Synthetic, Nondegradable Diadenosine Polyphosphates and Diinosine Polyphosphates: Their Effects on Insulin-Secreting Cells and Cultured Vascular Smooth Muscle Cells

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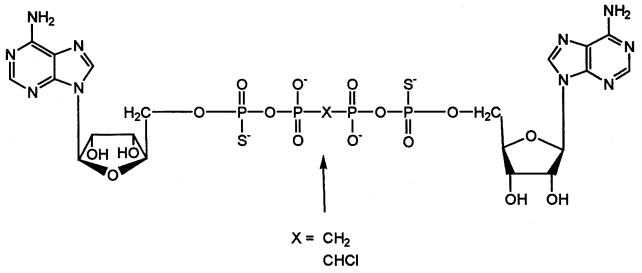
Received October 22, 2001

Diadenosine polyphosphates show a dissimilarity between their effects in static and perifusion experiments with respect to insulin release that may be due to degradation of the compounds. The aim was to investigate two nondegradable compounds of bisphosphorothioates containing a methylene or chloromethylene group (namely, diadenosine 5′,5‴-(P^1,P^4-dithio-P^2,P^3-methylene)tetraphosphate and diadenosine 5′,5‴-(P^1,P^4-dithio-P^2,P^3-chloromethylene)tetraphosphate), as mixtures of three or four diastereomers. Owing to their modified structures, these compounds are resistant to degradation (ectophosphodiesterases, diphosphohydrolases, and phosphorylases). Both compounds tested were minimally degraded (2%) even after 16 h when incubated with insulin-secreting (INS-1) cells. Additionally, diinosine polyphosphates (Ip_5I and Ip_6I), putative antagonists of diadenosine polyphosphates, were tested. By use of [^{3}H]Ap_4A, saturable binding sites for both diadenosine polyphosphate analogues were found in INS-1 cells, 3T3 preadipocyte cells, and vascular smooth muscle cells (VSMC) and for both Ip_5I and Ip_6I in INS-1 cells. The synthesized diadenosine polyphosphate analogues have the same affinity as Ap_4A, whereas Ip_5I and Ip_6I inhibit binding at higher concentrations (10−100 μM). Insulin release was investigated in static experiments over 90 min in INS-1 cells. Insulin release was inhibited dose-dependently by both of the diadenosine polyphosphate analogues to the same degree as by Ap_4A. The glucose-induced insulin release curve was not shifted to the right. Both compounds inhibit insulin release only at high (insulin stimulatory) glucose concentrations, e.g., 5.6 mM glucose. Ip_5I and Ip_6I antagonized Ap_5A-mediated inhibition of insulin release. [^{3}H]Thymidine incorporation into VSMC was not influenced by either synthetic diadenosine polyphosphate analogue, indicating that Ap_4A does not act by itself in this case but (active) degradation products mediate the effect. The data indicate the following. (1) Since nondegradable compounds inhibit insulin release as well as Ap_4A, it is Ap_4A itself and not any of its degradation products that induces this effect. (2) Diadenosine polyphosphate effects on cell proliferation are mediated via a degradation product in contrast to their effect on insulin release. (3) Ip_5I and Ip_6I act like antagonists. Both synthetic analogues and diinosine polyphosphates are valuable tools for diabetes research.

Introduction

Diadenosine polyphosphates (Ap_3A, Ap_4A (Figure 1), Ap_5A, and Ap_6A) belong to a group of ubiquitous compounds formed by two adenosine molecules bridged by three to six phosphates. They are present/stored in, for example, 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[1,2] or even to be important for development of essential hypertension.[3]

Binding sites for diadenosine polyphosphates have already been shown in various cells such as heart cells,[4] brain cells,[5,6] liver cells,[7] insulin-secreting cells,[8] and rat pancreatic islets.[8] In some cases, binding sites resemble adenosine receptors[9] (heart) or either adenosine or P_2 receptors (guinea pig vas deferens,[10] follicular oocytes,[11] heart,[12] kidney,[13] and insulin-secreting (INS-1) cells[8]).



Ap_4A: X = O; S = O

Figure 1. Structure of two stable analogues of diadenosine tetraphosphate (Ap_4A, P^1,P^4-bis(5′-adenosyl)tetraphosphate): Ap(s)pCH_2pp(s)A (diastereomeric mixture), diadenosine 5′,5‴-(P^1,P^4-dithio-P^2,P^3-methylene)tetraphosphate; Ap(s)pCHClpp(s)A (diastereomeric mixture), diadenosine 5′,5‴-(P^1,P^4-dithio-P^2,P^3-chloromethylene)tetraphosphate.

The intracellular presence of the diadenosine polyphosphates Ap_3A and Ap_4A was recently shown for rat pancreatic islets.[14] A role as second messenger was

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10.1021/jm011070z CCC: $25.00
Published on Web 03/18/2003

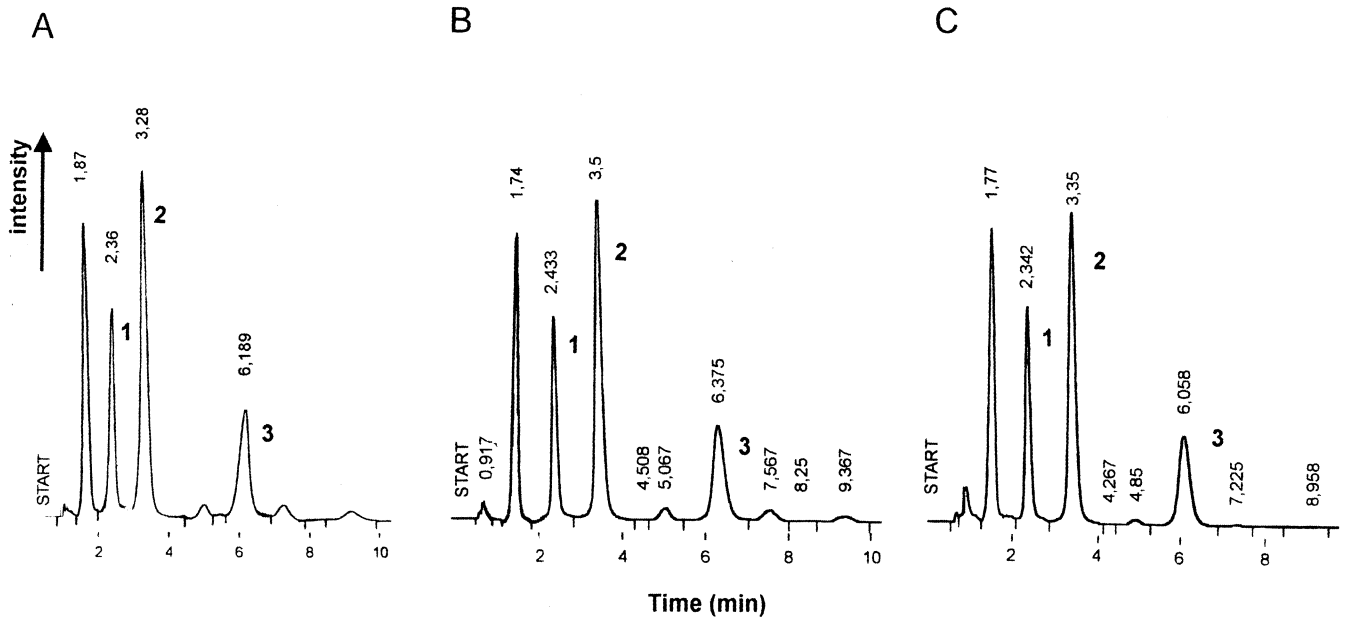

Figure 2. Purity and degradation of Ap(s)pCH$_2$pp(s)A tested by HPLC (Lichrosorb, 5 μm; solvent, KH_2PO_4 100 mM/9% methanol, pH 6.3; velocity, 1.5 mL/min; detector, UV 260 nm). The compound was incubated for 0 h (A), 1 h (B), and 16 h (C) in the presence of crude INS-1 cell homogenates. The peak areas of AMP running in front were used as an internal standard to correct the peak area values of the diastereomers. **1** = (*Sp*,*Sp*); **2** = (*Rp*,*Sp*); **3** = (*Rp*,*Rp*).

suggested because their concentration increased with increasing glucose concentrations and because they are effective inhibitors of the K_{ATP} channels using excised membrane patches.[14] According to investigations of INS-1 cells, it was speculated that on one hand Ap_4A stimulates insulin release by itself or by being metabolized to an intracellular degradation product such as ATP (closure of ATP-dependent K^+ channels). On the other hand, it was suggested that Ap_4A inhibits insulin release by the inhibitory degradation product adenosine. Both assumptions have been demonstrated under specific conditions. One reason for the controversial results may be that highly degradable diadenosine polyphosphates were used until now. The question was not addressed whether stable (nondegradable) and modified compounds exert the same or diverging effects. The aim of the present study was to investigate whether synthetic diadenosine polyphosphate analogues and inosine polyphosphates act on insulin-secreting cells and other cells via specific receptors and whether they modulate insulin release of INS-1 cells and cell proliferation of vascular smooth muscle cells (VSMC). VSMC tone and proliferation is under the control of various compounds including diadenosine polyphosphates.[15,16] Potent antagonists for Ap_4A effects are useful for investigations[17] and were therefore included in one experiment. Our studies indicate that diadenosine polyphosphate analogues act via specific receptors and inhibit insulin release directly rather than depending on having been degraded outside the cell. However, its effect on cell proliferation is dependent on its degradation. Ip_nIs are antagonists of the Ap_5A effect on insulin release.

Results

Purity and Stability of Diadenosine Polyphosphate Analogues during Cell Contact. For testing the purity and stability of both synthetic compounds Ap(s)pCH$_2$pp(s)A and Ap(s)pCHClpp(s)A (50 μL of a 1 mM solution), INS-1 cells were incubated for either 1 or 16 h at 37 °C (Figures 2 and 3). At the end of either incubation, an amount of 50 μL of 10 μM AMP was added as an internal standard. Three and four stereoisomers of the synthetic compounds were separated, respectively; the AMP standard was running in front. The peak areas were corrected for the percent recovery of the AMP peak. The original compound Ap(s)pCHClpp(s)A was 95.9% pure. After 1 and 16 h of incubation, the combined areas were 95.2% and 94.9%. The ratios for the four peaks were 1:0.7953:1.1190:1.0120 (0 h) and 1:0.8147:1.1632:1.0388 (1 h) and 1:0.7904:1.0669:0.9585 (16 h). We therefore considered the substance Ap(s)pCHClpp(s)A sufficiently pure and stable. The corresponding values for Ap(s)pCH$_2$pp(s)A were 92.05, 88.82, and 83.34. The ratios for the three peaks were 1:2.2138:1.1210, 1:2.2028:1.0986, and 1:1.9787:0.9580. The substance was marginally less pure. The same results were obtained when other cells, i.e., 3T3 preadipocyte cells, were used (data not shown). The elution profile of thiophosphate analogues of nucleotides invariably consists of *Sp* stereoisomers eluting before *Rp* stereoisomers, which is generally observed. The existence of three and four stereoisomers respectively for the abovementioned compounds is as expected. The existence of two thiophosphate centers in CH$_2$ compound leads to three diastereomers, namely, (*Rp*,*Rp*), (*Rp*, *Sp*), and (*Sp*,*Sp*), and these are present in the approximate ratio of 1:2:1. However, in the Ap(s)pCHClpp(s)A compound the stereochemical character of the central CHCl group depends on the stereochemistry of its two phosphorus ligands. It is prochiral when the two phosphorus ligands are stereochemically equivalent (*Rp*,*Rp* and *Sp*,*Sp*) but becomes stereogenic when the two phosphorus ligands are nonequivalent (*Rp*,*Sp*) (see Abbreviations). The resulting four diastereoisomers are formed in approximately 1:1:1:1 ratio. In practice and in accord with general observations that *Rp* α-phosphorothioate nu-

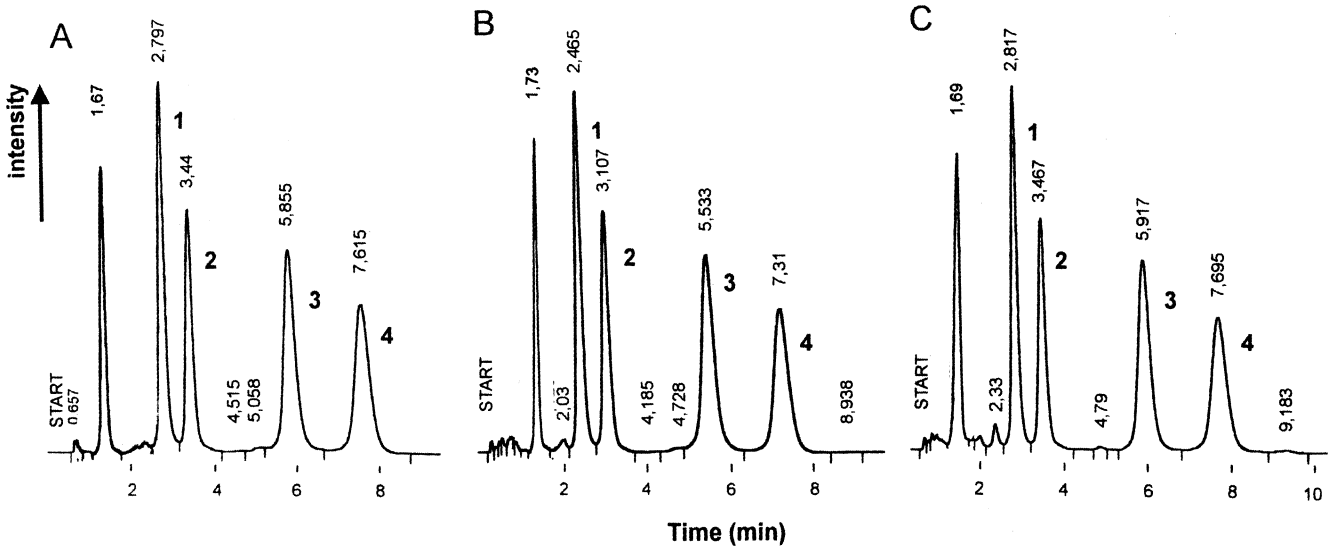

Figure 3. Purity and degradation of Ap(s)pCHClpp(s)A tested by HPLC (Lichrosorb, 5 μm; solvent, KH_2PO_4 100 mM/9% methanol, pH 6.3; velocity, 1.5 mL/min; detector, UV 260 nm). The compound was incubated for 0 h (A), 1 h (B), and 16 h (C) in the presence of crude INS-1 cell homogenates. The peak areas of AMP running in front were used as an internal standard to correct the peak area values of the diastereomers. **1** = (*Sp*,*S*′p); **2** and **3** = (*Rp*,*S*′p) and (*R*′p,*Sp*). Note that the assignment of **2** and **3** is arbitrary because no enzyme degradation can discriminate these diastereomers because of prochirality at the CHCl center. **4** = (*Rp*,*Rp*).

Table 1. [^{3}H]Ap_4A Degradation in the Presence of Ap_4A, Ap(s)pCH_2pp(s)A, Ap(s)pCHClpp(s)A, and INS-1 Cell Membranes[a]

addition	radioactive compound				
	Ap_4A	ATP	ADP	AMP	adenosine
control	95.3	0.82	1.97	0.47	0.61
none	52.4	8.4	11.3	5.3	22.4
Ap_4A	70.0	4.0	7.8	0.8	15.1
Ap(s)pCH_2pp(s)A	54.9	8.2	9.3		19.4
Ap(s)pCHClpp(s)A	61.3		8.8		14.3

[a] INS-1 cell membranes were incubated for 10 min at 37 °C in 90 μL of KRH buffer containing 0.31 μM [^{3}H]Ap_4A and either 30 μM Ap_4A, Ap(s)pCH_2pp(s)A, or Ap(s)pCHClpp(s)A. Samples from the medium were chromatographed by TLC as described in the Experimental Section. "Control" means [^{3}H]Ap_4A without incubation. "None" means no addition. Results are expressed as the percent of compound radioactivity in the presence of added substance. Each value represents the mean ± SE of two separate experiments.

cleosides elute before their *S*p stereoisomers, (*R*p,*R*p) eluted before the (*R*p,*S*p) and (*S*p,*R*p) isomers while the (*S*p,*S*p) stereoisomer eluted last. We are not able to define the stereochemistry of isomers **2** and **3** by the elution pattern.

Degradation Experiments Using [^{3}H]Ap_4A (Interaction with Ap_4A). Since the compounds themselves were hardly degraded, the question arose as to whether they may act indirectly by inhibiting the degradation of Ap_4A. This information is necessary for further in vivo experiments.

Degradation of [^{3}H]Ap_4A was determined in the incubation medium of INS-1 cell membranes. Table 1 shows the effect of [^{3}H]Ap_4A degradation over 10 min in the medium while incubating INS-1 cell membranes. [^{3}H]Ap_4A was degraded with a half-life of about 12 min (data not shown). The major degradation product was adenosine (Table 1). The degradation was slightly diminished by either diastereomeric mixture, indicating that only negligible amounts of their in vivo effects may result from increasing the effect of endogenous physiological Ap_4A.

Table 2. [^{3}H]Ap_4A Degradation in Rat Plasma in the Presence of Ap(s)pCH_2pp(s)A and Ap(s)pCHClpp(s)A[a]

addition	radioactive compound				
	Ap_4A	ATP	ADP	AMP	adenosine
control	95.3	0.82	1.97	0.47	0.61
none (saline)	21.5	42.1	22.3	2.4	<1
Ap(s)pCH_2pp(s)A	31.6	37.6	27.4	1.98	<1
Ap(s)pCHClpp(s)A	26.5	40.5	30.6	2.31	<1

[a] An amount of 40 μL of heparinized rat plasma was incubated with 10 nM [^{3}H]Ap_4A for 15 min in the presence of 3 μL of saline or 3 μL of a 1 mM solution of Ap(s)pCH_2pp(s)A or Ap(s)pCHClpp(s)A. "Control" means [^{3}H]Ap_4A without incubation. "None" means addition of saline. Results are expressed as the percent of compound radioactivity in the presence of added substance. Each value represents the mean ± SE of two separate experiments.

[^{3}H]Ap_4A degradation in plasma was tested next. Heparinized rat plasma was used to identify the stability of Ap_4A in the presence of both Ap(s)pCH_2pp(s)A and Ap(s)pCHClpp(s)A. The degradation half-life was about 22 min (data not shown). After 15 min, almost no degradation inhibition of [^{3}H]Ap4A in plasma by either compound was detected (Table 2).

Binding/Inhibition Experiments Using INS-1, 3T3 Cells, and VSMC. Binding experiments with [^{3}H]Ap_4A were performed using a particulate fraction of INS-1, 3T3 cells, or VSMC. Incubation was terminated under steady-state conditions (30, 10, or 5 min, respectively) and was performed at 22 °C. Nonspecific binding determined in the presence of 0.48 mM unlabeled Ap_4A was 20% of added radioactivity. Ap_4A, ATP, Ap(s)pCH_2pp(s)A, and Ap(s)pCHClpp(s)A were able to inhibit [^{3}H]Ap_4A binding to INS-1 cells, 3T3 cells, and VSMC (Figure 4). Inhibition of [^{3}H]Ap_4A binding was sigmoidal in either case and was essentially complete over 5 orders of magnitude (at least 0.01 μM to 1 mM). The rank order of potency showed that both analogues were identical or slightly more potent (with respect to 3T3 cells). The IC_{50} values calculated after log−log transformation of displacement curves in 3T3

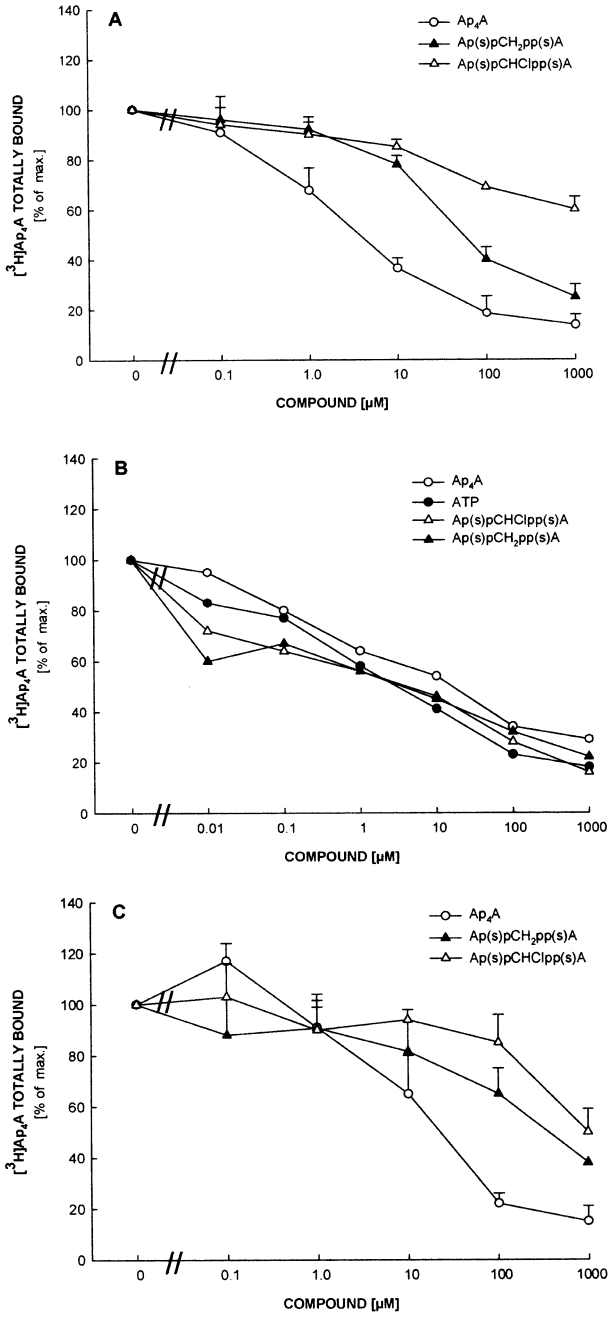

Figure 4. Inhibition of [^{3}H]Ap$_4$A binding to a particulate fraction of INS-1, 3T3, and VSMC cells by various diadenosine polyphosphates and stable Ap$_4$A analogues. (A) A particulate fraction of INS-1 cells was incubated for 30 min at 22 °C in 90 μL of KRH buffer containing 5.6 mM glucose, 104 nM [^{3}H]Ap$_4$A, and increasing concentrations of various diadenosine polyphosphates. (B) 3T3 cells were incubated for 10 min, and (C) VSMC cells were incubated for 5 min. Results are expressed as the percent of maximum totally bound radioactivity. Each value represents the mean ± SEM of three to seven separate experiments.

cells were 3.1 and 3.8 μM (analogues) compared to 17 μM (Ap$_4$A).

Insulin Secretion. The biological effect (insulin secretion) of either analogue was tested in a static incubation system using INS-1 cells. Ap$_4$A, Ap(s)pCH$_2$-pp(s)A, and Ap(s)pCHClpp(s)A dose-dependently decreased insulin release in the presence of 5.6 mM glucose (Figure 5). At the highest concentration tested

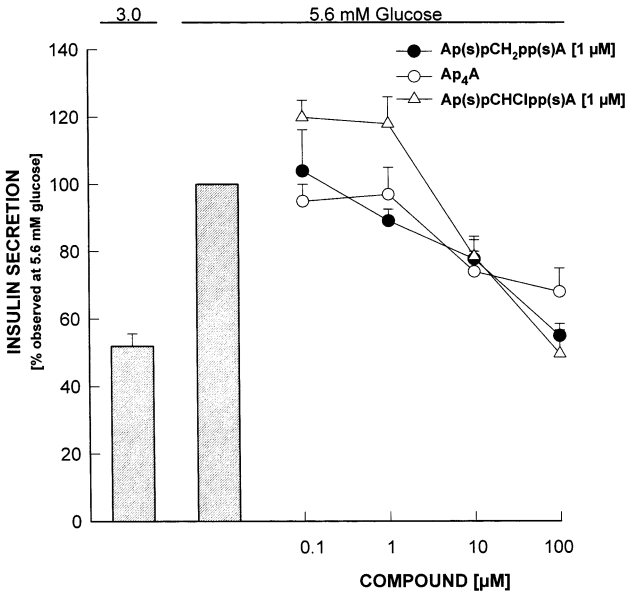

Figure 5. Inhibition of insulin release from INS-1 cells by Ap(s)pCH$_2$pp(s)A and Ap(s)pCHClpp(s)A. Cells were incubated for 90 min at 37 °C in 1 mL of KRH buffer containing 3.0 or 5.6 mM glucose with or without increasing concentrations of the compounds, as indicated in the figure. Results are expressed as the percent secretion in the presence of 5.6 mM glucose. Each value represents the mean ± SEM of three separate experiments.

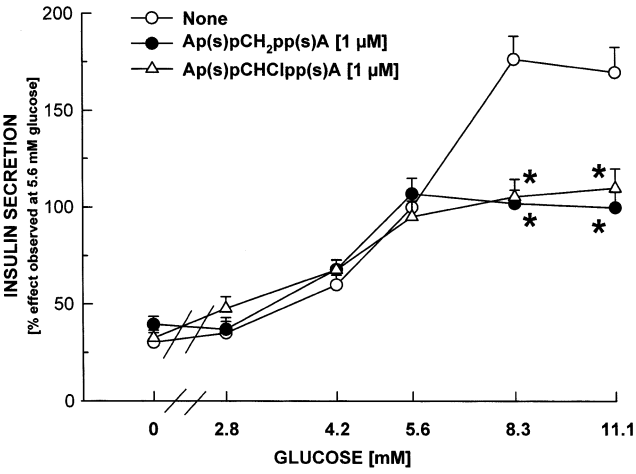

Figure 6. Modulation of the effect of Ap(s)pCH$_2$pp(s)A and Ap(s)pCHClpp(s)A on insulin response curves to glucose by Ap(s)pCH$_2$pp(s)A and Ap(s)pCHClpp(s)A. Cells were incubated for 90 min at 37 °C in 1 mL of KRH buffer containing various glucose concentrations in the absence and presence of 1 μM Ap(s)pCH$_2$pp(s)A and Ap(s)pCHClpp(s)A. Results are expressed as the percent secretion in the presence of 5.6 mM glucose. Each value represents the mean ± SEM of three separate experiments. The asterisk (*) represents $p < 0.05$ vs control.

(100 μM), the compounds reduced the glucose-induced insulin secretion to 77.8%, 51.3%, and 55.7%, respectively. Thus, both compounds mimic the effect of Ap$_4$A.

Next it was tested whether the sensitivity of the cells was reduced by both compounds, i.e., whether there was a rightward shift of the glucose-induced insulin response curve. As is shown in Figure 6, there was no rightward shift but an inhibition of insulin secretion only at higher (stimulatory) glucose concentrations mediated by either compound in rather the same way.

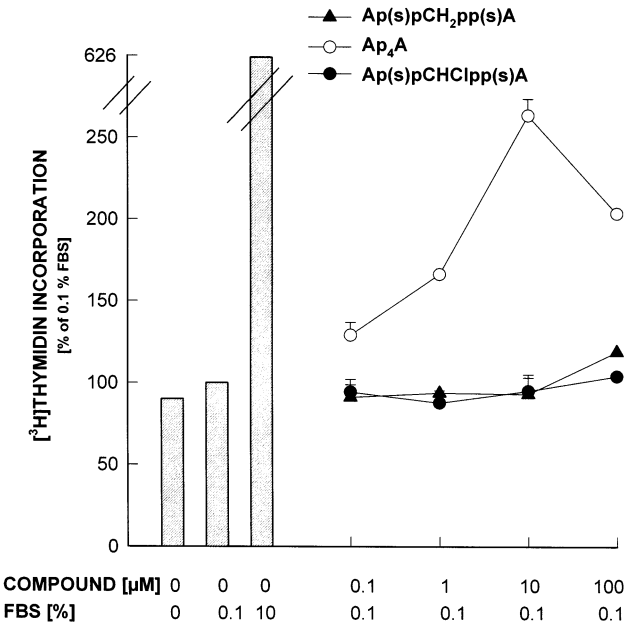

Figure 7. Effects of Ap(s)pCH$_2$pp(s)A and Ap(s)pCHClpp(s)A on [^{3}H]thymidine incorporation into VSMC. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2/95% air in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS). After 72 h of culture, the medium was removed and cells were incubated for 48 h in serum-free medium containing various concentrations of Ap(s)pCH$_2$pp(s)A and Ap(s)pCHClpp(s)A. An amount of 5 μCi [^{3}H]thymidine/well was added, and the incubation was carried out for another 5 h. The percentage change of [^{3}H]thymidine incorporation compared to untreated cells (control) was determined. Data are normalized to 100 (control, absence of either compound) in the presence of 0.1% FBS. Each values represents the mean ± SEM of five independent experiments. The asterisk (∗) represents $p < 0.05$ vs control in each group.

Since diadenosine polyphosphates were able to inhibit specific [^{3}H]Ap$_4$A binding to vascular smooth muscle cells (VSMC)[18] and mediate VSMC proliferation,[19] the aim was to investigate the effect of Ap$_4$A, Ap(s)pCH$_2$-pp(s)A, and Ap(s)pCHClpp(s)A on [^{3}H]thymidine incorporation. Figure 7 shows that both compounds have no effect in contrast to Ap$_4$A. Controls were performed using a high concentration of fetal bovine serum (FBS) (10%).

Diinosine Polyphosphates. Binding experiments with [^{3}H]Ap$_4$A were performed using a particulate fraction of INS-1 cells. Incubation was terminated under steady-state conditions (30 min) and performed at 22 °C. Nonspecific binding determined in the presence of 0.48 mM unlabeled Ap$_4$A was 20% of the added radioactivity. Ap$_4$A, Ap$_5$A, and at much higher concentrations Ip$_5$I and Ip$_6$I were able to inhibit [^{3}H]Ap$_4$A binding (Figure 8). No full inhibition by both the diinosine polyphosphates was established, since higher concentrations could not be used.

The biological effect (insulin release) of either diinosine polyphosphate was tested in a static incubation system using INS-1 cells. A sample of 10 μM Ap$_5$A inhibited the glucose-modulated insulin release (Figure 9). This inhibition was reversed by Ip$_5$I in a concentration-dependent manner. This reversion was neither influenced by the Ca channel blocker verapamil (50 μM) nor influenced by the potassium channel opener diazoxide (100 μM) (Figure 10).

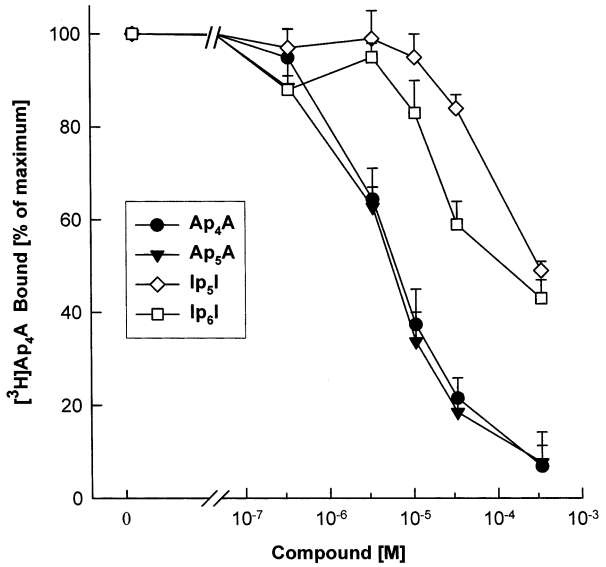

Figure 8. Inhibition of [^{3}H]Ap$_4$A binding to a particulate fraction of INS-1 cells by two diadenosine polyphosphates and two inosine polyphosphates. A particulate fraction of INS-1 cells were incubated for 30 min at 22 °C in 90 μL of KRH buffer containing 5.6 mM glucose, 104 nM [^{3}H]Ap$_4$A, and increasing concentrations of Ap$_4$A, Ap$_5$A, Ip$_5$I, and Ip$_6$I. Results are expressed as the percent of maximum totally bound radioactivity. Each value represents the mean ± SEM of three to seven separate experiments.

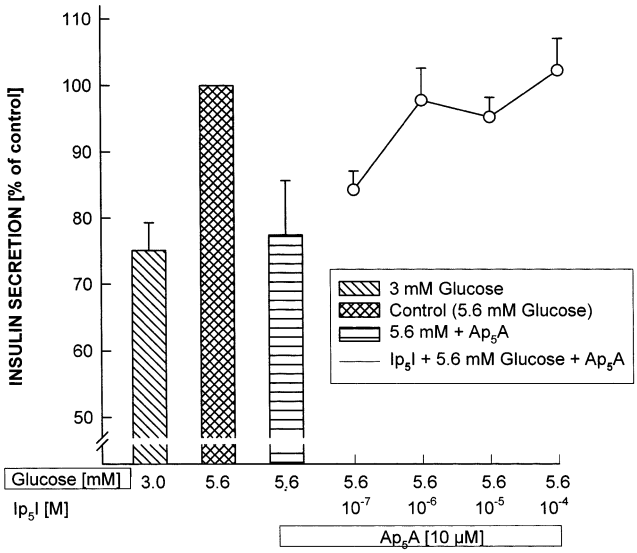

Figure 9. Modulation of the effect of Ap$_5$A on insulin response curves by Ip$_5$I. Cells were incubated for 90 min at 37 °C in 1 mL of KRH buffer containing 3.0 or 5.6 mM glucose with or without 10 μM Ap$_5$A plus increasing concentrations of Ip$_5$I. Results are expressed as the percent insulin secretion in the presence of 5.6 mM glucose. Each value represents the mean ± SEM of three separate experiments.

Discussion

In an earlier investigation,[8] it was not possible to discriminate whether the original compound Ap$_n$A or one of its degradation products such as ATP, ADP, AMP, adenosine, inosine, and others was responsible for the effects on insulin release, since the half-life of the compounds is 5−12 min. Diadenosine polyphosphates can easily be degraded by an asymmetrically acting NP$_4$N hydrolase (Ap$_4$A → ATP + AMP) or by a symmetrically acting NP$_4$N hydrolase (e.g., in *E. coli*) (Ap$_4$A

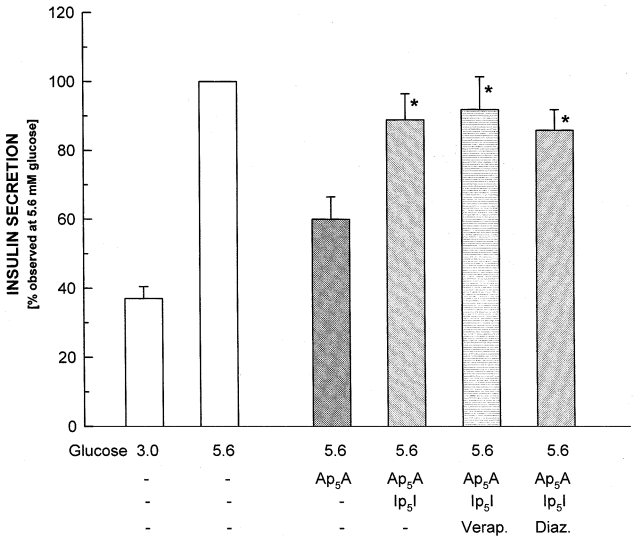

Figure 10. Effect of modulatory compounds (verapamil and diazoxide) on the Ip_5I-mediated reversal of the Ap_5A-mediated inhibition of insulin release from INS-1 cells. Cells were incubated for 90 min at 37 °C in 1 mL of KRH buffer containing 3.0 or 5.6 mM glucose with or without 10 μM Ap_5A plus 10 μM Ip_5I. In the Ip_5I experiments, either 50 μM verapamil or 100 μM diazoxide was included. Results are expressed as the percent insulin secretion in the presence of 5.6 mM glucose. Each value represents the mean ± SEM of six separate experiments. The asterisk (∗) represents $p < 0.05$ vs Ap_5A effect alone.

→ ADP + ADP) or modified by a NP_4N phosphorylase (Ap_4A → ATP + ADP) or degraded by phosphodiesterases (Ap_4A → ATP + AMP). These degradation processes make it impossible to evaluate the real effects of physiological diadenosine polyphosphates. It was therefore mandatory to confirm or contradict earlier results by using stable compounds and to include an antagonistic compound.

Our data show that the used compounds Ap(s)pCH_2pp(s)A and Ap(s)pCHClpp(s)A are rather pure and consist of the three and four expected stereoisomers, respectively, due to the nonstereoselective synthesis. The degradation is negligible, even when investigated in the incubation medium over 16 h. It is therefore most likely that the effects of the original diadenosine polyphosphate analogues and not that of a degradation product are observed. The data obtained in our system substantiate those earlier published[20] with respect to low degradation. The three diastereomers of Ap(s)pCH_2pp(s)A behaved as follows. The *S,S* form is resistant to degradation, and the *R,R* and *R,S* forms are degraded 40 times more slowly than Ap_4A.[23] All the diastereomers have been shown to be competitive inhibitors of the asymmetrically acting (5′-adenosyl)tetraphosphatase in the micromolar range.[20]

Degradation is not obvious even in plasma, making the compounds interesting for later in vivo investigations, and they do not act indirectly by influencing Ap_4A degradation, an endogenous compound. The effects of the synthesized compounds when investigated in vivo in the future are not due to the indirect effect of inhibiting an endogenous diadenosine polyphosphate like Ap_4A.

Binding/Inhibition of [^{3}H]Ap_4A Binding. Both compounds show an inhibition of binding of [^{3}H]Ap_4A to the Ap_4A binding site in INS-1, 3T3, and VSMC cells. As already demonstrated in other systems, diadenosine polyphosphates act via specific surface receptors.[5−8,18]

Biological Effects (Insulin Release, Cell Proliferation). Interestingly, both compounds reduce glucose-induced insulin release. The inhibition of insulin secretion by both compounds resembles what was shown for the unmodified physiological diadenosine polyphosphates,[8] indicating that this inhibition is not primarily mediated by a degradation product. Both compounds do not shift the response curve to the right. This can be interpreted in the way that there is no intracellular insensitivity to the glucose-mediated signaling in the insulin-secreting cell; there is only an inhibition of insulin release at distinct high glucose concentrations. The short-term increase in insulin release induced by Ap_4A as was observed recently[8] is therefore probably not an effect of the (nondegraded) compound itself but an early effect of intracellular ATP generated by this compound. Interestingly, Nakae et al.[21] showed that coronary effects of Ap_4A are caused at least indirectly by opening K_{ATP} channels. In fact, an opening of K_{ATP} channels would fit with the inhibition of insulin secretion observed in our studies.

The concentrations used in our experiments are in the physiological range. Other groups calculated high extracellular concentrations of Ap_4As. 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 1 μM could result.[22] Extracellular concentrations of 27 μM can be estimated for diadenosine polyphosphates after secretion from chromaffin cells.[23,24] The concentration in blood (resulting from storage in platelets) can be estimated to be 0.14−0.3 μM (recalculated from data of refs 25 and 26). The extracellular concentration was shown to be increased in vivo by oxidants or metabolic stress.[27] These concentrations are in the range of half-maximal inhibition of binding in our experiments, even for the synthetic compounds.

The data on insulin release are in contrast to another biological effect, i.e., the data on proliferation. In contrast to Ap_4A, the synthesized compounds show no effect, indicating that the proliferative effect of the physiological diadenosine polyphosphates is mediated by any of its degradation products. ATP is such a candidate.[16]

Diinosine Polyphosphates (Ip_5I, Ip_6I). Both Ip_5I and Ip_6I inhibit [^{3}H]Ap_4A binding to INS-1 cells. This effect is less prominent compared to Ap_4A and Ap_5A; i.e., the affinity of these compounds is lower than that of the endogenous diadenosine polyphosphates.

In Figure 9, the effects of Ip_5I on Ap_5A-modulated insulin release are shown. A 10 μM sample of Ap_5A reduces the glucose (5.6 mM) effect. This inhibitory effect is antagonized by Ip_5I. This inhibition is not directly mediated by an influence on Ca channels (verapamil experiment) or by K_{ATP} channels (diazoxide experiment). Ip_5I and Ip_6I were recently described as antagonists of the Ap_4A binding site by abolishing Ap_4A effects on Ca^{2+} responses in synaptosomes.[17] The postulated involved receptors were $P2X_1$ or $P2X_3$.[17] Diinosine polyphosphates are antagonists of the insulin

inhibitory effect of Ap_5A and therefore of interest in diabetes research.

In conclusion, our data indicate the presence of binding sites for both Ap_4A analogues (Ap(s)pCH_2pp(s)A and Ap(s)pCHClpp(s)A). This clarifies the situation because the compounds are stable over at least 16 h in the incubation medium. Interestingly, there is no major degradation of both compounds in rat blood. Thus, there is the first opportunity to investigate these compounds in long-term experiments in vivo. Since the analogues in vitro show a similar binding profile compared to physiological diadenosine polyphosphates, in vivo experiments may resemble the true effect of diadenosine polyphosphates because there are no perturbing effects of degradation products. The analogues do not inhibit nucleotidases. Therefore, in the event that these analogues were to be evaluated through in vivo experiments, the results would be caused solely by them rather than be complicated by degradation of Ap_4A. The effect on cell proliferation must be mediated by one or more of their degradation products because the stable analogues are ineffective. Diinosine polyphosphates are valuable antagonists of Ap_4A effects. All compounds tested are valuable tools for diabetes research.

Experimental Section

The diadenosine polyphosphates Ap_4A and ATP were from Sigma (Deisenhofen, Germany). [^{3}H]Ap_4A labeled in the adenosine part of the molecule (specific activity of 237 GBq/mmol) was purchased from Amersham (Braunschweig, 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) [Methyl-^{3}H]thymidine was from Amersham Pharmacia Biotech, Freiburg, Germany. The mixture of three diasteromers for diadenosine 5′,5‴-(P^1,P^4-dithio-P^2,P^3-methylene)tetraphosphate and the mixture of four diasteromers for its chloromethylene analogue were synthesized as already described[28] (Figure 1). The chemistry of preparation and the characteristics of the key Ap_4A analogues, especially Ap(s)pCHClpp(s)A, were published.[28] Ap(s)pCH_2pp(s)A is made in the same way. Diinosine polyphosphates were synthesized by enzymatic degradation of the corresponding diadenosine polyphosphates (e.g., Ap_5A to Ip_5I) by 5′-adenylic deaminase and purification by reverse-phase chromatography as described.[17] All other compounds (analytical grade) were from Baker (Griesheim, Germany) or from Merck (Darmstadt, Germany).

Cell Cultures. 1. 3T3 Cells. 3T3 preadipocyte cells were cultured in 75 cm^2 culture flasks (2×10^5 cells/20 mL) or 24-well culture plates (approximately 5×10^3 cells well^{-1} mL^{-1}) 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% (v/v) 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.

2. INS-1 Cells. INS-1 cells generously provided by Dr. C. B. Wollheim (Geneva, Switzerland) were grown in monolayer cultures (75 cm^2 culture flasks (5×10^6 cells/20 mL) or 24-well culture plates (1.5×10^5 cells well^{-1} mL^{-1}) in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), 2 mM glutamine, 1 mM pyruvate, 50 μM mercaptoethanol, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin and were cultured at 37 °C in humidified 5% CO_2/95% air.

3. VSMC (Vascular Smooth Muscle Cells). A primary culture of vascular smooth muscle cells was prepared essentially as described by Meyer-Lehnert and Schrier.[29] In brief, aortas and inferior vena cavas were removed from two to four female Sprague−Dawley rats under aseptic conditions and were placed in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. By use of a binocular microscope, the adventitia and outer media were stripped off. The vessels were opened by a longitudinal cut, and the intima was removed by scraping. The vessels were placed in fresh Dulbecco's modified Eagle's medium that included supplements and were minced. After incubation for 2 weeks, the tissues were disrupted and centrifuged gently, and the dissociated cells were then resuspended in Dulbecco's modified Eagle's medium that included supplements. The resulting suspension was then plated into 75 cm^2 flasks and cultured at 37 °C in humidified 5% CO_2/95% air until cells reached confluence (7−10 days). Vascular smooth muscle cells were passaged at least five times before use. Cell viability was tested by exclusion of trypan blue (>95%).

Purity/Stability of Ap(s)pCH_2pp(s)A and Ap(s)pCHClpp(s)A. The compounds were analyzed by HPLC using Hibar Lichrospher or Lichrosorb RP-18 columns (5 μm). The solvent was 100 mM KH_2PO_4, 9% methanol, Aqua bidest, pH 6.4. The injection volume was 20 μL, and the velocity was 1.5 mL/min. The compounds were detected at 260 nm. All diastereomers could be separated under these conditions. As generally observed, *R*p diastereomers eluted earlier than *S*p diastereomers.[30]

Binding/Inhibition Experiments. 1. 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 were homogenized with a Dounce homogenizer (Braun, Melsungen, Germany). The particulate fraction (2600*g*) was washed with KRH buffer and resuspended in KRH buffer containing 5.6 mM glucose (400 μL/flask). An amount of 30 μL of this suspension was incubated at 22 °C with 104 nM [^{3}H]Ap_4A with or without increasing concentrations of unlabeled Ap_4A, the diadenosine polyphosphate analogues, or Ip_nIs. The final volume was 90 μL. To determine the nonspecific binding, the incubation was performed in the presence of 0.48 mM unlabeled Ap_4A. The incubation was terminated after 30 min by cooling (4 °C), centrifuging at high speed, and washing twice with ice-cold KRH buffer. The pellet was lysed with 50 μL of 0.5% SDS and counted in a scintillation counter (TRI CARB 300 CD, Packard, Frankfurt, Germany) by using Quickscint 212, Zinsser Analytic (Frankfurt, Germany).

2. Modifications for 3T3 Cells. Incubation was performed for 15 min at room temperature while vortexing several times. Results are the mean values from at least three or two independent crude homogenate preparations in duplicate probes in each case.

3. Modifications for VSMC and INS-1 Cell Membrane Proteins. Half confluent cells (grown in 75 cm^2 culture flasks for 6 days) were washed with phosphate-buffered saline (PBS). Buffer 1 (50 mM Tris-HCl, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM benzamidine, 0.1 mM PMSF, 0.01% bacitracin, 0.002% soybean trypsin inhibitor, pH 7.4) was added, and cells were harvested by scraping. After spinning, cells were resuspended in buffer 1 and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 620*g* for 5 min at 4 °C. The pellet was discarded, and the supernatant was further centrifuged at 100000*g* for 1 h. The pellet was resuspended in buffer 1 to give a protein concentration of 1 mg/mL, kept on ice, and used immediately. An amount of 25 μL of this solution/well, with or without increasing concentrations of unlabeled Ap_4A, and the diadenosine polyphosphate analogues were incubated with [^{3}H]Ap_4A in a 96-well filter plate (Millipore) at 22 °C for 10 min. The incubation was stopped by washing twice with ice-cold PBS using a Millipore vacuum tool. The dried filters with the labeled compound were punched into scintillation vials and counted in a scintillation counter.

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 mM 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 [^{3}H]Ap$_4$A Degradation Products. To determine whether the synthetic compounds inhibit degradation of [^{3}H]Ap$_4$A, the original radioactively labeled compound and its degradation products were determined using thin-layer chromatography. Membranes from half confluent INS-1 cells were incubated for 10 min in KRH buffer containing 5.6 mM glucose and 0.31 μM [^{3}H]Ap$_4$A. An aliquot was taken from the incubation medium, mixed with the appropriate unlabeled standards (ATP, ADP, Ap$_4$A, AMP, inosine, and adenosine), transferred onto TLC sheets (aluminum sheets precoated with silica gel, Merck, Darmstadt, Germany), and developed for 2 h in dioxane/concentrated ammonia/water (6:1:4). After 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 the following: Ap$_4$A, 83.5%; ADP, 7.2%; adenosine, 5.8%; ATP, 3.6%. The same type of experiment was performed using rat plasma instead of INS-1 cell membranes.

[^{3}H]Thymidine Incorporation.[15] VSMC cells were grown in 24-well plates for 3 days until they were subconfluent (complete medium including 10% FBS). From the fourth day, they were incubated for 72 h in a serum-free medium to keep them quiescent. Then the test compounds (0.1−100 μM) dissolved in a medium containing 0.1% FBS were added. After 19 h, 0.5 μCi [^{3}H]thymidine was added. The incubation was performed for 5 h. Thereafter, cells were transferred on ice and sucked from the medium, and 1 mL of methanol was added for 10 min (increase of cell attachment). Cells were rinsed twice with PBS buffer and thrice with ice-cold 0.3 N TCA. Cells were lysed with 250 μL of 0.3 N NaOH (30 min, 37 °C). Radioactivity (incorporated into DNA) present in samples of the extracts was measured using a scintillation counter.

Statistics. Results are shown as the mean ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) (RS/1 statistics pack, BBN Software Products Corp.) followed by a post hoc test (Newman Keuls). A p value of less than 0.05 was considered significant.

Acknowledgment. We thank Dr. C. B. Wollheim, Geneva, for supplying INS-1 cells, and the Hoechst AG (Germany) for supplying (mono-^{125}I-Tyr A^{14})porcine insulin. We thank Dr. H. Schlüter (Herne, Germany) for purification of diadenosine polyphosphates and for supplying Ip$_5$I and Ip$_6$I. This research was supported by the Foundation "Das Zuckerkranke Kind", Germany, and the Deutsche Forschungsgemeinschaft, Germany (Grant Ve 90/11-1). We thank the BBSRC for their financial support.

Appendix

Abbreviations. Ap$_4$A, diadenosine tetraphosphate; Ap(s)pCH$_2$pp(s)A, mixture of three diastereomers (*R*p*R*p), (*S*p*S*p), and (*R*p*S*p) diadenosine 5′,5‴-(*P*^{1},*P*^{4}-dithio-*P*^{2},*P*^{3}-methylene)tetraphosphate; Ap(s)pCHClpp(s)A, mixture of four diastereomers (*R*p*R*p), (*S*p*S*p), (*R*p,*R*-(CHCl),*S*p), and (*R*p, *S*(CHCl),*S*p) diadenosine 5′,5‴-(*P*^{1},*P*^{4}-dithio-*P*^{2},*P*^{3}-chloromethylene)tetraphosphate; Ip$_5$I, diinosine pentaphosphate; Ip$_6$I, diinosine hexaphosphate

The detailed stereochemical situation is that for Ap(s)pCH2pp(s)A, the central methylene group is prochiral, and so there are only the three stereoisomers. These are accurately described as (*R*p,*R*p), (*R*p,*S*p), and (*S*p,*S*p). However, for Ap(s)pCHClpp(s)A, the central CHCl group is truly stereogenic, resulting in the existence of four stereoisomers. The nomenclature describes the four isomers according to the principles set out in "Stereochemistry of Organic Componds" by E. L. Eliel and S. H. Wilen (McGraw Hill, London and New York, 1994), pages 124 and 125. In this case, the two phosphorus ligands of the central CHCl group are either homotopic ((*R*p,*R*p) and (*S*p,*S*p), giving two stereoisomers) or diastereoisomeric ((*R*p,*S*p), also giving two stereoisomers). Thus, the four isomers are correctly named Ap(s)(*R*p)pCHClpp(s)(*R*p)A, Ap(s)(*R*p)pC(*R*)-HClpp(s)(*S*p)A, Ap(s)(*R*p)pC(*S*)HClpp(s)(*S*p)A, and Ap(s)-(*S*p)pCHClpp(s)(*S*p)A. A further precise stereochemical description requires application of the rule "like precedes unlike". In practice, in the absence of a detailed X-ray structure, we cannot distinguish between the second and third isomers, although we have done so for the first and last stereoisomers on the basis of enzyme degradation patterns.

References

(1) Schlüter, H.; Offers, E.; Brüggemann, G.; van der Giet, M.; Tepel, M.; Nordhoff, E.; Karas, M.; Spieker, C.; Witzel, H.; Zidek, W. Diadenosine phosphates and the physiological control of blood pressure. *Nature* **1994**, *367*, 187−88.

(2) Davies, G.; MacAllister, R. J.; Bogle, R. G.; Vallance, P. Effects of diadenosine polyphosphates on human umbilical vessels: novel platelet-derived vasoconstrictors. *Br. J. Clin. Pharmacol.* **1995**, *40*, 170−172.

(3) Schlüter, H.; Tepel, M.; Zidek, W. Vascular actions of diadenosine polyphosphates. *J. Auton. Pharmacol.* **1996**, *16* (6), 357−362.

(4) Walker, J.; Bossman, P.; Lackey, B. R.; Zimmermann, J. K.: Dimmick, M. A.; Hilderman, R. H. The adenosine 5′,5‴,P$_1$,P$_4$-tetraphosphate receptor is at the cell surface of heart cells. *Biochemistry* **1993**, *32*, 14009−14014.

(5) Hilderman, R. H.; Martin, M.; Zimmerman, J. K.; Pivorun, E. B. Identification of a unique membrane receptor for adenosine 5′,5‴-P^1,P^4-tetraphosphate. *J. Biol. Chem.* **1991**, *266* (11), 6915−6918.

(6) Rodriguez-Pascual, F.; Cortes, R.; Torres, M.; Palacios, J. M.; Miras-Portugal, M. T. Distribution of [^{3}H]diadenosine tetraphosphate binding sites in rat brain. *Neuroscience* **1997**, *77*, 247−255.

(7) Edgecombe, M.; McLennan, A. G.; Fisher, M. J. Characterization of the binding of diadenosine 5′,5″-P^1,P^4-tetraphosphate (Ap$_4$A) to rat liver cell membranes. *Biochem. J.* **1996**, *314*, 687−693.

(8) Verspohl, E. J.; Johannwille, B. Diadenosine polyphosphates in insulin-secreting cells: Interaction with specific receptors and degradation. *Diabetes* **1998**, *47*, 1727−1734.

(9) Vahlensieck, U.; Boknik, P.; Knapp, J.; Linck, B.; Müller, F. U.; Neumann, J.; Herzig, S.; Schlüter, H.; Zidek, W.; Deng, M. C.; Scheld, H. H.; Schmitz, W. Negative chronotropic and inotropic effects by diadenosine hexaphosphate (Ap$_6$A) via A$_1$-adenosine receptors. *Br. J. Pharmacol.* **1996**, *119*, 835−844.

(10) Hoyle, C. H. V.; Postorino, A.; Burnstock, G. Pre- and postjunctional effects of diadenosine polyphosphates in the guinea-pig vas deferens. *J. Pharm. Pharmacol.* **1995**, *47*, 926−931.

(11) Pintor, J.; King, B. F.; Ziganshin, A. U.; Miras-Portugal, M. T.; Burnstock, J. Diadenosine polyphosphate-activated inward and outward currents in follicular oocytes of *Xenopus laevis*. *Life Sci.* **1996**, *59* (12), 179−184.

(12) Hoyle, C. H. V.; Ziganshin, A. U.; Pintor, J.; Burnstock, G. The activation of P$_1$- and P$_2$-purinoceptors in the guinea-pig left atrium by diadenosine polyphosphates. *Br. J. Pharmacol.* **1996**, *118*, 1294−1300.

(13) Van der Giet, M.; Khattab, M.; Börgel, J.; Schlüter, H.; Zidek, W. Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br. J. Pharmacol.* **1997**, *120*, 1453−1460.

(14) Ripoll, C.; Martin, F.; Rovira, J. M.; Pintor, J.; Miras-Portugal, M. T.; Soria, B. Diadenosine polyphosphates. A novel class of glucose-induced intracellular messengers in the pancreatic β-cell. *Diabetes* **1996**, *45*, 1431−1434.

(15) Yu, S. M.; Chen, S. F.; Lau, Y. T.; Yang, C. M.; Chen, J. C. Mechanism of extracellular ATP-induced proliferation of vascular smooth muscle cells. *Mol. Pharmacol.* **1996**, *50*, 1000−1009.

(16) Erlinge, D. Extracellular ATP: a growth factor for vascular smooth muscle cells. *Gen. Pharmacol.* **1998**, *31*, 1−8.

(17) King, B. F.; Liu, M.; Pintor, J.; Gualix, J.; Miras-Portugal, M. T.; Burnstock, G. Diinosine pentaphosphate (Ip_5I) is a potent antagonist at recombinant rat P_{2X1} receptors. *Br. J. Pharmacol.* **1999**, *128*, 981−988.
(18) Verspohl, E. J.; Johannwille, B.; Kaiserling-Buddemeier, I.; Schlüter, H.; Hagemann, J. Diadenosine polyphosphates in cultured vascular smooth-muscle cells and endothelium cells—their interaction with specific receptors and their degradation. *J. Pharm. Pharmacol.* **1999**, *51*, 1175−1181.
(19) Hagemann, J.; Schlüter, H.; Verspohl, E. J. Long-term effects of diadenosine polyphosphates on proliferation of vascular smooth muscle cells (VSMC) of rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2001**, *363* (Suppl.), 130.
(20) Blackburn, G. M.; Taylor, G. E.; Thatcher, G. R.; Prescott, M.; McLennan, A. G. Synthesis and resistance to enzymic hydrolysis of stereochemically defined phosphonate and thiophosphate analogs of P^1,P^4-bis(5′-adenosyl)tetraphosphate. *Nucleic Acids Res.* **1987**, *15*, 6991−7004.
(21) Nakae, I.; Takahashi, M.; Takaoka, A.; Liu, Q.; Matsumoto, T.; Amano, M.; Sekine, A.; Nakajima, H.; Kinoshita, M. Coronary effects of diadenosine tetraphosphate resemble those of adenosine in anaesthetized pigs: involvement of ATP-sensitive potassium channels. *J. Cardiovasc. Pharmacol.* **1996**, *28*, 124−133.
(22) Ogilvie, A. Extracellular functions for Ap_nA. In *Ap_4A and Other Dinucleoside Polyphosphates*; McLennan, A. G., Ed.; CRC Press: Boca Raton, FL, 1992; pp 230−273.
(23) Pintor, J.; Torres, M.; Miras-Portugal, M. T. Carbachol induced release of diadenosine polyphosphates—Ap_4A and Ap_5A—from perfused bovine adrenal medulla and isolated chromaffin cells. *Life Sci.* **1991**, *48*, 2317−2324.
(24) Miras-Portugal, M. T.; Pintor, J.; Castro, E.; Rodriguez-Pascual, F.; Torres, M. Diadenosine polyphosphates from neuro-secretory granules: the search for receptors, signals and function. In *Cell Signal Transduction, Second Messengers, and Protein Phosphorylation in Health and Disease*; Municio, A. M., Miras-Portugal, M. T., Eds.; Plenum Press: New York, 1994.
(25) Lüthje, J.; Miller, D.; Ogilvie, A. Unproportionally high amounts of diadenosine triphosphate (Ap_3A) and diadenosine tetraphosphate (Ap_4A) in heavy platelets. *Blutalkohol* **1987**, *54*, 193−200.
(26) Floodgard, H.; Klenlow, H. Abundant amounts of diadenosine 5′,5‴-P^1,P^4-tetraphosphate are present and releasable, but metabolically inactive, in human platelets. *Biochem. J.* **1982**, *208*, 737−742.
(27) Varshavsky, A. Diadenosine 5′,5′-P^1,P^4-tetraphosphate: a pleiotropically acting alarmone? *Cell* **1983**, *34*, 711−712.
(28) Chan, S. W.; Gallo, S. J.; Kim, B. K.; Guo, M. J.; Blackburn, G. M.; Zamecnik, P. C. P^1,P^4-dithio-P^2,P^3-monochloromethylene diadenosine 5′,5‴-P^1,P^4-tetraphosphate: a novel antiplatelet agent. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4034−4039.
(29) Meyer-Lehnert, H.; Schrier, R. W. Potential mechanism of cyclosporine A-induced vascular smooth muscle contraction. *Hypertension* **1989**, *13*, 352−360.
(30) Stec, W. J.; Zon, G.; Uznanski, B. Reversed-phase high-performance liquid chromatographic separation of diasteromecirc phosphorothioate analogues of oligodeoxyribonucleotides and other backbone-modified congeners of DNA. *J. Chromatogr.* **1985**, *326*, 263−280.

JM011070Z