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Interleukin-2 Increases Activity of Sarcoplasmic Reticulum Ca^{2+}-ATPase, but Decreases Its Sensitivity to Calcium in Rat Cardiomyocytes

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Running title: interleukin-2 and calcium handling in rat heart

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ABBREVIATIONS: IL-2, interleukin-2; SR, sarcoplasmic reticulum; EDTA, di-sodium ethylenediaminetetraacetate dehydrate; NorBNI, nor-binaltorphimine; PTX, pertussis toxin; TG, thapsigargin; PLC, phospholipase C; PKC, protein kinase C; cAMP, cyclic adenosine monophosphate; AC, adenylyl cyclase; TEA, tetraethylamonium; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium concentration; EGTA, ethyleneglycol-bis-(beta-aminoethylether)-N,N,N',N'-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MOPS, 3-(N-morpholino) propane-sulfonic acid

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ABSTRACT

In order to further explore the role of interleukin-2 (IL-2) in cardiac function, we investigated its effects on the intracellular calcium transient and the activity of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase in rat cardiomyocytes. IL-2 (200 U/ml) decreased the amplitude of electrically-stimulated and caffeine-induced $[Ca^{2+}]_i$ transients in ventricular myocytes, but increased the end-diastolic calcium level. IL-2 did not affect the sarcolemmal L-type Ca^{2+} channel activity. The activity of SR Ca^{2+}-ATPase from IL-2 treated hearts increased in a dose dependent manner, but the sarcolemmal Ca^{2+}-ATPase activity did not change. After incubation of SR with ATP, the activity of SR Ca^{2+}-ATPase from IL-2 treated hearts increased much more than that in the control group. The responsiveness of SR Ca^{2+}-ATPase from IL-2-perfused hearts to the free calcium concentration was inhibited. The Ca^{2+} uptake and Ca^{2+} content were reduced in the SR vesicles prepared from IL-2 treated rat heart. Pretreatment with the κ–opioid receptor antagonist NorBNI (10 nmol/L) attenuated the effect of IL-2 on the SR Ca^{2+}-ATPase activity, SR Ca^{2+} uptake and Ca^{2+} content. The activity of Ca^{2+}-ATPase in SR isolated from untreated hearts did not change when IL-2 and SR were co-incubated. Thus, we conclude that the decreased calcium transient induced by IL-2 results from reduced SR calcium release, which is due to decreased SR Ca^{2+} uptake mediated by cardiac κ–opioid receptors, but not from reduced activity of the sarcolemmal L-type calcium channel.

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Current evidence suggests that the process of contraction in heart muscle is mainly initiated by the action potential activating L-type channels to cause an influx of Ca^{2+}. The voltage-dependent Ca^{2+} influx induces Ca^{2+} release across the sarcoplasmic reticulum (SR) by a process known as Ca^{2+}-induced Ca^{2+} release (Fabiato, 1983). Together, both sources of Ca^{2+} initiate contraction (Barry and Bridge, 1993; Sipido and Wier, 1991). Relaxation takes place as a result of two main systems that act to decrease the cytoplasmic Ca^{2+} concentration. The SR Ca^{2+}-ATPase pumps Ca^{2+} back into the SR ready for release at the next beat, and the sarcolemmal Na^{+}/Ca^{2+} exchanger extrudes the Ca^{2+} that entered the cell. Although other mechanisms (the sarcolemmal Ca^{2+}-ATPase and the mitochondria) are involved in the maintenance of a low cytoplasmic Ca^{2+} concentration, the proteins that perform the major roles on a beat-to-beat basis are the SR Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger (Bers, 2000).

The Ca^{2+}-ATPase of the SR catalyses the most important step in relaxation by coupling cleavage of ATP to transport of two Ca^{2+} into the SR lumen (Ikemoto, 1982; Inesi, 1985). This process comprises an ordered sequence of elementary events consisting of phosphorylation and dephosphorylation reactions of the enzyme as well as a conformational oscillation that exposes its Ca^{2+} binding moiety either at the cytoplasmic or the luminal side. A dysfunction of SR Ca^{2+}-ATPase has been proposed as a contributing factor to the development of cardiovascular diseases in which cytokines are involved, such as genetic hypertension (Nomura et al., 1997), ischemia and reperfusion injury, myocardial stunning, and heart failure (Zucchi and Ronca-Testoni, 1997).

Interleukin-2 (IL-2), one of the important cytokines, is generally produced by activated helper T lymphocytes and stimulates proliferation and effector functions in various cells of the immune

system. IL-2 is also known to have cardiac effects. A negative inotropic effect of IL-2 has been reported in hamster papillary muscles (Finkel et al., 1992) and isolated ventricular myocytes (Cao et al., 2002). Associated with the change in contractility profile are alterations in intracellular calcium regulation. In IL-2 treated cardiomyocytes, Ca^{2+} regulation is altered, manifesting as a smaller cytoplasmic Ca^{2+} transient and elevated end-diastolic calcium level (Cao et al., 2002). However, very little information is available on IL-2 induced changes in calcium handling. It is known that a decreased intracellular calcium transient might be accounted for by reduced influx of extracellular calcium and/or reduced calcium release from SR as a result of low calcium content. Further, the increased end-diastolic calcium level might result from reduced calcium reuptake by SR Ca^{2+}-ATPase or decreased calcium extrusion through the sarcolemmal Na^{+}/Ca^{2+} exchanger. It has been demonstrated that the negative inotropic effect and decreased calcium transient induced by IL-2 are mediated by the cardiac sarcolemmal κ-opioid receptor (Cao et al., 2002). So the opioid receptor may be involved in changes of calcium handling induced by IL-2. Therefore we hypothesized that the alterations of intracellular calcium handling in active and resting cardiomyocytes during IL-2 challenge may result from abnormalities of SR Ca^{2+}-ATPase activity by activating the sarcolemmal κ-opioid receptor.

Therefore, the aim of the present study was to investigate whether IL-2 alters SR Ca^{2+}-ATPase activity and/or the properties of the contractile apparatus in rat hearts and, if so, by which possible mechanisms. In particular, the activity of SR Ca^{2+}-ATPase was examined with special emphasis on the κ-opioid receptor mediated pathways.

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Materials and Methods

All procedures used in this study were approved by the Ethics Committee for the Use of Experimental Animals in Zhejiang University.

Preparation of isolated ventricular myocytes

Ventricular myocytes from adult male Sprague-Dawley (SD) rats (250.21±10.56 g) were isolated by enzymatic dissociation (Farmer et al., 1983). The hearts were excised, cannulated via the aorta, attached to a Langendorff apparatus, and perfused with a 100% oxygenated, non-recirculating, Ca^{2+}-free Tyrode's solution (pH 7.2) containing (mmol/L): NaCl 100.0, KCl 10.0, KH_2PO_4 1.2, $MgSO_4$ 5.0, glucose 20.0, and MOPS 10.0. Then the perfusion solution was switched to a 100% oxygenated recirculated low Ca^{2+} (50 μmol/L) Tyrode's solution containing 0.03% collagenase and 1% bovine serum albumin (BSA) for 10 min. The ventricles were cut, minced, and gently triturated with a pipette in the low Ca^{2+} Tyrode's solution containing BSA at 37°C for 10 min. The cells were filtered through 200-μm nylon mesh and resuspended in the Tyrode's solution in which the Ca^{2+} concentration was gradually increased to 1.25 mmol/L over 40 min. Only rod-shaped cells with clear cross-striations were used for experiments.

Intracellular calcium recording

Intracellular calcium was monitored using the fluorescent dye fura-2-AM. The acetoxymethyl ester form of fura-2 was added to a suspension of cells. After incubation with 1 μmol/L fura-2-AM at room temperature for 30 min, the cells were washed 3 times with fresh Krebs-Henseleit (K-H) solution containing 1% BSA. K-H solution contained (mmol/L): NaCl 118.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 1.25, glucose 10.0. Fluorescence was measured on an Olympus inverted microscope equipped with a fluorometer system (T.I.L.L., Germany). A small

aliquot of fura-2-loaded cells was placed on the glass bottom of a chamber and then perfused with K-H buffer with a gas phase of 95% O_2 /5% CO_2. The Ca^{2+}-dependent fura-2 signal was obtained by illuminating at 340 and 380 nm and recording the emitted light at 510 nm. The background fluorescence was automatically subtracted. As in previous studies in other laboratories (Ventura et al., 1992);(Kotsanas et al., 2000) we used the fluorescence ratio at the two wavelengths, which is believed to accurately represent the $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ transient was induced by supra-threshold stimulation at 0.2 Hz delivered through two platinum field-stimulation electrodes in the bathing fluid.

Measurement of sarcolemmal Ca^{2+} channel activity using Mn^{2+} influx

Mn^{2+} quench experiments were performed in Ca^{2+} medium containing 0.2 mmol/L $MnCl_2$ by continuously recording the excitation signals at 360 nm and the emission signal at 510 nm at 1 ms intervals (Merritt et al., 1989). Freshly isolated, quiescent cardiomyocytes preloaded with fura-2-AM were perfused continuously with an incubation buffer at a flow rate of 5 ml/min at room temperature. Incubation buffer (pH 7.4) contained (mmol/L): NaCl 130.0, KCl 4.7, KH_2PO_4 0.1, $MgSO_4$ 1.2, $CaCl_2$ 0.1, HEPES 10.0, glucose 10.0. After 12 min perfusion with continuous electrical stimulation, the perfusion was switched to buffer supplemented with 0.2 mmol/L $MnCl_2$ and the electrical stimulation was stopped. After 75 s, during which the basal quench was determined, electrical stimulation (1 Hz) was initiated to activate Mn^{2+} quench of fura-2 via voltage-sensitive Ca^{2+} channels. Drugs were added 12 min before the initiation of Mn^{2+} quench acquisition, except for isoproterenol, which was added 85 s before the Mn^{2+} perfusion was initiated. Ca^{2+} channel activity was monitored as the rate of fura-2 quench that was activated by electrical pacing of the cardiomyocytes and could be blocked by nifedipine.

Whole cell L-type calcium current measurements

Myocytes were placed in a superfusion chamber and superfused at 2 ml/min, using a physiological solution of the following composition (mmol/L): NaCl 135.0, CsCl 5.0, $MgCl_2$ 1.0, $CaCl_2$ 1.5, HEPES 10.0, Glucose 10.0, pH 7.4. Myocytes were then patch clamped in the whole cell configuration, using a patch pipette (resistance 2~2.5 MΩ) filled with the following solution (mmol/L): CsCl 135, $MgCl_2$ 3.0, HEPES 10.0, EGTA 10.0, cAMP 0.5, MgATP 2.0, pH 7.3. Calcium current recordings were measured using Axopatch 200B amplifier/pClamp7 software. Fast sodium currents were eliminated using a pre-pulse to –40 mV for 40 ms prior to the test pulse.

Isolated perfused rat heart preparation

Male SD rats were used. Immediately after stunning and decapitation, the heart was rapidly removed and mounted to a Langendorff apparatus. The heart was retrogradely perfused with a 95%O_2/5%CO_2 equilibrated K-H solution via the aorta at a constant velocity of 10 ml/min. The heart was allowed to stabilize for 10 min before the experiment commenced. The heart was perfused for 10 min with IL-2 at 10, 40, 200 or 800 U/ml, and then was quickly removed from the apparatus after each experiment, frozen in liquid nitrogen, and stored at -80^{o}C.

Preparation of SR from rat heart

Sarcoplasmic reticulum was prepared according to the methods of Jones as modified by Kodavanti et al. (Jones, 1979; Kodavanti et al., 1990; Pande et al., 1998). The whole hearts were transferred to ice-cold homogenizing medium containing (in mmol/L) Na_2HPO_4 50, Na_2EDTA 10, and NaF 25, pH 7.4. The ventricles were minced and placed in 10 ml ice-cold homogenizing medium. The minced tissue was homogenized in three bursts of 15 s each. An additional 5 ml of homogenizing medium was added and the homogenate was sedimented twice for 20 min at 14,000

× g. The supernatant was re-centrifuged at 45,000 × g for 30 min. The pellet obtained after this centrifugation, consisting of crude membrane vesicles (SR), was suspended in storage buffer (30 mmol/L histidine, 0.25 mol/L sucrose, 10 mmol/L EDTA, and 10 mmol/L NaF, pH 7.4) to a final concentration of 30-40 mg/ml protein and stored at -80^{o}C until used.

Cardiac SR fractions were used for measurement of the activity of SR Ca^{2+}-ATPase. All experiments were carried out in triplicate. Each sample contained pooled ventricular tissue from five hearts.

Preparation of sarcolemma from rat heart

Sarcolemma was prepared from rat ventricles by the methods of Watanabe et al. and Woo et al. (Watanabe et al., 1988; Woo et al., 2001). The ventricles were minced in 10 ml 0.6 mol/L sucrose in 10 mmol/L imidazole-HCl buffer, pH 7.0 and homogenized on ice for 30 s. The homogenate was centrifuged at 12,000 × g for 30 min at 4^{o}C. The supernatant was diluted with 120 ml KCl/MOPS buffer (0.16 mol/L KCl, 20 mmol/L MOPS, pH 7.4) and centrifuged at 96,000 × g for 1 h at 4^{o}C. The pellet was resuspended in 30% sucrose in 0.1 mol/L Tris-HCl, pH 8.3, containing 0.3 mol/L KCl and 50 mmol/L sodium pyrophosphate. KCl/MOPS buffer was layered on top of this suspension in an ultracentrifuge tube. The tube was centrifuged at 96,000 × g for 1 h at 4^{o}C. The white band at the interface between the KCl/MOPS and the 30% sucrose was recovered and diluted with 3 volumes of KCl/MOPS buffer and then centrifuged at 96,000 × g for 30 min at 4^{o}C. The pellet was resuspended in 1.5 ml of 0.25 mol/L sucrose in 30 mmol/L imidazole-HCl, pH 7.4. The isolated sarcolemma, 40-50 mg/ml, was frozen in aliquots in liquid nitrogen and stored at -80^{o}C.

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Protein determination

Protein content of the sarcoplasmic reticulum and membrane was determined by the method of Lowry et al. (Lowry et al., 1951) with BSA as the standard.

Measurement of Ca^{2+}-ATPase activity

The activity of Ca^{2+}-ATPase was determined with a kit (Nanjing Jiancheng Co., China) by measuring the inorganic phosphate (Pi) liberated from ATP hydrolysis (Kodavanti et al., 1990). Ca^{2+}-ATPase activity was assayed in a medium containing histidine 50 mmol/L (pH 7.0), $MgCl_2$ 3 mmol/L, KCl 100 mol/L, sodium azide 5 mmol/L, ATP 3 mmol/L and $CaCl_2$ 50 μmol/L (Pande et al., 1998). Cardiac SR membranes were added to the reaction mixture at a final concentration of 20-25 μg of protein per ml, pre-incubated for 10 min at 37^{o}C and the reaction was initiated by the addition of ATP. The ATP hydrolysis that occurred in the absence of Ca^{2+} (1 mmol/L EGTA) was subtracted in order to determine the activity of Ca^{2+}-stimulated ATPase. Ouabain was added fresh to a final concentration of 1 mmol/L in the media which remained unchanged throughout the incubation. Mitochondrial contamination was assessed by determining the activity of azide-sensitive ATPase, i.e., that activity inhibited by 5 mmol/L sodium azide (Lindemann et al., 1983). The Ca^{2+}-ATPase activity of 7~9 μmoles Pi/mg protein per hour reported in this study is very close to previous published values (Pande et al., 1998). The lower activity value may be due to the fact that we used a crude SR preparation as compared to semipurified fractions reported by others.

Measurement of SR Ca^{2+} uptake

Fura-2 free acid was used to continuously monitor the ATP-dependent Ca^{2+} uptake according to the method described previously (Kargacin and Kargacin, 1994). Freshly prepared cardiac SR

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vesicles were used for uptake studies immediately after resuspension in assay buffer (in mmol/L: KCl 100, oxalate 10, $MgCl_2$5, HEPES 25, EGTA 0.05; pH 7.0). Reactions were performed in a final volume of 2 ml with 250 µg vesicle preparation, 5 mmol/L ATP and an initial [Ca^{2+}] of 1.5 µmol/L as assessed by the fluorescence ratio from fura-2 (10 µmol/L). Uptake reactions were initiated by the addition of Ca^{2+} to a cuvette containing the SR vesicles. The solution in the cuvette was constantly stirred to insure adequate mixing. The fluorescence ratio due to excitation at 340 nm and 380 nm was measured at 510 nm emission using a fluorescence spectrophotometer (RF-1501, Shimadzu, Japan). To inhibit the Ca^{2+} release through ryanodine receptors (RyR) on SR, the experimental medium was supplemented with 10 µmol/L ruthenium red. All experiments were done at room temperature. Single exponential decays were fitted to the [Ca^{2+}] records using Clampfit 8.0 (Axon Instruments, Inc., USA).

Determination of Ca^{2+} content in preparations of SR vesicles

Samples of SR vesicles were digested with 2 ml 1N H_2NO_3 for 24 hours. The digestant was crystal clear. Using a distilled water-0.1% lanthanum chloride-containing diluent, the digestant was diluted 1:50. This was to prevent chemical interference from phosphate ions. Absorbance of aliquots of the extracts was measured by atomic absorption using a flame photometer (Hitachi 180-50, Japan) in the following conditions: range-UV (i.e. 422.7 nm), slit-2.6 nm, fuel-air-acetylene oxidizing flame. An external calibration curve was constructed using an aqueous solution of $CaCl_2$.

Assay of cyclic AMP

After treatment, the hearts were immediately removed from the Langendorff apparatus and placed in liquid nitrogen. Then, these preparations were preserved at -80^{o}C until use. About 250

mg of myocardium was obtained from the ventricles. The myocardium was homogenized in buffer containing 4 mmol/L EDTA (to prevent enzymatic degradation of cAMP), followed by heating for several minutes in a boiling water bath to coagulate the protein. The extracts were centrifuged at 12,000 rpm for 15 min. The cAMP in the supernatant was assayed following the indications of the manufacturer (Chinese Academy of Atomic Sciences).

Experimental protocols

Protocol 1: Effect of IL-2 on the $[Ca^{2+}]_i$ transient induced by electrical stimulation and caffeine

Changes in the field-stimulated $[Ca^{2+}]_i$ transient were determined by treating isolated ventricular myocytes with 200 U/ml IL-2 for 10 min. The frequency of field stimulation was 0.2 Hz.

To evaluate the Ca^{2+} loading state, caffeine was used to release Ca^{2+} from SR. Baseline values for $[Ca^{2+}]_i$ were measured in individual, field-stimulated myocytes for 1.5 min. Then the cells were perfused with or without IL-2 (200 U/ml) for 10 min. Field stimulation of the myocyte was discontinued, and caffeine (20 mmol/L) was applied to the cells 15 seconds later.

Protocol 2: Effect of IL-2 on sarcolemmal L-type calcium channel activity

The effect of IL-2 on sarcolemmal L-type calcium channel activity was determined by the whole cell patch clamp technique. Myocytes were perfused with 200 U/ml IL-2 for 10 min, then data were collected. To confirm the effect of IL-2 on L-type calcium channel activity in the patch clamp experiments, we performed Mn^{2+} quench experiments in isolated ventricular myocytes treated with IL-2 at 200 U/ml for 10 min. The Mn^{2+} quench experiments were also performed in the different conditions as follows: No $MnCl_2$, cells were electrically stimulated without added

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$MnCl_2$; No stimulation, $MnCl_2$ was added but cells were not electrically stimulated; Nifedipine, 10 μmol/L; Isoproterenol, 1 μmol/L.

Protocol 3: Dose-dependent effect of IL-2 on the activity of SR Ca^{2+}-ATPase

To assess the effect of IL-2 on SR Ca^{2+}-ATPase activity, SR fractions were prepared from rat hearts that had been perfused with IL-2 (10, 40, 200, or 800 U/ml) for 10 min. Furthermore, to quantify the effect of IL-2 on isolated SR, the SR fractions prepared from untreated rat hearts were incubated with IL-2 (10, 40, 200, or 800 U/ml) for 10 min before measuring the activity of Ca^{2+}-ATPase.

The effect of IL-2 on sarcolemmal Ca^{2+}-ATPase activity was assessed in sarcolemma from rat hearts perfused with IL-2 (10, 40, 200, or 800 U/ml) for 10 min.

Protocol 4: Effect of IL-2 on the activity of SR Ca^{2+}ATPase of rat heart as a function of ATP or Ca^{2+}

We also tested the effect of IL-2 (200 U/ml) on ATP and Ca^{2+} activation of SR Ca^{2+}-ATPase to understand more about its effects on the characteristics of the enzyme. Ca^{2+}-ATPase activity was determined as a function of ATP (0.1, 0.2, 0.5, 1.0, 2.0 or 4.0 mmol/L) as well as Ca^{2+} (1, 2.5, 5, 10, 20 or 40 μmol/L) concentrations.

Protocol 5: Effect of IL-2 on the activity of SR Ca^{2+}-ATPase of rat heart pretreated with NorBNI

In order to investigate whether exposure to IL-2 causes changes in SR Ca^{2+}-ATPase activity via the opioid receptor pathway, the effect of IL-2 on SR Ca^{2+}-ATPase of rat hearts pretreated with an opioid receptor antagonist was examined. The isolated rat hearts were perfused with the specific κ-opioid receptor antagonist, NorBNI (10 nmol/L) for 10 min (Wu et al., 1999), then IL-2

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(200 U/ml) was added to the perfusion buffer for 10 min.

Protocol 6: Effect of IL-2 on SR Ca^{2+} uptake and Ca^{2+} content

To verify the output of the SR Ca^{2+}-ATPase activation, the effect of IL-2 on SR Ca^{2+} uptake and SR Ca^{2+} content was determined. The rat hearts were exposed to IL-2 at 200 U/ml for 10 min with or without pretreatment of NorBNI (10 nmol/L) for 10 min, and then harvested for preparation of SR.

Chemicals

Collagenase (type I), bovine serum albumin (BSA), 3-[N-morpholino]propane-sulfonic acid (MOPS), taurine, fura-2-acetoxymethyl ester (fura-2-AM), fluo-2 free acid, ruthenium red, isoproterenol, nifedipine, tetraethylamonium (TEA), thapsigargin, nor-binaltorphimine (NorBNI), MgATP, cAMP, and ATP were purchased from Sigma (USA). Interleukin-2 (IL-2) was purchased from Shanghai Huaxin High Biotechnology Inc. The cyclic AMP kit was provided by the Chinese Academy of Atomic Sciences. A stock solution (100,000 U/ml) of IL-2 was prepared in distilled water. Different aliquots (1~10 μL) of the test solution were added to the reaction mixture to obtain the desired final concentrations of 10, 40, 200 and 800 U/ml.

Statistical analysis

Values presented here are means ± standard deviation of means (SD). Statistical comparisons were performed by Student's *t*-test, except for the dose-response data, which were analyzed by One-way analysis of variance and Dunnett's test. Differences of $p<0.05$ are regarded as significant.

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Results

Effect of IL-2 on intracellular calcium transients induced by electrical field stimulation and caffeine

Perfusion with 200 U/ml IL-2 significantly reduced the amplitude of the electrically stimulated $[Ca^{2+}]_i$ transient, which indicates the release of Ca^{2+} during E-C coupling (Yew et al., 1998). The basal fluorescence ratio of fura-2 loaded myocytes, an indication of the end-diastolic Ca^{2+} level during E-C coupling (Yew et al., 1998), was significantly increased in IL-2 treated isolated myocytes (Figure 1).

The decay of the electrically-induced $[Ca^{2+}]_i$ transients was measured to determine the uptake of Ca^{2+} by SR. The rate of the decay in the electrically-induced $[Ca^{2+}]_i$ transient of myocytes as indicated by the time constant of transient decay, tau (τ) (Trafford et al., 2001), was prolonged 28.02% in IL-2 treated myocytes over that in control myocytes (Figure 1).

We evaluated the effect of IL-2 on the Ca^{2+} stored in the SR by measuring the caffeine-induced $[Ca^{2+}]_i$ transient. As Figure 2 shows, after perfusion with 200 U/ml IL-2 for 10 min, the amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient decreased significantly.

Effect of IL-2 on sarcolemmal L-type calcium channel activity

In order to investigate whether the decrease in the $[Ca^{2+}]_i$ transient induced by IL-2 was through the sarcolemmal Ca^{2+} channel, we used the whole cell patch clamp technique to test the effect of IL-2 on the calcium current. In 16 cells, perfusion with 200 U/ml IL-2 for 10 min had no significant effect on the whole cell L-type calcium current (Figure 3).

We used Mn^{2+} as a Ca^{2+} surrogate to pass through the Ca^{2+} channel and quench fura-2 fluorescence essentially stoichiometrically at physiological $[Ca^{2+}]_i$. The rate of quenching is

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directly proportional to the net influx across the sarcolemma (Merritt et al., 1989). The rate of fluorescence loss was slow in the absence of $MnCl_2$. Addition of Mn^{2+} resulted in a quench rate of 5.5%/min in cells maintained without electrical stimulation. This increased to 17%/min during electrical stimulation at 1 Hz (p<0.01). In the presence of nifedipine (10 μmol/L) there was no activation of the quench rate by electrical stimulation, producing a quench rate similar to that observed in the absence of electrical stimulation. This indicates that the basal Mn^{2+} quench in the absence of electrical stimulation is primarily independent of voltage-operated Ca^{2+} channels. When the Ca^{2+} channels were activated by exposing the cardiomyocytes to isoproterenol (1 μmol/L), there was a significant increase in the electrical stimulation-dependent component of the Mn^{2+} quench of fura-2, which initially increased 8-fold over control (p<0.01).

Pretreatment of myocytes with IL-2 at 200 U/ml did not elicit a significant alteration in the initial rate of Mn^{2+} quench activated by electrical stimulation compared with control (p>0.05) (Table 1).

Effect of IL-2 on Ca^{2+}-ATPase activity of rat heart SR

Ca^{2+}-ATPase activity was measured by an optical assay in crude SR extracted from control and IL-2-treated rat hearts. The specific Ca^{2+}-ATPase inhibitor thapsigargin (TG) depressed Ca^{2+}-ATPase activity concentration-dependently in preparations from the control rat myocardium (Figure 4A).

In contrast, when rat hearts were perfused with IL-2 (10, 40, 200 or 800 U/ml), the activity of SR Ca^{2+}-ATPase increased in a dose-dependent manner (Figure 4B).

Effect of IL-2 on Ca^{2+}-ATPase activity as a function of ATP

Ca^{2+}-ATPase activity was determined as a function of ATP concentration in the SR from

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control and IL-2 perfused hearts. After incubating the SR with 0.1 to 4 mmol/L ATP, the activity of Ca^{2+}-ATPase increased dose-dependently in the control group. In the SR from IL-2-perfused hearts, a dose-dependent response to ATP was also observed; but the activity of Ca^{2+}-ATPase was considerably elevated over that in the control group (Figure 5).

Effect of IL-2 on Ca^{2+}-ATPase activity as a function of calcium

The activity of Ca^{2+}-ATPase activity as a function of free Ca^{2+} concentration was determined in the SR from untreated and IL-2 (200 U/ml) perfused rat hearts. The data in Figure 6A show that the activity of SR Ca^{2+}-ATPase in untreated hearts increased in a dose-dependent manner, while the activity in IL-2-perfused hearts did not rise significantly as the free calcium concentrations increased, remaining below control values at 10, 20 and 40μmol/L of free [Ca^{2+}].

Effect of IL-2 on Ca^{2+}-ATPase activity of rat hearts pretreated with NorBNI

After pretreatment with the specific κ–opioid receptor antagonist NorBNI (10 nmol/L) for 10 min, IL-2 perfusion in the isolated heart failed to induce changes in the activity of SR Ca^{2+}-ATPase. Treatment with NorBNI (10 nmol/L) alone did not significantly alter the activity of SR Ca^{2+}-ATPase (Figure 6B).

As Figure 5 shows, after pretreatment with NorBNI (10 nmol/L) for 10 min, IL-2 failed to elevate the SR Ca^{2+}-ATPase activity as a function of ATP. Similarly, the effect of IL-2 on the SR Ca^{2+}-ATPase activity as a function of free calcium was also attenuated by pretreatment with NorBNI (Figure 6A). Exposure to Nor-BNI (10 nmol/L) alone did not significantly alter the activity of SR Ca^{2+}-ATPase as a function of ATP or free calcium (data not shown).

Effect of IL-2 on the activity of Ca^{2+}-ATPase in isolated SR

We also tested the effect of IL-2 on the activity of Ca^{2+}-ATPase in isolated SR from untreated

hearts. The isolated SR was incubated with IL-2 at 10, 40, 200 and 800 U/ml. IL-2 did not affect Ca^{2+}-ATPase activity over this range of concentrations. Ca^{2+}-ATPase activity in the isolated SR was determined as a function of ATP concentration. After incubation with 0.1 to 4 mmol/L ATP, the activity of Ca^{2+}-ATPase of isolated SR increased dose-dependently, and there was no significant difference between the control and IL-2 treated SR vesicles (data not shown).

Effect of IL-2 on SR Ca^{2+} uptake and SR Ca^{2+} content

To examine the effects of IL-2 on Ca^{2+} uptake by the SR Ca^{2+}-ATPase, we performed Ca^{2+} uptake measurements fluorometrically in isolated cardiac SR using fura-2. As illustrated in Figure 7, free calcium concentration outside of the SR declined much more slowly in the SR isolated from IL-2 perfused rat heart. The decay constant in the presence of IL-2 was approximately half that of the control. Pretreatment with NorBNI (10 nmol/L) for 10 min attenuated the effect of IL-2.

To confirm the results from cardiac SR Ca^{2+} uptake, we determined the SR Ca^{2+} content using atomic absorption spectrophotometry. As shown in Figure 8, the Ca^{2+} content in cardiac SR prepared from IL-2 treated rat heart was less than that in control rat heart. Pretreatment with NorBNI attenuated the decrease of SR Ca^{2+} content by IL-2.

Effect of IL-2 on intracellular cAMP levels in the isolated rat heart

To determine further whether a cAMP-dependent pathway was involved in the intracellular calcium-related events induced by IL-2, we studied the effect of IL-2 on intracellular cAMP concentrations in isolated rat heart treated with IL-2 at 200U/ml. The cytosolic cAMP level in the ventricles treated with IL-2 was decreased compared with control. The reduction of cAMP by IL-2 was abolished by 10 nmol/L NorBNI (Figure 9).

Effect of IL-2 on the activity of sarcolemmal Ca^{2+}-ATPase

In a separate series of experiment, we measured the activity of sarcolemmal Ca^{2+}-ATPase in the rat heart to determine whether IL-2 affects both the sarcolemmal and the SR Ca^{2+}-ATPase

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activity. The hearts were perfused with IL-2 at 10, 40, 200 and 800 U/ml, and then the sarcolemma was isolated. IL-2 over this range of concentrations did not affect the activity of sarcolemmal Ca^{2+}ATPase (IL-2 0, 10, 40, 200 and 800 U/ml: 2.94±0.12, 2.88±0.11, 2.83±0.10, 2.96±0.14 and 2.88±0.10 μmoles Pi/mg protein/hr, respectively).

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Discussion

The findings reported here show that perfusion of the rat heart with IL-2 increased the activity of SR Ca^{2+}-ATPase dose-dependently, and that this effect is mediated, at least in part, via the cardiac κ-opioid receptor pathway. Although treatment with IL-2 can increase the activity of SR Ca^{2+}-ATPase as a function of ATP concentration, IL-2 acts mainly by reducing the sensitivity of SR Ca^{2+}-ATPase to free calcium, which is also mediated via κ-opioid receptors; as a result, SR Ca^{2+} uptake and SR Ca^{2+} content are reduced.

In previous studies, it was shown that IL-2 has a negative inotropic effect in isolated ventricular papillary muscle (Finkel et al., 1992) and cardiomyocytes, accompanied by a reduction of intracellular calcium transients (Cao et al., 2002). In the present study, IL-2 decreased the electrically stimulated calcium transient as well as caffeine-induced calcium release from SR, which indicates that IL-2 affects the intracellular calcium handling system. The decreased calcium release from SR induced by caffeine may be caused by inhibition of SR Ca^{2+}-ATPase, which results in reduced reuptake and refilling of calcium in SR. Reduction of extracellular calcium entry could result in a decrease of cytoplasmic free calcium level, which would then reduce caffeine-induced calcium release. But we found that the activity of sarcolemmal L-type calcium channels, as determined by whole cell patch clamp and Mn^{2+} quench experiments, was not altered by IL-2 treatment. So it seems that the reduced intracellular calcium transient induced by IL-2 is a consequence of decreased releasable calcium in cardiomyocyte SR, mainly as a result of decreased reuptake of calcium into the SR. In the present study, it was demonstrated that Ca^{2+} uptake and Ca^{2+} content of the SR prepared from IL-2 treated rat heart were markedly decreased, which suggests that the SR Ca^{2+} uptake mechanism is a likely target of IL-2 in cardiac muscle. This result

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is consistent with the observation of delayed decay of the electrical stimulated $[Ca^{2+}]_i$ calcium transient by IL-2. In the present study, we found that IL-2 increased the activity of SR Ca^{2+}-ATPase dose-dependently, and the increased activity was a function of ATP concentration. These results seem paradoxical when compared with the observation that IL-2 decreased SR Ca^{2+} uptake. In another series of experiments, we found that IL-2 decreased the sensitivity of SR Ca^{2+}-ATPase to calcium, which may reduce the efficacy of SR Ca^{2+}-ATPase, thus lowering the calcium transport rate and the loading/refilling of the SR with calcium. Therefore, the summation of two opposing effects induced by IL-2, i.e., increased SR Ca^{2+}-ATPase activity and decreased responsiveness of SR Ca^{2+}-ATPase to calcium, reduces the calcium reuptake into SR. That the latter effect is probably dominant in the cardiac action of IL-2 is supported by the experiments on SR Ca^{2+} uptake and SR Ca^{2+} content.

An interesting matter for discussion arises when considering sites at which IL-2 acts in cardiomyocytes. Incubation of IL-2 with isolated SR from untreated hearts had no effect on the SR Ca^{2+}-ATPase activity, while perfusion with IL-2 in isolated hearts increased the SR Ca^{2+}-ATPase activity, suggesting that the cell membrane may participate in the effect of IL-2 on SR Ca^{2+}-ATPase activity. Furthermore, we found no effect of IL-2 on the sarcolemmal Ca^{2+}-ATPase, providing evidence that this is not a target in the cardiac effect of IL-2.

Further support for the above findings are provided by the results of pretreatment with opioid receptor antagonists. Rat hearts perfused with a single dose of NorBNI, a selective κ-opioid receptor antagonist, showed a significant reduction of the activation of SR Ca^{2+}-ATPase as well as the responsiveness to calcium of IL-2, which indicates the participation of sarcolemmal opioid receptors. The complex interactions between the classical cytokines and opioids are manifest

within the nervous and immune systems. For example, in the rat central nervous system, anti-opioid sera inhibit the central analgesic effect of IL-2 (Jiang et al., 2000). Furthermore, in a previous study, we found that cardiac κ-opioid receptors mediate the effect of IL-2 on cell contraction and intracellular calcium transients (Cao et al., 2002). The present study is the first report to establish a pharmacological connection between SR Ca^{2+}-ATPase activity and cardiac sarcolemmal κ-opioid receptors.

Sarcoplasmic reticulum is the major intracellular Ca^{2+} store. Most of the Ca^{2+} that activates the contractile process is released from the SR store of calcium that is removed from the cytosol during diastole. SR Ca^{2+} uptake is mediated by the SR Ca^{2+}-ATPase (Lompre et al., 1994; Misquitta et al., 1999), whose activity is regulated by the small, 52-amino-acid protein phosphalamban, of which no subtypes are known (Simmerman and Jones, 1998). In its basal unphosphorylated state, phospholamban inhibits the SR Ca^{2+}-ATPase by decreasing its affinity for Ca^{2+} (Hasenfuss et al., 1997; James et al., 1989; Voss et al., 1994), whereas in its phosphorylated state, it enhances SR Ca^{2+}-ATPase activity (James et al., 1989) via an increase in its affinity for calcium without changing the maximum reaction rate of the enzyme (Luo et al., 1994; Odermatt et al., 1996). Phosphorylation of myocardial phospholamban is catalysed by several enzymes, including calcium/calmodulin-dependent protein kinase and cAMP-dependent protein kinase A, with distinct sites of phosphorylation by each kinase (Lompre et al., 1994; Lompre, 1998). In the present study, we found that treatment with IL-2 at 200U/ml decreased the intracellular cyclic AMP concentrations in the isolated rat heart, which suggests that cyclic AMP is a target component of IL-2 in the heart. It is also known that cardiac κ-opioid receptor stimulation inhibits adenylyl cyclase via Gi/o proteins, leading to a decreased intracellular cAMP level (Zhang and

Wong, 1998). Combined with the result in the present study that IL-2 decreased the sensitivity of SR Ca^{2+}-ATPase to free calcium, this indicates that the activation of κ-opioid receptor by IL-2 results in a decreased intracellular cAMP level, which may be responsible for reduced sensitivity of SR Ca^{2+}-ATPase to calcium by affecting the degree of cAMP-dependent phosphorylation of phospholamban.

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Footnotes

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Legends for figures

Figure 1 Effect of interleukin-2 (IL-2) on the systolic amplitude and the decay of the electrically stimulated $[Ca^{2+}]_i$ transient and the end-diastolic Ca^{2+} level of isolated ventricular myocytes. A, typical recordings of the electrically stimulated Ca^{2+} transient in control and IL-2-treated rat myocytes. B, pooled data of mean normalized electrically stimulated $[Ca^{2+}]_i$ transient amplitude, end-diastolic Ca^{2+} level and time constant obtained from control and IL-2-treated groups

(*n*=14 per group)

** $p<0.01$ compared with control group

Figure 2 Effect of interleukin-2 (IL-2) on the caffeine-induced Ca^{2+} transient. A, typical recordings of the caffeine-induced Ca^{2+} transient in control and IL-2-treated myocytes. B, pooled data of mean amplitude of caffeine-induced calcium transient in control and IL-2-treated myocytes

(*n*=15 per group)

** $p<0.01$ compared with control group

Figure 3 Effect of interleukin-2 (IL-2) on the L-type calcium current of isolated rat ventricular myocytes. A, typical example of L-type calcium current recording. B: pooled data of L-type calcium currents in control and IL-2 (200 U/ml) groups.

(*n*=10 and 16 in control and IL-2 group, respectively)

$p=0.09$ IL-2 group vs. control group; $p=0.24$ IL-2 group vs. washout

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Figure 4 Dose-response curve of Ca^{2+}-ATPase activity

A, dose-response curve of SR Ca^{2+}-ATPase activity to thapsigargin (TG). B, dose-response relationship of Ca^{2+}-ATPase activity in the SR from rat hearts perfused with interleukin-2 (IL-2) (*n*=24 in each group)

** $p<0.01$ compared with concentration 0 (in A) and control group (in B)

Figure 5 Dependence of Ca^{2+}-ATPase activity in SR from untreated or interleukin-2 (IL-2, 200 U/ml) or NorBNI (10 nmol/L)+IL-2 perfused rat hearts on ATP concentration. SR vesicles were incubated in assay media whose ATP concentrations varied between 0.1 and 4 mmol/L (*n*=24 in each group)

** $p<0.01$ compared with values in absence of IL-2 (-IL-2)

†† $p<0.01$ compared with values in presence of IL-2 (+IL-2)

Figure 6 Effect of interleukin-2 (IL-2, 200 U/ml) on the activity of SR Ca^{2+}-ATPase

A, dose-response curves of SR Ca^{2+}-ATPase activity to free Ca^{2+} concentrations in untreated or interleukin-2 (IL-2, 200 U/ml) or NorBNI (10 nmol/L)+IL-2 perfused rat hearts. B, effect of IL-2 (200 U/ml) on the activity of SR Ca^{2+}-ATPase from untreated and NorBNI (10 nmol/L)-pretreated/treated hearts (*n*=24 in each group)

** $p<0.01$ compared with value of 0 μmol/L (in A), and with value in absence of IL-2 (-IL-2) (in B)

†† $p<0.01$ compared with values in absence of IL-2 (-IL-2)

# $p<0.05$, ## $p<0.01$ compared with values in presence of IL-2 (+IL-2)

Figure 7 Effect of interleukin-2 (IL-2, 200 U/ml) on Ca^{2+} uptake by cardiac SR vesicles

A, cardiac SR vesicles (250 μg SR protein) prepared from a control rat were administered 1.5 μmol/L $CaCl_2$. B, SR vesicles prepared from an isolated rat heart perfused with IL-2 (200 U/ml) for 10 min. C, SR vesicles prepared from an isolated rat heart pretreated with norBNI (10 nmol/L) for 10 min followed by perfusion with IL-2 (200 U/ml) for 10 min. D, averaged (means±SD) time constants of Ca^{2+} uptake in control, IL-2 and NorBNI+IL-2 groups (7, 8 and 8 independent measurements, respectively)

** $p<0.01$ compared with control

†† $p<0.01$ compared with IL-2

Figure 8 Effect of interleukin-2 (IL-2, 200 U/ml) on Ca^{2+} content in cardiac SR vesicles from untreated and NorBNI (10 nmol/L)-pretreated rat hearts

(n=8 in each group)

* $p<0.05$ compared with control

† $p<0.05$ compared with IL-2

Figure 9 Effect of interleukin-2 (IL-2, 200 U/ml) on intracellular cAMP levels in the isolated rat hearts pretreated with or without NorBNI (10 nmol/L)

(n=8 in each group)

** $p<0.01$ compared with control

† $p<0.05$ compared with IL-2

Table 1 Effect of IL-2 on sarcolemmal Mn^{2+} influx

Groups	*n*	Mn^{2+} quench rate (fold increase over basal)
Control	20	2.6±0.2
IL-2 (200 U/ml)	20	2.5±0.4
Nifedipine (10 μmol/L)	7	0±0**
Isoproterenol (1 μmol/L)	7	22.1±1.9**

All data expressed as the fold increase above the basal Mn^{2+} quench rate to initiation of the electrical stimulation
** P<0.01 compared with control group

Figure 1

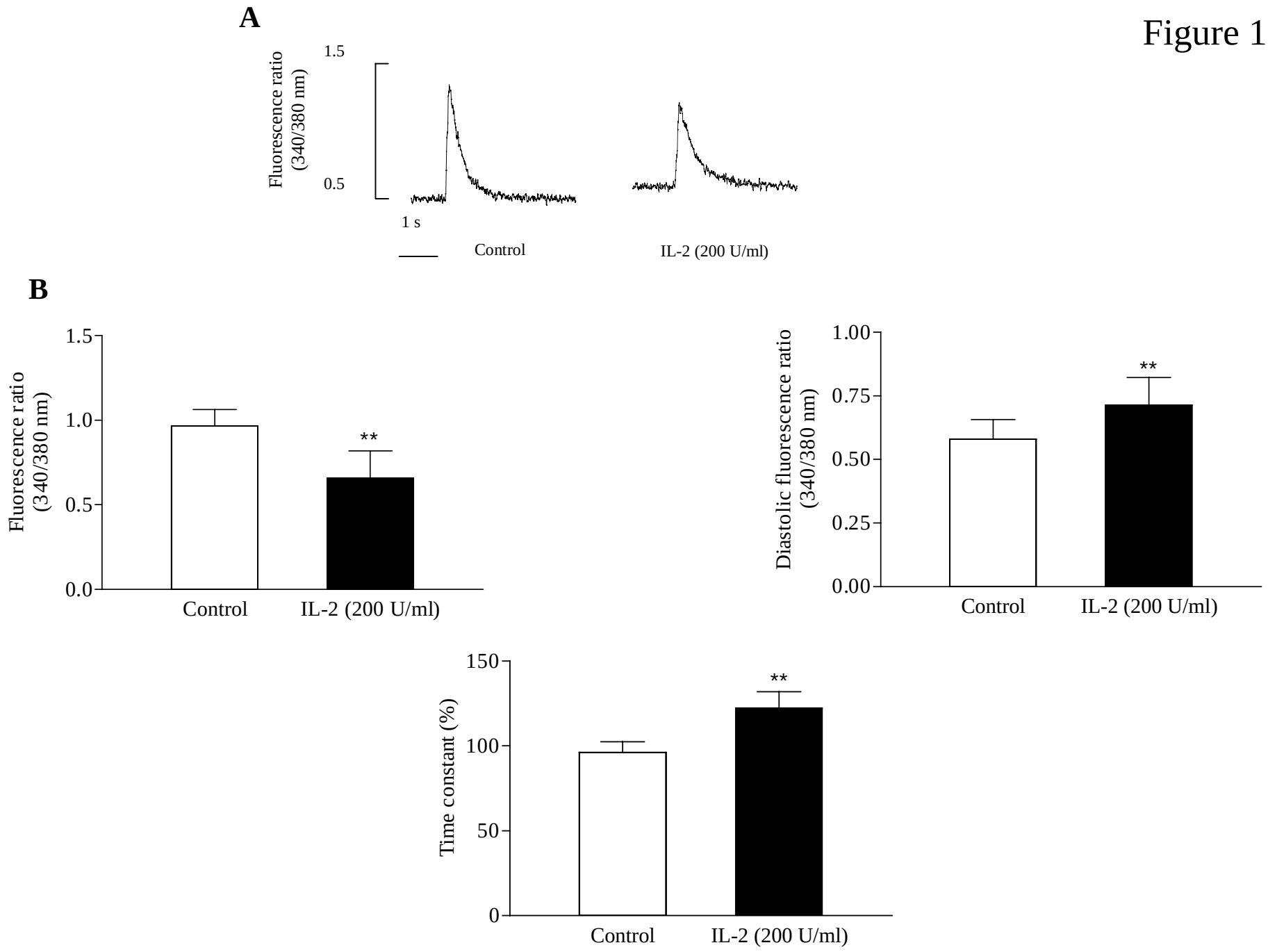
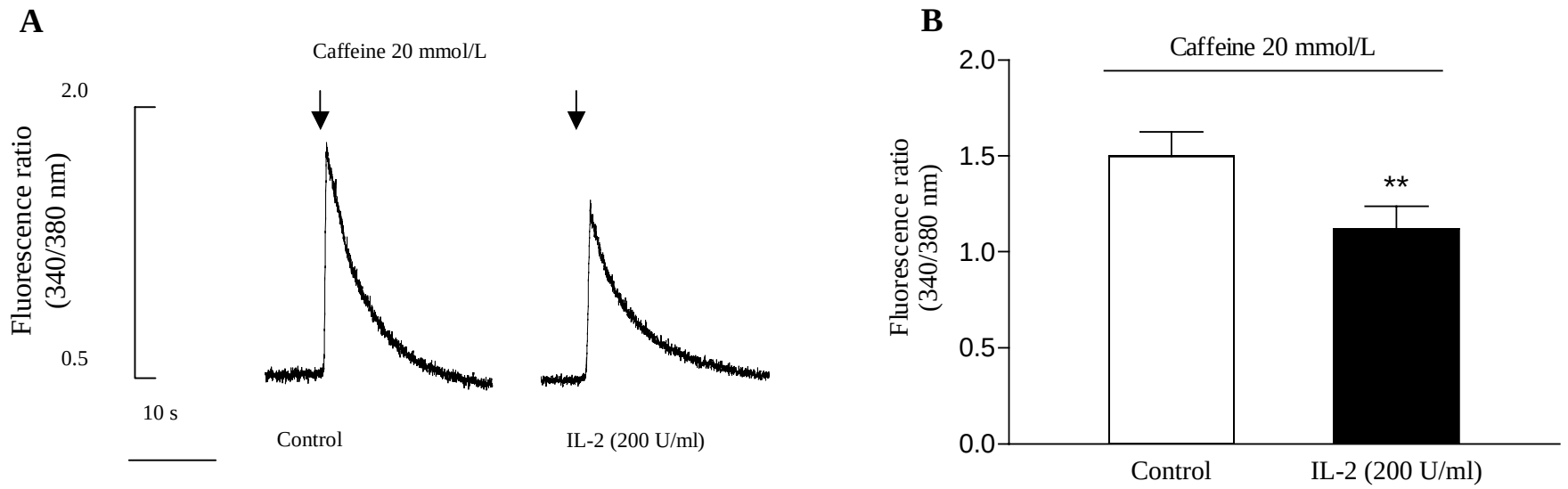


Figure 2



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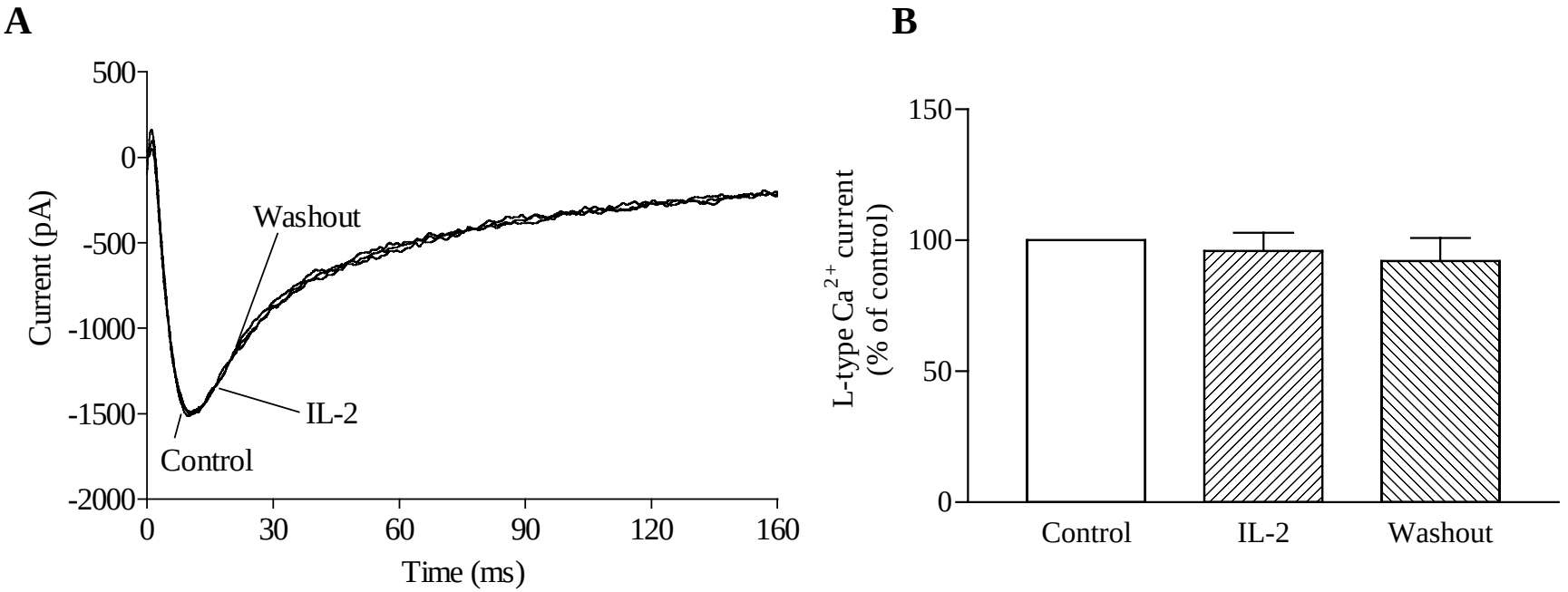


Figure 4

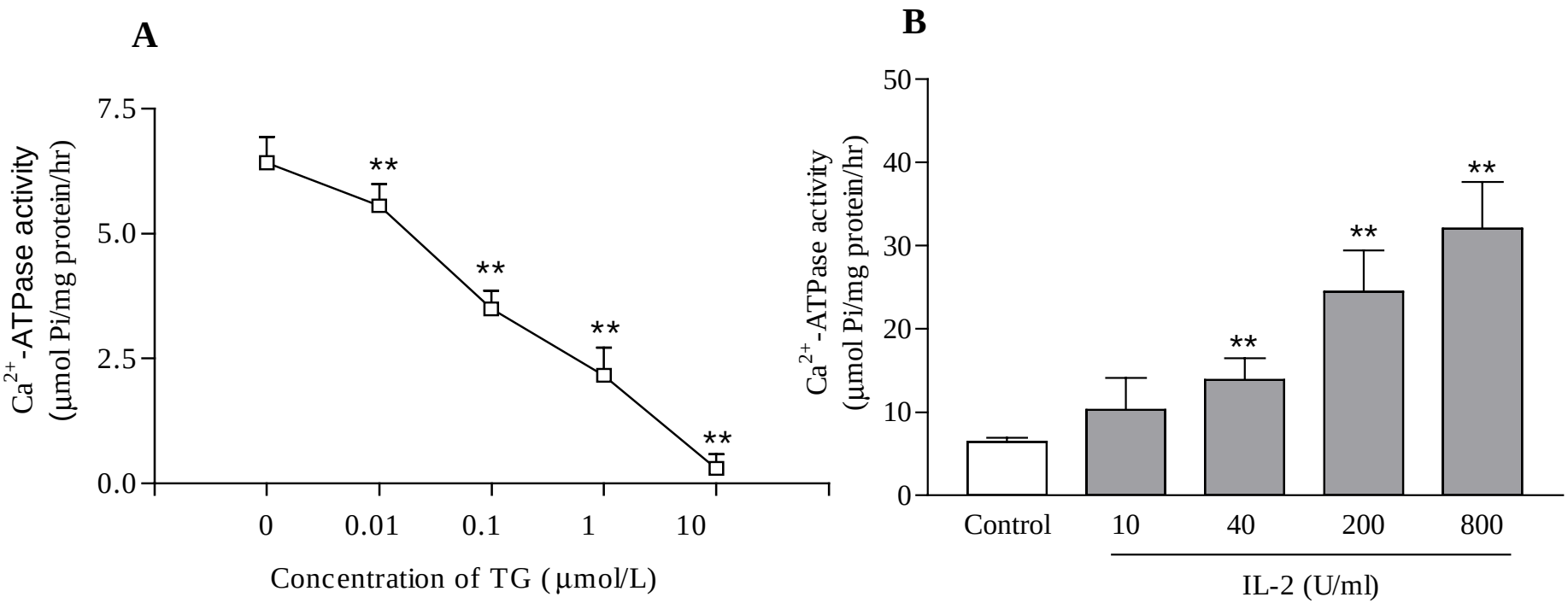


Figure 5

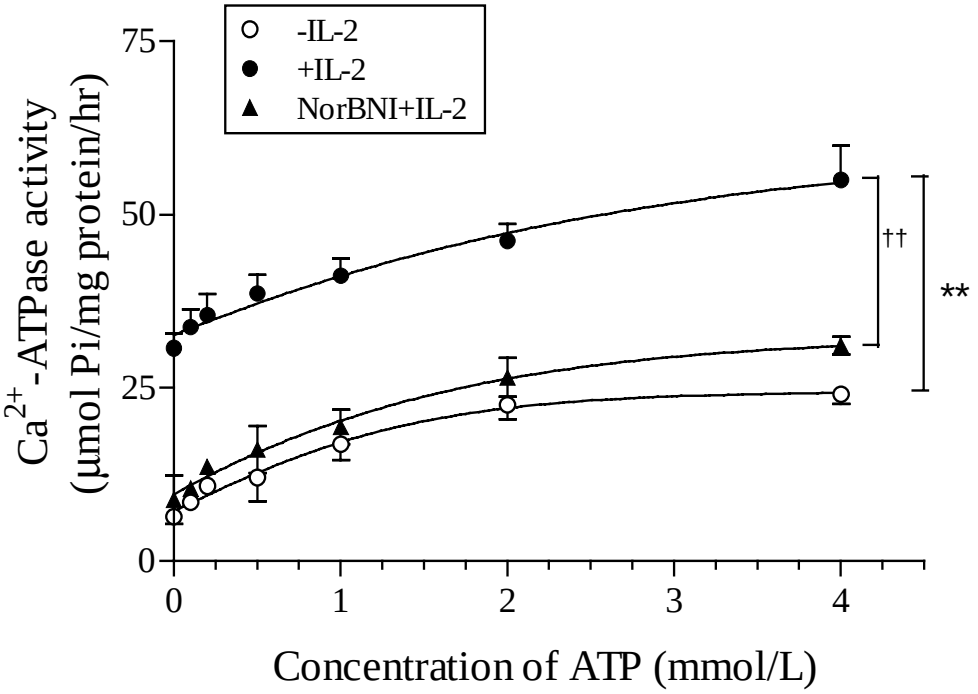


Figure 6

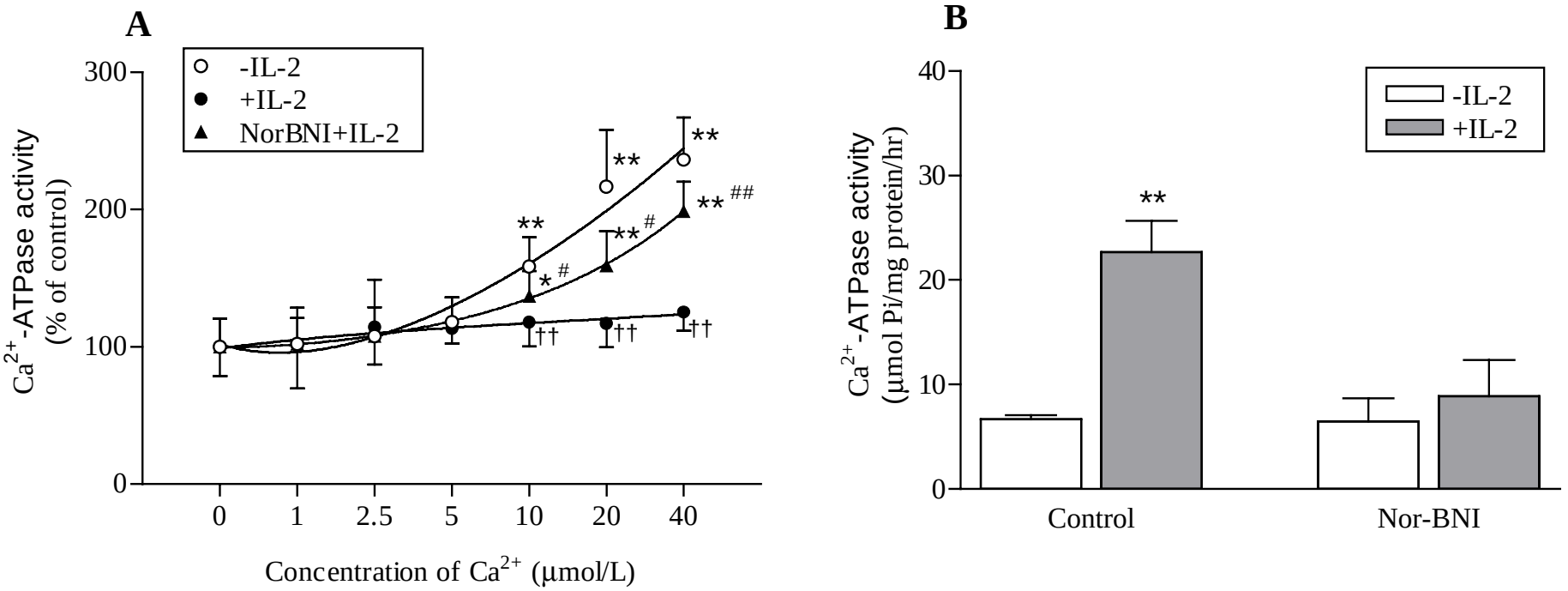
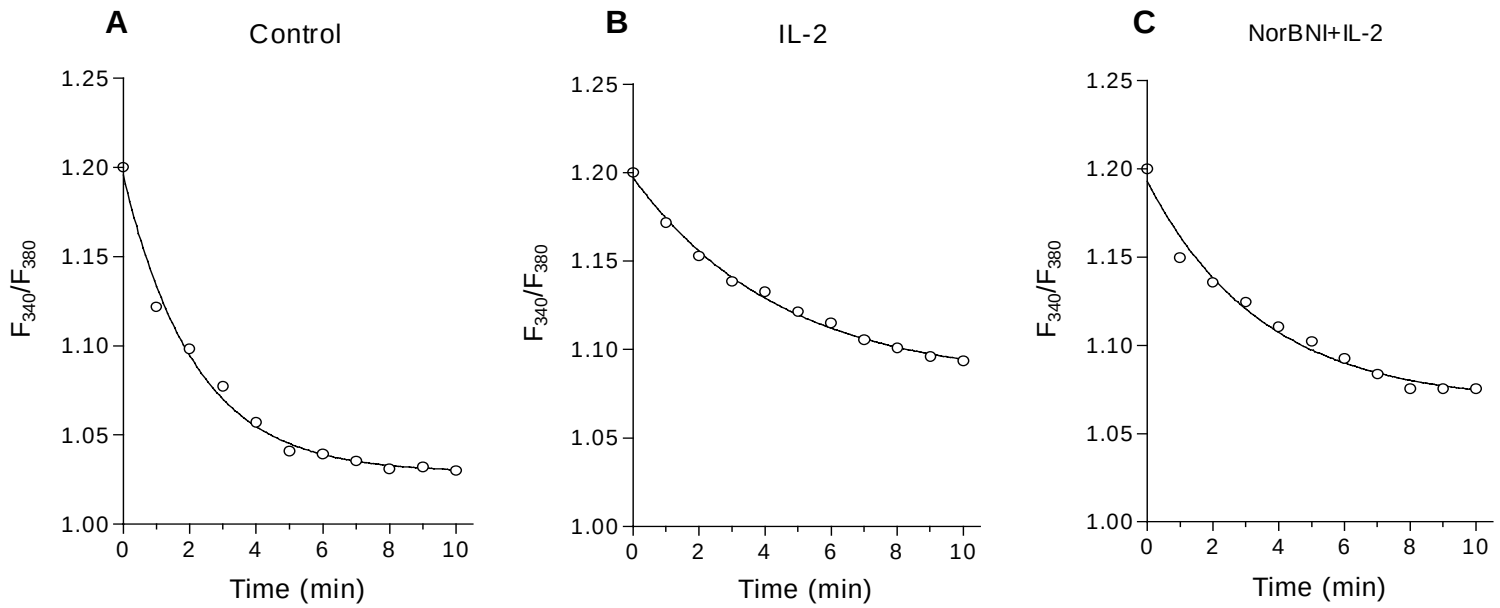


Figure 7



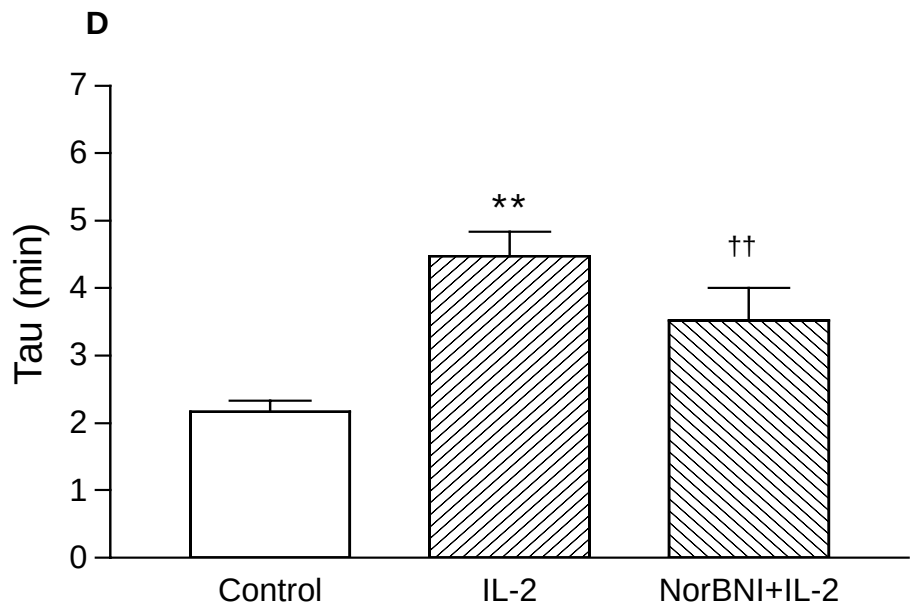


Figure 8

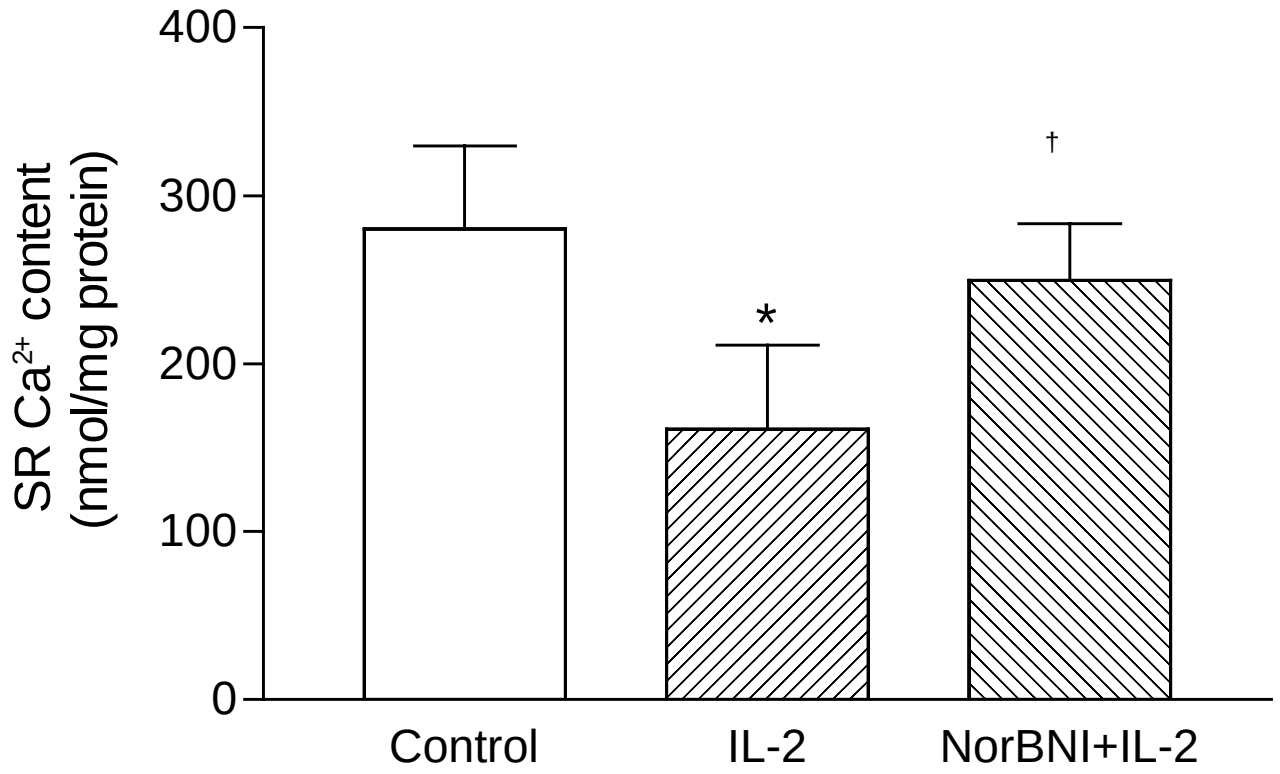


Figure 9