptors may cause cardiac dysfunction at least partially by decreasing basal TT LTCC activity in isoproterenol-treated mice. It is, however, necessary to confirm the present finding in more clinically relevant models of heart failure, such as those induced by pressure/volume overload or myocardial infarction, in future.

It is uncertain whether $G_{i/o}$ was activated by ligand-independent (i.e. basal) or -dependent activity of β_2-adrenergic and M_2-muscarinic receptors in isoproterenol-treated mice because ICI118,551 and atropine exert inverse agonism under some conditions (Nelson et al., 2006; Zhou et al., 1999). In animal models of heart failure and patients with idiopathic dilated cardiomyopathy, the expression level of M_2-muscaric receptors is increased (Le Guludec et al., 1997; Vatner et al., 1996). It is also known that β_2-adrenergic receptors become more efficiently coupled with $G_{i/o}$ in a failing than normal heart (Daaka et al., 1997; Liu et al., 2009; Paur et al.). These changes of M_2-muscarinic and β_2-adrenergic receptors chronically increase $G_{i/o}$ activity independently of their ligands. Indeed, we found that abnormal LTCC activities persisted at least for several hours in isolated myocytes of isoproterenol-treated mice in the absence of any G protein-coupled receptor agonists (Horiuchi-

Hirose et al., 2011; Kashihara et al., 2012). On the other hand, it is also possible that β_2-adrenergic and M_2-muscarinic receptors were activated by their ligands *in vivo*. Although isoproterenol administrated to mice must have stimulated β_2-adrenergic receptors, adrenaline secreted from the adrenal medulla would also have activated β_2-adrenergic receptors because its plasma concentration increases in heart failure (Kaye et al., 1995; Paur et al.). It is somewhat surprising that acetylcholine acting on M_2-muscarinic receptors worsens heart failure because many experimental studies have demonstrated that vagal stimulation has beneficial effects on heart failure (Kishi, 2012). Recently, Kanazawa et al. reported that the cardiac sympathetic nervous system exhibits cholinergic transdifferentiation in heart failure (Kanazawa et al., 2010). Thus, one possibility is that such aberrant acetylcholine might cause deteriorating effects on failing cardiac myocytes. Probably, both ligand-independent and -dependent activities of β_2-adrenergic and M_2-muscarinic receptors cause abnormal LTCC activities and cardiac function under excessive β_1-adrenergic stimulation in heart failure.

$G_{i/o}$ inhibits adenylyl cyclase (Sunahara et al., 1996). Thus, ICI118,551 and atropine might have improved cardiac function by increasing cAMP concentration in myocytes; however, neither mice treated with isoproterenol plus ICI118,551 nor atropine exhibited significantly higher HR than mice treated with isoproterenol alone (Table 2), contrary to this speculation. Moreover, we previously found that a selective protein kinase A inhibitor, H-89 did not affect SS or TT LTCC

activity in ventricular myocytes of mice chronically treated with isoproterenol plus pertussis toxin, indicating that the chronic inhibition of $G_{i/o}$ did not significantly increase cAMP concentration in cardiac myocytes (Kashihara et al., 2012). We propose that ICI118,551 and atropine increased cardiac function of isoproterenol-treated mice by normalizing the abnormal effects of PPs on basal LTCC activity independently of protein kinase A (Fig. 5). ICI118,551 and atropine each separately substantially reversed the effect of isoproterenol on many parameters measured in this study: i.e. these drug effects were not additive. This was probably because β_2-adrenergic and M_2-muscarinic receptors shared $G_{i/o}$ proteins and PPs as a common pathway. Although Liu and Hoffmann reported that activation of A_1-adenosine receptors lead to PP2A-induced dephosphorylation of phospholamban and troponin T in cardiac myocytes, we did not see significant effects of DPCPX on LTCC activity (Liu and Hofmann, 2003). PP2A has many different subunit compositions and, depending on the composition, different subcellular localization and different regulation (Janssens and Goris, 2001). Thus, we speculate that PP2A associated with LTCC may not be regulated by A_1-adenosine receptors in cardiac myocytes.

5. Conclusion

To summarize, for the first time, we found that chronic activation of $G_{i/o}$ through β_2-adrenergic and M_2-muscarinic, but not A_1-adenosine receptors compromises cardiac contractility at least partially by altering basal TT LTCC activity in heart failure. Thus, selective antagonists of β_2-adrenergic and M_2-muscarinic receptors may be useful in the treatment of chronic heart failure with excessive β-adrenergic stimulation; however, it is known that increased $G_{i/o}$ activity is not only harmful but also protective of a failing heart because it prevents excessive G_s/cAMP/protein kinase A stimulation. Therefore, it would be desirous to use these antagonists in heart failure in conjunction with β_1-adrenergic blockers that suppress G_s activity.

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References

Bers, D.M., 2008. Heart failure. In: Bers, D.M. (Ed.), Excitation-Contraction Coupling and Cardiac Contractile Force. Springer, Dordrecht, The Netherlands, pp. 316-323.

Daaka, Y., Luttrell, L.M., Lefkowitz, R.J., 1997. Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature 390, 88-91.

El-Armouche, A., Zolk, O., Rau, T., Eschenhagen, T., 2003. Inhibitory G-proteins and their role in desensitization of the adenylyl cyclase pathway in heart failure. Cardiovasc Res 60, 478-487.

Engelhardt, S., Hein, L., Wiesmann, F., Lohse, M.J., 1999. Progressive hypertrophy and heart failure in beta1-adrenergic receptor transgenic mice. Proc Natl Acad Sci U S A 96, 7059-7064.

Go, A.S., Mozaffarian, D., Roger, V.L., Benjamin, E.J., Berry, J.D., Borden, W.B., Bravata, D.M., Dai, S., Ford, E.S., Fox, C.S., Franco, S., Fullerton, H.J., Gillespie, C., Hailpern, S.M., Heit, J.A., Howard, V.J., Huffman, M.D., Kissela, B.M., Kittner, S.J., Lackland, D.T., Lichtman, J.H., Lisabeth, L.D., Magid, D., Marcus, G.M., Marelli, A., Matchar, D.B., McGuire, D.K., Mohler, E.R., Moy, C.S., Mussolino, M.E., Nichol, G., Paynter, N.P., Schreiner, P.J., Sorlie, P.D., Stein, J., Turan, T.N., Virani, S.S., Wong, N.D., Woo, D., Turner, M.B., 2013. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. Circulation 127, e6-e245.

Herzig, S., Meier, A., Pfeiffer, M., Neumann, J., 1995. Stimulation of protein phosphatases as a mechanism of the muscarinic-receptor-mediated inhibition of cardiac L-type Ca^{2+} channels. Pflugers Arch 429, 531-538.

Horiuchi-Hirose, M., Kashihara, T., Nakada, T., Kurebayashi, N., Shimojo, H., Shibazaki, T., Sheng, X., Yano, S., Hirose, M., Hongo, M., Sakurai, T., Moriizumi, T., Ueda, H., Yamada, M., 2011. Decrease in the density of t-tubular L-type Ca^{2+} channel currents in failing ventricular myocytes. Am J Physiol Heart Circ Physiol 300, H978-988.

Janssens, V., Goris, J., 2001. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. The Biochemical journal 353, 417-439.

Jumrussirikul, P., Dinerman, J., Dawson, T.M., Dawson, V.L., Ekelund, U., Georgakopoulos, D., Schramm, L.P., Calkins, H., Snyder, S.H., Hare, J.M., Berger, R.D., 1998. Interaction between neuronal nitric oxide synthase and inhibitory G protein activity in heart rate regulation in conscious mice. The Journal of clinical investigation 102, 1279-1285.

Kanazawa, H., Ieda, M., Kimura, K., Arai, T., Kawaguchi-Manabe, H., Matsuhashi, T., Endo, J., Sano, M., Kawakami, T., Kimura, T., Monkawa, T., Hayashi, M., Iwanami, A., Okano, H., Okada, Y., Ishibashi-Ueda, H., Ogawa, S., Fukuda, K., 2010. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling

cytokines in rodents. The Journal of clinical investigation 120, 408-421.

Kashihara, T., Nakada, T., Gomi, S., Hirose, M., Yamada, M., 2013. The molecular mechanism by which chronic and excessive β1 adrenergic stimulation remodels ventricular excitation contraction coupling. Journal of Physiological Sciences 63, S94.

Kashihara, T., Nakada, T., Shimojo, H., Horiuchi-Hirose, M., Gomi, S., Shibazaki, T., Sheng, X., Hirose, M., Hongo, M., Yamada, M., 2012. Chronic receptor-mediated activation of $G_{i/o}$ proteins alters basal t-tubular and sarcolemmal L-type Ca^{2+} channel activity through phosphatases in heart failure. Am J Physiol Heart Circ Physiol 302, H1645-1654.

Kawai, M., Hussain, M., Orchard, C.H., 1999. Excitation-contraction coupling in rat ventricular myocytes after formamide-induced detubulation. Am J Physiol 277, H603-609.

Kaye, D.M., Lefkovits, J., Cox, H., Lambert, G., Jennings, G., Turner, A., Esler, M.D., 1995. Regional epinephrine kinetics in human heart failure: evidence for extra-adrenal, nonneural release. Am J Physiol 269, H182-188.

Ke, Y., Wang, L., Pyle, W.G., de Tombe, P.P., Solaro, R.J., 2004. Intracellular localization and functional effects of P21-activated kinase-1 (Pak1) in cardiac myocytes. Circulation research 94, 194-200.

Kishi, T., 2012. Heart failure as an autonomic nervous system dysfunction. Journal of cardiology 59, 117-122.

Koeppen, M., Eckle, T., Eltzschig, H.K., 2009. Selective deletion of the A_1 adenosine receptor abolishes heart-rate slowing effects of intravascular adenosine in vivo. PloS one 4, e6784.

Le Guludec, D., Cohen-Solal, A., Delforge, J., Delahaye, N., Syrota, A., Merlet, P., 1997. Increased myocardial muscarinic receptor density in idiopathic dilated cardiomyopathy: an *in vivo* PET study. Circulation 96, 3416-3422.

Liu, Q., Hofmann, P.A., 2003. Modulation of protein phosphatase 2a by adenosine A_1 receptors in cardiomyocytes: role for p38 MAPK. Am J Physiol Heart Circ Physiol 285, H97-103.

Liu, R., Ramani, B., Soto, D., De Arcangelis, V., Xiang, Y., 2009. Agonist dose-dependent phosphorylation by protein kinase A and G protein-coupled receptor kinase regulates beta2 adrenoceptor coupling to G_i proteins in cardiomyocytes. The Journal of biological chemistry 284, 32279-32287.

Mann, D.L., 2008. Pathophysiology of heart failure. In: Libby, P., Bonow, R.O., Mann. D.L., Zipes, D.P., Braunwald, E. (Ed.), Heart Diseases. Saunders Elsevier, Philadelphia, pp. 541-560.

McDonald, T.F., Pelzer, S., Trautwein, W., Pelzer, D.J., 1994. Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. Physiol Rev 74, 365-507.

Mukherjee, R., Spinale, F.G., 1998. L-type calcium channel abundance and function with cardiac hypertrophy and failure: a review. J Mol Cell Cardiol 30, 1899-1916.

Nelson, C.P., Nahorski, S.R., Challiss, R.A., 2006. Constitutive activity and inverse agonism at the

M_2 muscarinic acetylcholine receptor. The Journal of pharmacology and experimental therapeutics 316, 279-288.

Osadchii, O.E., 2007. Cardiac hypertrophy induced by sustained beta-adrenoreceptor activation: pathophysiological aspects. Heart Fail Rev 12, 66-86.

Paur, H., Wright, P.T., Sikkel, M.B., Tranter, M.H., Mansfield, C., O'Gara, P., Stuckey, D.J., Nikolaev, V.O., Diakonov, I., Pannell, L., Gong, H., Sun, H., Peters, N.S., Petrou, M., Zheng, Z., Gorelik, J., Lyon, A.R., Harding, S.E., High levels of circulating epinephrine trigger apical cardiodepression in a $beta_2$-adrenergic receptor/G_i-dependent manner: a new model of Takotsubo cardiomyopathy. Circulation 126, 697-706.

Rockman, H.A., Koch, W.J., Lefkowitz, R.J., 2002. Seven-transmembrane-spanning receptors and heart function. Nature 415, 206-212.

Sunahara, R.K., Dessauer, C.W., Gilman, A.G., 1996. Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol 36, 461-480.

Uechi, M., Asai, K., Osaka, M., Smith, A., Sato, N., Wagner, T.E., Ishikawa, Y., Hayakawa, H., Vatner, D.E., Shannon, R.P., Homcy, C.J., Vatner, S.F., 1998. Depressed heart rate variability and arterial baroreflex in conscious transgenic mice with overexpression of cardiac $G_{s\alpha}$. Circulation research 82, 416-423.

Vatner, D.E., Sato, N., Galper, J.B., Vatner, S.F., 1996. Physiological and biochemical evidence for

coordinate increases in muscarinic receptors and G_i during pacing-induced heart failure. Circulation 94, 102-107.

Xiao, R.P., Zhu, W., Zheng, M., Cao, C., Zhang, Y., Lakatta, E.G., Han, Q., 2006. Subtype-specific $alpha_1$- and beta-adrenoceptor signaling in the heart. Trends Pharmacol Sci 27, 330-337.

Yamada, M., Ohta, K., Niwa, A., Tsujino, N., Nakada, T., Hirose, M., 2008. Contribution of L-type Ca^{2+} channels to early afterdepolarizations induced by I_{Kr} and I_{Ks} channel suppression in guinea pig ventricular myocytes. J Membr Biol 222, 151-166.

Zhou, Y.Y., Cheng, H., Song, L.S., Wang, D., Lakatta, E.G., Xiao, R.P., 1999. Spontaneous $beta_2$-adrenergic signaling fails to modulate L-type Ca^{2+} current in mouse ventricular myocytes. Molecular pharmacology 56, 485-493.

Zhu, M., Gach, A.A., Liu, G., Xu, X., Lim, C.C., Zhang, J.X., Mao, L., Chuprun, K., Koch, W.J., Liao, R., Koren, G., Blaxall, B.C., Mende, U., 2008. Enhanced calcium cycling and contractile function in transgenic hearts expressing constitutively active $G_{\alpha o}$* protein. Am J Physiol Heart Circ Physiol 294, H1335-1347.

Figure Legends

Fig. 1. Effect of antagonists on cardiac hypertrophy and failure in mice chronically treated with isoproterenol. A: low-magnification histological photographs of the ventricular cross section of hearts stained with hematoxylin-eosin. Original magnification ×1.25. B: left ventricular wall thickness (LVWT), left ventricular chamber diameter (LVCD), heart weight (HW)-to-tibial length (TL) ratio (HW/TL), and lung weight (LW)-to-tibial length ratio (LW/TL). Graphs show the mean ± S.E.M; n = 6-10 for each group. CONT, control; ISO, isoproterenol; ICI, ICI118,551; ATR, atropine. *P<0.05 vs. CONT; #P<0.05 vs. ISO.

Fig. 2. Effect of antagonists on cardiac function assessed with ultrasound cardiogram in mice chronically treated with isoproterenol. A: representative M-mode ultrasound cardiogram traces. EDD, end-diastolic diameter; ESD, end-systolic diameter. B: fractional shortening (FS). Graphs show the mean ± S.E.M; n = 6-10 for each group. CONT, control; ISO, isoproterenol; ICI, ICI118,551; ATR, atropine. *P<0.05 vs. CONT; #P<0.05 vs. ISO.

Fig. 3. Effect of antagonists on basal left-ventricular hemodynamic parameters of mice chronically treated with isoproterenol. A: representative traces of left ventricular pressure (LVP) and dP/dt (in mmHg/s). B: the maximal and minimum rates of LVP development (dP/dt_{max} and

dP/dt_{min}, respectively). C: left ventricular end-diastolic pressure (LVEDP). Graphs show the mean ± S.E.M; n = 5 for each group. CONT, control; ISO, isoproterenol; ICI, ICI118,551; ATR, atropine. *P<0.05 vs. CONT; #P<0.05 vs. ISO.

Fig. 4. Effect of antagonists on L-type Ca^{2+} channel (LTCC) current densities in whole cell, surface sarcolemmal, and t-tubular membranes in mice chronically treated with isoproterenol. A: representative L-type Ca^{2+} channel (LTCC) currents at 0 mV in CONT, ISO, ISO + ICI, ISO + ATR, and ISO + DPCPX myocytes with (broken lines) and without detubulation (solid lines). B: peak current-voltage relationships of LTCC currents in CONT, ISO, ISO + ICI, ISO + ATR, and ISO + DPCPX myocytes with (■) and without detubulation (○). C: LTCC current density at 0 mV in whole-cell (WC), surface sarcolemmal (SS) and t-tubular (TT) membranes in CONT, ISO, ISO + ICI, ISO + ATR and ISO + DPCPX myocytes. Graphs show the mean ± S.E.M; n = 6-8 for each group. CONT, control; ISO, isoproterenol; ICI, ICI118,551; ATR, atropine. *P<0.05 vs. CONT; #P<0.05 vs. ISO

Fig. 5. Effect of a protein phosphatase 1 and 2A inhibitor, okadaic acid, and a selective protein phosphatase 2A inhibitor, fostriecin on L-type Ca^{2+} channel (LTCC) current densities in whole-cell (WC), surface sarcolemmal (SS), and t-tubular membranes (TT). A and B: LTCC current

density at 0 mV in WC, SS, and TT in CONT, ISO, ISO + ICI, ISO + ATR, and ISO + DPCPX myocytes in the presence of okadaic acid (1 μM) (A) or fostriecin (1 μM) (B). Graphs show the mean ± S.E.M; n = 5-6 for each group. CONT, control; ISO, isoproterenol; ICI, ICI118,551; ATR, atropine. $^{*}P<0.05$ vs. CONT; $^{\#}P<0.05$ vs. ISO.

Table 1: The peak LTCC current amplitude at 0 mV and the membrane capacitance of non-detubulated (ND) and detubulated (D) myocytes.

	I_{Ca} at 0 mV (nA)	Membrane capacitance (pF)
CONT		
ND	1.38±0.06	168.8±6.6
D	0.53±0.03[a]	117.1±4.3[a]
ISO		
ND	1.60±0.11	229.7±9.5
D	1.11±0.06[a]	159.4±8.2[a]
ISO + ICI		
ND	1.91±0.12	226.3±7.4
D	0.76±0.07[a]	150.0±6.8[a]
ISO + ATR		
ND	1.78±0.06	227.3±6.8
D	0.78±0.06[a]	156.7±5.7[a]
ISO + DPCPX		
ND	1.80±0.12	239.0±5.4
D	1.16±0.08[a]	166.1±3.5[a]

Values are the mean ± S.E.M. N = 6-8 for each group. CONT, control, ISO, isoproterenol; ICI, ICI118,551; ATR, atropine. [a] $P < 0.05$ vs. ND.

Table 2: Effect of chronic administration of receptor agonists and/or inverse agonists on the heart rate and mean systemic blood pressure of mice.

	CONT	ISO	ISO + ICI	ISO + ATR	ISO + DPCPX
Heat rate, beats/min					
Before	665 ± 10	658 ± 12	654 ± 10	650 ± 10	653 ± 14
1 wk later	673 ± 6	596 ± 4[a]	635 ± 13	613 ± 15[a]	589 ± 7[a]
2 wk later	669 ± 7	551 ± 10[a]	569 ± 12[a]	583 ± 17[a]	544 ± 13[a]
3 wk later	660 ± 11	546 ± 5[a]	560 ± 10[a]	559 ± 15[a]	534 ± 7[a]
Mean systemic blood pressure, mmHg					
Before	81.2 ± 2.8	81.0 ± 2.1	80.7 ± 1.9	80.9 ± 1.6	83.7 ± 1.9
1 wk later	80.6 ± 1.2	73.1 ± 1.3[a]	80.0 ± 1.5[b]	76.6 ± 1.1	72.7 ± 3.1[a]
2 wk later	79.6 ± 0.5	66.8 ± 2.9[a]	75.3 ± 1.1[b]	76.4 ± 1.4[b]	68.8 ± 1.6[a]
3 wk later	80.4 ± 0.9	64.7 ± 1.2[a]	76.4 ± 1.0[b]	71.8 ± 1.5[a,b]	68.3 ± 1.6[a]

Values are the mean ± S.E.M. N = 6 for all groups. CONT, control; ISO, isoproterenol; ICI, ICI118,551; ATR, atropine. [a] $P < 0.05$ vs. CONT; [b] $P < 0.05$ vs. ISO.

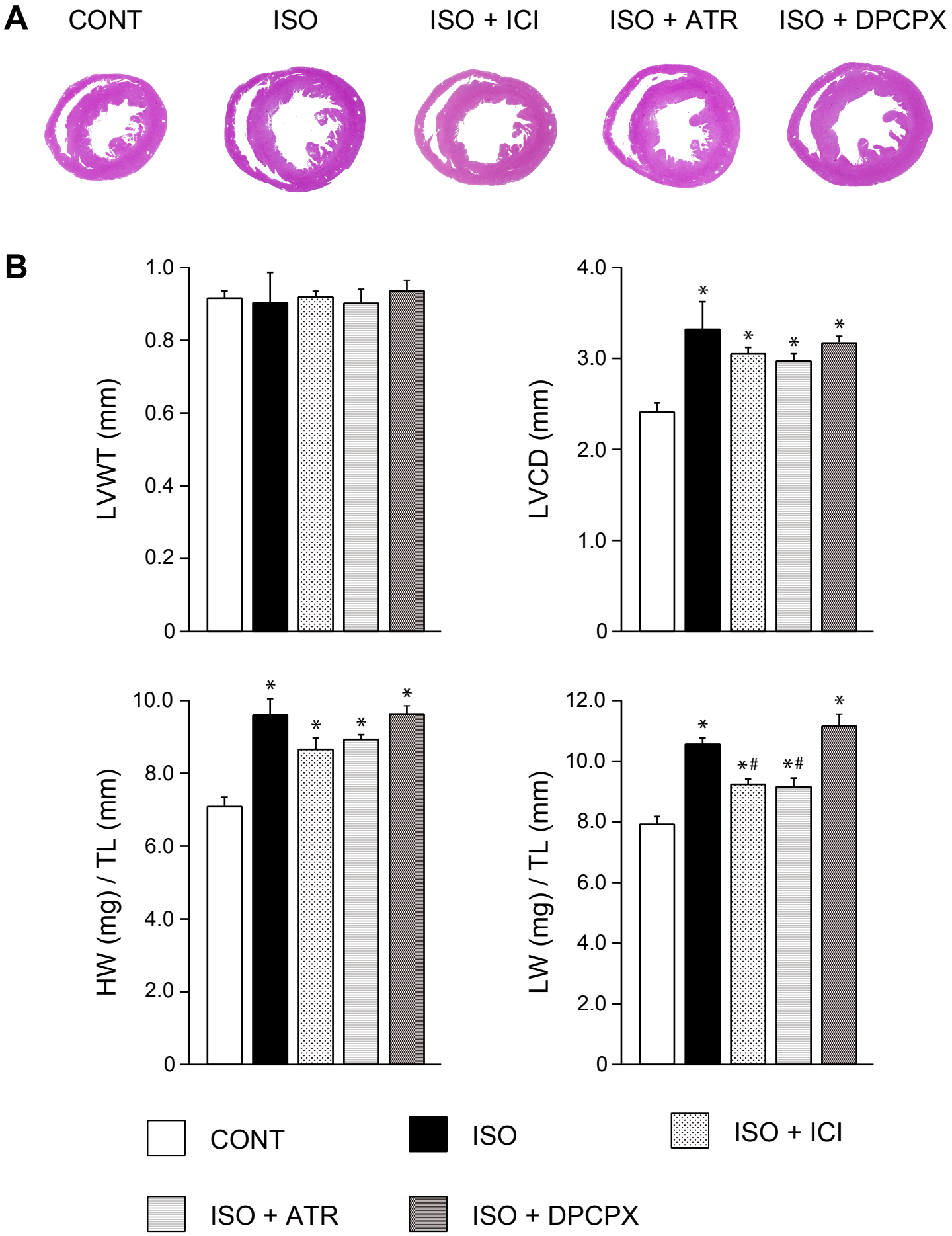

A
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX
B
LVWT (mm)
LVCD (mm)
HW (mg) / TL (mm)
LW (mg) / TL (mm)
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX

Fig. 1

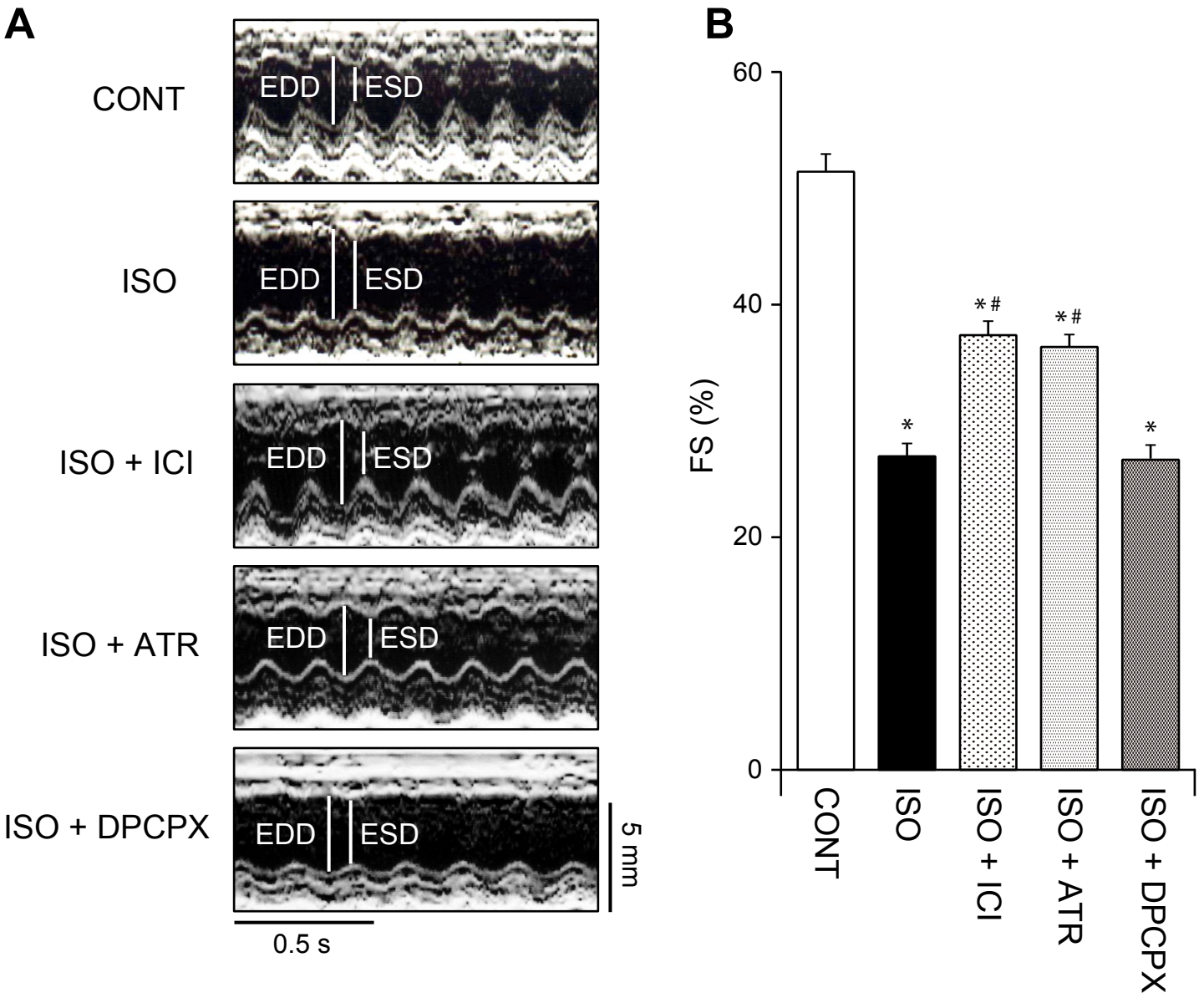

A
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX
EDD
ESD
5 mm
0.5 s
B
FS (%)
60
40
20
0
*
*#
*#
*
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX

Fig. 2

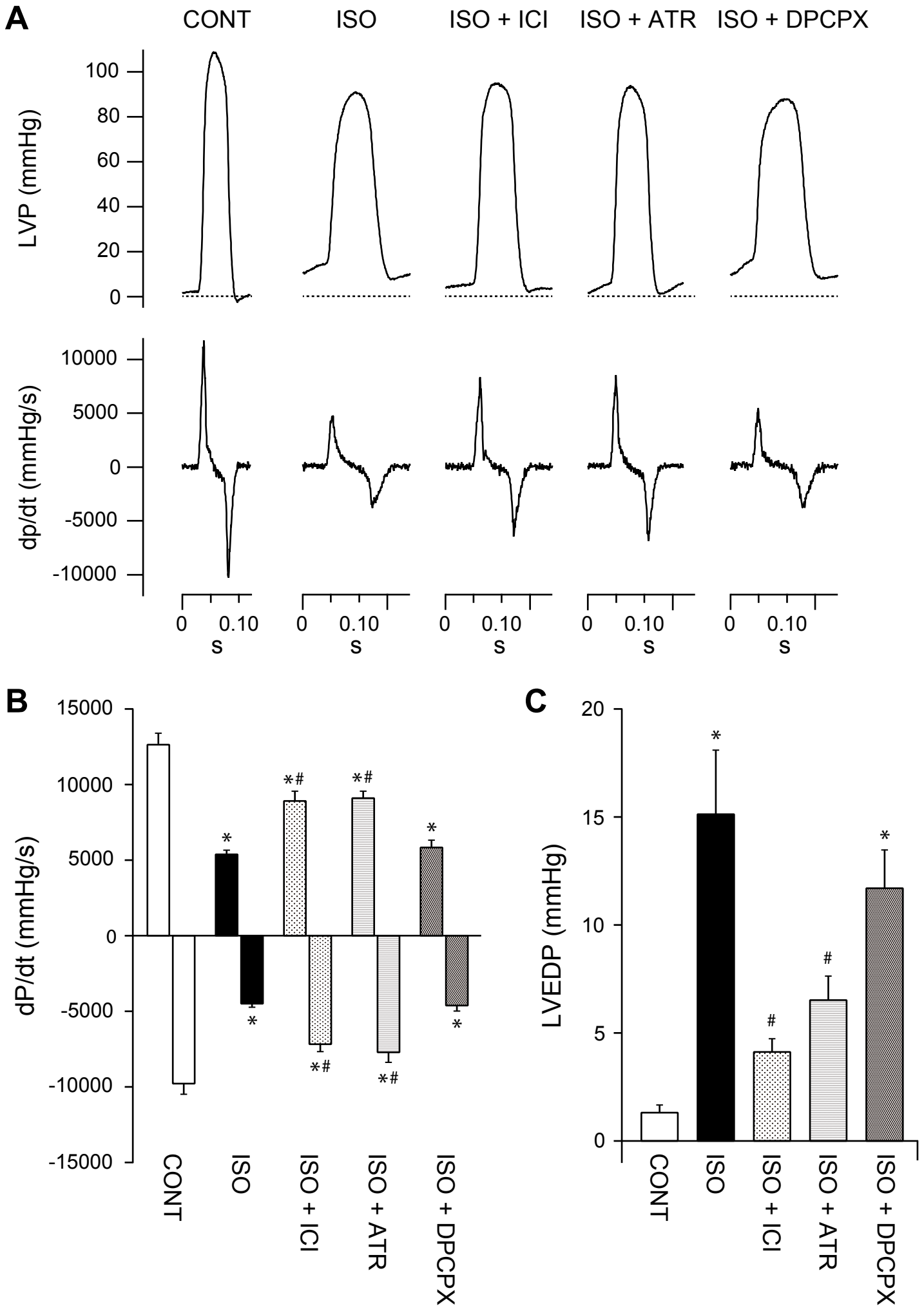

A
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX
LVP (mmHg)
100
80
60
40
20
0
dp/dt (mmHg/s)
10000
5000
0
-5000
-10000
0
0.10
s
B
dP/dt (mmHg/s)
15000
10000
5000
0
-5000
-10000
-15000
*
*#
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX
C
LVEDP (mmHg)
20
15
10
5
0
*
#

Fig. 3

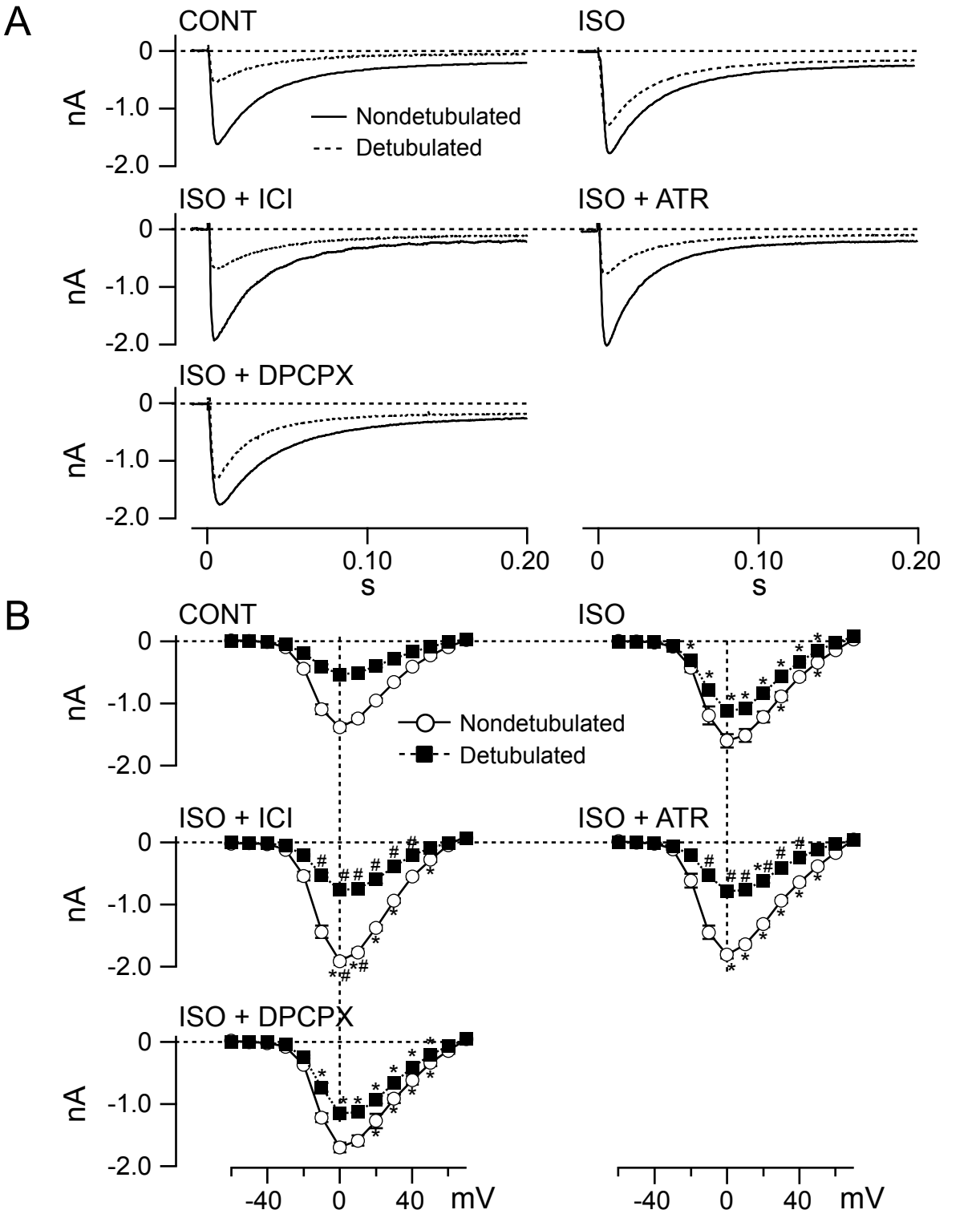

A
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX
Nondetubulated
Detubulated
nA
0
-1.0
-2.0
0.10
0.20
s
B
-40
40
mV
C
LTCC Current Density (pA/pF)
-20
-15
-10
-5
WC
SS
TT
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX

Fig. 4

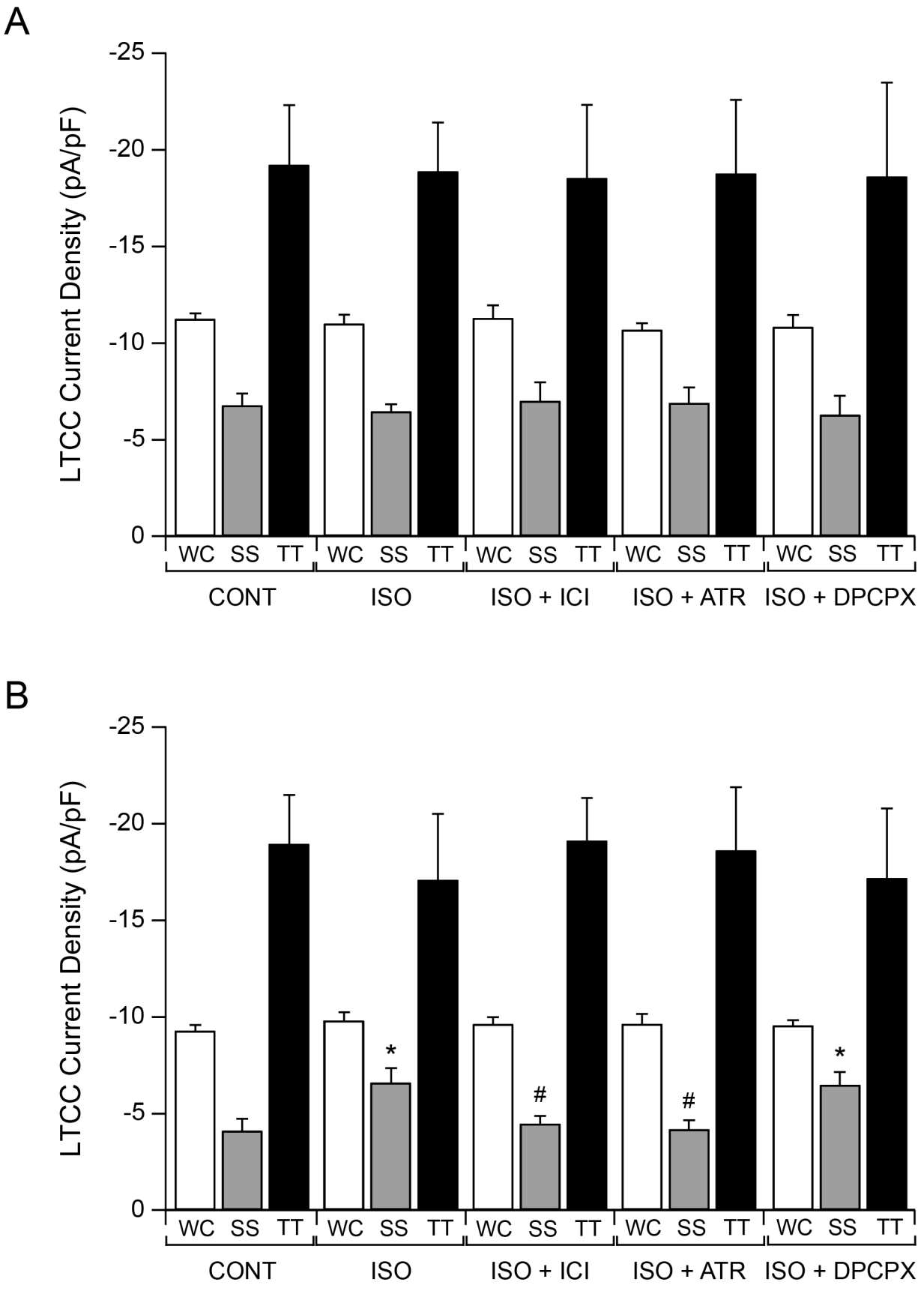
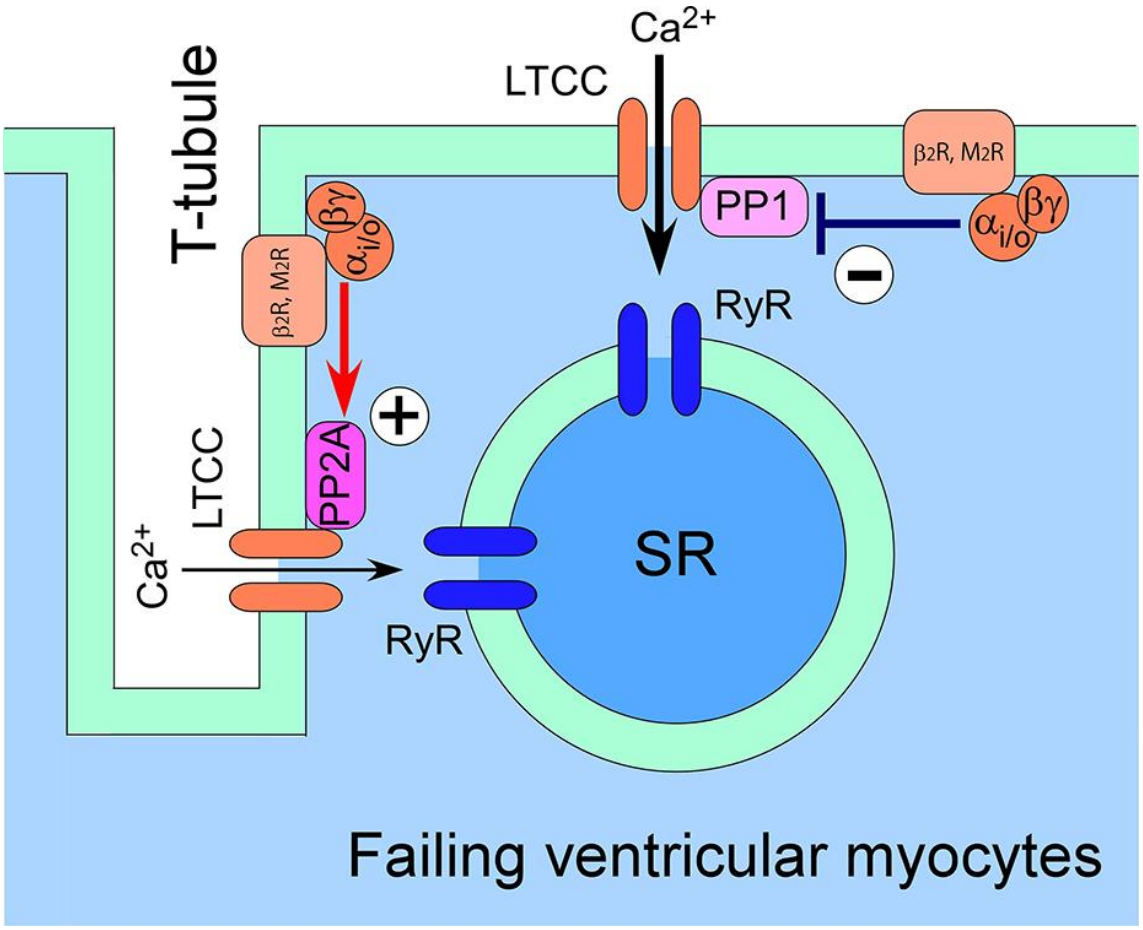

A
LTCC Current Density (pA/pF)
-25
-20
-15
-10
-5
0
WC SS TT WC SS TT WC SS TT WC SS TT WC SS TT
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX
B
LTCC Current Density (pA/pF)
-25
-20
-15
-10
-5
0
*
#
#
*
WC SS TT WC SS TT WC SS TT WC SS TT WC SS TT
CONT
ISO
ISO + ICI
ISO + ATR
ISO + DPCPX

Fig. 5



Graphical abstract