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EXTRACELLULAR ATP PROMOTES CELLULAR GROWTH OF GLOMERULAR MESANGIAL CELLS MEDIATED VIA PHOSPHOLIPASE C

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SUMMARY: The present study was undertaken to determine whether extracellular ATP promotes cellular growth of glomerular mesangial cells. ATP increased inositol 1,4,5-trisphosphate (IP_3) production and cellular free calcium concentration ($[Ca^{2+}]i$) in a dose-dependent manner. None of ADP, AMP or adenosine caused an increase in IP_3 production or $[Ca^{2+}]i$ mobilization. Also, ATP activated mitogen-activated protein (MAP) kinase and ^3H-thymidine incorporation and increased the absorbance by colorimetric assay in a dose-dependent manner. Again, either of ADP, AMP or adenosine had no effect. These results indicate that extracellular ATP binds to P_2 purinergic receptors and activates phospholipase C in glomerular mesangial cells. Such a signal transduction promotes cellular growth of mesangium. © 1994 Academic Press, Inc.

Intracellular nucleotides play fundamental roles in energy metabolism, nucleic acid synthesis and enzyme regulation. Also, extracellular ATP has been known to exert significant biological actions on various kinds of cells (1 - 4). In glomerular mesangial cells and homologous cells of vascular smooth muscle extracellular ATP binds to ATP-sensitive receptors, P_2 purinergic receptors and activates phospholipase C, resulting in a breakdown of phosphatidylinositol and a mobilization of cellular free calcium ($[Ca^{2+}]i$) (5 - 7).

Various vasoconstrictor hormones and cytokines have been clarified to promote cellular growth of glomerular mesangial cells and vascular smooth muscle cells, as well as cell contraction (8 - 10). Their mitogenic effects are now focused in physiological and pathophysiological conditions. The fact that ATP potentiates the actions of several growth factors in fibroblast cells (11, 12) raises the possibility that it might have a mitogenic action.

The present study was undertaken to determine whether extracellular ATP promotes cellular growth of glomerular mesangial cells. We therefore studied whether ATP activates mitogen-activated protein (MAP) kinase activity and thymidine incorporation in the cultured rat glomerular mesangial cells.

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MATERIALS AND METHODS

Cell culture

The experimental procedure was similar to that described in our previous report (13), modified from the method of Kreisberg and Karnovsky (14). Male Sprague-Dawley rats weighing 150 - 175 g were used. Kidneys were removed under sterile condition, and cortical tissues were cut away from the medulla. They were minced with physiological saline solution (PSS: 140 mM NaCl, 4.6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4) by a sharp razor blade, and then the minced renal cortical tissues were incubated with 3 ml PSS containing collagenase (1 mg/ml; Worthington Biochemicals, Freehold, N. J.) for 60 min at 37°C. After centrifuging the tubes at 500 g for 4 min at room temperature, the pellets were resuspended with Dulbecco's Modified Eagle's Medium (Flow Laboratories, McLean, VA) supplemented with 20% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The dispersed glomeruli were harvested into 35x10-mm plastic dishes with the medium and kept in a humidified incubator at 37°C under 95% air and 5% CO_2.

After the cultured cells were confluent, they were subcultured using Ca^{2+}-free and Mg^{2+}-free Hank's solution containing 0.025% trypsin and 0.01% EDTA. The dispersed cells were collected into culture tubes and centrifuged at 500 g for 5 min at room temperature. The pellets were resuspended in Dulbecco's Modified Eagle's Medium containing 20% fetal bovine serum, penicillin and streptomycin and cultured in a humidified incubator. The culture cells at 3-10th passages were subjected to the following studies on days 7 - 10 of the subculture. For measurement of $[Ca^{2+}]i$ the cells were cultured on thin glass slides (13-mm in diameter; Matsunami Kogyo Co., Osaka, Japan). The cells were grown on 35x10-mm dishes to study inositol 1,4,5-trisphosphate (IP_3) production and MAP kinase activity. Also, the cells were cultured on 24-well tissue culture clusters to measure ^3H-thymidine incorporation.

Measurement of $[Ca^{2+}]i$

The experimental procedure was similar to that used in our previous studies (15, 16). The cells were rinsed twice with 1 ml PSS and loaded with 5 µM fura-2/AM (Dojin Biochemicals, Kumamoto, Japan) in a volume of 0.25 ml for 60 min at 37°C. After aspiration of the fura-2/AM solution, the glass slide was rinsed and then placed in a 1x1-cm quartz cuvette with the aid of a special holder in a fluorescence spectrophotometer (CAF-100, Japan Spectroscopic Co., Tokyo, Japan). The dual wavelength excitation method for measurement of fura-2 fluorescence was used. The fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in the ratio mode. The effectors were added after a stable fluorescence signal (R) was achieved. ATP, ADP, AMP and adenosine were used. From the ratio of fluorescence at 340 and 380 nm, the $[Ca^{2+}]i$ was determined as described by Grynkiewicz et al (17), using the following expression: $[Ca^{2+}]i$ (nM) = Kd x [(R - Rmin)/(Rmax - R)]x β, where R is the ratio of fluorescence of the sample at 340 and 380 nm, and Rmax and Rmin were determined by treating the cells with 5 x 10^{-5} M digitonin and 1 x 10^{-2} M $MnCl_2$, respectively. The term β is the ratio of fluorescence of fura-2 at 380 nm in zero and saturating Ca^{2+} concentrations. Kd is the dissociation constant of fura-2 for Ca^{2+}, assumed to be 224 nM at 37°C (17).

Measurement of IP_3

The experimental procedure was similar to that described in the previous study (10, 18). The cells were washed twice with 2 ml of inositol-free Dulbecco's Modified Eagle's Medium and incubated with 1 ml inositol-free Dulbecco's Modified Eagle's Medium containing 5 µCi/ml myo-^3H-inositol (specific activity, 19.1 Ci/mmol, Amersham, Buckinghamshire, U. K.) in a humidified incubator for 24 hrs. At the time of the experiment, the cells were rinsed twice with 2 ml PSS and then incubated with 0.8 ml PSS containing 1 x 10^{-6} - 1 x 10^{-4} M ATP, ADP and AMP for 10 sec. The reaction was stopped by the addition of 0.2 ml 50% trichloroacetic acid, and then the cells were collected using Eppendorf chips. The suspensions containing the disrupted cells were centrifuged using a microcentrifuger. The supernatants were washed 5 times with ether and the water-soluble fractions were brought to pH 7.0 using 1N NaOH and stored at -20 °C until the analysis. The pellets were dissolved with 1 ml 0.1N NaOH containing 1% SDS and stored at 4°C for protein assay. The water-soluble fractions were applied to columns containing 1 ml of Dowex (1-x8, formate form, Muromachi Kagaku, Tokyo, Japan) and serially eluted ten times with 2-ml aliquots of H_2O, Borax (5 mM disodium tetraborate, 60 mM sodium formate), 0.2, 0.4 and 1.0 M ammonium formate in 0.1 M formic acid. This maneuver separated inositol, glycerophosphatidylinositol, inositol-1-phosphate, inositol bisphosphate and IP_3, respectively. IP_3 fractions include 1,3,4-IP_3, 1,4,5-IP_3 and 1,3,4,5-inositol tetrakisphosphate. Samples were collected into scintillation counter vials and counted using a liquid scintillation counter (Aloka LSC-671, Tokyo, Japan).

MAP kinase assay

The experimental procedure was similar to that described in our previous study (10). Cells grown on 35x10-mm plastic dishes were incubated with serum-free Dulbecco's Modified Eagle's Medium 24 hrs before the start of experiments. The cells were rinsed twice with 2 ml PSS. The cells were incubated for 10 min with 1 ml ATP, ADP, AMP, adenosine or phorbol-12-myristate-13-acetate (PMA; Sigma). After aspiration of the effector solutions, the cells were exposed to 0.5 ml of the solution containing 20 mM Tris, 5.6 mM β-glycerophosphate, 10 mM EGTA, 10 mM $MgCl_2$, 0.1 mM NaF, 2 mM DTT, 1 mM $NaVO_3$, 20 ug/ml aprotinin and 1 mM PMSF, pH 7.5. Thereafter, the dishes were immediately put on dry ice. The extracts were collected into microcentrifuge tubes by using Eppendorf chips. After centrifugation, the supernatants were transferred to plastic tubes and kept at -20°C until the time of assay.

Glass tubes containing 60 ul of the assay mixture [40 mM Tris, 40 mM $MgCl_2$, 2.5 mg/ml myelin basic protein (Sigma), 0.5 mM ATP and 0.5 uCi ^{32}P-γATP (specific activity, >10 Ci/mmol; Amersham) were incubated at 25°C for 15 min. Then 40 ul of samples were added and the mixtures were incubated for an additional 10 min at 25°C. The mixtures were transferred on glass microfiber filters (Whatman 2.4-cm GF/C; Whatman, Maidstone, England) by using Eppendorf chips. The filters were then put into ice-cold 10% trichloroacetic acid containing 50 mM sodium pyrophosphate, and shaked gently for 20 min. This maneuver was repeated 4 times. The filters were immersed in ice-cold ethanol for 20 min. After the filters were exposed to diethylether, they were put into counting vials containing 10 ml scintillation solution. The radioactivity was counted by using Aloka liquid scintillation counter. Also, cellular protein was measured by the method of Lowry et al (19).

Measurement of thymidine incorporation

The experimental procedure was similar to that described previously (10). At confluency, the cells were synchronized to the quiescent state by incubation in 1 ml serum-free Dulbecco's Modified Eagle's Medium. After 24 hrs, cells were exposed to the varying stimuli which were dissolved in the serum-free Dulbecco's Modified Eagle's Medium. The medium contained ^{3}H-thymidine (1 µCi/well; specific activity, 80.8 Ci/mmol; New England Nuclear, Wilmington, DE). The stimuli included ATP, ADP, AMP and adenosine. The cells were incubated with 1 ml of the effector for an additional 48 hrs in the humidified incubator. Thereafter, the cells were rinsed four times with PSS and immersed with 0.5 ml of 0.1N NaOH containing 1% sodium dodecylsulfate. They were collected into counting vials by Eppendorf chips and then added 10 ml scintillation solution. The radioactivity was measured using a liquid scintillation counter. Cells grown on several wells of 24-well tissue culture clusters were collected in a same manner and protein contents were measured by the method of Lowry et al (19).

Colorimetric MTT assay

Cell growth was estimated by a modified version of the colorimetric MTT assay of Mosmann (20, 21). The culture cells were suspended at a number of 4 x 10^4/ml and 50 µl of the suspension was plated into a 96-well tissue culture cluster. After cells were incubated in a humidified incubator for 72 h, the stimulants were added and further the cells were incubated for an additional 48 h. 20 µl of a sterilized 5 mg/ml MTT (Sigma) solution was added to each culture well. After 5 h of incubation, 100 ul of 10% sodium dodecylsulfate solution was added to the wells and mixed thoroughly to dissolve the dark blue crystals. Plates were read using a microplate reader (model 3550; Bio-Rad) at a wavelength of 595 nm.

Statistics

All values of IP_3, $[Ca^{2+}]i$, MAP kinase activity, ^{3}H-thymidine incorporation and absorbance at OD 595 nm were analysed by a multiple variance and Student's t-test. A P value less than 0.05 was considered significant.

RESULTS

Figure 1 shows the effect of ATP on $[Ca^{2+}]i$ in the cultured rat glomerular mesangial cells. ATP increased $[Ca^{2+}]i$ in a dose-dependent manner. When the cells were exposed to 1 x 10^{-4} M ATP, $[Ca^{2+}]i$ rised to 479.1 $\pm$ 42.0 from 113.1 $\pm$ 5.9 nM ($P<0.01$). However, there was no change in $[Ca^{2+}]i$ in response to ADP, AMP or adenosine (Table 1).

ATP-induced increase in IP_3 production in the cultured rat glomerular mesangial cells is shown in Fig. 2. ATP produced IP_3 in a dose-dependent manner. IP_3 increased to 8083 $\pm$ 292

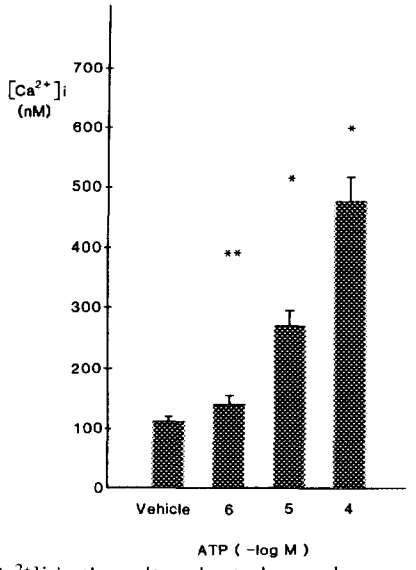

Fig. 1. ATP increases $[Ca^{2+}]i$ in the cultured rat glomerular mesangial cells. *P<0.01 and **P<0.05 vs the vehicle. Values are means ± SEM, n = 6.

from 3828 ± 261 CPM/mg protein when the cells were treated with 1 x 10^{-4} M ATP. Either of ADP or AMP did not cause an increase in IP_3 production (Table 1).

Figure 3 shows the activation of MAP kinase in the cultured rat glomerular mesangial cells. As shown in Figs. 1 and 2, ATP also dose-dependently activated MAP kinase. The 1 x 10^{-4} M ATP-activated MAP kinase was apparently similar to that activated by 1 x 10^{-6} M PMA. However, ADP, AMP and adenosine had no effect on MAP kinase activity (Table 1).

Table 1. Effects of ADP, AMP and adenosine on IP_3 production, $[Ca^{2+}]i$ mobilization, MAP kinase activation and ^3H-thymidine incorporation in the cultured rat glomerular mesangial cells

	n	Vehicle	1 x 10^{-4} M ADP	1 x 10^{-4} M AMP	1 x 10^{-4} M Adenosine
IP_3 (CPM/mg protein)	3	3828±261	4267±749	4605±712	
$[Ca^{2+}]i$ (nM)	6	113.1±5.9	107.8±7.3	106.8±8.9	104.8±9.2
MAP kinase (pmol/mg protein)	6	13.4±0.6	16.5±0.3	14.4±0.5	13.9±0.7
^3H-thymidine incorporation (x10^4 CPM/ mg protein)	4	5.15±0.39	5.65±0.38	6.60±0.27	5.96±0.45

Values are means ± SEM.

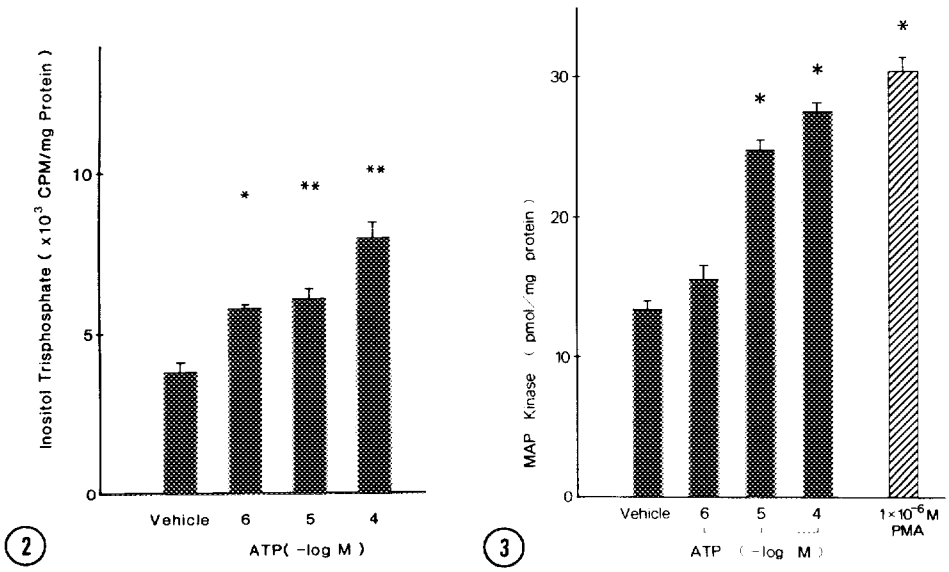

Fig. 2. ATP stimulates IP_3 production in the cultured rat glomerular mesangial cells. *P<0.05 and **P<0.01 vs the vehicle. Values are means ± SEM, n = 3.

Fig. 3. Activation by ATP and PMA of MAP kinase in the cultured rat glomerular mesangial cells. *P<0.01 vs the vehicle. Values are means ± SEM, n = 6.

Fig. 4 shows the stimulation by ATP of ^{3}H-thymidine incorporation in the cultured rat glomerular mesangial cells. ATP dose-dependently increased ^{3}H-thymidine incorporation, but ADP, AMP or adenosine did not (Table 1). 1 x 10^{-4} M ATP caused a 1.7-fold increase in ^{3}H-thymidine incorporation.

MTT incorporation was performed in the presence of various concentrations of ATP. ATP significantly increased the absorbance at OD 595 nm (vehicle, 0.35 ± 0.02; 1 x 10^{-7} M ATP, 0.44 ± 0.01; 1 x 10^{-6} M ATP, 0.45 ± 0.01; 1 x 10^{-5} M ATP, 0.48 ± 0.01; 1 x 10^{-4} M ATP, 0.51 ± 0.03, n = 4, P<0.01), but none of ADP, AMP or adenosine stimulated cell proliferation (1 x 10^{-4} M ADP, 0.38 ± 0.03; 1 x 10^{-4} M AMP, 0.38 ± 0.01; and 1 x 10^{-4} M adenosine, 0.40 ± 0.02, n = 4, N. S.).

DISCUSSION

Extracellular ATP at micromolar concentrations stimulated the hydrolysis of phosphatidylinositol and the mobilization of $[Ca^{2+}]i$ in rat glomerular mesangial cells in culture. However, extracellular ADP, AMP or adenosine did not affect IP_3 formation and $[Ca^{2+}]i$ mobilization. The action of ATP is mediated through ATP-sensitive receptors, which are most likely to belong to the superfamilies of G protein-coupled receptors, termed P_{2y} or P_{2u} purinergic receptors (7). Otherwise, it is known that there are two other ATP receptors, namely P_{2x} and P_{2z} purinergic receptors. They couple to the ligand-gated ion channels and cause the depolarization of plasma membrane, producing cellular Ca^{2+} influx (7).

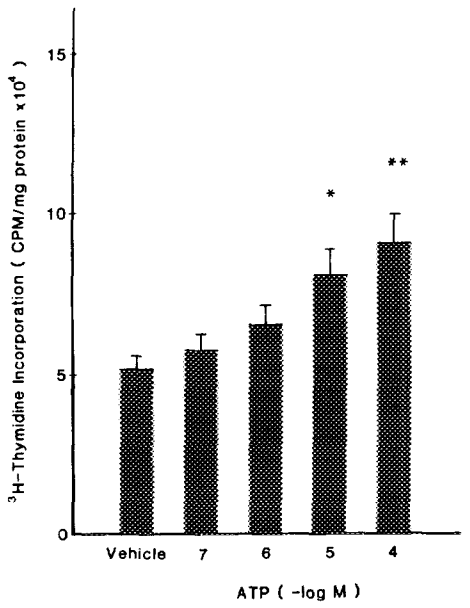

Fig. 4. Stimulation by ATP of ^{3}H-thymidine incorporation into the cultured rat glomerular mesangial cells. *P<0.05 and **P<0.01 vs the vehicle. Values are means ± SEM, n = 4.

The present study further demonstrated that ATP accelerated cellular growth, which was determined by thymidine incorporation, MAP kinase activation and colorimetric assay. ATP stimulated ^{3}H-thymidine incorporation and MAP kinase activation in a dose-dependent manner. However, none of ADP, AMP or adenosine had a significant induction of ^{3}H-thymidine incorporation and MAP kinase activation. Therefore, ATP-induced these two parameters are also dependent on P_2 purinergic receptors. MAP kinase is a downstream of protein kinase C in cellular signal transduction (22). In fact, in glomerular mesangial cells, PMA, an activator of protein kinase C, markedly activated MAP kinase. We reported that a vasoactive hormone arginine vasopressin also produces cellular growth in glomerular mesangial cells, which depends on V_1 receptors and phosphatidylinositol metabolism (10). This is supported by the finding that MAP kinase is not activated by AVP in renal papillary collecting tubule cells where the action of AVP is mediated via V_2 receptors and cAMP pathways (unpublished observation). These results indicate that extracellular ATP binds to P_2 purinergic receptors and activates phospholipase C in glomerular mesangial cells. Such a signal transduction promotes cellular growth of mesangium.

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