P_{2u} purinoceptor regulation of mucin secretion in SPOC1 cells, a goblet cell line from the airways

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The SPOC1 cell, a novel goblet cell line derived from rat trachea, was tested for its ability to exhibit regulated mucin secretion in response to purinergic (P_2) agonists. High-molecular-mass glycoconjugates (HMMGs) purified by CsCl-density-gradient centrifugation had a buoyant density of 1.45 g/ml. The purified HMMG material exhibited a single major band with an apparent molecular mass of greater than 1000 kDa in SDS/polyacrylamide gels stained with silver or blotted and stained with soya-bean agglutinin. [^{3}H]HMMG was resistant to proteoglycan-degrading enzymes, but was susceptible to neuraminidase. The HMMG was approx. 91% carbohydrate by weight, and the glycosides were O-linked. The HMMG amino acid composition was enriched in Ser and Thr (sum 27%). Thus SPOC1-cell HMMG possess the characteristics of mucin. Mucin secretion by SPOC1 cells, grown on permeable supports and perfused luminally, was stimulated by ATP, UTP and adenosine 5′-[γ-thio]triphosphate (100 μM) 4–5-fold over a baseline of 4 ng/min. The three dose–effect relations were nearly identical ($K_{0.5} \sim 4$ μM). SPOC1 cells grown on plastic and rat tracheal epithelial primary cells responded similarly to ATP and/or UTP. SPOC1 cells failed to respond to other purinergic agonists, either luminally or serosally, and consequently seem to possess an apical membrane P_{2u} purinoceptor. SPOC1-cell total RNA was probed for P_{2u} purinoceptor mRNA. Using conserved primers for both reverse transcriptase and PCR, a single band of the predicted size was observed, which had a nucleotide base sequence identical with the rat P_{2u} purinoceptor mRNA. Thus SPOC1 cells secrete mucin under the control of a P_{2u} purinoceptor; they should prove useful in dissecting the associated cellular regulatory pathways.

INTRODUCTION

Mucus formation in the airways results from the secretion of high-molecular-mass mucin glycoproteins by specialized goblet cells in the superficial epithelium and mucous cells in the submucosal glands. Although mucin hypersecretion and goblet/mucous cell metaplasia represent a major clinical manifestation of chronic obstructive airways disease (cystic fibrosis, bronchiectasis, chronic bronchitis, emphysema, and asthma) [1], very little mechanistic information is available at the cellular and molecular levels regarding the regulation of these processes. A major effort over the past several years has been directed at the development and study of primary cell cultures and epithelial explants of the airway superficial epithelium [2,3]. From studies with such experimental models, it is now known that mucin secretion is regulated by purinergic agonists [2,4,5] and inflammatory mediators such as prostaglandin $F_{2\alpha}$ [6] and platelet-activating factor [7,8]. Attempts at further delineating the regulatory mechanisms involved have been generally hampered by the heterocellular nature of most airway cell culture systems.

The recent development of the SPOC1 cell line [9,10] may be a significant advancement in the availability of culture models suitable for experimental efforts directed at studying the regulatory pathways governing mucin secretion. The SPOC1 cell was derived from spontaneously immortalized rat tracheal epithelial cell secondary cultures [9]; the cells assume a goblet cell phenotype when grown in tracheal xenografts [10]. The cell line has been shown to be diploid with minor and stable alterations of chromosomes 1, 3 and 6, and it has reduced requirements for peptide growth factors [9]. The secretory granules of SPOC1 cells grown in tracheal xenografts possess dense cores and are positive for a rat tracheal mucin-specific monoclonal antibody (RTE11 mAb) [10]. When grown in culture, SPOC1 cells develop a transepithelial electrical potential difference and resistance indicative of a polarized epithelium [9], and the cultures immunostain with RTE11 mAb and spontaneously secrete a glycoconjugate which reacts with the mAb [10]. Thus SPOC1 cells possess a number of important characteristics of goblet cells.

In this study we first sought to determine the suitability of the SPOC1 cell line for exploring the agonist regulation of goblet cell mucin secretion. Whether the high-molecular-mass glycoconjugate (HMMG) secreted by SPOC1 cells is a mucin was tested by purifying and characterizing the secretory product. That a P_2 purinoceptor regulates mucin secretion in the SPOC1 cell was tested by challenging perfused cultures with hallmark nucleotide agonists. Recent data for human airway epithelial explants suggested that the specific identity of this goblet cell purinoceptor is a P_{2u} subtype [5]. Thus we also sought to use the pure population of cells offered by the SPOC1 cell line to test this hypothesis by probing total RNA for the P_{2u} purinoceptor mRNA [11,12].

EXPERIMENTAL

Materials

Culture medium was purchased from Gibco/BRL (Gaithersburg, MD, U.S.A.) and the supplements from Collaborative Research (Bedford, MA, U.S.A.). The other chemicals used were purchased

Abbreviations used: HMMG, high-molecular-mass glycoconjugate; SBA, soya-bean agglutinin; mAb, monoclonal antibody; TCol, Transwell-COL 24 mm support; DMEM, Dulbecco's modified Eagle's medium; AB/PAS, Alcian Blue/periodic acid/Schiff reagent; ATP[S], adenosine 5′-[γ-thio]triphosphate; BCA, bicinchoninic acid. ELLA, enzyme-linked lectin assay; RT, reverse transcriptase; RTE, rat tracheal epithelial.

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from Sigma Chemical Co. (St. Louis, MO, U.S.A.), unless otherwise specified.

Cell culture

SPOC1 cells, passages 8–15, were seeded on Costar (Cambridge, MA, U.S.A.) Transwell-COL 24 mm supports (TCol) at a density of 120000 cells/support, in 12-well cluster plates at 40000 cells/well, or in 75 and 162 cm^2 tissue culture flasks (Costar T75 and T162) at 0.5×10^6 or 1.0×10^6 cells/flask respectively. The cells were grown in an F12/Dulbecco's modified Eagle's medium (DMEM)-based culture medium developed for primary cell cultures of rat tracheal epithelial cells that has also been shown to support the growth and differentiation of SPOC1 cells [10,13]. Briefly, the medium was supplemented with 30 mM Hepes, 6.5 mM L-glutamine, 10 μg/ml insulin, 0.1 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 5 μg/ml transferrin, 50 μM phosphoethanolamine, 80 μM ethanolamine, 25 ng/ml epidermal growth factor, 1 % (v/v) bovine pituitary extract, 3 mg/ml BSA (essentially globulin-free; Sigma no. A7638), 50 units/ml penicillin, and 50 μg/ml streptomycin. In addition, with the exception of SPOC1 cells grown solely for passaging, the medium was supplemented with 10 nM retinoic acid. The cultures were used for experiments on days 6–12 after confluence, when cellular Alcian Blue/periodic acid/Schiff reagent (AB/PAS)-positive material was plentiful [10]. Primary cultures of rat tracheal epithelial cells were prepared and grown on TCols as previously described [13].

Histology

SPOC1 cell cultures grown on TCols were fixed in 2 % glutaraldehyde + 2 % paraformaldehyde, embedded in methacrylate, and sectioned to a thickness of 5 μm. The sections were stained with Richardson's stain [14] or by the AB/PAS procedure.

Purification of SPOC1-cell HMMG

HMMG was purified by dual CsCl-gradient centrifugation [15] from material obtained from solubilized cells. SPOC1 cell cultures grown for 6 days after confluence in T162 flasks were removed by a 30 min exposure to PBS containing 10 mM EDTA (pH 7.4). The cells were centrifuged (1500 ***g***; 10 min; 4 °C), and the pellet was weighed and solubilized in 6 M guanidinium chloride (Gibco/BRL) containing 5 mM EDTA and 0.1 mM PMSF. After an overnight incubation with constant stirring at 4 °C, the material was centrifuged at 1500 ***g*** for 30 min at 4 °C to remove cellular debris. The solubilized material was prepared for density-gradient centrifugation by adding CsCl (Fisher Scientific, Pittsburgh, PA, U.S.A.), guanidinium chloride, and PBS to an initial density of 1.40 g/ml and 4 M guanidinium chloride. The solution was centrifuged for 3 days in a Beckman L7-55 ultracentrifuge equipped with a 50.2 Ti rotor at 150000 ***g*** (36000 rev./min) and 15 °C. Each tube was divided into 16 2.5 ml fractions which were weighed for density, and assessed for DNA (A_{260}) and sialic acid [16]. Sialic acid-positive fractions were pooled and dialysed against 0.2 M guanidinium chloride in PBS overnight at 4 °C. After adjusting the density of the solution to 1.50 g/ml with CsCl, the material was centrifuged a second time, as above, to separate mucins from residual DNA. The sialic acid-containing fractions were pooled and dialysed against deionized water. The HMMG content of this final solution was determined by weighing the material obtained after drying a known volume (85 °C; 36 h).

PAGE and lectin blotting

Samples of HMMG prepared in Laemmli buffer were subjected to SDS/PAGE, under reducing conditions. HMMg (2–5 μg) was loaded into the wells of 4–12 % gradient polyacrylamide gels (Novex, San Diego, CA, U.S.A.) and electrophoresed at 100 V for 1 h. The resulting gels were stained with silver [17] (Silver Stain Plut Kit; Bio-Rad) or PAS [18], or the samples therein were transferred to a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore, Bedford, MA, U.S.A.) at 25 °C for 2 h under a constant current of 200 mA in transfer buffer (192 mM glycine, 25 mM Tris, 20 % methanol, pH 8.3). Blotted membranes were washed in PBS + 0.05 % Tween-20, blocked by incubation in 0.1 % gelatin in PBS overnight, and then incubated in 0.1 μg/ml horseradish peroxidase-conjugated soya-bean agglutinin (SBA) for 1 h at room temperature. After being washed, the blots were developed with an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, U.S.A.).

[^{3}H]HMMG labelling, enzymic digestion and Sepharose CL-4B column chromatography

SPOC1 cell cultures grown on plastic were exposed to [^{3}H]glucosamine (1–5 μCi/ml; specific radioactivity 40 Ci/mmol) for 24 h. After removal of the label and a thorough wash with F12/DMEM, the cells were either solubilized in guanidinium chloride and the HMMG was purified by density-gradient centrifugation, or exposed to adenosine 5′-[γ-thio]guanosine (ATP[S]) (100 μM) for 1 h (see Figure 7) and the medium collected, centrifuged at 1500 ***g***, and the supernatant stored at 4 °C. These materials were subjected to enzymic digestion in a 0.1 M sodium acetate or Tris/acetate buffer with its pH and [Ca^{2+}] optimally adjusted for the respective enzyme, as follows: hyaluronidase, 10 units/ml, pH 6.1, for 20 h at 37 °C; heparinase III, 5 units/ml, pH 7.0, 1 mM Ca^{2+}, for 5 h at 42 °C; chondroitinase ABC, 0.2 unit/ml, pH 7.4, for 5 h at 37 °C; and neuraminidase, 0.6 unit/ml, pH 7.4 for 4 h at 40 °C. Each enzyme was obtained fresh from the manufacturer and used immediately. Each digestion was stopped by boiling, the sample was cooled to room temperature and diluted with an equal volume of 2-fold concentrated PBS. A 1 ml portion of each sample was chromatographed at 0.33 ml/min on a Sepharose CL-4B (Pharmacia/LKB, Uppsala, Sweden) column (1 cm × 50 cm). In all, 45 1 ml fractions were collected and aliquots thereof were mixed with 5 ml of Scintiverse (Fisher) and assessed for radioactivity in a Wallac 1410 (Gaithersburg, MD, U.S.A.) liquid-scintillation counter.

Thiol reduction and alkylation, β-elimination and proteolysis

Culture medium containing [^{3}H]HMMG was recovered from SPOC1 cells that had been grown on plastic, labelled overnight with [^{3}H]glucosamine, washed and exposed to ATP[S] as above. The susceptibility of the [^{3}H]HMMG to disulphide bond reduction was tested by incubating the material in 2.5 % (v/v) 2-mercaptoethanol/1 % SDS for 5 min at 100 °C, pH 8.6. The material was then alkylated by an overnight incubation with 0.4 M iodoacetamide in the dark at 4 °C, following which it was exhaustively dialysed against distilled water and analysed by Sepharose CL-4B chromatography. The presence of *O*-glycosides in the [^{3}H]HMMG was tested by (non-reductive) β-elimination: the material was incubated in 50 mM NaOH at 45 °C for 16 h, and then neutralized with acetic acid and chromatographed on Sepharose CL-4B. The susceptibility of the [^{3}H]HMMG to proteolysis was tested by digesting [^{3}H]HMMG with trypsin

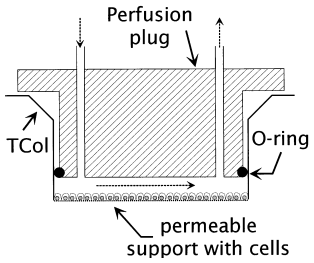

Figure 1 Perfusion of SPOC1 cells grown on permeable supports

For SPOC1 cell cultures grown on TCol inserts, the 'perfusion plug' depicted, fabricated from Delrin, was pressed into the cup-like portion of the insert, such that the O-ring seal created a closed compartment on the luminal side of the culture. Dotted arrows indicate the perfusion path. There was a nominal 2 mm clearance between the bottom of the plug and the permeable support.

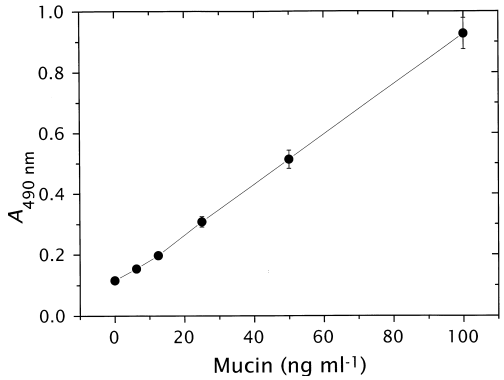

Figure 2 Standard curve for an ELLA using SBA and purified SPOC1 mucin

The ELLA utilized horseradish peroxidase-conjugated SBA and purified SPOC1 mucin (ng dry wt/ml) and were performed on 96-well microtitre plates. The data are expressed as means $\pm$ S.E.M. ($n = 3$).

(100 μg/ml) for 5 h at 37 °C after dialysis against a 0.1 M Tris/HCl buffer, pH 8.0. Both native and reduced/alkylated [^{3}H]HMMG preparations were so digested, after which the materials were boiled to inactivate the protease and analysed by Sepharose CL-4B chromatography.

Protein determinations

The protein content of purified SPOC1 HMMG was determined after deglycosylation of a 100 μg sample by β-elimination (above), followed by a 24 h dialysis (10 kDa cut-off) against distilled water. The protein content of the material was then determined in parallel samples using the bicinchoninic acid procedure (BCA Protein Assay kit, Pierce Chemical Co., Rockford, IL, U.S.A.) and the procedure of Lowry et al. [19], in each case using BSA as the standard.

Amino acid analysis

Samples were hydrolysed, after flushing with N_2, in 6 M HCl under vacuum at 115 °C for 24 h. The hydrolysates were analysed for their amino acid compositions on a Varian 5560 liquid chromatograph configured as an amino acid analyser (AA911 column; Interaction), using ninhydrin with colour development in a stainless-steel reaction coil. Temperature was maintained at 135 $\pm$ 0.05 °C with a thermostatically controlled silicone oil bath. The ninhydrin colour was monitored at 570 nm, except for proline which was determined at 440 nm.

SPOC1 cell perfusion and enzyme-linked lectin assay (ELLA)

SPOC1 cells grown on TCols were perfused luminally using the 'perfusion plug' depicted in Figure 1. TCols, with perfusion plugs inserted, were placed in a six-well cluster plate containing F12/DMEM. A similar perfusion plug was used for SPOC1 cells grown in 12-well cluster plates. In both cases, the cluster plates were placed in a water bath at 35 °C, and the affluent perfusion tubing was submerged to preheat the perfusion fluid (F12/DMEM). The cultures were perfused using a multichannel peristaltic pump (Masterflex; Cole Parmer, Chicago, IL, U.S.A.) at 0.2 ml/min, and the perfusate was collected with a multichannel fraction collector (IC-200; Buchler Instruments, Lenexa, KN, U.S.A.). The perfusion solutions were continuously gassed with 95 % O_2/5 % CO_2, and the water bath was covered and vigorously gassed with the same mixture; the pH of the perfusate was 7.4 at its entry into and exit from the chamber. Luminal addition of agonists was achieved by quickly shifting the afferent tubing to a new reservoir. Serosal addition of agonists to cultures grown on TCols was achieved by direct addition to the medium bathing the bottom of the culture.

Collected fractions were assessed for mucin content by ELLA. Samples of volume 100 μl were bound to 96-well high-binding microtitre plates (Costar no. 3590) overnight at 4 °C, or for 2 h at 37 °C. The plates were washed with PBS containing 0.05 % Tween 20 and 0.02 % thimerosol, and incubated with 1–5 μg/ml horseradish peroxidase-conjugated SBA for 1 h at 37 °C. The plates were washed and developed by incubating in them 0.04 % (w/v) substrate (*o*-phenylenediamine in 0.0175 M citrate/phosphate buffer, pH 5.0, containing 0.01 % H_2O_2). The reaction was stopped after 15 min by the addition of 4 M H_2SO_4, and the A_{490} was determined in a microtitre plate reader (Dynatech model MR5000, Chantilly, VA, U.S.A.). Absorbance was converted into ng of mucin from a standard curve (Figure 2) using purified SPOC1 HMMG (see Figure 4, right), one of which was constructed for each microtitre plate assayed.

Reverse transcriptase (RT)-PCR

RNA was isolated from SPOC1 cells, rat lung and rat colon by CsCl-density-gradient centrifugation [20]. Primers for the RT reaction and PCR were designed against regions of the P_{2u} purinoceptor gene which are highly conserved between human [12] and mouse [11]. A cocktail, 1 μM each, of three gene-specific primers (CTCCTCTGAGCTAAGTCCATCGTGC, GGGCTCTGCTGTGTCCTCTCTGAGCC and GGAGTCCTGCTACTCTTGGGACAG) was used for the RT reaction, using 8 μg of total RNA. The RT reaction was followed immediately by 35 cycles of hot-start PCR, as per the kit instructions (cDNA Cycle Kit; Invitrogen, San Diego, CA, U.S.A.). The forward and reverse primers had the following respective sequences: CTGGAATAGCACCATCAATGG and GAAGGTGGTGGACAAAGTAGAGCAC. The PCR products were analysed by ethidium bromide staining after electrophoresis in a 2 % agarose gel. Fragments were purified from positive bands on Qiaex beads (Qiagen Corporation, Chatsworth, CA, U.S.A.), and were subcloned into the T-tailed pCR vector (Invitrogen). Minipreps of transformed colonies were prescreened by *Eco*RI digestion to verify the presence and size of the inserts, and were sequenced with the Sequenase kit (United States Biochemical, Cleveland,

OH, U.S.A.). Sequence data were analysed with the Genetics Computer Group software (GCG, Madison, WI, U.S.A.).

RESULTS

SPOC1

AB.PAS staining

As shown previously by Randell et al. [10], SPOC1 cells grown for 6–12 days after confluence of TCols possess a multilayered morphology (Figure 3). A layer of cuboidal cells resides along the substratum with one to two layers lying above. Only the cells in these outer layers contain AB/PAS-positive material in their apical poles; cells in the basal layer are AB/PAS-negative.

HMMG purification

The HMMG from solubilized SPOC1 cells was localized as a well-formed peak of sialic acid content after the first density-gradient centrifugation (Figure 4). Four sialic acid-positive fractions were pooled as indicated, and, after dialysis against 0.2 M guanidinium chloride, the material was subjected to a second density-gradient centrifugation. This second centrifugation yielded a sharp peak in sialic acid content that was well separated from DNA. The densities of the two pooled fractions were 1.46 and 1.44 g/ml. This purified HMMG was dialysed exhaustively against distilled water, and subjected to further analysis. The results of three such purifications using different passages of cells were similar, yielding 0.79 ± 0.09 mg dry weight of HMMG/g wet weight of SPOC1 cells (mean $\pm$ S.D.).

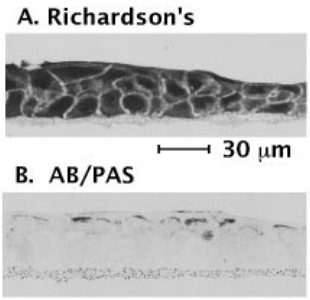

Figure 3 Light microscopy of SPOC1 cells grown on TCols

Sections (8 μm thick) of specimens 6 days after confluence were stained for general morphology with Richardson's stain (**A**) or for carbohydrate-bearing structures with AB/PAS stain (**B**).

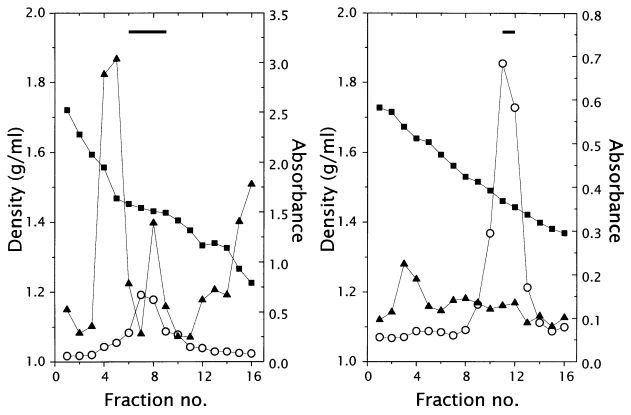

Figure 4 Isopycnic density-gradient centrifugation of SPOC1 HMMG in CsCl

Left, results of the first centrifugation, with 4 M guanidinium chloride and the starting density adjusted with CsCl to 1.40 g/ml. Right, results of the second centrifugation: 0.2 M guanidinium chloride and starting density, 1.50 g/ml. The 2.5 ml fractions were assessed for density (■), DNA (A_{260}; ▲) and sialic acid (A_{630}; ○). The horizontal bars in each panel indicate fractions pooled for subsequent analysis or use.

SDS/PAGE analysis of SPOC1 HMMG

The purified HMMG was analysed by electrophoresis in 4–12 % polyacrylamide gradient gels. Figure 5 shows the results of one analysis in which the purified cell-associated HMMG was compared with the material released into the cell culture medium after a 30 min maximal stimulation with ATP[S] (100 μM; see below). Duplicate lanes were silver-stained, or blotted and probed with the *N*-acetylgalactosamine-specific lectin, SBA. The purified cell-associated HMMG resolved as two bands in both staining protocols with apparent molecular masses greater than 1000 kDa and 265 kDa. The former was contained entirely within the stacking gel. In the SBA blot, this band stained intensely relative to the weakly staining 265 kDa band. In the silver-stained gel, by contrast, the two bands had approximately the same staining intensity. The strong SBA, but weak silver, staining of the $>$ 1000 kDa band is consistent with the material being a glycoprotein, i.e. extensive carbohydrate linkages shield the inner apoprotein from the silver stain, but interact strongly with the lectin. In support of this notion, in gels stained with PAS reagent, only the $>$ 1000 kDa band was identifiable (results not shown).

Culture medium from SPOC1 cells stimulated with ATP[S] for 30 min and applied to the gel neat contained the $>$ 1000 kDa material in both the silver-stained gel and the SBA blot. The 265 kDa band did not resolve well in the SBA blot, and in the silver-stained gel numerous additional bands were apparent at molecular masses of 265 kDa and below. From these analyses, it appears that HMMG material is released from SPOC1 cells by solubilization that has a mobility on SDS/PAGE similar to the material secreted by the cells. Importantly, of the numerous proteins secreted by SPOC1 cells only the HMMG material interacts with SBA.

Enzymic digestion, thiol reduction and β-elimination of HMMG, and Sepharose CL-4B column chromatography

To determine whether the purified HMMG material and other materials released into culture medium were comprised of proteoglycan or mucin, SPOC1 cells were labelled by exposure to [^{3}H]glucosamine and the materials collected were exposed to enzymes that hydrolyse specific carbohydrate linkages. Figure 6(A) shows the results for cell-associated [^{3}H]HMMG obtained by solubilization of SPOC1 cells and purified by density-gradient centrifugation. The profile of undigested HMMG (Figure 6A, top) shows that nearly all of the radioactivity was eluted in the void volume (V_0) of the column, as expected. In addition, digestion of the material with neuraminidase caused a significant shift in the elution profile, such that one-third to one-half of the total radioactivity was eluted at volumes greater than V_O, including a large symmetrical peak at the total column volume (V_T). This result signified that terminal sialic acid was labelled by the exposure to [^{3}H]glucosamine and was released by neuraminidase. None of the other enzymes tested had significant effects on the purified [^{3}H]HMMG: the radioactivity of material exposed to heparinase III, hyaluronidase and chondroitinase ABC was eluted in V_O, similar to the control (Figure 6A, bottom).

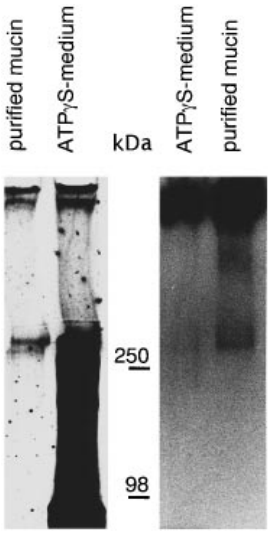

Figure 5 Electrophoresis of SPOC1 HMMG in 4–12% gradient polyacrylamide gels under reducing conditions

Duplicate lanes of a gel were either silver-stained (left) or blotted and probed with the SBA lectin (right). The outer lanes in each case contained HMMG (mucin) purified by density-gradient centrifugation, whereas the inner lanes contained culture medium from SPOC1 cells stimulated with ATP[S] (ATPγS). Positions of molecular-mass markers (kDa) are indicated.

Table 1 Amino acid composition of SPOC1 mucin

Amino acid	Content (mol/1000 residues)
Asx	79.7
Thr	132.5
Ser	143.7
Glx	138.3
Pro	68.0
Gly	81.5
Ala	34.3
Val	56.5
Cys*	11.0
Met	11.2
Ilu	31.5
Leu	67.3
Tyr	18.1
Phe	26.0
His	29.6
Lys	37.8
Arg	30.2

* Cys was measured as cystine; thus this value represents a minimum of 22 mol of Cys (half)/1000 residues.

The HMMG material released into the culture medium by ATP[S]-stimulated SPOC1 cells was also subjected to enzymic digestion. As shown in Figure 6(B), the undigested control medium yielded an elution profile similar to that seen for purified HMMG (Figure 6A), with the exception that a secondary peak at V_T was present which presumably represents residual [^{3}H]glucosamine. There was no discernible effect on the secreted HMMG of digestion with heparinase III, hyaluronidase and chondroitinase ABC: the elution profiles from these digestions essentially overlaid that of the control.

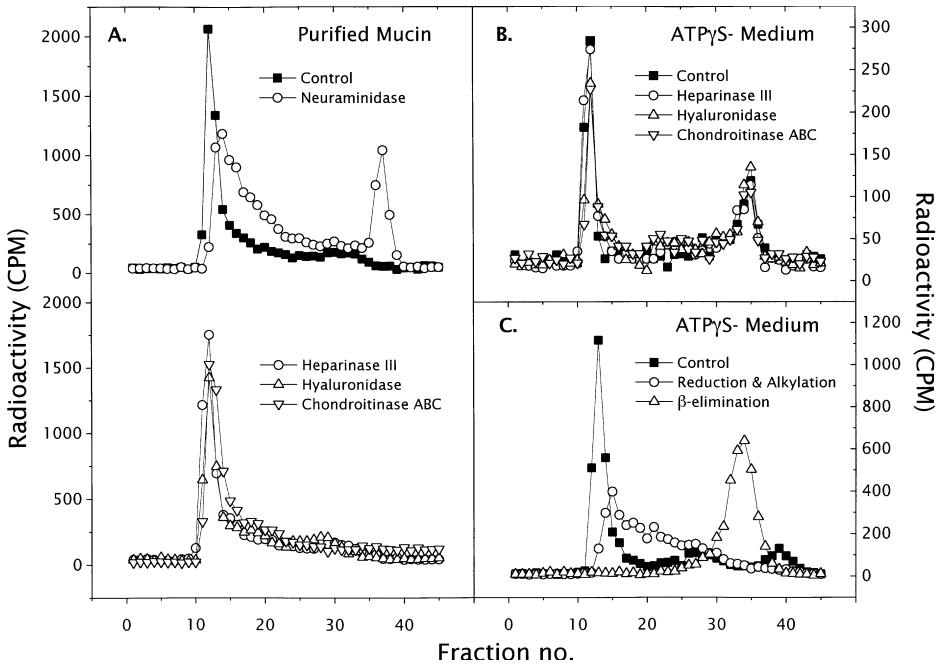

Figure 6 Sepharose CL-4B column chromatography

(**A**) Elution profiles of [^{3}H]glucosamine-labelled HMMG purified by density-gradient centrifugation. Top, control, or untreated, mucin and mucin digested with neuraminidase; bottom, mucins digested with proteoglycan-degrading enzymes. (**B**) and (**C**) Elution profiles of culture medium from [^{3}H]glucosamine-labelled cells stimulated with ATP[S] ATPγS. (**B**) Control medium and medium digested with proteoglycan-degrading enzymes; (**C**) control medium and medium after thiol reduction and alkylation or after β-elimination. Note that HMMG challenged with proteoglycan-degrading enzymes (**A**, bottom, and **B**) was boiled in a manner similar to that subjected to thiol reduction (**C**). The column was repacked for each experiment.

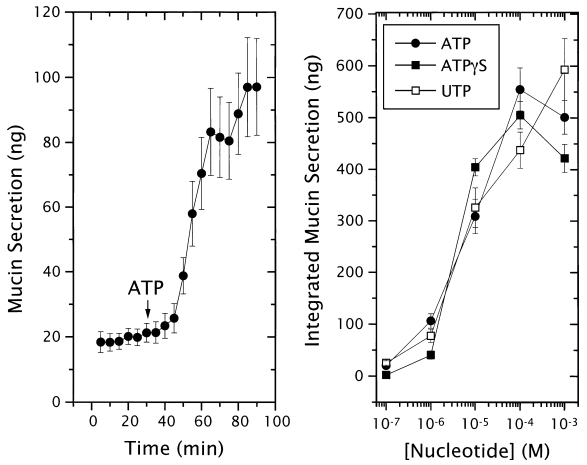

Figure 7 Effects of purinergic stimulation on mucin secretion by SPOC1 cells

Left, time course of the response to ATP (100 μM) by SPOC1 cells grown on TCols. The cultures were perfused luminally with F12/DMEM, fractions were collected at 5 min intervals, and ATP was applied to the perfusion bath at the time indicated by the arrow. The delay in the mucin-secretory response is due largely to the perfusion system. Right, dose–effect relations for three purinergic agonists, ATP (●), ATP[S] (■) and UTP (□). The data are presented as the suprabasal mucin-secretory response integrated over a 1 h exposure to agonist. In both panels, each point is represented as the mean ± S.E.M. of six SPOC1 cell cultures from two passages.

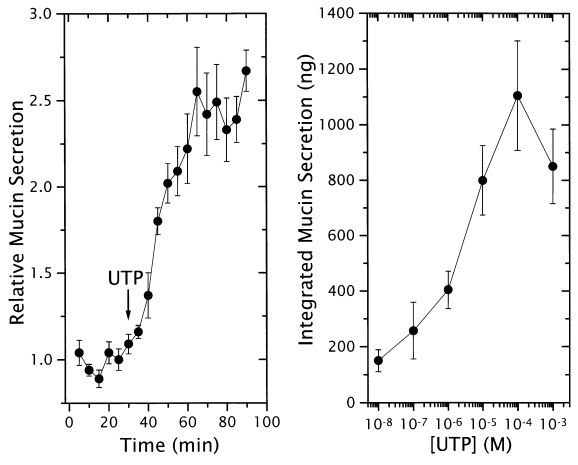

Figure 8 Effects of UTP on RTE primary cell cultures

Left, time course of the response to the addition of UTP (100 μM) to the luminal perfusion fluid of TCol cultures. UTP was added at the time indicated by the arrow. Right, dose–effect relations for UTP on the suprabasal integrated mucin-secretory response. In each panel, each point is represented as the mean ± S.E.M. of six cultures from two different preparations.

As a final test for the presence of proteoglycan materials released from ATP[S]-stimulated SPOC1 cells, the medium [^{3}H]HMMG material isolated in the V_O fractions was subjected to CsCl-density-gradient centrifugation. This analysis yielded a symmetrical peak of radioactivity centring on a density of 1.44 g/ml where mucin would be expected to distribute. No other peak of radioactivity was located within the gradient (results not shown).

Native mucins are generally extensively cross-linked via disulphide bonds formed between cysteine residues localized to the N- and C-termini of the macromolecules [21–23]. The presence of such disulphide linkages in SPOC1 HMMG was tested by thiol reduction and alkylation followed by chromatography on Sepharose CL-4B. As shown in Figure 6(C), the material subjected to this procedure was eluted from the column over a wide range of included volumes, indicating that thiol reduction and alkylation successfully cleared disulphide linkages between individual HMMG molecules.

To determine whether the HMMG contained *O*-glycosidic linkages as would be expected of mucins, material labelled with [^{3}H]glucosamine was subjected to non-reductive-β-elimination. Figure 6(C) shows that this procedure caused essentially all of the labelled material to be eluted from the column in a wide symmetrical peak centred on a volume near V_T. This result indicates that most of the carbohydrate associated with the HMMG is O-linked.

Digestion of [^{3}H]HMMG with trypsin, whether in the native form or after reduction and alkylation, yielded a broad heterodisperse population of glycopeptides, as judged by Sepharose CL-4B chromatography (results not shown). For reduced and alkylated mucins, trypsin digestion shifted all of the radioactivity out of the void volume, whereas with control mucins approx. 10 % of the material was eluted in the void volume. These results were similar to those obtained by others with a wide variety of purified mucins [24].

Protein content of SPOC1 HMMG

The protein content of SPOC1 HMMG was determined using two independent procedures after deglycosylation of a 100 μg sample of purified material. The value yielded by the BCA procedure was 9.4 μg of protein, and that by the Lowry procedure was 9.1 μg of protein. Hence, by these measures, the protein content of SPOC1 HMMG is approximately 9 %, leaving the carbohydrate content to be approximately 91 %.

Amino acid analysis

HMMG purified by density-gradient centrifugation was assessed for amino acid composition. Relative to the 5 % content expected from a purely random distribution of each amino acid, the results (Table 1) indicated that the material was rich in Ser and Thr, with the two residues accounting for 27 % of the total amino acids. The HMMG was also enriched in Gly and Glx, and was deficient in Met, Cys, Tyr. Since it is likely that only the disulphide-bonded form of Cys (cysteine) survived the acid hydrolysis of the HMMG preparatory to amino acid composition analysis, the 11 mol of Cys/1000 residues reported represents a minimum of 22 mol of cysteine/1000 residues.

As discussed fully below, the HMMG secreted by SPOC1 cells has the characteristics expected of a mucin, and shall hereafter be referenced as such.

Purinergic stimulation of mucin secretion

In SPOC1 cells grown on TCols and perfused luminally with F12/DMEM, a basal release of mucin was observed at approx. 4 ng/ml. On luminal exposure to a maximal dose of ATP (100 μM), mucin secretion was stimulated 4–5-fold over a period of 20–30 min (Figure 7). Histological analysis of cultures after stimulation revealed that the overall appearance of the SPOC1 cells was nominally unchanged compared with non-stimulated controls (results not shown). That the relatively slow onset of the mucin-secretory response is not an artifact of the SPOC1 cell line was tested by conducting comparable experiments on rat tracheal epithelial (RTE) primary cell cultures. The baseline rates of

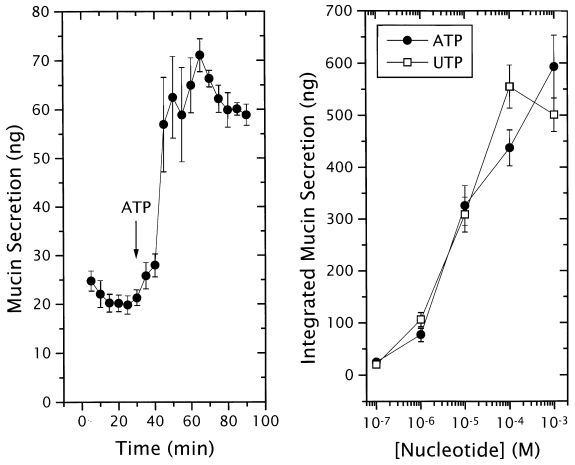

Figure 9 Response of SPOC1 cells grown on plastic to purinergic agonists

The SPOC1 cells were grown in 12-well cluster plates and perfused in a study similar to one shown in Figure 7. Left, either ATP or UTP (100 μM) was added at the arrow. Right, dose–effect relations for ATP and UTP on the suprabasal integrated mucin-secretory response. In each panel, each point is the mean $\pm$ S.E.M. and represents six cultures from two different passages.

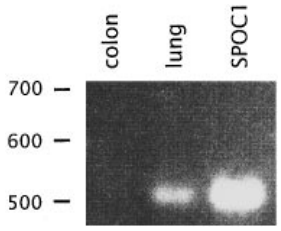

Figure 10 Ethidium bromide staining of products from RT-PCR for the P_{2u} purinoceptor

Total RNA from fresh colon and lung and from SPOC1 cells was subjected to an RT reaction with primers specific to human P_{2u} purinoceptor mRNA and then to PCR with primers to conserved regions of the gene. One-half of the resulting DNA products from each reaction were applied to the 2% agarose gel shown.

mucin secretion by the primary cell cultures from two different preparations were 5.0 ± 0.3 and 23.1 ± 0.8 ng/min. Because of this difference, the data were expressed relative to the mean baseline rate. As shown in Figure 8, the time course of the relative secretory response of the primary cell cultures to UTP was very similar to the response of SPOC1 cells to ATP, and there was about a 2.5-fold stimulation over baseline.

ATP, ATP[S] and UTP were all effective secretagogues for mucin secretion by SPOC1 cells. As Figure 7 shows, the dose–effect relationships for these agonists were virtually identical, and yielded an apparent $K_{0.5}$ for the mucin-secretory response of approx. 4 μM. The apparent affinity of UTP in eliciting mucin secretion by RTA primary cell cultures was also about 4 μM. The effectiveness of ATP[S] and UTP in eliciting mucin secretion by SPOC1 and RTE primary cell cultures is consistent with their possession of an apical membrane P_{2u} purinoceptor. To test whether other apical membrane purinoceptors are expressed by SPOC1 cells, TCol cultures were challenged with luminal applications of adenosine and 2-methylthio-ATP, hallmark P_1 and P_{2y} purinoceptor agonists respectively [25,26]. These agonists had no effect on mucin release, and the cultures responded normally to a subsequent challenge with UTP (results not shown).

Whether a permeable support was essential for the support of regulated SPOC1 cell mucin secretion was tested by perfusing cultures grown on plastic (12-well plates). Figure 9 shows the results of two studies in which cultures were challenged with ATP or UTP. In each case, the cells responded to the purinergic challenge with approximately the same time course and apparent affinity as observed for SPOC1 cultures grown on TCols (compare with Figure 7).

In other experiments, the response of SPOC1 TCol cultures to *serosal* applications of purinergic agonists was assessed. No response was observed, in multiple experiments ($n \geqslant 3$), with 100 μM adenosine, ATP, UTP, ATP[S] or 2-methylthio-ATP (results not shown). In each experiment, after the serosal test challenge, the cultures were challenged luminally with ATP or UTP, and in each case a normal mucin-secretory response was elicited. It therefore appears that P_{2u} purinoceptors are selectively localized to the apical membrane in SPOC1 cells, and that other types of purinoceptor are absent from both membranes.

Identification of P_{2u} purinoceptor

The data presented above and from other studies [2,4,5] suggest that the P_{2u} purinoceptor is the receptor that accounts for the response of goblet cells to purinergic agonists. To test this possibility, RT-PCR was used to probe SPOC1 cell total RNA for the presence of the P_{2u} purinoceptor. A single band of the predicted size (502 bp) was obtained for both SPOC1 cells and fresh rat lung (Figure 10), with the SPOC1 band being much more intense than that from lung. Rat colon total RNA was negative for P_{2u} purinoceptor mRNA, but an identical sample tested for another mRNA as part of another study was positive (CFTR; results not shown). The PCR product from SPOC1 RNA was subcloned and the partial nucleotide base sequence (80 bases) obtained was identical with the published sequence for the rat P_{2u} purinoceptor mRNA [27].

DISCUSSION

HMMG characterization

The HMMG purified from solubilized SPOC1 cells can be identified by multiple criteria as a mucin [21–23,28,29]. The material (i) possesses a molecular mass greater than 1000 kDa (Figure 5), (ii) is within the range of other mucins with a buoyant density of 1.45 g/ml (Figure 4), (iii) reacts positively with PAS and has an affinity for a lectin (SBA) that binds *N*-acetylgalactosamine, an amino sugar characteristic of mucins (Figure 5), (iv) is susceptible to digestion by neuraminidase and resistant to proteoglycan-degrading enzymes (Figures 6A and 6B), (v) contains intermolecular disulphide bonds which are responsible for the non-reduced material being eluted in the void volume of a Sepharose CL-4B column (Figure 6C), (vi) is primarily carbohydrate in content (91%) which is O-linked to the apomucin protein (Figure 6C) and (vii) has an amino acid composition enriched in Thr, Ser and Gly (Table 1). Thus SPOC1 cells not only possess the overall appearance of goblet cells when grown in tracheal xenografts and stain with AB/PAS and RTE11 mAb when grown in culture [10], but they also contain HMMGs having the biochemical characteristics of mucin. This material also appears to be secreted by SPOC1 cells under the stimulation of purinergic agonists since the high-molecular-mass material detected in the culture medium bathing stimulated cells, when compared with cell-associated mucin, has

similar (i) silver- and SBA-staining patterns on SDS/PAGE (Figure 5), (ii) resistances to proteoglycan-degrading enzymes Figure 6) and (iii) buoyant densities (1.44 and 1.45 g/ml respectively.

We note that in the chromatogram in Figure 6(C) thiol reduction and alkylation of culture medium harvested from SPOC1 cells stimulated with ATP[S] caused the mucins therein to be eluted over a broad range of void and included volumes. In the SBA blot of the same material (Figure 5), however, most of the lectin-positive material was present as a narrow zone in the stacking gel. At least two potential explanations exist for this apparent inconsistency. The Sepharose CL-4B column and the 4–12 % gradient gel used for electrophoresis do not have identical separation profiles, which may have resulted in a relatively compressed pattern on the gel. The SBA lectin used to probe the blot for HMMG also may detect only a subset of the total carbohydrate materials labelled with [^{3}H]glucosamine. Were the ratio of lectin-detectable to total carbohydrate residues to differ in the mucin species of different molecular mass that resulted from thiol reduction, the two means of detection would have yielded different signal profiles.

Three characteristics of the purified SPOC1 mucin and/or its secretion merit comment. First, SDS/PAGE analysis of mucin yielded both a silver- and an SBA-stained band of protein with an apparent molecular mass of 265 kDa, whereas the Sepharose CL-4B column elution profile of purified radiolabelled mucin showed no peaks intermediate between V_O and V_T. The most likely explanation of this difference is that the PAGE was performed under reducing conditions, and that during non-reducing conditions of the CL-4B column chromatography the 265 kDa protein remained associated with mucin, presumably through disulphide linkages. This notion is supported by the observation (Figure 6C) that thiol reduction and alkylation caused the HMMG to be eluted from a CL-4B column over a wide range of included volumes. It is possible that this extraneous 265 kDa band is a cross-linking glycoprotein, similar to the putative 'link' glycopeptide that has been hypothesized for gastrointestinal tract mucins; however, it is more than twice the size of the 118 kDa 'link' glycoprotein [30]. Its size (265 kDa) is within the range that might be expected for a mucin aproprotein. Thus a poorly glycosylated mucin that exists as a minor component of the secretory product is another possibility. For the purposes of this study, the quantity of the material is too small relative to that comprising the $>$ 1000 kDa mucin (Figure 5) to compromise the conclusions made herein.

Second, from other studies on cultured cells, proteoglycan materials would not have been an unexpected component of the culture medium removed from SPOC1 cells [29]. Our observation, however, was the apparent absence of such material: not only was the medium HMMG from ATP[S]-stimulated cells resistant to digestion by proteoglycan-degrading enzymes (Figure 6C), but the void-volume material resolved as a single peak with a buoyant density of 1.44 g/ml by density-gradient ultracentrifugation (results not shown). A likely explanation for the apparent lack of proteoglycans in the culture medium is that these proteins would be expected to be part of the extracellular adherence mechanism. Thus such proteoglycans that escaped to the medium would probably have been removed by the thorough washing applied to the cultures before the 30 min incubation with ATP[S]. The apparent absence of proteoglycans from the HMMG material is therefore most likely due to its absence from the secretory mucin pool rather than to a complete lack of proteoglycan secretion by SPOC1 cells. One beneficial effect of the lack of HMMGs other than mucin in the secretory product released by purinergic agonists was that it allowed an unambiguous and highly sensitive assay for mucin to be developed, using SBA as the binding agent in an ELLA (Figure 2).

Third, the amino acid composition of SPOC1 mucin (Table 1) is similar to that of other mucins in containing relatively high amounts of Ser and Thr [22,28]; however, it is significantly different from the composition of mucin secreted by RTE primary cultures [13]. Specifically, SPOC1 mucin has less Pro and Gly, and more Thr and Glx, than is found in HMMG from RTE primary cultures. One possible reason for these differences is that different techniques were used for the purification and determination of the amino acid composition of the mucin studied. Also, the high Gly content of RTE primary mucin may be due to an overestimation by interference with glucosamine, whereas we corrected for this possibility. A more likely reason is that RTE primary and SPOC1 cells express different HMMGs; the rat airways are likely to secrete a variety of mucins, of which RTE primary and SPOC1 cells may express different subsets. Lastly, non-mucin HMMG may be expressed and secreted by RTE primary cells [13]. In either case, because of the difficulties inherent in the characterization of mucins, the apparent differences between the secretory products of SPOC1 and RTE primary cells are most likely to be resolved after the genes encoding the secreted HMMGs are identified.

Purinergic regulation of mucin secretion

The mucin-secretory responses of perfused SPOC1 and RTE primary cell cultures to the purinergic agonists ATP, ATP[S] and UTP were similar (Figures 7 and 8). In each case, mucin secretion was stimulated over a period of 20–30 min with 4–5 and 2.5-fold stimulations over baseline rates of secretion respectively. In native goblet cells observed by video microscopy, we have observed mucin granule *exocytosis* to commence essentially immediately on ATP stimulation [2]. One possible reason for the apparent slow onset of secretion when assessed instead by biochemical determination of mucins released into a perfusate is that time is necessary for the mucins to hydrate and solubilize after exocytosis [31]. To allow for this possibility, the dose–effect relationships for purinergic stimulation of mucin secretion were determined as the integrated response, above baseline, over a period of 60 min (Figures 7–9).

Both the pattern of responses by SPOC1 and RTE primary cell cultures to, and their affinities for, hallmark purinergic agonists indicated a P_{2u} purinoceptor [25,26]. In both cases, the cells responded with similar time courses and kinetics to ATP and UTP (Figures 7 and 8). SPOC1 cells in addition responded similarly to ATP[S], but did not respond to either the P_{2y} purinoceptor agonist, 2 methylthio-ATP, or the P_1 agonist, adenosine. The apparent affinities for ATP, ATP[S] and UTP in eliciting a mucin-secretory response were in the low micromolar range, as appropriate for P_{2u} purinoceptor agonists [25,26]. In their pattern of agonist-induced responses, SPOC1 and RTE primary cells are similar to goblet cells in primary cultures of hamster tracheal epithelial cells [4] and those in explanted native human and canine superficial epithelia [2,5]. In addition, SPOC1 cells are similar to human and canine native goblet cells in not responding to luminal challenges with other purinergic agonists [2,5]. Thus by simple pharmacological criteria both the SPOC1 and RTE primary cells possess an apical membrane P_{2u} purinoceptor, and the SPOC1 cell apparently possesses no other apical membrane purinoceptor.

An important practical observation was that SPOC1 cells respond to purinergic agonists with a stimulation of mucin secretion equally well whether grown on permeable supports (TCols) or plastic (compare Figures 7 and 8). There were no

functional differences between cells grown on the two substrata in either the time course of the development of the mucin-secretory response to, or the apparent affinity of, luminal challenges with purinergic agonists. This observation is in distinct contrast with primary cell cultures that achieve an acceptable degree of secretory cell differentiation only when grown on permeable supports and generally on a substratum of collagen or other extracellular matrix protein [3,13,32].

SPOC1 cells differ from native goblet cells in lacking the ability to respond to serosal purinergic agonists. We have shown that goblet cells in native explanted epithelium respond by mucin granule exocytosis to serosal challenges with ATP, the non-hydrolysable 5′-adenosine imidodiphosphate, ADP and adenosine [2]. Of the many possible scenarios that could explain these differences, the only one in which SPOC1 and native goblet cells possess identical regulatory pathways holds the serosal responses in native tissues to be indirect and due to a primary stimulation of luminally directed ATP secretion by other cells in the epithelium. This local luminal ATP secretion would then stimulate mucin secretion by interacting with the goblet cell apical membrane P_{2u} purinoceptor.

P_{2u} purinoceptor mRNA

The extracellular actions of ATP in the non-vascular aspect of the lung were first appreciated for the stimulation of surfactant secretion by alveolar type II cells [33], of transepithelial ion and fluid transport in the airways [34,35], and of goblet cell mucin secretion [2,4,5]. The pharmacology of these responses proved not to match those of known purinoceptors, however, but instead indicated a novel receptor at which ATP and UTP were equipotent [34,36]. The P_{2u} purinoceptor, originally cloned and sequenced from mouse neuroblastoma cells [11], allowed the subsequent cloning of the gene from a human airway cell line [12] and rat alveolar type II cells [27]. The P_{2u} purinoceptors from the three species share a 97% identity and are expressed in a wide variety of tissues. The finding in this study that a P_{2u} purinoceptor mRNA is expressed in SPOC1 cells (Figure 10) is fully consistent with the pharmacology of purinergic stimulation of mucin secretion by globlet cells, namely that the mucin-secretory response is stimulated equally by ATP and UTP (Figure 7; [2,4,5]). No purinoceptor has been identified at the protein level in any cell type, so this demonstration in goblet cells must await the necessary reagents. The P_{2u} purinoceptor is the first receptor to be specifically identified at the mRNA level in a mucin-secreting cell of any type.

The SPOC1 cell line

As discussed above, SPOC1 cells grown in culture possess many physiological and pharmacological aspects relevant to 'normal' goblet cell function. On the other hand, the cells do not exhibit the morphological features of typical goblet cells, except when grown in tracheal xenografts [10]. SPOC1 cells in culture exhibit AB/PAS-positive material in the other cell layers, but they are cuboidal and express keratins that are normally found in other epithelial cell types. They also do not express K18 which is characteristically expressed in columnar epithelial cells [9]. Nonetheless, in providing a relatively pure population of secretory cells, for which a basal-like cell is the only major 'contaminant', the SPOC1 cell may offer a unique opportunity for dissection of the regulatory pathways controlling mucin secretion in the airways.

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