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Extracellular ATP and UTP activation of phospholipase D is mediated by protein kinase C-ε in rat renal mesangial cells

[1]Josef Pfeilschifter & Claire Merriweather

Department of Pharmacology, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

1 We have studied whether a nucleotide receptor mediates the effects of extracellular ATP and UTP on phosphatidylcholine metabolism in rat cultured glomerular mesangial cells.

2 ATP and UTP stimulated a biphasic 1,2-diacylglycerol (DAG) formation in [^{3}H]-arachidonic acid-labelled mesangial cells. In contrast, in cells labelled with [^{3}H]-myristic acid, a tracer that preferentially marks phosphatidylcholine, both nucleotides induced a delayed monophasic production of DAG with a concomitant increase in phosphatidic acid and choline formation.

3 A phospholipase D-mediated phosphatidylcholine hydrolysis was further suggested by the observation that ATP and UTP stimulate the accumulation of phosphatidylethanol, when ethanol was added to mesangial cells.

4 The rank order of potency of a series of nucleotide analogues for stimulation of phosphatidylethanol formation was UTP = ATP > ITP > ATPγS > βγ-imido-ATP = ADP > 2-methylthio-ATP = βγ-methylene-ATP = ADPβS, while AMP, adenosine, CTP and GTP were inactive, indicating the presence of a nucleotide receptor.

5 Elevation of cytosolic free Ca^{2+} by the calcium ionophore A23187 (1 μM) or the Ca^{2+}-ATPase inhibitor, thapsigargin (200 nM) slightly increased phosphatidylethanol formation. However, chelation of cytosolic Ca^{2+} with high concentrations of Quin 2 did not attenuate ATP- and UTP-induced phosphatidylethanol production, thus suggesting that Ca^{2+} is not crucially involved in agonist-stimulated phospholipase D activation.

6 The protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), but not the biologically inactive 4α-phorbol 12,13-didecanoate, increased phospholipase D activity in mesangial cells, suggesting that PKC may mediate nucleotide-induced phosphatidylcholine hydrolysis.

7 Down-regulation of PKC-α and -δ isoenzymes by 8 h PMA treatment still resulted in full phospholipase D activation. In contrast, a 24 h treatment of mesangial cells with PMA, a regimen that also causes depletion of PKC-ε, markedly attenuated nucleotide-evoked phosphatidylethanol formation. In addition, the selective PKC inhibitor, calphostin C attenuated ATP- and UTP-induced phosphatidylethanol production.

8 In summary, these data suggest that extracellular ATP and UTP use a common nucleotide receptor to activate phospholipase D-mediated phosphatidylcholine hydrolysis. Stimulation of phospholipase D appears to involve the PKC-ε isoenzyme, activated by DAG derived from phosphoinositide hydrolysis by phospholipase C.

Keywords: Phospholipase D; nucleotide receptors; renal mesangial cells; protein kinase C

Introduction

Extracellular adenine nucleotides have an important impact on a wide range of physiological responses of cells (Gordon, 1986). These effects of extracellular adenosine 5'-triphosphate (ATP) on cells appear to be mediated through specific P_2-type purinoceptors (Burnstock, 1978). In 1985, Burnstock & Kennedy proposed a further subclassification of P_2-purinoceptors into P_{2x} and P_{2y} subtypes on the basis of agonist potency order and functional responses. Later, additional subtypes of P_2-purinoceptors have been described on platelets (P_{2T}-type) and on mast cells (P_{2Z}-type) (Gordon, 1986). Extracellular ATP has been demonstrated to couple via P_2-purinoceptor to polyphosphoinositide degrading phospholipase C in hepatocytes (Charest *et al.*, 1985), endothelial cells (Pirotton *et al.*, 1987), HL-60 cells (Cockcroft & Stutchfield, 1989), macrophages (Pfeilschifter *et al.*, 1989) and various other cell types (for review see El-Moatassim *et al.*, 1992). In the kidney, ATP has been reported to stimulate the inositol lipid signalling cascade and subsequent Ca^{2+} mobilization in mesangial cells (Pfeilschifter, 1990a,b; Schulze-Lohoff *et al.*, 1992; Pavenstädt *et al.*, 1993), glomerular epithelial cells (Pavenstädt *et al.*, 1992) and MDCK cells (Paulmichl *et al.*, 1991).

More recently another nucleotide, UTP, was shown to be involved in the regulation of diverse cell functions (for review see Seifert & Schulz, 1989). Häussinger *et al.* (1987) and von Kügelgen *et al.* (1987) were the first to suggest that the action of UTP could involve a receptor distinct from the P_2-purinoceptor in rat liver. However, in certain other tissues, ATP and UTP may use a common nucleotide receptor for triggering their biological responses (Pfeilschifter, 1990b; Davidson *et al.*, 1990; for review see O'Connor, 1992). This latter receptor type appears to be distinct from previously described P_2-purinoceptor subtypes. Using rat mesangial cells, we have previously demonstrated that UTP and ATP stimulated phosphoinositide turnover, and that responses to both agonists were affected equally well by pertussis toxin and phorbol ester, non-additive at maximal concentrations, similarly attenuated by reactive blue 2 and displayed cross-desensitization. These features are all consistent with the hypothesis that ATP and UTP activate a common nucleotide receptor (Pfeilschifter, 1990b).

Another important signalling pathway appears to act exclusively on phosphatidylcholine in response to a wide variety of agonists and thus generates phosphatidic acid and

[1] Author for correspondence.

1,2-diacylglycerol (DAG) from lipid sources other than phosphoinositides (for review see Billah & Anthes, 1990). In mesangial cells, platelet-derived growth factor (Pfeilschifter & Hosang, 1991), angiotensin II (Pfeilschifter *et al.*, 1992), endothelin (Kester *et al.*, 1992) and vasopressin (Troyer *et al.*, 1992) have been reported to stimulate phosphatidylcholine hydrolysis by phospholipase D activity. In the present study we have investigated the effects of ATP and UTP on phosphatidylcholine hydrolysis. We present evidence that ATP and UTP activate with similar potencies and characteristics phospholipase D-mediated phosphatidylcholine turnover, thus suggesting that a common nucleotide receptor not only triggers phosphoinositide, but also phosphatidylcholine signalling cascades in rat mesangial cells.

Methods

Cell culture

Rat glomerular mesangial cells were cultured as described previously (Pfeilschifter *et al.*, 1984). In a second step, single cells were cloned by limited dilution using 96-micro-well plates. Clones with apparent mesangial cell morphology were used for further processing. The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which are considered to be specific for myogenic cells (Travo *et al.*, 1982), positive staining for Thy 1.1 antigen, negative staining for factor VIII-related antigen and cytokeratin excluded endothelial and epithelial contaminations, respectively. The generation of inositol trisphosphate upon activation of the angiotensin II AT_1 receptor was used as a functional criterion for characterizing the cloned cell line (Pfeilschifter, 1990c). The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 u ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and bovine insulin at 0.66 u ml^{-1} (Sigma).

Extraction and separation of diacylglycerol

Mesangial cells were labelled for 24 h with [^{3}H]-arachidonic acid (1.0 $\mu Ci\ ml^{-1}$; specific activity 240 Ci $mmol^{-1}$) or [^{3}H]-myristic acid (2.0 $\mu Ci\ ml^{-1}$; specific activity 54 Ci $mmol^{-1}$) in RPMI 1640 containing 10% fetal calf serum. The lipid extraction was performed according to Bligh & Dyer (1959). For separation of DAG the lipid extracts were dissolved in chloroform/methanol and spotted onto thin layer chromatography (t.l.c.) plates (precoated silica gel 60 with concentration zone, 0.25 mm thick). The chromatographs were developed in one dimension using n-heptane/diethyl ether/acetic acid (75:25:4, by vol.) as described by Pfeilschifter *et al.* (1984).

Assay of phospholipase D-catalyzed phosphatidylcholine hydrolysis

Nucleotide stimulated phosphatidylcholine hydrolysis was measured in cells prelabelled for 40 h with [methyl-^{3}H]-choline (5 $\mu Ci\ ml^{-1}$; specific activity 76 Ci $mmol^{-1}$) or for 24 h with [^{3}H]-myristic acid (2.0 $\mu Ci\ ml^{-1}$; specific activity 54 Ci $mmol^{-1}$). Prelabelled confluent cells were washed several times with medium to remove unincorporated label. The cells were incubated for a further 1 h. Ethanol was added to cell monolayers prelabelled with [^{3}H]-myristic acid 5 min prior to the addition of ATP or UTP. Incubations were terminated by collecting the medium and adding ice-cold methanol to the cells. After harvesting the cells from the dishes with a rubber policeman, lipids were extracted according to Bligh & Dyer (1959). Radioactivity in aliquots of medium, aqueous phase and chloroform phase was determined. Lipid extracts were separated by t.l.c., developed with either the top phase of ethyl acetate /2,2,4-trimethyl pentane/acetic acid/water (13:2:3:10, by vol.) for separation of phosphatidylethanol and phosphatidic acid or with chloroform/methanol/acetic acid/water (100:30:35:3, by vol.), for separation of phospholipids. Aqueous cell extracts and media were separated by ion-exchange chromatography on Dowex 50-WH^+ columns exactly as described by Cook & Wakelam (1989). Separation of aqueous extracts was cross-checked by a t.l.c. procedure modified from that described by Pritchard & Vance (1981) on silica gel G plates, developed with 0.5% NaCl/methanol/concentrated NH_4OH (10:10:1, by vol).

Statistical analysis

Statistical analysis was performed by one way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

Chemicals

[^{3}H]-choline and [^{3}H]-myristic acid were purchased from Amersham International, UK; [^{3}H]-arachidonic acid was from Du Pont de Nemours International, Regensdorf, Switzerland; ATP ATPγS, ADP, AMP, UTP, GTP, CTP, ITP, adenosine, cell culture media and nutrients were from Boehringer-Mannheim, Rotkreuz, Switzerland; βγ-imido-ATP, βγ-methylene-ATP and ADPβS were from Fluka Chemie, Buchs, Switzerland; 2-methylthio-ATP and calphostin C were from Ciba-Geigy Ltd., Basel, Switzerland; suramin was from Sigma Chemicals, Buchs, Switzerland; and phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate were from Calbiochem, Lucerne, Switzerland.

T.l.c. plates and all other chemicals used were from Merck, Darmstadt, Germany.

Results

ATP and UTP stimulate a biphasic DAG formation

Previously we have shown that [^{3}H]-myristic acid is selectively incorporated into phosphatidylcholine, whereas [^{3}H]-archidonic acid is equally distributed in phosphatidylcholine, phosphatidylethanolamine and phosphoinositides (Pfeilschifter *et al.*, 1992). Stimulation of mesangial cells with ATP (100 μM) or UTP (100 μM) increased the labelling of DAG, irrespective of the tracer used. The production of DAG was biphasic in [^{3}H]-arachidonic acid-labelled cells, with a first peak occurring at 20 s after the exposure to either ATP or UTP and a second peak at 5 min (Figure 1a). The first peak corresponds to the formation of inositol 1,4,5-trisphosphate (Pfeilschifter 1990a,b). DAG formation, therefore, may derive from phospholipase C-induced phosphoinositide hydrolysis. The second DAG peak occurred after inositol 1,4,5-trisphosphate levels had returned to control values. The time course of ATP- and UTP-stimulated DAG production in [^{3}H]-myristic acid-labelled cells is depicted in Figure 1b. DAG formation started to increase at 1 min and reached a maximum at 10 min. ATP- and UTP-induced production of DAG was dose-dependent as shown in Figure 2. There was no significant difference in the DAG response to ATP and UTP stimulation as regards the time-course and potency of both nucleotides, although UTP tended to be slightly more potent than ATP. In parallel to the nucleotide-triggered DAG formation there was a marked stimulation of phosphatidic acid generation (Figure 3).

ATP and UTP activate phospholipase D

Mesangial cells were labelled with [^{3}H]-choline and phosphatidylcholine hydrolysis was studied by analysis of the hydrophilic degradation products. Exposure of the cells to ATP (100 μM) or UTP (100 μM) caused a strong increase in choline production (Figure 4). There was no significant in-

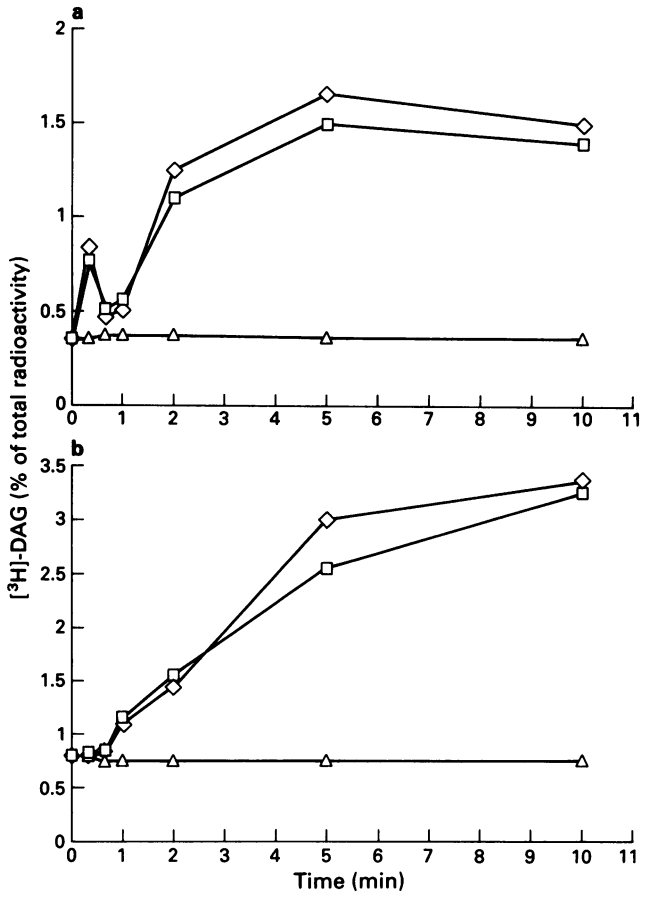

Figure 1 Time course of ATP and UTP-stimulated 1,2-diacylglycerol (DAG) formation in mesangial cells. Confluent cells were labelled with either [^{3}H]-arachidonic acid (a) or [^{3}H]-myristic acid (b) for 24 h. Cultures were then stimulated with ATP (100 μM, □), UTP (100 μM, ◇) or vehicle (△) for the indicated time periods. [^{3}H]-DAG formation was determined as described in Methods. Data are expressed as percentage of total incorporated radioactivity and are means of four experiments; the s.d. ranged from 5–12%.

crease in [^{3}H]-choline phosphate or in [^{3}H]-glycerophosphocholine upon stimulation of cells with ATP or UTP (data not shown). These data suggest that extracellular nucleotides activate a phospholipase D in mesangial cells, which degrades phosphatidylcholine. Phospholipase D can be more specifically assessed by its ability to catalyze a phosphatidyl transfer reaction in which ethanol or other primary alcohols act as the phosphatidyl moiety acceptor, thus generating phosphatidylethanol (Billah & Anthes, 1990). In the absence of ethanol, ATP and UTP stimulated the formation of phosphatidic acid and DAG (Figure 5). In the presence of 1% ethanol, ATP and UTP triggered the production of phosphatidylethanol, paralleled by a concomitant decline in phosphatidic acid and DAG accumulation (Figure 5). Obviously, both nucleotides triggered phospholipase D activation to a comparable extent. Preincubation of the cells with the putative P_2-receptor antagonist, suramin, dose-dependently attenuated both ATP- and UTP-induced phosphatidylethanol formation (Figure 6).

Table 1 shows the specificity for nucleotides and one nucleoside (100 μM each) in stimulating the production of phosphatidylethanol in mesangial cells. ITP, ATPγS, ADP and βγ-imido-ATP potently stimulated phosphatidylethanol production. βγ-Methylene-ATP, 2-methylthio-ATP and ADPβS had only a weak effect on phosphatidylethanol formation, while AMP, CTP, GTP and adenosine were completely ineffective in this capacity. The rank order of potency of the nucleotide analogues is compatible with a (common)

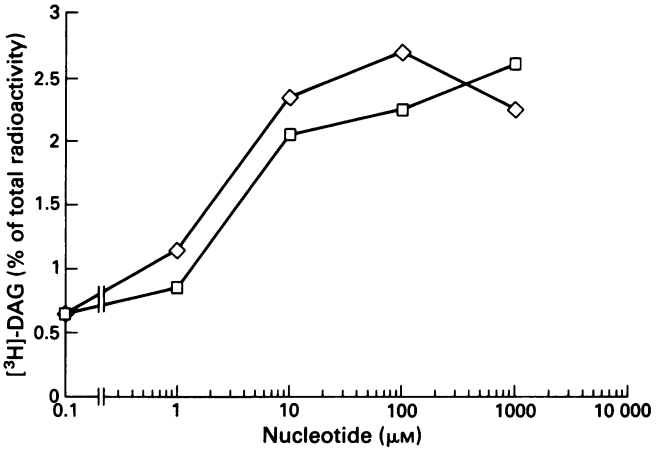

Figure 2 Dose-response curve of ATP- and UTP-stimulated 1,2-diacylglycerol (DAG) formation in mesangial cells. Confluent cells were labelled with [^{3}H]-myristic acid for 24 h and then stimulated with the indicated concentrations of ATP (□) or UTP (◇) for 10 min. [^{3}H]-DAG formation was determined as described in Methods. Data are expressed as percentage of total incorporated radioactivity and are means of four experiments; the s.d. ranged from 4–18%.

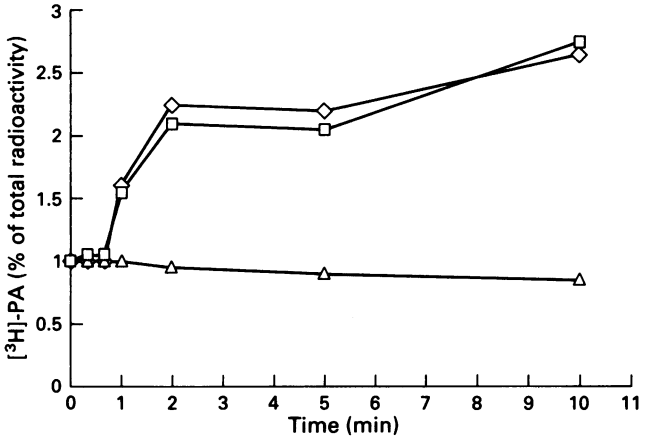

Figure 3 Time course of ATP- and UTP- stimulated phosphatidic acid (PA) formation in mesangial cells. Confluent cells were labelled with [^{3}H]-myristic acid for 24 h and then stimulated with ATP (100 μM, □), UTP (100 μM, ◇) or vehicle (△) for the indicated time periods. [^{3}H]-PA formation was determined as described in Methods. Data are expressed as percentage of total incorporated radioactivity and are means of four experiments; the s.d. ranged from 6–17%.

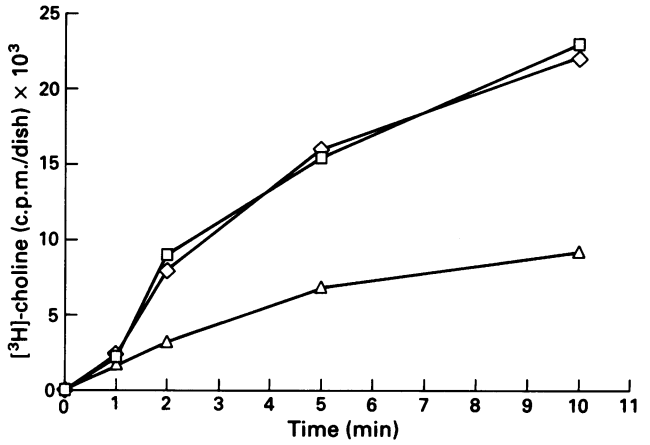

Figure 4 Time course of ATP- and UTP-stimulated choline formation in mesangial cells. Confluent cells were labelled for 40 h with [^{3}H]-choline. Cultures were then stimulated with ATP (100 μM, □), UTP (100 μM, ◇), or vehicle (△) for the indicated time periods. [^{3}H]-choline formation was determined in the culture supernatant. Results are means of four experiments, the s.d. ranged from 6–17%.

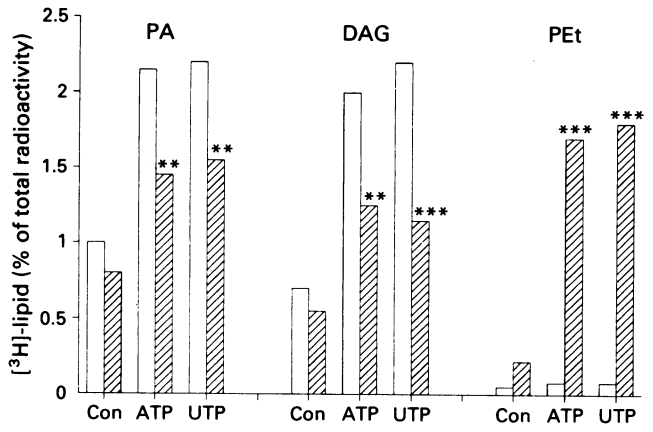

Figure 5 Effect of ATP and UTP on phosphatidic acid (PA), 1,2-diacylglycerol (DAG) and phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled with [^{3}H]-myristic acid and then exposed to ATP (100 μM), UTP (100 μM) or vehicle (Con) for 10 min, either in the presence (hatched columns) or absence (open columns) of 1% ethanol. [^{3}H]-phosphatidic acid (PA), [^{3}H]-phosphatidylethanol (PEt) and [^{3}H]-DAG formation was determined as described in Methods. Data are expressed as percentage of total incorporated radioactivity and are means of four experiments; the s.d. ranged from 3–13%. Significant differences from corresponding control (without ethanol): $^{**}P<0.01$ and $^{***}P<0.001$, ANOVA.

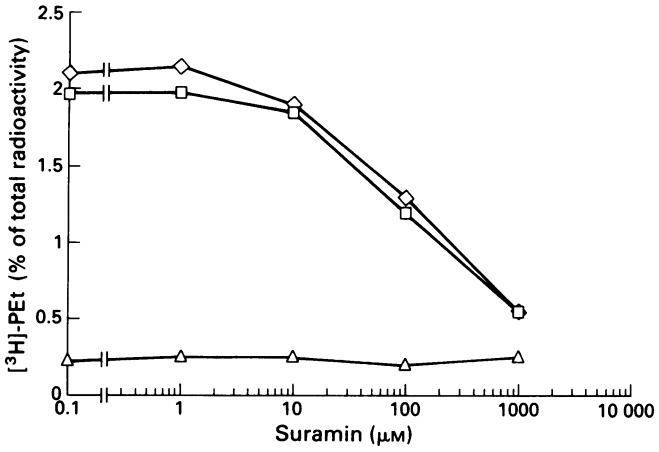

Figure 6 Effect of suramin on ATP- and UTP-stimulated phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]-myristic acid and pretreated for 30 min with the indicated concentrations of suramin. Cultures were then stimulated with ATP (100 μM, □), UTP (100 μM, ◇) or vehicle (△) for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in Methods. Results are means of four experiments; s.d. ranged from 5–18%.

nucleotide receptor-mediated phospholipase D activation. Further evidence for this suggestion was provided by the lack of additivity of maximal doses of ATP and UTP on phosphatidylethanol formation (Table 1).

Roles of calcium and protein kinase C (PKC) in ATP and UTP-stimulated phospholipase D activation

It has been previously demonstrated that ATP and UTP evoke an increased inositol 1,4,5-trisphosphate and DAG generation from phosphatidylinositol 4,5-bisphosphate which trigger Ca^{2+} mobilization and PKC activation, respectively (Pfeilschifter, 1990a,b). We were, therefore, interested to investigate what role, if any, the increased intracellular Ca^{2+} and PKC play in ATP- and UTP-stimulated phospholipase D-mediated phosphatidylcholine hydrolysis. The requirement of Ca^{2+} for phospholipase D activity was examined using the

Table 1 Effects of different nucleotides and one nucleoside on phosphatidylethanol (PEt) formation in mesangial cells

Addition	*PEt* (% of total radioactivity)
Control	0.21 ± 0.05
ATP	1.85 ± 0.19***
UTP	1.97 ± 0.21***
ITP	1.64 ± 0.09***
CTP	0.30 ± 0.07
GTP	0.30 ± 0.06
ADP	1.14 ± 0.11***
AMP	0.24 ± 0.03
Adenosine	0.19 ± 0.05
ATPγS	1.42 ± 0.14***
βγ-imido-ATP	1.12 ± 0.10***
βγ-methylene-ATP	0.57 ± 0.09
2-methylthio-ATP	0.64 ± 0.08*
ADPβS	0.53 ± 0.07
ATP + UTP	1.98 ± 0.22***

Confluent mesangial cells were incubated for 24 h with [^{3}H]-myristic acid and then exposed to the different nucleotides or one nucleoside (100 μM each) for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in Methods. Results are means ± s.d. of four experiments.
Significant differences from control: $^{*}P<0.05$ and $^{***}P<0.001$; ANOVA.

Ca^{2+} ionophore, A23187, the Ca^{2+} ATPase inhibitor, thapsigargin and by chelation of intracellular Ca^{2+} with high concentrations of Quin 2. As shown in Figure 7, A23187 (1 μM) and thapsigargin (200 nM) slightly increased phophatidylethanol formation. However, chelation of cytosolic Ca^{2+} with high doses of the Ca^{2+} buffer, Quin 2, did not attenuate ATP- or UTP-stimulated phosphatidylethanol formation, thus suggesting that an increase in cytosolic free Ca^{2+} is not essential for phospholipase D activation by nucleotides in mesangial cells.

The possible involvement of PKC in the nucleotide stimulation of phospholipase D activity was examined using phorbol esters, PKC down-regulation experiments, as well as a specific inhibitor of PKC. As shown in Figure 8, the PKC activator PMA caused a dose-dependent increase in phosphatidylethanol production. In contrast, the biologically inactive phorbol ester, 4α-phorbol 12,13-didecanoate, did not

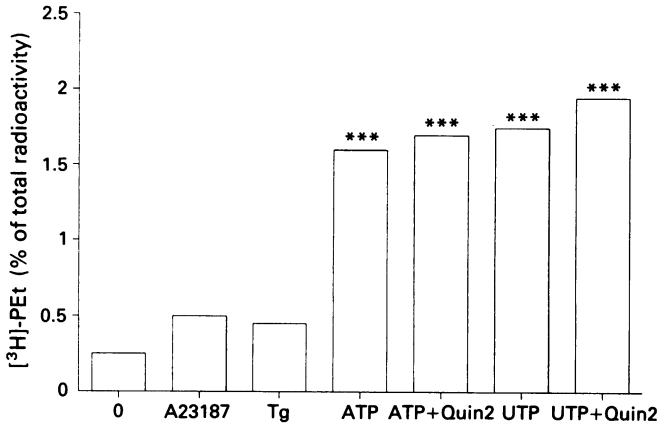

Figure 7 Effects of Ca^{2+} on phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]-myristic acid and pretreated for 60 min with 100 μM Quin 2/AM where indicated. Cultures were then stimulated with ATP (100 μM), UTP (100 μM), the Ca^{2+} ionophore A23187 (1 μM) or the Ca^{2+}-ATPase inhibitor, thapsigargin (Tg, 200 nM) for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in Methods. Results are means of four experiments; s.d. ranged from 4–11%. Significant differences from control: $^{***}P<0.001$; ANOVA.

activate phospholipase D (Figure 8). Furthermore, PKC down-regulation by prolonged PMA treatment (24 h) blocked ATP- and UTP-stimulated phosphatidylethanol synthesis (Figure 9). However, a 8 h treatment with PMA that causes depletion of α- and δ-isoenzymes of PKC in mesangial cells (Huwiler *et al.*, 1991; 1992) was not sufficient to eliminate nucleotide-induced phospholipase D activation (Figure 9). It was necessary to preincubate the cells for 24 h with PMA, a regimen that also causes depletion of PKC-ε (Huwiler *et al.*, 1991), to prevent completely the responses to ATP and UTP (Figure 9). Moreover, the selective PKC inhibitor, calphostin C (1 μM) (Kobayashi *et al.*, 1989) attenuated ATP- and UTP-stimulated phosphatidylethanol formation (Figure 10).

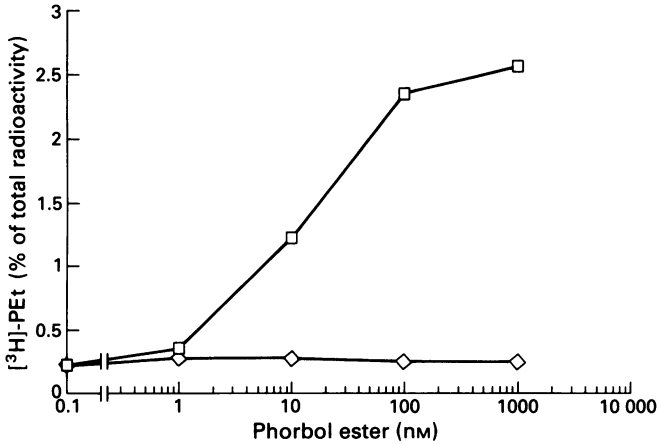

Figure 8 Dose-response curve of phorbol ester-stimulated phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]-myristic acid and then stimulated with the indicated concentrations of phorbol 12-myristate 13-acetate (PMA, □), or 4α-phorbol 12,13-didecanoate (◇) for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in Methods. Results are means of four experiments; s.d. ranged from 3–9%.

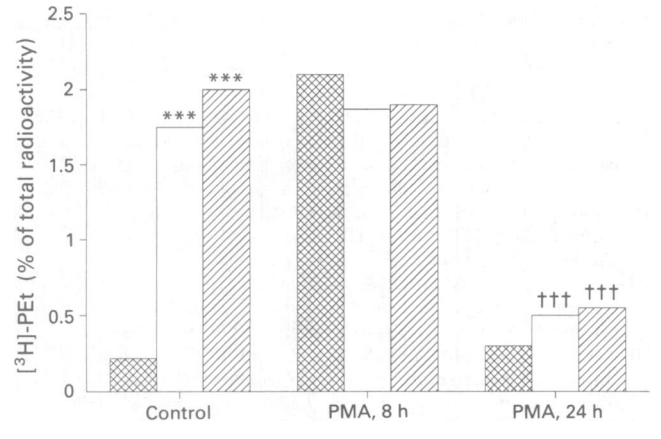

Figure 9 Effects of protein kinase C (PKC) down-regulation on ATP- and UTP-stimulated phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]-myristic acid and either non-pretreated (control) or pretreated for 8 h or 24 h with phorbol 12-myristate 13-acetate (PMA, 500 nM) as indicated. Cultures were then washed and stimulated with ATP (100 μM, open columns), UTP (100 μM, hatched columns) or vehicle (cross-hatched columns) for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in Methods. Results are means of four experiments; s.d. ranged from 5–19%. Significant differences from corresponding control (without stimulation): ***$P<0.001$; ANOVA. Significant differences from corresponding control ATP and UTP stimulation (without PMA pretreatment): †††$P<0.001$; ANOVA.

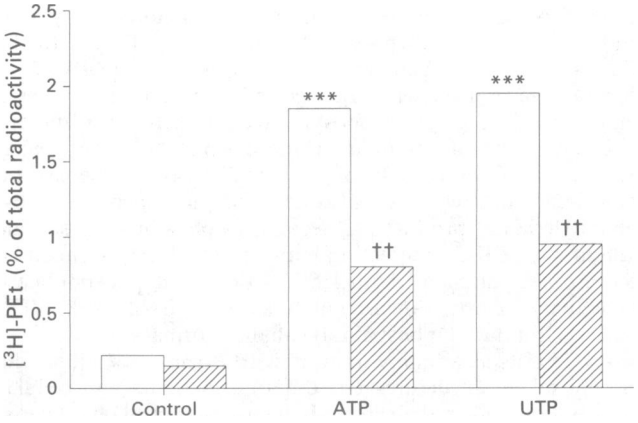

Figure 10 Effect of calphostin C on ATP- and UTP-stimulated phosphatidylethanol (PEt) formation in mesangial cells. Confluent cells were labelled for 24 h with [^{3}H]-myristic acid and pretreated for 30 min with calphosin C (1 μM, hatched columns) or vehicle (open columns). Cultures were then stimulated with ATP (100 μM), UTP (100 μM) or vehicle for 10 min in the presence of 1% ethanol. Lipid extraction and separation was performed as described in Methods. Results are means of four experiments; s.d. ranged from 7–14%. Significant differences from corresponding control: ***$P<0.001$; ANOVA. Significant differences from corresponding ATP or UTP stimulation in the absence of inhibitor. ††$P<0.01$, ANOVA.

Discussion

The presence of a nucleotide receptor with pharmacological characteristics different from those classically reported for a P_2-purinoceptor have been described first for sheep pituitary cells (Davidson *et al.*, 1990) and rat renal mesangial cells (Pfeilschifter, 1990b). ATP and UTP display similar potencies in activating the inositol lipid signalling cascade and subsequent Ca^{2+} mobilization whereas classical P_{2y} agonists (2-methylthio-ATP, ADPβS) and P_{2x} agonists (α,β-methylene-ATP; β,γ-methylene-ATP) were either inactive or showed only minor activity (Pfeilschifter, 1990b; Davidson *et al.*, 1990). The presence of this putative nucleotide receptor has also been described for human airway epithelial cells (Brown *et al.*, 1991), rat hepatocytes (Keppens *et al.*, 1992), the neuronal cell line N1E-115 (Iredale *et al.*, 1992), PC-12 phaeochromocytoma cells (Murrin & Boarder, 1992; Raha *et al.*, 1993) and a variety of other cell types (for review see O'Connor, 1992).

In the present study we have shown that ATP and UTP activate a phosphatidylcholine degrading phospholipase D in mesangial cells. Again, ATP and UTP displayed comparable potencies and characteristics in triggering this important signalling cascade, consistent with the hypothesis that ATP and UTP act via a common nucleotide receptor.

Phosphatidylcholine hydrolysis stimulated by ATP and UTP has also been demonstrated in endothelial cells (Purkiss & Boarder, 1992). Ultimate proof of a common nucleotide receptor mediating the described functional cell responses requires the development of convincing antagonists for either P_2-purinoceptors or the proposed nucleotide and pyrimidinoceptors.

We also have addressed the question concerning the pathways leading to activation of phospholipase D. There is substantial evidence for a role of Ca^{2+} and PKC in the activation of phospholipase D, indicating that activation of this enzyme is secondary to phosphoinositide hydrolysis (Billah & Anthes, 1990). In mesangial cells Ca^{2+} mobilization by the Ca^{2+} ionophore, A23187, and the Ca^{2+}-ATPase inhibitor, thapsigagrin, is able to trigger a small increase in phospholipase D activity. However, chelation of intracellular Ca^{2+} with high concentrations of Quin 2 did not affect ATP- and UTP-induced phospholipase D activation, indicating that agonist stimulation of the enzyme does not crucially

depend on Ca^{2+}. In this connection it is noteworthy that endothelin-stimulated phospholipase D was found to be Ca^{2+}-independent in mesangial cells (Kester *et al.*, 1992). In contrast, several lines of evidence indicate that PKC plays a major role in regulating phospholipase D activity in mesangial cells. First, the tumour promoting phorbol ester, PMA, activated phosphatidylcholine metabolism, whereas a biological inactive phorbol ester did not increase phosphatidylethanol production. Second, calphostin C, a specific inhibitor of PKC, interacting with the regulatory domain of the enzyme, attenuated nucleotide-stimulated phospholipase D activity. Third, down-regulation of PKC blocked ATP- and UTP-induced phosphatidylethanol formation.

PKC exists as a family of at least 10 isoenzymes, all having closely related structures but differing in tissue and cellular distribution and in their individual enzymological characteristics (Nishizuka, 1992). By immunoblot analysis we have shown previously that mesangial cells express four PKC isoenzymes, PKC-α, -δ, -ε and -ζ (Huwiler *et al.*, 1991; 1992; 1993). No PKC-β, -γ and -η isoforms were detected. On exposure to PMA, these isoenzymes displayed distinctly different down-regulation kinetics. An 8 h treatment with PMA was sufficient to deplete mesangial cells completely of PKC-α and -δ isotypes and a 24 h incubation with PMA was necessary to down-regulate PKC-ε. In contrast, PMA treatment for 24 h did not induce any depletion of PKC-ζ (Huwiler *et al.*, 1991; 1992; 1993). By comparing the down-regulation kinetics of the mesangial cell PKC isoenzymes with the time course of removal of the specific cellular functions, we have suggested that PKC-α and -δ may negatively regulate phosphoinositide turnover in response to angiotensin II (Huwiler *et al.*, 1991; 1992; Ochsner *et al.*, 1993) whereas PKC-ε is a candidate for regulation of phospholipase A_2 and prostaglandin synthesis in mesangial cells (Huwiler *et al.*, 1991). The data in Figure 9 show that an 8 h treatment with PMA still caused full activation of phospholipase D in mesangial cells, thus clearly ruling out a contribution of PKC-α or -δ isoenzymes to this cell response. In order to prevent ATP- and UTP-stimulated phosphatidylethanol formation, a 24 h incubation with PMA was required, suggesting that PKC-ε may not only trigger phospholipase A_2, but also phosphatidylcholine-specific phospholiase D. PKC-ε is a typical representative of the Ca^{2+}-independent group B isoenzymes (Stabel & Parker, 1991). In this connection it is worth noting that inhibitors of PKC which display a selectivity for the Ca^{2+}-dependent group A isoenzymes as compared to the Ca^{2+}-independent group B isotypes, such as staurosporine (McGlynn *et al.*, 1992), K-252a (Gschwendt *et al.*, 1989) or the specific PKC inhibitor CGP 41251 (Mate, B.M., Meyer, T., Stabel, S., Jaken, S., Fabbro, D. & Hynes, N.E., personal communication), did not inhibit ATP- or UTP-induced phospholipase D activation (Pfeilschifter & Merriweather, unpublished observations). Failure to achieve inhibition of phospholipase D with PKC inhibitors has led to the suggestion that phorbol ester may act in part through a PKC-independent mechanism (for review, see Billah & Anthes, 1990). Our observations could provide an alternative explanation for some of these reports. However, one should be aware that there may be a cell type-specific link between certain PKC isoenzymes and phosphilipase D activation. Overexpression of $PKC\beta_1$ in fibroblasts has been reported to enhance phospholipase D activity after PMA or endothelin-1 stimulation (Pai *et al.*, 1991a,b). On the other hand, DAG derived from phospholipase D-mediated phosphatidylcholine hydrolysis has been shown to activate selectively PKC-β in interferon-α-activated leucocytes (Pfeffer *et al.*, 1990) and PKC-ζ in activated *Xenopus* oocytes (Dominguez *et al.*, 1992). Thus, PKC-mediated activation of phospholipase D and the subsequent generation of DAG may provide a positive feedback loop to sustain PKC activation in cells.

In summary, our data suggest that ATP and UTP use a common nucleotide receptor to activate a phophatidylcholine-specific phospholipase D in mesangial cells. Furthermore, we provide evidence for a role of PKC-ε in mediating this nucleotide-induced cell response.

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References

BILLAH, M.M. & ANTHES, J.C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.*, **269**, 281–291.

BLIGH, E.G. & DYER, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**, 911–917.

BROWN, H.A., LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1991). Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5'-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.*, **40**, 648–655.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptors. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*. ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

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

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

COCKCROFT, S. & STUTCHFIELD, J. (1989). ATP stimulates secretion in human neutrophils and HL60 cells via a pertussis toxin-sensitive guanine nucleotide-binding protein coupled to phospholipase C. *FEBS Lett.*, **245**, 25–29.

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

DOMINGUEZ, I., DIAZ-MECO, M.T., MUNICIO, M.M., BERRA, E., GARCIA DE HERREROS, A., CORNET, M.E., SANZ, L. & MOSCAT, J. (1992). Evidence for a role of protein kinase C ζ subspecies in maturation of *Xenopus laevis* oocytes. *Mol. Cell. Biol.*, **12**, 3776–3783.

EL-MOATASSIM, C., DORNAND, J. & MANI, J.C. (1992). Extracellular ATP and cell signalling. *Biochim. Biophys. Acta*, **1134**, 31–45.

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

GSCHWENDT, M., LEIBERSPERGER, H. & MARKS, F. (1989). Differentiative action of K252a on protein kinase C and a calcium-unresponsive, phorbol ester/phospholipid-activated protein kinase. *Biochem. Biophys. Res. Commun.*, **164**, 974–982.

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

HUWILER, A., FABBRO, D. & PFEILSCHIFTER, J. (1991). Possible regulatory functions of protein kinas C-α and -ε isoenzymes in rat renal mesangial cells. *Biochem. J.*, **279**, 441–445.

HUWILER, A., FABBRO, D., STABEL, S. & PFEILSCHIFTER, J. (1992). Immunocharacterization of δ- and ζ-isoenzymes of protein kinase C in rat renal mesangial cells. *FEBS Lett.*, **300**, 259–262.

HUWILER, A., SCHULZE-LOHOFF, E., FABBRO, D. & PFEILSCHIFTER, J. (1993). Immunocharacterization of protein kinase C isoenzymes in rat kidney glomeruli, and cultured glomerular epithelial and mesangial cells. *Exp. Nephrol.*, **1**, 19–25.

IREDALE, P.A., MARTIN, K.F., ALEXANDER, S.P.H., HILL, S.J. & KENDALL, D.A. (1992). Inositol 1,4,5-trisphosphate generation and calcium mobilization via activation of an atypical P_2 receptor in the neuronal cell line, N1E-115. *Br. J. Pharmacol.*, **107**, 1083–1087.

KEPPENS, S., VANDEKERCKHOVE, A. & DE WULF, H. (1992). Extracellular ATP and UTP exert similar effects on rat isolated hepatocytes. *Br. J. Pharmacol.*, **105**, 475–479.

KESTER, M., SIMONSON, M.S., MCDERMOTT, R.G., BALDI, E. & DUNN, M.J. (1992). Endothelium stimulates phosphatidic acid formation in cultured rat mesangial cells: role of a protein kinase C-regulated phospholipase D. *J. Cell. Physiol.*, **150**, 578–585.

KOBAYASHI, E., NAKANO, H., MORIMOTO, M., TAMAOKI, T. (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.*, **156**, 548–553.

MCGLYNN, E., LIEBETANZ, J., REUTENER, S., WOOD, J., LYDON, N.B., HOFSTETTER, H., VANEK, M., MEYER, T. & FABBRO, D. (1992). Expression and partial characterization of rat protein kinase C-δ and protein kinase C-ζ in insect cells using recombinant baculovirus. *J. Cell Biochem.*, **49**, 239–250.

MURRIN, R.J.A. & BOARDER, M.R. (1991). Neuronal 'nucleotide' receptor linked to phospholipase C and phospholipase D? Stimulation of PC12 cells by ATP analogues and UTP. *Mol. Pharmacol.*, **41**, 561–568.

NISHIZUKA, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*, **258**, 607–614.

OCHSNER, M., HUWILER, A., FLECK, T. & PFEILSCHIFTER, J. (1993). Protein kinase C inhibitors potentiate angiotensin II-induced phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization in renal mesangial cells. *Eur. J. Pharmacol., Mol. Pharmacol.* (in press).

O'CONNOR, S.E. (1992). Recent development in the classification and functional significance of receptors for ATP and UTP, evidence for nucleotide receptors. *Life Sci.*, **50**, 1657–1664.

PAI, J.-K., DOBEK, E.A. & BISHOP, W.R. (1991a). Endothelin-1 activates phospholipase D and thymidine incorporation in fibroblasts overexpressing protein kinase. $C_{\beta 1}$. *Cell Reg.*, **2**, 897–903.

PAI, J.-K., PACHTER, J.A., WEINSTEIN, I.B. & BISHOP, W.R. (1991b). Overexpression of protein kinase Cβ1 enhances phospholipase D activity and diacylglycerol formation in phorbol ester-stimulated rat fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 598–602.

PAULMICHL, M., PFEILSCHIFTER, J. WÖLL, E. & LANG, F. (1991). Cellular mechanisms of ATP-induced hyperpolarization in renal epitheloid MDCK-cells. *J. Cell. Physiol.*, **147**, 68–75.

PAVENSTÄDT, H., SPÄTH, M. SCHLUNCK, G., NAUCK, M., FISCHER, R., WANNER, C. & SCHOLLMEYER, P. (1992). Effect of nucleotides on the cytosolic free calcium activity and inositol phosphate formation in human glomerular epithelial cells. *Br. J. Pharmacol.*, **107**, 189–195.

PAVENSTÄDT, H., GLOY, J., LEIPZIGER, J. KLÄR, B., PFEILSCHIFTER, J., SCHOLLMEYER, P. & GREGER, R. (1993). Effects of extracellular ATP on contraction, cytosolic calcium activity, membrane voltage and ion currents of rat mesangial cells in primary culture. *Br. J. Pharmacol.*, (in press).

PFEFFER, L.M., STRULOVICI, B. & SALTIEL, A.R. (1990). Interferon-α selectively activates the β isoform of protein kinase C through phosphatidylcholine hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 6537–6541.

PFEILSCHIFTER, J. (1990a). Extracellular ATP stimulates polyphosphoinositide hydrolysis and prostaglandin synthesis in rat mesangial cells. *Cell. Signal.*, **2**, 129–138.

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

PFEILSCHIFTER, J. (1990c). Angiotensin II B-type receptor mediates phosphoinositide hydrolysis in mesangial cells. *Eur. J. Pharmacol.*, **184**, 201–202.

PFEILSCHIFTER, J. & HOSANG, M. (1991). Effects of homo- and heterodimeric isoforms of PDGF on signalling events in rat renal mesangial cells. *Cell. Signal.*, **3**, 413–424.

PFEILSCHIFTER, J., KURTZ, A. & BAUER, C. (1984). Activation of phospholipase C and prostaglandin synthesis by (arginine) vasopressin in cultures of rat renal mesangial cells. *Biochem. J.*, **223**, 855–859.

PFEILSCHIFTER, J., THÜRING, B. & FESTA, F. (1989). Extracellular ATP stimulates poly (inositol phospholipid) hydrolysis and eicosanoid synthesis in mouse peritoneal macrophages in culture. *Eur. J. Biochem.*, **186**, 509–513.

PFEILSCHIFTER, J., HUWILER, A., MERRIWEATHER, C. & BRINER, V.A. (1992). Angiotensin II stimulation of phospholipase D in rat renal mesangial cells is mediated by the AT_1 receptor subtype. *Eur. J. Pharmacol. Mol. Pharmacol.*, **225**, 57–62.

PIROTTON, S., RASPE, E., DEMOLLE, D., ERNEUX, C. & BOEYNAEMS, J.-M. (1987). Involvement of inositol 1,4,5-trisphosphate and calcium in the action of adenine nucleotides on aortic endothelial cells. *J. Biol. Chem.*, **262**, 17461–17466.

PRITCHARD, P.H. & VANCE, D.E. (1981). Choline metabolism and phosphatidylcholine biosynthesis in cultured rat hepatocytes. *Biochem. J.*, **196**, 261–267.

PURKISS, J.R. & BOARDER, M.R. (1992). Stimulation of phosphatidate synthesis in endothelial cells in response to P_2-receptor activation. *Biochem. J.*, **287**, 31–36.

RAHA, S., DE SOUZA, L.R. & REED, J.K. (1993). Intracellular signalling by nucleotide receptors in PC12 pheochromocytoma cells. *J. Cell. Physiol.*, **154**, 623–630.

SCHULZE-LOHOFF, E., ZANNER, S., OGILVIE, A. & STERZEL, R.B. (1992). Extracellular ATP stimulates proliferation of cultured mesangial cells via P_2-purinergic receptors. *Am. J. Physiol.*, **263**, F374–F383.

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

STABEL, S. & PARKER, P.J. (1991). Protein kinase C. *Pharmacol. Ther.*, **51**, 712–795.

TRAVO, P., WEBER, K. & OSBORN, M. (1982). Co-existence of vimentin and desmin type intermediate filaments in a subpopulation of adult rat vascular smooth muscle cells growing in primary culture. *Exp. Cell. Res.*, **139**, 87–94.

TROYER, D.A., GONZALEZ, O.F. PADILLA, R.M. & KREISBERG, J.I. (1992). Vasopressin and phorbol ester-stimulated phosphatidylcholine metabolism in mesangial cells. *Am. J. Physiol.*, **262**, F185–F191.

VON KÜGELGEN, I., HÄUSSINGER, D. & STARKE, K. (1987). Evidence for a vasonconstriction-mediating receptor for UTP, distinct from the P_2 purine receptor, in rabbit ear artery. *Arch. Pharmacol.*, **336**, 556–560.

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