JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 1639–1645

Received December 23, 2005
Accepted August 21, 2006
DOI 10.1211/jpp.58.12.0011
ISSN 0022-3573

The impact of adenosine and A_{2B} receptors on glucose homoeostasis

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Abstract

Adenosine and adenosine receptor antagonists are involved in glucose homoeostasis. The participating receptors are not known, mainly due to a lack of specific agonists and antagonists, but are reasonable targets for anti-diabetic therapy. The stable, albeit nonselective, adenosine analogue NECA (5′-*N*-ethylcarboxamidoadenosine) (10 μM) reduced glucose-stimulated insulin release from INS-1 cells. This was mimicked by A_1-(CHA), A_{2A}-(CGS-21680) and A_3-receptor agonists (Cl-IB-MECA). Two newly synthesized A_{2B}-receptor antagonists, PSB-53 and PSB-1115, counteracted the inhibitory effect of NECA. These in-vitro effects were mirrored by in-vivo data with respect to CHA, CGS and Cl-IB-MECA. Distinct concentrations of either PSB-53 or PSB-1115 reversed the decrease in plasma insulin induced by NECA. This was not mimicked by a corresponding change in blood glucose. The effect of PSB-1115 was also obvious in diabetic GotoKakizaki rats: plasma insulin was increased whereas blood glucose was unchanged. During most experiments the effects on blood glucose were not impressive probably because of the physiologically necessary homoeostasis. The adenosine levels were not different in normal Wistar rats and in diabetic GotoKakzaki rats. Altogether the A_{2B}-receptor antagonists showed an anti-diabetic potential mainly by increasing plasma insulin levels under conditions when the adenosine tonus was elevated in-vivo and increased insulin release in-vitro.

Introduction

Adenosine, an endogenous nucleoside, participates in the regulation of glucose homoeostasis. It increases glycogenolysis and gluconeogenesis (Gonzalez-Benitez et al 2002), decreases insulin secretion (Hillaire-Buys et al 1987, 1994; Bertrand et al 1989) and stimulates glucagon secretion (Chapal et al 1985). Unselective antagonists of adenosine receptors, like pentoxifylline and aminophylline (Corssmit et al 1994; Arias et al 2001), exhibit a lowered glucose production and an improved insulin secretion. An involvement of adenosine in the generation of diabetes mellitus can not be excluded. Adenosine mediates its effects via four different receptor subtypes. Activation of the A_1 and A_3 receptor decreases cAMP levels because of their coupling to an inhibitory G protein (G_i), while the A_{2A} and A_{2B} subtypes are associated with a stimulatory G protein (G_s), which increases cAMP levels. All four receptor subtypes are also coupled to a G_i protein and activate the phospholipase C. The A_1 and A_{2A} receptor further activate K^+ channels, while the A_1-receptor subtype inhibits N-type Ca^{++} channels.

At present it is far from clear which of these adenosine receptor subtypes mediate glucose homoeostasis. An involvement in glucose production and insulin secretion is shown for the A_1 receptor (Gonzalez-Benitez et al 2002) as well as for the A_2 receptor (Bartrons et al 1984; Buxton et al 1987). Which subtype of the A_2 receptor (A_{2A} or A_{2B}) is involved is hard to elucidate because until recently specific pharmacological tools were lacking. In this study we used two newly synthesized A_{2B}-receptor antagonists, PSB-1115 (1-propyl-8-*p*-sulfophenylxanthine) and PSB-53 (1-butyl-8-*p*-carboxyphenylxanthine; UPAC name: 4-(1-butyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purine-8-yl)benzoic acid) (Hayallah et al 2002) to describe the possible involvement of the A_{2B} adenosine receptors in the generation and therapy of diabetes mellitus. Binding studies with the two antagonists on INS-1 cells have shown the selectivity of PSB-1115 and PSB-53 to the A_{2B}-receptor subtype (Bertarelli et al 2006), which was supported by biological tests with these compounds (Abo-Salem et al 2004). The participation of this receptor subtype is already known because an increase in

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Acknowledgements: We thank Aventis, Germany for generously supplying 125I-labeled porcine insulin and thank K. Bauer for skilful technical assistance and Dr F. Begrow for preparation of the manuscript.

insulin sensitivity and glucose tolerance was shown in mice after the administration of the A_{2B}-receptor antagonist ATW-GW8 (LaNouel et al 2002). In addition the effects of NECA (5′-*N*-ethylcarboxamidoadenosine), a stable albeit nonselective adenosine analogue, on hepatic glucose production was shown to be more potent than the effects of selective A_1-, A_{2A}- or A_3-receptor agonists (Yasuda et al 2003). Vice versa, the NECA effects could not be fully antagonized by selective A_1 A_{2A} or A_3 antagonists (Harada et al 2001).

In this study we tried to find out more about the possible role of adenosine in the generation of diabetes mellitus and the receptor subtypes being involved. We, therefore, used two A_{2B}-selective antagonists as new pharmacological tools (PSB-1115 and PSB-53). We further investigated whether an increased adenosine tonus coincides with a diabetic situation.

Materials and Methods

Chemicals

NECA, CHA (*N*6–3-cyclohexyladenosine), Cl-IB-MECA (2-chloro-*N*6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide) and CGS-21680 (2-{[*p*-(2-carboxyethyl)-phenethyl]amino}-5′-*N*-ethylcarboxamidoadenosine) were from Sigma-Aldrich (Germany). PSB-1115 and PSB-53 were synthesized in the laboratory of Dr C.-E. Mueller (Bonn, Germany). Structurally, PSB-53 and PSB-1115 are simple phenylxanthine derivatives. PSB-53 is substituted with an n-butyl group at N1, and the 8-phenyl ring is substituted in the *para*-position with a carboxylate function. In contrast to A_{2B}-antagonists with anilide structure, PSB-53 can be expected to be chemically and enzymatically rather stable (Kim et al 2002; Yan et al 2006). Due to its polar character it is expected not to penetrate well into the central nervous system, but to be mainly peripherally active. ^{125}I-Insulin was from Aventis (Germany); insulin antibodies raised in guinea-pigs and anti-guinea-pig IgG from goats were from Linco Research (USA). Glukoquant was from Roche Diagnostics (Mannheim, Germany).

INS-1 cell culture and incubation

INS-1 cells (Asfari et al 1992) generously provided by Dr C. Wollheim (Geneva, Switzerland) were plated at a density of 5×10^5 cells/mL and grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate, 50 μM mercaptoethanol, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin.

Insulin secretion and radioimmunoassay

INS-1 cells were plated at 1.5×10^5 cells/well in 24-well plates and cultured for 5 days. Cells were washed three times with Krebs–Ringer buffer containing 10 mM HEPES and 0.5% bovine serum albumin (KRH buffer). The cells were incubated with KRH buffer containing 5.6 mM glucose in the presence or absence of the respective compound for 90 min. Insulin release into the medium was determined by a radioimmunoassay using rat insulin as a standard, (mono-^{125}I-Tyr^{A14})-porcine insulin as the labelled compound and anti-insulin antibodies. The intra-assay and interassay variability was 4.2 and 9.8%, respectively. Each test compound had been checked for non-interference with the insulin radioimmunoassay. It should be noted that the antibody does not discriminate between rat, porcine or human insulin.

Animals

Either Wistar rats from a local strain or GotoKakizaki rats were used. The latter strain rather resembles a type 2 diabetic (Goto et al 1976). All animal studies were approved by the German animal welfare committee (G37/2004 of the University of Muenster).

In-vivo experiments (blood collection)

Indicated compounds were given to the rats during urethane anaesthesia (1.8–2.0 g kg^{-1}) via a vena jugularis catheter. NECA was administered in doses of 0.308 and 0.031 mg kg^{-1}. Adenosine receptor ligands CHA, CGS-21680 and CI-IB-MECA were given in doses of 2.45, 3.75 and 3.81 μg kg^{-1}, respectively. The A_{2B} antagonists PSB-1115 and PSB-53 were tested in two doses. PSB-1115 was given in concentrations of 2.45 μg kg^{-1} and 7.5 mg kg^{-1} and PSB-53 in concentrations of 2.29 μg kg^{-1} and 0.75 mg kg^{-1}. All compounds were tested against 1.0 mg kg^{-1} saline (0.9%). In experiments with NECA pretreatment, following substances were given 15 min after NECA. Blood was collected by a retrobulbar procedure 5, 10, 15, 20, 35, 55 and 65 min after application. All calculations of theoretical plasma levels achieved by the applied compounds were performed on the basis that animals and man possess an average blood volume of 7% of body weight.

Assays for glucose and insulin

Blood glucose was determined by the glucose oxidase method (Glucoquant). Under assay conditions glucose calibration curves were linear over the range 0–200 mg dL^{-1}. The absorbance of the enzymatic products was determined using a photometer UV-160A (334 nm; Shimadzu (Germany). The measurement of the samples (duplicates) was performed after 10 min.

Plasma insulin concentrations were assayed with a radioimmunoassay using rat insulin as a standard, (mono-^{125}I-Tyr^{A14})-porcine insulin as the labelled compound and anti-insulin antibodies. For calibration rat insulin between 0.39 and 25 μU/100 μL was used. There was no direct interference of the tested compounds with the radioimmunoassay.

Determination of plasma adenosine levels

Adenosine levels were determined using a combination of HPLC and mass spectrometry. To 360 μL heparinized blood 40 μL stopping solution containing Dilazap was added to inhibit adenosine uptake into erythrocytes, erythro-9-(2-hydroxy-3-nonyl)adenine to inhibit adenosine deaminase and indomethacin to inhibit nucleotide release from thrombocytes. Spiked ethenoadenosine (10 μg mL^{-1}) served as internal standard. Proteins were precipitated by 20 μL TCA (50%) and

the samples were neutralized after centrifugation. Peaks were separated by a standard HPLC procedure using a RP18 column.

Statistics

Results are shown as means ± s.e.m. Statistical significance was determined using one-way analysis of variance (RS/1 statistics pack; BBN Software Products Corp.) followed by a post-hoc test (Newman Keuls). $P < 0.05$ was considered significant.

Results

To discover by which receptor subtype(s) the adenosine effect on inhibition of insulin release is mediated, we first tested the effects of agonists and antagonists of adenosine receptor subtypes on insulin release of INS-1 cells.

NECA, a stable adenosine analogue, decreased glucose-induced insulin secretion from INS-1 cells by nearly 20% (Figure 1). Incubation of the INS-1 cells with selective A_1-, A_{2A}- and A_3-adenosine receptor agonists decreased insulin release in a concentration-dependent manner (Figure 1). The decrease by CGS-21680 as an A_{2A}-selective agonist appeared to be slightly more effective (−34%) than the effect of the A_1-selective agonist CHA (−18%) and the A_3 agonist Cl-IB-MECA (−23%); the tendency of the effects, however, was the same for all three substances.

To find out what kind of effects are mediated by the A_{2B}-receptor subtype, newly synthesized selective A_{2B}-adenosine receptor antagonists were used. Note that a selective agonist of A_{2B} receptors is not available. The antagonists PSB-1115 and PSB-53 had no influence on insulin secretion on their own (up to 10^{-5} M, data not shown). Both A_{2B}-receptor antagonists were able to completely antagonize the inhibitory effect of NECA (10 μM) on insulin secretion in a concentration-dependent manner (Figure 2). There was no major difference between the two antagonists.

Next we tried to find out whether the agonists and antagonists show the same effects on insulin in-vivo. Additionally we investigated their effects on blood glucose. NECA was administered in doses of 0.031 and 0.308 mg kg^{-1} so that a substance level of 0.1 and 1 μM NECA in blood was calculated to be reached. Blood glucose only tended to be increased by the adenosine analogue NECA and lacked statistical significance compared with the saline effect (data not shown). In contrast a NECA effect on plasma insulin was obvious: there was a significant reduction in plasma insulin levels by the NECA-treatment compared with the saline control group (Figure 3A, B).

Whether this marginal increase in blood glucose and the significant reduction of plasma insulin by NECA could be assigned to a special adenosine receptor subtype was investigated by using the selective adenosine receptor ligands CHA, CGS-21680 and Cl-IB-MECA. The dosing was chosen to achieve a plasma level of 0.1 μM for each substance. None of the agonists for the A_1-, A_{2B}- and A_3-adenosine receptor (CHA, CGS-21680 and Cl-IB-MECA) showed an increase in blood glucose (data not shown); while CHA as an A_1 agonist did not show any effect, the A_{2A}- and A_3-adenosine receptor agonists CGS-21680 and Cl-IB-MECA showed a tendency to even decrease the blood glucose levels compared with the saline control (data not shown). While the saline bolus caused a short-lived increase in plasma insulin, CHA, CGS-21680 and Cl-IB-MECA decreased the insulin levels within the first 15 min (Figure 4). After this time the insulin levels in the CGS-21680 and Cl-IB-MECA group re-increased, while the plasma insulin in the CHA group further decreased continuously (Figure 4).

After testing the selective agonists of the A_1, A_{2A} and A_3 adenosine receptor, the effects mediated by the A_{2B}-adenosine receptor antagonists on blood glucose and plasma insulin

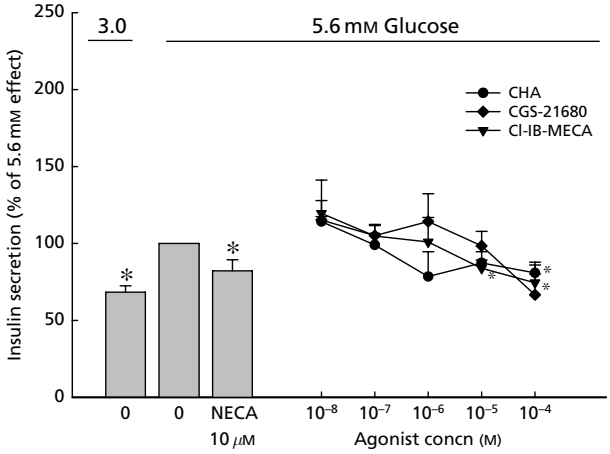

Figure 1 Effects of NECA and various adenosine receptor agonists on glucose-induced insulin secretion. INS-1 cells were incubated with 3.0 or 5.6 mM glucose for 90 min in the presence of either 10 μM NECA or increasing concentrations of CHA, CGS-21680 or Cl-IB-MECA. Released insulin was determined by RIA. Results are shown as mean ± s.e.m. of 4 independent experiments run in triplicates. *$P < 0.05$ vs the stimulatory effect of 5.6 mM glucose alone.

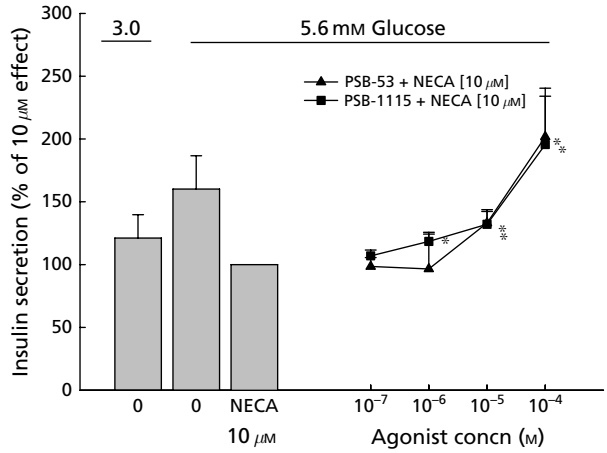

Figure 2 Effects of NECA and various adenosine receptor antagonists on glucose-induced insulin secretion. INS-1 cells were incubated with 3.0 or 5.6 mM glucose for 90 min in the presence of either 10 μM NECA alone or combined with increasing concentrations of PSB-53 or PSB-1115. Released insulin was determined by RIA. Results are shown as mean ± s.e.m. of 3 or 4 independent experiments run in triplicates. *$P < 0.05$ vs the effect of NECA alone.

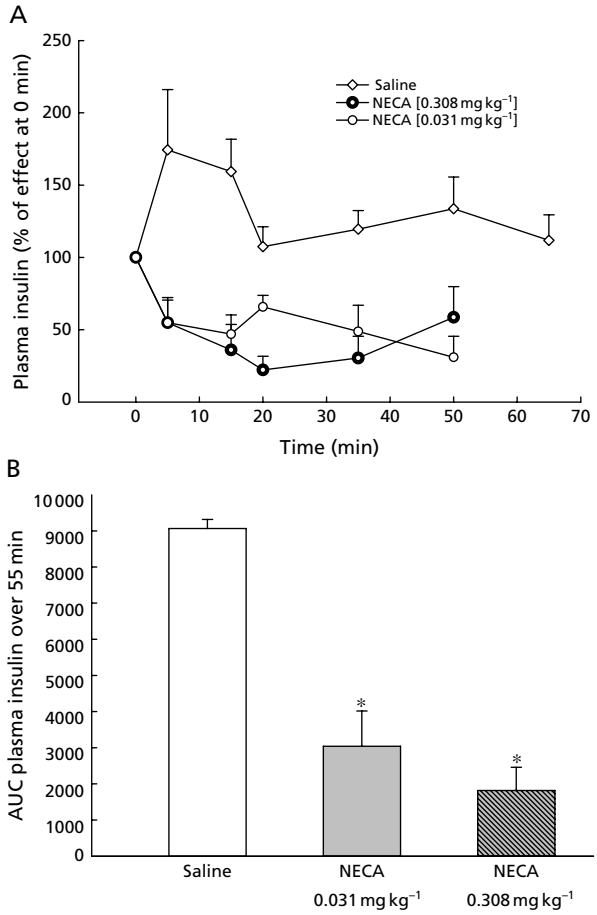

Figure 3 Effect of NECA on plasma insulin in rats. NECA (0.031 or 0.309 mg kg^{-1}) or saline were infused via bolus injection and blood was drawn at the indicated time points. Plasma insulin was determined by RIA. Values are normalized to zero (basal value directly before bolus injection) (A). The basal values are in the range of 10.7 and 12.2 μU mL^{-1}. The AUCs over time (50 min) are shown in Figure 3B. Mean ± s.e., 8–14 independent experiments; *$P < 0.05$ vs control (saline).

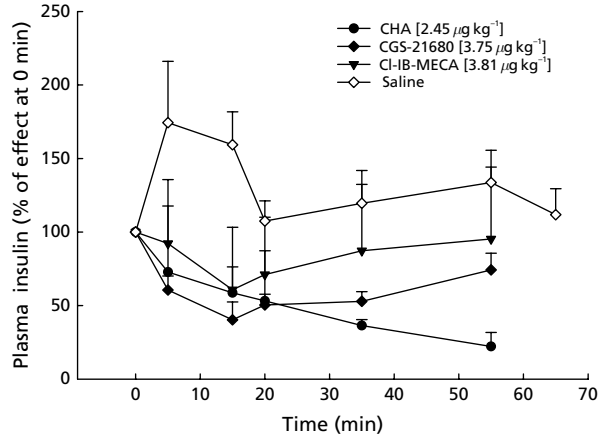

Figure 4 Effect of three different adenosine receptor agonists on plasma insulin in rats. CHA 2.45 μg kg^{-1}, CGS-21680 3.75 μg kg^{-1} and Cl-IB-MECA 3.81 μg kg^{-1} or saline were infused via bolus injection and blood was drawn at the indicated time points. Plasma insulin was determined by RIA. Values are normalized to zero (basal value directly before bolus injection). The basal values are in the range of 10.7 and 12.2 μU mL^{-1}. Mean ± s.e., 3 independent experiments.

levels were investigated. As already mentioned, there has been no selective A_{2B} agonist available yet. Little is known about the effective doses of the two A_{2B} antagonists, PSB-1115 and PSB-53, which were applied using two different concentrations to the rats. The smaller dose should reach a plasma level of 1 μM for either antagonist. This dose was chosen in relation to the K_d values of PSB-1115 and PSB-53 (Hayallah et al 2002). The higher doses of PSB-1115 should theoretically increase the plasma levels up to 307 μM whereas for PSB-53 a theoretical plasma level of 33 μM should be reached.

The smaller dose of PSB-1115 decreased the blood glucose level to a small, not significant, extent while the higher dose did not show any effect compared with that after a saline bolus (data not shown). PSB-53 decreased blood glucose at both concentrations. Neither substance PSB-1115 nor PSB-53 had an effect on the plasma insulin levels (data not shown). The reason why the effects on blood glucose and plasma insulin of the A_{2B} antagonists were not so strong could possibly be the lack of an agonistic adenosine tonus: the compounds themselves have no intrinsic activity. In further experiments we therefore increased the adenosine tonus by pretreating the rats with a bolus of NECA, which results in increased blood glucose levels and decreased plasma insulin. Fifteen minutes after the NECA bolus we administered the A_{2B} antagonists PSB-1115 and PSB-53 to the rats and collected blood samples 5, 20, 40 and 50 min after the second substance bolus. While the increased blood glucose levels were not affected by any of the various doses of PSB-1115 (Figure 5A), the NECA-elevated blood glucose was even increased after administration of PSB-53 (Figure 5B). The influence of the two A_{2B} antagonists on plasma insulin content was investigated. After the insulin levels were decreased by NECA administration of PSB-1115 a dose of 7.5 mg kg^{-1} increased the plasma insulin content for at least 50 min (Figure 6A). The smaller dose of PSB-1115 (2.45 μg kg^{-1}) did not show an effect. The A_{2B}-receptor antagonist PSB-53 antagonized the NECA-induced decrease in plasma insulin only when a small dose of 2.29 μg kg^{-1} was used (Figure 6B); the higher dose of 0.75 mg kg^{-1} lacked an effect.

Due to the fact that a possible increased adenosine tonus could be responsible for the disturbed glucose homoeostasis in type 2 diabetes, the influence of the A_{2B} antagonist PSB-1115 on blood glucose levels and plasma insulin content in diabetic GotoKakizaki rats (GK rats) was investigated next. PSB-1115 was administered at a dose of 7.5 mg kg^{-1} and blood glucose and insulin content were measured using the same protocol as before for normal Wistar rats. There was no influence of the A_{2B}-receptor antagonist on blood glucose level compared with the saline-treated rats; saline served as control. In both groups the glucose levels increased slightly over a period of at least 65 min (Figure 7A). In contrast, PSB-1115 increased the plasma insulin levels for at least 20 min (Figure 7B).

To clarify whether there is indeed an increased plasma level of adenosine responsible for the disturbed glucose homoeostasis we compared the plasma adenosine levels of

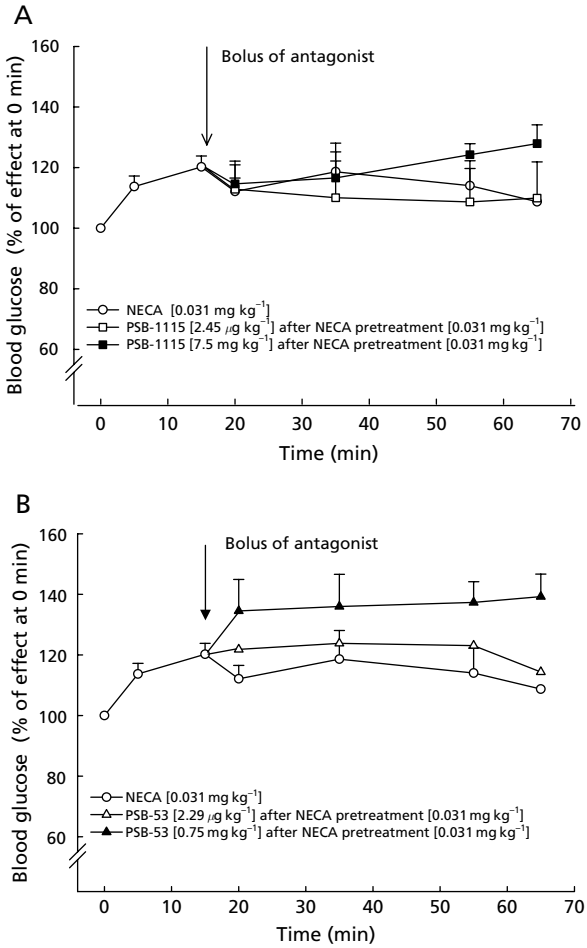

Figure 5 Effect of NECA in combination with two A_{2B}-receptor antagonists on blood glucose in rats. At 0 time point 0.031 mg kg^{-1} of NECA was given as a bolus. After 15 min either 2.45 or 7500 μg kg^{-1} of PSB-1115 (A) or 2.29 and 750 μg kg^{-1} of PSB-53 (B) were infused via bolus injection and blood was drawn at the indicated time points. Blood glucose was determined enzymatically. Values are normalized to zero (basal value directly before bolus injection of NECA). The basal values are in the range of 83–102 mg dL^{-1}. Mean ± s.e., 3 or 4 independent experiments.

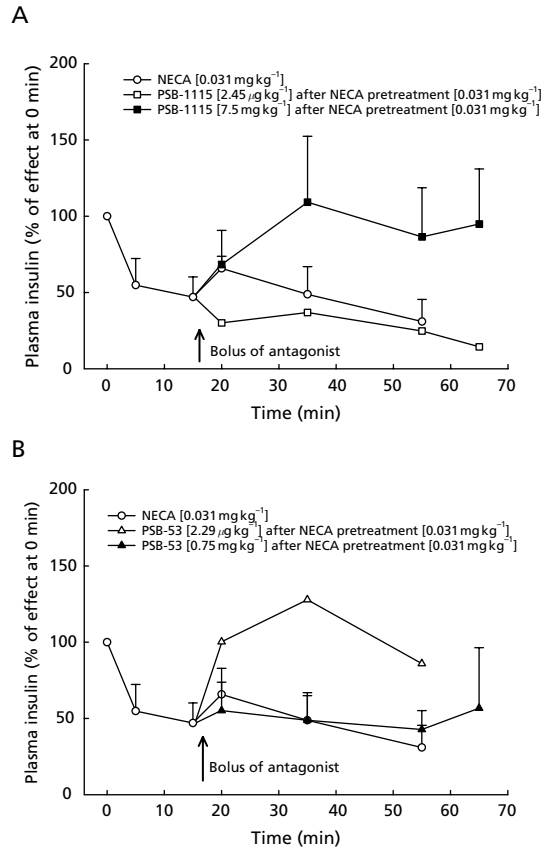

Figure 6 Effect of NECA in combination with two A_{2B}-receptor antagonists on plasma insulin in rats. At 0 time point 0.031 mg kg^{-1} of NECA was given as a bolus. After 15 min either 2.45 or 7500 μg kg^{-1} of PSB-1115 (A) or 2.29 and 750 μg kg^{-1} of PSB-53 (B) were infused via bolus injection and blood was drawn at the indicated time points. Plasma insulin was determined by RIA. Values are normalized to zero (basal value directly before bolus injection of NECA). The basal values are in the range of 10.7 and 12.2 μU mL^{-1}. Mean ± s.e., 3 or 4 independent experiments.

healthy Wistar rats with those of diabetic GK rats. However, the plasma adenosine content in Wistar rats was even a little higher than that in diabetic GK rats; difference between the two animal species lacked statistical significance (data not shown).

Discussion

It is yet not known by which receptor subtype adenosine as an endogenous purine nucleotide mediates an inhibitory effect on insulin secretion. To find out which kind of receptor subtype is responsible for the effects of adenosine on glucose or insulin metabolism, we tested effects of non-selective and selective adenosine receptor agonists and antagonists on INS-1 cells and on rats in-vivo.

The in-vitro experiments with INS-1 cells showed a decrease in insulin secretion by 10 μM NECA. Other groups displayed dual effects dependent on the NECA dose: low concentrations (0.01–50 μM) reduced whereas high concentrations (100–500 μM) stimulated insulin release (Bacher et al 1982; Bertrand et al 1989). Hypothetically these differences depend on the varying concentration-dependent affinity of adenosine to its receptor subtypes (e.g. its affinity to the A_1-receptor subtype is much higher than to the A_2 receptor subtypes). When the nucleoside interacts with the A_1 receptor, cAMP levels will decrease, which could explain a decrease in insulin secretion. When, however, the A_2 receptors are affected by higher concentrations of adenosine, the amount of cAMP will increase and insulin release should be expected to rise. We tested this hypothesis by using selective adenosine receptor agonists, but were not able to confirm this hypothesis: while CHA and Cl-IB-MECA as selective A_1- and A_3-receptor agonists should decrease the cAMP levels and CGS-21680 as a A_{2A} agonist should elevate cAMP levels with respective effects on insulin release, all receptor agonists decreased

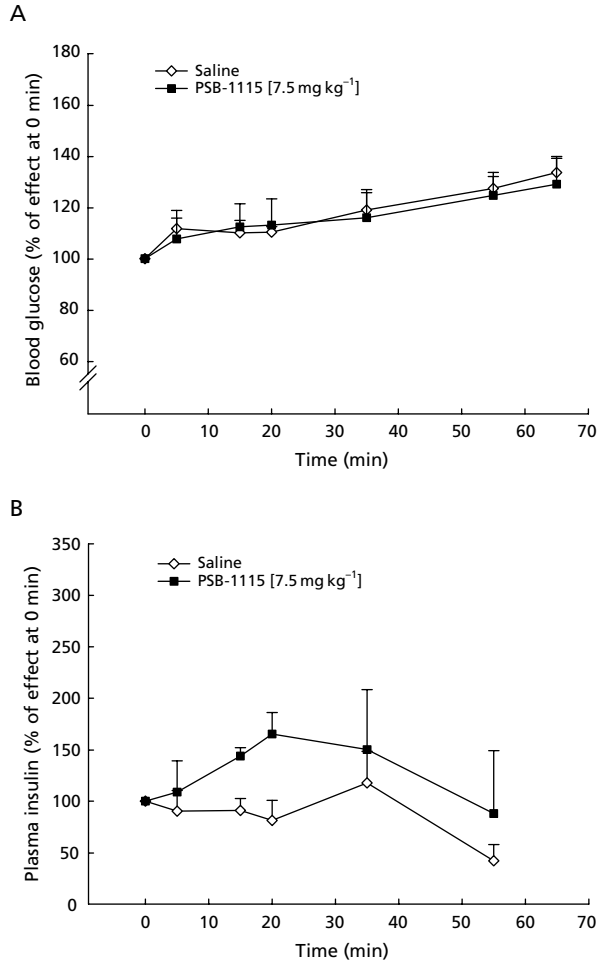

Figure 7 Effect of PSB-1115 on blood glucose (A) and plasma insulin (B). PSB-1115, 7.5 mg kg^{-1}, was infused via bolus injection and blood was drawn at the indicated time points. Plasma insulin was determined by RIA and blood glucose enzymatically. Values are normalized to zero (basal value directly before bolus injection of the compound). The basal values are in the range of 10.7 and 12.2 μU mL^{-1} (insulin) and 83–102 mg dL^{-1} (glucose). Mean ± s.e., 5 independent experiments.

insulin secretion to a similar extent. It is assumed that other second messenger systems than cAMP are possibly involved in the mechanism of insulin secretion under these conditions. In addition to this even the new PSB-1115 and PSB-53 (Hayallah et al 2002) as antagonists of the G_s-coupled A_{2B} receptor increased the NECA-induced insulin release.

Next, in-vivo experiments were performed. A NECA bolus increased blood glucose levels and decreased plasma insulin concentrations. The overall NECA effect might be explained by the receptor distribution: the A_1 receptor is highly expressed at pancreatic islets, whereas a high amount of A_2 receptors was found in liver (Arias et al 2001).

CHA, CGS-21680 and Cl-IB-MECA, in the same way as NECA, decreased plasma insulin levels after a bolus injection. There was no difference between agonists of G_i- (A_1, A_3) or G_s-coupled receptors. Other groups showed an inhibition of insulin secretion by the A_1-adenosine receptor agonist (Bertrand et al 1989; Hillaire-Buys et al 1994).

PSB-1115 and PSB-53, as new selective A_{2B} antagonists, showed a tendency to decrease blood glucose and to increase plasma insulin (data not shown). In general, antagonists should lack a pharmacological effect. The observed slight effect, however, could possibly be due to a small basal adenosine tonus. The rats, therefore, were pretreated with NECA to increase blood glucose levels and reduce plasma insulin before the antagonists PSB-1115 and PSB-53 were added. Neither compound antagonized the NECA-induced increase in blood glucose while they were both able to counteract the reduced plasma insulin levels.

The overall small effects on blood glucose are possibly due to the physiological importance of glucose homoeostasis. During all experiments the plasma insulin levels were much more sensitive to influence.

The A_{2B} antagonist PSB-1115 was also tested in diabetic GotoKakizaki rats. While the substance had no effect on blood glucose levels, it increased the plasma insulin for at least 20 min. The adenosine tonus might not be of major importance with respect to plasma insulin, since there was no difference in the adenosine levels between Wistar and GotoKakizaki rats. An involvement of A_{2B} adenosine receptors in glucose homeostasis was also found by LaNouel et al (2002): administration of the selective A_{2B}-receptor antagonist ATL-GW8 to mice showed an increased insulin sensitivity and glucose tolerance.

In this study we tried to find out by which adenosine receptor subtypes the adenosine effect on glucose homoeostasis is mediated. There is a discrepancy between the theory that an increase in cAMP levels by agonists of the A_2 receptors causes elevated glucose and insulin levels and an antagonist would be effective vice versa. Our results show that there must be further second messenger systems or ligand-gated ion channels being involved, which could fit our results. Another result of our investigations is that elevated adenosine levels do not seem to be responsible for the disturbed glucose metabolism in diabetics, especially with respect to the model GotoKakizaki rat. On the other hand the newly synthesized A_{2B} antagonists PSB-1115 and PSB-53 showed anti-diabetic potential indicated by decreasing blood glucose levels and increasing plasma insulin levels in Wistar rats at distinct doses, and elevated plasma insulin in diabetic GK rats in-vivo and increased insulin release in-vitro.

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