Neuropeptide regulation of secretion and inflammation in human airway gland serous cells

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ABSTRACT Airway submucosal gland serous cells are sites of expression of the cystic fibrosis transmembrane conductance regulator (CFTR) and are important for fluid secretion in conducting airways. To elucidate how neuropeptides regulate serous cells, we tested if human nasal turbinate serous cells secrete bicarbonate (HCO_3^-), important for mucus polymerisation and antimicrobial peptide function, during stimulation with cAMP-elevating vasoactive intestinal peptide (VIP) and if this requires CFTR. Serous cells stimulated with VIP exhibited a ∼15–20% cAMP-dependent decrease in cell volume and a ∼0.15 unit decrease in intracellular pH (pH_i), reflecting activation of Cl^- and HCO_3^- secretion, respectively. HCO_3^- secretion was directly dependent on CFTR and was absent in cells from CF patients. In contrast, neuropeptide Y (NPY) reduced VIP-evoked cAMP increases, CFTR activation, and Cl^-/HCO_3^- secretion. Culture of primary serous cells in a model that maintained a serous phenotype confirmed the activating and inhibiting effects of VIP and NPY, respectively, on fluid and HCO_3^- secretion. Moreover, VIP enhanced antimicrobial peptide secretion and antimicrobial efficacy of secretions while NPY reduced antimicrobial efficacy. In contrast, NPY enhanced cytokine release while VIP reduced cytokine release through a mechanism requiring CFTR. As levels of VIP and NPY are up-regulated in diseases like allergy, asthma, and chronic rhinosinusitis, the balance of these two peptides in the airway may control mucus rheology and inflammatory responses in serous cells. Furthermore, the loss of CFTR conductance in serous cells may contribute to CF pathophysiology by increasing serous cells inflammatory responses in addition to directly impairing Cl^- and HCO_3^- secretion.

Introduction

Several obstructive airway diseases share phenotypes of thickened mucus, including chronic rhinosinusitis (CRS), cystic fibrosis (CF), asthma and COPD [1–3]. From nasal turbinate down to small bronchi, a large percentage of airway–surface liquid (ASL) and mucus is generated by submucosal exocrine glands [3]. Gland serous cells are sites of expression of the CF transmembrane conductance regulator (CFTR) [3]. Observations of occluded gland ducts and gland hypertrophy, hyperplasia and infection in CF [3] suggest that defects in CFTR-dependent serous secretion contribute to CF pathology. Intact CF glands secrete less fluid in response to cAMP-elevating agonists such as vasoactive intestinal peptide (VIP) compared with non-CF glands [3]. Gland hypertrophy, duct plugging, and excess mucus are also observed in COPD and asthma [3], with gland hypertrophy more common in fatal asthma [4].

Bicarbonate (HCO_3^-) secretion by serous cells facilitates polymerisation of mucins secreted by more proximal mucous cells [5] (supplementary figure S1a). HCO_3^- is also critical to efficacy of antimicrobial peptides secreted by serous cells [3]. However, mechanisms by which serous cells secrete HCO_3^- are unknown. Identifying these mechanisms may yield insights into pathophysiology of CF and other diseases exhibiting altered mucus secretion or rheology. We previously studied nasal serous cell HCO_3^- secretion during cholinergic-induced secretion [6], which is largely intact in CF [3], as it is mediated by the Ca^{2+}-activated Cl^- and HCO_3^- channel (CaCC) TMEM16A [7]. Our initial goal was to directly test if serous cells also secrete HCO_3^- during VIP stimulation, if this occurs through CFTR, and if TMEM16A could substitute during loss of CFTR.

A further goal was to understand how VIP and neuropeptide Y (NPY), both upregulated in inflammatory airway diseases, interact to control airway secretion. Parasympathetic VIPergic [8, 9] and NPYergic [10, 11] neurons exist in the respiratory tract and may be increased in mucosa from patients with allergic rhinitis [12] or irritative toxic rhinitis [13]. Some nerves co-express VIP and NPY [14], including in the proximity of glands [15, 16]. VIP and NPY are found in the pedicle of nasal polyps, suggesting they may play a role in polyp formation [17]. Activated macrophages [18] or epithelial cells [19] can also make NPY, perhaps because NPY has direct antimicrobial effects [20]. Elevated NPY in asthma [21] may link psychological stress with allergic asthma exacerbations [22]. Mice lacking NPY or NPY1R have reduced allergic airway inflammation [23]. Other studies outside the airway suggest NPY increases T helper (Th)2 responses [24, 25]. NPY and NPYR1 expression are elevated in mouse lungs after influenza, and knockout of NPY reduced disease severity and interleukin (IL)-6 levels [18]. Allergic rhinitis patients have nasal secretions with elevated concentrations of VIP compared with control individuals at baseline [26] and during allergen challenge [27].

A recent review highlighted the need for elucidation of neuropeptide regulation of submucosal glands in obstructive lung diseases [28]. The role of VIP as a cAMP-dependent activator of secretion is established [3], but the role of NPY is unclear. A cocktail of NPY and noradrenaline inhibited cultured tracheal gland cell glycoprotein secretion [29], and NPY inhibited bulk mucus secretion in ferret trachea [30], but there is little mechanistic data for if/how NPY affects serous cells. NPY receptors are often G_i-coupled and could reduce cAMP responses to G_s-coupled VIP receptors [31], reducing secretion by lowering protein kinase A (PKA) activation of CFTR. Moreover, VIP and NPY are immunomodulators [32] and may regulate gland cytokine secretion.

We examined effects of VIP and NPY in primary serous cells isolated from human nasal glands. Cells were studied acutely as well as in an air–liquid interface (ALI) culture model that retained expression of serous cell markers, facilitating polarised studies and co-culture with human macrophages. Results below contribute to our understanding of airway serous cells and the role of CFTR in both secretion and inflammation, suggesting therapeutic targets for obstructive airway diseases.

Methods
Experimental procedures

Isolation of primary serous acinar cells, immunofluorescence and live cell imaging of cell volume, pH_i, and Cl^- was carried out as described [6, 33]. Culture of gland serous cells was carried out as described [34]. ASL height, ASL pH measurements, ELISAs and bacterial assays were carried out as reported [27, 35]. More detailed methods and reagents used provided in the supplementary materials.

Study approval

Tissue was acquired in accordance with the University of Pennsylvania guidelines regarding residual clinical material in research (IRB protocol #800614), the US Department of Health and Human Services Title 45 CFR 46.116, and the Declaration of Helsinki. Turbinate samples from 42 non-CF and nine CF patients (seven ΔF508/ΔF508, one ΔF508/G542X, and one ΔF508/E585X) were used (supplementary table S1).

Statistics

Data were analysed in GraphPad Prism. Multiple comparisons used 1-way ANOVA with Bonferroni (preselected pairwise comparisons), Dunnett's (comparisons to a control set) or Tukey–Kramer (comparison of all values) post-tests. A p-value <0.05 was considered significant. All data are mean±SEM from independent experiments using cells from ⩾4 patients. Minimal patient-to-patient variability was observed beyond effects of CFTR genotype, as described in the supplementary material. Data points in each figure represent independent experiments, some of which used separate cell cultures that originated from the same patient (common in studies using ALI cultures). In this case, an equal number of independent experiments, typically two, was performed using cells from each patient to ensure that cells from each patient were equally represented to prevent cells from any one patient skewing results.

Results

VIP stimulates Cl⁻ and HCO₃⁻ secretion through CFTR

Submucosal gland acinar cells (supplementary figure S1b) were isolated from human middle turbinate [33]. Serous acini exhibited secretory-granule immunofluorescence for lysozyme (supplementary figure S1c), basolateral immunofluorescence for VIP receptors (supplementary figure S1d and e), and apical immunofluorescence for TMEM16A and CFTR (supplementary figure S1f and g) as described [6, 7, 33]. Secretion was studied in isolated serous cells using simultaneous differential interference contrast (DIC) measurement of cell volume and quantitative fluorescence microscopy of indicator dyes to measure the concentrations of ions involved in driving secretion (Cl^-/HCO_3^-), a technique pioneered in salivary cells adapted for serous cells [6, 7, 33].

Epithelial fluid secretion is driven largely by Cl^-. Serous cell shrinkage during agonist stimulation reflects efflux of cellular K^+ and Cl^- upon activation of secretion and movement of osmotically obliged water. Cell swelling upon removal of agonist reflects solute uptake *via* mechanisms that sustain secretion, such as the $Na^+K^+2Cl^-$ co-transporter NKCC1 [7] (figure 1a). Human nasal serous cells shrank by ∼20% when stimulated with cAMP-elevating agonists forskolin or VIP (figure 1b), as previously reported [33].

Shrinkage was accompanied by transient acidification of intracellular pH (pH_i) followed by more sustained alkalinisation (figure 1c and d). Agonist-evoked acidification was absent in HCO_3^--free media (supplementary figure S2a–c), and the secondary alkalinisation was blocked with inhibition of the $Na^+HCO_3^-$ co-transporter (NBC; supplementary figure S2d). Thus, the transient acidification reflects HCO_3^- efflux during activation of secretion, while the alkalinisation reflects activation of NBC, sustaining HCO_3^- secretion by keeping intracellular $[HCO_3^-]$ high. This is similar to cholinergic-evoked serous cell acidification and subsequent alkalinisation by Na^+/H^+ exchangers (NHEs) [6], but reveals an important mechanistic difference between cAMP and Ca^{2+} pathways.

Acidification was blocked by eliminating the driving forces for conductive HCO_3^- efflux using ion substitution (supplementary figure S2e), suggesting acidification is mediated by an ion channel. Forskolin- or VIP-induced shrinkage and acidification were absent in cells from CF patients (figure 1c and d), and were inhibited by CFTR inhibitor $CFTR_{inh}172$ or K^+ channel inhibitors clofilium and clotrimazole in non-CF cells (figure 1e and f), demonstrating a requirement for both CFTR and counterion K^+ efflux. VIP-evoked responses were not reduced by TMEM16A inhibitors niflumic acid (NFA), $T16A_{inh}$-A01, $CaCC_{inh}$-A01, or 4,4'-diisothiocyanato-2,2'-silbenedisulfonic acid (DIDS) (figure 1f). Representative traces are in supplementary figure S3a and b.

In contrast, carbachol (CCh), which activates Ca^{2+}-driven secretion [7, 33], stimulated shrinkage and acidification blocked by TMEM16A inhibitors NFA, $T16A_{inh}$-A01, $CaCC_{inh}$-A01, or DIDS (figure 1g). CCh-induced secretion was intact in CF cells (figure 1h). Pharmacological activation of TMEM16A (E_{act}) restored secretion responses to VIP in CF cells (figure 1h). Representative traces are in supplementary figure S3c and d. The concentration of E_{act} used here had no acute (2–3 min) effects on intracellular calcium in these cells (supplementary figure S3e)

Thus, cAMP elevation activates both Cl^- and HCO_3^- secretion directly through CFTR (figure 1i), and targeting TMEM16A might restore HCO_3^- secretion in CF glands. We found no evidence for Cl^-/HCO_3^- exchanger-mediated HCO_3^- efflux (supplementary figure S4), agreeing with data showing Calu-3 cells [36], a bronchial line frequently used as a serous cell surrogate, secrete HCO_3^- mainly through CFTR while Cl^-/HCO_3^- exchanger pendrin (SLC26A4) is more important in surface epithelial cells. These data show a fundamental defect in HCO_3^- secretion in CF cells caused directly by loss of CFTR conductance, which may be restored directly by pharmacological CFTR correction.

NPY reduces CFTR-mediated fluid and HCO₃⁻ secretion during VIP stimulation

Calu-3s express high amounts of NPY1R relative to other airway lines (supplementary tables S2 and S3). We thus tested for NPYRs in primary serous cells. We observed no secretory responses to NPY, but the
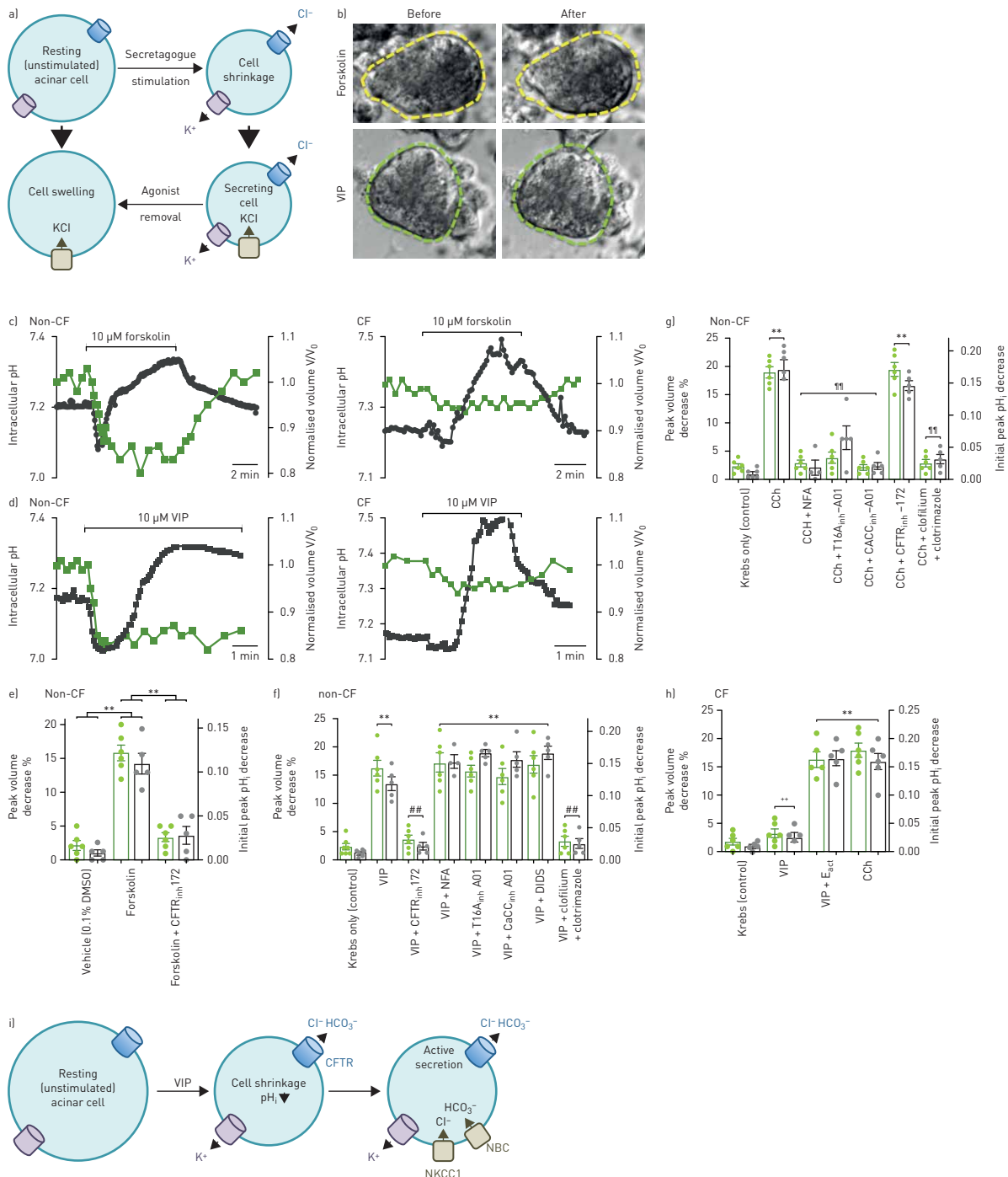
FIGURE 1 cAMP stimulation of human nasal serous cells results in cystic fibrosis transmembrane receptor (CFTR)-dependent Cl^- and HCO_3^- secretion. a) Diagram showing use of cell volume measurements to track fluid secretion, primarily driven by Cl^-, which was combined with simultaneous measurement of pH_i to track HCO_3^- secretion. b) Non-cystic fibrosis (CF) serous cells stimulated with adenylyl cyclase-activating forskolin (top) or G_s-coupled receptor agonist vasoactive intestinal peptide (VIP) (bottom) exhibited ~15% shrinkage reflecting the activation of fluid secretion. c and d) In cells from non-CF patients, forskolin (c) or VIP (d) induced shrinkage (~15%; green) accompanied by a transient decrease in pH_i (~0.1–0.15 unit; grey) followed by a sustained secondary alkalinisation. CF cells exhibited markedly reduced shrinkage and acidification; subsequent alkalinisation was intact. e–h) Bar graphs showing peak shrinkage (green) and acidification (grey) in non-CF (e–g) and CF (h) cells. Forskolin-induced shrinkage and acidification was inhibited by $CFTR_{inh}172$ (10 μM) (e). VIP-induced shrinkage and acidification was inhibited by $CFTR_{inh}172$ and K^+ channel inhibitors clofilium and clotrimazole (30 μM each) (f). Ca^{2+}-activated Cl^- channel inhibitors NFA (100 μM), $T16A_{inh}$-A01 (10 μM), $CaCC_{inh}$-A01 (10 μM) or 4,4'-diisothiocyanostilbene-2–2''-disulfonic acid (DIDS; 1 mM) had no effect on VIP-induced responses (f) but blocked carbachol (CCh; 100 μM) responses. CF cells exhibited minimal responses to VIP but intact responses to CCh. VIP responses were restored by TMEM16A-activator E_{act} (25 μM). All experiments done at 37°C with 5% CO_2/25 mM HCO_3^-. Data in e–h are mean±SEM of 5–8 individual experiments from ⩾4 individual patients (1–2 experiments per patient). Significances determined by one-way ANOVA, Bonferroni posttest. i) Diagram showing activation of serous cell secretion by VIP, with Cl^- and HCO_3^- efflux through CFTR (apically localised in intact glands) causing a decrease in cell volume and pH_i. Influx of Cl^- though NKCC1 and influx of HCO_3^- through NBC (both basolaterally localised in intact glands) maintains the driving force for Cl^- and HCO_3^- efflux during sustained secretion. **: $p<0.01$ versus control; ##: $p<0.01$ versus VIP; ¶¶: $p<0.01$ versus CCh; ++: $p<0.01$ versus non-CF.

magnitudes of VIP-evoked acidification and shrinkage were reduced with NPY (figure 2a and b). We hypothesised that G_i-coupled NPYRs might blunt the magnitude of VIP-evoked cAMP increases, reducing CFTR activation. We measured Cl^- permeability using 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ), a dye quenched by Cl^- but not by NO_3^-. Substitution of extracellular Cl^- for NO_3^- results in decreased intracellular $[Cl^-]$ and the resulting rate of SPQ fluorescence increase is roughly equivalent to anion permeability [7, 33, 35]. In the presence of VIP, fluorescence rapidly increased upon NO_3^- substitution. This was reduced by ∼50% by NPY (figure 2c and d). In the presence of $CFTR_{inh}172$, anion permeability was almost completely reduced and NPY had no effect (figure 2d). Even after 24 h stimulation with NPY, isolated serous cells exhibited reduced VIP-activated Cl^- permeability during acute VIP stimulation (supplementary figure S5).

CFTR is activated by PKA downstream of cAMP. We imaged real-time cAMP changes using an mNeonGreen cAMP biosensor (cADDis) [37]. VIP induced a rapid, reversible increase in cAMP blocked by VIPR antagonist $VIP_{(6-28)}$ (figure 3a and b). The cAMP increase was independent of Ca^{2+} (figure 3c). There was no difference in cAMP increases in non-CF or CF cells (supplementary figure S6), in contrast to previous hypotheses of defective cAMP signalling in CF [38]. However, NPY reduced VIP-evoked cAMP responses (figure 3d and e); the NPY effects were eliminated with NPY1R antagonist BIBO 3304 or pertussis toxin (PTX), which inactivates G_i proteins (figure 3d and e). Thus, NPY blunts CFTR-mediated Cl^-, HCO_3^-, and fluid secretion by reducing cAMP signalling.

NPY and VIP have opposing effects on Cl^- and HCO_3^- secretion in primary cultures of serous cells

To facilitate polarised studies, we used culture methods that preserved a serous phenotype [34]. Serous cells cultured at the ALI expressed markers Muc7, VIPR1, VIPR2, lysozyme, NKCC1 and α_1-antitrypsin, as well as both NPY1R and NPY4R (figure 4a–c). Lysozyme, Muc7, VIPR1 and VIPR2 were detected by immunofluorescence in serous cells (figure 4d and e) and Calu-3 cells (supplementary figure S7 and S8). ELISA and qPCR confirmed that serous cultures expressed Muc7 but not Muc5AC or Muc5B (supplementary figure S9). Serous ALIs also expressed functional CFTR. Apical substitution of Cl^- for NO_3^- led to a decrease in $[Cl^-]_i$ that was enhanced by VIP, blocked by $CFTR_{inh}172$, and blunted by NPY (figure 5a). Similar to the aforementioned freshly isolated cells, TMEM16A inhibitors did not affect VIP-activated Cl^- permeability (figure 5a). ALIs were resistant to viral expression of cADDis, but steady-state cAMP levels were measured 5 min after stimulation with VIP±NPY. NPY reduced cAMP increases, and this was abrogated by PTX (figure 5b).

ASL was labelled with Texas red dextran to track fluid secretion. VIP increased ASL height; this was inhibited by NKCC1 inhibitor bumetanide, PKA inhibitor H89, or $VIP_{(6-28)}$ (figure 5c and d), supporting that this reflected fluid secretion. NPY inhibited VIP-induced secretion (figure 5c and d) and isoproterenol-induced secretion (figure 5d), but not Ca^{2+}-activated CCh-induced secretion (figure 5d), showing effects of NPY were specific for cAMP. To test if phagocyte-produced NPY could produce these effects, we used primary human monocyte-derived macrophages primed with phorbol myristate acetate (PMA), which produce NPY (supplementary figure S10). Serous ALI transwells were transferred into plates above the macrophages with 24 h macrophage-conditioned media on the basolateral side. Addition of VIP increased ASL height, but this was reduced in the presence of PMA-primed macrophages, and this effect was reversed by BIBO 3304 (figure 5e).

To track HCO_3^- secretion, ASL was labelled with seminaptharhodafluor (SNARF)-1-dextran sonicated in perfluorocarbon, allowing measurement of pH in physiological ASL with no addition of aqueous fluid [39]. Steady-state unstimulated ASL pH (pH_{ASL}) was 7.2±0.04, equivalent to ∼15 mM HCO_3^- at 5% CO_2 ($[HCO_3^-]_i$=1.2 mM×$10^{pH-6.1}$); pH_{ASL} was reduced by NBC inhibitor DNDS (6.9±0.06; 7.6 mM HCO_3^-) but not with NPY alone (figure 5f). VIP increased pH_{ASL} (7.6±0.04; 38 mM HCO_3^-), suggesting VIP stimulated HCO_3^- secretion; increased pH_{ASL} was reduced by NPY (7.3±0.05) or DNDS (7.1±0.03). Effects of NPY were blocked by PTX. NPY similarly inhibited forskolin and isoproterenol (figure 5f). Note that with increased ASL volume (figure 5c) and buffering capacity, actual secreted HCO_3^- would be larger than changes in $[HCO_3^-]$.

Serous ALIs were incubated with unstimulated or PMA-stimulated macrophages as previously stated and steady-state pH_{ASL} was measured 2 h later; pH_{ASL} was unchanged by unstimulated macrophages, but PMA-stimulated macrophages reduced pH_{ASL} (figure 5g). This was inhibited by BIBO 3304. Addition of VIP increased pH_{ASL} (figure 5g). Effects observed were verified using a real-time HCO_3^- secretion assay, which also confirmed secretion was dependent on apical CFTR (supplementary figure S11).

Type 2 inflammation was suggested to upregulate Cl^-/HCO_3^- exchanger pendrin in airway surface epithelial cells [40, 41]. We examined if NPY or IL-13 induced altered expression of pendrin or Cl^- channels in primary serous cells, perhaps shifting serous cells away from CFTR toward a more
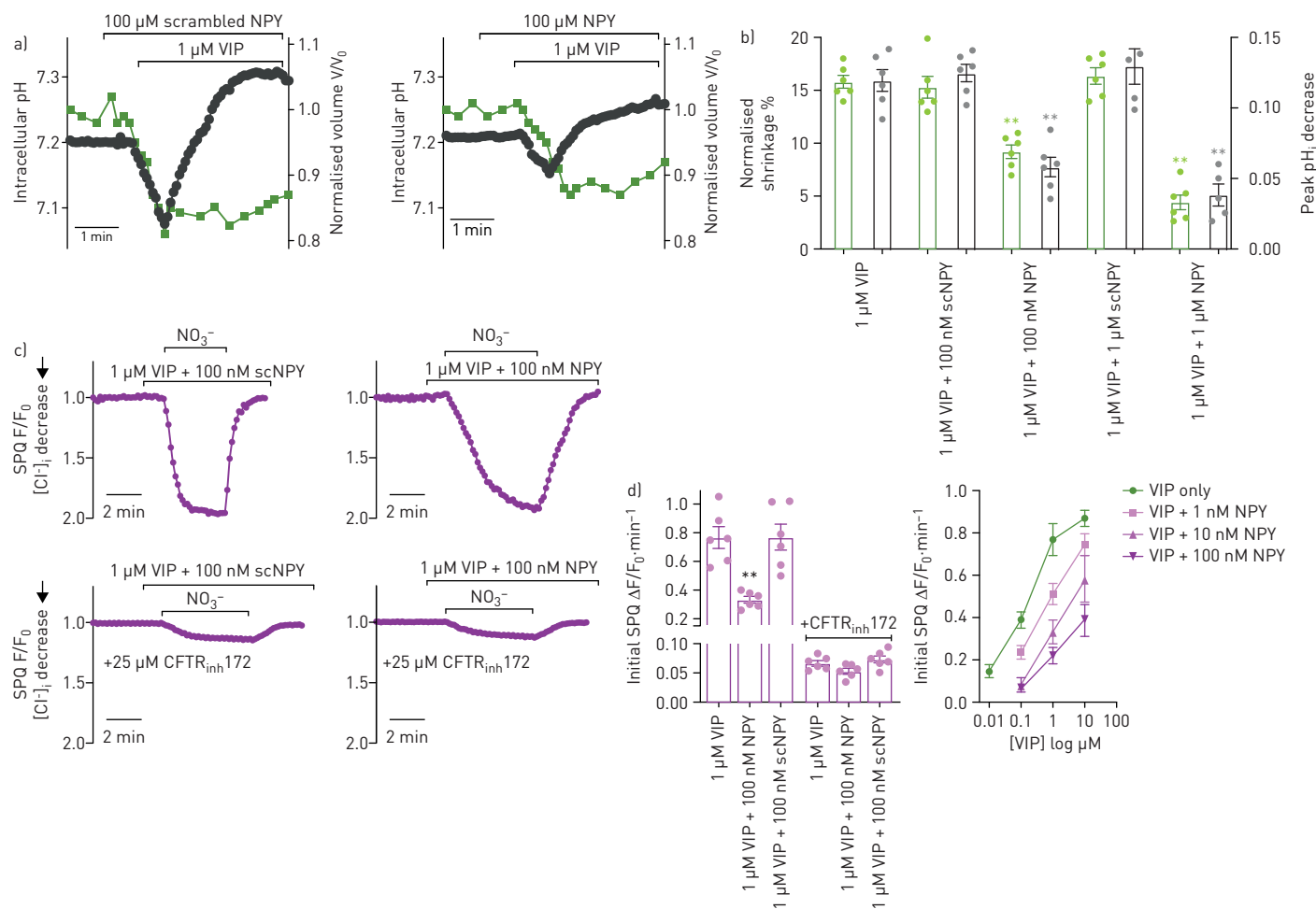
FIGURE 2 Neuropeptide Y (NPY) reduces secretory responses to vasoactive intestinal peptide (VIP) by reducing anion efflux though cystic fibrosis transmembrane receptor (CFTR) in primary nasal gland serous cells. a) Representative traces showing cell volume (green) and pH_i (grey) in cells stimulated with VIP in the presence of scrambled NPY (scNPY; left) or NPY (right). b) Bar graph showing peak responses. Cells stimulated with VIP in the presence of NPY exhibited reduced shrinkage (Cl^- secretion) and initial acidification (HCO_3^- secretion). Significance determined by 1-way ANOVA with Dunnett's post-test (VIP only as control group). **: $p<0.01$ *versus* control. c) Representative NO_3^- substitution experiments showing changes in 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) fluorescence with substitution of extracellular Cl^- for NO_3^-, which causes a decrease in $[Cl^-]_i$ and change in SPQ fluorescence. The rate of fluorescence change reflects the relative plasma membrane anion permeability. A downward deflection equals a decrease in $[Cl^-]_i$. d) Left is bar graph of initial rate of SPQ fluorescence change after VIP stimulation, which was inhibited by NPY but not scNPY. In the presence of $CFTR_{inh}172$ (10 µM), rates of SPQ fluorescence change were reduced ~10-fold and there was no effect of NPY. Right shows rates of SPQ fluorescence change over a range of VIP and NPY concentrations, showing dose dependency of VIP activation of anion permeability and NPY inhibition of anion permeability. Significance determined by 1-way ANOVA with Bonferroni post-test; a and c show representative traces, while b and d show mean±SEM from ⩾6 experiments using cells from ⩾3 patients (⩾2 experiments per patient), with **: $p<0.01$ *versus* VIP only.
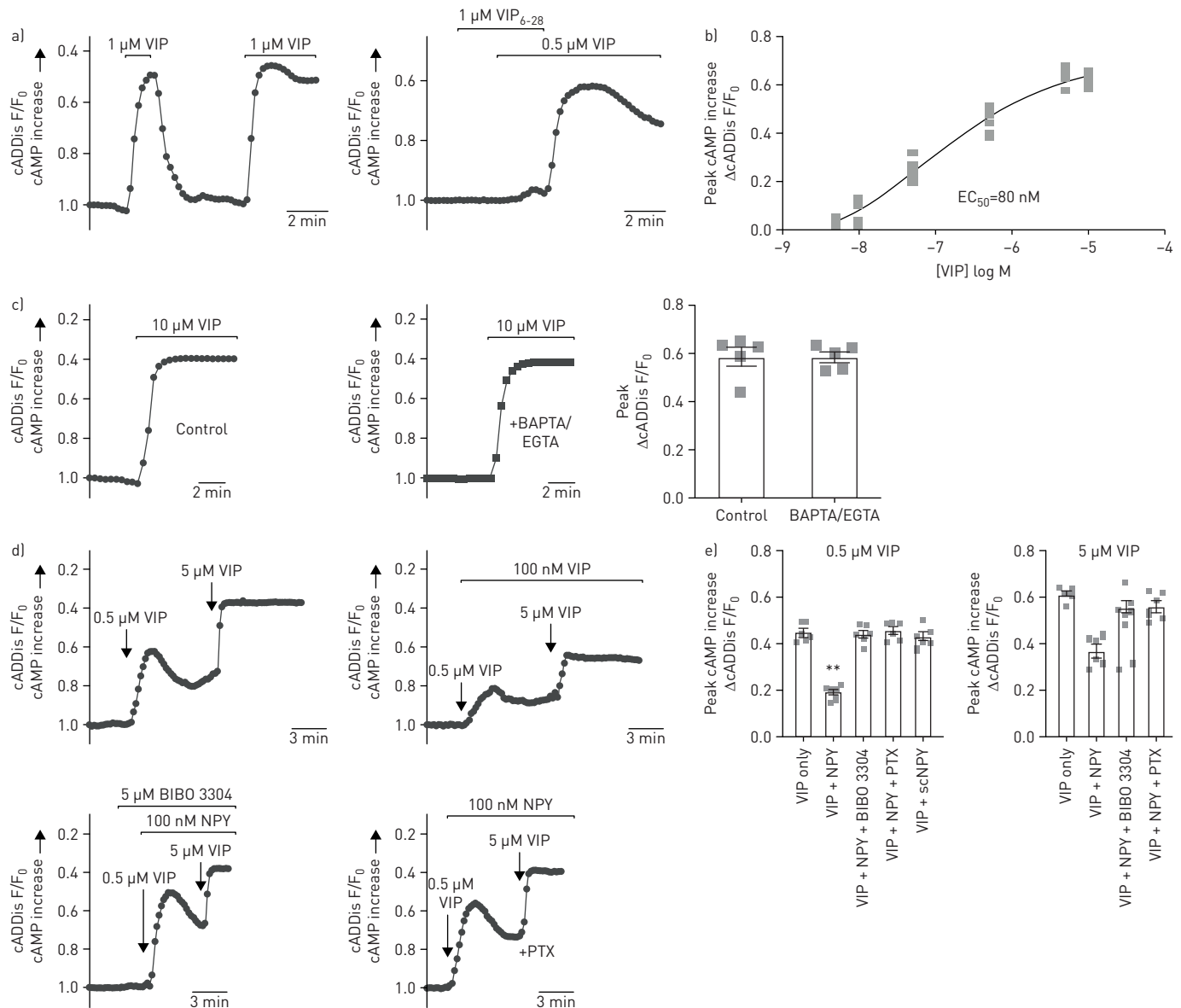
FIGURE 3 Neuropeptide Y (NPY) inhibits vasoactive intestinal peptide (VIP)-induced cAMP increases in primary nasal gland serous cells. a) Representative traces of cADDis fluorescence (upward deflection of trace=increase in cAMP) showing reversible VIP-activated cAMP increases blocked by VIP receptor antagonist VIP_{6-28}. b) Dose–response showing peak cADDis fluorescence changes with VIP. Each data point is a separate experiment; graph shows data from at $\geqslant$3 serous cells from $\geqslant$3 patients (1 experiment per patient) for each [VIP]. c) Representative traces and bar graph showing intact cADDis responses with calcium chelation by 10 µM BAPTA-AM loading (30 min) and stimulation in solution containing no added calcium+1 mM EGTA. Bar graph shows mean±SEM of 5 experiments using cells from 5 different patients (1 experiment per patient). No significant difference by Student's t-test. d) Peak cAMP responses to 0.5 µM and 5 µM VIP (top left) were inhibited by NPY (top right); NPY reduction of cAMP responses were abolished by NPY1R antagonist BIBO 3304 (5 µM; bottom left) or pertussis toxin (PTX; 6 hrs. pretreatment). e) Bar graphs showing peak responses (mean±SEM) from experiments as in D at two different [VIP]; data points from $\geqslant$6 experiments using cells from at least three patients ($\geqslant$2 experiments per patient). Significance determined by 1-way ANOVA with Dunnett's post test (VIP only as control); **: $p < 0.01$ versus VIP only.

TMEM16A- and/or pendrin-dominated secretory phenotype. However, there was no change in *SLC26A4* (encoding pendrin), *ANO1* (encoding TMEM16A) or *CFTR* expression in primary serous ALIs after 24 h IL-13 or NPY (supplementary figure S12). Up-regulation of pendrin was observed in surface epithelial cells (supplementary figure S12), fitting a lack of a role for pendrin in serous cells [36].

VIP acutely increases antimicrobial secretions and bactericidal activity while NPY reduces it

Carbonate and/or HCO_3^- have been reported to enhance antimicrobial activity of airway secretions [42]. We observed a small effect of HCO_3^- on antimicrobial activity of secretions produced by Calu-3 cells
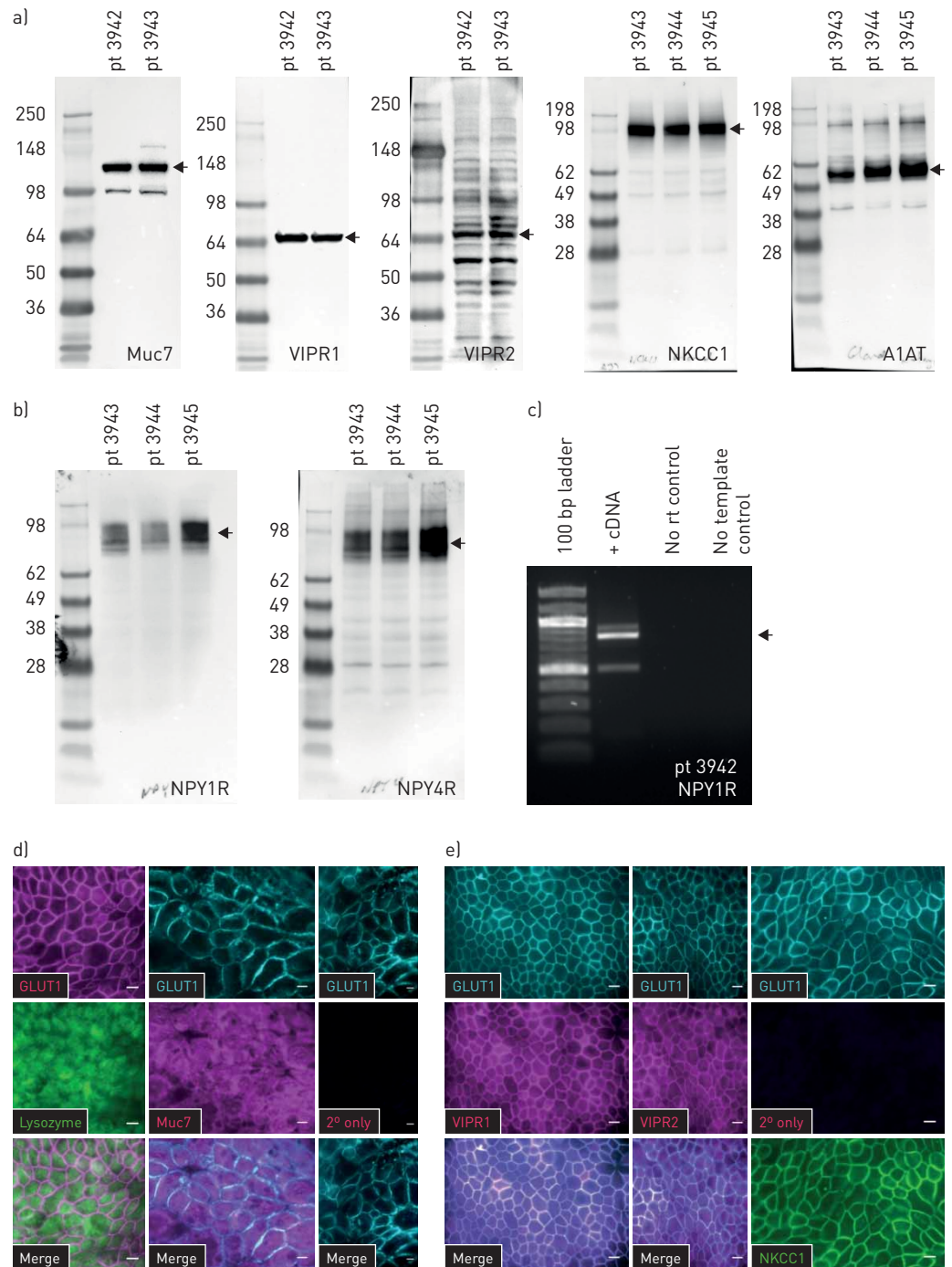
FIGURE 4 Expression of serous cell markers by primary nasal serous ALI cultures. a) Acinar cells isolated from middle turbinate were cultured and subject to Western blot for serous cell markers Muc7, vasoactive intestinal peptide (VIP) receptors VIPR1 and VIPR2, NKCC1 and α_1-antitrypsin (α_1AT). Results from cultures from 2–3 patients are shown, representative of results observed from at least three independent experiments. b) Western blot for NPY1R and NPY4R (running at the molecular weight of a dimer due to unboiled samples; see supplementary methods) in cultures from three patients (representative blot from three independent experiments). c) Representative rtPCR showing expression of mRNA for NPY1R in serous ALI; results representative of three independent experiments from three patients. d) Fixed cultures were immunostained for serous markers lysozyme and Muc7, which showed punctate cytoplasmic staining similar to serous-like secretory granules. e) Immunocytochemistry for VIPR and VIPR2 revealed lateral membrane staining similar to GLUT1 and NKCC1. All images in d and e are representative of cultures from ⩾3 separate patients. Scale bars are 20 µm.
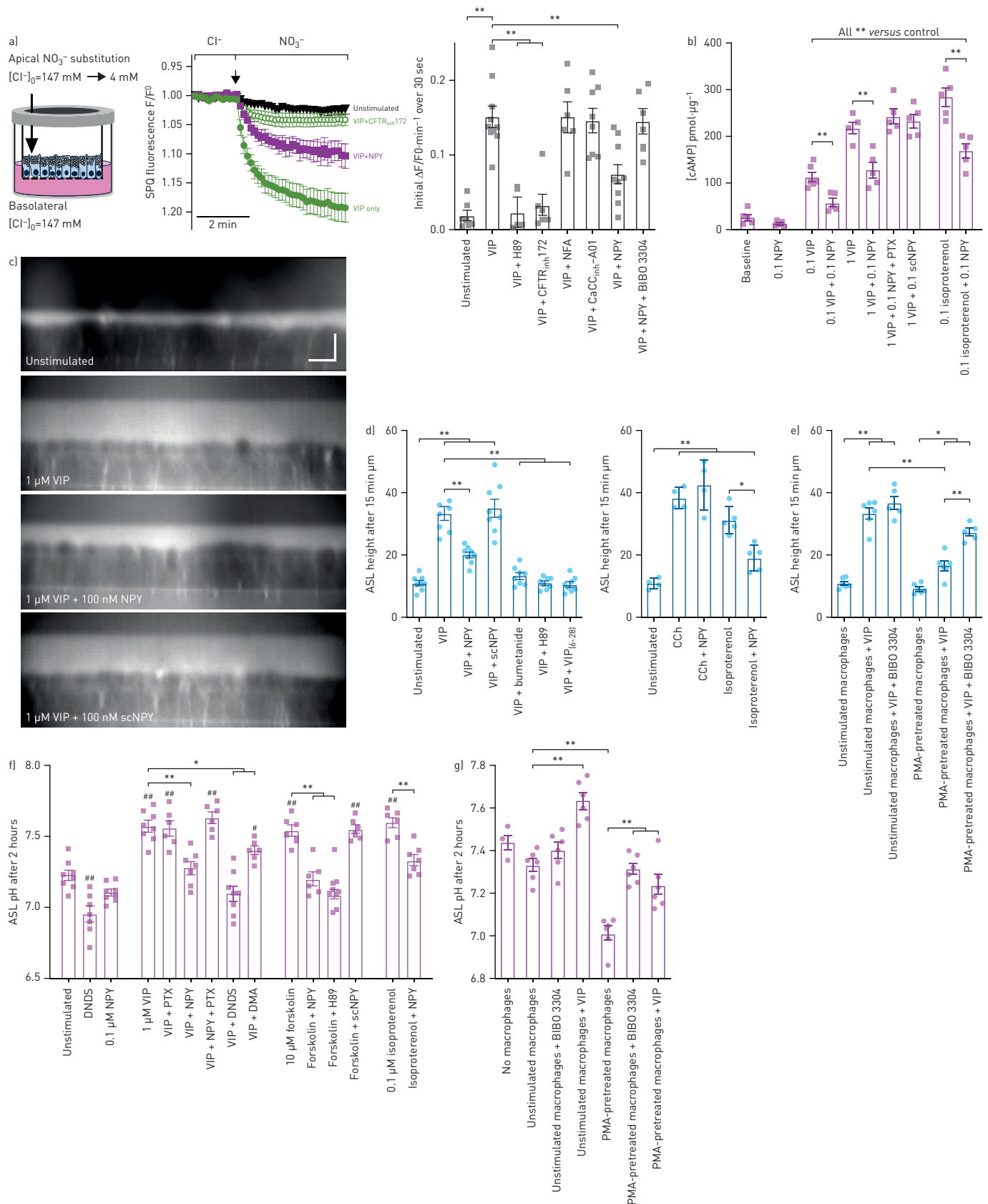
FIGURE 5 Modulation of fluid and HCO_3^- secretion by vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) in serous air–liquid interface (ALI) cultures. a) Apical NO_3^- substitution experiments (representative traces, left) and rates of SPQ change (bar graph, right) during stimulation with VIP (1 µM)±NPY (100 nM) in the presence or absence of indicated inhibitors. b) ELISA results from steady-state cAMP measurements during

stimulation with VIP or isoproterenol±NPY or scrambled NPY (scNPY). Concentrations shown are µM. c) Representative orthogonal slices of Texas red dextran-labelled airway surface liquid (ASL) in primary serous ALIs; scale bar is 10 µm in both x and z. d) ASL height after 15 min basolateral stimulation as indicated. e) Primary human macrophages were incubated for 24 hrs with or without phorbol myristate acetate (PMA), followed by washing to remove PMA and further 24 h incubation in phenol red-free media alone. ASL height was measured in ALIs incubated in the presence of the macrophages and macrophage-conditioned media with basolateral compounds added as indicated. f) ASL pH (pH$_{ASL}$) measured using SNARF-1-dextran in cultures stimulated as indicated for 2 h. As SNARF-1 is ratiometric, it is insensitive to changes in volume. Concentrations shown are µM. g) pH$_{ASL}$ in ALIs incubated in the presence of macrophages and macrophage-conditioned media as in e with basolateral compounds added as indicated. All bar graphs show mean±SEM of ⩾6 independent experiments using ALI cultures from ⩾3 patients (⩾2 cultures per patient). Significance in each bar graph determined by one-way ANOVA with Bonferroni posttest; *: $p<0.05$ and **: $p<0.01$ versus bracketed groups; and ##: $p<0.05$ and #: $p<0.01$ in f versus unstimulated conditions.

(supplementary figure S13). However, we hypothesised that NPY might have more profound effects through inhibition of both HCO_3^- secretion via CFTR and reduction of serous cell antimicrobial peptide secretion, likely mediated by a combination of constitutive and regulated vesicular release driven by both Ca^{2+} and cAMP, as in salivary and pancreatic exocrine acinar cells [43, 44]. Reductions of cAMP by NPY may lower antimicrobial secretion independent of effects on CFTR function.

Forskolin and VIP both increased secretion of serous cell antimicrobials lysozyme, Muc7 and β-defensin 1 (hβD1) over 2 h as measured by ELISA; this was reduced by NPY (figure 6a). There was no effect of inhibition of CFTR or TMEM16A on secretion of these antimicrobials (supplementary figure S14a). Neither lysozyme nor hβD1 secretion was reduced in cultures derived from CF patients (supplementary figure S14b). NPY had no effect on CCh-activated secretion of lysozyme or hβD1 over 2 h (supplementary figure S14c), supporting a specific effect of NPY on cAMP. Fitting with increased antimicrobial peptide secretion, VIP acutely increased the antibacterial effects of serous ASL washings against clinical isolates of *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA; figure 6b and c). NPY blunted the effects of VIP (figure 6b and c). A fluorescent live–dead staining of *P. aeruginosa* confirmed reduced bactericidal efficacy of NPY+VIP-stimulated ASL (supplementary figure S15).

Neither VIP nor NPY alone had longer-term (24–48 hrs) effects on expression of hβD1 by qPCR (supplementary figure S16a) despite increased secretion over 48 h (supplementary figure s16b). lipopolysaccharide (LPS) treatment up-regulated hβD2 expression and secretion (supplementary figure S16a–c), but this was not significantly affected by NPY or VIP. Lysozyme expression was significantly increased by VIP at 24 h (supplementary figure S16d), with lysozyme and Muc7 secretion also increased at 24 h (supplementary figure S16e). VIP-treated cultures exhibited more bactericidal ASL even at 48 h, reduced by NPY even in the presence of LPS (supplementary figure S16f). Thus, more chronic VIP and NPY stimulation can have longer term effects on serous cell antimicrobials.

NPY is pro-inflammatory

We hypothesised that airway gland cytokine secretion may be modulated by VIP and/or NPY. We first focussed on epithelial cell-derived cytokines involved in asthma and allergy, where alterations of VIP and/or NPY have been reported. In serous ALIs, IL-6, tumour necrosis factor (TNF)α, IL-1β, and granulocyte–macrophage colony-stimulating factor (GM-CSF) release were increased after 48 h treatment with toll-like receptor 4 (TLR4) activator LPS, TLR3 activator poly(I:C), TLR2 activator lipotechoic acid (LTA), TNFα or type 2 cytokines (IL-4+IL-13; supplementary figure S17a–d). While NPY or VIP had no effect alone on IL-6, TNFα, or GM-CSF release, NPY increased IL-1β release ~2-fold at baseline. NPY also increased IL-1β mRNA at 4 h (supplementary figure S17e)

NPY also potentiated release of these cytokines in combination with LPS, LTA, IL-4+IL-13, and TNF-α (supplementary figure S17a–d). NPY also enhanced LPS-induced IL-6 and IL-8 mRNA as well as IL-13- or TNF-α-induced GM-CSF mRNA at 4 h (supplementary figure S17f–g). NPY effects were blocked by PTX, implicating G$_i$ signalling (supplementary figure S17a–d). In contrast, VIP reduced cytokine secretion 25–50%; these reductions were eliminated by NPY (supplementary figure S17a–d). Co-stimulation with IL-4+IL-13 increased cytokines in response to either poly(I:C) or LPS, and this was enhanced further by NPY (supplementary figure S18a), suggesting that NPY is pro-inflammatory even within the context of Th2 inflammation observed in airway diseases like asthma.

NPY also enhanced cytokine release in response to heat-killed clinical isolates of *P. aeruginosa* and MRSA (figure 7a–c), which likely activate TLRs as previously mentioned. To validate results from cultured ALIs, we incubated freshly dissociated serous cells with TNFα or poly(I:C)±NPY; NPY enhanced cytokine secretion (supplementary figure S18b), confirming NPY is pro-inflammatory and may contribute to increased inflammation in airway diseases like asthma.
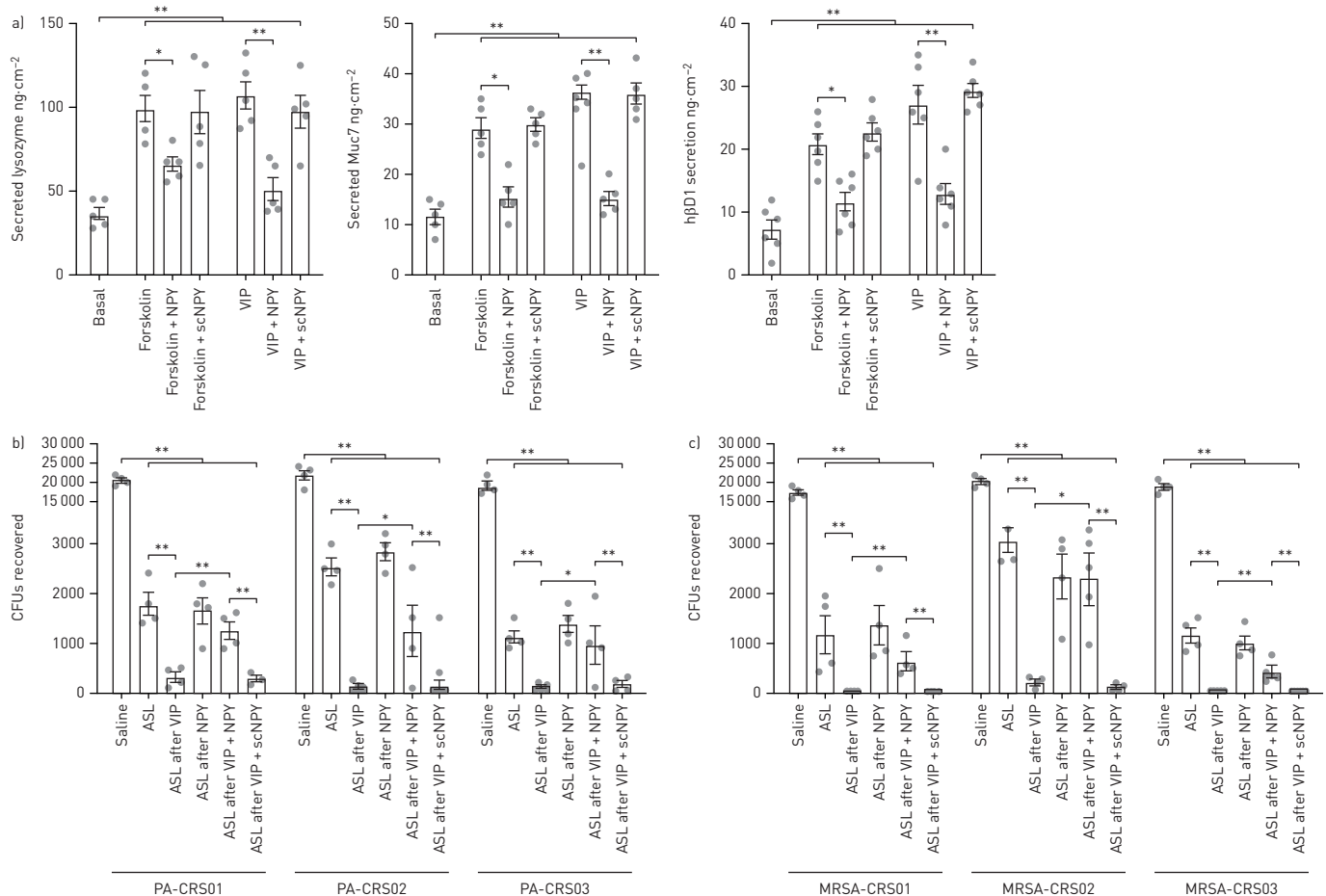
FIGURE 6 Antimicrobial peptide secretion and antibacterial efficacy of serous cells secretions are acutely enhanced by vasoactive intestinal peptide (VIP) but reduced by neuropeptide Y (NPY). a) air–liquid interface cultures (ALIs)were stimulated basolaterally (2 hrs) with forskolin (10 µM) or VIP (1 µM)±NPY (100 nM) or scrambled NPY (scNPY; 100 nM) as indicated. ASL was collected by washing the apical surface with 25% saline and assayed for lysozyme, Muc7, and hβD1 by ELISA. Results are mean±SEM from ⩾3 ALIs from ⩾3 individual patients (one ALI per patient). b-c) ASL from similar experiments was mixed with *Pseudomonas aeruginosa* (b) or MRSA (c) isolated from chronic rhinosinusitis (CRS) patients followed by incubation (2 hrs; 37°C; 5% CO$_2$), dilution, and plating for colony forming unit counting. Bar graphs show mean±SEM of ⩾5 experiments using ALIs from⩾3 different patients. **: p<0.01 and *: p<0.05 between bracketed groups. Significance determined by one-way ANOVA with Bonferroni post-test.

Anti-inflammatory effects of VIP require functional CFTR conductance; TMEM16A can substitute

In airway cells, Cl$^-$ conductance may be anti-inflammatory [45, 46], with increased [Cl$^-$]$_i$ promoting inflammation [47]. This may have implications for CF; in serous cells stimulated with VIP, [Cl$^-$]$_i$ may be higher in CF cells due to lack of CFTR-mediated efflux. We tested if CFTR contributes to anti-inflammatory effects of VIP using NPY to increase release of IL-1β, a cytokine upregulated in the lungs of some children with CF [48, 49]. NPY-induced IL-1β was not altered by CFTR$_{inh}$172 or TMEM16A activator E$_{act}$ (figure 7d). However, VIP reduced IL-1β by >50% (figure 7d). CFTR$_{inh}$172 reversed the effect of VIP, while E$_{act}$ restored effects of VIP, and the effect of E$_{act}$ was further reversed with CaCC$_{inh}$-A01 (figure 7d). We saw similar results when serous cells were stimulated with heat-killed *P. aeruginosa*. VIP reduced release of GM-CSF and IL-6, another cytokine involved in early CF inflammation [48]; this was blocked by CFTR$_{inh}$172 but subsequently restored by E$_{act}$ (figure 7e).

Thus, CFTR is required for anti-inflammatory effects of VIP, but TMEM16A can substitute. However, activation of Cl$^-$ conductance by E$_{act}$ was not sufficient for anti-inflammatory effects in the absence of VIP (figure 7e), likely because a reduction in [Cl$^-$]$_i$ requires counter-ion (K$^+$) flux activated downstream of secretagogues [33] but not activated by E$_{act}$ alone. Supporting this, K$^+$ channel activator 1-ethyl-2-benzimidazolinone (1-EBIO) [7] in combination with E$_{act}$ was anti-inflammatory in serous cells (figure 7e). VIP also reduced *P. aeruginosa*-induced release of IL-8 (figure 7f), another cytokine upregulated in CF [48, 49].
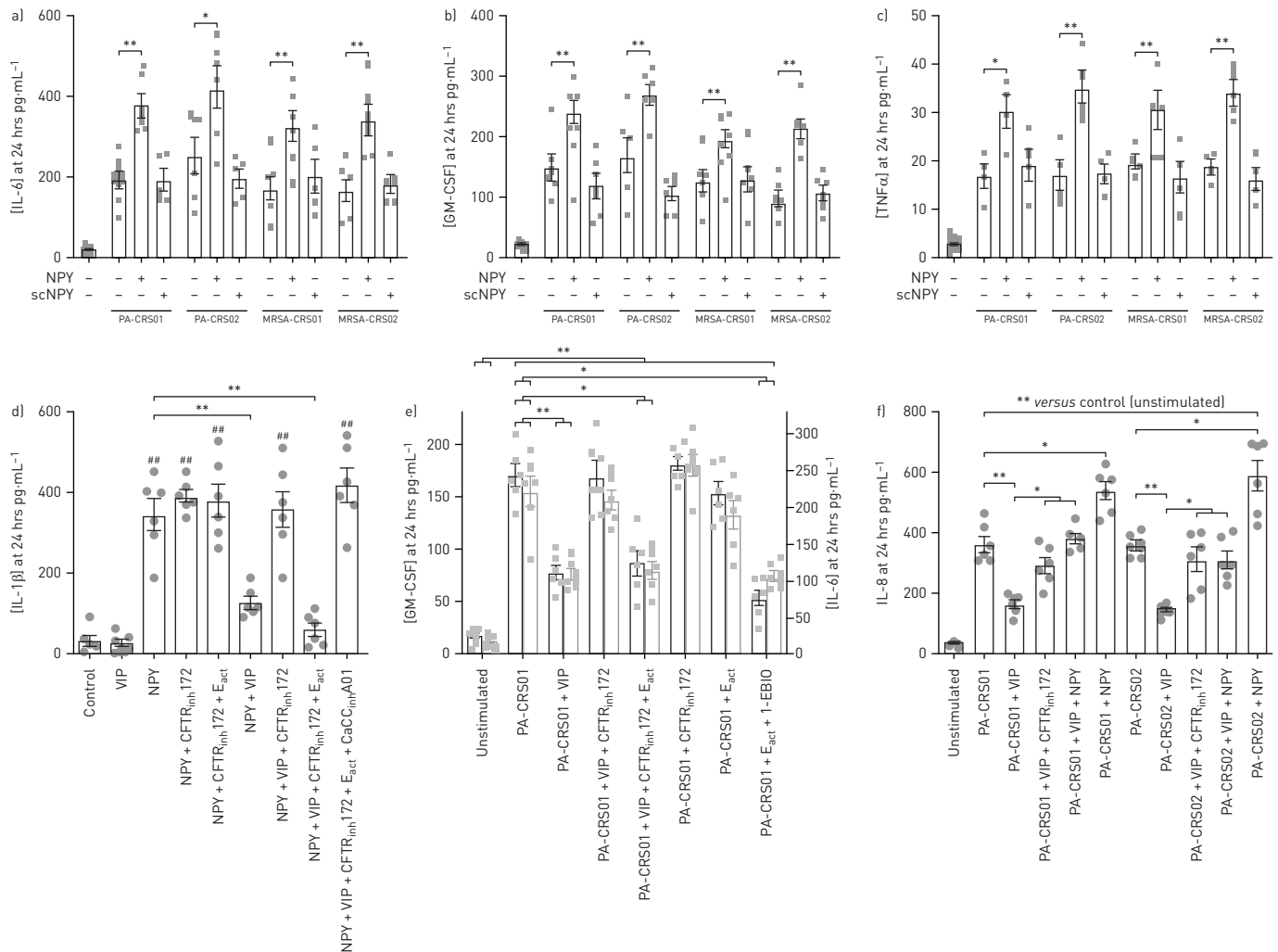
FIGURE 7 Serous cell cytokine secretion in response to bacteria is increased by neuropeptide Y (NPY) and decreased by vasoactive intestinal peptide (VIP) in a cystic fibrosis transmembrane conductance regulator (CFTR)-dependent manner. a-c) Primary serous cell air–liquid interface cultures (ALIs) were treated apically with heat-killed bacteria, followed by 24 h incubation±basolateral NPY (100 nM) or scrambled NPY (scNPY; 100 nM). Basolateral media was collected for quantification of interleukin (IL)-6 (a), granulocyte–macrophage colony-stimulating factor (GM-CSF) (b), and tumour necrosis factor (TNF)-α (c). Bar graphs shown mean±SEM of ⩾5 experiments using cells grown from ⩾3 different patients. d) Primary serous ALIs were treated basolaterally with VIP (100 μM) and/or NPY (100 nM) and treated apically with CFTR inhibitor CFTR$_{inh}$172 (15 μM), TMEM16A activator E$_{act}$ (15 μM), and/or TMEM16A inhibitor CaCC$_{inh}$-A01 (15 μM). Basolateral media was collected after 24 hrs. and assayed for IL-1β. e-f) Primary serous ALIs were treated apically with heat-killed *Pseudomonas aeruginosa*, followed by 24 hrs incubation ±basolateral NPY and/or VIP as well as ±apical CFTR$_{inh}$172 and/or E$_{act}$ and/or 1-EBIO (150 μM). Basolateral media was collected and assayed for GM-CSF (e), IL-6 (e), or IL-8 (f) by ELISA. Significance by one-way ANOVA with Bonferroni post-test comparing the three bars for each separate strain in a-c and comparing bracketed bars in d-e. **: p<0.01; *: p<0.05.

Discussion

This study suggests serous cells secrete HCO_3^- in addition to Cl^- during VIPergic stimulation directly through CFTR (figure 8a). NPY impairs both VIPergic fluid and antimicrobial peptide secretion by reducing cAMP signalling (figure 8b). The novel inverse relationship between NPY and VIP in the regulation of secretion suggests that the balance of these neuropeptides affects mucus rheology by promoting or inhibiting Cl^- and HCO_3^- secretion from serous cells, which control the hydration of mucins secreted by more proximal gland mucous cells.

This study reveals several insights relevant to CF pathogenesis (figure 8c). We found no evidence of Cl^-/HCO_3^- exchange (*e.g.* pendrin) activity in serous cells, suggesting loss of CFTR function directly contributes to impaired HCO_3^- secretion. Targeting CFTR *via* correctors and/or potentiators would restore serous cell HCO_3^- and Cl^- secretion independent of other proteins. Moreover, cAMP signalling was intact in CF cells, suggesting that appropriate pharmacological correction of mutant CFTR could restore secretion in response to the appropriate endogenous physiological stimuli (*e.g.* VIP). In CF patients that cannot benefit from CFTR correction (*e.g.* premature stop codon mutations), activation of TMEM16A
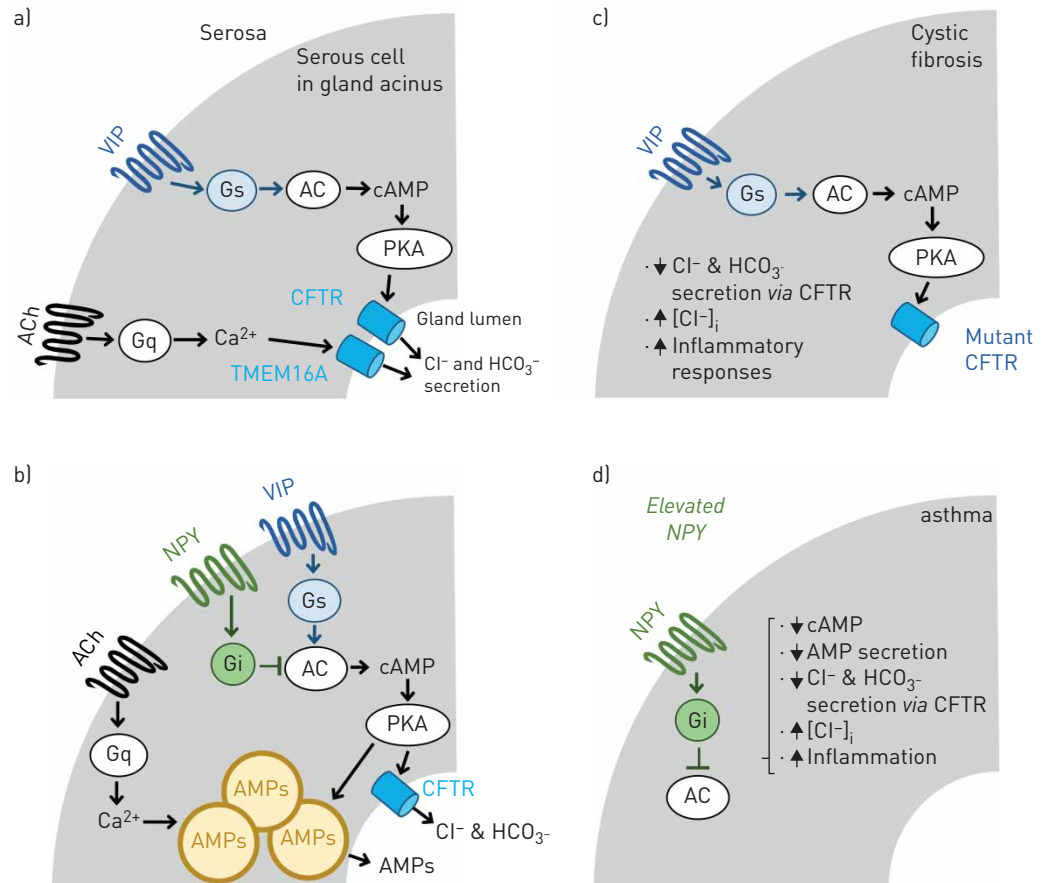
FIGURE 8 Model of vasoactive intestinal peptide (VIP)-ergic and neuropeptide Y (NPY)-ergic regulation of serous cell function and implications for airway diseases. a) Activation of VIP receptors on serous cells allows $G\alpha_S$ (Gs) activation of adenylyl cyclase (AC), elevation of cAMP, and Cl^- and HCO_3^- efflux through protein kinase A (PKA)-activated cystic fibrosis transmembrane conductance regulator (CFTR). In contrast, activation of muscarinic receptors by acetylcholine (ACh) stimulates $G\alpha_q$ (Gq)-dependent calcium (Ca^{2+}) elevation and Cl^-/HCO_3^- secretion through TMEM16A (based on data here and [3, 7]). While regulated by two independent pathways, our data suggest CFTR and TMEM16A are functionally equivalent anion efflux pathways. A result of Cl^- efflux (and parallel K^+ efflux, not shown [7]) is a reduction intracellular Cl^- concentration ($[Cl^-]_i$) b) NPY receptors, in contrast, activate $G\alpha_i$ (Gi) proteins to inhibit AC and reduce VIP-activated cAMP responses. This blunts both the CFTR-mediated anion secretion as well as VIP-activated antimicrobial peptide (AMP) secretion, likely vesicular secretion. NPY has no effects on cholinergic anion or AMP secretion because it is driven by Ca^{2+} rather than cAMP. c) We hypothesise that, in cystic fibrosis, VIP cannot elicit anion and fluid secretion via CFTR. Also, since $[Cl^-]_i$ remains elevated, inflammatory responses may be increased. d) In asthma, elevated NPY may increase Gi activity to blunt cAMP responses downstream of VIP, reducing CFTR activity and fluid secretion as well as vesicular AMP secretion. NPY also has pro-inflammatory effects.

could also restore Cl^-/HCO_3^- efflux. As CFTR may regulate other channels and transporters like the epithelial Na^+ channel or pendrin in surface epithelial cells [36, 40, 41], TMEM16A activation may not fully replace CFTR. However, this study and previous work [6] suggest TMEM16A can support levels of Cl^- and HCO_3^- efflux from serous cells equivalent to CFTR.

These results also reveal potential pathophysiological mechanisms in obstructive airway diseases like asthma (figure 8d). Elevated VIP in allergic rhinitis may promote watery serous secretions through elevated fluid and HCO_3^- secretion to thin mucus. However, under conditions of increased NPY (e.g. in asthma), the ability of VIP to stimulate fluid and HCO_3^- secretion through CFTR and antimicrobial peptide secretion is impaired due to a blunting of cAMP signalling. NPY also decreases airway ciliary beat frequency [50], which may further impair mucociliary clearance and innate defence. Increased inflammation via elevated NPY may exacerbate these effects. In summary, our data suggest that in some asthma, COPD or CRS patients, NPYR1 antagonists may be useful to thin secreted mucus, enhance antimicrobial secretion, and/or reduce inflammation.

While elevated NPY has not been reported in CF lungs, one study did suggest elevated NPY in olfactory epithelium of CFTR knockout mice [51]. It may be possible that NPY plays a role in a subset of CF

patients; skewing toward at Th17 or Th2 profile may be a risk factor for *P. aeruginosa* infection in CF lungs [52]. NPY might be elevated in these patients. NPY would not be expected to substantially affect ion transport in CF serous cells as the entire pathway is already absent due to loss of CFTR function; however, NPY could still reduce antimicrobial secretion or promote inflammation. A role for NPY in CF lungs and potential therapeutic value of NPYR inhibition requires further investigation. Regardless of NPY's relevance to CF pathophysiology, increased mucus viscosity in CF and asthma may share, at least in part, common mechanisms of reduced CFTR function. In CF, this is *via* direct CFTR mutation. In asthma, reduced CFTR-mediated secretion due to elevated NPY and blunted cAMP signalling may contribute to poorly hydrated gland mucus independent of direct CFTR defects.

The important contribution of exocrine cells to inflammation is established in parotid and pancreatic acini within the context of Sjögren's syndrome and pancreatitis, respectively [53]. However, this is less studied in the airway. Bronchial gland volume may be $\geqslant$50-fold the volume of goblet cells [3]. Gland acini are likely significant contributors of cytokines [54], particularly when gland hypertrophy and hyperplasia occur during COPD and asthma [3]. Our data support previous observations [45, 46] that the Cl^- channel activity of CFTR is anti-inflammatory during VIP stimulation, and may contribute to hyperinflammatory phenotypes reported in CF [45, 46]. Exocrine acinar cells accumulate Cl^- above electrochemical equilibrium ($\geqslant$65 mM $[Cl^-]_i$) [7] to support their dedicated fluid-secreting role. VIP stimulation lowers $[Cl^-]_i$ ($\sim$30 mM) in serous cells *via* KCl efflux through CFTR and K^+ channels. Changes in serous cell intracellular $[Cl^-]$ during stimulation are likely greater than the changes in surface epithelial cells, where resting intracellular $[Cl^-]_i$ is lower ($\leqslant$40 mM). Thus, the pro-inflammatory effects of elevated $[Cl^-]_i$ may be more pronounced in serous cells, increasing inflammation in the absence of CFTR function. Similar to Cl^- and HCO_3^- secretion, our data suggest that activation of TMEM16A could compensate for loss of CFTR, suggesting possible anti-inflammatory benefit to targeting TMEM16A in glands of patients who cannot utilise CFTR potentiator/corrector therapies.

Interestingly, NPY increased cytokine production during co-stimulation with TLR agonists, but it had less effect on β-defensin 2, which is also regulated by TLR-stimulated nuclear factor (NF)κB. This may suggest that NPY potentiates TLR-induced cytokine secretion *via* a non-NFκB mechanism, supported by the observation that NPY alone did not induce IL-6 or IL-8. Future work is needed to more fully dissect out the molecular details of NPYR signalling pathway in airway cells, including activated transcription factors.

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