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Research Article

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Regulation of Rat Mesangial Cell Growth by Diadenosine Phosphates

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Abstract

The newly recognized human endogenous vasoconstrictive dinucleotides, diadenosine pentaphosphate (AP_5A) and diadenosine hexaphosphate (AP_6A), were tested for growth stimulatory effects in rat mesangial cells (MC). Both AP_5A and AP_6A stimulated growth in micromolar concentrations. The growth stimulatory effect exceeded that of ATP, α,β-methylene ATP, adenosine 5′-O-(3-thio)triphosphate and UTP. Both diadenosine phosphates potentiated the growth response to platelet-derived growth factor, but not to insulin-like growth factor-1. To further elucidate the site of action in the cell cycle, RNA and protein synthesis were assessed. AP_5 and AP_6A stimulated protein synthesis, but not RNA formation. Furthermore, both agents increased cytosolic free Ca^{2+} concentration. It is concluded that AP_5A and AP_6A may play a regulatory role in MC growth as progression factors and possibly modify MC proliferation in glomerular disease. (*J. Clin. Invest.* 1995. 95:2862–2867.)
Key words: mesangial cells • diadenosine phosphates • PDGF • IGF-1 • cytosolic free Ca^{2+}

Introduction

In inflammatory glomerular diseases and in glomerulosclerosis one of the central features is the proliferation of mesangial cells (MC).[1] The mechanisms of MC proliferation are incompletely understood, but there is a growing body of information linking specific growth factors such as the platelet-derived growth factor (PDGF) or basic fibroblast growth factor to glomerular proliferative diseases (1–3). Glomerular MC can both produce and react to various polypeptide growth factors. Thereby MC activate inflammatory processes and promote MC proliferation, acting both in a paracrine and autocrine fashion (4, 5).

Recently, diadenosine pentaphosphate (AP_5A) and diadenosine hexaphosphate (AP_6A) have been described as novel endogenous vasopressor agents (6). Generally, diadenosine phosphates have been found in prokaryonts, in animals including mammalian organisms and in man (7). In mammals mainly the actions of diadenosine triphosphate and diadenosine tetraphosphate have been characterized. Both compounds are potent coronary and mesenterial vasodilators (8, 9). Furthermore, changes in the intracellular concentration of diadenosine tetraphosphate may be involved in cellular proliferation (10–12). As to the type of receptors, there are divergent findings in literature. Hilderman et al. identified a unique receptor for diadenosine phosphates (13, 14), but on the other hand a subtype of purinergic receptors, the $P2_x$ receptor, may be activated by diadenosine phosphates (15, 16).

Since a number of vasopressor hormones, such as angiotensin II, vasopressin and endothelin, also act as growth factors on vascular smooth muscle cells or MC (17, 18), it was tested in the present study, whether also AP_5A and AP_6A stimulate growth of MC. Furthermore, both dinucleotides were tested for interactions with polypeptide growth factors.

Methods

Animals and culture of glomerular mesangial cells. 3-mo-old male Wistar-Kyoto rats (WKY) were fed a standard pellet diet and water ad libitum. According to previously described methods (17, 19–21) whole glomeruli were isolated by serial sieving of minced renal cortical tissue from blood-free rat kidneys. Intact glomeruli without tubular contamination were digested in collagenase (type CLS4, 184 U/ml) under continuous gentle shaking at 37°C for 30 min according to a modified technique by Striker and Striker (21). This procedure results in digestion of the glomerular basement membrane and in shedding of epithelial cells (17). After repeated centrifugation and washing, primary cultures of glomerular MC were obtained from outgrowths of remnant glomeruli incubated in MC culture medium (CM) supplemented with 15% (vol/vol) fetal calf serum (FCS; Boehringer Mannheim, Germany) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The MC culture medium consisted of RPMI 1640 medium (Biochrom, Berlin, Germany), supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM L-glutamine (GIBCO, Eggenstein, Germany), 1 ml/dl non-essential amino acids, and heat-inactivated (56°C, 30 min) FCS as indicated. Until the third passage the MC culture medium also contained 5 μg/ml bovine insulin, 5 mg/ml transferrin, and 5 ng/ml sodium selenit (22). The medium was changed initially after 24 h and then every 2–3 d. Homogenous MC outgrowths were observed after 5–7 d and reached confluency within 2–3 wk. Pure MC cultures were identified by stellate shaped cells growing in interwoven layers by morphological characterization and positive immunofluorescent staining for myosin, MHC I antigens and extracellular type IV collagen and fibronectin. Staining for myosin was performed with indirect immunofluorescence microscopy using rabbit immunoglobulin (IgG) directed to smooth muscle cell-specific myosin and fluorescein isothiocyanate-conjugated goat IgG to rabbit IgG. These studies showed uniformly strong positive staining of distinct longitudinal fibrils in all observed cells, which is a staining pattern considered to be indicative for MC (20). MC did not stain for rat common leucocyte antigen, cytokeratin, and factor VIII associated antigen, thus excluding the presence of epithelial cells or endothelial cells (21, 23). Confluent monolayers were passaged by washing MC with physiological salt solution (PSS, containing [in mM] NaCl 135, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 5.5, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [Hepes] 10, pH 7.4) with addition of trypsin (0.125% wt/vol) and ethylene diamine tetraacetic acic (0.01% wt/vol) for 5 min at 37°C under gentle agitation before MC culture medium containing

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1. *Abbreviations used in this paper:* CM, culture medium; MC, mesangial cells.

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10% FCS was filled up. The concentration of FCS was reduced to 10% after the second passage and experiments were carried out using MC between the fourth and sixth passage. Cells were free of mycoplasma as determined by repeated staining with 4'6'-diamidine-2'-phenylindole dihydrochloride (Boehringer Mannheim). Prior to each experiment, the medium was changed from 10% to 0.5% FCS for 3 d to induce cellular quiescence, leaving the MC in a resting state, the G_0/G_1 phase (24).

Cell proliferation assays. MC were subcultured in 96-well dishes (Falcon Labware, Cockeysville, MD) at a density of 5×10^4 cells/ml and kept in CM with 2.5% FCS for 24 h to reach a subconfluent monolayer. After that, the cells were growth arrested for 72 h with RPMI 1640 plus 0.5% fetal calf serum without affecting cell adherence to culture wells or viability as checked by trypan blue vital dye exclusion. Quiescent MC were then exposed to fresh CM with 0.5% FCS with and without the tested agonists for another 48 h incubation period. For the last 6 h MC were pulsed with 2 μCi/ml [^{3}H]thymidine (specific activity 5.0 Ci/mmol), washed extensively with medium, trypsinized, and harvested onto filter paper by an automated cell harvester. Incorporated counts were measured by a liquid scintillation counter. Additional experiments for estimation of cell growth were performed by counting MC numbers microscopically and by measuring [^{3}H]thymidine incorporation into acid-precipitable material. For these experiments, MC were seeded out in 24-well culture dishes at a density of 5×10^4 cells/ml and incubated for identical periods as described above. After the last 6 h of [^{3}H]thymidine pulse (2 μCi/ml), the cells were washed with phosphate-buffered saline containing an excess of unlabeled thymidine and solubilized in 1 ml 0.1% sodium dodecyl sulfate (SDS) before bovine serum albumin (BSA; 10 μl, 10%) was added as a carrier protein. Precipitation on ice was performed with 200 μl 20% trichloroacetic acid (TCA) overnight. TCA-precipitable material was pelleted by centrifugation at 2,000 *g*, dissolved in 0.5 M NaOH, and transferred for liquid scintillation counting. All experiments gave results comparable to the aforementioned method.

Determination of cell cycle. The cell cycle of MC was determined by flow-cytometric analysis. Monolayer cultures were trypsinized, centrifuged at 400 *g* for 5 min at 4°C, and the pellet was washed in culture medium without FCS. Further disaggregation with trypsin, enzymatic digestion of RNA, and staining of isolated nuclei with propidium iodide were carried out using a set of reagents (Cycle Test Plus DNA Reagent Kit; Becton Dickinson, Erembodegem, Belgium) according to the instructions of the manufacturer. DNA content was measured using a FACScan® flow cytometer (Becton Dickinson). The percentage of cells in each phase of the cell cycle was estimated with an analysis model calculating the S phase from a rectangle (RFIT model).

Determination of RNA and protein synthesis. Subconfluent MC (5×10^4 cells/ml) were growth arrested in 24-well dishes by culturing them for 72 h in CM with 0.5% FCS. Thereafter, culture supernatants were exchanged by fresh CM with 0.5% FCS with and without agonists for a further 6–48 h culture period. The MC were pulsed during the last 6 h with 2 μCi/ml [^{3}H]uridine (specific activity 27 Ci/mmol) for determination of RNA synthesis or 2 μCi/ml [^{3}H]leucine (specific activity 42 Ci/mmol) for quantitation of protein synthesis, as described earlier (25). After pulsing, the cells were extensively washed with warm phosphate-buffered saline, lysed in 1 ml SDS (0.1%), and transferred after supplementation of 10 μl 10% BSA into an Eppendorf tube for TCA precipitation (200 μl, 20%) overnight on ice. The acid-precipitable material was washed by centrifugation, dissolved in 0.5 M NaOH, and counted using a liquid scintillation counter.

Measurements of cytosolic free calcium concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ in MC was measured using the calcium-sensitive fluorescent dye fura2 according to previously described methods (22, 26, 27) using a Spectrofluorophotometer RF-5001 PC (Shimadzu, Tokyo, Japan) with intracellular calcium measurement software (Shimadzu). Light source was a 150 W xenon lamp with ozone self-dissociation function. At the end of each measurement 1 mM digitonin, then 5 mM ethyleneglycol-bis-(aminoethylether)-tetraacetic acid (EGTA) were sequentially added to determine the maximum (Rmax) and the minimum (Rmin) of the 340/380 nm excitation fluorescence ratio. Control experiments confirmed that further increase of digitonin or EGTA concentration had no effect on Rmax or Rmin, respectively. $[Ca^{2+}]_i$ was calculated according to the equation reported by Grynkiewicz et al. (28). Cytosolic fura2 concentration was estimated to be < 100 μM by comparing the fluorescence of a standard solution of fura2 free acid in the presence of unloaded cells. The autofluorescence of the agonists used was negligible.

Purification of the diadenosine phosphates AP_5A and AP_6A. AP_5A and AP_6A were used after purification of the preparations obtained from Sigma. The HPLC equipment consisted of a L-6200 gradient pump (Merck, Darmstadt, Germany), coupled to a Rheodyne injector (Latek, Heidelberg, Germany) an UV-HPLC detector (Lambda-Max 481; Waters), a two-channel compensation recorder (Pharmacia Biosystems, Freiburg, Germany) and a RediFrac fraction collector (Pharmacia Biosystems, Freiburg, Germany). A Mono Q HR 5/5 (Cl^--form) anion-exchange column from Pharmacia (Freiburg, Germany) and a Lichrospher RP select B reversed phase column (4 × 250 mm) from Merck (Darmstadt, Germany) were used. HPLC-grade water and HPLC-grade acetonitrile were from Baker (Groß-Gerau, Germany). All other chemicals were from Sigma.

10 mg of AP_5A resp. AP_6A, dissolved in 1 ml of eluent A, were purified with an anion-exchange column. Eluent A was made up of 10 mM K_2HPO_4 (pH 8) in water and eluent B of 10 mM K_2HPO_4 (pH 8) with 1 M NaCl. The following gradient was run: 0–11 min: 100% A, 11–21 min: 0–15% B, 21–71 min: 15–40% B. The main UV-absorbing peak (measured at 254 nm) was collected and concentrated to dryness in a Speed-Vac concentrator.

Next the sample was dissolved in 1 ml eluent C (40 mM triethylammonium acetate in water) and chromatographed on a reversed phase column. Elution was performed from 0 to 20 min with 100% eluent C, with a linear gradient in 4 min from 0 to 2% eluent D (80% acetonitrile), in 46 min from 2 to 7% eluent D, in 6 min from 7 to 60% eluent D and in 1 min from 60 to 100% eluent D with a flow-rate of 0.5 ml/min. The main UV-absorbing peak was collected and the amount of the purified dinucleotide was calculated after UV-spectroscopic measurement of the absorbance of the sample. The fraction was divided into fractions containing 1 mg of the dinucleotide and then concentrated to dryness in a Speed-Vac concentrator. The purified samples were analyzed using matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) as described below. After purification, MALDI-MS revealed a single mass peak of 917 and 997 D, which could be attributed to AP_5A or AP_6A, respectively.

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS). MALDI MS was performed as described earlier (6, 29). A reflector-type time-of-flight mass spectrometer, equipped with a nitrogen laser (337 nm, pulselength 4 ns) was used for ion generation and mass analysis.

Reagents. Recombinant human PDGF B/B and IGF-1 expressed by *E. coli* were obtained from Boehringer Mannheim (Germany). Antisera for immunohistochemistry were purchased from Dako (Hamburg, Germany), ICN (Eschwege, Germany) and Sigma. Polyclonal rabbit antibody to PDGF B/B was from Genzyme (lot No. 84654; Cambridge, MA) and administered in an amount of 5 μg/ng PDGF. Tritium-labeled thymidine, uridine, and leucine were from Amersham Buchler (Braunschweig, Germany), fura-2 acetoxymethylester from Calbiochem (La Jolla, CA). α,β methylene adenosine triphosphate (α,β methylene ATP), ATP and uridine triphosphate (UTP) were from Sigma, adenosine 5'-O-(3-thio)triphosphate (ATP γ S) from Boehringer Mannheim.

Statistical analysis. Data are presented as mean±SD. Where error bars do not appear on figures, errors are within the symbol size. Original tracings shown were computed by locally weighted scatterplot smoothing (GraphPad Software Inc., San Diego, CA). Results were tested for statistical significance using analysis of variance (computer software SPSS for windows, Chicago, IL).

Results

Effects of AP_5A and AP_6A on MC proliferation. AP_5A and AP_6A induced a dose-dependent increase in DNA synthesis in

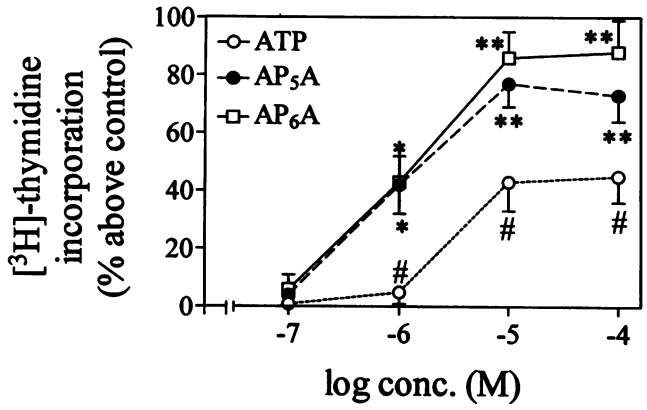

Figure 1. [^{3}H]Thymidine incorporation (in % above control) of mesangial cells stimulated by adenosine triphosphate (ATP), diadenosine pentaphosphate (AP_5A), and diadenosine hexaphosphate (AP_6A). Abscissa: log of concentrations in M. Data are means±SD from five independent experiments with 10 cultures each. $*P < 0.05$; $**P < 0.01$, each compared to control; $^{\#}P < 0.05$ compared with AP_5A and AP_6A.

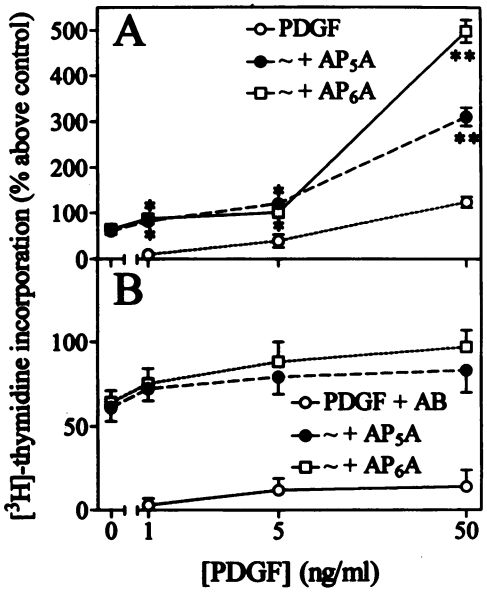

Figure 2. Effect of diadenosine phosphates and platelet-derived growth factor (PDGF) on [^{3}H]thymidine incorporation (in % above control) of mesangial cells. (*A*) Effect of PDGF, PDGF plus 10^{-5} M diadenosine pentaphosphate (+*AP$_5$A*), and PDGF plus 10^{-5} M diadenosine hexaphosphate (+*AP$_6$A*). (*B*) Effect of PDGF plus PDGF antibody (PDGF+AB), PDGF plus PDGF antibody plus 10^{-5} M diadenosine pentaphosphate (+*AP$_5$A*), and PDGF plus PDGF antibody plus 10^{-5} M diadenosine hexaphosphate (+*AP$_6$A*). Abscissa: PDGF concentration. Data are means±SD from four independent experiments with 10 cultures each. $*P < 0.01$; $**P < 0.001$, each compared with PDGF alone.

quiescent MC as determined by [^{3}H]thymidine uptake (Fig. 1). The maximum effect was obtained at diadenosine phosphate concentrations of 10^{-5} M, which induced an increase of MC proliferation 77±8% above control for AP_5A and of 86±9% above control for AP_6A (both $P < 0.01$). Growth responses to AP_5A and AP_6A were similar. Compared with ATP, which is a well characterized mitogenic cofactor for MC, AP_5A and AP_6A induced a significantly elevated MC proliferation at concentrations between 10^{-6} and 10^{-4} M (both $P < 0.05$). Counting MC after application of AP_5A and AP_6A microscopically (Table I) gave similar results for MC proliferation as [^{3}H]thymidine incorporation studies, so that MC growth responses to agonists were documented by [^{3}H]thymidine uptake in the following experiments.

Potentiation of PDGF-induced MC proliferation by AP_5A and AP_6A. Because PDGF is one of the most potent growth factors for MC, we examined AP_5A and AP_6A for cooperative mitogenicity with PDGF. As depicted in Fig. 2 *A*, PDGF B/B exerted a concentration-dependent mitogenic effect on MC between 1 and 50 ng/ml. Together with AP_5A or AP_6A (both 10^{-5} M) an additive effect on MC growth was noted for 1 ng/ml and 5 ng/ml PDGF. At 50 ng/ml PDGF, additional treatment of MC with AP_5A or AP_6A induced a potentiated proliferation (PDGF alone 124±12% increase of proliferation above control; AP_5A 79±8% increase; AP_6A 85±10% increase; PDGF plus AP_5A 311±20% increase; PDGF plus AP_6A 498±25% increase, both $P < 0.001$ compared with PDGF alone). When a PDGF antibody was added together with the diadenosine phosphates and PDGF, the synergistic effect was abolished, and only the growth stimulatory effect of the diadenosine phosphates remained (Fig. 2 *B*). The different action of PDGF and diadenosine phosphates was also reflected in the findings of cell cycle analysis performed after a 18 h culture (Fig. 3). In the presence

Table I. Mesangial Cell Growth after Stimulation by Diadenosine Pentaphosphate and Diadenosine Hexaphosphate (AP_5A and AP_6A) Counted by Light Microscopy

	Mesangial cell counts (*10^4/ml)	
Control	6.9±0.7	
Concentration (M)	AP_5A	AP_6A
10^{-7}	7.2±0.8	7.0±0.6
10^{-6}	8.8±0.4*	8.9±0.7*
10^{-5}	11.1±1.0*	12.3±1.1*
10^{-4}	11.0±1.2*	12.5±1.2*

Data are means±SD (n = 9). * $P < 0.05$ compared with control.

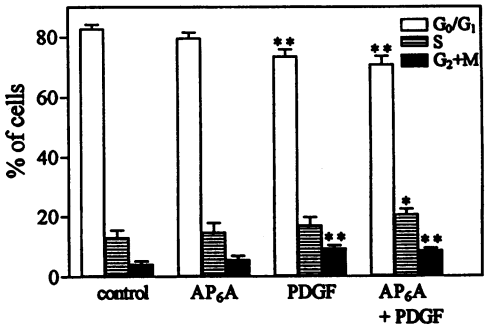

Figure 3. Determination of the cell cycle in mesangial cells, treated with medium (*control*), with 10^{-5} M diadenosine hexaphosphate (*AP$_6$A*), 50 ng/ml platelet-derived growth factor (*PDGF*) and the combination of both (*AP$_6$A + PDGF*) for 18 h. Data are means±SD from five independent cultures. $*P < 0.05$; $**P < 0.01$, compared with control.

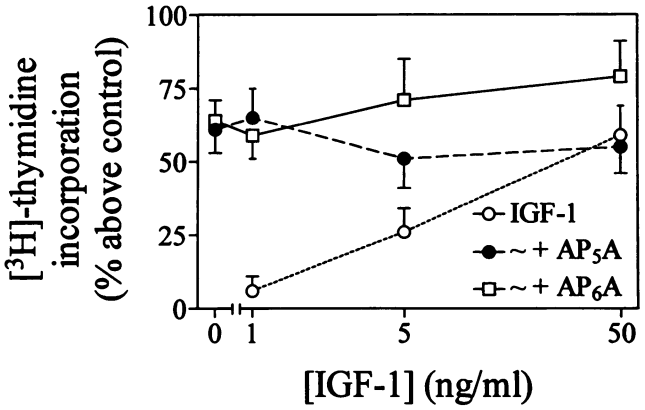

Figure 4. [^{3}H]thymidine incorporation (in % above control) of mesangial cells stimulated by insulin-like growth factor-1 (*IGF-1*), IGF-1 plus 10^{-5} M diadenosine pentaphosphate (*+AP$_5$A*), and IGF-1 plus 10^{-5} M diadenosine hexaphosphate (*+AP$_6$A*). Abscissa: IGF-1 concentration. Data are means±SD from four independent experiments with 10 cultures each.

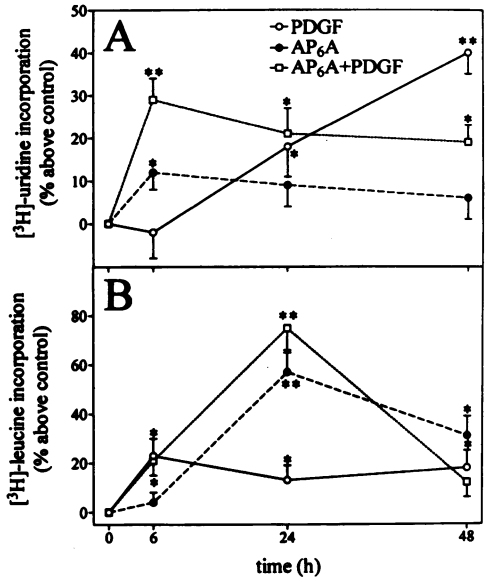

Figure 5. [^{3}H]Uridine incorporation (*A*) and [^{3}H]leucine incorporation (*B*) of mesangial cells stimulated by 10^{-5} M diadenosine hexaphosphate (*AP$_6$A*), 50 ng/ml platelet-derived growth factor (*PDGF*), and PDGF plus AP$_6$A in the above mentioned concentrations over 48 h. Data are means±SD from 12 cultures. $*P < 0.05$; $**P < 0.01$, each compared with control medium.

of 50 ng/ml PDGF, a progression to G_2 and M phase was noted in the initially largely quiescent cells, whereas AP$_5$A (data not shown) or AP$_6$A (each 10^{-5} M) alone did not cause similar changes. By a simultaneous exposure of MC with PDGF and AP$_6$A, progression to the S and G_2/M phase was accelerated compared to PDGF treatment alone.

Effects of IGF-1 in combination with AP$_5$A and AP$_6$A on MC growth. Since PDGF has been shown to act as a competence factor on the initial phase of the cell cycle of MC, we also analysed the cooperation between AP$_5$A or AP$_6$A and the growth factor IGF-1. As shown in Fig. 4, IGF-1 alone exerted a concentration-dependent increase of MC [^{3}H]thymidine uptake between 1 and 50 ng/ml. However, together with AP$_5$A or AP$_6$A (both 10^{-5} M) no additive or synergistic effects of IGF-1 on MC proliferation were observed, and the growth responses were similar to those with AP$_5$A or AP$_6$A alone.

RNA and protein synthesis by AP$_5$A and AP$_6$A. To further characterize the growth stimulatory effects of diadenosine phosphates, besides DNA also RNA and overall protein synthesis in response to AP$_6$A and PDGF were studied. As shown in Fig. 5 *A*, 10^{-5} M AP$_6$A had only minor effects on the overall RNA synthesis. Similar effects were also noted with AP$_5$A (data not shown). In contrast to RNA synthesis, the same concentrations of AP$_6$A induced a significant rise in overall protein synthesis of MC, which exceeded the effect of 50 ng/ml PDGF (Fig. 5 *B*).

Effects of ATP, α,β-methylene ATP, ATP γ S, and UTP in cooperation with AP$_5$A or AP$_6$A on DNA synthesis by MC. As depicted in Fig. 6, ATP, the P_{2x} receptor agonist a,β-methylene ATP, the P_{2y} receptor agonist ATP g S and UTP, each in concentrations of 10^{-5} M, which were maximally effective, exerted a significant increase in DNA synthesis by MC compared with medium control. Compared to the four above mentioned agonists, AP$_5$A and AP$_6$A exerted a significantly elevated growth response of MC at concentrations of 10^{-5} M ($P < 0.05$). Simultaneous treatment with AP$_5$A or AP$_6$A and one of the above mentioned nucleotides did not further enhance the [^{3}H]-thymidine uptake and only gave mitogenic responses similar to those of the diadenosine phosphates alone.

Measurements of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). As shown in Fig. 7, 10^{-5} M AP$_6$A increased $[Ca^{2+}]_i$ by about 100 nM. Significant effects on $[Ca^{2+}]_i$ were also observed with 10^{-6} M. Compared with other agonists such as angiotensin II, AP$_6$A increased $[Ca^{2+}]_i$ less rapidly, but elicited a long-lasting $[Ca^{2+}]_i$ elevation, which persisted for ~ 5 min. AP$_5$A elicited similar effects as AP$_6$A (Fig. 7 *B*). In Ca^{2+} free medium, AP$_5$A and AP$_6$A did not significantly affect $[Ca^{2+}]_i$ (data not shown).

Discussion

The results show that both AP$_5$A and AP$_6$A potently stimulate the growth of MC and potentiate the effect of PDGF. It is not

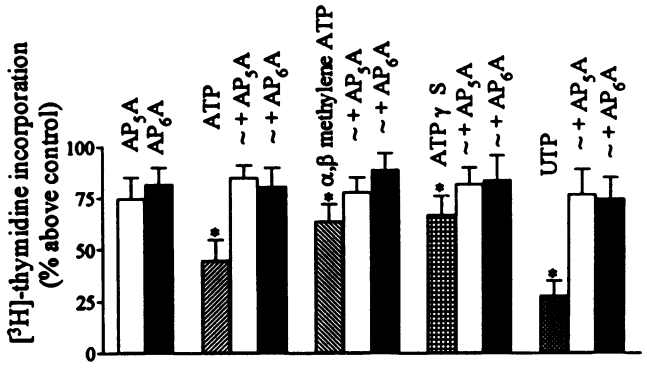

Figure 6. [^{3}H]Thymidine incorporation of mesangial cells stimulated by 10^{-5} M diadenosine pentaphosphate (AP$_5$A), 10^{-5} M diadenosine hexaphosphate (*AP$_6$A*), 10^{-5} M adenosine triphosphate (*ATP*), 10^{-5} M α,β-methylene ATP, 10^{-5} M adenosine 5′-O-(3-thio)triphosphate (ATP g S) and 10^{-5} M uridine triphosphate (*UTP*), each alone and together with 10^{-5} M AP$_5$A or AP$_6$A (*~ +AP$_5$A*, *~ +AP$_6$A*). Data are means±SD from three independent experiments with 10 cultures each. $*P < 0.05$ compared with the effect of additional administration of AP$_5$A or AP$_6$A and of AP$_5$A or AP$_6$A alone.

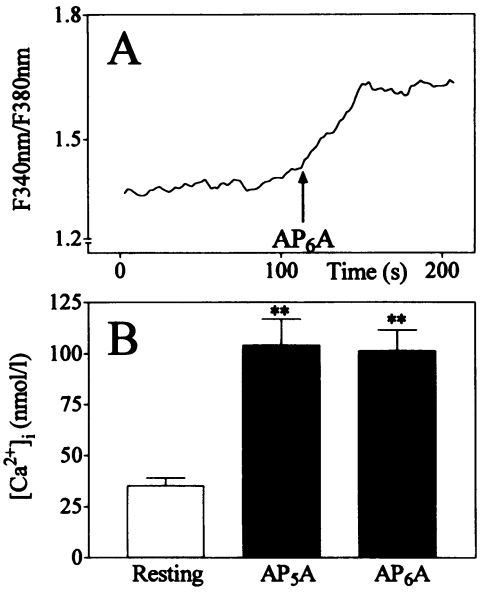

Figure 7. Effect of 10^{-5} M diadenosine pentaphosphate (AP_5A) or diadenosine hexaphosphate (AP_6A) on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). (*A*) Representative tracing out of 22 similar experiments showing the effect of 10^{-5} M AP_6A on $[Ca^{2+}]_i$. Ordinate: ratio of fluorescence with 340 nm and 380 nm excitation (F340 nm/F380 nm). (*B*) Resting $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ induced by AP_5A or AP_6A (each 10^{-5} M). Data are means±SD (n = 22 each). $^{**}P < 0.01$ compared with resting $[Ca^{2+}]_i$.

clear which receptors are activated by the diadenosine phosphates. Apparently, similar effects on growth are elicited by both α,β methylene ATP and ATP γ S, selective agonists of the P_{2x} and P_{2y} subtype of purinergic receptors (30). However, both agonists stimulated growth in a less degree compared with the diadenosine phosphates. This finding suggests that the diadenosine phosphates do not selectively activate either the P_{2x} or the P_{2y} receptor subtype. Furthermore, there are important differences between the growth stimulatory effects of diadenosine phosphates and ATP, which is known to stimulate growth in both MC and vascular smooth muscle cells (31–33). The maximal growth stimulatory effect of diadenosine phosphates exceeds that of ATP, suggesting that different receptors may be activated by diadenosine phosphates and ATP. Possibly, the selective diadenosine phosphate and ATP. Possibly, the selective diadenosine phosphate receptor described by Hilderman et al. in brain and myocardial tissue (13, 14) is the primary target of these agents.

AP_5A and AP_6A have been found in human platelets (6) and may also occur in other human tissues. Therefore the growth stimulatory effects of these substances could play a role in the development of glomerular damage in human disease such as glomerulonephritis or glomerulosclerosis. In addition to the action of polypeptide growth factors such as PDGF or basic fibroblast growth factor (5, 34), the diadenosine phosphates locally released by platelets could stimulate MC proliferation. Compared with ATP, the diadenosine phosphates are more potent growth factors, and from estimations of their concentrations in platelets, micromolar concentrations could be achieved by platelet aggregation (6). It has been shown that AP_5A and AP_6A are released nearly completely by thrombin-induced platelet aggregation (35).

Besides the growth stimulatory effect, both AP_5A and AP_6A potentiate the effect of PDGF, but not of IGF-1, on MC growth. Since PDGF may play a pivotal role in MC proliferation in response to glomerular damage, the synergistic effect of diadenosine phosphates may also be of importance. It may be speculated that the local concentration of diadenosine phosphates may modulate the effects of PDGF. Thus, the local diadenosine phosphate concentration could also indirectly determine mesangial cell proliferation in glomerular disease.

Interestingly, the effect of IGF-1 is not potentiated by AP_5A and AP_6A. This suggests that diadenosine phosphates and IGF-1 have the same site of action in the cell cycle as progression factors. It is known that IGF-1 stimulates the progression of late G_0/G_1 to the initiation of DNA synthesis, which requires protein synthesis, but not de novo RNA formation (36, 37). Similarly, diadenosine phosphates exerted much stronger effects on protein compared with RNA synthesis of MC. In contrast, the competence factor PDGF stimulated DNA synthesis in the initial phase of the cell cycle, before the diadenosine phosphates were active. The different mechanisms of growth stimulation may explain, why the action of PDGF, but not of IGF-1, can be potentiated by diadenosine phosphates.

The results furthermore show that diadenosine phosphates increase $[Ca^{2+}]_i$ in MC. In various cell types, it has been proposed that cellular Ca^{2+} handling and proliferation are closely linked (38). However, it is known that some growth factors stimulate growth without changing $[Ca^{2+}]_i$ (39). Therefore the changes in $[Ca^{2+}]_i$ induced by diadenosine phosphates may be more important in the regulation of MC contraction than of MC growth.

In summary, AP_5A and AP_6A, newly recognized endogenous vasoconstrictive dinucleotides, may play a role in the regulation of MC growth under physiological and pathological conditions.

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