University of Münster, Institute of Pharmaceutical Chemistry, Department of Pharmacology, Germany

# Diadenosine tetraphosphate ($Ap_4A$) induces a diabetogenic situation: its impact on blood glucose, plasma insulin, gluconeogenesis, glucose uptake and GLUT-4 transporters

E. J. VERSPOHL, N. HOHMEIER, M. LEMPKA

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*Prof. Dr. E. J. Verspohl, Dept. Pharmacology, Inst. Pharmaceutical Chemistry, Hittorfstr. 58–62, D-48149 Münster, Germany*
*verspoh@uni-muenster.de*

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Diadenosine polyphosphates such as $Ap_4A$ are physiologically released compounds for which both receptors as well as a role as second messengers for influencing insulin release have been shown. So far little is known about their pathophysiological impact on diabetes with respect to blood glucose and plasma insulin, glucose production via gluconeogenesis, glucose uptake and GLUT-4 expression. Rats given an intravenous bolus of $Ap_4A$ (0.75 mg/kg) developed a rapid and dramatic increase in blood glucose. Plasma insulin was only transiently increased (for 4 min), but did not follow the normally stimulatory effect of the elevated blood glucose. A bolus of 25 μg $Ap_4A$ quickly increased glucose release from perfused rat liver. Glucose uptake was reduced in 3T3 adipocytes. Reduced amounts of translocated GLUT-4 were found in 3T3 cell membranes incubated with 10 μM $Ap_4A$. Thus, $Ap_4A$ itself induces a diabetic situation which is likely to be mediated by an increase in gluconeogenesis and/or an insulin resistance caused by a decrease in GLUT-4 and an attenuation of glucose uptake.

## 1. Introduction

Diadenosine polyphosphates (e.g. $Ap_3A$, $Ap_4A$, $Ap_5A$ and $Ap_6A$) belong to a group of ubiquitous occurring compounds formed by two adenosine molecules bridged by three to six phosphates. They are stored in e.g. platelets [1–3], chromaffin cells [4, 5] and neuronal cells [6, 7] by whom they are released into the extracellular space and circulation. All of them are suggested to be involved in blood pressure regulation [3, 8, 9] or to be a potential candidate for the development of essential hypertension [3, 10]. Binding sites for various diadenosine polyphosphates have already been demonstrated in various cells, such as heart [11, 12], brain [13, 14], liver [15] and INS-1 cells [16]. In a quickly responding perfusion system insulin release is increased by diadenosine polyphosphates ($Ap_3A$, $Ap_4A$, $Ap_5A$, $Ap_6A$) [16]; in contrast, in a long-term, static incubation they inhibit insulin release possibly due to a degradation compound [16]. The intracellular presence of the diadenosine polyphosphates $Ap_3A$ and $Ap_4A$ and their role as second messenger was recently shown in pancreatic islets [17]. It was the aim of the present study to investigate whether application of the diadenosine polyphosphate $Ap_4A$ leads to pathophysiological consequences with respect to glucose metabolism *in vivo* and, thus, could be a diabetogenic factor. The source of changes in glucose levels (gluconeogenesis or insulin resistance) was investigated using

- *in vitro* assays for glucose uptake in 3T3-L1 cells and for the glucose transporter GLUT-4,
- *in situ* liver perfusion experiments and
- primary hepatocytes and HEP-G2 cells with respect to glucose release.

## 2. Investigations and results

### *2.1. Purity of $Ap_4A$*

To exclude the possibility that the action of $Ap_4A$ is due to contamination with ATP, a purified $Ap_4A$ sample and a purified sample with 1% ATP added were analyzed by HPLC. The purity of $Ap_4A$ was analyzed by gradient elution on a reversed-phase column, Porous R2/H (100 × 2.1 mm I.D., Perseptive Biosystems, Wiesbaden, Germany). The column temperature was ambient (22 ± 1 °C). Eluents A and B used for the gradient elution were 2 mM tetrabutylammonium hydrogensulfate in a phosphate buffer (10 mM $K_2HPO_4$, pH 6.8) and water-acetonitrile (20 : 80, v/v). The mobile phase was pumped at a flow rate of 300 μl/min by a high pressure gradient pump system (Bai, Bensheim-Auerbach, Germany). The column eluate was monitored with a variable wavelength UV detector at 254 nm (759 A, Absorbence Detector, Applied Biosystems, Bensheim, Germany). The sample was mixed with eluent A. The following gradient was used: 0–0.8 min: 100% eluent A, 0.8–4.8 min: 0–10% B, 4.8–16.8 min: 10–22% eluent B, 16.8–17.4 min: 22–50% eluent B. Data were recorded and processed with a Chromjet integrator (TSP instruments, Bai, Bensheim-Auerbach, Germany). A typical chromatogram of the purified compound is shown in

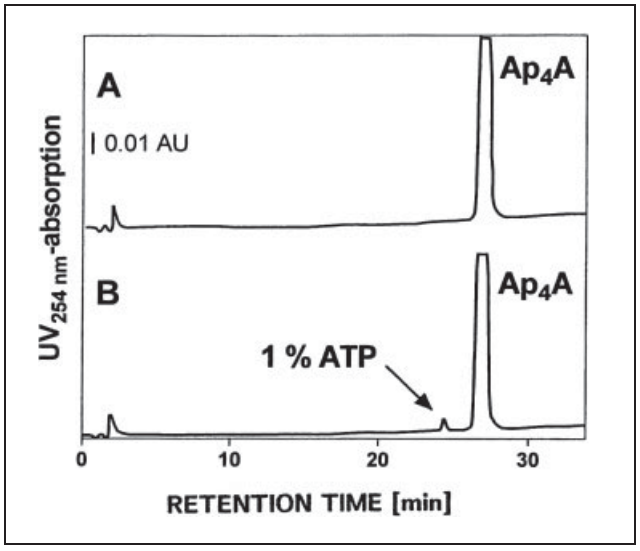


Fig. 1: Chromatograms of either purified $Ap_4A$ alone (A) or of purified $Ap_4A$ with 1% ATP added (B). A: sample of 10 mg $Ap_4A$ (A) or 10 mg $Ap_4A$ plus 100 μg ATP were used. Column: Reversed phase column (Poros R2/H, 100 × 2.1 mm I.D.). Eluent A: 2 mM tetrabutylammonium hydrogensulfate in a phosphate buffer (10 mM $K_2HPO_4$, pH 6.8); eluent B: water-acetonitrile (20 : 80%, v/v). Gradient: 0–0.8 min: 100% eluent A, 0.8–4.8 min: 0–10% B (80% acetonitrile), 4.8–16.8 min: 10–22% eluent B, 16.8–17.4 min: 22–50% eluent B. Flow-rate: 300 μl/min

Fig. 1. Degradation products which possibly falsify the results were less than 0.2%. In the chromatogram of the purified $Ap_4A$ in Fig. 1A no ATP can be detected. The detection limit of ATP is 20 μg. Therefore, the highest possible amount of ATP as contamination in our purified $Ap_4A$ is smaller than 20 μg (0.2%).

### *2.2. Blood glucose and plasma insulin*

When 0.75 mg/kg $Ap_4A$ is given intravenously by a bolus injection, the blood glucose levels increase rapidly and dramatically (Fig. 2); they stay elevated during investigation time of 45 min. Saline (control) has no significant effect (Fig. 2). Degradation products of $Ap_4A$ (see below) such as ATP or adenosine have a lower or no effect, respectively (Fig. 2).

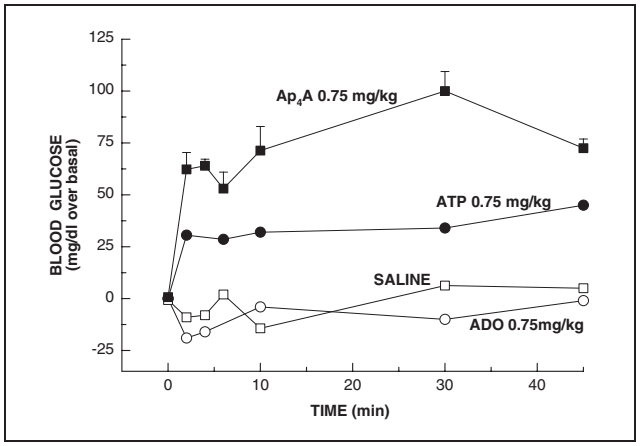


Fig. 2: Effect of $Ap_4A$, ATP and ADO (adenosine) on blood glucose in rats. 0.75 mg/kg $Ap_4A$ or saline or ADP or ATP were infused via bolus injection and blood was drawn at the indicated time points. Values are normalized to zero (basal value directly prior to bolus injection). Mean ± SE, 3 independent experiments; *$p < 0.05$ vs. control (saline)

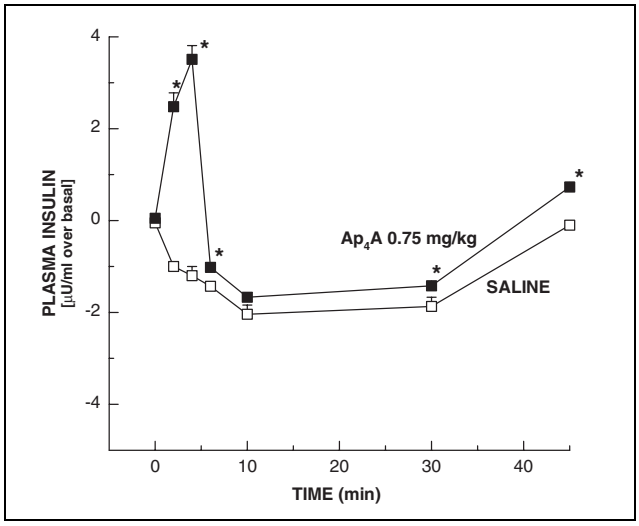


Fig. 3: Effect of $Ap_4A$ on plasma insulin in rats. 0.75 mg/kg $Ap_4A$ or saline was infused via bolus injection and blood was drawn at the indicated time points. Values are normalized to zero (basal value directly prior to bolus injection). The basal values are in the range of 10.7 and 12.2 μU/ml. Mean ± SE, 3 independent experiments; *$p < 0.05$ vs. control (saline)

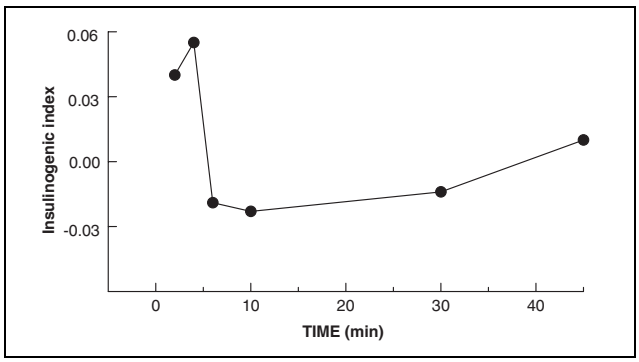


Fig. 4: Effect of $Ap_4A$ on the insulinogenic index in rats. The insulinogenic index is indicative of the sensitivity of the cells for glucose. A ratio of Δ plasma insulin to Δ blood glucose (insulinogenic index) is derived from data shown in Figs. 1 and 2; Three independent experiments were performed

In the same experiment plasma insulin levels were determined as well. They significantly increase at the very beginning but are no longer increased thereafter (Fig. 3).
The effect on insulinogenic index is shown in Fig. 4. The insulinogenic index is the ratio of Δ plasma insulin to Δ blood glucose; it indicates the sensitivity of the insulin secreting system towards glucose. Data for calculation are derived from results shown in Figs. 2 and 3. In response to the $Ap_4A$ bolus injection the insulinogenic index is increased in the first 4 min and decreased thereafter (Fig. 4).

### *2.3. Glucose production*

Fig. 5 shows the effect of $Ap_4A$ and of controls (glucagon and saline) on glucose release from the perfused rat liver *in situ* within 5 and 10 min (integrated values). A bolus of 25 μg $Ap_4A$ induces a release within the first 5 min; even at a time point between 5 and 10 min there is an effect above that of saline. The effect of glucagon (standard compound) is much greater than that of $Ap_4A$. The calculated increase glucose concentration was 4.7 μM per min and g liver tissue (the average liver tissue weight was 12 g). 0.9 nM glucagon (the most effective compound) exhibited an increase by 19.1 μM/min/g liver tissue.

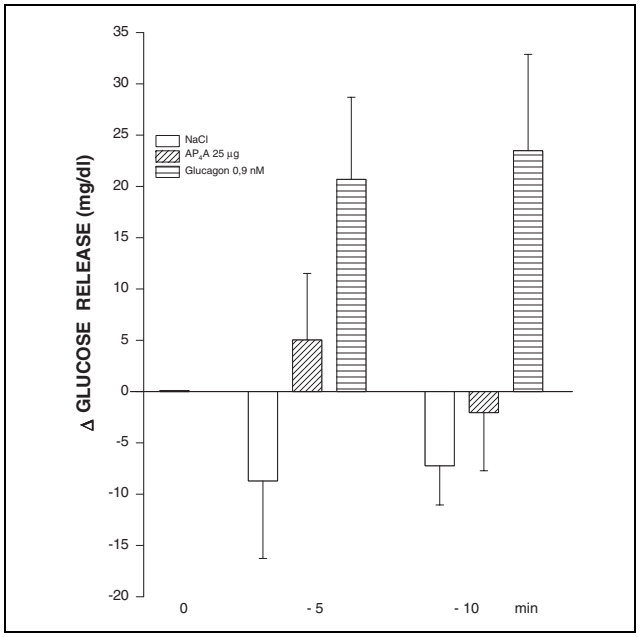


Fig. 5: Effects of $Ap_4A$ on glucose release from rat liver *in situ*. Rat liver was perfused with a constant pressure with a carbogen saturated Krebs-Henseleit buffer at 37 °C. All compounds including the controls (saline and glucagon) were applicated as a bolus (dose as indicated in the figure). Glucose was determined in the perfusate every minute over 20 min. Glucose concentrations from the effluent during the preperfusion period (0 min) were normalized to zero. The integrated values (AUCs, area under the curves) over the next 5 (−5 min) and another 5 minutes (−10 min) are shown as delta glucose release (glucose concentration in the effluent as mg/dl). Saline and glucagon (0.9 nM) were added as negative and positive controls. Mean ± SEM of four separate experiments; data were presented as accumulated data over 5 and 10 min

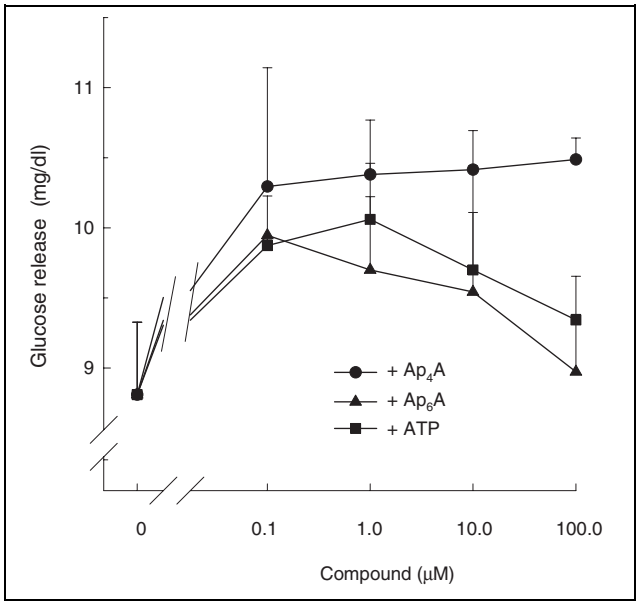


Fig. 6: Effects of $Ap_4A$, $Ap_6A$ and ATP on ascorbate-stimulated glucose release from HEPG2 cells. HEPG2 cells were preincubated at 37 °C for 2 h in glucose deprived KRH buffer. The medium was then changed for 3 h to an ascorbate (250 µM) but no glucose containing KRH buffer. Increasing concentrations of $Ap_4A$, $Ap_6A$ and ATP were added. Incubation was stopped by cooling the samples on ice; glucose was determined from the supernatant (200 µl). Mean ± SEM of 3–7 separate experiments

Fig. 6 shows the effect of diadenosine polyphosphates ($Ap_4A$ and $Ap_6A$) and ATP on glucose production from HEPG2 cells in the presence of 250 µM ascorbate (a highly stimulatory concentration). The data indicate an effect of all compounds; the effect of $Ap_4A$ is more pronounced than that of $Ap_6A$ and ATP. The effect of $Ap_4A$, therefore, cannot be mediated by its degradation product ATP.

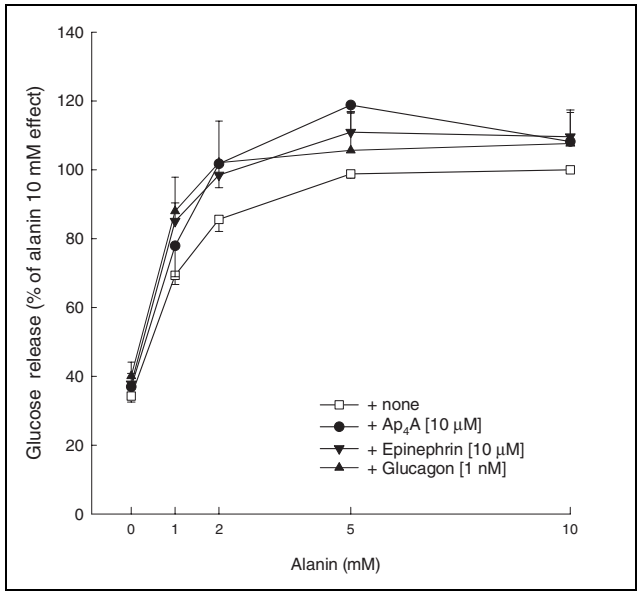


Fig. 7: Effects of $Ap_4A$, epinephrine and glucagon on the alanine-stimulated glucose release from primary hepatocytes. Collagenase isolated hepatocytes from 24 h fasted rats were incubateded ($0.8 \times 10^6$ cells/1ml KRH buffer) at 37 °C with test compounds and increasing concentrations of alanin for 30 min. After centrifugation of the cells at 4 °C the glucose concentration was determined in the supernatant. Data are presented as % of glucose release mediated by 10 mM alanin. Mean ± SEM of 3–7 separate experiments

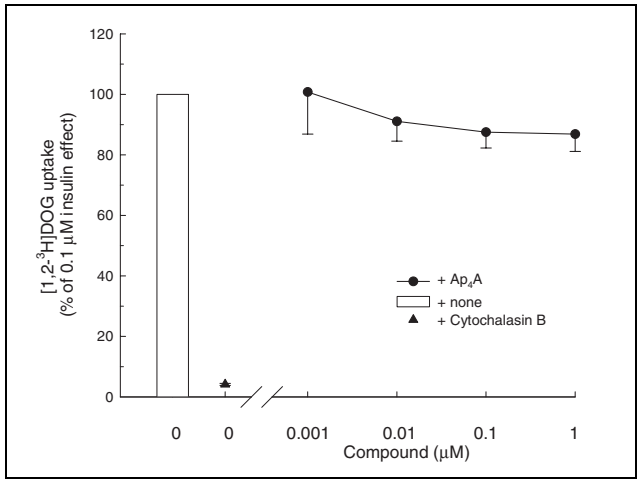


Fig. 8: Effect of $Ap_4A$ on insulin-stimulated [1,2-$^3$H]2-deoxyglucose (DOG) uptake by adipocytes having been differentiated from 3T3-L1 preadipocytes. After 3 h of preincubation cells were incubated with 100 nM insulin in the presence of either 10 µM cytochalasin B, or 10 µM $AP_4A$ for 20 min followed by an incubation with radioactively labelled deoxyglucose for 10 min. Glucose uptake was terminated by sucking off the medium and washing the cells with ice-cold glucose containing medium. Radioactivity of lysed cells (0.5% SDS) was counted. Mean ± SEM of 3–5 separate experiments

Fig. 7 shows the effect of $Ap_4A$ on glucose release from primary hepatocytes in the presence of alanin. The effect of 10 µM $Ap_4A$ was more pronounced than that of control.

### *2.4. Glucose uptake and GLUT-4 transporters*

Fig. 8 shows the effect of $Ap_4A$ on glucose uptake by 3T3-L1 cells. 1 µM $Ap_4A$ has a tendency to inhibit glucose uptake within 10 min.

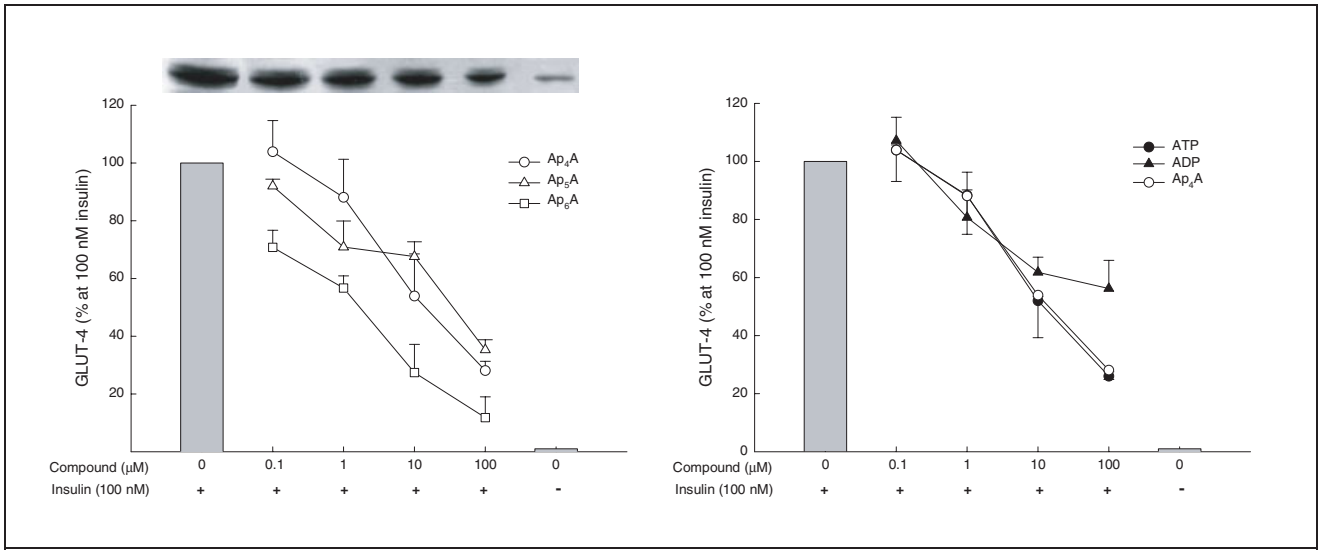


Fig. 9: Effect of $Ap_4A$, $Ap_5A$ and $Ap_6A$ (left panel) and of ATP, ADP (right panel) on GLUT-4 in the plasma membrane of 3T3 preadipocytes. 3T3 preadipocyte membrane fractions were prepared from non-insulin – and insulin (100 nM) stimulated cells in the presence or absence of 10 μM of either compound, their proteins resolved by SDS-PAGE and immunoblotted using specific antibodies against GLUT-4 (1 : 1000 dilution). Mean ± SEM of 4 separate experiments; on top the result of one representative experiment (autoradiography)

In Fig. 9 the effect of diadenosine polyphosphates and their degradation products on GLUT-4 transporters is shown. 100 nM insulin exhibits an increase in total GLUT-4 protein in 3T3-L1 cells. This increase is dose-dependently diminished by diadenosine polyphosphate used for 30 min with $Ap_6A$ being more effective than the other compounds (left panel); 10 μM $Ap_4A$ leads to a down-regulation by 43% of insulin-induced GLUT-4 protein levels translocated to the membranes. The degradation products ATP and ADP were effective as well (right panel).

## 3. Discussion

### *3.1. Diabetic situation*

The overall blood glucose concentration and the diabetic situation were not yet investigated *in vivo* with respect to $Ap_4A$ though this compound was described two decades ago. Intravenously given $Ap_4A$, a physiological compound, led to a metabolic deterioration in rats: There was a huge increase in blood glucose which persisted for a long period (up to 5 h). This tremendous *in vivo* increase in glucose was not counterbalanced by increased insulin levels except for the first 4 min. The physiological compound $Ap_4A$, therefore, may be a diabetogenic factor. This initial increase in plasma insulin levels fits to the short-lived insulin release which was recently shown in perifusion studies using INS-1 cells [16]. However, the situation gets worse after 4 min indicated by a low insulin level and a decreased insulinogenic index.

### *3.2. In situ- and in vitro investigations on glucose release*

There is not only an insensitivity of the insulin secretory system towards glucose (low insulinogenic index), but there must be a source of the high amounts of blood glucose. Therefore, the increase in glucose production on the one hand and glucose uptake and GLUT-4 concentration in a target tissue on the other hand have been investigated.

Gluconeogenesis and glycogenolysis are possible mechanisms of glucose production. Three different models were used to investigate this possibility. We employed an assay offering ascorbate (a gluconeogenetic precursor) to HEPG2 cells, a hepatoma cell line. $Ap_4A$ increased glucose release at a high ascorbate concentration. Using another model a release of glucose from the *in situ* perfused rat liver is obvious albeit it is one quarter of that of the leading, i.e. most effective compound glucagon. Though this effect lasted only 10 min and levelled off thereafter, this is presumably not in contrast to the observed *in vivo* bolus result with a longer lasting effects. This is because the elevated glucose levels in the in vivo experiment could not be eliminated since they were not followed by a plasma insulin release and since insulin levels were too low. In a third model a gluconeogenetic effect was shown for $Ap_4A$ when alanin was used (Fig. 7) whereas gluconeogenesis from lactat/pyruvate was not affected (data not shown). The rat liver perfusion model (which is probably more sensitive than HEPG2 cells) turned out to be an appropriate model. Altogether an increase of glucose release by $Ap_4A$ was observed.

Our data corroborate those of others: In a perfused liver model [17] $Ap_4A$ increased glucose output in a concentration-dependent way: The extra glucose output was 1.54 μmol/g per min. An increased gluconeogenesis was also observed in kidney (isolated rat proximal tubules) [18]. ATP as well is able to increase glucose release from liver [19, 20]; the effect of this very short-lived degradation product of $Ap_4A$ does not explain and cannot hold for the long lasting effect of $Ap_4A$ in our in vivo experiments albeit it corroborates data of an *in vitro* model (glycogen phosphorylase) [21].

### *3.3. Glucose uptake and GLUT-4 protein in adipocyte membranes*

Another possibility for an increase in blood glucose would be an inhibition of glucose uptake. This was investigated in adipocytes using a combination of $Ap_4A$ and insulin. There was a marginal inhibition which was only obvious in 10 min experiments. Similar results have been shown in primary rat cardiomyocytes [22] though it has to be admitted that these cells are not the most important target cells for insulin. The degradation product ATP may be involved since ATP itself inhibits glucose uptake [22].

More prominent is the effect on GLUT-4 transporters. Our findings in 3T3-L1 cells demonstrate that application of $Ap_4A$ leads to a decrease in GLUT-4 content of plasma

cell membranes, which therefore might contribute to the rise in plasma glucose levels. Thus the increase in gluconeogenesis and the decrease in GLUT-4 protein may participate in the increase in rat blood glucose levels in addition to the low plasma insulin levels.

In conclusion: $Ap_4A$ though being a short-lived physiological compound deteriorates metabolism in rats *in vivo*. Blood glucose is elevated which is not counterbalanced by an increased insulin release (*in vivo* data in this study and *in vitro* data published earlier [16]). This leads to a diabetic situation. The compound, therefore, could be a diabetogenic factor and may add to the pathogenesis of type 2 diabetes. Several data hint at an increase in gluconeogenesis and at insulin resistance (decreased GLUT-4). The results are interesting since diadenosine polyphosphates were already shown to have an impact on blood pressure and since the link between hypertension and diabetes is not yet understood.

## 4. Experimental

### *4.1. Chemicals*

Urethane was from Merck (Darmstadt, Germany). ATP, ADP and adenosine were from Sigma (Deisendorf, Germany). Diadenosine tetraphosphate ($Ap_4A$), $Ap_5A$, $Ap_6A$ were from Sigma as well, but chromatographically purified according to Heidenreich et al. [23] with a result shown in Fig. 1. Heparin-Na-25000 was from Ratiopharm (Ulm, Germany). Rat insulin was from Novo Nordisk (Bagsvaerd, Denmark), (mono-$^{125}$I-Tyr $A^{14}$)-porcine insulin from Hoechst (Frankfurt, Germany) and anti-insulin antibodies were from Linco (St. Louis, USA). $Ap_6A$ was provided by Schlüter, University of Berlin, Germany. All other compounds (analytical grade) were from Baker (Griesheim, Germany) or from Merck (Darmstadt, Germany).

### *4.2. Animals*

Wistar rats of either sex weighing 250–350 g were used. The animals were housed at 22 °C with a 12 h light/dark cycle (lights on at 6:00 h). A standard pellet diet and tap water were available *ad libitum*.

### *4.3. Bolus injection of $Ap_4A$*

Rats were anaesthetized with urethane (1.6 to 2.0 g/kg b.w.). One jugular vein was cannulated for the $Ap_4A$ bolus injection. Blood was drawn by the retrobulbar technique. The basal plasma insulin levels prior to infusion were in the range of 6–14 μU/ml, and those of glucose were in the range of 6.1 to 7.2 mM (non fasted rats). At time point 0 a bolus (10 s) of an $Ap_4A$ solution (neutral pH) was applied intravenously. Blood samples (7 drops) were collected into chilled tubes filled with 150 I.U. heparin-Na at time points-1, 2, 4, 6, 10, 30, and 45 min. The blood sampling procedure did not take longer than 20 s. Glucose was separated by centrifugation and was used immediately for the glucose determination or was kept frozen at −20 °C until the insulin assay was performed.

0.75 mg $Ap_4A$ per kg body weight were applied as a bolus in order to obtain rather physiological blood concentrations. Assuming that 7.5% of body weight being blood, the initial concentration of 0.75 mg/kg $Ap_4A$ (m.w. 836) in 75 ml blood (per 1 kg rat) will be approx. 12 μM. Since $Ap_4A$ is a hydrophilic compound its quick distribution into the extracellular space is expected leading to even 3 times lower concentrations compared to a distribution only in the blood. On the one hand this is rather in the range of the recently published $IC_{50}$ of $Ap_4A$ for binding sites of INS-1 cell homogenates [16]. On the other hand assuming a complete release from platelet dense granules, an extracellular concentration of about 100 μM is possible and after distribution in blood a concentration of 0.5 to 1 μM was estimated by the group of Ogilvie [2, 17, 24]. Extracellular concentrations of 27 μM were calculated for diadenosine polyphosphates after secretion from chromaffin cells [25, 26]. The concentration for one-half maximal inhibition of $K_{ATP}$ channels was shown to be 17 μM $Ap_4A$ [27].

### *4.4. Glucose release from perfused rat liver (in situ)*

Rat liver was prepared according to a technique described by Sugano et al. [28]. The liver was perfused *in situ* without recirculation of the perfusate (i.e. modified Krebs-Henseleit buffer containing NaCl 119 mM, KCl 4.74 mM, $KH_2PO_4$ 1.16 mM, $MgSO_4 \times 7\ H_2O$ 1.16 mM, glucose 11.5 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.0 mM, MOPS 3.0 mM, BSA RIA grade 0.5%, Na-taurocholate 0.02 mM). Perfusion lasted for about 15 min after having been equilibrated for at least 30 min. Liver cell viability (AST- and γ-GT activity, bile flow) as well as perfusion conditions (flux and volume of the perfusate) and $CO_2/O_2$ and potassium concentration in the perfusing buffer were constantly controlled. Results are calculated as integrated values (AUCs, area under the curves) over the next 5 (−5 min) and another 5 min (−10 min) and are given as delta glucose release (glucose concentration in the effluent as mg/dl).

### *4.5. Glucose release from primary hepatocytes of rats*

Rats were fasted for 24 h prior to the isolation of cells. Rats were anaesthetized by intraperitoneal injection of diazepam (1 mg/kg body weight) and ketamin (50 mg/kg body weight) and parenchymal hepatocytes were isolated by recirculating collagenase perfusion method *in situ* (collagenase CLS type I, 40 mg/dl, appromimately 30 ml/min) as described by Katz et al. [29]. Cell viability was estimated by trypan blue exclusion test (more than 90%). Viable hepatocytes were further purified by centrifugating through a Percoll® gradient. Purified cells were suspended in Krebs-Ringer buffer (NaCl 130 mM, KCl 5 mM, $Na_2HPO_4$ 10 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.3 mM), pH 7.35 containing 0.15% BSA. They were stored on ice until the experiments started (maximally for 2 h). Incubation buffer containing substrates, hormones and agents to be tested were prepared in tubes. Incubation was started by addition of hepatocytes (final concentration $0.8 \times 10^6$ cells/ml). Experiments were carried out at 37 °C while cells were kept in suspension by rotatory shaking (135 strokes/min). Glucose release was stopped by cooling the samples on ice and further on by centrifugation (2 min/4 °C/4000 rpm). Sample volumes (200 μl) of the supernatant were collected and frozen (−20 °C). Glucose was determined by using a commercial kit based on the hexokinase method (Glucoquant® Kit MPR3 Roche Diagnostics).

### *4.6. HEPG2 cell culture*

HEPG2 hepatoma cells were cultured in 75 $cm^2$ culture flasks ($0.7 \times 10^5$ cells/20 ml) or 24-well culture plates (approximately $8 \times 10^3$ cells/well/1ml) at 37 °C in a humidified atmosphere of 5% $CO_2$/95% air. They were grown in monolayer cultures in DME medium supplemented with 10% fetal bovine serum (v/v), 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The medium was changed once on the third day of culture and additionally the day prior to the experiment.

### *4.7. 3T3 cells cell culture*

3T3 Preadipocyte cells were cultured in 75 $cm^2$ culture flasks ($2 \times 10^5$ cells/20 ml) or 24-well culture plates (approximately $5 \times 10^3$ cells/well/1 ml) at 37 °C in a humidified atmosphere of 5% $CO_2$/95% air. They were grown in monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The medium was changed once on the fourth day of culture and additionally the day prior to the experiment.

### *4.8. Assays for glucose and insulin*

Blood glucose was determined by the glucose oxidase method. Employing Glucoquant® from Boehringer-Mannheim (Mannheim, Germany). Under assay conditions glucose calibration curves were linear over the range of 0.05–15 mg/dl. Plasma insulin concentrations were assayed with a radioimmunoassay using rat insulin as a standard, (mono-$^{125}$I-Tyr $A^{14}$)-porcine insulin as the labeled compound and anti-insulin antibodies.

### *4.9. Insulinogenic index*

The insulinogenic index is the ratio between the changes in plasma insulin and blood glucose levels (Δ insulin/Δ glucose) and is derived from data shown in other figures as indicated in the legends. The insulinogenic index indicates the sensitivity of the insulin secretory system to glucose and should normally not vary.

### *4.10. Glucose uptake*

The uptake of [1,2-$^3$H]2-deoxy-D-glucose by 3T3-L1 adipocytes was performed according to Harrison et al. [30]. Briefly: adipocytes were incubated 3 h prior to the main experiment in a Krebs-Ringer buffer without BSA and FBS, followed by a preincubation with or without 100 nM insulin in the presence of the indicated test compounds for 20 min (total volume of 300 μl). The main incubation was started by the addition of [1,2-$^3$H]2-Deoxy-D-glucose (final concentration 0.1 mM [1 μCi/ml]) for 10 min at 37 °C and stopped by washing with ice-cold glucose (5.6 mM) medium. The radioactivity of SDS (0.5%) lysed cells was counted in a scintillation counter (see above). Nonspecific uptake in the presence of cytochalasin B was subtracted.

### *4.11. GLUT-4 protein isolation and Western blotting*

Half-confluent 3T3-L1 cells were incubated with $Ap_4A$ (10 μM) for 30 min. Membranes were obtained by scraping off the cells into a homogenization buffer containing 50 mM TrisHCl, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM benzamidine, 0.01% bacitracin, 0.002% soybean trypsin in-

hibitor and 100 μM PMSF. The cells were then disrupted by sonication (3 × 30 s) on ice. The homogenate was first centrifuged at 600 × g to remove unbroken cells and nuclei. The resulting supernatant was centrifuged at 100,000 × g for 60 min in an ultracentrifuge. After centrifugation, the crude membranes were solubilized for 60 min at 4 °C in lysis buffer containing 5% (m/V) SDS, 80 mM Tris, pH 6.8, 5 mM EDTA, 0.5 mM PMSF. This homogenate was taken as plasma membrane fraction. Protein concentration was determined by the BCA Protein Assay Kit (Pierce; Rockford, USA). Proteins were resolved by SDS-Page on 10% SDS-polyacrylamide gels according to Laemmli and transferred to nitrocellulose sheets in an electrophoretic transfer cell (Trans Blot® SD from Bio-Rad) using a transfer buffer supplemented with SDS. Membranes were blocked overnight at 4 °C in blocking buffer (5% (m/V) BSA, 0.05% (m/V) Tween 20 in PBS buffer). Nitrocellulose membranes were incubated with specific antibodies against GLUT-4. A secondary biotinylated antibody reacted with streptavidin-peroxidase. The bands obtained after interaction with a peroxidase substrate were identified by enhanced chemiluminescence, using X-ray films and quantified by laser densitometry and expressed as percent of control.

### *4.12. Statistics*

For statistical evaluation multiple comparisons of means were carried out by one-way analysis of variance followed by a post-hoc test (Newman-Keuls-test).

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