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Blockade of swelling-induced chloride channels by phenol derivatives

Martin Gschwentner, §Andreas Jungwirth, Sabine Hofer, *Ewald Wöll, *Markus Ritter, Alex Susanna, Andreas Schmarda, †Gilbert Reibnegger, Germar M. Pinggera, Markus Leitinger, §Julian Frick, Peter Deetjen & [1]Markus Paulmichl

Department of Physiology, University of Innsbruck, Fritz-Pregl-Str. 3, A-6020 Innsbruck, Austria; §Department of Urology, SLKA, Müllner-Hauptstr. 20, A-5020 Salzburg, Austria; *Department of Internal Medicine, University of Innsbruck, Anichstr. 35, A-6020 Innsbruck, Austria and †Department of Chemistry and Pregl Laboratories, University of Graz, Harrachgasse 21, A-8010 Graz, Austria

1 In NIH3T3 fibroblasts, the chloride channel involved in regulatory volume decrease (RVD) was identified as I_{Cln}, a protein isolated from a cDNA library derived from Madin Darby canine kidney (MDCK) cells. I_{Cln} expressed in *Xenopus laevis* oocytes gives rise to an outwardly rectifying chloride current, sensitive to the extracellular addition of nucleotides and the known chloride channel blockers, DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid) and NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid). We set out to study whether substances structurally similar to NPPB are able to interfere with RVD.

2 RVD in NIH3T3 fibroblasts and MDCK cells is temperature-dependent.

3 RVD, the swelling-dependent chloride current and the depolarization seen after reducing extracellular osmolarity can be blocked by gossypol and NDGA (nordihydroguaiaretic acid), both structurally related to NPPB.

4 The cyclic AMP-dependent chloride current elicited in CaCo cells is less sensitive to the two substances tested while the calcium-activated chloride current in fibroblasts is insensitive.

5 The binding site for the two phenol derivatives onto I_{Cln} seems to be distinct but closely related to the nucleotide binding site identified as G x G x G, a glycine repeat located at the predicted outer mouth of the I_{Cln} channel protein.

Keywords: Fibroblasts; MDCK; volume regulation; gossypol; NDGA; patch clamp; chloride currents; I_{Cln}

Introduction

The simultaneous stimulation of potassium and chloride channels enables cells to lose potassium and chloride together with water thus reducing their volume after swelling. The chloride channels involved in regulatory volume decrease (RVD) of fibroblasts belong to a specialized class of proteins named I_{Cln}, initially cloned from a kidney cell line. I_{Cln} was cloned using mRNA isolated from Madin Darby canine kidney (MDCK) epithelial cells (Paulmichl *et al.*, 1992; 1993). Overexpression of the I_{Cln} protein in *Xenopus laevis* oocytes leads to an outwardly rectifying chloride current (Paulmichl *et al.*, 1992) which reverses at ≈ -30 mV, i.e. close to the reversal potential for chloride in oocytes under the conditions chosen (Paulmichl *et al.*, 1992). The current shows no inactivation at potentials up to +40 mV. At potentials more positive than +40 mV a voltage-dependent inactivation of I_{Cln} is observed. I_{Cln} can be blocked by nucleotides added to the extracellular fluid. Mutation of a putative extracellular nucleotide binding site of the I_{Cln} protein (the glycine repeat GsGLG; G = glycine, S = serine and L = leucine) dramatically reduces the ability of nucleotides to block I_{Cln} (Paulmichl *et al.*, 1992; 1993). These experiments as well as those showing the reduction of the endogenous swelling-induced chloride current in NIH3T3 fibroblasts (I_{Cl}) after incubating the cells with antisense oligodeoxynucleotides complementary to I_{Cln} mRNA (Gschwentner *et al.*, 1995a) point to the fact that I_{Cln} is the chloride channel activated after swelling rather than a regulator for an endogenous channel protein (Krapivinsky *et al.*, 1994). I_{Cln} expressed in oocytes (Paulmichl *et al.*, 1992) as well as I_{Cl} and RVD induced in fibroblasts (Gschwentner *et al.*, 1995a) after reducing extracellular osmolarity can be inhibited by the extracellular addition of 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), a substance known to block different chloride channels (Wangemann *et al.*, 1986; Kunzelmann *et al.*, 1989) in a variety of cells.

The aim of the present study was to investigate, if phenol derivatives with a molecular structure close to that of NPPB are able to impede swelling-induced chloride channels, and if the binding region for these substances is related to the putative nucleotide binding site, a glycine repeat at the extracellular side of the channel protein. These experiments could help in obtaining a more precise picture of the binding sites involved in regulation and blocking of this crucial class of ion channels and in developing highly specific blockers.

Methods

Electrophysiology and cell culture

Fibroblasts, CaCo and MDCK cells were grown on glass cover slips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, at 37°C, 5% CO_2/95% air and measured 24 to 48 h after splitting and reculturing the cells. Measurements of the cell membrane potential difference (PD) in NIH3T3 fibroblasts were made in the presence of an extracellular solution (composition in mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.5, $MgCl_2$ 2.5, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10, mannitol 50, pH 7.2 (adjusted with NaOH; real osmolality 305 ± 0.5 mM kg^{-1}) and changes of extracellular osmolarity were made by omitting mannitol.

[1] Author for correspondence.

Where indicated, the concentration of KCl was increased to 20 mM in exchange for NaCl. Cover slips with attached subconfluent fibroblasts were mounted in a perfusion chamber which allowed rapid fluid exchange (chamber volume 0.1 ml; perfusion rate 20 ml min^{-1}). During the experiments the cells were kept at room temperature (RT = 20°C–22°C) or 37°C (35°C–37°C) as stated. For intracellular recordings glass microelectrodes manufactured with a Flaming/Brown P-87 puller (Sutter Instruments, U.S.A.) were filled with 1 M KCl. The electrodes had resistances of about 100 MΩ and tip potentials of ≤5 mV. Signals were amplified and recorded on a chart recorder (BBC, Austria). The membrane potential was measured differentially between the intracellular voltage recording electrode and an extracellular 3 M KCl agar bridge.

For whole-cell voltage clamp experiments the method described by Marty & Neher (1983) was chosen to measure swelling-induced, calcium- or adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-activated chloride currents in isolated NIH3T3 fibroblasts or CaCo cells. These experiments were made at RT and at 37°C where indicated. Bath and pipette solutions were chosen to enable chloride current measurements which were only accepted with series resistances of 2.5–8 MΩ (pipette resistances were 1.5–3 MΩ). The isotonic extracellular solution used was composed of (in mM): NaCl 125, $CaCl_2$ 2.5, $MgCl_2$ 2.5, HEPES 10, mannitol 50, pH 7.2 (adjusted with NaOH; real osmolality 305 ± 0.5 mM kg^{-1}). Where indicated NaCl was changed to KCl. To reduce extracellular osmolarity, mannitol was omitted. One to two minutes after confirming whole-cell configuration hypotonic conditions were established and the activated chloride current measured. The current measurements were made by calculating the mean current over the entire interval of 500 ms for voltages up to +40 mV. For inactivating currents the current was measured at a fixed time point (10 ms) after the corresponding voltage step which was conducted using a 10-s interval. Fast exchange of the bath solution was obtained with a perfusion system having a flow rate of 10 ml min^{-1} and a bath volume of ≈250 μl. The filling solution of the patch pipette was (in mM): CsCl 125, $MgCl_2$ 5, ethyleneglycol-*bis* (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) 11, raffinose 50, HEPES 10, pH 7.2 (adjusted with CsOH; real osmolality 329 ± 0.5 mM kg^{-1}). The patch pipette filling solution used for measuring the calcium-dependent chloride current was (in mM): CsCl 125, $MgCl_2$ 5, EGTA 0.5, raffinose 59.3, HEPES 10, $CaCl_2$ 0.3, pH 7.2 (adjusted with CsOH; real osmolality 315 ± 1.0 mM kg^{-1}). For data acquisition and analysis an EPC-9 (HEKA, Germany) and an Axopatch 200A (Axon Instruments, U.S.A.) amplifier were used, controlled by an Atari and Apple computer running the appropriate software for driving the amplifier and REVIEW for analysis (Instrutech, U.S.A.). All current measurements were filtered at 1 kHz (analog 4-pole BESSEL).

Cell volume measurements

Cell volume measurements were made at 30 s intervals (Gschwentner *et al.*, 1995a; Paulmichl *et al.*, 1989; Wöll *et al.*, 1993) with a Casy-1 model TT (Schärfe, Germany). Absolute cell volume was calculated from the median of the cell volume distribution curves with latex beads used as calibration standards. During measurement cells were kept at either RT or 34°C–37°C as indicated. The isotonic extracellular solution for volume measurements in MDCK cells was (in mM): NaCl 65, mannitol 100, KCl 5.4, $MgCl_2$ 0.8, $CaCl_2$ 1.2, glucose 5, NaH_2PO_4 0.2, Na_2HPO_4 0.8, $NaHCO_3$ 20, equilibrated with 5% CO_2/95% O_2, pH 7.4 (real osmolality 273 ± 0.5 mM kg^{-1}). To reduce extracellular osmolarity the isotonic solution was diluted with a solution composed as above but without mannitol. For volume measurements in NIH3T3 fibroblasts the isotonic extracellular solution was (in mM): NaCl 115, KCl 5.4, $MgCl_2$ 0.8, $CaCl_2$ 1.2, glucose 5, NaH_2PO_4 0.2, Na_2HPO_4 0.8, $NaHCO_3$ 20, equilibrated with 5% CO_2/95% O_2, pH 7.4 (real osmolality 264 ± 1.4 mM kg^{-1}). To reduce extracellular osmolarity the isotonic solution was diluted with a solution composed as above but without NaCl to lead to a final reduction of NaCl of 35 mM. Final osmolality of the different solutions was measured with a vapor pressure osmometer (Wescor, U.S.A.).

Chloride fluorescence measurements

For fluorescence measurements the cells were superfused with a solution containing (in mM): Na-gluconate 131, K-gluconate 5.4, $MgSO_4$ 0.8, Ca-gluconate 1.2, NaH_2PO_4 1, glucose 5.5, Tris 5, pH adjusted to 7.4 (real osmolality 263 ± 1.3 mM kg^{-1}; Gschwentner *et al.*, 1995b). For the hypotonic solution Na-gluconate was reduced to 91 mM. At the points indicated (arrows in Figure 5a and b) the hypotonic extracellular fluid was changed to a solution containing 150 mM KSCN (real osmolality 267 ± 1.0 mM kg^{-1}). The decrease of fluorescence is proportional to P_{Cl} since SCN^- is able to quench 6-methoxy-*N*-ethylquinolinium iodide (MEQ) fluorescence more effectively than chloride (Verkman, 1990; Bubien *et al.*, 1990; Paulmichl *et al.*, 1993; Gschwentner *et al.*, 1995b) and I_{Cl} is more permeable to SCN^- and reduced by only ≈40% within the first 180 s after restoring normal osmolarity (Gschwentner *et al.*, 1995b; Wöll *et al.*, 1995). Fluorescence measurements were made under an inverted microscope equipped for epifluorescence and photometry. Light from a xenon arc lamp was directed through a gray filter (nominal transmission 0.3%) and a 340 ± 10 nm interference filter, deflected by a dichroic mirror and directed through the objective. The emitted light was directed through a 420 nm cutoff filter to a cooled photomultiplier tube for photon counting (Paulmichl & Lang, 1988). For loading the cells with MEQ the positively charged non-permeable MEQ is reduced to the non-polar cell-permeant 6-methoxy-*N*-ethyl-1,2-dihydroquinolinium iodide (diH-MEQ; (Biwersi & Verkman, 1991)) prior to the experiment. The fibroblasts were incubated for 10 min in a 5 μM diH-MEQ solution (37°C, 5% CO_2/95% air), washed with PBS and incubated in DMEM (37°C, 5% CO_2/95% air) for another 10 min prior to measurement.

Statistical analysis

Where applicable data are expressed as arithmetic means ± standard error of the mean (s.e.mean). Statistical analysis was made by *t* test where appropriate. Significant difference was assumed at $P<0.05$ and is indicated by an asterisk (*).

Results

Measurements of the membrane potential of NIH3T3 fibroblasts after reduction of extracellular osmolarity in the presence and absence of extracellular gossypol

In the presence of an isotonic solution containing mannitol (see Methods) the cell membrane potential difference (PD) of NIH3T3 fibroblasts averaged −35.7 ± 3.5 mV (37°C; $n=12$). Increasing the extracellular potassium concentration from 5.4 mM to 20 mM led to a significant and reversible depolarization by +7.13 ± 1.0 mV ($n=4$) from a resting potential of −33.1 ± 4.7 mV to −26.0 ± 3.9 mV ($n=4$). In contrast, at room temperature (RT) the resting membrane potential was significantly depolarized (−13.8 ± 0.8 mV; $n=16$). Increasing the extracellular potassium concentration (from 5.4 to 20 mM) under these conditions caused a significantly reduced reversible depolarization of only +2.25 ± 0.4 mV ($n=6$). These experiments indicate that the depolarized PD observed at RT might be the result of a reduced potassium conductance.

Decreasing extracellular osmolarity by 50 mM (omitting mannitol, see Methods) produced a significant reversible depolarization of PD by +7.4 ± 0.2 mV ($n=5$; 37°C). Addition of gossypol at a concentration of 0.5 μM to the isotonic extracellular fluid (37°C) caused no significant change in PD (0.0 ± 1.0 mV; $n=5$). In the presence of gossypol the depolar-

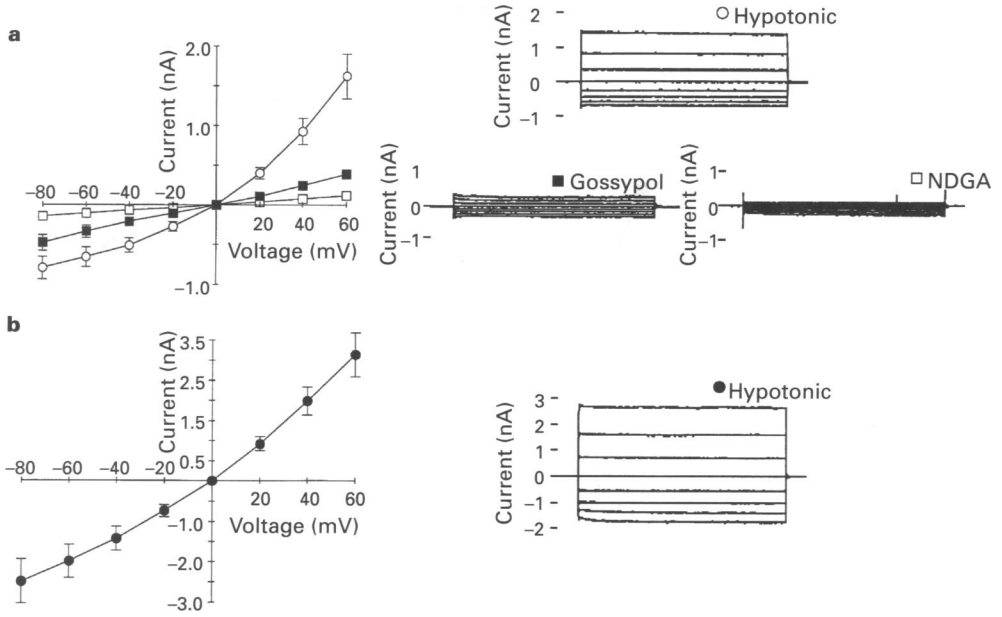

Figure 1 Current voltage curves of I_{Cl} elicited after reducing extracellular osmolarity. (a) I/V plots shown were made at RT in the presence and absence of gossypol and NDGA. Current-voltage curve of the swelling-induced chloride current in the absence ($n=11$; ○) and presence ($n=4$; ■) of 10 μM gossypol or 10 μM NDGA ($n=5$; □). I_{Cl} is outwardly rectifying and gossypol or NDGA are able to inhibit both current directions. Original tracings to the corresponding I/V plots are given using the respective symbols. (b) I/V plot and corresponding original tracing of I_{Cl} are shown at 37°C. The holding potentials were 0 mV and repetitive voltage steps (500 ms) were made from −80 mV to +60 mV in +20 mV increments (pulse interval 5 s).

izing effect after reducing extracellular osmolarity was abolished (+2.2±2.3 mV; $n=5$) but could again be elicited after removal of gossypol. At RT the reduction of extracellular osmolarity led, in contrast to measurements at 37°C, to a slight but significant and reversible hyperpolarization of −3.0±0.3 mV ($n=10$). Under these conditions in the presence of gossypol (10 μM) reduction of extracellular osmolarity did not produce a significant change of the PD (−0.1±0.2 mV; $n=3$) which could again be elicited after the removal of the blocker.

Whole-cell current measurements of the swelling-induced chloride current in NIH3T3 fibroblasts

Whole-cell current measurements were carried out at RT and at 37°C where indicated. As shown in Figure 1, the swelling-induced chloride current is outwardly rectifying and shows a significantly higher amplitude at 37°C than at RT. At +40 mV the current was +803±60 pA ($n=55$) at RT and +1740±170 pA ($n=31$) at 37°C. The current elicited was chloride-selective as shown by ion substitution experiments and the use of blockers known to block chloride currents (Gschwentner *et al.*, 1995a). Substitution of extracellular sodium with potassium did not change significantly the reversal potential of the current (0±3.8 mV, $n=8$, in the presence of sodium and +8.3±8.0 mV, $n=7$, in the presence of potassium). Substitution of chloride with different anions produced an order of selectivity of $SCN^- > I^- > Br^- > Cl^-$ > gluconate (Wöll *et al.*, 1995). As stated below, RVD can only be measured at 37°C probably because of the inadequate membrane potential at RT (see above and Discussion), which does not drive the chloride flux necessary for RVD. Under voltage clamp conditions at RT, the membrane potential was experimentally controlled, thus allowing observation of the swelling-induced chloride current.

The effect of gossypol on the swelling-induced chloride current in NIH3T3 fibroblasts

Whole-cell current measurements showed that gossypol added to the extracellular fluid was able to block the chloride current (I_{Cl}) activated in fibroblasts after reducing extracellular osmolarity by 50 mosM. As shown in Figure 2a, the half maximal concentration required to block I_{Cl} (IC_{50}) was ≈6 μM at RT and ≈2 μM at 37°C. Figure 1a shows the current/voltage relation of I_{Cl} in the absence and in the presence (1 to 2 min) of 10 μM gossypol. The cells were held at 0 mV and voltage steps made from −80 mV to +60 mV in +20 mV increments. At a potential of +40 mV the chloride current was +803±60 pA ($n=55$) and was reduced to +267±35 pA ($n=14$) in the presence of 10 μM gossypol. The current measurements were taken 1–3 min after changing the extracellular gossypol concentration. As shown in Figure 3, addition of 10 μM gossypol to the extracellular fluid caused a significant reduction of I_{Cl} within seconds. The effect of gossypol was totally reversible if the drug was removed within the first 2 min after its addition. Prolonged (>2 min) presence of the drug produced an only partial reversibility of the block, probably due to the high lipophilicity of the substance.

The effect of NDGA on the swelling-induced chloride current in NIH3T3 fibroblasts

As shown in Figure 2b nordihydroguaiaretic acid (NDGA) was able to impede directly the swelling-induced chloride current in fibroblasts. Measured at a potential of +40 mV the swelling-induced chloride current was diminished from +803±60 pA ($n=55$) to +92±15 pA ($n=11$) in the presence of 10 μM NDGA. The half maximal concentration needed to block I_{Cl} was ≈4 μM at RT and ≈1 μM at 37°C. Similar to the experiments with gossypol, the inward as well as the outward currents elicited after reduction of extracellular osmolarity vanished 1 to 2 min after the addition of NDGA to the extracellular fluid (Figure 1). The blocking of I_{Cl} could be observed within seconds after the addition of 10 μM NDGA (Figure 3). Prolonged (>2 min) presence of the drug produced only a partial reversibility of the block most likely due to the high lipophilicity of the substance.

The effect of NDGA and gossypol on the swelling-induced chloride current restrained by the nucleotide thymidine diphosphate (TDP)

In order to investigate a potential interaction between the nucleotide binding site and the presumed binding domains on

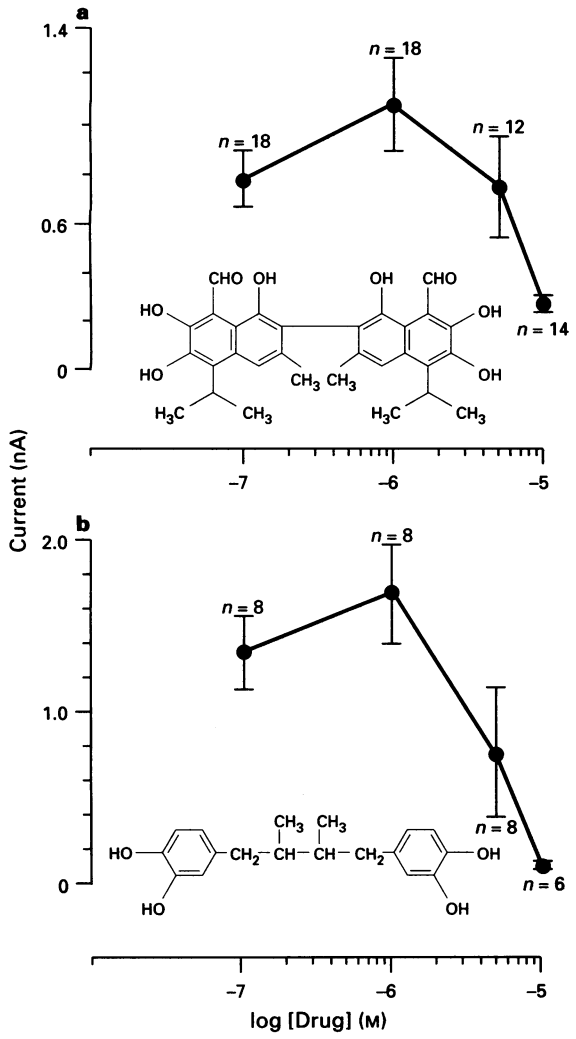

Figure 2 The effect of gossypol and NDGA on the swelling-induced chloride current (I_{Cl}) measured in NIH3T3 fibroblasts. Cumulative dose-response curve showing the inhibition of I_{Cl} in the presence of various gossypol (a) and NDGA (b) concentrations. The half maximal concentration needed for blocking I_{Cl} (IC_{50}) is for gossypol $\approx 6\,\mu M$ and for NDGA $\approx 4\,\mu M$ at RT. The insets depict the molecular structures of gossypol and NDGA. The holding potential was 0 mV and repetitive voltage steps were made to +40 mV.

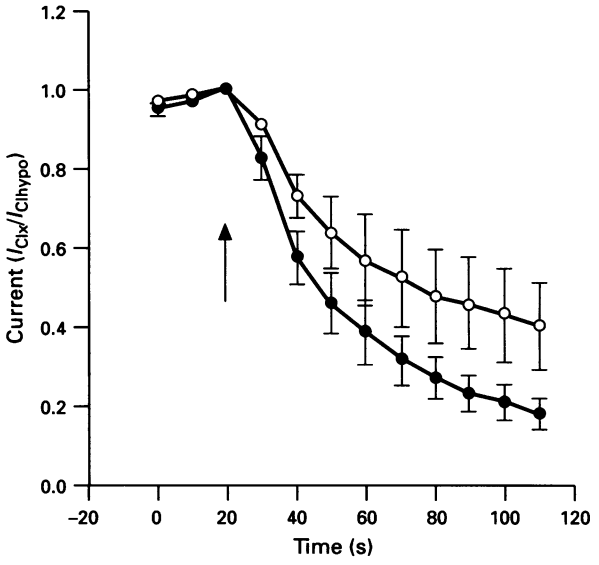

Figure 3 The effect of gossypol (○) and NDGA (●) (arrow) on I_{Cl} is very fast. In the first seconds after addition of gossypol ($n=5$) or NDGA ($n=10$) a significant reduction of I_{Cl} can be observed. The holding potential was 0 mV and repetitive voltage steps were made to +40 mV.

the chloride channel protein for the phenol derivatives tested we adopted an experimental protocol described in an earlier paper (Gschwentner *et al.*, 1995b) where we have shown that the very effective block of I_{Cl} in NIH3T3 fibroblasts effected by the nucleoside analogues AZT or acyclovir can be abolished by TDP or uridine, nucleotides not *per se* effective in blocking I_{Cl} (the concentrations tested ranged from 1 μM to 0.1 mM). We concluded from those experiments that TDP and uridine competitively block the nucleoside analogue-induced inhibition of I_{Cl}. Here we set out to test if a similar interaction can be observed between TDP and the phenol derivatives. As shown in Figure 4a the gossypol-induced block of I_{Cl} was totally eliminated if 100 μM TDP were present before (1 to 2 min) and during the addition of 10 μM gossypol. After washing out the TDP/gossypol mixture the readdition of gossypol alone was again effective. In contrast, TDP was not able to circumvent the NDGA block (Figure 4b). However, as shown in Figure 4c the onset of the NDGA (10 μM)-induced block of I_{Cl} was significantly retarded in the presence of 100 μM TDP. These experiments suggest that the corresponding binding site(s) for gossypol and NDGA are distinct from but closely related to the nucleotide binding site identified as a glycine repeat located at the predicted outer mouth of the pore. However, the binding sites of gossypol and NDGA are identical or at least functionally so close that binding to the respective sites is not independent. This hypothesis is further supported by experiments in which gossypol and NDGA were added simultaneously. In a different set of experiments at the concentrations tested gossypol produced a significant block of the swelling-dependent chloride current of $63.3 \pm 5.6\%$ ($n=4$) and NDGA to a significant block of $86.4 \pm 6.6\%$ ($n=4$). Both substances added simultaneously led to a significant block of $72.2 \pm 11.5\%$ ($n=8$) which was not significantly different from the values obtained in the presence of gossypol or NDGA alone.

Gossypol and NDGA impede the swelling-induced chloride flux

The halide-sensitive fluorescent dye, 6-methoxy-N-ethylquinolinium iodide (MEQ) was used to measure chloride permeability in single NIH3T3 fibroblasts. Quenching of the MEQ signal was determined by addition of SCN^- to the extracellular fluid after exposing the cells to a hypotonic solution. As shown in Figure 5a, 1 min after the addition of SCN^- the emitted MEQ signal was quenched by $99.4 \pm 4.1\%$ ($n=8$) in the absence of the blocker. Blocking the chloride channels by addition of gossypol in a concentration of 10 μM to the extracellular solution significantly reduced the quenched MEQ signal to $82.3 \pm 6.0\%$ ($n=9$). Similar results were obtained with 10 μM NDGA. After addition of SCN^- and a 1 min time interval, the reduction of the fluorescence signal was $100.3 \pm 2.3\%$ ($n=8$; Figure 5b) under control conditions, whereas in the presence of 10 μM NDGA the signal was only reduced to $82.2 \pm 5.6\%$ ($n=6$; Figure 5b).

Effect of gossypol on regulatory volume decrease (RVD)

Reducing extracellular osmolarity by 70 or 95 mosM (37°C; see Methods) caused swelling of MDCK cells and NIH3T3 fibroblasts followed by a decrease in cell volume towards values seen before extracellular osmolarity was reduced (Figure 6a and b). Five minutes after peak swelling, the cell volume (ΔCV_5) was reduced by -184 ± 18 fl ($n=9$) in MDCK cells and -188 ± 58 fl ($n=10$) in NIH3T3 fibroblasts. In the presence of 0.1 μM gossypol, RVD was significantly reduced in MDCK cells as shown by a ΔCV_5 of -120 ± 17 fl ($n=5$; Figure 6a). Addition of 1 or 10 μM gossypol annihilated the observed RVD in MDCK cells and caused a further increase of

the volume ($\Delta CV_5 = +164 \pm 9$ fl ($n=5$) and $+222 \pm 39$ fl ($n=5$), respectively; Figure 6a). In NIH3T3 fibroblasts RVD was abolished at a gossypol concentration of 5 μM as seen by the further increase of the cell volume to $+100 \pm 60$ fl ($n=7$; Figure 6b). At RT neither MDCK nor NIH3T3 fibroblasts were able to exhibit significant RVD (ΔCV_5 amounts to $+2 \pm 38$ fl, $n=6$ and -20 ± 17 fl, $n=7$, respectively) pointing to the fact that 37°C conditions are mandatory for RVD in these cells normally grown at that temperature. In 3 out of 6 measurements in MDCK cells and 3 out of 7 measurements in NIH3T3 fibroblasts a further increase of the cell volume under hypotonic conditions can be observed at RT with peak ΔCV_5 values amounting to +156 fl and +42 fl in MDCK cells and NIH3T3 fibroblasts, respectively.

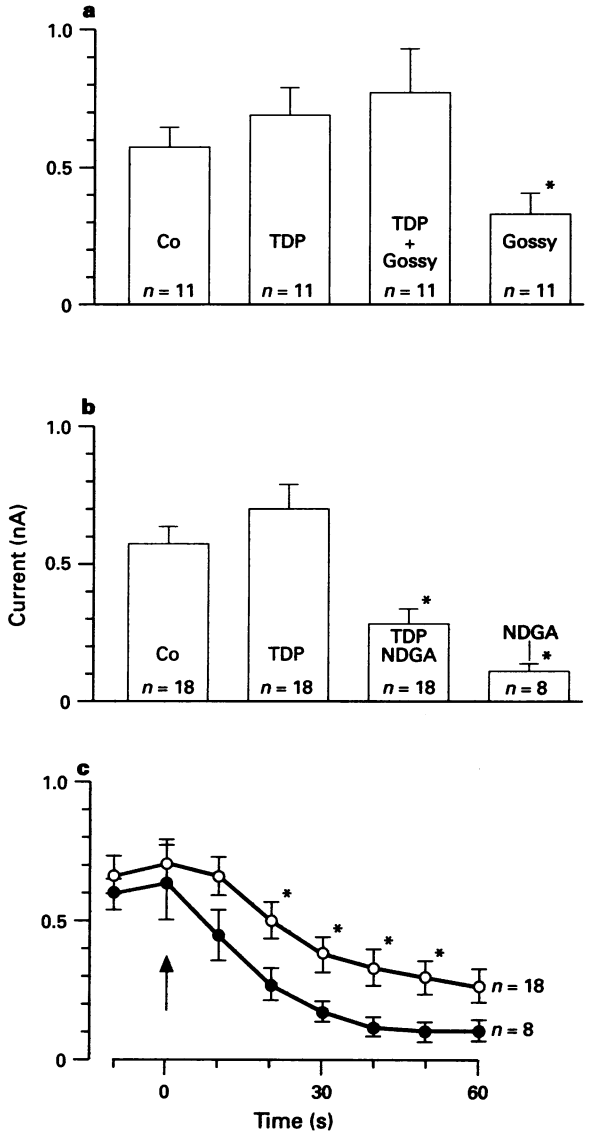

Figure 4 The effect of gossypol and NDGA on I_{Cl} can be modified by the nucleotide thymidine diphosphate (TDP). (a) TDP in a concentration of 100 μM does not lead to a significant change of the swelling-induced chloride current in NIH3T3 fibroblasts. In the presence of 100 μM TDP the blocking effect of 10 μM gossypol (Gossy) is abolished. After washing out the TDP/gossypol mixture, the readdition of 10 μM gossypol alone significantly reduced I_{Cl}. (b) Despite the presence of 100 μM TDP the addition of NDGA leads to a significant reduction of I_{Cl}. However, the time-course of inhibition is significantly impaired (c; at the time point indicated by the arrow NDGA in the presence (○) or absence (●) of TDP was added). The holding potential was at 0 mV and repetitive voltage steps were made to +40 mV. All current measurements were made 2 min after the corresponding drug addition. The current under hypotonic conditions is marked as Co.

The effect of gossypol and NDGA on the cyclic AMP-dependent chloride current I_{ClcAMP} elicited in CaCo cells

The effect of the phenol derivatives was studied in whole-cell configuration in a human colonic cancer cell line (CaCo). Before stimulating I_{ClcAMP} a current of $+75.3 \pm 11.2$ pA (+40 mV; $n=19$) could be measured. After addition of 0.5 mM dibutyryl-cyclic AMP, 0.1 mM isobutylmethylxanthine (IBMX) and 10 μM forskolin a cyclic AMP-dependent chloride current of $+537.9 \pm 84.9$ pA (+40 mV; $n=19$) was provoked. The addition of 10 μM gossypol reduced the current by only $44.1 \pm 13\%$ ($n=8$). Similarly, the addition of 10 μM NDGA reduced the current by only $27.8 \pm 13\%$ ($n=10$).

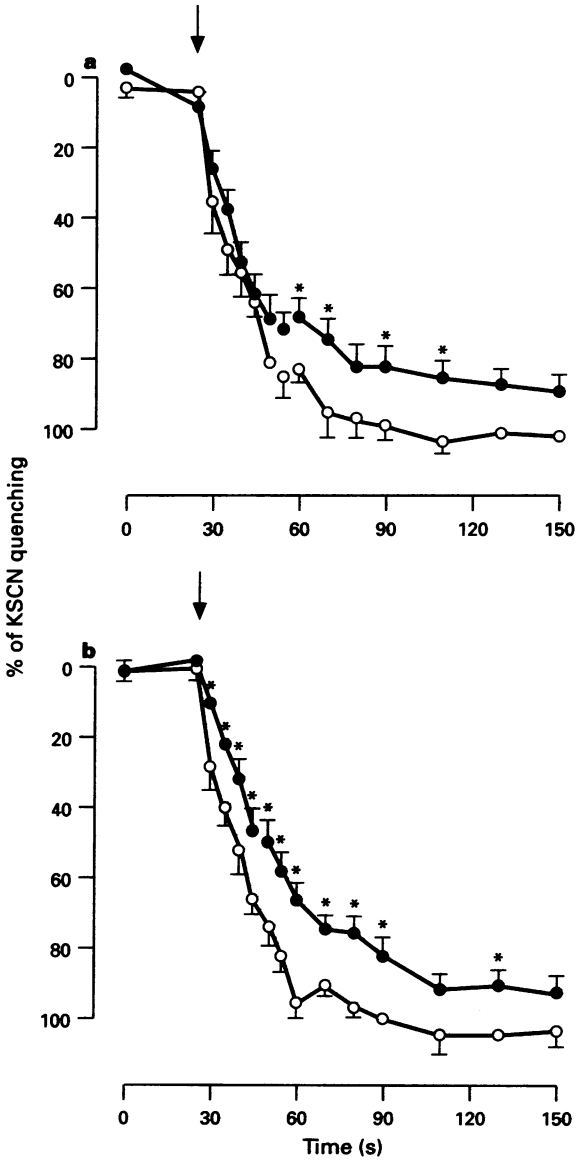

Figure 5 Measurements of chloride permeability (P_{Cl}) in NIH3T3 fibroblasts in the absence (control, ○) and presence of 10 μM gossypol (●) (a) or 10 μM NDGA (●) (b). Independent measurements of 8 cells under control conditions, 9 cells in the presence of gossypol and 6 cells in the presence of NDGA are shown. For the normalization of the fluorescence values the mean value of the data-sample-points (time window 1 min) prior to the addition of KSCN (arrow) was taken as unity.

The effect of gossypol and NDGA on the calcium-dependent chloride current elicited in NIH3T3 fibroblasts

In NIH3T3 fibroblasts the addition of the calcium ionophore ionomycin elicited a chloride current ranging from $+40\pm5$ pA ($n=56$) in the absence to $+1567\pm157$ pA ($n=50$) in the presence of the ionophore at a concentration of 1 μM (the holding potential was at 0 mV and repetitive voltage steps were made to +40 mV for 500 ms; Figure 7). Both phenol derivatives gossypol (10 μM) and NDGA (10 μM), did not significantly change the calcium-dependent chloride ($I_{ClCa^{2+}}$) current

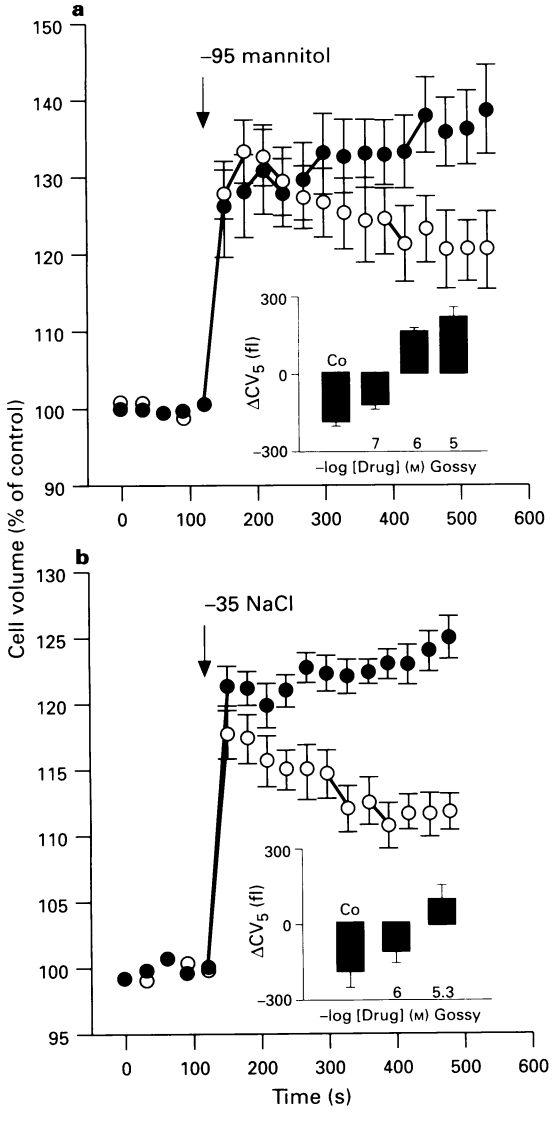

Figure 6 Volume measurements in the absence and presence of gossypol in (a) MDCK cells and (b) NIH3T3 fibroblasts at 37°C. (a) Volume measurements were made in MDCK cells in the absence (○) and presence (●) of 1 μM gossypol added to the extracellular solution. Reduction of osmolarity (95 mosM mannitol) is indicated by the arrow. The inset indicates the change of cell volume within 5 min (ΔCV_5 in fl) after swelling under control conditions (Co) and in the presence of gossypol at the concentrations indicated. (b) Same experimental protocol as in (a). Measurements were carried out in NIH3T3 fibroblasts using 5 μM gossypol and osmolarity was reduced by omitting 35 mM NaCl in the extracellular solution. The inset indicates the change of cell volume within 5 min (ΔCV_5 in fl) after swelling under control conditions (Co) and in the presence of gossypol at the concentrations indicated. The s.e.mean values of the control-data-points are smaller than the symbols.

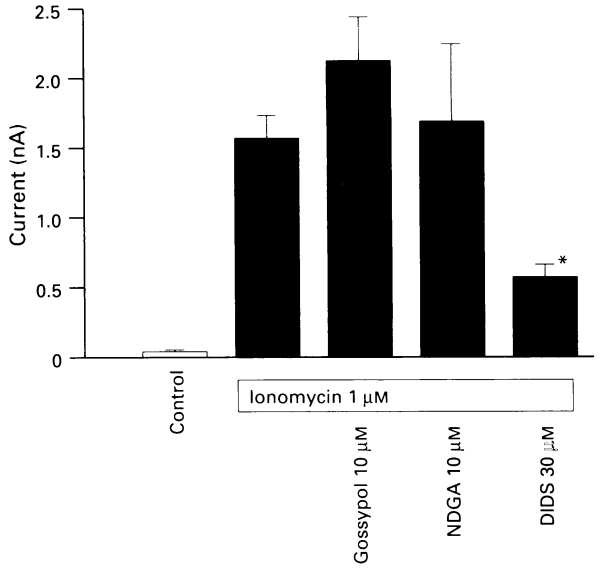

Figure 7 Summary of the experiments testing the effect of gossypol on the calcium-induced chloride current in NIH3T3 fibroblasts. Before stimulation (Control) a chloride current of $+40\pm5$ pA ($n=56$) can be measured. Addition of 1 μM ionomycin leads to a chloride current of $+1567\pm157$ pA ($n=50$) which is not significantly changed by the addition of 10 μM gossypol ($+2115\pm318$ pA; $n=22$) or 10 μM NDGA ($+1685\pm550$ pA; $n=10$). In contrast, 30 μM DIDS reduces the calcium-activated chloride current to $+569\pm88$ pA ($n=9$).

($+2115\pm318$ pA ($n=22$; at +40 mV) and $+1685\pm550$ pA ($n=10$; at +40 mV), respectively). In contrast, DIDS, in a concentration of 30 μM, significantly reduced $I_{ClCa^{2+}}$ to $+569\pm88$ pA ($n=9$; at +40 mV).

Discussion

Chloride channels are imperative for the survival and/or specific function of most cells (Lang *et al.*, 1993; Strange & Jackson, 1995). Substances able to block anion channels like anthracene-9-COOH (A9C), 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) or 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) do not discriminate between swelling-, cyclic AMP- or calcium-dependent chloride channels. On the other hand, specific targeting of only one class of these channel families would be desirable for therapeutic reasons as well as for the biophysical characterization of these important proteins. We set out to investigate the interaction of phenol derivatives, which are structurally related to A9C and NPPB, with I_{Cln}, a cloned swelling-dependent chloride channel (Paulmichl *et al.*, 1992; 1993; Gschwentner *et al.*, 1994). To this end the endogenous swelling-induced chloride channel in NIH3T3 fibroblasts which was identified as I_{Cln} was examined (Gschwentner *et al.*, 1995a). Several structurally related blockers are so far known to be able to inhibit chloride channels. A9C was shown to block chloride channels in muscle cells (Palade & Barchi, 1977) and in epithelial cells (Oberleithner *et al.*, 1983) at millimolar concentrations. Greger and coworkers demonstrated that in epithelial cells, NPPB added to the extracellular fluid is three orders of magnitude more potent than A9C (Wangemann *et al.*, 1986). Ochratoxin A was also demonstrated to be able to block chloride channels in epithelial cells and shows a structural similarity to NPPB (Wöll *et al.*, 1993; Gekle *et al.*, 1993). A9C and NPPB are capable of blocking I_{Cln} expressed in oocytes. Similarly, the endogenous swelling-induced chloride currents in fibroblasts and MDCK cells are sensitive to NPPB (Paulmichl *et al.*, 1993; Gschwentner *et al.*, 1995a). In order to obtain a more precise

picture of the molecular properties required to block I_{Cln} we investigated additional molecules with a close structural relation to A9C and NPPB. NDGA as well as gossypol are able to block the swelling-induced depolarization of the membrane potential seen in fibroblasts or MDCK cells (see also, Paulmichl *et al.*, 1989) as well as the chloride flux across the cell membrane of swollen fibroblasts as indicated by fluorescence-optical measurements. The depolarization of PD is believed to be predominantly due to the activation of chloride channels after reducing extracellular osmolarity (Lang *et al.*, 1986; Paulmichl *et al.*, 1989; Weiss & Lang, 1992). The voltage difference between the actual membrane potential and the equilibrium potential for chloride provides the driving force for chloride to leave the cell. The smaller the difference between the two potentials the smaller the contribution of chloride channels to affecting RVD. It is important, in this regard, that NIH3T3 fibroblasts as well as MDCK cells (not shown) show a markedly reduced membrane potential at RT. At RT we can show that in fibroblasts, reduction of extracellular osmolarity leads to a hyperpolarization instead of the depolarization seen at 37°C. An explanation for these findings could be that at RT the resting membrane potential in fibroblasts is depolarized below the equilibrium potential for chloride. Hence, activating chloride channels under these conditions does not lead to a loss of chloride and a successive shrinking of the cells but, on the contrary, depending on the size of the depolarization at RT with regard to the chloride equilibrium potential, abolished RVD or influx of chloride and water and additional swelling of the cells as shown by the volume measurements.

The experiments lead to the conclusion that gossypol and NDGA are able to block directly the swelling-induced chloride channels. For gossypol a similar mechanism was proposed by Strange and collaborators (Strange *et al.*, 1993; McManus *et al.*, 1994). As shown in Figure 3 NDGA added to the extracellular fluid is able to block the swelling-activated chloride current within seconds. It is, therefore, very unlikely that inhibition of lipoxygenase by NDGA (Lambert *et al.*, 1987) or gossypol (Hamasaki & Tai, 1985) is responsible for the observed effect, since the time needed to induce leukotriene production after reduction of extracellular osmolarity in Ehrlich cells is 5 to 10 min (Lambert *et al.*, 1987). Furthermore, NDGA as well as gossypol impede the cyclic AMP-dependent chloride currents (at albeit higher concentrations, see below) which is independent of the leukotriene metabolism. A tight link between NDGA-lipoxygenase and RVD (Doroshenko, 1991; Mastracola *et al.*, 1991; Mochizuki *et al.*, 1992), potassium channels (Piomelli *et al.*, 1987; Kim *et al.*, 1989; Kurachi *et al.*, 1989), calcium channels (Peppelenbosch *et al.*, 1992) and sodium channels (Cantiello *et al.*, 1990) was also proposed. The present experiments suggest that the blocking effect of NDGA on the swelling-induced chloride channels could also be explained by a direct interaction of NDGA with the channel itself as was shown for calcium channels (Sgaragli *et al.*, 1993; Wang *et al.*, 1993).

It is important to mention that the swelling-activated chloride channels are more sensitive to NDGA and gossypol than the cyclic AMP-activated chloride channels in CaCo cells (Tien *et al.*, 1994) or the calcium-activated chloride current in fibroblasts. Addition of gossypol or NDGA in a concentration of 10 μM to the extracellular solution reduces the cyclic AMP-dependent chloride current by 44% or 28%, respectively. In contrast (see above), the swelling-dependent chloride current is almost completely blocked under these conditions (Figures 1–3). Increasing the intracellular calcium concentration in NIH3T3 fibroblasts by adding 1 μM ionomycin to the extracellular solution leads to the induction of a marked calcium-dependent chloride current which is insensitive to 10 μM gossypol or NDGA. However, DIDS, in a concentration of 30 μM, significantly reduces this current (Figure 7).

A major finding in this study is a potentially close physical relationship between the nucleotide binding domain of the I_{Cln} channel protein and the binding sites for the phenol derivatives. As mentioned above, using site-directed mutagenesis we were able to demonstrate that a glycine repeat at the predicted outer mouth of the channel is responsible for blocking I_{Cln} expressed in oocytes as observed after adding nucleotides to the extracellular solution. The concentration needed for effecting a more than 50% block (IC_{50}) of the I_{Cln} current was found to be higher than 1 mM. The nucleoside analogues AZT and acyclovir block with an IC_{50} of ≈ 30 μM and ≈ 10 μM, and, as shown in an earlier study, their block can be effectively diminished by the simultaneous addition of TDP or uridine to the extracellular solution. As a possible explanation we suggested that TDP as well as uridine competitively block the binding and consecutive inhibition of the swelling-induced chloride current effected by the nucleoside analogues (Gschwentner *et al.*, 1995b). As shown in this study, the binding and blocking of the swelling-induced chloride current by phenol derivatives can similarly be impeded by TDP. Whereas the effect of gossypol is abolished in the simultaneous presence of TDP, the interaction of TDP with NDGA is much weaker and can only be shown in the kinetics of the onset of the block. It remains to be clarified where the important structures are within the gossypol molecule to make the interaction with the nucleotide binding site more pronounced compared to NDGA. However, the as yet unidentified binding site(s) of these derivatives and their close relatives like NPPB or A9C seems to be in close proximity to the nucleotide binding site in I_{Cln}. Mutagenesis studies are necessary to identify precisely the amino acids responsible for the binding of these structures, leading to the possible development of a clear picture of the three-dimensional structure of the binding domain which is paramount for developing new, more effective and/or selective blockers.

In conclusion, gossypol and NDGA inhibit the depolarization and RVD seen after reducing extracellular osmolarity in NIH3T3 fibroblasts or MDCK cells. Both substances are able to block directly swelling-induced chloride channels in fibroblasts. This inhibitory effect is not restricted to the swelling-induced chloride currents but can also be encountered with the cyclic AMP-dependent chloride currents elicited in CaCo cells, though at higher concentrations, pointing to the fact that by modifying the molecules tested a more selective blocker for a single chloride channel family might be found.

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