Glitazones exert multiple effects on beta-cell stimulus-secretion coupling

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Abstract:

Aims/hypothesis: Earlier studies suggest that glitazones exert beneficial effects in patients with type 2 diabetes (T2DM) by directly affecting insulin secretion of beta-cells besides improving the effectiveness of insulin in peripheral tissues. The effects of glitazones on stimulus-secretion coupling (SSC) are poorly understood. Methods: We tested the influence of troglitazone and pioglitazone on different parameters of SSC including insulin secretion (RIA), cell membrane potential $V_m$, various ion currents (patch-clamp), mitochondrial membrane potential $\Delta \Psi$, and cytosolic Ca$^{2+}$ concentration (fluorescence). Results: Troglitazone exerted stimulatory, inhibitory or no effects on insulin secretion depending on the drug and glucose concentration. It depolarized the mitochondrial membrane potential $\Delta \Psi$, thus lowering ATP production which results in opening of $K_{ATP}$ channels and reduced insulin secretion. However, it also exerted direct inhibitory effects on $K_{ATP}$ channels that can explain enhanced insulin secretion. Troglitazone also inhibited the currents through voltage-dependent Ca$^{2+}$ and $K^+$ channels. Pioglitazone was less effective than troglitazone on all parameters tested. The effects of both glitazones were markedly reduced in the presence of BSA. Conclusions: Glitazones exert multiple actions on beta-cell SSC which have to be considered as undesired side effects because the influence of these compounds on beta-cells is not controllable. The final effect on insulin secretion depends on many parameters including the actual glucose and drug concentration, protein binding of the drug and the drug by itself. Troglitazone and pioglitazone differ in their influence on SSC. It can be assumed that the effects of pioglitazone on beta-cells are negligible under $in vivo$ conditions.
Introduction:

Glitazones (thiazolidinediones) are used as oral drugs in the treatment of type-2 diabetes mellitus (T2DM). They exert manifold effects on lipid and glucose metabolism. Their positive actions on glycemic control are attributed to activation of the gamma-subtype of peroxisome proliferator-activated receptors (PPARs) leading to increased insulin sensitivity of peripheral tissues (Gross and Staels, 2007). Many studies have shown that patients with type-2 diabetes mellitus (T2DM) can profit from glitazones alone or in combination with other oral antidiabetic drugs (DeFronzo et al., 2010; Gastaldelli et al., 2007; Hanefeld et al., 2006; Kahn et al., 2011; Zinman et al., 2010). Interestingly, the recently published CANOE trial (Zinman et al., 2010) showed that low-dose combination therapy with rosiglitazone and metformin can at least delay the onset of overt T2DM without severe adverse effects. Both, human and animal studies suggest that glitazones protect against glucolipotoxicity which impairs beta-cell function. Glitazones are supposed to enhance beta-cell mass by reducing the rate of beta-cell apoptosis and/or increasing beta-cell proliferation (Campbell and Mariz, 2007; Decker et al., 2008; Kanda et al., 2010; Kawasaki et al., 2005). Interestingly, in mice such effects seem to be more prominent in obese than in lean animals (Kanda et al., 2010). A positive influence of glitazones on beta-cell mass may ameliorate the capacity of islets to secrete insulin. However, animal studies have suggested that glitazones also directly affect beta-cell stimulus-secretion coupling (SSC) (Bollheimer et al., 2003; Chang et al., 2009; Lamontagne et al., 2009; Lee et al., 1996; Masuda et al., 1995; Ohtani et al., 1998; Sunaga et al., 1999; Wang et al., 2007). Thus, it has been speculated that the beneficial effects of glitazones on glycemic control in T2DM patients result in part from improvement of beta-cell function (DeFronzo et al., 2010; Gastaldelli et al., 2007; Kahn et al., 2011). In animal
models of diabetes allowing to study the molecular mechanisms of drug actions in more detail, effects of glitazones on glucose homeostasis and beta-cell function have been confirmed (Ishida et al., 2004; Kawashima et al., 2011; Ogawa et al., 1999; Shimabukuro et al., 1998). As in other tissues glitazones may activate PPARgamma that has been shown to be expressed in beta-cells (Dubois et al., 2000; Welters et al., 2004). The AMP-activated protein kinase (AMPK) which is a key regulator of cell metabolism (Carling et al., 2011) with a special role in beta-cells (Düfer et al., 2010; Rutter et al., 2003) has been considered as another target of glitazones (Chang et al., 2009; Lamontagne et al., 2009; Wang et al., 2007). It has been reported that a glitazone without PPAR affinity enhances glucose-stimulated insulin secretion suggesting that the effect is mediated by AMPK (Zhang et al., 2009). However, taken as a whole the reported effects of glitazones on insulin secretion are inconsistent (Bollheimer et al., 2003; Lamontagne et al., 2009; Masuda et al., 1995; Ohtani et al., 1998). Probably they depend on the compound that has been tested, the drug concentrations, time period of application, animal species, etc. Moreover, detailed studies are lacking about the parameters of SSC that are affected by glitazones. The present paper aimed to identify conditions under which glitazones interfere with beta-cell SSC, the molecular targets of the interactions, and possible differences between the actions of distinct glitazones.
Methods and Materials

Cell and islet preparation. Experiments were performed using islets, clusters or single pancreatic beta-cells isolated from fed C57Bl/6 mice (Charles River, Sulzfeld, Germany) killed by CO₂. Islets were isolated by collagenase digestion. For generation of clusters or single cells islets were dispersed in Ca²⁺-free medium and cultured up to 4 days in RPMI 1640 medium (11.1 mM glucose) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Plant, 1988). The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and German laws were followed.

Solutions and chemicals. $V_m$ and perforated patch K$_{ATP}$ current recordings were done with amphotericin B (250 µg/ml) in the pipette solution which contained (in mM): 10 KCl, 10 NaCl, 70 K₂SO₄, 4 MgCl₂, 2 CaCl₂, 10 EGTA, 5 HEPES, pH 7.15 adjusted with KOH. Standard whole-cell recordings of K$_{ATP}$ currents, voltage-dependent K⁺ currents, and excised i/o K$_{ATP}$ current measurements were performed with a pipette solution containing (in mM): 130 KCl, 4 MgCl₂, 2 CaCl₂, 10 EGTA, 0.65 Na₂ATP, 20 HEPES, pH 7.15, adjusted with KOH. The bath solution for whole-cell current measurements, and determination of [Ca²⁺]ₖ, NAD(P)H autofluorescence and $\Delta \Psi$ contained (in mM): 140 NaCl, 5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 0.5 glucose or as indicated, pH 7.4 adjusted with NaOH. For measurements of voltage-dependent K⁺ currents, 0.1 mM tolbutamide was added to the bath solution. For K$_{ATP}$ current registration in the inside/out patch configuration the following bath solution was used (in mM): 130 KCl, 1.5 MgCl₂, 5 EGTA, 10 HEPES, 0.5 glucose, pH adjusted to 7.2 with KOH. Ca²⁺ currents in the perforated-patch mode were determined with a pipette solution composed of (mM): 70 Cs₂SO₄, 10 NaCl, 10 KCl, 7 MgCl₂, 10 HEPES, 250 µg/ml amphotericin B, pH 7.15. Bath solution contained (in mM): 115 NaCl, 1.2
MgCl$_2$, 10 CaCl$_2$, 20 TEACl, 10 HEPES, 0.1 tolbutamide, 15 glucose, pH 7.4. The incubation medium for determining insulin secretion contained (in mM): 122 NaCl, 4.8 KCl, 2.5 CaCl$_2$, 1.1 MgCl$_2$, 10 HEPES, pH 7.4.

Pioglitazone was purchased from Molekula (Gillingham, UK). Fura-2AM, rhodamine 123, RPMI 1640 medium, and penicillin/streptomycin were obtained from Invitrogen (Karlsruhe, Germany). All other chemicals were purchased from Sigma (Taufkirchen, Germany) and Merck (Darmstadt, Germany) in the purest form available.

**Patch-clamp recordings.** Electrophysiology was performed with single beta-cells or small clusters of cells. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, March-Hugstetten, Germany). Membrane currents and potentials were recorded with an EPC-9 patch-clamp amplifier using "Pulse" software (HEKA, Lambrecht, Germany). Perforated-patch and conventional whole-cell K$_{ATP}$ currents were measured at a holding potential of -70 mV and during 300 ms voltage pulses to -80 mV and -60 mV at 15 s intervals. Single K$_{ATP}$ channel currents were recorded at a holding potential of -50 mV. Channel activity of the single channels is given as NP$_o$, determined as a product of open probability (P$_o$) calculated by point-by-point analysis and the number of active channels in the patch (N). Currents through voltage-dependent K$^+$ channels have been registered in the conventional whole-cell mode by 150 ms pulses from -70 to 0 mV applied every 15 s. Currents through L-type Ca$^{2+}$ channels were elicited by 50 ms pulses from -70 to 0 mV in the perforated-patch mode.

**Measurement of [Ca$^{2+}$]$_o$, $\Delta \Psi$, and NAD(P)H.** Experiments were performed with single cells or small clusters at 37 °C. [Ca$^{2+}$]$_o$ was measured by the fura-2 method according to Grynkiewicz et al. (Grynkiewicz et al., 1985) using equipment and software from TILL photonics (Gräfelfing, Germany). The cells were loaded with fura-2AM (5 µM)
for 30 min at 37 °C. Intracellular fura-2 was excited alternately at 340 nm or 380 nm by means of an oscillating diffraction grating. The emitted light was filtered (LP515 nm) and measured by a digital camera. The ratio of the emitted light intensity at 340nm/380nm excitation was used to calculate \([\text{Ca}^{2+}]_c\) according to an in vitro calibration with fura-2 K⁺-salt. For determination of \(\Delta \Psi\) Rhodamine 123 (Rh123) fluorescence was excited at 480 nm and the intensity of the emitted light was measured and given in arbitrary units (a.u.). Cells were loaded with Rh123 (10 µg/ml) for 10 min at 37°C. An increase of Rh123 fluorescence corresponds to a decrease in mitochondrial membrane potential (Duchen et al., 1993; Krippeit-Drews et al., 2000). NAD(P)H autofluorescence was detected at 360 nm excitation wavelength.

**Measurement of insulin secretion.** After preparation islets were kept overnight in medium with 11.1 mM glucose. To determine insulin secretion, batches of 5 islets were incubated for 60 min at 37 °C with the indicated substances. In experiments without BSA, albumin was added to achieve a final concentration of 0.5% after the 60 min incubation period prior to collection of the supernatant to minimize loss of secreted insulin due to unspecific binding to the vessel wall. We performed a series of control experiments with 0.1% BSA present throughout static incubation. Insulin secretion in 3 and 15 mM glucose did not differ from that without BSA (G3/zero BSA: 0.11±0.04 vs. G3/0.1% BSA: 0.16±0.12 ng insulin/(islet*h)), ns; G15/zero BSA: 2.5±0.5 vs. G15/0.1% BSA: 2.7±0.5 ng insulin/(islet*h)), ns; n=4 for each condition).

Insulin was determined by radioimmunoassay using rat insulin (Crystal Chem. Inc., USA) as the standard.

**Presentation of results.** Electrophysiological and \([\text{Ca}^{2+}]_c\) experiments are illustrated by recordings representative of the indicated number of experiments carried out with different cells. At least two different cell preparations were used for each series of
experiments. Where possible means ± SEM are given in the text for the indicated number of experiments (n). The statistical significance of differences between means was assessed by a one sample $t$ test or Student’s $t$ test for paired values when two samples were compared; multiple comparisons were made by ANOVA followed by Student-Newman-Keuls test. A $P$ value of less than 0.05 was considered significant.
Results

Influence of glitazones on insulin secretion

In order to investigate whether glitazones directly influence beta-cell function we tested the glucose and concentration dependency of troglitazone on insulin secretion of mouse islets. We studied the effects of troglitazone in BSA-free (Fig. 1A) and BSA-containing media (Fig. 1B) to elucidate whether discrepancies in the literature about the action of glitazones on beta-cells can, at least in part, be explained by differences in protein binding of the drugs under different experimental conditions. Regardless of BSA troglitazone (1 or 10 µM) had no effect on basal insulin secretion (3 mM glucose) or at the threshold for glucose-induced insulin secretion (8 mM glucose) (Fig. 1A,B). At a stimulatory glucose concentration (15 mM) 1 µM troglitazone increased insulin release in the absence of BSA while 10 and 50 µM exerted an inhibitory effect (Fig. 1A). In the presence of BSA 1 µM troglitazone did not affect insulin secretion induced by 15 mM glucose while 10 and 50 µM clearly augmented it (Fig. 1B). We compared the effects of troglitazone to those of pioglitazone. In BSA-free medium pioglitazone at 1 or 10 µM was ineffective at all glucose concentration tested, only the high concentration of 50 µM stimulated glucose-induced insulin secretion (Fig. 1C). The results show that the influence of glitazones on insulin secretion depends on binding to plasma proteins. In contrast to troglitazone pioglitazone did not diminish insulin secretion at the drug concentrations tested. To investigate whether the stimulatory effect of pioglitazone involves paracrine effects of hormones secreted by alpha- and delta-cells we measured insulin release in the presence of tetrodotoxin (0.1 µg/ml) and SNX482 (100 nM) to block glucagon and somatostatin release, respectively (MacDonald et al., 2007; Zhang et al., 2007). The pioglitazone-induced enhancement of glucose-stimulated insulin release persisted in
the presence of the two toxins (G15 + pioglitazone (50 µM): 3.7±0.7 ng insulin/(islet*h) vs. G15 + pioglitazone, tetrodotoxin and SNX482: 3.4±0.3 ng insulin/(islet*h), ns, n=3, not shown).

**Action of glitazones on plasma membrane potential $V_m$ and $K_{ATP}$ channels in metabolically intact beta-cells**

To further analyse the mechanisms underlying the dual action of glitazones on insulin secretion we measured $K_{ATP}$ currents and $V_m$. In the presence of 3 mM glucose 1 µM troglitazone markedly increased the $K_{ATP}$ current measured with the perforated-patch configuration of the patch-clamp technique (Fig. 2 A upper trace and B) but has no influence on $V_m$ (Fig. 2A lower trace and C). The addition of 1 µM troglitazone at a stimulating glucose concentration (15 mM) led initially ($T_{1 ini}$) to an increase of the $K_{ATP}$ current (Fig. 2D upper trace and E) and an analogous membrane hyperpolarization (Fig. 2D lower trace and F). In the continuous presence of 1 µM troglitazone the current slightly decreased again (Fig. 2D upper trace and E; $T_{1 late}$ measured after 10 min with troglitazone) and accordingly $V_m$ depolarized (Fig. 2D lower trace and F), however, it stayed far below the threshold for the initiation of action potentials. With 10 µM troglitazone the biphasic effect was much more pronounced. Directly after addition of troglitazone ($T_{10 ini}$) the $K_{ATP}$ current was increased (Fig. 3A upper trace and B) and $V_m$ hyperpolarized (Fig. 3A lower trace and C) compatible with inhibition of insulin secretion. During the application of 10 µM troglitazone ($T_{10 late}$) $K_{ATP}$ current and $V_m$ came back to the initial values in 15 mM glucose (Fig. 3A,B,C). However, action potentials remained suppressed and thus it seems unlikely that insulin secretion recovers. Comparison to pioglitazone revealed that 1 µM of the glitazone in the presence of 15 mM glucose slightly hyperpolarized
$V_m$ (from $-47\pm2$ mV to $-56\pm3$ mV ($n=8$, $P\leq0.05$) but this was not sufficient to repolarize $V_m$ below the threshold potential for the opening of voltage-dependent $Ca^{2+}$ channels (Fig. 4A). However, the spike frequency was clearly diminished from 225±18 min$^{-1}$ to 125±31 min$^{-1}$ ($n=8$, $P\leq0.05$). At a glucose concentration of 7 mM electrical activity starts which is indicated by the low spike frequency (Fig. 4B). At this glucose concentration 1 µM pioglitazone was able to repolarize $V_m$ from -48±3 mV to -59±3 mV below the threshold potential for the opening of L-type $Ca^{2+}$ channels leading to suppression of spike activity ($n=7$, $P\leq0.05$) (Fig. 4B). With 10 µM pioglitazone we observed a similar effect as with 1 µM troglitazone. The $K_{ATP}$ current measured with the perforated patch-technique increased from 1.5±0.4 pA to 18.4±2.7 pA ($n=7$, $P\leq0.001$) and accordingly $V_m$ hyperpolarized from -47±2 mV to -73±2 mV and thus spike activity was completely suppressed ($n=9$; $P\leq0.001$) (Fig. 4C). Repeat of these experiments in the presence of 0.3 % BSA to increase protein binding of the glitazone revealed that the effect was almost completely blocked under these conditions (Fig. 4D). Only a slight hyperpolarization from -42±5 to -45±5 mV was observed.

Opening of $K_{ATP}$ channels with consecutive hyperpolarization of $V_m$ can explain the inhibitory effect of troglitazone on glucose-induced insulin secretion. Stimulation of insulin secretion which was observed with both glitazones tested must be due to other mechanisms. To further evaluate how glitazones affect $K_{ATP}$ channel activity we investigated whether glitazones interfere with ATP production.

**Effects of glitazones on mitochondrial membrane potential $\Delta \Psi$**

As a measure for ATP production we determined the mitochondrial membrane potential $\Delta \Psi$ that strictly correlates with mitochondrial ATP synthesis. An increase in
the glucose concentration from 0.5 to 15 mM hyperpolarized ΔΨ due to the rapid onset of ATP production which is accompanied by diminished Rh 123 fluorescence (Fig. 5A,C). Troglitazone in the presence of 15 mM glucose dose-dependently depolarized ΔΨ, i.e. it inhibited ATP production (Fig. 5A,C). The data are summarized in Fig. 5B,D. 10 µM pioglitazone slightly but significantly decreased ΔΨ (Fig. 5E,F). In contrast to troglitazone 1 µM pioglitazone did not influence ΔΨ (877±75 a.u. in 15 mM glucose vs. 911±89 a.u. in 15 mM glucose + pioglitazone, n=5, n.s.) (data not shown). To test whether the decrease in ATP synthesis induced by the depolarization of ΔΨ during application of 1 µM troglitazone is large enough to hyperpolarize Vₘ below the threshold for Ca²⁺ action potentials mitochondrial activity was blocked by NaN₃, an inhibitor of cytochrome a₃ (Misler et al., 1992). Fig. 5G,H shows that 600 µM NaN₃ depolarized ΔΨ to the same extent as 1 µM troglitazone. The reduction of mitochondrial activity abrogated electrical activity and hyperpolarized Vₘ in the presence of 15 mM glucose by -21±3 mV (ns vs. troglitazone-induced hyperpolarization of -25±3 mV, compare Fig. 2F and 5I). Mitochondrial depolarization was accompanied by accumulation of NAD(P)H (Fig. 6A). Comparative experiments with troglitazone and NaN₃ revealed that the rise in NAD(P)H autofluorescence induced by 10 µM of the glitazone was similar to the effect of blocking complex IV of the respiratory chains by 5 mM NaN₃ (data are summarized in Fig. 6B). The action of troglitazone on mitochondrial activity can thus explain the inhibitory effect of the drug on insulin secretion. To further investigate the mechanisms underlying the contradictory effects of glitazones on insulin secretion we tested the influence of the compounds on [Ca²⁺]c and several ion channels.
Effects of glitazones on cytosolic Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_c\) and L-type Ca\(^{2+}\) current \((\text{Ca}_V)\)

In the presence of 15 mM glucose \([\text{Ca}^{2+}]_c\) elicited typical oscillations between 116±8 nM (basal values) and 547±18 nM (peak values). The addition of 1 µM troglitazone stopped these fluctuations and \([\text{Ca}^{2+}]_c\) amounted to 118±10 nM (n=5, \(P \leq 0.001\) compared to peak values) (Fig. 7A). We next evaluated whether this effect can be attributed to the inhibition of Ca\(^{2+}\) channels by glitazones. Troglitazone dose-dependently diminished the L-type Ca\(^{2+}\) current \((\text{Ca}_V)\) measured with the perforated-patch technique (Fig. 7B,C). 10 µM pioglitazone did not significantly alter the \(\text{Ca}_V\) current (Fig. 7D). It was -59±5 pA before and -56±5 pA after application of pioglitazone (n=5, n.s.).

Influence of glitazones on voltage-dependent K\(^{+}\) currents \((\text{K}_V)\)

10 µM troglitazone markedly reduced the \(\text{K}_V\) current measured in the standard whole-cell configuration (394±44 pA without vs. 63±15 pA with troglitazone (n=5, \(P \leq 0.01\)) (Fig. 8A) while the same concentration of pioglitazone only marginally diminished the \(\text{K}_V\) current from 259±41 pA to 205±43 pA (n=5, \(P \leq 0.01\)) (Fig. 8B).

Direct effects of glitazones on K\(_{\text{ATP}}\) channels

Experiments performed in the inside/out configuration revealed that 10 µM troglitazone completely suppressed the \(K_{\text{ATP}}\) single channel current within 2 to 3 min after application (Fig. 9A). Accordingly, the \(K_{\text{ATP}}\) current measured in the standard whole-cell configuration was almost completely suppressed with 10 µM troglitazone (Fig. 9B,D) and clearly reduced with 1 µM of the drug (Fig. 9C,E). In contrast, 10 µM pioglitazone had only a moderate inhibitory effect on the \(K_{\text{ATP}}\) current amplitude when
applied in the standard whole-cell configuration, i.e. it reduced the current amplitude from 194±22 pA to 133±28 pA (n=4, P≤0.05) (Fig. 9F,G).

**Long-term effects of troglitazone on insulin secretion**

Our data showed that acute application of glitazones interferes with numerous targets involved in regulation of insulin release. In addition, it is well known that glitazones interact with nuclear receptors and thus they might influence beta-cell function via additional pathways, e.g. gene transcription, during "chronic" application. Therefore, we compared the influence of troglitazone on insulin release observed after short-term incubation (Fig. 1B) to a prolonged exposure time. Fig. 10 illustrates that, analogous to the 60 min incubation period, 24 h treatment of the islets with 10 µM troglitazone did not affect insulin release in the presence of 8 mM glucose but enhanced secretion stimulated by 15 mM glucose. In contrast, 50 µM troglitazone that were stimulatory when given for a short time reduced glucose-evoked secretion to ~50 %. The inhibitory effect was comparable to that observed with 10 µM troglitazone in the absence of BSA (see Fig. 1A).
**Discussion**

**Insulin secretion**

Recent studies suggest that glitazones have multiple effects on beta-cells besides their insulin sensitizing actions in peripheral tissues (Campbell and Mariz, 2007). Several papers demonstrate that glitazones affect insulin secretion but the results are controversial. Stimulatory and inhibitory effects of troglitazone, rosiglitazone and pioglitazone have been reported for insulin-secreting tumor cells and primary rodent beta-cells (Blumentrath et al., 2001; Bollheimer et al., 2003; Ishida et al., 2004; Kawashima et al., 2011; Kim et al., 2008; Lamontagne et al., 2009; Masuda et al., 1995; Ohtani et al., 1998; Wang et al., 2007). Contradictory results seem mainly to depend on the concentration used and on differences provoked by acute or prolonged application of the drugs. Our important contribution to this complex situation is the finding that the inhibitory effect of troglitazone in the absence of BSA was reversed to a stimulatory one in the presence of BSA, i.e. that the direction of the glitazone effects on beta-cells strongly depends on plasma protein binding (see Fig. 1). Obviously, binding to BSA lowers the free troglitazone concentration so far that 10 and even 50 µM lead to stimulation whereas these concentrations exert strong inhibition without BSA. Consequently, 1 µM troglitazone that stimulated insulin secretion in BSA-free medium was without effect in the presence of BSA. This hypothesis is further supported by our long-term experiments showing that 50 µM troglitazone in BSA-containing medium inhibit insulin release when applied for 24 h.

With respect to stimulation of insulin secretion pioglitazone was less effective than troglitazone. In BSA-free solution 50 µM of the drug were needed to affect insulin secretion. We could show that altered hormone secretion from alpha- and delta-cells seems not to be involved in the stimulatory effect of pioglitazone. In contrast to
troglitazone, a reversion from stimulation to inhibition of secretion with higher drug concentration was not observed with pioglitazone. The high plasma protein binding of both glitazones can explain the differences observed in insulin secretion with and without BSA. However, differences in plasma protein binding which is higher for troglitazone (~99.9%, (Shibukawa et al., 1995)) than for pioglitazone (97 to 99%, (Budde et al., 2003; Christensen et al., 2005)) can hardly account for the discrepancy in the effectiveness of pioglitazone and troglitazone on insulin secretion. This observation may be due to different accumulation in beta-cells or to different intrinsic effectiveness of the drugs. The free fraction of pioglitazone in the plasma is < 3% and seems to be similar in animals and humans (Eckland and Danhof, 2000). In humans, $c_{\text{max}}$ after a single oral dose of 30 mg pioglitazone amounts to 1.1±0.3 µg/ml (Wittayalertpanya et al., 2006) which is around 3 µM and thus in the range we have used in our experiments. Interestingly, the pharmacokinetics of pioglitazone in patients with diabetes is similar to those in healthy volunteers (Eckland and Danhof, 2000).

Glitazone targets within beta-cells

Our study shows that glitazones affect multiple targets in beta-cells including $\Delta \Psi$, $V_m$, $K_{\text{ATP}}$, $K_V$ and $C_{\text{av}}$ channels. An earlier paper by Lamontagne and coworkers describes a pioglitazone-evoked depolarization of $\Delta \Psi$ and reduction of ATP content (Lamontagne et al., 2009). With troglitazone we found a dose-dependent depolarization of $\Delta \Psi$, pioglitazone was less effective. A depolarization in $\Delta \Psi$ results in inhibition of ATP production which should in turn open $K_{\text{ATP}}$ channels and hyperpolarize $V_m$. In the perforated-patch configuration with intact cell metabolism the depolarization of $\Delta \Psi$ by troglitazone and pioglitazone was indeed followed by an
increase in the $K_{\text{ATP}}$ current amplitude and hyperpolarization of $V_m$ leading to the rapid drop in $[\text{Ca}^{2+}]_c$. Experiments with the cytochrome a$_3$ inhibitor NaN$_3$ confirmed that the extent of mitochondrial depolarization induced by the glitazones led to a drop in ATP synthesis large enough for $V_m$ hyperpolarization (Fig. 5G-I). Glitazone-induced mitochondrial depolarization was accompanied by accumulation of NAD(P)H (Fig. 6). This indicates that the effect on $\Delta\Psi$ cannot be explained by mitochondrial uncoupling which would lead to a decrease in NAD(P)H (Dellinger et al., 1998; Krippeit-Drews et al., 2000). One possible explanation for the rise in NAD(P)H could be a direct inhibition of respiratory chains. It has been shown e.g. for rotenone, NaN$_3$ and oligomycin, respectively, which inhibit complex I, IV, and V that blocking respiratory chains at diverse stages results in NAD(P)H accumulation. As our experiments showed a troglitazone-induced rise in NAD(P)H direct interaction with respiratory chains might be the reason for inhibition of ATP production. However, further studies are necessary to confirm this hypothesis. Note that the hyperpolarizing effect of pioglitazone on $V_m$ was markedly reduced in the presence of BSA (compare Fig. 4 C and D). Altogether, these data show that inhibitory but not stimulatory actions of glitazones on insulin secretion can be explained by their effects on mitochondrial metabolism. With troglitazone we could demonstrate that both effects, increase in $K_{\text{ATP}}$ current amplitude and $V_m$ hyperpolarization were transient, i.e. the current amplitude started to fall and $V_m$ to depolarize in the continued presence of the drug. The effect is highly pronounced with 10 µM troglitazone (see Fig. 3) where $V_m$ depolarizes above the threshold for activation of L-type Ca$^{2+}$ channels after prolonged application of the drug. However, no Ca$^{2+}$ action potentials were induced. This biphasic action that occurred despite sustained depolarization of $\Delta\Psi$ points to a delayed direct inhibition of $K_{\text{ATP}}$ current that overlays the initial
metabolism-dependent activation. The assumption is confirmed by the observation that troglitazone inhibited the $K_{ATP}$ current in experiments performed in the standard whole-cell configuration without intact metabolism or in cell-free patches. Whereas the reduction in $K_{ATP}$ current and concomitant depolarization of $V_m$ is only moderate with 1-10 µM pioglitazone or with 1 µM troglitazone, $K_{ATP}$ channels were completely blocked by 10 µM troglitazone. An inhibition of $K_{ATP}$ current of beta-cells or insulin-secreting cell lines by glitazones is in agreement with findings of several other groups (Chang et al., 2009; Lee et al., 1996; Shimomura et al., 2004; Sunaga et al., 1999). However, this direct interaction of troglitazone with $K_{ATP}$ channels does obviously not result in stimulation of insulin secretion. This complex scenario can be summarized as follows: with low concentrations of troglitazone direct $K_{ATP}$ channel block is not large enough to overcome the inhibitory drug effect on mitochondrial ATP synthesis. High concentrations of the drug depolarize $V_m$ despite reduced ATP production. However, as under this condition L-type Ca$^{2+}$ channels are almost completely blocked membrane depolarization cannot induce any rise in $[Ca^{2+}]_c$ that is required for insulin release. We suggest that the stimulatory effects of glitazones on insulin secretion cannot be explained by effects on stimulus-secretion coupling, i.e. direct inhibition of $K_{ATP}$ channels by glitazones is an epiphenomenon and the stimulatory effects on secretion are not caused by any influence on the triggering pathway. Unfortunately, in other studies that describe inhibition of $K_{ATP}$ channels in insulin-secreting cells measurements of insulin secretion are lacking (Chang et al., 2009; Lee et al., 1996; Sunaga et al., 1999). In one paper a slight augmentation of insulin secretion is observed with 100 µM troglitazone but the IC$_{50}$ value for inhibition of $K_{ATP}$ channels is 2.1 µM, i.e. the direct link between decrease in $K_{ATP}$ channel activity and increase of insulin secretion remains questionable. To complicate the situation
glitazones also directly influenced $K_v$ currents in addition to the direct inhibitory effect on $K_{ATP}$ current and the indirect activating one on $K_{ATP}$ channels via metabolism. One may speculate that inhibition of $K_v$ current depolarizes $V_m$ and thus contributes to the stimulatory effects of glitazones on insulin secretion. However, we have shown recently that genetic ablation of BK channels which also affect action potentials has the contrary effect, i.e. it inhibits insulin secretion (Düfer et al., 2011). Thus, the effects of $K^+$ channel blockade on insulin secretion depend on the specific role of each $K^+$ channel type in beta-cell function and thus inhibition of whole-cell $K_v$ current cannot be taken as a measure for insulin release.

Troglitazone was more effective in single cells or cell clusters than in islets. Insulin secretion e.g. is activated by 1 µM troglitazone but inhibited at 10 µM while $[Ca^{2+}]_c$ is already reduced to basal values at 1 µM of the drug (compare Fig. 1A to 8A). This discrepancy may be explained by different diffusion barriers and distances of islets and single cells.

**Comparison of glitazone effects on SSC with effects of the AMPK activator AICAR**

In a recent study we have tested the effects of the specific AMPK activator 5-aminomidazole carboxamide riboside (AICAR) on beta-cell SSC (Düfer et al., 2010). With AICAR insulin secretion was enhanced between 8 and 15 mM glucose. The effect could be attributed to inhibition of $K_{ATP}$ current, depolarization of $V_m$ and increase of $[Ca^{2+}]_c$. Importantly, in contrast to the glitazones AICAR did not affect $\Delta \Psi$ or NAD(P)H. Thus, it is unlikely that the stimulatory effects of glitazones on $K_{ATP}$ current are due to AMPK activation. Similarly, it is implausible that the suppression of $K_{ATP}$ current by glitazones is mediated by AMPK because the inhibitory effects were
Effects of glitazones in beta-cells vs. side effects

In principle it is desirable that an oral antidiabetic drug affects peripheral tissues and beta-cells concomitantly to reduce the insulin demand of the body and adapt insulin secretion to the need. However, as shown in this paper glitazones act on many different targets in beta-cells which exert opposed effects on insulin secretion. Thus, the effects of glitazones on beta-cells are not controllable and the fact that they act on targets in beta-cells has to be considered as undesired side effects. The alterations of SSC described here occur very rapidly illustrating that glitazones interact with beta-cell function through multiple non-genomic pathways. At present, we cannot exclude that additional genomic effects contribute to the dose-dependent change in insulin release observed after long-term treatment.

Due to assured deterioration of myocardial infarction (Lincoff et al., 2007; Nissen and Wolski, 2007) FDA restricted prescription of rosiglitazone to healthcare providers and patients enrolling in a certain medical access programme whereas the drug was withdrawn from the European market. For pioglitazone cardiac morbidity and mortality seem to be unaffected or even slightly reduced (Hughes et al., 2012; Lincoff et al., 2007) (Abbas et al., 2012). Troglitazone has been withdrawn from the market because of severe liver damage (Yokoi, 2010) which seems not to occur with other glitazones including pioglitazone (Shah and Mudaliar, 2010). As several studies show that pioglitazone is associated with increased risk of bladder cancer (Colmers et al., 2012; Zhu et al., 2012) it should not be prescribed to newly diagnosed diabetic
patients any more but only retained in those who are already known to clearly benefit from PPARgamma activating drugs.

Nevertheless, special ethnic groups or patients with distinct comorbidities may profit from pioglitazone. Two ongoing placebo-controlled phase IV studies evaluate whether patients with T2DM and non-alcoholic fatty liver disease (NAFLD) benefit from treatment with pioglitazone (Cusi, 2012). Other studies confirm beneficial effects of pioglitazone in patients suffering from NAFLD or diabetic patients with increased liver fat (Belfort et al., 2006; Sathyanarayana et al., 2011; Shah et al., 2011; Smith and Adams, 2011). A recent meta-analysis suggests that pioglitazone is more appropriate than metformin to reduce hyperinsulinemia and insulin resistance in patients with polycystic ovary syndrome (Du et al., 2012). In patients with impaired glucose tolerance pioglitazone is reported to induce regression of coronary atherosclerotic plaques probably due to reduced inflammation, increased adiponectin concentration, and improved endothelial function (Yang et al., 2012). In addition, animal studies describe a positive effect of glitazones on pancreatitis (Pini et al., 2012; Wan et al., 2012). Together, these data emphasize that the signalling pathways activated by glitazones are still interesting drug targets. Recently, it has been shown that glitazones activate PPARgamma by inhibiting Cdk5-mediated phosphorylation of the transcription factor (Choi et al., 2011). In addition, a selective non-thiazolidinedione PPARgamma agonist that seems not to share the adverse side effects of glitazones has reached phase II clinical trial (Dunn et al., 2011). As our data illustrate that drugs interacting with PPARgamma severely affect beta-cells, further studies are necessary to investigate whether compounds directly or indirectly targeting this nuclear receptor have less side effects than glitazones and do not interfere with beta-cell function. At present, clinical evaluation of glitazone efficacy
mainly focuses on insulin resistance. According to our data, pancreatic secretory capacity should also be assessed during long-term treatment, especially in patients with the afore-mentioned indications receiving a combination therapy with other drugs affecting the endocrine pancreas.

Since pioglitazone 1) has unlike troglitazone no inhibitory effects on insulin secretion, 2) exerts significant effects on beta-cell SSC \textit{in vitro} only at concentrations above 10 \(\mu\text{M}\) (this study and (Lamontagne et al., 2009)), and 3) has a protein binding rate of \(\sim 99\%\) (Christensen et al., 2005) it is assumed that adverse side effects on beta-cell SSC \textit{in vivo} are negligible. One pilot study tested the effects of one acute dose of rosiglitazone on beta-cell function and insulin sensitivity in healthy volunteers under hyperglycaemic clamp conditions (Farret et al., 2007). They found a rapid increase in insulin sensitivity and insulin clearance but not on secretion supporting our conclusion that the newer generation of glitazones does not critically affect beta-cell SSC.

Conclusion: Glitazones interact \textit{in vitro} with several targets in beta-cells which can lead – depending on the effectiveness of the glitazone in beta-cells and protein binding – to undesired effects on insulin secretion \textit{in vivo}.
Authorship contributions:

Participated in research design: Düfer, Krippeit-Drews, and Drews.

Conducted experiments: Noack, Edalat, and Düfer.

Contributed new reagents or analytic tools: N.A.

Performed data analysis: Noack, Edalat, Düfer, Krippeit-Drews, and Drews.

Wrote or contributed to the writing of the manuscript: Düfer, Krippeit-Drews, and Drews.
References


Colmers IN, Bowker SL, Majumdar SR and Johnson JA (2012) Use of thiazolidinediones and the risk of bladder cancer among people with type 2 diabetes: a meta-analysis. CMAJ.


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Figure Legends:

Fig. 1 Effects of glitazones on insulin secretion. A,B: Effects of 1, 10, and 50 µM troglitazone in the absence (A) and presence of BSA (0.5%) (B) at different glucose concentrations. C: Effects of 1, 10, and 50 µM pioglitazone on insulin secretion at different glucose concentrations. Experiments were performed with whole islets. *P≤0.05, **P≤0.01, ***P≤0.001, ##P≤0.01, different to G3, ### P≤0.001, different to G3

Fig. 2 Influence of 1 µM troglitazone on V_m and K_ATP current in metabolically intact beta-cells. A,D: Typical recordings of the K_ATP current (upper traces) in 3 mM glucose (G3) (A) and 15 mM glucose (G15) (D) and V_m (lower traces) in 3 mM glucose (A) and 15 mM glucose (D) showing the effects of 1 µM troglitazone (T1). The horizontal bar between the traces indicates the time intervals of current registrations in the voltage-clamp mode (VC) and V_m registrations in the current-clamp mode (CC). B and E present the summarized data for the K_ATP current amplitude, C and F the summarized data for V_m in 3 and 15 mM glucose, respectively and after addition of troglitazone. E,F: T1_init: data taken direct after application of troglitazone, T1_late: data obtained 10 min after application of troglitazone. Experiments were performed with single cells or cell clusters, *P≤0.05

Fig. 3 Action of 10 µM troglitazone on V_m and K_ATP current in metabolically intact beta-cells. A: Typical recordings of the K_ATP current (upper trace) and V_m (lower trace) in 15 mM glucose showing the effects of 10 µM troglitazone (T10). B and C present the summarized data for the K_ATP current amplitude and for V_m. T10_init: data taken direct after application of troglitazone, T10_late: data obtained 10 min after
application of troglitazone. Experiments were performed with single cells or cell clusters, \( *P \leq 0.05 \)

Fig. 4 Influence of pioglitazone on \( V_m \). Typical recordings in the absence and presence of BSA. A: 1 \( \mu \)M pioglitazone (P1) in 15 mM glucose. B: 1 \( \mu \)M pioglitazone at the threshold concentration of 7 mM glucose. C,D: Effects of 10 \( \mu \)M pioglitazone in 15 mM glucose in the absence (C) and presence (D) of BSA (0.5\%). The upper trace in (C) shows \( \text{K}_{\text{ATP}} \) current measured in the voltage-clamp mode (VC), the lower one \( V_m \) registered in the current-clamp mode (CC). Experiments were performed with single cells or cell clusters.

Fig. 5 Actions of troglitazone and pioglitazone on the mitochondrial membrane potential \( \Delta \Psi \). A,C,E: Typical recordings of \( \Delta \Psi \) in the presence of 1 \( \mu \)M (A) and 10 \( \mu \)M (C) troglitazone and 10 \( \mu \)M pioglitazone (E). G: Comparison of the effect of 1 \( \mu \)M troglitazone and 600 \( \mu \)M \( \text{NaN}_3 \), respectively, on \( \Delta \Psi \) in the presence of 15 mM glucose. I: Hyperpolarization of \( V_m \) that was induced by 600 \( \mu \)M \( \text{NaN}_3 \). Experiments were performed in the perforated-patch configuration. A,C,E,G: At the beginning of each experiment the glucose concentration was increased from 0.5 to 15 mM. The augmentation of glucose resulted in a decrease of the rhodamine fluorescence which reflects a hyperpolarization of \( \Delta \Psi \) and thus an increase in ATP production. At the end of each experiment FCCP (1 \( \mu \)M) was added to estimate the maximal depolarization of \( \Delta \Psi \). B,D,F,H: Quantitative analysis of the data for the respective experiments shown in A,C,E,G. The fluorescence signal is given as a.u. before and in the presence of the particular drug in A,C and E. In G the diagram compares the degree
of depolarization induced by NaN₃ or troglitazone. Experiments were performed with single cells or cell clusters, *P ≤ 0.05, ***P ≤ 0.001

Fig. 6 Effect of troglitazone on NAD(P)H accumulation. A: Representative recording showing the glucose-induced rise in NAD(P)H and the influence of 10 µM troglitazone and 5 mM NaN₃, respectively. B: Summary of the glucose-, glitazone- and NaN₃-induced elevation of NAD(P)H fluorescence. Experiments were performed with single cells or cell clusters. ***P ≤ 0.01, ### P ≤ 0.001, different to G0.5

Fig. 7 Influence of glitazones on [Ca²⁺]ᵢ and voltage-dependent Ca²⁺ current (Caᵥ). A: Typical measurement of [Ca²⁺]ᵢ in the presence of 1 µM troglitazone. B,D: Typical recordings of Caᵥ current showing the addition of 10 µM troglitazone (B) and pioglitazone (D). The insets show the respective currents at a and b at an extended time scale. C: Quantitative analysis of the experiments in which the effects of 1 and 10 µM troglitazone on Caᵥ current have been tested. The current amplitude was determined before switching to troglitazone and in the steady-state after addition of the glitazone. Experiments were performed with single cells or cell clusters, *P ≤ 0.05

Fig. 8 Action of glitazones on the voltage-dependent K⁺ current (Kᵥ). A,B: Traces showing the typical effects of 10 µM troglitazone (A) and pioglitazone (B) on Kᵥ current. The insets show the respective currents at a and b at an extended time scale. Experiments were performed with single cells or cell clusters.

Fig. 9 Effects of troglitazone and pioglitazone on the Kₐₜₚ current in cells with disrupted metabolism. A) Typical recording showing the effect of 10 µM troglitazone
on the single channel current in an i/o patch. 0: Closed state, 1,2: open states. B,C,F: Typical recordings representing the effects of 10 µM (B) and 1 µM (C) troglitazone and 10 µM pioglitazone (F) on the K$_{ATP}$ current measured in the standard whole-cell configuration. The insets show the respective currents at a and b at an extended time scale. D,E: Summarization for the experiments shown in B and C, respectively. The current amplitude was determined before switching to troglitazone and in the steady-state after addition of the glitazone. *P≤0.05, **P≤0.01

Fig. 10 Changes in insulin secretion induced by prolonged exposure to troglitazone. Whole islets were treated with 10 and 50 µM troglitazone, respectively, in medium containing 11.1 mM glucose and 10% fetal serum albumin for 24 h. Thereafter, insulin release was determined (0.5% BSA). **P≤0.01, ### P≤0.001, different to G3
**Fig. 1**

**A**

- BSA

**B**

+ BSA

**C**

- BSA

**glucose (mM)**
- 3 3 3 8 8 8 15 15 15 15

**troglitazone (µM)**
- 1 10 - 1 10 - 1 10 50

**insulin secretion ng/(h * islet)**

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Fig. 2

A

B

C

D

E

F
Fig. 3

A

G15  T10

I_{K_{ATP}} (pA)

V_m (mV)

1 min

B

G15  T10_{init}  T10_{late}

I_{K_{ATP}} (pA)

C

G15  T10_{init}  T10_{late}

V_m (mV)
Fig. 4

A

B

C

D

G15

G7

G15 BSA

P1

P1

P10

P10

$V_m$ (mV)

$V_m$ (mV)

$V_m$ (mV)

$V_m$ (mV)

2 min 4 min

1 min

1 min

1 min

1 min

-60 -20 20

-80 -20

-20

-80

-80

-80

0 30

VC CC VC CC VC CC

VC CC VC CC

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Fig. 5

A

\[ \Delta \Psi \text{ (a.u.)} \]

B

\[ \Delta \Psi \text{ (a.u.)} \]

C

\[ \Delta \Psi \text{ (a.u.)} \]

D

\[ \Delta \Psi \text{ (a.u.)} \]

E

\[ \Delta \Psi \text{ (a.u.)} \]

F

\[ \Delta \Psi \text{ (a.u.)} \]

G

\[ \Delta \Psi \text{ (a.u.)} \]

H

\[ \Delta \Psi \text{ (a.u.)} \]

I

\[ V_m \text{ (mV)} \]
Fig. 6

A

G0.5  G15  T10  NaN3

5 min

NADPH (a.u.)

B

Increase in NADPH (a.u.)

G0.5  G15  T10  NaN3

20  20  20  20

***  ***  ***

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Fig. 7

A

B

C

D

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Fig. 8

A

control

T10

0.05 s

100 pA

1 min

300

I_{K_v} (pA)

B

control

P10

0.05 s

100 pA

1 min

300

I_{K_v} (pA)
Fig. 10

Insulin secretion (ng/h · islet) as a function of glucose concentration (mM) and troglitazone concentration (µM) in the presence of BSA.

- Glucose concentrations: 3, 8, 15 mM
- Troglitazone concentrations: 10, 50 µM

**Significant difference (p < 0.01)**

### Key Points:
- BSA increases insulin secretion at all glucose and troglitazone concentrations.
- Troglitazone potentiates the effect of BSA at 15 mM glucose.
- Significant differences are observed between treatments with and without BSA at all glucose and troglitazone concentrations.