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Long-Term IL-2 Incubation-Induced *L-type Calcium* Channels Activation in Rat Ventricle Cardiomyocytes

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Abstract
The following study examined the impact of IL-2 on Ca^{2+} channel activity in the event of several hours' incubation in IL-2. The right ventricle free wall for action potential measurements was isolated and perfused with Tyrode solution. The whole-cell voltage clamp experiments were performed on enzymatically isolated single cardiomyocytes. The whole-cell voltage clamp recording of Ca^{2+} currents was performed using the Cs^{+}-based pipette and bath solutions. The protocol with depolarizing prepulse (− 40 mV) was used to inactivate both Na^{+} current and Ca^{2+} *T-type* current. The *L-type* Ca^{2+} current was elicited by a series of 250 ms depolarizing square pulses with 10 mV increments. At the 15th minute of continuous recording, the peak density at 0 mV was -3.036 ± 0.3015 pA/pF under IL-2 and -3.008 ± 0.3452 pA/pF in control conditions. The IL-2 in moderate concentration (1 ng/mL) has no acute effects on $I_{Ca.L}$ in rat ventricular cells. In contrast, to the lack of acute effects, the long-term incubation with IL-2 (2 h or more) produced a prominent enhancement of Ca^{2+} *L-type* current. In rat, ventricular myocardium IL-2 (1 ng/mL) produced a very gradual prolongation of subendocardial APs which reached a maximal extent after 3–4 h of treatment. The patch clamp study shows an IL-2-induced $I_{Ca.L}$ current activation, while the action potential studies on multicellular ventricular preparations suggest an IL-2-induced *L-type* Ca^{2+} channel participation in the development of AP.

Keywords Interleukin 2 · *L-type* Ca^{2+} current ($I_{Ca.L}$) · Action potential duration (APD) · Right ventricle · Heart · Rat

Introduction

IL-2 plays a central role in the immune system by stimulating and coordinating immune reactions [1]. Recently published data show that circulating IL-2 is actively involved in the pathophysiology of dilated idiopathic cardiomyopathy and myocarditis [2–4]. Several experiments reported that IL-2 has a negative ionotropic effect in a variety of cardiac muscle preparations including whole heart, isolated papillary muscles, and myocytes [5, 6]. Also, a large majority of published data indicate IL-2-mediated myocardial signalization via regulation of intracellular [Ca^{2+}] [7–12]. It is interesting that the intracellular [Ca^{2+}] on the other hand is directly related to the function of the *L-type* Ca^{2+} channels, which are often targeted by various cytokines [9–11]. *L-type* Ca^{2+} channels as the voltage-dependent channels are usually activated in response to membrane depolarization, leading to Ca^{2+} entry and contraction. Additionally, a significant number of recent findings indicate that IL-2 action can be linked to *L-type* Ca^{2+} channel through the activation of other ion channels that cause membrane depolarization and therefore increase *L-type* Ca^{2+} channel activity [13]. In general, the IL-2 action mechanism involves immediate response [14], or a delayed response that lasts from hours to days, depending on the production of secondary mediators [15].

Based on the above-mentioned information, we hypothesize that the way in which IL-2 mobilizes *L-type* Ca^{2+} channels for regulation of contractile force in ventricular preparations requires more time (because of its already established

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action on the expression of different intracellular players). At the same time, these were the main reasons why we decided to investigate the long-term effect of IL-2. To investigate this hypothesis, the contraction force was measured in intact IL-2-treated ventricular preparations and *L-type* Ca^{2+} currents ($I_{Ca.L}$) were measured in isolated IL-2-treated atrial cardiomyocytes. In subsequent experiments, in order to obtain better picture about the mechanisms of induced changes in *L-type* Ca^{2+} channel activity as a consequence of long-term IL-2 incubation, biophysical characterization of their kinetics was performed.

Materials and Methods

Animals

All animal experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health (8th edition, 2011). The experimental protocol was approved by the Bioethics Committee of the Moscow State University. Male outbred white rats weighing 200–250 g ($n = 32$) were kept in the animal house for 4 weeks under a 12:12 h light:dark period in standard T4 cages before the experiment and fed ad libitum.

Isolation of Cardiac Multicellular Preparations and Microelectrode Recordings

Rats were anesthetized with an intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine. Heparin (1000 U/kg) was added to the anesthetic solution to prevent blood coagulation in the coronary vessels of the excised hearts. The chest was opened, and the heart was rapidly excised and rinsed with a Tyrode solution that contained (in mmol/L) NaCl 118, KCl 2.7, NaH_2PO_4 2.2, $MgCl_2$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 25, and glucose 11, bubbled with carbogen gas (95% O_2 + 5% CO_2) at pH 7.4. The right ventricle free wall was isolated, pinned endocardial side up to the bottom of the experimental chamber (3 mL), and supplied with a Tyrode solution of 10 mL/min (37.5 °C). Since the preparations of the ventricular myocardium lacked intrinsic pacemaker activity, they were paced throughout the experiment with a pair of silver, Teflon-coated electrodes (pacing rate − 5 Hz, pulse duration − 2 ms, pulse amplitude − 2 times the threshold).

After an hour of equilibration, the transmembrane potentials were recorded from the endocardial surface of the preparations with sharp glass microelectrodes (30–45 MΩ) filled with 3 mol/L KCl, which were connected to a high-input impedance amplifier IE-210 (Warner Instrument Corp. Brunswick, CT, USA). The signal was digitized and analyzed using specific software (PowerLab 4/30, LabChart Pro V7, Australia). The stable impalements were maintained throughout the entire period of the IL-2 application. The changes in the resting potential, action potential (AP) amplitude, and AP duration at 50 and 90% of repolarization (APD50 and APD90) were determined [14–19].

Isolated Myocyte Preparation

The previously described cell isolation procedure was used with slight modifications [11]. Also, the rat hearts were isolated as described in the previous section. The hearts were attached to the Langendorff apparatus for retrograde perfusion with a Ca^{2+}-free solution containing (in mmol/L) NaCl 120, KCl 5.4, $MgSO_4 \cdot 7H_2O$ 5, Na-pyruvate 5, glucose 20, taurine 20, and Hepes 10 at pH of 7.4 adjusted with NaOH. After an initial perfusion period of 5 min with Ca^{2+}-free solution, the hearts were perfused for 18–20 min with the same solution and supplemented with type II collagenase (0.5 mg/mL), type XIV protease (0.08 mg/mL), and 20 µmol/L $CaCl_2$. The perfusate was continuously bubbled with carbogen, and the temperature was equilibrated at 37 °C. Finally, the ventricles were separated, chopped, and gently triturated to release the cells into a standard Kraftbrühe medium [11].

The suspension of the cells obtained from each of the hearts was split into 2 parts and incubated for 6 h. The first half (control) was stored in a normal Kraftbrühe medium [11]. The second was stored in the same medium, containing 1 ng/mL of IL-2. Recordings of the $I_{Ca.L}$ currents were started after the first hour of incubation.

Electrophysiological Experiments

The whole-cell voltage clamp recordings of $I_{Ca.L}$ currents were performed using an Axopatch 200B (Molecular Devices, Sunnyvale, CA, USA) amplifier. The myocytes were superfused in a small recording chamber (RC-26; Warner Instrument Corp, Brunswick, CT, USA; volume 150 µL) and mounted on an inverted microscope with an external K^+ or Cs^+-based solution at room temperature (23 ± 0.5 °C). The first solution was used while obtaining the whole-cell configuration and contained (in mmol/L) 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, and 10 Hepes, with pH adjusted to 7.4 at 20 °C with NaOH. The Cs^+-based solution was used for the measurement of $I_{Ca.L}$ currents and had the same composition, excluding KCl, which was substituted with equimolar CsCl. 4-Aminopyridine (4-AP), (2 mmol/L), was added to this solution to eliminate potassium currents (I_{to} and I_{Kur}).

Patch pipettes with a mean resistance of 2.18 ± 0.22 MΩ were pulled from borosilicate glass (Sutter Instrument, CA, USA) using a Model P-97 puller (Sutter Instrument,

CA, USA). The pipettes were filled with a Cs^+-based electrode solution containing (in mmol/L) CsCl 130, TEA 15, $MgCl_2$ 1, oxaloacetate 5, EGTA 5, Mg-ATP 5, Mg-GTP 0.03, and HEPES 10, with pH adjusted to 7.2 with CsOH. The pipette capacitance was compensated after obtaining a seal with a resistance > 2 GΩ. The whole-cell capacitance and access resistance were completely compensated using the amplifier's manual controls after gaining access to the cell interior. The mean cell capacitance was 84 ± 10.2 pF, and the mean access resistance was 10.8 ± 3.8 MΩ. In order to obtain current densities, the peak currents were normalized by cell capacitance. Steady-state activation or inactivation was obtained by conventional protocols, and the corresponding curves of $I_{Ca.L}$ were fitted with a Boltzmann equation of the following form: $I/I_{max} = 1/\{1 + \exp[(V - V_{1/2})/k]\}$. I is the Ca^{2+} current, I_{max} is the maximal amplitude of Ca^{2+} current, V is the voltage of the conditioning pulse, $V_{1/2}$ is the potential of half-activation or inactivation, and k is the slope factor. For each separate cell, the data were fitted to the Boltzmann distribution: $V_{1/2}$ and the slope were compared and used to generate a continuous curve that fitted the average normalized data. In the course of time recovery from inactivation of $I_{Ca.L}$, the equation: $y = y_0 + A_1[1 - \exp(-x/t_1)] + A_2[1 - \exp(-x/t_2)]$ was used for fitting the normalized data from 6 cells, where x is the time, while A_1 and A_2 represent the proportion of recovery accounted for the time constants t_1 and t_2, respectively [20].

Drugs

Collagenase type II was purchased from Worthington (Lakewood, NJ, USA), IL-2 was obtained from Invitrogen (Carlsbad, CA, USA), and Nifedipine from Tocris Bioscience. All other compounds were purchased from Sigma (St. Louis, MO, USA).

Statistics

All the data in the text and figures except the original recordings are presented as a means ± S.E.M. for n experiments. Analysis of variance (ANOVA) or unpaired t test was used to compare the control group of preparations/cells versus the group treated with IL-2. $p < 0.05$, was noted as a level of statistical significance. The normality of data arrays was checked using the Shapiro–Wilk test.

Results

Long-Term Incubation with IL-2 Enhances $I_{Ca.L}$ Currents in Ventricular Myocytes

$I_{Ca.L}$ was recorded by using Cs^+-based pipette and bath solutions, in conjunction with the addition of tetra-ethyl ammonium (TEA) to the pipette solution and 4-AP to the external solution, which was allowed to exclude K^+ currents. The protocol with depolarizing prepulse (− 40 mV) was used to inactivate the Na^+ and Ca^{2+} currents. The current was elicited by a series of 250 ms depolarizing square pulses with 10 mV increments.

The recorded Ca^{2+} current had a maximum peak density at 0 mV and was sensitive to nifedipine (10 µmol/L). Therefore, it was identified as $I_{Ca.L}$ current. The current demonstrated minimum rundown, and peak current density after 30 min of continuous recording was not < 80% of the control level. In the cells incubated for 1 h in control conditions, application of IL-2 (1 ng/mL) for 15 min failed to affect $I_{Ca.L}$ (Fig. 1). At the 15th minute of continuous recording, the peak density at 0 mV was − 3.036 ± 0.3015 pA/pF under IL-2 and − 3.008 ± 0.345 pA/pF in control conditions. Thus, IL-2 in moderate concentrations (1 ng/mL) has no acute effects on $I_{Ca.L}$ in rat ventricular cells.

To evaluate the long-term effects of IL-2, a comparison of $I_{Ca.L}$ was recorded 1 min after obtaining a whole-cell configuration in the myocytes incubated up to 6 h with

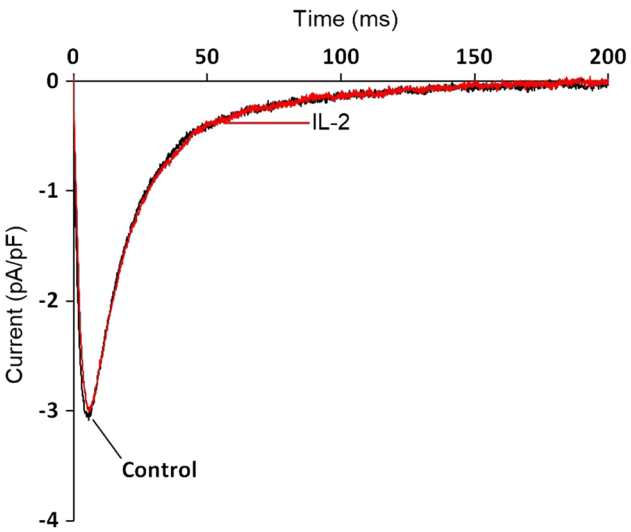

Fig. 1 The short-term application of IL-2 (1 ng/mL) failed to affect the *L-type* Ca^{2+} current in rat ventricular myocytes. Original traces of $I_{Ca.L}$ elicited by square-pulse depolarization of 0 mV in control conditions (black) and after 15 min of IL-2 application (red) are compared. The traces are from 1 representative cardiomyocyte. (Color figure online)

IL-2 (1 ng/mL) in the control Kraftbrühe medium (Fig. 2). While in the latter group of cells, $I_{Ca.L}$ amplitude remained stable during incubation (Fig. 2b), $I_{Ca.L}$ demonstrated a significant increase in the IL-2-treated cells. The maximal $I_{Ca.L}$ values were observed at the fourth hour of incubation (Fig. 2c) when the current amplitude at 0 mV was 2.3 times higher than at the start of the incubation. At the 5th and 6th hours, the current tended to decrease but was still much higher than in the control group of myocytes. Therefore, in contrast to the lack of acute effects, the long-term incubation with IL-2 (2 h or more) produced a prominent enhancement of the $I_{Ca.L}$ current. IL-2 did not significantly affect the activation properties of the $I_{Ca.L}$. Half-activation potential ($V_{1/2}$) and slope factor (k) were (-17.2 ± 0.4) mV and (2.3 ± 0.3) under control conditions, and (-20.9 ± 0.5) mV and (2.1 ± 0.3) after 6 h in IL-2 (Fig. 3a, $p > 0.05$). On the other side, IL-2 shifted the half-inactivation potential ($V_{1/2}$) from (-35.7 ± 1.2) to (-22.8 ± 1.5) mV, while k was not affected $(4.4 \pm 0.3$ vs $4.6 \pm 1.1)$ (Fig. 3b, $p < 0.05$).

In the course of time recovery from inactivation of $I_{Ca.L}$, IL-2 (1 ng/mL) did not change significantly the half time recovery of $I_{Ca.L}$ inactivation (47.6 vs 46.1 ms, Fig. 4, $p > 0.05$), even after 6 h of incubation in IL (1 ng/mL).

Effects of IL-2 Long-Term Incubation on the APD in Rat Ventricular Myocardium

The APs recorded from the subendocardial layer of right ventricular wall preparations were 28.6 ± 3.08 ms in length at 50% of repolarization and 59 ± 3.64 ms at 90% of repolarization. During the first 10 min of the IL-2 application (1 ng/mL), no significant alterations of the AP duration or other parameters were observed. Therefore, the AP waveform was analyzed between 1 and 6 h from the IL-2 application. To reduce the consumption of IL-2, the preparations were superfused in an encircled system with 100 mL of the same oxygenated Tyrode solution containing the drug. The control group of the preparations was superfused in the same conditions without IL-2.

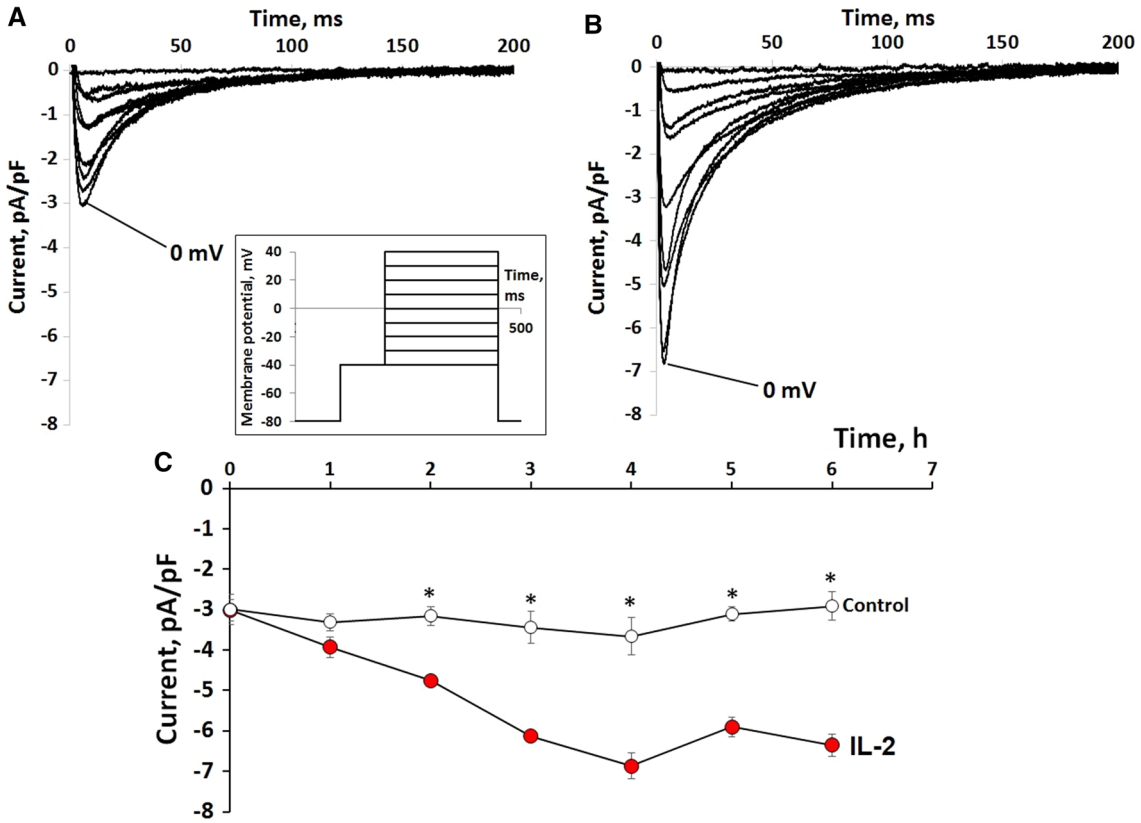

Fig. 2 Changes in the $I_{Ca.L}$ induced by long-term incubation with IL-2 (1 ng/mL) in rat ventricular cardiomyocytes. **a, b** The original traces of $I_{Ca.L}$ were recorded in control (**a**) or after 6 h of incubation in IL-2 (1 ng/mL) containing Kraftbrühe medium (**b**). (**a**, **b** recordings are from two different representative cells.) The $I_{Ca.L}$ was elicited by 250 ms depolarizing test pulses for 9 different potentials by 10 mV steps between −40 and 40 mV, which was preceded by the depolarizing prepulse to −40 mV from the holding potential of −80 mV (see inset). **c** Time course of changes in the $I_{Ca.L}$ peak amplitude was measured at 0 mV in the control (empty circles, $n = 6$ for each time point), IL-2 incubated (filled circles, $n = 6$ for each time point). The cells were obtained from 6 rat hearts. *Note significant differences between the 2 groups, unpaired t test, $p < 0.05$

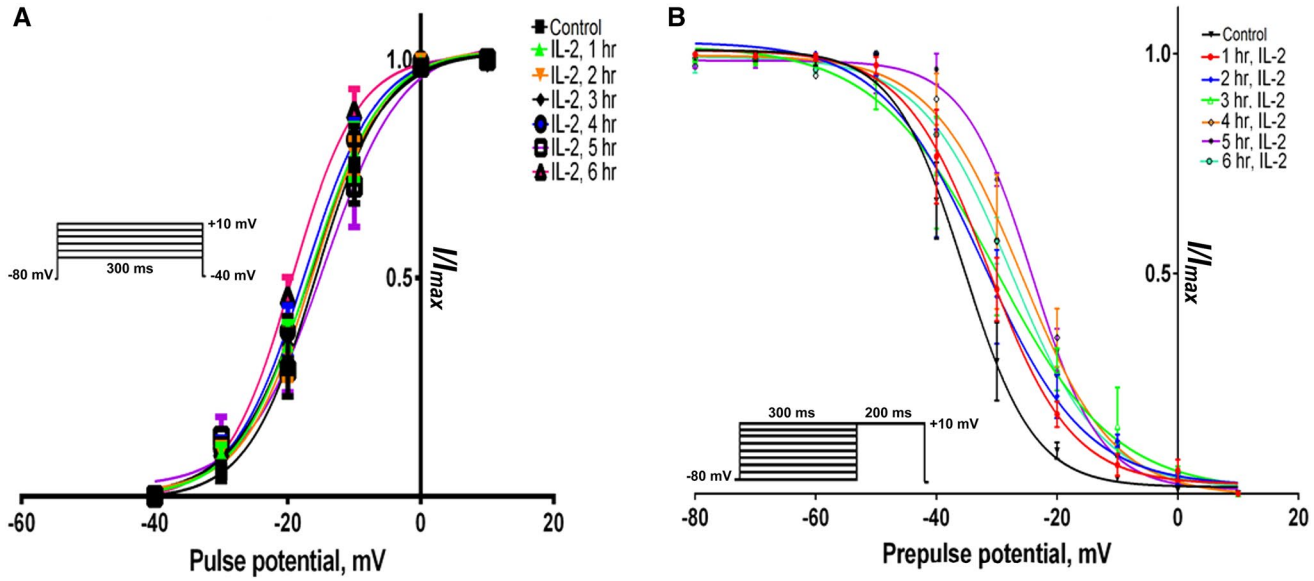

Fig. 3 Steady-state activation and inactivation of $I_{Ca.L}$ in the absence and presence of IL-2 (1 ng/mL). Protocols are given in the insets. **a** Half-activation potential ($V_{1/2}$) and slope factor (k) were (-17.2 ± 0.4) mV and (2.3 ± 0.3) under control conditions, and (-20.9 ± 0.5) mV and (2.1 ± 0.3) after 6 h in IL-2 ($n = 7$ cells from 5 hearts, $p > 0.05$). **b** Steady-state inactivation was determined by a double-pulse protocol. Half-inactivation potential ($V_{1/2}$) and slope factor (k) were (-35.7 ± 1.2) mV and (4.4 ± 0.3) under control conditions and (-22.8 ± 1.5) mV and (4.6 ± 1.1) after 6 h in IL-2. ($n = 6$ cells from 4 hearts, $p < 0.05$.)

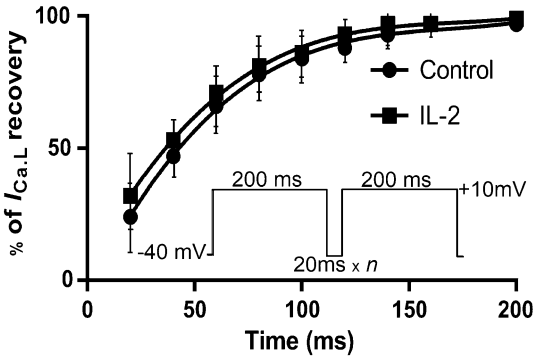

Fig. 4 Time recovery from inactivation of $I_{Ca.L}$ in the absence and presence of IL-2 (1 ng/mL). Depolarization protocols from -40 to $+10$ mV with a duration of 200 ms and various inter-pulse (of 20 ms) durations were applied (in the inset). IL-2 incubation induced insignificant shifts in half recovery time of *L-type* Ca^{2+} currents from 47.6 ms under control conditions (open circles) to 46.1 ms in cells incubate for 6 h in 1 ng/mL IL-2 (filled circles), ($n = 6$ cells, $p > 0.05$). In the cells incubated for 1, 2, 3, 4, and 5 h, shift in time recovery from inactivation was also insignificant ($p > 0.05$, data not shown)

IL-2 induced a pronounced increase in APD90, which became significant versus the control group of preparations, starting from the third hour of IL-2 application (Fig. 5a, c). The APD50 in the IL-2-treated group tended to prolong, but with an insignificant difference (Fig. 5b). Starting from the 4th hour of incubation, the APD90 and to a lesser extent the APD50 decreased in both groups, and the velocity of this secondary APDs shortening did not differ between the groups. In the rat ventricular myocardium, IL-2 (1 ng/mL) produced a very gradual prolongation of subendocardial APDs, which reached the maximum extent after the 3rd to 4th hour of treatment. The prolongation was more prominent at the final repolarization stage.

The Role of the L-type Ca^{2+} Channels in the Regulation of the APD

Employment of nifedipine (10 μmol/L) as a highly specific *L-type* Ca^{2+} channel antagonist not only stopped the APD50 and APD90 prolongation but also induced very significant shortening even in ventricular preparations exposed to the long-term effect of IL-2 (Fig. 5d). In the presence of nifedipine (10 μmol/L), IL-2 did not induce any change in APD50 and APD90 even after the 5th or 6th hour of perfusion (Fig. 5d).

Discussion

Cao et al. [8] have shown that IL-2-induced retention of the intracellular [Ca^{2+}] within the ventricular cardiomyocytes is supported by the sarcoplasmic reticulum (SR), which takes up over 90% of the intracellular [Ca^{2+}], while the remaining 10% can be extruded by a Na^+/Ca^{2+} exchanger [14]. The fact that the $I_{Ca.L}$ current did not change in the first 2 h of exposure to IL-2 suggests that the equilibrium between the Ca^{2+} released from the SR and the Ca^{2+} resequestered back

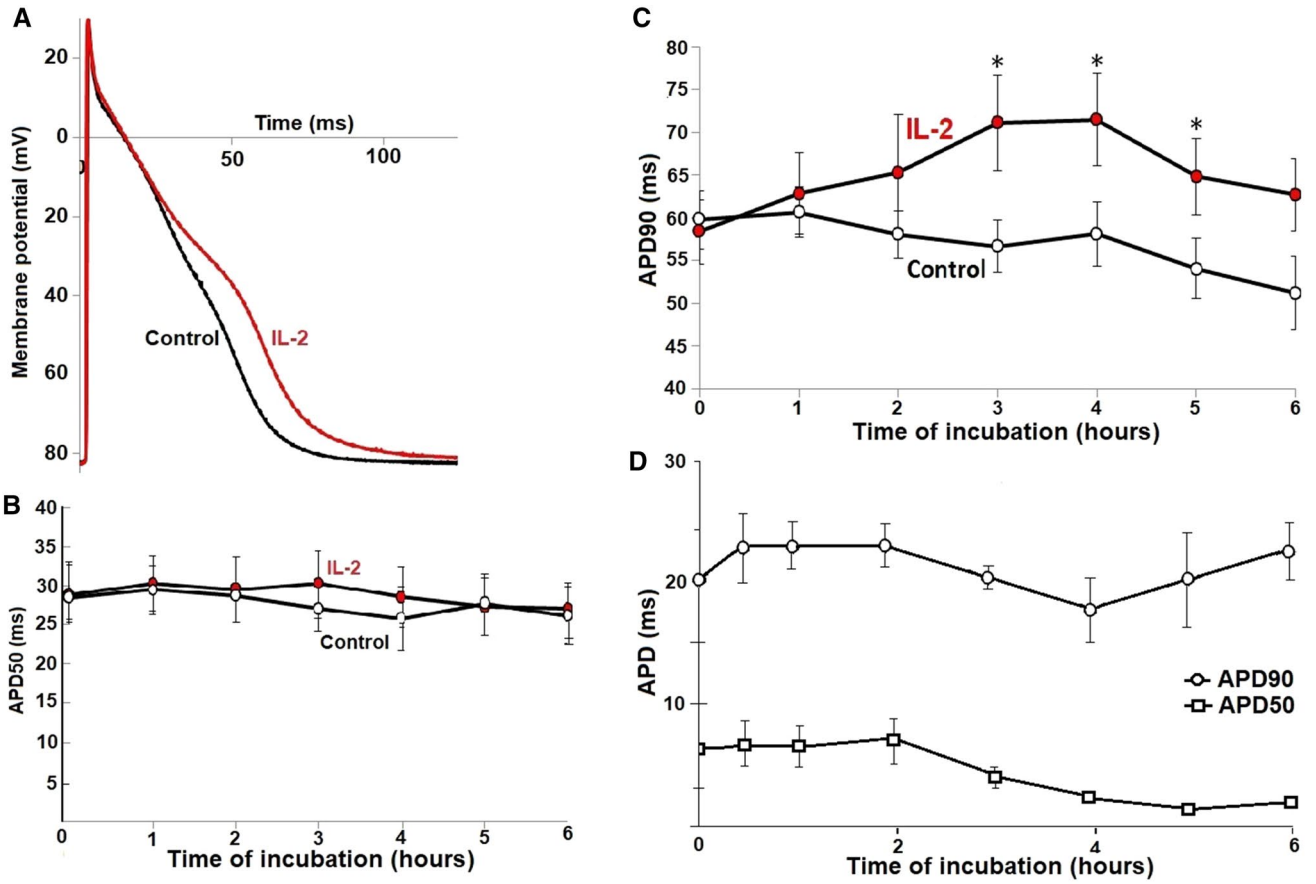

Fig. 5 Changes in the AP waveform induced by IL-2 in the preparations of rat ventricular myocardium. **a** Original traces of AP recorded in control conditions before the addition of the drugs and after 3 h of IL-2 (1 ng/mL) application. **b, c** Time course of changes in the APD measured at 50% (**b**) and 90% (**c**) of the repolarization level in the control ($n=6$) and treated with IL-2 ($n=6$) preparations. **d** Time course of changes in APD measured in the presence of nifedipine (10 µmol/L) ($n=6$). *Indicates a significant difference between the two groups, $p<0.05$

into the SR is probably balanced by extrusion via the Na^+/Ca^{2+} exchanger. In addition, the curves of steady-state inactivation showed that prolonged incubation in IL-2 shifted the half-inactivation potential to the right. It appears that IL-2 eroded the steady-state inactivation of $I_{Ca.L}$, which suggests that prolonged incubation in IL-2 fosters mechanisms that keep *L-type* Ca^{2+} channels in a prolonged active state. This is in agreement with the previous observation concerning the action of IL-2 upon maintenance of intracellular $[Ca^{2+}]$ homeostasis via κ-opioid receptors [7–9]. Actually, Cao et al. [9] have shown that IL-2 induces activation of the κ-opioid receptors by opening the mitochondrial K_{ATP} channels and activation of the protein kinase C (PKC) [21], which in turn can activate other effectors [21]. According to the results obtained in this study, one such effector could be *L-type* Ca^{2+} channels, which were affected by long-term incubation in IL-2.

In the present study, APD90 was almost identical in control and IL-2 groups (in the first hour of exposure) at 1.8 mmol/L extracellular $[Ca^{2+}]$, indicating that the short-term IL-2 (1 ng/mL) exposure did not result in any significant changes in APD90. In our previous study with IL-2 [14], it was shown that APD90 was changed after short-term exposure to IL-2 by using extremely high doses of IL-2 (50 ng/mL), a finding which confirms the dose-dependent effect of the IL-2. In fact, we and others have shown that its effect on APD90 is mainly dependent on Ca^{2+}-handling mechanisms in the SR [14, 22–25]. In this study, prolonged effects of IL-2 showed that *L-type* Ca^{2+} channels play a significant role in the prolongation of action potential of ventricular preparations. Taking into account that recent evidence suggests that most of the biologic effects of IL-2 are mediated by PKC [21], one can suggest that long-term effects of IL-2 on calcium movement probably involve a modulatory effect on PKC-mediated signal transduction. Therefore, it seems that PKC involvement in the examined IL-2 signaling needs further attention and will be addressed in our next study.

In conclusion, obtained data from action potential studies on rat ventricular multicellular preparations suggest an IL-2-induced *L-type* Ca^{2+} channel participation in the development of AP, while the patch clamp study implies IL-2-induced $I_{Ca.L}$ activation during prolonged exposition. A detailed picture of the activation of these signal pathways and their associations with intracellular [Ca^{2+}] dynamics remain unclear and need further attention.

Limitation

Taking into account the long-term incubation of the investigated cells, one of the basic limitations of this study was the unsuspected role of accumulated free radicals (O_2^-) on the whole onset of molecular events. Evidence of the involvement of accumulated oxygen free radicals in the prolongation of APD90 in rat ventricular myocytes has also been shown [26]. This is not the case in the short-term exposure, since an IL-2-induced O_2^- leak from the ventricular mitochondria is probably neutralized by the cardiomyocytes' superoxide dismutase. We believe that superoxide dismutase activity with time decreased due to increased O_2^- accumulation, which is the reason for the prolongation of APD90.

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Author Contributions All authors contributed toward data analysis, drafting, and revising the paper and agree to be accountable for all aspects of the work.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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