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Extracellular ATP and UTP exert similar effects on rat isolated hepatocytes

[1]Stefaan Keppens, Ann Vandekerckhove & Henri De Wulf

Afdeling Biochemie, Campus Gasthuisberg, Faculteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

1 Extracellular UTP and ATP show obvious similarities in their control of several metabolic functions of rat isolated hepatocytes.

2 They have a similar time-course and concentration-dependency for the activation of glycogen phosphorylase, the generation of inositol trisphosphate (IP_3), the inhibition of glycogen synthase and the lowering of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels.

3 There is a similar synergism of the nucleotides with glucagon in activating phosphorylase.

4 They undergo a similar inhibition by phorbol myristic acid of their glycogenolytic effect.

5 The ATP and UTP effect on IP_3 levels are not additive.

6 It is tentatively concluded that UTP and ATP use a common receptor.

Keywords: ATP; UTP; rat hepatocytes; glycogen metabolism

Introduction

Extracellular adenine nucleotides regulate many important cell functions in several different tissues through an interaction with P_2-purinoceptors, which can be subdivided into P_{2X} and P_{2Y} receptors according to the rank order of potency of several ATP analogues (Burnstock & Kennedy, 1985). P_{2Y}-purinoceptors are characterized by the rank order of potency: 2-methylthio-ATP $\gg$ ATP = ADP > α,β-methylene-ATP. P_{2X}-receptors are more specific for α,β-methylene-ATP than for ATP and 2-methylthio-ATP. Moreover the P_{2X} effect of ATP can be desensitized by pretreatment with α,β-methylene-ATP (see Gordon, 1986, for a review).

UTP and other uracyl nucleotides also regulate various functions in many different cell types, as reviewed by Seifert & Schultz (1989). These authors presented evidence that in several tissues and cell types the pyrimidine nucleotides exert their effects by interacting with a specific pyrimidinoceptor rather than by interacting with the purinoceptor. This view has recently been refined by O'Conner *et al.* (1991). Taking into account all nucleotide effects these authors propose that the nucleotide receptors can be subdivided into three classes: P_{2Y}-purinoceptors with 2-methylthio-ATP $\gg$ ATP = ADP > UTP, 'Nucleotide' receptors with UTP = ATP > ADP > 2-methylthio-ATP and a 'mixed' type of receptor with 2-methylthio-ATP > ATP = UTP = ADP as their respective rank order of potencies. The relative densities and coupling affinities of these receptors can, according to the authors, vary considerably from tissue to tissue.

Rat liver parenchymal cells possess purinoceptors activated by extracellular ATP. Binding of the nucleotide to these receptors leads to the activation of phospholipase C, generating diacylglycerol and inositol-(1,4,5)-trisphosphate (IP_3). IP_3 induces a rise in cytosolic calcium (Berridge & Irvine, 1989) leading to the activation of glycogen phosphorylase, which is responsible for glycogen breakdown. Diacylglycerol stimulates the activity of protein kinase C which results, among other effects, in the inactivation of glycogen synthase, in combination with the inhibition by calcium of glycogen synthase phosphatase (Mvumbi *et al.*, 1985).

Based on the rank order of the glycogenolytic potency of different ATP analogues, we (Keppens & De Wulf, 1986) and others (Gordon, 1986) have proposed that the purinoceptor involved in glycogenolysis belongs to the P_{2Y}-subclass in the nomenclature of Burnstock & Kennedy (1985). However, our results recently obtained with 2-methylthio-ATP (Keppens & De Wulf, 1991) suggest some heterogeneity for the P_{2Y}-purinoceptor. Indeed although 2-methylthio-ATP has been shown to be a more potent glycogenolytic agonist than ATP, some other effects were not compatible with the existence of a common receptor for both nucleotides: (a) 2-methylthio-ATP shows little affinity for the P_{2Y}-receptor characterized with ATPα[^{35}S]; (b) 2-methylthio-ATP has little effect on the levels of IP_3, and (c) it has no effect on the levels of cyclic AMP increased previously by glucagon (Keppens & De Wulf, 1991).

So far, the only information about effects of UTP on liver comes from the work of Häussinger *et al.* (1987, 1988). These authors used the perfused rat liver to study the effects of both ATP and UTP and reported a different time-course for the glucose release from the liver on perfusion with either ATP or UTP. Differences in portal vein pressure, K^+ uptake, calcium and thromboxane release and in oxygen consumption were also observed in their studies. Although they suggested that the effects of ATP were mediated by P_{2Y}-purinoceptors and those of UTP by a different receptor (Häussinger *et al.*, 1987) they were conscious of the fact that there is a complex interaction between hepatic parenchymal and non-parenchymal cells (Häussinger *et al.*, 1988).

The present study compares several effects of ATP and UTP at the level of the liver parenchymal cell, avoiding contribution by other substances, possibly released by ATP or UTP from non-parenchymal cells. The aims of this study were to substantiate the glycogenolytic effect of extracellular UTP on hepatocytes and to compare these effects of UTP with those induced by ATP.

Methods

Adult male Wistar-strain albino rats (200–250 g body wt.) fed *ad libitum* with a standard laboratory chow were used.

Isolation and incubation of the hepatocytes were done as previously described (Vandenheede *et al.*, 1976). Briefly, the liver was removed from the animal, cleared of blood and then perfused at 37°C for about 10 min with a Krebs-Henseleit bicarbonate buffer without calcium. Subsequently, collagenase (30 mg 100 ml^{-1} of perfusion buffer) and calcium (2.5 mM) were added and the perfusion was continued for another 25–30 min. The hepatocytes were harvested and incubated at 37°C (or at 10°C when indicated) in a Krebs-Henseleit buffer containing 10 mM glucose in closed plastic vials saturated with 95% O_2, 5% CO_2 (v/v). Cyclic AMP and IP_3 concentrations were

[1] Author for correspondence.

determined with a competitive protein-binding technique, the former based on the procedure described by Gilman (1970), using an assay kit from the Radiochemical Centre (Amersham, Bucks., U.K.) and the latter based on the method described by Bredt *et al.* (1989) with slight modifications. Cerebellar membranes, known to contain high amounts of specific IP_3 binding protein (Worley *et al.*, 1987) were prepared by homogenization and repetitive centrifugation of rabbit cerebellum. The incubation medium contained about 50 μg of these membranes, 1 nM [^{3}H]-IP_3 and 10 μl cell extract in a final volume of 100 μl. Bound and free ligand were separated after 10 min of incubation by filtration through Whatman GF/A filters, which were washed three times with 7 ml 50 mM Tris-HCl (pH 8.4) and 1 mM EDTA. Radioactivity was then determined by liquid scintillation counting.

UTP and ATP were from Sigma Chemical Co., St. Louis, MO, U.S.A.; [^{3}H]-inositol-(1,4,5)-trisphosphate was from Amersham International, Amersham, Bucks., U.K.; glucagon was from Novo Laboratories, Copenhagen, Denmark. Suramin was a gift from Bayer Belgium, Pharma division, Brussels, Belgium.

The curve fitting programme 'Enzfitter' (Leatherbarrow, 1987) was from Elsevier Biosoft (Cambridge, U.K.) and was used to fit the experimental data to a Michaelean-type of equation (concentration-dependent effects).

Results

To compare the effects of ATP and UTP we measured their capacity to activate glycogen phosphorylase, to increase IP_3 levels, to inhibit the rise in cyclic AMP levels after glucagon and to inactivate glycogen synthase.

First we determined the kinetics of these effects of ATP and UTP (Figure 1). The activation of glycogen phosphorylase by 5 μM UTP and ATP was rapid, reached maximal levels of phosphorylase a within 20 s and declined afterwards. The effect of 10 μM ATP and UTP on the levels of IP_3 was even more rapid and transient than the activation of phosphorylase, reaching maximal levels after 5 s. Finally, the inactivation of glycogen synthase by 5 μM ATP and UTP and the cyclic AMP-lowering effect of 1 mM ATP and UTP were maximal within 1 min and again no differences between ATP and UTP were seen.

Next we determined the concentration-dependencies of these four parameters. Figure 2 illustrates the effects of increasing concentrations of ATP and of UTP on the activation of phosphorylase (at 20 s), on the increase of IP_3 levels (at 5 s), on the decrease of cyclic AMP levels (at 1 min) and on the inactivation of synthase (at 1 min). The curves on the figure and the concentrations at which half-maximal effects were obtained (*K*-values, see below) were computer-generated by applying a Michaelean-type of equation to the data. The data show a complete similarity between ATP and UTP effects on the liver parenchymal cell. Indeed, both nucleotides were equipotent in activating phosphorylase ($K_a = 1.1\,\mu$M), increasing the levels of IP_3 ($K_{IP} = 40\,\mu$M), lowering the cyclic AMP levels ($K_c = 15\,\mu$M) and inactivating synthase ($K_s = 5\,\mu$M).

In order to compare further ATP and UTP effects on the liver parenchymal cell, we checked whether UTP, like ATP, could act synergistically with glucagon. For ATP it has already been shown that glucagon increases the ability of ATP to augment the levels of cytosolic calcium (Charest *et al.*, 1985). Figure 3 illustrates that a synergism can also be demonstrated by measuring the activation of phosphorylase at low temperatures (10°C) where there is only a very moderate effect of glucagon and of ATP or UTP separately. The combination of either ATP or UTP with glucagon resulted in a pronounced and similar activation of phosphorylase, presumably due to an increase in the cytosolic calcium levels.

We next used the P_2-purinoceptor antagonist, suramin (Dunn & Blakely, 1988) and procion blue, a P_{2Y}-antagonist (Burnstock & Warland, 1987). Figure 4 shows that the glycogenolytic effect of ATP, UTP or vasopressin was not inhibited by low concentrations (20 μM) of either P_2-antagonist. Figure 4 further illustrates that the inhibitory effect of higher concentrations of the antagonists (400 μM) is non-specific since the glycogenolytic effect of vasopressin (Figure 4c) was equally well counteracted. Even the glycogenolytic effect of glucagon was inhibited by these high concentrations of suramin and procion-blue (not shown).

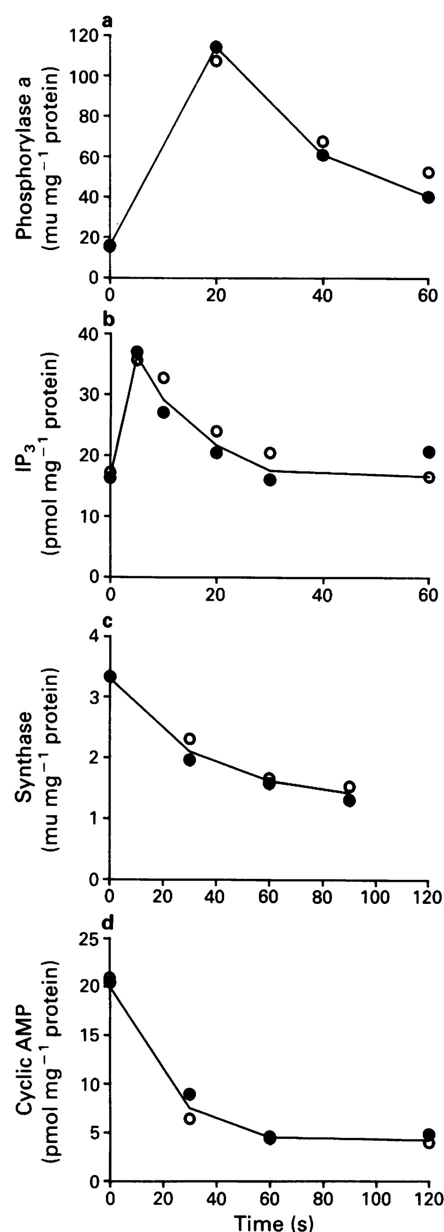

Figure 1 Comparison of the time-dependent activation of glycogen phosphorylase, increase of inositol trisphosphate (IP_3) levels, inactivation of glycogen synthase and inhibition of the rise in cyclic AMP levels. Hepatocytes were pre-incubated for 20–30 min in the presence of 10 mM glucose and were then challenged with ATP (○) or UTP (●) (both at 5 μM) for the effects on phosphorylase and synthase, at 10 μM for the increase in IP_3 levels and at 1 mM for lowering cyclic AMP levels. Samples were taken at the indicated times. (a) Phosphorylase activation; (b) IP_3 increase; (c) inactivation of synthase and (d) lowering of cyclic AMP levels in hepatocytes previously treated for 1 min with 20 nM glucagon. Data shown are representative of 3–5 independent experiments.

To check further the possibility that UTP (or ATP) might interact with a P_{2X} type of receptor, we used α,β-methylene-ATP, which, in other tissues, is able to induce a desensitization of the P_{2X}-mediated effect (Burnstock & Kennedy, 1985). Pretreatment of the hepatocytes for 5 min with 200 μM

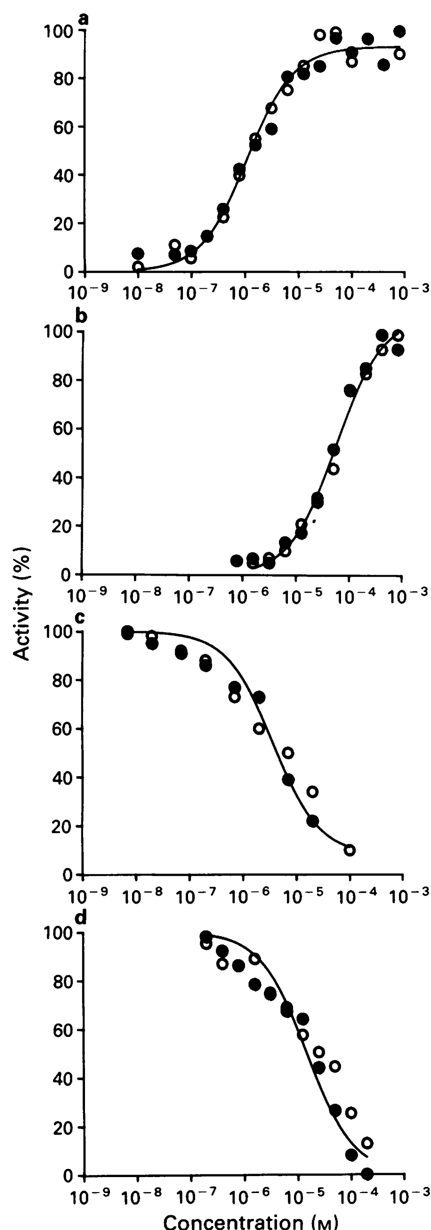

Figure 2 Comparison of the concentration-dependencies of ATP and UTP for the activation of glycogen phosphorylase, the increase in inositol trisphosphate (IP_3) levels, the inactivation of glycogen synthase and the inhibition of the rise in cyclic AMP levels. Hepatocytes were pre-incubated with 10 mM glucose for 20–30 min and then challenged with increasing concentrations of either ATP (○) or UTP (●). (a) Phosphorylase *a* levels after 20 s; (b) IP_3 levels after 5 s; (c) synthase *a* levels after 1 min and (d) cyclic AMP levels after 1 min (glucagon (20 nM) was added 1 min prior to the addition of the nucleotides). Data, expressed as percentage changes from control values, are mean values of at least 5 independent experiments; s.e.mean did not exceed 10%.

of α,β-methylene-ATP did not desensitize the glycogenolytic effect of UTP (or of ATP).

The effect of both agonists on phosphorylase activation was equally counteracted by phorbol myristic acid. Indeed, pretreatment of the hepatocytes for 5 min with 1.6 μM of the phorbol ester increased the K_a value 2 to 3 fold.

Finally we tested whether the ATP and UTP effects were additive. Since the activation of glycogen phosphorylase, the lowering of cyclic AMP levels after glucagon and the inactivation of synthase were all maximal, we looked for a possible additivity of ATP and UTP effects on the levels of IP_3. Figure 5 shows the IP_3 levels obtained after addition of supramaximal concentrations of ATP, UTP or of vasopressin either alone or in combination with each other. It is clear that the effect of ATP and UTP on the levels of IP_3 were not additive, in contrast to the additive effects of these nucleotides with vasopressin.

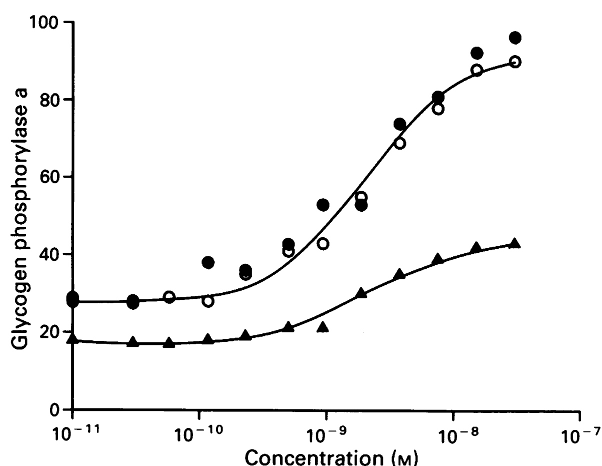

Figure 3 Synergistic effect between ATP or UTP and glucagon on the activation of glycogen phosphorylase. Hepatocytes were pre-incubated for 20 min at 37°C with 10 mM glucose. They were then brought to 10°C and kept at this temperature for at least 15 min before adding increasing concentrations of glucagon either alone (▲) or with 5 μM ATP (○) or UTP (●). Samples were taken 10 min later for the determination of the levels of phosphorylase *a*.

Discussion

It is clear from these data that ATP and UTP exert very similar effects on isolated hepatocytes. Indeed, both the time- and concentration-dependent activation of phosphorylase by UTP and ATP are completely identical (Figure 1, see also Keppens & De Wulf, 1985, and Charest *et al.*, 1985). Since the activation of phosphorylase by ATP has been shown to be linked to increased levels of IP_3 (Charest *et al.*, 1985) a similar situation might exist for UTP. Figures 1 and 2 indeed show a similar pattern of IP_3 generation after ATP and UTP. The time- and concentration-dependence for the increase in IP_3 levels after ATP, although measured in a different way, is similar to the results presented by Charest and co-workers (1985). Indeed they also reported a very rapid (maximal after 5 s) effect of ATP on the levels of IP_3 with a comparable concentration-dependence. The observation that higher concentrations of the nucleotides are needed to increase IP_3 levels ($K_{IP} = 50\,\mu$M) than to activate phosphorylase ($K_a = 1.1\,\mu$M) is in accordance with what is known for other cyclic AMP-independent, calcium-mediated agonists, for example vasopressin, for which the K_{IP} is about 50 fold higher than the K_a (Lynch *et al.*, 1985). A very small increase in IP_3 seems to be sufficient to initiate the release of calcium ions into the cytosol leading eventually to the activation of phosphorylase. Since overall effects of UTP and ATP on IP_3 levels are identical a very similar interaction of ATP and UTP with phospholipase C is suggested. In addition to these similarities, both nucleotides also inactivate glycogen synthase and lower the levels of cyclic AMP in an identical way (Figure 2). Furthermore they are, in combination with glucagon, equally potent in synergistically activating glycogen phosphorylase (Figure 3). Four other experiments further illustrate the similarity between ATP and UTP. First, phorbol myristic acid, previously shown to increase the K_a for the activation of phosphorylase by ATP (Keppens & De Wulf, 1991) equally increases the K_a for the activation of phosphorylase by UTP. Second, α,β-methylene-ATP, described as a P_{2X}-desensitizing agent (Burnstock & Kennedy, 1985) has no effect on the glycogenolytic potency of either UTP or ATP. Third, the use of a P_2-antagonist

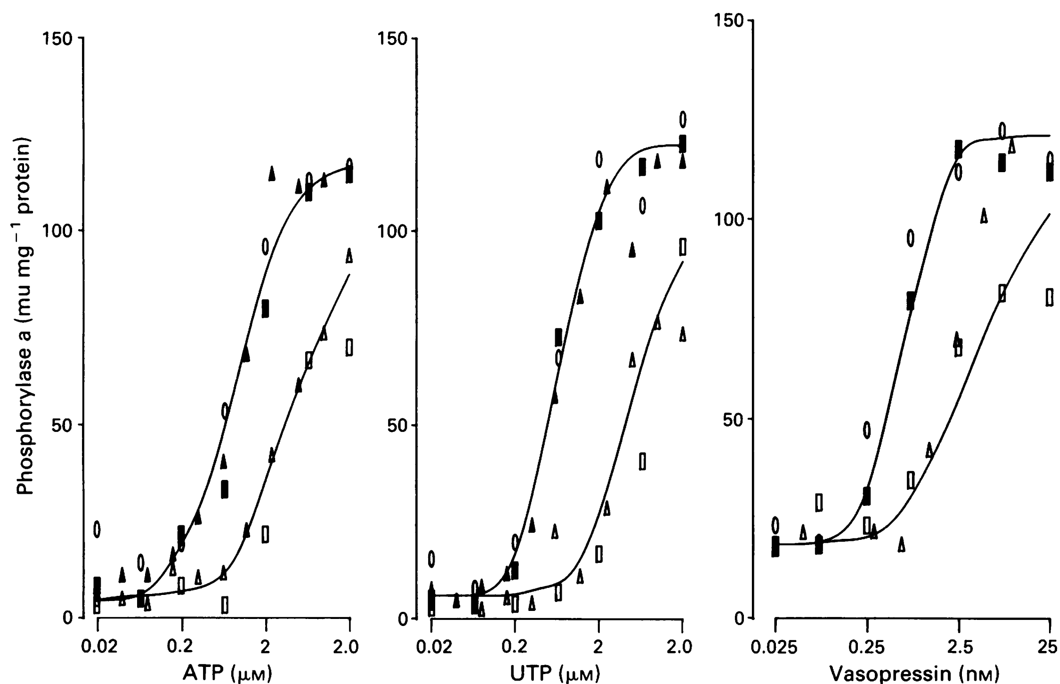

Figure 4 The effect of suramin and procion-blue on the glycogenolytic potency of ATP, UTP and vasopressin Hepatocytes were pre-incubated for 20 min at 37°C with 10 mM glucose. They were then further incubated as such (control, ○) or challenged with suramin (20 μM ■, 400 μM □) or procion blue (20 μM ▲, 400 μM △). One min later the cells were treated with the indicated concentrations of ATP (a), UTP (b) or vasopressin (c). The activity of glycogen phosphorylase was determined 20 s after the addition of ATP and of UTP, or 1 min after vasopressin. Data shown are from one representative experiment.

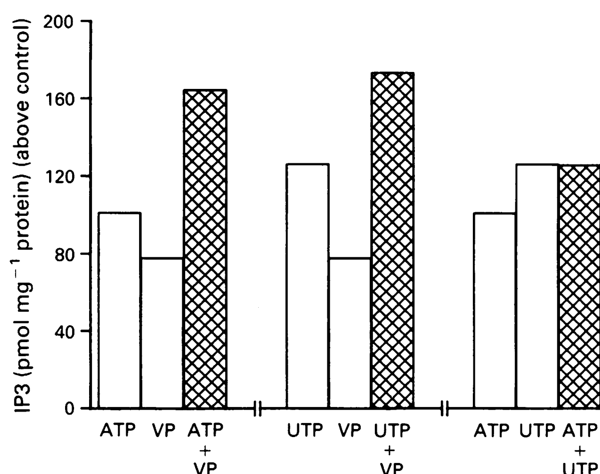

Figure 5 The non-additivity of the effects of ATP and UTP on the levels of inositol trisphosphate (IP_3). Hepatocytes were pre-incubated for 20 min at 37°C with 10 mM glucose. They were then challenged with supramaximal concentrations of ATP (1 mM), UTP (1 mM) or vasopressin (1 μM), either alone or in combination with each other. IP_3 levels were estimated 5 s later. Data shown are the means of two independent experiments, done in duplicate. Control value for IP_3 was 18.1 pmol mg^{-1} of protein.

(suramin) and a P_{2Y}-antagonist (procion blue) did not succeed in discriminating between the effects of ATP and UTP on glycogenolysis. Finally, there was no additivity of the effect of ATP and UTP on the levels of IP_3, in clear contrast to the additivity of vasopressin with either ATP or UTP (Figure 5). The overall conclusion to be drawn from these data is that no difference exists between the effects of ATP and UTP. In the light of the recently proposed receptor sub-classification by O'Conner et al. (1991) the liver would belong to those tissues with a heterogeneous 'mixed' receptor population, characterized by the rank order of glycogenolytic potency: 2-methylthio-ATP > ATP = UTP = ADP.

Our data showing a complete similarity between the effects of ATP and UTP on the liver parenchymal cell do not necessarily contradict the results reported by the group of Häussinger (1987, 1988) using the liver perfusion technique. They speculated that the differences observed between ATP and UTP might be due to their respective capacities to induce the release of thromboxane from non-parenchymal cells (Häussinger et al., 1988). Thromboxane on its own induces glycogenolysis in hepatocytes, thereby obscuring a direct effect of the nucleotides on the parenchymal cell. Our results clearly show that with a suspension of parenchymal cells, no difference between ATP and UTP is detected, suggesting a close similarity (or identity) between the ATP and UTP receptors. These data are in accord with those of Pfeilschifter (1990) on the effects of extracellular ATP and UTP on rat renal mesangial cells and those of Davidson and co-workers (1990) on pituitary cells. The authors concluded that there are probably no separate purino- and pyrimidino-ceptors on these cells. A similar situation might therefore exist for the liver parenchymal cells with ATP and UTP using a common receptor.

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References

BERRIDGE, M.J. & IRVINE, R.F. (1989). Inositol phosphates and cell signalling. *Nature*, **341**, 197–205.

BREDT, D.S., MOUREY, R.J. & SNYDER, S.H. (1989). A simple, sensitive, and specific radioreceptor assay for inositol 1,4,5-trisphosphate in biological tissues. *Biochem. Biophys. Res. Commun.*, **159**, 976–982.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK. G. & WARLAND, J.J.I. (1987). P_2-purinoceptors of two subtypes in the rabbit mesenteric artery: Reactive blue 2 selec-

tively inhibits responses mediated via the P_{2Y}- but not the P_{2X}-purinoceptor. *Br. J. Pharmacol.*, **90**, 383–391.

CHAREST, R., BLACKMORE, P.F. & EXTON, J.H. (1985). Characterization of responses of isolated hepatocytes to ATP and ADP. *J. Biol. Chem.*, **260**, 15789–15794.

DAVIDSON, J.S., WAKEFIELD, I.K., SOHNIUS, U., VAN DER MERWE, P.A. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phospholipase-C in pituitary cells. *Endocrinol.*, **126**, 80–87.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

GILMAN, A.G. (1970). A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc. Natl. Acad. Sci. U.S.A.*, **67**, 305–312.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

HÄUSSINGER, D., BUSSHARDT, E., STEHLE, T., STOLL, B., WETTSTEIN, M. & GEROK, W. (1988). Stimulation of thromboxane release by extracellular UTP and ATP from perfused rat liver. *Eur. J. Biochem.*, **178**, 249–256.

HÄUSSINGER, D., STEHLE, T. & GEROK, W. (1987). Actions of extracellular ATP and UTP in perfused rat liver. *Eur. J. Biochem.*, **167**, 65–71.

KEPPENS, S. & DE WULF, H. (1985). P_2-purinergic control of liver glycogenolysis. *Biochem. J.*, **231**, 797–799.

KEPPENS, S. & DE WULF, H. (1986). Characterization of the liver P_2-purinoceptor involved in the activation of glycogen phosphorylase. *Biochem. J.*, **240**, 367–371.

KEPPENS, S. & DE WULF, H. (1991). Characterization of the biological effects of 2-methylthio-ATP on rat hepatocytes: clear-cut differences with ATP. *Br. J. Pharmacol.*, **104**, 301–304.

LEATHERBARROW, R.J. (1987). *Enzfitter, a Non-Linear Regression Data Analysisprogram for the IBM PC.* Amsterdam, The Netherlands: Elsevier Science Publishers BV.

LYNCH, C.J., BLACKMORE, P.F., CHAREST, R. & EXTON, J.H. (1985). The relationships between receptor binding capacity for norepinephrine, angiotensin II, and vasopressin and release of inositol trisphosphate, Ca^{++} mobilization and phosphorylase activation in rat liver. *Mol. Pharmacol.*, **28**, 93–99.

MVUMBI, L., BOLLEN, M. & STALMANS, W. (1985). Calcium and glycogen act synergistically as inhibitors of hepatic glycogen-synthase phosphatase. *Biochem. J.*, **232**, 697–704.

O'CONNER, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PFEILSCHIFTER, J. (1990). Comparison of extracellular ATP and UTP signalling in rat renal mesangial cells. *Biochem. J.*, **272**, 469–472.

SEIFERT, R. & SCHULTZ, G. (1989). Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracyl nucleotides. *Trends Pharmacol. Sci.*, **10**, 365–369.

VANDENHEEDE, J.R., KEPPENS, S. & DE WULF, H. (1976). The activation of liver phosphorylase *b* kinase by glucagon. *FEBS Lett.*, **61**, 213–217.

WORLEY, P.F., BARABAN, J.M., SUPATTAPONE, S., WILSON, V.S. & SNYDER, S.H. (1987). Characterization of inositol trisphosphate receptor binding in brain. *J. Biol. Chem.*, **262**, 12132–12136.

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