Effects of tumor necrosis factor-alpha on calcium movement in rat ventricular myocytes[1]

LI Xiao-Qiang, ZHAO Ming-Gao, MEI Qi-Bing[2], ZHANG Yan-Feng,
CAO Wei, WANG Hai-Fang, CHEN Dan[3], CUI Yi

Department of Pharmacology, [3]Electron Microscopy Center, The Fourth Military Medical University, Xi-an 710032, China

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ABSTRACT

AIM: To study the effects of tumor necrosis factor-alpha (TNF-α) on calcium movement in rat ventricular myocytes. **METHODS:** Intracellular free Ca^{2+} concentration was measured with calcium fluorescent probe Fluo-3/AM and laser confocal microscope. L-type calcium current ($I_{Ca,L}$) was recorded with the whole-cell configuration of the patch-clamp techniques. **RESULTS:** At 2, 20 and 200 µg/L, TNF-α was found to increase intracellular free Ca^{2+} concentration in a dose-dependent manner illustrated by the increment of calcium fluorescence density with laser confocal microscope. Nicardipine 0.5 µmol/L slightly attenuated TNF-α-induced response. When the cardiac myocytes were exposed to caffeine (100 mmol/L) for 30 min, TNF-α failed to induce any change of intracellular free calcium. However, it was found that TNF-α inhibited $I_{Ca,L}$ in whole-cell patch-clamp experiments. At 2, 20, and 200 µg/L, TNF-α decreased peak $I_{Ca,L}$ by 3.9 % (-5.1 pA/pF±0.3 pA/pF *vs* -4.9 pA/pF±0.2 pA/pF, *n*=9, *P*>0.05), 15.7 % (-5.1 pA/pF±0.3 pA/pF *vs* -4.3 pA/pF±0.3 pA/pF, *n*=9, *P*<0.05) and 19.6 % (-5.1 pA/pF±0.3 pA/pF *vs* -4.1 pA/pF±0.4 pA/pF, *n*=9, *P*<0.01), respectively. It shifted the steady-state inactivation curve of $I_{Ca,L}$ to the left ($V_{1/2}$ shifted from -28.7 mV±0.3 mV to -37.8 mV±1.4 mV, *n*=7, *P*<0.05), while it took no effects on steady-state activation and recovery from inactivation. **CONCLUSION:** TNF-α inhibited $I_{Ca,L}$ in rat ventricular myocytes, while increasing the intercellular free Ca^{2+} level due to the release of Ca^{2+} from intracellular stores.

INTRODUCTION

TNF-α is a potentially powerful anti-neoplastic substance. In the heart it is produced by both cardiac myocytes and resident macrophages under conditions of cardiac stress, and is thought to be responsible for many of the untoward manifestations of cardiac diseases. When expressed in large amounts, it can pro-duce widespread deleterious effects, such as cytostatic and cytotoxic activity[1]. Clinical evidence demonstrated that TNF-α induced left ventricular dysfunction, acute pulmonary edema, and congestive cardiomyopathy[2]. Calcium ion plays important physiological roles, includ-ing mediation of cell contraction, secretion, protein phosphorylation, and gene transcription. In the cardiac myocyte, how does TNF-α affect calcium movement? In this aspect, there is not a categorical and uniform conclusion at present. To answer this question, the present experiments were designed to investigate the effects of TNF-α on calcium movement in rat ven-tricular myocytes.

MATERIALS AND METHODS

Detection of intracellular free Ca^{2+} concentration ([Ca^{2+}]$_i$) by laser confocal microscopy Reproducible single cell cultures were obtained by the procedure described previously[3,4]. Sprague-Dawley rats aging 1-3 d (provided by the Experimental Animal Center of Fourth Military Medical University, Grade II, Certificate No C98008) were used. Cardiomyocytes were cultured in MEM (Eagle's) culture medium containing 10 % fetal bovine serum in 5-mm culture plates with cell density of 1×10^4 L^{-1}. Cells were incubated at 37 °C in humidified air with 5 % CO$_2$ for 2 d. On the d 3, cardiomyocytes were rinsed with D-Hanks' solution for three times and then incubated in D-Hanks' solution containing Fluo 3-acetoxymethyl ester (Fluo-3/AM) 10 μmol/L at 37 °C for 30 min. The cells were then washed with D-Hanks' solution to remove the extracellular Fluo-3/AM. The fluorescence was detected with a MRC-1024 laser scanning confocal microscope (BIO-RAD Int, USA). An argon laser was used to excite Fluo-3 at 488 nm and emit at 526 nm. Systolic [Ca^{2+}]$_i$ changes were shown by fluorescence intensity (FI). TNF-α (Sigma) was a kindly gift from Dr WANG Hui in Biotechnology Center, Administration of Science Research, Fourth Military Medical University, and was dissolved in the modified Tyrode's solution.

Whole-cell patch-clamp techniques Five-week-old male Sprague-Dawley rats (provided by the Experimental Animal Center of Fourth Military Medical University, Grade II, Certificate No C98008) weighing 160-180 g were used.

The single myocyte was isolated from left ventricle of adult rats as described previously[3,5]. In brief, the rats were anesthetized with pentobarbital sodium (30 mg/kg, ip) and anticoagulated with heparin sodium (300 U/kg, iv). The heart was rapidly excised and mounted on a Langendorff apparatus. It was perfused conversely via the aorta for 5 min with a modified Tyrode's solution (in mmol/L: NaCl 144; KCl 4, CaCl$_2$ 1.8, MgCl$_2$ 0.5, NaH$_2$PO$_4$ 0.33, glucose 5.5, and HEPES 5.5, adjust pH to 7.4 with NaOH) equilibrated with 100 % O$_2$ at 37 °C at a rate of 5 to 10 mL/min, followed for 5 min with Ca^{2+}-free Tyrode's solution (omitting CaCl$_2$ from Tyrode's solution). The heart was then perfused with 0.1 % collagenase (type I, Sigma) dissolved in Ca^{2+}-free Tyrode's solution until the solution flowed freely (15 to 25 min). Then left ventricular free wall was dissected and gently blowed in a Kraftbruhe

(KB) solution (in mmol/L: KOH 70, KCl 40, *L*-glutamic acid 50, taurine 20, KH$_2$PO$_4$ 10, MgCl$_2$ 0.5, glucose 11, egtazic acid 0.5, and HEPES 10, adjusted pH to 7.4 with KOH). The isolated myocytes were stored in the KB solution at 4 °C and studied within 12 h after isolation.

Rod-shaped noncontracting cells with clear striations and resting potential of at least -75 mV were used. At room temperature (18-22 °C), currents were recorded by the gigaohm seal patch-clamp technique in whole-cell configuration with a CEZ 2300 amplifier (Nihon Kohden). The resistance of patch pipette ranged from 4 to 6 MΩ, when filled with the pipette solution. Pipette capacitance and series resistance were compensated to minimize the duration of capacitive currents. The run-down of $I_{Ca,L}$ averaged 18 % over 15 min. All the recordings of $I_{Ca,L}$ were, therefore, performed at 15 min after the establishment of a gigaohm seal. The current signal was sampled directly into a computer and analyzed by using pClamp software (version 7.0, AXON instruments Inc). For the recording of $I_{Ca,L}$, the external solution was composed of (in mmol/L) NaCl 137 or choline chloride 137 (for activation and inactivation curves), MgCl$_2$ 0.5, CaCl$_2$ 1.8, HEPES 5, glucose 10, and CsCl 4.6 (pH 7.3 with NaOH). The pipette solution was composed of (in mmol/L) CsCl 140, MgCl$_2$ 0.5, Na$_2$ATP 4, egtazic acid 1, HEPES 5, and glucose 5.5 (pH 7.2 with CsOH). TNF-α (Sigma) was dissolved in the modified Tyrode's solution. Solutions were gased with 95 % O$_2$ and 5 % CO$_2$.

To obtain current-voltage (*I/V*) curves, $I_{Ca,L}$ was recorded by applying 300 ms depolarizing pulses at a test potential ranging from -40 mV to +50 mV in 10-mV steps from a holding potential of -40 mV at an interval of 5 s (0.2 Hz). To study steady-state activation of $I_{Ca,L}$, cells were maintained at a holding potential of -80 mV. $I_{Ca,L}$ was elicited by depolarizing the cells to test potentials from -40 to +10 mV in 10 mV increments for 300 ms. A double-pulse protocol was used to determine the steady-state inactivation curves. Cells were clamped at a holding potential of -80 mV for 300 ms to a range of potentials from -80 mV to +10 mV (10-mV steps), then stepped to +10 mV for 200 ms at 1-s intervals. The time dependence of $I_{Ca,L}$ recovery from inactivation was determined by using a double-pulse protocol: two depolarizing pulses to +10 mV with varying interpulse intervals (times of 20 ms) were applied from a holding potential of -40 mV every 5 s. The extent of recovery at each interpulse interval was obtained by expressing the amplitude of $I_{Ca,L}$.

Data were expressed as mean±SD and the statistical significance of differences was estimated according to *t*-test for grouped observations.

RESULTS

Effect of TNF-α on intracellular free Ca²⁺ of myocardium Myocytes loaded with Fluo-3/AM beat synchronously in Hanks' solution. TNF-α 20 μg/L increased fluorescence intensity of $[Ca^{2+}]_i$ at least for 1 min (Fig 1) and increased it in a dose-dependent manner (Fig 2A). This increment was slightly attenuated by adding dihydropyridine calcium antagonist, nicardipine 0.5 μmol/L (Fig 2 B), or replacing the extracellular Hanks' solution with D-Hanks' solution, showing that L-type calcium channels took the minor effects of TNF-α on $[Ca^{2+}]_i$ metabolism. When the cardiac myocytes were exposed to caffeine (100 mmol/L) for 30 min, TNF-α 2, 20 μg/L failed to induce any changes of intracellular free Ca²⁺ (Fig 2C).

Effect of TNF-α on L-type calcium current ($I_{Ca,L}$) in rat ventricular myocytes The cell membrane capacitance (C_m) was 150 pF±25 pF (*n*=27 from 16 rats). The threshold for the activation of $I_{Ca,L}$ and the potential of peak current were -40 mV and 0 mV respectively at the holding potential of -40 mV. TNF-α 2, 20, and 200 μg/L decreased peak $I_{Ca,L}$ density (the amplitude normalize to cell membrane capacitance) at the test potential of 0 mV by 3.9 % (-5.1 pA/pF±0.3 pA/pF

vs -4.9 pA/pF±0.2 pA/pF), 15.7 % (-5.1 pA/pF±0.3 pA/pF *vs* -4.3 pA/pF±0.3 pA/pF) and 19.6 % (-5.1 pA/pF±0.3 pA/pF *vs* -4.1 pA/pF±0.4 pA/pF) respectively (Fig 3). At the same time, TNF-α did not change the threshold (-40 mV) of the activation of $I_{Ca,L}$ and the potential (0 mV) of peak current.

Effect of TNF-α on steady-state activation and inactivation kinetics of $I_{Ca,L}$ Steady-state activation or inactivation were obtained by conventional protocols, and the corresponding curves of $I_{Ca,L}$ were fitted with Boltzman equation of the following form: $I/I_{max}= 1/\{1+EXP[(V-V_{1/2})/\kappa]\}$. I is the calcium current, I_{max} is the maximal amplitude of calcium current, V is the voltage of conditioning pulse, $V_{1/2}$ is the potential of half activation or inactivation and κ is the slope factor. For each individual cell, data were fitted to the Boltzmann distribution of the form: $V_{1/2}$ and slope were compared and used to generate a continuous curve that fitted the average normalized data. TNF-α 200 μg/L did not markedly influence activation properties. Half activation potential ($V_{1/2}$) and slope factor (κ) were (-24.1±0.6) mV and (2.0±0.3) under control conditions, and at (-24.2±0.5) mV and (2.2±0.3) in the presence of TNF-α 200 μg/L (Fig 4A, *P*>0.05). Steady-state inactivation was determined by a double-pulse protocol. TNF-α 200 μg/L shifted half inactivation potential ($V_{1/2}$) from (-28.7±0.3) mV to (-37.8±1.4) mV, and slope factor (κ) was not affected (5.5±0.3 *vs* 5.7±1.2, Fig 4B, *P*<0.05).
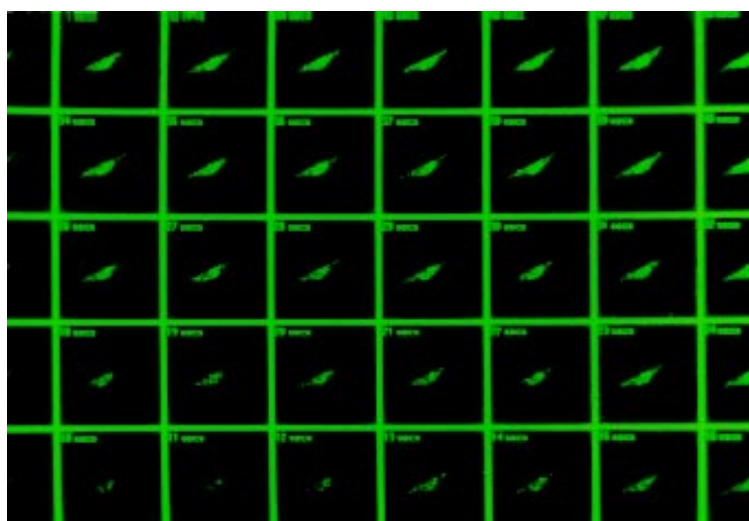

Fig 1. Fluorescence intensity dynamic process of intracellular free Ca²⁺ (Microscopic analysis of the intracellular calcium increase) in neonatal rat ventricular myocytes in the presence of TNF-α 20 μg/L. The first two lattices of the last line display fluorescence intensity of the myocyte in the absence of TNF-α. TNF-α 20 μg/L was added at the third lattice of the last line. Fluo-3-loaded neonate rat ventricular myocyte was recorded every 1 s from left to right and from down to up using laser confocal microscope system. Fluorescence intensity was seen to increase with the time at least for 60 s.
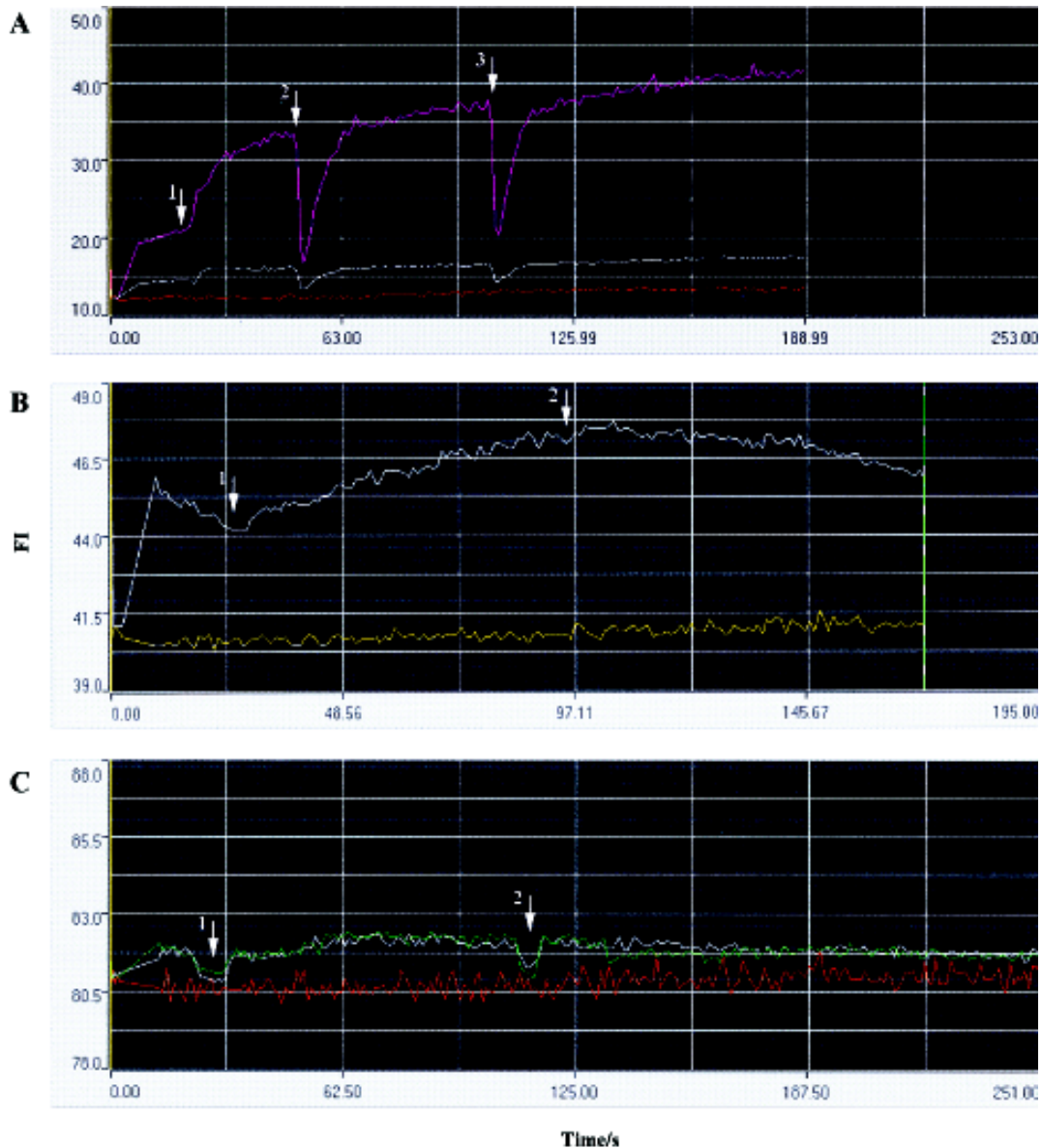
Fig 2. The dynamic process of fluorescence intensity in neonatal rat ventricular myocytes. The lowest curve in each graph stands for the basal fluorescence intensity used as control background. The other ones showed the dynamic process of fluorescence intensity changing with the drugs addition. A: arrow 1, 2, and 3 show the time of adding TNF-α 2, 20, and 200 μg/L respectively. B: arrow 1 and 2 show the time of adding TNF-α 2 μg/L and nicardipine (0.5 μmol/L). C: when the myocytes were exposed to caffeine 100 mmol/L for 30 min, adding TNF-α 2 μg/L (arrow 1), 20 μg/L (arrow 2) failed to induce any changes of fluorescence intensity (two curves show the responses of two cells respectively).

The time dependence of $I_{Ca,L}$ recovery from inactivation In the course of $I_{Ca,L}$ recovery from inactivation, the normalized data from 6 myocytes were fitted by a biexponential function according to the equation: $y=y_0+A_1[1-\exp(-x/t_1)]+A_2[1-\exp(-x/t_2)]$, where x is the time, A_1 and A_2 represent the proportion of recovery accounted for by the time constants t_1 and t_2 respectively. TNF-α 200 μg/L did not affect the half recovery time of $I_{Ca,L}$ from inactivation (46.7 ms *vs* 47.9 ms, Fig 5, $P>0.05$).

DISCUSSION

At the end of 19th century William Coley, a New York surgeon, was the first to describe necrosis of the tumor induced by bacterial toxins[6]. In 1975, a protein responsible for the induction of this process was identified and called TNF-α. Since its discovery, the understanding of the roles of TNF-α in human biology and diseases has grown. In the heart, both myocardial macrophages and cardiac myocytes themselves can
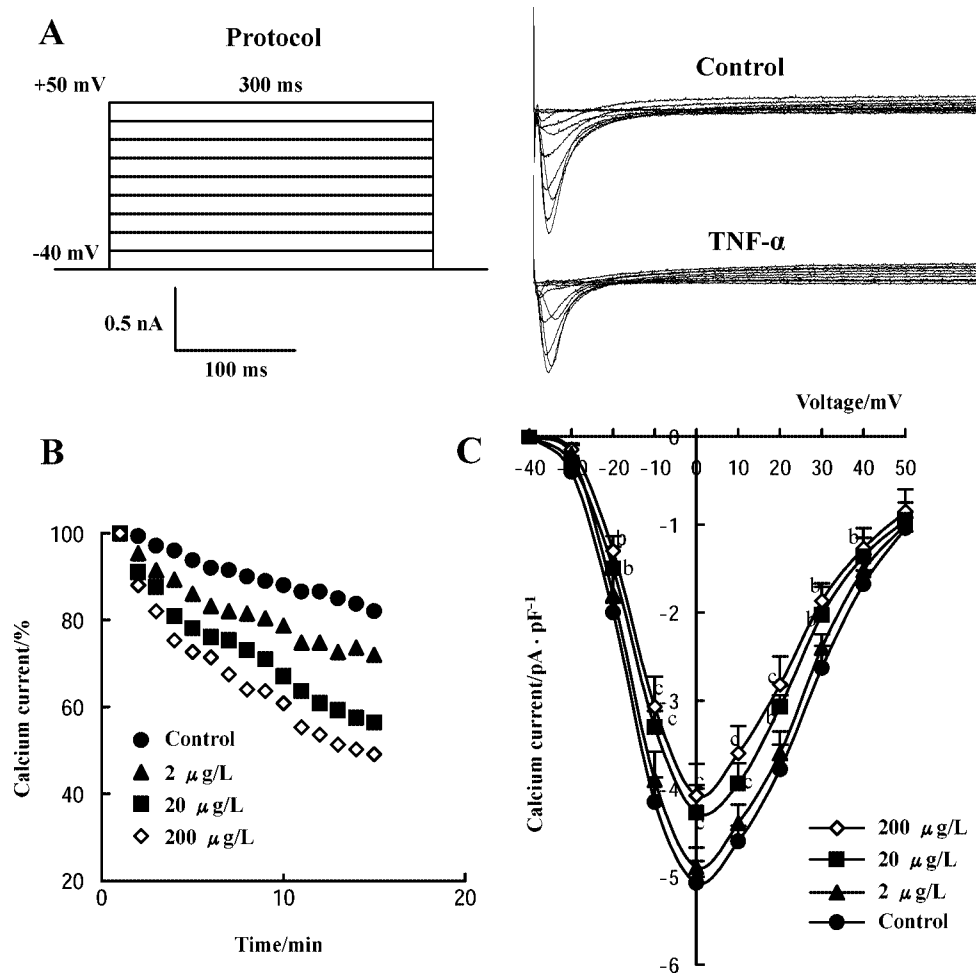
**Fig 3. Effects of TNF-α on $I_{Ca,L}$ in rat ventricular myocytes. A: protocol and original current recording. B: rundown of $I_{Ca,L}$ with the time and the effects TNF-α 2, 20, 200 µg/L on $I_{Ca,L}$. C: the *I-V* relationship of $I_{Ca,L}$ in ventricular myocytes in the absence and presence of TNF-α. TNF-α 2 µg/L, *n*=9 cells from 9 hearts. TNF-α 20 µg/L, *n*=9 cells from 9 hearts. TNF-α 200 µg/L, *n*=8 cells from 9 hearts. Mean±SD. [b]$P<0.05$, [c]$P<0.01$ *vs* control.**

synthesize TNF-α. Accumulating evidence indicates that myocardial TNF-α is an autocrine contributor to myocardial dysfunction and cardiomyocyte death in a variety of experimental and clinical conditions, including ischemia-reperfusion injury, sepsis, chronic heart failure, viral myocarditis, and cardiac allograft rejection[7]. So, the spectrum of biological activities for TNF-α is not limited to cytotoxic effects but rather TNF-α exerts pleiotropic effects[8].

Although the precise role of TNF-α in the heart is not known, the elaboration of TNF-α in cardiac pathophysiological contexts suggests that TNF-α may play a pathogenetic role in above-mentioned diseases. TNF-α is involved in the regulation of normal tissue homeostasis affecting cell proliferation, differentiation, and death[9]. Patients with HF have been shown increased levels of TNF-α in the myocardium[10]. However, the mechanisms by which this pleiotropic cytokine alters cardiac

mechanical function remain unclear. It is reported that Ca^{2+} is also involved in TNF-α-mediated cell damage through the activation of proteases[11].
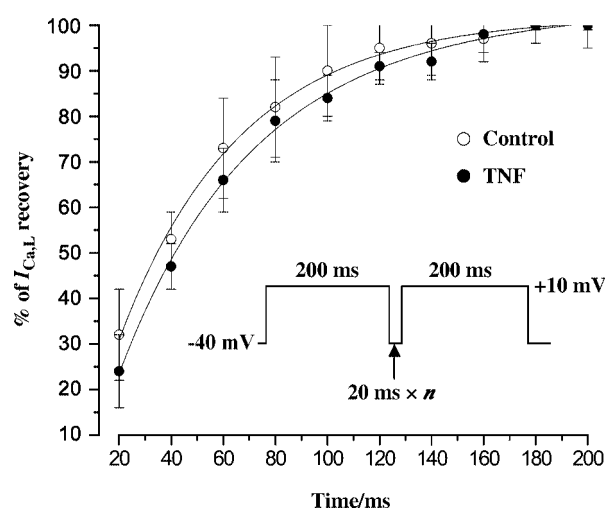
As a ubiquitous intracellular second messenger in the signal transduction pathways, Ca^{2+} plays a pivotal role in many biological processes, including muscle contraction, gene regulation, enzymatic reaction, cell injury and apoptosis[12]. Therefore, the effects of TNF-α on calcium movement probably mediate some cellular functions. But, there is not a categorical and uniform conclusion of TNF-α's effect on calcium movement in the cardiac myocyte at present. It has been reported that TNF-α inhibited cardiac L-type Ca^{2+} channel current $(I_{Ca,L})$[13] and decreased peak systolic $[Ca^{2+}]_i$[14]. Whereas Amadou *et al* reported that at a low concentration TNF-α produced a 40 % increase and at a high concentration TNF-α evoked a biphasic effect comprising an initial positive effect peaking at 5 min, fol-
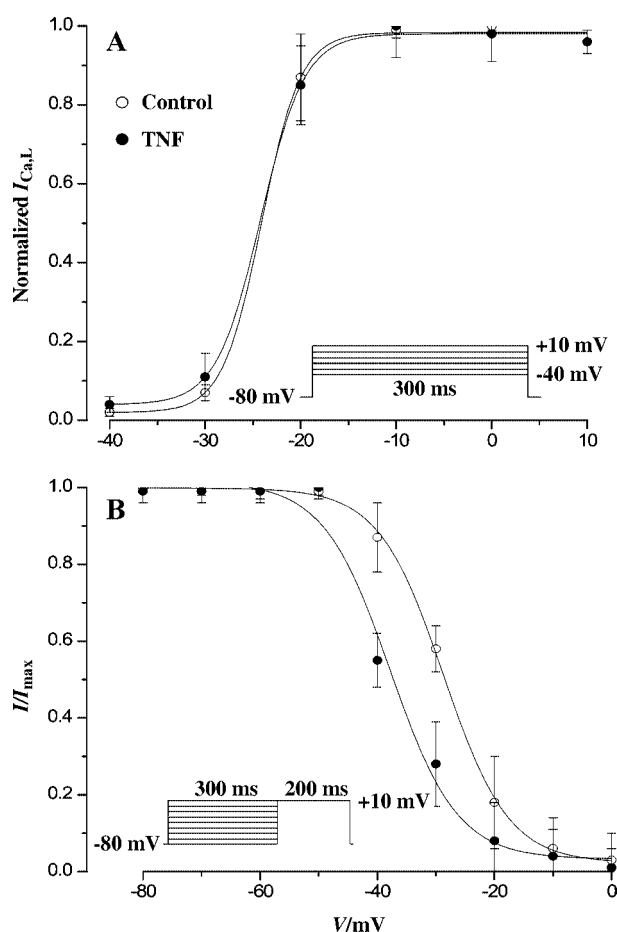
Fig 4. Steady-state activation and inactivation and time-dependent recovery of $I_{Ca,L}$ in the absence and presence of TNF-α 200 μg/L. Protocols are given in the insets. A: Half activation potential ($V_{1/2}$) and slope factor (κ) were (-24.1± 0.6) mV and (2.0±0.3) in control (open symbols), and (-24.2± 0.5) mV and (2.2±0.3) in the presence of TNF-α 200 μg/L (closed symbols) (n=6 cells from 5 hearts, [a]P>0.05). B: Steady-state inactivation was determined by a double-pulse protocol. n=7 cells from 6 hearts.

Fig 5. Recovery time curves from inactivation of $I_{Ca,L}$. The cells were depolarized from -40 mV to +10mV with a duration of 200 ms and various interpulse durations (times of 20 ms) were applied. Half recovery time of $I_{Ca,L}$ shifted from 46.7 ms under control conditions (open symbols) to 47.9 ms in the presence of TNF-α 200 μg/L (closed symbols), (n=5 cells from 5 hearts, [a]P>0.05).

lowed by a sustained negative effect[15].

In this study, we found that TNF-α 2, 20, and 200 μg/L, increased intracellular free Ca^{2+} concentration significantly in a dose-dependent manner in the cardiac myocytes. When sarcoplasmic reticulum (SR) calcium store was exhausted by caffeine, TNF-α failed to induce any changes of intracellular free calcium. Moreover, The whole-cell configuration of the patch-clamp indicated TNF-α inhibited cardiac L-type calcium channel current ($I_{Ca,L}$). It suggests that TNF-α-induced increment of Ca^{2+} concentration be due to calcium releasing from the sarcoplasmic reticulum (SR). This process is independent of voltage-dependent L-type Ca^{2+} channels. In addition, inactivation curves

showed TNF-α 200 μg/L shifted half inactivation potential to the left. So, TNF-α accelerated steady-state inactivation of $I_{Ca,L}$. It suggests that the effect of TNF-α on inactivation state be stronger than on activation state of L-type calcium channel.

These phenomena indicate TNF-α must participate in other signal transduction gateways to induce intracellular free Ca^{2+} release from the sarcoplasmic reticulum (SR) and inhibit Ca^{2+} influx feedback in ventricular myocytes of adult rats. Now growing evidence suggests that most of the biologic effects of TNF-α are mediated by the p55 receptor or tumor necrosis factor receptor 1 (TNFR1)[16]. The effects of TNF-α on calcium movement appear to involve a modulatory effect on G-protein-mediated signal transduction via its TNFR1 receptor. The specific target could be at the level of either G-protein or phospholipase C.

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