EXPERIMENTAL STUDY

Inhibition of insulin secretion via distinct signaling pathways in α_2-adrenoceptor knockout mice

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Abstract

Objective: Adrenaline inhibits insulin secretion through activation of α_2-adrenoceptors (ARs). These receptors are linked to pertussis toxin-sensitive G proteins. Agonist binding leads to inhibition of adenylyl cyclase, inhibition of Ca^{2+} channels and activation of K^+ channels. Recently, three distinct subtypes of α_2-AR were described, α_2A-AR, α_2B-AR and α_2C-AR. At present, it is unknown which of these α_2-AR subtype(s) may regulate insulin secretion. We used mice deficient in α_2-ARs to analyze the coupling and role of individual α_2-AR subtypes in insulin-secreting β cells.

Methods: The inhibitory effect of adrenaline on insulin secretion was measured in freshly isolated and cultured wild type (wt) and α_2-AR knockout (KO) mouse islets in order to examine the receptor subtypes which mediate adrenaline-induced inhibition of insulin secretion. Adenylyl cyclase activity was measured in isolated cultured islets. Membrane potential was measured using the amphotericin B permeabilized patch clamp method in isolated and cultured single islet cells.

Results: In wt, α_2A- and α_2C-AR KO mouse islets, adrenaline, 1 μmol/l, inhibited secretion by 83, 80 and 100% respectively. In contrast, in α_2A/2C-AR double KO mouse islets, adrenaline had no effect on stimulated secretion indicating that both α_2A-AR and α_2C-AR, but not α_2B-AR, are functionally expressed in mouse islets. Surprisingly, glucose (16.7 mmol/l)-induced secretion in the presence of 1 μmol/l forskolin was greatly impaired in α_2A-AR KO islets. However, when cAMP levels were increased further by the combination of forskolin (5 μmol/l) and 3-isobutyl-1-methylxanthine (100 μmol/l), secretion was stimulated 2.7-fold (8.5-fold in wt islets). Adrenaline lowered the concentration of cAMP in wt and α_2C-AR KO mouse islets by 74%. Adrenaline also hyperpolarized wt and α_2C-AR KO β cells. In contrast, adrenaline did not inhibit adenylyl cyclase in islets of α_2A-AR KO mice, nor did it hyperpolarize α_2A-AR KO β cells.

Conclusion: Adrenaline inhibits insulin release through α_2A- and α_2C-ARs via distinct intracellular signaling pathways.

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Introduction

Islets of Langerhans are innervated by the sympathetic nervous system (1, 2). In addition, a fine capillary network passes through these micro-organs (3), which enables catecholamines to interfere directly with hormone secretion from the islets. Studies with specific α_2- and β-adrenoceptor (AR) agonists and antagonists revealed that insulin secretion could be influenced by activation of all three groups of ARs (4). Whereas selective activation of α_1- and β-ARs stimulates secretion, activation of α_2-AR inhibits secretion (4). Physiologically predominant, however, is the inhibition of secretion through the activation of α_2-AR. These receptors are coupled to pertussis toxin (PTX)-sensitive G proteins in all cell types examined and their activation results in inhibition of adenylyl cyclase (5, 6). The correlation of inhibition of cAMP production and secretion was contested by a variety of experiments and groups, provoking the search for other mechanisms (7–10). Since insulin secretion is initiated by an elevation of cytosolic Ca^{2+} mainly through Ca^{2+} influx via depolarization-activated Ca^{2+} channels, inhibition of this signal by α_2-AR activation may be the mechanism of inhibition of insulin release. In fact, inhibitors of insulin secretion repolarize β cells and lower [Ca^{2+}], through direct G protein-mediated regulation of ion channels (11–13). The observation that ionomycin-induced secretion and secretion from permeabilized cells bypassing ion channel regulation is inhibited by adrenaline, galanin and somatostatin in a PTX-sensitive manner leads to the suggestion that additional effects distal to the generation of second messengers mediate inhibition (10, 14–17).
Three $\alpha_2$-AR subtypes, $\alpha_{2A}$-, $\alpha_{2B}$-, $\alpha_{2C}$-AR, were identified by molecular cloning from several species (18, 19). All three receptors modulate several intracellular signaling cascades including inhibition of adenyl cyclase, stimulation of phospholipase D, stimulation of mitogen-activated protein kinases, stimulation of $K^+$ currents and inhibition of $Ca^{2+}$ currents. The significance of these signaling pathways for individual physiological functions of $\alpha_2$-ARs is largely unknown at present. $\alpha_2$-ARs differ greatly in their tissue distribution and their distinct expression patterns may suggest different physiological functions of these subtypes (for review see 20).

Several mouse lines have been established by gene targeting which do not express functional $\alpha_2$-ARs (21–23). All of these mice developed apparently normally, although mice lacking $\alpha_{2B}$-ARs were not born at the expected Mendelian ratios, indicating that this receptor may play a role during embryonic development (23, 24).

The pharmacological identity of $\alpha_2$-adrenergic effects in islets is identical with $\alpha_2$-AR characteristics, i.e. a high affinity to clonidine and $\alpha_2$-yohimbine and a low affinity to prazosin (25). However, it is not known whether adrenaline exerts its different effects through the same or different isoforms of $\alpha_2$-ARs in insulin-secreting cells. Using RT-PCR, both $\alpha_{2A}$-AR and $\alpha_{2C}$-AR mRNA were found in a purified $\beta$ cell preparation, which, however, still contains some non-$\beta$ cells (26).

Sympathetic nerve activation also regulates glucagon and somatostatin secretion, hormones synthesized and secreted from $\alpha$ and $\delta$ cells within the islets of Langerhans. Glucagon activates, somatostatin inhibitors insulin secretion. Regulation of these paracrine effects through cell specific ARs remains unknown. Several studies on glucagon secretion demonstrated activation by $\beta$-AR agonists (27, 28). However, it has also been suggested that $\alpha_2$-AR activation stimulates or inhibits stimulated glucagon secretion (29–32).

The use of knockout (KO) mice missing one AR subtype makes it possible to understand the expression pattern of ARs and to analyze its specific role in cell function (33). We demonstrate here that the activation of two subtypes of ARs inhibits insulin secretion. Both receptors, $\alpha_{2A}$- and $\alpha_{2C}$-AR, are expressed in islet cells. The $\alpha_{2A}$-AR is expressed in insulin-secreting cells, inhibits adenyl cyclase and hyperpolarizes the cells. The activation of $\alpha_{2C}$-AR may be expressed on other cell types of the islets, since no direct effect on $\beta$ cell membrane potential and adenyl cyclase was observed.

Materials and methods

Animals, isolation of islets and cell preparation

The generation and genotyping of mice lacking $\alpha_{2A}$-, $\alpha_{2C}$- or both $\alpha_{2A}$- and $\alpha_{2C}$-ARs has been described previously (22, 23, 34). Mice were maintained on a C57BL6/j genetic background (fourth generation of backcrossing) and mice were maintained in a specified pathogen-free facility. Regular chow (Altromin, Laage, Germany) and water were supplied freely. For this study, wild type (wt) and $\alpha_2$-AR-deficient mice were used at 2–4 months of age. All animal procedures were approved by the responsible university and government authorities.

Mice carrying a targeted deletion of the genes encoding $\alpha_{2A}$-, $\alpha_{2C}$- or both, $\alpha_{2A}/\alpha_{2C}$-AR, were genotyped by PCR of genomic DNA using the following primers for $\alpha_{2A}$-AR: $\alpha_{2AF}$, 5'-GCTCCACTAGTTCTAGC; $\alpha_{2BR}$, 5'-TCTCATCCGCTCCACCTCA. $\alpha_{2B}$ and $\alpha_{2F}$ amplified a 320 bp fragment of the endogenous allele, whereas $\alpha_{2B}$ and $\alpha_{2F}$ produced a 212 bp fragment of the targeted $\alpha_{2B}$-AR KO allele. A wt fragment of 230 bp and the corresponding $\alpha_{2C}$ KO fragment of 250 bp were amplified with $\alpha_{2C}$-F, 5'-CATCTGCTTCCCTCTAG; $\alpha_{2C}$R, 5'-TCTCATCCGGCTCCACCTCA; and $\alpha_{2C}$F, 5'-GGGAGAGCATGAGCGAGAT; and $\alpha_{2C}$R, 5'-GGGAAGACAATAGC-

Measurement of insulin secretion

Freshly isolated and purified islets were preincubated for 1 h at 37°C in incubation buffer containing (in mmol/l): 140 NaCl, 5.6 KCl, 1.2 MgCl₂, 2.6 CaCl₂, 2.8 glucose, 10 Heps, pH 7.4 and 5 g/l BSA (fraction V; Sigma). Thereafter, batches of five islets/0.5 ml were incubated for 30 min at 37°C in the presence of test substances as indicated for each experiment. Insulin released into the supernatant and insulin content of the islets after acid/ethanol (1.5%/75% (v/v)) extraction was measured by RIA as described previously (9). Cultured islets were kept overnight (18–20 h) in RPMI 1640 supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 1 mmol/l Heps, 1 mmol/l Na-pyruvate, 10 µmol/l $\beta$-mercaptoethanol (Sigma) and antibiotics for 2–5 days prior to the experiment.
antibiotics. PTX was added whenever indicated at a concentrations of 100 ng/ml to the culture medium.

**Patch clamp experiments**

Cover slips with attached cells were mounted in a bath chamber on the stage of an inverted microscope (Zeiss, Jena, Germany) and superfused (2 ml/min) with a solution containing (in mmol/l) 140 NaCl, 5.6 KCl, 1.2 MgCl2, 2.6 CaCl2, 2.8 glucose and 10 Hepes, pH 7.4 at room temperature. The patch clamp pipettes (Clark Medical, Reading, Berks, UK) were pulled with a DMZ-Universal Puller (Zeitz, Augsburg, Germany) and had a resistance of 4 – 6 MΩ when filled with pipette solution containing in (mmol/l): 30 KCl, 95 K-gluconate, 1 MgCl2, 1.2 NaH2PO4, 4.8 Na2HPO4 and 2 mg/ml amphotericin B, pH 7.2. Membrane voltage was measured continuously using the current-clamp mode of the patch-clamp amplifier (U Fro¨be and R Busche, Freiburg, Germany) and was displayed by a pen recorder.

**Measurement of adenylyl cyclase**

After overnight culture, isolated islets were preincubated for 1 h in Krebs–Ringer buffered solution containing 2.8 mmol/l glucose followed by 20 min incubation in the presence of test substances as indicated. The supernatant was removed and cellular cAMP was extracted by boiling of the islets in 0.05 M Na-acetate buffer, pH 6.2 for 20 min. The extracted cAMP was measured after acetylation using the Amersham EIA Kit protocol (Amersham Biosciences, Freiburg, Germany).

**Statistical analysis**

Data are presented as means±S.E.M. Student’s unpaired t-test was used whenever possible. P values < 0.05 were accepted to indicate statistical significance.

**Results**

**Regulation of insulin secretion in freshly isolated islets from α2-AR KO mice**

To analyze the α2-AR subtype mediating adrenaline-induced inhibition of insulin secretion, freshly isolated islets from wt, α2c- and α2a-AR KO mice were incubated with 16.7 mmol/l glucose and 1 μmol/l forskolin for 30 min (Fig. 1A). Forskolin was added since activation of adenylyl cyclase is known to potentiate secretion. Glucose (16.7 mmol/l) alone significantly increased insulin release from freshly isolated islets of wt mice (from 0.74±0.08 ng insulin/five islets to 1.62±0.19 ng insulin/five islets (n = 7) after 60 min). However, insulin secretion was not stimulated by glucose in α2-AR KO mice islets (data not shown). This observation suggests that freshly isolated islets of α2-AR KO mice are glucose-insensitive, which made it impossible to analyze adrenaline effects in KO islets stimulated with glucose alone. Compared with rat islets, glucose poorly activates secretion in freshly isolated mouse islet preparations (34). As can be seen in Fig. 1A, secretion from wt islets was activated 4-fold by 16.7 mmol/l glucose and 1 μmol/l forskolin. Secretion, increased 8.5-fold with 16.7 mmol/l glucose, 5 μmol/l forskolin and 100 μmol/l IBMX, was inhibited by 1 μmol/l adrenaline by 83%. In α2a-AR KO mouse islets, secretion was not stimulated by 16.7 mmol/l glucose and 1 μmol/l forskolin. However, secretion was stimulated 2.7-fold by 16.7 mmol/l glucose in the presence of 5 μmol/l forskolin and 100 μmol/l IBMX, which was inhibited by 80% by 1 μmol/l adrenaline. In α2c-AR KO mouse islets, secretion was stimulated 2.7-fold by 16.7 mmol/l glucose and 1 μmol/l forskolin and completely inhibited by adrenaline. *Significant (P < 0.05) stimulation of secretion over basal release at 2.8 mmol/l glucose; #Significant (P < 0.05) inhibition of secretion by adrenaline. (B) Insulin content of isolated islets from wt (n = 5 preparations), α2a (n = 5) and α2c mice (n = 6) was not significantly different.
incubated with 16.7 mmol/l glucose and higher concentrations of forskolin, 5 μmol/l, and in the presence of 100 μmol/l 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, which prevents degradation of cAMP secretion from islets of α2A-AR KO mice was stimulated, although to a much lesser extent than secretion of wt islets (2.7- and 8.5-fold, respectively). Impaired insulin release in α2A-AR KO mouse islets was not caused by decreased insulin content (Fig. 1B). Isolated islets of wt, α2A-AR KO and α2C-AR KO mice contained 72 ± 13 ng insulin/islet (n = 5), 78 ± 11 ng insulin/islet (n = 5) and 59 ± 10 ng insulin/islet (n = 6) respectively.

Adrenaline, 1 μmol/l, inhibited stimulation by 83, 80 and 100% in wt and α2A-AR KO and α2C-AR KO mouse islets respectively. These observations indicate that targeted deletion of a single AR subtype is not sufficient to abolish adrenaline-mediated inhibition of insulin release. However, in mice missing both α2A- and α2C-AR subtypes, adrenaline had no effect on stimulated secretion (Fig. 2).

**Regulation of insulin secretion in cultured islets of α2-AR KO mice**

In the following, the PTX sensitivity of adrenaline effects in α2-AR KO mice islets was examined. Isolated islets were cultured overnight since shorter treatments by PTX resulted in insufficient blockade of PTX-sensitive G proteins. After overnight culture, isolated islets of wt mice secreted significantly more insulin (13% of content, Fig. 2A and B) than freshly isolated islets (4.5% of content, Fig. 1A). After PTX treatment, stimulated secretion increased even further. As consequence of increased insulin secretion, insulin content decreased. Compared with freshly isolated islets, insulin content was significantly reduced to 46% in wt islets after overnight tissue culture in high-glucose medium and after PTX treatment further to 22% in wt, to 40% in α2A-AR and to 61% in α2C-AR KO mice islets (Fig. 3).

Overnight culture completely restored insulin secretion from α2A-AR KO mouse islets, which was not significantly different from secretion of wt and α2A/2C-AR KO mouse islets (Fig. 2A and B). Under these conditions, adrenaline, 1 μmol/l, did not inhibit secretion in α2A/2C-AR KO mice islets (Fig. 2B). Adrenaline-induced inhibition, however, was also attenuated in α2A-AR KO mouse islets (40% compared with 80% inhibition of secretion in wt islets, Fig. 2A). After PTX treatment, adrenaline-mediated effects on secretion were abolished, indicating that PTX-sensitive G proteins mediate the effects (Fig. 2A and B). These results further indicate that both receptor subtypes, α2A-AR and α2C-AR, are functionally expressed in islets.

**Inhibition of adenylyl cyclase in α2-AR KO mouse islets**

To analyze the inhibitory coupling of α2A-AR and α2C-AR to adenylyl cyclase, cAMP concentrations
Effects of α₂-AR activation on membrane potential

To test which α₂-AR subtype mediates the adrenaline-induced hyperpolarization, we measured membrane potential using the perforated patch clamp technique in isolated, cultured mouse islet cells. Under resting conditions, membrane potential of α₂A-AR KO mice islets was −55 ± 2 mV (n = 17). Glucose depolarized the α₂A-AR KO cells to −35 ± 2 mV (n = 18) and induced action potentials in all cells examined (data not shown). Similar results were obtained with wt and α₂C-AR KO cells (Fig. 5). In wt islet cells, adrenaline elicited the expected reversible hyperpolarization (Fig. 5A). In contrast, in α₂A-AR KO mouse islet cells, adrenaline was without effect on membrane potential whereas somatostatin hyperpolarized the cells (Fig. 5B). However, adrenaline inhibited action potentials and hyperpolarized α₂C-AR KO islet cells (Fig. 5C). These data indicate that α₂A-AR but not α₂C-AR activation leads to a hyperpolarization of pancreatic β cells. Since adrenaline inhibited insulin secretion in α₂A-AR KO mouse islets, these results suggest that adrenaline inhibition of insulin secretion does not depend on inhibition of adenylyl cyclase (Fig. 4) or hyperpolarization (Fig. 5) of insulin-secreting cells.

Discussion

This study reveals that two α₂-AR subtypes, α₂A-AR and α₂C-AR, mediate adrenaline-induced inhibition of insulin secretion, since only in mice missing both receptor subtypes was the adrenaline effect abolished. This observation suggests that the third subtype, the α₂B-AR, is not involved in the regulation of insulin secretion in mouse islets.

Previously, pharmacological characterizations of α₂-AR-mediated inhibition of insulin secretion and catecholamine binding to insulin-secreting cells corroborated the expression of α₂A-ARs (25). The analysis of mRNA from purified β cells revealed that α₂A-AR and α₂C-AR are expressed in rat insulin-secreting cells (26). Here, we show that α₂A-ARs are coupled to the inhibition of adenylyl cyclase in mouse islets (Fig. 4). Adrenaline did not inhibit adenylyl cyclase through α₂C-AR. The α₂A-AR is also the sole α₂-AR subtype mediating adrenaline-induced hyperpolarization of islet cells (Fig. 5). Thus, it is not surprising that inhibition of insulin secretion was impaired in cultured α₂A-AR KO mouse islets (Fig. 3). Activation of α₂C-AR did not affect the membrane potential of

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**Figure 3** Insulin content of islets after culture and PTX treatment. Insulin content of wt, α₂A-AR and α₂C-AR KO mice islets was reduced to 46, 64 and 97% respectively, after culture and to 22, 40 and 61% of freshly isolated islets respectively, after PTX treatment (*P < 0.05 when compared with freshly isolated islets, Fig. 1B).

**Figure 4** Regulation of adenylyl cyclase activity in wt and α₂-AR KO mouse islets. Isolated islets were cultured and cellular cAMP concentrations measured as described under Materials and methods. Islets were incubated for 10 min in the presence of 16.7 mmol/l glucose, adenylyl cyclase activity was stimulated by 5 μmol/l forskolin. Somatostatin and β-AR receptors were inhibited by 1 μmol/l cyclosomatostatin (cyclo(7-aminoheptanoyl-Phe- ß-Trp-Lys-Th)) and 10 μmol/l propranolol respectively. Degradation of cAMP was blocked by 100 μmol/l IBMX. Adrenaline, 1 μmol/l, lowered cAMP accumulation in wt and in α₂C-AR KO mouse islets by 74%, but had no effect in α₂A-AR KO islets. Shown are means ± S.E.M. of three to six observations from two independent experiments. *P < 0.05, increase of cAMP concentration measured as described under Materials and methods.
Figure 5 Effect of adrenaline on membrane potential in stimulated islet cells of α-AR KO mice. Membrane voltage was measured in isolated islet cells as described under Materials and methods. (A) In wt islet cells, adrenaline, 1 μmol/l, hyperpolarized the cell to −60 mV. (B) In cells missing the α₂A-AR, adrenaline had no effect on membrane voltage. Somatostatin, 1 μmol/l, hyperpolarized the cell. (C) In cells missing the α₂C-AR, adrenaline and somatostatin hyperpolarized the cells to −60 and −61 mV respectively. Bar graphs represent mean ± S.E.M. for n = 4 (A), n = 9 (B) and n = 13 (C) cells for adrenaline; n = 1 (A), n = 9 (B) and n = 3 (C) for somatostatin. The membrane voltage scale is valid for both the trace on the left side and the bar graph on the right side. *Significant effects of adrenaline or somatostatin.
glucose-depolarized islet cells, suggesting that the receptors are not coupled to ion channels or are not expressed in β cells. However, both, α\textsubscript{2A}- and α\textsubscript{2C}-AR, when stably expressed in HEK293 cells, have been found to activate G protein-activated inwardly rectifying K\textsuperscript+ channels although with different activation and deactivation kinetics (35). These results indicate that the α\textsubscript{2A}-AR is the main receptor in β cells and mediates hyperpolarization, inhibition of adenylcyclase and inhibition of secretion. However, despite the lack of effects on membrane potential and adenylcyclase, adrenaline still significantly lowered insulin release from α\textsubscript{2A-AR} KO mouse islets (Figs 1 and 2A). Thus, several possibilities remain to explain the inhibitory effect of the α\textsubscript{2C-AR} subtype on insulin release. First, the α\textsubscript{2C-AR} may not be expressed in insulin-secreting β cells but rather in α and δ cells, which are known to influence insulin release via glucagon and somatostatin respectively. In fact, stimulated glucagon secretion was inhibited by α\textsubscript{2C-AR} activation (29–32). Whether decreased glucagon secretion is responsible for the inhibition of insulin release in α\textsubscript{2A-AR} KO mice is not known. However, it is unlikely since insulin secretion was induced in the presence of 16.7 mmol/l glucose, a condition at which glucagon secretion is already suppressed. Moreover, in purified islet cells Schuit & Pipeleers (28) were unable to demonstrate α\textsubscript{2C}-AR-mediated effects in non-β cells. Alternatively, α\textsubscript{2C-ARs} in pancreatic β cells may inhibit insulin secretion through a non-hyperpolarizing, non-cAMP-lowering signaling pathway. Indeed, we showed previously that adrenaline inhibits insulin release distal to the modification of second messengers like Ca\textsuperscript{2+} and cAMP by a yet unknown mechanism (10).

Our results further extend previous findings that α\textsubscript{2A-} and α\textsubscript{2C-ARs} are not only essential feedback regulators for neurotransmission (36) but are also involved in controlling endocrine secretion, e.g. insulin release. In contrast, the third α\textsubscript{2-AR} subtype, α\textsubscript{2B}, does not play a major role in the regulation of secretion but may have its most important function in angiogenesis of the placenta during embryonic development (37).

It is very surprising that stimulation of secretion was impaired in α\textsubscript{2A-AR} KO mouse islets. We expected a facilitation of secretion due to genetic deletion of an important inhibitory pathway. The observation that secretion was restored after culture lead us to conclude that inhibitory pathways are activated in freshly isolated islets. It has been shown that blood noradrenaline concentrations in α\textsubscript{2A-AR} KO mice are 2-fold increased compared with wt mice due to the lack of neuronal feed back inhibition (21). Increased noradrenaline concentrations released from sympathetic nerve endings within the islets may inhibit insulin secretion, especially after killing mice by cervical dislocation prior to the isolation of islets.

ARs are not the sole receptors which activate PTX-sensitive G proteins in insulin-secreting cells. Other receptors for important physiological inhibitors, such as somatostatin, galanin, and pancreastatin, are coupled to PTX-sensitive G proteins and have been shown to inhibit insulin secretion (12, 13, 38). Since somatostatin is synthesized and stored in δ cells of the islets of Langerhans, it exerts paracrine effects and may contribute to the inhibition of glucose-induced insulin secretion in isolated islets.

The inhibitory pathways play an important role in the regulation of insulin secretion in mice. These pathways have been demonstrated in other species, including man. Pheochromocytoma has been attributed as a cause for non-insulin-dependent diabetes mellitus and diabetic patients suffer from increased catecholamine release (39, 40). These pathways are therefore important targets for antidiabetic drugs (41).

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