Opioid receptor-dependent modulation of insulin-release in pancreatic beta-cells

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Abstract

In this study we aimed to look at the effects of opioid-receptor selective agonists and antagonists on insulin secretion in the pancreatic β-cell line RIN-5F. Cells were treated for 24 hours with 1μM of selective agonists (DAMGO for MOP, DPDPE for DOP, U50488 for KOP) in the presence or absence of 10μM of selective antagonists (CTOP for MOP, naltindole for DOP, norBNI for KOP). An enzyme-based immunoassay was used to detect the amount of insulin in the supernatant, using a standard curve generated from known concentrations of rat insulin. A trypan blue viability assay was performed to assess the toxicity of each treatment and the secreted insulin was expressed as per 10^6 viable cells. Treatment with DAMGO or DPDPE caused an increase in secreted insulin by 94.2% and 76.3% respectively, compared to the non-treated controls. Co-treatment of DAMGO and CTOP was able to cancel out the agonists’ effect. However, CTOP itself was able to increase insulin secretion by 72% when compared to control. These results suggest that opioid-induced insulin secretion may be based on G-protein independent mechanisms. In addition, none of the KOP-receptor selective ligands tested here were able to significantly affect insulin secretion compared to control, whereas naltindole was highly toxic to pancreatic β-cells since it induced maximum cell death. Collectively, these findings suggest that MOP and DOP receptor-binding opioids might be more relevant in increasing insulin secretion from pancreatic β-cells than KOP receptor ligands.

1 Introduction

Diabetes mellitus is a metabolic disease that is characterised by a disturbance in glucose and insulin homeostasis. Insulin is excreted centrally by the nervous system and locally by the pancreatic β-cells in response to plasma glucose levels and other endocrine signals. Diabetes is clinically divided in two types: Type 1 (T1DM) results from failure of the pancreas’ ability to produce enough insulin to regulate the body’s metabolism, mainly due to an autoimmune degeneration of pancreatic β-cells. Type 2 (T2DM) manifests as an initial phase of cell desensitisation to prolong insulin secretion (called insulin resistance) and a second phase of declined insulin secretion, a manifestation which is linked to excessive plasma glucose levels and increased body weight. Based on this classification, one can conclude that glucose and insulin are key players in diabetes mellitus since their dysregulation, along with other endocrine substances such as glucagon released from pancreatic alpha-cells, play a catalytic role in the development and the progression of diabetes.

Insulin secretion, as shown by Ammala et al., is triggered by glucose treatment in the same manner as the repetitive electrical stimulations, through a calcium-dependent exocytosis. However, since the glucose effect on pancreatic cells follows a step-wise format, insulin secretion follows a characteristic biphasic time-dependent format, with a first phase of secretion within 10–15 mins, followed by a slower second phase. This biphasic insulin secretion is important in understanding opioid-induced insulin secretion. Since impaired insulin secretion from pancreatic β-cells is a major factor in the pathology of both types of diabetes mellitus, it is only rational that many studies have focused on investigating the effect of different drugs on this process. Although there are a number of regulators of insulin secretion, such as plasma glucose levels, circulating hormones, paracrine and autocrine signals, the main player in the regulation of β-cell secretion is insulin itself, since it has been reviewed in maintenance and regulation of β-cell function, another reason why insulin secretion is such a critical factor in the pathophysiology of diabetes.
Patients with diabetes mellitus may develop different types of painful neuropathies. About 30% of diabetic patients experience neuropathic pain due to nerve injury induced by diabetes and about 20% of patients will develop chronic pain at some point during their life. Although opioids like morphine and fentanyl are considered ideal medications for medium-severe pain, their efficacy in diabetic neuropathy is as limited as in other types of neuropathic pain. Nevertheless, the combination of opioids with non-opioid drugs that are effective in neuropathic pain (such as α2 adrenoceptor agonists and serotonin/noradrenaline reuptake inhibitors) has proven to offer certain therapeutic advantages, while it is being used in cases of diabetic neuropathies where first-line treatment with anticonvulsants or antidepressants was ineffective.

Opium has been used for thousands of years due to its various therapeutic and recreational properties (i.e. analgesia, euphoria, drowsiness). However, it was only after the discovery of the three classical opioid receptor types (mu; MOP, delta; DOP, kappa; KOP) and their endogenous ligands (endomorphins, enkephalins and dynorphins respectively) that we increased our understanding for the opioid system and made opioid drugs one of the few classes of compounds that have been studied for so long. Opioid receptors are found in a wide variety of tissues, including the hypothalamic-pituitary system, while their role and effect in endocrine regulation has been well-studied during the last decade. All opioid receptors (MOP and DOP primarily, KOP in less extent) have been shown to be expressed in the pancreas and liver, while endogenous opioid peptides and their receptors have been shown to be present in pancreatic α- and β-cells.

Nevertheless, the first published work on the identification of endogenous opioids in the pancreas gave rise to studies that focused on the ambiguous mechanisms involved in the endogenous opioid-mediated glucose and insulin homeostasis. First showed that morphine and met-enkephalin were able to stimulate glucose-induced insulin release in isolated pancreatic islets, an effect that was observed to develop rapidly, representing the first phase of pancreatic insulin release and which could be blocked by naloxone pre-treatment and therefore, showing an opioid-receptor mediated effect. A number of studies that followed confirmed the results of Green et al either directly or indirectly by showing a decrease in insulin secretion by administration of naloxone in healthy humans, which highlighted the role of endogenous opioids on pancreatic function.

Today, opioid receptor targeting has been shown to be effective in improving hypoglycemia, as well as stimulating insulin secretion. Therefore, although more than 30 years have passed since the first studies on the endocrine effects of opioids, there hasn't been sufficient progress towards a clear understanding about the role of opioid receptors and endogenous opioids in insulin secretion, as reflected by the small volume of published work on this area of research compared to the length of period studied. Part of the reason for this delay in progress, is the considerable controversy that exists in the literature regarding the contribution of the three classical opioid receptors and their ligands to the mechanisms and pathways mediating glucose-induced insulin secretion. A number of studies have shown a stimulatory effect of opioid agonists, while others have shown the opposite inhibitory effect.

A major hypothesis for this inconsistency is based on the different methodologies employed and the different models used in detecting insulin levels. The insulin signaling system is autoregulated through different homeostatic mechanisms that involve glucose and glucagon secretion (among others). It is therefore most likely that observations of insulin secretion using high-complexity models (e.g. animal studies, perfused organs, mixed-cells tissues etc) would differ from observations performed in primary β-cells or β-cell lines. Many in vitro studies that we discuss later in this manuscript, have used isolated pancreatic islets to study glucose-induced insulin secretion, although these isolates include different types of cells (α-cells, β-cells, γ-cells) that produce different signals under certain conditions (e.g. glucagon, insulin, neuropeptide Y, amyloid peptide, ATP, GABA and other peptides) which can contribute differentially to the final insulin secretion. This heterogeneity may qualify studies using β-cells in particular (the main cells secreting insulin in pancreas) as more accurate in terms of drug-screening on insulin secretion.

Nevertheless, a major gap in the literature is the investigation of the individual role of the three classical opioid receptors in insulin secretion, using receptor-selective agonists and antagonists. Interestingly, no one looked at the effects of selective activation of MOP, DOP and KOP receptors in glucose-mediated insulin release in a β-cells line. Detailed investigation into the role of the different opioid receptors involved in opioid-mediated insulin secretion and glucose homeostasis may lead to the identification of new target strategies in diabetes therapy. A major step towards this aim is to understand the individual role of the classical opioid receptors in insulin secretion.

Here we report the effect of different opioid receptor-selective ligands, agonists and antagonists, to glucose-induced insulin release in a rat pancreatic cell line. In this study, we are using the well-characterised rat pancreatic β-cell line, RIN-5F, which releases insulin in glucose-containing media.

2 Methods

2.1 Materials

The pancreatic β-cell line (RIN-5F) was purchased from the American Type Culture Collection (ATCC® CRL-2058™; P.O. Box 1549 Manassas, VA 20108 USA). The insulin immunoassay kit was

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purchased by Merck Millipore (EZRMI-13K; Massachusetts 01821, USA). All tissue culture media and supplements were obtained from Life Technologies (GIBCO®, Paisley, U.K.). [D-Pen2]-enkephalin (DPDPE), nor-binaltorphimine (nor-BNI) and the D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) were purchased by Abcam Biochemicals. The [D-Ala2 N-Me-Phe4 Gly5-ol]-enkephalin (DAMGO) was purchased by AdipoGen. Naltrindole hydrochloride and the trypan blue solution (0.4%, 100 ml/bottle) were purchased by Sigma-Aldrich. All other reagents used were purchased with the highest quality available.

2.2 Cell culture and maintenance

Cells were cultured and maintained in a similar way as previously described. Briefly, RIN-5F rat pancreatic β-cells were grown in 25cm² flasks in RPMI-1640 media containing L-glutamine, substituted with 10% heat-inactivated fetal calf serum, 100 IU ml⁻¹ penicillin, and 100 mg ml⁻¹ streptomycin. Stock cultures were grown at 37°C with 5% CO₂, 95% humidity and were subcultured twice a week as required using phosphate buffered saline and trypsin/0.5mM EDTA. Cells were used for experiments as they approached confluence. An appropriate volume of cells was seeded in the wells of a 96-well plate, in the presence or absence of drugs in a total volume of 200μl and were incubated for 24 hours. Prior to the main experiment, the cell number-dependent basal insulin secretion levels of RIN-5F cells (1x10⁴, 2x10⁵ and 4x10⁶ cells/well) were determined.

2.3 Drug treatment

Cells were treated with a selective opioid-receptor agonist at 1μM final concentration (DAMGO; a MOP agonist, DPDPE; a DOP agonist, U50488; a KOP agonist) to determine their effect on insulin secretion (n=4-5 wells per ligand). A combination treatment of an opioid agonist plus the respective opioid-receptor antagonist at 10μM final concentration (CTOP; a MOP antagonist, naltrexone; a DOP antagonist, nBNI; a KOP antagonist) was used in some wells to determine if the potential effect of the agonist was receptor-mediated (n=4-5 per group). Antagonists where used at a higher concentration than the agonist in order to ensure sufficient displacement of the agonist from their receptor binding site. In addition, cells in some wells were also treated with an antagonist alone (n=4-5 per ligand), to determine the effect of these ligands on insulin secretion. Cells that were not treated with any compound were used as a negative control.

2.4 Immunoassay for insulin detection

An enzyme-based immunoassay (sandwich ELISA) was used to detect the amount of insulin secreted by RIN-5F cells in the presence or absence of test compounds, according to the manufacturer’s instructions. In brief, after incubation of cells with test compounds for 24 hours, a supernatant sample of 10μl was taken from all wells and transferred to a microtiter plate pre-coated with a monoclonal mouse anti-rat insulin antibody. The captured insulin was then detected by a second, biotinylated polyclonal anti-insulin antibody. Unbound materials were thoroughly washed away with manufacturer’s washing buffer prior to the binding of streptavidin-horseradish peroxidase to the immobilized biotinylated antibodies. Another step of thorough washing of free enzyme conjugates followed before the final quantification of immobilized antibody-enzyme conjugates. The quantification was performed by monitoring the horseradish peroxidase activity in the presence of its substrate (3,3′,5,5′-tetramethylbenzidine).

The enzyme activity was measured by spectrophotometry at 450 nm, corrected from the absorbance of 590nm, after the acidification of formed products with 0.3M HCL. Since the increase in absorbance is directly proportional to the amount of captured insulin in the samples, the latter was derived by interpolation from a reference curve generated within each performed assay using reference standards of known concentrations of rat insulin. The dose-response curve produced by the internal standards was fitted to a sigmoidal 4-parameter logistic equation according to the manufacturer’s instructions by plotting the corrected absorbance at 450nm minus 590nm, against the concentration of rat insulin standards.

2.5 Viability assay

A Trypan blue exclusion assay was used to determine the viability of the pancreatic cells in the presence of test compounds, according to the Strober method. Trypan blue is impermeable to intact healthy cells but can enter cells with a compromised cell membrane creating an intracellular blue staining that can be detected by light-microscopy. Trypan blue staining can be therefore used as a marker of cell death. Cells treated for 24 hours with test compounds were trypsinized, stained with 0.04% trypan blue. Before the number of stained cells versus the total number of cells were counted using a haemocytometer under a light microscope. Viability was defined as viable cells per well.

2.6 Data analysis

Concentration–response curves were analysed by linear regression or by a 4-parameter sigmoidal fit, as appropriate. GraphPad Prism V6.0 software (San Diego, CA, USA) was used for statistical analysis. Students t-test and analysis of variance (ANOVA) with multiple comparison testing were used as required and as described in the table and figure legends (minimum significance set at p<0.05). Data is presented as mean ± SD from (n) number of experiments as indicated in the respective figure or table legends.
3 Results

3.1 Optimization of insulin detection

RIN-5F cells physiologically secrete insulin when incubated in an appropriate glucose-contained growth medium. Since the amount of secreted insulin/well is dependent on cell numbers, we first optimized cell numbers per well for a 96 well format to obtain absorbance values that fall within the range of values of the standard curve. Three different concentrations were used (1x10^4; 2x10^4 and 4x10^4 cells/well) with five parallel wells per group.

Known amounts of insulin (0, 0.5, 1, 2, 5, 10 ng/ml) were used in triplicates as internal standards to produce a standard curve (Figure 1), which was initially fitted using linear regression analysis (R^2 = 0.9898), confirming the linearity of the internal standards (Figure 1A). Subsequently, the same data was fitted more efficiently to a sigmoidal 4-parameter logistic equation (Figure 1B; R^2 0.9990, EC50 5.62, HS 0.23), which did not produce any significant normality of residuals (D’Agostino & Pearson’s test; p=0.583). These data confirmed the manufacturer’s suggestion for using the sigmoidal fitted curve for interpolation of data derived from samples. Absorbance values from the three cell concentrations tested were plotted against the derived insulin values from the sigmoidal standard curve (Figure 1C). Although all three groups produced large amounts of secreted insulin (>20 ng/ml) using 10^4 cells per well produced the least variability (variation coefficient 2.80%) and a mean absorbance value of 2.65, which translated to 23.8 ng/ml of insulin per well. Since the minimum acceptable density for optimum growth of the RIN-5F cells in a 96-well is 10^4, this cell density was chosen to be used for all subsequent experiments. The rationale was that the collected samples of these wells would be diluted 4 times before processing by ELISA and therefore the secreted basal insulin value would be expected to fall within the EC50 of the curve (e.g. 23.8 ng.ml^1 x 4 = 5.95 = EC50 5.62).

3.2 Total secreted insulin after opioid treatment

After determining the optimal cell density numbers per well, we measured insulin secretion of cells seeded and treated with test compounds. An internal standard curve was generated within this assay and analyzed using both analysis methods (linear regression and the 4-parameter sigmoidal logistic equation; Figure 2A, B). In agreement with the previous data, the sigmoidal standard curve provided a better fit compared to a linear fit and was therefore used to interpolate the absorbance values produced by the samples.

After taking into account the dilution factor used to dilute the samples (x4), the concentration of secreted insulin by the seeded cells was calculated and expressed as a bar-chart (Figure 2C). Compared to the untreated control, treatment of cells with the selective MOP receptor ligands DAMGO, CTOP and combination of both, significantly increased insulin secretion. Treatment with the DOP receptor specific agonist DPDPE also significantly increased insulin secretion. A more peculiar relationship was observed for the KOP receptor, where the selective agonist U50488 and the antagonist nBNI did not elicit any effects, while conversely their combination significantly increased insulin secretion (Figure 2C). Finally, naltrindole-treated cells (with or without DPDPE) showed a highly significant decrease of insulin secretion, reflecting the high-toxicity of naltrindole that we previously observed in non-pancreatic cells (non-published observation).

3.3 Secreted insulin / cell numbers at endpoint

Since the test compounds can potentially affect cellular viability and proliferative capacity, which is likely to affect the extent of secreted insulin, it is important to account for these potential effects when evaluating the stimulatory or inhibitory effects of drug-induced insulin secretion. We therefore, measured cellular viability using trypan blue (TB) exclusion, immediately after the collection of the supernatant. Since the assay provides information on the number of dead and alive cells in each treated well, it allows the expression of our total results as a ratio of secreted insulin/live cells at endpoint. Taking the number of live cells in the non-treated wells as a baseline, we calculated the live cells per treated well. Different opioid treatments differentially affected cell viability and proliferation over the same time period (Table 1). Consistent with our preliminary results (unpublished), TB staining revealed that naltrindole was highly toxic to pancreatic RIN-5F cells, with no viable cells after 24-hour incubation with this DOP-selective antagonist.

After determining the number of viable cells per well following 24 hours of treatment, we standardized the insulin concentrations on cell numbers (Figure 3). The insulin values of Figure 3 are shown in Table 2 as mean insulin secretion and as percentage increase or decrease compared to the baseline control. Untreated control cells produced insulin levels of 2.24 (±0.85) ng/ml/10^5 cells, while in comparison the MOP receptor agonist DAMGO nearly doubled the measured insulin levels (12.12 ±0.81 ng/ml/10^5 cells; a 94.2% increase compared to control). In parallel, the DOP receptor agonist DPDPE also significantly stimulated insulin secretion (11.00 ±2.55 ng/ml/10^5 cells; a 76.3% increase compared to control). Interestingly, compared to DPDPE the MOP receptor antagonist CTOP on the other hand, also prolonged insulin secretion (10.73 ±3.11 ng/ml/10^5 cells; a 72% increase). Co-treatment of CTOP and DAMGO was able to reduce the effect of their individual administration, although showing a trend towards increased insulin secretion, which was not significant from control. Contrary to the MOP and DOP ligands, none of the KOP agonist and antagonist tested significantly affected insulin levels, despite a small trend towards higher insulin levels.
Figure 1 Optimization of the insulin detection method using an enzyme-based immunoassay (ELISA). A standard curve was generated by using known amounts of insulin (0, 0.5, 1, 2, 5, 10 ng/ml) in triplicates which were fitted using linear regression analysis (A), confirming the linearity of the internal standards. The same data were fitted more efficiently into a sigmoidal 4-parameter logistic equation (B) which did not produce any significant normality of residuals (D’Agostino & Pearson’s test; p=0.583). Three different cell densities (1x10^5, 2x10^5 and 4x10^5) were seeded in individual wells and the corresponding standard curve was used to calculate the total amount of secreted insulin per well (C). Absorbance was measured at 450nm corrected for the absorbance at 590nm. All data are shown as mean ± SD from n=4-5.

Figure 2 Detection of total insulin secreted in the well, using an enzyme-based immunoassay (ELISA) and reading absorbance at at 450nm minus 590nm. A standard curve was generated by using known amounts of insulin (0, 0.5, 1, 2, 3.8, 5, 7.8, 10 ng/ml) in triplicates which were fitted using linear regression analysis (A) and a sigmoidal 4-parameter logistic equation (B); the latter was used to calculate the total insulin concentration in wells treated or non-treated with opioid-receptor selective ligands (C). One-Way analysis of variance (ANOVA) was used with a Sidak multiple comparison test (* p<0.005 and ** p<0.0005 compared to non-treated control; # p<0.0001 compared to DPDPE). All data are shown as mean ± SD from n=4-5.

Finally, since naltrindole completely killed the treated cells over a period of 24 hours, the ratio of insulin release/cell number could not be determined. However, the total insulin reduction in the naltrindole-treatment shows that naltrindole’s toxicity is so rapid that it inhibited the basal glucose-induced insulin excretion. Also, the increase of insulin seen with DPDPE was significantly abolished by naltrindole co-treatment (3.02 ±0.48 ng/ml/10^5 cells; a 51.6% decrease) due to naltrindole’s toxicity, but it was not zero. DPDPE’s concentration in the co-treatment group was 10% of that of naltrindole and was still able to contain some of naltrindole’s toxicity, insinuating a competitive behavior for both actions (DPDPE’s insulin stimulation and naltrindole’s toxicity) which most likely is mediated through the DOP receptor.

4 Discussion

Opioids represent an important drug class in diabetic pharmacotherapy due to their role in diabetic neuropathy and their complex and under-explored endocrine effects. Although opioids are not considered very effective as a stand-alone treatment for diabetic neuropathic pain, their strong analgesic effect offers a potential co-administration solution to drug-resistant and persistent neuropathic pain44. In addition, the remarkable wide range of tissues that express the classical opioid receptors constitutes the basis of their non-analgesic side effects, some of which are currently explored for conditions other than pain45-47. The expression of opioid receptors in pancreas and liver47 initiated early studies to perceive the role of endogenous opioids in processes associated with the endocrine and autocrine system49, such as glucose homeostasis50 and insulin...
release\textsuperscript{40}, as well as in related conditions such as diabetes\textsuperscript{51}, obesity\textsuperscript{22}, exercise-associated autonomic failure\textsuperscript{53} and hypoglycemia-associated failure\textsuperscript{30} among others.

Table 1 Cell viability data from cells treated with different opioid ligands compared to non-treated control using a trypan-blue exclusion assay. Results are expressed as a mean number of cells viable per well after 24h treatment (± SD from n=4-5). One-Way analysis of variance (ANOVA) was used with a Sidak multiple comparison test (* p<0.05 and ** p<0.005 compared to non-treated control).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of viable cells per well (x10\textsuperscript{3}; mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ligand (control)</td>
<td>520 ±80</td>
</tr>
<tr>
<td>DAMGO</td>
<td>346 ±166</td>
</tr>
<tr>
<td>DAMGO + CTOP</td>
<td>520 ±153</td>
</tr>
<tr>
<td>CTOP</td>
<td>410 ±167</td>
</tr>
<tr>
<td>DPDPE</td>
<td>370 ±9</td>
</tr>
<tr>
<td>DPDPE + naltrindole</td>
<td>120 ±15 *</td>
</tr>
<tr>
<td>Naltrindole</td>
<td>0 **</td>
</tr>
<tr>
<td>U50488</td>
<td>430 ±92</td>
</tr>
<tr>
<td>U50488 + nBNI</td>
<td>530 ±92</td>
</tr>
<tr>
<td>nBNI</td>
<td>320 ±16</td>
</tr>
</tbody>
</table>

Table 2 Secreted insulin concentration per viable cells after treatment with opioid-receptor selective ligands, compared to basal (non-treated control). Concentration data are extracted from Figure 3 and are shown as percentage of significant change from the control. One-Way analysis of variance (ANOVA) was used with a Holme-Sidak multiple comparison test (* p<0.05 and ** p<0.005 compared to non-treated control; # p<0.05 compared to DAMGO and ## p<0.005 compared to DPDPE). All data are shown as mean ± SD from n=4-5.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Secreted insulin (mean ng/ml/10\textsuperscript{3} viable cells ± SD)</th>
<th>Significant % change from control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ligand (control)</td>
<td>6.24 ±0.85</td>
<td>-</td>
</tr>
<tr>
<td>DAMGO</td>
<td>12.12 ±0.81</td>
<td>+ 94.2 **</td>
</tr>
<tr>
<td>DAMGO + CTOP</td>
<td>8.06 ±2.17</td>
<td>- #</td>
</tr>
<tr>
<td>CTOP</td>
<td>10.73 ±3.11</td>
<td>+ 72.6 *</td>
</tr>
<tr>
<td>DPDPE</td>
<td>11.00 ±2.55</td>
<td>+ 76.3 *</td>
</tr>
<tr>
<td>DPDPE + naltrindole</td>
<td>3.02 ±0.48</td>
<td>- 51.6 ##</td>
</tr>
<tr>
<td>Naltrindole</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>U50488</td>
<td>8.43 ±2.49</td>
<td>-</td>
</tr>
<tr>
<td>U50488 + nBNI</td>
<td>7.68 ±1.53</td>
<td>-</td>
</tr>
<tr>
<td>nBNI</td>
<td>8.96 ±0.70</td>
<td>-</td>
</tr>
</tbody>
</table>

The majority of these studies on opioids and insulin secretion have investigated the effect of endogenous opioid ligands (e.g. enkephalins, endomorphins) or clinically used opioids (e.g. morphine, fentanyl) on insulin secretion in isolated pancreatic islets. To the best of our knowledge there are no current data in the literature regarding opioid receptor-specific agonists & antagonists and their effect on insulin secretion in a single study. In this manuscript we provide the first data for MOP, DOP and KOP selective agonists and antagonists in terms of their effect on insulin secretion in pancreatic β-cells (Figure 4).

Figure 3 Detection of insulin secreted per 10\textsuperscript{3} viable cells by using an enzyme-based immunoassay (ELISA) and reading absorbance at 450nm minus 590nm. The chart was produced by combining the total insulin data per well from Figure 2C and the cell viability data from Table 1. One-Way analysis of variance (ANOVA) was used with a Holme-Sidak multiple comparison test (* p<0.05 and ** p<0.005 compared to non-treated control; # p<0.05 compared to DAMGO and ## p<0.005 compared to DPDPE). All data are shown as mean ± SD from n=4-5.

Since insulin plays a central role in glucose homeostasis, in pancreas autocrine function and in diabetes, the effect of opioid ligands (endogenous and synthetic) on glucose-induced and glucose-independent insulin secretion has attracted significant attention. However, the convoluted relationship between different feedback systems in pancreatic endo-/autocrine function, as well as the different pharmacology of the three classical opioid receptor types, has created some inconsistency in the literature and a certain level of complexity in the interpretation of published data. The main question posed was: do opioids increase or decrease glucose-induced insulin secretion? Answering this question is not a simple task, given the different cell types in the pancreas, the effect of glucagon and other peptides on pancreatic function, the cross-talk between liver and pancreas on glucose homeostasis and the central nervous system effect on the pancreas (e.g. hormonal secretion and opioidergic neuronal activation).
Our outcomes provide preliminary evidence that MOP and DOP selective ligands DAMGO and DPDPE respectively can stimulate overall glucose-induced insulin secretion from β-cells, measured after 24 hours of treatment; a time-period which would include both phases of insulin’s biphasic secretion. These data fit with the majority of the previous studies on opioid-mediated insulin release, which show a stimulatory effect of opioids as described in the introduction section above.

Similarly to Qian et al. who used an MTT viability assay to assess whether opioid effect cellular toxicity of the opioid concentrations used, we used the TB viability assay to account for the potentially different effects of the different opioids used.

In addition, we have observed that the MOP-selective antagonist CTOP also significantly increased insulin secretion in our cells, which clearly shows that a MOP receptor antagonist can also increase insulin secretion similarly to MOP receptor agonist like DAMGO. This result insinuates that the effect of increased insulin secretion is either mediated through a non-opioid receptor mechanism or through a G-protein-activation independent mechanism. However, the reduced effect on insulin release by the co-treatment of DAMGO and CTOP compared to individual treatment presents a case of antisynergism which insinuates an opioid-receptor mediated mechanism, although further investigation is needed to draw solid conclusions.

In this manuscript we also show the insignificant contribution of the KOP receptor-specific opioids to insulin secretion, as shown by the non-significant change of insulin secretion by the KOP receptor agonist U50488 and the KOP receptor antagonist norBNI. Finally, we show in this manuscript that the DOP receptor selective antagonist naltrindole possesses a significant toxicity to β-cells, which seems to be partially attenuated by the co-administration of the DOP receptor selective agonist DPDPE. Whether this effect of DPDPE is interpreted as a protection against naltrindole’s toxicity, or simply a result of a competition for DOP receptor binding, the conclusion drawn is similar.

Since our data suggest that insulin release can be increased by both agonists and antagonists of the MOP and DOP receptors (e.g. DAMGO, DPDPE, CTOP), it intrigued us to hypothesize an opioid mediated insulin secretion which is dependent on opioid receptor-binding but being independent from G-protein activation. This hypothesis fits with a study by Green et al. that ascertained a cAMP-independent mechanism of opioid-induced stimulation of insulin secretion, as well as with data from Olianas et al. who observed a cAMP-independent mechanism of DPDPE-induced glucose uptake. DPDPE and DAMGO are both analogues of enkephalin (Tyr-Gly-Gly-Phe-Met/Leu) that possess different selectivity on opioid receptors (DOP and MOP receptors respectively), while CTOP is also a tyrosine and penicillamine containing peptide. It is therefore possible that these structurally similar ligands bind to MOP or DOP receptors and cause a cAMP-independent intracellular reaction that causes insulin-containing vesicles in β-cells to release their insulin.

Our data suggest that the KOP receptor targeted ligands U50488 and norBNI did not produce a significant insulin release compared to control. These two ligands are structurally very different from the rest of the opioids tested since they are not peptides. In addition, although it is questionable whether KOP receptors are expressed in pancreatic islets, higher concentrations of these ligands need to be tested in the future to check whether there is an effect on insulin...
secretion. However, in a recent study by Shang et al, treatment of STZ-mice with U50488 reduced hyperglycemia but did not affect plasma insulin levels, which shows that KOP receptors may play a role in glucose-homeostasis rather than insulin secretion per se.

5 Conclusion

Our study furnishes evidence of the opioid-induced increase of insulin secretion, by receptor selective agonists and antagonists, which implies that the role of opioid receptors in insulin secretion in β-cells is more complicated than G-protein dependent. Moreover, investigation into the molecular pathways that are involved downstream of the opioid receptor after ligand binding is needed to clarify the role of opioid receptors on insulin secretion. Understanding these pathways may provide an insight in novel pancreatic mechanisms of insulin secretion and the identification of novel drug targets for diabetes.

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7 Competing interests

None

8 Author’s contributions

ND and NG conceived and designed the experiments. AP carried out the literature review, conducted all experiments, collected and analyzed the data. All authors provided input in the interpretation of the data. All authors participated equally in the drafting of the manuscript. All authors have read and approved the final version of the manuscript.

9 References


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