Influence of Interleukin –2 on Ca^{2+} Handling in Rat Ventricular Myocytes

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Abstract

In the present study we examined the effect of interleukin-2 (IL-2) on cardiomyocyte calcium handling. The effects of steady-state and transient changes in stimulation frequency on the intracellular Ca^{2+} transient were investigated in isolated ventricular myocytes by spectrofluorometry. In the steady state (0.2 Hz) IL-2 (200 U/ml) decreased the amplitude and prolonged the decay of the electrically stimulated and caffeine-induced Ca^{2+} transients. At 1.25 mM $[Ca^{2+}]_o$, when the stimulation frequency increased from 0.2 to 1.0 Hz, diastolic Ca^{2+} level and peak $[Ca^{2+}]_i$ as well as the amplitude of the transient increased. The positive frequency relationships of the peak and amplitude (peak minus diastolic) $[Ca^{2+}]_i$ were blunted in the IL-2-treated myocytes and this was not normalized by increasing $[Ca^{2+}]_o$ to 2.5 mM. IL-2 inhibited the frequency relationship of caffeine-induced Ca^{2+} release. Blockade of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase with thapsigargin resulted in a significant reduction of the amplitude/frequency relationship of the transient similar to that induced by IL-2. The restitutions were not different between control and IL-2 groups at 1.25 mM $[Ca^{2+}]_o$, which was slowed in

IL-2-treated myocytes when $[Ca^{2+}]_o$ was increased to 2.5 mM. There was no difference in the recirculation fraction between control and IL-2-treated myocytes at both 1.25 and 2.5 mM $[Ca^{2+}]_o$. Perfusion with IL-2 (200~1000 U/ml) for 10 min had no significant effect on the whole-cell L-type calcium current. It is concluded that the cardiac effect of IL-2 on calcium handling relates to depression of SR function.

Key words: Frequency relationship; Interleukin-2; Intracellular Ca^{2+}; Myocytes

Introduction

Compelling evidence now exists that circulating interleukin-2 (IL-2) is actively involved in the pathophysiology of myocarditis and idiopathic dilated cardiomyopathy {Koike 1989 44 /id}{Marriott, Goldman, et al. 1996 45 /id}{Matsumori, Yamada, et al. 1994 43 /id}. In patients with myocarditis and cardiomyopathy, the serum level of IL-2 increases {Matsumori, Yamada, et al. 1994 43 /id}. It has been demonstrated that heart is the target organ of IL-2. Several experiments showed that IL-2 has a negative inotropic effect in a variety of cardiac muscle preparations including whole heart, isolated papillary muscles, and myocytes {Cao, Xia, et al. 2002 257 /id}{Finkel, Oddis, et al. 1992 19 /id}{Weisensee, Bereiter-Hahn, et al. 1993 20 /id}. Clinical data also showed that IL-2 therapy depresses cardiac contractility in patients {Tio, Nieken, et al. 2000 46 /id}. The question arising from these findings is whether the inotropic effect of IL-2 on heart may be related to a substantial alteration in intracellular Ca^{2+} handling.

Increased heart rate during exercise enhances cardiac output directly, and by the effect of this increased rate on myocardial performance. The latter, termed contraction-frequency relation, strength-interval relation, or Treppe phenomenon was first observed by Bowditch in the isolated frog heart {Bowditch 1871 47 /id}. Recent studies demonstrated that the contraction-frequency relationship is also present in isolated myocardium and cardiomyocytes {Borzak, Murphy, et al. 1991 49 /id}{Frampton, Harrison, et al. 1991 50 /id}{Frampton, Orchard, et al. 1991 51 /id}{Layland & Kentish 1999 48 /id}{Kotsanas, Delbridge, et al. 2000 7 /id}. The steady-state contraction-frequency relationship is an intrinsic property of cardiac muscle that reflects the time-dependency of the mechanisms that make Ca^{2+} available to the myofilaments on a

beat-to-beat basis. Mechanical restitution and post-rest potentiation are the two manifestations of the contraction-frequency relationship under non-steady-state conditions. The former describes the recovery of contractile force as a function of the interval between contractions, while the latter, post-rest potentiation, describes the increase in contractile force that occurs when stimulation is recommenced following a prolonged pause. The decline of force following post-rest potentiation has been used to estimate the fraction of Ca^{2+} that is sequestered by the sarcoplasmic reticulum (SR), termed the recirculation fraction (RF) {Bers 1991 52 /id}. The contraction-frequency relationship has been attributed to a parallel change in intracellular Ca^{2+} transients {Schillinger, Lehnart, et al. 1998 293 /id}, so the steady-state contraction-frequency relationship, mechanical restitution and post-rest potentiation have been widely used as tools for investigating myocardial Ca^{2+} handling.

Most reports demonstrated that under normal conditions most mammalian hearts exhibit a positive contraction-frequency relationship, including rat cardiac muscle {Frampton, Harrison, et al. 1991 50 /id}{Frampton, Orchard, et al. 1991 51 /id}{Layland & Kentish 1999 48 /id}{Schouten & ter Keurs 1986 53 /id}. However, it was found that in models of severe cardiac hypertrophy and failure this relationship was severely blunted or even reversed {Brooks, Bing, et al. 1994 56 /id}{Ezzaher, el Houda, et al. 1992 55 /id}{Mulieri, Hasenfuss, et al. 1992 54 /id} and appeared to arise from defects in intracellular Ca^{2+} regulation. In addition, mechanical restitution and post-rest potentiation are also sensitive indicators of myocardial Ca^{2+} handling. In failing human and canine hearts, mechanical restitution is slowed {Cooper, Fry, et al. 1992 57 /id}{Mulieri, Hasenfuss, et al. 1992 54 /id}{Prabhu & Freeman 1995 58 /id}, while the recirculation fraction is reduced in hypertrophied rabbit and failing human heart {Kotsanas,

Holroyd, et al. 1996 1 /id} {Seed, Noble, et al. 1984 59 /id}.

Apart from the inotropic effect of IL-2, there is at present no information on whether IL-2 induces derangements in cellular Ca^{2+} handling and alters the mechanical restitution or the recirculation fraction in myocardium. Therefore, we investigated changes of the intracellular Ca^{2+} transient in IL-2-treated rat ventricular myocytes in response to both steady-state and transient alterations in stimulation frequency. We found that the positive Ca^{2+} transient-frequency relationship was blunted and even reversed, and this was not normalized by increasing extracellular [Ca^{2+}] to 2.5 mM. The restitution of the [Ca^{2+}]$_i$ transient was slowed at 2.5 mM extracellular [Ca^{2+}]. Although the evidence indicates depressed SR Ca^{2+} uptake, the relative amount of Ca^{2+} recirculating in IL-2-treated cells remained unaltered.

Materials and Methods

Isolation of ventricular myocytes

Single cardiac myocytes from male Sprague-Dawley rats (weighing 242.52±9.82 g) were prepared by enzymatic dissociation during retrograde perfusion of the heart using a modified Langendorff technique. All procedures used in this study were approved by the Ethics Committee for the Use of Experimental Animals. Immediately after stunning and decapitation, the heart was rapidly removed and rinsed in ice-cold Ca^{2+}-free Tyrode's solution. The heart was perfused with 100% oxygenated, non-recirculating Ca^{2+}-free Tyrode's solution. Then the perfusion solution was switched to 100% oxygenated recirculated low Ca^{2+} (50 μM) 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℃ 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 mM in 40 min.

Intracellular calcium transient recording

Intracellular calcium transients were determined by a spectrofluorometric method using the dye fura-2-AM as the Ca^{2+} indicator. After stabilization, isolated myocytes were incubated with 5 μM fura-2-AM at room temperature for 30 min. 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 solution under a gas phase of 95% O_2 / 5% CO_2. The myocytes selected for the study were

rod-shaped with clear striations. The Ca^{2+}-dependent fura-2 signal was obtained by illuminating at 340 and 380 nm and recording the emitted light at 510 nm. Background fluorescence was automatically subtracted. As in previous studies in other laboratories {Kotsanas, Delbridge, et al. 2000 7 /id}{Ventura, Spurgeon, et al. 1992 62 /id}, we calculated the fluorescence ratio at the two wavelengths, which is believed to accurately represent the intracellular calcium concentration ($[Ca^{2+}]_i$). The Ca^{2+} transient was induced by supra-threshold field stimulation at 0.2 Hz delivered through two platinum electrodes in the bathing fluid.

Whole-cell calcium current measurements

Sarcolemmal calcium currents were recorded with the whole-cell patch-clamp technique. Myocytes were superfused at 2 ml/min, using a physiological solution containing (mM) 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 (mM): CsCl 135, $MgCl_2$ 3.0, HEPES 10.0, EGTA 10.0, cAMP 0.5, MgATP 2.0, pH 7.4. Membrane potential was controlled with a patch-clamp amplifier (Axopatch 200B) and membrane currents were sampled by a 12-bit A/D converter (Digidata 1200B). Fast sodium currents were eliminated using a pre-pulse to –40 mV for 40 ms prior to the test pulse. Voltage-clamp protocols, data acquisition, and data storage utilized pClamp 7.0 and current analysis was performed by Clampfit 8.0.

Experimental protocols

All experiments were performed at room temperature (22 °C). The extracellular Ca^{2+}

concentration ($[Ca^{2+}]_o$) was 1.25 mM, except when stated to be 2.5 mM. Data were collected during steady-state conditions and were assessed at the completion of each protocol. By this means the $[Ca^{2+}]_i$-frequency relationship, restitution and recirculation fraction were studied in each heart.

Determination of stimulation frequency effects on $[Ca^{2+}]_i$

After 5-10 min of stabilization at 0.2 Hz, the myocytes were stimulated at 0.2, 0.4, 0.6, 0.8, and 1.0 Hz. Following each change in frequency, cells were allowed 3 min for their contractile response to stabilize. To investigate the influence of IL-2 on the $[Ca^{2+}]$i-frequency relationship, stimulation frequencies of isolated myocytes were increased stepwise after addition of IL-2. Myocytes were exposed to IL-2 for 10 min by exchanging the superfusion buffer containing IL-2 200 U/ml.

Assessment of SR Ca^{2+} load

The SR Ca^{2+} load after different frequencies of stimulation was assessed by rapid application of caffeine (20 mM) in the presence or absence of IL-2. Baseline values for $[Ca^{2+}]_i$ were measured in individual, field-stimulated myocytes for 1.5 min. Then the cells were perfused by a solution with or without IL-2 for 10 min. Field stimulation of the myocyte was discontinued, and then caffeine (20 mM) was applied to the bath. This elicited a caffeine contracture. The amplitude of the fura-2 fluorescence ratio in response to caffeine application was used as an index of SR Ca^{2+} content. Preliminary experiments confirmed a previous report {Frampton, Harrison, et al. 1991 50 /id}that varying the time of caffeine application after the last stimulation had little effect on the

size of the caffeine-induced rise in $[Ca^{2+}]_i$.

Determination of restitution of Ca^{2+} transient

In order to investigate whether exposure to IL-2 causes changes in the restitution of the Ca^{2+} transient, responses to 200 U/ml IL-2 were examined. We used the restitution measurement protocol described by Kotsanas et al.{Kotsanas, Holroyd, et al. 1996 1 /id}. Cells were stimulated at 0.2 Hz, and when a steady state had been achieved, a single test stimulus was delivered either 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2, 3, 4, or 5 s following a steady-state contraction. These stimuli were controlled by a computer installed with the MedLab system. To maintain a steady state, cells were allowed to stabilize for 3 min between each experimental run.

Determination of recirculation fraction of Ca^{2+} transient

Cells were stimulated at 1.0 Hz until the amplitude of the Ca^{2+} transient reached a steady state. Then, the stimulation was stopped for 30 s and recommenced at 0.2, 0.4, or 0.8 Hz. Cells were allowed to stabilize for 5 min at 1.0 Hz between experimental runs. The recirculation fraction (RF) was determined as the slope of the linear relationship between the amplitudes of post-rest contractions *n* and *n-1*.

Chemicals and solutions

Collagenase (type I), bovine serum albumin (BSA), 3-[N-morpholino]propane-sulfonic acid (MOPS), taurine, thapsigargin, and fura-2-acetoxymethyl ester were purchased from Sigma Chemical Co. Interleukin-2 (IL-2) was purchased from Shanghai Huaxin High Biotechnology Inc.

Ca^{2+}-free Tyrode's solution contained (mM): NaCl 100.0, KCl 10.0, KH_2PO_4 1.2, $MgSO_4$ 5.0, glucose 20.0, MOPS 10.0; pH was adjusted with KOH to 7.2.

Krebs-Henseleit (K-H) buffer contained (mM): 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 (pH 7.4).

Statistical analysis

Values presented here are means±standard deviation of means from *n* individual cells investigated in separate experiments. One-way analysis of variance and Dunnett's test were employed to determine the differences among groups. Student's *t*-test was performed for paired samples. Differences were considered significant at the $P<0.05$ level.

Results

Effect of IL-2 on the intracellular calcium transient induced by electrical stimulation and caffeine

As shown in Fig. 1, IL-2 at 200 U/ml decreased the peak of the intracellular calcium transient and increased the diastolic Ca^{2+} level. In order to compare differences in the falling phase of the calcium transient, a single exponential function was fitted to the declining phase at 0.2 Hz. This revealed that the time constant of the $[Ca^{2+}]_i$ decline (τ) was significantly greater in the IL-2 treated group than in the control group.

In Fig 2, IL-2 at 200 U/ml decreased the amplitude of the caffeine induced calcium transient. In order to determine the abilities of the Na^+/Ca^{2+} exchanger to lower cytosolic free Ca^{2+}, the decay of the caffeine induced $[Ca^{2+}]_i$ transient was made. The rate of the decay in the caffeine induced $[Ca^{2+}]_i$ transient of myocytes as indicated by the time constant (τ) of transient decay was 3171.1±271.9 ms in the control, while the value in IL-2 group was significant prolonged by 21.1% .

Effect of IL-2 on sarcolemmal calcium current

We used the whole-cell patch-clamp technique to test the effect of IL-2 on the calcium current. As shown in Fig 3, perfusion with 200, 500, and 1000 U/ml IL-2 for 10 min had no significant effect on the whole-cell L-type calcium current. Furthermore, IL-2 at 1000 U/ml did not alter the calcium current of the myocyte in the presence of isoproterenol (0.1 μM).

Effect of IL-2 on frequency dependence of Ca^{2+} transient

The effects of frequency over the range 0.2 - 1.0 Hz at 1.25 mM extracellular [Ca^{2+}] are summarized in Fig. 4 A-C. Increasing stimulation frequency consistently resulted in an increase in diastolic and peak F340/380 levels in the control group, showing a positive $[Ca^{2+}]_i$-frequency relationship in cardiomyocytes. After pretreatment with IL-2 at 200 U/ml for 10 min, the diastolic $F_{340/380}$ level increased and the peak $F_{340/380}$ level decreased relative to the control group. Although the positive frequency dependence of diastolic and peak F340/380 levels remained after pretreatment with IL-2, the amplitude calculated from the difference between these parameters showed a negative frequency dependence.

In order to investigate whether increasing SR content could reverse the IL-2-dependent decrease in amplitude, we measured the $[Ca^{2+}]_i$ - frequency relationship in control and IL-2 pretreated myocytes in 2.5 mM extracellular [Ca^{2+}]. These experiments were performed on a different population of cells to those used for the 1.25 mM extracellular [Ca^{2+}] experiments, and the results are summarized in Fig. 4 D-F. In control myocytes, the positive $[Ca^{2+}]_i$ - frequency relationship was maintained, with a significant frequency-related increase in each parameter of the Ca^{2+} transient. Following pretreatment with IL-2, the alterations of the diastolic and peak $F_{340/380}$ levels of cardiomyocytes in 2.5 mM extracellular [Ca^{2+}] were similar to those in 1.25 mM extracellular [Ca^{2+}], but the changes in the diastolic $F_{340/380}$ levels were less than those of the control group, which indicates that at 2.5 mM extracellular [Ca^{2+}], the positive frequency dependence of diastolic and peak $F_{340/380}$ levels after pretreatment with IL-2 still existed. The

frequency dependent relationship of the amplitude was attenuated.

Effect of IL-2 on frequency dependence of caffeine-induced Ca^{2+} release

In order to investigate whether IL-2 influences the calcium load in the sarcoplasmic reticulum of cardiomyocytes, the frequency dependence of caffeine-induced calcium release at 1.25 mM extracellular [Ca^{2+}] was determined. In control myocytes, the positive frequency dependence of caffeine-induced calcium release was observed, i.e., the release of Ca^{2+} produced by rapid caffeine application increased as the preceding stimulation frequency was increased at the range of 0.2-1.0 Hz. After pretreatment with IL-2 at 200 U/ml for 10 min, the increase of caffeine-induced Ca^{2+} release with increasing stimulation frequency was attenuated (Fig. 5).

Effect of thapsigargin on frequency dependence of Ca^{2+} transient

Thapsigargin, a selective inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) pump in cardiac myocytes {Janczewski & Lakatta 1993 30 /id}{Wrzosek, Schneider, et al. 1992 29 /id}, blocks the SERCA2a and therefore prevents re-uptake of cytosolic Ca^{2+} into the sarcoplasmic reticulum. The consequence of this process is the removal of the SR as a functional organelle involved in intracellular Ca^{2+} cycling. In this series of experiments there was a marked and significant reduction in the frequency-dependence of the amplitude after treatment with 1 μM thapsigargin for 10 min (Fig. 6). This is consistent with a central role for the SR and SERCA2a in the regulation of the frequency dependence. After pretreatment with IL-2 at 200 U/ml in the presence or absence of thapsigargin, the frequency dependence of the amplitude was very similar to that of thapsigargin alone.

Effect of IL-2 on restitution of the intracellular Ca^{2+} transient

To investigate whether slower recovery of SR Ca^{2+} release between contractions might contribute to the reduced peak and amplitude of the Ca^{2+} transient after IL-2 treatment, we looked at the rate of recovery (restitution) of the Ca^{2+} transient in response to a premature stimulation (i.e. simulating extrasystole). The method used in the experiments is illustrated using examples of original recordings in Fig. 7. For these experiments, the cells were stimulated at 0.2 Hz, and when a steady state had been reached, a single test stimulus was delivered at a variable interval following a steady-state contraction. The extrasystolic interval was varied over the range 500 to 5000 ms, and immediately after the extrasystole the intercontraction interval was returned to normal.

Fig. 7 shows the Ca^{2+} transients recorded in control and IL-2 treated myocytes as the extrasystolic interval was varied. The shortest interval at which a stimulus elicited a contractile response was 500 ms. As the extrasystolic interval was lengthened, the amplitude of the Ca^{2+} transient of the extrasystole progressively increased. The amplitude of the Ca^{2+} transient of the first contraction after the extrasystole was potentiated and decayed toward the steady state over the ensuing four to five contractions.

The recovery of the Ca^{2+} transient of the extrasystole conducted at 1.25 and 2.5 mM extracellular [Ca^{2+}] is summarized in Fig. 7 (E, F). The restitution curves between the control and IL-2 groups at 1.25 mM extracellular [Ca^{2+}] were not different. In control myocytes, restitution of

the Ca^{2+} transient was well fitted by a double exponential function with a time constant of 330 ms. In the IL-2 group the recovery of the Ca^{2+} transient was virtually identical to that of the control group and occurred with a time constant of 346 ms. The restitution curve of the Ca^{2+} transient in controls was significantly different from that of the IL-2 group at 2.5 mM extracellular [Ca^{2+}]. In control myocytes, the time constant of recovery of the Ca^{2+} transient was 226 ms compared to 318 ms in IL-2 group.

Effect of IL-2 on recirculation fraction of intracellular Ca^{2+}

The recirculation fraction is the fraction of Ca^{2+} released from the SR during each Ca^{2+} transient that is resequestered back into the SR. Calcium uptake by the SR loads the SR with Ca^{2+} between contractions, while Na^{+}/Ca^{2+} exchange moves Ca^{2+} across the sarcolemma and out of the cell. A change in the rate of SR Ca^{2+} uptake or Na^{+}/Ca^{2+} exchange might therefore alter the balance between the amount of Ca^{2+} recirculating within the cell and the amount extruded out of the cell.

In order to investigate whether these fractions are altered in IL-2 treated rat myocytes, we designed the next series of experiments. Fig. 8 (A) shows a recording from a control rat myocyte demonstrating the protocol used. As shown in this example, the cell was stimulated at 1 Hz until a steady state was reached. Stimulation was stopped for 30s and then recommenced at 0.2 Hz. The amplitude of the Ca^{2+} transients following the rest period was potentiated, and monotonically declined to a new steady state that was determined by the post-rest stimulation frequency. In previous studies the decline of cell shortening or twitch force following post-rest potentiation has

been interpreted in terms of a loss of Ca^{2+} from the cell, or conversely, the force of successive contractions reflects the fraction of Ca^{2+} sequestered by the SR and therefore recirculating within the cell {ter Keurs HEDJ. 1992 40 /id}.

The decay of post-rest force showed an exponential process {ter Keurs HEDJ. 1992 40 /id}. Therefore, during the period when force is declining, the force of successive contractions decreases by a constant fraction and the ratio of the force of successive contractions should be the same. If the force of post-rest contraction *n* is plotted as a function of the force of post-rest contraction *n-1*, the data points should lie on the same line, the slope of which will be the recirculation fraction. In Fig 8 (B) we show this same analysis applied to the amplitude of the Ca^{2+} transients for this particular cell. It is clear that the relationship between the amplitude of successive post-rest transients is linear. In this particular cell the RF was 0.94.

Fig. 8 (C) shows the data for control and IL-2 treated cells obtained at 1.25 mM extracellular $[Ca^{2+}]$. There was no difference in the RFs between control and IL-2 treated cells. In the control group the value of RF was 0.94 (r^2=0.91), in the IL-2 group the value of RF was 0.95 (r^2=0.98), while the value for pooled data from control and IL-2 groups was 1.057 (r^2=0.98). The similarity between control and IL-2 groups was also obtained at 2.5 mM extracellular $[Ca^{2+}]$ (data not shown).

Discussion

Effect of IL-2 on the $[Ca^{2+}]_i$-frequency relationship

The cardiac effects of IL-2 have been investigated in several studies in different preparations {Finkel, Oddis, et al. 1992 19 /id}{Sobotka, McMannis, et al. 1990 130 /id}{Weisensee, Bereiter-Hahn, et al. 1993 20 /id}. In hamster papillary muscle and isolated perfused rat heart, IL-2 had negative inotropic effects {Finkel, Oddis, et al. 1992 19 /id}{Sobotka, McMannis, et al. 1990 130 /id}. In a previous study, we found that IL-2 decreased the contractility as well as the intracellular calcium transient of isolated rat ventricular myocytes {Cao, Xia, et al. 2002 257 /id}, which established a direct relationship between contractility and the intracellular calcium transient in a single cardiac myocyte. In the present study, we demonstrated that IL-2 reduced the amplitude of the Ca^{2+} transient, and increased the diastolic calcium level and the time constant of the Ca^{2+} transient under conditions of steady-state stimulation, indicating abnormal calcium dynamics in the myocyte treated with IL-2. It is well know that the influx of Ca^{2+} through L-type Ca^{2+} channels and Ca-induced Ca^{2+} release initiate contraction in cardiomyocytes {Barry & Bridge 1993 295 /id}{Bers 1991 52 /id}{Fabiato 1983 294 /id}. The decreased amplitude of the Ca^{2+} transient most probably reflects reduced SR Ca^{2+} release or Ca^{2+} influx. Relaxation takes place as a result of two main systems decreasing 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 via the Ca^{2+} channels{Bers 1991 52 /id}. The increase of the diastolic calcium level and time constant after treatment with IL-2 presumably reflects slower

clearance of cytosolic calcium during relaxation. We speculated that IL-2 could disturb calcium handling of rat ventricular myocytes.

Under the experiment conditions, at 1.25 and 2.5 mM $[Ca^{2+}]_o$, rat ventricular myocytes demonstrated positive frequency dependence over the frequency range investigated. The amplitude of the Ca^{2+} transient increased with frequency (Fig. 4). The positive frequency dependence can be attributed to an increase in SR Ca^{2+} load with increasing stimulation frequency, because the release of Ca^{2+} produced by rapid caffeine application increased as the preceding stimulation frequency was increased from 0.2 to 1.0 Hz. This positive $[Ca^{2+}]_i$-frequency relationship observed in rat ventricular myocytes is similar to those reported in most other mammalian species {Frampton, Harrison, et al. 1991 50 /id}{Layland & Kentish 1999 48 /id}{Money-Kyrle, Davies, et al. 1998 61 /id}.

Frequency-dependence may result from complex interactions among numerous frequency-dependent processes. With regard to the SR, these can be grouped into mechanisms that increase SR Ca^{2+} release and those that limit SR Ca^{2+} content and release. The major factor tending to enhance SR Ca^{2+} release is the increased Ca^{2+} loading. Raising the stimulation frequency increases Ca^{2+} entry per unit time and also increases Na^+ entry via I_{Na}, thereby raising cytosolic $[Na^+]$ and promoting Ca^{2+} influx and decreasing Ca^{2+} efflux, via Na^+/Ca^{2+} exchange. The increased $[Ca^{2+}]_i$ promotes SR Ca^{2+} loading, so that more Ca^{2+} is available for release {Bassani, Yuan, et al. 1995 26 /id}{Satoh, Blatter, et al. 1997 27 /id}.The factors tending to limit SR release are the rate-dependent abbreviation of the action potential, which limits the time and magnitude of

triggered Ca^{2+} influx, and the rate of Ca^{2+} cycling by the SR, which depends in part on the activity of the SR Ca^{2+} pump and the recovery from inactivation of the SR Ca^{2+} release channels. As seen in Fig 4, IL-2 disturbed the $[Ca^{2+}]_i$-frequency relationship of rat myocytes, which may have resulted from changes in one or more of the above mechanisms. In our previous study, the action potential duration decreased in IL-2 treated rat papillary muscles {Lin, Xia, et al. 2002 258 /id}, indicating limitation of SR Ca^{2+} loading and release with IL-2 treatment. In the present study, the depression of caffeine induced Ca^{2+} release in rat myocytes perfused with IL-2 confirmed that the decrease of SR Ca^{2+} release resulted in the depression of the $[Ca^{2+}]_i$-frequency relationship. Furthermore, we investigated the frequency relationship with inhibition of SERCA2a by perfusing the cells with its inhibitor, thapsigargin. We found that the effect of thapsigargin was similar to that of IL-2 on the frequency relationship of the amplitude of the Ca^{2+} transient of rat myocytes, suggesting that, at least, a decrease in SEACA2a activity may be one of the underlying mechanisms of the depression of the frequency response by IL-2.

If the depressed response to stimulation frequency simply reflected reduced SR Ca^{2+} stores in the IL-2-treated rat myocytes, then it might be expected that the relationship would be normalized if SR Ca^{2+} stores could be restored to levels similar to those in control rat myocytes. It is known that the SR Ca^{2+} stores of rat cardiac muscle are nearly saturated when the extracellular $[Ca^{2+}]$ is elevated to 2.5 mM {Fabiato 1981 28 /id}. So we investigated the frequency relationship under such conditions and found that the positive $[Ca^{2+}]_i$ - frequency relationship was still evident in control myocytes. In contrast, the frequency response of the Ca^{2+} transient amplitude in IL-2-treated myocytes was partly attenuated at 2.5 mM $[Ca^{2+}]_o$ compared with that at 1.25mM

$[Ca^{2+}]_o$, which may be due to the increase of the SR Ca^{2+} content. So, considered together with the result of caffeine-induced calcium release, the difference between the control and IL-2-treated myocytes at 2.5 mM $[Ca^{2+}]_o$, although somewhat reduced, was still significant, suggesting the reduced SR Ca^{2+} release in IL-2-treated myocytes may be more complex than simply a normal SR containing less Ca^{2+}.

Diastolic $[Ca^{2+}]_i$, the $[Ca^{2+}]_i$ at the end of the interstimulation interval, was also influenced by frequency. Increasing the stimulation frequency increased the diastolic fluorescence ratio, presumably because Ca^{2+} influx exceeded the amount of Ca^{2+} that could be removed by SR uptake or by extrusion mechanisms during the reduced interval. Because in rat ventricle the SR Ca^{2+}-ATPase removes 92% of the Ca^{2+} from the cytosol{Hove-Madsen & Bers 1993 31 /id}, the further increase of diastolic $[Ca^{2+}]_i$ in the IL-2-treated myocytes with increasing frequency indicated that the uptake of the Ca^{2+} by the SR Ca^{2+}-ATPase is limited compared with the control group. This result is in accordance with the findings of the effect of thapsigargin on the frequency relationship of Ca^{2+} transient amplitude. These confirmed that IL-2 treatment might affect the function of the SR Ca^{2+}-ATPase. Measurement of SR Ca^{2+}-uptake and SERCA2a activity /protein level will be necessary to fully characterize this effect of IL-2.

Effect of IL-2 on restitution of Ca^{2+} transient

The action potential duration and the force of contraction of cardiac muscle vary with the proceeding stimulation interval. In regularly-stimulated rat heart, an extra stimulus interpolated soon after a steady-state contraction elicits an action potential that is shorter in duration, and a

twitch of markedly less force than the control. The gradual restoration of the action potential duration and contractile force during diastole are called restitution {Bass 1975 23 /id}{Boyett & Jewell 1978 24 /id}{Seed & Walker 1988 25 /id}. This reflects the time-dependent recovery of SR Ca^{2+} release {Wier & Yue 1986 32 /id}.

Restitution of the Ca^{2+} transient was determined by inserting extra stimuli during periods of steady-state stimulation. The mechanism of restitution has been explained in terms of (1) release of Ca^{2+} from the SR in proportion to the Ca^{2+} current (I_{Ca}) {Trautwein, McDonald, et al. 1975 63 /id}, (2) slow replenishment of the releasable Ca^{2+} store of SR {Antoni, Jacob, et al. 1969 64 /id}{Edman & Johannsson 1976 65 /id}, (3) time-dependent recovery of the SR release channel from inactivation {Fabiato 1985 66 /id}, and (4) a slow reduction in activity of the SR calcium pump during diastole {Schouten 1990 67 /id}. The restitution primarily manifests the beat interval-dependent Ca^{2+}-handling properties of the SR {Yue, Burkhoff, et al. 1985 39 /id}. In the present study, the restitution curves for $F_{340/380}$ were nearly identical in the control and IL-2 groups at 1.25 mM $[Ca^{2+}]_o$, indicating that IL-2 treatment did not result in any significant contribution to the restitution. The rate of restitution of the Ca^{2+} transient, reflecting the time course of SR Ca^{2+} cycling, was unchanged. It is possible that any decline in SR Ca^{2+}-ATPase activity may be compensated by increased Ca^{2+} extrusion through Na^{+}/Ca^{2+} exchange. Furthermore, when the extracellular $[Ca^{2+}]$ was increased to 2.5 mM, the rate of restitution in the IL-2 group was slower than that in the control group. The slower rate of restitution under elevated extracellular calcium concentration most likely arises from a reduced driving force for Ca^{2+} efflux through Na^{+}/Ca^{2+} exchange.

In addition, Vornanen et al. considered that redistribution of intracellularly stored calcium is complete by at least the latter half of the period of mechanical restitution and that the rate of mechanical restitution is controlled by the rate of recovery of I_{Ca} from inactivation {Vornanen & Shepherd 1997 37 /id}. Changes in SR Ca^{2+}-ATPase are most likely to account for the frequency-dependent potentiation rather than the mechanical restitution. Neither the calcium influx via the L-type calcium channel in normal cardiomyocytes nor the increased calcium influx stimulated by isoproterenol were affected by IL-2. So we suggest that relatively constant activity of I_{Ca} after IL-2 treatment contributes to the phenomena in which no changes of restitution was observed in IL-2-treated myocytes at 1.25 mM $[Ca^{2+}]_o$.

Effect of IL-2 on recirculation fraction of intracellular Ca^{2+}

In the classical concept, the recirculation fraction (RF) is the fraction of Ca^{2+} released from the SR during each Ca^{2+} transient that is resequestered back into the SR. It is usually estimated from the decay of force when contractility is transiently enhanced following either post-extrasystolic or post-rest potentiation {ter Keurs HEDJ. 1992 40 /id}{Ragnarsdottir, Wohlfart, et al. 1982 38 /id}. A linear relationship exists between the force of one potentiated contraction and the preceding contraction. The recirculation fraction is reflected by the slope of the linear relationship. In the present study, we used the post-rest potentiation to estimate the RF of rat myocytes. In agreement with other studies {Kotsanas, Delbridge, et al. 2000 7 /id}, we found that stimulation following a 30-sec rest period produced near maximal potentiation of the amplitude of the first post-rest calcium transient.

By analyzing the data from control myocytes, we found a strong linear relationship between the amplitudes of successive post-rest transients when the extracellular [Ca^{2+}] was 1.25 mM. The mean value of the RF was 0.94, which agrees well with the value of rat cardiomyocytes measured in other laboratories {Bassani, Bassani, et al. 1994 42 /id}{Ravens, Mahl, et al. 1996 41 /id}. The RF of IL-2-treated myocytes was not significantly from controls. When extracellular [Ca^{2+}] was elevated to 2.5 mM, the RF was also not significantly different between control and IL-2-treated groups (data not shown).

Two processes are important in removing Ca^{2+} from the cytosol on a beat-to-beat basis. In rat cardiac myocytes, the SR Ca^{2+} ATPase and the sarcolemmal Na^{+}/Ca^{2+} exchanger compete for intracellular Ca^{2+}, with the SR Ca^{2+}ATPase being the dominant pathway {Hove-Madsen & Bers 1993 31 /id}. The SR takes up over 90 percent of the intracellular Ca^{2+} which is thus retained within the cell, while the Na^{+}/Ca^{2+} exchanger extrudes the remaining Ca^{2+}. Changes in the relative activities of these pathways would be expected to alter the fraction of Ca^{2+} cycling between the cytosol and the SR. The fact I_{Ca} did not change in rat myocytes treated with 200-1000 U/ml IL-2 suggested that the capacity of the SR for Ca^{2+} uptake and the release may be reduced by IL-2 treatment. Furthermore, the RF may be also be reduced by IL-2. But there was little difference between the RFs in the two groups. So the most plausible explanation is that there must be a corresponding decrease in the amount of Ca^{2+} lost from the cell, which results in the unchanged RF. Two mechanisms may be involved. The first is the decreased activity of the Na^{+}/Ca^{2+} exchanger, which is the major route for Ca^{2+} efflux. The second is the elevated level of

intracellular [Na^+], which in turn reduces Ca^{2+} extrusion through Na^+/Ca^{2+} exchange. The prolonged decay of caffeine induced calcium transient in IL-2 treated myocytes indicated that IL-2 could decrease the activity of the Na^+/Ca^{2+} exchanger. So further study is needed to determine the activity of the Na^+/Ca^{2+} exchanger and the intracellular Na^+ concentration to uncover the mechanisms by which IL-2 affects cardiac calcium homeostasis.

In mass Ca^{2+} dynamics, the RF is exclusively related to the SR Ca^{2+} pump, and the 1-RF is related predominantly to sarcolemmal Na^+/Ca^{2+} exchange coupled with the Na^+-K^+ pump {Shimizu, Araki, et al. 1998 218 /id}{Tada & Katz 1982 219 /id}. As mentioned above, alterations of SR Ca^{2+} release rate and intracellular sodium levels may induce changes in RF, which were not taken into account in the classical RF concept. Cytosol calcium removal is closely related to the SR Ca^{2+} uptake via the SR Ca^{2+} pump and the Ca^{2+} efflux via Na^+/Ca^{2+} exchange coupled with the Na^+-K^+ pump as well as via the sarcolemmal Ca^{2+} pump, which can also affect the value of the RF. Therefore, the original RF concept needs to be modified when considering the calcium dynamics in cardiomyocytes.

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