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Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacotherapy

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ABSTRACT **The Cl^- secretory pathway that is defective in cystic fibrosis (CF) can be bypassed by an alternative pathway for Cl^- transport that is activated by extracellular nucleotides. Accordingly, the P_2 receptor that mediates this effect is a therapeutic target for improving Cl^- secretion in CF patients. In this paper, we report the sequence and functional expression of a cDNA cloned from human airway epithelial (CF/T43) cells that encodes a protein with properties of a P_{2U} nucleotide receptor. With a retrovirus system, the human airway clone was stably expressed in 1321N1 astrocytoma cells, a human cell line unresponsive to extracellular nucleotides. Studies of inositol phosphate accumulation and intracellular Ca^{2+} mobilization induced by extracellular nucleotides in 1321N1 cells expressing the receptor identified this clone as the target receptor in human airway epithelia. In addition, we independently isolated an identical cDNA from human colonic epithelial (HT-29) cells, indicating that this is the same P_{2U} receptor that has been functionally identified in other human tissues. Expression of the human P_{2U} receptor (HP2U) in 1321N1 cells revealed evidence for autocrine ATP release and stimulation of transduced receptors. Thus, HP2U expression in the 1321N1 cell line will be useful for studying autocrine regulatory mechanisms and in screening of potential therapeutic drugs.**

The transepithelial movement of fluid is coupled to the transport of Cl^- and other electrolytes, and in airway tissue the regulation of this activity is vital to normal function. Defective airway epithelial Cl^- secretion is a principal characteristic of cystic fibrosis (CF) (1). This defect contributes to the development of dehydrated mucus, which obstructs airways and compromises lung function. Acting as signaling molecules, extracellular 5′-nucleotides elicit diverse responses in a variety of tissues (2–4). In airway epithelia, ATP and UTP activate an alternative, non-CF transmembrane conductance regulator (CFTR)-dependent Cl^- conductance (5), raising the possibility that nucleotides may be used therapeutically to induce Cl^- secretion in the airways of individuals with CF (6, 7). Extracellular ATP and UTP also stimulate mucus secretion by goblet cells (8, 9) *in vitro*, and excessive activation of this pathway *in vivo* may contribute to hypersecretion in chronic bronchitis. Isolation and molecular characterization of the receptor for extracellular nucleotides present in human airway and other epithelia will permit studies on the expression of this receptor in normal and diseased tissues and facilitate identification of drugs for therapy.

Recently, a murine P_{2U} receptor cDNA was cloned from neuroblastoma–glioma hybrid (NG108-15) cells (10) which, when expressed in K562 human leukemia cells (11), encodes a 53-kDa protein that exhibits pharmacological and signaling properties similar to a P_{2U} nucleotide receptor that regulates ion transport in human airway (6, 12) and intestinal (13) epithelia. We used the murine P_{2U} receptor amino acid sequence to isolate human P_{2U} receptor cDNAs from CF/T43 (human airway) and HT-29 (human colonic) epithelial cell libraries. The human cDNAs are identical[§] and were functionally characterized by expression in human cells that lack endogenous P_{2U} receptor responses. The 1321N1 astrocytoma cell expression system, in addition to being useful for the pharmacological characterization of retrovirally transduced cDNA, constitutes a possible model for the previously postulated autocrine P_2 receptor regulation of cell function (4).

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing. Degenerate oligonucleotide primers [5′-AATGG(C/A/G)AC(C/T/A)TGGGA(G/A)-GG(G/A)GA(C/T)GA(A/G)-3′ and 5′-GACGTG(C/G)-AA(A/G)GGCAG(A/C)(A/C)AGC(A/T)GAGGGCGA-A-3′] based on the murine P_{2U} receptor sequence (10) were used in low-stringency PCR to amplify products from a cDNA library constructed in λUni-ZAP XR (Stratagene) from CF/T43 cell poly(A)$^+$ RNA. Products were cloned into pCR II (Invitrogen) and screened by Southern blot using probe P263, a random primer-labeled partial cDNA (corresponding to amino acids 8–276) generated by high-stringency PCR amplification of the murine P_{2U} receptor clone.

A cloned PCR product of about 500 bases (probe D9) that hybridized with probe P263 was labeled by random priming and used to screen the CF/T43 cDNA library. Hybridization conditions were as described below for Northern and Southern blots. Phage from one plaque, which hybridized strongly with probe D9, was purified by additional screening and pBluescript SK(−) (Stratagene) was rescued by *in vivo* excision. In parallel, an HT-29 cDNA library prepared in λgt10 was screened with probe P263, and insert from a positive plaque-purified phage was subcloned into the *Not* I site of pBluescript. Plasmid DNAs were purified by CsCl gradient centrifugation (14), and both strands of the two clones were sequenced by dideoxy chain termination (Sequenase version 2.0; United States Biochemical).

Heterologous Expression. A retroviral vector-containing plasmid, pLHP2USN, was constructed by insertion of the cloned CF/T43 cDNA into the *Eco*RI and *Xho* I sites of pLXSN (15). An amphotrophic packaging cell line, PA317, was used to produce the LHP2USN retroviral vector and a control vector containing only the neomycin-resistance

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Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}; HP2U, human P_{2U} receptor.

[§]The sequence reported in this paper has been deposited in the GenBank database (accession no. V07225).

(*neo*^{r}) gene (LN) (15). Human astrocytoma cells (1321N1) generously provided by T. K. Harden (University of North Carolina, Chapel Hill) were infected with LHP2USN or LN (2 hr with Polybrene at 4 μg/ml) and, after 48 hr, selected with G418 (600 μg/ml; Life Technologies, Grand Island, NY). To assay for receptor activity, the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) was measured in confluent cells on coverslips by fura-2 fluorescence with microspectrofluorimetry (16). Measurement of cell inositol phosphate formation was as described (12). The cloned HT-29 cDNA was transiently expressed and characterized in K562 cells as described (11).

Northern and Southern Blots. Total cell RNA and poly(A)$^+$ RNA were isolated by standard procedures or purchased commercially (Clontech). Human and mouse genomic DNAs were extracted from cultured cells (14) and digested with restriction enzyme (5 units/μg of DNA). Northern and Southern blots were prepared (14) and UV-crosslinked (Stratagene). Prehybridization and hybridization with cDNA probes were performed using QuikHyb (Stratagene) according to the manufacturer's instructions, except that 0.2× standard saline citrate (SSC)/0.1% SDS was used for the high-stringency wash. Autoradiographs were prepared with Kodak XAR film and one intensifying screen at −80°C.

RESULTS AND DISCUSSION

Full-length clones obtained by screening human CF/T43 and HT-29 cDNA libraries were found by sequence analysis to contain identical cDNA inserts. The amino acid sequence deduced from an 1128-bp open reading frame (Fig. 1) bears substantial similarity (89% identity) to the mouse P_{2U} receptor sequence reported by Lustig *et al.* (10), with the majority of the sequence differences concentrated in the carboxyl-terminal region. The HP2U sequence is considerably less similar to the chicken P_{2Y} purinoceptor (37% identity) described by Webb *et al.* (17). The HP2U sequence exhibits structural features typical of the family of G protein-coupled receptors and common to both the chicken and murine P_2 receptor clones: (*i*) seven hydrophobic domains, (*ii*) consensus N-linked glycosylation sequences near the amino terminus, (*iii*) a number of residues highly conserved among G protein-coupled receptors (e.g., Asn^{51}, Asp^{79}, Cys^{106}, and Cys^{183}), and (*iv*) potential phosphorylation sites in the third intracellular and carboxyl-terminal domains. Like the mouse and chicken P_2 receptors, the HP2U sequence is more closely related to the cloned G protein-coupled receptors for peptide hormones than to those for adenosine and cAMP.

A retroviral system was used to obtain stable expression of the CF/T43 HP2U clone in 1321N1 cells, a human astrocytoma cell line that is unresponsive to classical P_2 receptor agonists. The effect of extracellular nucleotides on $[Ca^{2+}]_i$ was assessed in CF/T43 cells, uninfected 1321N1 cells, and 1321N1 cells infected with retroviral vector containing either the airway HP2U cDNA (LHP2USN) or no insert (LN). Exposure of CF/T43 cells to extracellular UTP (0.1 mM) in Ca^{2+}-containing medium resulted in an initial rapid increase in $[Ca^{2+}]_i$ which relaxed to a plateau (Fig. 2*A*). Application of UTP to CF/T43 cells in Ca^{2+}-free medium induced a rapid increase in $[Ca^{2+}]_i$ which returned to baseline without a plateau. Extracellular UTP had no effect on $[Ca^{2+}]_i$ in uninfected 1321N1 cells (data not shown) or LN-infected cells, whereas carbachol elicited large responses. The $[Ca^{2+}]_i$ response to UTP in 1321N1 cells expressing the LHP2USN vector was similar to that of CF/T43 cells in both Ca^{2+}-free and Ca^{2+}-containing media. In each experiment, the addition of ATP produced the same result observed with UTP (data not shown).

To examine the pharmacological specificity of the $[Ca^{2+}]_i$ response in 1321N1 cells expressing the HP2U clone, concentration–effect curves were generated for UTP, ATP, and

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      CGGCACGAGGCACCCCGAGAGGAGAAGCGCAGCGCAGTGGCGAGAGGAGCCCCTTGTGGC    60
      AGCAGCACTACCTGCCCAGAAAAATGCTGGAGGCTGGGCGTGGCCCCAGGCCTGGGGACC   120
      TGTTTTTCCTGTTTCCCGCAGAGTTCCCTGCAGCCCGGTCCAGGTCCAGGCGTGTGCATT   180
      CATGAGTGAGGAACCCGTGCAGGCGCTGAGCATCCTGACCTGGAGAGCAGGGGCTGGTCA   240

      GGGCGATGGCAGCAGACCTGGGCCCCTGGAATGACACCATCAATGGCACCTGGGATGGGG   300
  1       M  A  A  D  L  G  P  W  N  D  T  I  N  G  T  W  D  G

      ATGAGCTGGGCTACAGGTGCCGCTTCAACGAGGACTTCAAGTACGTGCTGCTGCCTGTGT   360
 19   D  E  L  G  Y  R  C  R  F  N  E  D  F  K  Y  V  L  L  P  V

      CCTACGGCGTGGTGTGCGTGCTTGGGCTGTGTCTGAACGCCGTGGGCCTCTACATCTTCT   420
 39   S  Y  G  V  V  C  V  L  G  L  C  L  N  A  V  G  L  Y  I  F

      TGTGCCGCCTCAAGACCTGGAATGCGTCCACCACATATATGTTCCACCTGGCTGTGTCTG   480
 59   L  C  R  L  K  T  W  N  A  S  T  T  Y  M  F  H  L  A  V  S

      ATGCACTGTATGCGGCCTCCCTGCCGCTGCTGGTCTATTACTACGCCCGCGGCGACCACT   540
 79   D  A  L  Y  A  A  S  L  P  L  L  V  Y  Y  Y  A  R  G  D  H

      GGCCCTTCAGCACGGTGCTCTGCAAGCTGGTGCGCTTCCTCTTCTACACCAACCTTTACT   600
 99   W  P  F  S  T  V  L  C  K  L  V  R  F  L  F  Y  T  N  L  Y

      GCAGCATCCTCTTCCTCACCTGCATCAGCGTGCACCGGTGTCTGGGCGTCTTACGACCTC   660
119   C  S  I  L  F  L  T  C  I  S  V  H  R  C  L  G  V  L  R  P

      TGCGCTCCCTGCGCTGGGGCCGGGCCCGCTACGCTCGCCGGGTGGCCGGGGCCGTGTGGG   720
139   L  R  S  L  R  W  G  R  A  R  Y  A  R  R  V  A  G  A  V  W

      TGTTGGTGCTGGCCTGCCAGGCCCCCGTGCTCTACTTTGTCACCACCAGCGCGCGCGGGC   780
159   V  L  V  L  A  C  Q  A  P  V  L  Y  F  V  T  T  S  A  R  G

      CGCTAACCTGCCACGACACCTCGGCACCCGAGCTCTTCAGCCGCTTCGTGGCCTACAGCT   840
179   P  L  T  C  H  D  T  S  A  P  E  L  F  S  R  F  V  A  Y  S

      CAGTCATGCTGGGCCTGCTCTTCGCGGTGCCCTTTGCCGTCATCCTTGTCTGTTACGTGC   900
199   S  V  M  L  G  L  L  F  A  V  P  F  A  V  I  L  V  C  Y  V

      TCATGGCTCGGCGACTGCTAAAGCCAGCCTACGGGACCTCGGGCGGCCTGCCTAGGGCCA   960
219   L  M  A  R  R  L  L  K  P  A  Y  G  T  S  G  G  L  P  R  A

      AGCGCAAGTCCGTGCGCACCATCGCCGTGGTGCTGGCTGTCTTCGCCCTCTGCTTCCTGC  1020
239   K  R  K  S  V  R  T  I  A  V  V  L  A  V  F  A  L  C  F  L

      CATTCCACGTCACCCGCACCCTCTACTACTCCTTCCGCTCGCTGGACCTCAGCTGCCACA  1080
259   P  F  H  V  T  R  T  L  Y  Y  S  F  R  S  L  D  L  S  C  H

      CCCTCAACGCCATCAACATGGCCTACAAGGTTACCCGGCTGGCCAGTGCTAACAGTTGCC  1140
279   T  L  N  A  I  N  M  A  Y  K  V  T  R  L  A  S  A  N  S  C

      TTGACCCCGTGCTCTACTTCCTGGCTGGGCAGAGGCTCGTACGCTTTGCCCGAGATGCCA  1200
299   L  D  P  V  L  Y  F  L  A  G  Q  R  L  V  R  F  A  R  D  A

      AGCCACCCACTGGCCCCAGCCCTGCCACCCCGGCTCGCCGCACGCTGGGCCTGCGCAGAT  1260
319   K  P  T  G  P  S  P  A  T  P  A  R  R  T  L  G  L  R  R

      CCGACAGAACTGACATGCAGAGGATAGGAGATGTGTTGGGCAGCAGTGAGGACTCTAGGC  1320
339   S  D  R  T  D  M  Q  R  I  G  D  V  L  G  S  S  E  D  S  R

      GGACAGAGTCCACGCCGGCTGGTAGCGAGAACACTAAGGACATTCGGCTGTAGGAGCAGA  1380
359   R  T  E  S  T  P  A  G  S  E  N  T  K  D  I  R  L  *

      ACACTTCAGCCTGTGCAGGTTTATATTGGGAAGCTGTAGAGGACCAGGACTTGTGCAGAC  1440
      GCCACAGTCTCCCCAGATATGGACCATCAGTGACTCATGCTGGATGACCCCATGCTCCGT  1500
      CATTTGACAGGGGCTCAGGATATTCACTCTGTGGTCCAGAGTCAACTGTTCCCATAACCC  1560
      CTAGTCATCGTTTGTGTGTATAAGTTGGGGGAATTAAGTTTCAAGAAAGGCAAGAGCTCA  1620
      AGGTCAATGACACCCCTGGCCTGACTCCCATGCAAGTAGCTGGCTGTACTGCCAAGGTAC  1680
      CTAGGTTGGAGTCCAGCCTAATCAAGTCAAATGGAGAAACAGGCCCAGAGAGGAAGGTGG  1740
      CTTACCAAGATCACATACCAGAGTCTGGAGCTGAGCTACCTGGGGTGGGGCCAAGTCAC  1800
      AGGTTGGCCAGAAAACCCTGGTAAGTAATGAGGGCTGAGTTTGCACAGTGGTCTGGAATG  1860
      GACTGGGTGCCACGGTGGACTTAGCTCTGAGGAGTACCCCCAGCCCAAAGATGAACATC  1920
      TGGGGACTAATATCATAGACCCATCTGGAGGCTCCCATGGGCTAGGAGCCAGTGTGAGGC  1980
      TGTAACTTATACTAAAGGTTGTGTTGCCTGCTAAAAAAAAAAAAAAAAAA            2030
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FIG. 1. Human P_{2U} receptor (HP2U) nucleotide and deduced amino acid sequence. The putative transmembrane domains are underlined.

structural analogues of ATP selective for P_{2X} (adenosine 5′-[α,β-methylene]triphosphate) and P_{2Y} (2-methylthio-ATP) receptors (Fig. 2*B*). UTP and ATP were nearly equipotent, with UTP exhibiting slightly more efficacy, while 2-methylthio-ATP and adenosine 5′-[α,β-methylene]triphosphate had little effect. Similar concentration–effect curves were generated for UTP and ATP in K562 cells, a cell line that also lacks endogenous P_{2U} receptors (11), transiently transfected with HP2U (data not shown). Clearly, the agonist specificities most closely fit the pharmacological classification of the P_{2U} receptor (18).

Pretreatment of CF/T43 and HP2U-1321N1 cells with pertussis toxin inhibited ATP-stimulated intracellular Ca^{2+} mobilization in both cell types by 20–30% (Fig. 2*C*). These results are consistent with the partial inhibition by pertussis toxin on inositol phosphate accumulation induced by ATP or UTP in CF/T43 cells (12) and on P_{2U} receptor responses in other systems (11, 19). The pertussis toxin sensitivity of HP2U-1321N1 cells and the observation that UTP or ATP increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} are consistent with the predicted coupling of HP2U to phospholipase C via a G protein. To test this possibility more directly, we measured inositol phosphate formation.

P_{2U} receptor-activated Ca^{2+} responses are linked to activation of inositolphospholipid hydrolysis in various cells (4,

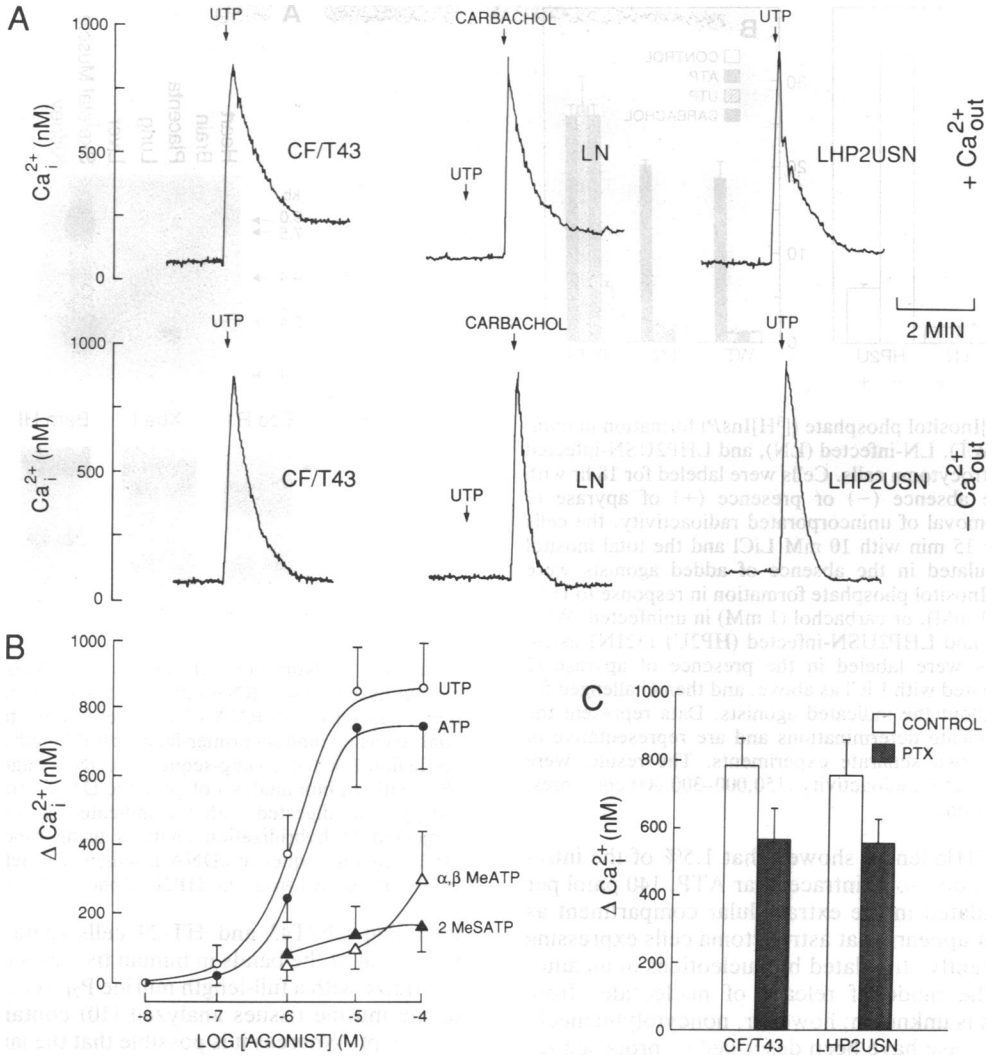

FIG. 2. (*A*) $[Ca^{2+}]_i$ response to UTP (0.1 mM) and/or carbachol (0.1 mM) in CF/T43 cells and in LN-infected and LHP2USN-infected 1321N1 astrocytoma (LN- and LHP2USN-1321N1, respectively) cells bathed in Ca^{2+}-containing (1.3 mM Ca^{2+}; upper row) or Ca^{2+}-free (1.0 mM EGTA; lower row) NaCl/Ringer solutions. In the presence of extracellular Ca^{2+}, basal $[Ca^{2+}]_i$ averaged 72 ± 8 nM (mean ± SEM, n = 32), 67 ± 11 nM (n = 30), and 68 ± 5 nM (n = 123) in CF/T43, LN-1321N1, and LHP2USN-1321N1 cells, respectively. In Ca^{2+}-free solution, the mean basal $[Ca^{2+}]_i$ values were 65 ± 7 nM (n = 18), 64 ± 7 nM (n = 23), and 63 ± 6 nM (n = 22) in CF/T43, LN-1321N1, and LHP2USN-1321N1 cells, respectively. There was no significant difference in basal $[Ca^{2+}]_i$ ($P > 0.05$; Student's t test) between the different cell preparations or between cells bathed with Ca^{2+}-containing medium and cells bathed with Ca^{2+}-free medium. In the presence of extracellular Ca^{2+} the mean change in $[Ca^{2+}]_i$ (peak − basal value) in response to UTP was 823 ± 145 nM (n = 7) and 881 ± 119 nM (n = 10) in CF/T43 and LHP2USN-1321N1 cells, respectively. In Ca^{2+}-free Ringer solution, the mean change in $[Ca^{2+}]_i$ was 807 ± 123 nM (n = 7) and 867 ± 112 nM (n = 8) in CF/T43 and LHP2USN-1321N1 cells, respectively. There was no significant difference in the mean change in $[Ca^{2+}]_i$ in response to UTP between CF/T43 cells and LHP2USN-1321N1 cells ($P > 0.05$) in either the presence or absence of extracellular Ca^{2+}. In the presence and absence of extracellular Ca^{2+}, carbachol induced mean changes in $[Ca^{2+}]_i$ of 863 ± 132 nM (n = 10) and 834 ± 141 nM (n = 8), respectively, in LN-1321N1 cells. These values do not differ significantly ($P > 0.05$) from the UTP-elicited $[Ca^{2+}]_i$ changes in CF/T43 and LHP2USN-1321N1 cells. (*B*) Concentration–effect relationships of different purine and pyrimidine compounds on changes in $[Ca^{2+}]_i$ (ΔCa_i^{2+}; peak − basal values) in LHP2USN-infected 1321N1 cells bathed with Ca^{2+}-containing NaCl/Ringer solution. Each point is the mean ± SEM for five or more separate experiments. α,β MeATP, adenosine 5′-[α,β-methylene]triphosphate; 2MeSATP, 2-methylthio-ATP. (*C*) Effects of pertussis toxin (PTX; 10 ng/ml for 24 hr) on ATP (0.1 mM)-induced changes in $[Ca^{2+}]_i$ (ΔCa_i^{2+}) in CF/T43 and LHP2USN-1321N1 cells bathed with Ca^{2+}-containing NaCl/Ringer solution. For control and PTX-treated CF/T43 cells, the average change in $[Ca^{2+}]_i$ was 788 ± 84 nM (n = 14) and 568 ± 88 nM (n = 14), respectively. For control and PTX-treated LHP2USN-infected 1321N1 cells, the average change in $[Ca^{2+}]_i$ was 765 ± 116 nM (n = 12) and 558 ± 71 nM (n = 8), respectively. For both PTX-treated CF/T43 and LHP2USN-1321N1 cells, the average change in $[Ca^{2+}]_i$ was significantly lower ($P < 0.05$) than in control cells.

20, 21), and inositol phosphate formation was detected in HP2U-1321N1 cells incubated with UTP and ATP (Fig. 3). In our initial studies, very high levels of inositol phosphates were found to accumulate in HP2U-1321N1 cells that had not been exposed to exogenously added nucleotides (Fig. 3*A*). One possible explanation for the higher basal levels—i.e., intrinsic, agonist-independent (constitutive) receptor activation—is not supported by the large, reproducible $[Ca^{2+}]_i$ modulations observed in response to nucleotide addition (Fig. 2). Therefore, we hypothesized that during the lengthy labeling period the 1321N1 cells released 5′-nucleotides into the medium in quantities sufficient to "self-activate" the expressed receptor. To test this notion, the phosphatase apyrase was added to the medium during cell labeling to metabolize extracellular ATP. The inclusion of apyrase resulted in a reduction of baseline inositol phosphate levels to values near those of controls (Fig. 3*A*). Further, preliminary HPLC analysis (22) of medium bathing HP2U-1321N1 cells

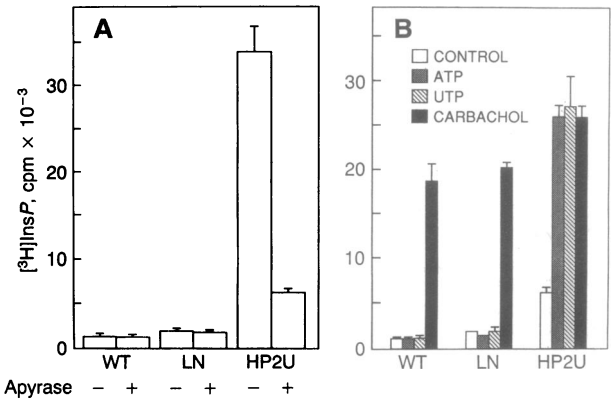

FIG. 3. (*A*) [^{3}H]Inositol phosphate ([^{3}H]Ins*P*) formation in uninfected (wild type, WT), LN-infected (LN), and LHP2USN-infected (HP2U) 1321N1 astrocytoma cells. Cells were labeled for 18 hr with [^{3}H]inositol in the absence (−) or presence (+) of apyrase (2 units/ml). After removal of unincorporated radioactivity, the cells were incubated for 15 min with 10 mM LiCl and the total inositol phosphates accumulated in the absence of added agonists were measured. (*B*) [^{3}H]Inositol phosphate formation in response to UTP (0.1 mM), ATP (0.1 mM), or carbachol (1 mM) in uninfected (WT), LN-infected (LN), and LHP2USN-infected (HP2U) 1321N1 astrocytoma cells. Cells were labeled in the presence of apyrase (2 units/ml), preincubated with LiCl as above, and then challenged for 15 min with or without the indicated agonists. Data represent the mean ± SD of triplicate determinations and are representative of results obtained in two separate experiments. The results were normalized with the total radioactivity (150,000–300,000 cpm) present in the lipid fraction.

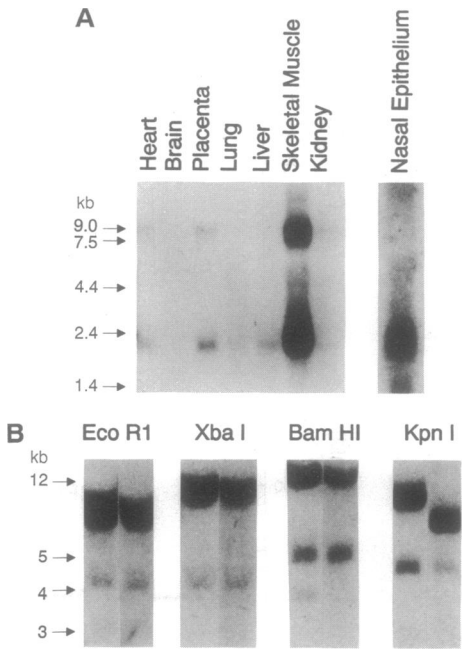

FIG. 4. (*A*) Northern blot analysis of RNAs isolated from various human tissues. Total RNA (20 μg; primary cultures of nasal epithelium) and poly(A)$^+$ RNA (2 μg) were subjected to hybridization analysis using random primer-labeled full-length HP2U cDNA (nasal epithelium) or the coding-sequence cDNA fragment D9 as probes. (*B*) Southern blot analysis of genomic DNA. Human genomic DNA (10 μg) was digested with the indicated restriction enzymes and subjected to hybridization analysis using random primer-labeled HP2U coding-sequence cDNA fragment D9 (left lanes) or a *Kpn* I fragment excised from the HP2U clone (right lanes) as probes.

prelabeled with [^{3}H]adenine showed that 1.5% of the intracellular [^{3}H]ATP pool (total intracellular ATP, 140 pmol per 10^6 cells) accumulated in the extracellular compartment as [^{3}H]ATP. Thus, it appears that astrocytoma cells expressing HP2U are persistently stimulated by nucleotides in an autocrine fashion. The mode of release of nucleotides from astrocytoma cells is unknown; however, noncytolytic mechanisms of ATP release have been described or proposed for a variety of neuronal, secretory, and other cell types (reviewed in ref. 4). Since many of the cell types that release ATP also endogenously express nucleotide receptors, expression of the P_{2U} receptor in 1321N1 cells may have rendered this physiologically important regulatory mechanism accessible to further study.

The addition of apyrase to the medium facilitated comparison of agonist-induced inositol phosphate accumulation in control and LHP2USN-infected cells. Accordingly, experiments for the characterization of HP2U were performed on cells pretreated with apyrase during the labeling procedure. Consistent with activation by HP2U of phospholipase C, ATP and UTP increased inositol phosphate accumulation in 1321N1 cells expressing LHP2USN but not in uninfected or LN-infected controls (Fig. 3*B*).

P_{2U} receptors have been functionally described in various human organs and cell types (4). As shown in Fig. 4*A*, P_{2U} receptor mRNA is widely distributed in human tissue, including the heart, liver, lung, and kidney, as reported for the mouse (10), and placenta and skeletal muscle. Consistent with studies showing P_{2U} receptor-mediated modulation of epithelial ion transport, mRNA was detected in kidney proximal-tubule cells and the salivary gland duct cell line HSG-PA (data not shown) and in primary cultures of nasal epithelium, a tissue representative of the therapeutic site in airways for P_{2U} receptor regulation of ion transport (6, 7).

Some human tissues, including nasal and proximal-tubule epithelia and liver, express only a 2.1-kb mRNA; however, as many as three mRNAs were observed (additional bands at 7.5 kb and/or 9 kb) in other human tissues and cell lines, including CF/T43 and HT-29 cells (data not shown). Although all of the bands in human tissues were found to cross hybridize with a full-length murine P_{2U} receptor cDNA, none of the murine tissues analyzed (10) contain more than one transcript. Whereas it is possible that the larger transcripts in human tissues and cells represent unprocessed forms of the 2.1-kb mRNA, the relative abundance of the hybridizing bands suggests that the larger RNAs are as stable as the smaller RNA and, therefore, may not represent an intermediate step in processing. Further, significant amounts of the larger RNAs were retained on an oligo(dT)-cellulose column, indicating that these mRNAs are polyadenylylated. If all of the bands represent fully processed mRNAs, they may represent alternatively processed forms of the same gene or products of different genes. Southern blot analysis of human genomic DNA was performed to explore the possibility that more than one gene for P_{2U} receptors exist in the human genome.

Human genomic DNA was digested to completion with the restriction enzymes *Eco*RI, *Xba* I, *Bam*HI, and *Kpn* I. Among these, only *Kpn* I will cut in the HP2U cDNA. Under stringent conditions, the full-length HP2U cDNA hybridized to two or more fragments in all genomic DNA digests (data not shown). Since the multiple bands in the *Eco*RI, *Xba* I, and *Bam*HI digests may result either from intronic sequence in a single gene or from hybridization to distinct genes, all digests were reprobed with two nonoverlapping probes: (*i*) probe D9, an ≈500-bp portion of the P_{2U} receptor coding sequence (Fig. 4*B*, left lanes) or (*ii*) probe UT3P, a *Kpn* I fragment of HP2U, consisting of the 3′-most 350 bases of untranslated cDNA (Fig. 4*B*, right lanes). These probes recognized the same bands in DNA cut by *Eco*RI and *Xba* I (Fig. 4*B*), indicating that the multiple bands hybridizing to full-length HP2U cDNA in these digests do not result from a restriction site located in an intron of a single gene. In the *Bam*HI digest,

both probes hybridized strongly with the same large band, but one of the two smaller, less intensely hybridizing bands recognized by D9 was not recognized by UT3P. Consistent with the presence of a *Kpn* I site separating the sequences recognized by the two probes, the *Kpn* I digest contained two different, strongly hybridizing bands, one recognized by D9 and the other recognized by UT3P. However, a smaller, less intensely hybridizing band in the *Kpn* I digest was recognized by both D9 and UT3P, suggesting that a *Kpn* I site is not situated between the regions in this restriction fragment that hybridize with the probes. These findings suggest that the bands recognized by HP2U probes in restriction digests of human genomic DNA arise from two structurally similar genes.

In summary, HP2U represents a P_2-type nucleotide receptor isolated from human sources. The results of Northern analysis and the fact that identical cDNAs encoding the receptor were cloned independently from both airway and colonic epithelial cells attest to its wide distribution in human tissues. In some human tissues, the cloned cDNA hybridizes with multiple mRNA transcripts. The mechanism by which these transcripts are generated, including a possible correlation with the multiple hybridizing restriction fragments in human genomic DNA, remains to be definitively established. Heterologous expression of the receptor in one of the few cell lines that lack a response to extracellular nucleotides demonstrates that the clone encodes a protein possessing the functional properties of a UTP/ATP-selective, Ca^{2+}-mobilizing receptor coupled to G proteins and phospholipase effector enzymes—i.e., a P_{2U} receptor. In addition, expression of HP2U in 1321N1 astrocytoma cells unexpectedly revealed autocrine feedback on the expressed receptor by nucleotides released from the cells. Further characterization of this phenomenon may provide important clues concerning mechanisms of cellular homeostasis. Most importantly, the availability of both the cloned human airway receptor and a specific, robust system for its expression will greatly facilitate identification of drugs that will for safety reasons most likely be based on pyrimidines (UTP) rather than purines (23, 24) for the treatment of CF and possibly other major human disorders.

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