

Chapter 3: Role and Mechanism of Subcellular Ca²⁺ Distribution in the Action of Two Inotropic Agents with Different Toxicity

M Alemanni, M Rocchetti, D Re, A Zaza

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Abstract

Istaroxime is a novel inotropic agent that inhibits Na⁺/K⁺ ATPase and stimulates sarcoplasmic reticulum (SR^{*}) Ca²⁺ ATPase (SERCA) 2, with a lower pro-arrhythmic effect than digoxin. The differences in the direct and indirect modulation of ryanodine receptor (RyR) activity by the two drugs were investigated. The increase of twitch amplitude of ventricular myocytes was analysed to evaluate the inotropic effect and the toxicity of the two drugs in mouse. Accumulation of cytosolic Ca²⁺, SR Ca²⁺ leak and the rate of SR reloading during Na⁺/Ca²⁺ exchanger blockade after caffeine-induced depletion were evaluated through the measurement of cytosolic Ca²⁺ and membrane current. Both compounds increased cell twitch in a concentration-dependent fashion. The exposure of cells to the equi-

* Abbreviations used in the chapter: AC - aftercontraction; a.u. - arbitrary units; [Ca²⁺]_{cyt} - cytosolic Ca²⁺ concentration; CICR - Ca²⁺-induced Ca²⁺ release; C_m - membrane electrical capacitance; NCX - Na⁺/Ca²⁺ exchanger; RyR - ryanodine receptor; SERCA - sarco(endo)plasmic reticulum Ca²⁺ ATPase; SR - sarcoplasmic reticulum; τ_{decay} - time constant of decay

inotropic concentrations of the drugs associated to the highest degree of inotropism achieved showed a marked increase of aftercontraction incidence with digoxin but not with istaroxime. Digoxin induced a rapid accumulation of resting Ca^{2+} while istaroxime did not alter resting Ca^{2+} levels. However, both drugs left the SR Ca^{2+} leakage unaffected. Moreover, istaroxime enhanced the SR Ca^{2+} reuptake rate, improved Ca^{2+} -induced Ca^{2+} release gain and accelerated the decay kinetics of Ca^{2+} transients. The different toxicity observed between the two drugs seems to be related to an accumulation of cytosolic Ca^{2+} induced by digoxin which does not derive from a direct modulation of RyR activity but which may in turn affect it. SERCA modulation observed with istaroxime might contribute to a better Ca^{2+} compartmentalization and thus reduce the associated toxicity.

Introduction

Despite well established efficacy of anti-remodelling agents in the management of heart failure, inotropic support may still be required both in the acute and chronic stages of the disease. Such a need is unmet by the drugs currently available, which fail to improve the long term clinical outcome because of an increased risk of life threatening arrhythmias^{1,2}. This prompts the search for positive inotropic agents with a more favourable balance between inotropic and pro-arrhythmic effects (safety profile). Istaroxime is a novel inotropic agent that, according to evidence from in vitro and in vivo studies, has a substantially better safety profile than digoxin³⁻⁵. As digoxin, istaroxime inhibits the Na^+/K^+ pump, which may contribute to its inotropic effect, but, unlike digoxin, it also stimulates Ca^{2+} uptake by

the sarcoplasmic reticulum (SR), an effect fully preserved in the failing heart⁶ and potentially accounting for the favourable safety profile. Although previous studies showed that istaroxime stimulates the Sarco/Endoplasmic Reticulum Ca²⁺ ATPase (SERCA) 2 activity^{5;6}, they did not rule out the possibility that direct inhibition of ryanodine receptor (RyR) opening may contribute to its peculiar effect on SR function. On the other hand, RyR facilitation might have a role in the large pro-arrhythmic effect of digoxin. Characterization of the mechanism underlying the different toxicity of istaroxime and digoxin is of general interest as it can provide clues to improve inotropic interventions and identify strategies for antiarrhythmic therapy under conditions of dysfunctional Ca²⁺ handling, such as heart failure.

The present study aims to assess the role of direct RyR channel modulation in determining the difference in toxicity between istaroxime and digoxin. At variance with previous ones, the present study has been carried out on murine myocardium, in which the role of SR in Ca²⁺ handling is prominent⁷. To this end, key evidence on mechanisms of drug action coming from previous studies in guinea-pigs has been reproduced in murine myocytes, thus allowing to test the drug for its species-specificity. The major finding of this work is that the two drugs does not modulate RyR activity directly, but they differently modulate cytosolic Ca²⁺ ([Ca²⁺]_{cyt}), which in turn affects RyR activity.

Materials

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health

(NIH Publication No. 85-23, revised 1996) and to the guidelines for animal care endorsed by the hosting institution.

Myocytes isolation and recording solutions

Adult mouse ventricular myocytes were isolated as previously described⁸ with minor modifications. Briefly, adult female C57BL/6J (Harlan Italy, S. Pietro al Natisone, Italy) mice were anaesthetized with ketamine/heparine (125 mg kg⁻¹/12500 U.I. kg⁻¹ I.M.). Hearts were quickly excised, mounted on Lagendorff apparatus and perfused at 37°C with a Ca²⁺-free solution until the perfusate was blood free. After perfusion with a solution containing collagenase type II (248 U/ml, Worthington, Lakewood, NJ) for 3.5 min, ventricles were minced and gently stirred in the same collagenase-containing solution for 5 min more. Cells were then filtered and centrifuged for 2 min at 10 g. The cell pellet was suspended in a 0.5 mM Ca²⁺ solution and then centrifuged again at the same speed. Finally, the cell pellet was resuspended in a solution containing 1 mM Ca²⁺ and stored at 4°C. All cells were used within 8 hrs from isolation.

Myocytes suspension was placed in a 30 mm Petri dish on the stage of an inverted microscope (Nikon Eclipse TE200, Tokio, Japan). A thermostated manifold, allowing for fast (electronically timed) solution switch, was used for cell superfusion. During measurements, myocytes were superfused at 2 ml/min with solutions specific for each experimental protocol, as detailed in Table 1. Measurements were performed at 35.0 ± 0.5°C with the exception of the estimation of SR Ca²⁺ leak, which was conducted at room temperature. Temperature

Table 1 *List of extracellular and internal solutions employed.*

	Sol 1	Sol 2	Sol 3	Sol 4	Intracellular solution
NaCl	140	140	-	-	-
LiCl	-	-	140	140	-
CaCl ₂	2	-	-	1	0.2
EGTA	-	-	10	-	0.5
KCl	4	4	4	4	23
MgCl ₂	1	1	1	1	3
HEPES	5	5	5	5	5
Glucose	10	10	10	10	-
Aspartate-K ⁺					110
GTP-Na ⁺	-	-	-	-	0.4
ATP-Na ⁺	-	-	-	-	5
Creatine phosphate-Na ⁺	-	-	-	-	5
pH	7.4	7.4	7.4	7.4	7.3

Concentrations are expressed in mM. Extracellular solutions employed during electrophysiological recordings had 2 mM 4-aminopyridine and 0.1 mM BaCl₂ added to block I_{to} and other K⁺ currents. For the SR reloading protocol the internal solution was Na⁺-free (all Na⁺ salts replaced with either K⁺ or Tris salts).

was monitored at the pipette tip with a fast-response digital thermometer (Physitemp Instruments, Clifton, NJ).

Electrophysiological techniques

The composition of experimental solutions, identified in the following text by number (e.g. solution 1) is detailed in Table 1. Ventricular myocytes were voltage-clamped in the whole-cell configuration, membrane currents were recorded by means of an Axopatch 200-A amplifier (Molecular Devices, Sunnyvale, CA). The average value of series resistance was $6.8 \pm 0.4 \text{ M}\Omega$ ($n = 25$ cells). Current signals were filtered at 2 kHz and digitized at 5 kHz (Axon Digidata 1200, Molecular Devices). Trace acquisition and analysis were controlled by a dedicated software (pClamp 8.0; Molecular Devices).

Measurement of intracellular Ca^{2+} and mechanical activity

Myocytes were incubated in solution 1 (Table 1) for 45 min with the membrane-permeable dye Fluo4-AM (10 μM) and then washed for 30 min to allow deesterification. Fluo4 emission was collected through a 535 nm band pass filter, converted to voltage, low-pass filtered (200 Hz) and digitized at 2 kHz. $[\text{Ca}^{2+}]_{\text{cyt}}$ was calculated from the signal after further low-pass digital filtering (FFT, 100 Hz) and subtraction of background luminescence and expressed in fluorescence arbitrary units (a.u.). Fluo4 was selected because of the high signal/noise ratio required for Ca^{2+} leak measurements. Fluo4 non-ratiometric signal does not allow reliable quantification of absolute Ca^{2+} levels;

accordingly, the experiments are entirely based on the detection of changes in Ca^{2+} levels relative to the control condition.

Mechanical activity was measured in intact myocytes as unloaded cell-shortening (twitch) triggered by field electrical stimulation at 2 Hz. Cell motion was monitored by a video edge detector (Crescent electronics, Sandy, UT) connected to a fast (non-interlaced) camera with a sampling rate of 200 frames/sec. The amplitude of unloaded cell shortening provides an estimate of myocardial contractility which, albeit rough, is adequate to quantify drug effects for the purpose of the present study.

Experimental protocols

Drug-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ accumulation

Increased $[\text{Ca}^{2+}]_{\text{cyt}}$ levels may contribute to enhance RyR opening, thereby facilitating spontaneous Ca^{2+} release and the resulting arrhythmias. This protocol aimed to test whether, at concentrations producing similar increases in cell contractility, but with markedly different proarrhythmic effect, istaroxime and digoxin differ in their tendency to increase $[\text{Ca}^{2+}]_{\text{cyt}}$ at diastolic potential. Experiments were performed in Fluo4 loaded myocytes under patch-clamp, to allow simultaneous measurement of $[\text{Ca}^{2+}]_{\text{i}}$ and membrane current. Because modulation of all membrane fluxes contributing to $[\text{Ca}^{2+}]_{\text{cyt}}$ levels was relevant in this setting, experiments were performed under physiological ionic conditions (solution 1; Table 1) and minimal intracellular Ca^{2+} buffering. Myocytes were loaded by a train of five depolarizing pulses (-40 to 0 mV, 400 ms, 0.5 Hz) and then clamped at an holding potential of -80 mV. After Fluo4 signal stabilization,

either istaroxime (20 μM) or digoxin (150 μM) were applied and $[\text{Ca}^{2+}]_{\text{cyt}}$ and membrane current were monitored for at least 2 min before delivering a 10 mM caffeine pulse to estimate total SR Ca^{2+} content. Drug-induced changes in fluorescence were expressed as percent of control values to correct for differences in probe loading. The amount of Ca^{2+} released by the SR (i.e. total Ca^{2+} content in moles) was estimated from the integral of the inward current elicited by the caffeine pulse, which reflects the charge transported by NCX, by assuming a 3:1 $\text{Na}^+/\text{Ca}^{2+}$ transport stoichiometry, and normalized to cytosolic volume (computed as cell capacitance (C_m)/9.94)⁹.

SR leak-load relationship

The probability of spontaneous (untriggered) RyR opening can be estimated from the abolition of Ca^{2+} outflow from the SR (leak) induced by the RyR blocker tetracaine^{10;11}. The open probability of RyR depends on both cytosolic and SR luminal Ca^{2+} levels^{12;13}; therefore, to assess a direct modulation of intrinsic RyR properties by either drugs, Ca^{2+} levels in both these compartments must be known (measured or controlled). Accordingly, Ca^{2+} leak and SR Ca^{2+} content (load) were measured in every cell under each experimental condition to obtain leak-load relationships; RyR intrinsic properties are defined by the shape and steepness of such relationships. To prevent Ca^{2+} fluxes from compartments other than the SR, measurements were performed under Ca^{2+} - and Na^+ -free conditions (solution 3; Table 1) which completely inhibit ion movements through sarcolemmal channels and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). Such conditions also

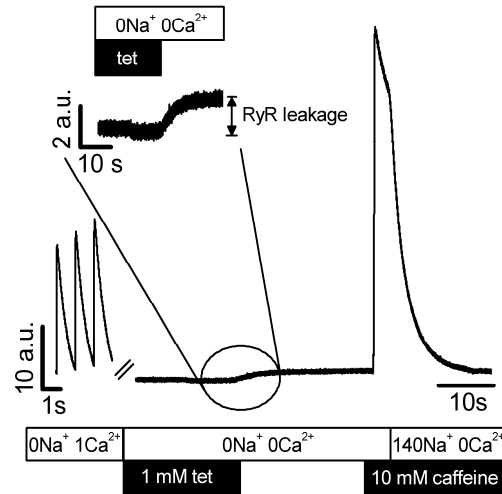


Figure 1 *Representative fluorescence recording depicting the protocol employed to measure SR Ca²⁺ leak.* The SR Ca²⁺ load was graded by stimulating the cell in a solution devoid of Na⁺ (solution 4, Table 1) and stopping the stimulation at different time points. In this condition the Ca²⁺ transients did not reach steady state. Once stimulation was stopped, a switch to a solution Na⁺- and Ca²⁺-free (solution 3, Table1) plus 1 mM tetracaine was performed. Tetracaine was washed out after the signal stabilized for 30 seconds; SR Ca²⁺ load was quantified by a pulse of 10 mM caffeine. In the inset the increase of fluorescence following tetracaine wash-out, which is a direct measurement of leakage, is magnified five times.

eliminate $[Ca^{2+}]_{cyt}$ changes indirectly resulting from drug-induced Na^+/K^+ pump inhibition, a crucial requirement in the present study. Tetracaine-sensitive SR Ca^{2+} leak was quantified in Fluo4-loaded intact myocytes with a protocol devised by Curran et al¹⁰. As illustrated in Fig. 1, SR was initially depleted from Ca^{2+} by a 10 mM caffeine pulse (in 140 mM Na^+ , 0 mM Ca^{2+}) and subsequently loaded by trains of electrical pulses (1 Hz) in the presence of 1 mM Ca^{2+} . Under NCX inhibition, Ca^{2+} progressively accumulates during the electrical stimulation¹⁴; thus, SR Ca^{2+} content could be modulated over a wide range, as required by the construction of leak-load relationships, by applying stimulation trains of different duration. Tetracaine (1 mM) was applied immediately after stopping stimulation and $[Ca^{2+}]_{cyt}$ was allowed to achieve a steady level. Tetracaine was then discontinued and SR Ca^{2+} leak was measured as the resulting increase in fluorescence (at steady-state). Finally, SR Ca^{2+} load was estimated within the same myocyte from the amplitude of the Ca^{2+} transient induced by a further caffeine pulse. To estimate Ca^{2+} -independent quench of probe fluorescence by tetracaine, the latter was applied in Na^+ - and Ca^{2+} -free conditions (solution 3; Table 1) in the presence of 30 μ M ryanodine and 50 μ M cyclopiazonic acid to block all Ca^{2+} movements across membranes. Under such conditions tetracaine inhibited Fluo4 signal by 7.2% (n = 12 cells); this value was used to correct all SR leak measurements.

SR Ca²⁺ uptake function

Previous functional studies in guinea-pig myocytes showed that 4 μ M istaroxime, but not digoxin, enhanced Ca²⁺ uptake by the SR⁵. On the other hand, in biochemical assays enhancement of SERCA2 activity was maximal at intermediate istaroxime concentrations, with inhibition at very high ones⁵. Therefore, interpretation of the present results required to verify istaroxime effect on SR Ca²⁺ uptake in murine myocytes and at the relevant concentrations. The protocol used to this end, identical to that previously applied to guinea-pig myocytes^{5,6}, involves the analysis of Ca²⁺ transients and membrane current during voltage-triggered (400 ms steps from -40 to 0 mV at 0.25 Hz applied in the presence of 1 mM Ca²⁺) loading of the SR after caffeine-induced depletion (an outline of the protocol is shown in Fig. 5A). To rule out the contribution of NCX, measurements were performed under Na⁺-free conditions (solution 4; table 1). The amplitude of the Ca²⁺ transients elicited by each voltage pulse is a function of SR Ca²⁺ content and can be normalized to I_{Ca} amplitude to estimate the "gain" of the release mechanism (CICR-Gain)⁹. The time constant of Ca²⁺ decay (τ_{decay}), estimated by monoexponential fitting of the Ca²⁺ signal in each step, reflects SR Ca²⁺ uptake rate. The analysis of these parameters for each step of the loading train provides information on the ability of the SR to uptake Ca²⁺ in the presence of a wide range of luminal Ca²⁺ concentrations, thus providing a very sensitive measure of SR function⁶.

Reagents

Stock Fluo4-AM solution (1 mM in dry dimethyl sulfoxide) was purchased from Molecular Probes (Carlsbad, CA) and it was diluted in NT; istaroxime (PST2744; for chemical structure see ref^{3;4}) was synthesized at Prassis Sigma-Tau (Settimo Milanese, Italy) and dissolved in water; ryanodine was purchased from Calbiochem (Darmstadt, Germany). All other reagents were from Sigma-Aldrich (St. Luis, MO).

Statistical analysis

Data are presented as mean \pm SE. Aftercontraction incidence was compared with χ^2 -test. Individual means were compared by employing paired and unpaired Student's *t*-test as appropriate. Group comparisons were tested with two-way ANOVA with Bonferroni correction. Statistical significance was defined as $p < 0.05$ (NS, non significant). The least square method was used for non linear fitting. Sample size (number of cells, obtained from at least three hearts) is specified for each experimental condition in the figure legends.

Results

Concentration dependency of inotropic and toxic effects

Inotropy was defined as the increase in twitch amplitude measured after the achievement of steady-state at each drug concentration. Toxicity was defined as the appearance of "aftercontractions" (AC, unstimulated twitches), corresponding to spontaneous Ca^{2+} release events. Whenever a drug induced AC (Fig. 2A, indicated by the

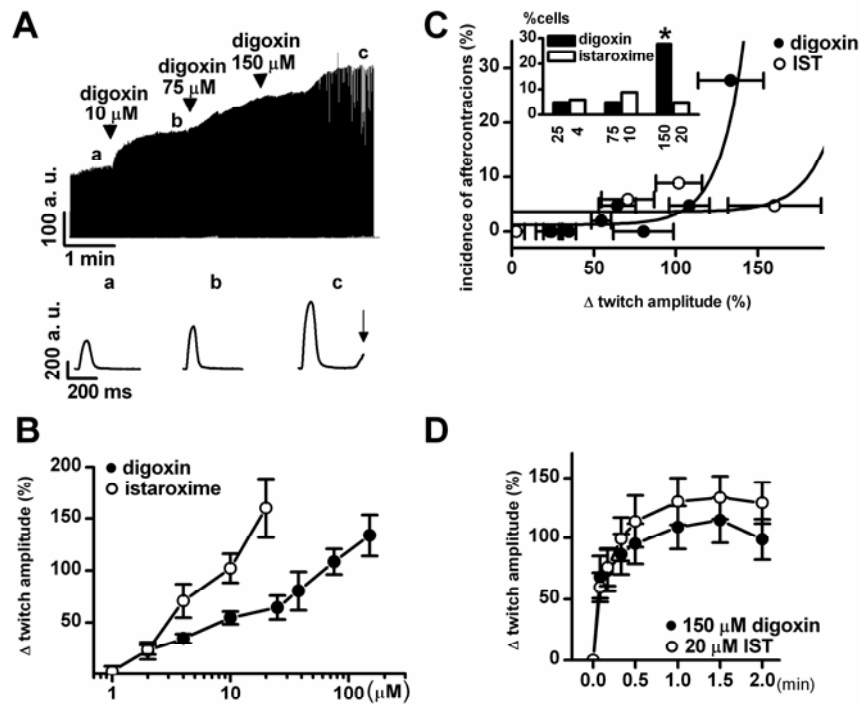


Figure 2 Comparison of the inotropy and the toxicity of istaroxime and digoxin.

A, example of AC induced by digoxin. Arrowheads indicate the time when increasing concentrations of the drug were added. Samples of the top trace at an expanded time scale are shown below (a-c). The arrow on trace c indicates AC. B, percentage increase in twitch amplitude from control (before drug application) value plotted versus istaroxime or digoxin concentration. A total of 127 cells from 14 hearts was used, with a minimum of 17 cells at each drug concentration. C, Percentage of cell developing AC as a function of the inotropic effect. Solid lines represent best fit of data points with an arbitrary function. The incidence of AC at different pairs of equi-inotropic drug concentrations is shown in the inset (* $p < 0.05$). D, rate of onset of the inotropic effect for equi-inotropic drug concentrations (20 μM istaroxime or 150 μM digoxin), shown as percentage increase in twitch amplitude plotted versus time ($n \geq 10$ cells per point).

arrow), inotropy was defined from the largest twitch amplitude achieved prior to their onset.

Istaroxime and digoxin increased twitch amplitude in a concentration-dependent fashion (Fig. 2B); potency was higher for istaroxime than for digoxin (EC_{50} 4 μ M vs 25 μ M, istaroxime vs digoxin). Whereas AC were seldom observed during istaroxime exposure at all concentrations, a sharp increase in their incidence was observed at the largest digoxin concentration tested (150 μ M). Figure 2C shows AC incidence plotted against the corresponding increase in twitch amplitude achieved at each drug concentration. The only pair of drug concentrations with similar inotropic effect but significantly different AC incidence was 20 μ M istaroxime (AC in 1 of 21 myocytes) and 150 μ M digoxin (AC in 5 of 18 myocytes; $p < 0.05$) (Fig. 2C, inset). Accordingly, this concentration pair was selected for the other experiments of the present work, aimed to clarify the mechanism of the differential toxicity. At these concentrations, the rate of onset of the inotropic effect was similar between istaroxime and digoxin and, in cells that did not develop AC, reached a plateau after approximately 1 minute of exposure (Fig. 2D). AC ensued after 69.5 ± 11.9 sec ($n = 5$) of exposure to 150 μ M digoxin.

Drug-induced Ca^{2+} accumulation

Prior to drug superfusion myocytes in istaroxime and digoxin groups had similar $[Ca^{2+}]_{cyt}$ (11.31 ± 2.81 a.u. vs 10.94 ± 1.13 a.u.; NS). Whereas challenge with 150 μ M digoxin was immediately followed

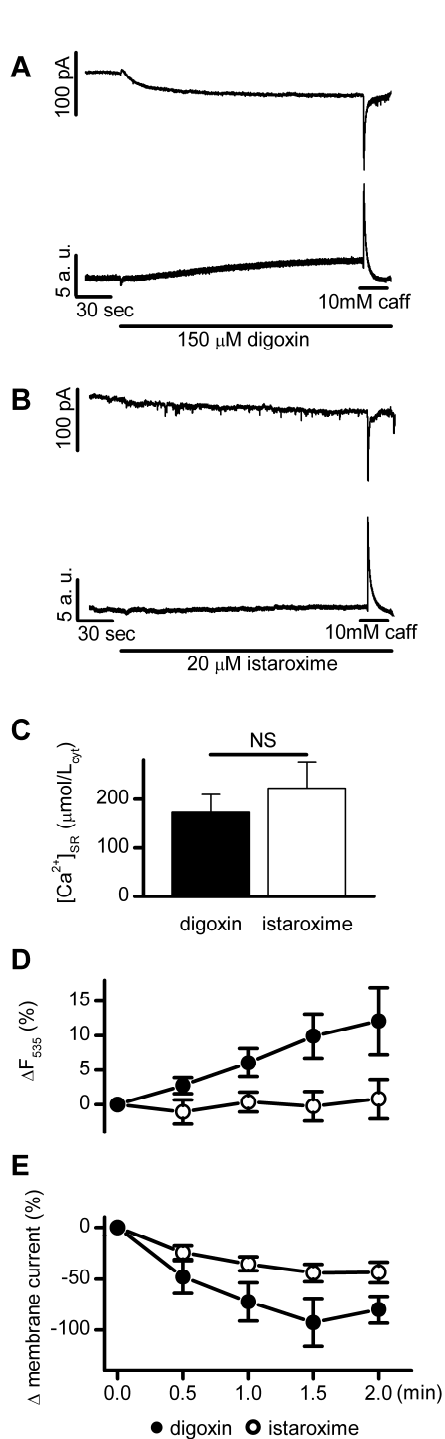


Figure 3 Modulation of $[Ca^{2+}]_i$ by istaroxime and digoxin. A and B, representative traces of membrane current (upper panels) and $[Ca^{2+}]_{cyt}$ (lower panels) changes after the exposition of either 150 μ M digoxin (A) or 20 μ M istaroxime (B). At the end of the recordings, 10 mM caffeine was applied to assess the SR Ca^{2+} load. C, SR Ca^{2+} load determined as the average amount of Ca^{2+} extruded through NCX (150 μ M digoxin: n = 5, 20 μ M istaroxime: n = 6 cells). D, percentage change of $[Ca^{2+}]_{cyt}$ as a function of time during the exposure to either compound. E, percentage change of membrane current as a function of time during the exposure to either compound (n \geq 5 cells per point).

by a progressive increase in $[Ca^{2+}]_{cyt}$ (example in Fig. 3A), the latter remained unchanged during over 2 minutes of exposure to 20 μ M istaroxime (example in Fig. 3B). Analysis of average $[Ca^{2+}]_{cyt}$ at 0.5 minute intervals (Fig. 3D) shows that the increment was significantly steeper for digoxin ($p < 0.05$ at ANOVA). Both agents induced an inward shift of membrane current (examples in Fig 3A and B), which was more pronounced for digoxin (Fig 4E ; ANOVA, $p < 0.05$). In spite of the larger accumulation of $[Ca^{2+}]_{cyt}$ observed with digoxin, SR Ca^{2+} content measured at the end of drug exposure was similar between the two agents (Fig 3C). This suggests that the distribution of Ca^{2+} between subcellular compartments, rather than overall myocyte Ca^{2+} content, may be differently modulated by istaroxime and digoxin. The following set of experiments addresses the question of whether the different subcellular Ca^{2+} distribution may be explained by direct modulation of RyR properties.

Effects on the SR leak-load relationship

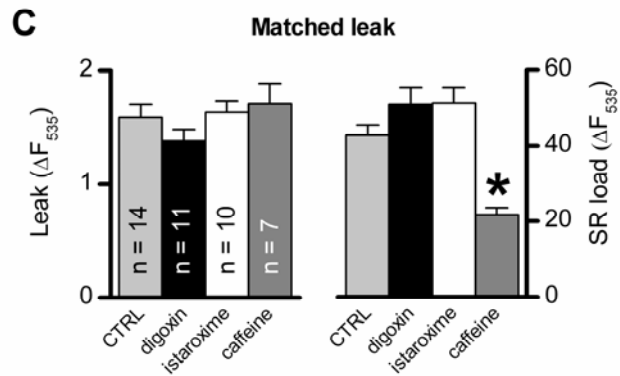
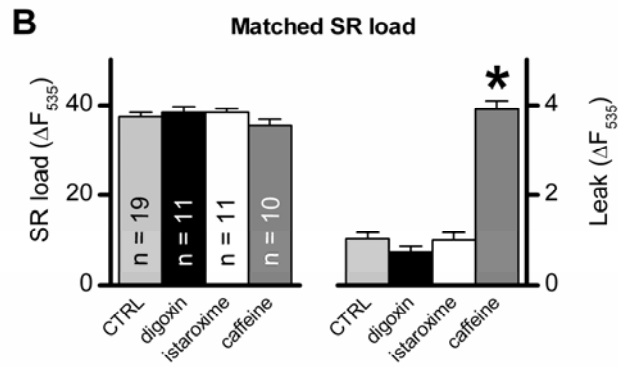
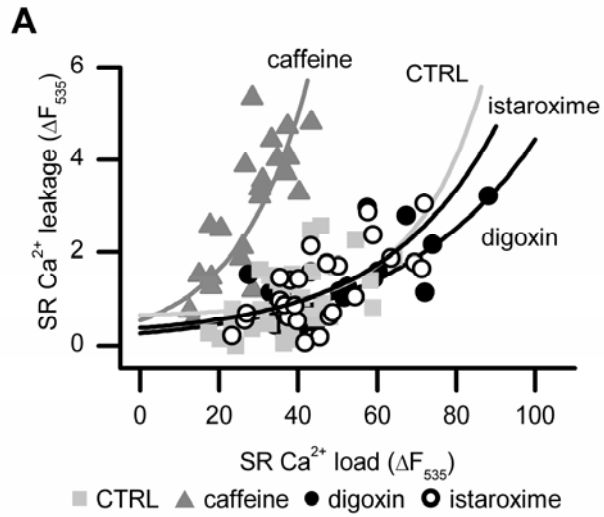
This set of experiments compares the effects of 20 μ M istaroxime and 150 μ M digoxin on the SR leak-load relationship. The effect of a caffeine concentration (0.5 mM) known to induce a moderate increase in RyR open probability¹⁵ was also evaluated as a positive control. Figure 4A shows that whereas the steepness of the leak-load relationship was obviously increased by 0.5 mM caffeine, neither istaroxime nor digoxin had measurable effects. In order to submit the observation to statistical analysis, leak was compared between myocytes in each treatment group having comparable SR Ca^{2+} load, as

shown by the left panel in Figure 4B. The corresponding leak values, shown on the right panel of the same figure, exceeded control ones only for caffeine-treated myocytes ($p < 0.05$ for caffeine vs control), but were not altered by either istaroxime or digoxin. Complementary analysis, shown in figure 4C, was performed by comparing SR Ca^{2+} loads between myocytes in each treatment group displaying similar leak, as shown in the left panel of Figure 4C. Mean SR Ca^{2+} load was significantly lowered only in the caffeine group ($p < 0.05$ for caffeine vs control), thus confirming the previous results. These observations argue against a direct modulation of RyR function by either 20 μM istaroxime or 150 μM digoxin, thus suggesting istaroxime-induced stimulation of active SR Ca^{2+} uptake as the mechanism of the different subcellular Ca^{2+} distribution induced by the two agents. The following set of experiments aimed to test whether such istaroxime action, previously observed in guinea-pig myocytes, occurs also in murine myocardium at the concentration relevant to the present study.

Modulation of SR Ca^{2+} uptake function

The upper traces in the example of figure 5B show that following SR Ca^{2+} depletion (by 10 mM caffeine), Ca^{2+} transient amplitude and relaxation rate progressively increased at each step of the reloading train. Such an increment, relatively shallow in control, was markedly enhanced by 20 μM istaroxime. Lower traces in the same example show that inward current, mostly representing I_{CaL} under these conditions, progressively decreased at each step, a change enhanced by istaroxime. Statistics of Ca^{2+} transient amplitude, gain of the

Figure 4 Comparison of the effects of istaroxime and digoxin on the SR Ca^{2+} leakage. A, leak-load relationships of cells field-stimulated at 1 Hz in Na^+ -free solution (solution 4, Table 1) (CTRL: n = 33, 500 μ M caffeine: n = 23, 150 μ M digoxin: n = 22, 20 μ M istaroxime: n = 26 cells). Solid lines represent best fit of data points with a monoexponential function. B, statistical analysis of the data in A. When cells with comparable SR load were pooled for each group, an higher mean leak was associated only to caffeine. C, converse analysis of the one in B. When cells with comparable leak were pooled for each group, a lower mean SR load was associated only to caffeine. Sample size showed in each histogram; * $p < 0.05$ vs CTRL.



release mechanism (Ca^{2+} -induced Ca^{2+} release [CICR] gain), and time constant of Ca^{2+} decay (τ_{decay}) along the loading protocol are shown in figure 5C-E. In the presence of istaroxime the increments of Ca^{2+} transient amplitude (Fig. 5C), and CICR gain (Fig. 5D) were significantly steeper ($p < 0.05$ at ANOVA). τ_{decay} decreased along the loading train (Fig. 5E), conceivably reflecting allosteric enhancement of SERCA2 function as the magnitude of Ca^{2+} transient increased¹⁶; istaroxime markedly reduced τ_{decay} throughout the loading train ($p < 0.05$ at ANOVA). To test whether istaroxime modulation of τ_{decay} was secondary to increased $[\text{Ca}^{2+}]_{\text{cyt}}$, we compared its effect between transients displaying high and low peak Ca^{2+} values. The results of this analysis (Fig. 5F) show that istaroxime reduced τ_{decay} independently of the level of Ca^{2+} at which relaxation started.

Discussion

The main findings of this study can be summarized as follows: 1) in murine myocytes concentration-dependency of inotropic vs toxic effects differs between istaroxime and digoxin, as previously described in guinea-pig myocytes; 2) the different safety profile between istaroxime and digoxin is associated with a stronger tendency of the latter to induce $[\text{Ca}^{2+}]_{\text{cyt}}$ accumulation; 3) the difference in $[\text{Ca}^{2+}]_{\text{cyt}}$ accumulation may reflect different Ca^{2+} distribution between SR and cytosolic compartments; 4) drug-induced changes in such distribution cannot be attributed to direct modulation of RyR function, which was not affected by either istaroxime or digoxin; 5)

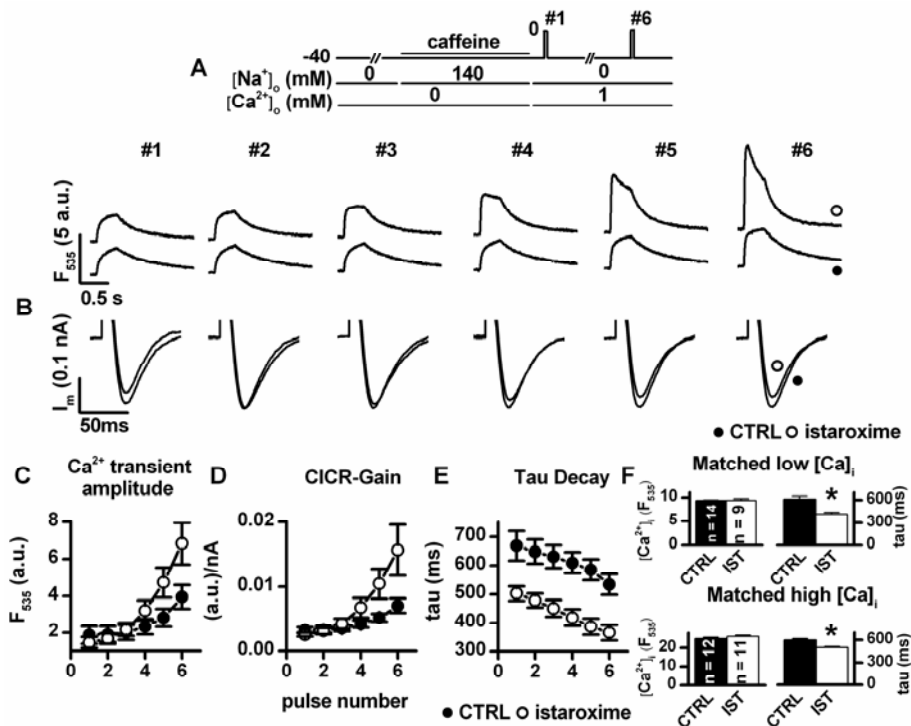


Figure 5 *Modulation of SERCA activity by istaroxime.* A, outline of the experimental protocol (see *Materials*). B, representative traces of [Ca²⁺]_{cyt} (upper panels) and membrane current (lower panels) recorded during SR reloading after caffeine-induced SR depletion in CTRL and during exposure to 20 μM istaroxime; the recordings were performed in the absence of NCX function (Na⁺-free extracellular and internal solutions, see Table 1). C-E, average values of Ca²⁺ transient parameters measured during each pulse (1-6) of the stimulation train in CTRL and with 20 μM istaroxime. n ≥ 8 cells per point. F, Ca²⁺ transients from the two conditions pooled in order to have a comparable [Ca²⁺]_{cyt} at the beginning of the decay. The average time constants were reduced in the presence of 20 μM istaroxime with both a low and a high [Ca²⁺]_{cyt} (sample size showed in each histogram; * p < 0.05).

enhancement of SR Ca^{2+} uptake function by istaroxime, previously observed in guinea-pig myocardium, is present also in murine one.

Toxicity vs inotropy

As in guinea-pig myocytes³, the inotropic effect that could be achieved without inducing aftercontractions was larger with istaroxime as compared to digoxin. However, concentration-dependency of effects showed some interesting differences between the two species. Whereas istaroxime inotropic potency was similar to that observed in guinea-pig myocytes, digoxin one was considerably lower³, thus resulting in a reversed potency ratio (istaroxime > digoxin). Reduced Na^+/K^+ pump sensitivity to inhibition by cardiac steroids has been reported in rodents¹⁷, therefore lower digoxin potency was expected. Why istaroxime preserved its potency instead is less obvious and might suggest that the two agents bind to distinct sites on the Na^+/K^+ pump. An alternative explanation is provided by the notion that SR Ca^{2+} transport, enhanced by istaroxime only, plays a larger role in rodent's excitation-contraction coupling¹⁸. A more robust SR function in rodents might also explain why AC were associated only with major digoxin inotropy in mouse myocytes (Fig. 2C), whereas they appeared even at threshold digoxin concentrations in guinea-pig ones³. Inotropic effects of both istaroxime and digoxin (at equi-inotropic concentrations) achieved a steady-state in roughly 1 minute (Fig. 2D), thus suggesting that the difference in toxicity between the two agents was not a matter of time.

Mechanism of the difference in toxicity

The appearance of AC reflects "instability" of the SR Ca^{2+} store, resulting in spontaneous autoregenerative Ca^{2+} release (Ca^{2+} waves). The factors governing store stability include Ca^{2+} activities in the cytosol and SR lumen, and intrinsic RyR properties^{12;13;19}. At concentrations leading to similar inotropy but different toxicity (i.e. AC incidence), $[\text{Ca}^{2+}]_{\text{cyt}}$ accumulation at diastolic potentials was remarkably faster with digoxin than with istaroxime. This suggests that the same level of inotropy may be associated with higher $[\text{Ca}^{2+}]_{\text{cyt}}$ in the case of digoxin; a finding that may justify facilitation of AC. Indeed, elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ increases RyR open probability and may be crucial to the genesis of spontaneous autoregenerative Ca^{2+} release, a view supported by the finding that the incidence of AC correlates with $[\text{Ca}^{2+}]_{\text{cyt}}$ levels, rather than with SR Ca^{2+} content^{20;21}.

Once established that digoxin led to larger $[\text{Ca}^{2+}]_{\text{cyt}}$ accumulation as compared to istaroxime, the mechanism of such a difference needs to be discussed. Firstly, in spite of the similar inotropic effect, digoxin might still block Na^+/K^+ pump more than istaroxime. However, if associated with unchanged SR function, stronger Na^+/K^+ pump inhibition would be expected to impact on both $[\text{Ca}^{2+}]_{\text{cyt}}$ and SR Ca^{2+} contents. As SR Ca^{2+} content was similar after exposure to either istaroxime or digoxin (Fig. 3C), stronger Na^+/K^+ pump inhibition is an unlikely cause of differential modulation of $[\text{Ca}^{2+}]_{\text{cyt}}$. A better explanation may be provided by a difference between istaroxime and digoxin in the modulation of Ca^{2+} distribution between the cytosolic and SR compartments. Experiments in guinea-pig myocytes showed that istaroxime may enhance SR Ca^{2+} uptake, an effect confirmed in

the setting of the present study and consistent with istaroxime-induced stimulation of SERCA2 activity^{5,6} and Fig. 5. However, direct modulation of RyR function might contribute to change SR Ca²⁺ uptake and, at the same time, directly affect the probability of AC occurrence^{15;22}. This hypothesis was ruled out in the present study by the complete lack of effect of both istaroxime and digoxin on the SR leak-load relationship. Nevertheless, it has been recently reported that digitoxin, a compound structurally similar to digoxin, may form Ca²⁺-permeant channels into biological membranes²³. Since measurement of tetracaine-sensitive Ca²⁺ leak evaluates RyR-dependent Ca²⁺ efflux only, we cannot rule out that an increase in RyR-independent Ca²⁺ permeability might contribute to subcellular Ca²⁺ redistribution in the presence of digoxin.

Difference with previous studies

The present finding that even high digoxin concentrations did not affect the SR leak-load relationship is apparently in contrast with the report of digoxin-induced increase in the open probability of purified RyR proteins incorporated in lipid bilayers²⁴ and with our own observation that digoxin enhanced Ca²⁺ leak in purified cardiac microsomes⁵. However, whether cardiac steroids can directly affect RyR activity in intact myocytes is controversial. Nishio *et al.* reported that ouabain exerted inotropic effects also in Na⁺-free conditions, i.e. independently of Na⁺/K⁺ pump inhibition²⁵, thus suggesting direct RyR sensitization by the drug. However, Altamirano *et al.* found that the inotropic effects of several cardiotonic steroids, including digoxin, was completely prevented by removal of extracellular Na⁺ (ref. ²⁶).

Ouabain has been reported to increase basal cytosolic Ca^{2+} while decreasing Ca^{2+} spark frequency, possibly as a consequence of leak-induced SR depletion²⁷. While this suggests enhancement of RyR open probability by ouabain, it is important to stress that both sparks and non-spark Ca^{2+} release contribute to SR Ca^{2+} leak and may affect subcellular Ca^{2+} distribution¹¹. Therefore, the latter observation is not necessarily in contrast with the present findings.

Conclusions and practical implications

From the present finding we conclude that the lower pro-arrhythmic risk of istaroxime as compared to digoxin, may result from enhanced confinement of intracellular Ca^{2+} to the SR compartment. The latter is likely to reflect istaroxime-induced SERCA2 stimulation, rather than direct modulation (positive or negative) of RyR properties by either drug.

Irrespective of the underlying mechanism, the present findings support the view that enhanced compartmentalization of Ca^{2+} within the SR may reduce the pro-arrhythmic risk associated with increments of total cellular Ca^{2+} . This view is apparently challenged by the notion that RyR opening is facilitated by high luminal Ca^{2+} concentrations and by the observation that interventions increasing SR Ca^{2+} content (enhanced cAMP/PKA signalling, SERCA2 overexpression etc.) may increase the likelihood of spontaneous Ca^{2+} release and related arrhythmias^{22,28}. The two views may probably be reconciled by considering that Ca^{2+} store stability may ultimately depend on the extent of SR Ca^{2+} accumulation and on the concomitant changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ levels. This suggests that enhancement of SR Ca^{2+} uptake

may increase the safety of inotropic interventions only if its extent can be finely regulated.

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