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Role for the TRPV1 Channel in Insulin Secretion from Pancreatic Beta Cells

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Abstract Transient receptor potential channels have been put forward as regulators of insulin secretion. A role for the TRPV1 ion channel in insulin secretion has been suggested in pancreatic beta cell lines. We explored whether TRPV1 is functionally expressed in RINm5F and primary beta cells from neonate and adult rats. We examined if capsaicin could activate cationic non-selective currents. Our results show that TRPV1 channels are not functional in insulin-secreting cells, since capsaicin did not produce current activation, not even under culture conditions known to induce the expression of other ion channels in these cells. Although TRPV1 channels seem to be irrelevant for the physiology of isolated beta cells, they may play a role in glucose homeostasis acting through the nerve fibers that regulate islet function. At the physiological level, we observed that *Trpv1*$^{-/-}$ mice presented lower fasting insulin levels than their wild-type littermates, however, we did not find differences between these experimental groups nor in the glucose tolerance test or in the insulin secretion. However, we did find that the *Trpv1*$^{-/-}$ mice exhibited a higher insulin sensitivity compared to their wild-type counterparts. Our results demonstrate that TRPV1 does not contribute to glucose-induced insulin secretion in beta cells as was previously thought, but it is possible that it may control insulin sensitivity.

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Introduction

Glucose-stimulated insulin secretion depends on several ion channels that regulate the intracellular Ca^{2+} concentration and the exocytosis of insulin-containing granules in pancreatic beta cells (Hiriart and Aguilar-Bryan 2008). Recently, several transient receptor potential (TRP) channels have been reported in insulin-secreting cells (Islam 2011), which may directly participate in hormone release or function as a link between other signaling molecules and insulin secretion (Diaz-Garcia et al. 2013). The activity of some TRP channels is essential for proper glucose homeostasis, resulting in severe glucose intolerance when these channels are missing (Colsoul et al. 2010; Uchida et al. 2011) or acting as modulators of the responsiveness of peripheral tissues to the circulating insulin levels (Zhang et al. 2012).

The vanilloid member 1 of the family of transient receptor potential (TRPV1) channels has been related to the physiology of insulin-secreting cells; however, its role in this process is still a matter of controversy (Akiba et al. 2004; Jabin Fagelskiold et al. 2012; Mogi et al. 2013). TRPV1 is a polymodal stimuli sensor, which can be activated by temperatures above 41 °C, acid extracellular and basic intracellular pH, and by the binding of exogenous and endogenous ligands (Morales-Lazaro et al. 2013). This channel is expressed in the peripheral and central nervous systems, as well as in other non-neural tissues, playing a conspicuous role in nociceptive and inflammatory processes (Jara-Oseguera et al. 2008).

Indeed, some light has been shed on the role of TRPV1 on the metabolic status of mammalian organisms since it

was described as a regulator of glucagon-like peptide-1 (GLP-1) secretion (Wang et al. 2012), adipogenesis (Zhang et al. 2007), and neuronal excitability from the paraventricular nucleus that innervates the liver and controls glucose production (Gao et al. 2012). Furthermore, the administration of a TRPV1 antagonist in *ob/ob* mice, a model of type 2 diabetes mellitus (T2DM) and insulin resistance, reversed the elevated blood glucose and insulin levels of fasted animals, and improved glucose tolerance (Tanaka et al. 2011). It has been also reported that the elimination of capsaicin-sensitive fibers in Zucker diabetic fatty rats, another model of T2DM, prevents deterioration of glucose homeostasis, as observed by a better glucose tolerance and reduced glycated hemoglobin, which is indicative of insulin resistance (Gram et al. 2007). Moreover, *Trpv1*$^{-/-}$ mice are resistant to the high-fat diet-induced metabolic syndrome, and although their weight gain is similar to that of wild-type animals, they show reduced circulating pro-inflammatory cytokines, systolic blood pressure, and glucose intolerance (Marshall et al. 2012).

It has been suggested that TRPV1 is functionally expressed in rat insulinomas such as RINm5F cells and that it is present in the cell membranes of rodent primary endocrine cells from the pancreas, although no activation has been observed in native pancreatic beta cells (Akiba et al. 2004; Jabin Fagelskiold et al. 2012). This channel has also been related to the regulation of insulin secretion and islet inflammation through fibers that innervate pancreatic islets and also to the presence of insulin resistance by yet unraveled mechanisms (Razavi et al. 2006). However, the question of whether TRPV1 plays an important role in the development and/or physiology of normal native insulin-secreting cells needs further clarification.

In an effort to determine whether TRPV1 plays a role in pancreatic beta cells, here we studied glucose-stimulated insulin secretion in primary beta cells from wild-type (C57BL/6J) and *Trpv1*$^{-/-}$ adult mice and used electrophysiological and biochemical techniques to explore the presence of TRPV1 in primary beta cells from neonate and adult rats, as well as in the rat insulinoma cell line RINm5F. Finally, we explored the possible effect of beta-adrenergic blockade on insulin sensitivity of wild-type animals and *Trpv1*$^{-/-}$ mice. Our results shed light on the controversy of whether TRPV1 is expressed in primary pancreatic beta cells, as well as on its role in insulin resistance.

Material and Methods

Materials

Reagents were obtained from the following sources: collagenase P from Roche; insulin (Humulin®) from Eli Lilly y Compañía de México S.A de C.V.; nerve growth factor (NGF) 2.5S from Millipore; heparin (Inhepar®) from PiSA® Farmaceutica; bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), poly-L-lysine, trypsin, propranolol, and buffer salts were purchased from Sigma. Tissue culture dishes were from Corning; fetal bovine serum (FBS) from Equitech-BIO; Hank's balanced salt solution (HBSS), RPMI 1640 medium, and penicillin–streptomycin–amphotericin B solution from Life Technologies.

Experimental Animals

All methods used in this study were approved by the Animal Care Committee of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. Animal care was performed according to the *"International Guiding Principles for Biomedical Research Involving Animals", Council for International Organizations of Medical Sciences, 2010.*

Wistar rats were obtained from the local animal facility, maintained in a 12:12-h light-dark cycle (06:00–18:00), and allowed *ad libitum* access to standard laboratory rat diet and tap water.

Wild-type (WT) C57BL/6J and TRPV1 *knockout* (*Trpv1*$^{-/-}$) mice (Caterina et al. 2000) were obtained from The Jackson Laboratory and the latter were genotyped as previously reported (Nieto-Posadas et al. 2012).

Morphometric Analysis

Body weights from mice were measured using an analytical balance (Compax CX-6000). The sizes (nose to base of tail in supine position) and abdominal circumferences from mice were measured using a flexometer (Seretide® Diskus®), and the body mass index (BMI) was calculated.

Culture of Pancreatic Beta cells

Previous to surgery, rats or mice were anesthetized with an intraperitoneal pentobarbital sodium (40 mg/kg) injection and after pancreas dissections were performed, animals were euthanized by cervical dislocation.

Pancreatic beta cells were obtained following previously used techniques (Diaz-Garcia et al. 2012). Briefly, pancreatic islets were isolated and separated from the acinar tissue by collagenase digestion and a Ficoll gradient centrifugation; clean islets were then handpicked. Islet cells were dissociated by incubating them in a shaker bath for 10 min at 37 °C in calcium-free Spinner solution, with 15.6 mM glucose, 0.5 % BSA, and 0.01 % trypsin, followed by mechanical disruption. Single cells were cultured in RPMI-1640 medium, with or without NGF (50 ng/ml),

supplemented with 1 % FBS, 200 units/ml penicillin G, 200 mg/ml streptomycin, 0.5 mg/ml amphotericin B, and 200 mM L-glutamine. Neonate rat beta cells were cultured with NGF for two days, while adult rat beta cells were cultured in the presence of the growth factor for four days.

The rat insulinoma RINm5F (ATCC) was grown in RPMI 1640 medium supplemented 10 % FBS, 200 units/ml penicillin G, 200 mg/ml streptomycin, 0.5 mg/ml amphotericin B, and 200 mM L-glutamine. In experiments with NGF, the FBS was lowered to 1 % and cells were maintained for 2 days. Cell cultures were maintained at 37 °C in 5 % CO_2 and in 95 % air-humidified atmosphere.

Plasma Insulin Measurements

Fasted animals were anesthetized, and bled through the caudal vena cava using a sterile syringe. Central blood was gently placed in heparinized tubes and centrifuged at 8,870×*g* and 4–6 °C during 19 min, using an Eppendorf centrifuge 5415R. The supernatants were recovered and stored at −70 °C before use. Insulin levels in plasma were measured using an Ultrasensitive Rat Insulin Elisa kit (Mercodia Cat. 10–1173), following the manufacturer's instructions.

Intraperitoneal Glucose (IPGTT) and Insulin Tolerance Tests (ITT)

Glucose (2 g/kg body weight) or insulin (0.2 U/kg body weight) was injected intraperitoneally into overnight (12 h) fasted WT and TRPV1$^{-/-}$ mice. The total volume of inoculation was 10 µl/g body weight. Samples were obtained via tail bleeding. Blood glucose levels were measured at 0 (before injections) and 15, 30, 60, 90, 120, and 180 min after glucose or insulin administration, using a commercial blood glucose meter (Accu-Chek Active, Roche). For studying the effect of beta-adrenergic blockade on insulin sensitivity, a control insulin tolerance test was performed in each group. Two days later, mice were injected with propranolol (5 mg/kg body weight) and fasted during 12 h. A second insulin tolerance test was performed, inoculating the mice with a similar bolus of insulin plus a second dose of propranolol. A propranolol stock solution (10 mg/ml) was prepared in ethanol and 0.5 µl/g body weight of this solution plus 9.5 µl/g of a physiological saline solution were injected in mice.

Reverse Hemolytic Plaque Assay (RHPA)

To measure insulin secretion by individual beta cells from mice, RHPA (Neill and Frawley 1983) was performed as described previously (Hiriart and Ramirez-Medeles 1991). Briefly, after 1-2 day in culture, islet cells were detached from culture dishes, and equal volumes of islet cells were mixed with *Staphylococcus aureus* protein A–coated sheep erythrocytes, introduced to Cunningham chambers previously treated with poly-L-lysine to promote cell attachment, and incubated for 1 h.

Experiments were carried out at 5.6 or 15.6 mM glucose, 5.6 mM glucose in high KCl (40 mM) HBSS, and 5.6 mM plus NGF (50 ng/ml) in the presence of an insulin antiserum. Stimulating media were then replaced with HBSS with guinea-pig complement. Insulin release was revealed by the presence of hemolytic plaques around the mouse secreting cells, which result from the complement-mediated lysis of erythrocytes bearing insulin–anti-insulin complexes bound to protein A.

Each coverslip was scanned with a Leika Micro Dissection System 6000 (version 6.4.1.2887) coupled to a Hitachi HV-D20 camera, and the immunoplaque areas were measured with the aid of the image analyzer software ImageJ from NIH, USA (http://rsb.info.nih.gov/ij). Three independent experiments were performed by duplicate and an average of 150 cells was analyzed per replicate in each experimental condition, in each culture. The percentages of mouse insulin-secreting cells were also determined and the overall glucose-induced secretory activity of beta cells was calculated as a secretion index, by multiplying the average immunoplaque area by the fraction of plaque-forming cells.

Electrophysiology

Whole cell patch-clamp recordings (Hamill et al. 1981) were performed in RINm5F cells and isolated beta cells from adult and neonate rats, at room temperature (21–23 °C) using an Axopatch 200B amplifier (Axon Instruments). Patch electrodes were pulled from Kimax-51 capillary tubes (Kimble Glass) and had a tip resistance of 2 - 4 MOhms. Electrode tips were coated with Sylgard (Dow Corning) or dental wax to reduce pipette stray capacitance. After the whole cell configuration was obtained, series resistance was compensated by approximately a 50–70 % resulting in a voltage error ≤2 mV, and only recordings with stable gigaseals were analyzed. Cell capacitances were 6.4 ± 0.4 pF ($N = 16$) and 5.9 ± 0.4 pF ($N = 12$) for pancreatic beta cells from adult and neonate rats, respectively. Recordings were filtered at 2 kHz and digitized at a sampling frequency of 10 kHz. Pulse generation and data acquisition were performed with the PClamp software version 8 (Axon Instruments). Cationic non-selective (CAN) currents were elicited by pulse protocols of 250 ms long, 10 mV voltage steps, from −120 to +80 mV, starting from a holding potential of −80 mV. Current to voltage relationships were calculated by averaging the last 50 ms of each recording obtained by pulse protocols and plotted as a function of the membrane potential.

The extracellular solution used for recordings in rat beta cells was similar to the previously reported for cationic non-selective currents in beta cells (Diaz-Garcia et al. 2013), which contained (mM): 140 NaCl, 10 HEPES, 5 $MgCl_2$, 10 Glucose and 100 nM TTX; pH 7.4 and 290–300 mOsm/kg. The intracellular solution contained (mM): 140 NaCl, 10 HEPES, and 2 EDTA; pH 7.2 and 275–280 mOsm/kg.

For recordings in RINm5F cells, the pipette solution contained (mM): 100 CsAsp, 10 CsCl, 5 CsF, 20 NaCl, 10 HEPES, and 2.5 BAPTA; pH 7.2 and 275–280 mOsm/kg.

Western Blot

The total proteins extracts were obtained from cells suspended in lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 % NP-40) containing protease inhibitors (Roche) and phosphatases inhibitors (1 mM $NaVO_4$ and 10 mM NaF). After centrifugation, the supernatants were collected and quantified by the bicinchoninic acid method. Proteins were separated on 10 % SDS-PAGE gels and transferred to PVDF membranes (Millipore). Membranes were blocked with 6 % of dry milk in PBS/0.05 % Tween-20 and incubated overnight with the indicated antibody. For TRPV1 detection, the VR1 (P-19) antibody (Santa Cruz Biotechnology, Inc) and the anti-Rat TRPV1 extracellular antibody (Alomone Labs) were used. Detection of GAPDH (6C5, Millipore) was used as a load control and detected with the chemiluminiscence reagent system (Amersham Bioscience). For TRPV1 surface determination, plasma membrane proteins were isolated from RINm5F and HEK293 cells transiently expressing TRPV1 (as positive control) using the Pierce Cell Surface isolation, according to the manufacturer's instructions.

RT-PCR and Sequencing

Total RNA was isolated from RINm5F cells, primary cultures of rat beta cells and rat DRG neurons (positive control) using TRIzol reagent (Invitrogen), following the manufacturer's instructions; 2.5 µg of total RNA was reverse transcribed using random Hexamers (Invitrogen) and the SuperScript^{TM}III reverse transcriptase (Invitrogen), following the supplier's protocol. The PCR was carried out with the oligonucleotides indicated in Table 1 in a total volume of 50 µl containing 5 µl of cDNA solution, 1× PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 0.2 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 30 pmol of each primer, and 2.5 U of Taq DNA polymerase (Invitrogen) on a Thermal cycle for 35 cycles. For the sequencing reaction, the PCR bands were purified with a gel extraction kit (Promega) and 100 ng of purified DNA was sequenced with the Big Dye^{R}Terminator v3.1 Cycle Sequencing kit using 5 pmol of the antisense PCR1 primer or the antisense PCR2 primer.

Data Analysis

Data in each experimental group were analyzed for normality using a Kolmogorov-Smirnov test. In case of failure for normality, data were subjected to a square-root transformation (Latham 1978; Osborne 2010) and tested again. If normality was achieved before or after transformation, an unpaired *t Student's* test was performed for comparisons, applying a Welch correction in case of variance heterogeneity. If data could not be adjusted to a Gaussian distribution after transformation, a non-parametric Mann-Whitney test was chosen for analysis, using GraphPad InStat version 3.00 (GraphPad Software). Graphics were constructed using Origin version 7 (OriginLab Corporation).

Results

Do TRPV1 Channels Play a Role in Insulin Secretion?

It has been previously reported that insulin secretion by the RINm5F rat insulinoma cell line is stimulated by the application of capsaicin at concentrations well below (1 nM) (Akiba et al. 2004) the ones required to activate half of the response (EC_{50}) of TRPV1 (near 300 nM) (Nieto-Posadas et al. 2011) but not by higher concentrations of this agonist of TRPV1 channels (Akiba et al. 2004). Although the only capsaicin concentration tested in this other study that stimulated insulin secretion from RINm5F cells was that of 1 nM (Akiba et al. 2004), it was indeed suggested that the expression and/or function of TRPV1 may play an important role in the physiology of these cells. Nonetheless, a physiologically relevant role for TRPV1 in primary pancreatic beta cell cultures has not been previously addressed. In order to characterize the role of TRPV1 and determine its functional properties in a model of native pancreatic beta cells, we explored insulin secretion from primary cultures of single beta cells from WT and *Trpv1*$^{-/-}$ mice. Beta cells were incubated with 5.6 or 15.6 mM glucose (that resemble basal and stimulating concentrations of the secretagogue); 40 mM K^+ (that depolarizes beta cells and bypasses the metabolic phase of the glucose-induced insulin secretion process), and NGF (50 ng/ml) in HBSS with 5.6 mM glucose (to try to sensitize TRPV1 channels (Stein et al. 2006) if they were present in these cells).

To our surprise, in our experiments, using the RHPA technique to evaluate insulin secretion from isolated single beta cells from WT and *Trpv1*$^{-/-}$ mice (Fig. 1a), we

Table 1 PCR conditions

Product name	Size (bp)	Rat mRNA TRPV1 localization	Primer sequence (5′–3′)	Temp alignment (°C)
PCR1	1,085	Exon 2 to Exon 7	sensePCR1: CCACATCTTCACTACCAGGAGTCGTACCCG antisensePCR1: CGATCACCTCCAGAACCGAG	59.3
PCR2	1,055	Exon 7 to Exon 15	sensePCR2: CTCGGTTCTGGAGGTGATCG antisensePCR2: CCACCCTGAAACACCACCGGTAGTCATCCT	57.5
PCR1.1	663	Exon 2 to Exon 5	sensePCR1.1: ATGGAACAACGGGCTAGCTTAGACT antisense PCR1.1: CACCAAGAGGGTCACCAGCGTCATGTTCCG	60
PCR2.1	314	Exon 14 to Exon 16	sensePCR2.1: AGGATGACTACCGGTGGTGTTTCAGGGTGG antisensePCR2.1: TTATTTCTCCCCTGGGACCATGGAATC	59.8

The table summarizes amplicon sizes, exons comprised in the amplification products, primer sequences and temperatures of alignment in each PCR

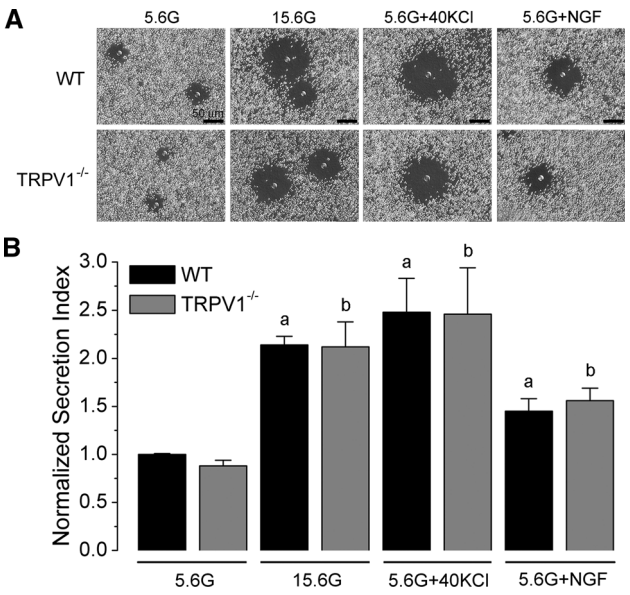

Fig. 1 Insulin secretion does not differ between isolated beta cells from WT and *Trpv1*$^{-/-}$ mice. **a** Representative micrographs obtained by a RHPA, by incubating with 5.6 and 15.6 mM glucose alone (5.6G and 15.6G, respectively), or with 5.6 mM glucose plus 40 mM KCl (5.6G+40KCl) or 50 ng/ml NGF (5.6G+NGF), in the presence of an insulin antibody and complement. Insulin secretion is proportional to the immunoplaque areas (*dark regions* around beta cells). *Scale bar* 50 µm. **b** Normalized secretion indexes for beta cells from WT and *Trpv1*$^{-/-}$ in each condition. *Bars* represent mean ± SEM of three different experiments by duplicate, except for experiments with NGF (two different cultures by duplicate) where at least 150 cells were measured per chamber. $^{a}p < 0.05$ and $^{b}p < 0.05$ for comparison between basal and stimulated insulin secretion for WT and *Trpv1*$^{-/-}$ mice, respectively

observed that cells from both animal groups exhibit a similar secretion index at basal glucose conditions, while these parameters increase more than twofold with 15.6 mM glucose and 40 mM KCl, and nearly by 50 % in the presence of NGF (Fig. 1b), without differences between cells from both types of animals in each condition. These data have a two-pronged implication: (1) Primary pancreatic beta cells do not express functional TRPV1 channels or (2) this channel is not important for insulin secretion.

Pancreatic Beta cells from Neonate or Adult Rats do not Express Functional TRPV1 Channels

The presence of TRPV1 mRNA has been previously shown for islet cells and the expression of TRPV1 protein in the pancreas has been suggested using whole-organ extracts (Akiba et al. 2004). In an effort to solve the conundrum of whether functional TRPV1 channels were present in isolated pancreatic beta cells from primary cultures, we used capsaicin (Morales-Lazaro et al. 2013) to probe for the presence of cationic non-selective currents (CAN) in cells from adult and neonate rats. We failed to see the activation of TRPV1-mediated currents (Fig. 2a). We were only able to observe, from the current to voltage (IV) relationships, currents with an outward rectification and a reversal potential close to 0 mV (Fig. 2a), as usually occurs in recordings from CAN currents from native beta cells using symmetrical Na^{+} concentrations (Colsoul et al. 2010) but these were not different before and after the application of capsaicin.

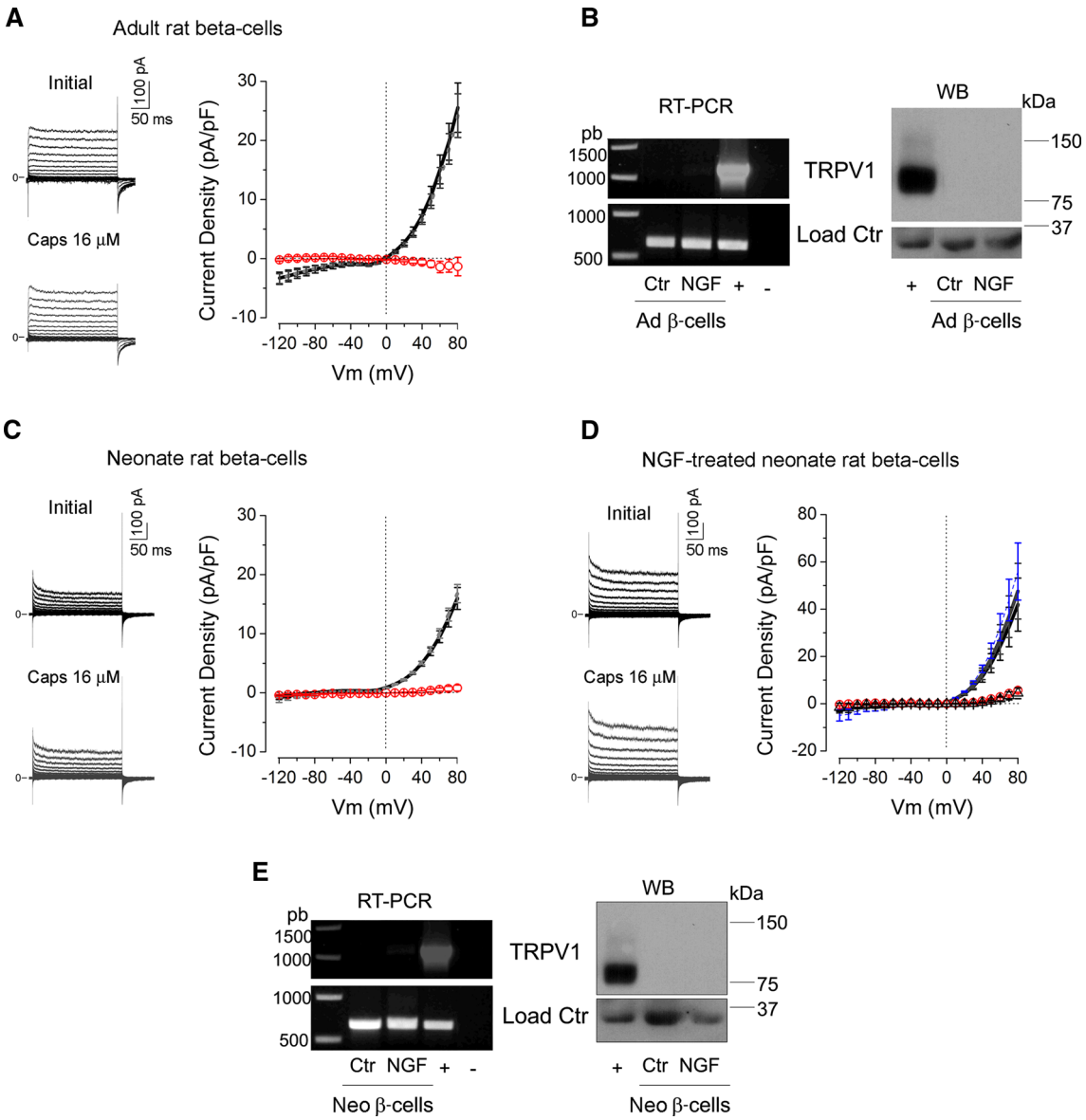

Fig. 2 Pancreatic beta cells do not express functional TRPV1 channels. **a** The left panel shows representative current traces of an adult rat beta cell before (Control) and after application of capsaicin (Caps 16 µM). IV relationships of Control (*black line*) and Caps 16 µM-treated (*gray line*) adult rat beta cells ($N = 16$) are overlapped. The subtraction of the initial IV curve from the IV curve after capsaicin application (open circles) exhibits values near zero for all the membrane potentials tested. **b** RT-PCR products (*left*), using the pair of oligonucleotides for PCR2 in control (Ctr) and NGF-treated adult rat beta cells (Ad β-cells). Total RNA from DRG neurons was used as a positive control (+). A PCR reaction without template cDNA was used as negative control (−) Western blots (WB, *right*) of total protein from adult rat beta cells (40 µg). Total protein (10 µg) from DRG neurons was used as a positive control for TRPV1 detection and GAPDH was used as load control (Load Ctr). **c** Current traces and IV relationships of neonate rat beta cells ($N = 5$) before (Control, *black line*) and after application of Caps 16 µM (*gray line*). **d** Same representation as in (**c**) for neonate rat beta cells which were treated with NGF ($N = 4$). The dotted line represents the IV relationships of NGF-treated neonate rat beta cells after application of vehicle (0.4 % ethanol in the bath solution, $N = 3$). Subtractions of the initial IV relationship from the IV relationship after capsaicin (*open circles*) or vehicle application (*black open triangles*) are also shown. **e** RT-PCR in total RNA from neonate rat beta cells (Neo β-cells). WB of total protein from neonate (20 µg) beta cells untreated (Ctr) or treated with NGF (Color figure online)

These data are in good accordance with our results obtained using the RT-PCR method where amplification products corresponding to TRPV1 transcripts were not observed in the primary cultures of beta cells from adult rats (Fig. 2b, left panel). Previous experiments from our group have shown that incubation of pancreatic beta cells with nerve growth factor (NGF), a molecule that is synthesized and secreted by these cells, can promote the expression of ion channels (Rosenbaum et al. 1998; Vidaltamayo et al. 2002). To determine if NGF could promote

a similar effect on TRPV1 expression, we cultured cells during 4 days with this factor and found that a weak signal corresponding to the putative size of the TRPV1 mRNA became detectable by PCR (Fig. 2b, left panel). To determine if the small amount of mRNA detected in NGF-treated cells could be translated into protein, the total protein levels for TRPV1 were then analyzed by Western blot (WB), using an antibody against the N-terminal domain of TRPV1. Positive signals, however, were not observed in extracts of total proteins from control and NGF-treated beta cells from adult rats (Fig. 2b, right panel), indicating that NGF does not induce the expression of TRPV1 protein.

Functional TRPV1 channels have been reported in insulin-secreting cell lines and tumors, which are dedifferentiated cells (Jabin Fagelskiold et al. 2012; Mergler et al. 2012). Moreover, some ion channels are expressed in cells during different developmental stages (Wang et al. 2013; Zsiros et al. 2009). In an effort to uncover a possible role for TRPV1 as a marker of immature pancreatic beta cells, which may differentially express some ion channels compared with those present in adult cells (Jermendy et al., 2011), the effect of capsaicin was analyzed on currents of pancreatic beta cells from neonate rats. In these experiments, capsaicin (16 μM) also failed to produce the activation of TRPV1-mediated currents (Fig. 2c). Finally, it has been demonstrated that NGF potentiates TRPV1 currents in heterologous and native-expression systems (Stein et al. 2006) and improves the excitability of neonate beta cells, partly, by increasing the expression of voltage-gated Ca^{2+} channels (Navarro-Tableros et al. 2007b). Thus, we incubated neonate beta cells for 48 h with NGF and after this time the cells exhibited no response to capsaicin (Fig. 2d) either, as observed from the indistinguishable experiments with cells before and after vehicle application (Fig. 2d, dotted line and black open triangles, right panel). Accordingly, when we performed experiments to determine the mRNA levels for TRPV1, the results obtained using RT-PCR to study mRNA levels and our biochemical assays to assess the total protein (WB) levels of TRPV1 in neonate beta cells in the presence or absence of NGF, showed that mRNA for TRPV1 is faintly present and that the protein is not produced in pancreatic beta cells, contrasting the result obtained with DRG neurons (Fig. 2e), which natively express this ion channel. This is similar to what we observed in the aforementioned experiments in primary cultures from islet cells of adult rats (Fig. 2b).

TRPV1 is Not Localized to the Plasma Membrane of RINm5F Cells

The expression of TRPV1 has been previously suggested in RINm5F cells by revealing the presence of mRNA for the channel (Akiba et al. 2004). In our experiments, we also detected RT-PCR amplification products for TRPV1 mRNA from RINm5F cells (Fig. 3a, left panel) similar to what we observe with the NGF-treated primary pancreatic beta cells, as described above in Fig.2e. However, the presence of mRNA does not ensure that a complete form of the channel or an alternative splice-variant is not encoded (Schumacher et al. 2000; Vos et al. 2006; Wang et al. 2004). The RT-PCR amplification products we obtained using several pairs of oligonucleotides (see "Methods"), were sequenced and then aligned to the reported TRPV1 mRNA sequence (*Rattus norvegicus* Trpv1 mRNA GI: 14010882). The percentage of similarity between our sequenced RINm5F cell-TRPV1 product and the expected Trpv1 transcript was of 99.9 %.

The latter result excludes that the expression of a negative dominant splice-variant form of Trpv1 (i.e., lacking exon 7) could account for the absence of capsaicin responsiveness in RINm5F cells, as has been described in other cells (Vos et al. 2006). Even though the mRNA coding for *Trpv1* is present in the cells, the protein is not necessarily produced and/or exported to the plasma membrane. In this regard, a weak band corresponding to TRPV1 was observed in extracts of total proteins from RINm5F cells. However, we could not detect TRPV1 in membrane-only protein extracts from these cells, despite of a protein load of approximately 20-fold as compared to the amount of the positive control loaded (Fig. 3a, right panel).

RINm5F cells also expresses receptors to NGF (Polak et al. 1993). However, under our experimental conditions, we could not observe activation of capsaicin-evoked currents in NGF-treated RINm5F cells (Fig. 3b), which shows that functional TRPV1 channels are not found in the RINm5F insulinoma, even after a long-term incubation with NGF for 48 h (Fig. 3c).

TRPV1 Regulates Insulin Sensitivity Through the Adrenergic Tone

The aforementioned data support the notion that TRPV1 does not participate in insulin secretion by pancreatic beta cells since it is simply not functionally expressed in these cells. However, some of our experimental evidence points to a possible role of TRPV1 in glucose homeostasis. On one hand, the average body weight and size of $Trpv1^{-/-}$ mice were decreased in more than 18 and 5 %, respectively, as compared to their WT littermates, and on the other hand, the abdominal circumference presented a modest, but statistically significant, reduction of around 5 % in $Trpv1^{-/-}$ animals (Fig. 4a). Although the body mass index did not differ between both experimental groups (3.29 ± 0.11 and 3.08 ± 0.05 kg/m^2 for WT and $Trpv1^{-/-}$ mice, respectively, $p = 0.1014$), these data

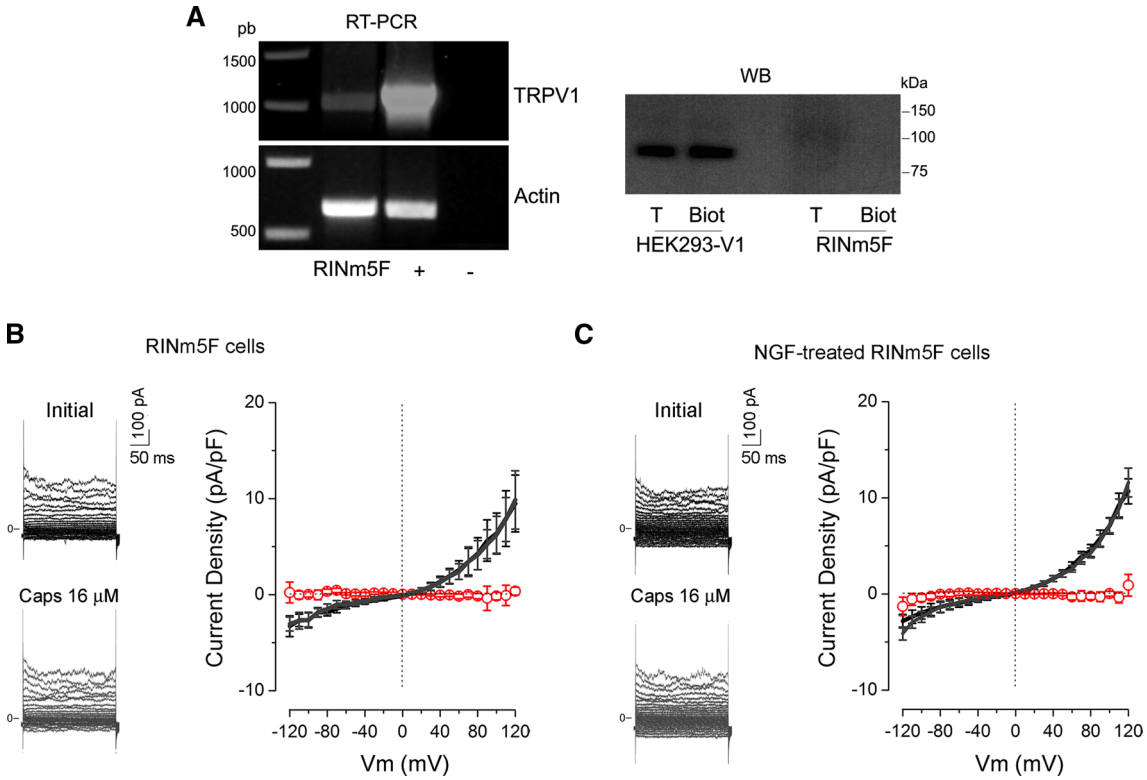

Fig. 3 TRPV1 is not-expressed in the plasma membrane of RINm5F cells. **a** RT-PCR products (*left*), using the pair of oligonucleotides for PCR2. Total RNA from DRG neurons was used as a positive control (+) and a PCR reaction without template cDNA was used as negative control (−). The right panel shows total (T, 80 µg) and biotinylated (Biot) proteins obtained from RINm5F and HEK293 cells expressing TRPV1 (positive control). **b** The *upper panel* shows current traces of a RINm5F cell before (Control) and after application of Caps 16 µM. The lower panel shows the IV relationships of Control (*black line*) and Caps 16 µM-treated (*gray line*) RINm5F cells ($N = 3$). Subtraction of the initial currents from the currents after capsaicin application (*open circles*), exhibiting an IV curve with values near zero for all the membrane potentials tested. **c** Same representations as in (**b**) for RINm5F cells which were treated with NGF ($N = 5$) (Color figure online)

indicated that *Trpv1*$^{-/-}$ mice presented a tendency to be leaner and potentially different from WT animals.

It has been described that insulin levels can be reduced by antagonizing the TRPV1 activity in fasted obese mice (Tanaka et al. 2011). Thus, we performed insulin measurements in WT and *Trpv1*$^{-/-}$ mice to determine if the absence of TRPV1 channels impacts on the circulating insulin levels of non-obese animals. We found that they were highly variable in WT animals (95 % confidence interval 0.0917–0.7925, $N = 14$), but not in *Trpv1*$^{-/-}$ mice (95 % confidence interval 0.1007–0.1639, $N = 13$). Indeed, fasted *Trpv1*$^{-/-}$ animals presented a linear 3.3-fold reduction in plasmatic insulin levels with respect to WT mice (Fig. 4b) and surprisingly their glucose tolerance curves overlapped (Fig. 4c).

Considering that insulin levels were different between WT and *Trpv1*$^{-/-}$ mice, we decided to perform an intraperitoneal glucose tolerance test to analyze how this phenomenom impacts glucose homeostasis in *Trpv1*$^{-/-}$ mice. We chose the intraperitoneal instead of the oral pathway because it is known that *Trpv1*$^{-/-}$ mice show an altered oral glucose tolerance test as TRPV1 regulates GLP-1 secretion in the gut (Wang et al. 2012), which could result in difficulties in the interpretation of the data. Moreover, with the intraperitoneal administration of glucose, it was possible to bypass the absorptive processes in the intestines as well as the potential differences in incretin release and incretin-mediated enhancement of insulin secretion between WT and *Trpv1*$^{-/-}$ mice.

Interestingly, glucose levels of fasted animals were similar in both groups and they increased after an intraperitoneal glucose challenge of 2 g/kg of body weight. In these experiments, blood glucose levels peaked at 15 min and basal levels recovered at the end of the test. Both the peak glucose and the decay to the basal level reflect the balance between glucose-induced insulin release and glucose uptake by peripheral tissues in response to the secreted insulin. It is expected that a reduction in the circulating insulin could account for an impaired glucose homeostasis, yielding hyperglycemic and glucose-intolerant animals. However, *Trpv1*$^{-/-}$ mice show a normal glucose homeostasis, which could be explained by a higher responsiveness to insulin actions.

Insulin sensitivity was further explored through an insulin tolerance test by administering an intraperitoneal

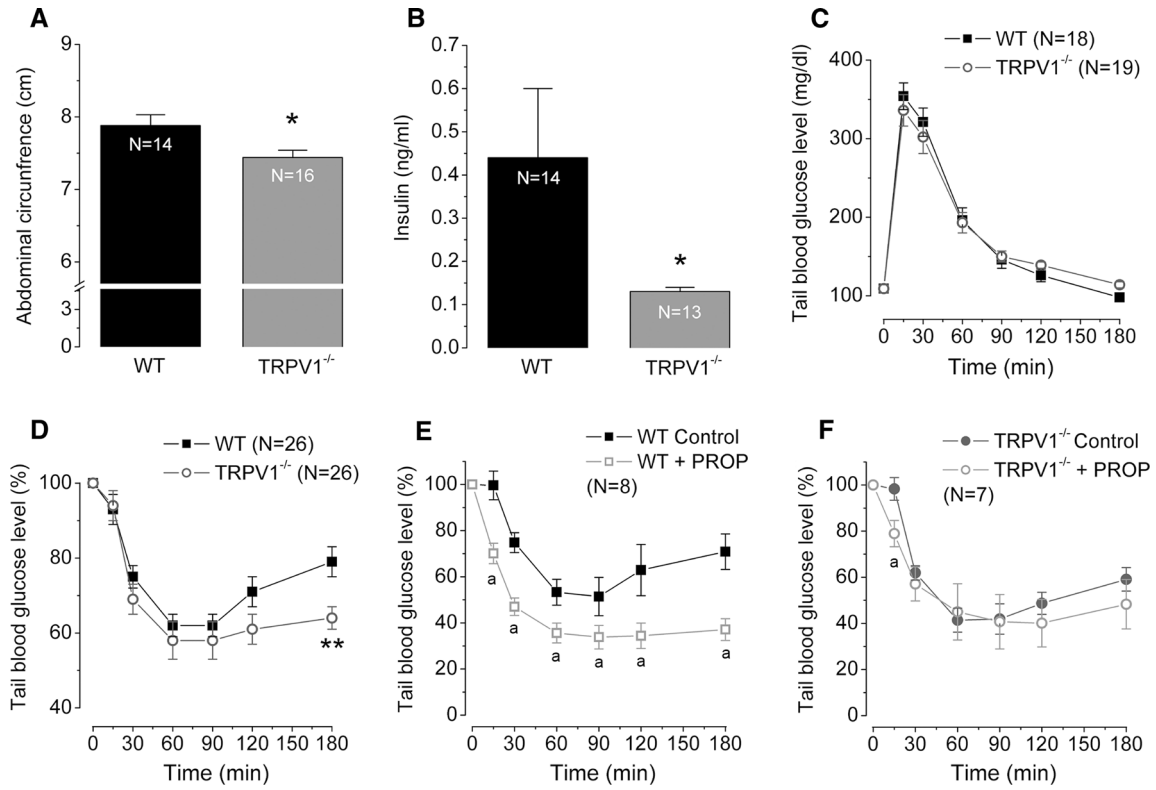

Fig. 4 Absence of TRPV1 prevents the increase of insulin sensitivity after blockade of beta-adrenergic receptors. **a** *Trpv1*$^{-/-}$ mice show a reduced abdominal circumference and fasting blood insulin levels **b** with respect to WT animals. **c** Both groups showed a similar IPGTT. **d** Insulin administered intraperitoneally in *Trpv1*$^{-/-}$ mice caused a significant decrease in blood glucose levels with respect to WT animals. **e** Glucose levels were significantly reduced at all times in an ITT in WT animals after treatment with propranolol. **f** Treatment with propranolol caused a reduction in glucose levels only at 15 min in *Trpv1*$^{-/-}$ mice. $*p < 0.05$ and $**p < 0.01$ for comparisons between WT and *Trpv1*$^{-/-}$ mice. $^{a}p < 0.05$ for comparisons between control and propranolol-treated groups

insulin dose of 0.2 U/kg, which resulted in a 40 % decrease of the blood glucose levels to a plateau at 60–90 min. After this, glucose levels partially recovered at different rates, which were more prominent in WT mice (79 % of basal) and that led to a significant 15 % reduction in the average blood glucose level of *Trpv1*$^{-/-}$ animals at 180 min (Fig. 4d). The sustained effect of insulin in lowering the blood glucose levels of *Trpv1*$^{-/-}$ mice was evidenced in an increased insulin sensitivity of these animals in comparison to their WT littermates.

It is known that beta-adrenoceptors are expressed both in pancreatic beta cells and insulin-responsive tissues, and their genetic ablation causes increased insulin sensitivity and diminished glucose-stimulated insulin release (Asensio et al. 2005). Finally, in an effort to study the mechanisms by which TRPV1 function could affect glucose homeostasis, we used propanolol, a blocker of beta-adrenoceptors, to determine if the sympathetic tone was contributing to the differences in insulin sensitivity between WT and *Trpv1*$^{-/-}$ mice. We analyzed its effects on insulin tolerance tests, since it is known that propranolol ameliorates the catecholamine-dependent insulin unresponsiveness in skeletal muscle from diabetic rats (Bostrom et al. 1989). Treatment with propranolol caused an increase in the blood glucose levels of fasted animals, 103 ± 8 versus 165 ± 9 mg/dl in WT ($p < 0.01$, $N = 8$) and 119 ± 12 versus 176 ± 12 mg/dl in *Trpv1*$^{-/-}$ mice ($p < 0.01$, $N = 7$) indicating a mild glucose intolerance due to a reduced insulin release by pancreatic beta cells (probably due to the blockade of beta-2 adrenoceptors), as occurs in models of genetic ablation of beta-adrenoceptors (Asensio et al. 2005).

We also performed insulin tolerance tests in WT and *Trpv1*$^{-/-}$ mice before and after propranolol treatment. To compare between treated and non-treated animals in both groups, all measurements were normalized by the levels of glucose obtained under fasted conditions and changes after insulin administration were expressed as percentages. Our results show that the propranolol-treated group of WT mice exhibited a robust reduction in glucose levels with respect to control animals at all times. At 180 min, the glucose levels of propranolol-treated WT mice were decreased as compared to the control animals by $\sim$30 % (Fig. 4e). On the contrary, the effect of propranolol was almost absent in *Trpv1*$^{-/-}$ mice, causing a significant decrease of glucose

levels only at 15 min, and following a similar time course from 30 to 180 min (Fig. 4f). The latter indicates that *Trpv1*$^{-/-}$ mice are characterized by a reduced beta-adrenergic responsiveness, which could account for the modest effect of propranolol in these animals, compared to the robust increase in insulin sensitivity observed in WT mice.

Discussion

Since the year 2004, some studies have suggested that TRPV1 may be functionally relevant for insulin secretion by pancreatic beta cells (Akiba et al. 2004; Waluk et al. 2012; Yang et al. 2011). However, these studies have not unequivocally assessed for the presence of TRPV1 channels: they did not measure the direct activity of TRPV1 channels and/or they have not used completely unambiguous pharmacological tools. Here, we detailed the possible methodological reasons that may originate the contrasting outcomes from the previous studies with respect to our own work.

For example, the study conducted by Akiba et al. (2004), who detected Trpv1 transcripts in RINm5F cells, did not test for the presence and stability of the putative Trpv1 membrane protein. Although Akiba et al. (2004) observed staining for TRPV1 in islet cells, the antibody was not competed with a blocking peptide to disregard spurious signals (Akiba et al. 2004), as we observed in our experiments with certain antibodies (data not shown).

In the study of Akiba et al. the effect of capsaicin on insulin secretion was analyzed after an hour of incubation with this agonist. Furthermore, Akiba et al. observed that the most prominent effect of capsaicin in RINm5F cells was achieved at a concentration of 1 nM, which is far from the reported EC_{50} of TRPV1 activation by this ligand ($\approx$300 nM) (Nieto-Posadas et al. 2011). During prolonged periods of time with capsaicin, the channel not only desensitizes in the presence of extracellular Ca^{2+} (Touska et al. 2011) but it also is endocytosed (Sanz-Salvador et al. 2012). Moreover, it is not known if a long-term application of capsaicin could affect beta cell physiology as occurs in other tissues where it causes oxidative stress and apoptosis by TRPV1-independent mechanisms (Hail 2003). Besides, inferences on insulin secretion were made by the authors using pharmacological approaches that could be misleading (i.e., capsazepine could also modulate other channels present in beta cells such as low- and high-voltage activated Ca^{2+} channels (Castillo et al. 2007; Docherty et al. 1997).

Other authors have proposed an effect of low concentrations of capsaicin (0.1–1 nM) on the stiffness of membranes from insulin-secreting cells (Yang et al. 2011). Moreover, changes in the biophysical properties of plasma membrane do not necessarily reflect TRPV1-channel activation, as it is also known that capsaicin affects membrane fluidity and stability (Hail 2003).

Recently, Fagelskiold et al. have reported specific TRPV1 signals in protein extracts from INS-1E and human islets using Western blot analysis (Jabin Fagelskiold et al. 2012). The presence of functional TRPV1 channels in INS-1E cells has been demonstrated using microfluorimetric Ca^{2+} measurements and whole-cell patch clamp recordings (Jabin Fagelskiold et al. 2012). However, these authors could not detect capsaicin-evoked microfluorimetric signals in primary pancreatic beta cells from rats and humans (Jabin Fagelskiold et al. 2012). Moreover, no electrophysiological recordings were performed in these cells in order to provide an improved resolution of TRPV1 activity by allowing the study of channel-mediated currents (Neher 1992).

The information available from other studies raises issues that are unresolved and that we aimed to answer in the present study: Are TRPV1 channels relevant to the glucose-induced insulin secretion process? Are TRPV1 channels functionally present in the plasma membrane of beta cells? Could TRPV1 channels be differentially expressed, or induced, in beta cells at an early developmental stage? And finally, do TRPV1 channels play a role in insulin homeostasis?

In an effort to clarify the role of TRPV1 in insulin secretion, we have used here a combination of experimental tools to assess how TRPV1 may regulate insulin secretion. To corroborate if TRPV1 was relevant to insulin secretion, *Trpv1*$^{-/-}$ mice were used as a model of genetic deletion. Our results from the RHPA indicate that the absence of TRPV1 neither affected the basal nor the glucose-stimulated insulin secretion, meaning that TRPV1 does not play an intrinsic role in beta cells during insulin release. Furthermore, the genetic ablation of TRPV1 is not responsible for a change in the expression of other voltage-gated ion channels resulting in undetectable impairment of insulin-granule exocytosis in response to high KCl-induced depolarization. This evidence also suggests that TRPV1 channels are irrelevant for vesicle trafficking in beta cells, as opposed to certain neurons where TRPV1 regulates the exocytosis and vesicle recycling (Goswami et al. 2010; Morenilla-Palao et al. 2004). Moreover, the ability of NGF to sensitize beta cells and promote insulin secretion was preserved in both groups, indicating that the regulatory pathway, along with the metabolic, ionic, and exocytotic machineries of beta cells remain intact even when TRPV1 is absent.

If TRPV1 channels were present in these cells, we would expect to detect the expression of its transcripts, protein and/or agonist-induced currents, as has been the case for TRPA1 channels in RINm5F and primary rat beta

cells (Cao et al. 2012). However, as evidenced from our electrophysiological experiments, this study shows that TRPV1 is not functionally expressed in primary pancreatic beta cells or the rat insulinoma RINm5F, even when the latter was incubated with NGF during 2 days, which is known to promote the expression of ion channels in beta cells (Navarro-Tableros et al. 2007a). Although we found detectable levels of Trpv1 mRNA in long-term NGF-treated adult and neonate beta cells, Western blot experiments confirmed the lack of expression of TRPV1 in these cells. On the other hand, RINm5F cells expressed detectable levels of mRNA for TRPV1, but the resulting protein does not reach the plasma membrane. Our evidence indicates that the previous reports on TRPV1 activity in RINm5F cells are not due to the expression of functional channels, and more importantly, that TRPV1-expressing cell lines do not adequately reflect the ion channel composition of native beta cells.

In our study, *Trpv1*$^{-/-}$ mice were normoglycemic, coinciding with the results of Marshall and colleagues (Marshall et al. 2012). As *Trpv1*$^{-/-}$ mice exhibited increased insulin sensitivities as compared to that of the WT animals, this could compensate for the diminished insulin levels in the blood of the animals. Our results regarding insulin sensitivity are also in agreement with those of Razavi and colleagues (Razavi et al. 2006), however, our results do contrast theirs in that they observed an improved glucose clearance in *Trpv1*$^{-/-}$ animals and we did not. There are some issues that could account for this difference. Razavi et al. used younger mice (5 animals of 6–8 week-old per group, vs. 18–19 animals of 8–10 week-old in our study) and they also obtained higher glucose levels at the beginning of the glucose tolerance test ($\approx$10 mM or 180 mg/dl), which suggests that their fasting process was not similar to our protocol, in which fasted glucose levels of 109 mg/dl were achieved.

Comparisons between *Trpv1*$^{-/-}$ and wild-type mice regarding body weight measurements have resulted in divergent outcomes (reviewed in Ahern 2013). For instance, Caterina et al. described that *Trpv1*$^{-/-}$ mice did not exhibit alterations in the body weight with respect to their wild-type littermates (Caterina et al. 2000), which is in agreement with other reports (Luo et al. 2012; Pabbidi et al. 2008). Other authors, however, have reported body mass gain in aging *Trpv1*$^{-/-}$ mice (Garami et al. 2011; Wanner et al. 2011) or a reduced body mass in *Trpv1*$^{-/-}$ with respect to WT mice (Taylor et al. 2008). Under our experimental conditions, we observed a reduction in the body weight of *Trpv1*$^{-/-}$ mice and a tendency to a decreased body mass index.

We focused on the fact that the absence of TRPV1 increases insulin sensitivity, since it could be an important target in the treatment of metabolic diseases. Furthermore, it has been reported that capsaicin-treatment denervated animals exhibit a reduction of the circulating epinephrine and norepinephrine levels in response to a hypoglycemic challenge (Fujita et al. 2007). Thus, we hypothesized that a reduced adrenergic tone in *Trpv1*$^{-/-}$ mice could be in part responsible for the increased insulin sensitivity so we used propranolol to block beta-adrenergic receptors.

The most outstanding outcome from these experiments was that blockade of beta-adrenoceptors in normal mice causes an increment in insulin sensitivity in peripheral tissues. In the *Trpv1*$^{-/-}$ mice, however, propranolol could not enhance insulin actions any further, which suggests that the adrenergic tone was already diminished in these animals. This compensating mechanism which combines a decreased insulin resistance and circulating insulin levels also occurs in *Trpm2*$^{-/-}$ and *Trpm8*$^{-/-}$ mice, resembling the phenotype that we observed in the present study for the *Trpv1*$^{-/-}$ mice (McCoy et al. 2013; Zhang et al. 2012).

In the present study, we referred to the phenomenon of "increased insulin sensitivity" when we observed that intraperitoneally administered exogenous insulin produced an enhanced glucose-lowering effect. Although an oversensitization of peripheral tissues (like skeletal muscle and adipocytes) to insulin actions is a plausible explanation for the evidence here shown, other mechanisms could be responsible for the reduction of glucose levels during the insulin tolerance test. Alterations in insulin activity and thus in glucose homeostasis, could also be related to the changes in the function of the insulin degrading enzyme (Abdul-Hay et al. 2011), insulin clearance by the renal system (Rubenstein and Spitz 1968), the ability to modulate the hepatic glucose production and/or the signaling of the counter-regulatory hormone glucagon (Cherrington 2005; Liang et al. 2004).

Our work shows that TRPV1 channels are not functional in pancreatic beta cells and supports a role for the TRPV1 channel in the control of insulin sensitivity in peripheral tissues, through the connection between TRPV1-positive sensitive fibers and the adrenergic tone. In light of our results, we propose that although TRPV1 does not intrinsically play a role in pancreatic beta cell physiology per se, it could be relevant to understand some of the peripheral complications characteristic of diabetes mellitus, related to autonomic neuropathies.

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