Diadenosine Polyphosphates in Insulin-Secreting Cells

Interaction With Specific Receptors and Degradation

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A role of diadenosine polyphosphates as second messengers was suggested for insulin-secreting cells. It has not yet been investigated whether specific receptors for these compounds exist and how these extracellular compounds and their degradation products may contribute to insulin release. Specific saturable binding sites for diadenosine polyphosphates exist in INS-1 cells and rat pancreatic islets. In INS-1 cells, the rank order of diadenosine polyphosphates displacing [^{3}H]Ap$_4$A from binding sites was Ap$_4$A = Ap$_5$A >Ap$_3$A = Ap$_6$A. Binding was specific, since suramin was not able to displace the binding; adenosine, ATP, UTP, α,β-methylene ATP, β,γ-methylene ATP, ADP-βS, 2-methylthio ATP, and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) were able to displace [^{3}H]Ap$_4$A from its binding sites. Insulin release was investigated in INS-1 cells. Perifusion experiments showed an increase in insulin release stimulated by the diadenosine polyphosphates in the presence of 8.3 mmol/l glucose; in static incubations (90 min), however, insulin release was inhibited dose dependently by the four diadenosine polyphosphates. This discrepancy might be due to the instability of the compounds. [^{3}H]Ap$_4$A was degraded in the extracellular medium to mainly adenosine and low concentrations of ATP, ADP, AMP, and inosine (half-maximal degradation after 25 min). The insulin stimulatory effect is due to the original compounds (acute perifusion experiments), and the insulin inhibitory effect (static incubation experiments) is due to the production of inhibitory compounds, such as adenosine, in the medium. Small amounts of intact [^{3}H]Ap$_4$A, but mainly [^{3}H]ATP, accumulated in the cells within 20 min. The uptake of labeled compounds is dependent on an intact metabolism and intact receptor internalization. This data indicates that *1*) specific bindings sites for diadenosine polyphosphates exist in INS-1 cells and rat pancreatic islets mediating insulin release; *2*) the receptors involved in INS-1 cells may be diadenosine polyphosphate receptors, albeit others, such as P$_{2X}$-receptors, cannot be ruled out; and *3*) diadenosine polyphosphates, and mainly their degradation products in the extracellular space, are to a high degree accumulated within cells with unknown function. Thus, diadenosine polyphosphates are worth being investigated more closely in physiological and pathophysiological terms. ***Diabetes*** **47:1727–1734, 1998**

Diadenosine polyphosphates (Ap$_3$A, Ap$_4$A, Ap$_5$A, and Ap$_6$A) belong to a group of ubiquitous compounds formed by two adenosine molecules bridged by three to six phosphates. They are present/stored in, e.g., dense granules of platelets, chromaffin cells, and neuronal cells, and they are released into the extracellular space. Some of them are suggested to be involved in blood pressure regulation (1–3) or even to be important for the development of essential hypertension (4). Binding sites for various diadenosine polyphosphates have already been shown in various cells, such as heart (5,6), brain (7,8), and liver (9).

The intracellular presence of the diadenosine polyphosphates Ap$_3$A and Ap$_4$A was recently shown for rat pancreatic islets (10). A role as second messengers was suggested, since their concentration increased with raising glucose concentrations and since they are effective inhibitors of the K$_{ATP}$ channels using excised membrane patches (10). Ventricular K$_{ATP}$ channels are inhibited by diadenosine polyphosphates as well (11–13). Whereas in these in vitro studies, the diadenosine polyphosphate content is increased by various maneuvers, not addressed was the question of whether high amounts being secreted into the blood, e.g., from platelets or chromaffin cells, interact with specific, possibly yet undefined, receptors on insulin-secreting cells or are simply accumulated as the original compound or its degradation product within target cells. It was the aim of the present study to investigate whether diadenosine polyphosphates act on insulin-secreting cells via specific receptors and whether they or their degradation products modulate insulin release. Our studies indicate that diadenosine polyphosphates act via specific receptors and mediate insulin release when the original compounds are not degraded outside the cell.

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KRH buffer, Krebs-Ringer buffer containing 10 mmol/l HEPES and 0.5% bovine serum albumin; α,β-meATP, α,β-methylene ATP; β,γ-meATP, β,γ-methylene ATP; 2-MeSATP, 2-methylthio ATP; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; TLC, thin-layer chromatography.

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RESEARCH DESIGN AND METHODS

Diadenosine polyphosphates, ATP, UTP, ADP, AMP, α,β-methylene ATP (α,β-meATP), β,γ-methylene ATP (β,γ-meATP), ADP-βS, adenosine, inosine, and concanavalin A were from Sigma (Deisenhofen, Germany); pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and suramin were supplied by Dr. Lambrecht (Frankfurt, Germany); and 2-methylthio ATP (2-MeSATP) was from ICN (Eschwege, Germany). $[^3H]Ap_4A$ labeled in the adenosine part of the molecule (spec. act. 237 GBq/mmol) was purchased from Amersham (Braunschweig, Germany). Tritiated samples were counted in a scintillation counter (Tri Carb 300 CD; Packard, Frankfurt, Germany) by using Quickscint 212 (Zinsser Analytic, Frankfurt, Germany). Cells were homogenized with a Dounce homogenizer (Braun, Melsungen, Germany). Rat insulin was from Novo Nordisk (Bagsvaerd, Denmark); (mono-^{125}I-Tyr A^{14})-porcine insulin was from Hoechst (Frankfurt, Germany); and anti-insulin antibodies were from Linco (St. Louis, MO). All other compounds (analytical grade) were from Baker (Griesheim, Germany) or Merck (Darmstadt, Germany).

Cell culture. INS-1 cells (14), generously provided by Dr. C.B. Wollheim (Geneva, Switzerland), were grown in monolayer cultures in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 10 mmol/l N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), 2 mmol/l glutamine, 1 mmol/l pyruvate, 50 µmol/l mercaptoethanol, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin.

HL-60 and K-562 cells were grown in suspension culture using RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin.

Uptake experiments

Uptake by INS-1 cells. Half-confluent cells (grown in microwells for 5 or 6 days) were washed twice with ice-cold Krebs-Ringer buffer containing 10 mmol/l HEPES and 0.5% bovine serum albumin, pH 7.35 (KRH buffer), and then incubated for up to 24 h at 22°C in 300 µl KRH buffer containing 31 nmol/l $[^3H]Ap_4A$ and 5.6 mmol/l glucose. In some experiments, 2 mmol/l sodium cyanide or 0.25 mg/ml concanavalin A were added during a preincubation period of 45 min at 37°C and during the main incubation. Incubation was terminated at the indicated time points by washing the cells twice with ice-cold KRH buffer. Samples from the supernatant were counted to determine the total amount of radioactivity. Cells were lysed with 400 µl 0.5% sodium dodecyl sulfate (SDS) for 30 min at 37°C, and the wells were washed with another 300 µl SDS. The combined samples were counted in a scintillation counter after adding 5 ml of scintillation cocktail.

Uptake by HL-60 and K-562 cells. The procedure was the same except that cells were grown in suspension in 75-cm^2 culture flasks for 2 days, centrifuged (100*g*, 8 min), washed twice with ice-cold KRH buffer, and resuspended in KRH buffer containing 5.6 mmol/l glucose.

Binding experiments with INS-1 cells. Half-confluent cells (grown in 75-cm^2 culture flasks for 6 days) were washed with KRH buffer and harvested by scraping. Cells were resuspended in KRH buffer after spinning and homogenized with a Dounce homogenizer. The particulate fraction (2,600*g*) was washed with KRH buffer and resuspended in KRH buffer containing 5.6 mmol/l glucose (400 µl/flask). Then, 30 µl of this suspension was incubated at 22°C with 104 nmol/l $[^3H]Ap_4A$ with or without increasing concentrations of either various unlabeled diadenosine polyphosphates or purinoceptor or adenosine receptor agonists. The final volume was 90 µl. To determine the nonspecific binding, the incubation was performed in the presence of 0.48 mmol/l unlabeled Ap_4A. The incubation was terminated at the indicated time points by cooling (4°C), centrifuging at high speed, and washing twice with ice-cold KRH buffer. The pellet was finally lysed with 50 µl 0.5% SDS and counted as described under uptake experiments.

Binding experiments with rat pancreatic islet homogenates. Isolation of rat pancreatic islets was performed as described by Lacy and Kostianovsky (15), with slight modifications (16), using the collagenase method. Isolated islets were homogenized with 1 ml KRH buffer with 0.5% albumin in a dounce homogenizer. The pellet was obtained at 20,000*g* (20 min) and was resuspended in 300 ml of the same buffer. The main binding experiments were as described for INS-1 cell homogenates.

Perifusion studies. INS-1 cells were grown on perforated glass coverslips for 5 days in RPMI medium as described above. The perifusion medium was KRH buffer, pH 7.35. The basal medium contained 3.0 mmol/l glucose, and the test medium contained 8.3 mmol/l glucose. The medium reservoirs were maintained at 37°C, and the perfusion mediums were not recycled. Perifusion flow rate was adjusted to 0.5 ml/min. In the first 10 min, the cells on coverslips were perifused with basal medium and were allowed to equilibrate under constant perifusion flow rate. In the following 20 min, designated in the figures as –20 to 0 min, five samples were collected to determine the basal insulin secretion. At zero time, cells were perifused with test medium, e.g., containing Ap_4A.

Insulin release. To measure insulin release, half-confluent INS-1 cells grown in microwells were incubated for 90 min at 37°C in KRH buffer containing 3.0 or 8.3 mmol/l glucose with or without increasing concentrations of diadenosine polyphosphates. Insulin released into the medium was 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.

Determination of $[^3H]Ap_4A$ degradation products. The original radioactively labeled compound and its degradation products were determined in the extracellular and the intracellular space of INS-1 cells using thin-layer chromatography (TLC). Half-confluent cells were incubated for up to 90 min in KRH buffer containing 5.6 mmol/l glucose and 0.31 µmol/l $[^3H]Ap_4A$. At individual time points, as indicated in the figure legends, aliquots were taken from the incubation medium, mixed with the appropriate unlabeled standards (ATP, ADP, Ap_4A, AMP, inosine, or adenosine), and transferred onto TLC sheets (aluminum sheets precoated with silica gel; Merck), developed for 2 h in dioxane:ammonia:water (6:1:4). Chromatography spots of unlabeled standards were visualized under ultraviolet light and cut out. Their radioactivity was counted in a liquid scintillation counter as described before. The content (purity) of the original labeled material was: Ap_4A 83.5%, ADP 7.2%, adenosine 5.8%, and ATP 3.6%.

Degradation within cells. INS-1 cells were incubated for 20 min in KRH buffer containing 5.6 mmol/l glucose with 1.56 µmol/l $[^3H]Ap_4A$. Cells were washed twice with ice-cold KRH buffer. After 0 or 10 min of incubation in KRH buffer containing 5.6 mmol/l glucose, cells were cooled to –70°C for 30 min, then proteins were precipitated with 0.6 M $HClO_4$. After centrifugation, the supernatant was neutralized with KOH, and aliquots were transferred onto TLC sheets. The chromatography was carried out as described before.

RESULTS

Binding experiments. Binding experiments were performed using a particulate fraction of INS-1 cells. Binding of $[^3H]Ap_4A$ to INS-1 cells is shown in Fig. 1. The binding was rapid, with a one-half maximal binding occurring after 4 min and a steady-state binding occurring at 30 min. After this time, bound radioactivity was ~12% of total radioactivity. Nonspecific binding (determined in the presence of 0.48 mmol/l unlabeled Ap_4A) ranged from 3 to 4% of added radioactivity and was already subtracted. Further experiments were performed under steady-state conditions with a 30-min incubation period.

Binding experiments were also performed using rat pancreatic islet homogenates as shown in Fig. 2. The binding was rapid, with a one-half maximal binding occurring after 8 min and a steady-state binding occurring at 30 min. Nonspecific binding (determined in the presence of 0.48 mmol/l unlabeled Ap_4A) was ~4% of added radioactivity and was already subtracted.

All tested diadenosine polyphosphates were able to inhibit specific $[^3H]Ap_4A$ binding to INS-1 cells (Fig. 3). Inhibition of specific $[^3H]Ap_4A$ binding by unlabeled Ap_3A, Ap_4A, Ap_5A, and Ap_6A was sigmoidal and was essentially complete over three orders of magnitude (0.32 µmol/l to 0.32 mmol/l). The rank order of potency of the diadenosine polyphosphates was $Ap_4A = Ap_5A > Ap_3A = Ap_6A$. The IC_{50} (concentration for one-half maximal inhibition of K_{ATP} channels) values calculated after logit-log transformation of displacement curves were 1.3×10^{-5}, 8.5×10^{-6}, 6.0×10^{-6}, and 1.4×10^{-5} mol/l for Ap_3A, Ap_4A, Ap_5A, and Ap_6A, respectively (plots not shown).

As shown in Fig. 4, each of the tested purinoceptor agonists and adenosine receptor agonists was able to inhibit specific $[^3H]Ap_4A$ binding to INS-1 cells. The P_2 receptor agonist ATP and the P_1 receptor agonist adenosine were potent inhibitors of $[^3H]Ap_4A$ binding, as potent as Ap_4A. UTP, which is an agonist at various P_2 receptor subtypes, primarily the P_{2U} receptor, competed with $[^3H]Ap_4A$ with less potency than ATP (Fig. 4*A*). The displacing activities of various P_{2X} and P_{2Y} agonists are shown in Fig. 4*B*: the P_{2X} agonists α,β-meATP and β,γ-meATP, the P_{2X} and P_{2Y} agonist 2-MeSATP, and the P_{2Y} agonist ADP-βS inhibited specific $[^3H]Ap_4A$ binding. Thus, the binding site did not clearly discriminate between these com-

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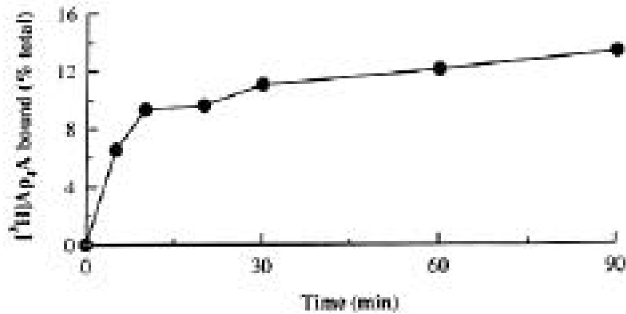

FIG. 1. Time course of [^{3}H]Ap$_4$A binding to a particulate fraction of INS-1 cells. The particulate fraction of INS-1 cells was incubated for up to 90 min at 22°C in 90 µl KRH buffer containing 5.6 mmol/l glucose and 104 nmol/l [^{3}H]Ap$_4$A. Results are expressed as percent bound of total radioactivity. Each value represents the mean of two experiments.

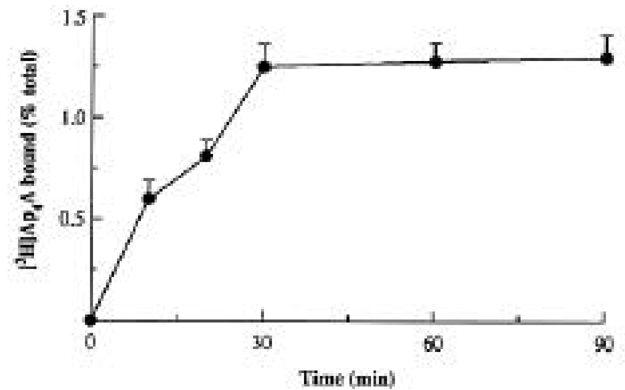

FIG. 2. Time course of [^{3}H]Ap$_4$A binding to islet cell homogenates. The particulate fraction of rat pancreatic islets was incubated for up to 90 min at 22°C in 90 ml KRH buffer containing 5.6 mmol/l glucose and 104 nmol/l [^{3}H]Ap$_4$A. Results are expressed as percent bound of total radioactivity. Each value represents the mean of four experiments.

pounds. Nevertheless, ATP, Ap$_4$A, and Ap$_5$A seem to be the most potent inhibitors of [^{3}H]Ap$_4$A binding to INS-1 cells.

Figure 5 shows the effect of the P$_2$ receptor antagonist suramin and the P$_{2X}$ receptor antagonist PPADS on specific [^{3}H]Ap$_4$A binding. Suramin did not displace specific binding at all concentrations tested, whereas PPADS was able to inhibit [^{3}H]Ap$_4$A binding with a more than one order of magnitude lower potency than Ap$_4$A.

Next, the biological effect of diadenosine polyphosphates was tested. In a perifusion system, all diadenosine polyphosphates (Ap$_{3/4/5/6}$A) increased insulin release in the presence of 8.3 mmol/l glucose (Fig. 6). Both the initial peak and the second phase of insulin secretion were elevated significantly by Ap$_3$A, Ap$_4$A, and Ap$_5$A; the Ap$_6$A effect lacked statistical significance. The insulin secretory potency during both phases of secretion is summarized for all diadenosine polyphosphates in Fig. 6*B*.

When a static incubation system was used, however, all diadenosine polyphosphates dose-dependently decreased insulin release in the presence of 8.3 mmol/l glucose (Fig. 7). At the highest concentration tested (10 µmol/l), Ap$_3$A, Ap$_4$A, Ap$_5$A, and Ap$_6$A reduced the glucose-induced insulin secretion to 79.2, 77.8, 84.3, and 82.3%, respectively.

Uptake experiments. The uptake by INS-1 cells of [^{3}H]-labeled compounds at 22°C was linear from 0 to 4 h when [^{3}H]Ap$_4$A was used, with a one-half maximal uptake occurring after 2 h and maximum uptake occurring at 6 h (Fig. 8*A*). At 6 h, the maximum uptake was 42% of total radioactivity, indicating an accumulation. Closely similar data were found when other cells, such as the leukemia cell lines HL-60 and K-562, were used (data not shown).

Next, the effects of various inhibitors of [^{3}H]-labeled compounds uptake were tested. The effects of sodium cyanide (an inhibitor of the oxidative phosphorylation) and concanavalin A (an inhibitor of receptor internalization) were examined at various temperatures. Lowering temperature from 22 to 4°C led to a dramatic decrease of [^{3}H] uptake (Fig. 8*B*). In addition, concanavalin A at either temperature (22 or 4°C) led to a considerable decrease in the uptake of [^{3}H]-labeled compounds. Cyanide, tested at 22°C, was also able to reduce uptake (Fig. 8*B*).

Degradation experiments. Degradation of [^{3}H]Ap$_4$A was determined in the incubation medium, as well as within INS-1 cells. Figure 9*A* shows the effect of degradation over 90 min in the medium while incubating INS-1 cells. [^{3}H]Ap$_4$A was degraded with a half-life of ~22 min. The major degradation product was adenosine. Figure 9*B* shows [^{3}H]Ap$_4$A and its intracellular degradation products. Only small amounts of [^{3}H]Ap$_4$A were found, and the main concentration within the cells consists of [^{3}H]ATP.

DISCUSSION

In a recent paper, diadenosine polyphosphates have been proposed to possess cellular functions in insulin-secreting cells as a new type of second messenger (10). However, diadenosine polyphosphates are known to be ubiquitously present outside the cells. In addition to a second messenger function, extracellular receptor effects cannot be excluded. This possibility was investigated in the present study.

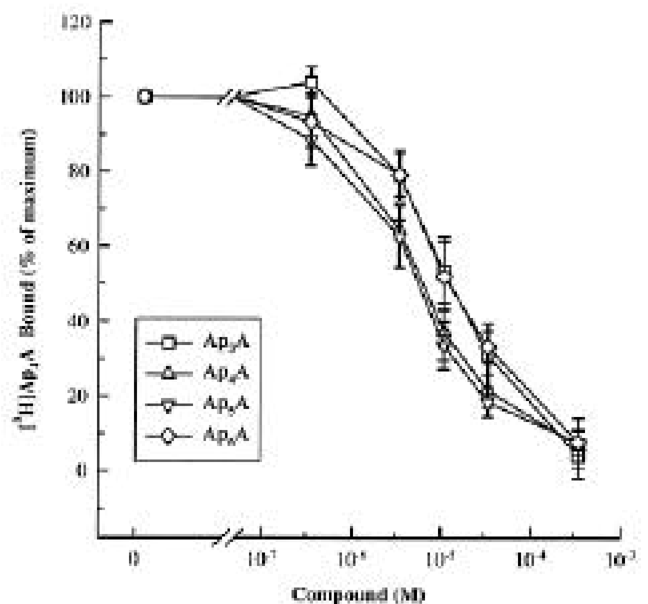

FIG. 3. Inhibition of [^{3}H]Ap$_4$A binding to a particulate fraction of INS-1 cells by various diadenosine polyphosphates. The particulate fraction of INS-1 cells was incubated for 30 min at 22°C in 90 µl KRH buffer containing 5.6 mmol/l glucose, 104 nmol/l [^{3}H]Ap$_4$A, and increasing concentrations of various diadenosine polyphosphates. Results are expressed as percent of maximum bound radioactivity. Each value represents the mean ± SE of three to seven experiments.

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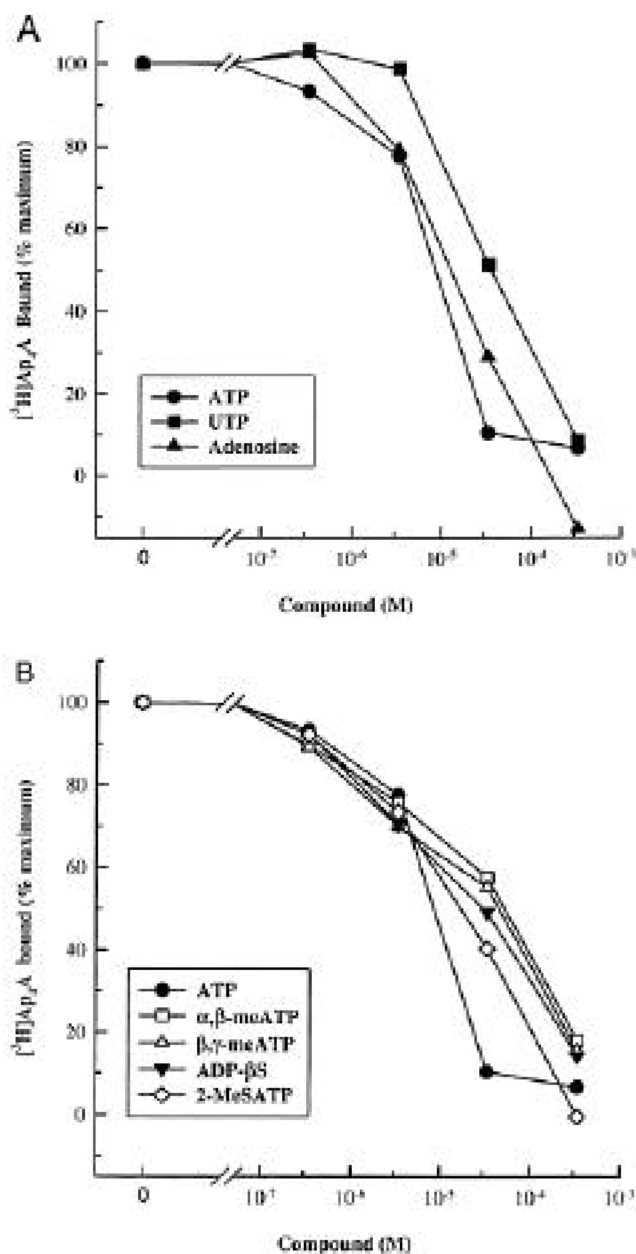

FIG. 4. Inhibition of $[^3H]Ap_4A$ binding to a particulate fraction of INS-1 cells by purinoceptor and adenosine receptor agonists. The particulate fraction of INS-1 cells was incubated for 30 min at 22°C in 90 µl KRH buffer containing 5.6 mmol/l glucose, 104 nmol/l $[^3H]Ap_4A$, and increasing concentrations of P_1- and P_2-receptor agonists (*A*) or P_{2X}- and P_{2Y}-receptor agonists (*B*). Results are expressed as percent of maximum bound radioactivity. Each value represents the mean of two experiments.

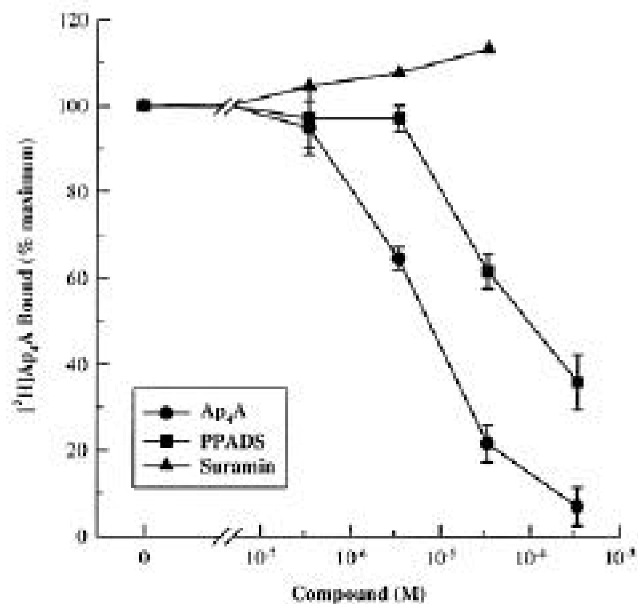

FIG. 5. Inhibition of $[^3H]Ap_4A$ binding to a particulate fraction of INS-1 cells by purinoceptor antagonists. The particulate fraction of INS-1 cells was incubated for 30 min at 22°C in 90 µl KRH buffer containing 5.6 mmol/l glucose, 104 nmol/l $[^3H]Ap_4A$, and increasing concentrations of the purinoceptor antagonists suramin and PPADS. Results are expressed as percent of maximum bound radioactivity. Each value represents the mean ± SE of two or three experiments.

Binding sites for labeled Ap_4A are present because its binding is time-dependent and saturable both in the insulin-secreting cell line INS-1 and in isolated rat pancreatic islets with similar kinetics. Additionally, binding is specific because suramin, a compound not structurally related to diadenosine polyphosphates, in contrast to various diadenosine polyphosphates, does not displace $[^3H]Ap_4A$ from its binding sites.

The concentration for one-half maximal inhibition of K_{ATP} channels was shown to be 1.7×10^{-5} mol/l for Ap_4A (10), which fits well with the concentrations required for one-half maximal inhibition of $[^3H]Ap_4A$ binding in our experiments: 1.3×10^{-5}, 8.5×10^{-6}, 6.0×10^{-6}, and 1.4×10^{-5} mol/l for Ap_3A, Ap_4A, Ap_5A, and Ap_6A, respectively. The rank order for inhibition of binding was $Ap_4A = Ap_5A > Ap_3A = Ap_6A$. Thus, our data indicate the presence of diadenosine polyphosphate receptors in INS-1 cells that poorly discriminate between the various diadenosine polyphosphates.

It is already known that in other systems, diadenosine polyphosphates act via specific surface receptors. Interestingly, Nakae et al. (17) showed that the coronary effects of Ap_4A are caused, at least indirectly, by the opening of K_{ATP} channels. In pigs, Ap_4A increased coronary blood flow and decreased systemic blood pressure. Schlüter et al. (4) found a decrease of blood pressure after injection of Ap_4A as well. In fact, an opening of K_{ATP} channels would fit with the inhibition of insulin secretion observed in our studies.

Physiological concentrations were probably used in our experiments. Other groups calculated high extracellular concentrations: assuming a complete release from platelet dense granules, an extracellular concentration of ~100 µmol/l is possible, and after distribution in blood, a concentration of 1 µmol/l could result (18). Extracellular concentrations of 27 µmol/l can be estimated for diadenosine polyphosphates after secretion from chromaffin cells (19,20). The concentration in blood (resulting from storage in platelets) can be estimated to be 0.14–0.3 µmol/l (recalculated from the data of Lüthje et al. [21] and Floodgard and Klenlow [22]). The extracellular concentration was shown to be increased in vivo by oxidants or metabolic stress (23). These concentrations are in the range of half-maximal inhibition of binding in our experiments. Thus, besides being produced by pancreatic β-cells as second messengers (10), effects of diadenosine polyphosphates from various sources acting on β-cells are likely to contribute to a major degree to the effects of

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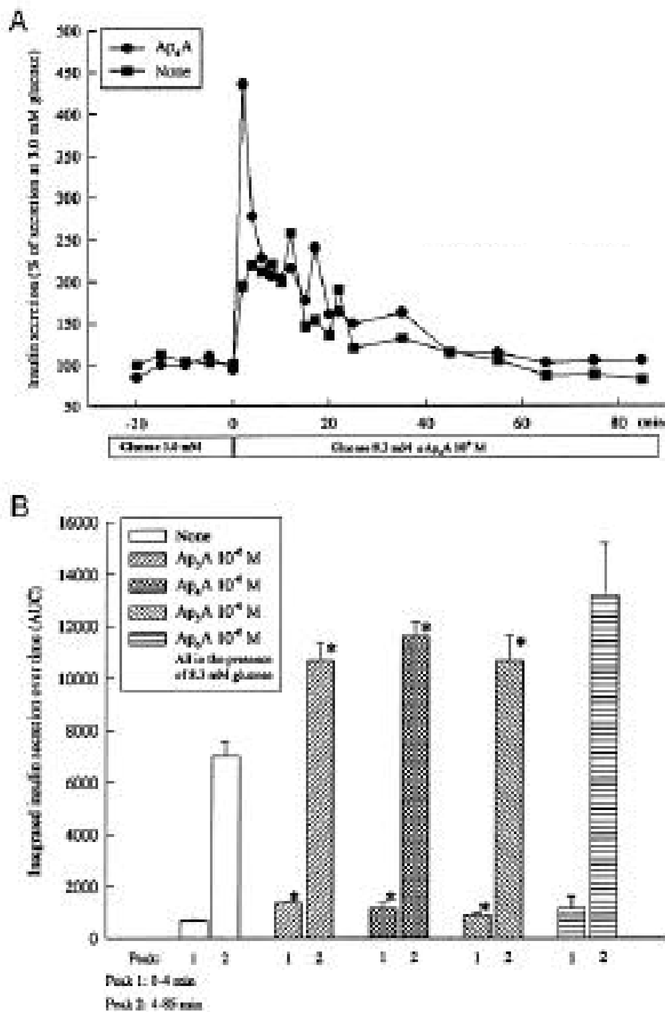

FIG. 6. Effect of Ap_4A on insulin release from perifused INS-1 cells. Cells on cover slips were perifused with 3.0 mmol/l glucose (control period) and then with 8.3 mmol/l glucose in the absence or presence of 1 mmol/l Ap_4A for 85 min. All data are normalized to 100% (control period). *A* shows a typical experiment with respect to Ap_4A. *B* summarizes the integrated area under the insulin release curve for all Ap_nAs. The mean ± SE of three to five separate experiments is shown. **P* < 0.05 vs. absence of Ap_4A.

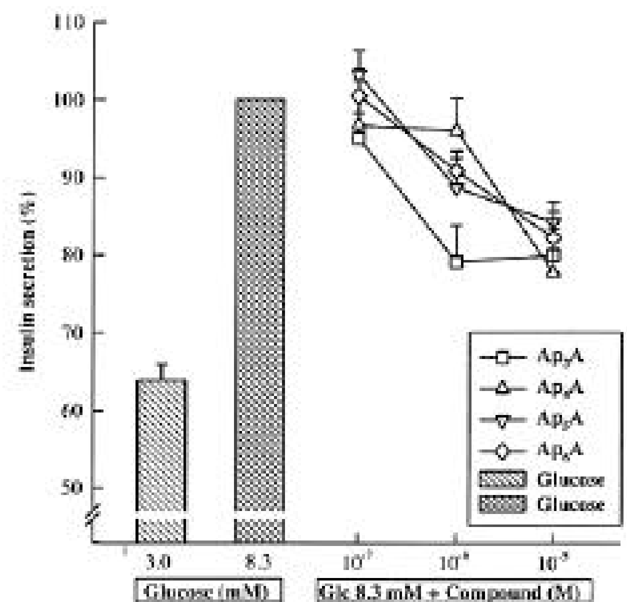

FIG. 7. Inhibition of insulin release from INS-1 cells by diadenosine polyphosphates. Cells were incubated for 90 min at 37°C in 1 ml KRH buffer containing 3.0 or 8.3 mmol/l glucose with or without increasing concentrations of diadenosine polyphosphates, as indicated in the figure. Results are expressed as percent secretion in the presence of 8.3 mmol/l glucose. Each value represents the mean ± SE of 9–15 experiments.

diadenosine polyphosphates in vivo. These high and regulated concentrations of diadenosine polyphosphates in blood imply an important function due to signaling via specific receptors on β-cells.

From the perifusion experiments (acute effects), an insulin stimulatory effect can be derived. This effect fits to the proposed intracellular effect as a second messenger for which a closure of K_{ATP} channels has been described (10) that should lead to insulin release. In contrast, when long-term effects (90 min) in static incubations were studied, an inhibition of insulin release was observed. An explanation for this discrepancy could be that diadenosine polyphosphates are labile compounds that are easily degraded.

More difficult to handle is the question with respect to the consequences of degradation. Whereas some low, albeit possibly significant, concentrations of inosine, ATP, ADP, and AMP show up in the incubation medium during a 90-min incubation with labeled Ap_4A, there is a significant rise in adenosine. Thus, the direct effect on insulin release (stimulation) is mainly due to an effect of intact Ap_4A; later effects (after 30 min) on insulin release during a static incubation >90 min (inhibitory effects) may account for the degradation compound adenosine, which is well known for its insulin-inhibiting effect (24). The measured binding is due to Ap_4A and not adenosine; this is because binding was saturated within 30 min, although the degradation product adenosine is still produced.

The question is whether Ap_4A is only synthesized within the cells or is accumulated from extracellular sources. In fact, it is clearly shown that high amounts of [^{3}H]-labeled compounds derived from labeled [^{3}H]Ap_4A are taken up by the insulin-secreting cell line INS-1. The uptake is half-maximal within 120 min, is efficient (up to 40% of the compound), and is saturable. This uptake is not restricted to or unique for INS-1 cells, since other cells such as HL-60 or K-562 show the same behavior in qualitative terms. The fact that lowering the temperature or adding cyanide impairs the uptake dramatically is compatible with the view that an active process is involved in the uptake for which an intact metabolism of cells is a prerequisite. The uptake of tritiateded compounds by INS-1 cells was hampered as well by concanavalin A, which is known to impair receptor internalization. This implies that a possible membrane-bound receptor is present on the surface of these cells and is being internalized. From the data (Fig. 9*B*), it cannot be decided whether the amounts of [^{3}H]Ap_4A are highly accumulated as intact compounds being rapidly degraded within the cells or whether uptake of already degraded products takes place.

The answer to the question of which type of receptor on INS-1 cells Ap_4A binds to is not easy. The receptor does not clearly discriminate between various diadenosine polyphosphates. Therefore, a specific diadenosine polyphosphate

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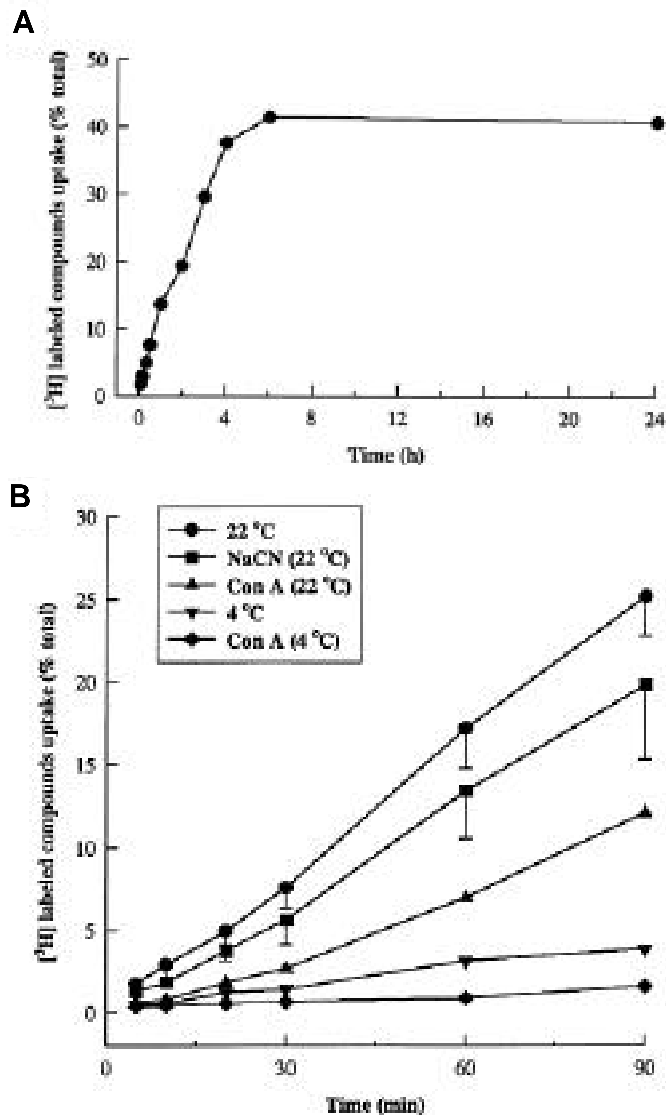

FIG. 8. Time course of [^{3}H]Ap$_4$A uptake by INS-1 cells. *A*: Cells were incubated for up to 24 h at 22°C with 300 µl KRH buffer containing 5.6 mmol/l glucose and 31 nmol/l [^{3}H]Ap$_4$A. *B*: Cells were preincubated for 45 min at 37°C in 270 µl buffer containing 5.6 mmol/l glucose with or without 2 mmol/l NaCN or 0.25 mg/ml concanavalin A. Cells were then incubated for up to 90 min with 31 nmol/l [^{3}H]Ap$_4$A at 22 and 4°C, respectively, as indicated in the figure. Results are expressed as percent uptake of total radioactivity. Each value represents the mean ± SE of two or three experiments.

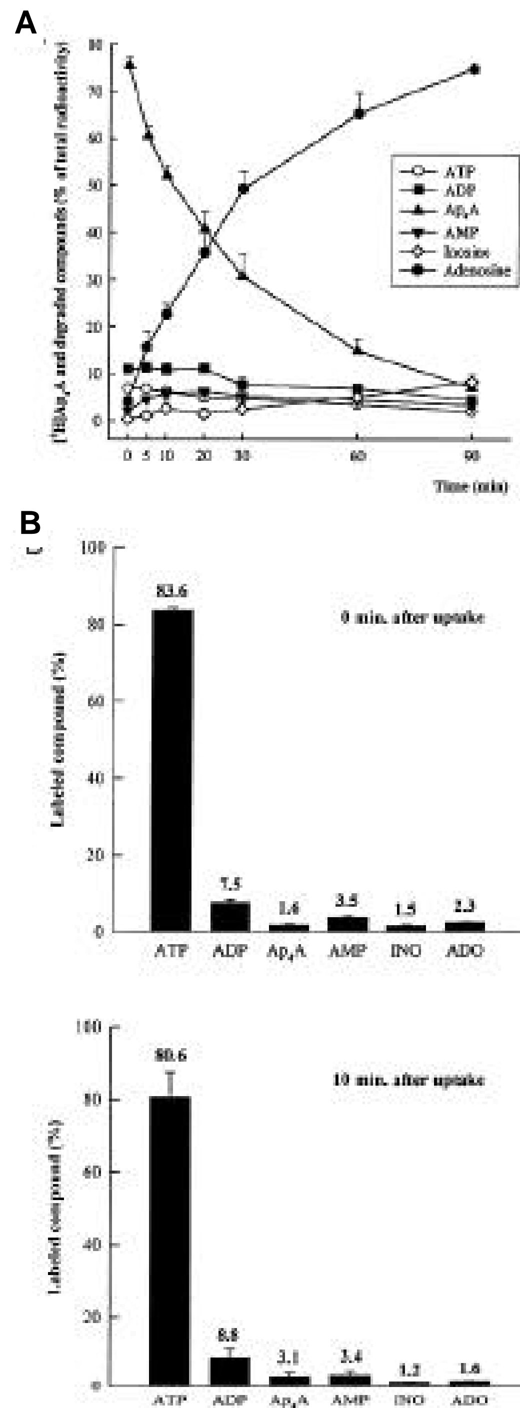

FIG. 9. Degradation of [^{3}H]Ap$_4$A. Degradation was investigated in the incubation medium (*A*) and within INS-1 cells after 20 min of uptake (*B*). *A*: Cells were incubated with 310 nmol/l [^{3}H]Ap$_4$A in KRH medium (5.6 mmol/l glucose). At the indicated time points, samples from the medium were chromatographed as described in METHODS. The mean ± SE of three separate experiments is shown. *B*: Cells were incubated for 20 min with 1.56 µmol/l [^{3}H]Ap$_4$A and degraded products within cells were determined at 0 and 10 min after uptake. The mean ± SE of three separate experiments is shown.

receptor could be involved. Binding sites for diadenosine polyphosphates have been shown in various cells, such as heart (5,6), brain (7,8), and liver (9). In some cases, binding sites resemble adenosine receptors (heart [6]) or either adenosine or P$_2$ receptors (guinea pig vas deferens [25], follicular oocytes [26], heart [27], kidney [28]). In contrast to [^{3}H]Ap$_4$A displacement studies in these cell types, e.g., rat liver cell plasma membrane preparations (9) or mouse brain membrane homogenates (7), where adenosine did not compete with [^{3}H]Ap$_4$A binding, adenosine was an effective inhibitor of [^{3}H]Ap$_4$A binding in INS-1 cells. More detailed studies are necessary to show whether the Ap$_n$A binding site in INS-1 cells is an adenosine binding site as it was shown to be for heart (6).

In our study, purinergic receptor agonists were able to inhibit [^{3}H]Ap$_4$A binding to membrane preparations of INS-1 cells, as well. In follicular oocytes Pintor et al. (26) showed that inward currents were mediated by a suramin-sensitive P$_2$

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purinoceptor (possibly P_{2U} receptor) with a potency order of Ap_4A >ATP > Ap_3A > Ap_5A and that outward currents were mediated by a theophylline-sensitive P_1 purinoceptor with a potency order of Ap_2A >ATP > Ap_4A = Ap_5A = Ap_6A >Ap_3A. This rank order was not obvious in INS-1 cells, and because the binding site in INS-1 cells does not discriminate between the diadenosine polyphosphates with high selectivity, it is unlikely that a P_2 receptor, in addition to a diadenosine polyphosphate receptor, is involved.

A receptor that is specific only for adenine dinucleotides and insensitive to ATP, termed P_4 (29–31), was characterized in rat brain synaptosomes and in deermouse brain (32). The characteristics of the receptor found in INS-1 cells are not in accordance with the characteristics of this P_4 receptor (29) because in INS-1 cells, ATP interacts with the receptor, while a P_4 receptor should be insensitive to ATP.

Pintor et al. (33) showed the existence of a P_2 receptor in rat brain synaptic terminals that exhibits specificity for diadenosine polyphosphates and possesses a pharmacological profile inconsistent with any known purinoceptor subtype: Ap_4A >ADP-βS > 5′-adenylyl-imidodiphosphate (AMP-PNP) > α,β-meATP. For this receptor termed P_{2D}, a broad selectivity for dinucleotides can be expected that is in accordance with our displacement data in INS-1 cells; however, their displacement rank order is different from ours.

Interactions of diadenosine polyphosphates with the P_{2X} receptor subtype have been shown for fibroblasts (34), urinary bladder (35), mesangial cells (36), and vas deferens (37). The involvement of a P_{2X} receptor cannot be excluded for INS-1 cells, since PPADS, which is selective for P_{2X} receptors, competed with [^{3}H]Ap_4A binding in our experiments.

In conclusion, our data clearly indicate the presence of specific receptors for diadenosine polyphosphates in insulin-secreting cells mediating increase of insulin release. Degradation products, such as adenosine, demonstrated in the extracellular and intracellular space of cells may account for the inhibitory effect on glucose-induced insulin release during long-term experiments. The type of receptor is not clear but may be a specific diadenosine polyphosphate receptor or a P_{2X} receptor. The exact role of diadenosine polyphosphates in physiological and pathophysiological terms is worthy of being evaluated next.

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Author Queries (please see Q in margin and underlined text)

No running head was supplied. If this one is not acceptable, please provide running head.

Q1: **Please provide first names for both authors.**
Q2: **Change to “stimulated by the diadenosine polyphosphates” OK? If not, please reword for clarity.**
Q3: Change to sentence beginning “Our studies indicate…” OK? If not, please reword for clarity.
Q4: **<<Au: Addition of “islet” correct here?>**
Q5: <<Au: Changes to sentence beginning “The pellet was obtained…” OK? If not, please reword for clarity.> Q6: <<Au: “cells were perifused with” OK for “it was switched to the”? If not, please reword for clarity.>
Q7: <<Au: “after this” as meant?>
Q8: <<Au: OK to add “carried out”?>
Q9: <<Au: “islet” correct for “cell” here?>
Q10: <<Au: “and” OK for “as well as” here?
Q11: ><<Au: “closely similar” OK for “rather identical”? Would you rather say “almost identical”? Please advise.>
Q12: <<Au: “and” OK for “whereas” here?>
Q13: **<<Au: Fig. 4 legend: Please indicate more clearly what the difference between *A* and *B* is.>**
Q14: Changes to sentence beginning “The concentration for one-half…” OK? If not, please reword for clarity.>
Q15: <<Au: “It is already known that” OK for “Already”?>
Q16: **<<Au: “area under the insulin release curve” OK for insulin release AUC”? If not, please spell out AUC.><<Au: You use “Ap_nA” here and “Ap_xA” in the text. Would you like to choose one? Please advise.> <<Au: It is not clear whether the inset in Fig. 6*A* is a repeat of part of the data in *B*. If so, the inset should be removed. If not, please provide a clearer explanation of the inset.>**
Q17: <<Au: “close to” OK for “rather”?>
Q18: <<Au: “which is” as meant?>
Q19: <<Au: Changes to sentence beginning “This rank order was not….” OK? If not, please reword for clarity.>

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