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# Characterization of cAMP dependent CFTR-chloride channels in human tracheal gland cells

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Human tracheal gland cells are believed to be a major site at the origin of cystic fibrosis. Since this disease is due to mutations in a protein called CFTR, we looked for the activity of CFTR in human tracheal gland cells in culture. We have identified CFTR-like chloride-selective channels as having a linear current–voltage relationship and unitary conductance of 7 pS in these cells In cell-attached patches, theophylline (1 mM), IBMX (1 mM), or a cocktail of dibutyryl cAMP (1 mM) and IBMX (0.1 mM) promoted the opening of channels. The unitary current had a reversal potential close to the cell resting potential. Replacement of choline by $K^+$ or $Na^+$ in the pipette solution was without effect on the current–voltage relationship, the reversal potential or the unitary conductance, which is consistent with the chloride selectivity of the channel. Channels were always found clustered and their opening probability was not noticeably dependent on membrane potential. This work therefore represents the first observation of a CFTR-like channel activity in submucosal gland cells.

Human tracheal gland cell; CFTR; Chloride channel; Patch-clamp

## 1. INTRODUCTION

Human mucociliary clearance involved in the pulmonary defence against dust and bacteria is dependent on the thickness of the surface fluid layer and therefore on the transepithelial chloride and fluid transport [1]. Airway epithelium is composed of the surface epithelial cells and a complex network of tracheal submucosal glands. The lumens of these glands join the airway surface through ducts. The three main cell types in the surface epithelium (goblet, clara and serous cells), all contribute to mucus secretion, however the mucous and serous cells of the submucosal glands are the major source [2]. A more detailed understanding of the physiological function and regulation of $Cl^-$ channels in human airway cells is important regarding their implication in cystic fibrosis [3]. Cystic fibrosis (CF), the most common fatal genetic disease in caucasians, is an exocrinopathology in which lung disease caused by hypersecretion of dehydrated mucus leading to chronic bacterial infection, is the main cause of death [4]. The CF gene encodes a protein named CFTR (cystic fibrosis transmembrane conductance regulator) which is involved in the transport of chloride ions [5]. Recently, several groups have provided evidence that CFTR is a cAMP-regulated low conductance $Cl^-$ channel [6–10].

In pulmonary tissues, both intracellular $Ca^{2+}$ and cAMP have been implicated in $Cl^-$ secretion [11,12], and it is assumed that separate $Cl^-$ channels are activated by these two regulatory pathways [13]. CF causes a decrease in cAMP-dependent [14,15] but not calcium-dependent regulation of chloride transport in the airway [14]. Various $Cl^-$ channels have been identified in tracheal surface epithelial cells [16,17], including CFTR-$Cl^-$ channels in immortalized human airway cell lines [18] but there have been no single channel studies reported for submucosal gland cells, although these cells are believed to be the major site of mucus secretion in the airway epithelium [19–21]. Furthermore, in situ hybridization and immunocytochemistry indicate that CFTR expression is far higher in submucosal glands than in other airway cells [21]. Until now, CFTR chloride channels have not been described in human pulmonary cells from submucosal gland cells.

Recently, primary culture methods for human tracheobronchial gland cells (HTG cells) have been developed [22,23]. Cultured confluent HTG cells become highly polarized and responsive to cholinergic and adrenergic agonists [24], and synthesize bronchial inhibitor (BrI), a serous secretory marker, as well as high molecular weight glycoproteins [25]. Furthermore, HTG cells in culture have been shown to transport $Cl^-$ ions in response to bradykinin, alpha and beta adrenergic and especially cholinergic agonists [26]. This newly

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developed culture model could therefore provide a useful tool for characterizing the channels involved in $Cl^-$ secretion by submucosal gland cells. A preliminary report concerning this work has been presented in abstract form [27] at the Sixth North American Cystic Fibrosis Conference (Washington, DC).

## 2. MATERIALS AND METHODS

### 2.1. *Culture of human tracheal gland cells*

Isolation and culture of HTG cells were performed as previously described [22] with some modifications, i.e. cells were grown on type I collagen coated 24-well plates in Dulbecco's Modified Eagle's/Ham's F12 Mixture (DMEM/F12) supplemented with 1% Ultroser G and 3 $\mu$M epinephrine to provide optimized growth and differentiation in culture. Ultroser G was from IBF (Villeneuve la Garenne, France). Tissue culture flasks and plates were manufactured by Falcon and distributed by Becton Dickinson France S.A. (Le Pont de Claix, France).

### 2.2. *Polymerase chain reaction (PCR)*

RNA isolation was performed on HTG cells following the technique of Chirgwin [28]. RT-PCR amplification was performed using the Gene AMP RNA PCR kit (Perkin Elmer Cetus). The PCR experment was performed using the following oligonucleotide synthetic primers: 5'-CACTCCTCTTCAAGACAAA-3' (exon 15) and 5'-CTGGATGAAGTCAAATATGG-3' (exon 16) and at primer annealing temperature of 50°C. The amplification was performed for 35 cycles.

### 2.3. *Patch-clamp recordings*

Currents were amplified using a LIST EPC-7 patch-clamp amplifier (filter setting 3 kHz and low pass filtered at 2–5 kHz using a 6-pole Bessel filter). Currents were continuously recorded on Digital-Audio-Tapes (DAT, Biologie Meylan, France). Stored data were further digitized at 1–5 kHz and transferred to an Olivetti M28PC computer. For analysis, recordings were low pass filtered at 100–200 Hz. Patch pipettes were formed in three steps by pulling soft glass tubes (Clark Electromedical) on a Brown-Flaming micropipette puller (model P-87 Sutter Instrument Company, USA). The pipette resistance ranged from 8 to 15 M$\Omega$. Voltage refers to the bath with respect to the patch pipette. Outward currents are displayed upwardly. Dashed lines indicate current levels. The zero-current baseline when the channels are in the closed state is indicated by 'c'. The data analysis was performed using software developed in our laboratory. Current amplitude histograms were fitted to Gaussian curves. Reversal potentials and conductance data were obtained by least square regression analysis. The pipette solution contained (in mM): 150 KCl (or 150 NaCl or 150 choline chloride), 1 $CaCl_2$, 1 $MgCl_2$ and 5 Tris (pH 7.6). NaCl-rich saline was used to perfuse the cells. Theophylline (1,3-dimethyl-xanthine), IBMX (3-isobutyl-1-methyl-xanthine), db-cAMP (dibutyryl cAMP) were added to the bath saline at the final concentration indicated in the text. Experiments were performed at room temperature. All chemicals were from Sigma.

## 3. RESULTS

There is now increasing evidence that submucosal gland cells are an important target for the disease cystic fibrosis. Indeed, in the human airway in vivo, through in situ hybridization and immunochemical methods, it has been shown that there is a high expression of CFTR especially in the distal component of the glands, i.e. the serous gland cells [21]. Since HTG cells are of serous type [22], we checked that HTG cells express CFTR

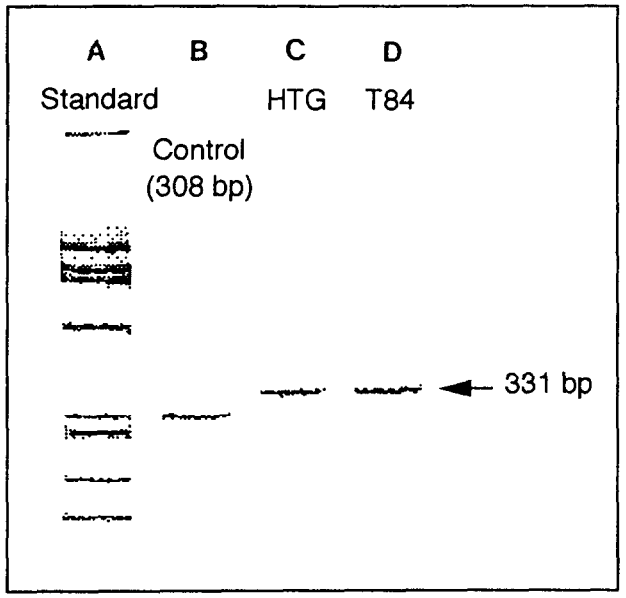


Fig. 1. RT-PCR analysis of the presence of CFTR mRNA in HTG cells. Lane A: $\Phi$X174/*Hae*III molecular weight marker. Lane B: 308 bp fragment, positive control of RT-PCR. Lane C: 331 bp fragment from a HTG cell culture. Lane D: 331 bp fragment from T84 cell culture.

mRNA when cultured by using cDNA-PCR amplification and polyacrylamide gel electrophoresis. Results revealed that HTG cells do express CFTR mRNA (Fig. 1, lane C). Control mRNA expression was also performed using (i) T84 cell line (Fig. 1, lane D), known to express a high level of CFTR protein [5], and (ii) RNA of known base pair number (308 bp) (Fig. 1, lane B). Since it has been demonstrated that CFTR is associated to a cAMP-dependent low conductance $Cl^-$ channel [6–10] we have searched for the presence of CFTR $Cl^-$ channels in the membrane of the human tracheo-bronchial gland cells using the patch-clamp technique.

Patch-clamp experiments were performed on HTG cells after they had formed a confluent monolayer. HTG cells in culture are highly polarized (M. Merten, unpublished data). The membrane which was directly accessible to patch-clamp electrodes was thus likely to be well differentiated and to contain the channels normally located in the apical membrane. Sealing of the patch pipette to the membrane was successful in less than 30% of the trials, presumably because submucosal gland cells secrete high molecular weight sulphated glycoproteins [25] and produce a mucus-like deposit onto the cells, which might have prevented the cellular membrane from sealing to the patch pipette. Once obtained, gigaohm seals remained stable for periods ranging from 5 to 45 min. Single channel currents were detected in 32 successful experiments performed on HTG cells.

In addition to the low conductance chloride channel

described below, we found cation-selective channels, outwardly rectifying chloride channels and high conductance (> 200 pS) anion-selective channels. These two $Cl^-$ channels were only observed in excised inside-out patches.

When HTG cells were bathed in NaCl saline and studied in the cell-attached configuration, no spontaneous low conductance $Cl^-$ channel activity was observed (0/30) (Fig. 2A). Low conductance $Cl^-$ channels were however induced in 46% of the patches obtained in this study (14/32) when the cells were stimulated by adding theophylline (1 mM), IBMX (1 mM), or a cocktail of db-cAMP (1 mM) and IBMX (0.1 mM) to the bath. Activation by db-cAMP and IBMX of low conductance $Cl^-$ channels ($n = 4$) is illustrated in Fig. 2B. Prior to the addition of the activators, no channel activity was recorded for periods as long as 20 min (Fig. 2B, upper trace). Channels became active within 2 min when stimulants were added (Fig. 2B, lower trace). Low conductance $Cl^-$ channels were always observed in clusters containing a variable number ($3.6 \pm 1$, $n = 14$) of identical channels. Binomial analysis of the channel opening distribution showed that clustered channels behaved independently with an identical opening probability (Fig. 2C). We activated low conductance $Cl^-$ channels by using either theophylline ($n = 6$, Fig. 2D) or IBMX ($n = 4$, data not shown). We also used two other protocols to tentatively activate low conductance $Cl^-$ channels in cell-attached patches. The calcium ionophore ionomycin (5 $\mu$M) was used to increase the intracellular calcium concentration (this was checked in experiments using Fluo 3, data not shown). The histogram in Fig. 2A illustrates that ionomycin-treated cell-attached patches ($n = 5$) did not contain low conductance $Cl^-$ channels. In addition, voltage steps which have been found to effectively unblock quiescent outward rectifier chloride channels in patches excised from several epithelia (e.g. [29]) failed to activate low conductance channels under the cell-attached conditions.

The properties of the low conductance channels were therefore deduced from the 14 experiments in which channel activity was induced by procedures that increased intracellular cAMP. Representative patch current traces from a HTG cell activated by theophylline are shown in Fig. 3A. This patch contained three channels which were active at both depolarized (positive) and hyperpolarized (negative) potentials. The channels had a linear current–voltage relationship and the cur-

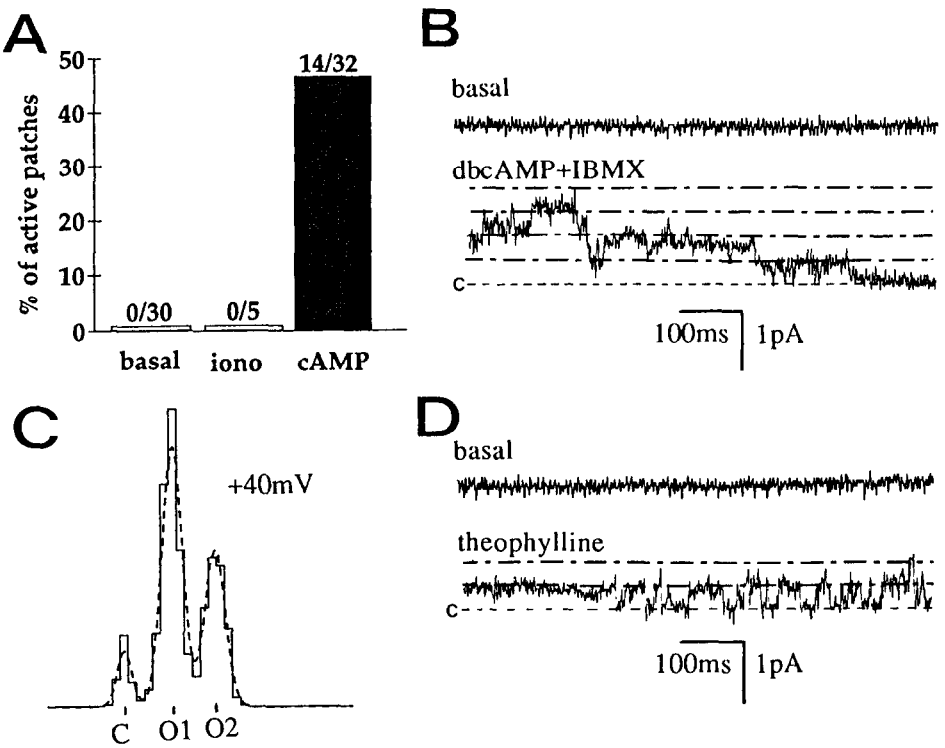


Fig. 2. (A) Number of cell-attached patches containing active low-conductance chloride channels in non-stimulated (basal), ionomycin- (iono, 5 $\mu$M) or cAMP-stimulated HTG cells. (B) Activation of low conductance chloride channels by db-cAMP and IBMX. Patch potential: +60 mV. When the HTG cells were bathed in a NaCl saline (basal conditions), there was no channel activity in the patch for up to 10 min continuous recordings. Addition of db-cAMP (1 mM) and IBMX (0.1 mM) to the bath caused channels to open within 2 min. The pipette contained choline chloride saline. (C) Amplitude histogram (continuous trace) fitted to Gaussian curves. Current recorded at +40 mV showing three equally spaced peaks corresponding to the opening of one and two (O1 and O2) channels (unitary conductance: 0.35 pA, Po = 0.6). C (close) refers to the null current baseline. (D) Activation of low conductance $Cl^-$ channels by theophylline (1 mM). Prior to the addition of the xanthine derivative to the bath NaCl saline (basal condition), no channel activity was recorded. The pipette was filled with choline chloride saline. In this and following figures, voltages refer to the actual patch potential from the cell resting level.

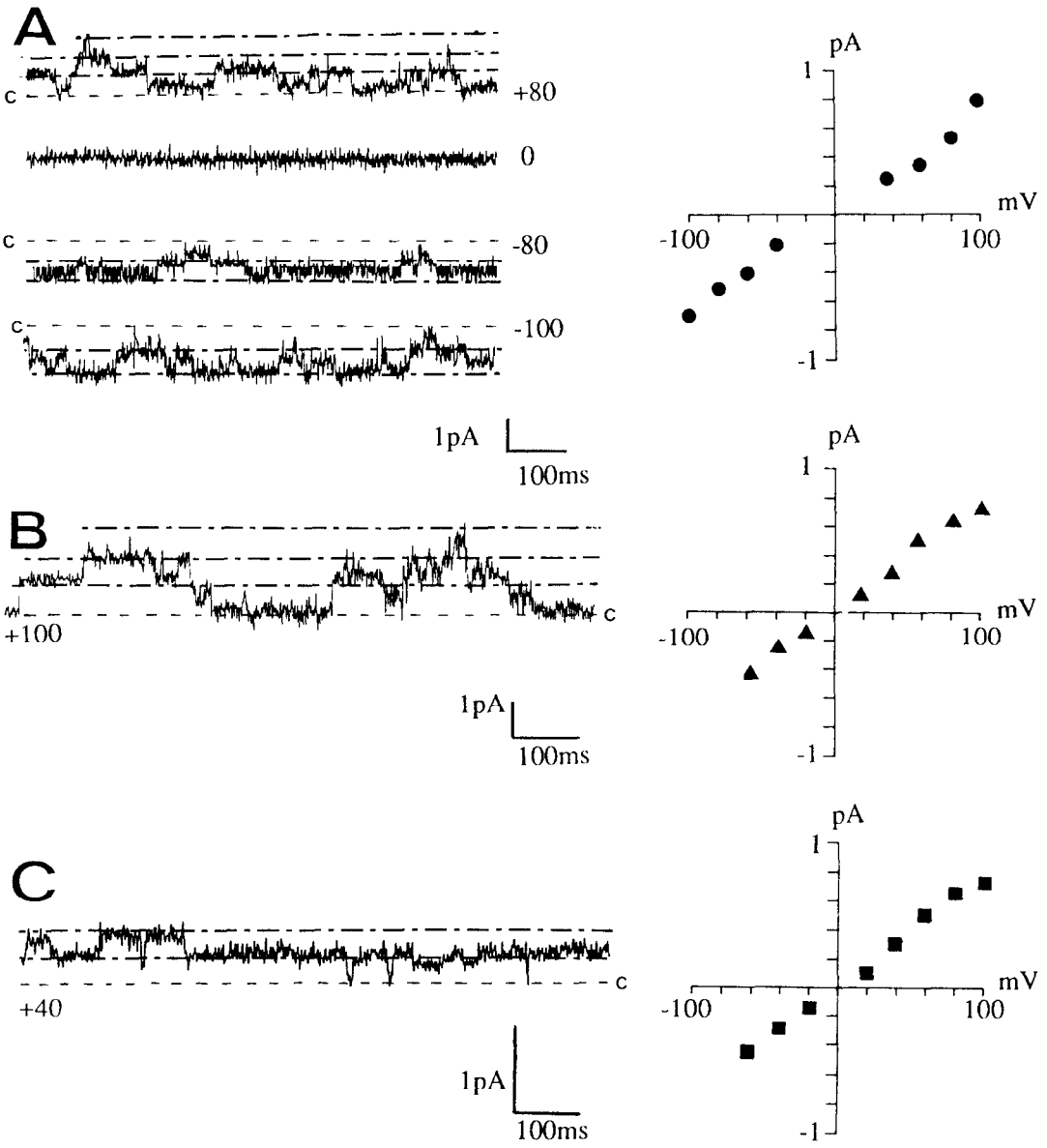


Fig. 3. (A) Representative single channel currents from theophylline (1 mM)-induced low conductance chloride channels recorded at the indicated potentials in a cell-attached patch. The pipette contained the KCl saline. Diagram corresponding unitary current–voltage relationship (B,C) Low conductance chloride channels and corresponding I V relationships with pipettes filled with choline chloride (B) or NaCl saline (C). Single channel conductances were 7.2 and 6.8 pS, respectively. In both ionic conditions, the reversal potential was close to zero mV. The bath contained db-cAMP (1 mM) and IBMX (0.1 mM) in (B) and IBMX (1 mM) in (C). Patch potentials: +100 mV in B and +40 mV in (C)

rent reverse direction at a potential close to the cell resting membrane potential (0 mV applied to the patch pipette). The reversal potential did not change when the patch pipette was filled with either KCl (Fig. 3A, $n = 6$), choline chloride (Fig. 3B, $n = 4$) or NaCl (Fig. 3C, $n = 4$), indicating that the channel was mainly selective for chloride over choline, $K^+$ and $Na^+$. The unitary conductance, deduced from the slope of the I–V relationship, was $7 \pm 0.8$ pS ($n = 14$). The opening probability of cAMP-activated cell-attached channels does not noticeably vary with the applied potential (see Fig. 3).

Patches containing cAMP-activated low conductance $Cl^-$ channels were excised in the inside-out configurations. Within 2 min after the excision we observed a progressive decrease in the channel activity. The deactivated excised channel could not be reactivated by pulsing the voltage to high positive or negative values.

## 4. DISCUSSION

The low conductance $Cl^-$ channel in HTG cells has a unitary conductance of 7 pS in cell-attached patches

of cAMP-treated cells and a linear I–V relationship. The properties of this channel are consistent with those of CFTR-related $Cl^-$ selective channels found in immortalized airway cell lines [18], human pancreatic duct [30], thyroid [31] and T84 cells [32]. Expression of the cystic fibrosis gene produced a cAMP-regulated $Cl^-$ channel with a cell-attached conductance of 8 pS in the non-epithelial Sf9 cells [6], 4 pS in Vero cells [9], 9 pS in CHO cells [8] and 10 pS in HeLa cells [7]. Recently, Bear and collaborators [10] demonstrated that incorporation of the purified CFTR protein in artificial bilayer produced a similar low conductance $Cl^-$ channel that is regulated by protein kinase A (PKA). Since we found CFTR expression in HTG cells, we believe that the 7 pS $Cl^-$ channel in HTG cells is the CFTR-channel. Recently, using immortalized human airway cell lines, Haws et al. [18] identified a linear 6 pS $Cl^-$ channel activated by forskolin in cell-attached patches. This channel has properties closely related to those of the channel described in this paper.

In airway cells, mutations in the CFTR gene lead to an imbalance in ion and fluid transport and ultimately to abnormal mucus secretion. Several lines of evidence suggest that submucosal gland cells are a major primary site of the disease cystic fibrosis. Firstly, these ducts are already impacted at birth before the occurrence of complications from chronic respiratory infections. Secondly, chronic infections and obstruction of gland ducts are early clinical hallmarks of neonates with CF [33]. Thirdly, submucosal cells from CF patients concentrate intracellular chloride [34]. Finally, cystic fibrosis HTG cells in culture show a constitutive hypersecretion of macromolecules which is up to 50-fold that in normal cells, and are hyporesponsive to agonists [35]. As mentioned above, the highest expression of CFTR is found in submucosal gland cells whereas CFTR is almost undetectable in airway surface lining cells [20,21]. The presence of normal CFTR mRNA and of CFTR-like $Cl^-$ channels in the human tracheo-bronchial gland cells is therefore consistent with the idea that submucosal gland cells are a major target for the disease.

As far as we know, the present data constitute the first report on single channel recording in submucosal gland cells. Our data show that CFTR mRNA and CFTR-chloride channels are present in cultured HTG cells. Mucus secretion occurred in a dehydrated form in the airway tract. The precise site of hydration is still uncertain but its location would influence the viscosity and thus the mucus clearance [1]. With a better hydratation occurring in the gland itself, the secretory products should be easily evacuated in the broncho-tracheal lumen. Since hydratation is at least partly consecutive to a chloride-dependent water transport, the presence of the CFTR-$Cl^-$ channel and its activation by cAMP in HTG cells is of primary importance in the understanding of the regulation of the hydratation process of the human airway mucus.

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