British Journal of Pharmacology (2005) 144, 1037–1050
www.nature.com/bjp

Vasotocin and vasopressin stimulation of the chloride secretion in the human bronchial epithelial cell line, 16HBE14o-

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1 Effects of neuropeptides of the vasopressin family on Cl^- secretion have not yet been reported in lung. Using the 16HBE14o- bronchial epithelial cell line, we investigated their action on Cl^- secretion.

2 In symmetrical Cl^- solutions, basolateral application of arginine vasotocin (AVT), oxytocin or isotocin induced a transient I_{sc} stimulation (I_{peak}), whereas arginine vasopressin (AVP) did not. The effects of different Cl^- channel blockers and of a protein kinase C (PKC) inhibitor suggest that CFTR is involved in I_{peak}. The calcium-activated K^+ channel (SK4) and the Cl^-/HCO_3^- exchanger favor the driving force for AVT-mediated Cl^- secretion. The antagonists of V1a (SR49059)- and V1b (SSR149415)-receptors blocked I_{peak}, while SR121463B, a V2 receptor antagonist, did not. These results point to the stimulation of a V1-like receptor mediating I_{peak} and presenting an efficacy order, AVT > oxytocin > isotocin ≫ AVP.

3 When a serosal to mucosal Cl^- gradient was applied, AVT and AVP both stimulated I_{sc} according to a biphasic profile, I_{peak} being followed by a plateau phase ($I_{plateau}$). The pharmacology of $I_{plateau}$ suggests that CFTR channels are involved and that $Na^+/K^+/2Cl^-$ is the only transporter associated with $I_{plateau}$. dDAVP, a V2 receptor agonist-induced $I_{plateau}$ with the same potency as AVP, suggesting the involvement of V2 receptors in the AVP-induced $I_{plateau}$. V2 receptors are present on both opposite membranes, while V1-like receptors are mainly expressed on the basolateral membranes. RT–PCR experiments show the expression of V1a, V1b, V2 and vasopressin-activated calcium-mobilizing (VACM) receptors mRNAs.
British Journal of Pharmacology (2005) **144,** 1037–1050. doi:10.1038/sj.bjp.0706103
Published online 31 January 2005

Keywords: AVT; AVP; V1; V2; SK4; CFTR; bronchial epithelium; 16HBE14o-

Abbreviations: ASL, airway surface liquid; AVP, arginine vasopressin; AVT, arginine vasotocin; CaCC, Ca^{2+}-activated Cl^- channels; camp, adenosine-8-^{3}H3′, 5′-cyclic monophosphate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; $CFTR_{inh}$-172, 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone; dDAVP, [deamino-Cys[1], D-Arg[8]]-vasopressin; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, disodium salt; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid, disodium salt; MEM, modified Eagle's medium; NFA, 2-(3-[Trifluoromethyl]anilino)nicotinic acid; NMDG, *N*-methyl-D-glucamine; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; PACAP-27, pituitary adenylate cyclase-activating peptide; PAF, platelet-activating factor; PKA, protein kinase A; PKC, protein kinase C; NO, nitric oxide; SR49059, (2*S*)1-[(2*R*,3*S*)-5-chloro-3-(2-chloro-phenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]pyrrolidine-2-carboxamide; SR121463B, (1-[4(N-*tert*-butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy) cyclohexane]indoline-2-one, phosphate monohydrate, *cis*-isomer; SSR149415, (2*S*,4*R*)-1-[-5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-4-hydroxy-*N*,*N*-dimethyl-2-pyrrolidine carboxamide; TPA, tetrapentylammonium bromide; VACM, vasopressin-activated calcium-mobilizing; VIP, vasoactive intestinal polypeptide

Introduction

The volume and composition of airway surface liquid (ASL) bathing airway epithelia are modulated by coordinated, active and passive ion and water transports. The thickness and composition of ASL was found to be of major importance since it determines the efficiency of airway mucus clearance (Matsui *et al*., 1998; Tarran *et al*., 2001). Alteration of these parameters may be at the origin of severe pathologies. For example, the human genetic disease, cystic fibrosis (CF) results from a defective cystic fibrosis transmembrane conductance regulator (CFTR). The resulting ion transport disorders in CF epithelia diminish ASL volume, causing a decrease in mucociliary clearance (Matsui *et al*., 1998; Knowles & Boucher, 2002).

Airway epithelia synthesize and release several bioactive agents that regulate their functions in an autocrine and/or paracrine way. For example, eicosanoids (Widdicombe *et al*., 1989), endothelin (McKay *et al*., 1991), platelet-activating factor (PAF) (Tamaoki *et al*., 1991), nucleotides (Pratt *et al*., 1986; Knowles *et al*., 1991) and nitric oxid (NO) (Tamaoki *et al*., 1995) have been found to alter ion secretion and/or ciliary motility (Mason *et al*., 1991, Tamaoki *et al*., 1992; McCoy *et al*., 1995; Blouquit *et al*., 2003). Furthermore, several lung cell lines including 16HBE14o- have been found to

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Published online 31 January 2005

secrete arginine vasopressin (AVP) (Campling *et al*., 1995; Tamaoki *et al*., 1998a, b) and several vasopressin receptors have been described in rat lung (Tahara *et al*., 1998; Ceremuga *et al*., 2001).While few studies concern the role of AVP in the lung, some functions have been associated with this peptide. For instance, AVP stimulates ciliary motility of rabbit tracheal epithelium, an effect being mediated through the V1b receptor (Tamaoki *et al*., 1998b). AVP was also shown to inhibit liquid secretion in the ovine fetal lung by enhancing Na^+ absorption (Hooper *et al*., 1993; Perks *et al*., 1993). Interestingly, a recent study by Dérand *et al*. (2004) on the Calu-3 cell line, a model of serous cells of the bronchial tissue, shows the activation of CFTR-dependent Cl^- secretion by other neuropeptides, the vasoactive intestinal polypeptide (VIP) and the pituitary adenylate cyclase-activating peptide (PACAP-27), through VPAC1 receptors.

In this study, we assessed the effects of neuropeptides of the vasopressin family on Cl^- secretion in 16HBE14o- cells. The 16HBE14o- cell line has been immortalized from the human bronchial epithelium and has been reported to form a differentiated epithelium presenting tight junctions and maintain cAMP- and Ca^{2+}-dependent Cl currents (Cozens *et al*., 1994). These cells possess high levels of CFTR mRNA as well as mRNA coding for the α-subunit of the epithelial sodium channel (ENaC), which could be associated with amiloride-sensitive currents depending on culture conditions (Kunzelmann *et al*., 1996). In addition, the 16HBE14o- cell line presents a Ca^{2+}-dependent activation of Cl^- and K^+ conductances by several agonists (nucleotides, histamine, bradykinin, isoproterenol) (Cozens *et al*., 1994; Koslowsky *et al*., 1994; Mall *et al*., 2000; Walsh *et al*., 2000; Abraham *et al*., 2004). In 16HBE14o- cells, we show a stimulatory effect on Cl^- secretion of arginine vasotocin (AVT), AVP and other neuropeptides belonging to this family. We also intended to characterize the underlying ion transport mechanisms as well as the neuropeptide receptors involved in this process.

Methods

Cell culture

The cell line 16HBE14o- was a generous gift of Dr D.C. Gruenert (Colchester, Vermont, U.S.A.). This cell line is derived from bronchial surface epithelial cells, immortalized by the SV40 T-antigen (Cozens *et al*., 1994). Cells were cultured on plastic flasks coated with fibronectin (BD Biosciences, MA, U.S.A.)-collagen (Cohesion, CA, U.S.A.) and bovine serum albumin (Sigma Chemical Co., MO, U.S.A.). Cells were kept in a humidified atmosphere of 5% CO_2/95% air at 37°C and grown in modified Eagle's medium (MEM, Invitrogen Ltd, U.K.) containing (in $g\,l^{-1}$): L-glutamine (0.292), D-glucose (1), $NaHCO_3$ (2.2) supplemented with 10% fetal bovine serum (Dominique Dutscher S.A., France). To prevent bacterial contamination, a mixture of penicillin and streptomycin ($100\,U\,ml^{-1}$ each) was added. For most experiments, 16HBE14o- cells were grown on permeable supports (Transwell-Clear, Corning Inc, NY, U.S.A.) coated with fibronectin-collagen, for 8–10 days after they reached confluence.

Measurements of short-circuit current and epithelial resistance

Cell monolayers were mounted in home-made Ussing chambers as described previously (Bernard *et al*., 2003). Bath solutions were gassed with 5% CO_2 to adjust the pH to 7.4 and all experiments were performed at 37°C in a humidified atmosphere of 5% CO_2/95% air. The Ussing chamber was connected to an automatic voltage clamp (Physiologic Instruments, VCC-600, TX, U.S.A.) and measurements of short-circuit current (I_{sc}) were made in Ringer solution containing (in mM): 120 NaCl, 5 KCl, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$ and 11.1 D-glucose. For some experiments, the apical Ringer solution was changed to a Ringer solution with a reduced Cl^- concentration (10 mM) in order to apply a serosal to mucosal Cl^- gradient. This solution was obtained by substituting NaCl by equimolar Na-gluconate. In anion substitution experiments (i.e. Cl^--free solution), equimolar Na-gluconate, K-gluconate and Ca-gluconate replaced NaCl, KCl and $CaCl_2$, respectively. The Ca^{2+} concentration was increased to 5 mM to compensate the Ca^{2+}-buffering capacity of gluconate. HCO_3^--containing solutions were equilibrated with a humidified atmosphere of 5% CO_2/95% air. In the HCO_3^--free solution, 24.8 mM Na-gluconate replaced $NaHCO_3$ and 10 mM HEPES buffered the solution, the pH being adjusted to 7.4 with 1 N NaOH solution. Acetazolamide (1 mM) was added in the HCO_3^--free solution to limit endogenous cell HCO_3^- production. After mounting cell monolayers in the Ussing chamber, an equilibration period of 10 min allowed the stabilization of the resting I_{sc}. The transepithelial resistance (Rt) was measured by applying (1 s) bipolar 1 mV voltage pulses every 60 s and was calculated according to Ohms law ($Rt = \Delta Vt/\Delta I$).

^{86}Rb effluxes

Cell monolayers grown on permeable supports were loaded with ^{86}Rb ($37\,kBq\,ml^{-1}$) for 3 h from the basal side in a humidified atmosphere of 5% CO_2/95% air at 37°C. After three rapid (15 s) washing steps with 'cold' Ringer solution, the ^{86}Rb effluxes were followed in open circuit conditions by sampling the basal bathing solutions at regular time periods (every 10 s). The apical bathing solution was also sampled at the end of the experiment. At the end of experiments, cell monolayers were lysed with a NaOH (1 N) solution for 4 h in order to measure the remaining radioactivity. Then, the sampled radioactivity was measured after addition of 4 ml liquid scintillation fluid (ACS, Amersham Corporation, IL, U.S.A.) in a liquid scintillation counter (Packard Instruments, U.S.A.). Efflux rates were calculated as the percentage (%) per min of ^{86}Rb loss into the medium relative to total ^{86}Rb contained in the monolayer at the beginning of the time period measured $((c.p.m._x - c.p.m._{(x+1)})/c.p.m._x \times 100$, where x and $x+1$ represent successive time points) and plotted as a function of time. Owing to the large cell K^+ pool, reliable ^{86}Rb efflux measurements could be measured during long time periods; after 10 min of ^{86}Rb efflux, 85% of the initial ^{86}Rb load was still present in nontreated cells. The medium radioactivity at the end of the experiment was at least 20 times larger than the background level. Considering the rapid time sampling (10 s), only basolateral ^{86}Rb effluxes were measured. In the experiments reported below, the basolateral

^{86}Rb loss was six times larger than the apical ^{86}Rb loss consistent with our previous study (Bernard *et al*., 2003). Nevertheless, the apical ^{86}Rb loss was taken into account to calculate the percentage (%) per min of ^{86}Rb loss into the medium relative to total ^{86}Rb contained in the monolayer.

RNA isolation and reverse transcriptase/polymerase chain reaction (RT–PCR)

Total RNA was isolated from cells grown on permeant supports (Transwell-Clear, Corning Inc., NY, U.S.A.) using an RNAeasy Mini Kit (Qiagen GmbH, Germany) and was reverse transcribed using oligo-dT primers during 1 h at 37°C (Superscript First-Strand cDNA Synthesis System for RT–PCR, Invitrogen Ltd, U.K.). Primer sequences and expected product length for hV1a, hV1b, hV2, hVACM and hβ-actin are given in Table 1. For all PCR reactions: denaturation 94°C/30 s; annealing 56°C/30 s; extension 72°C/60 s; 40 cycles; recombinant *Taq* DNA polymerase was from Invitrogen Ltd, U.K. PCR products were analyzed on a 1.7% agarose gel using double-stranded DNA fragments from 100 bp to 12 kb as standard (1 kb Plus DNA Ladder, Invitrogen Ltd, U.K.). After purification (QIAquick gel extraction kit, Qiagen Gmbh, Germany), sequences of the PCR products were confirmed by sequencing (Genome express, Meylan, France).

Chemicals

Chloride channel blockers: DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, disodium salt), DNDS (4,4′-dinitrostilbene-2,2′-disulfonic acid), disodium salt, glybenclamide (*N*-p-{2-(5-chloro-2-methoxybenzamido)ethyl}benzenesulfonyl-*N*′-cyclohexylurea), NFA (2-(3-[Trifluoromethyl]anilino)nicotinic acid) and NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid) were purchased from Sigma (Sigma Chemical Co., MO, U.S.A.); $CFTR_{inh}$-172 (3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone) was a gift of Dr Verkman (San Francisco, U.S.A.). Potassium channel blockers: TPA (tetrapentylammonium bromide) was purchased from Fluka (Fluka Chemie GmbH, Switzerland), chromanol 293B was a generous gift from Dr J. Pünter (Aventis Pharma Deutschland GmbH, Germany), clotrimazole (1-(oChloroα,αdiphenylbenzyl)imidazole), forskolin and charybdotoxin were purchased from Sigma (Sigma Chemical Co., MO, U.S.A.). Others: [Arg^8]-Vasopressin, [Arg^8]-Vasotocin, [deamino-Cys^1, D-Arg^8]-Vasopressin (dDAVP), oxytocin, isotocin, H-89, chelerythrine chloride and clofilium tosylate were purchased from Sigma (Sigma Chemical Co., MO, U.S.A.). The nonpeptidic V1a antagonist, SR49059 ((2*S*)1-[(2*R*, 3*S*)-5-chloro-3-(2-chloro-phenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]-pyrrolidine-2-carboxamide), the nonpeptidic V1b antagonist, SSR149415 ((2*S*,4*R*)-1-[-5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-4-hydroxy-*N*,*N*-dimethyl-2-pyrrolidine carboxamide) and the nonpeptidic V2 antagonist, SR121463B (1-[4(*N*-*tert*-butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy) cyclohexane]indoline-2-one, phosphate monohydrate; *cis*-isomer) were from Sanofi-Synthelabo Recherche (Toulouse, France). All reagents were prepared as >1000-fold stock solutions. TPA, charybdotoxin, AVP, AVT and dDAVP were dissolved in water and all others chemicals in DMSO.

Data analysis

All data are presented as mean ± s.e.m., where *n* indicates the number of experiments. Paired or unpaired Student's *t*-test was used and a *P*-value <0.05 was chosen to indicate statistical significance. Statistical analysis was carried oute by the GraphPAD software version 1.13 (Christiane Mo, University of Montreal, Canada).

The dose–response curves were fitted with a single Hill equation:

$$Y = Y_{max}/(1 + (EC_{50})/X)^{n_H}),$$

Table 1 Primers used in RT–PCR to determine expression of hV1a, hV1b, hV2 and hVACM Receptor mRNA in 16HBE14o- cells

Gen	*Acc. number*			*Primer sequence*	*Position*	*Tm (°C)*	*Expected product length (bp)*
hV1a	AF101725	Pair 1	Forward	5′-CAAATGTGCTGGGACATCACCTAC-3′	322–345	68.3	869
			Reverse	5′-ACTGTTTGTTGGGCTTCGATTGTT-3′	1168–1191	68.3	
		Pair 2	Forward	5′-AACATCTGGTGCAACGTCC-3′	724–742	63.7	352
			Reverse	5′-CAGTCTTGAAGGAGATGGCC-3′	1057–1076	63.7	
hV1b	AF101726	Pair 1	Forward	5′-TCAGCCTCCCTCAAGTCTTCATTT-3′	491–514	67.9	780
			Reverse	5′-AAGATGATGGTCTCAGCGG-3′	1253–1271	62.9	
		Pair 2	Forward	5′-CCTGGCTATCTTCGTTCTGC-3′	612–631	63.7	621
			Reverse	5′-CTCCAAGTCCCTTGGTGACTCTTC-3′	1210–1233	68.1	
hV2	AF101727	Pair 1	Forward	5′-CTCTCCATAGTCTTTGTGGCTGTGG-3	130–154	68.7	986
			Reverse	5′-TCACGATGAAGTGTCCTTGG-3′	1097–1116	63.8	
		Pair 2	Forward	5′-ATTCATGCCAGTCTGGTGC-3′	694–712	63.8	140
			Reverse	5′-GACCACAATCACTAGCGTCATCCTC-3′	810–834	68.7	
hVACM-1	NM003478		Forward	5′-AGCACTCATGGAATGCTGTG-3′	777–796	64.0	444
			Reverse	5′-CAGCTCAGGGCATTTTGATT-3′	1202–1221	64.0	
hβ-actin	X00351		Forward	5′-CTGTGCTATCCCTGTACGCCTC-3′	413–434	66.8	436
			Reverse	5′-CATGATGGAGTTGAAGGTAGTTTCG-3′	849–825	66.4	

where Y is the normalized peak amplitude (%), Y_{max} is the maximum normalized peak amplitude (%), EC_{50} is the concentration required to obtain the half-maximum peak amplitude (nM), X is the concentration of the agonist (nM) and n_H the Hill coefficient. Fits were made using Origin software version 5.0 (Microcal, Northampton, U.S.A.).

Results

AVT stimulates chloride secretion in 16HBE14o- cells

As reported previously (Cozens *et al*., 1994), 16HBE14o- cells grown on permeant supports form a polarized and tight monolayer. In resting conditions, cell monolayers displayed a transepithelial resistance (Rt) of $1140 \pm 60\ \Omega cm^{-2}$ and a short-circuit current (I_{sc}) of $0.8 \pm 0.1\ \mu A\,cm^{-2}$ ($n = 44$). Basal application of 1 μM AVT (a vasopressin ortholog of non-mammalian vertebrates) transiently enhanced I_{sc} (I_{peak}, $13.3 \pm 0.7\ \mu A\,cm^{-2}$) in less than 1 min (Figure 1a and d) and simultaneously reduced Rt ($844 \pm 107\ \Omega cm^{-2}$). The peak was followed by a stabilization of I_{sc} and Rt to values not different from initial ones (I_{sc}, $0.6 \pm 0.1\ \mu A\,cm^{-2}$ and Rt $1104 \pm 56\ \Omega cm^{-2}$ after 5 min of AVT application). The EC_{50} of this effect was 63 ± 24 nM (Figure 1b).

We also tested other neuropeptides belonging to the vasopressin family on I_{sc}. Surprisingly, basolateral application of 1 μM AVP had no significant stimulatory effect on I_{sc} (Figure 1c and d). Conversely to AVP, basal application of 1 μM isotocin (a fish vasopressin ortholog) or that of 1 μM oxytocin induced a transient I_{sc} stimulation having a similar profile to that found with AVT (illustration not shown). The EC_{50} of this effect was of 550 ± 81 nM ($n = 3$ for each concentration tested) and of 221 ± 84 nM ($n = 3$–7 for each concentration tested) for isotocin and for oxytocin, respectively. Therefore, the deduced affinity sequence for the neuropeptide receptor (expressed as the I_{peak} amplitude in response to peptide application) was AVT > oxytocin > isotocin $\gg$ AVP.

Since no sodium transport could be detected in 16HBE14o- cells (Bernard *et al*., 2003), it was likely that the observed conductance was due to anion secretion. Cl^- or bicarbonate was therefore substituted in the experimental medium by gluconate or Cl^-, respectively, to examine the conductance of these anions (Figure 2a). When using Cl^--free solutions (both sides), I_{peak} was reduced by $96.8 \pm 0.6\%$ ($P < 0.001$, $n = 3$). HCO_3^- substitution (both sides) also markedly affected the AVT-induced I_{peak}, which was reduced by $82.6 \pm 5.8\%$ ($P < 0.001$, $n = 5$). This inhibition was larger than that expected for an anion channel presenting similar permeabilities to HCO_3^- and Cl^-. Thus, if the AVT-induced I_{peak} mainly corresponds to Cl^- secretion, the pronounced I_{sc} inhibition observed in absence of HCO_3^- may indicate that an additional HCO_3^--dependent step is involved in anion secretion (see below and Discussion).

Pharmacology of channels and transporters involved in the AVT-induced I_{peak}

A pharmacological approach was then designed to characterize the AVT-induced I_{sc} response in order to identify the underlying channels and/or transporters involved. NPPB (100 μM), DIDS (300 μM), glybenclamide (300 μM) or NFA (300 μM) was added to the apical bathing solution 10 min before AVT application. As shown in Figure 2b, the AVT-stimulated I_{peak} was blocked by NPPB ($87.8 \pm 5.8\%$ of inhibition; $P < 0.001$, $n = 4$), glybenclamide ($67.9 \pm 4.7\%$ of

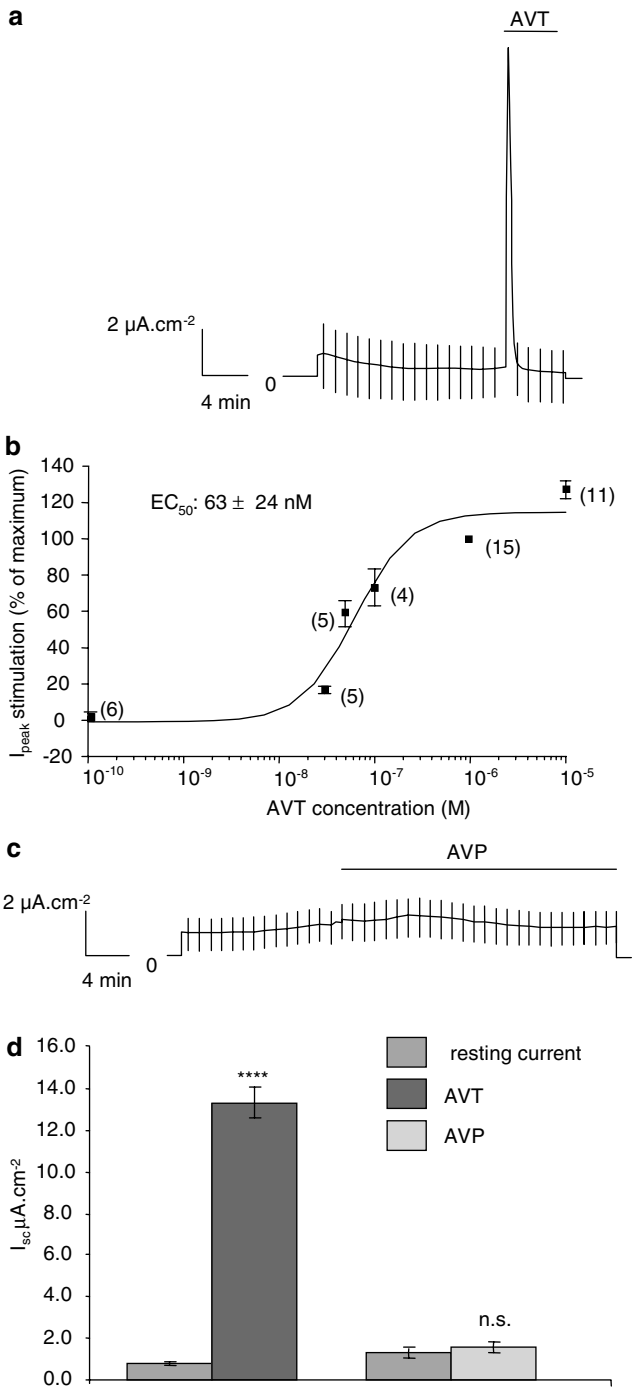


Figure 1 Effect of AVT and AVP on I_{sc} in 16HBE14o- cells. (a) Typical recording of the effect of basolateral AVT (1 μM) application induces a fast and transient I_{sc} increase (I_{peak}). (b) Dose–response curve for the AVT-induced I_{peak}. Effective concentration for half-maximal I_{sc} response, EC_{50} after basolateral AVT application; number of experiments in brackets. (c) Typical recording of the effect of basolateral application of 1 μM AVP had no effect on I_{sc} in cells bathed in symmetrical Cl^- media. (d) Comparison of the effects of AVT and AVP to I_{sc}. Each peptide was added to the basolateral side at a 1 μM concentration. Student's t-test; $n = 44$ for AVT and $n = 4$ for AVP; ****$P < 0.001$ compared to the stabilized I_{sc} before peptide addition; n.s. indicates not significant.

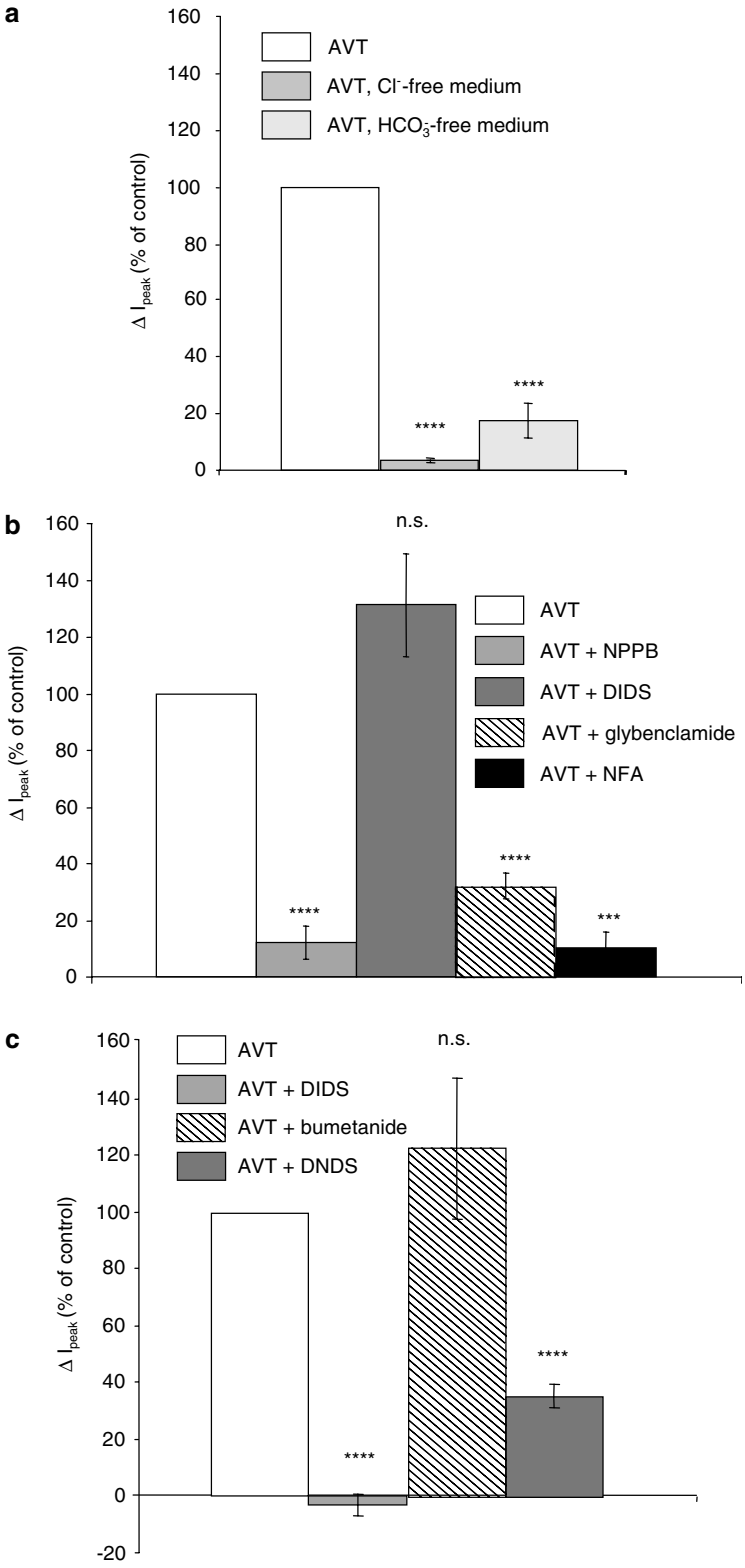


Figure 2 Pharmacological characterization of the AVT-induced I_{peak}. (a) Effect of Cl^- ($n=3$) or HCO_3^- ($n=5$) substitution on the AVT-induced I_{sc} stimulation (ΔI_{peak} is the increase in I_{sc}). Significance by Student's *t*-test: $^{****}P<0.001$ compared with a Cl^-- and HCO_3^--containing medium. (b) Effect of 100 μM NPPB ($n=4$), 300 μM DIDS ($n=4$), 300 μM glybenclamide ($n=4$), 300 μM NFA ($n=3$) on ΔI_{peak}. Drugs were added to the apical side 10 min before basolateral application of 1 μM AVT. (c) Effect of 300 μM DIDS ($n=4$), 3 mM DNDS ($n=7$), 50 μM bumetanide ($n=5$) on ΔI_{peak}. Drugs were added to the basolateral side 10 min before 1 μM AVT application (basolateral side). Significance by Student's *t*-test: $^{***}P<0.005$, $^{****}P<0.001$ compared with cells stimulated only with AVT; n.s. indicates not significant.

inhibition; $P<0.001$, $n=4$) and NFA ($89.7\pm5.5\%$ of inhibition; $P<0.01$, $n=3$), but was not affected by DIDS ($31.2\pm18.2\%$ of increase, n.s., $n=4$).

We further tested the effect of the protein kinase A (PKA) inhibitor, H-89 or the protein kinase C (PKC) inhibitor, chelerythrine chloride on the AVT-induced I_{peak} response. Each agent was added on both sides of the epithelium 1 h before AVT application. I_{peak} was reduced by $4.9\pm6.4\%$ (n.s., $n=4$) and by $35.5\pm9.3\%$ ($P<0.05$, $n=6$) following 10 μM H-89 and 2 μM chelerythrine chloride pretreatment, respectively.

Several ion transporters (the $Na^+/K^+/2Cl^-$, the $Na^+nHCO_3^-$ cotransporters and the Cl^-/HCO_3^- exchanger) located on basolateral membranes of airway epithelia cells have been suggested to participate in transepithelial anion transport. These transporters mediate Cl^- or HCO_3^- uptake across the basolateral membrane in order to facilitate anion diffusion through apical membrane (Haas, 1994; Devor *et al*., 1999; Romero & Boron, 1999; Loffing *et al*., 2000). To define the transporters possibly involved in the AVT-induced I_{peak}, we tested the effect of basolateral application of DIDS or bumetanide to block the Cl^-/HCO_3^- exchanger or the $Na^+/K^+/2Cl^-$ cotransporter, respectively (Figure 2c). Basal application of DIDS (300 μM) completely blocked I_{peak} ($102.7\pm4.1\%$ of inhibition; $P<0.001$, $n=4$) while bumetanide (50 μM) was ineffective ($23.0\pm24.7\%$ of increase, n.s., $n=5$) We also tested the effect of DNDS, an agent known to block the $Na^+nHCO_3^-$ cotransporter (Romero *et al*., 1997) and the anion exchanger (Kenney & Kaplan, 1988; Devor *et al*., 1999). DNDS (3 mM) blocked I_{peak} by $63.9\pm4.0\%$; $P<0.001$, $n=7$. To distinguish between an effect of DNDS on the $Na^+nHCO_3^-$ cotransporter or on the anion exchanger, we performed additional experiments in which Na^+ was substituted by *N*-methyl-D-glucamine (NMDG) and choline. The absence of Na^+ in the medium did not suppress the AVT-induced I_{peak}. Conversely, using Na^+-free solutions, a three-fold increase in the AVT-induced I_{peak} was observed ($309.1\pm62.8\%$ increase, $P<0.01$, $n=5$). Thus, the inhibitory effect of DNDS on AVT-induced I_{peak} was unlikely due to the inhibition of the $Na^+nHCO_3^-$ transporter. We can therefore conclude from these experiments that the Cl^-/HCO_3^- exchanger plays a major role in the AVT-induced I_{peak}, while the $Na^+/K^+/2Cl^-$ and $Na^+nHCO_3^-$ cotransporters are not involved.

A V1-like receptor mediates the AVT-induced I_{peak}

To characterize the receptor(s) involved in I_{peak}, we tested several agonists and antagonists of V1 and V2 receptors. The V1a antagonist, SR49059 (100 nM) and the V1b antagonist, SSR149415 (100 nM) (Serradeil-Le Gal *et al*., 1993; Serradeil-Le Gal *et al*., 2002) totally blocked I_{peak} induced by 1 μM AVT, while the V2 antagonist SR121436B (100 nM) (Huang *et al*., 2000) was ineffective (Figure 3). The determined IC_{50} for SR49059 and for SSR149415, between a 0.1–100 nM concentration range, were of 16 ± 3 nM ($n=3$–7 for different concentrations tested) and 16 ± 11 nM ($n=3$–8 for different concentrations tested), respectively, and did not allow us to discriminate between a V1a- or a V1b-receptor. The V2 receptor agonist, dDAVP, did not stimulate I_{sc} (I_{sc} of 0.8 ± 0.1 and of 0.9 ± 0.1 $\mu A\,cm^{-2}$ before and after 1 μM dDAVP application, n.s., $n=4$) confirming the lack of V2 receptors implication.

The neuropeptide action through V1-type receptors is mediated by activating phospholipase C, which in turn stimulates phosphatidylinositol turnover to increase intracellular calcium (Ca_i). The possible implication of a V1 receptor stimulation by AVT was assessed by measuring the activity of SK4 channels (Ca^{2+}-activated K^+ channels), which have been previously found to mediate the K_{Ca} current in airway epithelia including 16HBE14o- cells (Bernard *et al*., 2003). In this aim, ^{86}Rb effluxes through 16HBE14o- cell monolayers were followed as a function of time (with a short sampling time of 10 s periods). After a 50 s-control period, basolateral application of 1 μM AVT application induced a transient stimulation of ^{86}Rb effluxes (two-fold increase, $P<0.05$, $n=6$), which was not observed when epithelia were previously treated with 10 μM clotrimazole, a specific SK4 channel inhibitor (Ishii *et al*., 1997) (Figure 4a). In addition, clotrimazole (10 μM) and two other K_{Ca} channel inhibitors, TPA (1 mM) and charybdotoxin (100 nM), inhibited ($P<0.001$, $n=4$) the AVT-induced I_{peak}, confirming the opening of SK4 channels upon AVT application (Figure 4b). Furthermore, the transient AVT-stimulated ^{86}Rb effluxes were largely blocked by applying the SR49059 compound (100 nM) on the basal side of cell monolayers (AVT-induced ^{86}Rb effluxes increased by 2.8 ± 0.6-fold and by 1.4 ± 0.3-fold for nontreated and SR49059-treated cells, maximum stimulation taken at 35 s, $P<0.001$, $n=4$). The inhibitory effect of clotrimazole (and other K_{Ca} blockers) on the AVT-induced I_{peak} or ^{86}Rb effluxes is in agreement with the involvement of a V1-like receptor known to elevate intracellular calcium upon agonist stimulation.

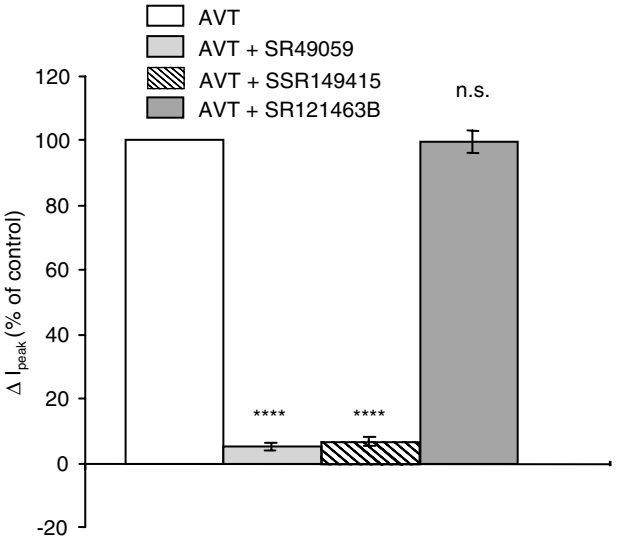


Figure 3 Effects of SR49059, SR121463B and of SSR149415 on the AVT-induced I_{peak}. The V1a antagonist SR49059 (100 nM) and the V1b antagonist SSR149415 (100 nM) blocked I_{peak}, while the V2 antagonist SR121436B (100 nM) was ineffective. SR49059 ($n=5$), SR121463B ($n=4$) and SSR149415 ($n=5$) were added to the basolateral bathing medium 15 min before basolateral application of 1 μM AVT. Significance by Student's *t*-test: ****$P<0.001$ compared with cells stimulated only with AVT; n.s. indicates not significant.

Evidence for the presence of a second receptor mediating the stimulation of Cl^- *secretion*

It has been previously reported in 16HBE14o- cells that Cl^- is at equilibrium at the apical cell membrane and that the generation of a Cl^- gradient (by lowering apical Cl^- concentration) was required to observe a cAMP-dependent

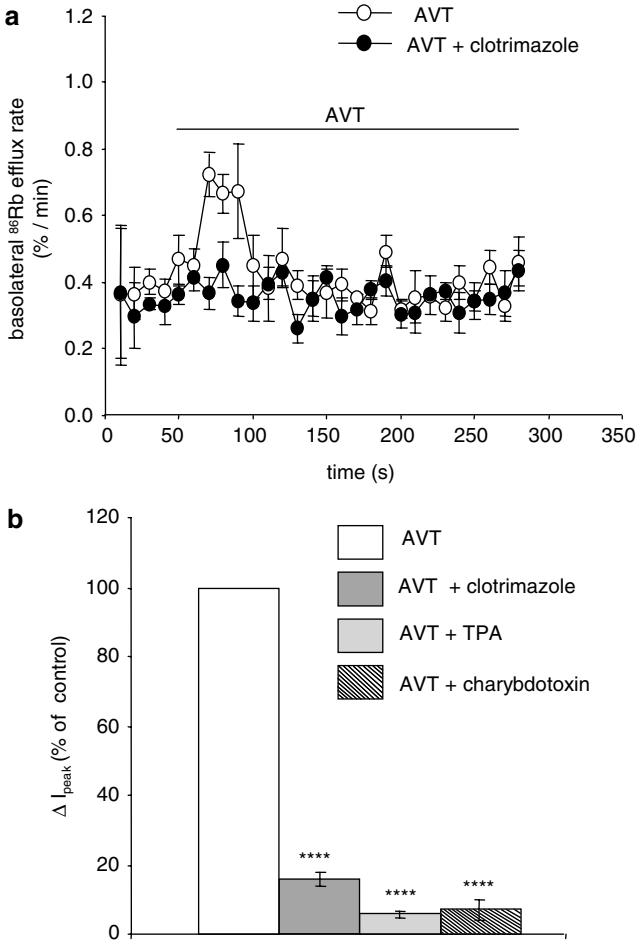


Figure 4 AVT stimulates ^{86}Rb fluxes in 16HBE14o- cells through SK4 channels. (a) After 50 s, AVT (1 μM, basolateral side) induces a transient increase of ^{86}Rb effluxes measured through basolateral membranes ($n=6$). Basolateral application of clotrimazole (10 μM; $n=6$) blocks the response. (b) Effects of clotrimazole (10 μM; $n=4$), TPA (1 mM; $n=4$) and charybdotoxin (100 nM; $n=4$) on the AVT-induced I_{peak}. ΔI_{peak} is the increase in I_{sc} after basolateral application of 1 μM AVT. Significance by Student's t-test: ****$P<0.001$ compared with cells not treated with a K^+ blocker; $n=4$.

stimulation of I_{sc} by opening CFTR channels (Cozens *et al.*, 1994). We therefore investigated the effect of the previously tested neuropeptides in these experimental conditions. Application of a Cl^- gradient through the epithelium increased I_{sc} from 1.5 ± 0.3 to 14.5 ± 0.8 μA cm^{-2} ($n=21$; see Figure 5a for a typical experiment). In all, 74% of this current was blocked by anion channel inhibitors indicating a major contribution of a transcellular pathway (Bernard *et al.*, 2003). Subsequent basolateral application of 1 μM AVT further transiently stimulated I_{sc} to 24.4 ± 1.4 μA cm^{-2}. This initial stimulation (I_{peak}) was similar to that described previously in symmetrical Cl^- conditions. However, the AVT-induced I_{peak} was followed by the development of a sustained second I_{sc} increase reaching 17.1 ± 1.1 μA cm^{-2} ($I_{plateau}$). This value was significantly larger ($P<0.001$) than I_{sc} before AVT application (14.5 ± 0.8 μA cm^{-2}). Means of I_{sc} changes after AVT application are given in Figure 5c.

Considering the presence of a second phase of I_{sc} stimulation after AVT application in the presence of a Cl^- gradient, we investigated the effect of AVP in these experimental conditions. As reported in Figure 5b, basolateral application of 1 μM AVP also stimulated I_{sc} from 13.7 ± 0.4 to 17.3 ± 0.7 μA cm^{-2} ($n=37$, $P<0.001$). This transient phase was followed by a second phase of I_{sc} stimulation, $I_{plateau}$, which reached 17.4 ± 0.7 μA cm^{-2}, after 15 min, a value significantly different ($P<0.001$) from I_{sc} before AVP application (13.7 ± 0.4 μA cm^{-2}). I_{peak} was four times larger for AVT than for AVP ($P<0.001$) and the plateau phase ($I_{plateau}$) was slightly larger with AVP than AVT ($P<0.05$), (Figure 5c). We hypothesized from these experiments that two distinct receptors are involved in the neuropeptide I_{sc} stimulation. I_{peak} would correspond to the stimulation of a V1-like receptor having a larger affinity for AVT than AVP. $I_{plateau}$ would correspond to the stimulation of a second receptor. The EC_{50} determined for the stimulation of $I_{plateau}$ by AVP was of 54 ± 10 nM (Figure 5d).

Pharmacology of channels and transporters implicated in $I_{plateau}$

The effect of anion channel inhibitors on $I_{plateau}$ mediated by 1 μM AVP (basal application) in the presence of a Cl^- gradient is shown in Figure 6a. $I_{plateau}$ was blocked by apical application of 100 μM NPPB (80.0 ± 2.4% of inhibition; $P<0.001$, $n=4$), 300 μM DIDS (33.1 ± 7.4% of inhibition; $P<0.01$, $n=4$), 300 μM glybenclamide (90.3 ± 2.8% of inhibition; $P<0.001$, $n=4$) and 300 μM NFA (79.3 ± 8.7% of inhibition; $P<0.005$, $n=4$).

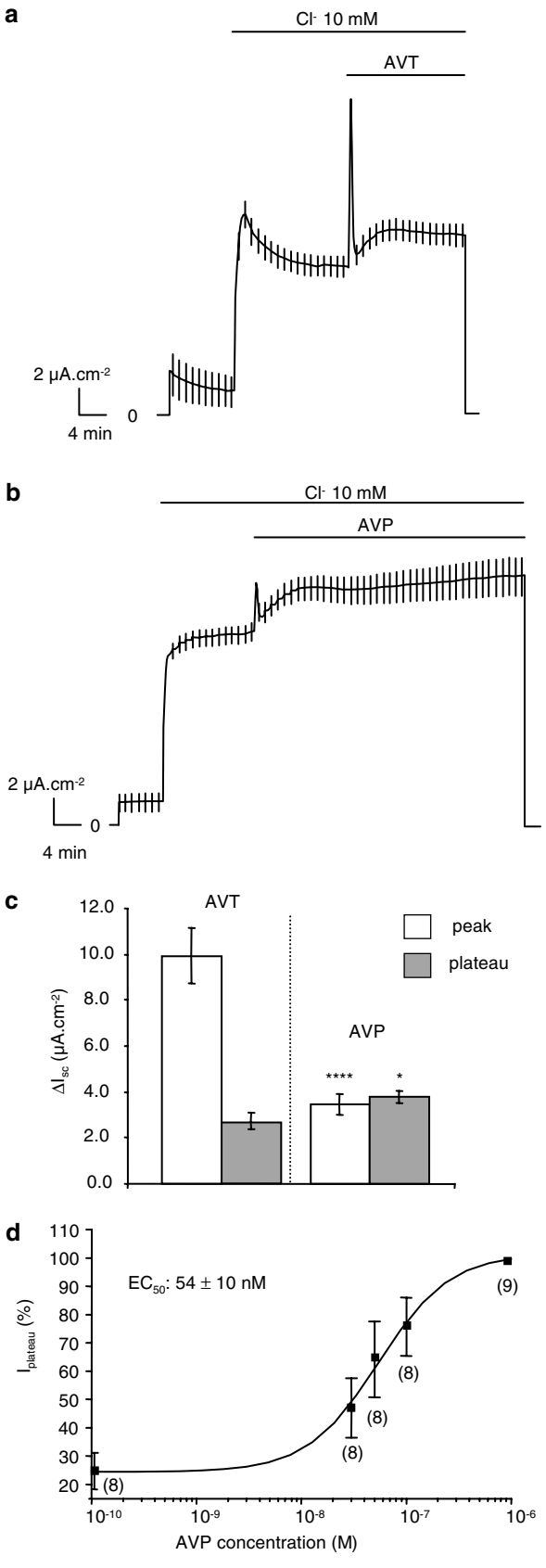
We further studied the effect of PKA and PKC inhibitors on the AVP-induced response. In all, 10 μM H-89 or 2 μM chelerythrine chloride were added on both sides of cell monolayers 1 h before AVP application (1 μM, basal side). $I_{plateau}$ was reduced by 53.9 ± 11.2% ($P<0.01$, $n=4$) and by 39.1 ± 12.4% ($P<0.01$, $n=5$) following H-89 and chelerythrine chloride pretreatment, respectively (Figure 6b).

Furthermore, we investigated the effect of 20 μM forskolin, an agent known to increase cellular cAMP, on the AVT-mediated I_{peak} and $I_{plateau}$ amplitudes. AVT (1 μM) was preferred to AVP since inducing a large I_{peak} response and a comparable $I_{plateau}$ response (see above). Experiments were performed by first applying AVT (Figure 7a) or forskolin (Figure 7b). The AVT-mediated I_{peak} was not changed by applying forskolin before AVT. However, the AVT-mediated $I_{plateau}$ was suppressed by applying forskolin before AVT ($P<0.001$, $n=8$). We also observed that the forskolin I_{sc} response was smaller ($P<0.01$, $n=8$) when this agent was applied after AVT, suggesting that the neuropeptide had already increased cellular cAMP.

In addition, we tested the effect of 2 μM $CFTR_{inh}$-172 (Ma *et al.*, 2002) on the AVT-stimulated currents, the inhibitor being added 15 min before AVT, in the presence of a Cl^- gradient. $CFTR_{inh}$-172 blocked 75.4 ± 3.8% of I_{peak} ($n=4$, $P<0.001$) and $82.6\pm4.7$of $I_{plateau}$ ($n=4$, $P<0.0001$).

The SK4 inhibitor, clotrimazole, failed to block $I_{plateau}$ induced by AVP application (Figure 8a). This result differs from that observed previously with the AVT-induced I_{peak}, which was largely blocked by clotrimazole (see Figure 5b). Chromanol 293B, described as a specific inhibitor of the KCNQ1/KCNE3 complex, which mediates K_{cAMP} currents in airway epithelial cells (Mall *et al.*, 2000; Cowley & Linsdell, 2002), also failed to block $I_{plateau}$ (Figure 8a).

The nature of the basolateral entry pathway(s) for Cl^- involved in $I_{plateau}$ was assessed by blocking the $Na^+/K^+/2Cl^-$



or the Cl^-/HCO_3^- transporters. $I_{plateau}$ was inhibited by $53.1 \pm 6.3\%$ ($P<0.001$, $n=8$) and by $24.1 \pm 5.5\%$ ($P<0.05$, $n=5$) when 50 μM bumetanide or 300 μM DIDS were applied, respectively (Figure 8b). The inhibitory effect of these two agents is therefore different from that obtained on I_{peak} when the V1-like receptor was stimulated (see Figure 2c), suggesting that different transporters are involved in I_{peak} and $I_{plateau}$.

V2 receptors are involved in the AVP-induced $I_{plateau}$

The effect of V1 and V2 antagonists was investigated on the AVP-induced $I_{plateau}$. In this aim, 100 nM of theV1a antagonist SR49059 or 100 nM of the V2 antagonist SR121463B were

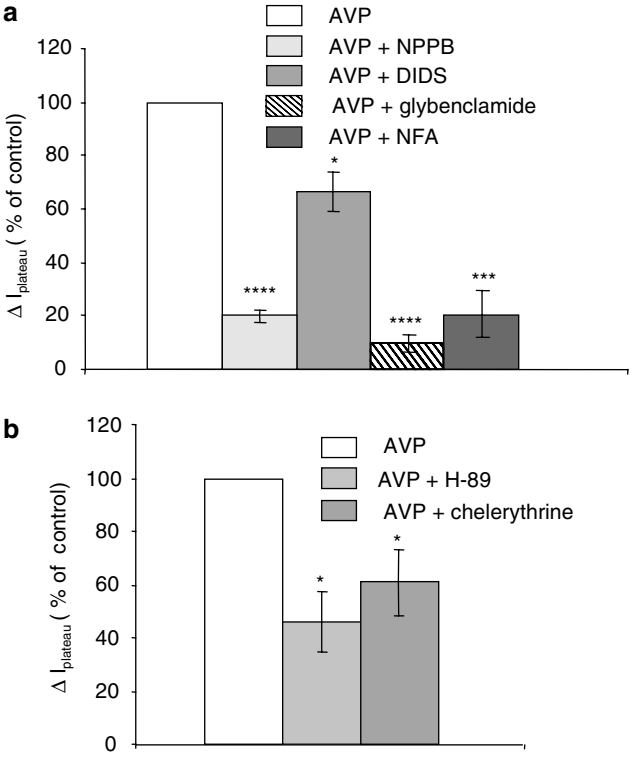


Figure 6 Pharmacology of AVP-induced $I_{plateau}$. A serosal to mucosal Cl^- gradient (130/10 mM) was applied across 16HBE14o- cell monolayers (see Figure 5b for typical experiment). (a) Effect of apical application of NPPB (100 μM; $n=4$), DIDS (300 μM; $n=4$), glybenclamide (300 μM; $n=4$) and NFA (300 μM; $n=4$) on $\Delta I_{plateau}$. Drugs were applied 10 min before basolateral application of 1 μM AVP. (b) Effect of H-89 (10 μM; $n=4$) and chelerythrine (2 μM; $n=5$) on $\Delta I_{plateau}$. Drugs were applied on both sides of the epithelium, 1 h before basolateral application of 1 μM AVP. Significance by Student's t-test: $^*P<0.01$; $^{***}P<0.005$, $^{****}P<0.001$ compared with cells stimulated with AVP only.

Figure 5 AVT or AVP induce a biphasic I_{sc} stimulation in 16HBE14o- cells. A serosal to mucosal-directed Cl^- gradient (130/10 mM) was applied across 16HBE14o- cell monolayers to favor Cl^- secretion. (a) After I_{sc} reached steady state, 1 μM AVT was added to the basolateral side and I_{sc} increased with a biphasic profile. (b) Similar experiment as in (a) performed with 1 μM AVP. (c) Mean $\pm$ s.e.m. of ΔI_{sc}; I_{peak} and $I_{plateau}$ resulting from AVP application ($n=37$) were compared to those resulting from AVT application ($n=21$). Significance by Student's t-test: $^{****}P<0.001$ and $^*P<0.05$. (d) Dose–response curve for AVP-induced $I_{plateau}$. Effective concentration for half-maximal response in $I_{plateau}$, EC_{50} after AVP application; number of experiments in brackets.

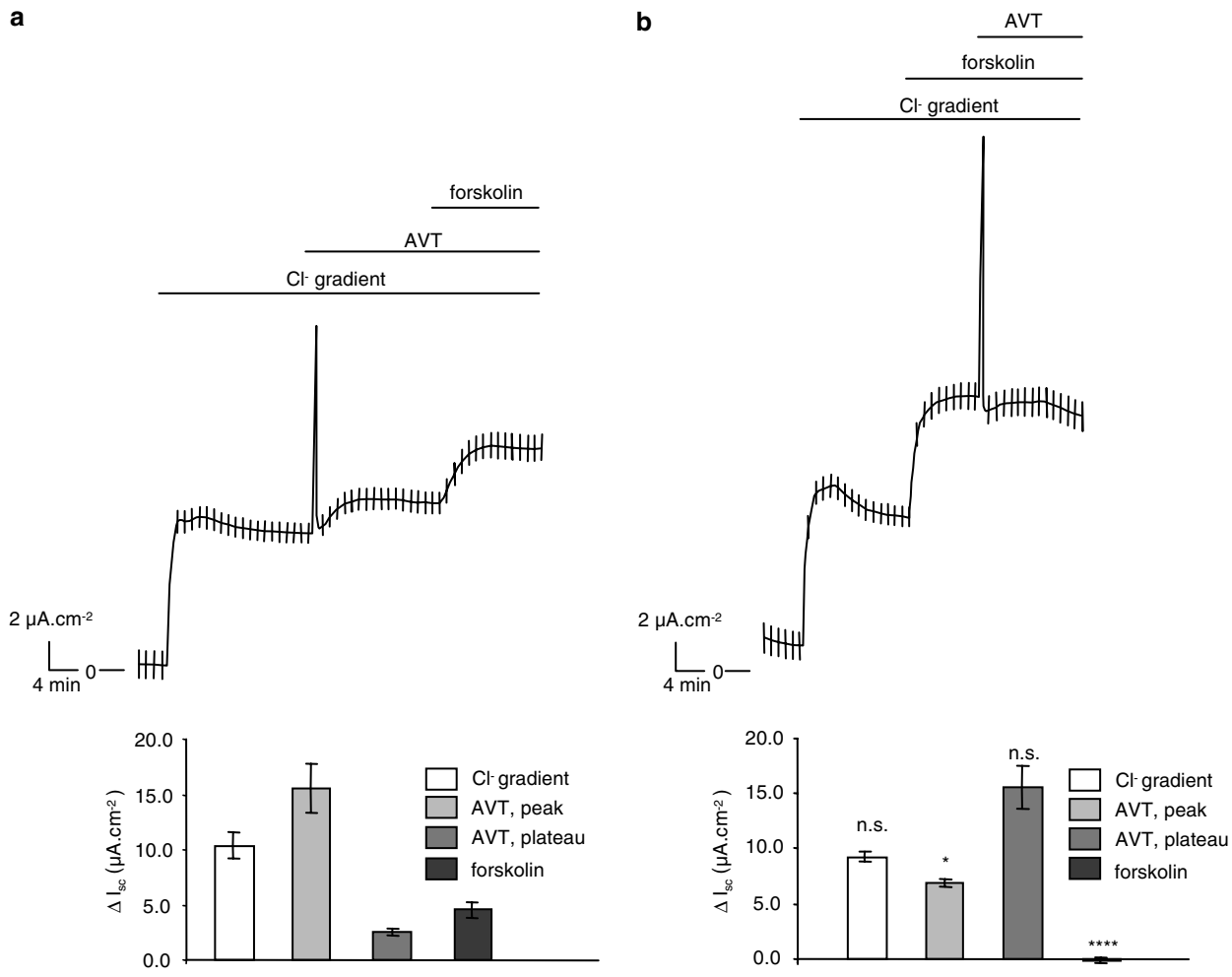


Figure 7 Effect of forskolin on the AVT-induced I_{sc}. A serosal to mucosal Cl^- gradient (130/10 mM) was applied across 16HBE14o- cell monolayers. (a) AVT (1 μM) was applied before forskolin (20 μM), $n=8$. (b) forskolin (20 μM) was applied before AVT (1 μM), $n=8$. Mean $\pm$ s.e.m. of AVT-induced ΔI_{sc} (I_{peak} and $I_{plateau}$) and the forskolin effect are given. Significance by Student's *t*-test: $^*P<0.01$; $^{****}P<0.001$ compared with cells stimulated with AVT first.

applied to the basal side of cell monolayers 15 min before AVP (1 μM) addition. SR121463B partially blocked the AVP-induced $I_{plateau}$ (42.8 $\pm$ 2.4% of inhibition, $n=4$, $P<0.001$), while SR49059 had no effect (Figure 9a). The V2 receptor agonist, dDAVP (30 nM), stimulated I_{sc} by 2.6 $\pm$ 0.4 μA cm^{-2} ($n=5$, $P<0.005$) and for a dDAVP concentration of 30 nM, $I_{plateau}$ was not different from that found with a similar concentration of AVP (89. 0 $\pm$ 15.0%, $n=5$, n.s.; Figure 9b). I_{peak} was never observed after dDAVP application (data not shown).

Altogether, these data indicate the presence of functional V2 receptors located on basolateral membranes of 16HBE14o- cells. Their stimulation by AVP, dDAVP or AVT leads to a sustained increase of chloride secretion ($I_{plateau}$).

Localization of AVT receptors

The presence of AVT receptors located on opposite membranes of the epithelium was assessed in the presence of a serosal to mucosal Cl^- gradient (a typical experiment is shown in Figure 10a). After stabilization of I_{sc} (10.3 $\pm$ 0.2 μA cm^{-2}, $v=6$), 1 μM AVT was added to the apical bathing media. A small but significant I_{peak} (ΔI_{sc} of 1.0 $\pm$ 0.2 μA cm^{-2}, $P<0.005$, $n=6$) was followed by a plateau phase (ΔI_{sc} of 0.9 $\pm$ 0.2 μA cm^{-2}, $P<0.005$, $n=6$). Subsequent addition of 1 μM AVT to the basal side induced a marked biphasic I_{sc} response: I_{peak} (ΔI_{sc} of 11.1 $\pm$ 1.2 μA cm^{-2}, $P<0.005$, $n=6$) followed by a plateau phase (ΔI_{sc} of 1.5 $\pm$ 0.1 μA cm^{-2}, $P<0.005$, $n=6$). These values of I_{peak} and $I_{plateau}$ are similar to those observed when AVT is added to the basolateral side without previous application on the apical side (see Figure 5a, c left panel). Basolateral AVT application induced a 10.7-fold larger I_{peak} than when applied apically (Figure 10b). Therefore, we propose that V1-like receptors mediating I_{peak} are preferentially located on basolateral membranes. In contrast, $I_{plateau}$ elicited by apical or basolateral AVT application were similar (1.6-fold larger for basal application), indicating that V2 receptors mediating $I_{plateau}$ are located on both membranes.

Presence of V1a, V1b, V2 and vasopressin-activated calcium-mobilizing (VACM) receptor mRNA in 16HBE14o- cells

mRNA expression for V1a, V1b and V2 receptors was analyzed in 16HBE14o- cells by RT–PCR using specific primers. The mRNA expression of the VACM receptor was also investigated since it was reported as a high-affinity

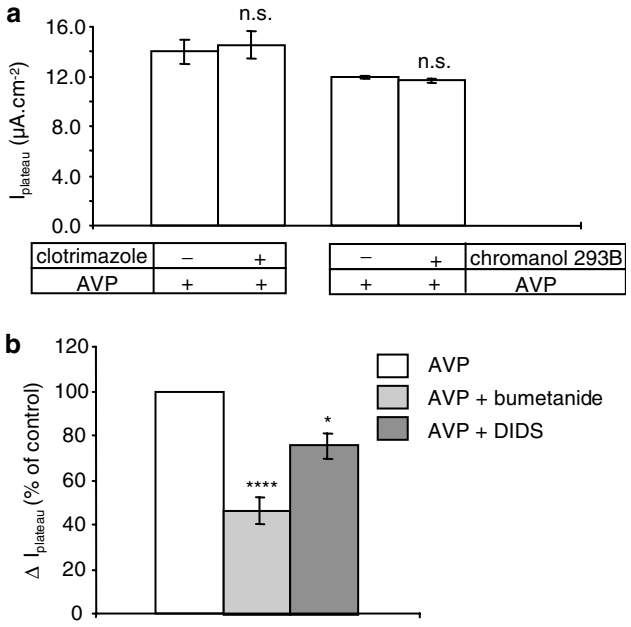


Figure 8 Effect of K^+ channel − or transporter − inhibitors on $I_{plateau}$. A serosal to mucosal Cl^- gradient (130/10 mM) was applied across 16HBE14o- cell monolayers. (a) Clotrimazole (10 μM, $n=4$) or chromanol 293B (100 μM, $n=4$) were added to the basolateral side of 16HBE14o- cells 10 min before basolateral application of 1 μM AVP. (b) Effect of bumetanide (100 μM, $n=8$) or DIDS (300 μM, $n=5$), on $\Delta I_{plateau}$. Drugs were added to the basolateral side of 16HBE14o- cells 10 min before basolateral application of 1 μM AVP. Significance by Student's t-test: $^*P<0.05$; $^{****}P<0.001$ compared with cells stimulated with AVP only.

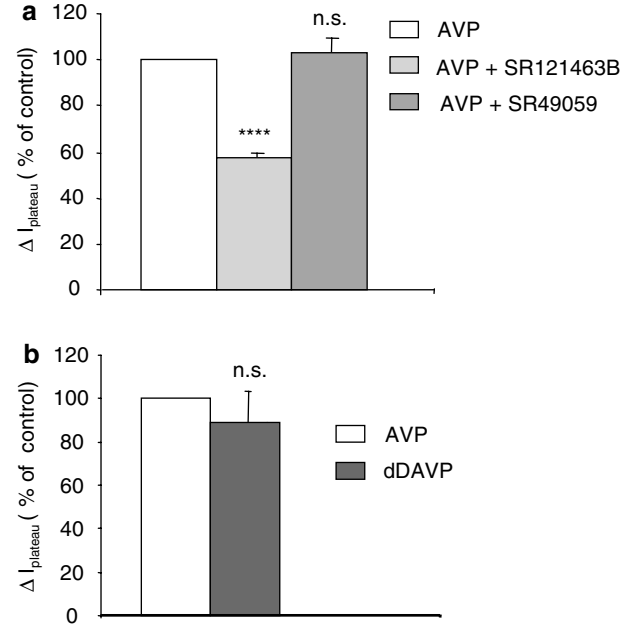


Figure 9 Effect of SR121463B, SR49059 and dDAVP on the AVP-induced $I_{plateau}$. A serosal to mucosal Cl^- gradient (130/10 mM) was applied across 16HBE14o- cell monolayers. (a) Effect of the V1a (SR49059; 100 nM; $n=4$) and V2 (SR121463B; 100 nM; $n=4$) antagonists on $I_{plateau}$. Antagonists were added 10 min before a basolateral application of 1 μM AVP. (b) Comparison of the effects of AVP and dDAVP on $I_{plateau}$. A submaximal concentration of 30 nM ($n=5$) was chosen for AVP and dDAVP. Significance by Student's t-test: $^{**}P<0.01$; $^{****}P<0.001$ compared with cells stimulated with AVP only.

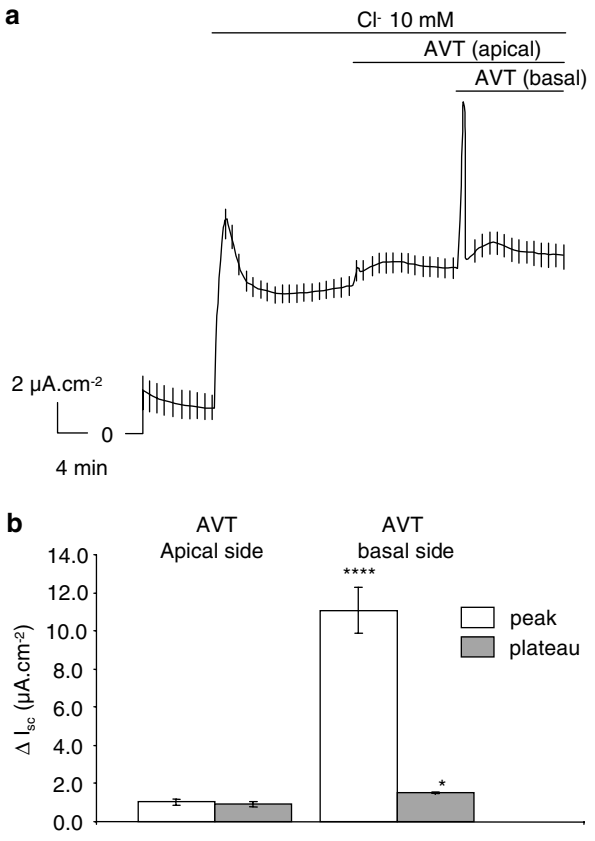


Figure 10 Membrane localization of neuropeptide receptors. (a) Typical recording of the stimulation of 1 μM AVT on I_{sc}. AVT was applied successively on the apical and basolateral side of the cells. (b) I_{peak} and $I_{plateau}$ resulting from basal side AVT application were compared to I_{peak} and $I_{plateau}$ resulting from apical side AVT application, $n=6$. Mean ± s.e. of ΔI_{sc}. Significance by Student's t-test: $^{****}P<0.0001$ and $^*P<0.05$.

receptor for AVP (Burnatowska-Hledin *et al*., 1995). Specific amplification products of the expected size were detected for all neuropeptide receptor mRNAs (Figure 11). Comparison of the sequenced PCR products with V1a, V1b, V2 and VACM published sequences (National Center for Biotechnology Information) confirmed the mRNA expression of these receptors in 16HBE14o- cells.

Discussion

The main new finding of this study concerns the stimulation of chloride secretion by neuropeptides belonging to the vasopressin family in a human bronchial cell line. Our study points to an implication of V1-like and V2 receptors mediating the stimulation of Cl^- secretion through their associated signaling pathways.

Nature of Cl^- channels involved in the AVT-stimulated Cl^- secretion (I_{peak})

Basolateral application of AVT was found to stimulate transiently I_{sc} in symmetrical Cl^--containing media. The stimulated current, I_{peak}, was identified as an anionic current by substituting Cl^- or HCO_3^-. This current was blocked by

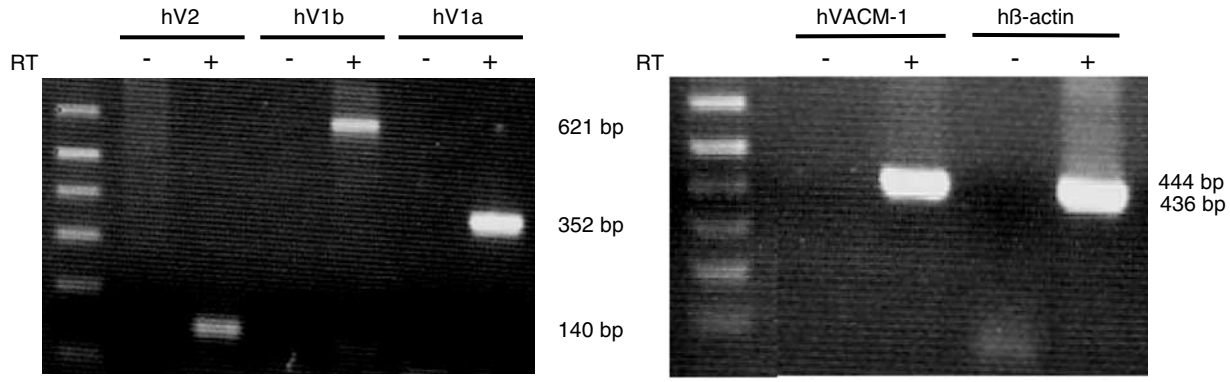


Figure 11 Detection of V1a, V1b, V2 and VCAM receptor mRNAs by RT–PCR analysis. Amplified PCR products generated using gene-specific primers (see Table 1) were separated on a 1.7% agarose gel and size markers were used to indicate the size of the amplified fragments. Markers (100–600 bp) are indicated in the first lane of the gel. (−) control without reverse transcriptase; (+) with reverse transcriptase. Human β-actin (hβ-actin) was used as a control.

apical application of glybenclamide, NPPB and NFA, but was not affected by DIDS. $CFTR_{inh}$-172, a specific CFTR inhibitor (Ma *et al*., 2002), also blocked I_{peak}. This pharmacological profile suggests the involvement of CFTR channels in the AVT-stimulated I_{peak} (Schultz *et al*., 1999) and rules out the implication of Ca^{2+}-activated Cl^- channels (CaCC) reported to be sensitive to DIDS (Ji *et al*., 1998; Atherton *et al*., 2003). Application of forskolin to elevate intracellular cAMP did not affect the amplitude of the AVT-stimulated I_{peak} and our attempts to block I_{peak} by H-89, a PKA inhibitor, failed. However, chelerytrin, a specific, but broad-spectrum inhibitor of PKC isozymes reduced I_{peak}. This last finding could be consistent with the reported direct activation of CFTR by PKC (Tabcharani *et al*., 1991). However, implication of other Cl^- channels than CFTR is not excluded.

Major participation of SK4 channels in the AVT-stimulated Cl^- secretion (I_{peak})

K^+ channels are involved in Cl^- secretion by generating a favorable electrochemical gradient for Cl^- exit in epithelial cells (Mc Cann & Welsh, 1990; Devor & Frizzell, 1993). In native airway cells (Devor *et al*., 2000; Mall *et al*., 2003) as in airway cell lines (Cowley & Linsdell 2002; Bernard *et al*., 2003), SK4 channels were shown to play a major role in the control of Ca^{2+}-activated Cl^- secretion, being preferentially located in basolateral membranes of 16HBE14o- cells (Bernard *et al*., 2003). We found that basal application of clotrimazole, a specific SK4 channel inhibitor (Ishii *et al*., 1997), and other K_{Ca} blockers, charybdotoxin (Joiner *et al*., 1997) and TPA, blocked the AVT-stimulated I_{peak}. In addition, AVT was found to transiently stimulate ^{86}Rb effluxes through basolateral membranes of 16HBE14o- cells, the stimulated ^{86}Rb effluxes being completely blocked by clotrimazole. Furthermore, the V1a antagonist, SR49059 blocked the AVT-stimulated ^{86}Rb effluxes. Taken together, these findings suggest the opening of SK4 channels following AVT application, most likely through an increase in intracellular calcium. The membrane potential hyperpolarization consecutive to the SK4 channel opening is expected to increase the driving force for Cl^- secretion.

Nature of the basolateral entry pathway involved in I_{peak}

The $Na^+/K^+/2Cl^-$, the $Na^+nHCO_3^-$ cotransporters and the Cl^-/HCO_3^- exchanger participate in the transepithelial anion transport by mediating Cl^- or HCO_3^- entry through the basolateral membrane of airway epithelia cells. We intended to determine which transporter(s) were involved in the AVT-induced chloride secretion (I_{peak}). Basal application of bumetanide had no effect on I_{peak} excluding a role of the $Na^+/K^+/2Cl^-$ cotransporter in AVT-induced currents. The disulfonic stilbenes (DIDS and DNDS) reduced I_{peak} but Na^+ substitution did not, ruling out the possible involvement of the $Na^+nHCO_3^-$ cotransporter. Conversely, Na^+ substitution further stimulated the AVT-induced I_{peak} and the AVT-induced ^{86}Rb fluxes (our unpublished results). A larger increase in intracellular calcium of cells bathed with a Na^+-free medium or an increased driving force for K^+ exit resulting from the change in Na^+/K^+ ATPase activity would represent some possibilities to explain this unexpected increase of the AVT-induced chloride secretion. HCO_3^- (24 mM) substitution markedly reduced ($82.6 \pm 5.8\%$ of inhibition) the AVT-induced I_{peak}. This large I_{sc} inhibition is unlikely due to the sole reduction of HCO_3^- transport through anion channels, since experiments were performed in the presence of 120 mM Cl^- and the relative HCO_3^- *versus* Cl^- conductance for the CFTR channel is in the range of 0.1–0.3 (Tabcharani *et al*., 1993; Poulsen *et al*., 1994). The inhibition of the AVT-induced current in the absence of HCO_3^- further supports a role of the Cl^-/HCO_3^- exchanger, sensitive to DIDS and DNDS, in the Cl^- entry through basolateral membranes required for the AVT-induced I_{peak}. It is relevant to mention that in mesangial cells from renal glomeruli, AVP has been found to stimulate the Cl^-/HCO_3^- exchanger (Ganz *et al*., 1989).

AVP also stimulates Cl^- secretion: nature of channels and transporters involved

Conversely to AVT, AVP did not stimulate I_{sc} when the epithelium was bathed in symmetrical Cl^- conditions. However, when the apical Cl^- concentration was lowered in order to facilitate Cl^- secretion, we observed a stimulation of I_{sc} after basolateral application of AVP. This response was biphasic,

composed of a transient peak (I_{peak}) followed by a sustained plateau phase ($I_{plateau}$). The same profile was elicited with AVT in asymmetrical Cl^- conditions. Therefore, the second I_{sc} phase ($I_{plateau}$) is unmasked by an experimental procedure that favors Cl^- secretion. The pharmacological characteristics of $I_{plateau}$ were close to those found for I_{peak} with a marked sensitivity to glybenclamide, NPPB and NFA and a minor effect of DIDS. $CFTR_{inh}$-172 blocked $I_{plateau}$ by 83%. In addition, H-89 blocked $I_{plateau}$ by 54% and chelerytrin blocked $I_{plateau}$ by 39%. These effects are consistent with the activation of CFTR channels by PKA and/or PKC (for a review, see Frizzell, 1999). This proposal is further supported by our findings that forskolin completely blocked the AVT-stimulated $I_{plateau}$. Considering the small effect of DIDS, we cannot exclude a minor participation of other channels, such as CaCC.

The development of $I_{plateau}$ was not associated with the opening of SK4 channels as found for I_{peak} since (i) clotrimazole had no effect on $I_{plateau}$ and (ii) the increase in ^{86}Rb effluxes (synchronous with I_{peak}) had recovered when $I_{plateau}$ was reached. The involvement of K_{cAMP} channels was also unlikely since chromanol 293B did not affect $I_{plateau}$. The effects of basolateral transporter inhibitors, bumetanide and DIDS, on $I_{plateau}$ were different from those found for I_{peak}. Bumetanide inhibited $I_{plateau}$ by 53% and DIDS by only 24%. This result contrasts with that observed for I_{peak}, which was totally blocked by DIDS but not by bumetanide. Thus, it appears that the $Na^+/K^+/2Cl^-$ cotransporter plays a role in $I_{plateau}$ by favoring Cl^- entry through basolateral membranes. It is known that the $Na^+/K^+/2Cl^-$ cotransporter is activated by an elevation of intracellular cAMP and/or a decrease of $[Cl^-]_i$ in secretory epithelia (reviewed in Haas & Forbusch, 2000) and by vasopressin in the thick ascending limb of the loop of Henle (Giménez & Forbush, 2003). Both an increase in cellular cAMP and/or a decrease in $[Cl^-]_i$ may participate in the stimulation of the $Na^+/K^+/2Cl^-$ cotransporter in response to AVP/AVT in 16HBE14o- cells.

Receptors involved in Cl^- stimulation in 16HBE14o- cells

Vasopressin receptors belong to the G-protein-coupled receptor family and are classified into two major types, V1 and V2 receptors, associated with different signaling pathways (Billah & Michell, 1979; Barberis & Tribollet, 1996). The V1-type vasopressin receptor has been molecularly subdivided into V1a (Morel *et al*., 1992) and V1b receptors (Sugimoto *et al*., 1994), which stimulate the Gq subunit of the heterotrimeric G-protein, leading to an increase in intracellular Ca^{2+} (Ca_i) (Thibonnier *et al*., 1994; Sugimoto *et al*., 1994). The vasopressin V2 receptor stimulates the Gs subunit of the heterotrimeric G-protein leading to an increase in intracellular cAMP (Birnbaumer *et al*., 1992; Lolait *et al*., 1992).

In order to characterize the receptors involved in the I_{sc} response to AVT or AVP, we tested agonists and antagonists of the V1 and V2 receptors. Our results point to the presence of both receptor classes in the 16HBE14o- cell line, each receptor being at the origin of one of the two I_{sc} stimulation phases, I_{peak} and $I_{plateau}$.

The main evidence for the involvement of a V1-like receptor in I_{peak} is the complete inhibition of I_{peak} by SR49059, a specific V1a receptor antagonist and by SSR149415, a specific V1b receptor anatgonist. In addition, I_{peak} was not affected by SR121463B, a V2 antagonist and I_{sc} was not stimulated by dDAVP, a V2 agonist. The increase of the SK4-mediated K^+ permeability by AVT also argues in favor of the presence of a V1-type receptor since SK4 channels are stimulated by a Ca_i increase, which is elicited by the stimulation of the V1 receptor. In this respect, the absence of effect of dDAVP on I_{peak} is in agreement with the absence of Ca_i increase reported in rabbit tracheal epithelium (Tamaoki *et al*., 1998b). We found in 16HBE14o- cells that the affinity sequence of the V1-like receptor was AVT > oxytocin > isotocin $\gg$ AVP. AVP had a poor effectiveness to induce I_{peak} in symmetrical Cl^- conditions, a transepithelial Cl^- gradient being required to observe a response (an approximate EC_{50} in this last condition was estimated to $35 \pm 14\,\mu M$). Conversely, AVT induced I_{peak} in both experimental conditions with an EC_{50} of 63 ± 24 nM. The AVT-mediated I_{peak} therefore seems to be mediated by a V1-like receptor, but presenting an efficacy order (relative to I_{sc} stimulation) and a sensitivity for both V1a-and V1b-antagonists unusual for a mammalian V1 receptor. Interestingly, a receptor for AVT has been cloned in the teleost fish, *Catostomus commersoni* (Mahlmann *et al*., 1994) presenting an EC_{50} of 13, 85 and of 485 nM for AVT, oxytocin and AVP, respectively. To our knowledge, such an efficacy order has not yet been described in human. Additional experiments are therefore necessary to further characterize this V1-like receptor.

The involvement of the V2 receptor in $I_{plateau}$ was supported by the following results: (i) SR121463B, the V2 antagonist, blocked $I_{plateau}$ (while SR49059, a specific V1a antagonist did not) and (ii) dDAVP, a V2 agonist, induced a stimulatory response of I_{sc} comparable to $I_{plateau}$. An opening of CFTR channels by a cAMP increase would be consistent with the V2 receptor-associated signaling pathway.

Our RT–PCR experiments identified the mRNAs of the three vasopressin receptors, V1a, V1b and V2 receptors and the mRNA of the VACM-1 receptor, a fourth AVP receptor (Burnatowska-Hledin *et al*., 1995) in 16HBE14o- cells. The VACM-1 receptor is distributed in a wide variety of tissues including the lung (Ceremuga *et al*., 2001). This calcium-mobilizing receptor presents a high affinity for AVP with a K_d of approximately 2 nM (Burnatowska-Hledin *et al*., 1995). It is unlikely that the VACM-1 receptor mediates one of the I_{sc} stimulations observed upon AVP/AVT stimulation in 16HBE14o- cells because AVP was found to present a weak efficacy on the V1-like receptor associated with I_{peak}. It is also unlikely that VACM-1 mediates $I_{plateau}$ since its development was not associated with SK4 channel stimulation (linked to a Ca_i increase).

AVT occurs throughout the vertebrate phylum, being present from primitive fish up to vertebrate, including birds (Lane *et al*., 1988; Mohr *et al*., 1995). In mammals, AVT has been replaced by AVP, but the presence of AVT has also been reported in the mammalian pineal gland (Goldstein, 1992). The lung is a target tissue for AVP and several functions have been associated with this peptide, namely ciliary motility and fluid clearance (Hooper *et al*., 1993; Perks *et al*., 1993; Tamaoki *et al*., 1998a, b; Norlin and Folkesson, 2001). Furthermore, several lung cell lines including 16HBE14o- have been found to secrete AVP (Campling *et al*., 1995; Tamaoki *et al*., 1998a, b), suggesting an autocrine or paracrine effect for this peptide.

In conclusion, this is the first report to demonstrate the stimulation of chloride secretion by AVT or AVP in a human

airway cell line. Two different receptors stimulate Cl^- secretion. V1-like receptors are located preferentially on the basolateral membranes, while V2 receptors are located on both opposite membranes of 16HBE14o- cells. The molecular characterization of the V1-like receptor found in our study remains to be undertaken considering its relative high AVT *versus* AVP affinity. Our observations must nevertheless be extended to native bronchia epithelia before postulating that peptides of the vasopressin family represent potential new agonists of Cl^- secretion in human airways where they may control the fluidity and the clearance of the airway surface liquid (ASL).

We thank Dr D.C. Gruenert for kindly providing the 16HBE14o-. We thank Dr Barberis (Montpellier, France) for fruitful discussion. We are grateful to Dr Serradeil-Le Gal (Sanofi-synthelabo, Toulouse, France) for kindly providing the SR49059 and SR121463B compounds. We also thank Dr S. Schmieder for critical reading of the manuscript. This work was supported by the CNRS, the MENRT and the Foundation 'Vaincre la Mucoviscidose' (Paris, France).

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(Received July 16, 2004
Revised October 7, 2004
Accepted November 19, 2004)