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Insensitivity of volume-sensitive chloride currents to chromones in human airway epithelial cells

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1 Chromones (sodium cromoglycate and sodium nedocromil) block cell swelling-activated Cl^- channels in NIH-3T3 fibroblasts and endothelial cells. This has led to hypothesize that cell volume regulation might be involved in asthma pathogenesis.

2 Using whole-cell patch-clamp experiments, we studied the effect of chromones on volume-sensitive Cl^- currents in transformed human tracheal epithelial cells (9HTEo-) and in primary cultures of human bronchial epithelial cells (BE).

3 Cl^- currents activated by hypotonic shock were poorly blocked by extracellular nedocromil or cromoglycate. The block was voltage-dependent since it was observed only at positive membrane potentials. At the concentration of 5 mM, the current inhibition by both chromones at +80 mV was about 40% for 9HTEo- and only 20% for BE.

4 Intracellular application of chromones elicited a voltage-independent inhibition in 9HTEo- cells. Under this condition, volume-sensitive Cl^- currents were reduced at all membrane potentials (60 and 45% inhibition by 2 mM nedocromil and cromoglycate respectively). In contrast intracellular chromones were ineffective in BE cells.

5 The relative refractoriness to chromones, in contrast with the high sensitivity shown by other Cl^- channels, suggests that the epithelial volume-sensitive Cl^- channel is not involved in asthma.

Keywords: Nedocromil sodium; sodium cromoglygate; Cl^- currents; asthma; cell volume regulation; airway epithelium

Introduction

Agents used for the prophylactic treatment of allergic asthma, namely sodium cromoglycate and sodium nedocromil, are able to block different Cl^- channels in epithelial and non epithelial cells. Sodium cromoglycate was found to block intermediate conductance Cl^- channels if applied to the cytoplasmic side of membrane patches excised from mast cells (Romanin *et al*., 1991) and HT_{29} colonic carcinoma cells (Reinsprecht *et al*., 1992). This compound and sodium nedocromil were also able, from the putative extracellular side, to inhibit an airway epithelial Cl^- channel reconstituted in planar phospholipid bilayers (Alton *et al*., 1996). Furthermore, whole-cell recordings, carried out on endothelial cells (Heinke *et al*., 1995) and NIH-3T3 fibroblasts (Gschwentner *et al*., 1996), have shown that chromones inhibit the volume-sensitive Cl^- current ($I_{Cl(vol)}$). This type of channel is essential for the adaptive cell volume decrease that follows a hypotonic shock.

The ion channel block caused by chromones has suggested that Cl^- transport may be in some way involved in the pathogenesis of bronchial asthma (Norris & Alton, 1996). In particular, the sensitivity of $I_{Cl(vol)}$ to nedocromil and cromoglycate has led to hypothesize that the process of cell volume regulation in airway epithelial cells is linked to the occurrence of asthmatic attacks. The involvement of Cl^- transport in asthma is also suggested by the recent mapping of a candidate $I_{Cl(vol)}$ gene (Nagl *et al*., 1996) in a chromosomal region where a locus for asthma has been localized (Daniels *et al*., 1996).

The gene(s) responsible for $I_{Cl(vol)}$ channels have not been clearly identified (Nilius *et al*., 1996). It has been recently claimed that I_{Cln}, which is a candidate gene for the Cl^- current in NIH-3T3 fibroblasts (Gschwentner *et al*., 1995), is not related to the volume-sensitive Cl^- channel endogenously expressed in other cells (Voets *et al*., 1996). Another candidate for this current in NIH-3T3 cells is CIC 3 (Duan *et al*., 1997). On the other hand, the swelling-induced current in endothelial cells (Heinke *et al*., 1995) has biophysical characteristics, i.e. lack of inactivation at positive membrane potentials and linear current-voltage relationship, which are different from those of volume-sensitive Cl^- currents in epithelial cells (Jackson & Strange, 1995; Kubo & Okada, 1992; Rasola *et al*., 1994). These indications suggest the existence of different types of Cl^- channels regulated by cell volume changes. We therefore decided to check whether chromones are able to block the $I_{Cl(vol)}$ of human airway epithelial cells. Our results show that sodium nedocromil and sodium cromoglycate partially inhibit $I_{Cl(vol)}$ only at elevated millimolar concentrations.

Methods

Cell culture

9HTEo- cells were obtained from human tracheal epithelium by transformation with the SV40 large T antigen (Gruenert *et al*., 1988). Cells were grown at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (Hyclone, Cramlington, U.K.) supplemented with 10% serum (Fetal Clone II, Hyclone), 2 mM glutamine, 100 units ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin. Human bronchial cells were cultured in serum-free medium prepared by mixing LHC (Biofluids, Rockeville, MD, U.S.A.), RPMI 1640 (Hyclone) and supplements as described (Galietta *et al*., 1998). For electrophysiological experiments, 9HTEo- and human bronchial epithelial cells (BE) were seeded on Petri

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dishes (25,000 cells per dish). Experiments were carried out in the first 3 days after plating in order to have small and isolated cells. The cell capacitance was in the range 10–30 pF.

To induce cell polarization, BE cells were seeded at high density on permeable supports (Transwell-COL, Costar, Cambridge, MA, U.S.A.) in DMEM/Ham's F12 (1:1) with 2% Fetal Clone II and supplements (Galietta *et al*., 1998). After 6 days, when bronchial monolayers developed a large transepithelial electrical resistance, filters were cut in pieces of about 1 cm^2. At the beginning of each experiment, a piece of filter was washed with Puck's saline A (Sigma, St. Louis, MO, U.S.A.) and mildly trypsinized for 1 min. This treatment was required to break junctions between cells and to allow giga-seal formation. The filter fragment was then mounted in a home-made microchamber for patch-clamp experiments.

Electrophysiology

Total membrane currents were measured in the whole-cell configuration of the patch-clamp technique (Hamill *et al*., 1981). Under this condition, the application of an extracellular hypotonic solution to 9HTEo- or BE cells results in activation of volume-sensitive Cl^- channels, as previously described (Rasola *et al*., 1994; Zegarra-Moran *et al*., 1997). Patch-clamp electrodes were prepared from borosylicate glass capillaries using a puller (List-Medical, Darmstadt, Germany). The microelectrode tip was fire polished with a microforge (List-Medical) to obtain an electrical resistance between 2 and 4 MΩ. A standard patch-clamp amplifier (EPC-7, List-Medical) was used to clamp the cells at different membrane potentials. Voltage stimulation and acquisition of resulting membrane currents were carried out with a 16-bit AD/DA converter (ITC-16, Instrutech) controlled by a personal computer (Mega/STE, Atari, Sunnyvale, CA, U.S.A.). The current signal was sampled at 2 kHz and filtered with an Ithaco 4302, 8-pole Bessel filter (Ithaca, NY, U.S.A.) at a cut-off frequency of 1 kHz.

Solutions

The intracellular solution which filled the patch micropipette was (in mM): 60 CsCl, 45 Cs_2SO_4, 1 $MgCl_2$, 0.5 [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA), 10 Na-N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (Na-HEPES), 45 mannitol (pH = 7.3; 277 mosm kg^{-1}). For experiments with BE cells, 2 mM adenosine 5′-triphosphate sodium salt (NaATP) was included in the pipette solution to avoid run down of swelling-activated Cl^- currents (unpublished results). The isotonic extracellular solution contained (in mM): 130 NaCl, 2 KCl, 1 KH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 10 Na-HEPES, 10 glucose, 20 mannitol (pH = 7.3; 295 mosm kg^{-1}). The hypotonic extracellular solution contained (in mM): 70 NaCl, 2 KCl, 1 KH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, 40.7 mannitol (pH 7.3; 206 mosm kg^{-1}). When chromones were added to extracellular hypotonic solutions, mannitol was properly reduced to maintain the osmolarity.

For the study with intracellular chromones the mannitol in the micropipette solution was replaced by isosmolar amounts of nedocromil sodium and sodium cromoglycate to obtain the desired chromone concentration.

Materials

Nedocromil sodium and sodium cromoglycate were from Fisons (Loughborough, U.K.). Sodium cromoglycate was 21 mM in water. Nedocromil sodium stock solution was prepared in water at a concentration of 1 M. All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Statistics

Experiments are shown as raw data or as mean ± standard error of the mean. The statistical significance of differences between groups of measures was evaluated with the Student's *t*-test for paired or unpaired data as appropriate.

Results

Application of the hypotonic extracellular solution to 9HTEo- and BE cells resulted in a slow increase of membrane conductance. The current reached a stable value after about 10 min. At steady-state, current-voltage relationships were determined by clamping the membrane at potentials in the range −100 to +120 mV. The currents activated by the hypotonic shock had the typical features of volume-sensitive Cl^- currents in 9HTEo-, BE and other epithelial cells (Kubo & Okada, 1992: Jackson & Strange, 1995; Rasola *et al*., 1994; Zegarra-Moran *et al*., 1997), i.e. inactivation at positive potentials and outwardly rectifying current-voltage relationship. Extracellular application of nedocromil sodium (NED) or sodium cromoglycate (CRG) at concentrations up to 2 mM had no effect on hypotonically-activated currents. Indeed, 5 mM of either compound was required to cause a significant inhibition (Figures 1 and 2). The onset and removal of block were rapidly obtained (1–2 min) upon application and wash out of NED and CRG. As shown in the current-voltage relationships of Figure 1, chromone effect on 9HTEo- cells was voltage-dependent since it was only observed at positive membrane potentials. At 5 mM, the current inhibition induced by NED and CRG at +80 mV was 44 ± 11% and 33 ± 7%, respectively ($n = 5$ for both conditions; Figure 2). The current in BE cells was even more refractory to chromones (Figure 2). At +80 mV the inhibition by NED and CRG was 17 ± 5% and 22 ± 5%, respectively ($n = 4$ for both compounds; Figure 2A) whereas at negative potentials no block was observed (Figure 2B).

We asked whether chromones could have a higher inhibitory effect if applied from the intracellular side. Accordingly, we included NED or CRG in the pipette solution at the concentration of 2 mM. After having established the whole-cell configuration, the cells were exposed to the hypotonic solution. In 9HTEo- cells, the maximal values of hypotonically-activated currents obtained with intracellular chromones were significantly smaller than those measured in their absence (Figure 3). The inhibition was equally present at all membrane potentials. Indeed, NED decreased the current by 55 ± 8% at −100 mV and by 54 ± 7% at +80 mV ($P < 0.05$, $n = 5$). With CRG, the inhibition was 50 ± 7% at −100 mV and 41 ± 9% at +80 mV ($P < 0.05$, $n = 4$). Intracellular chromones were ineffective at the concentration of 0.5 mM (not shown). The same experiments were repeated on BE cells. Intracellular chromones (0.5–2 mM) did not significantly reduce volume-sensitive Cl^- currents at membrane potentials in the range −100 to +120 mV (see Figure 3).

The chromone concentration necessary to partially block hypotonically-activated currents was greater than 1 mM, a value higher than that used to block similar currents in NIH-3T3 fibroblasts (Gschwentner *et al*., 1996). We hypothesized that Cl^- currents activated after hypotonic shock in airway epithelial cells were different from that in NIH-3T3 cells. To test

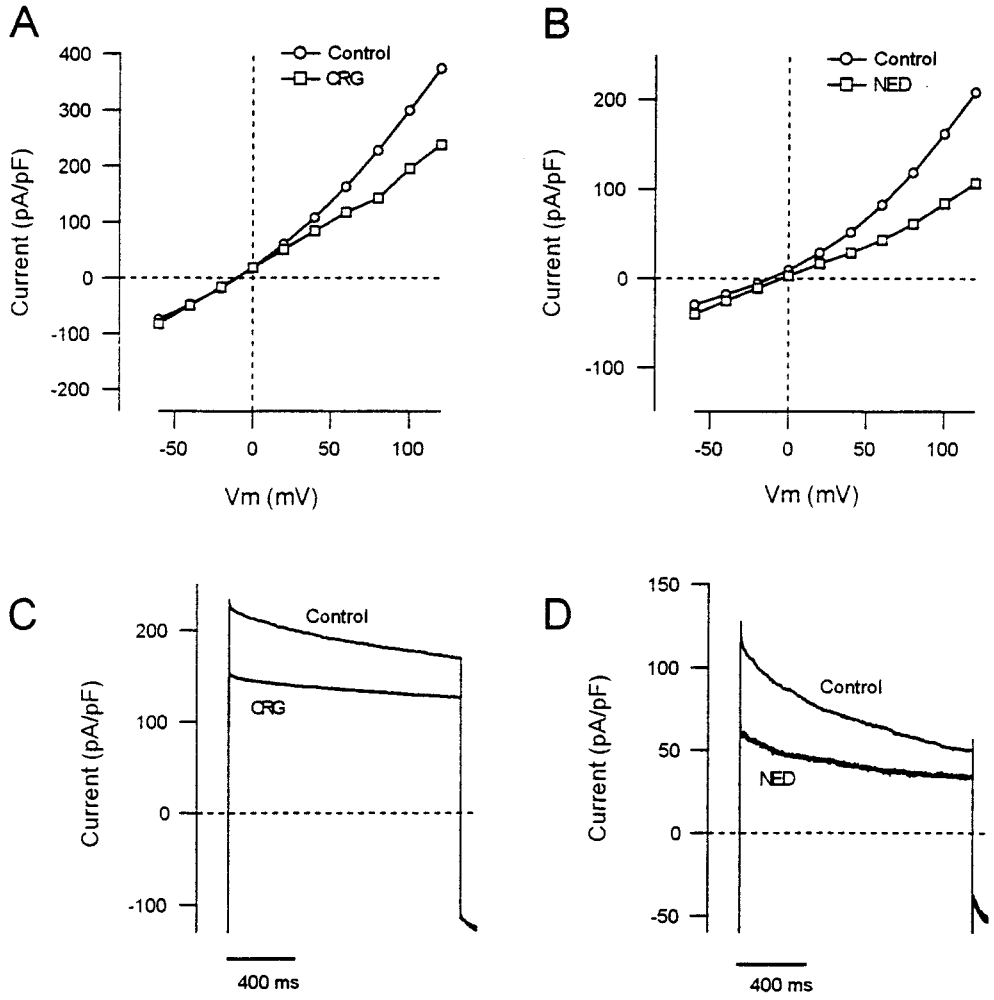

Figure 1 Current-voltage relationships of swelling-activated Cl^- currents evoked in 9HTEo- cells in control conditions and after the extracellular addition of 5 mM CRG (A), or NED (B). Data are from two representative experiments. Currents were elicited by voltage pulses to the indicated membrane potentials starting from a holding potential of −100 mV. The current values were corrected by the cell capacitance. C and D are traces from the same experiments of A and B, evoked at 80 mV in control conditions and with chromones.

this hypothesis we studied the guanosine 3′,5′-cyclic monophosphate (cyclic GMP) sensitivity of 9HTEo- currents. Indeed, a characteristic feature of volume-sensitive Cl^- currents in NIH-3T3 fibroblasts is that they are blocked by extracellular nucleotides in a dose-dependent way (Gschwentner *et al.*, 1995). For example, 1 mM cyclic GMP inhibits 80% of the current. In contrast, hypotonically-activated Cl^- currents in 9HTEo- were not affected by 1 mM cyclic GMP ($n=5$, not shown).

We asked whether the sensitivity of volume-sensitive Cl^- currents to chromones is affected by cell differentiation. Therefore, we did patch-clamp experiments on BE cells grown on permeable supports. Under these conditions, bronchial monolayers show characteristics of the airway epithelia *in vivo*, i.e. transepithelial electrical resistance and potential difference (Galietta *et al.*, 1998). A mild trypsinization was used to break the junctions between the cells. Membrane capacitance measurements indicated the absence of electrical coupling between cells. Application of hypotonic shock for up to 15–20 min did not elicit swelling-dependent currents ($n=4$, not shown). To assess whether trypsinization was responsible for the lack of volume-sensitive Cl^- currents in polarized BE cells, we did experiments on cells grown on Petri dishes and treated with the same trypsinization protocol. In these conditions, BE cells responded to hypotonic shock with activation of Cl^- currents of the same amplitude of non-trypsinized cells.

Discussion

Various types of Cl^- channels have been described in airway epithelial cells. Each channel type can be activated by a different stimulus such as cyclic AMP, intracellular Ca^{2+}, or cell swelling (Anderson *et al.*, 1992). Our experiments show that volume-sensitive Cl^- currents in human airway epithelial cells are relatively refractory to chromones. Indeed, millimolar concentrations of NED or CRG are required to cause a significant block from either the extracellular or intracellular side of the membrane in transformed human tracheal epithelial cells. Swelling-activated Cl^- currents in primary cultures of human bronchial epithelial cells were even less sensitive to both compounds. In particular, the intracellular effect was not observed. A much higher sensitivity to chromones, with IC_{50}'s of 15–19 μM, has been reported for intermediate conductance Cl^- channels in HT_{29} and mast cells in inside-out patch experiments (Romanin *et al.*, 1991; Reinsprecht *et al.*, 1992).

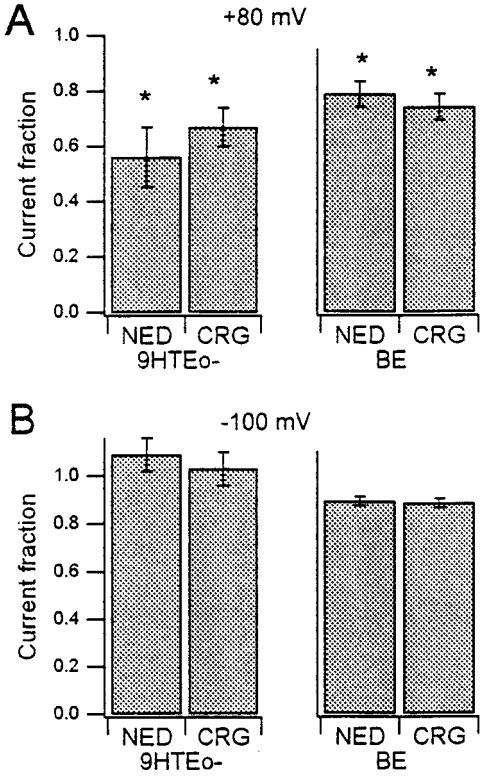

Figure 2 Inhibition of volume-sensitive Cl^- currents by 5 mM extracellular NED or CRG. The effect is reported as the current fraction remaining after chromone application in 9HTEo- and BE cells at two membrane potentials, 80 mV (A) and −100 mV (B). Bars represent the mean ± s.e.mean from 4–5 experiments for each condition. The asterisks indicate a statistically significant inhibition ($P<0.05$).

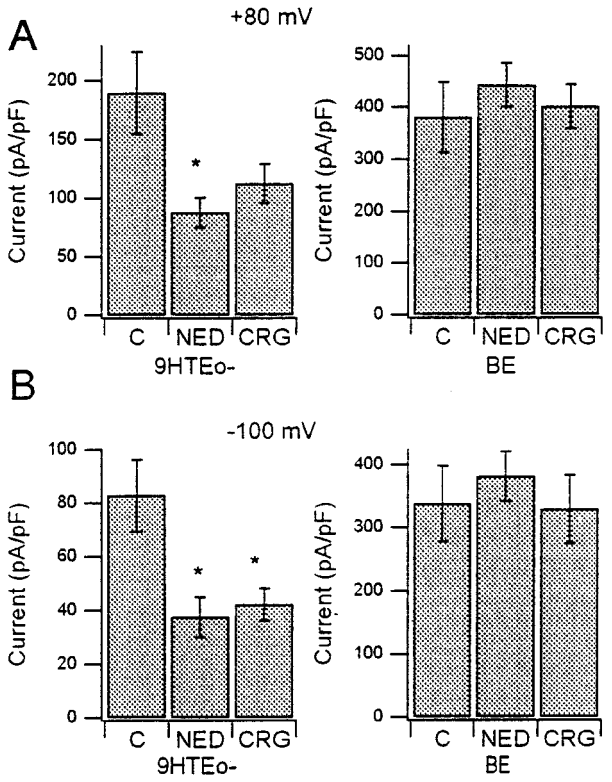

Figure 3 Volume-sensitive Cl^- currents elicited in the absence and in the presence of 2 mM intracellular NED or CRG. Values of membrane currents measured at 80 mV (A) and −100 mV (B) are reported after normalization for cell capacitance. Each bar represents the mean ± s.e.mean of 4–5 experiments. Asterisks indicate a statistically significant reduction of the current.

Micromolar concentrations of NED and CRG, particularly from the intracellular side, were also able to strongly block volume sensitive Cl^- currents in endothelial cells (Heinke *et al*., 1995). Furthermore, a Cl^- channel isolated from different epithelial cells, including 9HTEo-, and incorporated in lipid bilayers, is also blocked by NED at micromolar concentrations (Alton *et al*., 1996). These contrasting sensitivities could be due to different Cl^- channel types. For example, in endothelial cells (Heinke *et al*., 1995) the current activated by hypotonic shock is quite different from that of airway epithelial cells (Rasola *et al*., 1994) since its current-voltage relationship is linear and it lacks the typical inactivation at high positive membrane potentials. In addition, outwardly rectifying chloride channels, usually detected in single channel recordings, could represent an entity different from swelling-activated Cl^- channels (Krouse *et al*., 1994). More intriguing is the different sensitivity found in NIH-3T3 fibroblasts. In these cells (Gschwentner *et al*., 1996), the volume-sensitive current has biophysical characteristics, i.e. outward rectification and voltage-dependent inactivation, similar to those of 9HTEo- and BE cells (Rasola *et al*., 1994; Zegarra-Moran *et al*., 1997) but it is considerably more sensitive to extracellular NED ($IC_{50} \approx 100\ \mu M$). This discrepancy could imply that the channels underlying the macroscopic volume-sensitive Cl^- current in NIH-3T3 fibroblasts and airway epithelial cells are different. This hypothesis seems to be confirmed by the fact that the current in 9HTEo- cells is not blocked by extracellular cyclic GMP. Indeed, the nucleotide sensitivity is a typical feature of the hypotonically-activated current in NIH-3T3 cells (Gschwentner *et al*., 1995).

We were not able to induce activation of Cl^- currents by hypotonic shock in polarized bronchial cells. This lack of response does not seem to be due to the process of trypsinization since the currents of cells grown on plastic dishes were not affected by this treatment. Our results would suggest that airway epithelial cells loose the expression of volume-sensitive Cl^- channels upon polarization. This conclusion would be consistent with the observation that the expression of these channels is inversely related to cell differentiation (Voets *et al*., 1997).

In conclusion, it appears that sodium cromoglycate and sodium nedocromil have the ability to inhibit various Cl^- channels although with different potencies and mechanisms of block. Chromones have been found to block with high potency intermediate conductance Cl^- channels in various cells, including 9HTEo- (Romanin *et al*., 1991; Reinsprecht *et al*., 1992; Alton *et al*., 1996). Although these channels have some properties resembling volume-sensitive Cl^- channels, it is more probable that they represent different entities (Krouse *et al*., 1994). The relatively high concentrations required for the block in airway epithelial cells, compared to those used for other channels, and the lack of effect of extracellular chromones at physiological membrane potentials, argue against the possibility of epithelial $I_{Cl(vol)}$ being the target of chromones in asthma.

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