Chapter 4: Conclusions

The findings reported in Chapters 2 and 3 are the following: 1) the banding of the ascending aorta in guinea pig led to the development of heart hypertrophy and mild failure; 2) in this model the sarcoplasmic reticulum (SR*) Ca^{2+} content was modestly reduced (about -32% from the control value), however, when the SR load was very low, a depressed SR function was observed; 3) istaroxime (IST), an inhibitor of the Na^+/K^+ ATPase (NKA) and a positive modulator of the Sarco(Endo)plasmic Ca^{2+} ATPase (SERCA), was able to restore almost completely the SR Ca^{2+} content and the SR functionality, with a bigger effect when the SR load depression was more pronounced; 4) in SR microsomes derived from skeletal muscle IST failed to enhance Ca^{2+} uptake, at variance with what previously observed in cardiac vesicles[1]; 5) in healthy mouse ventricular myocytes IST retained its specific activity on SERCA as well as the lower pro-arrhythmogenic effect when compared to digoxin, like what previously observed in guinea pig ventricular myocytes[1;2]; 6) exposure of mouse cardiomyocytes to digoxin induced the accumulation of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$), which was absent during IST exposure; 7) both drugs failed to affect directly the ryanodine receptor (RyR)-mediated SR Ca^{2+} leakage.

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* Abbreviations used in the chapter: AoB - aortic banded; $[Ca^{2+}]_{cyt}$ - cytosolic Ca^{2+} concentration; EC-coupling - excitation contraction coupling; IST - istaroxime; NCX - Na^{2+}/Ca^{2+} exchanger; NKA - Na^+/K^+ ATPase; PLB - phospholamban; RyR - ryanodine receptor; SERCA - Sarco(Endo)plasmic Ca^{2+} ATPase; SR - sarcoplasmic reticulum

Effects of Istaroxime on a Guinea Pig Heart Failure Model

Characterization of a Model of Mild failure

12 weeks of pressure overload following aortic banding (AoB) induced in adult female guinea pigs a clear hypertrophic phenotype, as revealed by echocardiographic characterization performed by Micheletti et al[3] and by the increase in heart weight and cell capacitance (Chapter 2, table 2). The analysis of Ca^{2+} homeostasis in ventricular cardiomyocytes derived from the hypertrophied hearts confirmed a condition of mild failure: the SR Ca^{2+} content had a relatively small reduction that did not affect Ca^{2+} transient profile nor excitation-contraction (EC)-coupling gain. Additionally, Na^{+}/Ca^{2+} exchanger (NCX) activity was not changed (Chapter 2, figure 4), although the expression levels of the protein were not examined, and a previous characterization of this model showed unaltered SERCA expression (but increased phospholamban [PLB] expression in the monomeric form)[3]. Only under extreme experimental conditions, far from the normal physiological setting, with an empty SR and with NCX activity completely abolished, functional derangements of EC-coupling were detectable, in particular the reduced SERCA activity that should derive from the increased presence of monomeric PLB (Chapter 2, figure 2 and supplemental figure 2).

Overall, this model of heart failure seems to represent the early stages of the pathology, where the hypertrophy manages in compensating the demand for improved contraction generated by the increased afterload (the index event). The absence of mortality and the presence of lung congestion only in a minority of animals in

the aortic banded group suggests that the animals considered are in the asymptomatic phase of the pathology or in the first phases of overt heart failure. The characterization of animal models of heart failure at the beginning of the syndrome is particularly useful because of the lack of availability of human tissues at this stages (human failing hearts come from heart transplantations). For this reason, the analysis of the effects of IST on this model allows to test its effectiveness when abnormalities in Ca^{2+} handling are moderate, and the drug might act in a different way, or even be uneffective, with respect to the observations made in more severe models of heart failure[4-6].

Effects of Istaroxime

IST positive luso-inotropic effect in guinea pig ventricular myocytes was readily observable in control animals (sham group), like previously reported in cells from normal hearts[1]. This effect was more pronounced in hypertrophic cells. The effectiveness of IST application on cells from both AoB and sham groups was inversely correlated with the starting SR Ca^{2+} load, i.e. in cells with lower SR content the percentage increase during IST exposure was higher (Chapter 2, figure 5). This effect is not surprising: as the SR Ca^{2+} content increases, the pump approaches its thermodynamic balance, and a further increase becomes more difficult to achieve[7]. Consequently, when applied in cells with a lower SR content (like the cells from the AoB group), the drug can more easily improve SERCA activity and SR refilling. The functional derangements of the SR Ca^{2+} uptake present in the AoB group were restored to control levels by the application of istaroxime (Chapter 2, figure 6).

Additionally, IST affected NCX activity, as expected by the inhibition of NKA and the consequent increase of intracellular Na^+ as well as by the improved SERCA function induced by the drug[1;2;8].

Taken together, these data suggests that IST is capable to completely rescue the functional abnormalities in Ca^{2+} handling associated to this model of mild failure. SERCA stimulation surely contributes to the rescue, because the simple inhibition of NKA cannot restore the SR Ca^{2+} uptake function, as previously shown in cardiomyocytes from healthy hearts[1]. Moreover, a recent clinical trial conducted on patients presenting acute heart failure but with a good end-organ perfusion (to whom inotropic therapy is not routinely recommended) showed that IST was able to moderately improve the hemodynamic parameters, confirming these results[9;10].

Mechanism of Action of Istaroxime

How IST promotes SERCA activity is currently not known. In line of principle, IST might interact directly with SERCA, or rather affect the activity of a regulatory protein, like PLB[11] or the phosphatase inhibitor I-1 (ref. [12]). However, IST retains its modulatory activity in cardiac SR vesicles, making the hypothesis that the drug might interfere with a soluble modulator of SERCA, like I-1, unlikely[1]. Thus, the target of IST should be the pump itself, PLB, or their interaction. The inability of the drug to modulate SERCA activity in SR vesicles from skeletal muscle, which are devoid of PLB, suggests that the drug targets PLB disrupting the interaction of the inhibitor with SERCA.

Although further work is needed to verify this hypothesis, if confirmed it would indicate that the enhancement of SERCA activity by IST is tissue specific, since only the cardiac isoform of the pump is regulated by PLB[11;13], and that possible side effects derived from aspecific pump upregulation can be avoided.

Comparison of Istaroxime and Digoxin Effects on Cytosolic Ca^{2+}

In Chapter 3 the effects of IST and digoxin on $[Ca^{2+}]_{cyt}$ homeostasis were investigated in order to find differences that may account for their dissimilar pro-arrhythmic effect. The two drugs share a common target, NKA, but IST is also a modulator of SERCA activity, while some groups hypothesized that digoxin affects RyR activity[14-16]. Therefore, some alterations in Ca^{2+} handling might be expected to underlie their different toxicity.

The positive luso-inotropic action of IST, as well as its different toxicity when compared to digoxin, were confirmed in mouse (Chapter 3, figures 2 and 5): when tested at equi-inotropic concentrations (similar increase in cell contraction), digoxin induced aftercontractions, a potentially arrhythmogenic phenomenon, in the 26.3% of the cardiomyocytes tested, versus the 4.8% of cells exposed to IST. Rodents are less sensitive to cardiotonic steroids[17], however, a marked inotropic effect (an increase of about 150% of the cellular contraction) and a different pro-arrhythmicity were still present in mouse cardiomyocytes. Since EC-coupling between rodents and upper mammals shows significant variations, the alterations of Ca^{2+} homeostasis accounting for the different pro-arrhythmicity of the two drugs are not due to a species-specific

effect, but are involved in the normal process that governs Ca^{2+} waves and aftercontraction genesis, and thus are of more general interest.

When resting cardiomyocytes were exposed to digoxin, $[Ca^{2+}]_{cyt}$ begun to accumulate immediately (Chapter 3, figure 3). Several mechanisms could contribute to this effect. NCX is the primary Ca^{2+} influx and efflux mediator at rest[18], and the inhibition of NKA leads to a raise of intracellular Na^{+}, which in turn affects NCX activity[19]. Thus, $[Ca^{2+}]_{cyt}$ was raised both because of the SR Ca^{2+} leakage, which, in normal conditions, is partially extruded from the cell by NCX, and because of the possible Ca^{2+} influx mediated by NCX working in the reverse mode. Additionally, it has been suggested that digoxin can promote RyR activity, thereby increasing the SR Ca^{2+} leakage and contributing to $[Ca^{2+}]_{cyt}$ accumulation[14-16]. IST, on the other hand, did not produce appreciable changes of $[Ca^{2+}]_{cyt}$ over a period of time (2 minutes) larger than the one needed by digoxin to induce aftercontractions. In the same time span, both drugs achieved the maximal inotropic effect, therefore, the lack of $[Ca^{2+}]_{cyt}$ accumulation during exposure to IST cannot be attributed to a slower action of the drug. Given that both drugs affected NKA activity and thus led to a raise in intracellular Na^{+}, with a consequent reduction of NCX activity, three hypothesis can be made to explain this observation: (1) digoxin increased the SR Ca^{2+} leakage while IST did not; (2) IST promoted SERCA activity and compensated for the increase in $[Ca^{2+}]_{cyt}$ induced by the partially impaired NCX function; (3) a combination of the previous two hypothesis.

However, neither digoxin nor IST affected SR Ca^{2+} leakage directly (Chapter 3, figure 4). This result suggests that the $[Ca^{2+}]_{cyt}$ accumulation observed in resting cardiomyocytes was more likely the consequence of SERCA stimulation by IST. It is worth noting that digitoxin, a cardiotonic steroid structurally related to digoxin, creates Ca^{2+} selective channels in phospholipid bilayers[20]. On the other hand, the protocol employed for Ca^{2+} leakage measurement evaluated RyR-mediated leakage only[21;22]; the leakage due to the formation of Ca^{2+} channels in the SR membrane by digoxin itself would not be detectable. Thus, it is not possible, with these data, to rule out that a digoxin-mediated leak occurred.

The lack of a RyR-mediated leak induced by digoxin is in opposition with previous reports of an increase of RyR open probability observed in single channel recordings following digoxin application[16]. However, it is not clear whether cardiotonic steroids can directly affect RyR activity in a cardiomyocyte. In the absence of Na^{+}, ouabain, another cardiotonic steroid, was reported to increase Ca^{2+} transient amplitude as well as cell shortening[14], to decrease Ca^{2+} spark frequency and, in the same cells, to raise basal fluorescence, suggesting an increase of $[Ca^{2+}]_{cyt}$ (ref. [15]). Yet, it is important to stress that Ca^{2+} sparks, too, contribute to $[Ca^{2+}]_{cyt}$, and that the measurement of SR Ca^{2+} leakage reported in Chapter 3 takes into account both parameters[21;22]. Additionally, Altamirano et al[23] failed to observe any effect of many cardiotonic steroids, including digoxin, in Na^{+}-free conditions, in accordance with the results presented here.

To sum up, the results reported in Chapter 3 indicate that digoxin induced a $[Ca^{2+}]_{cyt}$ accumulation during resting conditions that was

not attributable to the enhancement of RyR-mediated SR Ca^{2+} leakage, while IST did not produced significant changes in $[Ca^{2+}]_{cyt}$, likely because of the stimulation of SERCA. Moreover, the increase of $[Ca^{2+}]_{cyt}$ following digoxin exposure seems to be the consequence of NKA inhibiton, although a digoxin-mediated Ca^{2+} influx cannot be ruled out.

Translational Considerations

The increase of SERCA activity to improve the SR function of the failing myocardium has been taken into account in the last decade as a positive inotropic intervention, mostly by means of gene transfer[13]. SERCA downregulation and the imbalance between the pump and NCX activity in Ca^{2+} homeostasis during heart failure has been proven in many animal models[24-26] and in man[27;28], SERCA downregulation worsens the recovery from a severe ischemic injury[29] and a mutation that renders PLB superinhibitory results in left ventricular dilation and contractile dysfunction[30]. Instead, SERCA overexpression has been shown to increase the force of contraction in cells from human failing hearts[31], to improve left-ventricular function in rats with heart failure[32], to favour the recovery from ischemic injury[33] and to protect against the worsening of heart failure following myocardial infarction[34]. However, excessive enhancement of SERCA activity leads to myocardial remodelling: mice expressing an isoform of the pump with higher affinity developed hypertrophy and diastolic dysfunction[35], while in man a truncated PLB, lacking the domain for the interaction with SERCA, induces dilated cardiomyopathy and heart failure[36].

SERCA plays a role also in the propagation of Ca^{2+} waves: SERCA inhibition slows Ca^{2+} wave propagation[37] and, as Ca^{2+} waves tend to reduce the SR load[38], they can be seen at steady state only if the SR Ca^{2+} uptake is enhanced[39]. Consequently, some concern about a possible pro-arrhythmic effect following SERCA stimulation has arisen. Nevertheless, there is no evidence that the simple SERCA stimulation induces Ca^{2+} waves: Ca^{2+} waves are obtained experimentally by increasing RyR activity with caffeine[38;39] or by raising extracellular Ca^{2+} in order to increase Ca^{2+} influx[37;40]. Instead, the inhibition of SERCA increases the probability of Ca^{2+} wave onset at a given SR load[40] and SERCA overexpression tends to reduce aftercontraction incidence[41] and suppresses alternans[42]. Accordingly, Chen et al[43] report that in the first 24 hours following myocardial infarction, when RyR activity is greatly enhanced[44] and arrhythmias spontaneously occur[45], the constitutive overexpression of SERCA favoured the onset of lethal arrhythmias, but after this period the overexpression of the pump improved the function of non-infarcted myocardium without inducing arrhythmias[43].

The experiments reported in Chapters 2 and 3 give further insight into the role of SERCA stimulation in the management of heart failure. The moderate stimulation of SERCA by IST effectively rescued a mild model of heart failure, as well as other, worse conditions of failure[4-6], like pump overexpression did[32;34] but without the risks related to an excessive stimulation of the pump[35]. Additionally, the effect of the drug was inversely related to the starting SR Ca^{2+} content, suggesting the possible existence of a safety margin (the SR content is increased but not over a certain

value, reducing the risks of SR Ca^{2+}overload) which would be more difficult to achieve by means of gene therapy.

The stimulation of SERCA keeps $[Ca^{2+}]_{cyt}$ low, a condition that seems to prevent the onset of arrhythmogenic events, at variance with digoxin, which induced $[Ca^{2+}]_{cyt}$ accumulation and was associated to a greater aftercontraction incidence. This conclusion is supported by the finding that in Purkinje fibers the toxic effect of cardiotonic steroids is always accompanied by a raise of $[Ca^{2+}]_{cyt}$ (ref. [46]), while cells that develop aftercontractions and cells that do not have a similar SR Ca^{2+} load[47;48], but in the latter case $[Ca^{2+}]_{cyt}$ is lower[47].

Overall, SERCA stimulation represents a promising tool in the management of heart failure, which could possibly be employed in a wider range of case because of its low arrhythmogenicity, in contrast with current inotropic interventions. Due to the possible risks of arrhythmic episodes ensuing from SERCA overstimulation, at the moment the moderate enhancement of the pump activity achieved by means of pharmacologic agents appears more attractive than gene therapy. In the end, however, only long term studies of SERCA stimulation effect on morbidity and mortality would be able to tell whether this intervention will improve the clinical outcome of heart failure patients.

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Appendix: List of Academic Contributions

Abstracts

- Rocchetti M, Alemanni M, Mostacciuolo G, Micheletti R, Zaza A. SERCA stimulation by PST2744 in guinea-pig with aortic constriction. EWGCCE annual meeting – Florence, September 22-24, 2006.
- Rocchetti M, Barassi P, Alemanni M, Mostacciuolo G, Micheletti R, Ferrari P, Zaza A. Istaroxime modulation of sarcoplasmic reticulum function in cardiac hypertrophy/failure. European Heart Failure Winter Research Meeting – Garmisch, January 24-27, 2007.
- Rocchetti M, Alemanni M, Mostacciuolo G, Ferrari P, Micheletti R, Zaza A. Rescue of sarcoplasmic reticulum function in a heart failure model. Azure Heart – Carry-le-Rouet, June 1-3, 2007.
- Chisci R, Rocchetti M, Menduni F, Alemanni M, Castoldi R, Cipolla L, Zaza A. SR Ca^{2+} pump (SERCA) stimulation by kinases PH-domain inhibitors. EWGCCE annual meeting – Manchester, September 7-8, 2007.
- Alemanni M, Rocchetti M, Mostacciuolo G, Jovenitti I, Zaza A. β-adrenergic modulation of RyR-mediated SR Ca^{2+} leak and spark frequency in murine ventricular myocytes. ISHR meeting (European section) – Nice, May 30-June 2, 2009
- Alemanni M, Rocchetti M, Re D, Zaza, A. Different modulation of resting calcium in mouse ventricular

myocytes by two positive inotropes. EXGCCE annual meeting – Cologne, September 17-19, 2009

Oral communications

- Alemanni M., Rocchetti M., Mostacciuolo G., Altomare C., Marangoni S., Zaza A. Istoraxime effects on SR calcium release in mouse ventricular myocytes. EWGCCE annual meeting – Madrid, September 18-20, 2008.
- Alemanni M, Rocchetti M, Mostacciuolo G, Zaza A. Calcium sparks: rilasci quantali di calcio dal reticolo sarcoplasmatico. Significato e modulazione. III Biosimposio del Dipartimento di Biotecnologie e Bioscienze (Università di Milano Bicocca) – Milano, 28 Novembre 2008

Publications

- Rocchetti M, Alemanni M, Mostacciuolo G, Barassi P, Altomare C, Chisci R, Micheletti R, Ferrari P, Zaza A. Modulation of sarcoplasmic reticulum function by PST2744 [Istaroxime; (E,Z,)-3-((2-Aminoethoxy)imino) androstane-6,17-dione hydrochloride)] in a pressure-overload heart failure model. 2008 J Pham Exp Therap 326, 957-965 (reported in chapter 2)
- Altomare C, Barile L, Marangoni S, Rocchetti M, Alemanni M, Mostacciuolo G, Giacomello A, Messina E, Zaza A. Caffeine-induced Ca^{2+} signalling as an index of cardiac progenitor cells differentiation. 2009 Circ Res [submitted]
- Alemanni M, Rocchetti R, Re D, Zaza A. Role and mechanism of subcellular Ca^{2+} distribution in the action of

two inotropic agents with different toxicity. 2009 J Mol Cell Cardiol [submitted] (reported in chapter 3)

Acknowledgements

There are tears in our eyes that cannot be wept if not as words, but writing them down can bring to us the same comfort that the others give when riding our cheeks. Two drops resemble one the other so much that once mingled it is no longer possible to tell them apart, but there is no tear similar to the others. There are tears shed for joy, and tears shed for pain, tears of sorrow and tears of relief. Some tears are silly, some mirror the best parts of us. Unfortunately, most of men are so afraid to weep a silly tear that end up avoiding them at all, loosing a part of their humanity.

There are many good reasons to shed a tear. The sadness of leaving fellows that no one will be ever able to rival with. The happiness when meeting the friends of a life. Things we managed to learn, things we too much easily forgot. Who grew us up and who nowadays loyally stands at our side notwithstanding the hurdles. The end of a love. The beginning of a new one. Difficult decisions. Death. Landscapes that will never leave our eyes, travels that will never leave our minds, words that will never leave our ears. The end of trouble times and the cry that ushers a new man. Chances we decided to lose; chances we lost anyway, despite our efforts.

Today, my tears are entirely for whom left me a month ago. A person that even last summer I thought she would have been here with me today to share my joy. She was among the ones who cuddled me, the ones who loved me first. She was with me from the very beginning of my life, taking care of me for so long that I

cannot recall a single moment without the thought of her coming into my mind.

From the teaching of how to tie my shoelaces to her presence nearby my bed when I was sick, year after year the connection between us grew so strong that a month ago I actually lost a beloved mother. Her gentleness, her simplicity, her complete dedication to every person she met in her life, her joy so plain that it never left place for a complaint were so astonishing that none of the ones who stood beside her casket could do anything but recall them.

I have no regrets, because in the only month of her life where the parts were reversed, the only time she needed care, instead of giving it, I was at her side, holding her with the same embrace she used twenty-seven years ago to welcome me. I knew a time would have come when these tears should have been wept, anyway, the ones that now fill my eyes are not tears of sorrow, they are not tears of despair. Mine, are tears of gratitude.

Milan, November 26, 2009