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σ Receptor ligands and imidazoline secretagogues mediate their insulin secretory effects by activating distinct receptor systems in isolated islets

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Abstract

The effects of two potent σ receptor agonists (+)-3-PPP ((*R*)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine) and DTG (*N*,*N*′-di-(*o*-tolyl)guanidine) on the insulin secretory responses in rat islets of Langerhans were investigated. Both σ receptor ligands were able to potentiate the insulin secretory response of islets incubated at 6 mM glucose, in a dose-dependent manner and were also able to reverse the effects of diazoxide on insulin release. When islets were treated with efaroxan, a well-characterised imidazoline insulin secretagogue, and either (+)-3-PPP or DTG together, there was an unexpected and profound absence of stimulation of insulin release as compared to when islets were incubated with each compound alone. Experiments performed with islets where there was desensitization of DTG/σ receptor or efaroxan/imidazoline binding site mediated responses suggest that at least two distinct receptor systems appear to be involved. The complex interactions of these two classes of drug require further investigation.

Keywords: Imidazoline receptor; Efaroxan; σ Receptor; Insulin secretion; DTG (*N*,*N*′-di-(*o*-tolyl)guanidine); (+)-3-PPP ((*R*)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine)

1. Introduction

Imidazoline insulin secretagogues, such as efaroxan and phentolamine, are thought to mediate their effects in the pancreatic β-cell primarily by inducing the closure of ATP-sensitive (K_{ATP}) K^+ channels (Schulz and Hasselblatt, 1989; Chan and Morgan, 1990; Chan et al., 1991; Chan, 1993; Plant and Henquin, 1990; Dunne, 1991; Jonas et al., 1992). The nature of the binding site responsible for this effect has still not been defined in detail but it appears to be 'atypical', in that it is pharmacologically distinct (Chan et al., 1994; Olmos et al., 1994; Zaitsev et al., 1996) from the imidazoline I_1 and I_2 receptors described in other tissues (Michel and Ernsberger, 1992; Regunathan and Reis, 1996). It is also unclear whether the imidazoline binding site forms part of the K_{ATP} channel itself or whether it is located on a separate protein. In this context, accumulating evidence indicates that the imidazoline binding site is not associated with the sulphonylurea receptor (which forms one of the two known subunits of the K_{ATP} channel) (Brown et al., 1993; Dunne et al., 1995; Ishida-Takayashi et al., 1996; Rustenbeck et al., 1997) and, as a consequence, it has been argued that the ion-conducting subunit (designated Kir6.2) probably contains the imidazoline binding domain (Proks and Ashcroft, 1997). This suggestion assumes particular significance in view of recent results which have been taken to indicate that a common feature of several ion channels may be the possession of a binding site for σ ligands. Occupation of this site (the phencyclidine (PCP)/σ 'receptor') is assumed to result in occlusion of the ion-conducting pore of these channels, causing a reduction in ion flow. Molderings et al. (1996) have shown that some imidazoline drugs can displace the binding of radioligands to σ sites on ion channels and Olmos et al. (1996) have provided evidence for a correlation between the effects of imidazolines on insulin secretion and their affinity for PCP/σ receptors.

Based on such observations, the hypothesis was advanced that the capacity of imidazoline compounds to stimulate insulin secretion may reflect their ability to occupy a PCP/σ binding site on the islet β-cell K_{ATP} channel, leading to occlusion of the ion-conducting pore. This would cause a reduction in the rate of K^+ efflux

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promoting membrane depolarisation, gating of voltage-sensitive calcium channels and enhancement of insulin release. However, we have recently presented preliminary evidence suggesting that this hypothesis may be an oversimplification since two compounds which display high affinity for PCP/σ binding sites associated with ion channels (phencyclidine and dizoclipine) are, at best, only weakly effective as insulin secretagogues (Chan et al., 1997).

In the present study, we have extended this work to examine more fully the effects of two further high affinity σ receptor ligands, DTG (*N,N′*-di-(*o*-tolyl)guanidine) and (+)-3-PPP ((*R*)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine hydrochloride), on insulin release from isolated rat islets. Moreover, we have studied the interactions between these compounds and imidazoline drugs to ascertain whether they share a common mechanism of action.

2. Materials and methods

2.1. Materials

Collagenase (type XI), (+)-3-PPP ((*R*)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine hydrochloride) and diazoxide were purchased from Sigma (Poole, Dorset, UK). DTG (*N,N′*-di-(*o*-tolyl)guanidine) was purchased from Tocris Cookson (Bristol, UK). Glibenclamide was a gift from SmithKline Beecham Pharmaceuticals (Welwyn, Herts., UK). Efaroxan was a gift from Reckitt and Colman Products (Kingston-upon-Hull, UK). [^{125}I]iodine and anti-bovine insulin antiserum (for radioimmunoassay, RIA) were from ICN Biomedicals. All other reagents were of analytical reagent grade.

2.2. Insulin secretion experiments

Rat islets of Langerhans were isolated by collagenase digestion (Montague and Taylor, 1968) from the pancreata of male Wistar rats (180–250 g body weight) allowed free access to food and water. The isolation medium was a bicarbonate-buffered physiological saline solution (Gey and Gey, 1936) containing 4 mM D-glucose and 1 mM $CaCl_2$. Islets were selected under a binocular dissecting microscope and were used within 2 h of isolation.

In secretion experiments, the saline solution was supplemented with 1 mg/ml bovine serum albumin (Type V). In static incubations, groups of three isolated islets were incubated in the presence of test reagents for 60 min at 37°C. After this time, samples of the medium were removed for measurement of their insulin content by radioimmunoassay.

For de-sensitization experiments, islets were cultured in RPMI-1640 culture media, supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, the antibiotics penicillin (400 IU/ml) and streptomycin (200 μg/ml) and test reagents, in a humidified atmosphere of air:CO_2 (95:5) for 18 h. After this time, the islets were washed in bicarbonate-buffered physiological saline. Following a pre-incubation period of 30 min in the physiological saline, the islets were then used in static incubation experiments.

2.3. Statistics

Data are presented as means ± S.E.M. The statistical significance of differences between means was assessed by analysis of variance followed by Tukey's multiple comparison test or by the Student's *t*-test for unpaired data when only two groups were compared.

3. Results

3.1. Secretory responses to DTG and 3-PPP

It has been shown previously that the insulin secretagogue activity of imidazolines, including efaroxan, is glucose-dependent. Therefore, in initial studies the effects of DTG and (+)-3-PPP were investigated in islets incubated with increasing concentrations of glucose. Islets responded to increasing glucose (0–20 mM) with the typical sigmoidal dose–response curve (Fig. 1A). The inclusion of each of the σ receptor ligands (100 μM) resulted in a further elevation of the secretory rate at intermediate glucose concentrations but neither DTG nor (+)-3-PPP enhanced secretion at a sub-threshold (0, 4 mM) or maximal (20 mM) glucose concentration. Fig. 1B shows the effect of increasing concentrations of (+)-3-PPP on insulin release in islets incubated at 6 mM glucose. At 10 μM, (+)-3-PPP induced a slight but not significant increase in the rate of insulin release. However, at 100 μM, a marked increase in the secretion rate was observed with (+)-3-PPP.

It is well established that imidazoline secretagogues are able to reverse the inhibitory effects of the K_{ATP} channel agonist, diazoxide, on glucose-induced insulin release (Chan and Morgan, 1990; Plant and Henquin, 1990). In accord with this, the insulin secretory response of islets incubated at 20 mM glucose was diminished by inclusion of 200 μM diazoxide and this response was overcome by 100 μM efaroxan (Fig. 2). (+)-3-PPP was also able to reverse the inhibition of glucose-induced secretion mediated by diazoxide, in a dose-dependent manner. The potency of this response was very similar to that seen previously with efaroxan (Chan and Morgan, 1990) in that the threshold concentration was approximately 10 μM and the $EC_{50} \sim 20$ μM (+)-3-PPP. Equivalent data were also obtained with DTG (not shown). These values for both direct stimulation of insulin release and the reversal of diazoxide-induced effects are considerably higher that estimates of the Ki of these σ receptor ligands for binding to σ sites.

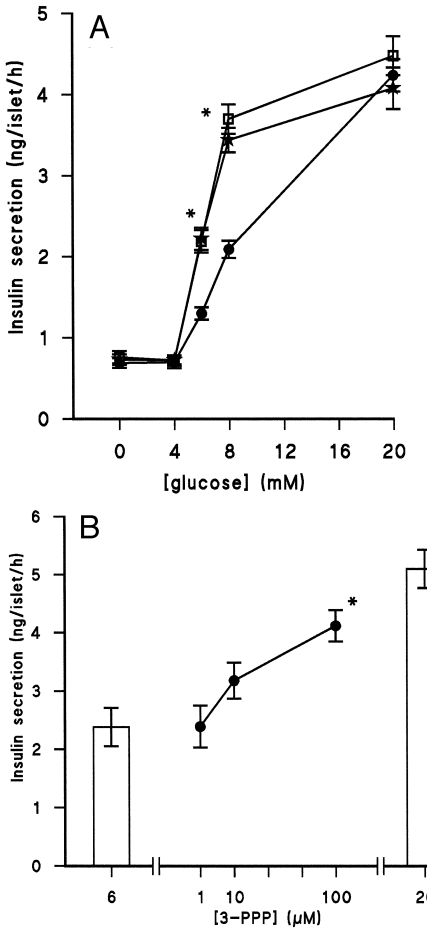

Fig. 1. The effect of DTG and (+)-3-PPP on glucose-stimulated insulin release. (a) Groups of three islets were incubated with increasing glucose concentrations (range 0–20 mM), in the absence (●) or presence of 100 μM DTG (□) or (+)-3-PPP (★). Following incubation at 37°C for 60 min, samples of the incubation medium were assayed for insulin. Data represent mean rates of insulin release ± S.E.M. for 22–27 observations. * $P < 0.001$, relative to absence of σ receptor ligand. (b) Groups of three islets were incubated at 6 mM glucose, with increasing concentrations of (+)-3-PPP (as indicated) or 20 mM glucose. The secretion rate was measured after 60 min of incubation at 37°C. Results are mean values ± S.E.M. for seven observations. * $P < 0.001$, relative to absence of (+)-3-PPP.

3.2. Interactions between σ receptor ligands and imidazolines

On the basis of the initial results presented above, it appears that the insulin secretory effects of DTG and (+)-3-PPP are very similar to those obtained with efaroxan. In order to investigate the relationship between efaroxan and the σ receptor ligands further, DTG and (+)-3-PPP were added to islets in the presence of a stimulatory concentration of efaroxan. Under these conditions, there was an unexpected loss of activity compared to when islets were incubated with either of the two groups of compound alone (Fig. 3). Thus, efaroxan alone, reversed the inhibitory effect of diazoxide, as did either DTG or (+)-3-PPP alone (Fig. 3). However, when efaroxan was present

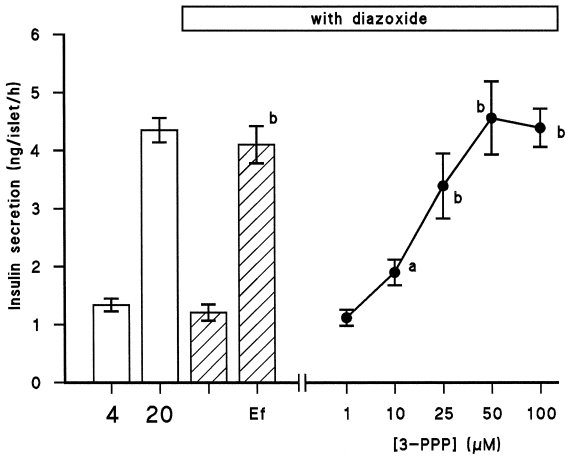

Fig. 2. Dose-dependent reversal of diazoxide-induced inhibition of insulin secretion by (+)-3-PPP. Isolated rat islets were incubated in the presence of 4 mM glucose, 20 mM glucose, 20 mM glucose plus 200 μM diazoxide (hatched bars), efaroxan (Ef, 100 μM) and increasing concentrations of (+)-3-PPP. The secretion rate was measured after 60 min of incubation at 37°C. Results are mean values ± S.E.M. for 14 observations. (a) $P < 0.05$; (b) $P < 0.001$, relative to 20 mM glucose plus diazoxide alone.

with either DTG or (+)-3-PPP, the response to each agent was abolished (Fig. 3).

3.3. Effect of functional de-sensitization on responses to efaroxan and σ receptor ligands in rat islets

Previous work has established that prolonged exposure (> 18 h) of islets to efaroxan leads to functional de-sensitization such that the islets no longer respond to the imida-

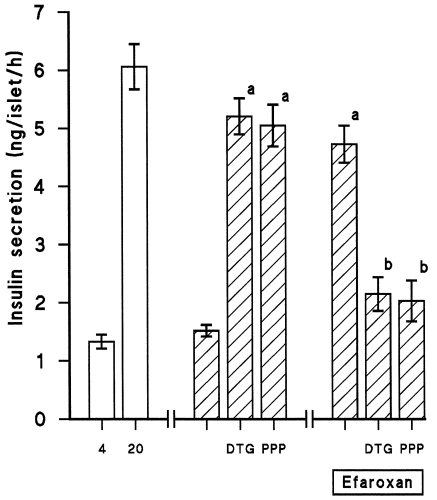

Fig. 3. Interactions between DTG and (+)-3-PPP with the imidazoline secretagogue agonist efaroxan and antagonist KU14R. Isolated rat islets were incubated in the presence of 4 mM glucose, 20 mM glucose, 20 mM glucose plus 200 μM diazoxide (hatched bars) and the following compounds: DTG, (+)-3-PPP, efaroxan, and KU14R, all at 100 μM. The secretion rate was measured after 60 min of incubation at 37°C. Results are mean rates ± S.E.M. for 14–21 observations. (a) $P < 0.001$, relative to 20 mM glucose plus diazoxide alone; (b) $P < 0.001$, relative to absence of efaroxan.

zoline upon subsequent re-exposure. We exploited this phenomenon to establish whether the responses to efaroxan and σ receptor ligands can be de-sensitized in parallel. Islets were first cultured in the presence of 100 μM efaroxan to achieve de-sensitization to the imidazoline. Fig. 4 (left panel) shows that control islets, cultured in media without efaroxan, responded to efaroxan, DTG and the combination of the two, as expected: each compound alone reversed the inhibitory effect of diazoxide but when administered together this effect was lost. Islets cultured in the presence of efaroxan (Fig. 4; right panel) were unresponsive to efaroxan on subsequent re-exposure, consistent with functional de-sensitization of the islet imidazoline receptor. However, in these islets, DTG was still able to reverse the inhibitory effect of diazoxide on insulin secretion. Moreover, co-incubation of efaroxan and DTG did not result in the loss of reversal of diazoxide's action on insulin release, rather the secretion rate of islets under these conditions was at a level similar to islets treated with DTG alone (Fig. 4; right panel). The sulphonylurea glibenclamide was highly effective at reversing the inhibition of secretion induced by diazoxide in these efaroxan-de-sensitized islets (Fig. 4; right). These results confirm that the imidazoline binding site and the sulphonylurea receptor are distinct but further suggest that the σ receptor and the imidazoline binding site can also be functionally discriminated.

It has been documented that σ receptors are also subject to down-regulation upon long term exposure to selective agonists (Bremer et al., 1989; Itzhak and Alerhand, 1989). In similar experiments to those described above, islets were cultured for 18 h in the presence of 100 μM DTG, and their secretory responses subsequently examined. In such islets, there was no loss of response to efaroxan, but the islets were refractory to DTG (Fig. 5). Moreover, the paradoxical loss of response to efaroxan in the presence of DTG was no longer evident in these islets (Fig. 5). Thus, these results support the view that the insulin secretory responses mediated by efaroxan and DTG must be regulated independently.

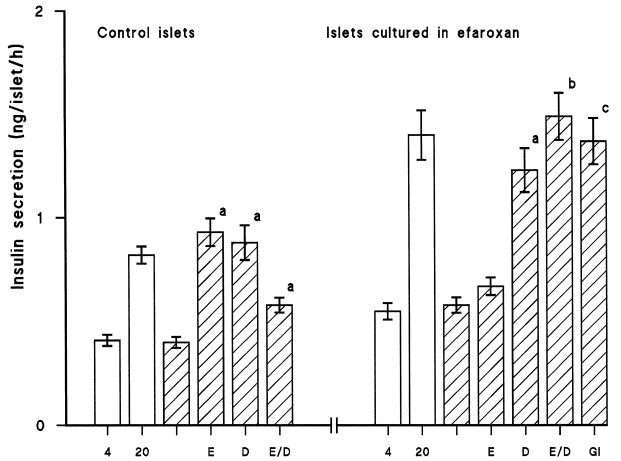

Fig. 4. Reversal of diazoxide-induced inhibition of insulin secretion after de-sensitization of efaroxan-mediated responses. Isolated islets were cultured for 18 h in media either in the absence (left panel, control) or presence of 100 μM efaroxan (right panel). Islets were washed and incubated in the presence of 4 mM glucose, 20 mM glucose or 20 mM glucose plus 200 μM diazoxide (hatched bars). Efaroxan (E, 100 μM), DTG (D, 100 μM) and glibenclamide (Gl, 1 μM) were included as shown. Insulin release was measured after 60 min incubation at 37°C. Results represent mean values ± S.E.M. for 19–25 observations. (a) $P <$ 0.001, relative to 20 mM glucose plus diazoxide alone; (b) $P < 0.001$, relative to DTG alone; (c) $P < 0.001$, relative to efaroxan alone.

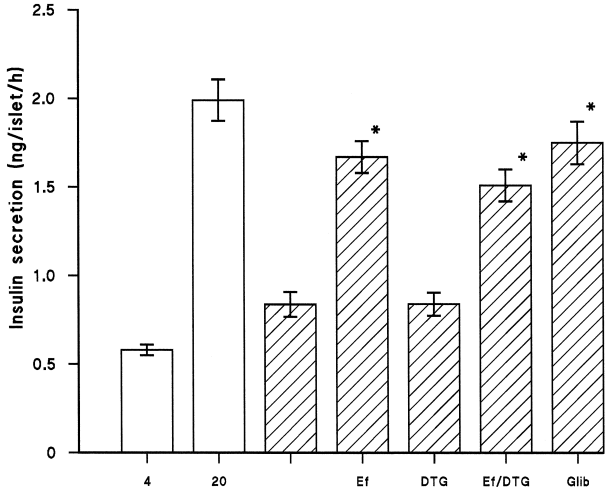

Fig. 5. Reversal of diazoxide-induced inhibition of insulin secretion after de-sensitization of DTG-mediated responses. Isolated islets were cultured for 18 h in media supplemented with 100 μM DTG. Islets were washed and incubated in the presence of 4 mM glucose, 20 mM glucose or 20 mM glucose plus 200 μM diazoxide (hatched bars). Efaroxan (Ef, 100 μM), DTG (DTG, 100 μM) and glibenclamide (Glib, 1 μM) were included as shown. Insulin release was measured after 60 min incubation at 37°C. Results represent mean values ± S.E.M. for 21–29 observations. * $P < 0.001$, relative to 20 mM glucose plus diazoxide alone.

4. Discussion

Considerable effort has been invested over the last decade to understand the functional roles of σ receptors and they have now been implicated in a diversity of physiological and pathophysiological processes (reviewed by Walker et al., 1990; Su, 1991). These include control of motor functions, neurotransmitter synthesis and release, regulation of smooth muscle contraction and neurodegeneration. In addition, we now show that certain σ receptor agonists (though not all) can stimulate insulin secretion from pancreatic β-cells. The molecular basis of this effect has not been defined in detail but some features of the response suggest that the mechanism may be similar to that responsible for stimulation of insulin secretion by efaroxan and other imidazoline ligands. This is significant in view of evidence that σ-2 receptors are associated with ion channels and that some imidazolines can displace the

binding of radioligands from σ binding sites (albeit with rather with low affinity) (Molderings et al., 1996; Olmos et al., 1996). Since imidazoline insulin secretagogues are known to reduce the rate of K^+ flux through K_{ATP} and Ca^{2+}-activated K^+ channels (Chan, 1993; Dunne et al., 1995), it is conceivable that imidazoline and σ receptors could be identical.

We have begun to examine the relationship between imidazoline receptors and σ binding sites in pancreatic islets and have obtained preliminary results which are not consistent with the view that σ receptor ligands and imidazoline compounds share a common mode of action in β-cells (Chan et al., 1997). The data obtained in the present study strengthen this conclusion. The most compelling evidence favouring a separation of their activities comes from the experiments in which imidazoline and/or σ receptor mediated responses were selectively de-sensitization by long term exposure to agonists. Down-regulation of receptors in response to long term agonist exposure has been widely reported for many receptor systems, and a number of observations have indicated that imidazoline sites can be regulated in this way (Olmos et al., 1992; Hamilton et al., 1993; Alemany et al., 1995). Previously, we have shown that those imidazolines which stimulate insulin release (e.g., efaroxan and phentolamine) are able to induce de-sensitization of imidazoline responses in islets (Chan et al., 1994). In confirmation of this, we observed that treatment of islets for 18 h with 100 μM efaroxan resulted in loss of responsiveness to this compound upon subsequent re-exposure (Fig. 4). Significantly, however, islets which no longer responded to efaroxan were still fully responsive to σ receptor ligands.

Like imidazoline receptors, σ receptors are also subject to regulation. For example, chronic administration of haloperidol to rodents results in decreased binding of σ receptor ligands, indicative of down-regulation of σ receptors (Bremer et al., 1989; Itzhak and Alerhand, 1989). In our experiments, culture of rat islets with DTG for 18 h resulted in down-regulation as judged by the failure of these islets to respond to DTG at the end of the culture period (Fig. 5). Despite this, they remained normally responsive to both glucose and diazoxide suggesting that there was no major perturbation of islet K^+ channel activity following long term DTG exposure. In DTG-desensitized islets, efaroxan still reversed the inhibitory effect of diazoxide, confirming that loss of σ receptor mediated responses was not accompanied by diminution of the response to imidazolines. Thus, the functional de-sensitization occurred in a homologous manner and was selective for σ receptor ligands.

Taken together, these results show that, in isolated rat islets, there is a clear distinction between functional responses controlled by σ receptor ligands and those regulated by imidazolines. It follows, therefore, that the effects of these two groups of agent cannot be due to occupation of identical binding sites since they are regulated differentially. This does not, however, imply that no functional relationship exists between imidazoline and σ binding sites in islets. Indeed, the evidence presented here points to the contrary. For example, we observed that addition of both a σ receptor ligand and an imidazoline compound to rat islets resulted in blockade of the response to each compound alone (Fig. 3). Thus, whereas efaroxan or DTG each reversed the inhibitory effect of diazoxide in islets, addition of both drugs together resulted in the total abrogation of this effect. This result was extremely unexpected and is difficult to explain on the basis of our current understanding. However, the observation that this paradoxical effect was lost in de-sensitized islets (irrespective of whether the de-sensitization was achieved with σ receptor ligands or imidazolines) suggests that it is mediated by the same receptors which are responsible for the insulin secretagogue activity of each group of compounds.

In spite of the evidence confirming that σ receptors and imidazoline receptors can be functionally discriminated in β-cells, it should also be emphasised that there are a number of important similarities between the actions of imidazolines and σ receptor ligands in rat islets. Thus, DTG and (+)-3-PPP stimulate insulin secretion from isolated rat islets in a glucose-dependent manner (Fig. 1) and they share, with imidazoline compounds, the ability to attenuate the inhibitory effect of diazoxide on glucose-induced insulin secretion (Figs. 2 and 3). A multi-site model, therefore, appears necessary in order to explain the mechanisms of action of imidazoline and σ receptor ligands in islets; and such a model could also be expanded to include sulphonylurea. It would then provide an explanation for the earlier finding that the imidazoline secretagogue antagonist, RX801080 (2-(2,3-dihydro-2-benzofuranyl)-2-imidazole hydrochloride), can inhibit insulin secretion induced by glibenclamide, under conditions when there was no change in [^{3}H]glibenclamide binding (Brown et al., 1993).

Further experiments will be required to test the validity of the two site model proposed here. However, it is clear that the existence of a single binding site which recognises both σ receptor ligands and imidazoline drugs, cannot adequately explain the interactions between these compounds in the pancreatic β-cell.

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