Dinucleotides as Growth-promoting Extracellular Mediators

PRESENCE OF DINUCLEOSIDE DIPHOSPHATES Ap2A, Ap2G, AND Gp2G IN RELEASABLE GRANULES OF PLATELETS

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Dinucleoside diphosphates, Ap2A, Ap2G, and Gp2G represent a new class of growth-promoting extracellular mediators, which are released from granules after activation of platelets. The presence of these substances was shown after purification from a platelet concentrate. The substances were identified by UV spectrometry, retention time comparison with authentic substances, matrix-assisted laser desorption/ionization mass spectrometry, and enzymatic analysis. Ap2A, Ap2G, and Gp2G have growth-stimulating effects on vascular smooth muscle cells in nanomolar concentrations as shown by [3H]thymidine incorporation measurements. The calculated EC50 (log M; mean ± S.E.) values were –6.07 ± 0.14 for Ap2A, –6.27 ± 0.25 for Ap2G, and –6.81 ± 0.44 for Gp2G. At least 61.5 ± 4.3% of the dinucleoside polyphosphates are released by platelet activation. The intraplatelet concentrations suggest that, in the close environment of a platelet thrombus, similar dinucleoside polyphosphate concentrations can be found in as platelets. Intraplatelet concentration can be estimated in the range of 1/20 to 1/100 of the concentration of ATP. In conclusion, Ap2A, Ap2G, and Gp2G derived from releasable granules of human platelets may play a regulatory role in vascular smooth muscle growth as growth-promoting mediators.

To further evaluate the pathogenesis of hypertension, there is a continued interest in the identification of novel endogenous compounds with growth-stimulating effects on vascular smooth muscle cells (VSMCs). In this context, novel endogenous dinucleotides have been recognized as powerful vasoactive messengers.

In the last decade dinucleoside polyphosphates received considerable attention in view of their multiple biological and pharmacological activities. Dinucleoside polyphosphates were identified in prokaryotic (1), eukaryotic, and mammalian cells (2). Di(adenosine-5') tri- and tetraphosphates (Ap3A, Ap4A) were the first dinucleoside polyphosphates to be identified in human platelets (3, 4). Di(adenosine-5') pentaphosphate (Ap5A) and di(adenosine-5') hexaphosphate (Ap6A) have been described as vasoconstrictive substances isolated from human platelets (5). Recently, di(adenosine-5') heptaphosphate (Ap7A) has been isolated in human platelets and has been postulated to play a role in the control of vascular tone (6). Furthermore, dinucleoside polyphosphates containing adenosine and guanosine (adenosine-5' oligophospho 5'-guanosines (ApG5-n) n = 5–6) or containing two guanosines (guanosine-5'-oligophospho 5'-guanosines (GpG5-n) n = 5–6) were identified in human platelets (7). ApG5 (n = 5–6) have a vasoconstrictive effect, whereas GpG5 do not affect vascular tone in the isolated perfused rat kidney. Both ApG5s and GpG5s (with n = 3–6) are growth-stimulating factors of VSMCs. Several purine receptor subtypes (P2 receptors) mediating the actions of dinucleoside polyphosphates have been established with different physiological effects (8, 9). The P2 receptors are divided into two families of ligand-gated ion channel and G protein-coupled receptors termed P2X and P2Y receptor, respectively. There are eight mammalian P2X receptors (P2X1–8) (10, 11) and five mammalian P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11) that have been cloned (9).

From this results the question arose as to whether P1,P2-dinucleoside diphosphates containing two adenosine or guanosine groups or an adenosine and a guanosine group also occur in humans. There is one report on the existence of diadenosine diphosphate (ApA3) isolated from human cardiac tissue (12). In contrast to ApA, the P1,P2-dinucleoside diphosphates ApG5 and GpG5 have not been described as endogenous substances so far in the literature.

Here the existence of diadenosine diphosphate (ApA3), adenosine guanosine diphosphate (ApG5), as well as diadenosine diphosphate (GpG5) in releasable granules of human platelets is shown for the first time and their growth-stimulating effect on cultured vascular smooth muscle cells is described.

MATERIALS AND METHODS

Chemicals

HPLC water (gradient grade) and acetonitrile were from Merck (Germany). All other substances were purchased from Sigma (Germany).

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Purification of Dinucleoside Diphosphates from Human Platelets

Dinucleoside diphosphates were isolated from human platelets unsuitable for transfusion. The platelets were suspended in an isotonic salt solution and centrifuged at 2500 × g for 5 min. The pellet was resuspended in an isotonic salt solution and centrifuged again (2500 × g, 5 min). The supernatant was aspirated, and the platelet pellet was frozen to −30 °C and rethawed in bidistilled water (step 1). Then the resulting suspension was deproteinized (step 2) with 0.06 M perchloric acid (final concentration). After adjusting the pH to 7.0 with 5 M KOH, the precipitated protein and KClO₄ were removed by centrifugation.

Chromatography

Preparative Reversed Phase Chromatography—Triethy lammonium acetate (TEAA) was added to the supernatant (final TEAA concentration 40 mM), and hydrophobic substances were concentrated on a C18 reversed phase column (Lichroprep, 310 × 65 mm, 40–65 μm, Merck, Germany) using 40 mM TEAA in water (eluent A; flow, 2 ml/min). After removing substances not binding to the column with aqueous 40 mM Tris (pH 9.5), the adsorbed substances were eluted with 20% acetonitrile in water (eluent B1). The elution was detected by measuring the UV absorption at 254 nm. The eluate was lyophilized and stored frozen at −80 °C (step 3).

Affinity Chromatography—The lyophilized eluate of the reversed phase chromatography was dissolved in 1 M ammonium acetate (eluent C; pH 9.5) and purified further with affinity chromatography (step 4). The affinity chromatography gel, phenylboronic acid coupled to a cation-exchange resin (BioRex 70, Bio-Rad), was synthesized according to Barnes (13). The affinity resin was packed into a glass column (150 × 20 mm) and equilibrated with 1 M NH₄Ac (pH 9.5; flow: 2 ml/min). The pH of the eluate from the reversed phase column was adjusted to pH 9.5 and loaded to the affinity column. The column was washed with 1 M NH₄Ac (pH 9.5) with a flow rate of 2 ml/min. Binding substances were eluted with 1 M HCl (eluent D). Fractions were monitored with an UV detector at 254 nm. The eluate was frozen and lyophilized.

Reversed Phase Chromatography—The eluate of the affinity chromatography was dissolved in 0.1 M K₂HPO₄ in water (pH 8.0, eluent F) and chromatographed by using an anion-exchanger (DEAE 5PW, 150 × 20 mm, 10 μM, Tosohaa, Japan) using 20 mM K₂HPO₄ in water, pH 8.0 (eluent F), and 20 mM K₂HPO₄ and 1 M NaCl (pH 8.0) (eluent G) in water using the following gradient: 0–10 min: 0–5% G; 10–15 min: 5–35% G; 105–110 min: 35–100% G; 110–120 min: 100% G; 120–121 min: 100%–0% G. The flow rate was 2.0 ml/min, and absorption was measured at 254 nm (step 6).

Reversed Phase Chromatography—Thereafter, each fraction of the reversed phase chromatography with a significant UV absorbance was lyophilized, dissolved in 0.2 ml of 20 mM KH₂PO₄ in water (pH 7.0, eluent F0) and chromatographed by using an anion-exchanger (DEAE 5PW, 150 × 20 mm, 10 μM, Tosohaa, Japan) using 20 mM KH₂PO₄ in water, pH 8.0 (eluent F0), and 20 mM KH₂PO₄ and 1 M NaCl (pH 8.0) (eluent G0) in water using the following gradient: 0–4 min: 0–4% H; 4–64 min: 4–11% H; 64–70 min: 11–70% H (flow, 0.5 ml/min; step 7). The resulting fractions were lyophilized and stored at −30 °C. Fractions corresponding to the main UV₂₅₄ absorbing peaks were rechromatographed (step 8) on the reversed phase column (conditions as in step 7).

Identification of the P₁,P₂-dinucleoside Diphosphates by Reversed Phase Chromatography

To test the fractions for homogeneity, a small part (1/1000) of the desalted and lyophilized fractions of the anion-exchange chromatography were chromatographed on a second reversed phase HPLC column (Pores, R ZH/2, 2.1 × 100 mm, Persepive Biosystems). The column was run in the gradient mode (flow rate, 300 μl/min) with 10 mM KH₂PO₄ and 2 mM tetrabutyl-ammonium hydrogen sulfate in water (eluent I) and 80% acetonitrile in water (eluent J; gradients: 0–30.5 min, 0–30% J; 30.5–31 min, 30–50% J; 31–34.5 min, 50% J; 34.5–35 min, 0% J). The elution was detected by measuring the UV absorption at 254 nm.

Matrix Assisted Laser Desorption/Ionization Mass Spectrometry

The desalted and lyophilized fractions of the anion-exchange chromatography were examined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (14). A reflectron-type time-of-flight mass spectrometer (Reflex III, Bruker-Franzen, Germany) was used according to Hillenkamp (14). The sample was mounted on an x, y, z movable stage allowing for irradiation of selected sample areas. In the analysis, sinapinic acid (Sigma Chemical Co.) with an emission wavelength of 337 nm and 3-ns pulse duration was used. The laser beam was focused to a diameter typically of 50 μm at an angle of 45° to the surface of the target. Microscopic sample observation was possible via a diachronic mirror in the beam path. 10–20 single spectra were accumulated for a better signal-to-noise ratio. The concentrations of the analyzed substances were 1–10 μM in double-distilled water. 1 μl of the analyzed fraction was mixed with 1 μl of the matrix solution. For this study, a solution of 50 mg/ml 3-hydroxypropionic acid was used. For calibration of the mass spectra, diadenosine hexaphosphate (Ap₆A) was used as external standard. The mixture was gently dried on an inert metal surface before introduction into the mass spectrometer. The mass accuracy was in the range of −0.05%.

UV Spectroscopy

The desalted, lyophilized fractions of the reversed phase chromatography (step 8) were dissolved in water (100 μl). To measure UV spectra at different pH the pH values of the solutions were adjusted to 3.0, 7.0, and 9.0 by 0.1 M HCl and 0.1 M NaOH, respectively. The UV absorbance of the fractions was determined by a UV-visible spectrometer (DU-600, Beckman) at wavelengths between 190 and 400 nm with a scan speed of 400 nm/min.

Platelet Activation by Thrombin and Purification of Dinucleoside Diphosphates AP₆A, AP₃G, GP₂G, and Serotonin from the Supernatant

Three platelet concentrates (each 200 ml; 10⁷ platelets/ml) were suspended in 600 ml of a buffer containing 0.14 M NaCl, 0.15 mM Tris-HCl. To prevent premature activation 0.35% (w/v) albumin was added to the buffer. The resuspended platelet concentrates were divided into three parts.

To test the release of the dinucleoside diphosphates, one aliquot was incubated with thrombin (0.05 units/ml) for 1 min. Preliminary experiments showed that fibrinogen binding in platelets did not exceed 2000 molecules/cell. After stimulation with thrombin, the fibrinogen binding rose 20- to 30-fold. Determination of fibrinogen was performed exactly as described previously (15). Moreover, the concentration of serotonin was determined in the supernatant. As control the second aliquot was not incubated with thrombin.

For purification of dinucleoside diphosphates AP₆A, AP₃G, and GP₂G from the supernatant, platelets were removed by centrifugation (4000 rpm, 4 °C, 10 min). The supernatant was deproteinized with 0.6 M (final concentration) perchloric acid and centrifuged (4000 rpm, 4 °C, 5 min). After adjusting the pH to 7.0 with 5 M KOH the precipitated proteins and KClO₄ were removed by centrifugation (4000 rpm, 4 °C, 5 min). The supernatants of both aliquots of the platelet concentrates were chromatographed according to chromatographic steps for the purification of dinucleoside diphosphates from platelets. Dinucleoside diphosphates AP₆A, AP₃G, and GP₂G were identified by retention time comparison with authentic substances as well as MALDI-MS.

For the measurement of the total endogenous serotonin content, a method described by Hervig et al. (16) was used. Briefly, 600 μl of the platelet concentrate as prepared above was mixed with 200 μl of 2.8 M perchloric acid solution containing dithiothreitol (40 mM) to precipitate the proteins. The precipitate was removed by centrifugation (8000 × g, 2 min), and 520 μl of the supernatant was neutralized with 130 μl of 3 M K₂HPO₄. The precipitated potassium perchlorate was removed by a second centrifugation (8000 × g, 2 min).

The supernatant was transferred and was directly analyzed by the reversed phase chromatographic method of Anderson et al. (17). 100 μl of the supernatant was injected onto a reversed phase column (Supersphere, 210 × 4.1 mm, 4 μm, Merck) eluted with 0.1 M phosphate buffer (pH 4.5) containing 250 μl/liter triethylamine, 150 μg/liter sodium octylsulfate, and 20% (v/v) methanol (flow rate, 0.5 ml/min). The fluorescence was detected using an SPS-920 intelligent fluorescence detector (Jasco) with excitation and emission wavelength settings of 285 and 350 nm, respectively. Quantification of serotonin was done by using a calibration curve.

For the measurement of the released serotonin after thrombin stimulation, a method described by Hervig et al. (16) was used. Briefly, 600
μl of the platelet concentrate as prepared above were incubated with 10 NIH units of thrombin (10 μl) for 10 min. After removing the platelet remnant by centrifugation (8000 × g, 30 s), 450 μl of supernatant was mixed with 150 μl of the perchloric acid/dithiothreitol solution and centrifuged again (8000 × g, 2 min). 400 μl of the supernatant was neutralized with 100 μl of a 3 M solution of K2HPO4, recentrifuged as above, and injected to the chromatography using the method described by Anderson et al. (17).

Synthesis and Chromatography of Authentic \( P_1P_2 \)-dinoside Diphosphates

In contrast to diadenosine diphosphate and diguanosine diphosphate, adenosine guanosine diphosphate was commercially not available. Therefore, synthesis of adenosine guanosine diphosphate was necessary to control the authenticity of the isolated substances. Adenosine guanosine diphosphate was synthesized and chromatographed following a study described elsewhere (18). Briefly, Ap2G was synthesized by mixing AMP (25 mM) and GMP (25 mM) as substrates in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.5 mM), HEPES (2 mM), and magnesium chloride (125 mM). The substances were dissolved in water, thoroughly mixed with a vortex mixer, and incubated at 37 °C at pH 6.5 for 48 h. The reaction mixture was concentrated on a preparative C18 reversed phase column (condition described above). The concentrate was displaced on a reversed phase column (carrier: 40 mM TEAA in water (eluuent A); displacer: 160 mM n-butanol (eluuent B), flow 100 μl/min). As a result of displacement chromatography, anion-exchange chromatography yielded baseline separated dinucleoside diphosphates (Ap2A, Ap2G, and Gp2G).

Commercially available diadenosine diphosphate and diguanosine diphosphate are contaminated with mononucleotides. Therefore, these \( P_1P_2 \)-dinoside diphosphates were purified by displacement chromatography using a reversed phase column (conditions as described above) before testing the authenticity of the isolated substances.

Enzymatic Cleavage Experiments

Aliquots of the fractions containing homogenous nucleotides from the reversed phase chromatography (steps 7 and 8 of the purification procedure), were incubated with enzymes as described in the following. The samples were dissolved: (a) in 20 μl 200 mM Tris buffer (pH 8.9) and incubated with 5′-nucleotide hydrolase (3 milliunits (mU); from Crotalus durissus, EC 3.1.15.1, from Roche Molecular Biochemicals, Germany, purified according to Sulkowski and Laskowski (19) for 9 min at 37 °C; (b) in 20 μl of 200 mM Tris and 20 mM EDTA buffer (pH 7.4) and incubated with 5′-nucleotide hydrolase (1 mU; from calf spleen, EC 3.1.16.1, from Roche Molecular Biochemicals, Germany) for 1 h at 37 °C; and (c) in 20 μl of 10 mM Tris, 1 mM ZnCl2, and 1 mM MgCl2 buffer (pH 8.0) and incubated with alkaline phosphatase (1 mU; EC 3.1.3.1, from calf intestinal mucosa, from Roche Molecular Biochemicals, Germany) for 1 h at 37 °C. The reaction was terminated by an ultrafiltration with a centrifuge filter (exclusion limit, 10 kDa). After filtration of the perchloric acid/dithiothreitol solution and recentrifugation (8000 × g, 2 min), 400 μl of eluent F was subjected to anion-exchange chromatography on a MiniQ column of 20 mm, 10 μm, triethanolamine, stimuli were added and cells were exposed to the stimulating agents for 20 h before 3 μCi/ml [3H]thymidine was added to the serum-free medium. Four hours later, experiments were terminated by aspirating the medium and subjecting the cultures to sequential washes with phosphate-buffered saline containing 1 mM CaCl2, 1 mM MgCl2, 10% trichloroacetic acid, and ethanol/ether (2:1, v/v). Acid-insoluble [3H]thymidine was extracted into 250-μl dishes with 0.5 M NaOH, and 100 μl of this solution was mixed with 5 ml of scintillant (Packard, Ultrimagold) and quantified using a liquid scintillation counter (Beckman LS 3801, Düsseldorf, Germany).

RESULTS

Human platelets were isolated (step 1) and deproteinated with perchloric acid (step 2), and the supernatant nucleotides were growth-arrested in 0.1% FCS for 48 h without affecting cell adhesion to culture wells. Quiescent VSMCs were then exposed to fresh medium and subjecting the cultures to sequential washes with phosphate-buffered saline containing 1 mM CaCl2, 1 mM MgCl2, 10% trichloroacetic acid, and ethanol/ether (2:1, v/v). Acid-insoluble [3H]thymidine was extracted into 250-μl dishes with 0.5 M NaOH, and 100 μl of this solution was mixed with 5 ml of scintillant (Packard, Ultrimagold) and quantified using a liquid scintillation counter (Beckman LS 3801, Düsseldorf, Germany).

Cell Proliferation Assay with Aortic Smooth Muscle Cells

Aortic smooth muscle cells (VSMCs) from normotensive Wistar-Kyoto rats were subcultured in 96-well dishes (Falcon) at a density of 5 × 104 cells/ml and kept in culture medium containing 10% fetal calf serum (FCS) to reach a subconfluent monolayer. After 24 h, the cells were growth-arrested in 0.1% FCS for 48 h without affecting cell adherence to culture wells. Quiescent VSMCs were then exposed to fresh culture medium with 0.1% FCS with and without the tested agonists for another 48-h incubation period. Cell proliferation was measured using the [3H]thymidine incorporation rate as described elsewhere (20). The viability of VSMCs was tested using trypan blue exclusion test. The viability was 95.2 ± 3.5% under control conditions and 93 ± 4.1% after stimulation with dinucleoside diphosphates.

Cell Proliferation Assay with Fibroblasts

Human skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository Institute for Medical Research (Camden, NJ) and cultured over several passages after detachment of the confluent cells with Puck’s Saline A physiological solution (21) containing 0.04%/0.02% EDTA buffer. The cells were allowed to grow as described for VSMCs.

Fibroblasts were seeded in 24-well culture plates and grown to confluence. Then the medium was replaced by serum-free medium consisting of a mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-10 medium (1:1). Following another 24-h cultivation in serum-free medium, stimuli were added and cells were exposed to the stimulating agents for 20 h before 3 μCi/ml [3H]thymidine was added to the serum-free medium. Four hours later, experiments were terminated by aspirating the medium and subjecting the cultures to sequential washes with phosphate-buffered saline containing 1 mM CaCl2, 1 mM MgCl2, 10% trichloroacetic acid, and ethanol/ether (2:1, v/v). Acid-insoluble [3H]thymidine was extracted into 250-μl dishes with 0.5 M NaOH, and 100 μl of this solution was mixed with 5 ml of scintillant (Packard, Ultrimagold) and quantified using a liquid scintillation counter (Beckman LS 3801, Düsseldorf, Germany).

RESULTS

Human platelets were isolated (step 1) and deproteinated with perchloric acid (step 2), and the supernatant nucleotides were growth-arrested in 0.1% FCS for 48 h without affecting cell adhesion to culture wells.
Ap2A, and Gp2G were separated because of hydrophobic interac-
tions. Incubation of the molecule with 3$\text{H}$-nucleotide hydrolase (calf spleen) and alkaline phosphatase.

**TABLE I**

Results of positive-ion PSD-Maldi mass spectra of the isolated fractions (A, adenine; A, adenosine; M, protonated parent ion; p, phosphate group, e.g. Ap$_2$A, adenosine tetraphosphate) (conditions: reflectron-type time-of-flight mass spectrometer (Reflex III, Bruker-
Franzen, Germany); concentration of the analysed substance: 1–10
$\mu\text{mol/l}$; matrix solution: 50 mg/ml 3-hydroxy-picolinic acid; emission
wavelength: 337 nm; pulse duration: 3 ns).

| Fragment ions | Ap$_2$A/Da | Ap$_2$G/Da | Gp$_2$G/Da |
|---------------|------------|------------|------------|
| A'            | 136        | 136        | 134        |
| G' -H$_2$O    | 232        | 152        | 152        |
| A -2H$_2$O    | 250        | 250        | 248        |
| G -2H$_2$O    | 330        | 330        | 346        |
| A -H$_2$O     | 348        | 348        | 364        |
| G -H$_2$O     | 428        | 428        | 444        |
| G -2H$_2$O    | 525        | 525        | 523        |
| M -O$_2$H$_2$O| 542        | 542        | 540        |
| M             | 677        | 677        | 709        |

were concentrated by ion-pair reversed phase chromatography
(step 3). In the following steps, isolation and identification of
dinucleoside diphosphates from human platelets is exemplified
for Gp$_2$G.

After mononucleotides were separated from dinucleotides by
affinity chromatography (13) (step 4) the desalted and lyophil-
eluted eluate (step 5) was fractionated by anion-exchange chro-
matography (step 6). The anion-exchange chromatogram is
shown in Fig. 1A. Although P$_1$P$_2$-dinucleoside diphosphates
have the same charge, P$_1$P$_2$-dinucleoside diphosphates Ap$_2$A,
Ap$_2$G, and Gp$_2$G were separated because of hydrophobic inter-
action between the anion-exchanger and the P$_1$P$_2$-dinucleoside
diphosphate.

Fractions of the anion-exchange chromatography with a signif-
icanent absorbance at 254 nm were separated by reversed
phase chromatography (step 7). In Fig. 1B the chromatogram of
the reversed phase chromatography is given. The substance
eluting at a retention time of 27 min was rechromatographed by
reversed phase chromatography (step 8) using the same
conditions as before (step 7).

In the last chromatographic step (step 8), a single UV peak
was obtained (Fig. 1C). The substance underlying this peak
was identified by the following results: (a) The substance chro-
matographed to homogeneity was analyzed by MALDI-PSD mass spectrometry revealing a molecular mass of 709.4 (Fig. 2A).
Each signal was assigned to a fragment of Gp$_2$G as shown
in Table I. The MALDI-PSD spectrum was completely identical
to that of authentic Gp$_2$G (14). (b) The UV spectrum of guanine
was obtained from the rechromatographed substance, includ-
ing the characteristic shift obtained by acidification to pH 3.0,
7.0, and 9.0 (Fig. 2B; Table II) (22). (c) The retention time of the
isolated fraction in step 8 was identical to that of authentic Gp$_2$G (15).
(d) Cleavage of the molecules with 3'-nucleotide hydrolase
(from C. durissus) yielded GMP, as evidenced by
MALDI mass spectra and by retention times identical with those of authentic Gp$_2$G. The cleavage pattern was identical to
that of synthetic Gp$_2$G. Incubation of the molecule with 3'-
nucleotide hydrolase (calf spleen) and alkaline phosphatase
yielded no cleavage products. The enzymatic cleavage experi-
ments demonstrate that the polyphosphate chain interconnects
the guanosines via phosphoester bonds with the 5'-oxygen of

the riboses (Fig. 2C).

In analogous manner also Ap$_2$A as well as Ap$_2$G were puri-
fied from human platelets and identified by the signal pattern of
the PSD-MALDI-MS fragmentations (Table I), enzymatic cleavage experiments, and UV spectroscopy (Table II).

Ap$_2$A, Ap$_2$G, and Gp$_2$G induced a dose-dependent increase in
DNA synthesis in vascular smooth muscle cells as determined by
[3H]thymidine uptake (Fig. 3). The bar labeled as control in
Fig. 3 represents the [3H]thymidine incorporation in cultures
without the stimuli. The maximum effect of Ap$_2$A was ob-
tained at a concentration of 10$^{-6}$ M, which induced an increase of
vascular smooth muscle cell proliferation of 225.9 ± 66.9% above
control, 168.6 ± 31.0% above control at a concentration of
10$^{-6}$ M for Ap$_2$G, 77.0 ± 13.3% above control at a concentra-
tion of 10$^{-6}$ M for Gp$_2$G, and 1175.0 ± 66.3% above control at
a concentration of 5 × 10$^{-9}$ M for PDGF. These data are means ±
S.E. from 10 independent experiments with 8 cultures. The raw
data of a characteristic series of measurements for each

![Fig. 2. Identification steps of the substance underlying the homoge-
nous fraction labeled in Fig. 1C as Gp$_2$G. A, positive-ion PSD-MALDI
mass spectrum (abscissa: relative mass/charge, m/z; ordinate:
relative intensity). Interpretation of the spectrum is given in Table I. B, UV
spectrum of the fraction (abscissa: wavelength/nm; ordinate: relative
intensity, arbitrary units). C, chromatogram of anion-exchange chro-
matography of the homogenous fraction with 5'-nucleotidase (con-
ditions: Minil PC 3.2/3 Amersham Pharmacia Biotech; eluent F: 10
mM K$_2$HPO$_4$, pH 7.0; eluent G: 20 mM K$_2$HPO$_4$, pH 7.0 with 1 mM NaCl;
gradient: 0–5 min, 0% G; 5–35 min, 0–40% G; 35–37 min, 40–100% G;
flow rate, 30 µl/min). Incubation with 3'-nucleotidase and alkaline
phosphatase yielded no hydrolysis products.

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**Graphical Data**

- **Fig. 1A**: Chromatogram showing the separation of dinucleoside diphosphates.
- **Fig. 1B**: UV spectrum of guanine with characteristic shifts at pH 3.0, 7.0, and 9.0.
- **Fig. 1C**: Single UV peak indicating the substance underlying the chromatogram.
- **Fig. 2A**: MALDI-PSD mass spectrum of Gp$_2$G.
- **Fig. 2B**: UV spectrum reflecting the characteristic shifts of guanine.
- **Fig. 2C**: Cleavage of Gp$_2$G by 3'-nucleotide hydrolase.

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**Legend**

- **A'**: Fragment of guanine.
- **G' -H$_2$O**: Fragment of guanosine.
- **A -2H$_2$O**: Fragment of adenosine.
- **G -2H$_2$O**: Fragment of guanosine.
- **A -H$_2$O**: Fragment of adenosine.
- **G -H$_2$O**: Fragment of guanosine.
- **G -2H$_2$O**: Fragment of guanosine.
- **M -O$_2$H$_2$O**: Fragment of monophosphate.
- **M**: Parent ion of Gp$_2$G.

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**Notes**

1. The enzymatic cleavage and UV spectroscopy results are consistent with the molecular mass determined by MALDI-PSD mass spectrometry.
2. The UV spectrum of guanine shows characteristic shifts at various pH conditions, indicating the presence of the dinucleoside diphosphate in the fraction.
3. The enzymatic cleavage with 3'-nucleotide hydrolase and alkaline phosphatase confirms the integrity of the polyphosphate chain.
dinucleoside diphosphates were the stimulants. Data are means 
6
19,570
6
2
(10
not significantly affect DNA synthesis of cultured fibroblasts
(control: 5,175 
6
S.E.). Characteristic data for 10
2
6 M Ap2A: 16,440 ± 1,621
(control: 5,175 ± 160); 10
6 M Ap2G: 8,453 ± 2,210
(control: 3,199 ± 335); 10
6 M Gp2G: 6,556 ± 429
(control: 3,986 ± 239)
(in cpm/well ± S.E.). Characteristic data for 10
2
2
5 M Ap2A: 178 ± 21; 10
5 M Ap2G: 221 ± 25; 10
5 M Gp2G: 229 ± 22; control: 172 ± 12 (in cpm/well ± S.E.).

After isolation and identification of P1,P2-dinucleoside diphosphates from human platelets, the question arose as to whether P1,P2-dinucleoside diphosphates are released in the extracellular space. Fig. 4 shows the anion-exchange chromatograms of a platelet suspension (condition: column, Mono Q PC 3.2/2. 32 × 2 mm, Amersham Pharmacia Biotech (Sweden); eluent A: 20 mM K2HPO4, pH 8.0; eluent B: 20 mM K2HPO4, 1 mM NaCl, pH 8.0. Gradient: 0–100 min, 0–15% B; 100–160 min, 15–40% B; 160–161 min, 40–100% B; 161–166 min, 100% B; flow rate, 0.5 ml/min) as well as matrix-assisted laser desorption/ionization mass spectrometry.

P1,P2-dinucleoside diphosphates. The intracellular amount of serotonin was 3.2 ± 0.5 attomol/platelet. In the supernatant of unstimulated platelets serotonin was not detectable. After platelet stimulation with thrombin the serotonin amount of supernatant was 2.2 ± 0.4 attomol/platelet, indicating that 68.7 ± 12.6% of the intracellular serotonin amount was released by thrombin stimulation. The comparable degree of secretion of P1,P2-dinucleoside diphosphates and serotonin suggests that both classes of agents are released in a quantitatively similar fashion.

**DISCUSSION**

The findings revealed that P1,P2-dinucleoside diphosphates Ap2A, Ap2G, and Gp2G are endogenous messengers of human platelets.

The results of the cell proliferation assay show that Ap2A, Ap2G, and Gp2G act as potent growth mediators of VSMCs. The maximum effect of Ap2A, Ap2G, and Gp2G on VSMC proliferation rate was about one order of magnitude less, and the threshold concentration was about one order of magnitude higher than for PDGF, indicating that the dinucleoside phosphates are weaker growth factors than PDGF. Nevertheless, it has to be kept in mind that the local concentrations of these nucleotides after platelet aggregation probably are much higher than the physiological PDGF concentrations.

The receptor-mediating vascular growth is not yet known, although a P2 purinoceptor subtype is most likely. Especially,
the P2Y4 purinoceptor may be considered, because ATP and GTP binding to this receptor cause similar mitogenic effects in VSMCs (23). At present, the growth-stimulating effect of dinucleoside diphosphates is only demonstrable in VSMCs. The growth of fibroblasts is not affected by dinucleoside diphosphates. This result may represent a different expression of purinoceptors on VSMCs and fibroblasts.

In contrast to Ap2G and Gp2G with n = 3–6 (7), Ap3A, Ap4A, and Gp2G do not potentiate the growth-stimulating effect of PDGF. Presently it is open to speculation whether this different behavior reflects activation of different purine receptor subtypes.

Because the P1, P2-dinucleoside diphosphates are released upon platelet activation, their growth-stimulating effect may contribute to that of PDGF and other growth mediators released from platelets. Therefore, together with known growth mediators, the described nucleotides may also participate in initiating atherosclerotic lesions.

Obviously, Ap3A, Ap4A, and Gp2G may exert their effects after release by platelet activation as is known for the dia-

denosine phosphates Ap2A, (n = 3–6) (3, 24) and for the ApGs and GpG with n = 3–6 (7).

From the intracellular amount of P1, P2-dinucleoside diphosphates in intact human platelets, the intracellular concentration of P1, P2-dinucleoside diphosphates in intact human platelets can be calculated as 0.1–0.4 mM (volume of a platelet: 5.2 fl (25)). In platelets, two pools of nucleotides have been demonstrated (26). One pool is utilized for the metabolic needs of the platelets. The second pool, the dense granules, is a storage pool, which can be released into the extracellular space. As demonstrated, serotonin and dinucleoside diphosphates Ap2A, Ap3A, and Gp2G are released in parallel, it can be assumed that dinucleoside diphosphates are costored in dense granula with serotonin. The concentration of P1, P2-dinucleoside diphosphates in the dense granula can be estimated to be 0.2–0.8 mM, assuming that 50% of total volume of human platelets constitutes dense granula (27).

The extracellular dinucleoside polyphosphate concentrations occurring after platelet activation depend on the extracellular volume of distribution. The intraplatelet concentrations suggest that, in the close environment of a platelet thrombus, similar dinucleoside polyphosphate concentrations can be found as in platelets. Therefore, the maximum extracellular concentration of P1, P2-dinucleoside diphosphates can be calculated as 0.2–0.8 mM in accordance to the concentration of P1, P2-dinucleoside diphosphates in dense granula. The minimum concentration can be correspondingly estimated as 0.1–0.4 μM in accordance with the concentration of P1, P2-dinucleoside diphosphates after the release into the surrounding blood volume of the platelets. These estimations demonstrate that the extracellular concentrations of P1, P2-dinucleoside diphosphates are sufficient for affecting the rate of proliferation of vascular smooth muscle cells.

How are these substances biosynthesized? The enzymes involved in synthesis of diadenosine polyphosphates are only partially known, and none of the known enzymes are described in human platelets (for review see Ref. 28). Aminoacyl-tRNA synthetases catalyze the formation of ApA and ApA (amino-

cyl-AMP + ADF → ApA, aminoacyl-AMP + ATP → ApA) (29). Adenosine 5′-monophosphate does not react with this enzyme (30), and therefore this type of enzymatic reaction cannot yield ApA. ApA phosphorylases are another class of diadenosine polyphosphate-synthesizing enzymes according to the following reaction ADP + ATP → ApA + P1. (29). Theoretically, the reaction of a diadenosine polyphosphate phosphorylase catalyzing the formation of ApA should be AMP + ADP → ApA + P1. Alternatively, a nonenzymatic synthesis may be considered. Given that mostly mononucleotides such as AMP are found together with biogenic amines such as catecholamines, the coexistence of both nucleotides and amines within the same subcellular localization may allow a nonenzy-
natic reaction generating diadenosine polyphosphates. From AMP and a biogenic amine a phosphoramide may be generated, which is a highly reactive intermediate. A further reaction with another AMP could then yield diadenosine phosphate (ApA). At present no definite answer can be given by which biochemical pathway P1, P2-dinucleoside diphosphates are synthesized in human platelets.

In conclusion, releasable granules of human platelets contain diadenosine polyphosphate (ApA), adenosine guanosine dinucleotide (ApG), and as diadenosine diphosphate (Gp2G), which are potent growth-stimulating mediators in vascular smooth muscle cells.

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