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Comparison of extracellular ATP and UTP signalling in rat renal mesangial cells

No indications for the involvement of separate purino- and pyrimidino-ceptors

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Extracellular ATP and UTP caused a rapid formation of InsP_3, with similar kinetics and dose-dependences. ITP also displayed strong agonistic properties in terms of InsP_3 production, whereas CTP was almost inactive. Pretreatment of the cells with pertussis toxin attenuated ATP- and UTP-stimulated InsP_3 generation to a comparable extent, indicating that both nucleotides couple to phospholipase C by a pertussis-toxin-sensitive G-protein. Short-term (15 min) treatment of the cells with phorbol 12-myristate 13-acetate (PMA) produced a dose-dependent inhibition of ATP- and UTP-induced InsP_3 formation. Furthermore, down-regulation of protein kinase C by long-term (24 h) exposure of the cells to PMA resulted in a comparable potentiation of phosphoinositide hydrolysis by both nucleotides. Preincubation of mesangial cells with ATP or UTP caused a pronounced cross-desensitization of subsequent nucleotide-stimulated InsP_3 production. ATP and UTP displayed no additivity in terms of InsP_3 formation, when used at maximally effective concentrations. In contrast, the peptide hormone angiotensin II interacted in an additive manner with either nucleotide in stimulating phosphoinositide hydrolysis. Reactive Blue 2, a putative P_{2y}-purinoceptor antagonist, caused a rightward shift of both the ATP and UTP dose–response curves. However, since 2-methylthio-ATP was only a partial agonist in stimulating InsP_3 formation, the mesangial-cell ATP receptor appears to be different from a classic P_{2y}-receptor. In summary, these results provide no evidence for separate purino- and pyrimidino-ceptors on mesangial cells. In contrast, ATP and UTP may use a common nucleotide receptor for transducing their signals in mesangial cells.

INTRODUCTION

Extracellular adenine nucleotides exert diverse regulatory functions on intact cells. ATP is secreted as an excitatory co-transmitter from perivascular nerves, stimulates secretion from mast cells and platelets, and has contractile or relaxant effects on many blood vessels [1,2]. These effects of extracellular ATP appear to be mediated through specific P_2-type purinergic receptors [1,2], which couple to phosphoinositide-degrading phospholipase C in hepatocytes [3,4], endothelial cells [5,6], Ehrlich ascites cells [7], HL-60 cells [8,9], macrophages [10], erythrocytes [11] and myocytes [12,13]. Mesangial cells have recently been shown to respond to extracellular ATP in terms of phosphoinositide hydrolysis and prostaglandin synthesis [14]. Mesangial cells are contractile cells and contribute to the regulation of the glomerular filtration rate by modulating the filtration surface area in the glomeruli [15,16]. In the rat kidney it has been shown that the renal vasoconstriction elicited by periarterial nerve stimulation is primarily due to release of a purinergic transmitter, probably ATP, at low physiological frequencies of stimulation [17]. Therefore, ATP co-secreted with noradrenaline may act on mesangial cells too, and contribute to the regulation of glomerular filtration. Besides adenine nucleotides, uracil nucleotides are involved in the regulation of diverse cell functions, and cause relaxation of guinea-pig trachea [18] and dilate intra- and extra-cranial arteries [19,20]. Furthermore, UTP contracts rat portal vein [21,22] and increases systemic blood pressure [21]. UTP is stored in granules of platelets [23] and is also present in appreciable amounts in liver, brain and kidney [24]. In a similar way to ATP, UTP has been found to stimulate phosphoinositide hydrolysis in endothelial cells [5,25], pituitary cells [26,27] and human epidermoid carcinoma A431 cells [28]. However, several differences that distinguish ATP and UTP actions have been reported, e.g. sensitivity to desensitization, potency order of nucleotides or sensitivity to pertussis toxin (for review see [29]). Häussinger and his colleagues were the first to suggest that the action of UTP could involve a receptor distinct from the purinergic P_2 receptor in rat liver [22,30]. In an isolated perfused rat liver model, both nucleotides produced distinct patterns of glucose output and K^+ uptake [22]. Furthermore, in HL-60 cells, UTP-induced NADPH oxidase activity was found to be more sensitive towards inhibition by pertussis toxin and activators of adenylate cyclase than was NADPH oxidase activity induced by ATP [31]. In addition, in HL-60 cells after differentiation toward a neutrophil-like cell-type, the extent of secretion induced by ATP remained unchanged, whereas that induced by UTP was markedly increased [32]. Recently, I demonstrated that different adenine nucleotides stimulate InsP_3 formation and prostaglandin E_2 synthesis in mesangial cells with a rank order of potency typically observed for P_{2y}-purinoceptors [14]. The reported findings on UTP prompted me to compare ATP and UTP signalling in mesangial cells.

MATERIALS AND METHODS

Chemicals

ATP, adenosine 5′-[γ-thio]triphosphate (ATP[S]), UTP, ITP and CTP were purchased from Boehringer, Mannheim, Germany; adenosine 5′-[αβ-methylene]triphosphate (pp[CH_2]pA) from Fluka Chemie, Buchs, Switzerland; 2-methylthio-ATP was from Ciba–Geigy Ltd., Basel, Switzerland; *myo*-[2-^{3}H]inositol was from Amersham International, Amersham, Bucks., U.K.; phorbol 12-myristate 13-acetate (PMA), pertussis toxin and

Abbreviations used: PMA, phorbol 12-myristate 13-acetate ('TPA'); ATP[S], adenosine 5′-[γ-thio]triphosphate; pp[CH_2]pA, adenosine 5′-[αβ-methylene]triphosphate.

angiotensin II were from Calbiochem, Lucerne, Switzerland; Reactive Blue 2 was from Sigma, Deisenhofen, Germany; all other chemicals used were from Merck, Darmstadt, Germany.

Cell culture

Cultivation of mesangial cells was performed as described previously [33]. The cells were grown in RPMI 1640 supplemented with 20% (v/v) fetal-calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and bovine insulin at 0.66 unit/ml (Sigma). Mesangial cells were characterized morphologically by phase-contrast microscopy, with positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells [34], and negative staining for Factor VIII-related antigen and cyto-keratin, excluding endothelial and epithelial contamination respectively. For the experiments, passages 9–29 of mesangial cells were used.

Determination of inositol phosphates

Confluent mesangial cells in 35 mm-diam. dishes were labelled for 72 h with *myo*-[2-^{3}H]inositol (10 μCi/ml) in RPMI 1640 free of inositol, containing 2% dialysed fetal-calf serum. After the labelling period the medium was removed and the cells were rinsed several times to remove free [^{3}H]inositol, and incubated for a further 1 h in fresh medium. After this procedure, mesangial cells were incubated in 1 ml of RPMI 1640 with or without the different nucleotides for the indicated time periods. The reaction was then terminated by rapid aspiration of the medium and addition of 1 ml of 15% (w/v) trichloroacetic acid. To extract inositol phosphates, the dishes were left on ice for 1 h, and the trichloroacetic acid was then removed with diethyl ether. The final extract was neutralized and applied to anion-exchange columns containing 1 ml of Dowex 1-X8 (100–200 mesh, formate form; Serva, Heidelberg, Germany). Free inositol and the inositol phosphates were eluted sequentially in accordance with Berridge [35] as described previously [36].

Statistics

Statistical analysis was by Student's t test, and a $P < 0.05$ was used as the criterion for statistical significance.

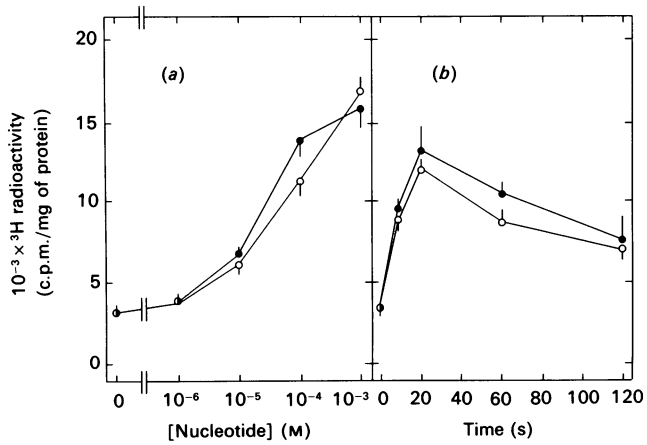

Fig. 1. Dose-dependence (*a*) and time course (*b*) of ATP- and UTP-stimulated InsP_3 formation in mesangial cells

Confluent mesangial cells were labelled with *myo*-[2-^{3}H]inositol and then stimulated with the indicated concentrations of ATP (○) or UTP (●) for 20 s (*a*), or were stimulated for the indicated time periods with 100 μM-ATP (○) or -UTP (●) (*b*). InsP_3 was separated as described in the Materials and methods section. Results are mean values ± S.E.M. ($n = 4$).

Table 1. Stimulation of InsP_3 formation by different nucleotides and inhibition by pertussis toxin

Confluent mesangial cells were prelabelled with *myo*-[2-^{3}H]inositol and preincubated with pertussis toxin (100 ng/ml) or vehicle for 15 h. Thereafter the cells were stimulated with the different nucleotides (100 μM each) for 20 s. InsP_3 was separated as described in the Materials and methods section. Results are mean values ± S.E.M. ($n = 4$).

Addition	InsP_3 (c.p.m./mg of protein)
Control	2420 ± 150
ATP	8430 ± 610
UTP	10200 ± 650
ITP	6930 ± 750
CTP	3910 ± 300
Pertussis toxin	2390 ± 170
ATP + pertussis toxin	6080 ± 370
UTP + pertussis toxin	6930 ± 540

RESULTS AND DISCUSSION

ATP and UTP stimulation of InsP_3 formation

Addition of ATP or UTP to mesangial cells evoked a rapid and dose-dependent production of InsP_3 as shown in Fig. 1. There was no significant difference in the InsP_3 response to ATP and UTP stimulation as regards the time-course and the potency of both nucleotides, although UTP tended to be slightly more potent than ATP. As shown previously, ATP caused a sequential formation of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 [14], and comparable results (not shown) were obtained for UTP. Another purine nucleotide, ITP, also stimulated InsP_3 generation, whereas the pyrimidine nucleotide CTP was almost inactive (Table 1). Treatment of mesangial cells with pertussis toxin (100 ng/ml) for 15 h partially attenuated InsP_3 formation in response to ATP or UTP (Table 1), suggesting that the receptors for both nucleotides couple to phospholipase C by pertussis-toxin-sensitive G-proteins. Short-term (15 min) preincubation of mesangial cells with PMA dose-dependently inhibited the ATP- and UTP-evoked InsP_3 generation (Fig. 2*a*). The biologically inactive 4α-phorbol 12,13-didecanoate had no inhibitory effect on nucleotide-induced InsP_3 production (results not shown). Down-regulation of protein kinase C by long-term (24 h) exposure of the cells to PMA removes the negative-feedback control on InsP_3 production exerted by the enzyme [37,38]. A 24 h pretreatment with PMA caused a dose-dependent potentiation of ATP- and UTP-stimulated InsP_3 generation (Fig. 2*b*), in a way similar to that reported for angiotensin II [38]. Obviously, protein kinase C exerts a strong feedback control on ATP- as well as UTP-induced phosphoinositide hydrolysis. From these results, it is obvious that ATP and UTP receptors couple to identical signal-transducing cascades.

Desensitization of ATP- and UTP-induced InsP_3 formation

Agonist-induced desensitization of receptor-mediated phosphoinositide signalling is an important regulatory phenomenon [39], and a homologous type of desensitization has been observed for angiotensin II-induced InsP_3 generation in mesangial cells [40]. A prolonged (2 h) exposure of mesangial cells to 250 μM-ATP or -UTP markedly attenuated or even completely abolished InsP_3 production in response to a second challenge with ATP or UTP (Table 2). There was a complete cross-desensitization for both nucleotides, as shown in Table 2. This was a specific effect on the ATP/UTP signalling system, since the response to angiotensin II was not affected (results not shown). The inactive

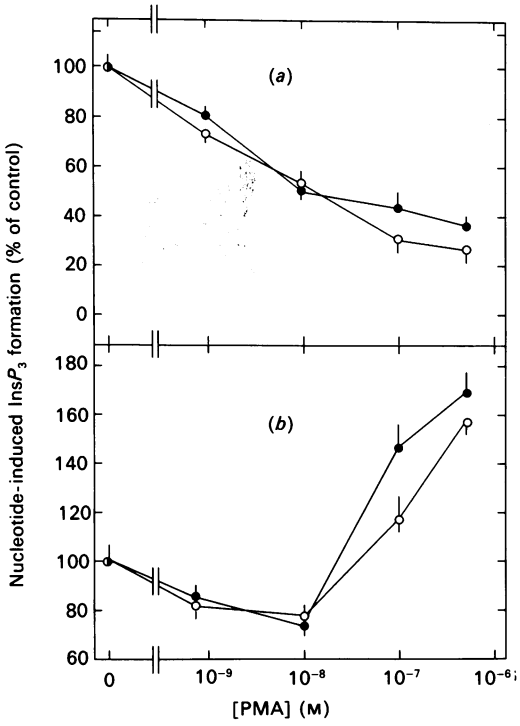

Fig. 2. Dose-dependence of PMA effects after short-term (*a*) and long-term (*b*) pretreatment on ATP- and UTP-induced InsP_3 formation

Confluent mesangial cells were labelled with *myo*-[2-^{3}H]inositol, pretreated with the indicated concentrations of PMA for 30 min (*a*) or 24 h (*b*) respectively, and then stimulated with ATP (100 μM) (○) or UTP (100 μM) (●) for 20 s. InsP_3 was separated as described in the Materials and methods section. Results are mean values ± S.E.M. ($n = 4$).

nucleotide, CTP, had no desensitizing effect on ATP- and UTP-evoked InsP_3 generation (Table 2). In contrast, ATP[S] potently inhibited ATP and UTP stimulation, whereas pp[CH_2]pA had only a moderate effect. Since the latter agonist has been reported selectively to desensitize P_{2x}-receptors [2], its weak activity indicates a P_{2y}- rather than a P_{2x}-receptor involvement, a conclusion that has also been drawn from the rank order of potency of a series of ATP analogues in mesangial cells [14]. The observed cross-desensitization between ATP and UTP does not necessarily exclude the possibility that the nucleotides act via different purino- and pyrimidino-ceptors, although all agonists investigated so far cause an exclusively homologous type of desensitization in mesangial cells [15,16,40].

No additivity of ATP- and UTP-induced InsP_3 formation

Further evidence for a common nucleotide receptor was provided by the lack of additivity of maximal doses of ATP and UTP on InsP_3 production (Fig. 3). Agonists that share identical signalling pathways should display additive effects when combined at sub-maximal concentrations, but not at maximal doses. Combined addition of maximal doses (1 mM) of ATP and UTP showed no additive effects on InsP_3 generation, as shown in Fig. 3. To exclude the possibility that the number of coupling G-proteins or the amount of phosphoinositide-specific phospholipase C was limiting the formation of InsP_3 in response to a combined stimulation with ATP and UTP, I investigated InsP_3 production in response to combinations of angiotensin II and either nucleotide. Separate additions of ATP (1 mM), UTP (1 mM) or angiotensin II (1 μM) caused a potent stimulation of InsP_3 formation, with 7.9 ± 0.4-, 9.0 ± 0.3- and 9.8 ± 0.7-fold increases above basal values respectively (means ± S.E.M., $n = 4$). Combined additions of ATP and angiotensin II (17.7 ± 1.5-fold) or UTP and angiotensin II (18.8 ± 1.1-fold) caused an additive generation of InsP_3. In contrast, combinations of ATP and UTP (10.8 ± 1.1-fold) did not demonstrate an additive formation of InsP_3 (see also Fig. 4). These results clearly exclude the possibility that a limiting number of G-proteins, or phospholipase C, or substrate depletion, is responsible for the lack of additivity of combinations of ATP and UTP. In contrast, the data suggest that it is the number of nucleotide receptors that limits the response, and that either nucleotide can fully occupy this common pool of receptors.

Table 2. Desensitization of ATP- and UTP-stimulated InsP_3 formation

Confluent mesangial cells were labelled with *myo*-[2-^{3}H]inositol and preincubated for 2 h with 250 μM of the indicated nucleotides. Thereafter the cells were stimulated with ATP (100 μM) or UTP (100 μM) for 20 s. InsP_3 was separated as described in the Materials and methods section. Results are mean values ± S.E.M. ($n = 4$).

Preincubation	Stimulation	InsP_3 (c.p.m./mg of protein)
None	None	2120 ± 260
	ATP	11580 ± 250
	UTP	12230 ± 490
ATP	None	2060 ± 230
	ATP	2130 ± 270
	UTP	2680 ± 180
UTP	None	2140 ± 190
	ATP	3570 ± 230
	UTP	4160 ± 250
CTP	None	2310 ± 190
	ATP	11950 ± 730
	UTP	11990 ± 680
ATP[S]	None	3100 ± 410
	ATP	3420 ± 320
	UTP	2680 ± 170
pp[CH_2]pA	None	2610 ± 210
	ATP	8810 ± 730
	UTP	8320 ± 410

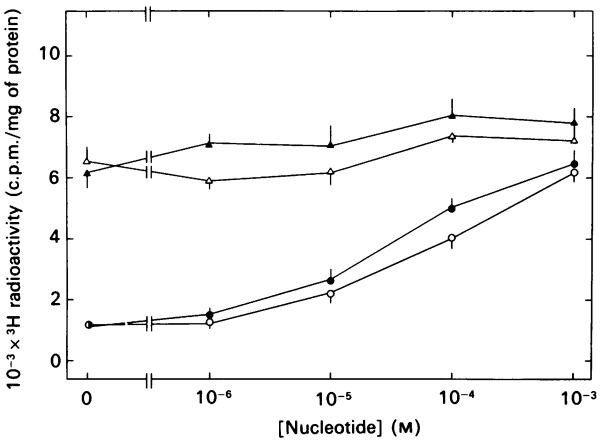

Fig. 3. Effects of single and combined additions of ATP and UTP on InsP_3 formation in mesangial cells

Confluent mesangial cells were labelled with *myo*-[2-^{3}H]inositol and then stimulated with the indicated concentrations of ATP (○), UTP (●), ATP + UTP (1 mM) (△) or UTP + ATP (1 mM) (▲) for 20 s. InsP_3 was separated as described in the Materials and methods section. Results are mean values ± S.E.M. ($n = 4$).

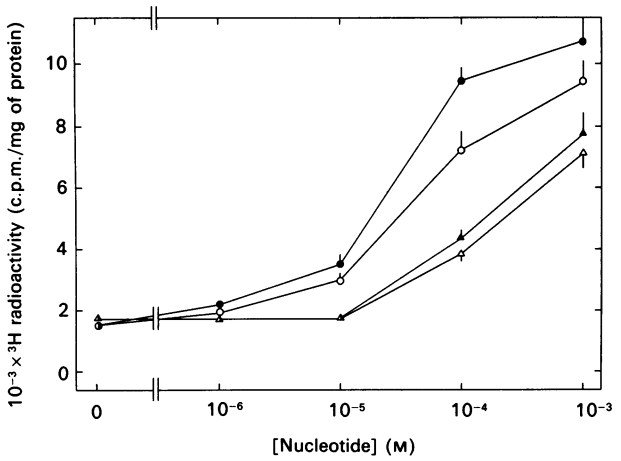

Fig. 4. Effect of Reactive Blue 2 on ATP- and UTP-stimulated InsP_3 formation in mesangial cells

Confluent mesangial cells were labelled with *myo*-[2-^{3}H]inositol, pretreated for 30 min with Reactive Blue 2 (50 μM) (△, ▲) or vehicle (○, ●) and then stimulated with the indicated concentrations of ATP (○, △) or UTP (●, ▲) for 20 s. InsP_3 was separated as described in the Materials and methods section. Results are mean values ± S.E.M. ($n = 4$).

Table 3. Effect of 2-methylthio-ATP on InsP_3 formation in mesangial cells

Confluent mesangial cells were labelled with *myo*-[2-^{3}H]inositol and stimulated with the indicated concentrations of ATP, UTP or 2-methylthio-ATP. InsP_3 was separated as described in the Materials and methods section. Results are mean values ± S.E.M. ($n = 4$).

	InsP_3 (c.p.m./mg of protein)		
Concn.	ATP	UTP	2-Methylthio-ATP
0	2290 ± 190	2290 ± 190	2290 ± 190
1 μM	2600 ± 170	2670 ± 180	3610 ± 160
10 μM	3850 ± 170	5300 ± 150	3810 ± 170
100 μM	14420 ± 1130	16680 ± 570	3920 ± 280
1 mM	18930 ± 1030	18420 ± 1450	4660 ± 470

Effect of Reactive Blue 2 on ATP- and UTP-induced InsP_3 formation

To characterize further the receptor subtype mediating ATP and UTP responses in mesangial cells, I used the putative P_{2y}-receptor antagonist Reactive Blue 2 [41,42]. As shown in Fig. 4, preincubation of mesangial cells with 50 μM Reactive Blue 2 shifted the dose–response curves for ATP and UTP to the right, indicating that both nucleotides mediate InsP_3 formation via a P_{2y}-purinoceptor-like nucleotide receptor. Furthermore, the P_{2y}-receptor agonist 2-methylthio-ATP [2,41,42] at 1 μM was more potent than ATP or UTP in stimulating InsP_3 production in mesangial cells (Table 3). However, 2-methylthio-ATP was much less effective than either ATP or UTP at higher concentrations (Table 3). A similar low apparent efficacy of 2-methylthio-ATP has been reported for prostacyclin synthesis in endothelial cells [25]. This may indicate a heterogeneity of nucleotide receptors that exceeds the present classification schemes. From the data in the present paper, there is no reason why ATP and UTP do not act at purinoceptors, which may more appropriately be called 'nucleotidoceptors'. The real problem, of course, lies in the lack of convincing antagonists for either P_2-purinoceptors or the proposed pyrimidinoceptors. Until these exist it will be hard to prove that separate receptors exist, and that uracil nucleotides are not acting via 'nucleotidoceptors'.

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