Studies on the role of disturbed Ca^{2+} homeostasis in the pathomechanism of the cardiac effects of experimental diabetes using conventional and novel experimental techniques

PhD Thesis

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Introduction

The Na^+/Ca^{2+} exchanger (NCX) plays a crucial role in cardiac electrophysiology *via* maintaining ionic distributions between the cytoplasm and the extracellular space, shaping the action potential and modulating the contractile activity of the heart *via* tight regulation of the cytoplasmic $[Ca^{2+}]$. Since the NCX is the primary transporter to extrude Ca^{2+} from the cells, both the Ca^{2+} content of the cardiomyocytes and magnitude and kinetics of the intracellular Ca^{2+}-transient during action potential are highly dependent on its expression level and functional activity. In spite of its critical function, a relatively selective pharmacological NCX inhibitor (SEA0400) has only recently become available, offering yet unexplored novel possibilities in studying NCX function and malfunction. In our first experimental study we aimed to evaluate the effects of selective, partial NCX inhibition by SEA0400 on Ca^{2+} handling in isolated canine ventricular myocytes.

Since the origin and progression of the pathomechanisms, leading to diabetes-induced cardiomyopathy, are poorly explored, monitoring diabetes induced changes in intracellular Ca^{2+} handling in cardiomyocytes in various functional states may help us to improve our rather limited understanding of the pathophysiology of diabetes-associated heart diseases. This improvement, in turn, may open much needed novel therapeutic avenues for more effective prevention and early treatment of cardiac complications in diabetic patients. In our second study we aimed to investigate in an experimental animal model of Type 1 diabetes the putative perturbations in NCX function, by monitoring shifts in intracellular $[Ca^{2+}]$, following the application of the selective NCX inhibitor SEA0400.

The final part of the present thesis describes a promising methodological work. Virus-mediated gene transfer has recently become an important tool for introduction of recombinant genes into cardiomyocytes, offering the potential to treat both rare and common cardiac disorders. In our third study we have developed a novel, pseudorabies virus vector (PRV)-based technique, which enables the targeted delivery of genetically encoded activity sensors into primary culture of isolated adult canine cardiomyocytes. This system has several advantageous features: 1) the virus enters the cells without destroying the intact physiological properties of the cells for a prolonged period; 2) the virus had no effect on the observed physiological properties. We have shown for the first time, that novel herpes virus-based vectors can efficiently transduce genes into non-dividing cardiac myocytes, offering an alternative approach for gene transfer in this fastidious experimental object.

Aims of the study

Unfortunately, however, there is no single best technique/method with which one can measure local intracellular [Ca^{2+}]. While each method for analyzing Ca^{2+} activity has certain advantages over the others, each also suffers significant drawbacks. Our first goal was to establish a simple, reliable experimental system for the measurement of Ca^{2+} activity, which can provide us with more detailed insights into the Ca^{2+} housekeeping of cardiomyocytes under physiological and pathophysiological conditions. Our second goal was to develop an alternative approach for Ca^{2+} measurement and to test the applicability of the novel technique in the experimental settings of basic cardiac electrophysiology by paying particular attention to the combination of genetically encoded Ca^{2+}-sensors with virus-based gene transfer.

In summary, the primary goals of the experimental work summarized in this thesis were, as follows:

1. To directly validate the NCX inhibitory effect of SEA0400 on caffeine-induced Ca^{2+} transients in canine cardiac myocytes with undisturbed Ca^{2+} handling.
2. To investigate the role of NCX in pathological shifts of Ca^{2+} handling in cardiac myocytes isolated from rabbits with experimental Type 1 diabetes.
3. To develop a novel, pseudorabies virus (PRV)-based method for targeted delivery of foreign genes into isolated adult cardiomyocytes in primary culture to facilitate future investigations of subcellular events underlying the patomechanism of the cardiac effects of diabetes

Summary of methods

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals. Rabbit and canine ventricular myocytes were produced by enzymatically dissociation on a modified Langendorff apparatus. In experimental type 1 diabetes experiment were used male New Zealand white rabbits and diabetes mellitus was induced by infusion of a single intravenous dose of alloxan.

Optical measurements of $[Ca^{2+}]_i$ transients in field stimulated myocytes were used a single wavelength calcium-sensitive fluorescent dye (Fluo-4) and stimulated at a constant frequency of 1 Hz and superfused with normal Tyrode solution at 37ºC. Optical signals were recorded by a photon counting photomultiplier module and sampled at 1 kHz. Simultaneous recording of cell shortening from both ends was determined by a video edge detection system.

For evaluation of the functionality of the transferred troponeon gene, calcium transients were monitored using a dual channel photon counting system in cardiomyocytes expressing troponeon. The troponeon was excited at 480 nm, fluorescence emission was recorded at 535 and 485nm. Changes in $[Ca2+]_i$ levels were characterized by the ratio of emitted fluorescence intensities obtained at 485 and 535 nm wavelengths, (FCITRINE 535 / FCFP 485) following correction for non-specific background fluorescence.

Results

The dynamics of $[Ca^{2+}]$ changes at the whole cell level and in well-defined subcellular compartments in excitable cells during the course of membrane depolarization can now be much better understood in the context of disease processes such as cardiac arrhythmias and heart failure or diabetes.

Recent research underlines that in addition to multiple functions, spatial and temporal separation of local Ca^{2+} signals even within the same cytosolic compartment may be crucial in normal cellular function. Indeed, it has been shown that disintegration of this complex spatiotemporal signaling system is characteristic during development of cardiac diseases, which emphasizes the needs of a more specific and targeted detection of local Ca^{2+} signals, in order to understand disease process in cardiac myocytes and also to explore new potential therapeutic targets. In this field, application of the FRET based methods can provide us with a useful tool in the Ca^{2+} signaling research.

1. Effect of SEA0400 on caffeine induced Ca^{2+} transients in canine cardiomyocytes

Earlier reports show that SEA0400 may have different efficacy in inhibiting NCX depending on the intracellular ionic composition. Specifically, high intracellular Ca^{2+} has been shown to counteract the blocking effect of SEA0400 on NCX. These results may question the use of SEA0400 as an applicable tool in the Ca^{2+} handling research. Therefore, in our first experiments we characterized the NCX inhibiting effect of SEA0400 in an experiment where a high and long lasting intracellular Ca^{2+} level is achieved by application of 10 mM caffeine. In this experimental setting the extrusion of Ca^{2+} from the cell is achieved only via the NCX, thus in this model the activity of NCX can be studied accurately. Under these conditions, inverse Ca^{2+} dependent NCX inhibiting effect of SEA0400 can be assumed to be maximal, thus in the experiments on the diabetic rabbit cells the actual degree of NCX block is presumably at least as large as in this caffeine experiment. According to our results, the effect of 1 μM SEA0400 on the rate of decay of the caffeine-induced Ca^{2+} transient was only a fraction of that observed with 10 mM $NiCl_2$ (20%) These results suggest that the NCX-blocking effect of SEA0400 may be relatively moderate when $[Ca^{2+}]_i$ is elevated. It must be noted that weak inhibition of NCX in our experiments is not necessarily a disadvantage, because higher degree of inhibition can easily result in accumulation of Ca^{2+} inside the cell, which would lead to cell hypercontraction or cell death, especially in case of cells from diabetic animals, where the Ca^{2+} homeostasis is already compromised.

2. Disturbed Ca^{2+} handling in type 1 diabetes

Our second aim was to investigate the altered Ca^{2+} handling of cardiac myocytes in a diabetic animal model, with special emphasis on possible contribution of NCX to altered Ca^{2+} handling during the disease process. Isolated diastolic dysfunction is observed in almost half of otherwise asymptomatic patients with well-controlled diabetes and thus may precede diastolic heart failure. Since the mechanisms underlying diastolic dysfunction observed in diabetic patients are not well understood, we tested the hypothesis that diastolic dysfunction may be associated with impaired myocardial Ca^{2+} handling, NCX and/or SERCA function in an animal model of type 1 diabetes.

Thus, our goal was to characterize the cellular events associated with diastolic dysfunction in experimental diabetes mellitus and to explore what role NCX may play in these pathological circumstances.

We were able to reproduce characteristic diabetic alterations, including increased diastolic Ca^{2+} levels as manifestation of diastolic dysfunction, and reduced cell shortening. However, despite of the obvious signs of disturbed Ca^{2+} handling, the differences between the diabetic and healthy cells were not very pronounced in our experiments. Obviously, this is a major limitation of the diabetes model we used, emphasizing the need for development of better experimental models and further extensive research in this field. Interestingly, in our rabbit model, at least at this stage of disease, the loss of contractile force was not associated with reduced intracellular Ca^{2+} transient. The increased Ca^{2+} transient seen in our experiments probably reflects a compensatory mechanism that helps to maintain cellular contractility. Alternatively, decrease of the Ca^{2+} sensitivity of the contractile machinery can also occur as a primary alteration during the disease process.

As one of the most characteristic change in diabetic cells, we found that relaxation of the Ca^{2+} transient was prolonged, which is in line with previous observations suggesting a diminished SERCA function as a consequence of diabetes. Inhibition of NCX by application of SEA0400 further prolonged the Ca^{2+} transient in diabetic, but not in normal cells, suggesting an increased role for NCX in the maintenance of Ca^{2+} homeostasis in diabetic cells. This is also supported by our other finding that SEA0400 increased the Ca^{2+} transient in the diabetic cells, but not in normal cells, indicating that diabetic cells are more sensitive to any perturbation of the Ca^{2+} cycling. On the other hand, SEA0400 also increased the contractile force in the diabetic cells, which may suggest a potential therapeutic possibility for NCX inhibition.

A difficult point in our results is the increased contractility observed in normal cells in response to SEA0400, which was apparently independent of the intracellular Ca^{2+} transient. It is very hard to interpret this finding, because it is generally accepted that in normal healthy myocytes the increase of the contractile force should be, at least in part, the consequence of the elevated Ca^{2+} transient. Although increased sensitivity of the contractile machinery to Ca^{2+} could be a possible explanation, it is hard to assume that this would occur following partial inhibition of NCX. A possible, though rather speculative explanation is that inhibition of NCX can also affect the local submembrane Ca^{2+} levels ($[Ca^{2+}]_{sm}$), which could in turn influence membrane bound enzyme systems involved in signaling cascades, leading to altered Ca^{2+} sensitivity of the contractile system. A possible role for such local $[Ca^{2+}]_{sm}$ changes in the regulation of cell physiology emphasizes the need for the development of novel methodology to detect Ca^{2+} in specific subcellular compartments.

3. Delivery of troponeon to cultured cardiomyocytes

Virus-mediated gene transfer methods have also become applicable experimental tools in cardiovascular research. However, in spite of the permanent improvements, techniques for introducing foreign genes to cultured adult cardiomyocytes suffer from substantial limitations, such as relatively low infection efficacy and/or cell surviving rate for the integration of transgenes be delivered; and vector associated cytotoxic effects directly affect a number of physiological properties of the cells. We opted for a canine model, since the dog have characteristic action potentials and ionic current similar to those in human. For testing the possible effects of our novel PRV vector and viral infection the transient outward potassium current was chosen because I_{to} is a relatively large current, and is present in all cells.

In our third study we demonstrated that pseudorabies virus vectors can effectively transduce cultured dog cardiomyocytes. The transferred foreign gene (troponeon) could be detected as early as 16 hours following infection. Furthermore, we have shown that infected cardiomyocytes well tolerate the presence of PRV vector, since their electrophysiological properties were not changed following the infection. Also, the survival of the cells suitable for electrophysiological studies was high enough even after 4 days, proving that the virus entering the cells did not cause any observable cytotoxic effects. In our study the virus did not affect the I_{to} current. I_{to} was present in all cells even after 72 hours of viral infection, and neither its density nor its kinetics were significantly different from those observed in control cells. In addition we found that the kinetics of intracellular Ca^{2+} transient was neither significantly different between infected and non-infected cells; and verified by FRET measurement the transferred troponeon gene was fully functional.

Conclusions and future perspectives

In conclusion, using our animal model of type I diabetes mellitus, we were able to reproduce the major characteristic alterations of the myocardial Ca^{2+} handling seen in diabetic patients, such as decreased force of contraction and deteriorated adaptation of the Ca^{2+} cycling in different experimental circumstances. Regarding the underlying mechanisms, we could demonstrate that, depending on the specific circumstances, the decreased SERCA and/or NCX function can play a significant role in these alterations. The moderate alterations that we generally saw in this model belong to the limitations of our study, together with the imperfection of the pharmacological NCX inhibition. Concerning this latter point, however, we demonstrated that SEA0400, the most widely used NCX inhibitor, can be applied when the experimental purpose does not require full NCX blockade. In many cases interpretation of our results invokes careful speculations, which underlines the need of development of more sophisticated Ca^{2+} imaging techniques, with which we can address more specific problems in the Ca^{2+} handling research. As a first step in this direction, we developed and tested a viral vector based gene delivery system that can be a useful tool in studying localized Ca^{2+} signals and other subcellular events or introduce siRNA for silencing ionic channel subunits underlying transmembrane ionic currents, helping to understand disease process at subcellular level in various pathological states, including diabetes.

Published literature related to PhD topic

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Conference presentations related to PhD topic

I. **Prorok J,** Tóth A, Iost N, Kovács PP, Kristóf AA, Tombácz D, Tóth J, Ördög B, Virág L, Papp JG, Varró A, Boldogkői Z.. Herpesvirus-mediated delivery of genetically encoded fluorescent Ca^{2+} sensor to adult canine cardiomyocytes. 32nd Meeting of the European Working Group on Cardiac and Cellular Electrophysiology, Madrid, Spain. 2008

II. **Prorok J., Nagy N.,** Kormos A., Acsai K., Papp Gy., Varró A., Tóth A. 2008 The effect of the NCX inhibitor SEA0400 is intracellular Ca^{2+} level dependent in canine ventricular myocytes. Cardiol. Hungarica, 2008, 38. Suppl.B: B20

II. **Prorok J**, Jost N, Kovács PP. Kristóf A, Tóth A, Ördög B, Boldogkői Z. 2007. Gene transfer into cardiac muscle cells with herpes virus. Cardiologica Hungarica, 37: Suppl A, A24.