

Section IV: Mechanism of Action of Nedocromil Sodium

I_{Cln} : A chloride channel paramount for cell volume regulation

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Cell volume regulation is a ubiquitous cell regulatory mechanism based on meticulously controlled ion transport mechanisms. Keeping the absolute volume constant seems to be of the highest priority for most cells and is achieved at the expense of altered intracellular ion concentrations. We have been able to demonstrate that I_{Clw} , a chloride channel cloned from epithelial cells, is paramount for the ability of swollen cells to regulate their volume back to that under resting conditions. A unique feature of I_{Cln} is the distinct sensitivity of these channels for nucleotides and nucleoside analogues added to the extracellular fluid. In addition, cromolyn sodium and nedocromil sodium, drugs used by patients with asthma, are able to impede the function of these channels. (J Allergy Clin Immunol 1996;98:S98-101.)

Key words: Chloride channel, cromolyn sodium, epithelial cell, fibroblast, nedocromil sodium, regulatory volume decrease

The simultaneous activation of different ion transporters and/or channels is crucial for cells to maintain their cytoplasmatic volume within the normal range that occurs under resting conditions. After cell shrinkage, most cells readjust their cytoplasmatic volume by the activation of transporters and channels that permit ions, together with water, to enter the cells, leading to cell swelling despite the continued presence of, for example, hypertonic extracellular medium.¹ In contrast, after cell swelling, the reduction of cell volume is achieved by the movement of solutes and water in the opposite direction.²⁻⁴ This movement of ions, which enables cells to shrink back to normal size after having gained volume, is effected by the precisely timed activation of potassium and chloride channels.⁴

Abbreviations used

AMP:	Adenosine monophosphate
AZT:	Azido thymidine
DIDS:	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
I_{Cln} :	A chloride channel cloned from epithelial cells
IC_{50} :	Concentration at which 50% inhibition occurs
MDCK:	Madin-Darby canine kidney cells
NDGA:	Nordihydroguaiaretic acid
NPPB:	5-nitro-2-(3-phenylpropylamino)-benzoic acid
RVD:	Regulatory volume decrease

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CHARACTERISTICS OF I_{Cln}

With use of the expression cloning technique, we have identified an epithelial chloride channel (I_{Cln}) that plays a critical role in regulatory volume decrease (RVD).⁵ The mRNA for the cloning process was isolated from Madin-Darby canine kidney (MDCK) cells, which display many of the features of cells in the distal portion of the nephron.⁶ However, after sequencing the cloned

DNA and developing suitable "probes" and antibodies, mRNA coding for I_{Cln} and/or the I_{Cln} protein could be identified in many different organs such as brain, lung, muscle, intestine, spleen, liver, kidney, and testis⁵ (Gschwentner M and Paulmichl M, unpublished results). After expressing the mRNA in *Xenopus laevis* oocytes, a characteristic current can be measured. The chloride current, identified by its reversal potential and pharmacologic characteristics, is strongly outwardly rectifying and shows a voltage-dependent inactivation at voltage steps (800 ms) from a holding potential of -70 mV to values greater than $+40$ mV.

Besides the known chloride channel blockers 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS),⁷ the observed currents can also be reduced by adding nucleotides to the extracellular fluid. This nucleotide dependence appears to be a unique feature not only of I_{Cln} expressed in oocytes but also of the endogenous I_{Cln} identified in NIH 3T3 fibroblasts, MDCK cells, the B-cell lymphoma cell line H9, tracheal epithelium, and cardiac cells, as well as chloride channels reconstituted in planar phospholipid bilayers.⁸⁻¹² Our proposed (computer-generated) model of the I_{Cln} protein revealed a potential nucleotide binding sequence at the predicted extracellular mouth of the pore (GxGxG where G is glycine and x is any other amino acid), which, mutated to AxGxG or AxAxA (A is alanine), leads to a dramatic reduction of the sensitivity of the expressed chloride currents for the nucleotide cyclic adenosine monophosphate (cAMP).⁵ Furthermore, the expressed mutations led to chloride currents that were totally resistant to inactivation at voltage potentials over $+40$ mV, indicating that the mutations occurred in close proximity to the voltage sensor of the ion channel itself. These findings⁹ suggest that I_{Cln} is indeed the channel itself and render the regulator hypothesis, as proposed by others,¹³ very unlikely.

Whereas nucleotides such as cAMP, guanosine triphosphate, cyclic guanosine monophosphate, or inositol triphosphate block the I_{Cln} expressed in oocytes by more than 50% (IC_{50}) at an extracellular concentration of 1 mmol/L (for adenosine triphosphate, adenosine diphosphate, AMP, and adenosine, higher concentrations are necessary for the same effect),⁵ nucleoside analogues such as azido thymidine (AZT) or acyclovir have IC_{50} values of 20 and 10 μ mol/L, respectively. These substances do not interfere with the cAMP-activated chloride current in colon carcinoma cells,

believed to be connected to the cystic fibrosis transmembrane regulator.⁹ It is most important to note that nucleotides such as thymidine diphosphate or uridine have no effect on I_{Cln} at concentrations up to 0.1 mmol/L. However, in the presence of these substances, AZT and acyclovir (both 0.1 mmol/L) are without any significant effect on the swelling-activated chloride current. These findings⁹ can be explained by thymidine diphosphate and uridine competitively blocking the inhibitory effect of the nucleoside analogues, which could, in turn, lead to the speculation that therapeutic concentrations of these drugs might safely be increased in this way without increasing their side effects. (The side effects seen in patients treated with AZT or acyclovir mainly involve neurotoxicity, nephrotoxicity, and impaired function of bone marrow stem cells, all of which could be explained by blockade of the swelling-induced chloride current in those cells.)

In addition to the above-mentioned NPPB and DIDS, another class of blockers for I_{Cln} comprises the phenol derivatives nordihydroguaiaretic acid (NDGA) and gossypol. These substances have IC_{50} values of 24 and 36 μ mol/L, respectively, and are known to interfere with the leukotriene pathway.^{14, 15} It was previously suggested that inhibition of 12-lipoxygenase by NDGA resulted in perturbation of RVD.¹⁴ Our experiments suggest a simpler model, in which NDGA directly interacts with the I_{Cln} protein and thus impairs RVD.^{3, 16}

The kinetics of I_{Cln} expressed in oocytes resemble those of the chloride currents that are responsible for RVD after cells are swollen in hypotonic medium. After transiently knocking out the I_{Cln} protein in NIH 3T3 fibroblasts by antisense oligodeoxynucleotides complementary to the sequence coding for I_{Cln} in MDCK cells, we demonstrated that I_{Cln} is the predominant channel involved in RVD.¹⁰

EFFECT OF NEDOCROMIL SODIUM AND CROMOLYN SODIUM

The whole-cell voltage clamp method¹⁷ was chosen to measure swelling-induced chloride currents in isolated NIH 3T3 fibroblasts and to investigate the effect of the benzo- γ -pyrone-2-carboxylate compounds cromolyn sodium and nedocromil sodium, which are structurally similar to the two phenol derivatives tested and mentioned previously. Fibroblasts were grown on glass cover slips in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, equilibrated with 95% humidified air and 5% CO_2 at 37°C and

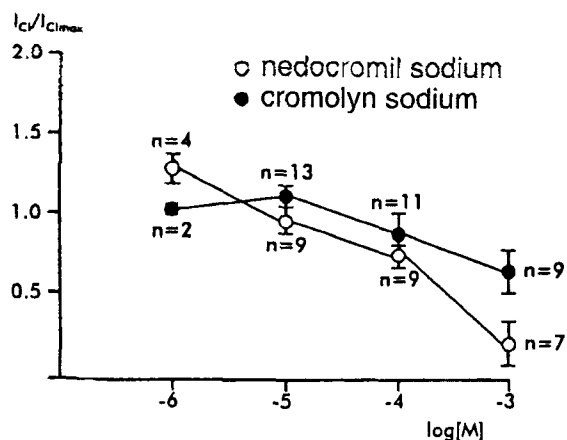


FIG. 1. The blocking effect of nedocromil sodium and cromolyn sodium on the swelling-induced chloride current in NIH 3T3 fibroblasts. The current is expressed as the current in the presence of the different blocker concentrations divided by the current immediately before adding the lowest blocker concentration (all measurements were made under hypotonic conditions). Where applicable, data are expressed as arithmetic means $\pm$ standard error of the mean. Statistical analysis was made by *t*-test where appropriate. A significant difference was assumed at $p < 0.05$.

measured 24 to 48 hours after splitting. All experiments were made at room temperature (20 to 22°C). Bath and pipette solutions were chosen to enable chloride current measurements. The isotonic extracellular solution used was composed of the following (in mmol/L): NaCl 125, CaCl₂ 2.5, MgCl₂ 2.5, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10, mannitol 50, adjusted to pH 7.2 with NaOH. To reduce extracellular osmolarity, mannitol was omitted. One to two minutes after confirming whole-cell configuration, hypotonic conditions were established and the activated outwardly rectifying chloride current was measured. Fast exchange of the bath solution was obtained with use of a perfusion system with a flow rate of 10 ml/min and a bath volume of ≈ 250 μ l. The filling solution of the patch pipette was (in mmol/L): CsCl 125, MgCl₂ 5, ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid 11, HEPES 10, raffinose 50, adjusted to pH 7.2 with CsOH. For data acquisition and analysis, an EPC-9 (HEKA, Lambrecht, Germany) and an Axopatch 200A (Axon Instruments, Inc., Foster City, Calif.) amplifier were used, controlled by an Atari (ATARI Corporation, Sunnyvale, Calif.) and an Apple computer (Apple Computer, Inc., Cupertino, Calif.) running the according software for driving the am-

plifier and REVIEW for analysis (Instrutech Corporation, Elmont, N.Y.). All current measurements were filtered at 1 kHz (analogue 4-pole BESSEL).

As shown in Fig. 1, both compounds were able to impair the swelling-induced chloride current in NIH 3T3 fibroblasts in a dose-dependent manner, with nedocromil sodium being significantly more potent ($IC_{50} \approx 0.1$ mmol/L compared with $IC_{50} \approx 1$ mmol/L for cromolyn sodium). However, it remains to be tested whether the beneficial effect of these drugs in the treatment of asthma involves direct inhibition of I_{Cln} . A similar inhibitory effect of these drugs at similar concentrations on chloride channels in different cell systems has been described by others.¹⁸

CONCLUSIONS

In conclusion, we demonstrated that I_{Cln} is the critical chloride channel involved in the regulation of cell volume after swelling. The chloride current evoked by I_{Cln} can be blocked by applying chloride channel blockers such as NPPB or DIDS, as well as by adding phenol derivatives or benzo- γ -pyrone-2-carboxylate derivatives to the extracellular fluid. A special feature of I_{Cln} appears to be the exquisite sensitivity of this channel for nucleotides and related structures from the extracellular side.

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