

Original Paper

Mechanisms Responsible for the Trophic Effect of Beta-Adrenoceptors on the I_{to} Current Density in Type 1 Diabetic Rat Cardiomyocytes

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Key Words

Repolarization • Adrenergic • Arrhythmia • Arrestin • G protein

Abstract

Background/Aims: In diabetic ventricular myocytes, transient outward potassium current (I_{to}) amplitude is severely reduced because of the impaired catecholamine release that characterizes diabetic autonomic neuropathy. Sympathetic nervous system exhibits a trophic effect on I_{to} since incubation of myocytes with noradrenaline restores current amplitude via beta-adrenoceptor (β AR) stimulation. Here, we investigate the intracellular signalling pathway through which incubation of diabetic cardiomyocytes with the β AR agonist isoproterenol recovers I_{to} amplitude to normal values. **Methods:** Experiments were performed in ventricular myocytes isolated from streptozotocin-diabetic rats. I_{to} current was recorded by using the patch-clamp technique. Kv4 channel expression was determined by immunofluorescence. Protein-protein interaction was determined by coimmunoprecipitation. **Results:** Stimulation of β AR activates first a $G_{\alpha s}$ protein, adenylyl cyclase and Protein Kinase A. PKA-phosphorylated receptor then switches to the $G_{\alpha i}$ protein. This leads to the activation of the β AR-Kinase-1 and further receptor phosphorylation and arrestin dependent internalization. The internalized receptor-arrestin complex recruits and activates cSrc and the MAPK cascade, where Ras, c-Raf1 and finally ERK1/2 mediate the increase in Kv4.2 and Kv4.3 protein abundance in the plasma membrane. **Conclusion:** β_2 AR stimulation activates a $G_{\alpha s}$ and $G_{\alpha i}$ protein dependent pathway where the ERK1/2 modulates the I_{to} current amplitude and the density of the Kv4.2 and Kv4.3 channels in the plasma membrane upon sympathetic stimulation in diabetic heart.

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Introduction

Autonomic neuropathy, including cardiac autonomic neuropathy is a common complication of chronic diabetes that affects noradrenaline turnover [1, 2]. Thus, in streptozotocin-induced type I diabetic rats the catecholamine content in the stellate ganglion, responsible for the sympathetic innervation of the heart, is dramatically reduced, suggesting that cardiac cells do not receive the appropriate noradrenaline supply [3, 4].

Another complication of diabetes mellitus is diabetic cardiomyopathy, a dysfunction characterized by severe defects in cardiac electromechanical properties whose clinical manifestation is a higher risk of arrhythmia [reviewed in 5]. The major electrical alterations observed in diabetic patients and in animal models are prolonged QT interval, lengthened action potential duration (APD) and reductions in potassium repolarizing currents [6, 7].

The transient outward potassium current (I_{to}) is responsible for the phase 1 of the cardiac action potential. In humans, I_{to} affects the shape and duration of the plateau phase and consequently modulates the action potential duration [reviewed in 8]. In rats, I_{to} is the major repolarizing current and therefore directly determines the APD. Thus, this current is a target for sympathetic regulation because of its impact on action potential duration. During sympathetic stimulation noradrenaline reduces I_{to} current amplitude in ventricular myocytes. This is an acute response mediated by the α 1-adrenoceptors and involves activation of the G α s-PKA pathway [9-11].

On the other hand, an intact sympathetic basal innervation is required in order to get functional cardiac ionic currents. In fact, I_{to} current amplitude is reduced in some disorders affecting the development or the activity of the sympathetic nervous system such as muscular dystrophy, Chagas' disease and diabetes mellitus [12-16].

Moreover, chronic incubation with noradrenaline, which mimics the restoration of sympathetic tone, usually reverses the reduction of I_{to} current amplitude in these diseases and also in reserpine-treated catecholamine-depleted rats [3, 17, 18]. In type I diabetes, incubation with noradrenaline restores I_{to} amplitude via activation of β -adrenoceptors (β ARs) and this sympathetic trophic effect may require an increase in the abundance of channel forming proteins in the cell membrane. Diabetes does not affect either the time constant recovery from inactivation or the voltage dependence of inactivation, but it accelerates the I_{to} current inactivation [19]. However, this effect is caused by the impairment in CaMKII-mediated regulation of the I_{to} current and it is not related to the β -adrenoceptor [20].

Although species variability exists, in rats the functional I_{to} channel is a heterotetramer formed by the Kv4.2 and Kv4.3 proteins [19]. In this work, we describe the intracellular signalling pathway connecting β -adrenoceptor (β AR) stimulation with an increase in Kv4.2 and Kv4.3 protein levels at the plasma membrane and the subsequent restoration of the native I_{to} current in diabetic cardiomyocytes.

Materials and Methods

The investigation complies with the Spanish (RD 1201/2005) and European (D2003/65/CE and R2007/526/CE) regulations for Care of Animals used in experimental and other research purposes, and was approved by the Ethics Committee for Animal Care of the University of the Basque Country (N $^{\circ}$ CEBA/45b/2010/CASISSAENZ).

Induction of type I diabetes

Young adult Sprague-Dawley rats (weighing 200-220 g) were anaesthetised with an intraperitoneal injection of chloral hydrate (3 ml kg $^{-1}$). Type I diabetes was induced by an intravenous injection of 65 mg/kg body weight of streptozotocin. All the experiments were performed 4 weeks after injection, when type I diabetes was well established confirmed by both symptoms and blood glucose concentrations measurements as previously described [4, 15]. Blood glucose levels were measured with an Acutrend Glucometer (Roche), and rose from 7.1 \pm 1.1 mmol/l in control conditions to 23.5 \pm 1 mmol/l after 4 weeks of diabetes induction.

Cardiomyocyte isolation

Healthy (control) and diabetic rats were anaesthetized by intraperitoneal injection of chloral hydrate (3 ml/kg). The hearts were removed and perfused with a Tyrode solution containing (in mmol/l): NaCl 118, KCl 5.4, NaHCO_3 24, MgCl_2 1.02, CaCl_2 1.8, NaH_2PO_4 0.42, dextrose 20 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) and protease Type XIV (0.03 mg/ml). Next, the hearts were perfused with a KB solution (in mmol/l): 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. Last, cardiomyocytes were obtained by mechanical agitation of the ventricles.

Incubation with inhibitors and activators

Aliquots of the KB solution containing the myocyte suspension were incubated for ~20 hours (18–24) at 4°C with either isoproterenol, dissolved in deionized water plus ascorbic acid, (Sigma Chemical Co.), vehicle, forskolin or isoproterenol plus each of the following compounds: 8-Br-cAMP, 2',3'-DDOA, FTP Inhibitor III, PD98059, H-89, β -ARK1 Inhibitor, PP2 (Calbiochem); choleric toxin (Gentaur); GW5074, pertussis-toxin or concanavalin A (Sigma Chemical Co.).

Electrophysiological recordings

Cells were transferred to a shallow chamber, mounted on the stage of an inverted microscope Olympus CK2. The cells were allowed to settle for at least 10 min before being superfused with the external bathing solution. For the experiments we used only Ca^{2+} -tolerant rod-shaped cells, with clear cross-striations and lacking any visible blebs on their surfaces. All experiments were performed at room temperature (20–22°C).

Ionic currents were recorded using the whole-cell configuration of the Patch-Clamp technique with an Axopatch 200B patch-clamp amplifier (Axon Instruments Inc.). Recording pipettes were obtained from borosilicate tubes (Sutter Instruments), and had a tip resistance of 1–3 M Ω when filled with the internal solution (in mmol/l): L-aspartic acid (potassium salt) 80, KH_2PO_4 10, MgSO_4 1, KCl 50, HEPES- K^+ 3, ATP- Na_2 3, EGTA- K^+ 10, adjusted to pH 7.2 with KOH.

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. Series resistances were compensated 80% in order to minimize voltage errors and were checked regularly throughout the experiment. The voltage-clamp experimental protocols were controlled with the "Clampex" program of the "pClamp 10.2" software (Axon Instruments Inc.). The external bathing solution was (in mmol/l): NaCl 86, MgCl_2 1, HEPES- Na^+ 10, KCl 4, CaCl_2 0.5, CoCl_2 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. Pulses were applied at a frequency of 0.1 Hz to ensure the full recovery of I_{to} from inactivation. The TEA-resistant time-independent I_{ss} was digitally subtracted and peak I_{to} minus I_{ss} were normalized to cell capacitance, and expressed as pA/pF.

Immunofluorescence

Freshly isolated ventricular cardiomyocytes were placed on tissue culture plates containing poly-L-lysine coated round coverslips. There, cells were incubated for 20 hours at 4°C with vehicle or isoproterenol. Cells in coverslips were then fixed in methanol during 10 min and washed with phosphate buffer before incubation for 1 hour at room temperature in blocking buffer (0.22% gelatine, 1% BSA, 0.1% Triton X-100 and 1% normal donkey serum in phosphate buffer). Primary anti-Kv4.2 and Kv4.3 (Santa Cruz Biotechnology) and secondary donkey anti-goat Alexa 488 (Invitrogen) antibodies diluted in blocking buffer were applied for 60 and 45 min, respectively. Coverslips were mounted in Mowiol reagent (Calbiochem) and analysed in a Leica LCS SP2 AOBs fluorescence microscope.

Immunoprecipitation

All procedures were performed at 4°C, in a homogenization buffer (HF) containing Tris-HCl 20 mmol/l pH 7.4, EDTA 1 mmol/l, and 2.5 $\mu\text{l/ml}$ of the Sigma Protease inhibitor cocktail (Sigma Chemical Co.). Myocytes were homogenized 1 minute on ice. Nuclei and debris were pelleted by centrifugation at 500g for 10 minutes. The protein content was determined by using the Bradford method [21].

Myocytic samples were solubilized in RIPA buffer (Tris-HCl 50 mmol/l pH 7.4, NaCl 150 mmol/l, EDTA 1 mmol/l, Igepal 0.5%, Sodium deoxycholate 1 %, 2.5 μ l/ml of the Sigma Protease inhibitor cocktail and the crosslinker DTBP 0.5 mg/ml). 1 mg of proteins were centrifuged at 25000g for 30 min. The pellet was resuspended in 150 μ l of RIPA buffer and incubated for 1 hour at 4°C. Samples were cleared by centrifugation at 15000g for 25 minutes and supernatants were incubated for 3 hours at 4°C with 40 μ l of protein G-sepharose. Next, samples were centrifuged 2 minutes at 4000g. 150 μ l of the supernatant were obtained and incubated overnight with 2 μ g of a mouse monoclonal anti- β Arrestin-1/2 antibody (Santa Cruz Biotechnology) in 140 μ l of RIPA at 4°C. 100 μ l of 50% protein G-sepharose were added and the mixture was incubated for 3 hours at 4°C. The beads were pelleted and washed three times in RIPA buffer at 4000g for 2 minutes. The bound proteins were eluted using 50 μ l of SDS sample buffer. The pellet from each immunoprecipitation experiment was probed with Arrestin as control, and with each one of the proteins of interest.

Western blot

Immunoprecipitation samples were fractionated on 10% SDS-polyacrylamide gels and transferred to Nitrocellulose membranes (Amersham Biosciences). Nitrocellulose membranes were blocked in TTBS solution (Tris-HCl 50 mmol/l pH 7.5, NaCl 150 mmol/l, Tween-20 0.05%) containing BSA 3%. Membranes were incubated with primary antibodies: rabbit polyclonal anti- β Arrestin-1/2 (1:200; Santa Cruz Biotechnology), chicken polyclonal anti- β_2 AR and mouse monoclonal anti-cSrc (1:200; AbCam). Secondary antibodies were as follows: donkey anti-rabbit IgG (1:5000, Amersham Biosciences), anti-chicken IgG (1:5000, GeneTex) and anti-mouse IgG (1:3300, Sigma Chemical Co.). Blots were developed using the West Pico chemiluminescence reagent (Pierce) and the bands were acquired with a Kodak Gel Logic 2200 and digitized with the Kodak Molecular Imaging 4.0.4 software (Eastman Kodak Company).

Data analysis

Current recordings were analyzed using the Clampfit program of pClamp 10.2 software (Axon Instruments Inc.). No leaks or capacitive subtractions were used during the experiment or during digital analysis, and cells with very slow capacitive transients or with evident current leak were eliminated.

Data are expressed as mean \pm SEM. Multiple comparisons were made using ANOVA and Bonferroni's method. A p value of <0.05 was considered as statistically significant.

Results

Long term incubation with isoproterenol restores I_{to} amplitude in diabetic cardiomyocytes

We have previously described that norepinephrine acutely reduces I_{to} current density in myocytes isolated from healthy rats and this effect is mediated by stimulation of α_1 -adrenoceptors [3, 9].

In a previous work we also showed that incubation with noradrenaline for 24 hours restored I_{to} current amplitude in diabetic cardiomyocytes. This effect was mediated by β ARs, as incubation with the β -antagonist propranolol prevented the effect, whereas the α -blocker prazosin did not [3]. To determine the appropriate concentration of β -agonist, we performed titration experiments, where I_{to} current was recorded after incubating the cells for 20 hours at 4°C with increasing concentrations of isoproterenol.

In Figure 1 we show that, as expected, cardiac myocytes isolated from diabetic rat hearts showed current densities significantly smaller than those obtained from healthy cells (13.9 \pm 1.0 vs. 21.9 \pm 1.1 pA/pF, n=25 and 23 respectively, p<0.0001). The concentration response relationship showed the classical bell shape of β -adrenergic receptors [22]. Incubation with isoproterenol 1 nM restored I_{to} density up to control values (23.8 \pm 2.3 pA/pF, n=13, p<0.0001 compared to diabetic cells). However, neither higher nor lower concentrations of the agonist fully recovered current amplitude (Fig. 1B, 1C). We therefore chose the 1 nM concentration for the subsequent experiments.

In a control experiment (Fig. 1C) we incubated healthy myocytes with 1 nM isoproterenol for 20 h and observed no effect on current amplitude (20.37 \pm 2.7 pA/pF, n=9) compared to

Fig. 1. Effect of increasing concentrations of isoproterenol on I_{to} current in diabetic cardiomyocytes. Representative recordings of I_{to} current amplitude (A and B) and current densities (C) in healthy and diabetic cardiomyocytes and in diabetic cells incubated with different concentrations of isoproterenol for 20 hours at 4°C. 1 nM isoproterenol restores I_{to} current amplitude in diabetic myocytes up to control values (#, * $p < 0.0001$ compared to healthy and untreated diabetic cells, respectively). General stimulation protocol and the scale for each group of experiments are also shown. I_{to} trace corresponding to the 1 nM isoproterenol group will be used as point of reference for subsequent comparisons.

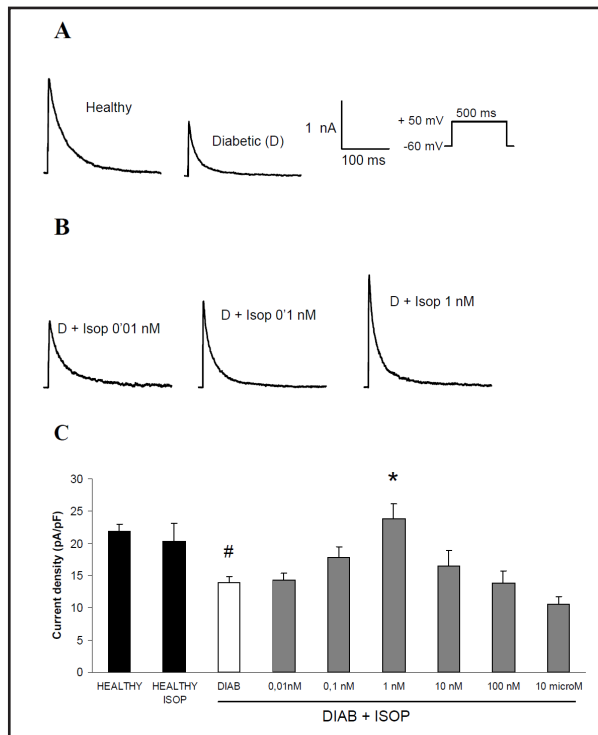
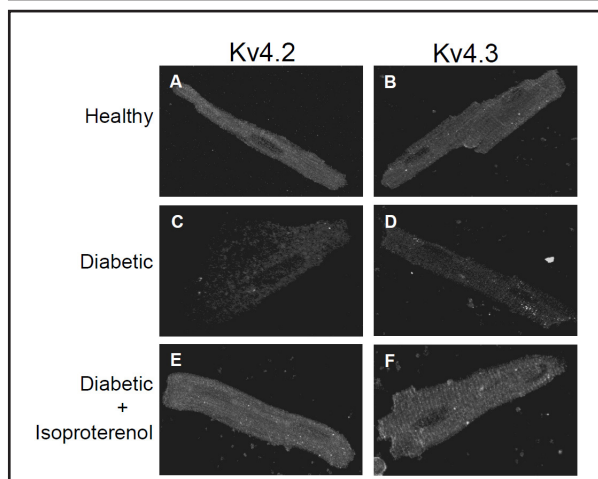


Fig. 2. Immunodetection of Kv4.2 and Kv4.3 protein in healthy, diabetic and isoproterenol-treated diabetic cardiomyocytes. Ventricular myocytes isolated from healthy and diabetic rats were incubated with or without isoproterenol 1 nM for 20 hours at 4°C and then stained as follows. Staining with anti-Kv4.2 antibody to healthy (A), diabetic (C) and isoproterenol-treated (E) cells. Staining with anti-Kv4.3 antibody to healthy cells (B), diabetic (D) and diabetic incubated with isoproterenol (F). Representative images of four experiments are shown.



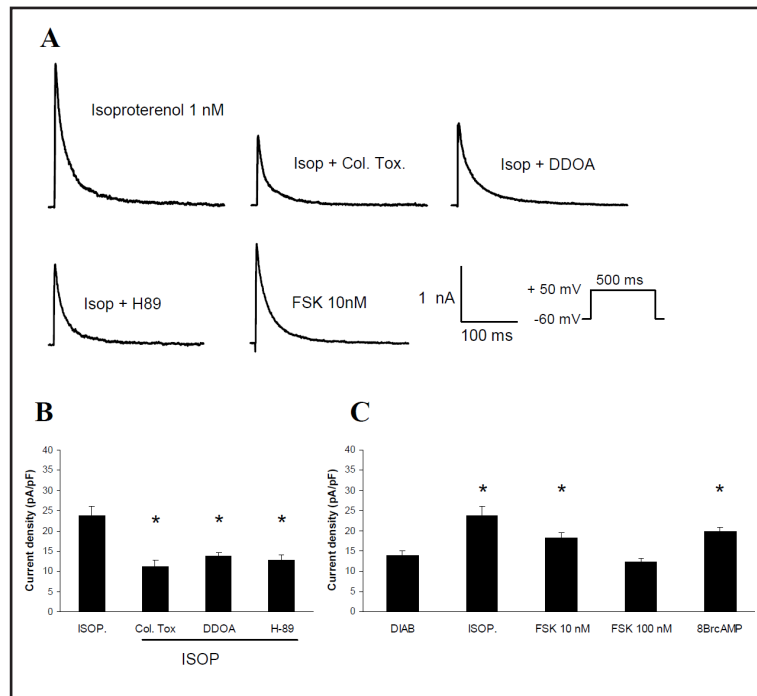
non incubated healthy cells (21.9 ± 1.1 pA/pF, n.s.). Therefore, unlike diabetic myocytes, I_{to} current in healthy cells does not respond to isoproterenol.

Diabetes reduces Kv4.2 and Kv4.3 protein abundance in the cell membrane and this effect is recovered by incubation with isoproterenol

One of the abnormalities associated with diabetic cardiomyopathy is a decreased expression of genes encoding potassium channels. The subsequent reduction in channel protein amount may be responsible for the reduced potassium currents and for the lengthening in action potential duration observed in the diabetic heart [7, 23]. Thus, downregulation of Kv2.1, Kv4.2 and more controversially Kv4.3 expression has been reported in rat ventricle in mild type I diabetes models [23, 24].

We tested whether diabetes reduces I_{to} channels levels in diabetic myocytes and if β AR stimulation could increase them. Cardiomyocytes isolated from healthy and diabetic rats were seeded in coverslips and incubated in the presence or absence of isoproterenol 1 nM for 20 hours. Cells were then stained with immunofluorescent antibodies against Kv4.2 and

Fig. 3. Effect of $G_{\alpha s}$ /AC pathway on isoproterenol-induced I_{to} current restoration. Representative traces (A) and current density values (B) of diabetic cardiomyocytes incubated for 20 hours at 4°C with isoproterenol 1 nM alone or together with either choleric toxin 3 μ g/ml, 2',3'Dideoxiadenosine (DDOA) 300 μ M or H89 100 nM. * $p < 0.001$ compared to isoproterenol-treated diabetic cells. (C) Current density values of diabetic cardiomyocytes incubated for 20 hours at 4°C with vehicle, isoproterenol 1 nM or forskolin 10 and 100 nM or 8Br-cAMP 10 μ M. * is $p < 0.001$ compared to diabetic cardiomyocytes.



Kv4.3 proteins and samples were analyzed by fluorescence microscopy. Kv4.2 and Kv4.3 channel abundance were reduced in diabetic compared to healthy myocytes. However, diabetic myocytes incubated with isoproterenol showed normal channel protein abundance, similar to healthy cells (Fig. 2).

These results indicate that β AR stimulation restores the amount of Kv4.2 and Kv4.3 proteins in the cell membrane and therefore restores I_{to} current density. The following experiments aimed to identify the functional signalling pathway that connects β AR activation with the restoration of Kv4.3 and Kv4.2 channels.

Isoproterenol activates $G_{\alpha s}$ -AC-cAMP-PKA pathway in diabetic cardiomyocytes

β ARs belong to the G protein-coupled receptor superfamily and the major subtypes expressed in the mammalian heart are β_1 and β_2 . β_1 is the most abundant beta subtype in the heart, but β_2 is not less physiologically relevant as it can, for instance, protect cardiac myocytes from cell death after ischemia and reperfusion injury [25]. Although they differ in physiological roles and pharmacological properties, both activate the classic $G_{\alpha s}$ -AC-cAMP-PKA pathway, whereas only β_2 activates also $G_{\alpha i}$ protein [reviewed in 26]. We tested the implication of the classic pathway in the trophic effect on I_{to} by incubating diabetic ventricular myocytes with isoproterenol together with specific blockers or vehicle. As Figure 3 shows, inhibition of either $G_{\alpha s}$ by choleric toxin or adenylyl cyclase by 2',3'-DDOA prevented the isoproterenol-induced restoration of I_{to} density (11.2 ± 1.5 pA/pF, $n=14$, $p < 0.0001$ and 13.7 ± 0.9 pA/pF, $n=13$, $p=0.0004$ respectively). Similar results were obtained after protein kinase A blockade by H-89 (12.8 ± 1.2 pA/pF, $n=18$, $p < 0.0001$).

To further test the involvement of the AC, we incubated diabetic cells with the adenylyl cyclase activator forskolin expecting that this would reproduce the isoproterenol-mediated effects. Interestingly, forskolin showed different results depending on the concentration used. The 1 nM concentration only slightly recovered the I_{to} density (14.7 ± 1.9 pA/pF, $n=13$, ns with respect to diabetics), 10 nM partially restored I_{to} density (18.2 ± 1.3 pA/pF, $n=24$, $p < 0.0001$ compared to diabetic cells) but, 100 nM had no effect on the current, and remained significantly reduced (12.3 ± 0.9 pA/pF, $n=15$, ns with respect to diabetics). One possible explanation of this paradox is that high concentrations of forskolin might inhibit downstream components of the pathway such as c-Raf1 [27], as will be discussed below.

Fig. 4. Effect of β AR internalization on isoproterenol-induced I_{to} current restoration in diabetic cells. Representative traces (A) and current density values (B) of diabetic cardiomyocytes incubated for 20 hours at 4°C with isoproterenol 1 nM alone; isoproterenol plus with either β -ARK1 Inhibitor 0.1 mM, Concanavalin A 150 μ g/ml or pertussis toxin 1 μ g; and 8BrcAMP 10 μ M together with pertussis toxin 1 μ g/ml. * is $p < 0.001$ compared to isoproterenol-treated diabetic cells.

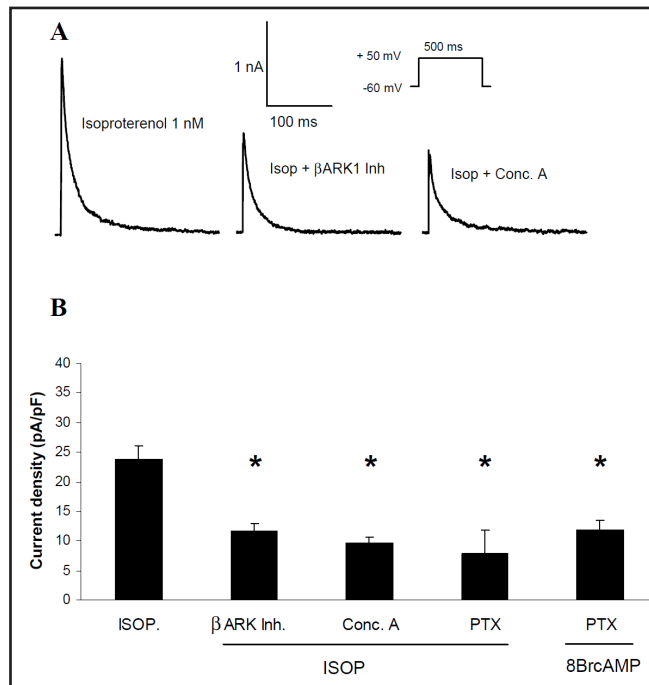
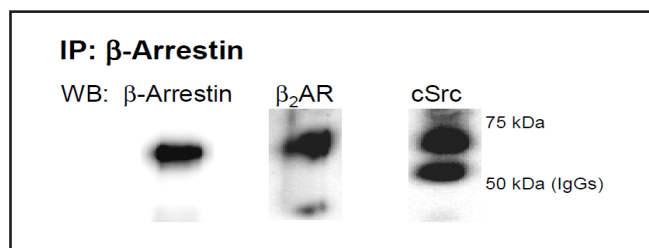


Fig. 5. Formation of the β_2 AR complex in stimulated diabetic ventricular myocytes. Ventricular myocytes isolated from diabetic hearts were stimulated for 5 minutes with isoproterenol 1 nM. After that, β -Arrestin was immunoprecipitated from myocytic samples with protein G-sepharose. The pellet from each immunoprecipitation experiment was probed with β -arrestin as control. β_2 AR and the downstream effector cSrc also coimmunoprecipitate with arrestin. Each image corresponds to representative western blots from 3 immunoprecipitation experiments. When possible, the western blotting antibodies were raised in different species than that used for immunoprecipitation to skip the IgGs band.

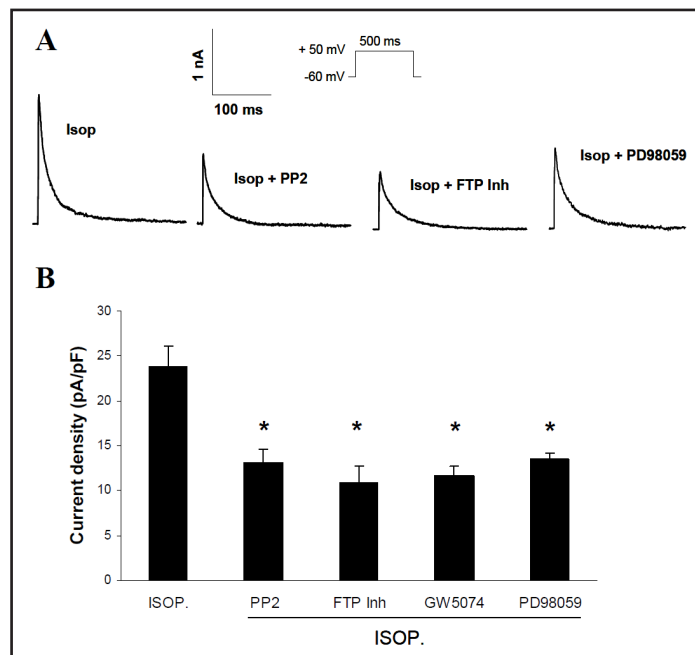


In order to avoid this experimental shortcoming, we used 8BrcAMP, a non-hydrolyzable analogue of cAMP. We incubated myocytes isolated from diabetic rats with 8BrcAMP 10 μ M and observed that it restores I_{to} density to control values (19.7 ± 1.1 pA/pF, $n=10$, n.s.).

G α i protein activation and receptor internalization are also required to restore I_{to} amplitude

After activating the $G\alpha_s$ -AC-PKA pathway, β_2 ARs can also couple to the pertussis-toxin-sensitive $G\alpha_i$ proteins [28]. Upon $G\alpha_i$ protein activation, the released $\beta\gamma$ subunits activate the β -adrenoceptor kinase 1 [29, 30]. This G protein-coupled receptor kinase (GRK2 or β -ARK1) phosphorylates the activated β AR, resulting in receptor desensitization and arrestin-dependent internalization. We wondered whether activation of $G\alpha_i$ protein, release of $\beta\gamma$ complex and further GRK-mediated phosphorylation and internalization of the β -AR were involved in the isoproterenol-mediated recovery of I_{to} current density (Fig. 4). To answer this question we first blocked the $G\alpha_i$ heterotrimeric protein activation using pertussis toxin and found that isoproterenol did not restore the I_{to} amplitude in diabetic cells (7.8 ± 4.1 pA/pF, $n=14$, $p=0.0027$). In addition, when $G\alpha_i$ protein is blocked by a 2h preincubation with PTX, incubation with 8BrcAMP for 20h does not recover the current density (11.8 ± 1.68 pA/pF, $n=12$, $p < 0.001$).

Fig. 6. Effect of cSrc/MAP kinase pathway on I_{to} current restoration. Representative traces (A) and current density values (B) of diabetic cardiomyocytes incubated for 20 hours at 4°C with isoproterenol 1 nM alone or together with either PP2 50 nM, FTP Inhibitor III 10 μ M, GW5074 1 μ M or PD98059 20 μ M. * is $p < 0.001$ compared to isoproterenol-treated diabetic cells.



We then inhibited the β AR phosphorylation by using β -ARK1 inhibitor. Again, we observed no restoration of I_{to} density in isoproterenol-treated diabetic myocytes (11.7 ± 1.3 pA/pF, $n=14$, $p < 0.0001$). Finally, we blocked the internalization of the phosphorylated β AR using the endocytosis inhibitor concanavalin A and obtained similar results (9.6 ± 1.0 pA/pF, $n=14$, $p=0.0002$). Taken consequently, these results indicate that both $G_{\alpha s}$ and $G_{\alpha i}$ pathways are required to increase the I_{to} current density in the diabetic myocardium upon isoproterenol treatment.

The β_2 AR-Arrestin-cSrc complex is functional in diabetic myocytes

Stimulation of MAP kinase pathway by β_2 ARs is mediated by the $\beta\gamma$ complexes from $G_{\alpha i}$ proteins through a pathway involving the recruitment of the tyrosine kinase cSrc by the β AR-Arrestin complex and the subsequent activation of the low-molecular-mass G protein Ras [31, 32]. As MAP kinase pathway increases the expression of certain proteins, one possibility is that in diabetic cardiac cells β AR stimulation leads to an increase on Kv4.2 and Kv4.3 channels in the cell membrane by activating Src-MAPK cascade.

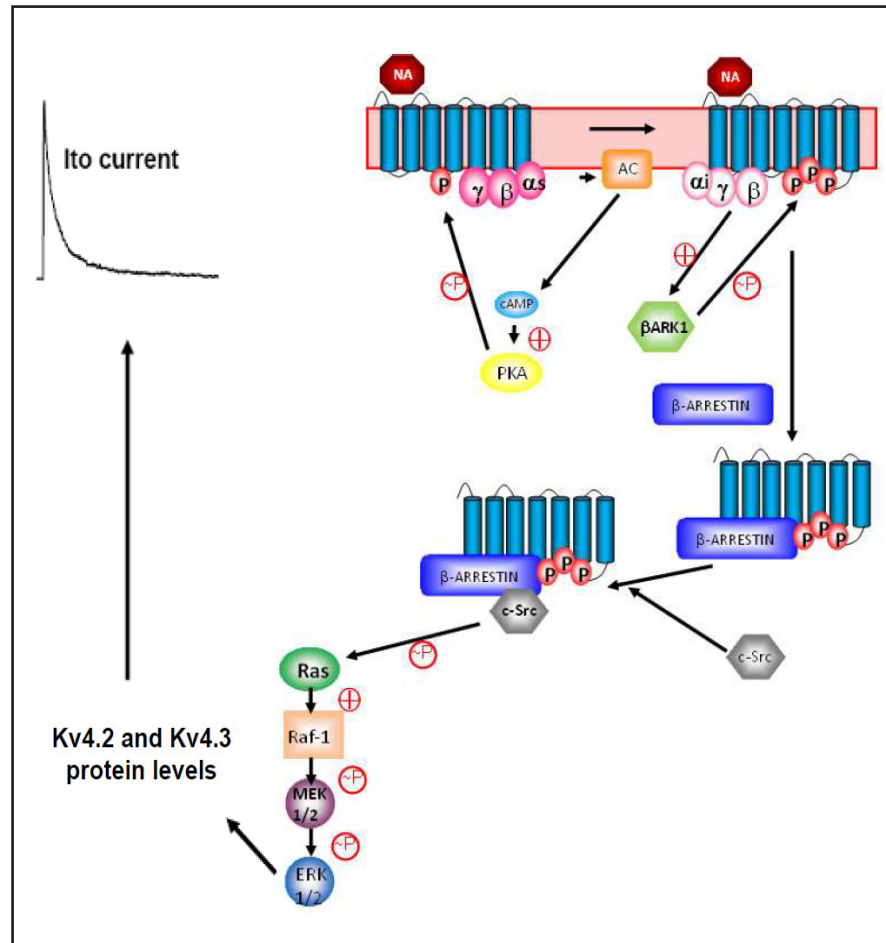
To confirm this hypothesis we tested the formation of the β_2 AR-Arrestin-cSrc complex. Diabetic myocytes were incubated with isoproterenol in order to activate the beta receptor and then Arrestin was immunoprecipitated. We performed incubations with isoproterenol for 0, 5, 10, 15, 20, 30 and 60 minutes, as well as for 18 hours. However, we only observed the β_2 receptor and the cSrc proteins in the pellet of the 5 minute incubation group (Fig. 5). Co-immunoprecipitation experiments in the rest of the groups were negative (data not shown) indicating the transient nature of the interaction.

Internalized β AR-cSrc complex activates MAPK pathway and thus restores I_{to} current

One of the possible targets of internalized β_2 AR is the Mitogen-Activated Protein (MAP) kinase pathway. MAP kinases can phosphorylate transcription factors and regulate transcription. In a classical MAP kinase pathway an external signal activates the MAP kinase kinase c-Raf-1. This phosphorylates the MAP kinase kinase MEK1/2 which then phosphorylates ERK1/2, the last cytoplasmic kinase in the cascade [reviewed in 33].

We tested this hypothesis by incubating the cells with isoproterenol while blocking the pathway at different levels. In a first group of experiments we used either the cSrc tyrosine kinase inhibitor PP2 or the Ras blocker FPT inhibitor III (13.1 ± 1.5 pA/pF, $n=9$, $p=0.0022$

Fig. 7. Summary of the transduction pathway connecting β AR stimulation with the restoration of I_{to} current amplitude in diabetic cardiomyocytes.



and 10.8 ± 1.9 pA/pF, $n=17$, $p<0.0001$ respectively). In the second group we inhibited the kinase activity of c-Raf-1 by GW5074 (11.7 ± 1.0 pA/pF, $n=20$, $p<0.0001$) or finally prevented ERK1/2 activation by PD98059 (13.5 ± 0.7 pA/pF, $n=44$, $p<0.0001$). Electrophysiological recordings show that incubation with each blocker prevented isoproterenol-induced I_{to} current restoration (Fig. 6).

Discussion

β -adrenoceptors are major regulators of cardiac physiology as they mediate catecholamine-induced inotropic, chronotropic and lusitropic responses. Therefore, β ARs transduction pathways have been extensively studied. However, our results describe for the first time the necessity of G_{α_s} , G_{α_i} and ERK activation for the isoproterenol-induced increase of Kv4.2 and Kv4.3 proteins in the cell membrane, providing a mechanism for the trophic effect of the sympathetic nervous system on I_{to} current in type I diabetes.

Dual coupling of β_2 ARs to G_{α_s} and G_{α_i} proteins have been described in cultured cells as well as in reconstituted systems [26, 34, 35]. Moreover, G_{α_s} to G_{α_i} switching mediates the isoproterenol-induced cardiac hypertrophy in neonatal rats [36]. Our results show that this dual coupling is also active in adult cardiomyocytes isolated from diabetic rats. Like in cardiac hypertrophy, incubation with isoproterenol activates first G_{α_s} and then G_{α_i} signalling pathways, leading to the activation of ERK kinase and, eventually, to an increase in Kv4 proteins in the plasma membrane in the type I diabetes.

Whereas some authors postulate that PKA-mediated β AR phosphorylation is a critical step for internalization, G protein switching and activation of ERK1/2 kinases [34, 35], others

propose that ERK activation is PKA-independent [37]. We have observed that adenylyl cyclase and PKA inhibition prevent isoproterenol-induced I_{to} restoration. This result indicates that protein kinase A is required for the increase in Kv4.2 and Kv4.3 protein levels. A reasonable explanation is that PKA phosphorylates the β AR, targets it for internalization and redirects the signalling pathway towards ERK activation. However, PKA might activate the MAP kinase pathway through a downstream mechanism different from β AR phosphorylation. This mechanism usually involves the PKA-dependent activation of Rap-1 thereby activating Src. However, Rap-1 is not found in ventricular myocytes. The alternative hypothesis is that two kinases, PKA and β -ARK1, contribute to phosphorylate and regulate the β AR. Consistent with this, we have found that isoproterenol also fails to restore I_{to} current density when the G protein receptor kinase β -ARK1 is inhibited.

Once activated, the $G\alpha_i$ protein releases its $\beta\gamma$ subunits activating ERK. However, the inhibition of AC activity by $G\alpha_i$ might contribute to this effect. In this regard, it has been reported that activation of PKA inhibits activation of c-Raf-1 [27, 38] and thus, increased concentration of cAMP inhibits MAP kinase activity. Therefore, inhibition of AC might relieve the inhibitory effect of PKA on Ras allowing the sequential activation of c-Raf-1 and the other members of the MAP kinase pathway. This is consistent with our results with the AC activator forskolin. In cells incubated with forskolin 10 nM but not 100 nM, the isoproterenol-induced restoration of I_{to} amplitude was complete. Thus, whereas the low concentration of forskolin (10 nM) could facilitate the G protein switching required for ERK activation, high concentrations (100 nM) might cause an exaggerated PKA activation that inactivates Raf1.

Taken together our results agree with the model proposed by Luttrell et al. [32] in cell cultures overexpressing the beta adrenoceptors. Thus, in diabetic cardiomyocytes β_2 AR stimulation activates the $G\alpha_s$ protein, adenylyl cyclase and PKA. The PKA-phosphorylated receptor then switches to a $G\alpha_i$ protein whose $\beta\gamma$ complex activates β -ARK1. Once dually phosphorylated by PKA and β -ARK1, the β_2 ARs internalizes in a process that requires Arrestin. The β_2 -Adrenoceptor-Arrestin complex recruits cSrc and activates Ras, which thereby activates MEK1/2 and finally ERK1/2. In our work, we found a physiological function for this intracellular pathway, where the MAPK cascade modulates the Kv4.2 and Kv4.3 protein levels in the cell membrane, and therefore I_{to} current amplitude, in response to sympathetic stimulation (Fig. 7).

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