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Identification of a Uridine Nucleotide-selective G-protein-linked Receptor That Activates Phospholipase C*

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Incubation of C6-2B rat glioma cells with UDP or UTP resulted in a time- and concentration-dependent increase in the accumulation of inositol phosphates. In contrast, ATP, ADP, and analogs of these nucleotides known to be effective agonists at P_{2U}-, P_{2X}-, P_{2Y}-, P_{2T}-, and P_{2Z}-purinergic receptors all had no effect on inositol phosphate levels in C6-2B cells. Pyrimidine nucleotides stimulated inositol phosphate accumulation with an order of potency of UDP > 5-BrUTP > UTP > dTDP > UDP glucose. $K_{0.5}$ values for UDP, 5-BrUTP, and UTP were 2.3 ± 0.5, 9 ± 3, and 57 ± 10 μM, respectively. A similar uridine nucleotide selectivity was observed for arachidonic acid release presumably occurring as a consequence of activation of phospholipase A_2. Cross-desensitization and additivity experiments indicated that UDP and UTP interact with the same population of receptors. The effect of uridine nucleotides on inositol phosphate accumulation was inhibited markedly by pretreatment of cells with pertussis toxin. UDP also caused a guanine nucleotide-dependent increase in inositol lipid hydrolysis in streptolysin-*O*-permeabilized cells. Taken together these results describe the existence of a novel uridine nucleotide receptor that is not activated by adenine nucleotides. This receptor is pharmacologically distinct from the previously described P_{2U}- and other P_2-purinergic receptors, and likely is a member of a new class of receptors for extracellular nucleotides.

Extracellular release of adenine nucleotides from neurons, platelets, and other tissues results in activation of a broad range of physiological responses (1, 2). Considerable progress has been made in the last decade in the pharmacological delineation of the cell surface receptors that are activated by extracellular ATP and ADP. These P_2-purinergic receptors include the P_{2X}-, P_{2Y}-, P_{2U}-, P_{2T}-, and P_{2Z}-receptors, which are selectively activated by ATP, ADP, or various analogs of these two adenine nucleotides (1–3). The one exception to the adenine nucleotide selectivity of these receptors is the so-called P_{2U}-purinergic receptor, which is not only activated by ATP and ATPγS,[1] but also is stimulated by the pyrimidine, UTP (4–8). Although care has been taken to prove that the P_{2U}-purinergic receptor that stimulates inositol lipid hydrolysis in many target tissues is a single receptor activated by both ATP and UTP (8, 9), this does not rule out the possibility that separate receptors for purines and pyrimidines occur. Indeed, studies measuring vascular smooth muscle responses (10–12) or metabolic effects in liver (13) and in neutrophils or HL-60 cells (14, 15) have led to the proposal that "pyrimidinergic" receptors exist (16). However, none of these have been unambiguously defined. A large amount of information has accumulated on the physiological release of ATP and ADP. However, outside of its storage in high concentrations in platelet storage granules, little is known for UTP concerning its potential storage, release, and activity as an extracellular molecule. The presence of P_{2U}-purinergic receptors that respond to extracellular UTP supports the idea that this is an important extracellular signaling molecule, and potentially provides avenues for studying the physiological regulation of extracellular UTP levels.

The inositol lipid signaling cascade is the major second messenger response known to be associated with activation of P_2-purinergic receptors (3). Both the P_{2Y}- and P_{2U}-purinergic receptors activate phospholipase C in a broad range of tissues. However, this is not the only signaling response regulated by these receptors. For example, activation of a P_2-purinergic receptor that appears to be distinct from the P_{2Y}-purinergic receptor that activates phospholipase C results in a decrease in intracellular cyclic AMP accumulation in some target tissues (17–20). It is also likely that there are other receptors for extracellular adenine and uridine nucleotides that have not been identified, and we report here that C6-2B glioma cells express a novel receptor that possesses a strict selectivity for uridine nucleotides. Activation of this receptor results in activation of phospholipase C through a guanine nucleotide-dependent pertussis toxin-sensitive pathway. The existence of a receptor that is activated by uridine nucleotides but not by adenine nucleotides adds support to the idea that extracellular UTP subserves an important cell signaling function.

EXPERIMENTAL PROCEDURES

Cell Culture and Subcloning—C6-2B and C6 rat glioma cells and HT29 human colonic carcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with DMEM and 5% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. To isolate homogeneous cell populations derived from single cloned cells, C6-2B cells were seeded on 150-cm^2 dishes at cell densities ranging from 10 to 1000 cells/dish. Attached isolated single cells were identified the following day, and compact clusters (apparently clonal) of dividing cells were isolated using 7-mm diameter cloning cylinders. The C6-2B cell clones were transferred to tissue culture flasks and numbered in the order of their isolation.

Inositol Phosphate Assay—Confluent cells grown on 12-well plastic plates (Costar, Cambridge, MA) were incubated for 18 h in 0.5 ml of

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[1] The abbreviations used are: ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); α,β-MeATP, α,β-methylene adenosine-5′-triphosphate; 5-BrUTP, 5-bromo-uridine-5′-triphosphate; 2-MeSATP, 2-methylthioadenosine triphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); HPLC, high perfomance liquid chromatography; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $Ins(1,3,4)P_3$, inositol 1,3,4-trisphosphate; $Ins(1,3,4,5)P_4$, inositol 1,3,4,5-tetrakisphosphate; DMEM, Dulbecco's modified Eagle's medium.

inositol-free DMEM containing 10 μCi/ml [^{3}H]inositol. The cells were washed twice with HEPES-buffered (pH 7.4) DMEM (H/DMEM) and preincubated with H/DMEM containing 10 mM LiCl for 15 min before challenge with agonists. In those experiments where desensitization was studied, the cells were preincubated with agonist for 20 min prior to addition of LiCl and rechallenged by agonist. Incubations were terminated by removal of the medium followed by addition of 5% trichloroacetic acid. The trichloroacetic acid extracts were neutralized by (3 × 2 ml) extractions with ethyl ether. Resolution of the individual inositol phosphates on Dowex AG1-X8 columns was performed as described previously (21).

Quantitation of Individual Inositol Phosphates by HPLC—Samples were prepared as described above except that the cells were labeled with 100 μCi of [^{3}H]inositol in 0.5 ml of DMEM. Individual inositol phosphates were resolved using a Whatman Partisil 10 strong anion-exchange column (Alltech Applied Science, Deerfield, IL) and an ammonium phosphate gradient as described previously (22). Radioactivity was monitored on-line with a Radiomatic Flo-one detector (Radiomatic Instruments, Tampa, FL).

Arachidonic Acid Release—Confluent cells grown on 12-well plastic plates were labeled for 6 h with 0.5 μCi of [^{3}H]arachidonic acid in 0.5 ml of DMEM. The cells were washed free of unincorporated radioactivity and challenged with agonists for 10 min. Released [^{3}H]arachidonic acid was measured in the cell supernatant as reported previously (23).

Assay of Phospholipase C Activity in Permeabilized Cells—[^{3}H]Inositol-prelabeled cells on 12-well plates were rinsed twice with phosphate-buffered saline and permeabilized by a 5-min incubation with 0.2 IU/ml streptolysin-*O* in assay buffer containing 0.424 mM $CaCl_2$, 3 mM $MgCl_2$, 2 mM EGTA, 115 mM KCl, 10 mM HEPES, and 10 mM LiCl (pH 7.4) as described previously (8). Streptolysin-*O* containing buffer was removed and the cells were incubated in 0.5 ml of assay buffer containing various additions. Assays were terminated by the addition of 0.25 ml of 15% trichloroacetic acid and [^{3}H]inositol phosphates were quantitated as described above.

Materials—*Myo*-[2-^{3}H]inositol (20 Ci/mmol) and [5,6,7,8,9,11, 12,14,-15-^{3}H]arachidonic acid (200 Ci/mmol) were purchased from American Radiolabelled Chemicals, Inc. (St. Louis, MO). Uridine, UMP, UDP, UTP, 5-BrUTP, UDP-glucose, ADP, ATP, dTTP, GDP, GTP, CDP, CTP, dUTP, dCTP, dGTP, and dATP were from Boehringer Mannheim. 2-MeSATP and α,β-MeATP were purchased from Research Biochemicals Inc. (Natick, MA). 2′-Deoxynucleotide diphosphates (dUDP, dTDP, and dCDP) were purchased from Sigma. Pertussis toxin was from List Biological Laboratories, Inc. (Cumsell, CA). Streptolysin-*O* was obtained from Murex Diagnostics Limited (Dartford, United Kingdom). Suramin was obtained from the Center for Disease Control (Atlanta, GA).

Data Analysis—Unless stated otherwise, experiments were performed with triplicate determinations that differed <10% from the mean. The data were considered statistically different at $p < 0.05$ using a paired Student's *t* test.

RESULTS

Incubation of C6-2B rat glioma cells with UDP resulted in a time-dependent increase in intracellular levels of inositol phosphates (Fig. 1). Incubation of these cells with UTP also elicited an inositol phosphate response that was temporally and quantitatively similar to the response observed with UDP. Inositol phosphate accumulation in the presence of either agonist was essentially maximal within 10 min, an effect that apparently is at least in part due to homologous desensitization of the receptor responding to the uridine nucleotides (see below). The time course of accumulation of inositol phosphates in the presence of the uridine nucleotides was similar to that observed with the muscarinic cholinergic receptor agonist carbachol; the maximal effect observed with carbachol was up to 2-fold greater than that with UDP or UTP (Fig. 1). In contrast to the stimulatory effect of the uridine nucleotides, incubation of C6-2B cells with ATP or ATPγS did not result in increases in inositol phosphate levels. To further characterize the effect of uridine nucleotides on inositol phosphate production, individual inositol phosphates in extracts from UDP-, carbachol-, and ATP-pretreated cells were resolved by HPLC. UDP and carbachol caused marked increases in Ins(1,4,5)P_3 and its immediate metabolic products (Table I and Fig. 2). Although these cells were labeled to high specific radioactivity with [^{3}H]inositol, ATP had no reproducible effect on accumulation of individual [^{3}H]inositol phosphates during a 20-s, 5-min, or 20-min incubation. The lack of effect of ATP was not due to hydrolysis of this nucleotide since greater than 95% of added [^{3}H]ATP (100 μM) was recovered unchanged after incubation in the presence of C6-2B cells for 20 min at 37 °C (data not shown). Under the same conditions greater than 95% of added [^{3}H]UTP also was recovered.

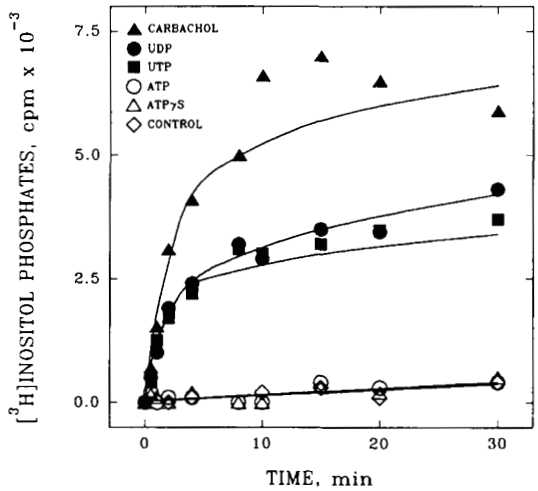

FIG. 1. **Time course of inositol phosphate formation in C6-2B glioma cells.** [^{3}H]Inositol-labeled C6-2B cells were challenged with 300 μM carbachol (▲), UTP (■), UDP (●), ATP (○), ATPγS (△), or vehicle (◇) for the indicated times. Incubations were terminated by addition of 5% trichloroacetic acid and total inositol phosphates were collected and measured as detailed under "Experimental Procedures." The amount of [^{3}H]inositol phosphates present at $t = 0$ (≈2000 cpm) was subtracted from the indicated values. Data represent the mean value of results obtained with triplicate assays that are representative of results from three different experiments. Error bars were omitted for the clarity of the figure.

TABLE I
Agonist-induced stimulation of inositol trisphosphate and tetrakisphosphate

[^{3}H]Inositol prelabeled C6-2B cells were challenged for either 20 s or 20 min with 300 μM ATP, UDP, or carbachol. Individual inositol phosphates were separated by HPLC as detailed under "Experimental Procedures." Data for levels of Ins(1,4,5)P_3, Ins(1,3,4)P_3, and Ins(1,3,4,5)P_4 are presented and are the mean ± S.D. of three determinations.

Addition	Ins(1,4,5)P_3	Ins(1,3,4)P_3	Ins(1,3,4,5)P_4
		cpm	
20 s			
None	3240 ± 280	86 ± 35	636 ± 286
ATP	3363 ± 300	120 ± 29	696 ± 12
UDP	2961 ± 293	752 ± 53	1819 ± 433
Carbachol	2516 ± 165	3166 ± 567	2788 ± 40
20 min			
None	2594 ± 206	119 ± 93	849 ± 316
ATP	2938 ± 645	122 ± 16	744 ± 23
UDP	3146 ± 609	749 ± 43	2433 ± 435
Carbachol	3738 ± 229	3075 ± 36	4205 ± 250

Observation of inositol phosphate responses to UDP and UTP but not to ATP suggested the existence of a receptor that had not been described previously. As such, the response to a wide range of nucleotides and to analogs of UTP, ATP, and ADP were tested (Fig. 3; Table II). The most potent agonist identified was UDP which stimulated inositol phosphate accumulation with a $K_{0.5}$ of 2.3 ± 0.5 μM (Fig. 3). However, 5-BrUTP ($K_{0.5}$ = 9 ± 3 μM) and UTP ($K_{0.5}$ = 57 ± 10 μM) also were full agonists. Small effects were observed with UDP-glucose but UMP or uridine had no effect. Similarly, 2′-deoxy-UTP had no effect on inositol phosphate accumulation. The pyrimidine dTTP caused a concentration-dependent activation of phospholipase C, but this only occurred at concentrations 100-fold greater than

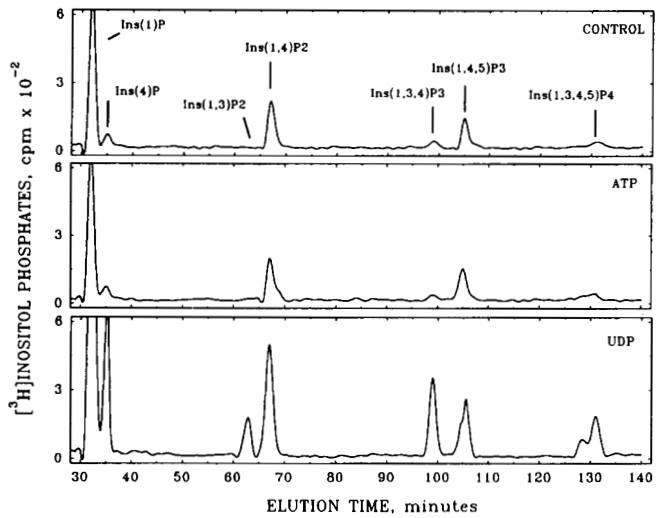

FIG. 2. **Effect of UDP and ATP on accumulation of individual [^{3}H]inositol phosphates.** [^{3}H]Inositol-prelabeled C6-2B glioma cells were challenged for 5 min with vehicle or with 300 μM UDP or ATP. Individual inositol phosphates were separated by HPLC and identified by co-elution with authentic standards as indicated. The data are representative of results obtained from two separate experiments carried out in duplicate.

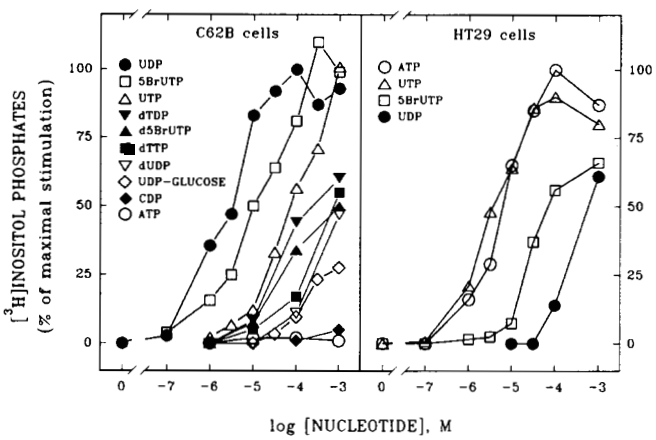

FIG. 3. **Concentration-dependent stimulation of inositol phosphate formation by nucleotides in C6-2B rat glioma and HT29 human colon carcinoma cells.** [^{3}H]Inositol-labeled cells were incubated for 20 min in the presence of the indicated agonists. *Left,* data for C6-2B glioma cells are presented. Individual inositol phosphate concentration-response curves represent the mean of three to six different experiments performed with triplicate samples each. The data are plotted as the percentage of the maximal response obtained in each experiment with 100 μM UDP which ranged from 7,000 to 21,000 cpm. *Right,* results for HT29 human colon carcinoma cells are presented. The data are the mean of triplicate determinations and are representative of results obtained in three experiments. Error bars were omitted for the clarity of the figure.

stimulatory concentrations of UDP. CTP and GTP had no effect. A wide range of compounds previously shown to be agonists at P_{2X}-, P_{2Y}-, P_{2T}-, and P_{2U}-purinergic receptors were tested. None of these compounds stimulated inositol lipid hydrolysis (Table II). For example, the potent P_{2Y}-receptor agonist 2-MeSATP, the potent P_{2X}-receptor agonist α,β-MeATP, the potent P_{2T}-receptor agonist 2-MeSADP, and the potent P_{2U}-receptor agonist ATPγS all had no effect on inositol phosphate accumulation. Similarly, the P_{2Y}-purinergic receptor agonist 2-MeSATP had no effect on individual inositol phosphates resolved by HPLC after challenge of cells for 20 s or 20 min (data not shown and Ref. 20). Finally, inositol phosphate responses in a cell line, HT29 human colon carcinoma cells, previously shown to express P_{2U}-

TABLE II

Effect of nucleotides on inositol phosphate formation in C6-2B cells

[^{3}H]Inositol-prelabeled cells were incubated for 20 min in the presence of the indicated additions (300 μM each). Inositol phosphates were measured as described under "Experimental Procedures." The data represent the mean ± S.E. from three to nine different experiments assayed with triplicate determinations.

Addition	cpm
None	1994 ± 167
UDP	6229 ± 376
5-BrUTP	5725 ± 333
UTP	5415 ± 162
dTDP	4166 ± 458
d5-BrUTP	3839 ± 179
dTTP	3568 ± 160
UDP-glucose	3317 ± 292
dUDP	3210 ± 360
dUTP	2127 ± 73
UMP	2201 ± 28
Uridine	1905 ± 11
Thymidine 5′-tetraphosphate	2198 ± 12
UTP 2′,3′-dialdehyde	2077 ± 38
UDP 2′,3′-dialdehyde	1983 ± 235
XTP	2074 ± 116
ATP	1885 ± 26
ATPγS	1903 ± 142
2MeSATP	2136 ± 107
2MeSADP	2008 ± 103
ADPβS	1956 ± 130
ADP	2110 ± 91
α,β-Methylene ATP	2013 ± 19
CTP	2098 ± 104
CDP	1914 ± 176
GTP	2183 ± 77
GTPγS	1933 ± 28
GDP	2092 ± 121
dATP	1900 ± 42
dGTP	1959 ± 272
dCTP	1678 ± 29
Carbachol	8481 ± 922

purinergic receptors were tested for comparison. As is illustrated in Fig. 3, *right,* ATP and UTP were effective agonists in HT29 cells under conditions where, in C6-2B cells, they were either less potent than UDP (UTP) or were without effect (ATP). UDP was approximately 100-fold less potent than UTP in HT29 cells.

Simultaneous challenge of C6-2B cells with UDP and carbachol resulted in inositol phosphate accumulation that was essentially the sum of the accumulations observed in the presence of either agonist alone (Fig. 4). In contrast, simultaneous challenge of cells with UTP and UDP resulted in no greater inositol phosphate accumulation than accumulation observed with either agonist alone, suggesting that these compounds activate the same receptor. ATP alone had no effect on inositol phosphate accumulation and no significant effect of ATP was observed when combined with UDP, UTP, or carbachol (Fig. 4).

Cross-desensitization experiments also were carried out to examine whether ATP interacts with the receptor that is activated by UDP and UTP. These experiments took advantage of the fact that receptor-stimulated accumulation of total [^{3}H]inositol phosphates is negligible in the absence of LiCl. Thus, cells were preincubated, *i.e.* desensitized, with agonist in the absence of LiCl and inositol phosphate accumulation in response to a second drug challenge was assessed by co-addition of agonist and LiCl. Preincubation of C6-2B cells with UDP or UTP for 20 min resulted in a marked decrease in subsequent responsiveness to both UTP and UDP (Fig. 5) but a much smaller decrease in response to carbachol. In contrast, preincubation of cells with ATP had no inhibitory effect on subsequent responsiveness to carbachol, UDP, or UTP. Taken together with the additivity experiments, these results indicate

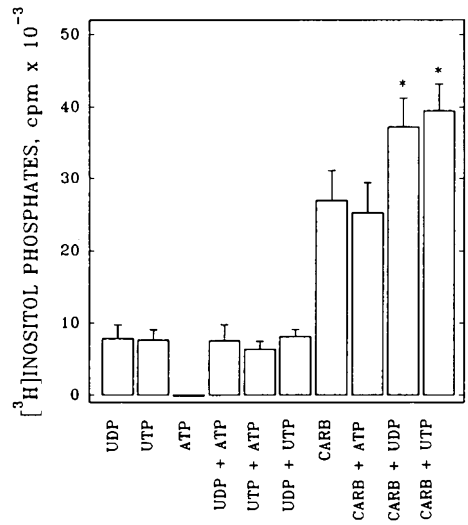

FIG. 4. **Additivity of nucleotide- and carbachol (*CARB*)-stimulated inositol phosphate formation in C6-2B cells.** [^{3}H]Inositol-labeled cells were incubated with the indicated agonists or combination of agonists (300 μM each) for 20 min, trichloroacetic acid (5%) was added, and inositol phosphate formation was quantitated as indicated under "Experimental Procedures." The amount of [^{3}H]inositol phosphates present at $t = 0$ (≈2300 cpm) was subtracted from the indicated values. The data are the mean ± S.D. of triplicate determinations. *, differed significantly ($p < 0.05$) from values determined with carbachol alone.

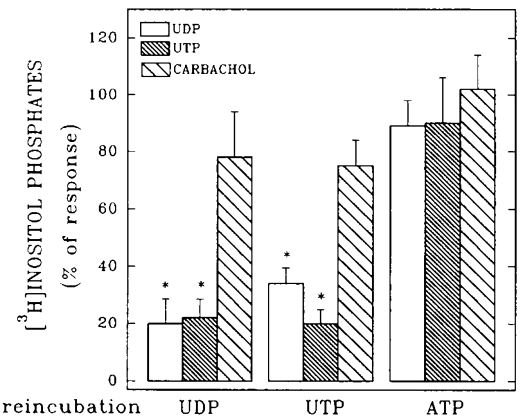

FIG. 5. **Desensitization of agonist-stimulated inositol phosphate formation in C6-2B cells.** [^{3}H]Inositol-labeled cells were preincubated for 20 min with 300 μM UDP, UTP, or ATP or in the absence of any drug. The cells were rapidly washed and incubated for an additional 10 min with 300 μM carbachol (*wide stripes*), 300 μM UDP (*open bar*), or 300 μM UTP (*narrow stripes*) in the presence of 10 mM LiCl. The results are plotted as the percent of agonist responses obtained with cells that were preincubated in the absence of any added drug. Data represent the mean ± S.D. of triplicate determinations. *, $p < 0.01$.

that UDP and UTP act at a common receptor at which ATP is not an effective ligand.

Reactive blue and suramin have been proposed to competitively inhibit adenine nucleotide receptors in a number of tissues (3). Experiments were carried out to examine the effect of reactive blue and suramin on UDP-stimulated inositol phosphate formation in C6-2B cells. Suramin (3–50 μM) caused a parallel shift to the right of the concentration-effect curve of UDP without affecting maximal response to UDP (Fig. 6; data not shown). Because suramin concentration in excess of 50 μM also resulted in inhibition of carbachol-stimulated inositol phosphate formation in C6-2B cells (data not shown), a detailed Schild analysis of the effect of suramin on UDP response was not performed. Unlike suramin, reactive blue (3–50 μM) caused

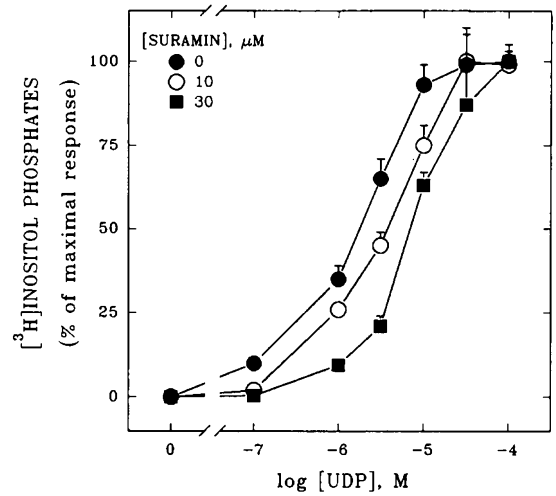

FIG. 6. **Inhibition of UDP-stimulated inositol phosphate formation by suramin.** [^{3}H]Inositol-labeled cells were preincubated for 5 min without or with the indicated concentration of suramin and challenged with UDP for an additional 15 min. The results are plotted as the percentage of the maximal response obtained with UDP at each given suramin concentration. The data represent the mean ± S.D. of results obtained in four different experiments.

a noncompetitive inhibition of UDP responses, as judged by decreases of the maximal effect of UDP with negligible changes of the concentration of agonist producing half-maximal effects (data not shown).

Receptor-mediated regulation of phospholipase C by a broad range of extracellular stimuli has been shown to involve G-proteins. At least two general mechanisms are apparently involved, one involving members of the pertussis toxin-insensitive G_q family of G-proteins (24) and one involving pertussis toxin-sensitive G_i or G_o (25–27). C6-2B glioma cells were incubated with various concentrations of pertussis toxin and responsiveness to carbachol and UDP were subsequently measured. Pretreatment of cells with pertussis toxin resulted in only small (0–20%) decreases in carbachol-stimulated inositol phosphate accumulation (Fig. 7, *A* and *B*). In contrast, UDP- and UTP-stimulated inositol phosphate accumulation was reduced by up to 80% by pretreatment of C6-2B cells with maximally effective concentrations of pertussis toxin. The effect of pertussis toxin was observed as a decrease in response to maximally effective concentrations of UDP with little or no change in apparent potency (Fig. 7*B*).

The data with pertussis toxin-treated cells suggested that the receptor for UDP coupled to a G-protein to regulate phospholipase C. This possibility was examined directly by measuring inositol lipid hydrolysis in streptolysin-*O*-permeabilized C6-2B cells. GTPγS produced a concentration-dependent increase in inositol phosphate formation (Fig. 8). Addition of UDP alone had no effect on enzyme activity. However, incubation of membranes with 100 μM UDP in the presence of increasing concentrations of GTPγS resulted in a marked stimulation over the effect observed with guanine nucleotide alone. The $K_{0.5}$ for UDP for augmentation of inositol phosphate formation in the presence of 30 μM GTPγS was approximately 20 μM (data not shown).

The data presented thus far indicate that a novel receptor for uridine nucleotides activates phospholipase C in C6-2B glioma cells. The possibility that this receptor also activates another second messenger signaling response was considered. Phospholipid pools were prelabeled with [^{3}H]arachidonate as described under "Experimental Procedures." Agonist-promoted release of [^{3}H]arachidonic acid, supposedly occurring as a consequence of activation of phospholipase A_2, was then studied. Incubation of

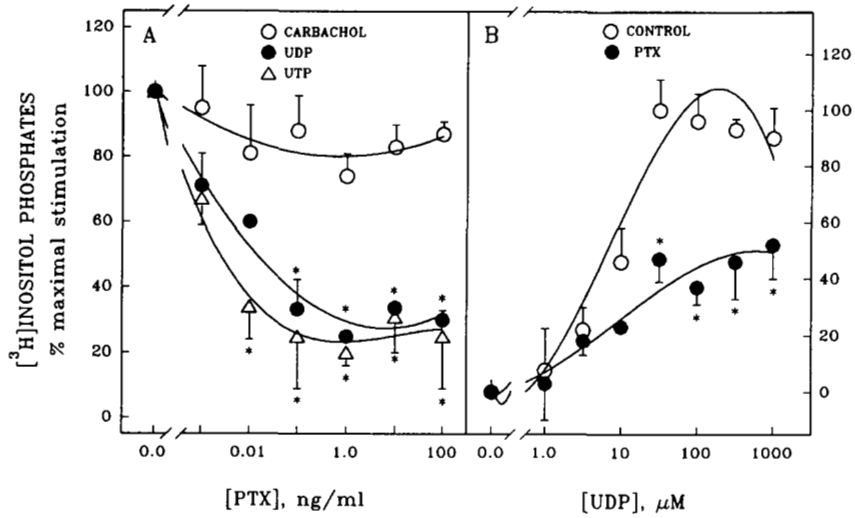

FIG. 7. **Effect of pertussis toxin (*PTX*) on inositol phosphate formation.** C6-2B cells were labeled for 18 h with 5 μCi of [^{3}H]inositol in 0.5 ml of inositol-free DMEM containing the indicated concentrations of pertussis toxin (*panel A*) or with 30 ng/ml pertussis toxin (*panel B*). The data are the mean ± S.D. of triplicate determinations. *Panel A*, the effects of 300 μM carbachol, UDP, and UTP on inositol phosphate accumulation were determined. The data are plotted as the percent of maximal stimulation observed with each drug in control cells. *Panel B*, the response to the indicated concentrations of UDP was quantitated in control cells or cells pretreated for 18 h with 30 ng/ml pertussis toxin. *, $p < 0.05$.

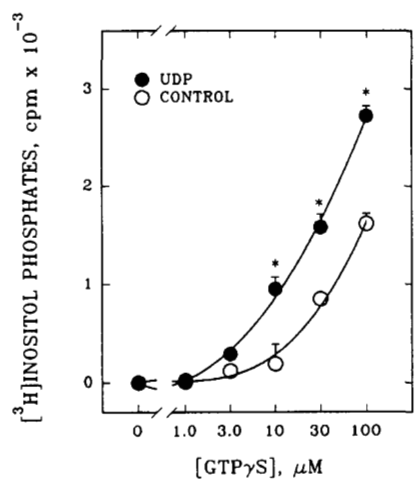

FIG. 8. **Guanine nucleotide-dependent inositol lipid hydrolysis in streptolysin-*O*-permeabilized C6-2B glioma cells.** C6-2B cells were permeabilized with streptolysin-*O* and [^{3}H]inositol phosphate accumulation was measured as described under "Experimental Procedures." Assays were in the presence of the indicated concentrations of GTPγS with (●) or without (○) 100 μM UDP. The data are the mean ± S.D. of triplicate determinations and the results are representative of those obtained in three separate experiments. *, $p < 0.01$.

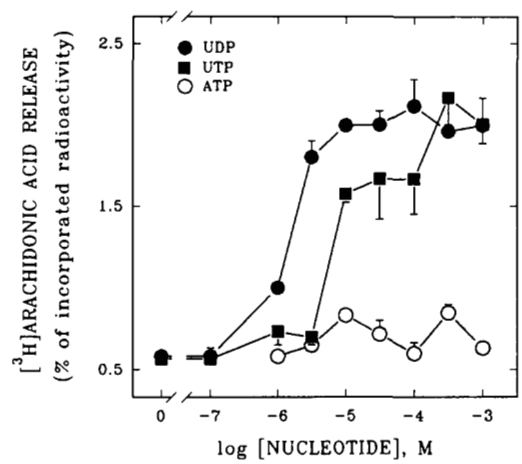

FIG. 9. **Receptor-stimulated [^{3}H]arachidonic acid release in C6-2B glioma cells.** [^{3}H]Arachidonic acid-prelabeled cells were incubated for 10 min with the indicated additions. [^{3}H]Arachidonic acid released into the medium was quantitated as described under "Experimental Procedures." The data are the mean ± S.D. of results obtained with triplicate determinations and are representative of results obtained in four separate experiments.

cells with UTP, UDP, or carbachol (Fig. 9; data not shown) all resulted in a concentration-dependent release of [^{3}H]arachidonic acid. The potency of these agonists was similar to that observed in measurements of [^{3}H]inositol phosphate accumulation. Negligible increases in [^{3}H]arachidonic acid release were observed in the presence of ATP.

Experiments were carried out to determine whether the uridine nucleotide-selective response could be observed in cells expanded from single cells cloned from the C6-2B cell line as described under "Experimental Procedures." Eight different subclones were isolated and their inositol phosphate responses measured. Seven of these subclones (Clones 1, 2, 3, 4, 6, 7, and 8) were morphologically similar to the parent cells that were previously studied in detail. Clone 5 was markedly different morphologically. These cells were much smaller and possessed many long processes. However, all eight of the subclones expressed receptors that responded to UDP with a marked increase in inositol phosphate accumulation (data for 5 clones are presented in Fig. 10, *left*). The inositol phosphate response to ATP was negligible in all of these subclones.

C6-2B rat glioma cells represent a subclone that was originally isolated from C6 glioma cells by de Vellis and Brooker (28). Inositol phosphate responses to nucleotides also were tested in C6 glioma cells that were obtained from the American Type Culture Collection. In contrast to the lack of response observed with C6-2B cells, ATP and ATPγS markedly stimulated inositol phosphate accumulation in C6 glioma cells (Fig. 10, *right*), and the pharmacological specificity for enhancement of inositol lipid hydrolysis in C6 glioma cells (ATP = UTP > ATPγS ≫ UDP) was similar to that observed in HT29 human colon carcinoma cells (Fig. 3, *right*) and for P_{2U}-purinergic receptors in many target cells. Carbachol, which markedly stimulated inositol phosphate accumulation in C6-2B glioma cells (Fig. 1), had no reproducible effect on accumulation in C6 glioma cells (Fig. 10, *right*).

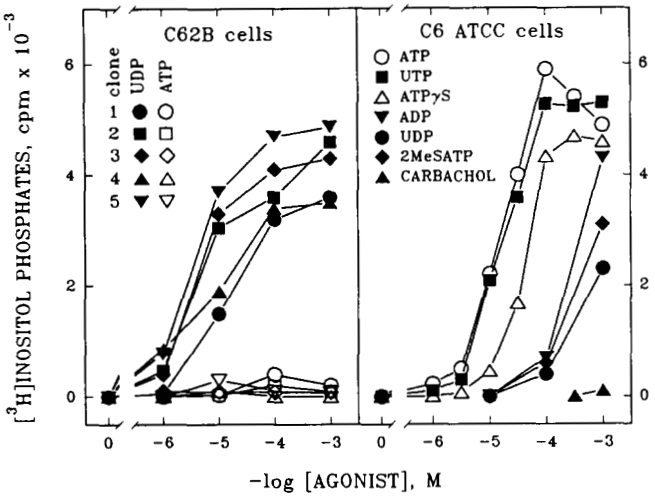

FIG. 10. **Effect of UDP and ATP on inositol phosphate formation in subclones of C6-2B cells and in C6 glioma cells obtained from the American Type Culture Collection.** *Left*, the effect of UDP (*filled symbols*) and ATP (*open symbols*) on inositol phosphate formation was determined with C6-2B cell populations expanded from five different cloned single cells as detailed under "Experimental Procedures." The data are the mean of triplicate determinations and are representative of results from two different experiments with each clone. *Right*, the effect of ATP, UTP, UDP, ATPγS, and carbachol on inositol phosphate accumulation was determined in C6 glioma cells obtained from the American Type Culture Collection.

DISCUSSION

A receptor that has not been described previously exists on C6-2B rat glioma cells. This receptor, which stimulates inositol lipid hydrolysis, shows a strict selectivity for uridine nucleotides and is activated only nominally or not at all by ATP, ADP, or analogs of these adenine nucleotides. This receptor can be clearly differentiated from the P_{2U}-purinergic receptor, which is found on many target tissues. The C6-2B cell receptor is activated by UDP, and UTP is a less potent full agonist. ATP and ATPγS have little or no effect. In contrast, ATP, ATPγS, and UTP are the most potent agonists at P_{2U}-purinergic receptors; UDP has been shown to be much less potent than UTP (Fig. 3, *right*, and Ref. 5) or to be without effect (8) at these receptors. Several different types of experiments including those examining additivity of responses or cross-desensitization also confirmed that the receptor on the C6-2B glioma cells does not recognize adenine nucleotides.

A full delineation of the structure-activity relationships for nucleotide-activated inositol phosphate formation in C6-2B cells was limited by the lack of availability of a wide range of pyrimidine nucleotides. UDP was the most potent and effective of the nucleotides studied. The presence of a γ-phosphate, *i.e.* UTP, resulted in a reduced potency of approximately 30-fold. Replacement of the γ-phosphate of UTP with glucose (UDP-glucose) resulted in further decreases of both potency and efficacy. However, increased potency relative to UTP was observed with substitution of bromo at position 5 of the pyrimidine ring of UTP (5-BrUTP). In contrast, substitution of a keto group for the $-NH_2$ at position 4 (CTP, CDP) resulted in total loss of activity. 2′-Deoxynucleotides have no effect on P_{2U}-purinergic receptors (8), and similarly, the receptor on C6-2B cells was not activated by dUTP at any concentration tested up to 1 mM. However, as was observed with 5-BrUTP, 5-bromo substitution of dUTP (2′-deoxy-5-BrUTP) resulted in an increase in activity. Some activity (~50% of maximal stimulation observed with UDP) was observed with dTTP and dTDP, which can be considered as 5-methyl analogs of dUTP and dUDP, respectively. Thus, substitution at position 5 of the pyrimidine ring with bromo or $-CH_3$ resulted in enhanced activity of the uridine triphosphate analogs. These results suggest that compounds such as 5-BrUDP or 5-MeUDP will be very potent agonists at this receptor.

Specific antagonists for receptors for extracellular nucleotides have not been identified. However, suramin and reactive blue, which have a broad range of pharmacological activities previously have been shown to competitively block P_2-purinergic receptors in a number of tissues (3). Suramin also blocks the receptor for uridine nucleotides on C6-2B glioma cells, although at higher concentrations an apparently nonspecific inhibition of the inositol lipid hydrolysis occurred.

Effects of extracellular uridine and uracil nucleotides have been reported previously. These responses range from relaxation or contraction of various smooth muscle preparations to inhibition of O_2 uptake, stimulation of glucose output, and enhancement of superoxide formation (16). Difficulties inherent in defining receptors for extracellular nucleotides on heterogeneous tissue preparations have compromised classification of these responses, but it is likely that many of these effects are mediated by P_{2U}-purinergic receptors. That is, a receptor that is activated by both UTP and ATP is involved. However, P_{2U}-purinergic receptors apparently do not completely account for the response to uridine nucleotides in peripheral tissues and Seifert and Schultz (16) have proposed the existence of a separate class of pyrimidinergic receptors. The pharmacological selectivities of responses in several vascular smooth muscle preparations were not the same as those of the P_{2U}-purinergic receptor (10–12), and in several cases the response also can be distinguished from that observed in C6-2B glioma cells since UMP, uridine, and CTP all were shown to produce vascular responses similar to those of UTP or UDP (29–33). Support for the existence of a receptor for UTP that is not activated by ATP also has come from studies in which responses to UTP and ATP in a heterogeneous tissue could be differentially blocked by pretreatment with supposed antagonists and by experiments in which cross-desensitization did not occur between the two nucleotides (10, 11, 34).

The data obtained with C6-2B glioma cells places the existence of a selective receptor for uridine nucleotides on firm ground. With the pharmacological specificity defined here in hand, it will be important to more clearly delineate the physiological role of this receptor in various tissues. These results together with the large amount of existing information on P_{2U}-purinergic receptors indicate that there are at least two different receptors that respond to extracellular uracil nucleotides. Relatively little is known about the regulation of the storage and release of these nucleotides, although platelet storage granules are a rich source of UTP and UTP is released upon platelet activation (16, 35). Resting levels of extracellular UTP in solid tissues are in the range of 1×10^{-6} M (16, 36). A multiplicity and widespread existence of receptors for uridine nucleotides and the presence of nucleotides at concentrations capable of activating these receptors suggests that the regulated release of uridine nucleotides will prove to be a physiologically important process.

The second messenger responses regulated by the receptor for uridine nucleotides on C6-2B glioma cells followed the general properties observed for many receptors including the muscarinic cholinergic receptor on the same cells. Streptolysin-*O*-permeabilized cells were used to illustrate directly that this receptor promotes guanine nucleotide-dependent activation of phospholipase C. The large sensitivity of the intact cell response to inactivation by pertussis toxin places these receptors into the subclass of receptors that couple to phospholipase C through G_i or G_o (25–27). Several recent reports suggest that activation of phospholipase C in this pathway occurs as a consequence of release of βγ-subunits from the involved G-protein

(37–40). Our assumption is that the activation of arachidonate release by this uridine nucleotide receptor is phospholipase A_2-mediated and occurs secondarily to activation of phospholipase C with subsequent Ca^{2+} mobilization and activation of protein kinase C; however, this has not been formally proven.

In addition to the uridine nucleotide-selective receptor, we have observed previously that activation of a P_{2Y}-purinergic receptor on C6-2B glioma cells results in inhibition of adenylylcyclase and a decrease in intracellular cyclic AMP levels (20). This receptor does not activate phospholipase C and thus is apparently different from the P_{2Y}-purinergic receptor(s) found on many target tissues which activates phospholipase C but does not regulate adenylylcyclase activity. Lin and Chuang (41) have reported that ATP stimulates inositol phosphate and Ca^{2+} accumulation in C6 glioma cells, and we have confirmed their results with C6 glioma cells obtained from the American Type Culture Collection (Fig. 10, *right*). In contrast, we have been unable to observe stimulation of inositol lipid hydrolysis by ATP or its analogs in C6-2B glioma cells, which are a subclone originally isolated from the RGC6 glioma cell line by deVellis and Brooker (28). We also isolated eight additional subclones from C6-2B glioma cells. All of these expressed a phospholipase C-linked receptor that was activated by UDP but not by ATP. Earlier studies on the β-adrenergic receptor/cyclic AMP system had revealed differences among the signaling properties of the various C6 cell (including C6-2B cells) subclones (42–45), and although carbachol markedly stimulated inositol phosphate accumulation in C6-2B glioma cells, no reproducible effect of muscarinic receptor stimulation was observed in C6 cells obtained from the American Type Culture Collection.

In summary, a phospholipase C-activating receptor for UDP and UTP has been identified on C6-2B rat glioma cells. Based on the properties of second messenger responses, this receptor must be structurally related to the superfamily of seven transmembrane-spanning G-protein-linked receptor proteins. The recently cloned P_{2U}- (46) and P_{2Y1}-purinergic receptors (47) have amino acid sequences that predict seven transmembrane spanning motifs. However, they express a low sequence homology with each other and suggest the existence of two new subclasses of G-protein-linked receptors. Our anticipation is that the receptor described here will fall structurally into a class of receptors with structures containing only low sequence homology to P_{2Y}-purinergic receptors and perhaps somewhat higher homology to the so-called P_{2U}-purinergic receptor(s) that is activated by both UTP and ATP. Since only uridine nucleotides are agonists at the receptor studied here, we propose that it be called a uridine nucleotide receptor. Molecular cloning of this uridine nucleotide receptor will be required to establish whether it is a member of a new class of receptor proteins or is a second member of the subfamily of P_{2U}-purinergic receptor-like proteins.

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