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cAMP-dependent activation of ion conductances in bronchial epithelial cells

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Abstract. The cAMP-dependent activation of Cl^- channels was studied in a bronchial epithelial cell line (16HBE14o-) in fast and slow whole-cell, and cell-attached patch-clamp experiments. The cells are known to express high levels of cystic fibrosis transmembrane conductance regulator mRNA and protein. Isoproterenol, forskolin and histamine (all 10 μmol/l) reversibly and significantly depolarized the membrane voltage (V_m) and increased the whole-cell Cl^- conductance significantly by 34.0 ± 0.9 ($n = 3$), 18.1 ± 2.7 ($n = 50$), and 25 ± 4.5 ($n = 37$) nS respectively. The effect of histamine was blocked by cimetidine (10 μmol, $n = 5$) but not by diphenhydramine (10 μmol/l, $n = 4$), which suggests binding of histamine to H_2 receptors. The forskolin-induced current was not inhibited significantly by 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (0.5 mmol/l, $n = 9$) nor glibenclamide (10 μmol/l, $n = 3$) and had an anion-permeability sequence of $Cl^- = Br^- > I^-$ ($n = 9$). In cell-attached recordings forskolin (10 μmol/l) increased the conductance of the patched membrane from 65.5 ± 13.6 pS to 150.8 ± 33.2 pS ($n = 30$). Although the conductance was increased significantly, clear ion-channel events occurring in parallel with the current activation were not detected in the cell-attached membrane. In 4 out of 30 cell-attached recordings single-channel currents were observed. These channels, with a single-channel conductance of about 6 pS, were already active before forskolin was added. No effect of forskolin on the channel amplitude, open probability or kinetics of these channels was observed. From these data we conclude that the cAMP-induced conductance increase in 16HBE14o- cells can be correlated with the activation of very small and not resolvable (probably less than 2 pS) Cl^- channels rather than with the activation of channels with a conductance of 6–10 pS.

Key words: Cl^- channels – Histamine – Forskolin – Isoproterenol – CFTR – Bronchial epithelial cells

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) [19, 30, 31] is regarded as a channel-forming protein. In a number of investigations, expression of CFTR protein was correlated with (a) the induction of a cAMP-dependent whole-cell Cl^- conductance and (b) the appearance of a 6- to 10-pS Cl^- channel [1, 3, 7, 18, 28, 29, 32]. In previous studies we also found expression of a cAMP-dependent Cl^- conductance in cells that were transfected with the wild-type sequence cDNA of CFTR [12, 21]. However, we and others did not find activation of 6- to 10-pS Cl^- channels in cells intrinsically expressing CFTR. Rather the activation of very small Cl^- channels with a single-channel conductance of 1–2 pS or less was suggested [9, 22, 24, 34].

In the present study we examined the effects of cAMP-dependent stimulation in an immortalized bronchial epithelial cell line (16HBE14o-) [4]. The cells grow as a polarized monolayer and, in contrast to most other respiratory cell lines, express high levels of CFTR mRNA and protein in quantities comparable to that present in the colonic carcinoma cell line T_{84}. Because of these properties and the fact that the cells are immortalized and available in large quantities, they are a very useful model for studying ion transport and the function of CFTR. Here we examine the activation of cAMP-dependent currents. In an accompanying paper we describe the effects of secretagogues that increase intracellular Ca^{2+} [20].

Materials and methods

Cell culture. The 16HBE14o- cell line was derived from a bronchial surface epithelium as described in a previous study [4]. The methods of cell culture are described in detail in the accompanying manuscript [20]. For most experiments, the cells were cultured on tissue-culture plasticware coated with fibronectin, collagen (Vitrogen; Collagen Corp. Palo Alto, Calif., USA) and bovine serum albumin and kept in an atmosphere of 5% CO_2 [11, 27]. For some experiments, cells were plated on vitrogen-gel-coated permeable

filters (Millipore, Bedford, Mass., USA) and grown with an air/liquid interface in a DMEM/F12 medium supplemented with 20 g/l ultroser G (Biotechnics, France), as described previously for primary cultures of human cells [4, 35]. Since very similar results were obtained in this study with both filter- and plastic-grown cells, the results were pooled.

Patch clamp. All studies were performed at 37° C. The methods for patch clamping whole cells are described in detail in the accompanying paper [20]. In brief, the patch pipettes (2–4 MΩ) were filled with a solution containing (in mmol/l) KCl 30, potassium gluconate 95, NaH_2PO_4 1.2, Na_2HPO_4 4.8, EGTA 1, $CaCl_2$ 0.73, $MgCl_2$ 1.0, D-glucose 5, ATP 1. The standard bath solution contained (in mmol/l) NaCl 145, K_2HPO_4 1.6, KH_2PO_4 0.4, $CaCl_2$ 1.3, $MgCl_2$ 1, D-glucose 5. A flowing KCl electrode served as a reference and appropriate corrections for the measured liquid-junction voltages were made. The pH was adjusted to 7.2. The Ca^{2+} activity in this solution was 0.1 μmol/l. In some experiments the cells were examined with the slow whole-cell method as described in a previous report [26]. Since results obtained in the fast and slow whole-cell methods were comparable, the data were pooled. The access conductance was controlled with a sinusoidal command voltage (800 Hz) in each experiment and was between 30 nS and 120 nS (for details cf. [20]). Appropriate corrections for the measured access conductance were made in order to obtain accurate whole-cell conductance measurements. Experiments with an access conductance below 30 nS were excluded from the analysis. The membrane voltage (V_m) of the cells was recorded continuously using the current-clamp mode of the patch-clamp amplifier and the whole-cell current was measured at regular intervals by clamping the membrane voltage to ± 30 mV in steps of 10 mV. The whole-cell conductance (G_m) was calculated from the measured whole-cell current (I) and the applied clamp voltage (V_c).

For cell-attached recordings smaller pipettes (5–8 MΩ) were used and filled with the standard extracellular solution described above. Currents and voltages were recorded continuously and displayed by a pen recorder. To minimise noise the high-gain range (50 mV/pA) of the patch-clamp amplifier (List LM-EPC 7, Darmstadt, Germany, or a similar instrument built at our institution) was used. For further analysis the current recordings obtained in cell-attached experiments were filtered at 400 Hz and digitised at 1.5 kHz. No attempts were made to quantify the variance of the stationary current fluctuations.

All compounds were of the highest available grade of purity. They were obtained from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany). Agonists were dissolved from a stock in standard bath solution and prepared fresh daily.

All data are presented as original recordings or as mean values ± SEM (n number of observations). Statistical analysis was performed according to Student's t-test. P values below 0.05 were accepted as indicating statistical significance.

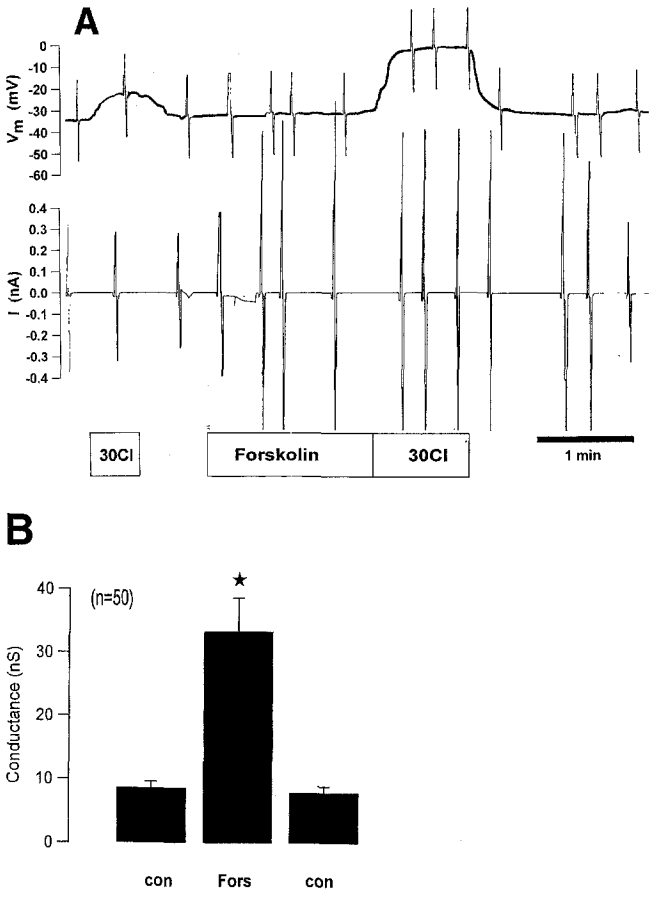

Fig. 1 A, B. Effect of forskolin (10 μmol/l) on bronchial epithelial cells (16HBE14o-). **A** The whole-cell current (I) and the membrane voltage (V_m) are plotted as functions of time. Note that the cell was monitored in zero-current mode for most of the time (V_m measurement). Once or twice per minute the amplifier was switched to voltage-clamp mode and V_m was clamped in the positive or negative direction in 10 mV increments (staircase on top of V_m recording in the *upper trace*). The corresponding currents are apparent in the *lower trace*. G_t was determined from the ΔI obtained from ± 30 mV. Forskolin reversibly increased the whole-cell current I (*lower recording*). Enhanced depolarization of V_m caused by a reduction of extracellular Cl^- concentration to 30 mmol/l (*30Cl*) was observed after stimulation with forskolin, indicating the activation of a Cl^- conductance. **B** Summary of the whole-cell conductances obtained in experiments described in **A**. ★ Significant difference

Results

Effect of forskolin and isoproterenol on whole-cell properties

Forskolin (10 μmol/l) slightly but significantly depolarized the cells by 5.9 ± 1.4 mV and reversibly increased the whole-cell conductance significantly from 8.6 ± 1.0 nS to 33.3 ± 5.3 nS (n = 50) (Fig. 1 A, B). Reduction of the extracellular Cl^- concentration to 30 mmol/l depolarized the membrane voltage (V_m) to a significantly greater degree after forskolin stimulation (17.6 ± 1.1 mV) as compared to the control state (7.1 ± 1.1 mV; n = 47; also cf. Fig. 1 A). The effect of forskolin was inhibited only slightly and not significantly, by 0.5 mmol/l 4,4′-diisothiocyanatostilbene-2,2′-sulphonic acid (ΔG = −5.4 ± 4.2 nS, n = 9) and was increased, albeit not significantly, by 0.5 mmol/l glibenclamide (ΔG = 4.0 ± 3.7 nS, n = 3).

When bath Cl^- was replaced by equimolar concentrations of Br^- in forskolin-stimulated cells there was no significant change in either V_m or whole-cell current (n = 3). In contrast, when Cl^- was replaced by I^-, V_m was significantly and reversibly depolarized by 6.3 ± 1.9 mV (n = 9) and the whole-cell conductance was reduced by 21.9 ± 5.1 nS (n = 9). From these experiments a permeability sequence of the cAMP-induced whole-cell Cl^- current of $Cl^- \approx Br^- > I^-$ can be deduced.

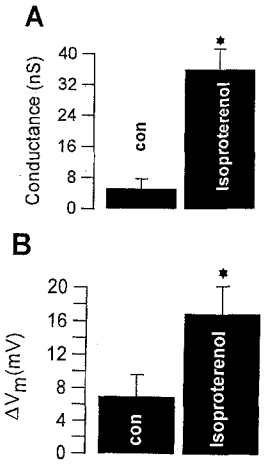

Fig. 2 A, B. Effect of isoproterenol (10 μmol/l) on bronchial epithelial cells (16HBE14o-). **A** Summary of the whole-cell conductances before and after stimulation with isoproterenol ($n = 3$). **B** Summary of the change of V_m (ΔV_m) due to a decrease of extracellular Cl^- concentration to 30 mmol/l before (*con*) and after stimulation with isoproterenol. ★ Significant difference

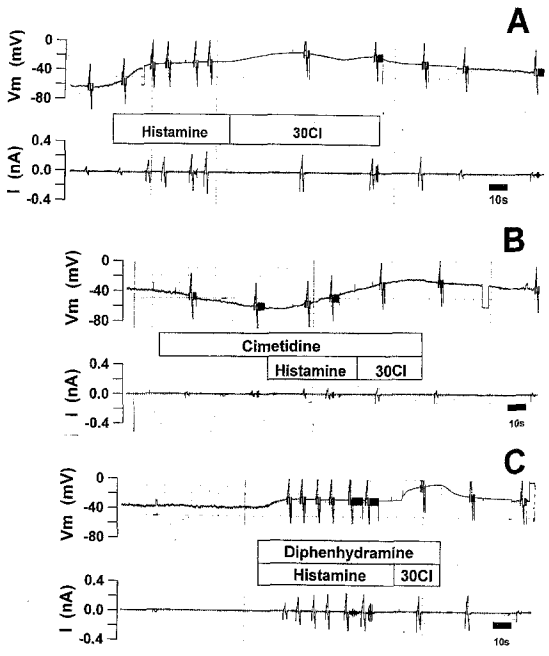

Fig. 3 A–C. Effect of histamine (10 μmol/l) on bronchial epithelial cells (16HBE14o-). All experiments were carried out on the same cell. The whole-cell current (I) and the membrane voltage (V_m) are plotted as functions of time. Note that the cell was monitored in zero-current mode for most of the time (V_m measurement). Once or twice per minute the amplifier was switched to voltage-clamp mode and V_m was clamped in a positive and negative direction in 10 mV increments (staircase on top of V_m recording in the *upper trace*). The corresponding currents are apparent in the *lower trace*. G_t was determined from the ΔI obtained from ± 30 mV. Depolarization of V_m by partial replacement of extracellular Cl^- in the bath (30 mmol/l, *30Cl*) indicates the magnitude of the fractional Cl^- conductance. **A** Histamine reversibly depolarized V_m and increased the whole-cell current I (*lower recording*). **B** In the presence of cimetidine (10 μmol/l) only a slow depolarization of V_m and only a slight activation of the whole-cell current were observed. **C** No inhibition of the histamine-induced whole-cell current was seen with diphenhydramine (10 μmol/l)

Isoproterenol (10 μmol/l), a compound acting primarily on β_1 receptors, had a biphasic effect on V_m of 16HBE14o- cells. V_m was depolarized and the whole-cell conductance was increased significantly from 2.7 ± 0.4 nS to 36.6 ± 0.3 nS ($n = 3$). In isoproterenol-stimulated cells the depolarization of V_m by a reduction of the Cl^- concentration in the bath to 30 mmol/l was increased significantly by 10.0 ± 2.8 mV ($n = 5$), indicating the activation of a Cl^- current (Fig. 2 A, B).

Effect of histamine

The effect of various concentrations of histamine was examined and a concentration-dependent activation of Cl^- currents was observed (Fig. 3 A and Fig. 4 A, B). The reduction of extracellular Cl^- concentration to 30 mmol/l depolarized V_m to a significantly larger degree after stimulation with histamine (16.1 ± 1.4 mV compared to 10.2 ± 1.9 mV, $n = 19$) indicating the activation of a Cl^- conductance. The time course of current activation was very similar to that by forskolin, suggesting that intracellular cAMP was increased by the binding of histamine to an H_2-type receptor. To verify this assumption, we examined the effects of the H_1 and H_2 inhibitors diphenhydramine and cimetidine (Fig. 3 B, C). Cimetidine (10 μmol/l) abolished the histamine-induced current almost completely ($n = 5$), whilst the same concentration of diphenhydramine was ineffective ($n = 4$). However, at 0.1 mmol/l diphenhydramine also blocked the current significantly.

Effects of cAMP-dependent stimulation in cell-attached membranes

In cell-attached experiments we stimulated the cell with forskolin in order to correlate possible single-channel events with the activation of the Cl^- conductance. Most of the cell-attached patches were already very active before stimulation, i.e. current noise was present that apparently resulted from channels already active in the membrane of non-stimulated cells (Fig. 5 C). However, we were not able to deduce a unique single-channel conductance from these traces. The current traces of the cell-attached patches did not change significantly upon stimulation with forskolin, although the overall (baseline) membrane current was increased reversibly (Fig. 5 A, B). In all 30 experiments we found no single-channel events of which the open probability correlated to the stimulation of the cells by forskolin, although the baseline membrane current was reversibly activated in cell-attached membranes (Fig. 5 A, B, D). In 4 out of these 30 cell-attached recordings resolvable single-channel currents were observed (Fig. 6 A, B). These channels, with a single-channel conductance of about 6–7 pS, were not activated by stimulation with forskolin but were already active before forskolin was applied. The original recording of Fig. 6 A is part of a continuous 15-min recording; it contains five single channels. P_o

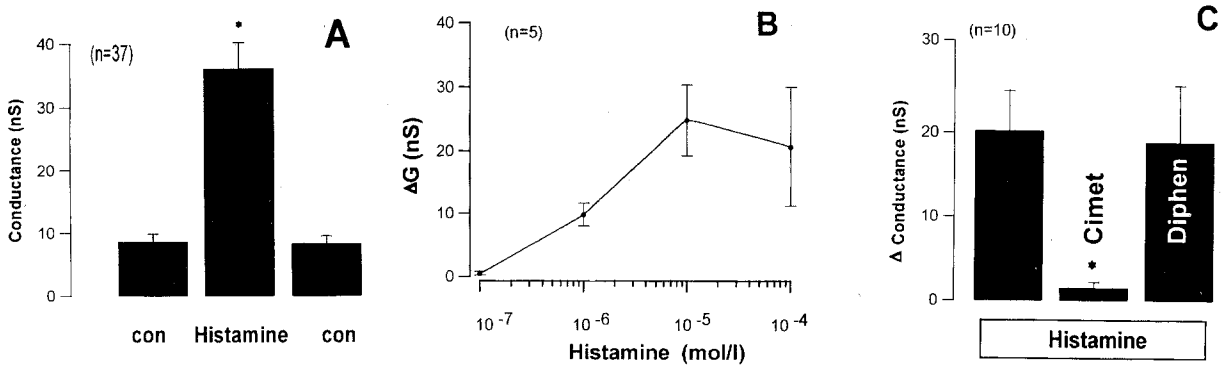

Fig. 4 A. Summary of the effect of histamine on the whole-cell conductances as described for Fig. 3 A. **B** Concentration/response curve for histamine-induced change in the whole-cell conductance (ΔG) of (16HBE14o-) cells. The half-maximal effect on ΔG is achieved at approximately 1.5 μmol/l. **C** Summary of the experiments with inhibitors of histamine receptors. Cimetidine but not diphenhydramine (both 10 μmol/l) inhibited the effect of histamine on the whole-cell conductance. ★ Significant difference

was determined as described previously [16]. No effect on the channel amplitude, open probability or kinetics of these channels was observed when the cells were stimulated with forskolin. P_o was 0.89 ± 0.2 in the control state and 0.88 ± 0.3 after forskolin.

Discussion

cAMP-dependent Cl^- currents were studied in the bronchial epithelial cell line 16HBE14o- [4]. In a previous study we reported polarized growth and high expression of CFTR message and protein in these cells [4]. In this study, we demonstrated that short-circuit currents in monolayers formed by 16HBE14o- cells as well as $^{36}Cl^-$ efflux can be activated by hormones increasing cAMP. Here, we characterized in more detail this CFTR-related, i.e. cAMP-dependent Cl^- conductance.

Whole-cell experiments indicate that these cells activate Cl^- channels when stimulated by forskolin, isoproterenol or histamine. The latter agonist appears to act by binding to a H_2 receptor with a subsequent increase in cytosolic cAMP, since cimetidine, but not diphenhydramine, blocked the effects of histamine. Very similar results were obtained in transepithelial measurements with filter-grown 16HBE14o- monolayers [20]. In this case, the increase in the equivalent short-circuit current (I_{sc}) after application of histamine to the apical side of the epithelium was also blocked by cimetidine. However, histamine applied basolaterally presumably bound to H_1 receptors and only transiently activated I_{sc} [19]. Both H_1 [13, 14] and H_2 [8] histamine receptors have been observed in airway cells, although the relative importance of the two different types is not clear [17]. The present results and those of an accompanying report are compatible with a polarized distribution of apical H_2 and basolateral H_1 receptors. Apical stimulation results in a sustained increase of NaCl secretion whilst basolateral stimulation results in a more transient current activation. Transient effects of histamine on I_{sc} and cytosolic Ca^{2+} mediated by H_1 receptors were also described previously [13, 14].

CFTR expression is correlated to the appearance of a cAMP-dependent Cl^- conductance in a variety of cells [2, 5, 18, 25]. cAMP-dependent stimulation of whole-cell Cl^- currents in epithelial cells has been attributed to the activation of CFTR Cl^- channels [1, 3, 15, 32, 33]. In these studies, the single-channel conductance of the CFTR channel was measured in the range of about 6–10 pS. However, we and others did not detect ion channels of this conductance that could be correlated to cAMP-dependent stimulation of colon carcinoma (HT_{29}, T_{84}) and respiratory epithelial cells in primary culture [6, 22, 23]. Rather, activation of very low-conductance Cl^- channels with a single-channel conductance of less than 4 pS was suggested [6, 9, 22].

In the present study we re-examined this issue and tried to discover the nature of the cAMP-activated Cl^- channel. We used a cell line that expresses high levels of CFTR and shows a clear whole-cell current activation following stimulation with cAMP-increasing agonists.

One might argue that activation of Cl^- channels was prevented in our previous [22, 24] and present cell-attached experiments because the physical state of the membrane within the patch pipette may, for instance, prevent the insertion of new vesicular membrane. This appears unlikely because the membrane conductance of these patches clearly increased more than twofold (by approximately 90 pS) after forskolin. In the present studies these changes in membrane current occurred within the expected time (20–60 s) after the application of forskolin and they were completely reversible when forskolin was removed. The resolution of the present experiments would have enabled us to see channels of more than 2 pS. Although we have not formally proven that this membrane conductance is one for Cl^-, this appears very likely in view of the whole-cell observations reported here. In the previous studies [22, 24], because of the use of small concentrations of nystatin [10], we were even able to prove that these membrane currents were correlated in time with the changes of the cell voltage. Therefore, we conclude that cAMP induced a Cl^- membrane conductance with an average value of 90 pS, but that the respective current events had no detectable current amplitude (i.e. $\leq$ 2 pS).

In another study [15] similar experiments were performed with the same cell line. As in the present study,

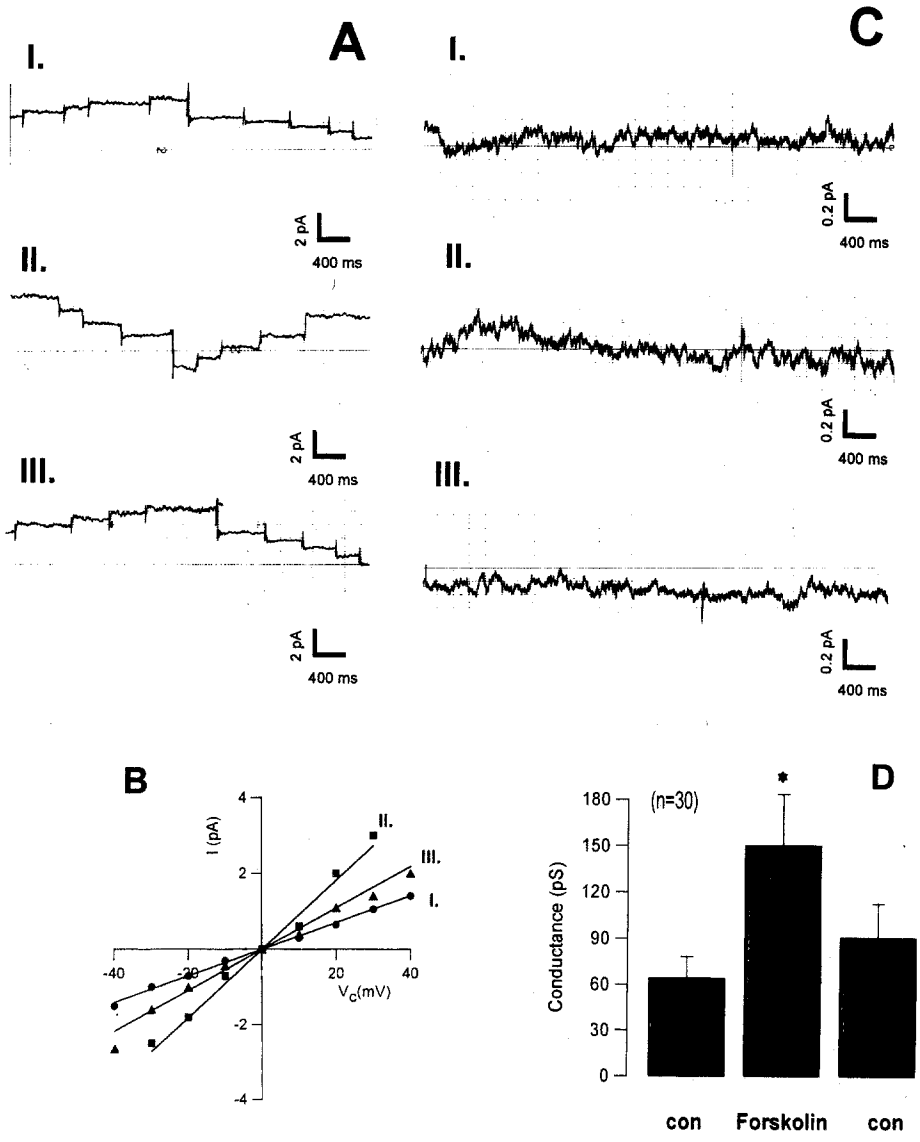

Fig. 5 A–D. Effect of forskolin (10 μmol/l) on cell-attached membrane patches of bronchial epithelial cells (16HBE14o-). **A** Original continuous recording of the current present in a cell-attached membrane before (*I*), during (*II*), and after (*III*) stimulation with forskolin (10 μmol/l). From a holding potential of −40 mV (cell negative), the membrane was clamped to ± 40 mV in steps of 10 mV. Note that the changes in membrane current per 10 mV step in clamp voltage (V_c) are bigger in *II*. **B** I/V_c curve obtained from the experiment shown in **A**. *I, II, III* correspond to the traces in **A**. Note that the current of the cell-attached membrane was reversibly increased by forskolin. **C** Ion currents through a cell-attached membrane at high gain. Although a current was activated in the cell-attached membrane, no resolvable single-ion-channel events were observed. The current trace was filtered at 400 Hz. *I, II, III* correspond to the traces in **A** and **B**. **D** Summary of experiments shown in **A** and **B**. ★ Significant difference

it was not possible to activate 6- to 10-pS CFTR channels in cell-attached patches by forskolin. However, preincubation of the cells with forskolin 5−60 min prior to the patch-clamp experiments apparently increased the numbers of active 6-pS channels from 0.6 to 3.0/patch in subsequent cell-attached recordings. The results from such unpaired experiments are difficult to interpret. Besides, this number of 3 (n) × 6 pS (single-channel conductance) hardly suffices to explain the large increases in whole-cell conductance observed in these cells (20 nS, cf. Fig. 1).

In the present experiments on four occasions (13% of all experiments in this series) we also observed channels with comparable single-channel conductances in cell-attached patches of non-stimulated cells. However, we could not detect further activation of these channels by stimulation of the cell with forskolin. Therefore, the appearance of a 6-pS channel did not correlate with cAMP stimulation. In contrast, we observed a reversible activation of the baseline membrane current in these and all other cell-attached membranes.

Taken together, these results are in agreement with those studies demonstrating that cAMP- and also Ca^{2+}-activated Cl^- channels in epithelial cells are of very small conductance [6, 22, 24, 34]. The possibility cannot be excluded at this stage that this class of cAMP-controlled Cl^- channels can expose a wide distribution of single-channel conductances with a mean value in the

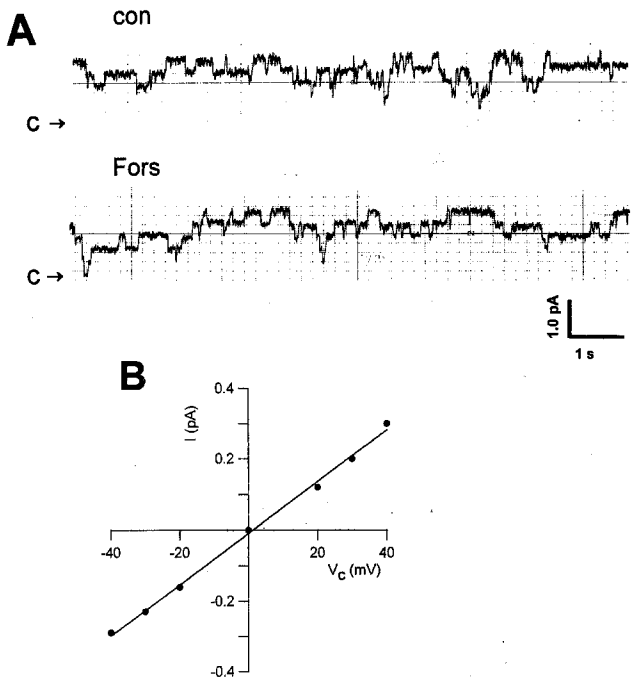

Fig. 6 A. Original recording of single-channel events in a cell-attached membrane of a bronchial epithelial cell (16HBE14o-). The clamp voltage was + 40 mV. *C* The zero-current level. The current recording was filtered at 400 Hz. Note that the open probability of the channels under control (*con*) conditions (P_o = 0.92) was not increased by 10 µmol/l forskolin (*Fors*; P_o = 0.81). **B** Current/voltage (I/V_c) relationship of the channels shown in **A**. The I/V_c curves were identical with and without forskolin

low picosiemens range and that the usual single-channel protocols are prone to pick up the population of "larger" channels selectively.

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