Am J Physiol Gastrointest Liver Physiol 291: G977–G986, 2006;
doi:10.1152/ajpgi.00041.2006.

Phospholipase D and extracellular signal-regulated kinase in hepatic stellate cells: effects of platelet-derived growth factor and extracellular nucleotides

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Submitted 23 January 2006; accepted in final form 1 May 2006

Benitez-Rajal, Joaquin, Maria-Jose Lorite, Alastair D. Burt, Christopher P. Day, and Michael G. Thompson. Phospholipase D and extracellular signal-regulated kinase in hepatic stellate cells: effects of platelet-derived growth factor and extracellular nucleotides. *Am J Physiol Gastrointest Liver Physiol* 291: G977–G986, 2006; doi:10.1152/ajpgi.00041.2006.—We have previously provided evidence suggesting that phosphatidic acid, possibly derived from the hydrolysis of phosphatidylcholine by phospholipase D (PLD), is involved in platelet-derived growth factor (PDGF)-mediated increases in extracellular signal-regulated kinase (ERK) activity and DNA synthesis in rat hepatic stellate cells (HSC), the primary fibrogenic cells of the liver. A recent study has shown the presence of P2Y nucleotide receptors on HSC that are coupled to contraction and synthesis of the matrix component, α_1-procollagen, leading to the suggestion that they may represent a new therapeutic target in the treatment of liver fibrosis. However, although extracellular nucleotides have been shown to stimulate both PLD and ERK, and to elicit proliferation of fibrogenic cells outside the liver, their effect on these parameters in HSC have not yet been investigated. PLD activity was determined by [^{3}H]choline release and [^{3}H]phosphatidylbutanol production, ERK activity by Western blotting, and DNA synthesis by [^{3}H]thymidine incorporation. We report here, for the first time in HSC, that extracellular nucleotides stimulate PLD activity and a sustained activation of ERK. However, in contrast to PDGF, nucleotides had negligible effects on DNA synthesis. Moreover, the effects of PDGF and nucleotides on PLD and ERK were not additive, suggesting activation of the same PLD isoform and pool of ERK. The data demonstrate that nucleotide-stimulated PLD and ERK activities are not coupled to DNA synthesis in HSC. Instead, these responses may be linked to other phenotypic changes associated with activated HSC such as increases in contraction, motility, or extracellular matrix deposition.

liver fibrosis; phosphatidic acid; DNA synthesis

HEPATIC STELLATE CELLS (HSC) are regarded as the principal cell type responsible for liver fibrosis. In response to injury, the HSC population expands and transforms or "activates" into myofibroblast-like cells characterized by increased proliferation, motility, contraction, and production of extracellular matrix components. In the case of chronic liver damage, this results in fibrosis and ultimately cirrhosis (12, 36). As a result, the intracellular signaling pathways regulating these events in HSC are the subject of intense study toward the ultimate goal of developing antifibrotic therapies based on their manipulation.

Platelet-derived growth factor (PDGF) is the most potent mitogen for HSC in vitro, and we have previously demonstrated that PDGF stimulates phosphatidic acid (PA) production in these cells (33). Moreover, the addition of exogenous PA to HSC elicits a sustained activated of extracellular signal-regulated kinase (ERK) and DNA synthesis in these cells, and inhibition of ERK abolishes both PA (33) and PDGF (21) effects on DNA synthesis.

There are two main routes through which PA can be derived: indirectly via the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC) and directly from phosphatidylcholine by the activation of phospholipase D (PLD). Although PDGF has been shown to activate PLC in HSC (29), there have been no reports of PLD activity in these cells to date. PLD exists as two main isoforms, PLD1 and PLD2, whose roles appear to be particularly relevant to activated HSC. They may be involved in the regulation of cell contraction/migration via effects on the cytoskeleton (18, 20), cell proliferation via activation of ERK (35), or production of extracellular matrix components (15). An important role for PLD is also suggested by the demonstration that, in vivo, coadministration of a purified preparation of PLD dramatically increased the onset of fibrosis/cirrhosis in the carbon tetrachloride model (26).

A recent investigation has shown the presence of P2Y nucleotide receptors in quiescent and activated rat HSC (9). Evidence now suggests that, after tissue injury, free nucleotides are released from damaged cells and exert paracrine effects on their target tissues (2, 6), including proliferation of fibrotic mesangial cells in the kidney (14). However, little is known regarding the role of nucleotides in HSC.

The aim of this study was to investigate our hypothesis that HSC contain a nucleotide-sensitive PLD activity and that this is coupled via ERK to DNA synthesis. We report here that nucleotides elicited PLD and ERK activity, but, unlike PDGF, had negligible effects on DNA synthesis. Moreover, the effects of PDGF and nucleotides on PLD and ERK were not additive, suggesting activation of the same PLD isoform and pool of ERK. The data demonstrate that nucleotide-stimulated PLD and ERK activities are not coupled to DNA synthesis in HSC. Instead, they may be linked to responses such as increases in motility, contraction, (39) or extracellular matrix production (9).

METHODS

Materials and Reagents

[Methyl-^{3}H]choline chloride (specific activity 85 Ci/mmol), [9,10-^{3}H]oleic acid (specific activity 10 Ci/mmol), [methyl-^{3}H]thymidine (specific activity 80 Ci/mmol), Hyperfilm MP, and enhanced chemi-

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luminescence Western blotting detection reagents were from Amersham International (Amersham, Bucks, UK). All materials for isolation of HSC were from previously defined sources (33), and tissue culture reagents were from Life Technologies (Paisley, UK). EN^{3}HANCE spray was from DuPont NEN (Stevenage, Herts, UK) and silica gel K6 thin-layer chromatography plates were obtained from Whatman (Maidstone, Kent, UK). Scintillation fluid (Ultima-Gold XR) was from Packard (Pangbourne, Berks, UK) and the nonradioactive MAP kinase assay kit was from New England Biolabs (UK) (Hitchin, Herts, UK). Dowex-50-WH$^+$, nucleotides, and phorbol ester were from Sigma (Poole, Dorset, UK). The polyclonal antibody for ERK 2 was from Santa Cruz, distributed by Insight Biotechnology (Wembley, UK). The RT-PCR and miniprep kits were from Quiagen (Crawley, UK), and the total RNA extraction kit was from Flowgen (Ashby-de-la-Zouch, UK).

Laboratory Animals

Male Sprague-Dawley rats weighing 450–550 g were supplied by Charles River (Margate, UK) and housed in group cages under conditions of controlled temperature and illumination. They were supplied laboratory chow and water ad libitum. All animal experiments were conducted under license and in accordance with current Home Office regulations.

Isolation and Culture of HSC

Cells were isolated by pronase and collagenase digestion as described previously (33), suspended in DMEM containing 20% fetal calf serum and antibiotics and seeded at ~10^6 cells/ml. Experiments were performed on 2-wk-old activated HSC that had been grown to confluence and passaged once.

PLD Activity

This was quantified by measuring both [^{3}H]choline release and [^{3}H]phosphatidylethanol (PtdEtOH) formation.

Choline release. This was performed essentially as in previous studies (25, 40). Briefly, before each experiment, cells were transferred to 3 ml of serum-free DMEM containing [methyl-^{3}H]choline (1.33 μCi/ml) for 24 h. At the end of this labeling period, the cells were washed and incubated in DMEM for 30 min. After a further wash, cells were then incubated with or without agonists in DMEM for 15 min or as specified in the figure legends. At the end of the incubation, the medium was removed from the dishes and to 0.8 ml of this were added 3 ml of ice-cold chloroform-methanol (1:2 vol/vol). After 30 min on ice, phases were separated by the addition of chloroform (1 ml) and water (1 ml). The upper (water-methanol)

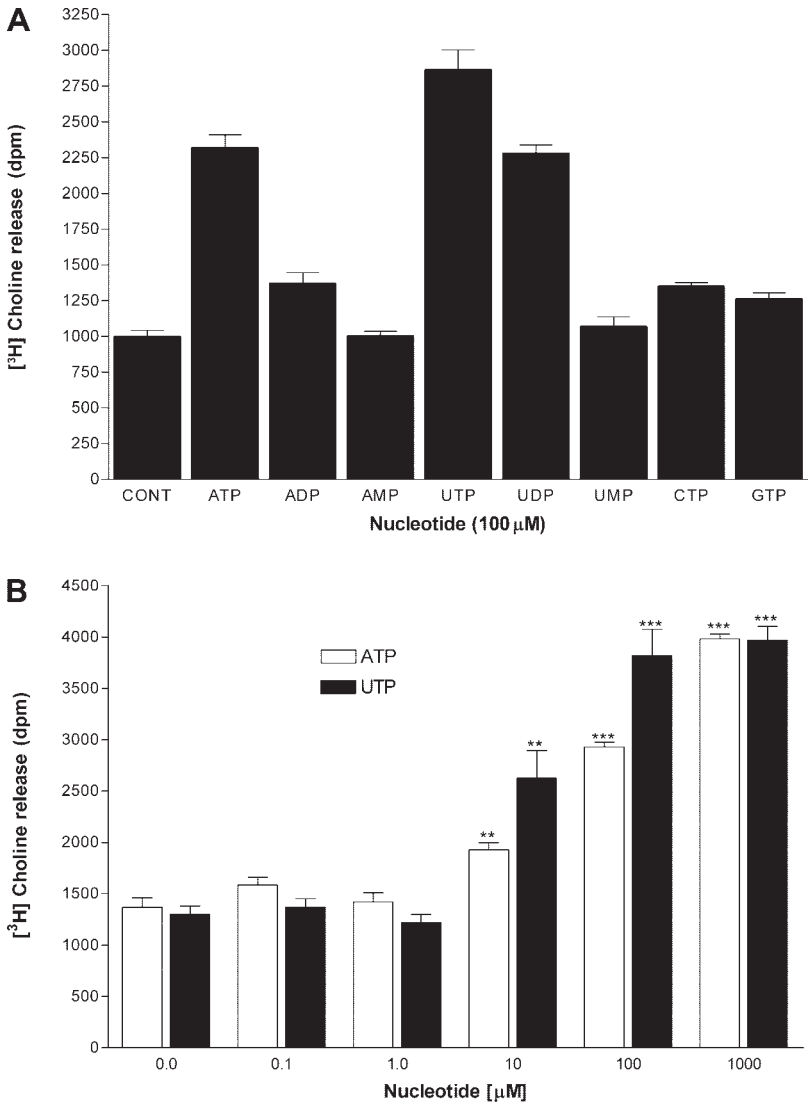

Fig. 1. Stimulation of [^{3}H]choline release by nucleotides in hepatic stellate cells (HSC). Cells were incubated for 24 h in serum-free medium with 1.33 μCi/ml [methyl-^{3}H]choline and then treated with either individual nucleotides (100 μM) (*A*) or increasing concentrations of ATP/UTP, for 15 min (*B*). CONT, control. [^{3}H]Choline release was measured as described in METHODS. Figures show results from a single experiment with each bar representing the mean ± SE of 3 dishes. Similar results were obtained in 2 other independent experiments. By Student's *t*-test, $^{**}P < 0.01$; $^{***}P < 0.001$.

phase was removed, and metabolites were separated by Dowex ion-exchange chromatography and subjected to scintillation counting.

PtdEtOH formation. This is the classic transphosphatidylation reaction used as a specific marker for PA produced by PLD activity (11). HSC were incubated in 3 ml of serum-free DMEM containing [9,10-^{3}H]oleic acid (1.33 μCi/ml) for 36 h. The cells were then washed to remove the label and incubated with agonist in the presence of 2% ethanol for 15 min or as specified in the figure legends. Phospholipids were then extracted, and [^{3}H]PtdEtOH was separated by thin-layer chromatography. After autoradiography, [^{3}H]PtdEtOH was measured by scintillation counting (41).

RT-PCR Analysis of PLD Isoenzymes

RNA was extracted from both primary nonpassaged and passaged 14-day HSC by use of a total RNA extraction kit. One microgram was used as a template for PCR using a single step RT-PCR kit at an annealing temperature of 60°C and 30 cycles. PLD 1 (U69550) and PLD 2 (D88672) sequences were obtained from the Gen Bank/EMBL Data Bank. The sequences of the upstream and downstream primers for the PLD isoenzymes were as follows: 5′-CAGCATCAGTAGAGTCTATG-3′ and 5′-TTTCCGTGAACCACAGAACC-3′ for PLD 1a and PLD 1b, respectively; 5′-CACTGCAGAAGATCGCCGC-3′ and 5′-GCCAAAGAACTGCTCCTCC-3′ for PLD 1; and 5′-ACTGTAACCCAGACGGAC-3′ and 5′-GCTGGCTGTGTGTCTGGC-3′ for PLD 2. PCR yielded products of the expected size that were then eluted from a 2% agarose gel, cloned into a pGEMT-easy vector system, and transformed into Novablue competent cells. Plasmid DNA was extracted with a miniprep kit and sequenced.

ERK Activity

Cells were incubated in 2 ml of serum-free DMEM for 24 h, washed and treated with or without agonist for 15 min or as specified in the figure legends. ERK activity was then extracted and assayed with a commercially available nonradioactive kit. Briefly, a monoclonal phospho-antibody to p44/42 ERK (threonine 202 and tyrosine 204) was used to selectively immunoprecipitate active ERK from cell lysates. The resulting immunoprecipitate was then used to phosphorylate Elk-1 fusion protein. Phosphorylation of Elk-1 at serine 383 was determined by Western blotting using a phospho-Elk-1 (serine 383) antibody.

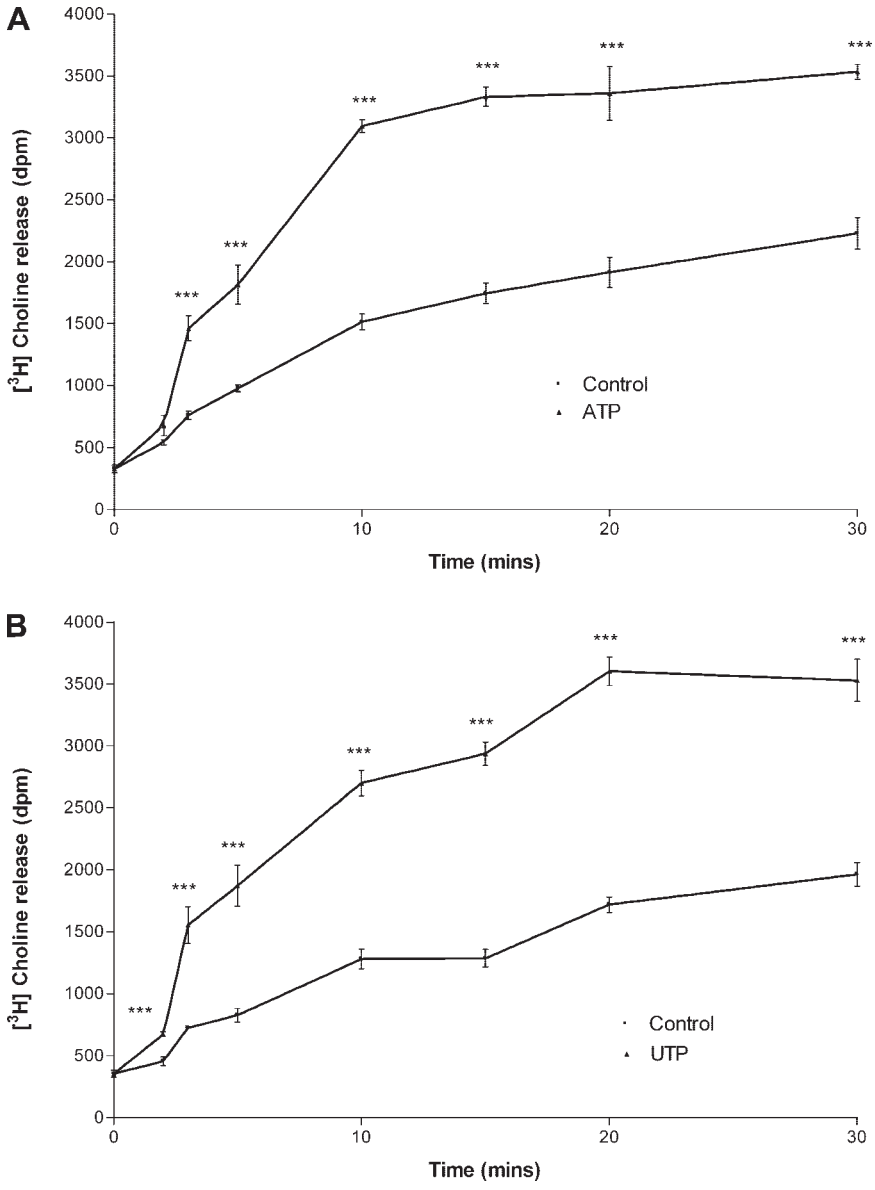

Fig. 2. Time-dependent stimulation of [^{3}H]choline release by ATP/UTP in HSC. Cells were incubated for 24 h in serum-free medium with 1.33 μCi/ml [methyl-^{3}H]choline and then treated with either ATP (*A*) or UTP (*B*) (100 μM) for times up to 30 min. [^{3}H]choline was extracted, separated by Dowex ion-exchange chromatography, and subjected to scintillation counting as described in METHODS. Figures show combined results from 2 experiments with each bar representing the mean ± SE of 6 dishes. By Student's *t*-test, ***$P < 0.001$.

To demonstrate efficient extraction of ERK, controls were performed by blotting nonimmunoprecipitated protein samples with an antibody to ERK 2 (33) and subsequent detection by chemiluminescence.

DNA Synthesis

This was performed essentially as described previously using HSC which had been incubated in serum-free DMEM for 24 h (33). Nucleotides were either added for 12 h in the presence of [^{3}H]thymidine or they were added for 24 h and radiolabel was present for either 24 h or the last 4 h. DNA was then extracted and radioactivity was determined by scintillation counting (33).

Statistical Analysis

Data for [^{3}H]choline, and [^{3}H]PtdEtOH are presented as mean ± SE where n refers to the number of experiments, all of which were performed on at least three occasions. Statistical analysis was performed by use of Student's t-test. Data for ERK activity are presented as a qualitatively representative experiment of three and were assessed by two-way ANOVA of logarithmically transformed data.

RESULTS

Nucleotides (100 μM) stimulated [^{3}H]choline release during a 15-min incubation. The largest responses were observed with UTP, UDP, and ATP (Fig. 1*A*). The effects of both ATP and UTP were concentration dependent with significant effects observed at 10 μM and above (Fig. 1*B*). The increase in [^{3}H]choline release elicited by both nucleotides was time dependent. An increased rate of [^{3}H]choline release was observed from ATP/UTP-treated cells for the first 10 min, after which [^{3}H]choline release from agonist and control cells increased in parallel (Fig. 2).

Nucleotides (100 μM) also elicited [^{3}H]PtdEtOH formation during a 15-min incubation (Fig. 3). The largest increases were again observed with UTP, UDP, and ATP. The effects of both ATP and UTP were concentration dependent (Fig. 4) and similar to the response observed for [^{3}H]choline release (Fig. 1*B*). The increase in [^{3}H]PtdEtOH formation elicited by ATP and UTP was also time dependent. Like the effect on [^{3}H]choline release (Fig. 2), an increased rate of [^{3}H]PtdEtOH formation was observed from agonist-treated cells for the first 10 min and this then plateaued (data not shown). Downregulation of PKC by 24 h preincubation with 1 μM phorbol ester (12-*O*-tetradecanoylphorbol 13-acetate) completely blocked ATP and UTP effects on [^{3}H]PtdEtOH formation. In a representative 15-min experiment, ATP or UTP increased [^{3}H]PtdEtOH formation from 3,279 ± 299 dpm to 11,904 ± 647 and 14,244 ± 1,017 dpm, respectively (both $P < 001$ vs. control, $n = 3$) in the absence of phorbol ester. In contrast, they had no significant effect (3,511 ± 180 vs. 3,487 ± 425 and 3,627 ± 399 dpm, respectively) in its presence. Analysis of the RT-PCR products showed the presence of two PLD 1 bands and a PLD 2 band of the appropriate size in both primary, nonpassaged and 2-wk-old passaged HSC (Fig. 5). Subsequent sequencing (data not shown) showed a high degree of homology to the published sequences for rat brain PLD 1 (97%) and PLD 2 (98%).

Nucleotides (100 μM) stimulated ERK activity during a 15-min incubation, and again the largest effects were detected with UTP, UDP, and ATP. Increases were also observed with CTP and GTP (data not shown), but AMP and UMP had no effect (Fig. 6, *A* and *B*). A protein-loading control demonstrated efficient extraction of ERK before immunoprecipitation (Fig. 6*C*). The stimulation of ERK by both ATP and UTP was concentration dependent (Fig. 7) and occurred over the same range as that observed for [^{3}H]choline release (Fig. 1*B*) and [^{3}H]PtdEtOH formation (Fig. 4). The responses to ATP and UTP

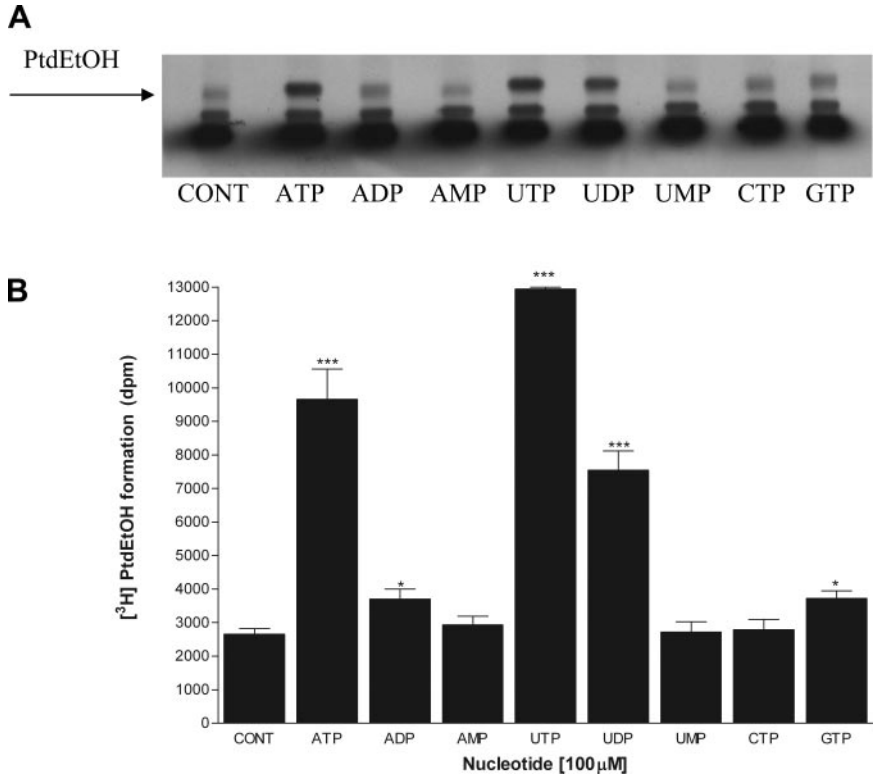

Fig. 3. Stimulation of [^{3}H]phosphatidylethanol ([^{3}H]PtdEtOH) production by nucleotides in HSC. Cells were incubated for 36 h in serum-free medium with 1.33 μCi/ml [9,10-^{3}H]oleic acid and then treated with nucleotides (100 μM) for 15 min. [^{3}H]PtdEtOH formation was assessed as described in METHODS. Figures show representative autoradiograph (*A*) and histogram (*B*) of results from a single experiment with each bar representing the mean ± SE of 3 dishes. Similar results were obtained in 2 other independent experiments. By Student's t-test, $^*P < 0.05$; $^{***}P < 0.001$.

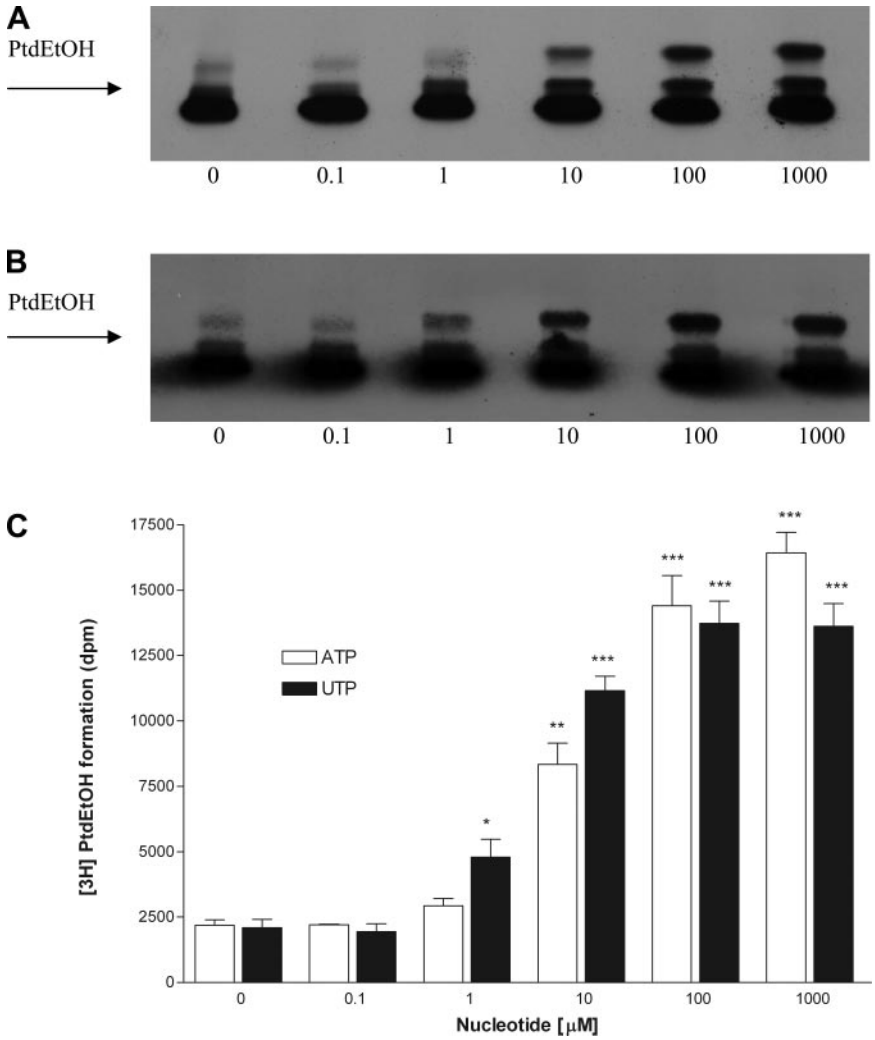

Fig. 4. Concentration-dependent stimulation of [^{3}H]PtdEtOH production by ATP and UTP in HSC. Cells were incubated for 36 h in serum-free medium with 1.33 μCi/ml [9,10-^{3}H]oleic acid and then treated with increasing concentrations of ATP or UTP for 15 min. [^{3}H]PtdEtOH formation was measured as described in METHODS. Figures show representative autoradiograph for ATP (*A*), representative autoradiograph for UTP (*B*), and histogram of results from a single experiment with each bar representing the mean ± SE of 3 dishes (*C*). Similar results were obtained in 2 other independent experiments. By Student's *t*-test, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

were also time dependent. ATP elicited a rapid stimulation of ERK, which had returned to basal levels by 60 min. In contrast, the response to UTP was considerably more sustained with 40–50% of the maximal response remaining after 120 min (Fig. 8).

When PDGF (100 ng/ml) and ATP or UTP (100 μM) were added simultaneously, neither nucleotide was able to significantly increase the effect of PDGF on ERK activity above that observed with PDGF alone (Fig. 9, *A* and *B*). Similarly, PDGF was unable to significantly increase the effect of ATP or UTP on [^{3}H]PtdEtOH activity above that observed with either nucleotide alone (Fig. 9*C*).

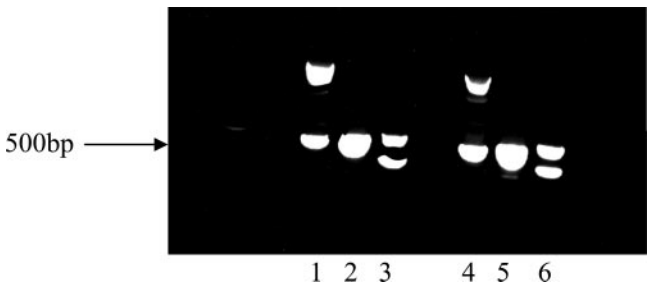

Fig. 5. Identification of mRNA encoding phospholipase D (PLD) 1 and PLD2 in HSC. Primers corresponding to the published sequences of rat brain PLD1 and PLD2 were synthesized and used for RT-PCR. Analysis of the RT-PCR products showed the presence of amplicons of the appropriate size which were subsequently sequenced. *1*) PLD 2: primary HSC; *2*) PLD 1: primary HSC; *3*) PLD1a and b: primary HSC; *4*) PLD 2: passaged HSC; *5*) PLD 1: passaged HSC; *6*) PLD1a and b: passaged HSC.

The addition of PDGF (100 ng/ml) to HSC significantly increased [^{3}H]thymidine incorporation into DNA during both 12- and 24-h incubations. For example, when radiolabel was added for the last 4 h of a 24 h incubation, [^{3}H]thymidine incorporation increased from 654 ± 108 to 2,187 ± 314 dpm/well ($P < 0.01$, $n = 4$). In contrast, we were unable to detect any significant effects of nucleotides on DNA synthesis regardless of whether they were added for 12 h in the presence of [^{3}H]thymidine or they were added for 24 h and radiolabel was present for either 24 h or the last 4 h (Table 1).

DISCUSSION

A study by Takemura and colleagues 10 years ago described a "nucleotide receptor" on HSC (39). However, the possibility of such receptors on these cells had not been further investigated until recently. Nucleotide receptors are classified as P2

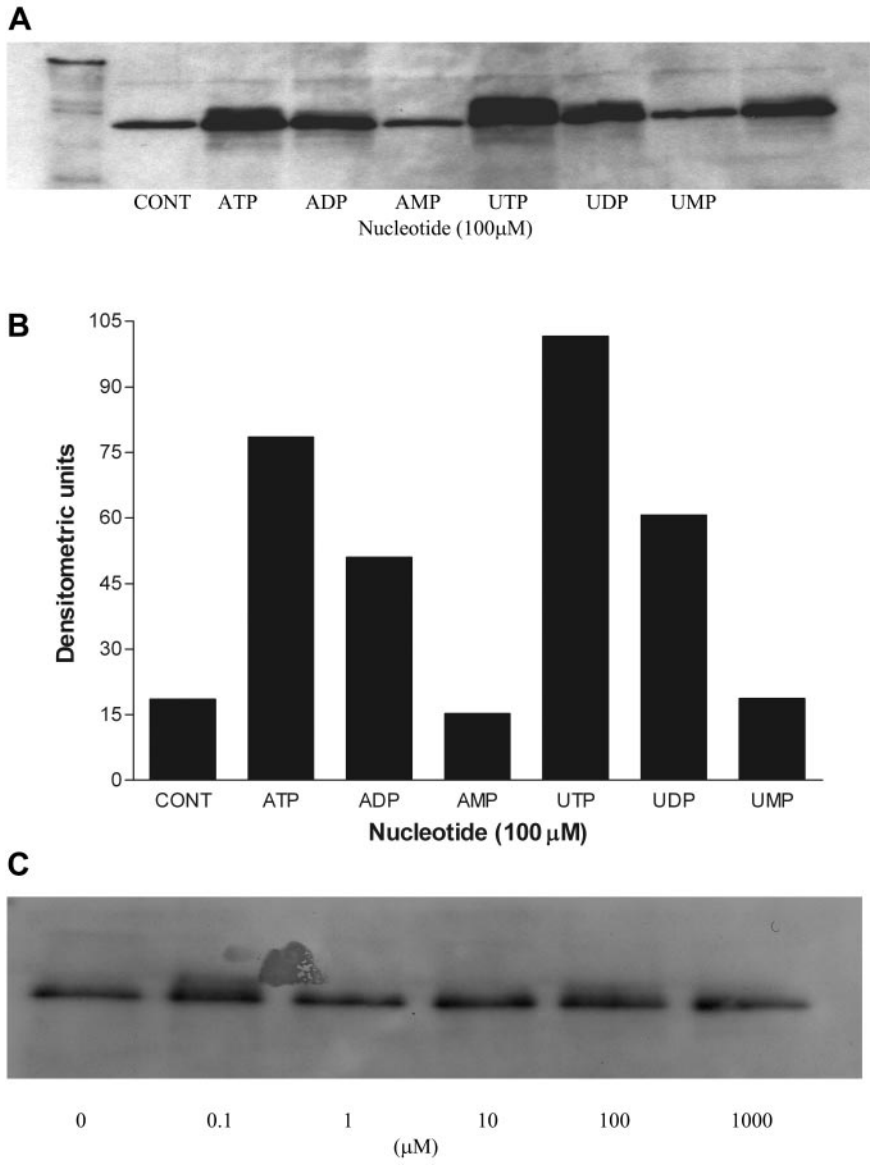

Fig. 6. Stimulation of ERK activity by nucleotides in HSC. Cells were incubated for 24 h in serum-free medium and then treated with nucleotides (100 μM) for 15 min. Active ERK was measured as described in METHODS. Figures show representative Western blot (*A*) and histogram (*B*) from a representative experiment of 3. A protein loading control generated by incubating cells with 0–1000 μM ATP for 15 min and then Western blotting nonimmunoprecipitated protein for total ERK 2 is also shown (*C*).

and are subdivided into ligand-gated P2X receptors and G protein-coupled P2Y receptors (2). Of the latter, four P2Y subtypes have been cloned in the rat, and these, with their ligand specificity, are $P2Y_1$ (ADP), $P2Y_2$ and $P2Y_4$ (ATP = UTP), and $P2Y_6$ (UDP) (32). A recent RT-PCR study demonstrated that primary, nonpassaged, *day 1* HSC expressed the $P2Y_2$ and $P2Y_4$ subtypes, whereas by *day 7* they expressed the $P2Y_1$ and $P2Y_6$ receptors (9). However, the observation that cells were activated by ATP at this time does suggest the presence of an additional receptor(s). These authors also demonstrated that nucleotide activation of PLC and the corresponding increase in intracellular calcium in passaged HSC are also mediated by P2Y receptors by showing that the effects were inhibited by the P2 receptor inhibitor, suramin, in the absence of extracellular calcium (9). In preliminary experiments, we were also able to reproduce these nucleotide effects on PLC in passaged, 14-day HSC (J. Benitez-Rajal, unpublished observation), and the responses we observed were qualitatively identical to those reported for PLC activation in primary, nonpassaged HSC (39). When taken with our finding of mRNA for PLD 1 and PLD 2 in primary and passaged cells, this demonstrates that these signaling pathways are equally applicable to both forms of cultured HSC. Our observation that the largest responses seen on *day 14* were observed with ATP and UTP suggests that $P2Y_2$ and/or $P2Y_4$ receptors were present at this time. $P2Y_2$ receptors, in particular, have been found both within, e.g., hepatocytes (7) and without the liver, e.g., renal mesangial cells (17).

As a result of tissue injury, free nucleotides are released from both platelets and damaged cells (36), and it is now generally assumed that localized concentrations in the high micromolar range can be reached at the cell surface (6).

The demonstration that nucleotides stimulate both [^{3}H]choline release and [^{3}H]PtdEtOH formation provides, for the first time, clear evidence of PLD activity in HSC. The ability of phorbol ester to inhibit nucleotide-mediated increases in PLD in HSC is similar to responses observed with these agents in both hepatocytes (13) and nonliver cells (e.g., Ref. 30). This is

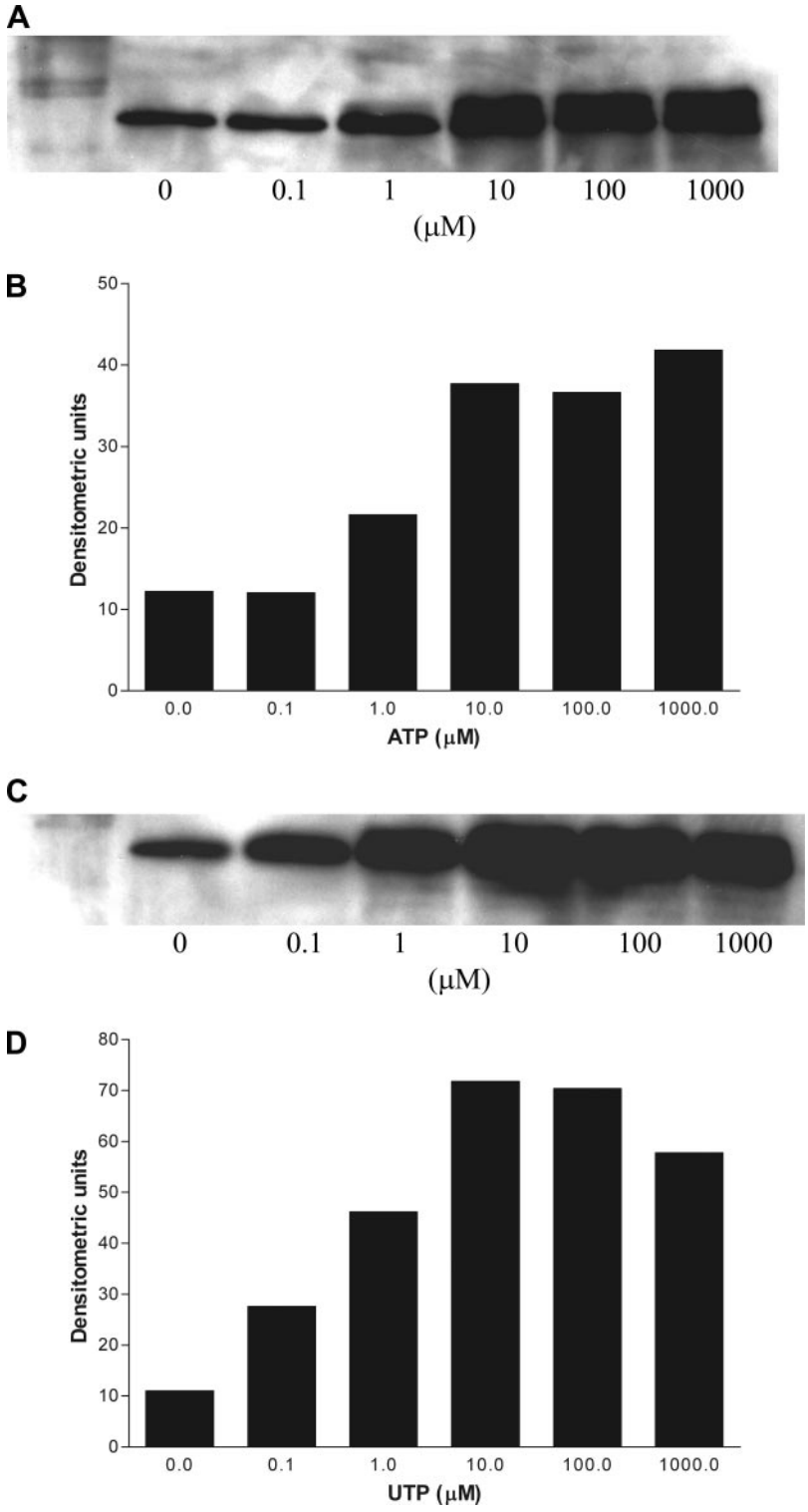

Fig. 7. Concentration-dependent stimulation of ERK activity by ATP and UTP in HSC. Cells were incubated for 24 h in serum-free medium and then treated with increasing concentrations of ATP or UTP for 15 min. Active ERK was determined as described in METHODS. Figures, which are from a representative experiment of 3, show representative Western blot (*A*) and histogram (*B*) for ATP and representative Western blot (*C*) and histogram (*D*) for UTP. Two-way ANOVA of logarithmically transformed data showed for ATP, $P < 0.05$ vs. concentration (*A*) and for UTP, $P < 0.01$ vs. concentration (*B*).

consistent with the notion that diacylglycerol generated by nucleotide-induced PLC-mediated hydrolysis of PIP_2 is required for PKC activation of PLD (1, 9). It is already well established that nucleotides stimulate PKC in other cell types (e.g., Ref. 10), but little is known regarding PKC in HSC. A previous study has shown that these cells contain the α, ϵ, δ, and ζ isoforms (31) and, although it would be predicted from a knowledge of their structure that α, ϵ, and δ would be subject to downregulation by phorbol ester and thus may play a role in the activation of PLD in response to nucleotides in HSC, all of these isoforms have been shown to be involved in receptor-mediated PLD activation (5, 24, 27, 28). The identity of the PKC isoform(s) mediating nucleotide effects on PLD in HSC requires further investigation.

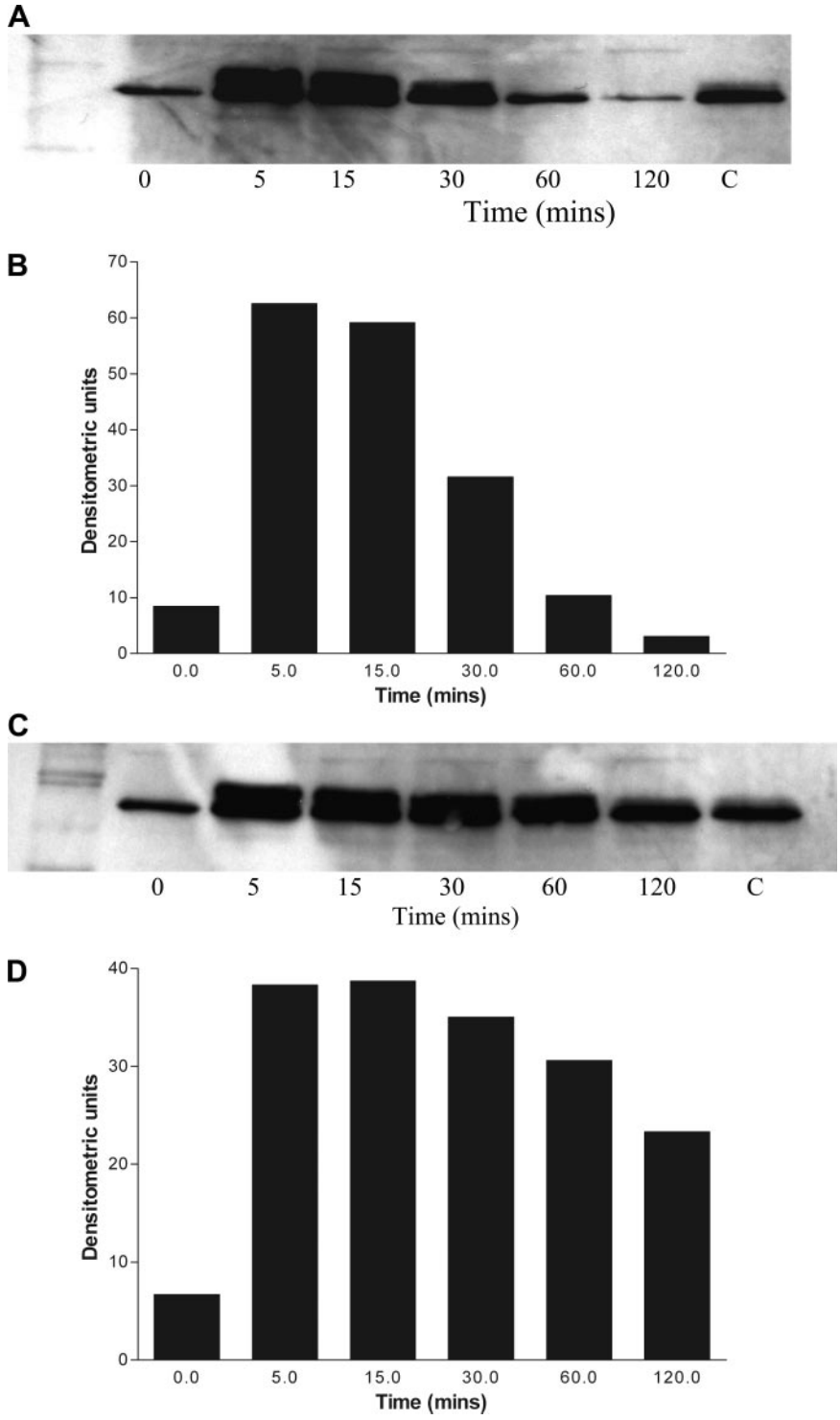

Fig. 8. Time-dependent stimulation of ERK activity by ATP and UTP in HSC. Cells were incubated for 24 h in serum-free medium and then treated with ATP or UTP (100 μM) for increasing times up to 30 min. Active ERK was assessed as described in METHODS. Figures, which are from a representative experiment of 3, show representative Western blot (*A*) and histogram (*B*) for ATP and representative Western blot (*C*) and histogram (*D*) for UTP. Two-way ANOVA of logarithmically transformed data showed for ATP, $P < 0.05$ vs. time (*A*) and for UTP, $P < 0.01$ vs. time (*B*). C, positive control.

Studies in a number of cell types have established an important role for the ERK cascade in cell proliferation (e.g., Ref. 4). We report here, for the first time, that nucleotides stimulate this pathway in HSC. Moreover, compared with the transient responses observed in some other cell types (e.g., Ref. 8, 42), the effects in HSC were sustained in a manner similar to that observed for PDGF, the major HSC mitogen in vitro (33). However, although nucleotides have been shown to stimulate DNA synthesis in fibrogenic cells outside the liver (14), they had no effect on HSC.

In a previous study with PDGF, we suggested that PA production may contribute to the sustained ERK signal and DNA synthesis in HSC (33). Recent investigations have unearthed two main PLD isoforms, PLD 1 and PLD2 (23), which have been implicated both in regulation of the actin cytoskeleton (18, 20) and DNA synthesis via ERK (35) and in the

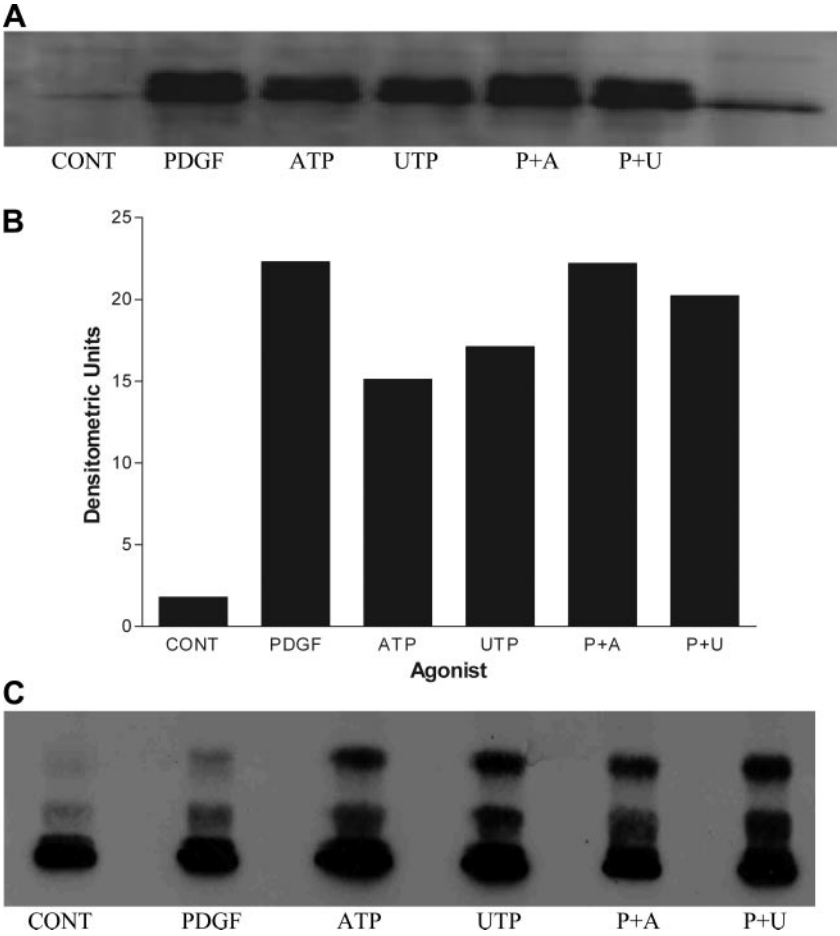

Fig. 9. Combined effects of PDGF and nucleotides on PLD and ERK activity in HSC. ERK activity or [^{3}H]PtdEtOH formation were measured as described in METHODS after treatment of cells with either PDGF (100 ng/ml), ATP, or UTP (100 μM) alone or in combination for 15 min. Figures show either a representative Western blot (*A*) and histogram (*B*) for ERK activity or an autoradiograph for [^{3}H]PtdEtOH formation (*C*) from a representative experiment of 3 in each case.

production of extracellular matrix proteins (15). We report here, for the first time, the presence of mRNA encoding both PLD isoforms in HSC. However, when PDGF and nucleotides were added simultaneously, we did not observe additive effects on [^{3}H]PtdEtOH formation, suggesting that the same isoform was being activated by both agonists. Similar observations also imply that PDGF and nucleotides stimulate the same pool of ERK in these cells. This supports a view that PLD/ERK are linked to a response common to both sets of agonists. Nucleotides elicit production of the matrix component α_1-procollagen and contraction of HSC (9, 39) and migration of other cell types, e.g., endothelial cells (19), although, as yet, there are no reports of their ability to stimulate this phenomenon in HSC. Like nucleotides, PDGF also has effects on matrix components (e.g., Ref. 3) and the cytoskeleton and is the most potent stimulus for migration in HSC (21), a response that is inhibited almost 60% by blockade of ERK activity (22). This suggests that, for PDGF, a fraction of ERK may translocate to the nucleus where it is involved in transcription, whereas cytoplasmic downstream targets for both PDGF and nucleotide-stimulated ERK may include myosin light-chain kinase, calpain, and focal adhesion kinase, all of which have been implicated in contraction and migration (16). Similarly, evidence linking PLD with the production of extracellular matrix components (15), cell contraction (e.g., Ref. 37) and migration (e.g., Ref. 38), its association with focal adhesions (18), and its role in actin stress fiber formation (19) suggests that this too may be linked to PDGF and nucleotide effects on the production of extracellular matrix components and/or the cytoskeleton.

Table 1. *Nucleotides (100 μM) have no effect on DNA synthesis in 14-day passaged HSC*

	Time, h		
	12 h label 12 h agonist	24 h label 24 h agonist	24 h label 4 h agonist
Control	2,205±116	2,861±252	419±72
ATP	2,029±178	3,062±146	555±40
UTP	1,830±90	2,437±116	317±23
CTP	1,806±185	2,413±130	347±24
GTP	2,360±174	2,617±93	375±32

Data are expressed as dpm/well (means ± SE, $n = 4$) and are from a single representative experiment of 3. Agonists were added at *time zero,* and [^{3}H]thymidine was given for either 12 h, 24 h, or the last 4 h of a 24-h incubation. DNA was then extracted and radioactivity determined by scintillation counting. HSC, hepatic stellate cells.

In summary, we have demonstrated, for the first time in HSC, the presence of PLD activity and have shown that extracellular nucleotides stimulate PLD and ERK but have no effect on DNA synthesis. We suggest that investigation of possible effects of nucleotides on HSC migration and a link between the PLD/ERK pathways and nucleotide-mediated effects on the cytoskeleton and production of extracellular matrix components in these cells would be a fruitful area for future research (34).

ACKNOWLEDGMENTS

J. Benitez-Rajal was supported by a Newcastle University Research Studentship and the laboratory was supported by The Newcastle Hospitals Trust. J. Benitez-Rajal is now at Chiltern International Ltd., Slough, Berkshire, UK, and M. J. Lorite is currently at Eli Lilly and Co., Madrid, Spain.

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