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H Schlüter, … , J Jankowski, W Zidek

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Research Article

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Adenosine(5′) Oligophospho-(5′) Guanosines and Guanosine(5′) Oligophospho-(5′) Guanosines in Human Platelets

Hartmut Schlüter,* Isolde Groβ,* Jürgen Bachmann,* Raimund Kaufmann,‡ Marcus van der Giet,* Martin Tepel,* Jerzy-Roch Nofer,¶ Gerd Assmann,¶ Michael Karas,‖ Joachim Jankowski,* and Walter Zidek*

**Med. Klinik I, Univ.-Klinik Marienhospital, University of Bochum, Hölkeskampring 40, 44625 Herne; ‡Institut für Lasermedizin, University of Düsseldorf, Universitätsstr. 1, 40001 Düsseldorf; ‖Institut für Biochemie, University of Münster, Wilhelm-Klemm-Str. 2, 48149 Münster; and ¶Institut für Klinische Chemie und Laboratoriumsmedizin, University of Münster, Albert-Schweitzer-Str. 33, 48129 Münster, Germany*

Abstract

We isolated and identified nucleoside(5′) oligophospho-(5′) nucleosides containing adenosine and guanosine (Ap_nG; n = 3–6) as well as diguanosine polyphosphates (Gp_nG; n = 3–6) in human platelets. For identification, UV spectrometry, matrix-assisted laser desorption/ionization, postsource decay matrix-assisted laser desorption/ionization mass spectrometry, and enzymatic cleavage experiments were used. The adenosine(5′) oligophospho-(5′) guanosines act as vasoconstrictors and growth factors. The diguanosine polyphosphates are potent modulators of growth in vascular smooth muscle cells, but do not affect vascular tone. (*J. Clin. Invest.* 1998. 101:682–688.) Key words: dinucleoside polyphosphates • platelets • growth factors • cytosolic free Ca^{2+} concentration • vascular smooth muscle cells

Introduction

It has become increasingly clear that nucleotide derivatives have numerous extracellular effects. A great number of nucleotide receptor subtypes has been established with different physiological effects (1). Among these, cellular growth stimulation has been observed. ATP, for example, has been shown to stimulate proliferation of vascular smooth muscle cells (VSMCs)[1] and glomerular mesangial cells (2–4). In addition, many vascular effects of nucleotides have been described that vary depending on the purinoceptor subtype activated (5). Recently, the diadenosine polyphosphates were found to be important regulators of vascular tone (6, 7) and to activate a receptor that is not sensitive to suramin, and probably represents a novel purinoceptor subtype (8). In this study, two further subclasses of dinucleoside polyphosphates are identified in human cells; namely adenosine(5′) oligophospho-(5′)guanosines (Ap_nG) and guanosine(5′) oligo(5′)guanosines (Gp_nG; n = 3–6). Furthermore, we attempted to elucidate for the first time the biological effects of these compounds. The action of the substances identified on the vasculature of the isolated perfused rat kidney and the ability to promote growth of vascular smooth muscle cells were also studied.

Methods

Purification of dinucleoside polyphosphates from human platelets. Dinucleoside polyphosphates were isolated from human platelets unsuitable for transfusion. The platelets were suspended in an isotonic salt solution and centrifuged at 2,500 *g* for 5 min. The pellet was resuspended in isotonic salt solution and centrifuged again (2,500 *g* for 5 min). The supernatant was aspirated, and the platelet pellet was frozen to −30°C and rethawed in bidistilled water. Then the resulting suspension was deproteinized (step 1) with 0.6 M perchloric acid (final concentration). After adjusting the pH to 7.0 with 5 M KOH, the precipitated proteins and $KClO_4$ were removed by centrifugation. 40 mM triethylammonium acetate was added to the supernatant (TEAA, final concentration), and the mixture was loaded to a preparative reversed phase column (step 2, LiChroprep RP-18 B; Merck, Darmstadt, Germany; equilibration and sample buffer: 40 mM TEAA in water; flow rate: 5 ml/min). By this procedure, dinucleoside polyphosphates are eluted from the reversed phase column where most hydrophobic constituents are bound. The lyophilized eluate, dissolved in 1 M ammonium acetate, pH 9.5, was loaded to a phenyl boronic acid gel (step 3) according to Barnes et al. (eluent: 1 mM HCl; flow rate: 1 ml/min; 9).

The lyophilized eluate from the phenyl boronic acid gel was chromatographed (step 4) on an anion exchange column (MonoQ h 5/5, Cl^--form; Pharmacia LKB Biotechnology, Piscataway, NJ; eluent A: 10 mM K_2HPO_4, pH 7; eluent B: 50 mM K_2HPO_4, pH 7 with 1 M NaCl; gradient, 0–10 min: 0–10% B; 10–60 min: 10–40% B; 60–70 min, 40–100% B; flow rate: 0.5 ml/min). Fractions were collected according to the UV absorbance profile (peak fractionation). Each fraction from the anion exchange column with a significant UV absorbance was further fractionated (step 5) with a reversed phase column (Superspher RP-18 end-capped, 250 × 4 mm; Merck; eluent A: 40 mM TEAA in water; eluent B: acetonitrile; gradient, 0–4 min: 0–4% B; 4–64 min, 4–11% B; 64–70 min, 11–70% B; flow rate: 0.5 ml/min). All fractions corresponding to the main UV_{254nm}-absorbing peaks were rechromatographed (step 6) on the reversed phase column (conditions as in step 5).

Postsource decay matrix-assisted laser desorption/ionization mass spectrometry (PSD-MALDI-MS). Sample preparation for PSD-MALDI-MS and configuration of the mass spectrometer was described earlier (10, 11).

Synthesis of adenosine(5′)oligophospho-(5′)guanosine and guanosine(5′)oligophospho-(5′)guanosine compounds Ap_nG, n = 3–6. Ap_nG (n = 3–6) were synthesized according to Ng and Orgel (12) using ADP, ATP, GDP, and GTP as substrates. The authors suggested

Address correspondence to Prof. W. Zidek, Med. Klinik I, Univ.-Klinik Marienhospital, University of Bochum, Hölkeskampring 40, 44625 Herne, Germany. Phone: +49 2323 499 1670; FAX: +49 2323 499 302.

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1. *Abbreviations used in this paper:* Ap_nG, adenosine(5′)oligophospho-(5′)guanosines; Gp_nG, guanosine(5′)oligophospho-(5′)guanosines; PSD-MALDI-MS, postsource decay matrix-assisted laser desorption/ionization mass spectrometry; TEAA, triethyl ammonium acetate; VSMCs, vascular smooth muscle cells.

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that the water-soluble carbodiimide attacks mononucleotides, forming *N*-phosphorylureas. The presence of Mg^{2+} catalyzes the nucleophilic attack of one phosphate anion on the activated derivative (*N*-phosphorylurea) resulting in dinucleosid polyphosphates (12). 10 mM of each nucleotide were mixed with 2.5 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma Chemical Co., St. Louis, MO) in an aqueous solution containing 2 M Hepes and 50 mM $MgCl_2$. The pH was adjusted to 6.5, and the reaction mixture was incubated for 24 h at 37°C. Then, the Ap_nG generated during the incubation were concentrated on a preparative C18 reversed-phase gel (Lichroprep A; Merck). The Ap_nG were eluted from the column with 30% acetonitrile, and the eluate was lyophilized. The lyophilizate was dissolved in 10 mM K_2HPO_4, pH 7 (eluent A), and was fractionated with an anion exchange column as described above (purification of dinucleoside polyphosphates from human platelets, step 4). Each fraction from the anion exchange column that showed a significant UV absorbance was further fractionated with a reversed phase column as described above (step 5) and rechromatographed if necessary as in step 6. The Ap_nGs were identified with MALDI-MS, with enzymatic cleavage with 5′-nucleotidase from Crotalus durissus and alkaline phosphatase (see enzymatic cleavage experiments). The enzymatic experiments were done to show that the dinucleoside polyphosphates contained 5′-5′ ribose phosphate esters.

Enzymatic cleavage experiments. 200-ng aliquots of the fractions from the anion exchange column, purified twice with reversed-phase chromatography (step 5 and 6 of the purification procedure), were incubated with enzymes as follows: the samples were dissolved (*a*) in 20 μl 200 mM Tris buffer (pH 8.9) and incubated with 5′-nucleotide hydrolase (3 mU; from Crotalus durissus, EC 3.1.15.1, from Boehringer Mannheim, Mannheim, Germany, purified according to Sulkowski & Laskowski [13] for 9 min at 37°C); (*b*) in 20 μl 200 mM Tris and 20 mM EDTA buffer (pH 7.4) and incubated with 3′-nucleotide hydrolase (1 mU; from calf spleen, EC 3.1.16.1; Boehringer Mannheim) for 1 h at 37°C; and (*c*) in 20 μl 10 mM Tris, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$ buffer (pH 8) and incubated with alkaline phosphatase (1 mU; EC 3.1.3.1 from calf intestinal mucosa; Boehringer Mannheim) for 1 h at 37°C. The reaction was terminated by ultrafiltration with a centrifuge filter (exclusion limit 10 kD). After filtration of the enzymatic cleavage products, the filtrate, dissolved in 980 μl eluent A, was subjected to the anion exchange chromatography on a MiniQ PC 3.2/3 (Pharmacia LKB Biotechnology; eluent A, 10 mM K_2HPO_4, pH 7; eluent B, 50 mM K_2HPO_4, pH 7 with 1 M NaCl; gradient, 0–3 min: 0% B; 3–20 min: 0–50% B; 20–21 min: 50–100% B; flow rate: 100 μl/min).

Cell proliferation assay. Vascular smooth muscle cells (VSMCs) from normotensive Wistar-Kyoto rats were subcultured in 96-well dishes (Falcon Labware, Cockeysville, MD) at a density of 5×10^4 cells/ml and kept in culture medium containing 10% FCS to reach a subconfluent monolayer. After 24 h, the cells were growth-arrested in 0.5% FCS for 48 h without affecting cell adherence to culture wells or viability as checked by Trypan blue vital dye exclusion. Quiescent VSMCs were then exposed to fresh culture medium with 0.5% FCS with and without the tested agonists for another 48-h incubation period. Cell proliferation was measured using the [^{3}H]thymidine incorporation rate as described elsewhere (14).

Measurements of perfusion pressure in the isolated perfused rat kidney. The effect of dinucleoside polyphosphates on vascular tone was evaluated in the isolated rat kidney perfused with a constant flow of 10 ml/min while perfusion pressure was continuously monitored. Details of the preparation have been given elsewhere (15).

Quantitation of dinucleoside polyphosphates. The fate of dinucleoside polyphosphates during their passage through the isolated perfused rat kidney was studied by collecting 10 ml of the effluate directly after each bolus injection of the individual dinucleoside polyphosphates (10 μM). For comparison, equal amounts of the nonhydrolyzable nucleotide α, β methylene ATP were injected.

Degradation of dinucleoside polyphosphates by VSMCs was measured by incubating subconfluent quiescent cells in 96-well plates (density of 5×10^4 cells/ml) washed three times with PBS and incubated with 10 μM dinucleoside polyphosphates in HBSS for different time periods.

To the effluate from the isolated perfused rat kidney and the supernatant from cell culture, respectively, perchloric acid (0.6 M final concentration) was added immediately. After removal of perchlorate by titrating the supernatants to pH 8 with KOH, centrifugation, and adding TEAA (final concentration, 40 mM), the samples were desalted with a reversed-phase column (effluate from the isolated perfused rat kidney: preparative reversed phase column as described in

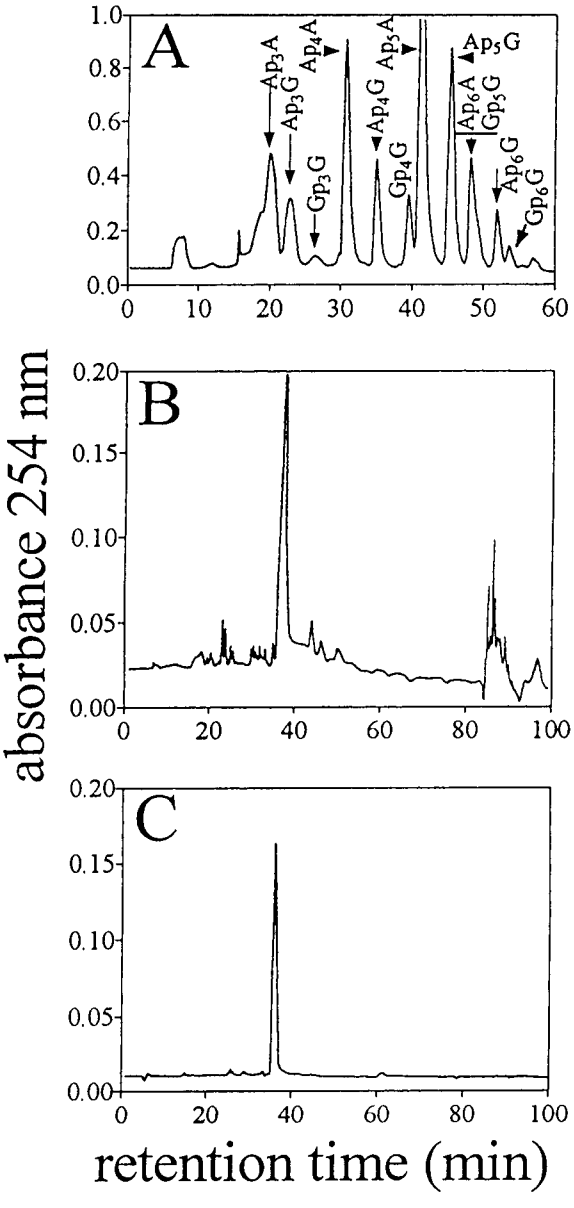

Figure 1. Chromatograms of the purification of the dinucleoside polyphosphates from a platelet extract. (*A*) Anion exchange chromatography of a platelet extract (step 4). The peaks in *A* are labeled with abbreviations of the substances identified in the respective fraction. The effluents of all chromatographies were peak-fractionated. (*B*) Typical reversed phase chromatography (step 5) of a fraction from the anion exchange column (fraction-labeled Ap_6G in *A*). (*C*) Rechromatography of the fraction corresponding to the UV254-nm absorbing peak at 36 min (step 6) with the reversed phase column. Ap_nA, adenosine(5′)oligophospho-(5′) adenosines.

step 2; supernatants: Superspher RP-18 end-capped, 250 × 4 mm; Merck; eluent A, 40 mM TEAA in water; eluent B, 20% acetonitrile). Chromatographic analysis was done with the anion exchange HPLC described under "enzymatic cleavage experiments". The intact dinucleoside polyphosphates and α, β methylene ATP were quantitated by integrating the respective UV peaks.

Incubation of the platelets with thrombin and purification of the dinucleoside polyphosphates from the supernatant. Platelets from three platelet concentrates, each from 2 l whole blood were suspended in 300 ml of a physiological salt solution and divided into two parts. The first part was incubated with thrombin (0.05 U/ml) for 1 min. After incubation, the platelets were removed by centrifugation (2,500 *g* for 5 min). The supernatant was deproteinized with perchloric acid. The second part was deproteinized with perchloric acid. The supernatants of both parts were chromatographed according to step 2 to step 6. The gradient of the anion exchange chromatography (step 4) was modified as follows: gradient, 0–10 min: 0–5% B; 10–100 min, 5–35% B; 100–105 min, 35–40% B; 105–110 min, 40–100% B. The purified substances were identified with PSD-MALDI-MS as described above. The concentrations of the dinucleoside polyphosphates were estimated plotting known concentrations of the respective substances against their UV absorbance at 254 nm. To estimate the recovery of the dinucleoside polyphosphates after extraction and chromatography, [^{3}H]Ap_4A (Amersham Corp., Arlington Heights, IL) was added to the untreated platelets.

As a control, the same experiment and the same chromatographic procedure as described above were performed except that instead of thrombin, an equal volume of physiological salt solution was added.

Results

In the following experiment, purification and identification of the dinucleoside polyphosphates from human platelets is exemplified for Ap_6G. Fig. 1 shows chromatographic purification of Ap_6G from human platelets. In the last chromatographic step, a single UV peak was obtained. The substance underlying this peak was identified by the following results: (*a*) MALDI-MS revealed a mass of 1013 D (Fig. 2). (*b*) After addition of 1 mM NaCl, MALDI-MS showed seven additional signals corresponding to masses of 1013 + $n \times 22$ D (n from 1 to 7, data not shown). This result indicates six negative charges in the molecule. (*c*) PSD-MALDI-MS revealed a fragmentation pattern that was identical with that of synthetic Ap_6G (Fig. 2). The fragmentation yields a pattern characteristic of each substance. Interpretation of the fragment ions is listed in Table I. (*d*) Cleavage of the molecule with 5′-nucleotide hydrolase (Crotalus durissus) yielded AMP and guanosine pentaphosphate as well as GMP and adenosine pentaphosphate, as evidenced by the retention times and UV spectra (Fig. 3). The cleavage pattern was identical with that of synthetic Ap_6G. (*e*) Incubation of the molecule with 3′-nucleotide hydrolase (calf spleen) and alkaline phosphatase yielded no cleavage products. The enzymatic cleavage experiments demonstrate that the polyphosphate chain interconnects the nucleosides adenosine and guanosine via phosphoester bonds with the 5′-oxygens of the riboses.

In an analogous manner, Ap_3G, Ap_4G, and Ap_5G as well as Gp_3G, Gp_4G, Gp_5G, and Gp_6G were purified and identified from the various anion exchange chromatography fractions indicated in Fig. 1 *A*. Table I presents the signal pattern of PSD-MALDI-MS fragmentation of the various dinucleoside

Table I. Masses of the Fragment Ions (in D) Obtained by PSD-MALDI Mass Spectrometry of Each Dinucleoside Polyphosphate Isolated

Fragment ions	Ap_3G	Gp_3G	Ap_4G	Gp_4G	Ap_5G	Gp_5G	Ap_6G	Gp_6G
A′	136		136		136		136	
G′	152	152	152	152	152	152	152	152
A − $2H_2O$	232		232		232		232	
G − $2H_2O$	248	248	248	248	248	248	248	248
Ap_1	348		348		348		348	
Gp_1	363	364	364	364	364	364	364	364
Ap_2 − H_2O	409		410					
Ap_2	428		427		427		428	
Gp_2	444	444	444	444	443	444	444	444
Ap_3 − H_2O			490		490		490	
Ap_3			507		508		508	
Gp_3			523	523	523	523	523	523
M − Gp_2	330		410		490	506	570	585
M − Ap_2					505		585	
M − Gp_1	410	425	490	506	569	586	650	665
M − Ap_1			505		585		666	
M − A			587		666		747	
M − G + H_2O	604	524	683	604	762	684	843	763
M − A′ − H_2O	621		701		780		861	
M − G′ − H_2O		620		701		780		859
M − G′	622	638	701	718	782	797	861	877
M − A′	638		717		797		877	
M	773	789	853	869	933	949	1013	1029

From the characteristic fragmentation patterns, the structure of each dinucleoside polyphosphate can be deduced. The dinucleoside polyphosphates are listed in the first line. The masses of the fragment ions obtained from each dinucleoside polyphosphate by PSD MALDI mass spectrometry are shown in the columns. The left column labeled "fragment ions" shows the fragments, which could be assigned to the masses obtained after fragmentation. *M*, protonated parent ion, *A′*, adenine, *G′*, guanine, A, adenosine, *G*, guanosine, *p*, phosphate group, e.g., Ap_3, adenosine triphosphate.

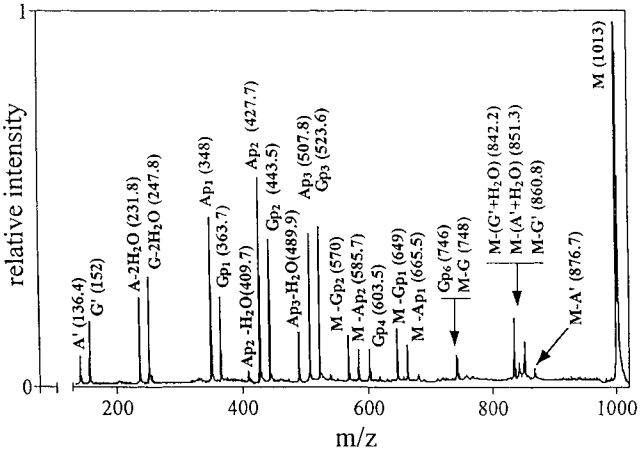

Figure 2. Mass spectrometric analysis of the purified fractions from platelets. Typical positive-ion mass spectrum measured with the PSD-MALDI-MS of the UV-absorbing fraction shown in Fig. 1 *C* (abscissa: relative mass/charge, m/z, z = 1; ordinate: relative intensity). The abbreviations used as labels of the fragments are explained in Table I.

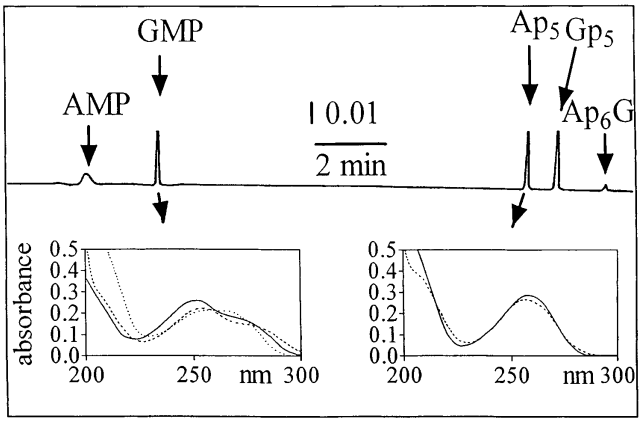

Figure 3. Anion exchange chromatography of the enzymatic cleavage products after incubation of the fraction labeled Ap_6G in Fig. 1 *C* with the 5′-nucleotide hydrolase. The *inserts* show the UV spectra of the fractions indicated by the *arrows* (*solid line*, pH 6.4 [water], *dashed lines*, pH 2 [0.01 M HCl], *dotted lines,* pH 11 (10 mM NaOH). The spectra correspond to those of adenosine and guanosine. The peaks labeled AMP and Gp_5 showed UV spectra identical with those of Ap_5 and GMP, respectively.

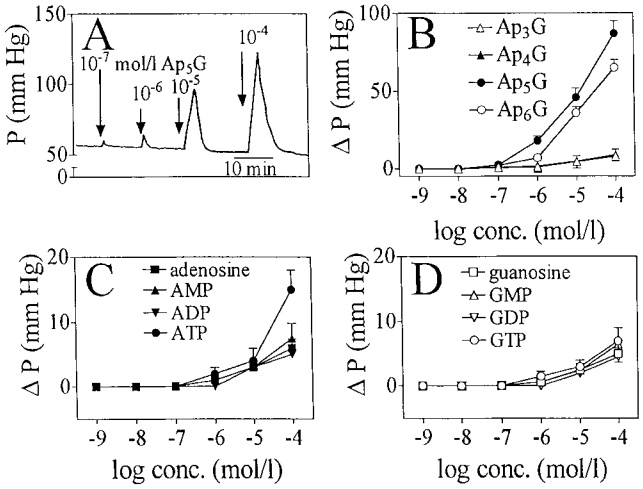

Figure 4. Effects of adenosine(5′)oligophospho-(5′)guanosines and guanosine(5′)oligophospho-(5′)guanosines (Ap_nG, Gp_nG, n = 3–6) as well as of potential degradation products on perfusion pressure in an isolated perfused rat kidney. Data are means±SD. (*A*) Changes in perfusion pressure *P* (ordinate) after bolus injections of 100 μl physiological salt solution containing the indicated concentrations of Ap_5G. (*B*) Concentration–response curves showing the changes in perfusion pressure (ΔP) after bolus injections of Ap_nGs as described in *A*. (*C*) Concentration–response curves showing the changes in perfusion pressure (ΔP) after bolus injections of adenosine, AMP, ADP, and ATP as described in *A*. (*D*) Concentration–response curves showing the changes in perfusion pressure (ΔP) after bolus injections of guanosine, GMP, GDP, and GTP as described in *A*.

polyphosphates. Table II lists the retention times of the dinucleoside polyphosphates and their cleavage products after incubating the different dinucleoside polyphosphates with the 5′-nucleotide hydrolase (Crotalus durissus). The alkaline phosphatase and the 3′-nucleotide hydrolase did not degrade any of the molecules.

What is the physiological significance of this family of compounds? Ap_5G and Ap_6G constrict the renal vasculature in concentrations $\geq 10^{-7}$ M (Fig. 4, *A* and *B*). The vasoconstrictive actions of these agents may also be elicited indirectly by their degradation products. To test this possibility, adenosine and guanosine containing mononucleotides as well as both nucleosides were examined. All potential degradation products showed much weaker vasoconstrictive effects than those of the Ap_5G and Ap_6G (Fig. 4, *C* and *D*), indicating that the vasoconstrictive action of the latter is direct rather than indirect. Ap_3G and Ap_4G were very weak vasoconstrictors, and Gp_nGs did not affect vascular tone (data not shown). Furthermore, both

Table II. Retention Time (min) of the Reaction Products After Incubation of the Different Fractions from the Platelet Extract with the 5′-Nucleotide Hydrolase (Crotalus Durissus)

	Ap_3G	Gp_3G	Ap_4G	Gp_4G	Ap_5G	Gp_5G	Ap_6G	Gp_6G	Standards
Ap_1	9.03		9.04		9.05		9.04		9.04
Ap_3			11.89						11.87
Gp_3			12.57	12.55					12.54
Ap_4					13.23				
Gp_1	10.16	10.18	10.17	10.16	10.16	10.18	10.17	10.17	10.16
Ap_2	10.63								10.65
Gp_2	11.15	11.16						11.13	13.25
Gp_4					13.91	13.90			13.93
Ap_5							15.32		15.33
Gp_5							15.69	15.73	15.70
Np_xN^P	12.07	12.91	13.94	14.73	15.63	16.05	16.57	17.40	
Np_xN^A	12.08	12.90	13.94	14.70	15.65	16.02	16.59	17.43	

The left column shows the reaction products that could be assigned to each peak according to its retention time. For comparison, the retention times of the authentic reaction products are shown in the right column, and those of the dinucleoside polyphosphates are shown in the last row. Np_xN^P, uncleaved fraction purified from platelets; Np_xN^A, uncleaved authentic dinucleoside polyphosphate.

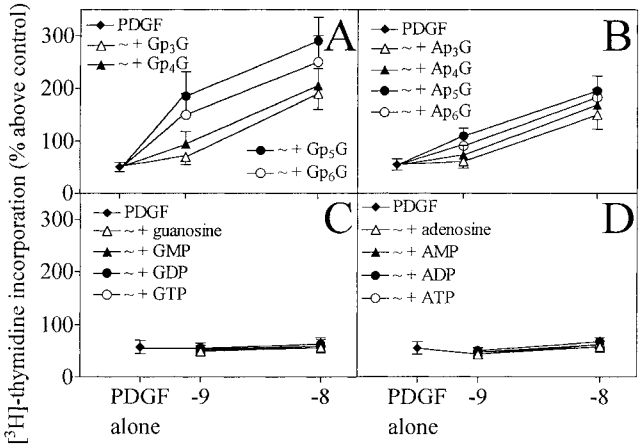

Figure 5. [^{3}H]Thymidine incorporation rate (ordinate) in % above control in VSMCs incubated with various concentrations of dinucleoside polyphosphates (*A*, Gp_nG; *B*, Ap_nG) and of potential degradation products (*C* and *D*) with 5 ng/ml PDGF. Growth stimulation with 5 ng/ml PDGF alone was plotted for comparison. Abscissae: log of concentration in M.

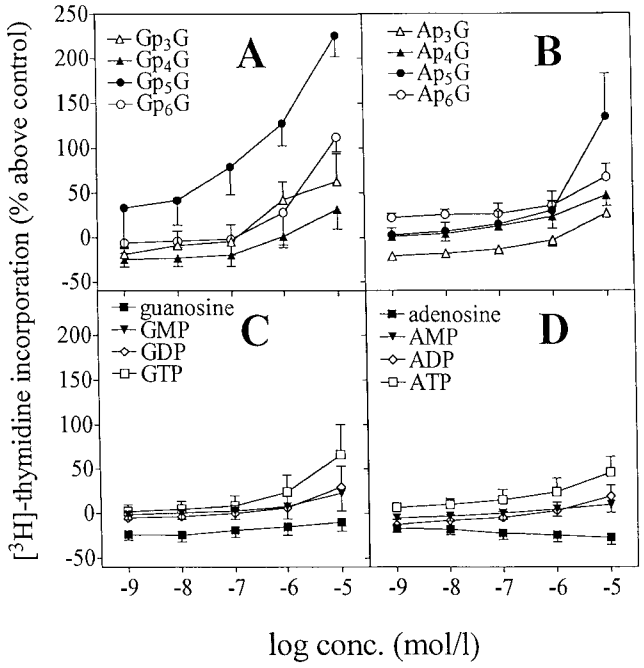

Figure 6. [^{3}H]Thymidine incorporation rate (ordinate) in % above control in VSMCs incubated with various concentrations of dinucleoside polyphosphates (*A*, Gp_nG; *B*, Ap_nG) and of potential degradation products (*C* and *D*) Abscissae: log of concentration in M.

the Gp_nGs and the Ap_nGs act as growth modulators together with the platelet-derived growth factor in VSMCs in concentrations $\geq 10^{-9}$ M (Fig. 5, *A* and *B*). As with vasoconstrictor effects, growth stimulation together with PDGF cannot be attributed to degradation products of the dinucleoside polyphosphates (Fig. 5, *C* and *D*). The growth stimulatory effects of the dinucleoside polyphosphates alone were apparent in concentrations $\geq$ 100 nM (Fig. 6, *A* and *B*). Gp_5G and, among Ap_nG, Ap_5G, appear to be most effective with respect to cell proliferation. The order of potency of the direct growth–promoting effect as well as of the PDGF-potentiating effect was $Gp_5G > Ap_5G > Gp_6G > Gp_3G >> Ap_6G > Ap_4G >> Gp_4G > Ap_3G$. The threshold concentrations for the direct growth stimulatory action were 10^{-7} M for Gp_5G, 10^{-6} M for Ap_5G, and 10^{-5} M for the other compounds. Indirect effects by degradation products could largely be excluded (Fig. 6, *C* and *D*).

To clarify the mechanism of action of the dinucleoside polyphosphates, it is of interest to know how fast these molecules are degraded by ectonucleotidases of the VSMCs. VSMCs were incubated with dinucleoside polyphosphates, and the concentrations of the dinucleoside polyphosphates were chromatographically quantitated ($n = 5$). Half-lives of the dinucleoside polyphosphates were 49±6 min (Ap_3G), 56±7 min (Ap_4G), 75±9 min (Ap_5G), 65±9 min (Ap_6G), 54±5 min (Gp_3G), 62±8 min (Gp_4G), 72±10 min (Gp_5G), and 69±9 min (Gp_6G).

Recoveries of the respective dinucleoside polyphosphates in the effluate from the isolated perfused kidney were 83.5±12.3% (Ap_3G), 79.4±9.7% (Ap_4G), 85.4±9.9% (Ap_5G), 81.0±10.3% (Ap_6G), 89.2±5.3% (Gp_3G), 87.3±8.7% (Gp_4G), 77.5±10.6% (Gp_5G), and 78.4±13.1% (Gp_6G) of the amount of α, β methylene ATP. In the effluate, no α, β methylene ADP was detected by chromatography.

Finally, the question arose whether the dinucleoside polyphosphates are released into the extracellular space, and whether by platelet aggregation extracellular concentrations (which affect VSMCs) are reached. Fig. 7 *A* depicts a chromatogram obtained from an untreated platelet suspension. Furthermore, the supernatant from an unstimulated platelet suspension was chromatographed. As shown in Fig. 7 *B*, the dinucleoside polyphosphates were not detectable in the supernatant from unstimulated platelets. Fig. 7 *C* shows a chromatogram obtained from the supernatant of a platelet suspension after stimulation with thrombin. After platelet aggregation, the dinucleoside polyphosphates described above were found in the supernatant. Comparison with Fig. 7 *A* reveals that at least 60% of the dinucleoside polyphosphates are released by platelet aggregation. It can be estimated from the UV peaks that extracellular concentrations in the range of 0.5–3 μM occur after thrombin stimulation.

Discussion

Ap_3G, Ap_4G, and Gp_4G have been found in bacteria such as *Escherichia coli*, in yeast, and in crustaceans (16–19). Furthermore, Ap_4G was isolated from *Physarum polycephalum*, rat

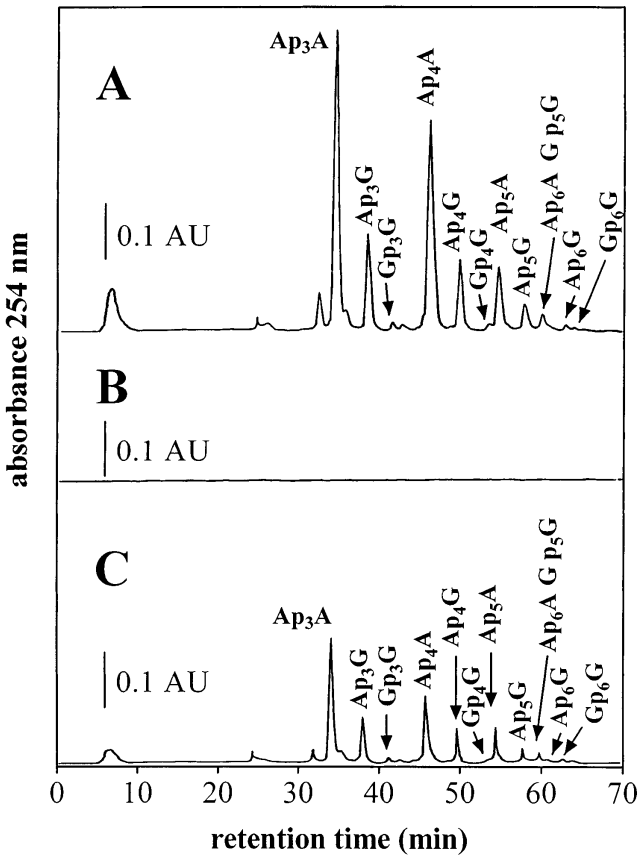

Figure 7. Anion exchange chromatography (according to step 4) of a platelet suspension (*A*), a supernatant from an unstimulated platelet suspension (*B*), and of a further supernatant from a platelet suspension aggregated with thrombin (*C*). Representative tracings out of five similar experiments are shown. The peaks in *A* and *C* are labeled with the substances identified in the respective fraction by MALDI-MS and retention times.

liver, and *Saccharomyces cerevisiae* (20), but the physiological role in these organisms has not yet been elucidated. To the best of our knowledge, these compounds have not so far been isolated from human tissue.

Four classes of enzymes are known to synthesize dinucleoside polyphosphates in vitro: the aminoacyl-tRNA synthetases, Ap_4A phosphorylases, guanylyltransferases, and luciferases (for review see reference 21). The aminoacyl-tRNA synthetases catalyze the reaction of aminoacyl-AMP with NDP (or NTP) resulting in Ap_3N (or Ap_4N) and the aminoacid (22).

A guanylyltransferase from yolk platelets of Artemia catalyzes the interaction of two molecules of GTP to form Gp_4G with the release of inorganic pyrophosphate (23). Gp_3G may be synthesized by this enzyme when GDP interacts with Gp_4G. In the presence of ADP and Gp_4G, this enzyme catalyzes the synthesis of Ap_3G (24). The pathways that have been described so far, however, are not capable of producing Ap_5G, Gp_5G, Gp_6G, or Ap_6G.

Dinucleoside polyphosphates are degraded by asymmetrical and symmetrical hydrolases and phosphorylases. The substrate specificity of these enzymes mainly depends on the number of phosphates of the dinucleoside polyphosphates. Phosphodiesterases, another group of dinucleoside polyphosphate–hydrolyzing enzymes have a broad substrate specificity (25). In blood, dinucleotides are primarily metabolized by plasma enzymes (26, 27).

The physiological concentrations of the dinucleoside polyphosphates in blood cannot be determined exactly. The concentration of Ap_nGs and Gp_nGs may be $\sim$ 1/200–1/500 that of ATP, which can easily be identified in chromatograms of platelet extracts. Furthermore, it is not known if these compounds occur in human cells apart from platelets.

The present experiments showed that Ap_nGs and Gp_nGs act both as growth factors in VSMCs and can modulate the action of a peptide growth factor. As is known for the diadenosine polyphosphates (28, 29), the Ap_nGs and Gp_nGs may also exert their effects after release by platelet aggregation. One hypothesis of atherogenesis is that the initial lesion is local endothelial damage followed by platelet adhesion and aggregation. This cascade causes the release of growth factors such as PDGF from platelets, which leads to VSMC proliferation. Apart from the known growth factors such as PDGF, it is clear that the Ap_nGs and Gp_nGs may also stimulate VSMC growth either directly or indirectly, thus modulating the effect of other growth factors.

It would seem that at least one adenosine moiety is required for vasoconstrictive action since the Ap_nGs both increase vascular tone and cellular growth, whereas the Gp_nGs do not influence vascular tone, affecting only VSMC growth. Thus, vasoconstriction and proliferation appear to be mediated by different receptors. The vasoconstrictive action of Ap_5G and Ap_6G appears to be a direct effect, since all potential degradation products are considerably less active than these dinucleoside polyphosphates. Direct action is further supported by the finding that the dinucleoside polyphosphates are recovered from the isolated perfused kidney to a similar degree as the nonhydrolyzable compound α, β methylene ATP. Furthermore, these substances show half-lives in the range of 1 h in cell culture. Nevertheless, degradation products may also modulate biological actions of the dinucleoside polyphosphates. The receptors involved in the vasoconstrictive action of Ap_nG may be the $P_{2\chi}$ purinoceptor, as this subtype also appears to mediate similar actions of Ap_nA on vascular tone (30). This concept is supported by the fact that the order of potency ($Ap_5G > Ap_6G > Ap_4G$) is comparable to that of the vasoconstrictive actions of diadenosine polyphosphates ($Ap_5A \geq Ap_6A \geq Ap_4A$) in rat mesenteric arteries (30). Nevertheless, at present a contribution of other P_2 purinoceptors or even P_1 purinoceptors cannot be excluded.

The receptor mediating vascular growth is not yet known. It may represent a novel P_2 purinoceptor subtype. Alternatively, it may be identical with the P_{2U}-purinoceptor, since ATP and GTP binding to this receptor cause similar mitogenic effects on VSMCs (31).

After isolation of the dinucleoside polyphosphates from platelets, their physiological relevance was examined. We were able to show that the dinucleoside polyphosphates are released by platelet aggregation with thrombin. Under these conditions, extracellular concentrations of dinucleoside polyphosphates occur, which, in view of the concentration response curves observed, appear to be capable of affecting VSMCs. It may therefore be assumed that the dinucleoside polyphosphates identified in this study may indeed play a role for the events occurring in the vascular wall secondary to platelet aggregation.

In conclusion, human platelets contain not only diadenosine polyphosphates (7), but also Ap_nGs and Gp_nGs (n = 3–6). These dinucleoside polyphosphates appear to form a large family of substances with different physiological actions that may play a role in regulation of organ perfusion and of vascular growth under physiological and pathological conditions.

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