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Nucleotide receptors in hepatic stellate cells of the rat

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Abstract When hepatic stellate cells were stimulated by UTP, ATP, or ADP, cellular levels of inositol phosphates significantly increased (UTP > ATP > ADP > 5′-*O*-(3-thiotriphosphate). Thirty min after incubation with 100 μM of UTP, ATP, or ADP, levels of inositol monophosphate increased to 1318 ± 116, 616 ± 87 and 591 ± 234% of control levels, respectively, with concomitant increase in the production of inositol trisphosphate and bisphosphate. These nucleotides transiently increased the $[Ca^{2+}]_i$ of fura-2-loaded stellate cells. Moreover, UTP, ATP, ADP and adenosine 5′-*O*-(3-thiotriphosphate) were able to induce contraction of stellate cells as detected using the silicone-rubber membrane method. These results suggested that hepatic stellate cells have nucleotide receptors which react predominantly with extracellular UTP and ATP and trigger the receptor-mediated contraction of the cells.

Key words: Stellate cell; Sinusoidal microcirculation; Nucleotide; Nucleotide receptor; Vasoconstriction

1. Introduction

Recent studies revealed that stellate cells (fat-storing Ito cells) play important roles in the pathogenesis of liver disease. These cells have retinyl palmitate-enriched lipid droplets and deliver retinol to extrahepatic tissues; they secrete extracellular matrix proteins and play a role in the pathogenesis of the liver fibrosis caused by in chronic hepatitis [1,2]. Since stellate cells have contractile desmin [3] and a-smooth-muscle actin [4] and encompass the external surface of the sinusoidal endothelial cells with their long cytoplasmic processes [5–7], contraction of these cells has been postulated to play a role in the regulation of sinusoidal microcirculation [8–11]. In fact, when stimulated by vasoactive compounds, such as eicosanoids [8,9] and endothelin-1 [10], cultured stellate cells undergo contraction.

Extracellular nucleotides are potent modulators of cardiovascular functions [12–14]. Some nucleotides increase the portal vein pressure [15] and affect metabolism in hepatic constituent cells [16]. Since ATP and related nucleotides are released from various cells, such as platelets and neurons, these nucleotides may bind to cell surface receptors and function as paracrine-like ligands. In fact, some cells have P2 type purinoceptors [12–14] with high affinity for these nucleotides; interaction of nucleotides with P2 receptors enhances the degradation of inositol phospholipid thereby generating second messengers, such as Ins*P*3 and diacylglycerol, which trigger Ca^{2+} mobilization and activate protein kinase-C [17,18].

To elucidate the role of extracellular nucleotides in hepatic sinusoidal cells, the effects of ATP and related nucleotides on perisinusoidal stellate cells were studied in vitro. The present report describes the binding of some nucleotides to nucleotide receptors on cultured stellate cells, which then increase the intracellular calcium concentration, enhance the metabolism of inositol phosphates, and induce contraction of these cells.

2. Materials and methods

2.1. Chemicals

ATP, ADP, AMP, adenosine, UTP, adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), α,β-methyleneadenosine 5′-triphosphate (AMP-CPP) and dimethylpolysiloxane (60,000 c.p. and 12,500 c.p.) were purchased from Sigma (St. Louis, MO). UDP, UMP, uridine, fura-2 acetoxymethylester (fura-2/AM), and collagenase were from Wako Pure Chemical Co. (Osaka). CTP and GTP were from Yamasa Co.(Chiba). 2-Methylthioadenosine 5′-triphosphate (2-MeSATP) was from Research Biochemicals Inc. (Natick, MA). Pronase E and DNase were from Merk (Darmstadt, Germany) and Boehringer Mannheim (Mannheim, Germany), respectively. *myo*-[2-^{3}H]Inositol (19 Ci/mmol) was from Amersham (Bucks., UK).

2.2. Preparation of stellate cells

Male Wistar rats, 450–500 g, were obtained from SLC (Shizuoka) and fed laboratory chow and water ad libitum. Stellate cells were prepared from the liver as described [8]. Briefly, the liver was perfused with a Ca^{2+}/Mg^{2+}-free Krebs–Ringer (KR) solution for 10 min at 37°C (10 ml/min) followed by collagenase digestion for 30 min at 37°C. The digested liver was excised, cut into small pieces and incubated in KR solution containing 0.1% pronase E and 20 μg/ml of DNase for 30 min at pH 7.3. The resulting suspension was filtered through a nylon mesh (150 μm in diameter). After centrifugation of the filtrate on an 8.2% Nycodenz cushion (1,400 × *g*, 4°C, 20 min), a stellate-cell-enriched fraction was obtained. The cells in the upper-whitish layer were washed by centrifugation (400 × *g* at 4°C for 10 min) and cultured in DMEM (GIBCO) supplemented with 10% fetal-bovine serum (Flow) and antibiotics (10^5 U/l penicillin G and 100 mg/l streptomycin) used for experiments.

2.3. Measurement of [^{3}H]inositol-phosphates

Stellate cells cultured in cell-culture plates (FALCON 3046) were radiolabeled with *myo*-[2-^{3}H]inositol (2 μCi/ml) for 48 h, washed three times with HEPES-PBS and incubated in the same buffer supplemented with 10 mM LiCl, 0.1% bovine serum albumin and 10 mM glucose for 10 min at 37°C. Reactions were started by adding various agents. At varying times after incubation, 2 ml of 5% trichloroacetic acid was added to each well. Trichloroacetic acid extracts were then washed four times with 5 ml each of diethyl ether. After adjusting the pH to 7.2, [^{3}H]Ins*P* species were separated by using 0.5 ml Bio-Rad AG1-X8 resin as described by Berridge et al. [19].

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Abbreviations: Ins*Ps*, total inositol phosphates; Ins*P*1, inositol 1-monophosphate; Ins*P*2, inositol (1,4)-bisphosphate; Ins*P*3, inositol (1,4,5)-trisphosphate; AMP-CPP, α,β-metyleneadenosine 5′-triphosphate; ATP-γ-S, adenosine 5′-*O*-(3-thiotriphosphate); DMEM, Dulbecco's modified Eagle's medium.

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2.4. Measurement of cytosolic Ca^{2+} using fura-2

Cells grown to confluent monolayers were loaded with fura-2 by incubation with 5 μM fura-2/AM for 120 min at 37°C. The fura-2-labeled cells were then removed from the culture dishes with 0.05% trypsin/EDTA. The cells were suspended in phosphate-buffer solution (pH 7.4) and fura-2-derived fluorescence was analyzed by a thermostated fluorescence spectrophotometer (Hitachi, F-2000) at 37°C. The concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was calculated as described [20].

2.5. Detection of cell contraction by silicone rubber membrane method

Silicone-rubber sheets were prepared by a modification of the method of Harris et al. [8,21]. Isolated stellate cells were plated on silicone rubber sheets containing 1.5 ml culture medium. After adding various agonists, wrinkle formation was monitored by phase-contrast microscopy (IMT-2, Olympus) using a video-camera system (FCD-10, Olympus). The extent of contraction was determined by counting the increase in the number of wrinkles around the cells observed.

2.6. Statistical analysis

Results are expressed throughout as arithmetic means ± S.E.M. Data were compared by analysis of variance (ANOVA).

3. Results

3.1. Inositol-phosphate formation in stellate cells

Upon incubation with 100 μM of either ATP, ADP, UTP, UDP, ATP-γ-S, CTP or GTP, cellular levels of Ins*Ps* (Ins*P* + Ins*P*2 + Ins*P*3) increased significantly (Fig. 1A). However, AMP, adenosine, UMP, uridine, AMP-CPP and 2MeSATP had negligible effect on cellular levels of Ins*Ps*. The effects of ATP, ADP, UTP and ATP-γ-S on inositol phosphate levels depended on the dose of these nucleotides (Fig. 1B); UTP was the most potent at low concentration (EC_{50} = 6 μM). ATP, ADP and UTP elevated the cellular level of Ins*P*3, transiently (~2 min). This was followed by the accumulation of Ins*P*2 and Ins*P*1 (Table 1).

3.2. Measurement of intracellular Ca^{2+} levels

Analysis using fura-2 revealed that intracellular concentration of free Ca^{2+} in unstimulated stellate cells was 299 ± 11 nM. When stimulated by 100 μM of UTP, ATP, ADP or ATP-γ-S, it increased by 405 ± 75, 340 ± 72, 332 ± 51, or 309 ± 41%, respectively. Fig. 2 depicts typical profiles of the change in $[Ca^{2+}]_i$ in ATP, UTP or ATP-γ-S-stimulated stellate cells. When stimulated with 100 μM of ATP, cellular $[Ca^{2+}]_i$ increased and reached its maximum after 31.7 ± 1.7 s and returned to the basal level within 197 ± 14 s. Adenosine, AMP, uridine, UMP, ATP-CPP and 2-MeSATP hardly induced any such change in cellular calcium levels.

3.3. Contraction of stellate cells

To elucidate the possible roles of extracellular nucleotides in regulating the hepatic sinusoidal circulation, the effect of various nucleotides on stellate cell contraction was examined by the silicone-rubber membrane method [8,10]. A marked contraction of stellate cells was induced by 100 μM of UTP, ATP, ADP, or ATP-γ-S as determined by the increase in the number of wrinkles formed on silicone membranes. Wrinkles were detected within 5 min and reached a maximum at 20 min after adding these nucleotides. The typical appearance of the wrin-

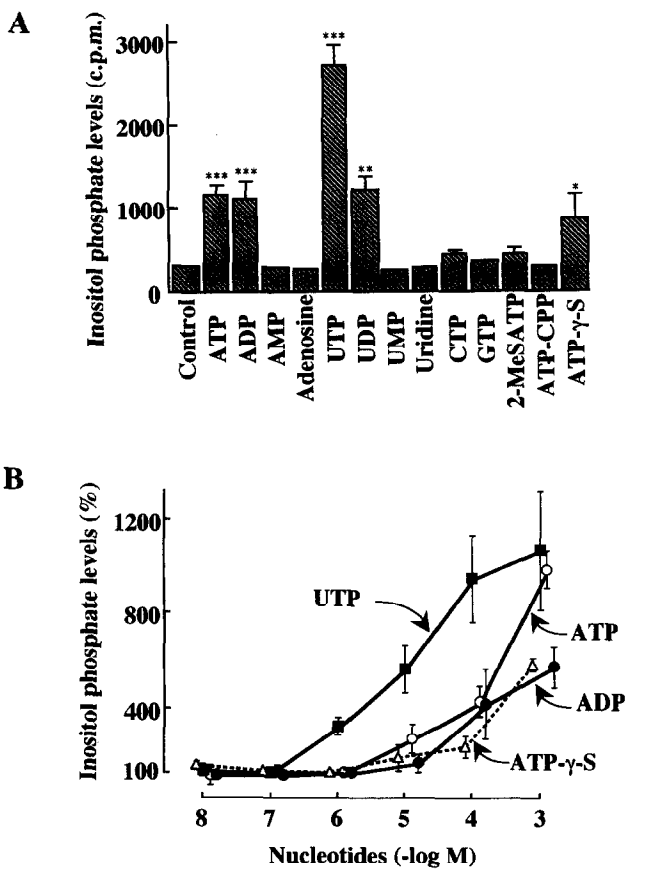

Fig. 1. Inositol phosphate accumulation in stellate cells stimulated with various nucleotides and nucleosides. (A) Cells labeled with *myo*-[2-^{3}H]inositol were stimulated with 100 μM of each agonist for 30 min in the presence of 15 mM LiCl. [^{3}H]Inositol phosphates produced were extracted and analyzed as described in the text. Data are expressed as mean ± S.E.M. (c.p.m.) of three different experiments. $^*P < 0.01$; $^{**}P < 0.005$; $^{***}P < 0.001$ as compared with control value. (B) Dose-response curves for the accumulation of inositol phosphates. Cells were incubated in the presence of various concentrations of ATP, ADP, UTP or ATP-γ-S for 30 min. Data shown are expressed as % of unstimulated control and represent mean ± S.E.M. derived from three different experiments at least.

Table 1
Accumulation of inositol phosphates in stimulated stellate cells

		0 min	1 min	2 min	5 min	10 min	30 min
UTP(100 μM)	Ins*P*1	300 ± 52	770 ± 166	672 ± 150	1237 ± 225	2490 ± 520	2878 ± 558
	Ins*P*2	78 ± 7	450 ± 106	338 ± 45	273 ± 20	353 ± 21	235 ± 34
	Ins*P*3	37 ± 5	111 ± 24	60 ± 13	90 ± 13	128 ± 8	86 ± 17
ATP(100 μM)	Ins*P*1	271 ± 52	262 ± 95	390 ± 41	802 ± 151	1387 ± 373	1551 ± 240
	Ins*P*2	127 ± 25	368 ± 61	465 ± 31	347 ± 22	236 ± 21	172 ± 30
	Ins*P*3	56 ± 9	71 ± 21	84 ± 22	97 ± 23	76 ± 8	108 ± 18

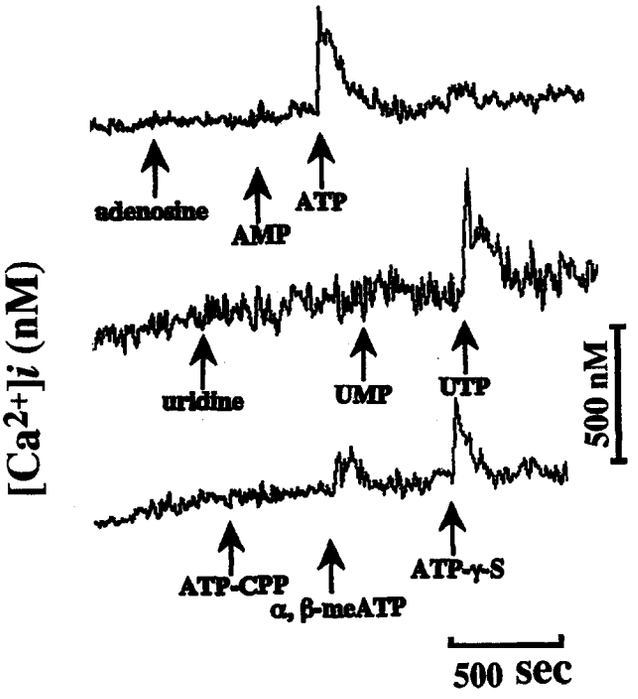

Fig. 2. Changes of $[Ca^{2+}]_i$ in stimulated stellate cells. Stellate cells (1.5×10^6/ml) were labeled with fura-2. The cells were transferred into a quartz cuvette and the cell-derived fluorescence intensity was monitored continuously using a fluorescence spectrophotometer.

kles is shown in Fig. 3. The effect of nucleotides was dose-dependent; as significantly greater contraction was induced by higher concentrations of 100 μM of ATP and UTP, and 1 mM of ADP.

4. Discussion

Extracellular nucleotides play important roles in platelet aggregation, neurotransmission, cardiac function and vascular relaxation [12–14]. Because nucleotides are released from injured cells and/or activated platelets, they may function as local mediators for controlling vascular resistance. Several types of nucleotides have been reported to increase the portal pressure and stimulate glycogen metabolism in the perfused rat liver [22]. Though extracellular nucleotides have been demonstrated to modulate the metabolism of primary-cultured hepatocytes, their effects on other constituent cells in the liver remain to be elucidated. Recent studies suggested that the contraction of stellate cells may play an important role in regulating of sinusoidal blood flow [8–11]. Kawada et al. [10] showed directly that both eicosanoids and endothelin-1 induce the contraction of stellate cells. The present work demonstrates that extracellular nucleotides, such as ATP, UTP and ADP, also induce the contraction of stellate cells.

The effects of extracellular nucleotides may be mediated by cell-surface purinoceptors that may be classified into two major groups, P1 and P2. P1-purinoceptors are specific for adenosine and activate adenylate cyclase, while P2-purinoceptors are specific for both ATP and ADP [12]. Based on the properties of their reactions with ATP and ATP analogs, P2 purinoceptors may be classified further into four subtypes, P2x, P2y, P2t and P2z [12]. However, various types of cells have been demonstrated to interact with UTP more strongly than with ATP through some receptor that does not correspond to any of these subtypes. This type of receptor has also been named a nucleotide receptor [14]. The nucleotide receptor is specific for agonists in the order of UTP > ATP = ATP-γ-S > 2-MeS ATP > AMP-CPP. The present study shows that stellate cells react preferentially with UTP and ATP as determined by Ins*P* metabolism. These results indicate that stellate cells have nucleotide receptors which are distinct from P2x purinoceptors. The properties of nucleotide receptors in stellate cells are similar to those described with human fibroblasts [23], HL-60 cells [24], hepatocytes [16], pituitary cells [25], neutrophils [26,27], and with Ehrich ascites cells [28].

The present study also demonstrates that both UTP- and ATP-sensitive cells induce the contraction of stellate cells. These results are consistent with the observation that UTP and ATP, but not adenosine, increase portal pressure by inducing contraction of the porto-hepatic vasculature in the perfused rat liver. Häussinger et al. [22] reported that the nucleotide-induced increase in portal vein pressure may be mediated by thromboxane A2 produced by Kupffer cells. However, the possibility that these nucleotides react directly with porto-sinusoidal vasculature should be considered because UTP and ATP induced the contraction of both stellate cells and portal vein (Minamiyama et al., unpublished observation).

The present study supports the hypothesis that extracellular

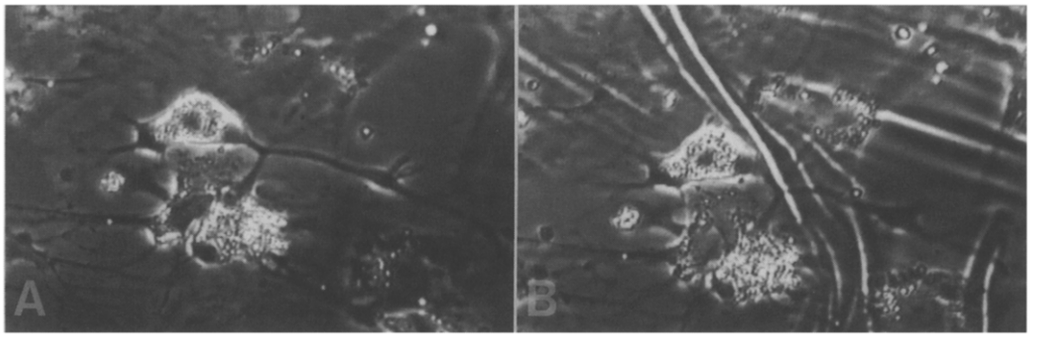

Fig. 3. Contraction of stellate cells stimulated by ATP. Stellate cells cultured on silicone-rubber sheets were incubated with ATP and the development of wrinkles in the sheet around the cells was monitored using a photo-contract microscope equipped with a video-camera system. (A) Basal state; (B) 20 min after addition of 100 μM ATP. Note that the stimulated cells underwent contraction as determined by the formation of wrinkles in the silicone membrane.

nucleotides function as local mediators of stellate cell contraction and regulate the hepatic sinusoidal microcirculation.

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