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URIDINE ENHANCES NEURITE OUTGROWTH IN NERVE GROWTH FACTOR-DIFFERENTIATED PHEOCHROMOCYTOMA CELLS

A. M. POOLER,[a] D. H. GUEZ,[a] R. BENEDICTUS[a] AND R. J. WURTMAN[a,b]*

[a]*Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, 45 Carleton Street, Cambridge, MA 02139, USA*

[b]*Division of Health Sciences and Technology, Harvard University–Massachusetts Institute of Technology, USA*

Abstract—During rapid cell growth the availability of phospholipid precursors like cytidine triphosphate and diacylglycerol can become limiting in the formation of key membrane constituents like phosphatidylcholine. Uridine, a normal plasma constituent, can be converted to cytidine triphosphate in phosphatidylcholine-12 cells and intact brain, and has been shown to produce a resulting increase in phosphatidylcholine synthesis. To determine whether treatments that elevate uridine availability also thereby augment membrane production, we exposed phosphatidylcholine-12 cells which had been differentiated by nerve growth factor to various concentrations of uridine, and measured the numbers of neurites the cells produced. After 4 but not 2 days uridine significantly and dose-dependently increased the number of neurites per cell. This increase was accompanied by increases in neurite branching and in levels of the neurite proteins neurofilaments M and neurofilament 70. Uridine treatment also increased intracellular levels of cytidine triphosphate, which suggests that uridine may affect neurite outgrowth by enhancing phosphatidylcholine synthesis. Uridine may also stimulate neuritogenesis by a second mechanism, since the increase in neurite outgrowth was mimicked by exposing the cells to uridine triphosphate, and could be blocked by various drugs known to antagonize P2Y receptors (suramin; Reactive Blue 2; pyridoxal-phosphate-6-azophenyl-2′,4′ disulfonic acid). Treatment of the cells with uridine or uridine triphosphate stimulated their accumulation of inositol phosphates, and this effect was also blocked by pyridoxal-phosphate-6-azophenyl-2′,4′ disulfonic acid. Moreover, degradation of nucleotides by apyrase blocked the stimulatory effect of uridine on neuritogenesis. Taken together these data indicate that uridine can regulate the output of neurites from differentiating phosphatidylcholine-12 cells, and suggest that it does so in two ways, i.e. both by acting through cytidine triphosphate as a precursor for phosphatidylcholine biosynthesis and through uridine triphosphate as an agonist for P2Y receptors.

*Correspondence to: R. J. Wurtman, MIT E25-604, 45 Carleton Street, Cambridge, MA 02139, USA. Tel: +1-617-253-6731; fax: +1-617-253-6882.
E-mail address: dick@mit.edu (R. J. Wurtman).

Abbreviations: CDP-choline, 5′-cytidine diphosphocholine; CTP, cytidine triphosphate; DAG, diacylglycerol; HBSS, Hanks' balanced salt solution; IP, inositol phosphates; IP3, inositol triphosphate; MEM, Minimal Essential Medium; NGF, nerve growth factor; PC, phosphatidylcholine; PC12, pheochromocytoma cells; PI, phosphatidylinositol; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′ disulfonic acid; RB-2, reactive blue 2; UTP, uridine triphosphate.

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The principal constituents of mammalian cell membranes are phosphatides, the most abundant of which is phosphatidylcholine (PC). PC biosynthesis is initiated by the phosphorylation of choline to form phosphocholine, which then combines with cytidine triphosphate (CTP) to form 5′-cytidine diphosphocholine (CDP-choline); this compound then reacts with diacylglycerol (DAG) to produce PC (Kennedy and Weiss, 1956). The rate at which cells form PC is affected by the availability of its precursors. Thus, uridine or cytidine increases CTP levels (Richardson et al., 2003); availability of CTP levels in turn can be rate-limiting in the syntheses of CDP-choline (Choy et al., 1980) and PC (Savci and Wurtman, 1995); and DAG levels can control the conversion of CDP-choline to PC (Araki and Wurtman, 1997).

CTP can be synthesized both from the phosphorylation of cytidine and from the pyrimidine nucleotide uridine triphosphate (UTP). Intracellular levels of UTP depend on those of free uridine (Wurtman et al., 2000). Recently, our laboratory demonstrated that treating undifferentiated rat pheochromocytoma (PC12) cells with uridine could increase intracellular levels of CTP, UTP and CDP-choline (Richardson et al., 2003). Hence, it seemed possible that uridine would also enhance the production of cellular membranes. Since exposing PC12 cells to nerve growth factor (NGF) stimulates both PC synthesis (Araki and Wurtman, 1997) and the extension of membrane-rich neurites, we hypothesized that increasing the availability of uridine might further promote neurite formation in these cells.

UTP produced in response to uridine treatment could also promote neuritogenesis by an additional mechanism unrelated to its role as a CTP precursor. Extracellular nucleotides can act as trophic factors for glial and neuronal cells (Rathbone et al., 1999), and UTP can stimulate intracellular signaling pathways by activating the pyrimidine-sensitive, G-protein-coupled P2Y nucleotide receptors (Gallagher and Salter, 2003; Moskvina et al., 2003). Antagonism of these P2Y receptors inhibits NGF-induced neurite outgrowth in PC12 cells (Arslan et al., 2000); however, it has not been determined whether their stimulation enhances neurite formation. Thus, we also investigated whether, if uridine was found to stimulate neurite outgrowth, this effect might also depend on activation of P2Y receptors.

The present study shows that uridine treatment significantly increases neurite outgrowth and branching in NGF-differentiated PC12 cells and that this effect apparently

involves both the CTP-mediated enhancement of PC synthesis and the activation of P2Y receptors.

EXPERIMENTAL PROCEDURES

Cell culture

PC12 cells were maintained in Minimal Essential Medium (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37 °C. Cells were differentiated for 2 or 4 days in medium containing 50 ng/ml mouse 2.5S NGF, and 1% fetal bovine serum, with or without test compounds. When test compounds were dissolved in a vehicle, the same amount of that vehicle was also added to the control group. PC12 cells that were not exposed to NGF are called undifferentiated. NGF and fetal bovine sera were obtained from Invitrogen. No animals were used in these experiments. Only cell lines were used.

Neurite outgrowth studies

PC12 cells were sparsely plated on collagen-coated 60 mm culture dishes in MEM containing 1% fetal bovine serum. The experimental treatments were as follows: uridine, UTP, cytidine, Reactive Blue 2 (RB-2), suramin, PPADS and apyrase Grade VII (Sigma, St. Louis, MO, USA). All treatments were added 24 h after plating. At the end of the treatment period, images were obtained with a phase-contrast Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany) microscope, using OpenLab software. Six digital images were captured for each dish, for a total of 18–24 images per treatment group. Approximately 300 cells were quantified for each treatment group for each experiment. Experiments were performed in triplicate unless otherwise noted. Quantification of neurites, including neurite branching and neurite length, was performed by one or usually more researchers blind to experimental treatments. Neurite length was measured using public domain NIH software Image J. Processes longer than the diameter of the cell body were counted as neurites, and only process-bearing cells were analyzed.

Western blot analysis

Following experimental treatments, the media were aspirated and cells were scraped in 100 μl lysis buffer which included the appropriate protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, San Diego, CA, USA). The total amount of protein in each sample was determined using the bicinchoninic acid assay (Sigma). Prior to gel electrophoresis, Bromphenol Blue (0.07%) was added to each sample. Equal amounts of protein were loaded and separated using SDS-PAGE (4–20%; Bio-Rad, Hercules, CA, USA). Proteins were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA), which were blocked in 5% bovine serum albumin and incubated overnight with the antibody of interest (rabbit anti-neurofilament M and mouse anti-neurofilament 70 [both Chemicon, Temecula, CA, USA]; rabbit anti-P2Y2 and anti-P2Y4 [Calbiochem]; or rabbit anti-P2Y6 [Novus Biologicals, Littleton, CO, USA]). Following this incubation, membranes were incubated for 1 h with the appropriate peroxidase-linked secondary antibody (Sigma). Protein-antibody complexes were detected and visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA) and Kodak X-AR film. Films were digitized using a Supervista S-12 scanner with a transparency adapter (UMAX Technologies, Freemont, CA, USA). Analysis was performed using the public domain NIH Image program available on the internet at http://rsb.info.NIH.gov/nih-image/.

Analysis of nucleotides

Levels of intracellular UTP and CTP were analyzed by HPLC using an anion-exchange column, as previously described (Richardson et al., 2003).

Immunocytochemistry

PC12 cells were treated as described above, except they were grown on 12 mm glass coverslips (A. Daigger and Co., Vernon Hills, IL, USA) coated with collagen. Proteins were visualized using immunofluorescence. Briefly, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, blocked in 10% normal goat serum, and incubated overnight in the appropriate antibodies (mouse anti-neurofilament 70, and either rabbit anti-P2Y2, rabbit anti-P2Y4 or rabbit anti-P2Y6). Cells were then incubated in fluorochrome-conjugated secondary antibodies for 1 h (goat anti-rabbit ALEXA 488 and goat anti-mouse ALEXA 568; Molecular Probes, Eugene, OR, USA) and mounted on glass slides with mounting media with or without DAPI (Vector Laboratories, Burlingame, CA, USA). Control antigens provided with the primary antibodies were used to ensure that immunostaining was specific. Digital images were obtained on a Zeiss Axioplan microscope with OpenLab software, using a Zeiss Plan-Neofluor 40× oil-immersion objective.

Metabolic labeling and phosphatidylinositol (PI) turnover analysis

Analysis of PI turnover was performed as described by Nitsch et al., (1997). Briefly, cells were labeled metabolically for 36 h with 1.25 μCi/dish of myo-[2-^{3}H]inositol (17.0 Ci/mmol; Amersham Biosciences) in serum-free MEM, washed twice with Hanks' balanced salt solution (HBSS), and treated for 15 min with 10 mM lithium chloride in HBSS. Drugs were added in the presence of 10 mM lithium for 60 min at 37 °C. Cells were lysed with ice-cold methanol, and lipids were removed by extraction with chloroform/methanol/water (2:2:1; by volume). Labeled water-soluble inositol phosphates (IP) were separated from free [^{3}H]inositol by ion-exchange chromatography, using AG 1-X8 columns (Bio-Rad), and 1 M ammonium formate and 0.1 M formic acid as eluent. Radioactivity was quantified by liquid scintillation spectrometry.

Data analysis

Data are presented as mean±S.E.M. Analysis of variance (ANOVA) was used to determine differences between groups (significance level, $P<0.05$). When differences were detected, means were separated using the Newman-Keuls multiple range test.

RESULTS

Extracellular uridine increases neurite outgrowth and neurite branching in NGF-differentiated PC12 cells

Undifferentiated PC12 cells did not sprout neurites (fewer than 1%), whether or not they were exposed to uridine (50 μM; data not shown). In contrast, NGF-differentiated cells both produced neurites and exhibited a significant enhancement in neurite number when exposed to uridine (50 μM, $P<0.01$; 100 or 200 μM, $P<0.001$) for 4 days (Fig. 1A–C). Shorter treatments (2 days) or lower uridine concentrations (10 μM or 25 μM) were ineffective, as was treatment with cytidine.

Exposure to uridine (50 μM for 4 days) significantly increased neurite branching ($P<0.01$; Fig. 1D), but had no effect on the average length of neurites (data not shown).

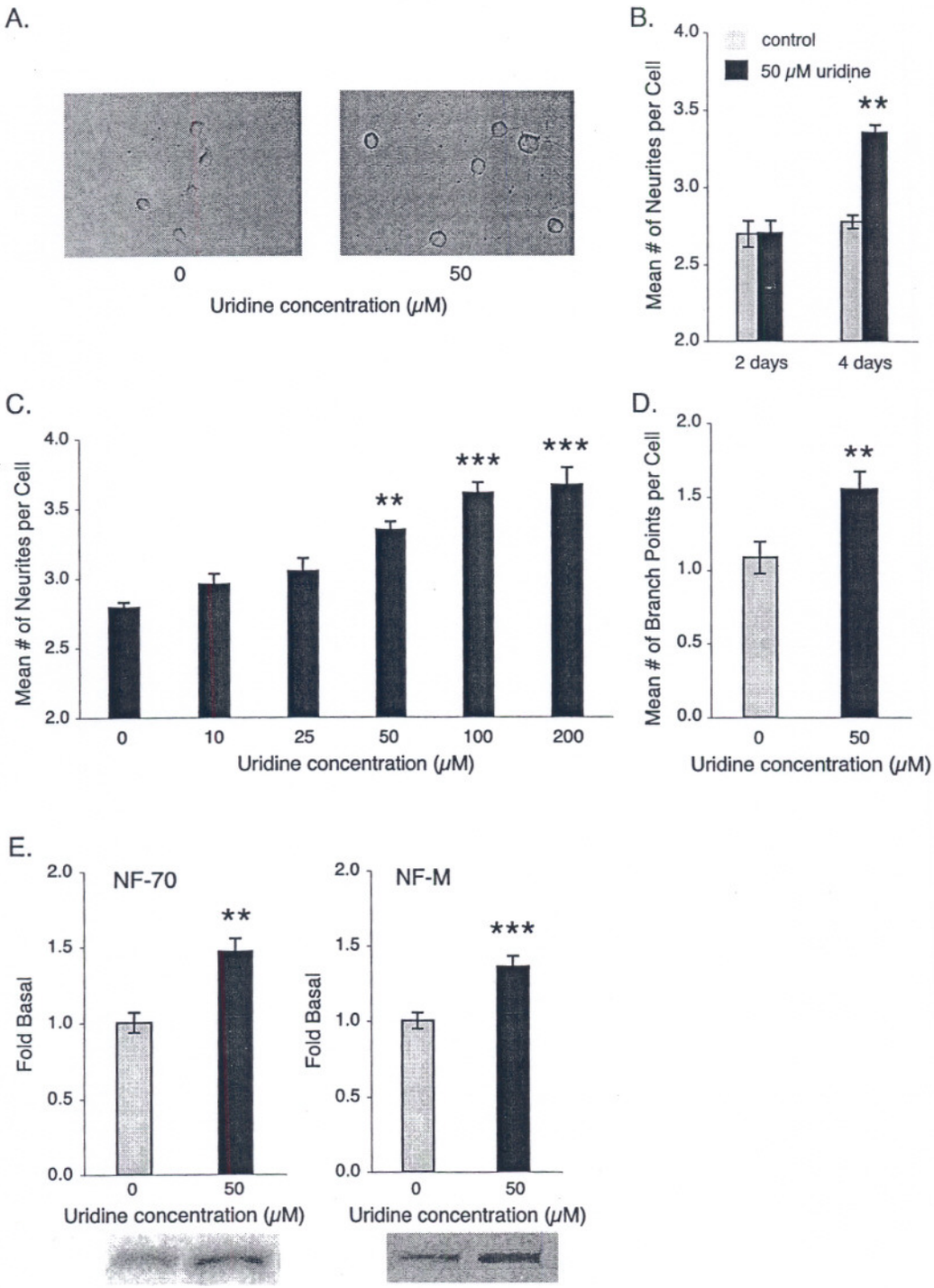

Fig. 1. Uridine treatment enhanced neurite outgrowth in differentiated PC12 cells. PC12 cells were cultured in MEM supplemented with 1% fetal bovine serum, with 50 ng/ml NGF and in the presence of different concentrations of uridine. (A) Differentiated PC12 cells in the absence (left image) or presence (right image) of uridine (50 μM, 4 day treatment). (B) After 2 or 4 days of treatment, the number of neurites per cell was scored. Uridine treatment significantly enhanced neurite formation after 4 days, but no effect was observed after 2 days. (C) Four-day uridine treatment (50, 100 and 200 μM) significantly increased the number of neurites produced during differentiation. (D) The number of branch points was quantified for each cell. Uridine treatment significantly increased the amount of neurite branching after 4 days, compared with branching observed in control cells. (E) Levels of the structural proteins neurofilament 70 and neurofilament M were determined using Western blotting. Treatment of differentiated PC12 cells with uridine (50 μM) for 4 days significantly increased the expression of these proteins relative to control. Values represent means±S.E.M. ** $P<0.01$, *** $P<0.001$ vs. control.

Uridine treatment enhances neurofilament expression in differentiated PC12 cells

Neurofilament proteins are highly enriched within neurites (Lee et al., 1982); hence an increase in neurite number might be expected to be associated with increased expression of neurofilament proteins. We measured neurofilament 70 (70 kD) and neurofilament M (145 kD) levels in differentiated PC12 cells using Western blotting, following 4 day treatment with uridine (50 μM) (Fig. 1E). Both neurofilament M and neurofilament 70 expression were significantly ($P<0.01$, $P<0.001$, respectively) increased following uridine treatment, compared with their levels in cells treated only with NGF. In undifferentiated cells, uridine treatment had no effect on levels of either neurofilament protein (data not shown).

Uridine increased intracellular UTP and CTP levels in differentiated PC12 cells

In undifferentiated PC12 cells, the addition of exogenous uridine increases intracellular UTP and CDP-choline levels (Richardson et al., 2003). To determine whether uridine also affects UTP or CTP levels in differentiated PC12 cells, we treated the cells for 2 days with NGF, with or without uridine, cytidine or UTP. Uridine (50 μM) significantly ($P<0.05$) increased both intracellular UTP and CTP levels

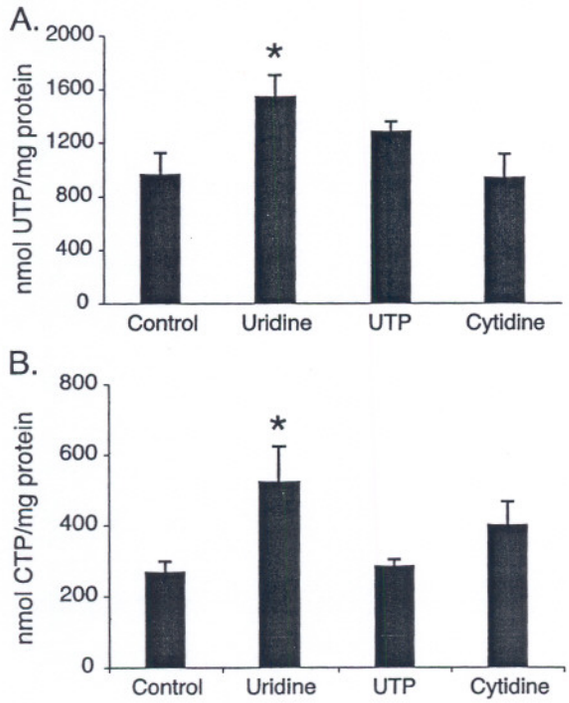

Fig. 2. Uridine treatment increased intracellular levels of UTP and CTP in PC12 cells differentiated with NGF (50 ng/ml). Levels were determined using HPLC. Two-day uridine treatment (50 μM) significantly increased A, intracellular UTP levels and B, intracellular CTP levels. Neither UTP (100 μM) nor cytidine (50 μM) had a significant affect on either UTP or CTP levels within the cells. Values represent means±S.E.M. * $P<0.05$ vs. control.

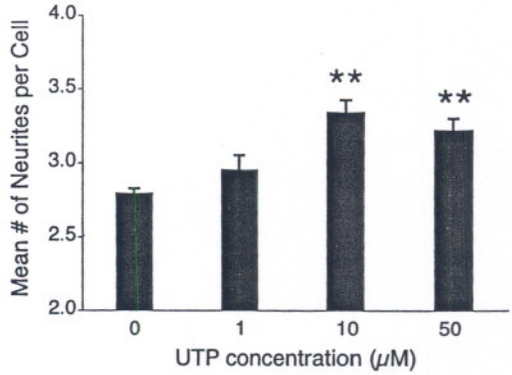

Fig. 3. UTP treatment increased neurite outgrowth. Treatment of PC12 cells for 4 days with NGF (50 ng/ml) and different concentrations of UTP significantly enhanced the number of neurites produced per cell, compared with control. Values represent means±S.E.M. ** $P<0.01$ vs. control.

(Fig. 2A, B, respectively) compared with levels in control cells. Exposure to UTP (100 μM) or cytidine (50 μM) had no effect.

UTP treatment increased neurite outgrowth in differentiated PC12 cells

Since uridine treatment increased UTP levels, and since PC12 cells reportedly contain UTP-sensitive G-protein-coupled receptors (Arslan et al., 2000), we determined whether exogenous uridine may also modulate neuritogenesis via a direct, receptor-mediated action of UTP, in addition to acting via CTP. Differentiated PC12 cells were exposed to various concentrations of UTP (Fig. 3), for 4 days. UTP (10 and 50 μM) was found to be more effective than uridine itself inasmuch as both enhanced neurite outgrowth.

P2Y2, P2Y4 and P2Y6 receptors were expressed in differentiated PC12 cells

Next, we investigated the mechanism by which extracellular UTP affects neurite outgrowth. UTP is an agonist of the pyrimidine-activated class of P2Y receptors, namely, P2Y2, P2Y4 and P2Y6 receptors. Previously, Arslan et al. (2000) demonstrated that differentiated PC12 cells contain mRNA for these P2Y receptors. To determine whether UTP-responsive P2Y receptor proteins are expressed in PC12 cells, we measured the levels of P2Y2, P2Y4 and P2Y6 receptors in undifferentiated cells, and cells exposed to NGF for 0–7 days. Expression of the P2Y2 receptor reached maximal levels after exposure to NGF for 3 days; these were significantly ($P<0.001$) higher than the levels observed in cells exposed for shorter periods. To visualize the expression and localization of the P2Y2, as well as the P2Y4 and P2Y6, receptors, we grew cells in the presence or absence of NGF for 4 days and then immunostained them for the neuritic marker neurofilament 70, and for P2Y2, P2Y4 or P2Y6 (Fig. 4B, left to right, respectively). For P2Y2 and P2Y4 visualization, control cultures were incubated with primary antibody plus a control antigen in

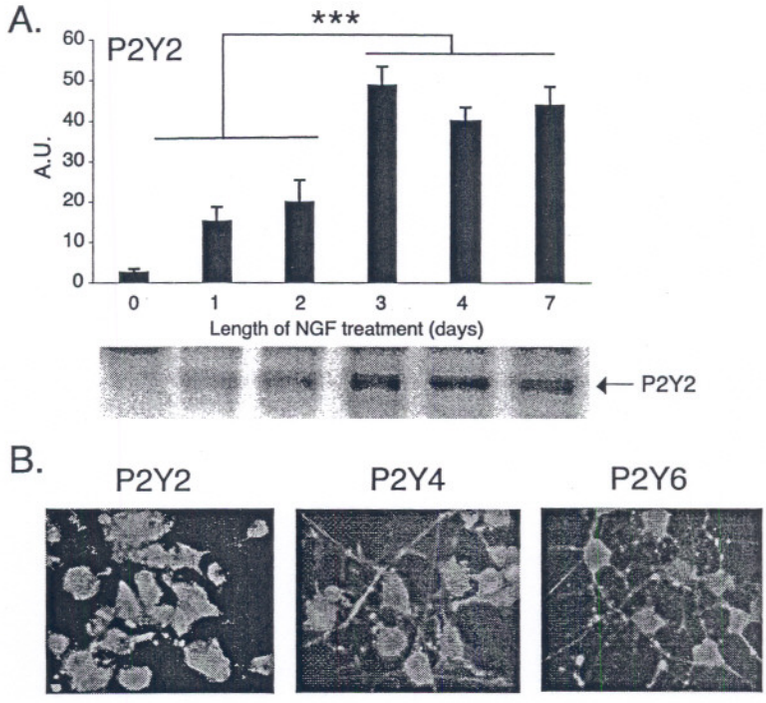

Fig. 4. NGF-differentiated PC12 cells express pyrimidine-sensitive P2Y receptors. (A) Cells were differentiated for various lengths of time. P2Y2 receptor expression was measured using Western blotting. After 3 days of differentiation, levels of P2Y2 receptor protein were significantly enhanced compared with fewer than 3 days of differentiation. (B) Following 4 days of NGF differentiation, cells were fixed and neurofilament 70 (red) and P2Y receptor (green) proteins were visualized using immunofluorescence. From left to right: P2Y2, P2Y4 and P2Y6. Values represent means±S.E.M. *** $P<0.001$ vs. control.

order to ensure that the immunostaining would be specific. Control antigen was not available for the P2Y6 receptor. All three receptors were highly expressed in differentiated PC12 cells (Fig. 4B), but undetectable in the undifferentiated cells (data not shown).

Antagonism of P2Y receptors inhibited the effect of uridine on NGF-induced neurite outgrowth

To determine whether P2Y2, P2Y4 or P2Y6 receptors participate in the stimulatory effect of uridine on neurite outgrowth, we incubated differentiated PC12 cells with uridine (100 μM) and a P2Y receptor antagonist, chosen from suramin (30 μM), pyridoxal-phosphate-6-azophenyl-2′,4′ disulfonic acid (PPADS; 30 μM) or RB-2 (10 μM) for 4 days. As before, uridine enhanced neurite outgrowth; each of the P2Y antagonists significantly ($P<0.05$ or 0.001) blocked this response (Fig. 5). It was not possible to determine which P2Y receptor or receptors (e.g. P2Y2, P2Y4 or P2Y6) mediate this effect, since no potent and selective antagonists exist at this time for individual pyrimidine-activated P2Y receptors. None of the P2Y receptor antagonists inhibited the uptake of uridine into the PC12 cells (data not shown).

Uridine and UTP stimulated IP formation

P2Y2, P2Y4 and P2Y6 receptors are positively coupled to the phospholipase C/DAG/inositol triphosphate (PLC/DAG/IP3) signaling pathway. To determine whether concentrations of uridine or UTP that promote neurite outgrowth also are able to activate these receptors, we exposed differentiated PC12 cells labeled with [^{3}H]-inositol to uridine (50 μM) or UTP (10, 100 μM) for 1 h, and then

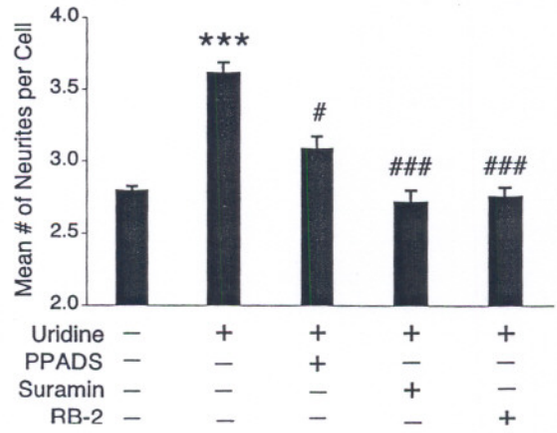

Fig. 5. P2Y receptor antagonists inhibited the effect of uridine on neurite outgrowth. Cells were incubated for 4 days in MEM containing 50 ng/ml NGF and with or without uridine (100 μM), the P2Y receptor antagonists PPADS (30 μM), suramin (30 μM) or RB-2 (10 μM). All three antagonists significantly blocked the stimulatory effect of uridine on neurite outgrowth. Values represent means±S.E.M. *** $P<0.001$ vs. control; # $P<0.05$, ### $P<0.001$ vs. uridine treatment.

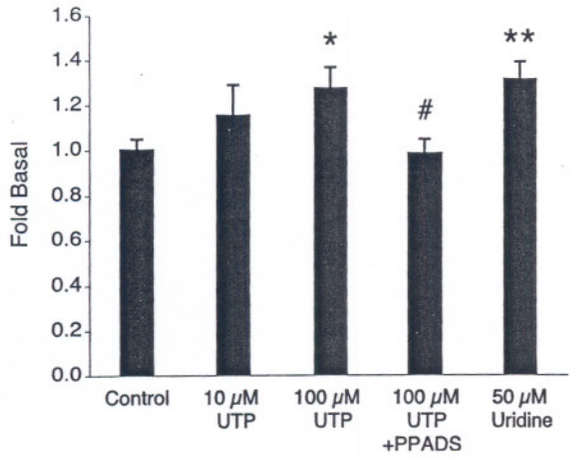

Fig. 6. PI turnover is stimulated by UTP and uridine. Differentiated cells were metabolically labeled with [^{3}H]inositol overnight, stimulated with UTP (10, 100 μM), uridine (50 μM) or UTP plus PPADS (100 μM) in the presence of 10 μM lithium, and radiolabeled IPs derived from PI breakdown were measured by scintillation counting. Both UTP (100 μM) and uridine (50 μM) significantly increased IP formation, and the effect of UTP was significantly blocked by co-incubation with the P2Y receptor antagonist PPADS. Values represent means±S.E.M. * $P<0.05$, ** $P<0.01$ vs. control; # $P<0.05$ vs. 100 μM UTP treatment.

measured radiolabeled IP formed as a result of [^{3}H]-PI breakdown (Fig. 6). IP formation was significantly increased by 100 μM UTP ($P<0.05$) or 50 μM uridine ($P<0.01$) treatment. The P2Y receptor antagonist PPADS (100 μM) significantly ($P<0.05$) blocked the effect of UTP on IP formation.

Apyrase inhibited the effect of uridine on neurite outgrowth

To determine whether uridine nucleotides are involved in uridine's effect on neurite outgrowth, we treated differentiated PC12 cells with uridine (50 μM) and apyrase (1 U/ml), which cleaves both tri- and diphosphate nucleotides. Degradation of nucleotides by apyrase blocked stimulation of neurite outgrowth by uridine ($P<0.05$); apyrase alone had no effect (Fig. 7).

DISCUSSION

These data show that uridine (50–200 μM) is able to enhance neurite outgrowth (Fig. 1B, C) and neurite branching (Fig. 1D) in NGF-differentiated PC12 cells. The increase in neuritogenesis is accompanied by increases in cellular levels of the proteins neurofilament M and neurofilament 70, which are enriched in neurites. Uridine treatment increases cellular levels of CTP and UTP in the differentiated PC12 cells (Fig. 2), suggesting that one mechanism by which it enhances neurite outgrowth involves promoting membrane biosynthesis. Treatment of the PC12 cells with low concentrations of exogenous UTP, which is thought not to enter cells, also significantly increases neuritogenesis (Fig. 3), suggesting that cell-surface UTP-sensitive receptors are also involved in uridine's enhancement of neurite outgrowth. Since the pyrimidine-sensitive P2Y2, P2Y4 and P2Y6 receptors were found to be present on differentiated PC12 cells (Fig. 4), and since several P2Y receptor antagonists blocked the stimulatory effect of uridine on neurite outgrowth (Fig. 5), this set of receptors might be the site at which UTP acts, a hypothesis supported by the finding that UTP (or uridine) also stimulated the formation of IP (Fig. 6), and that UTP's effect was blocked by a P2Y receptor antagonist. Furthermore, degradation of nucleotides (such as UTP) by apyrase abolished uridine's stimulatory effect on neuritogenesis (Fig. 7). Taken together, our data suggest that uridine promotes neurite outgrowth by two distinct mechanisms, 1) by increasing the availability of CTP, a limiting precursor in phosphatide biosynthesis (Savci and Wurtman, 1995), and 2) by stimulating a P2Y receptor-coupled signaling pathway. Uridine is not unique in playing multiple roles in the cell; for example, intracellular DAG, can control PC synthesis both by combining with the enzyme that couples it to CDP-choline (Araki and Wurtman, 1997) and by acting as a second messenger in the DAG/IP3 signaling pathway (Li and Wurtman, 1998). It is not presently possible to determine the relative contributions of each of uridine's two actions to neurite outgrowth.

Previous studies in our laboratory have demonstrated that the rate of formation of new cellular membrane is dependent upon the availability of such membrane precursors as UTP, CTP, CDP-choline and DAG. CTP and DAG levels can, respectively, regulate PC synthesis in brain slices (Savci and Wurtman, 1995) and in cell culture (Araki and Wurtman, 1997). Degradation of nucleotides by apyrase inhibited the stimulation of neurite outgrowth by uridine (Fig. 7), indicating that nucleotides, such as UTP and CTP, are necessary for uridine's effect on neuritogenesis. The present study suggests that increasing the availability of UTP and CTP can also increase PC synthesis, and thus can increase the formation of new cell membrane in the form of neurites.

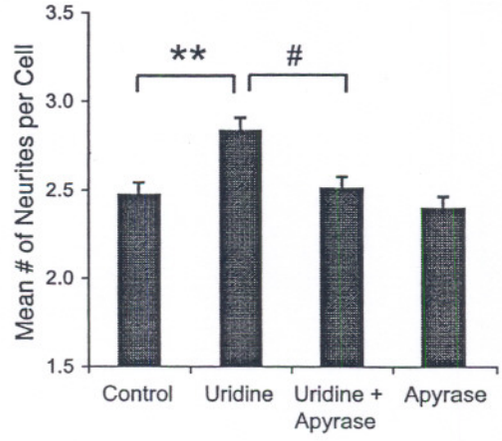

Fig. 7. Apyrase treatment significantly inhibited the effect of uridine on neurite outgrowth. PC12 cells were incubated for 4 days in MEM containing 50 ng/ml NGF and with or without uridine (50 μM) or apyrase (1 U/ml). Values represent means±S.E.M. ** $P<0.001$ vs. control; # $P<0.05$ vs. uridine treatment.

A growing body of evidence suggests that nucleotides and receptors for these compounds constitute signaling pathways in the brain. The P2 family of nucleotide receptors includes ionotropic P2X receptors and G-protein-coupled P2Y receptors (Communi et al., 2000). A subset of the P2Y receptors, namely the P2Y2, P2Y4 and P2Y6, are pyrimidine-sensitive (Ralevic and Burnstock, 1998), and their activation releases calcium from intracellular stores via the DAG/IP3 pathway (Arslan et al., 2000; Bofill-Cardona et al., 2000). The nucleotides and their receptors may have trophic effects in neurons (Liu et al., 2000), perhaps mediated by P2Y receptors, since increases in intracellular calcium levels have been associated with neuritogenesis (Gysbers et al., 2000). In NGF-differentiated PC12 cells, the addition of ATP or GTP enhances the percentage of cells that form neurites (D'Ambrosi et al., 2001; Gysbers et al., 2000), whereas inhibition of P2 receptors by antagonists prevents neuritogenesis (D'Ambrosi et al., 2000). Furthermore, a previous study suggested that UTP treatment also increases neurite sprouting (Gysbers and Rathbone, 1996), although the mechanism by which this occurred was not investigated. Another study (Silei et al., 2000) reported that uridine treatment increased the differentiation of neuroblastoma cells, but did not assess whether P2Y receptors were involved. In order to determine whether P2Y receptors participate in uridine's stimulation of neurite outgrowth in the present study, we treated differentiated PC12 cells with uridine and P2Y receptor antagonists, and found that the antagonists blocked uridine's effect (Fig. 5). Furthermore, uridine and UTP stimulated both neurite outgrowth (Figs. 1, 3) and IP formation (Fig. 6), and these effects could also be inhibited by P2Y receptor antagonists. Hence our data suggest that some proportion of the effects of uridine and UTP on neurite production is mediated by activating pyrimidine-sensitive P2Y receptors. It is not presently possible to determine which specific receptor (P2Y2, P2Y4 or P2Y6) or combination of receptors mediates this effect, since highly specific antagonists for individual P2Y receptors are not available.

Uridine has not been shown to bind directly to P2Y receptors, and its effects on these receptors probably require its conversion to extracellular UTP. The UTP could reach the receptors by release from the PC12 cells, or, alternatively, extracellular nucleotide diphosphokinases could form nucleotide triphosphates from uridine in the extracellular space (Lazarowski et al., 2000). Neurons, during normal cellular activity (Zhang et al., 2003), and astrocytes, following cell damage (Lazarowski et al., 1997), have been shown to release nucleotides (e.g. ATP) which can activate cell surface receptors (Koizumi et al., 2003). Therefore uridine could be taken up into the cell and converted to UTP, which would then be released into extracellular space where it could stimulate P2Y receptors. Since UTP is rapidly broken down following its release from the cell, its extracellular concentration is thus difficult to measure accurately (Lazarowski and Boucher, 2001). Regardless, the stimulation of IP accumulation caused by uridine (Fig. 6) suggests that, following uridine treatment, either one or both of the above mechanisms could operate to increase extracellular UTP levels, and thus to activate P2Y receptors. Moreover, the formation of IP3 requires activation of phospholipase C, an enzyme that may be involved in NGF-induced differentiation in PC12 cells (Rong et al., 2003).

When considering the possible role of uridine in neuron growth *in vivo*, it may be important to determine whether brain levels of uridine actually fluctuate, and the range of such fluctuations. In humans, consumption of CDP-choline causes dose-related increases in plasma uridine levels (Wurtman et al., 2000), suggesting that uridine levels, at least in blood, can be modulated *in vivo*. In gerbils, increases in blood uridine produced by consuming uridine monophosphate causes parallel changes in brain uridine levels (M. Cansev, C. J. Watkins, personal communication). Currently, no data are available on possible effects of short-term (e.g. eating) or long-term (e.g. development; aging) processes on brain uridine levels.

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ERRATUM TO "URIDINE ENHANCES NEURITE OUTGROWTH IN NERVE GROWTH FACTOR-DIFFERENTIATED PHEOCHROMOCYTOMA CELLS" [NEUROSCIENCE 134 (2005) 207–214]

A. M. POOLER,[a] D. H. GUEZ,[a] R. BENEDICTUS[a] AND R. J. WURTMAN[a,b]*

[a]*Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, 45 Carleton Street, Cambridge, MA 02139, USA*
[b]*Division of Health Sciences and Technology, Harvard University–Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

The publisher regrets that several changes requested by the author at the proof stage were not made:

In the title, "pheochromocytoma cells" should read "PC12 cells."

In lines 6, 10, and 33 of the Abstract, "phosphatidylcholine-12" should read "PC12."

In addition, in line 17 of the Abstract, "neurofilaments M" should read "neurofilament M."

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*Corresponding author. Fax: 1-617-253-6882.
E-mail address: dick@mit.edu (R. J. Wurtman).

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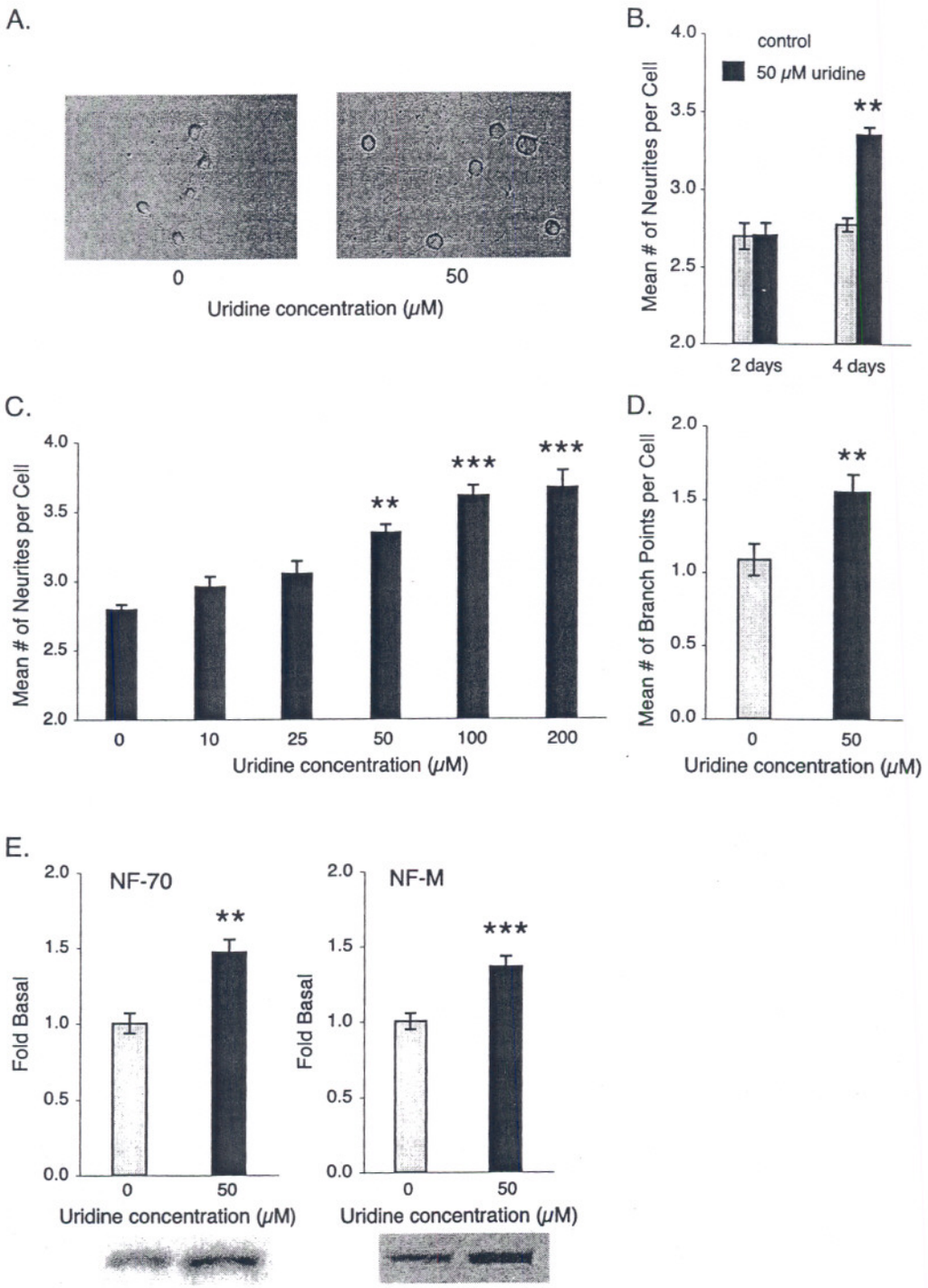

Fig. 1. Uridine treatment enhanced neurite outgrowth in differentiated PC12 cells. PC12 cells were cultured in MEM supplemented with 1% fetal bovine serum, with 50 ng/ml NGF and in the presence of different concentrations of uridine. (A) Differentiated PC12 cells in the absence (left image) or presence (right image) of uridine (50 μM, 4 day treatment). (B) After 2 or 4 days of treatment, the number of neurites per cell was scored. Uridine treatment significantly enhanced neurite formation after 4 days, but no effect was observed after 2 days. (C) Four-day uridine treatment (50, 100 and 200 μM) significantly increased the number of neurites produced during differentiation. (D) The number of branch points was quantified for each cell. Uridine treatment significantly increased the amount of neurite branching after 4 days, compared with branching observed in control cells. (E) Levels of the structural proteins neurofilament 70 and neurofilament M were determined using Western blotting. Treatment of differentiated PC12 cells with uridine (50 μM) for 4 days significantly increased the expression of these proteins relative to control. Values represent means±S.E.M. ** $P<0.01$, *** $P<0.001$ vs. control.