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Inhibition of volume-activated chloride currents in endothelial cells by chromones

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1 We have studied the effects of the reported chloride channel blocker, sodium cromoglycate, on volume-activated Cl^- currents in endothelial cells from bovine pulmonary artery by means of the whole-cell patch clamp technique. Cl^- currents were activated by challenging the cells with a hypotonic extracellular solution of 60% of the normal osmolarity.

2 Half maximal activation of the current at +95 mV occurred after exposure of the cells for 148 ± 10 s ($n=6$) to hypotonic solution (HTS). At the same membrane potential but in the presence of 100 μM sodium cromoglycate (disodium-1,3-*bis* (2'-carboxylate-chromone-5'-yloxy)-2-hydroxy-propane) activation was delayed (253 ± 25 s, $n=6$) and the maximal current amplitude was reduced to 63 ± 7% of the control ($n=13$).

3 In comparison, an equimolar concentration of NPPB (5-nitro-2(3-phenyl) propylamino-benzoic acid), another Cl^- channel blocker, completely blocked the volume-activated current in less than 20 s.

4 Sodium cromoglycate, applied at the time when the HTS-induced current was completely activated, dose-dependently inhibited this current with a concentration for half maximal inhibition of 310 ± 70 μM. Data for nedocromil sodium were not significantly different from those for sodium cromoglycate.

5 Sodium cromoglycate, loaded into the endothelial cells via the patch pipette in ruptured patches, resulted in a decline of the HTS activated current with a time course that was compatible with diffusion of the compound from the pipette into the cell. Intracellulary applied sodium cromoglycate was also more effective and at 50 μM caused a decrease in the amplitude of the current to 25 ± 6% ($n=10$) of the control current.

6 It is concluded that blockade of volume-activated Cl^- currents by extracellular sodium cromoglycate may be due to an intracellular action following its permeation across the cell membrane.

Keywords: Endothelial cells; volume-activated Cl^- currents; chromones

Introduction

It has been shown recently that Cl^- channels may play a substantial role in the control of secretion from mast cells stimulated by $Fc_\varepsilon RI$ clustering (Romanin *et al.*, 1991). These channels, as well as those in other cell types, are potently blocked by sodium cromoglycate, which may suggest that cromone-derivatives are specific blockers of anion channels with intermediate conductance (Reinspecht *et al.*, 1992).

Volume increase activates in many, if not all, non-excitable cells a Cl^- current which has been described in detail elsewhere (Nilius *et al.*, 1994a,c). In many cell types including endothelial cells from human umbilical vein and bovine pulmonary artery, this volume-sensitive current is mediated by activation of a supposedly small conductance channel (Nilius *et al.*, 1994a,c). These chloride channels are involved in the control of many cell functions, e.g. in the control of the cell volume, mitogenic activity (Hoffman & Simonsen, 1989; Lewis & Cahalan, 1990), and pH control (Parker, 1993). In endothelial cells they may also act as mechanosensors (Oike *et al.*, 1994).

It is therefore of interest to characterize the possible modulation of these Cl^- channels by sodium cromoglycate. This is particularly relevant because cell swelling might be involved in inflammation (Sorota, 1992) due to allergic challenges, on which both nedocromil sodium and sodium cromoglycate exert therapeutic activity.

Methods

We have used single endothelial cells from an established cell line from bovine pulmonary artery (cell line CPAE, ATCC CCL-209). Cells were grown in Medium 199, detached by exposure to 0.05% trypsin in a Ca^{2+} and Mg^{2+}-free solution, reseeded on gelatin coated cover slips, and kept in culture for two to four days before use. We used only non-confluent cells in our experiments.

Whole-cell membrane currents were measured either in nystatin perforation membrane patches or in ruputured patches. The latter were used for loading sodium cromoglycate into the cells. Currents were monitored with an EPC-7 (List Electronic, Germany) or an EPC-9 (Heka Electronics, Germany) patch clamp amplifier and sampled at 4 ms intervals (2048 points per record, filtered at 100 Hz). Holding potential was 0 mV. We used the following voltage protocols: (a) 10 s duration steps ranging from +100 mV to −140 mV and spaced by 20 mV were applied every 20 s; (b) a step to −80 mV for 0.6 s, followed by a step to −150 mV for 0.2 s and a 2.6 s linear voltage ramp to +100 mV. This protocol was repeated every 10 s. Membrane capacitance was directly measured by the EPC-9.

The standard extracellular solution was a Krebs solution, containing (in mM): NaCl 132, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 10, HEPES-NaOH 11.5, pH 7.3. Before challenging the cells with a hypotonic solution, bath perfusion was changed to a modified Krebs solution which contained (in mM): NaCl 94.6, KCl 4.2, $MgCl_2$ 0.9, $CaCl_2$ 1.1, glucose 8.2, HEPES 8.2, mannitol 81, pH 7.3 with NaOH. The osmolarity, as measured with a vapour pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 290 mOsm. Hypotonic solutions were obtained by omitting mannitol from the modified Krebs solution, resulting in an osmolarity of 209 mOsm. The normal pipette solution contained (in mM): CsCl 140, EGTA 0.5, HEPES 10, titrated with CsOH to pH 7.3. To minimize a possible contribution of Ca^{2+} activated currents, we applied in

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some experiments the following intracellular solution (in mM): CsCl 140, $MgCl_2$ 1, Na_2ATP 4, $CaCl_2$ 1, $MgCl_2$ 1, EGTA 4, HEPES 10, pH 7.3 with CsOH. The free Ca^{2+} concentration in this pipette solution is 50 nM, taking into account the chelating properties of EGTA and ATP. However, no differences were found in these experiments with the Ca^{2+} buffered solution. Cs^+ (140 mM) was used to prevent a contribution of K^+ currents to the volume-activated currents. For nystatin perforated patches, the tip of the pipette was filled by capillarity with nystatin free solution, and backfilled with a pipette solution that contained nystatin (0.4 mg ml^{-1}).

NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid, RBI, Natick, MA, U.S.A.) was used at a concentration of 100 μM. Sodium cromoglycate (disodium-1,3-*bis*(2'-carboxylate-chromone-5'-yloxy)-2-hydroxypropane) and nedocromil sodium (disodium 6,9-dihydro-9-ethyl-4,6-dioxo-10-propyl-*4H*-pyranol [3,2-g] quinoline-2,8-dicarboxylate) were applied from a 100 mM stock solution in distilled water at concentrations between 5 μM and 1 mM in the extracellular solution. By rupturing the patches, sodium cromoglycate (5 or 50 μM) diffused from the pipette into the cytoplasm.

Drugs were applied via a multi-barrelled pipette in the immediate vicinity of the cell connected to a microprocessor controlled system of magnetic valves. Experiments were performed at room temperature (20–22°C). Pooled data are given as the mean ± s.e.mean.

Results

Challenging endothelial cells with hypotonic solution activates a Cl^- current, that has been described in detail elsewhere (Nilius *et al.*, 1994a,b,c). Figure 1 shows the activation of this current with either the step or ramp voltage protocol described in the methods. Figure 1a shows the current responses upon 10 s voltage steps to values between −140 and +100 mV in increments of 20 mV in an isotonic extracellular solution. Only small and time-independent currents were elicited. Five minutes after challenging the cells with hypotonic solution (HTS), much larger currents could be evoked (Figure 1b). Figure 1c shows the voltage-dependence of the maximal current amplitude observed during each step. These *I-V* curves were similar to those obtained from the voltage ramp protocols, which correspond to the solid lines in Figure 1c. HTS-activated currents reversed close to the theoretical Cl^- equilibrium potential (+9 mV). The *I-V* curve of the HTS-activated current is nearly linear at these symmetrical Cl^- concentrations. In the remaining experiments, we have exclusively used voltage ramp protocols, and reconstructed the time course of the HTS-activated current at +90 and +100 mV.

Figure 2 shows the effects of two different Cl^- channel blockers on the HTS activated currents measured by the ramp protocol. Application of 100 μM NPPB induced a fast block of this current, which developed in less than 20 s and was completely reversible (Figure 2a, for more details, see Nilius *et al.*, 1994b). Figure 2b shows the current-voltage relationships measured before (1) and during stimulation with hypotonic solution (HTS) in the absence (2) or presence of 100 μM NPPB (3). The volume-activated current reversed at +6 mV, which is close to the theoretical equilibrium potential for Cl^- (+9 mV), and is almost completely blocked in the presence of NPPB at all voltages. Figure 2c shows from another cell the effect of 100 μM sodium cromoglycate (SCG) on the volume-activated current. In contrast with the effects of NPPB, the block induced by SCG and the recovery of the current upon washout proceeded much slower, i.e in the range of some 100 s. Current-voltage relationships of the volume-activated currents (Figure 2d) show a partial inhibition at all potentials. Thus, the effects of SCG were small and slow as compared to those of NPPB. Similar results were obtained by extracellular application of nedocromil sodium, an analogue of sodium cromoglycate (traces not shown).

Activation of the volume-activated current in the presence of SCG was slower than in control cells. Time for half maximal activation of the current was 148 ± 10 s ($n=6$) under control conditions, and 253 ± 25 s ($n=6$) in the presence of 100 μM sodium cromoglycate. Figure 3a-c show the concentration-dependence of the block by SCG. It can again be observed that the block develops slowly, and that the recovery of the current after wash out is also extremely slow. Figure 3d shows an experiment in which an endothelial cell was challenged three times with HTS. The cell was superfused with 100 μM sodium cromoglycate immediately after the first HTS challenge and exposed to HTS 5 minutes later. The activated current is clearly reduced. The original size of the current was not restored at 5 min after washout (Figure 3d).

The slow effects of even high concentrations of sodium cromoglycate prompted us to study the effects of intracellular sodium cromoglycate, applied via the patch pipette in ruptured patches. The amplitude of the HTS-activated current was consistently larger in ruptured patches than in nystatin perforated patches. Figure 4 shows an experiment in which an endothelial cell was loaded with 5 μM sodium cromoglycate via the patch pipette. Diffusion of sodium cromoglycate into the cell after breaking the membrane in the patch induced a time-dependent decrease of the HTS-activated current during subsequent challenges with HTS, which stabilized after approximately 14 min (Figure 4a). The time course of the development of the inhibtory effect could be estimated in 3 cells. The current reached a stationary level with a time constant of approximately 2.3 min (Figure 4b). This time constant is comparable with that of the diffusion of a fluorescence marker with a molecular weight of 832 (154 ± 23 s, Oike *et al.*, 1994). We therefore assume that diffusion is the rate limiting step for the development of the effect by intracellular application of sodium cromoglycate.

The inhibitory effect of intracellular sodium cromogylcate was also concentration-dependent. Figure 5a shows the activation of Cl^- current by HTS in the absence and in the presence of 5 or 50 μM sodium cromoglycate. Currents were obtained from three different cells, and expressed per unit cell capacitance.

To quantify the effects of sodium cromoglycate, we have expresed the curent amplitude in the presence of sodium cromoglycate as a fraction of that in its absence ($I_{Cl,norm}$), and represented these values as a function of the concentration of sodium cromoglycate or nedocromil sodium. The data points were fitted to the equation:

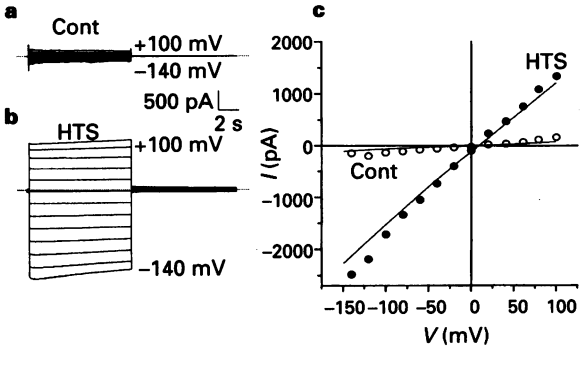

Figure 1 Current activation by challenging an endothelial cell with hypotonic solution. (a) Voltage steps from +100 mV to −140 mV and 20 mV spaced were applied every 20 s in normotonic solution. Pipette solution contained CsCl to minimize the contribution of K^+ currents. (b) Currents measured 3 min after superfusing the cells with hypotonic solution (HTS). The same protocol as in (a) was applied. (c) Current voltage relationships (full lines) obtained from linear voltage ramps from −150 to +100 mV applied before (Cont) and 3 min after superfusion with HTS. The symbols indicate the maximal currents during the voltage steps from (a) (○) and (b) (●). All data were obtained from the same cell.

$$I_{Cl,\,norm} = \frac{1}{1+[C]/K_I} \quad (1)$$

K_I represents the concentration for half maximal block; C is the concentration of sodium cromoglycate or nedocromil sodium. K_I, estimated from the best fit to the data points, was 310 ± 70 μM (Figure 5b). Thus, volume-activated Cl^- currents in endothelial cells are less sensitive to sodium cromoglycate than those in mast cells (Reinspecht *et al.*, 1992). In the latter experiments however, sodium cromoglycate was applied from the intracellular side to excised inside-out patches. From the data obtained by intracellular sodium cromoglycate loading of the cells (Figure 5b), it is evident that intracellular sodium cromoglycate exerts a much more potent block, with an estimated K_I value of 5–10 μM.

These experiments suggest that sodium cromoglycate may be slightly membrane-permeable leading to an effective inhibition of volume-activated Cl^- currents by some intracellular action. This view was supported by experiments in which we have incubated cells for 120 min with a concentration of 100 μM sodium cromoglycate. After this preincubation, HTS-activated currents (even in the absence of extracellular sodium cromoglycate) were smaller than in control cells: the current density was decreased from 37 ± 7 pA/pF in control cells ($n=7$) to 12 ± 3 pA/pF, ($n=3$) in the cells preincubated with 100 μM sodium cromoglycate for 120 min. This decrease is transient, and currents partially recovered after long periods of washout in sodium cromoglycate-free solution (21 ± 5 pA/pF ($n=3$) after 14 min), indicating that sodium cromoglycate might not be trapped in the cell.

Discussion

Cl^- channel blockers are important tools for studying the involvement of the channels in various cell functions, and also for the molecular biological characterization of these channels (Paulmichl *et al.*, 1993). It has been shown recently that *intracellular* application of the antiallergic drug, sodium cromoglycate, potently blocks intermediate conductance Cl^- channels (Reinspecht *et al.*, 1992) and in the same concentration-range also inhibits 5-hydroxytryptamine secretion by mast cells (Hemmerich *et al.*, 1991). Other Cl^- channel blockers, such as NPPB, also affect both mediator secretion and Cl^- channel activity in mast cells (Romanin *et al.*, 1991), but they are not very selective and also block other channels (see Nilius *et al.*, 1994c).

We were interested in the *extracellular* action of sodium cromoglycate in another well-described type of Cl^- current, i.e. the volume-activated Cl^- current (Nilius *et al.*, 1994a,b,c). This current plays an important role in volume regulation in a variety of cell types (Lewis & Cahalan, 1990). Cell swelling and thus volume regulation is likely to be involved in inflammation due to allergic challenges. In our experiments, on bovine pulmonary artery endothelial cells, whole cell Cl^- currents were measured under conditions in which no other currents are coactivated (block of K^+ currents by Cs^+ and buffering of the intracellular Ca^{2+} concentration at 50 nM). At variance with the supposedly exclusive intracellular effects of sodium cromoglycate on the immunologically activated intermediate Cl^- channel (Reinspecht *et al.*, 1992), we found a weak inhibition of the volume-activated Cl^- current in endothelial cells by extracellulary applied sodium cromoglycate and nedocromil

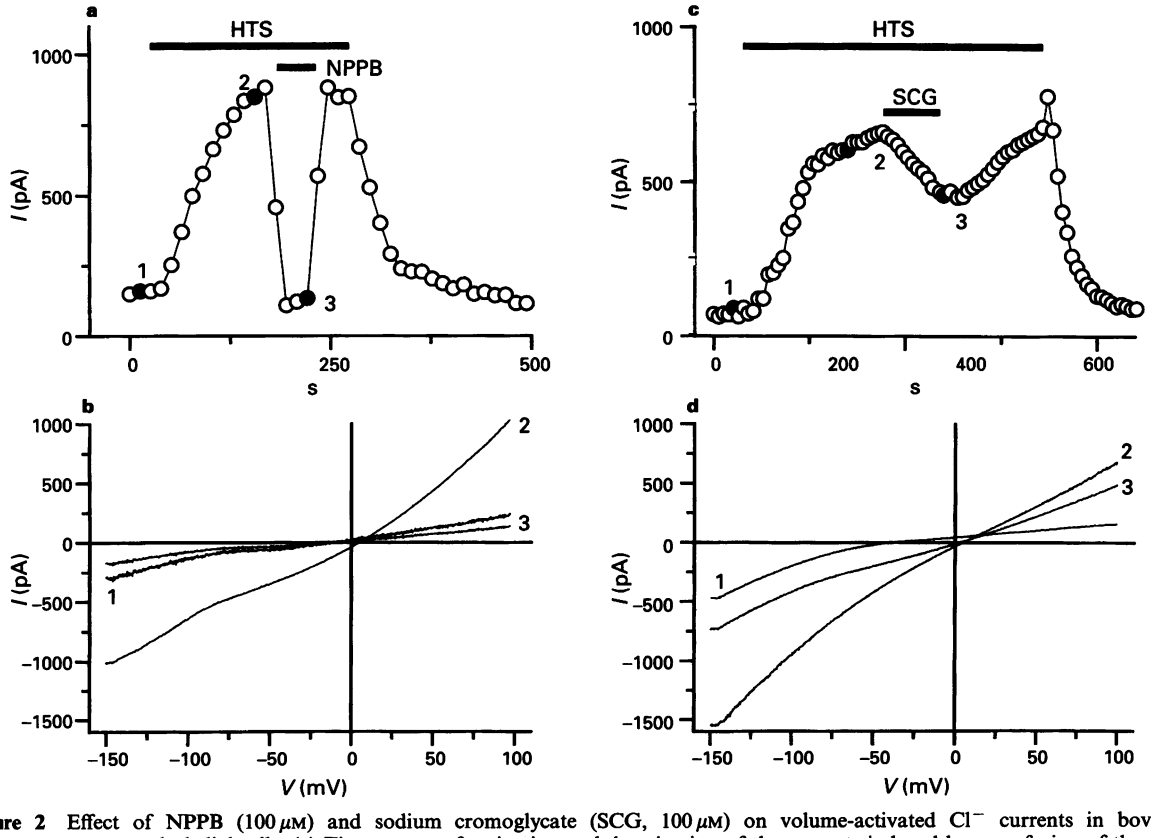

Figure 2 Effect of NPPB (100 μM) and sodium cromoglycate (SCG, 100 μM) on volume-activated Cl^- currents in bovine pulmonary artery endothelial cells. (a) Time course of activation and deactivation of the currents induced by superfusion of the cell with hypotonic solution (HTS). Data were obtained from a voltage window between +90 and +100 mV. Note the rapid and reversible inhibition of the HTS-induced current by NPPB (100 μM). (b) Current-voltage relationships of the volume-activated Cl^- current reconstructed from linear voltage ramps. The numbers indicate the time at which the currents were measured (1: control, 2: maximal HTS-induced current, 3: in the presence of NPPB). The reversal potential of the HTS-induced current is +7 mV (E_{Cl} = 9 mV). (c) Time course of the HTS-induced current and its block by 100 μM sodium cromoglycate (SCG). Same conditions as in (a). Note the slow and incomplete block of the current by sodium cromoglycate, as well as the slow recovery upon washout. (d) Current-voltage relationships before and during HTS, and the inhibition of the HTS-induced current by SCG.

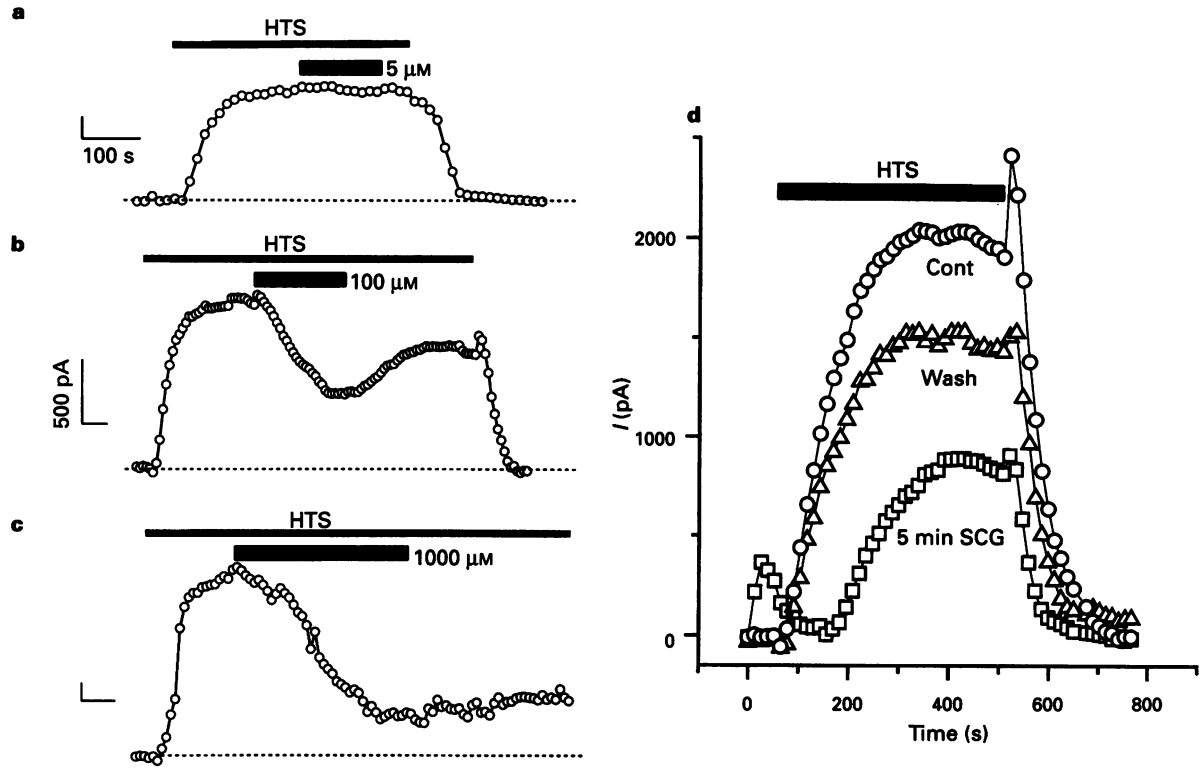

Figure 3 Effects of extracellularly applied sodium cromoglycate on the hypotonic solution (HTS) induced Cl^- current. (a)-(c) Inhibition of the volume-activated Cl^- current at various sodium cromoglycate (SCG) concentrations. Bars indicate the superfusion with HTS and 5, 100, 1000 μM sodium cromoglycate respectively. Background current before challenging the cells with HTS is subtracted. (d) Time course of HTS-activated current before (Cont), during (5 min SCG) and after (Wash) application of 100 μM sodium cromoglycate, applied after the first HTS application. The second challenge with HTS was applied 5 min after superfusion of the cell with sodium cromoglycate. After 5 min washout, the response of the cell to a third challenge with HTS was still reduced.

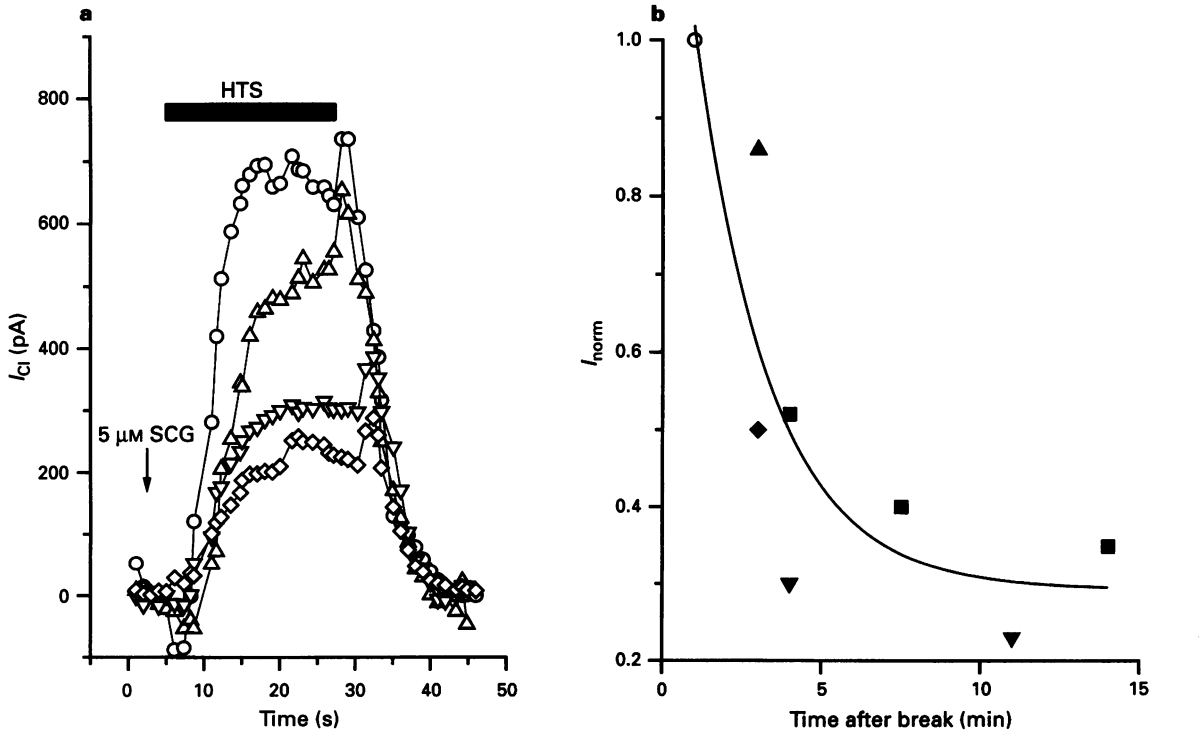

Figure 4 Effect of intracellular loading of an endothelial cell with 5 μM sodium cromoglycate (SCG). (a) Subsequent stimulations with hypotonic solutions (HTS) after breaking into the cell with sodium cromoglycate evoked gradually smaller responses, which were never observed under control conditions: (○) 1 min; (△) 4 min; (▽) 8 min; (◇) 14 min. The response to HTS stabilized 8–14 min after break in. (b) Maximal current responses normalized to the first response after breaking into the cell as a function of time after break in. Data from three cells, represented by different symbols. The solid line is an exponential fit with a time constant of 2.3 min.

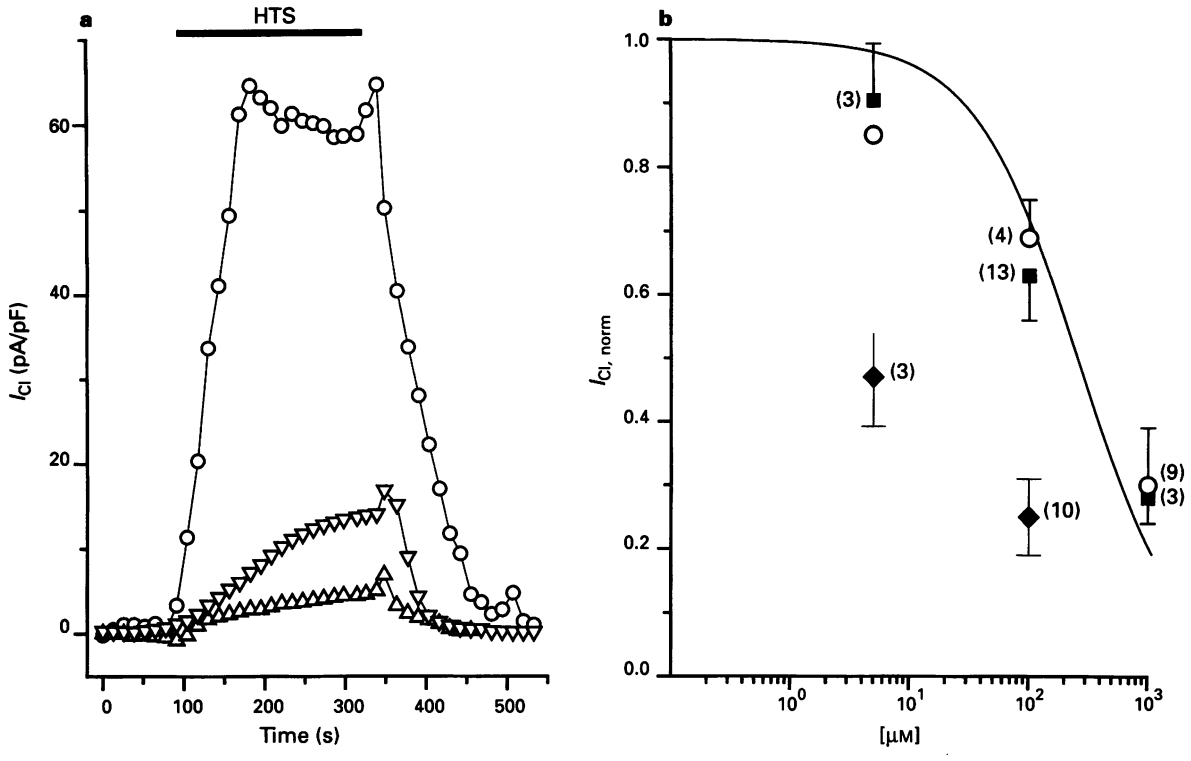

Figure 5 Concentration-dependence of the effects of intracellular and extracellular sodium cromoglycate. (a) HTS-activated current from three different cells under control conditions (○) and after intracellular loading with 5 (▽) and 50 μM (△) sodium cromoglycate. Currents are expressed per unit membrane capacitance (measured before HTS). Note the slower activation of the current in the presence of sodium cromoglycate. (b) Synopsis of the data with extracellular sodium cromoglycate (SCG ■) and nedocromil sodium (○), as well as those by intracellular loading with sodium cromoglycate (◆). For extracellular sodium cromoglycate, a K_I value of 310 μM was obtained (see text). The value for intracellular sodium cromoglycate is in the range of 5–10 μM, i.e. nearly 2 orders of magnitude smaller.

sodium. However, this effect appeared to be different from that of other well-described compounds like NPPB, tamoxifen, dideoxy-forskolin, DIDS (Nilius *et al.*, 1994a,b). The inhibition and recovery upon washout occurred in a time range of some 100 s. Half maximal inhibition occurred at much higher concentrations than that reported for the intracellular action, which had a K_I of 15 μM.

Because of the slow time course and the weaker blocking effects, we argue that, although it is a doubly-negatively charged compound which is very polar, a small fraction of non-charged sodium cromoglycate might enter the cell, and that the extracellular effects reflect a slow permeation of the compound across the cell membrane. To check whether intracellular sodium cromoglycate could also affect the HTS-activated Cl^- current in endothelial cells, we loaded the cells with sodium cromoglycate via the patch pipette. Inhibitory effects of sodium cromoglycate were more rapid and more pronounced in these experiments. If we assume a complete equilibration of sodium cromoglycate between pipette solution and cytoplasm, half maximal inhibition of the current occurs at a concentration about two orders of magnitude lower than that for extracellularly applied sodium cromoglycate. We therefore suggest that a small fraction of sodium cromoglycate might be non-charged, enter the cell and exert its inhibitory action from the cytoplasmic side. The possibility cannot be excluded however that this compound blocks from either side of the membrane with differing efficacies and at different sites.

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