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Evidence that UTP and ATP Regulate Phospholipase C through a Common Extracellular 5′-Nucleotide Receptor in Human Airway Epithelial Cells

H. ALEX BROWN, EDUARDO R. LAZAROWSKI, RICHARD C. BOUCHER, and T. KENDALL HARDEN

Curriculum in Neurobiology (H.A.B.) and Departments of Pharmacology (T.K.H.) and Medicine (E.R.L., R.C.B.), University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

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SUMMARY

Extracellular ATP and UTP produced a rapid accumulation of inositol phosphates in human airway epithelial cells (CF/T43). The order of agonist potencies for a series of nucleotide analogues differed markedly from that of the classically described P_{2X}- or P_{2Y}-purinergic receptors. UTP was the most potent agonist and was fully efficacious; ATP and adenosine-5′-*O*-(3-thiotriphosphate) were also full agonists. In contrast, 2-methylthio-ATP, adenosine-5′-*O*-(2-thiodiphosphate) and α,β-methylene-ATP were without effect. ADP and UDP had little or no effect at concentrations as high as 100 μM, and deoxyribose and dideoxyribose compounds were inactive. The effects of ATP and UTP were not additive, whereas bradykinin- or histamine-stimulated inositol phosphate production was additive with the effects of ATP or UTP. Preincubation of cells with either UTP or ATP resulted in a parallel loss of responsiveness to both agonists. Desensitization was specific for the response to nucleotides, because no ATP- or UTP-induced effect on the response to histamine or bradykinin was observed. Pertussis toxin treatment of CF/T43 cells produced a 30–40% decrease in the response to ATP or UTP, which correlated with the ADP-ribosylation of 41- and 43-kDa proteins. Bradykinin and histamine responses were not modified by pertussis toxin. Guanine nucleotides had little effect on the inositol phosphate response in intact CF/T43 cells at concentrations below 100 μM. However, in streptolysin-O-permeabilized cells GTPγS produced a concentration-dependent activation of inositol phosphate formation. UTP or ATP had little effect in permeabilized cells in the absence of guanine nucleotides but markedly increased inositol phosphate formation in the presence of guanine nucleotides. Taken together, these results suggest that UTP and ATP activate a 5′-nucleotide receptor on CF/T43 cells that is distinct from the classically defined P_{2X}- and P_{2Y}-purinergic receptors. Activation of phospholipase C by this receptor involves, at least in part, a guanine nucleotide-binding regulatory protein.

Extracellular adenine nucleotides and adenosine elicit a broad range of physiological responses in the central nervous system and peripheral tissues (1). Burnstock (2) proposed in 1978 that the biological effects of adenine nucleotides and adenosine are mediated by two major receptor types. Those activated by adenosine are called P_1-purinergic receptors, now subdivided into A_1 and A_2 adenosine receptors (3), and exhibit a potency order of adenosine > AMP ≫ ADP > ATP. Those activated by ATP or ADP are called P_2-purinergic receptors and exhibit a potency order of ATP = ADP ≫ AMP > adenosine. In 1985, Burnstock and Kennedy (4) proposed a further subdivision of P_2-purinergic receptors into P_{2X}-purinergic receptor and P_{2Y}-purinergic receptor subtypes. P_{2X}-purinergic receptors are activated with a potency order of α,β-MeATP > β,γ-MeATP > ATP = ADP > 2MeSATP. P_{2Y}-purinergic receptors are activated with a potency order of 2MeSATP > ADPβS > ATP = ADP > α,β-MeATP > β,γ-MeATP. A comprehensive classification of P_2-purinergic receptors has proven difficult, because no selective antagonists are available. However, other P_2-purinergic receptor subtypes apparently exist. For example, a receptor on platelets (1) is activated by ADP but not by ATP (P_{2T}), and very high concentrations of ATP have been proposed to permeabilize mast cells through a receptor, termed P_{2Z} (5). This permeabilization phenomenon has

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ABBREVIATIONS: α,β-MeATP, α,β-methylene-adenosine-5′-triphosphate; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); ATPαS, adenosine-5′-*O*-(1-thiotriphosphate); ADPβS, adenosine-5′-*O*-(2-thiodiphosphate); App(NH)p, adenylyl-imidodiphosphate; 2-MeSATP, 2-methylthioadenosine triphosphate; Br-UTP, 5-bromo-uridine-5′-triphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); GDPβS, guanosine-5′-*O*-(2-thiodiphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; HPLC, high performance liquid chromotography; $InsP_1$, inositol monophosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; $InsP_4$ inositol tetrakisphosphate; HBSS, Hanks' buffered saline solution; BzATP, (4-benzoyl)benzoyl acid adenosine-5′-triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; G protein, guanine nucleotide-binding protein.

been demonstrated to be specific for ATP and apparently is not relevant for the other nucleotides (6).

Extracellular ATP has been reported to activate the inositol lipid signaling system in numerous tissues (7–9). In some cases, this response has been shown to be mediated by a receptor exhibiting the pharmacological properties of a P_{2Y}-purinergic receptor (10, 11). Recently, it has become clear that the inositol phosphate response to ATP in a broad range of tissues occurs with an order of agonist potency distinct from that described above for purinergic receptor subtypes (12–16). It has been suggested that UTP activates this putative receptor (called the P_{2U}-purinergic receptor by Dubyak) (17), although this has yet to be unambiguously demonstrated, and several reports indicate that receptor-mediated responses to ATP and UTP can be differentiated in a number of target tissues (18–20). Seifert and Schultz (21) have proposed that the effects of ATP and UTP are mediated through distinct purinergic and pyrimidinergic receptors.

In the current study, we have characterized the inositol lipid signaling response to receptor activation by ATP or UTP on human airway epithelial cells (CF/T43). Structure-activity relationships for 5′-nucleotide analogues have been examined, and a number of experimental approaches have been taken to test whether ATP and UTP act at the same putative extracellular receptor.

Experimental Procedures

Materials. 2-D-*myo*-[^{3}H]Inositol was purchased from American Radiolabelled Chemicals Inc. (St. Louis, MO). [α-^{32}P]NAD was obtained from New England Nuclear-DuPont (Wilmington, DE). Pertussis toxin was from List Biological Laboratories INC (Campbell, CA). Lubrol-PX was obtained from Pierce (Rockford, IL). The 2′-deoxynucleotide triphosphates and the 2′,3′-dideoxynucleotide triphosphates were obtained from United States Biochemical Corp. (Cleveland, OH). BzATP was obtained from Sigma (St. Louis, MO) or was prepared by Dr. Jose Boyer in our laboratory. All of the nucleotides and their analogues, as well as the affinity-purified goat anti-rabbit IgG alkaline phosphatase, were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), with the exception of 2-MeSATP and α,β-MeATP, which were purchased from Research Biochemicals Inc. (Natick, MA). Keratinocyte growth medium was purchased from Clonetics (San Diego, CA). HBSS and inositol-free Dulbeccos modified Eagle's medium were from GIBCO (Grand Island, NY). Hormone and growth factor supplements were obtained from Research Products Inc. (San Diego, CA). Streptolysin-O was purchased from Burroughs-Wellcome Diagnostics (Greenville, NC). All other reagents and salts were purchased from Sigma.

Antisera. Dr. Allen Spiegel, at the National Institutes of Health, generously supplied antiserum EC, raised against the carboxyl-terminal sequence (KNNLKECGLY) of $G_{\alpha i3}$ (22), and antiserum AS, raised against the carboxyl-terminal sequence (KENLKDCGLF) of $G_{\alpha t}$, which recognizes the G protein subunits $G_{\alpha i1}$ and $G_{\alpha i2}$ (23). The antiserum Go/1, which recognizes $G_{\alpha o}$, was obtained from New England Nuclear-DuPont.

Cell culture. CF/T43 cells were grown on 12-well plates in keratinocyte growth medium plus bovine pituitary extract, at 37°, in an humidified atmosphere of 5% CO_2 and 95% air. During the course of these experiments, cells from passage 30–86 were used, with no appreciable changes in agonist potencies or efficacies.

Cell labeling and inositol phosphate assay. Confluent CF/T43 cells were incubated for 24 hr in inositol-free Dulbecco's modified Eagle's medium supplemented with 10 ng/ml epidermal growth factor, 3.75 μg/ml endothelial cell growth factor, 500 ng/ml hydrocortisone, 5 μg/ml insulin, and 10 μCi/ml [^{3}H]inositol. The cells were washed twice in incubation medium (HBSS supplemented with 25 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM LiCl) and incubated for 10 min before addition of agonists. In those experiments in which the rates of either desensitization or recovery were measured, the preincubation phase with LiCl was omitted. The assays were terminated by addition of cold 6.25% perchloric acid. Resolution of individual inositol phosphates on Dowex AG1-X8 columns was performed as previously described (24). The identity of individual inositol phosphates was confirmed by co-chromatography with known standards. In most experiments the inositol phosphates were separated into two fractions, an $InsP_1$ fraction and an inositol polyphosphate fraction consisting of $InsP_2$, $InsP_3$, and $InsP_4$. In all figures other than the time course (Fig. 1) and the HPLC traces (Fig. 2), the basal levels of inositol phosphates were subtracted from stimulated levels.

HPLC. Samples were prepared as described above, except that the cells were initially labeled with 100 μCi/ml [^{3}H]inositol. The cell extracts were loaded, via injector, onto a Whatman Partisil 10 strong anion exchange column (Alltech Applied Science, Deerfield, IL) and were separated using an ammonium phosphate gradient, as previously described (25). Radioactivity was monitored on-line with a Radiomatic Flo-one detector (Radiomatic Instruments, Tampa, FL). The generated [^{3}H]inositol compounds were identified by their coelution with standards of known [^{3}H]inositol phosphates.

Phospholipase C assays in permeabilized cells. [^{3}H]Inositol-labeled CF/T43 cells were rinsed three times in wash buffer (145 mM NaCl, 5 mM EGTA, pH 7.4) at 30°. Cells were then permeabilized by a 5-min incubation with 0.2 IU of 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 7.3. Under these conditions, the free calcium concentration was calculated to be 25 nM.[1] After a brief rinse, 0.5 ml of fresh assay buffer containing the indicated compounds was added to the permeabilized cells. Assays were terminated by the addition of 0.5 ml of 12.5% cold perchloric acid to the reaction mixture. [^{3}H]Inositol phosphates were resolved and quantitated as described above.

Membrane preparation. CF/T43 cells grown to confluence on Falcon 150- × 25-mm dishes were washed in ice-cold 145 mM NaCl, 5 mM HEPES, pH 7.4, and incubated for 20 min at 4° in a hypotonic lysis buffer (5 mM Tris, pH 7.4, 1 mM EGTA). The swollen cells were scraped and homogenized with a ground glass homogenizer. Nuclei and unbroken cells were separated by centrifugation at 320 × *g* for 12 min at 4°. The supernatant was collected and centrifuged at 17,000 × *g* for 20 min at 4°. The resultant pellet was resuspended in lysis buffer. The centrifugation at 17,000 × *g* was repeated, and the resultant pellet was resuspended in 10 mM HEPES, pH 7.4. Membranes were stored at −70° until used.

***In vitro* ADP-ribosylation and identification of G_α proteins.** ADP-ribosylation of CF/T43 cell membranes was performed essentially as previously described (26). Briefly, membranes were prepared, as indicated above, from control or pertussis toxin-treated cells. Unless stated otherwise, 20 μg of membranes were incubated for 30 min at 37° in a 100-μl final volume containing (final concentrations) 10 mM Tris, pH 7.6, 10 mM dithiothreitol, 0.5 mM ATP, 0.05 mM GTP, 1 mM EDTA, 0.25% Lubrol-PX, 6 μM β-NAD, 5 μCi of [α-^{32}P]NAD, and 10 μg/ml activated pertussis toxin. The toxin was preactivated by incubation with 20 mM dithiothreitol at 30° for 15 min. Reactions were terminated by addition of Laemmli sample buffer (27), and SDS-PAGE was carried out on a 8.5% polyacrylamide gel containing 8 M urea as described (26). Gels were washed with 25 mM Tris, pH 7.6, 192 mM glycine, 20% methanol, and proteins were transferred to a nitrocellulose membrane (Bio-Rad) at 100 mA for 14–16 hr. For immunodetection of G protein α subunits, nitrocellulose membranes were incubated with 3% bovine serum albumin in 20 mM Tris, pH 7.5, 500 mM NaCl, for 30 min and were subsequently incubated for 2 hr with a 1/500 dilution of primary antiserum and for 45 min with a 1/2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG. The Western blots were

[1] This was calculated using the calcium chelator software program Eqcal [L. Backman (1988)], distributed by Biosoft (Milltown, NJ).

developed using an alkaline phosphatase reaction, as described (22) (0.3 mg/ml *p*-nitro blue, 0.15 mg/ml 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt, in 0.1 M $NaHCO_3$, pH 9.8).

Presentation of data. The data for the intact cell inositol phosphate experiments are the means of duplicate or triplicate assays and are representative of multiple experiments. Data are usually expressed as percentages of the maximal response for a given experiment. Unless otherwise indicated, standard errors of data points varied from the mean by <10%.

Results

Activation of phospholipase C by 5′-nucleotide analogues. Incubation of CF/T43 cells with maximally effective concentrations of ATP, UTP, histamine, or bradykinin resulted in a rapid and time-dependent accumulation of inositol phosphates (Fig. 1). The maximal accumulation observed with ATP and UTP was similar and was routinely intermediate between accumulation observed with histamine or bradykinin. ADPβS (100 μM) produced little or no effect on inositol phosphate accumulation at times up to 60 min. The cessation of accumulation of inositol phosphates after 10 min of incubation with nucleotides cannot be explained by rapid degradation of the agonist, because addition of fresh nucleotide after 10 min did not produce further accumulation of inositol phosphates.

HPLC analysis of [^{3}H]inositol phosphates generated in CF/T43 cells in response to 100 μM ATP (Fig. 2) revealed a profile of species similar to those previously reported for many tissues. A 2.5-, 7.9-, 1.7-, and 7.6-fold increase occurred in levels of $Ins(1,4)P_2$, $Ins(1,3,4)P_3$, $Ins(1,4,5)P_3$, and $InsP_4$, respectively, during a 5-min challenge with ATP (Fig. 2). Similar increases in [^{3}H]inositol phosphates were observed in the presence of UTP, histamine, or bradykinin (data not shown).

To define the pharmacological specificity of the putative P_2-purinergic receptor in these cells, concentration-effect curves were generated for structural analogues of ATP and ADP and for other nucleotides (Fig. 3). UTP, ATP, and ATPγS stimulated inositol phosphate accumulation to the same extent and with $K_{0.5}$ values of 3, 10, and 30 μM, respectively (Fig. 3). In contrast, the P_{2Y}-purinergic receptor agonists 2MeSATP and ADPβS and the P_{2X}-purinergic receptor agonists α,β-MeATP and β,γ-MeATP had little or no effect on inositol phosphate accumulation (Fig. 3). Half-maximal concentrations were determined from concentration-effect curves for a number of 5′-nucleotide analogues. Some of these concentration-effect curves are also shown in Fig. 3. Several compounds (ITP, GTPγS, GTP, and 5-Br-UTP) produced maximal effects that were typically 70–90% of those observed for ATP, ATPγS, and UTP, with $K_{0.5}$ values of the compounds ranging from 30 to 100 μM. ADP, UDP, App(NH)p, CTP, ATPαS, UTPαS, adenosine, uridine, and uric acid produced little or no effect, even at concentrations as high as 100 μM. The difference in the

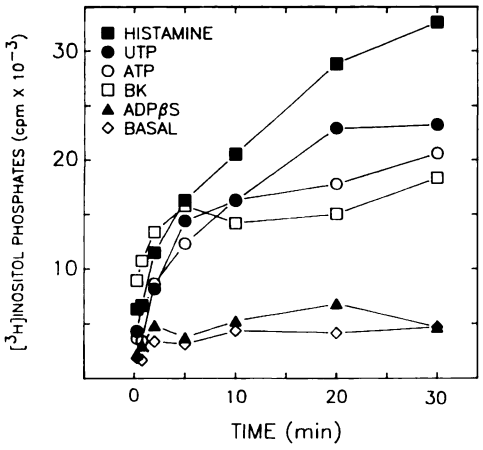


Fig. 1. Time course of inositol phosphate accumulation in CF/T43 cells. [^{3}H]Inositol-labeled CF/T43 cells were incubated with the indicated agonists at 37° in modified HBSS containing LiCl. The agonists were added at maximal concentrations [10 μM for bradykinin (*BK*) and 100 μM for all other compounds], and the inositol phosphates ($InsP_{1-4}$) were collected and separated as described in Experimental Procedures. The data are the mean values of triplicate assays and are representative of three similar experiments.

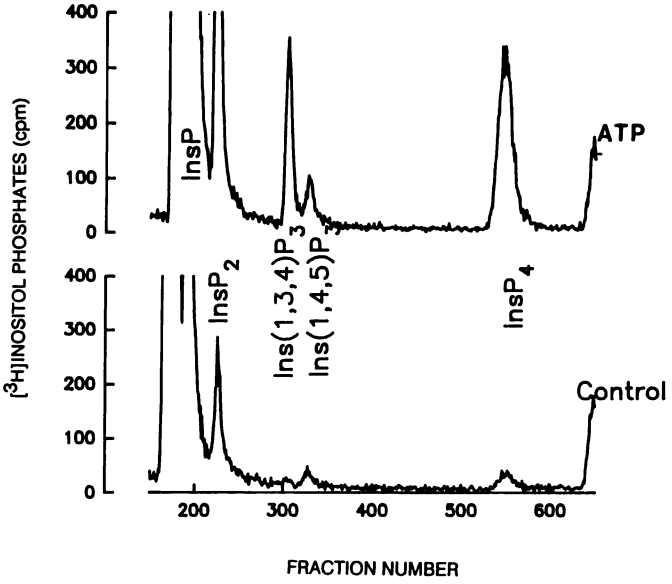


Fig. 2. HPLC analysis of [^{3}H]inositol-labeled material in CF/T43 cells. [^{3}H]Inositol-labeled CF/T43 cells were incubated at 37° for 5 min in either the presence (*upper*) or absence (*lower*) of 100 μM ATP. [^{3}H]Inositol-labeled compounds were separated on a Partisil-SAX HPLC column, as described in Experimental Procedures. The elution of known inositol phosphate standards is indicated. These results are representative of those obtained in duplicate samples for three experiments.

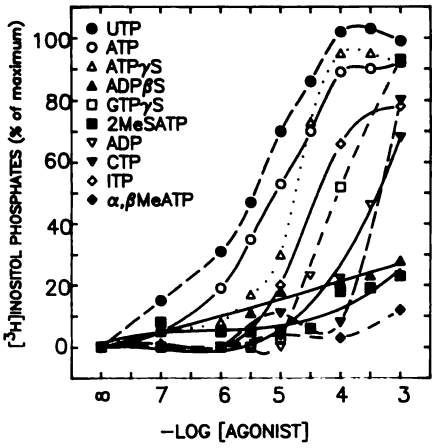


Fig. 3. Concentration-dependent stimulation of inositol phosphate formation by 5′-nucleotide analogues in CF/T43 cells. [^{3}H]Inositol-labeled cells were incubated in a modified HBSS, containing LiCl, for 5 min at 37°. Inositol phosphates were collected and separated on Dowex AG1-X8, as described in Experimental Procedures. Individual concentration-effect curves represent the mean of 2–12 different experiments, performed in either duplicate or triplicate assays, for each agonist. Data are expressed as a percentage of the maximal response obtained with UTP in the same experiment. The maximal $InsP_{2-4}$ production in 12 experiments for 100 μM UTP was 3500 ± 1710 cpm.

responses to agonists in CF/T43 cells, compared with the response described for the P_{2Y}-purinergic receptor, does not appear to be explained by cellular differences in rates of nucleotide hydrolysis. Using P_{2Y}-purinergic receptor-stimulated phospholipase C activity of turkey erythrocyte membranes as a bioassay, ADPβS and 2MeSATP were degraded by <5% over a 5-min incubation with CF/T43 cells (data not shown). Further, the same order of agonist potencies (i.e., UTP > ATP ≫ ADPβS > 2MeSATP) was observed when [^{3}H]inositol phosphates were measured after drug challenge of only 20 sec. We have observed similar pharmacological specificities for nucleotide-stimulated inositol phosphate production in primary cultures of human airway epithelial cells, A-431 carcinoma cells, and HT-29 colonic carcinoma cells (data not shown).

2′-Deoxyribose (dATP, dCTP, dGTP, and dTTP) and 2′,3′-dideoxyribose triphosphates (dideoxy-GTP and dideoxy-ATP) were ineffective in stimulating inositol phosphate production (data not shown). Further, addition of a (4-benzoyl)benzoyl acid substitution to the 3′-hydroxyl group of the ribose of ATP (BzATP) resulted in loss of capacity to stimulate inositol phosphate accumulation. This compound has been successfully used as an activator and a photoaffinity label for the P_{2Y}-purinergic receptor (28).

Evidence that ATP and UTP act at the same extracellular receptor. The responses of maximally effective concentrations of histamine and bradykinin were additive with the responses observed with either UTP or ATP (Fig. 4). In marked contrast, the effects of maximally effective concentrations of ATP and UTP in combination were not greater than those observed with either agonist alone.

The rapid cessation of inositol phosphate accumulation in the presence of ATP, illustrated in Fig. 1, suggests that agonist-induced desensitization is a prominent property of this ATP receptor. This phenomenon was investigated further, with the idea that, if ATP and UTP act through the same receptor, then cross-desensitization should occur between these two agonists. In the absence of LiCl, very little agonist-induced accumulation of inositol phosphates occurred in CF/T43 cells. Thus, cells were preincubated for various times with either UTP or ATPγS in the absence of LiCl and were rechallenged for 5 min with histamine, ATP, or UTP in the presence of 10 mM LiCl (Fig. 5). Preincubation with either UTP or ATPγS resulted in a concurrent loss of responsiveness to subsequent rechallenge with ATP or UTP. The time course and extent of loss of responsiveness were similar with each agonist. In contrast, the responses to histamine (Fig. 5) and bradykinin (data not shown) were not significantly altered by preincubation of CF/T43 cells with UTP or ATPγS. Although a 20-min preincubation of the cells with bradykinin (10 μM) resulted in a 40–50% loss of $InsP_{2-4}$ production in response to this agonist, no significant change in responsiveness to ATP or UTP was observed (control, ATP = 2568 cpm, UTP = 2216 cpm; after BK incubation, ATP = 2908 cpm, UTP = 2479 cpm).

The effects of preincubation with ATPγS or UTP on subsequent responsiveness to these two nucleotides were indistinguishable when concentration dependence was examined. As shown in Fig. 6, preincubation with either agonist resulted in a concentration-dependent shift to the right of the concentration-effect curve for ATPγS and for UTP and a decrease in the maximal response. To determine whether the loss of responsiveness to each agonist was reversible, CF/T43 cells were desensitized for 20 min with either 100 μM ATPγS or UTP in the absence of LiCl. As illustrated in Fig. 7, cells were washed free of agonist (100 μM ATPγS) and rechallenged with ATP, UTP, or histamine at the indicated times after removal of the desensitizing agonist. After an initial lag, responsiveness to ATP or UTP recovered to control levels over a 60-min period (Fig. 7). Similar results were obtained after preincubation with 100 μM UTP (data not shown). There was no appreciable change in histamine-stimulated inositol phosphate generation under the same conditions.

Potential involvement of a G protein. The possible involvement of a G protein in the action of ATP and UTP was

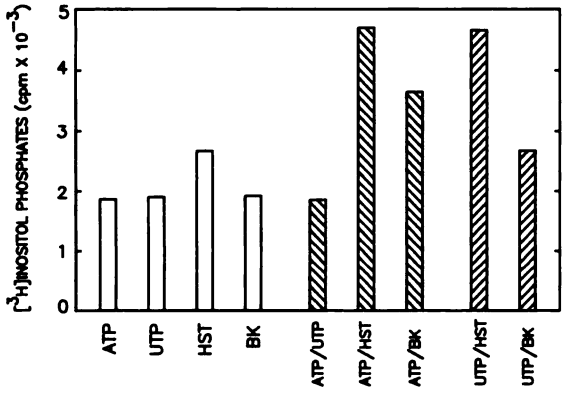


Fig. 4. Pharmacological additivity for the generation of inositol phosphates. Agonists were added either alone or simultaneously with another compound, as indicated, and incubated for 5 min at 37°. Inositol phosphates ($InsP_{2-4}$) were separated and quantitated as described in Experimental Procedures. ATP, UTP, and histamine (*HST*) were present at 100 μM and bradykinin (*BK*) at 10 μM. These concentrations were previously shown to produce a maximal stimulation. The data are from a typical experiment in which samples were assayed in triplicate, and the results are representative of three experiments.

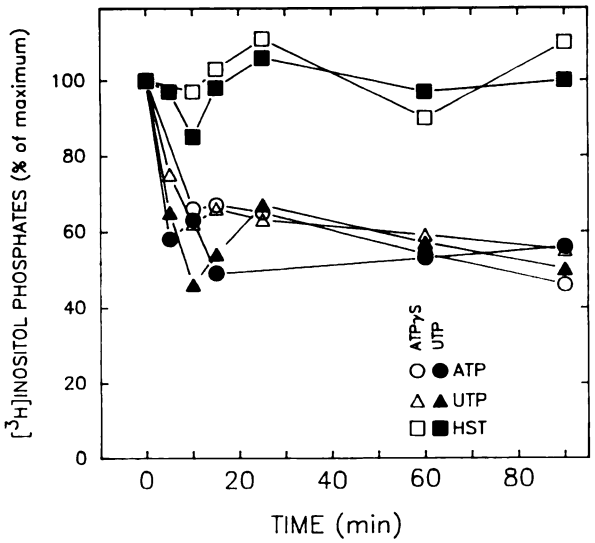


Fig. 5. Time course of desensitization of 5′-nucleotide receptor-stimulated inositol phosphate formation. [^{3}H]Inositol-labeled CF/T43 cells were preincubated in modified HBSS without LiCl, with either 100 μM ATPγS (*open symbols*) or 100 μM UTP (*filled symbols*), for the indicated times. Cells were then rapidly washed and transferred to fresh modified HBSS containing LiCl and a 100 μM concentration of the indicated agonists, ATP (*circles*), UTP (*triangles*), or histamine (*HST*) (*squares*). The cells were incubated for 5 min at 37°. [^{3}H]Inositol phosphates ($InsP_{2-4}$) were separated and quantitated as previously described. These data are expressed as a percentage of the maximal response (approximately 1500 cpm) and are representative of four experiments, in which assays were conducted in duplicate.

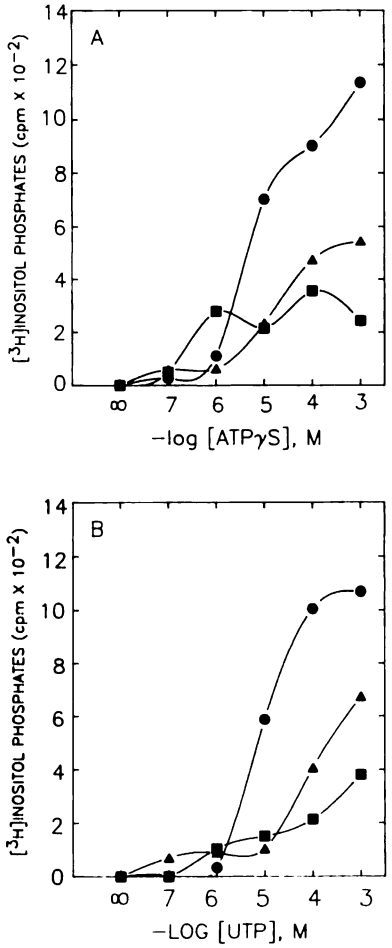


Fig. 6. Concentration-effect relationship for ATP- and UTP-stimulated inositol phosphate accumulation in desensitized CF/T43 cells. [^{3}H]Inositol-labeled cells were pretreated with either vehicle (●), 100 μM ATPγS (▲), or 100 μM UTP (■) in the absence of LiCl. After a 20-min incubation, the cells were rapidly washed twice in modified HBSS containing LiCl, and concentration-effect curves were generated for ATPγS (A) and UTP (B). [^{3}H]Inositol phosphates ($InsP_{2-4}$) were separated and quantitated as described in Experimental Procedures. Data are representative of results from two experiments, in which samples were assayed in duplicate.

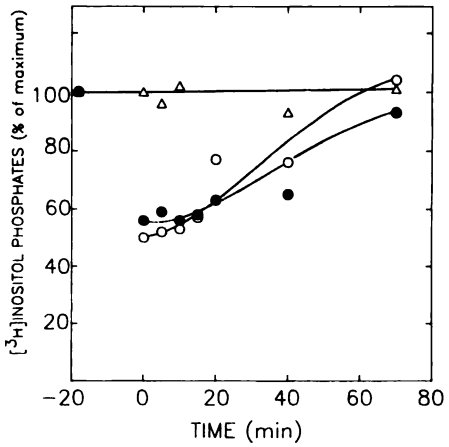


Fig. 7. Time course of recovery of nucleotide-stimulated [^{3}H]inositol phosphate production in intact CF/T43 cells following desensitization. [^{3}H]Inositol-labeled cells were preincubated for 20 min with 100 μM ATPγS in the absence of LiCl, as described in Experimental Procedures. At the indicated times, cells were rapidly washed twice in agonist-free, modified HBSS containing LiCl and were subsequently incubated for 5 min at 37° in the presence of 100 μM levels of the indicated agonist, i.e., histamine (Δ), ATP (○), or UTP (●). [^{3}H]Inositol phosphates ($InsP_{2-4}$) were collected and separated as described. These data are typical of results obtained in three experiments, in which samples were assayed in duplicate.

studied by examining nucleotide-stimulated inositol phosphate responses in CF/T43 cells permeabilized with streptolysin-O. As shown in Table 1, the generation of inositol phosphates was markedly enhanced by the hydrolysis-resistant guanine nucleotide analogue GTPγS. Preincubation with GDPβS (300 μM) produced a rightward shift in the GTPγS concentration-effect curve for phospholipase C activation (data not shown). The addition of UTP, ATPγS, histamine, and bradykinin in the absence of guanine nucleotide had little effect on phospholipase C activity. However, in the presence of 10 μM GTPγS all four compounds stimulated inositol phosphate production over that observed with GTPγS alone.

Inositol phosphate formation in the presence of GTPγS occurred with a time course exhibiting a considerable lag (Fig. 8). This lag in inositol phosphate accumulation was not observed when GTPγS and UTP or histamine were added simultaneously. The final rate observed with UTP plus GTPγS (5 min) was not significantly greater than that observed with GTPγS alone. The final rate was slightly enhanced in the presence of histamine plus GTPγS. GTP caused very small increases in [^{3}H]inositol phosphate accumulation. This activity was enhanced by UTP, ATP, and histamine but not by ADPβS

TABLE 1

CF/T43 streptolysin-O-permeabilized cells: Guanine nucleotide dependence of inositol phosphate response

Confluent CF/T43 cells were prelabeled with [^{3}H]inositol and permeabilized with 0.2 IU of streptolysin-O, as described in Experimental Procedures. After the permeabilized cells were washed, fresh assay buffer containing the indicated compounds (GTPγS and bradykinin at 10 μM and GTP, ATPγS, UTP, and histamine at 100 μM) was added. $InsP_{2-4}$ were measured in the presence of GTPγS after a 2-min incubation, whereas $InsP_{2-4}$ were measured after application of agonists in the presence and absence of GTP after a 5-min incubation at 30°. Data are expressed as cpm over basal. These data are means of values from two to five different experiments performed in duplicate for each agonist.

Agonist	$InsP_{2-4}$		
	Control (5 min)	GTP (5 min)	GTPγS (2 min)
		cpm	
None	0	355 ± 45	1308 ± 168
UTP	373 ± 72	835 ± 215	1733 ± 16
ATP	344 ± 71	755 ± 94	2404 ± 49
ATPγS	234 ± 141	3964 ± 1287	1805 ± 40
ADPβS	240 ± 40	479 ± 126	1189 ± 77
Bradykinin	98 ± 48	1794 ± 300	2276 ± 488
Histamine	373 ± 81	1251 ± 287	2838 ± 453
AlF_4^-	3537 ± 170		

(Table 1). ATPγS produced a much greater response in the presence of GTP than did UTP, ATP, or histamine in permeabilized cells. For example, the level of stimulation observed with ATPγS in the presence of GTP was similar to that in the presence of GTPγS alone. No such enhanced response was observed in intact cells after simultaneous application of GTP and ATPγS.

Effect of pertussis toxin on phospholipase C activity. The inositol phosphate response to both UTP and ATP was inhibited in a concentration-dependent manner by pretreatment of intact cells with pertussis toxin (Fig. 9A). The loss of response to UTP occurred in parallel with the loss of response to ATP, and the maximal loss of responsiveness to each nucleotide was 30–40%. In contrast to its effect on nucleotide-mediated stimulation, pertussis toxin had no effect on hista-

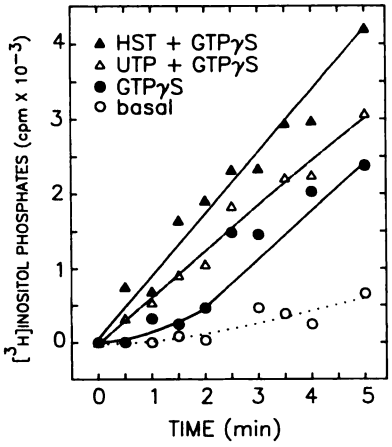
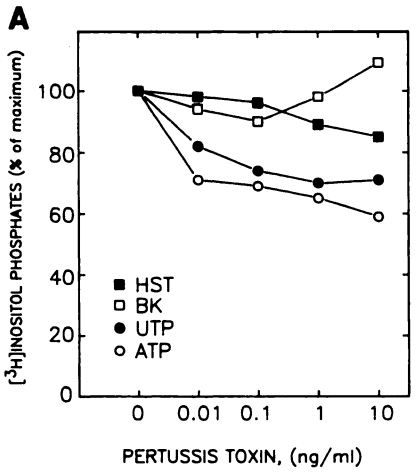


Fig. 8. Time course of guanine nucleotide stimulation of phospholipase C in permeabilized CF/T43 cells. Cells were permeabilized and washed as described in Experimental Procedures. The time course of [^{3}H]inositol phosphate accumulation was measured after the addition of assay buffer containing the indicated compounds, i.e., GTPγS (10 μM) in the absence and presence of agonists [100 μM UTP or histamine (*HST*)]. The reaction was terminated at the indicated times by the addition of 0.5 ml of 12.5% perchloric acid to the cells.



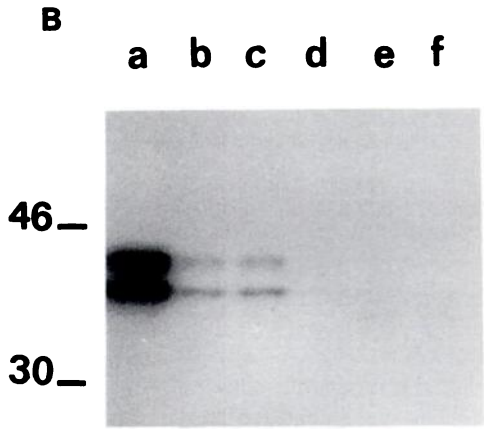


Fig. 9. Effect of pertussis toxin on phospholipase C activation in intact CF/T43 cells. [^{3}H]Inositol-labeled cells were treated with the indicated concentrations of pertussis toxin for 24 hr. A, The cells were challenged for 5 min with 100 μM ATP, UTP, or histamine (*HST*) or 10 μM bradykinin (*BK*), and inositol phosphates were separated and quantitated as described in Experimental Procedures. These data have been expressed as percentages of the maximal response for each agonist (UTP = 2800 cpm, ATP = 2400 cpm, histamine = 3600 cpm, and bradykinin = 1800 cpm) and represent the mean of results from three different experiments, in which samples were collected in duplicate. B, CF/T43 cells were incubated for 24 hr with the indicated concentrations of pertussis toxin, expressed in (ng/ml), 0 (*lanes a* and *f*), 0.01 (*lane b*), 0.1 (*lane c*), 1.0 (*lane d*), or 10 (*lane e*). Membranes prepared from these cells were incubated in the presence of 3 μCi of [^{32}P]NAD, with (*lanes a, b, c, d,* and *e*) or without (*lane f*) 10 μg of activated pertussis toxin, for 30 min at 37°. Proteins were separated by SDS/urea-PAGE, as described in Experimental Procedures. The autoradiograph shown is representative of four similar experiments with independent membrane preparations.

mine- or bradykinin-stimulated inositol phosphate production in this range of toxin concentrations.

Two species, of apparent M_r 41,000 and 43,000, were [^{32}P]ADP-ribosylated during the incubation of membranes with pertussis toxin and [^{32}P]NAD (Fig. 9B). Loss of capacity of pertussis toxin to catalyze *in vitro* labeling of these proteins occurred in membranes prepared from pertussis toxin-pretreated cells. The concentration dependence for this loss of *in vitro* labeling of both the 41- and 43-kDa species coincided with the concentration dependence for toxin-induced loss of response of phospholipase C to ATP and UTP.

To identify potential pertussis toxin-sensitive G proteins in CF/T43 cells, immunoblots were generated using antibodies selective for various G protein α subunits. The ADP-ribosylated species comigrated (Fig. 10, *lane a*) with proteins showing immunoreactivity with an antibody (AS) that recognizes $G_{\alpha i1}$ and $G_{\alpha i2}$ proteins (Fig. 10, *lane b*). A 43-kDa protein was also detected by antibody EC, which recognizes the $G_{\alpha i3}$ subunit (Fig. 10, *lane c*). An antibody raised against $G_{\alpha o}$ did not detect any immunoreactivity in CF/T43 membranes (Fig. 10, *lane d*).

Discussion

The airway epithelial cell line CF/T43 responds to extracellular nucleotides by production of intracellular inositol phosphates. The pharmacological properties of the CF/T43 nucleotide receptor that mediates this response are different from those classically described for P_2-purinergic receptor subtypes by Burnstock and Kennedy (4), Gordon (1), and others. Most notably, UTP is the most potent agonist at this receptor, whereas P_{2Y}-specific agonists 2MeSATP and ADPβS and the P_{2X}-specific agonists α,β-MeATP and β,γ-MeATP are without effect. The activity of UTP described here and elsewhere suggests the existence of a receptor type more appropriately referred to as a 5′-nucleotide receptor, rather than a purinergic receptor subtype (29, 30). This putative 5′-nucleotide receptor is apparently present in a variety of cell types and may be responsible for the effects of ATP on inositol lipid hydrolysis observed in many target tissues.

Based on the limited number of nucleotide analogues examined for capacity to increase inositol phosphate production in CF/T43 cells, a few observations have been made with regard to the structure-activity relationship between the nucleotide agonists and the putative receptor. (a) Full agonists at this receptor are 5′-nucleotide triphosphates; the presence of a triphosphate is a necessary, but not sufficient (e.g., CTP, TTP, and 2-MeSATP are inactive), requirement for full activation. (b) Substitution on phosphate groups alters the activity of the compounds for inositol phosphate production. The substitution of a sulfur for an oxygen in the α-position (e.g., ATPαS) renders the compound inactive, whereas substitution in the γ-position (e.g., ATPγS) only slightly reduces potency (Fig. 3). (c) Modification to the ribose moiety ablates the capacity to stimulate phospholipase C activity. (d) The 5′-nucleotide receptor discriminates between various nucleotide bases, but it is not

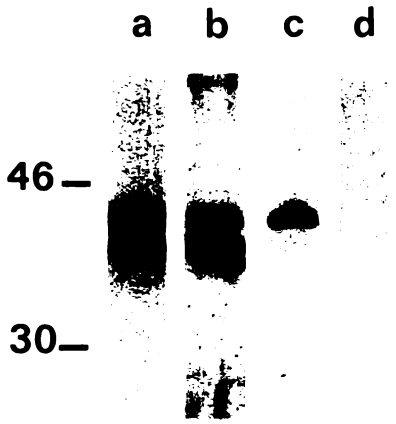


Fig. 10. Immunological analysis of G_α proteins in CF/T43 cell membranes. Membrane proteins (10 μg/lane) from CF/T43 cells were separated by SDS/urea-PAGE and transferred to nitrocellulose membranes, as described in Experimental Procedures. *Lane a*, autoradiograph of pertussis toxin-catalyzed [^{32}P]ADP ribosylation; *lane b*, an immunoblot against antisera AS; *lane c*, an immunoblot against antisera EC; *lane d*, an immunoblot against antisera GO/1. Nitrocellulose membranes were incubated with an anti-rabbit IgG secondary antibody, and proteins were localized by an alkaline phosphatase reaction. These data are representative of blots from two different preparations.

simply a purine versus pyrimidine discrimination. Both the pyrimidine base UTP and the purine base ATP are fully efficacious and potent agonists in CF/T43 cells. Other purine nucleotides, such as inosine, hypoxanthine, and guanine triphosphates, were moderately potent agonists in intact cell assays. The pyrimidine nucleotide CTP was essentially without effect on inositol phosphate generation.

The lack of apparent structural similarity between purine and pyrimidine nucleotide bases has raised questions as to whether the effects of ATP and UTP are mediated by a single extracellular nucleotide receptor. Despite the observation that ATP and UTP have similar biological effects in a variety of tissues, previous studies have led to the conclusion that their effects could be differentiated into pyrimidinergic and purinergic components (reviewed in Refs. 21 and 29). For example, Seifert *et al.* (18) noted that production of superoxide by NADPH oxidase in HL-60 cells and human neutrophils could be stimulated by both ATP and UTP. The response to UTP and to the peptide hormone f-Met-Leu-Phe was completely inhibited by preincubation with pertussis toxin (100 ng/ml), whereas stimulation by ATP was only partially inhibited at this concentration of toxin. Stutchfield and Cockcroft (19) reported that differentiation of HL-60 cells resulted in a marked increase in UTP- but not ATP-stimulated β-glucuronidase secretion. Further, von Kugelgen and Starke (20) observed that ATP and UTP elicit concentration-dependent vasoconstriction in rabbit basilar artery. However, their results suggest that cross-desensitization between ATP and UTP does not occur.

Results from a number of experiments reported here are consistent with the hypothesis that ATP and UTP act through the same 5′-nucleotide receptor. For example, the effects of ATP and UTP on inositol phosphate accumulation were additive with the effects of histamine or bradykinin receptor activation, but no additivity was observed between the effects of ATP and UTP. Preincubation of cells with ATPγS or ATP (data not shown) resulted in desensitization to the effects of subsequently added ATP or UTP, but no loss of response to histamine or bradykinin was observed. Similar results were obtained after preincubation of cells with UTP. The time courses of agonist-induced loss of responsiveness to ATP or UTP were identical, as were the time courses of recovery of responsiveness after transfer of cells to agonist-free medium. Finally, pretreatment of cells with pertussis toxin caused a coincidental toxin concentration-dependent loss of responsiveness to ATP and UTP, without affecting the capacity of histamine or bradykinin to stimulate inositol phosphate accumulation.

Inositol phosphate production provides a measure of receptor-effector function at the level of second messenger production, whereas Seifert *et al.* (18), Stutchfield and Cockcroft (19), and von Kugelgen and Starke (20) measured more distal responses to ATP and UTP. Perhaps therein lies the difference in their results and those reported here. Recently, Pfeilschifter (30) has also obtained data, with rat renal mesangial cells, consistent with the effects of ATP and UTP occurring through the same receptor. Although it was concluded that this response is mediated by a P_{2Y}-purinergic receptor (31), the nucleotide response in renal mesangial cells may more closely resemble the properties of the 5′-nucleotide receptor described here for CF/T43 cells. UTP has previously been shown not to interact with the P_{2Y}-purinergic receptor (32).

The dependence on guanine nucleotides for inositol phosphate production in permeabilized CF/T43 cells (Fig. 8 and Table 1) suggests the involvement of a G protein in ATP- and UTP-stimulated inositol phosphate formation. UTP increased the rate of activation, but not the final activity, of phospholipase C in the presence of GTPγS. This finding suggests that an increase in the rate of exchange of GTPγS for GDP on the involved G protein(s) is promoted by the agonist-occupied receptor. The inhibitory effect of pertussis toxin on ATP or UTP stimulation of phospholipase C in CF/T43 cells also suggests involvement of a G protein(s), although it was not possible, on the basis of pertussis toxin inhibition or immunoblots with G protein-sensitive antisera, to identify this G protein(s). These results are similar to those reported by Cowen *et al.* (33) for the effects of pertussis toxin on UTP stimulation in HL-60 cells. In each case, the inhibition induced by pertussis toxin was only partial. The explanation for only a partial inhibition of the inositol phosphate response remains unclear.

The response to ATPγS and GTP in permeabilized cells was much greater than expected, based upon the responses to other receptor agonists (ATP, UTP, and histamine). Recently, it has been suggested that a phosphate transfer catalyzed by the enzyme nucleotide diphosphate kinase could be involved in G protein function (34). The formation of GTPγS by a thiophosphate transfer from ATPγS could explain the results illustrated in Table 1. The significance of this observation is under investigation.

The human airway cell line (CF/T43) used in the present study to characterize the putative 5′-nucleotide receptor was derived from epithelial cells from a patient with the chronic pulmonary disease cystic fibrosis (35). In primary cultures of airway epithelial cells from both control and cystic fibrosis patients, the activation of this putative receptor appears to be linked to the activation of chloride secretion (36). The mechanism of this linkage is not known, but the potency and relative efficacies of the 5′-nucleotide analogues for stimulation of transepithelial chloride secretion, mobilization of calcium from internal stores as measured by fura-2 (36), and inositol phosphate production are reasonably well correlated. Recent *in vivo* studies have demonstrated that ATP and UTP are highly

effective Cl^- secretagogues, with similar EC_{50} values for changes in membrane potential difference after application to human nasal airway epithelia (37). Although the time course and magnitude of peak changes were distinct for control and cystic fibrosis subjects, the effects of ATP and UTP on changes in potential difference were indistinguishable in each respective group.

In summary, our data do not exclude the possibility that in some tissues distinct purinergic and pyrimidinergic receptors may exist. However, in CF/T43 cells, pharmacological studies suggest the existence of a 5′-nucleotide receptor that acts as a common extracellular site for the actions of ATP and UTP on inositol phosphate generation. This 5′-nucleotide receptor-regulated response appears to involve one or more G proteins. Development of a direct radioligand binding assay for this putative receptor will be an important next step in its identification.

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Send reprint requests to: T. Kendall Harden, Department of Pharmacology, CB 7365, 1119 FLOB, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7365.