Reduced Calmodulin Expression Accelerates Transient Outward Potassium Current Inactivation in Diabetic Rat Heart

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Key Words

I_{to} • Inactivation • CaMKII • Insulin • Cardiac myocyte

Abstract

Background/Aims: In myocytes from diabetic hearts, the reduction in the amplitude of the transient outward potassium current (I_{to}) and the acceleration of its inactivation contribute to the action potential duration lengthening. Whereas the reduced amplitude is attributable to a reduced support of trophic factors, the mechanism underlying the acceleration of inactivation remains unknown. Ca^{2+} /Calmodulin-dependent protein kinase II (CaMKII) modifies the inactivation kinetics of I_{to} . In this work we explored the role of CaMKII in the acceleration of I_{to} current inactivation observed in diabetic myocytes. **Methods:** We used patch-clamp and immunoblotting techniques in enzymatically-isolated myocytes from healthy and streptozotocin-induced diabetic rat hearts, and in blood samples from diabetic patients. **Results:** In control myocytes, inhibition of either calmodulin or CaMKII accelerated I_{to} current inactivation. However, in diabetic myocytes I_{to} inactivation was already accelerated, and did not respond to calmodulin or

CaMKII inhibition. Calmodulin protein abundance was significantly reduced in diabetic myocytes. Incubation of diabetic myocytes with insulin recovered calmodulin expression to normal values. A similar pattern of calmodulin expression appears in the blood of diabetic patients. Insulin treatment also restored I_{to} current inactivation kinetics as well as the responsiveness to regulation by calmodulin. **Conclusion:** Diabetes-induced acceleration of I_{to} current inactivation is due to a reduced effect of CaMKII on I_{to} channels as a result of a diabetes-induced reduction in calmodulin protein expression. A correct follow up of the insulin treatment could prevent this alteration.

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Introduction

Diabetes Mellitus (DM) is associated with long-term damage to and failure of various organs, including the heart. The QT interval, the corrected QT interval (QTc) and the QT dispersion (QTd) are prolonged in diabetic

patients when compared with age matched controls. These alterations could be the cause of the higher incidence of ventricular arrhythmias and sudden death that have been demonstrated in patients with diabetes mellitus. The electrocardiographic alterations are due to a DM-induced prolongation of cardiac action potential duration related to a decrease in K^+ repolarizing currents [reviewed in 1]. In ventricular cells, we can find different kinds of voltage-dependent potassium currents, such as transient outward (I_{to}), delayed rectifiers (I_K) and inward rectifiers (I_{K1}). Several studies demonstrated that one of the main causes for the action potential prolongation is a reduction in the I_{to} current density in diabetic myocytes, even at very early stages of the disease, although there is also a reduction in the delayed rectifier current densities. Finally, the inward rectifier current I_{K1} is not affected by diabetes [2-5].

I_{to} currents are generally classified in two different types, "fast" ($I_{to,f}$) and "slow" ($I_{to,s}$), with different recovery from inactivation time constants. A general consensus exists that while $I_{to,f}$ is mediated by Kv4.2 and/or Kv4.3 channels, $I_{to,s}$ is generated primarily by Kv1.4 channels, being the $I_{to,f}$ the predominant type in rat ventricle [6-9]. The transient outward K^+ current, I_{to} , is one of the main repolarizing currents in mammalian myocardium [reviewed in 9] and is regulated by different signaling pathways [10-12]. Type 1 diabetes reduces I_{to} current amplitude [2, 4, 5, 13], prolongs the current recovery kinetics [5] and accelerates I_{to} inactivation [2, 4]. Whereas the reduction in amplitude is attributable to a reduced basal support of different trophic factors [14-16], and the slower recovery kinetics is proposed to be related to a shift from Kv4.2/Kv4.3 ($I_{to,f}$) to Kv1.4 ($I_{to,s}$) channels [17], the mechanism underlying the accelerated I_{to} inactivation remains unknown. In the present work, we will study the possible causes for this accelerated inactivation.

I_{to} and Kv4.2/Kv4.3 current inactivation kinetics are modulated by CaMKII phosphorylation, either in heterologous expression system or in rat ventricular myocytes [18, 19]. It is also known that CaMKII modulates the inactivation kinetics of native I_{to} currents in murine colonic cells, human atrial and rat ventricular myocytes [18, 20, 21]. As CaMKII alters inactivation kinetics of I_{to} we wonder if this kinase could be involved in DM-induced acceleration of I_{to} inactivation. Because Ca^{2+} /Calmodulin-dependent protein kinase II slows down the inactivation kinetics of native I_{to} , we explored the hypothesis that the acceleration in I_{to} current inactivation found in diabetic myocytes could be caused by a defective regulation by CaMKII.

Materials and Methods

The investigation conforms to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. Sprague-Dawley rats were obtained from Harlan (Barcelona, Spain) and maintained in a temperature ($22 \pm 1^\circ\text{C}$) and humidity (50%)-controlled room with 12 h-12 h light-dark cycle (lights on at 08:00 h.) and given free access to standard rat chow diet and water. After 7 days of adaptation to these conditions, animals were randomly divided into 2 groups. The control group was 10-12 weeks old and the experimental group was 6-8 weeks old at the moment of type 1 diabetes induction. Experiments were carried out four weeks after type 1 diabetes induction, thus all the animals were 10-12 weeks old when the myocytes were isolated.

After anaesthetising the animals with an intraperitoneal injection of 6% chloral hydrate (3 ml Kg^{-1}) (Sigma, St. Louis, MO), the jugular vein was dissected to enable a slow injection of 65 mg Kg^{-1} body weight of streptozotocin. Following topical application of sulfathiazole to avoid possible infection, the wound was closed with 2-3 sutures. The animals in the control group were subjected to the same treatment, using only a citrate buffer vehicle, without streptozotocin. Experiments were performed 30 days after injection, when type 1 diabetes was stable and streptozotocin was totally cleared from the body. Blood samples were taken during the surgical procedure and immediately before heart extraction. Plasma glucose levels were measured with an Acutrend Glucometer (Roche), and averaged $165.5 \pm 7 \text{ mg dl}^{-1}$ ($9.1 \pm 0.3 \text{ mmol L}^{-1}$) in control and $651.5 \pm 14.8 \text{ mg dl}^{-1}$ ($36.1 \pm 0.8 \text{ mmol L}^{-1}$) in diabetic animals ($p < 0.0001$). Blood samples were taken always at the same time early in the morning, taking account that rats feed at that time, these blood glucose levels are postprandial levels.

Cell isolation

The animals were anaesthetized with chloral hydrate (3 ml kg^{-1} , IP) and killed by cervical dislocation. The hearts were removed and perfused with a Tyrode solution containing (in mmol L^{-1}): NaCl 118, KCl 5.4, NaHCO_3 24, MgCl_2 1.02, CaCl_2 1.8, NaH_2PO_4 0.42, dextrose 12 and taurine 20, bubbled with 95% O_2 and 5% CO_2 , pH 7.4 at 37°C , followed by the same solution without Ca^{2+} , and by the same nominally Ca^{2+} -free solution containing collagenase Type I (0.5 mg ml^{-1}), and protease Type XIV (0.03 mg ml^{-1}). The hearts were finally perfused with a KB solution [22] (in mmol L^{-1}): taurine 10, glutamic acid 70, creatine 0.5, succinic acid 5, dextrose 10, KH_2PO_4 10, KCl 20, HEPES-K 10, EGTA-K 0.2, adjusted to pH 7.4 with KOH. The right ventricle was dissected from the rest of the heart and single cells were obtained by mechanical agitation.

Current recordings

Experiments were performed at room temperature ($20\text{--}22^\circ\text{C}$) to isolate rapidly activating I_{to} current from capacitive current. Ionic currents were recorded using the whole-cell variation of the patch-clamp technique [23] with an Axopatch 200B patch-clamp amplifier (Axon Instruments Inc., Union City, CA). Recording pipettes were obtained from borosilicate glass

(Sutter Instruments, Novato, CA), and had a tip resistance of 1.5–3 M Ω when filled with the internal solution (in mmol L⁻¹): L-aspartic acid (potassium salt) 80, KH₂PO₄ 10, MgSO₄ 1, KCl 50, HEPES-K 5, ATP-Na₂ 3, EGTA-K 10, adjusted to pH 7.3 with KOH.

After the formation of the “Giga-seal” between the pipette and cell membrane, pipette capacitance was fully compensated electronically. Following the patch rupture, whole cell membrane capacitances were measured from integration of the capacitive transients elicited by voltage steps from -50 to -60 mV, which did not activate any time dependent membrane current. As previously described, there were no differences in membrane capacitances between the experimental groups [2, 15]. Series resistances were compensated 80% in order to minimize voltage errors and were checked regularly throughout the experiment to ensure that there were no variations with time. The voltage-clamp experimental protocols were controlled with the “Clampex” program of the “pClamp” software, Axon Instruments Inc.

The bathing solution was (in mmol L⁻¹): NaCl 86, MgCl₂ 1, HEPES-Na 10, KCl 4, CaCl₂ 0.5, CoCl₂ 2, dextrose 12, TEA-Cl 50, adjusted to pH 7.4 with NaOH. I_{to} was recorded applying depolarizing pulses to +50 mV, starting from a holding potential of -60 mV. The TEA-resistant time-independent I_{ss} was digitally subtracted.

Using this combination of protocols and solutions in this particular type of cells, myocytes from the right ventricle of rat heart, the current recorded is I_{to} , carried out by Kv4.2/Kv4.3 heterotetramers.

Perfusing the cells with KN93, a selective inhibitor of CaMKII [24], typically decreased the peak current amplitude and increased the rate of inactivation as already described [21]. These effects have been attributed in part to a direct action of KN93 on the extracellular side of K⁺ channels, acting as an open channel blocker [25]. This direct effect of the drug on the extracellular side of the channels can be avoided by adding KN93 and calmidazolium to the solution of the recording pipette [18, 26]. In these experimental conditions, KN93 and calmidazolium in the recording pipette have demonstrated similar selectivity and more potency than selective peptides in the recording pipette and than permeable forms of these peptides in the bathing solution [18].

Chemicals

Collagenase, protease and insulin were obtained from Sigma Chemical Co. (St. Louis, MO). Calmidazolium and KN93 were from Calbiochem (San Diego, CA).

Blood samples of diabetic patients

Blood samples were collected from healthy, well controlled diabetic (WCD) patients and poorly controlled diabetic (PCD) patients. Blood was centrifuged at 2000 g for 15 minutes and the cellular fraction collected for further experiments.

Western blot

All procedures were performed at 4°C in a homogenization buffer (HF) containing Tris-HCl 20 mmol L⁻¹ pH 7.4, EDTA 1 mmol L⁻¹, and 2.5 μ l ml⁻¹ of the Sigma Protease inhibitor cocktail

(Sigma Chemical Co.). Both myocytes and blood samples were homogenized 1 min on ice. Nuclei and debris were pelleted by centrifugation at 500g for 10 minutes. The supernatant was stored at -80°C. The protein content of all preparations was determined by using the Bradford method.

Samples were fractionated on 12 % SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilom P, Millipore, Billerica, MA). PVDF membranes were blocked in TTBS solution (Tris-HCl 50 mmol L⁻¹ pH 7.5, NaCl 150 mmol L⁻¹, Tween-20 0.05%) containing BSA 3%. Blots were incubated with a primary antibody anti-calmodulin (1:2000, Sigma Chemical Co., St. Louis, MO) or anti-CaMKII (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed using chemiluminescence (West pico, Pierce). The classic protein loading control actin is also reduced by diabetes (not shown). Thus, we cut the upper part of the PVDF membrane and used the Ponceau Red staining as control for equal protein loading.

Data analysis

Current traces were analyzed using the Clampfit program of pClamp software (Axon Instruments Inc.). Inactivating current trace was fitted to the equation $I(t) = A_{slow} * e^{-t/\tau_{slow}} + A_{fast} * e^{-t/\tau_{fast}}$ by using the Chebyshev polynomial fitting. The goodness of fit was estimated with the correlation coefficient of the fit, which ranged from 0.98 to 0.999. Western blot bands were analyzed with the Scion Image software (Scion Corporation, Frederick, MD). ANOVA and Dunnett's test were used for statistical analysis. The data are expressed as the mean $\pm$ S.E.M. A p value of less than 0.05 was considered to be statistically significant.

Results

Inhibition of CaMKII accelerates I_{to} inactivation in control myocytes

CaMKII has been shown to regulate the inactivation of I_{to} channels (Kv4.2/Kv4.3). Thus, pharmacological inhibition of calmodulin or CaMKII activity accelerates I_{to} inactivation [18–21]. We performed a first group of control experiments to recapitulate the effects of CaMKII inhibition on I_{to} inactivation in myocytes isolated from healthy animals. Consistent with these previous works, we found that inhibition of calmodulin (calmidazolium 10 μ mol L⁻¹) accelerated current inactivation (Fig 1A). I_{to} inactivation was fitted to a bi-exponential function ($I(t) = A_{slow} e^{-t/\tau_{slow}} + A_{fast} e^{-t/\tau_{fast}}$) in order to quantify time constants (τ) and relative amplitudes of the slow and fast inactivation components (A_{slow} and A_{fast} respectively). As shown in figures 1B and 1C, calmodulin inhibition did not affect the inactivation time constants τ_{fast} and τ_{slow} . However, it did increase the relative contribution of the rapid component of inactivation. To explore the role of CaMKII, we blocked the kinase activity with the specific

Fig. 1. CaMKII blockade accelerates I_{to} inactivation kinetics in rat cardiomyocytes. A) Superimposed I_{to} current recordings from two representative cardiomyocytes isolated from healthy rats with similar membrane capacitance; one recorded in control conditions and the other with 10 $\mu\text{mol L}^{-1}$ calmidazolium (CMZ) in the internal solution. I_{to} current was elicited by 500 ms depolarizing pulses to +50 mV from a holding potential of -60 mV. The TEA-resistant time-independent I_{ss} current was digitally subtracted for further analysis. Inset, I_{to} current amplitude in control and with CMZ or KN93 in the pipette solution. Panels B) and C) show I_{to} inactivation kinetic parameters from cells recorded in control conditions and cells recorded with 10 $\mu\text{mol L}^{-1}$ CMZ or 40 $\mu\text{mol L}^{-1}$ KN93 in the internal solution. Panel B) shows I_{to} inactivation time constants, τ_{fast} and τ_{slow} and Panel C) Relative contribution (%) of fast and slow components (A_{fast} and A_{slow} respectively) of I_{to} inactivation in the same current recordings. Inhibition of calmodulin or CaMKII accelerates I_{to} current inactivation through changes in the relative amplitudes of the fast and slow components. $n=11-14$. * $p<0.05$.

inhibitor KN93 40 $\mu\text{mol L}^{-1}$. Similar to results obtained with calmidazolium, kinase inhibition caused an increase in the relative amplitude of the fast component of inactivation without affecting the inactivation time constants. Thus, either calmodulin or CaMKII blockade increased the relative contribution of the fast component of inactivation (figure 1B, C).

It has been described that KN93 decreased the peak current amplitude and increased the rate of inactivation due in part to a direct open channel block acting on the extracellular side of K^+ channels [25]. We and others have described that it is possible to avoid this direct effect of the drug on the extracellular side of the channels by adding KN93 and calmidazolium to the solution of the recording pipette [18, 26]. Note that neither calmidazolium nor KN93 treatment affected I_{to} current amplitude (Fig 1A inset). These results are therefore consistent with previous reports.

CaMKII activity can not regulate I_{to} inactivation in diabetic cells

I_{to} current inactivation has been reported to be accelerated in ventricular myocytes isolated from diabetic animals [2, 4]. We speculated that the alteration in I_{to} inactivation found in diabetic cells could be due to a defective regulation by the CaMKII pathway. To test this hypothesis, we first analyzed the inactivation properties of I_{to} in diabetic cardiac myocytes. As expected, diabetes accelerated I_{to} current inactivation (figure 2A). Although

τ_{fast} and τ_{slow} were similar to that found in control cells, diabetes changed the relative amplitude of each component compared to cells isolated from healthy hearts (figure 2C and D). Diabetes reduced both amplitudes, A_{fast} and A_{slow} , because of the total current reduction, but the relative contribution of the fast component was increased in diabetic cells, consistent with an abnormally accelerated inactivation. Interestingly, diabetes-induced

Fig. 2. In diabetic cardiomyocytes I_{to} inactivation kinetics are not sensitive to CaMKII inhibition. A) Normalized I_{to} current recordings elicited using same protocol as in Figure 1 in a control and a diabetic myocyte. B) Normalized I_{to} current recordings elicited in a diabetic myocyte in control conditions and a diabetic myocyte with CMZ in the internal solution. C) I_{to} inactivation time constants from control myocytes (CONT) compared to those from diabetic myocytes recorded in control conditions and with $10 \mu\text{mol L}^{-1}$ CMZ or $40 \mu\text{mol L}^{-1}$ KN93 in the internal solution. Neither diabetes nor CMZ or KN93 affect current inactivation time constants. D) Relative contribution (%) of fast and slow components of I_{to} inactivation in the same recordings. The relative contribution of the fast component is increased in diabetic myocytes. I_{to} inactivation velocity loses its ability to respond to calmodulin or CaMKII inhibition in diabetic myocytes. $n=10-29$. * $p<0.05$, ** $p<0.01$.

increase in the fast component of inactivation was similar to the increase induced by calmodulin blockade or kinase inhibition in control cells (compare figures 2 and 1). We then tested the responsiveness of diabetic myocytes to CaMKII regulation by analyzing the I_{to} inactivation parameters after treatment of diabetic cells with either calmodulin or kinase inhibitors. Neither calmodulin nor CaMKII inhibition affected current inactivation in diabetic cells (figure 2B, C, D). These results support the hypothesis that I_{to} inactivation can not be regulated by CaMKII activity in insulin-dependent diabetic cells.

Insulin restores both calmodulin abundance and I_{to} inactivation properties in diabetic cells

Calmodulin gene expression has been reported to be regulated by insulin [27]. whether the impaired responsiveness of I_{to} channels to CaMKII regulation in insulin-dependent diabetic cells could be caused by a reduction in calmodulin abundance. We determined calmodulin protein expression in control myocytes, in myocytes isolated from diabetic rat hearts, and in myocytes isolated from diabetic hearts incubated with insulin. Insulin has been reported to significantly increase

Fig. 3. Insulin treatment of diabetic cardiomyocytes restores normal I_{to} inactivation kinetics and responsiveness to Calmodulin blockade. A) Western blot of calmodulin in control myocytes (CONT), diabetic myocytes (DBT) and diabetic myocytes incubated for 5 hours with $1 \mu\text{mol L}^{-1}$ insulin (DBT + INS). The Ponceau Red staining was used as control for equal protein loading. For quantitation, optical densities of calmodulin immunostained bands were measured and expressed as a percentage of control. Each column is the mean $\pm$ SEM of 10 experiments. *** $p < 0.001$. B) Normalized I_{to} current recordings elicited in a diabetic myocyte in control conditions and a diabetic myocyte incubated for 5 hours with $1 \mu\text{mol L}^{-1}$ insulin. Fitted curves are superimposed to each current trace. C) Relative amplitude of fast and slow components of I_{to} inactivation in the same current recordings. Insulin treatment of diabetic cardiomyocytes recovers CaM expression to normal values, restores I_{to} inactivation kinetics and also inactivation responsiveness to CaM blockade. $n = 8-29$. * $p < 0.05$.

protein expression after 2-4 hours of incubation in cultured diabetic myocytes [16], and calmodulin expression enhancement has been reported to be significant after 4 hours of culture in the presence of insulin [27]. Thus, myocytes were incubated with insulin $1 \mu\text{mol L}^{-1}$ for 5 hours at room temperature in KB medium. Control cells were maintained in the same conditions for the same time. Immunoblotting experiments showed that calmodulin protein expression was significantly reduced in diabetic myocytes and incubation with insulin efficiently restored calmodulin abundance (figure 3A). Insulin incubation of diabetic myocytes restored total I_{to} current density as previously described [28]. At the same time, insulin incubation also restored I_{to} inactivation kinetics to control values. Fast and slow time constants were unaffected but the relative amplitude of the fast component of inactivation was reduced to control values. Basically, insulin treatment did reverse the effects on I_{to} inactivation induced by diabetes. Moreover, insulin treatment also restored the I_{to} responsiveness to CaMKII regulation, since in diabetic cells incubated with insulin, calmodulin inhibition accelerated inactivation through an increase in the relative contribution of the fast component (figure 3B, C).

Taken together, these results indicate that the lack of insulin in type I diabetic cells results in a reduction of the amount of calmodulin. Presumably, this leads to the disruption of the CaMKII pathway and the subsequent misregulation of downstream targets like the I_{to} channels.

Another possible explanation is that the misregulation of the CaMKII pathway would not be due to a reduction

in the amount calmodulin, but in the kinase itself. To assess this possibility we performed additional immunoblotting assays in the same three experimental groups. Figure 4 shows that neither diabetes alone, nor insulin incubation of diabetic myocytes changed CaMKII protein expression with respect to control myocytes.

Fig. 4. Neither diabetes nor insulin treatment of diabetic cardiomyocytes affects CaMKII protein expression. Western blot of CaMKII in control myocytes (CONT), diabetic myocytes (DBT) and diabetic myocytes incubated for 5 hours with 1 $\mu\text{mol L}^{-1}$ insulin (DBT + INS). The Ponceau Red staining was used as control for equal protein loading. For quantitation, optical densities of CaMKII immunostained bands were measured and expressed as a percentage of control. Each column is the mean $\pm$ SEM of 10 experiments. *** $p < 0.001$.

Fig. 5. Diabetes reduces calmodulin expression also in diabetic patients. Western blot of calmodulin in blood samples from control (CONT), poorly controlled diabetic patients (PCD) and well controlled diabetic patients (WCD). The Ponceau Red staining was used as control for equal protein loading. For quantitation, optical densities of calmodulin immunostained bands were measured and expressed as a percentage of control. $n = 7-11$, ** $p < 0.01$.

Calmodulin expression is also impaired in diabetic patients

Blood samples were collected from healthy, well controlled diabetic (WCD) patients and poorly controlled diabetic (PCD) patients. Glycosylated hemoglobin (hb-g) increases in parallel with maintained glucose levels, and thus it is considered the best control of the insulin treatment in diabetic patients. We selected patients with a one year follow up every three months, and assigned to the “well controlled” group those with hb-g levels under 6.5 %, and to the “poorly controlled” group those with values over 6.5 %. The WCD group averaged 6.1 ± 0.2 %, and the PCD group averaged 9.1 ± 0.2 % ($n=17$, $p < 0.0001$).

We determined calmodulin protein expression in blood samples from healthy people, poorly controlled diabetics and well controlled diabetics. Immunoblotting experiments showed that calmodulin protein expression was reduced in PCD but not in WCD (figure 5).

Discussion

Here we show that in diabetic myocytes, I_{to} current inactivation is accelerated and it does not respond to

inhibition of either calmodulin or CaMKII. Whereas CaMKII protein expression is not modified either in diabetic or in diabetic myocytes incubated with insulin, calmodulin expression is significantly reduced in diabetic myocytes compared to control ones, and is fully restored to control values after incubation with insulin. Insulin treatment also restores both I_{to} current inactivation velocity and the ability of myocytes to respond to calmodulin inhibition. Collectively, our data provide a mechanism to explain the diabetes-induced acceleration of I_{to} inactivation.

Changes in I_{to} current expression and/or kinetics significantly affect action potential (AP) repolarization and consequently the magnitude and time course of $I_{Ca,L}$, Ca^{2+} loading of the sarcoplasmic reticulum (SR), SR Ca^{2+} release and contractility [6, 29]. Diabetes prolongs cardiac AP duration in ventricular myocytes related to a decrease in outward K^{+} currents, mainly the fast component of the transient outward potassium current (I_{to}) [2-4, 13]. All these alterations could explain the increased predisposition of type 1 diabetic patients to episodes of ventricular arrhythmia.

Both CaMKII blockade and diabetes accelerates I_{to} inactivation by an increase in the amplitude of the rapid component of inactivation. Previous studies have shown

that Kv4 channels inactivation involves multiple processes. The kinetic model developed to explain the macroscopic and microscopic behaviour of Kv4 channels assumes at least 6 different inactivated states of the channel. The inactivated state that originates from the open state is unstable, and the channels may inactivate from the preopen closed state. This model involves two inactivation components of the macroscopic current [30, 31]. In our case, if the channel phosphorylation by CaMKII facilitates the inactivation from the open state this would increase the amplitude of the slow component of inactivation. In any case, the objective of the present paper is not to describe the biophysical mechanisms of channel gating, but the physiological mechanism underlying the acceleration of the I_{to} current inactivation that takes place in the diabetic heart.

Besides, in the heart, the CaMKII signaling pathway regulates myocyte excitability and contractility in a very complex way affecting many different targets, including I_{to} [32, 33]. In human atrial and rat ventricular myocytes, phosphorylation of Kv4.2 and Kv4.3 channels, molecular determinants of I_{to} , by CaMKII slows down the inactivation kinetics [18, 21]. Our results show that blocking calmodulin or inhibiting CaMKII accelerated I_{to} inactivation kinetics agreeing with these previously published results. In diabetic myocytes, however, the I_{to} inactivation kinetics is already accelerated and the current does not respond to calmodulin or CaMKII inhibition, suggesting that the CaMKII can not regulate the channel.

The total calmodulin concentration is a limiting factor in the cell, of special importance for those targets that associate with calmodulin dynamically, including CaM-dependent protein kinases. Thus, formation of CaM-target complexes, such as CaMKII, in the cells is limited by CaM availability [34, 35]. CaM expression is regulated by insulin in a transcriptional and posttranscriptional manner. The CaM I gene has a promoter which contains three potential sp1 sites, at least two of them with demonstrated activity. Thus, insulin stimulates transcription of mRNA CaM expression and reduces RNA degradation [27, 36]. Insulin stimulates both the biosynthesis of transcription factor Sp1 and its O-linked *N*-acetylglucosaminylation (O-GlcNAcylation), which promotes nuclear localization of Sp1 and its ability to transactivate calmodulin (CaM) gene transcription [37]. Insulin deprivation leads to deficiency of sp1 transcription factor and CaM, and incubation of diabetic cells with insulin recovers sp1 levels and CaM expression [38, 39]. Calmodulin abundance is reduced in hepatocytes, fat cells, and vascular smooth muscle isolated from diabetic animals

[27, 38]. Our results indicate that calmodulin is also a limiting factor in the diabetic myocardium. Therefore, the dynamic association with CaM-dependent protein kinases as well as the regulation of downstream targets like I_{to} channel are expected to be impaired in the diabetic ventricle. In our work, incubation of diabetic myocytes with insulin restored calmodulin levels. Moreover, in diabetic myocytes incubated with insulin, I_{to} inactivation kinetics were similar to those obtained from control myocytes and become responsive to calmodulin inhibition. Besides, the absence of any change in CaMKII protein expression further reinforces the role of calmodulin in the impaired I_{to} regulation in diabetic myocytes.

A very interesting question is if a type 1 diabetic patient has the same alterations found in the rat model of type 1 diabetes, and if regularly using insulin patients would not exhibit these effects. We performed a group of experiments measuring calmodulin expression in blood samples collected from healthy people and insulin-treated diabetic patients. The most widely employed method to follow up the goodness of insulin treatment is the proportion of glycosylated hemoglobin, as it is increased with maintained high glucose levels, and decreased if glucose drops down. Thus, we divided diabetic patients into two groups, well controlled and poorly controlled in terms of Hb-g. In this set of experiments, calmodulin expression is reduced in blood samples from human diabetic patients. The reduction in calmodulin expression is higher in the animal model than in poorly controlled patients. However, it must be taken into account that diabetic animals received no insulin at all. In the well controlled group, this is the group of diabetic patients receiving the correct amount of insulin, calmodulin expression also recovers to control values. Thus, the results in the rat model can be at least partially extrapolated to human patients.

Limitations of the study: In the human ventricle the main repolarizing currents are the transient outward I_{to} , the slow delayed rectifier IK_s , the rapid delayed rectifier IK_r , and the inward rectifier IK_1 . This work is performed in an animal model (rat) which has only part of the cardiac K^+ repolarizing currents present in human heart (I_{to} and IK_1). Different works carried out in different animal models showed that IK_1 is not affected by diabetes, IK_s is slightly reduced, IK_r is slightly or no reduced, and I_{to} is reduced by half, being this last the main responsible for the action potential alterations found in diabetic hearts [2, 14, 40, 41]. However, based on the results obtained with I_{to} , we can expect that the intracellular regulation of the other channels would be impaired in diabetic hearts as well.

In conclusion, Diabetes induced acceleration of I_{to} current inactivation kinetics is due to a reduced effect of CaMKII on I_{to} channels as a consequence of a Diabetes-induced decrease in calmodulin levels. A correct follow up of the insulin treatment could prevent this alteration.

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