British Journal of Pharmacology (2004) 141, 698–708 © 2004 Nature Publishing Group All rights reserved 0007–1188/04 $25.00
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Activation of $VPAC_1$ receptors by VIP and PACAP-27 in human bronchial epithelial cells induces CFTR-dependent chloride secretion

[1,2]Renaud Dérand, [1,2]Alicia Montoni, [1]Laurence Bulteau-Pignoux, [1]Thierry Janet, [1]Bertrand Moreau, [1]Jean-Marc Muller & *,[1]Frédéric Becq

[1]Laboratoire des Biomembranes et Signalisation Cellulaire, UMR 6558 CNRS, Université de Poitiers, 40 Avenue du Recteur Pineau, 86022 Poitiers, France

1 In the human airway epithelium, VIP/PACAP receptors are distributed in nerve fibers and in epithelial cells but their role in transepithelial ion transport have not been reported. Here, we show that human bronchial epithelial Calu-3 cells expressed the $VPAC_1$ receptor subtype which shares similar high affinity for VIP and PACAP-27.

2 The stoichiometric binding parameters characterizing the ^{125}I-VIP and ^{125}I-PACAP-27 binding to these receptors were determined.

3 We found that VIP ($EC_{50} \approx 7.6$ nM) and PACAP-27 ($EC_{50} \approx 10$ nM) stimulated glibenclamide-sensitive and DIDS-insensitive iodide efflux in Calu-3 cells.

4 The protein kinase A (PKA) inhibitor, H-89 and the protein kinase C (PKC) inhibitor, chelerythrine chloride prevented activation by both peptides demonstrating that PKA and PKC are part of the signaling pathway. This profile corresponds to the pharmacological signature of CFTR.

5 In the cystic fibrosis airway epithelial IB3-1 cell lacking functional CFTR but expressing $VPAC_1$ receptors, neither VIP, PACAP-27 nor forskolin stimulated chloride transport.

6 Ussing chamber experiments demonstrated stimulation of CFTR-dependent short-circuit currents by VIP or PACAP-27 applied to the basolateral but not to the apical side of Calu-3 cells monolayers.

7 This study shows the stimulation in human bronchial epithelial cells of CFTR-dependent chloride secretion following activation by VIP and PACAP-27 of basolateral $VPAC_1$ receptors.
British Journal of Pharmacology (2004) **141,** 698–708. doi:10.1038/sj.bjp.0705597

Keywords: CFTR chloride channel; VIP; PACAP-27; VPAC1 receptor; Calu-3

Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FSK, forskolin; PAC_1, PACAP receptor; PACAP, pituitary adenylate cyclase-activating peptide; PHM, peptide histidine methionine; PKA, protein kinase A; PKC, protein kinase C; $VPAC_1$ and $VPAC_2$, VIP/PACAP receptors 1 and 2; VIP, vasoactive intestinal polypeptide

Introduction

The vasoactive intestinal polypeptide (VIP) is a 28-amino-acid neuropeptide of the inhibitory nonadrenergic, noncholinergic nervous system in mammalian airways (Maggi *et al*., 1995). VIP is a member of a super-family of structurally related peptides including glucagon, glucagon-like peptides, secretin, exendin, pituitary adenylate cyclase-activating peptide (PACAP) and others (Hoyle, 1998). PACAP exists in two amidated forms (PACAP-27 and PACAP-38) and has 68% sequence homology with VIP. Three types of seven transmembrane VIP/PACAP receptors coupled to G proteins have been cloned and characterized: they are named $VPAC_1$, $VPAC_2$ and PAC_1 (Sreedharan *et al*., 1991; Harmar *et al*., 1998; Laburthe & Couvineau, 2002). PAC_1 is the PACAP-specific receptor. $VPAC_1$ and $VPAC_2$ are common receptors for PACAP and VIP, coupled to adenylate cyclase (Laburthe & Couvineau, 2002). In the human lung, VIP/PACAP receptors have been identified by RT–PCR, autoradiographic ligand binding studies, immunohistochemical localization and distribution (Linden *et al*., 1997; Busto *et al*., 1999; Busto *et al*., 2000; Groneberg *et al*., 2001a).

In the digestive tract, VIP is one of the principal physiological regulator of water and electrolytes secretion, especially in the intestine (Waldman *et al*., 1977; Chang & Rao, 1991; Nguyen *et al*., 1992; Leung *et al*., 2001; Izu *et al*., 2002) and pancreas (Ito *et al*., 1998). One plasma membrane protein appears to orchestrate these regulated secretory processes: the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a chloride channel responsible for the transepithelial chloride transport in airway, exocrine pancreas, intestine and others (Gray *et al*., 1988; Riordan *et al*., 1989; Tabcharani *et al*., 1991; Gadsby & Nairn, 1999; Quinton, 1999). CFTR is physiologically regulated by various substances, including secretin and VIP in the intestine and the exocrine pancreas (Gray *et al*., 1988; Becq *et al*., 1993; Ameen *et al*., 1999). Its channel activity is controlled by protein

*Author for correspondence; E-mail: frederic.becq@univ-poitiers.fr
[2]Contributed equally to the work.
Advance online publication: 26 January 2004

kinase-dependent phosphorylation and is gated by ATP hydrolysis at two distinct domains (NBD 1 and 2) (Tabcharani *et al*., 1991; Gadsby & Nairn, 1999). The control by VIP and PACAP-27 of CFTR-dependent chloride transport have not been reported previously in the airway. Here, we report on the expression of basolateral $VPAC_1$ receptors in the human bronchial epithelial Calu-3 cells, and the stimulation by VIP and PACAP-27 of CFTR-mediated chloride transport.

Methods

Cell culture

Calu-3 (ATCC), a cell line of human pulmonary origin (Shen *et al*., 1994), was cultured at 37°C in 5% CO_2 and maintained in DMEM Ham's F12 Nutritif Mix (1 : 1) supplemented by 10% fetal calf serum and 1% antibiotics (50 IU ml^{-1} penicillin and 50 $\mu g\,ml^{-1}$ streptomycin). IB3-1 (delF508/W1282X) were generously given by Dr P. Zeitlin (Zeitlin *et al*., 1991) and routinely cultured at 37°C in 5% CO_2 incubators in LHC-8 medium (Biofluids, Inc., Rockville, MO, U.S.A.) supplemented with 10% fetal bovine serum and 100 IU ml^{-1} penicillin/streptomycin (Bulteau *et al*., 2000).

RT–PCR analysis

Total RNA from Calu-3 and IB3-1 cells was extracted according to the Trizol Reagent method (Life Technologies, InVitrogen). RT–PCR was performed on 2.0 μg of total RNA using Ready-to-Go RT–PCR beads (Amersham Biosciences). The protocol used was the one-step protocol provided by the manufacturer. Reverse transcription and amplification involved the following steps: 30 min at 42°C, 10 min at 95°C and 35 PCR cycles (1 min at 95°C, 1 min at 48°C, 2 min at 72°C) followed by 10 min at 72°C. The different sense and antisense primers used for the amplification of human $VPAC_1$, $VPAC_2$ and PAC_1 receptors were respectively: $VPAC_1$ receptor: sense primer, 5′-TGT TCT ACG GTT CTG TGA AGA-3′; antisense primer, 5′-AGC ACC CAT AAT CCT CAA AAT-3′; $VPAC_2$ receptor: sense primer, 5′-AGC AAA GCA GGA AAC ATA AGC-3′; antisense primer, 5′-TAG AGA ACG TCG TCC TTG ACC-3′; PAC_1 receptor: sense primer, 5′-CTC TGC TGG TGG AGA CCT TC-3′; antisense primer, 5′-CCA CAG AGC TGT GCT GTC AT-3′. The expected sizes of the amplified PCR fragments were 447, 302 and 161 bp, respectively. Positive controls have been performed for: $VPAC_1$ with human H9 lymphoblastoma; $VPAC_2$ and PAC_1 with human IMR32 neuroblastoma cell lines. Negative control consisted of heat-inactivated reverse transcriptase. The PCR fragments were controlled by sequencing (ABI Prism Big Dye Terminator Cycle Sequencing ready Reaction Kit, P.E. Applied Biosystems). The 1 kb molecular weight markers was from Life Technologies, InVitrogen.

Peptide radioiodination

For binding experiments, VIP and PACAP-27 were radioiodinated using the chloramine-T technique as previously described (Martin *et al*., 1986) with slight modifications. Briefly, 0.5 mCi of ^{125}I-Na solution (~5 μl, NEN, Boston, MA, U.S.A.) were mixed to 15 μl of a 10^{-4} M aqueous solution of VIP or PACAP-27 (Neosystem, France). The reaction was initiated by adding 25 μl of chloramine-T (Fluka, France) at 3 mg ml^{-1} in a 0.2 M sodium phosphate buffer (pH 7.6), then stopped after 1 min incubation at room temperature with 25 μl of sodium metabisulfite (2 mg ml^{-1} in 0.2 M sodium phosphate buffer, pH 7.6). The radioiodination mix was first eluted from C_{18} SEP PACK (Waters Associates, Ireland) with 85% acetonitrile in H_2O-0.1% trifluoroacetic acid (TFA), after a rinsing in 10 ml H_2O-0.1% TFA to remove the free radioiodine. The sample containing radioiodinated peptides was separated by reversed-phase HPLC (Spectraphysics, France), using a 5 μm VYDAC C_{18} column (Interchrom, France). Elution was conducted for 27 min with 0–85% linear gradient of acetonitrile in H_2O-0.1% TFA. Fractions corresponding to the monoiodinated forms (specific activity, 2200 mCi $mmol^{-1}$) of VIP or PACAP-27 typically giving high specific binding were pooled. Acetonitrile was evaporated under nitrogen and the resulting sample divided into aliquots and stored at −20°C.

Receptor binding studies

Binding studies were performed on intact nonpolarized cells, according to conditions previously reported (with slight modifications) (Muller *et al*., 1985). Briefly, 150,000 cells were seeded in 24-well dishes 3 days before experiments. Then, cells were incubated for 2 h at 13°C, in presence of 30 pM of ^{125}I-VIP or ^{125}I-PACAP-27 and increasing concentration of VIP or PACAP-27, in Dulbecco's modified Eagle medium with 15 mM HEPES (pH 7.4), containing 1% BSA, 0.1% bacitracin and 150 μM phenylmethansulfonide fluoride (PMSF). Binding reactions were stopped by cooling dishes on ice. Cells were rinsed three times with 1 ml cold PBS-0.1% BSA and lysed in 500 μl of 0.5 N NaOH. Radioactivity in the cell lysates was quantified using a γ counter (Cobra II, Packard, IL, U.S.A.). Fitting of the data by nonlinear regression was computed according to either a Hill or a two-site competition inhibition equation, using the Graphpad™ software.

Iodide efflux experiments

CFTR chloride channel activity was assayed by measuring the rate of iodide (^{125}I) efflux as previously described (Bulteau *et al*., 2000; Dormer *et al*., 2001). All experiments were performed at 37°C on nonpolarized cells cultured in 24-well plates to perform parallel experiments and comparison analysis. At the beginning of each experiment, cells were washed with efflux buffer containing (in mM): 137 NaCl, 5.36 KCl, 0.8 $MgCl_2$, 5.5 glucose and 10 HEPES, pH 7.4. Cells were then incubated in efflux buffer containing 1 μM KI and 1 μCi $Na^{125}I.ml^{-1}$ (NEN, Boston, MA, U.S.A.) for 30 min at 37°C to permit the ^{125}I to reach equilibrium. After washing cells with iodide-free efflux medium to remove extracellular ^{125}I, the loss of intracellular ^{125}I was determined by removing the medium with efflux buffer every 1 min for up to 10 min. The first four aliquots were used to establish a stable baseline in efflux buffer alone. A medium containing the test drug was used for the remaining aliquots. Residual radioactivity was extracted with 0.1 N NaOH, and determined using a gamma counter (Cobra II, Packard, IL, U.S.A.). The fraction of initial intracellular ^{125}I lost during each time point was determined and time-dependent rates of ^{125}I efflux were calculated from $\ln(^{125}I_{t1}/^{125}I_{t2})/(t_1-t_2)$, where $^{125}I_t$ is the intracellular ^{125}I at time

t, and t_1 and t_2 successive time points (Bulteau *et al*., 2000; Dormer *et al*., 2001). Curves were constructed by plotting rate (k) of ^{125}I *versus* time. Relative rates (R) were determined which correspond to: k_{peak}/k_{basal}. All comparisons were based on maximal values for the time-dependent rates excluding the points used to establish the baseline (Bulteau *et al*., 2000; Dormer *et al*., 2001). In experiments using the chloride transport inhibitors DIDS and glibenclamide or the protein kinase inhibitors H89 and chelerythrine chloride, these agents were present in the loading solution and in the efflux buffer.

Short-circuit current (I_{sc}) measurements

Calu-3 cells were seeded on Snapwell permeable supports (Corning Costar) at a density of 5×10^5 cells cm^{-2}. On day 1, the medium bathing the apical surface was removed to establish an air interface. After $\sim$10 days, the cells formed a confluent monolayer. Short-circuit current measurements were performed after an additional 14 days in culture. The inserts were mounted in a modified Ussing chamber (Phymep, France) filled on the basolateral side with 5 ml of a Krebs bicarbonate solution containing (in mM): 120 NaCl, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 25 $NaHCO_3$, 10 glucose. On the apical side, 10 mM mannitol was added instead of glucose to avoid activation of the apical electrogenic Na^+-glucose cotransporter (Singh *et al*., 1997). During the experiments, this solution was kept at 37°C and continuously bubbled with 5% CO_2, 95% air. The epithelium was short-circuited with a voltage-clamp (EC-825, Warner Instrument) connected to apical and basolateral chambers with Ag-AgCl electrodes. The junction potential difference and the fluid resistance between potential sensing electrodes were compensated. Because Calu-3 cells occasionally showed some amiloride-sensitive Na^+ current (Bulteau *et al*., 2000), all experiments were performed in the presence of 10 μM amiloride in the apical solution to remove Na^+ transport.

Chemicals

Forskolin, glibenclamide, DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), H-89 (*N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide), chelerythrine chloride (1,2-dimethoxy-*N*-methyl-[1,3]-benzodioxolo[5,6-c]phenantridinium chloride) were from Sigma Chemicals (St Louis, MO, U.S.A.). Neuropeptides were from Neosystem and Chloramine T from Fluka. All other products were from Sigma (St Louis, MO, U.S.A.), except αMEM and Dulbecco's MEM/Ham Nutritif Mix F12 from Fisher PAA and Gibco BRL. The vehicles for all neuropeptides (water) and for other drugs (dimethyl sulfoxide, DMSO, final DMSO concentration: 0.1%) have no effect on either binding, basal iodide efflux or transepithelial currents.

Statistics

Results are expressed as means ± s.e. of n observations. Sets of data were compared with either an analysis of variance (ANOVA) or Student's t-test. Differences were considered statistically significant when $P<0.05$. ns: nonsignificant difference, $*P<0.05$, $**P<0.01$, $***P<0.001$. All statistical tests were performed using GraphPad Prism version 3.0 for Windows (Graphpad Software, San Diego, CA, U.S.A.).

Results

Molecular identification of VIP/PACAP receptors in human Calu-3 and IB3-1 cells

To examine VIP/PACAP receptors mRNA expression in the human bronchial epithelial Calu-3 cells, we analyzed total RNA *via* reverse transcriptase–polymerase chain reaction (RT–PCR). We discriminated between $VPAC_1$, $VPAC_2$ and PAC_1 receptors variants using appropriate primers. Results shown in Figure 1 demonstrate the expression of only one type of receptors in Calu-3 cells; the $VPAC_1$ subtype (lane 2, compared to the positive and negative controls, lanes 1 and 3, respectively). We did not detect PCR products for $VPAC_2$ (lane 5) or PAC_1 (lane 8). Positive and negative controls for $VPAC_2$ and PAC_1 are shown in Figure 1, lanes 4, 6, 7, 9, respectively). The 447 bp PCR band sequence corresponds to that of the $VPAC_1$ receptor (data not shown).

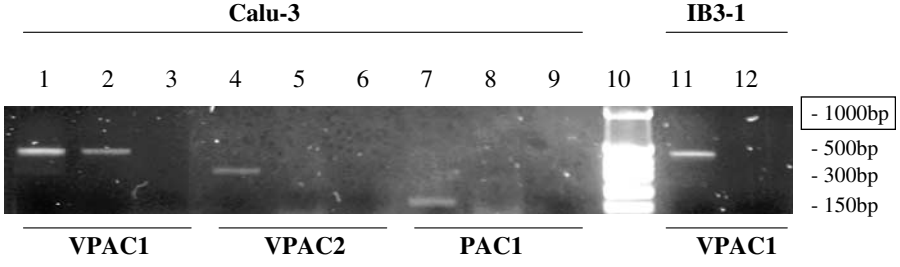

Figure 1 Analysis of the expression by RT–PCR of $VPAC_1$, $VPAC_2$ and PAC_1 receptors in the human bronchial epithelial non CF (Calu-3) and CF (IB3-1) cell lines. RT–PCR experiments for $VPAC_1$ (lane 1, Calu-3 and lane 11, IB3-1), $VPAC_2$ (lane 5, Calu-3), PAC_1 (lane 8, Calu-3) receptors. Positive controls correspond to experiments performed on human cell lines: lane 1 (H9 lymphoblastoma, $VPAC_1$); lane 4 (IMR32 neuroblastoma, $VPAC_2$); lane 7 (IMR32 neuroblastoma, PAC_1). Negative controls correspond to heat-inactivated reverse transcriptase: lane 3 (Calu-3, $VPAC_1$), lane 6 (Calu-3, $VPAC_2$), lane 9 (Calu-3, PAC1) and lane 12 (IB3-1, $VPAC_1$). Lane 10: 1 kb molecular weight markers. Total RNA extraction and RT–PCR experiments were performed as described in experimental procedures. Half of the final reactions (25 μl) were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The expected sizes are 447 bp ($VPAC_1$), 302 bp ($VPAC_2$) and 161 bp (PAC_1).

Pharmacological characterization of $VPAC_1$ receptors in Calu-3 cells

Analysis of the pharmacological properties of $VPAC_1$ receptors and of the VIP and PACAP-27 binding sites of Calu-3 cells have been performed with the following experiments. We measured inhibition of the radioligands ^{125}I-VIP or ^{125}I-PACAP-27 binding by unlabeled VIP, PACAP-27, PACAP-38 or secretin. Experiments in which the unlabeled competitor molecule was the same as the radioligand was used to obtain the typical pharmacological parameters: affinity (IC_{50}) and maximal binding capacity (B_{max}). These binding experiments were conducted on intact nonpolarized cells grown in 24-well culture dishes. High-affinity interaction was observed for both radioligands (Figure 2 and Table 1). The total specific binding was 682 c.p.m. for VIP and 627 c.p.m. for PACAP-27. The pharmacological profile of the ^{125}I-VIP binding sites was further analyzed with VIP, PACAP-27, PACAP-38 and secretin as competitors (Figure 2a and Table 1).

In Figure 2a, the maximal percentage (100%) corresponds to the absence of ^{125}I-VIP displacement by unlabeled peptides whereas 0% corresponds to total ^{125}I-VIP displacement by VIP. One single site was obtained for VIP binding ($IC_{50} = 1.1 \pm 0.34$ nM, $n = 8$; $B_{max} = 58{,}421$ c.p.m.). VIP and PACAP-27 have similar affinities ($IC_{50} = 1.1 \pm 0.34$ and 2.3 ± 0.66 nM, respectively, $n = 8$ each) and similar binding capacities for the VIP-binding sites. Different results were obtained with secretin and PACAP-38. The total ^{125}I-VIP displacement by secretin was below 0%, as shown in Figure 2a, which corresponds to an higher binding capacity for secretin on VIP binding sites as compared to VIP or PACAP-27. The corresponding affinity of secretin (calculated from Figure 2a) for VIP-binding sites was 26.8 ± 3.1 pM ($n = 8$). With PACAP-38, we found a lower affinity ($IC_{50} = 24.7 \pm 2.6$ nM, $n = 8$) but a higher binding capacity (as for secretin) when compared to VIP or PACAP-27 interactions (Figure 2a).

Similarly, the pharmacological profile of the ^{125}I-PACAP-27 binding sites was analyzed using the same four peptides (Figure 2b, c and Table 1). In Figure 2b, the maximal percentage (100%) corresponds to the absence of ^{125}I-PACAP-27 displacement by unlabeled peptides, whereas 0% corresponds to total ^{125}I-PACAP-27 displacement by PACAP-27. The competition–displacement curve for PACAP-27 (Figure 2b and c) was best described by a two-sites model: a high-affinity site ($IC_{50} = 5 \pm 0.52$ pM, $n = 8$; $B_{max} = 2108$ c.p.m.) and a low-affinity site ($IC_{50} = 1.6 \pm 0.61$ nM, $n = 8$; $B_{max} = 3297$ c.p.m.). VIP interacted with both binding sites ($IC_{50} = 8.3 \pm 1.4$ pM and 0.6 ± 0.16 nM, $n = 8$ each) although with a very low binding capacity (Figure 2b and c). A very modest interaction of PACAP-38 with ^{125}I-PACAP-27 binding sites could also be observed and no significant interaction of secretin with ^{125}I-PACAP-27 binding sites was found (Figure 2b and c). The different IC_{50} values corresponding to these profiles are summarized in Table 1 and the binding capacities values (B_{max} and number of sites per cell) can be found in Table 2.

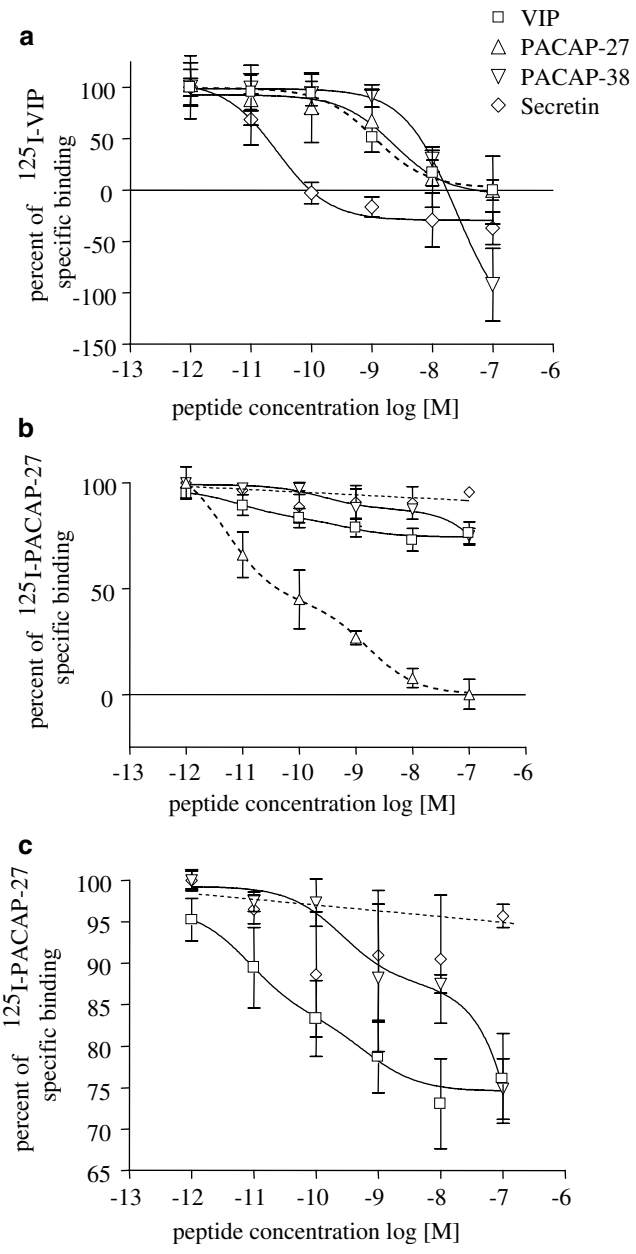

Figure 2 Competitive inhibition of the specific ^{125}I-VIP binding and ^{125}I-PACAP-27 in Calu-3 cells. (a) Cells were incubated for 2 h at 13°C in the presence of the radioligand and VIP (dotted curve), PACAP-27, PACAP-38 or secretin at the indicated concentrations. The maximal percentage (100%) corresponds to the absence of ^{125}I-VIP displacement by unlabeled peptides, whereas 0% corresponds to total ^{125}I-VIP displacement by VIP. Data are expressed in percent of specific ^{125}I-VIP binding and represent the mean ± s.e. ($n = 8$). (b) Cells were incubated for 2 h at 13°C in the presence of the radioligand and VIP, PACAP-27 (dotted curve), PACAP-38 or secretin at the indicated concentrations. The maximal percentage (100%) corresponds to the absence of ^{125}I-PACAP-27 displacement by unlabeled peptides whereas 0% corresponds to total ^{125}I-PACAP-27 displacement by PACAP-27. (c) Same as in (b) but at a different scale to enlarge the binding displacement curves corresponding to the three peptides presented in (a). Data are mean ± s.e. ($n = 8$) of percent of specific ^{125}I-PACAP-27 binding.

Activation of iodide efflux by VIP and PACAP-27 in Calu-3 cells

In digestive tissues, it is known that VIP/PACAP receptors regulate transepithelial chloride transport (Waldman *et al.*, 1977; Chang & Rao, 1991; Nguyen *et al.*, 1992; Leung *et al.*, 2001; Izu *et al.*, 2002). Therefore, we studied the activity of chloride channels in human airway epithelial Calu-3 cells in the

Table 1 Affinities of VIP and PACAP-27 binding sites

Ligands	*^{125}I-VIP displacement IC$_{50}$*	*^{125}I-PACAP-27 displacement IC$_{50}$*
VIP	1.1±0.34 nM	8.3±1.4 pM and 0.6±0.16 nM
PACAP-27	2.3±0.66 nM	5.0±0.52 pM and 1.6±0.61 nM
PACAP-38	24.7±2.6 nM	0.3±0.13 nM and 4.3±0.2 μM
Secretin	26.8±3.1 pM	

The binding parameters (IC_{50}) have been determined from experiments of competitive displacement of ^{125}I-VIP and ^{125}I-PACAP-27 by VIP and analogs in Calu-3 cells. Data are mean±s.e. of eight independent experiments.

Table 2 Binding parameters for VIP and PACAP-27 in Calu-3 cells

Radioligands	B_{max} (c.p.m.)		*Sites per cell*	
^{125}I-VIP	58,421		29,258	
^{125}I-PACAP-27	2108	3297	1058	2397

The B_{max} data in c.p.m. were obtained using GraphPad™ and the number of sites per cell were then calculated from these values. For PACAP-27 binding, the B_{max} values correspond to the high- (left) and low- (right) affinity binding sites. All binding experiments were carried out in 24-wells dishes containing about 300,000 cells.

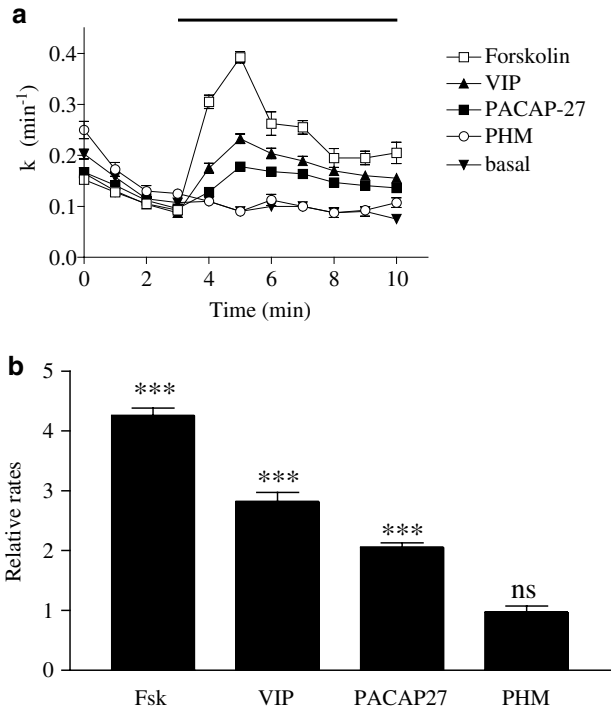

Figure 3 Iodide efflux from Calu-3 cells stimulated by forskolin, VIP, PACAP-27 and PHM. (a) Iodide efflux induced by forskolin, VIP, PACAP-27 and PHM. Note that no stimulation occurred with PHM as compared to the control (denoted basal). (b) Histograms showing the mean relative rates for each experimental condition as indicated below each bar. Concentrations used: forskolin (noted Fsk), 5 μM; VIP, PACAP-27 and PHM were all at 10 nM. In this and subsequent figures the presence of an agonist is indicated by an horizontal bar. Data are mean±s.e. for $n=6$. Some error bars are smaller than the symbol. Statistical analysis was performed for each agonist by comparison with the basal (no agonist added). ***$P<0.001$, ns: nonsignificant difference.

presence of VIP or PACAP-27. In addition, we evaluated the effect of the VIP-related peptide histidine methionine (PHM). We and others have previously shown that forskolin stimulates iodide efflux only through the opening of the CFTR chloride channels in Calu-3 cells (Haws *et al*., 1994; Shen *et al*., 1994; Bulteau *et al*., 2000). We first compared the effect of 10 nM VIP, PACAP-27, PHM to that of 5 μM forskolin as presented in Figure 3. This set of experiments revealed that iodide effluxes were stimulated by all agonists except PHM. The order of stimulation being forskolin > VIP > PACAP-27 > > PHM. We then investigated in more detail the VIP and PACAP-27 responses and constructed dose–response relationships from separate experiments. As shown in Figure 4a, no significant efflux was observed with very low concentration of VIP, that is, 0.03 nM. On the contrary, with concentrations of 0.1 nM up to 300 nM VIP (the highest concentration tested here), the efflux was dramatically stimulated. Two different effects were observed from these data. First, the peak amplitude increased dose-dependently. For example, the peak rates of efflux were 0.087±0.003, 0.129±0.01, 0.174±0.02 and 0.22±0.017 at 0.03, 0.3, 3 and 30 nM VIP, respectively ($n=8$ each). Second, the time-to-peak of efflux was shorter for high concentrations. For example, at 3 nM the time-to-peak occurred after 3 min post-VIP addition but within 1 min in the presence of 100 or 300 nM VIP. Figure 4a also shows averaged results from eight experiments using concentrations of VIP ranging from 0.03 to 300 nM from which we calculated the half-maximal effective concentration $EC_{50}=7.6\pm1.77$ nM ($n=8$). Similar experiments were performed with PACAP-27 as shown in Figure 4b. We obtained an EC_{50} of 10±1.34 nM ($n=8$). Although the two EC_{50} are not significantly different, the magnitude of the response obtained with both peptide indicates that VIP is slightly more potent than PACAP-27. These results show that VIP and PACAP-27 but not PHM stimulate anion transports in Calu-3 cells.

Glibenclamide but not DIDS inhibited VIP- and PACAP-27-mediated iodide efflux

The pharmacological profile for inhibition of VIP- and PACAP-27-mediated iodide efflux was then compared using different chloride channel inhibitors, that is, the sulfonylurea drug glibenclamide and the stilbene disulfonate derivative DIDS. These agents have been chosen because glibenclamide but not DIDS inhibits CFTR channel activity (Haws *et al*., 1994; Schultz *et al*., 1999; Bulteau *et al*., 2000). Both neuropeptides have been used at 10 nM; a concentration that approximates the EC_{50} determined from Figure 4. Our results show that in the presence of VIP (Figure 5b) or PACAP-27 (Figure 5c) the stimulation of the corresponding iodide efflux was inhibited by 100 μM glibenclamide but not by 200 μM DIDS when added from the extracellular side. A similar inhibitory profile was obtained with forskolin and is presented Figure 5a for comparison. The pharmacological signature of forskolin, VIP and PACAP-27 responses are similar as summarized Figure 5d, suggesting that both neuropeptides activate CFTR chloride channels *via* $VPAC_1$ receptors in human bronchial epithelial cells.

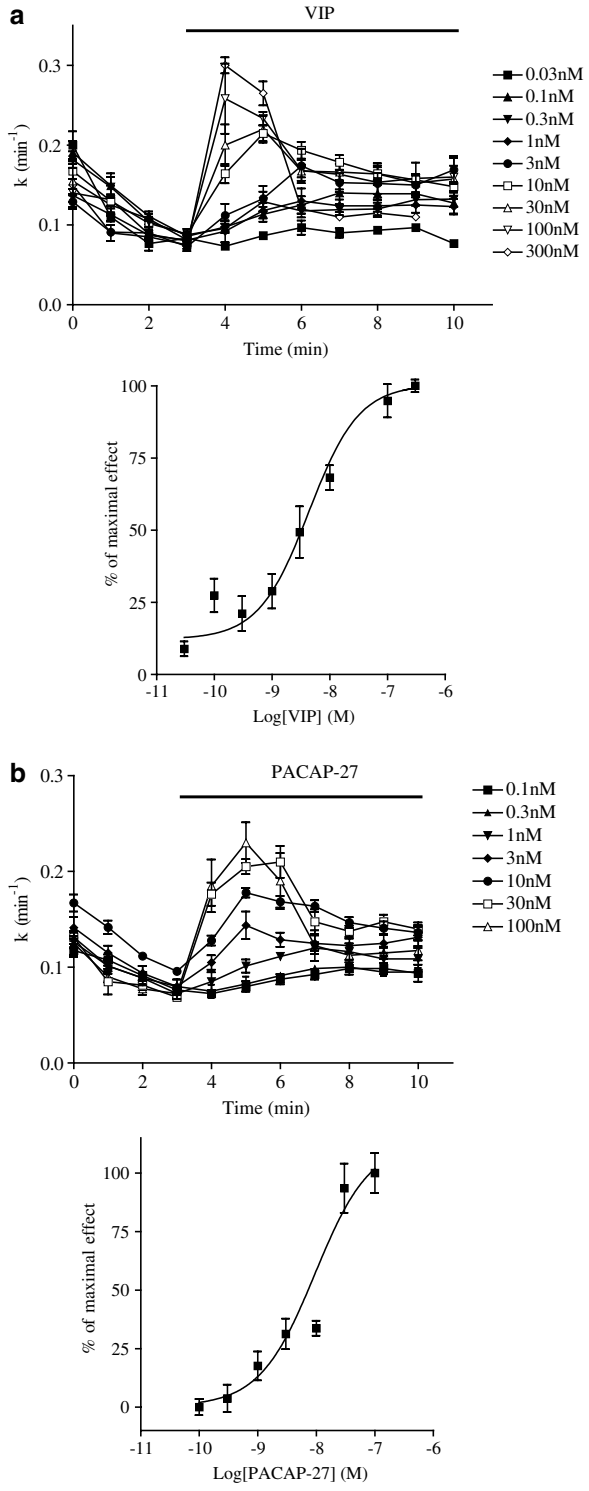

Figure 4 Dose–response relationships between the concentration of VIP and PACAP-27 and the stimulation of the iodide efflux in Calu-3 cells. (a) VIP-stimulated ^{125}I efflux at the concentration indicated. On the right, the percentage of maximal effect is plotted as function of the concentration of VIP. The half-maximal effective concentration EC_{50} was 7.6 ± 1.77 nM. (b) PACAP-27-stimulated ^{125}I efflux at the concentration indicated. The percentage of maximal effect plotted as function of the concentration of PACAP-27 is given. The half-maximal effective concentration EC_{50} was 10 ± 1.34 nM. Data are mean $\pm$ s.e. for $n = 8$. Some error bars are smaller than the symbol.

Effect of protein kinase inhibitors on VIP- and PACAP-27-mediated iodide efflux

Because CFTR chloride channel regulation is critically dependent on both protein kinases A and C (PKA and PKC, respectively) (reviewed in Gadsby & Nairn, 1999), we determined whether they are part of the signaling pathway linking $VPAC_1$ receptors to CFTR activity. We used the PKA inhibitor H-89 and the PKC inhibitor chelerythrine chloride. Our multiwell assay for chloride channel activity allowed parallel experiments and this part of the study was conducted including separately, forskolin as a control, VIP and PACAP-27 as agonists. We found 80% inhibition of the forskolin-induced CFTR-mediated efflux in the presence of H-89 and 50–60% inhibition with chelerythrine chloride (not shown). Results presented in Figure 6a show that 10 μM H-89 and 1 μM chelerythrine chloride inhibited the iodide efflux stimulated by 10 nM VIP ($n = 12$, $P < 0.05$). In the presence of inhibitors, the amplitude of the response was reduced and the time-to-peak longer than for control. Similar inhibition was found from 12 additional experiments with 10 nM PACAP-27 as shown in Figure 6b. A summary of the effect is provided in Figure 6c. The amplitude of the inhibition between either PKA or PKC inhibitors was not statistically significant, that is, both kinase inhibitors are equipotent in preventing the stimulation by VIP and PACAP-27.

Specificity of VIP and PACAP-27 towards CFTR

In this part of the study, we used the human airway epithelial IB3-1 cells (Zeitlin *et al*., 1991) derived from a CF patient (delF508/W1282X) because CFTR is absent from the plasma membrane due to its abnormal trafficking (Zeitlin *et al*., 1991; Dormer *et al*., 2001). However, calcium- and volume-dependent chloride channels are functional (Dormer *et al*., 2001). We analyzed the effect of VIP (Figure 7a) and PACAP-27 (Figure 7b) on CF airway epithelial IB3-1 cells and found that both peptides failed to stimulate iodide efflux even when added with 5 μM forskolin. Because we found expression of $VPAC_1$ receptors in IB3-1 cells by RT–PCR analysis (Figure 1, lane 11 compared to the negative control lane 12), these results cannot be attributed to an absence of the receptors but are explained by the absence of the channel itself.

Effect of VIP and PACAP-27 on the short-circuit current in Calu-3 cells

Finally, we performed Ussing chamber experiments to study the transepithelial chloride secretion in polarized Calu-3 cells exposed to VIP and PACAP-27. We grew Calu-3 cells monolayers on Costar snapwell permeable supports and measured I_{sc} across these monolayers bathed in standard Krebs bicarbonate solution at 37°C. As a control experiment, we exposed monolayers to 10 μM forskolin added on both apical and basolateral membranes (Figure 8a). The short-circuit current increases (ΔI_{sc}) was $31 \pm 9\ \mu A\,cm^{-2}$ ($n = 4$). Glibenclamide ($n = 3$, 500 μM apical) fully inhibited this response (Figure 8a) as expected from previous studies (Shen *et al*., 1994; Devor *et al*., 1999; Bulteau *et al*., 2000). Figure 8b presents an experiment in which we first added 100 nM VIP on the apical side and then on the basolateral side of the monolayer. No stimulation could be recorded when only the

apical side was exposed to VIP ($n=3$, Figure 8b, denoted AP). On the contrary, when added to the basolateral side (Figure 8b, denoted BL) a significant I_{sc} was increased ($\Delta I_{sc}=18\pm1.5$ $\mu A\,cm^{-2}$, $n=4$). A similar stimulation was observed with 100 nM PACAP-27 added on the basolateral side of three monolayers of Calu-3 cells ($\Delta I_{sc}=15\pm1.7$ $\mu A\,cm^{-2}$) which was fully inhibited by glibenclamide ($n=2$, 500 μM apical, not shown). These data demonstrate that VIP and PACAP-27 stimulate CFTR-dependent chloride transport in human bronchial epithelial cells through $VPAC_1$ receptors located on the basolateral membrane.

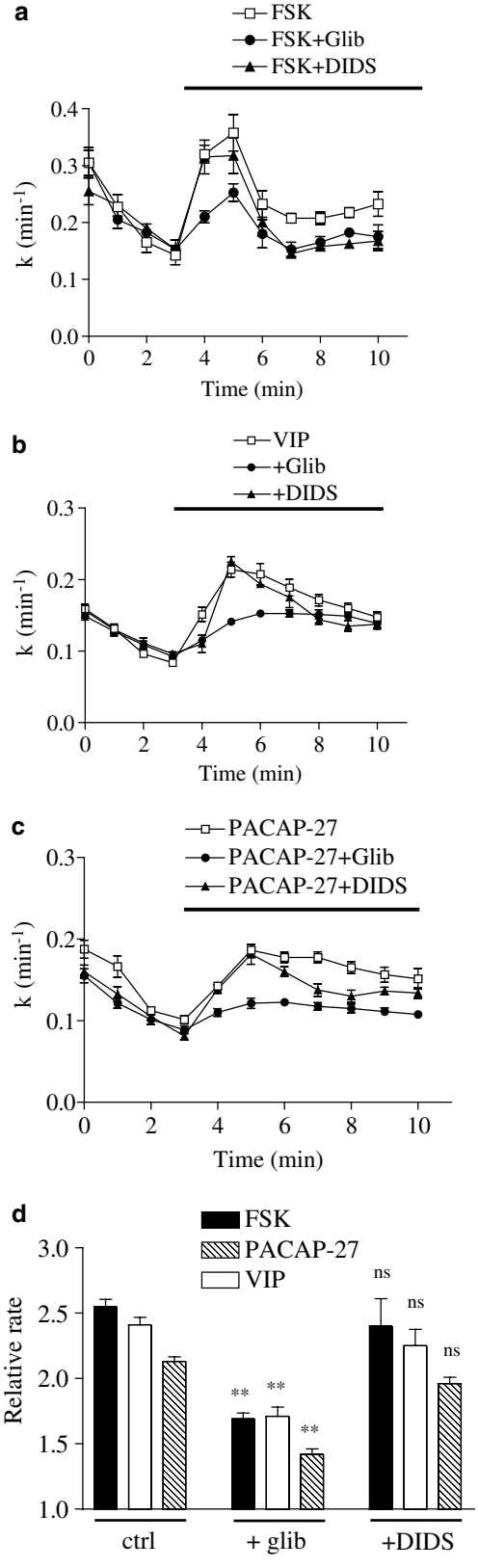

Figure 5 Pharmacological inhibitory profile of iodide efflux in Calu-3 cells. Forskolin (a, denoted FSK), VIP (b) and PACAP-27 (c) stimulated ^{125}I efflux with and without glibenclamide (denoted + glib) or DIDS, as indicated. Mean ± s.e. for each data point from six separate experiments. (d) Histograms presenting a summary of the relative rates obtained for experiments with forskolin, VIP and PACAP-27 in the absence or presence of glibenclamide (+glib) or DIDS (+DIDS). Concentrations used are: glib. 100 μM; DIDS 200 μM; FSK 10 μM; VIP 10 nM; PACAP-27 10 nM. Data are mean ± s.e. for $n=6$. Statistical analysis was done for a given agonist by comparison between experiments with or without inhibitor: ns: nonsignificant difference, $^{**}P<0.01$.

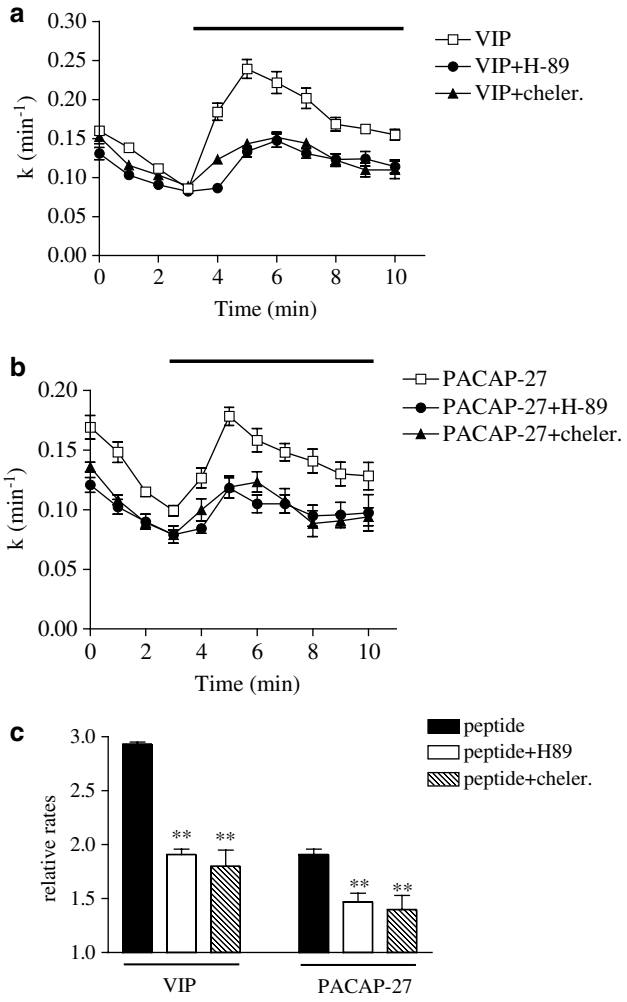

Figure 6 Effect of two different protein kinase inhibitors on VIP and PACAP-27 responses. VIP (a) and PACAP-27 (b) stimulated ^{125}I efflux with and without H89 and chelerythrine chloride. Data are mean ± s.e. for $n=12$ for each experimental condition. (c) Histograms presenting a summary of the relative rates obtained for these conditions Concentrations used are: H89 10 μM; chelerythrine chloride 1 μM; VIP 10 nM; PACAP-27 10 nM. Statistical analysis was done for a given agonist by comparison between experiments with or without inhibitor: nonsignificant difference, $^{**}P<0.01$.

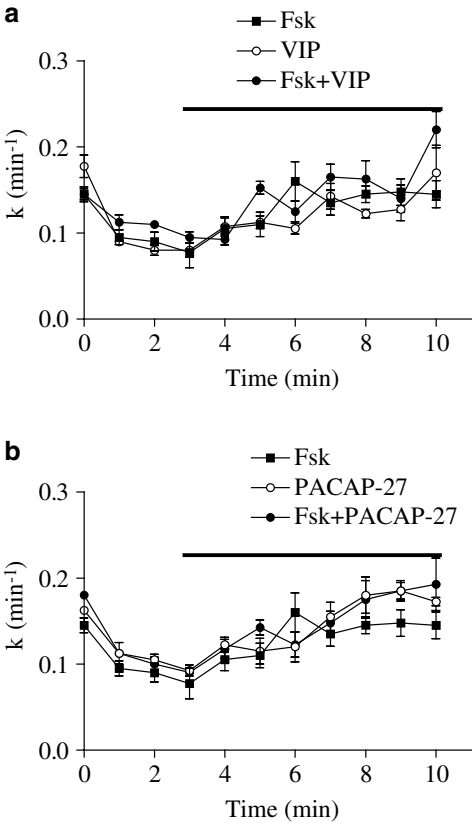

Figure 7 Effect of forskolin, VIP and PACAP-27 on the cystic fibrosis airway epithelial IB3-1 cell. Forskolin (Fsk, 5 μM), VIP 10 nM (a), and PACAP-27 10 nM (b) failed to stimulate ^{125}I efflux. Note that there is no additive effect. Data are mean $\pm$ s.e. for $n=4$ for each experimental condition.

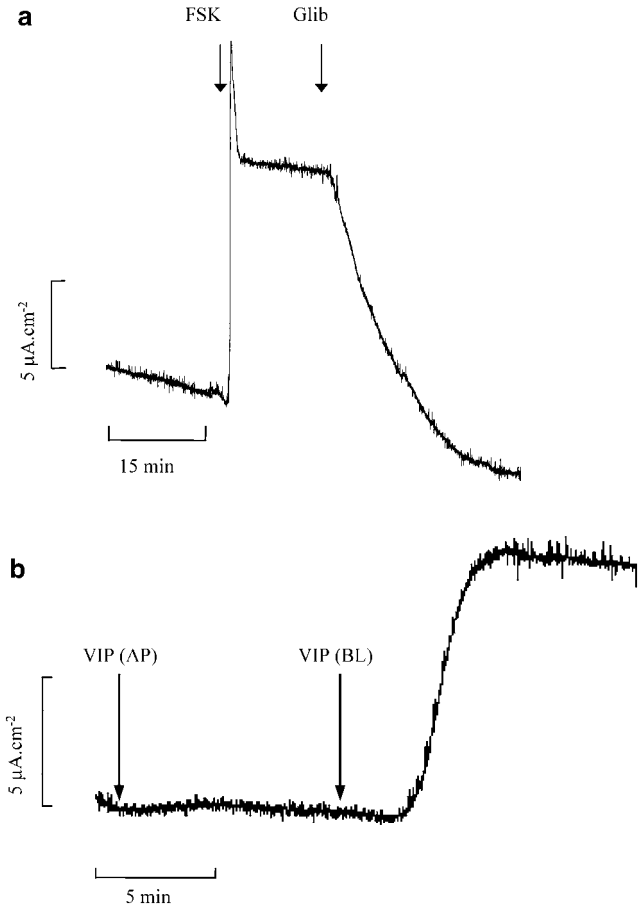

Figure 8 Effect of forskolin and VIP on short-circuit current (I_{sc}) in Calu-3 cells. (a) Stimulation of the short-circuit current (I_{sc} in μA cm^{-2}) by 10 μM forskolin added on both apical and basolateral membranes and its inhibition by 500 μM glibenclamide from the apical side of the monolayer. (b) Effect on I_{sc} of 100 nM VIP added on the apical (denoted AP) and then on the basolateral (denoted BL) side of Calu-3 monolayers. Note that only VIP applied basolaterally stimulated I_{sc}.

Discussion

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) are neurotransmitters of the inhibitory nonadrenergic, noncholinergic nervous system involved in a number of physiological and pathological conditions, mediated through $VPAC_1$ and $VPAC_2$ receptors and specific PACAP (PAC_1) receptors (reviewed in Laburthe & Couvineau, 2002). The present study shows that in human bronchial epithelial cells $VPAC_1$ receptors located on the basolateral membrane of cells, stimulate CFTR chloride channel activity and transepithelial chloride secretion. This conclusion comes from several observations that are presented here. First, the stimulation of efflux by either VIP or PACAP-27 is concentration-dependent with EC_{50} in the physiological nanomolar range. Second, the pharmacological profile for inhibition of VIP and PACAP-27-activated iodide efflux is similar to that of forskolin, that is, glibenclamide-sensitive but DIDS-insensitive. Third, the effects of two protein kinase inhibitors are similar for forskolin, VIP and PACAP-27, showing that both PKA and PKC are involved in the regulatory pathway bridging $VPAC_1$ receptor to the chloride transport response. Fourth, the experiments with the cystic fibrosis epithelial IB3-1 cells that also express $VPAC_1$ receptors clearly demonstrate that no other chloride channel except CFTR is activated by the neuropeptides. Fifth, the transepithelial chloride current is stimulated by forskolin, VIP and PACAP-27 and blocked by glibenclamide. Sixth, the stimulation of transepithelial chloride current occurs only when the neuropeptides (and thus the receptors) are on the basolateral side of the cells, an observation in perfect agreement with the basolateral location of VIP receptors in the digestive tract (Laburthe & Couvineau, 2002).

Properties of the $VPAC_1$ receptor in human bronchial epithelial cells

The molecular pharmacology and structure of the VIP/PACAP receptors have been broadly studied (see Laburthe & Couvineau, 2002 for a recent review). Data from competitive binding inhibition of ^{125}I-VIP or ^{125}I-PACAP-27 radiopeptides revealed that binding sites for both radiotracers are present in Calu-3 cells. The ^{125}I-VIP binding sites represented a single class of binding components. The pharmacological profile of the ^{125}I-VIP binding sites indicates that they share similar affinity for VIP and PACAP-27 (in the nM range),

which is a typical property of the polyvalent VIP/PACAP receptor $VPAC_1$. These receptors displayed a lower affinity for PACAP-38 and a surprisingly very high relative affinity for the VIP-related peptide secretin.

The ^{125}I-PACAP-27 binding data revealed a more complex pattern with two binding sites. The first one represents one-third of the sites with a very high affinity for PACAP-27 and the second (two-third of the sites) displayed a much lower affinity for PACAP-27 in the nanomolar range. We found an original pharmacological profile for ^{125}I-PACAP-27 binding sites. Only a minor part of them interacted with VIP or PACAP-38 (with a maximal 30% inhibition of the specific ^{125}I-PACAP binding) while no binding inhibition was observed in the presence of secretin. All the binding curves exhibited an irregular shape, again suggesting a complex interaction of the radiotracer with more than one binding species. At least two hypothesis could support such observation. For example, a very limited concentration of PAC_1 receptors (the so-called 'specific' PACAP receptor that poorly interacts with VIP) could be expressed on Calu-3 cell surface. Indeed, PAC_1 receptors were detected in normal human lung tissue but at a very low density (Busto *et al*., 2000). However, we do not favor this hypothesis since these receptors should then display a similar high affinity for both forms of PACAP in the nanomolar range, which was not observed in the present study. Alternatively, a subpartition of the $VPAC_1$ receptors that displays a particularly high affinity (in the pM range) and higher binding efficacy for PACAP-27 while they poorly interact with the other agonists, could exist.

VIP/PACAP receptors expression and cellular localization in human lung

The expression of VIP and PACAP receptors in the human airway have been reported in nerve fibers and in epithelial cells (Rogers, 2000; Groneberg *et al*., 2001a). All three receptors $VPAC_1$, $VPAC_2$ and PAC_1 have been identified in human lung (Busto *et al*., 1999). Of particular interest, localization of VIP-immunoreactive nerves as well as of VIP-binding sites has been found around airway submucosal glands (Dey *et al*., 1981; Carstairs & Barnes, 1986; Elgavish *et al*., 1989; Fischer *et al*., 1992). The distribution of $VPAC_2$ mRNA from an *in situ* hybridization study showed signals in submucosal gland cells that surrounded trachea and bronchi with a staining of both serous and mucous cells (Groneberg *et al*., 2001a). PAC_1 receptors appear to be expressed at a low density according to immunohistochemistry experiments on human lung section (Busto *et al*., 2000). $VPAC_1$ receptors were also localized to several pulmonary cell types in rat (Usdin *et al*., 1994) and human (Ichikawa *et al*., 1995) including macrophages, smooth muscle of pulmonary veins and airway epithelium from the trachea to the respiratory bronchioles. Similarly, in ferret, VIP receptors have been localized to nerves innervating submucosal glands (Ramnarine & Rogers, 1994; Dey *et al*., 1996). The human Calu-3 cells have the characteristics of serous glands cells, the major site of chloride secretion in the airway (Haws *et al*., 1994; Shen *et al*., 1994). Preliminary evidence suggested functional VIP/PACAP receptor in Calu-3 cells (Linden *et al*., 1997) but authors did not identify the receptor at a molecular level. We found in the present study that only the $VPAC_1$ subtype could be identify unequivocally in Calu-3 cells. Although expected, the polarized effect of the neuropeptides on cell monolayers mounted in Ussing chamber demonstrated the basolateral location of the $VPAC_1$ receptors.

Motor control of mucus and chloride secretion in human bronchial epithelial cells

In the airway, three neural pathways are responsible for the innervation of secretory cells: sympathetic (adrenergic), parasympathetic (cholinergic) and nonadrenergic, noncholinergic (NANC) systems (reviewed in Rogers, 2000). The principal neurotransmitters of the NANC system are VIP/PACAP, substance P, neurokinin A and calcitonin gene-related peptide. VIP and PACAP-27 exhibit many different activities in diverse target organs, which express high-affinity receptors (reviewed in Groneberg *et al*., 2001b). In the airway, increasing evidence recognized submucosal glands as a major player in airway defense, mucus and electrolyte secretion as well as in airway diseases such as cystic fibrosis (Haws *et al*., 1994; Shen *et al*., 1994; Pilewski & Frizzell, 1999; Joo *et al*., 2002a). Both mucus and electrolyte secretion are part of the mucociliary transport that clear the airway from pathogens. Impairment of mucociliary clearance led to stagnation of mucus in the airways, which causes airway obstruction and provides an ideal environment for bacterial and fungal growth as it is diagnosed in diseases like cystic fibrosis, chronic bronchitis and asthma (Rogers, 2000; Joo *et al*., 2002a). The implication of VIP in airway diseases is not clear. The density of VIP-positive nerves was found significantly higher in the glands of bronchitic than in nonbronchitic subjects (Lucchini *et al*., 1997) whereas in a second study (Chanez *et al*., 1998) no correlation between disease severity and the number of nerves was found in biopsies from patients suffering from asthma and chronic bronchitis. Finally, the density of airway VIP binding sites was found to be reduced in airway tissues from cystic fibrosis and asthma patients (Sharma *et al*., 1995). In submucosal glands, which are innervated by peptidergic nerves (Ramnarine & Rogers, 1994; Dey *et al*., 1996), a recent study demonstrated that VIP and forskolin stimulated sustained mucus secretion in pigs (Joo *et al*., 2002b). In tracheal submucosal glands of ferret as well as in ciliated and basal cells of dog tracheal mucosa, VIP stimulate cAMP cell production indicating expression of VIP receptors (Lazarus *et al*., 1986).

In conclusion, we have demonstrated that human bronchial Calu-3 epithelial cells expressed basolateral $VPAC_1$ receptors which interact with VIP or PACAP-27 causing activation of CFTR-mediated chloride secretion *via* a pathway in which protein kinase A and C are both involved. Our study therefore shows that as in the intestine (Waldman *et al*., 1977; Chang & Rao, 1991; Nguyen *et al*., 1992; Leung *et al*., 2001; Izu *et al*., 2002) CFTR is an important molecular target for the physiological action of VIP and PACAP-27 in the chloride transepithelial transport of human bronchial epithelial cells.

We thank Caroline Norez, Chantal Jougla, James Habrioux and Claire Guichard for expert technical assistance. This work was supported by a thesis fellowship to R.D., a post-doc grant to B.M. by the French association Vaincre La Mucoviscidose, a thesis fellowship by the Region Poitou-Charentes to A.M., and specific grants from VLM and CNRS.

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(Received July 28, 2003
Revised October 7, 2003
Accepted October 29, 2003)