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Effects of NEM on Voltage-activated Chloride Conductance in Toad Skin

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Abstract. The regulation of the voltage-activated chloride current conductance (G_{Cl}) in toad skin was investigated by the use of the SH reagents N-ethylmaleimide (NEM) and p-chloro-mercuricbenzenesulfonic acid PCMBS. This anion pathway is controlled by a voltage-sensitive gating regulator. Mucosal application of NEM decreased the voltage-activation in a time and concentration dependent manner, half-maximal inhibition being exerted at a concentration of 30 μM within 20 min. At concentrations higher than 100 μM, the voltage-activated G_{Cl} was near-completely and irreversibly inhibited in less than 10 min. Resting, deactivated conductance was essentially unaffected. NEM had no effect on active sodium transport (measured as I_{sc}) under conditions, which fully dissipated the voltage-activated G_{Cl}. After complete inhibition of the voltage-activated G_{Cl} with NEM, chloride conductance could still be stimulated by CPT-cAMP as in control tissues. Under these conditions, NEM at concentrations above 1 mM decreased G_{Cl} reversibly. Mucosal application of PCMBS at 500 μM inhibited the activated conductance by 35%, which was slightly reversible. Inhibition of voltage-activated G_{Cl}, which was observed after mucosal addition of the membrane-impermeable NEM analogue, eosin-5-maleimide, was completely reversible after washout. This suggests that the binding site for the maleimide is not accessible from the external face of the apical membrane. Brief application of NEM at lower concentrations (1–3 min, ≤100 μM) led to partial inhibition of G_{Cl}, followed by occasionally complete recovery upon washout of NEM. Recovery of voltage-activated G_{Cl} was progressively attenuated and eventually disappeared after subsequent brief applications of NEM. This could reflect recruitment of permeation/control sites from a finite pool. The data are discussed in the frame of a working model for the voltage-activated Cl^--pathway, that contains two principle components, i.e., an anion-selective permeation path which is controlled by regulatory protein(s).

Key words: cAMP — Theophylline — PCMBS — Na^+ transport — *Bufo viridis* — Graphical model

Introduction

Transport of chloride across amphibian skin is to a large extent passive. It involves a chloride-specific conductive pathway and must be localized in morphological structures other than the principal, sodium transporting cells of the epithelium (*see* Larsen, 1991; Katz & Nagel, 1994 for recent reviews). The path for chloride passage has to be equipped with the characteristic elements of membrane channels to determine the ion selectivity and to establish the specific permeation pattern. A particularly interesting property of the transepithelial chloride passage is a remarkable rectification, due to strong voltage-sensitivity of some structure, which is activated by serosa-positive clamp potentials; reversed polarity turns off the activated conductive state (Larsen & Kristensen, 1978). Site and nature of the subelements involved in the regulation of selectivity and permeability of the chloride path are unknown. Supposing that chloride transport occurs via membrane channels in the apical membrane of mitochondria-rich cells, a voltage-sensitive gating mechanism in these channels has been proposed (Willumsen & Larsen, 1986). Furthermore, control of this gate by cAMP has been suggested, mainly based on the observations that inhibitors of the phosphodiesterase as theophylline or IBMX increased the voltage-activated chloride conductance (Katz & Van Driessche, 1988, Kristensen & Larsen, 1978) and that the voltage-sensitivity of the conductive pathway was shifted to

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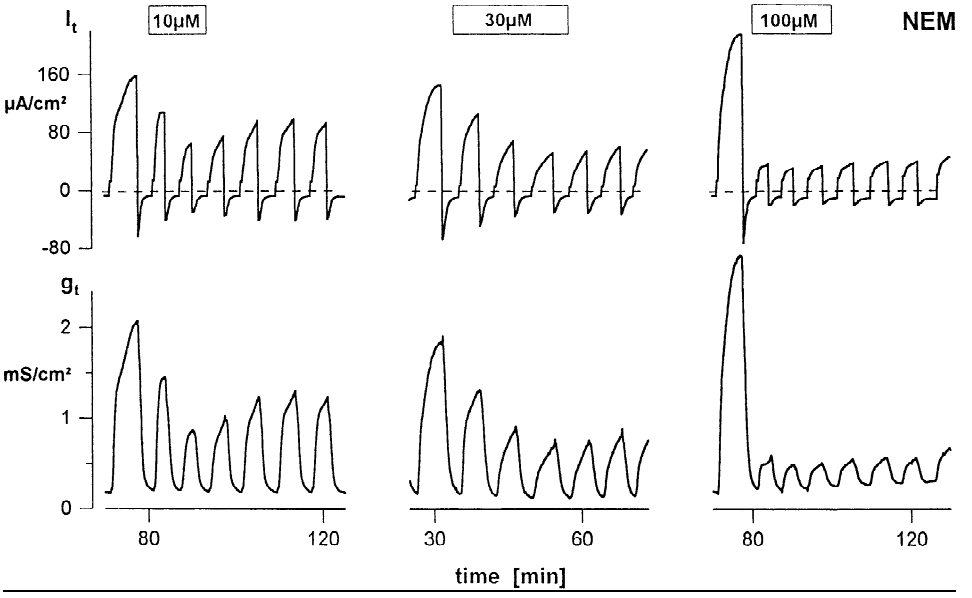

Fig. 1. Effect of mucosal application of NEM at three concentrations on the voltage-activated g_t of toad skin. Tissue pieces from the same animals were perfused with 10, 30, and 100 µM NEM on the mucosal side during the periods as indicated by the bars. In each case, NEM was added in the inactivated state, 1 min before a voltage perturbation. V_t was rhythmically changed between −30 and +80 mV from. Amiloride (10^{-5} M) was present throughout.

lower potential values by cAMP-analogues (Willumsen, Vestergaard & Larsen, 1992). Evidence from recent studies in our laboratory (Katz & Nagel, 1995) indicates that the effect of cAMP is more complex than previously proposed, eliminating the voltage-sensitivity of the path at low concentrations and progressively converting the permeation barrier to a permanently open conformation at higher levels. Our results furthermore suggest that the effects of inhibitors of the phosphodiesterase on chloride conductance are not related to the presumably induced increase in cellular cAMP.

In the present study, we have attempted to further characterize the voltage-activated chloride path with the aid of SH-group reactive agents as NEM and PCMBS. Although the diversity of potential reactions after these agents generally impede specific conclusions on affected sites and structures, hints on different components might be derived from the concentration dependency of effects. It will be shown that NEM can be used to analyze and distinguish between the proposed permeation elements of the chloride conductive pathway.

Materials and Methods

Toads (*Bufo viridis*) were collected in Israel. They were kept at the laboratory with free access to tap water and were fed mealworms every second week. The animals were sacrificed by doubly pithing, and abdominal skins were carefully dissected. They were mounted in a modified Ussing type chamber (Helman & Miller, 1971) and continually perfused on both sides with aerated Ringer solution of the following composition (in mM): Na^+ 115, K^+ 2.5, Ca^{2+} 1, Cl^- 117, Hepes 3.5; pH 7.6. Na^+ transport was eliminated by addition of amiloride (10^{-5} M) to the mucosal fluid.

Transepithelial current (I_t) and small pulse tissue conductance (g_t) were determined using a custom-made voltage clamp device with sample & hold circuitry (Nagel, Garcia-Diaz & Essig, 1983) connected via a 14 bit AD/DA-interface (Industrial I/O Card, Taipeh, Taiwan) to a PC-class computer. Records of I_t and g_t *vs.* time were produced from these files using standard graphics software (Origin, MicroCal Software, Northampton MA). Transepithelial potential difference (V_t) is referred to the mucosal side of the tissue. I_t is considered positive for anion flux from mucosa to serosa. Between voltage-activation by perturbation of V_t to +60 or +80 mV, the preparations were maintained at −30 mV to ensure deactivation of the voltage-sensitive Cl^--specific conductance, G_{Cl} (Larsen & Kristensen, 1978). Time constants for the activation of G_{Cl} after voltage perturbation were obtained from the regression line in semilogarithmic plots of g_t *vs.* time.

All chemicals used were of reagent grade. Eosin-5 maleimide was obtained from Molecular Probes, Oregon. Other drugs were purchased from Sigma. Experiments were performed at room temperature. Where appropriate, mean values are reported ±SD; significance of difference was calculated using Student *t*-test considering $2P < 0.05$ as significant.

Results

The effect of NEM on voltage-activated chloride conductance, G_{Cl}, was analyzed in tissues from more than 40 toads. Figure 1 shows the results a typical experiment illustrating the response of G_{Cl} on mucosal application of

NEM at three different concentrations. Voltage-activated chloride current was in each case induced repeatedly before the depicted period and had assumed constant pattern. Baseline, inactivated conductance at −30 mV was less than 0.2 mS/cm^2. After voltage perturbation to +80 mV, g_t started to increase immediately and approached steady-state values slightly above 2 mS/cm^2 within five min. NEM at concentrations of 10, 30 or 100 μM was applied from the mucosal side as indicated by the hatched bars. Voltage perturbation was done 1 min after addition of NEM and it is evident that activation of G_{Cl} was reduced at that time already in all cases. Essentially fully response was achieved at 100 μM within 5 min, whereas inhibition of g_{Cl} became progressively larger with time at the lower concentrations of NEM. In all cases, baseline conductance was not notably affected by NEM. In the depicted experiment, reversibility after washout of NEM was poor at the concentration of 10 μM and is hardly detectable at 30 and 100 μM. In other experiments, in particular at low concentrations and after brief application of NEM, certain recovery of G_{Cl} was observed (*see below*). With concentration of more than 200 μM NEM, the voltage-activated G_{Cl} was completely eliminated within 10–15 min and was not recovered when NEM was thereafter washed away (*not shown*).

Table 1 summarizes the mean values for the influence of NEM observed in 26 experiments. Inhibition of g_t was complete within 20 min at concentrations above 100 μM; response at concentrations below 50 μM was usually not complete before 40 min. Due to this combination of time and concentration dependence, it is not possible to obtain an absolute value for half maximal effect of NEM. On the average, NEM at a concentration of about 30 μM decreased the voltage-activated g_t by 50%. NEM at any concentration did not affect the baseline inactivated conductance at −30 mV. The time constant of conductance activation was slightly longer after NEM than in the corresponding control experiments (1.87 ± 0.87 min *vs.* 1.54 ± 0.46 min, $n = 14$), but the difference is not significant ($2P > 0.5$).

Application of NEM below 100 μM from the serosal side was ineffective during exposure times up to 60 min. At a concentration of 500 μM, NEM reduced g_t by about 50% within 20 min; with longer duration, however, and even more at higher concentrations, essentially all of the voltage-activated gain in g_t was dissipated by 500 μM serosal NEM.

Inhibition of Cl^--related conductance by NEM was limited to the voltage activated component as shown for 3 different experiments in Fig. 2. In the experiment depicted in the upper pannel, more than 80% of voltage-activated gain in g_t was lost after 25-min incubation with 500 μM NEM on the mucosal side. After washout of NEM, application of 300 μM CPT-cAMP from the serosal side led to a large increase in g_t, similar to the response in untreated control tissues. Likewise, the pattern of voltage induced change in g_t under the combined influence of NEM and CPT-cAMP were not different from that in tissues which were exposed to CPT-cAMP alone; in both cases, g_t increased almost instantaneously after voltage perturbation, which is fundamentally different from control conditions where activation of g_t occurs with discrete time dependency. Stimulation of baseline conductance with CPT-cAMP was possible also after preceding inhibition with higher concentrations of NEM, provided NEM was removed after the apical solution. This is shown in the middle panel of Fig. 2, where CPT-cAMP was ineffective with 2 mM NEM in the solution, but had the usual effect briefly after washout of the maleimide. The slow onset of the CPT-cAMP-induced stimulation might be due to delayed washout of the high concentration of NEM used. Finally, reversible inhibition of g_t was observed with concentrations above 1 mM NEM as shown in the lower panel of Fig. 2. The gain in baseline conductance during CPT-cAMP was comparable in tissue pieces whether they were exposed or not to low concentrations of NEM. Thus, g_t increased by 3.1 mS/cm^2 in untreated pieces (5 observations), whereas it increased by 2.5 mS/cm^2 in skin samples from the same animals pretreated with 50–200 μM NEM (11 observations).

The effects of increasing the intracellular level of cAMP with the phosphodiesterase inhibitor, theophylline were different from those after CPT-cAMP. This could be revealed after partial inhibition of G_{Cl} with NEM as shown for a typical experiment in Fig. 3. In this case, theophylline did not affect the baseline inactivated conductance; in other tissues, very slight increase in g_t could be observed. In contrast, the partly inhibited G_{Cl} is notably increased after addition of theophylline. The effect of theophylline was always fully reversible and, in relative terms, not different from the stimulatory action of the xanthine on G_{Cl} under control conditions.

Table 1. Effect of mucosal application of NEM on voltage-activated Cl^- conductance of toad skin

g_t^{-30mV} mS/cm^2	G_{Cl} mS/cm^2	NEM <20 μM	NEM <50 μM	NEM <200 μM	NEM 500 μM	NEM 1–2 mM
0.39 ±0.17 $n = 26$	2.87 ±1.85 $n = 26$	0.60 ±0.18 $n = 8$	0.30 ±0.19 $n = 11$	0.16 ±0.25 $n = 7$	0.12 ±0.09 $n = 6$	0.04 ±0.04 $n = 7$

The values of G_{Cl} represent the steady state increase of tissue conductance after voltage perturbation from −30 to +80 mV. Control values represent the average of 2–3 readings; experimental data were obtained 20–40 min after addition of NEM at the indicated concentration and are normalized to the respective values of G_{Cl} of the control period. Baseline inactivated tissue conductance at −30 mV was not notably affected by NEM. Values are given ±SD.

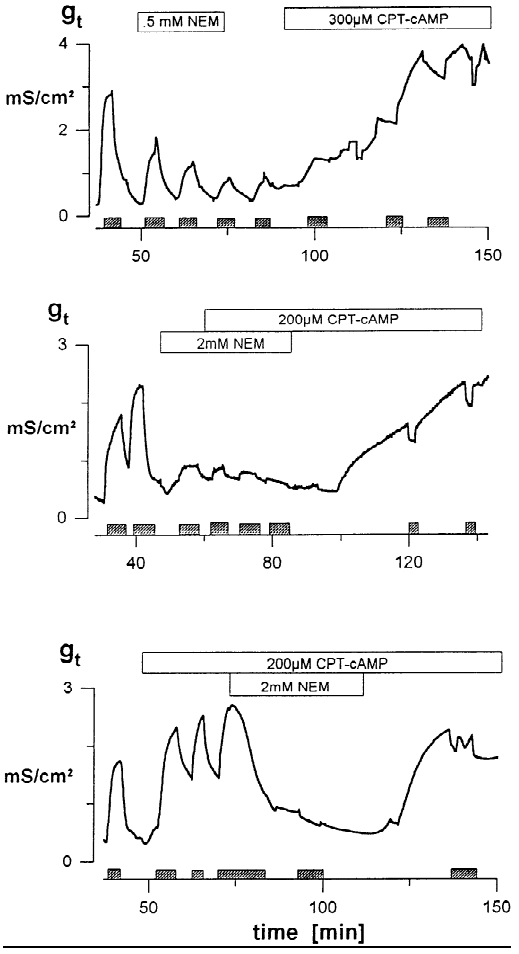

Fig. 2. Effect of serosal application of supramaximal concentrations of CPT-cAMP after preceding inhibition with NEM. Upper pannel: Effect of 300 µM CPT-cAMP on g_t after inhibition of voltage-activated G_{Cl} by 0.5 mM NEM with subsequent washout of the maleimide. Middle pannel: Effect of 200 µM CPT-cAMP on g_t during and after the presence of 2 mM NEM in the mucosal bath. Lower pannel: Effect of 2 mM mucosal NEM after stimulation of baseline conductance with CPT-cAMP in the serosal bath. Voltage sensitivity of g_t was not completely lost during CPT-cAMP alone, but completely lacking during and after washout of NEM. Voltage perturbations from the holds potential of −30 mV were done to +80 mV as indicated by the light gray bars. 10^{-5} M amiloride was present throughout. Note the instantaneous change in g_t in the presence of NEM and CPT-cAMP.

The effect of NEM on Na^+ transport of toad skin is shown in Fig. 4. After each application of NEM at the concentrations of 100 µM, a slight, albeit notable decrease in the magnitude of I_t at short-circuit (I_{sc}) was observed. However, the sensitivity of the Na^+ pathway to amiloride remained completely unchanged. In contrast to this moderate effect, voltage-activation of G_{Cl} as indicated by the response of I_t and g_t during voltage perturbation to +80 mV decreased drastically and was virtually eliminated briefly after addition of 100 µM NEM. As in the above experiments in amiloride-treated tissue, CPT-cAMP led to a large gain in G_{Cl} after NEM with little change in current. On the average of 4 experiments, the I_{sc} was reduced by less than 20% during application of NEM for 10 min at a concentration of 100 µM; G_{Cl} was, at this time, always completely eliminated. In 4 other experiments, where NEM was applied at concentrations between 10 and 50 µM, the I_{sc} remained essentially unchanged, whereas G_{Cl} strongly decreased. Above 1 mM, NEM caused incomplete and irreversible decrease of the I_{sc}. Under these conditions, the tissues were insensitivite to amiloride.

The voltage-conductance relationship of the chloride-specific conductive pathway under control conditions and after partial inhibition with NEM was analyzed in 6 experiments. Figure 5*a* shows the original record of a typical experiment. After a sequence of voltage perturbations under control conditions, 50 µM NEM was applied while V_t was maintained at 80 mV. During the incubation with NEM for 5 min, about 50% of the activated g_t was dissipated; after washout of NEM, g_t recovered slightly (*see below*) and remained then almost constant. Due to the reduced magnitude of g_t, the following series of voltage perturbations could be done up to V_t = 150 mV. The relation between V_t and g_t derived from these data is depicted in Fig. 5*b*; it indicates that only the maximal level but not the shape of the voltage-sensitivity of g_t was affected by NEM. Similar results were obtained in the other experiments of this kind.

When NEM was applied at lower concentrations and for brief periods of time, response pattern as shown for two typical experiments in Fig. 6 were obtained. The upper pannel shows that application of 100 µM NEM for 1 min during inactivated state reduced the subsequent activation of G_{Cl} to about 50%. Within 30 min, however, G_{Cl} returned almost to the level before NEM. A second treatment with 100 µM NEM for 1 min during the activated state decreased G_{Cl} as before but the recovery was now only incomplete. Subsequent application of NEM for 3 min lead to strong decrease of G_{Cl} with essentially no recovery. Similar observation from another experiment is shown in the lower pannel, where 50 µM NEM was applied for 2 min. In this case, recovery was lacking already after the second application of NEM. Partial reactivation of the inhibited G_{Cl} was observed in all experiments, if NEM at concentrations $\leqslant$100 µM was applied for brief periods of time (less than 5–6 min) and could occasionally be induced during several brief applications of NEM. No difference was apparent whether NEM was applied in the inactivated state or during voltage-activation. The magnitude of reactivation decreased with each consecutive addition and eventually disappeared. At higher concentrations of NEM and after incubation for more than 10 min, reactivation of G_{Cl} was never observed.

The effect of two membrane-impermeable SH-reactive agents, eosine-5-maleimide and PCMBS on voltage-activated G_{Cl} was tested in additional experi-

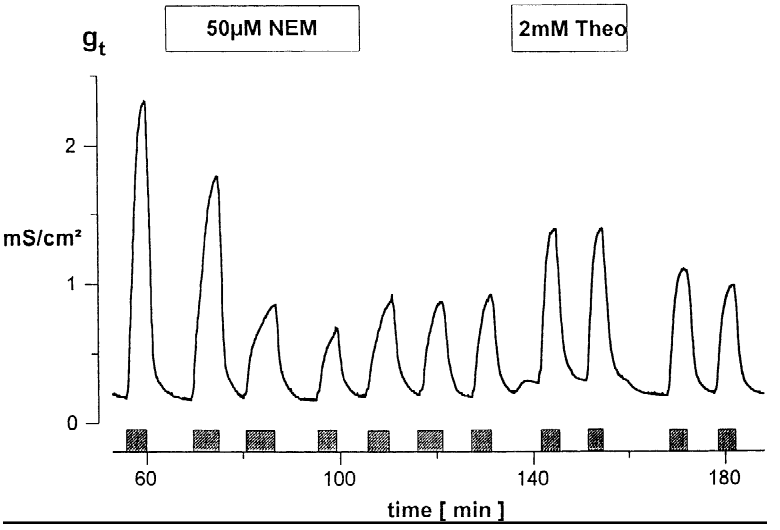

Fig. 3. Response of G_{Cl} on mucosal application of 2 mM theophylline after partial inhibition with NEM. Light gray bars indicate periods of voltage perturbation from −30 to +80 mV. Amiloride (10^{-5} M) was present throughout.

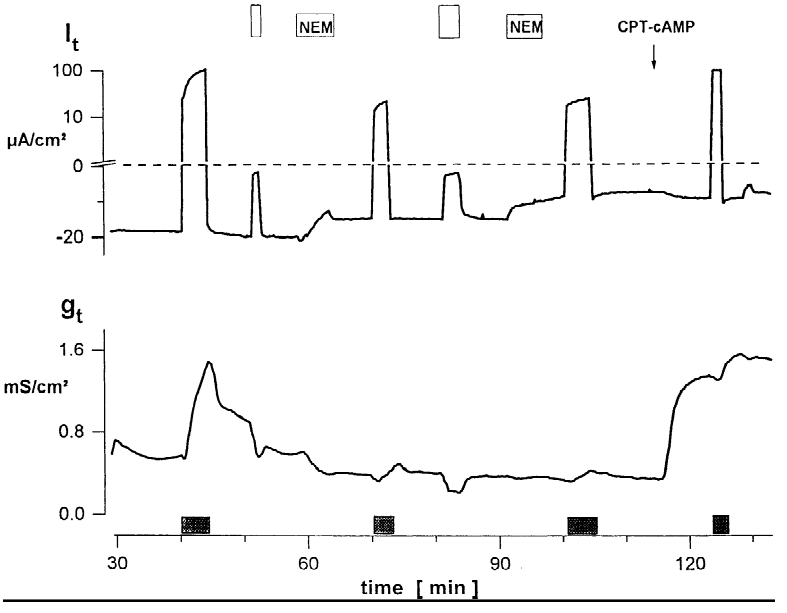

Fig. 4. Effect of mucosal application of 100 µM NEM (indicated by bars on top of the record) on Na^+ transport (I_t during short-circuit) and voltage activation of G_{Cl} in toad skin. Transepithelial voltage was altered between short-circuit and 80 mV, as indicated by the light gray bars. Mucosal application of 10^{-5} M amiloride is shown by the hollow bars. Note that positive values of I_t are plotted on a logarithmic scale to pronounce the presentation of negative values of I_t, i.e., the I_{sc}.

ments. The membrane-impermeable analogue of NEM, eosine-5-malemide (EMA) was used in 8 experiments. The typical experiment is shown in Fig. 7. EMA applied at a concentration of 100 µM from the mucosal side in the activated state, decreased G_{Cl} rapidly to values close to those at inactivation. After washout, G_{Cl} quickly returned, in this experiment, to about 70% of the control level. In other cases, reversibility was even more complete. The response on application of 100 µM NEM for 1 min is shown at the right hand of Fig. 7. Here, the less pronounced inhibition is only slowly and slightly reversible. In all other experiments with EMA between 20 and 200 µM at the apical side, inhibition of G_{Cl} ranged between 30% at 20 µM and almost 100% at 200 µM EMA. Recovery was prompt ranging between 70 and 100%. Progressive loss of activation after repeated addition of EMA was not observed. No effect could be detected after serosal addition of EMA at concentrations up to 200 µM. Mucosal application of 500 µM PCMBS decreased the activated G_{Cl} by 35% in 30 min (8 experiments). A slight recovery was obtained upon washout of PCMBS. Serosal application of 500 µM PCMBS slowly decreased G_{Cl}. Both SH-reactive agents had no detectable effect on the cAMP-induced G_{Cl}.

Discussion

Previous studies have shown that chloride movement across amphibian skin occurs mainly by electrodiffusion and must be cellularly separated from the sodium transporting compartment (for review *see* Larsen 1991; Katz

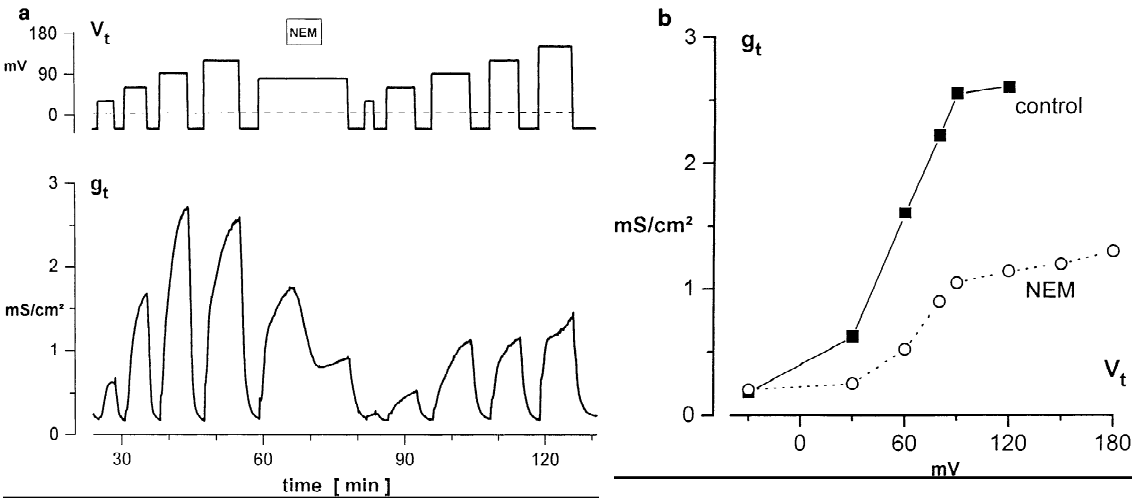

Fig. 5. Voltage sensitivity of the Cl^- pathway before and after brief application of 50 μM NEM from the mucosal side. (*a*) Reproduction of the original record, showing the transepithelial clamp potential and the tissue conductance. NEM was applied for 5 min and thereafter rapidly washed away. Na^+ transport was throughout blocked with 10^{-5} M amiloride (*b*) Relation between g_t and V_t from the data in (*a*).

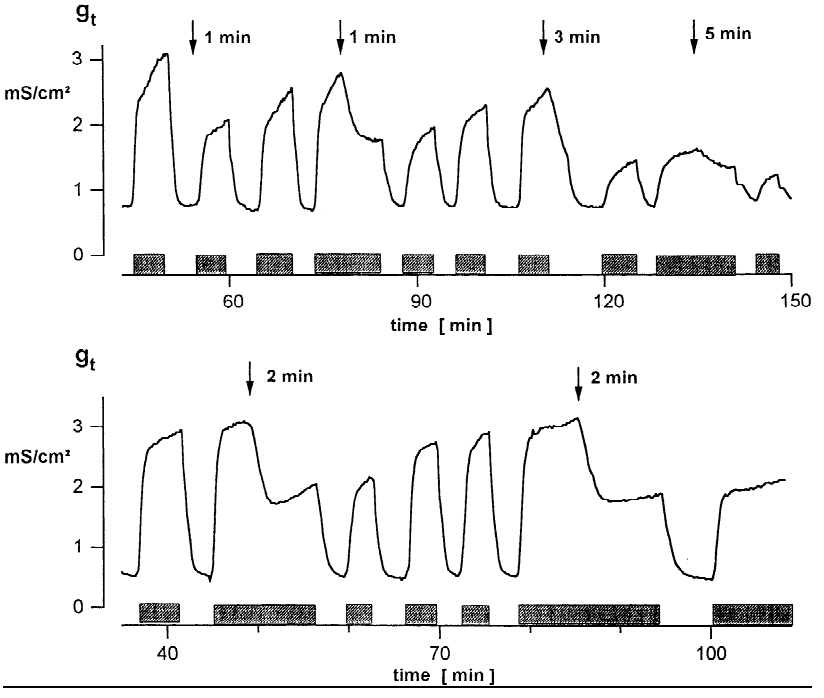

Fig. 6. Response of voltage-induced G_{Cl} on repeated, brief application of NEM at low concentration(50 or 100 μM, mucosal side). Voltage activation of G_{Cl}, which is initially completely regained after brief inhibition with NEM, remained depressed after repeated use of the SH-agent. Light gray bars below the record indicate periods of voltage perturbation to +80 mV from a hold voltage of −30 mV. NEM was added at the arrows for the indicated duration. Amiloride (10^{-5} M) was present throughout.

& Nagel, 1994). The voltage-activated chloride conductance pathway, G_{Cl} across amphibian skin, seems to be composed of two components, i.e., a conductive path (channel), which is controlled through a voltage-sensitive Cl^--dependent regulatory gating mechanism. In the present study we used NEM to investigate the mechanism of control of this pathway. A graphical model of this pathway, which is based on the one presented by Imai et al., (1994) for the putative Cl^--regulated channels in the loop of Henle, is depicted in Fig. 8. The model envisages a membrane protein, which forms the transmembrane anion conductive path. A voltage-sensitive controller (*V*) that can be affected by various agents, governs a gate (*G*), which is furthermore regulated through a cAMP-sensitive regulator (*R*). Voltage activation of the gate occurs with comparatively slow time constants (min) only by serosa positive voltages, i.e., under conditions which favor influx of the permeable anion into the path, and thus resembles the characteristics of inward rectifying Cl^- channels. The voltage-activated gating mechanism requires, in addition, the presence of Cl^- (or Br^-) on the external side of

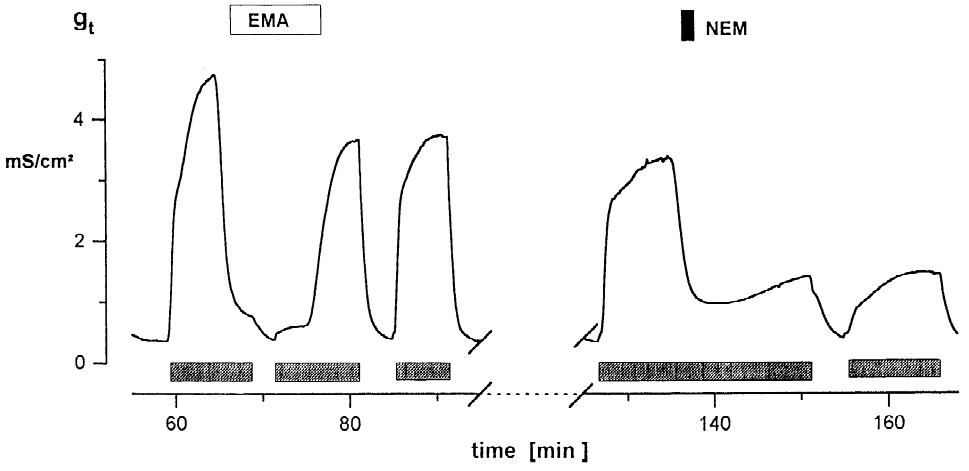

Fig. 7. Effect of 100 μM eosin-5-maleimide (EAM, hatched bar) at the mucosal side on voltage-induced G_{Cl} in toad skin (left panel) in comparison to the response of G_{Cl} on mucosal addition of 100 μM NEM (right panel, dark bar). Note the brief duration of NEM application. Light gray bars below the record indicate voltage perturbation from −30 to +80 mV. Amiloride (10^{-5} M) was present throughout.

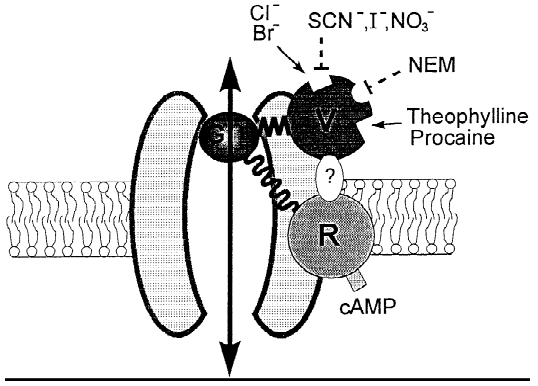

Fig. 8. Simplified graphical model of the voltage-activated Cl^- transport pathway of amphibian skin. A voltage-sensitive controller (*V*) governs a gate (*G*) of an anion-selective conductance path. Regulation of the pathway can be achieved by phosphorylation through a cAMP-sensitive regulator (*R*). The voltage sensor (*V*) may also be influenced by the cAMP-sensitive regulator (*R*). The voltage-sensitive controller requires the presence of Cl^- (or Br^-) at an external site. This is blocked by NO_3^-, SCN^- and I^-. The fully activated anionic path (CPT-cAMP stimulated) can conduct $Cl^- > Br^- > NO_3^- > I^-$. NEM at low concentration excerts its inhibitory effects on the voltage-sensitive controller (broker line arrow).

the tissue (Larsen & Kristensen, 1978). If Cl^- is displaced by certain other anions (NO_3^-, SCN^-, I^-) that have higher affinity for this site, the gate cannot be activated, and the pathway remains closed (Harck & Larsen, 1986; Katz & Nagel, 1995). The anion conductive path itself is a simple diffusive path and does not show any rectification. The cAMP-sensitive regulator was suggested to act as a physiological control of the voltage-sensitive site (Willumsen et al., 1992), but this interference seems to be more complex as higher concentrations of cAMP eliminated control of the anion conductance path by the voltage-sensor (Katz & Nagel, 1995). The maximal conductance obtained with cAMP did not differ significantly from the maximal value during voltage activation. Also, closer examination of Fig. 3*B* in Willumsen et al. (1992), shows in fact, that the time-dependent response was dissipated similarly in their preparation upon application of cAMP, although the maximal conductance did not increase significantly. Both observations are difficult to reconcile with physically separated voltage-activated and cAMP-induced chloride pathways, but rather suggest a single ionic channel with two regulatory sites. Anionic selectivity of the conductive path can be revealed only when the path is opened, either through maximal voltage activation, or in the presence of cAMP (Larsen, 1991; Katz & Nagel, 1994).

The experimental results of the present study can be explained in the constraints of the above model. In this simple interpretation we consider a single voltage-activated chloride pathway, where the inhibitory effects of NEM reveal two sites of regulation with drastically different affinity for this SH-specific group reagent. One of them, the voltage-sensitive controller ('*V*' in Fig. 8) of the gate, can be blocked by brief application at concentrations below 100 μM. In these conditions, NEM has virtually no inhibitory influence on the permeability of the conductive path after its transformation into the permanently open state in the presence of CPT-cAMP. The conductance-voltage relations indicate that NEM does not affect the sensitivity of the voltage sensor to voltage. Rather, the maximal level of conductance is decreased by the irreversible binding of NEM. This inhibition, however, can be overcome in the presence of CPT-cAMP, which eliminates the influence of the voltage sensor of this component (Katz & Nagel, 1995), presumably through phosphorylation of a distinct site, transforming

the path to a permanently open state. Inhibition in this conformation requires concentrations of NEM higher than 1 mM, but is often imperfect or occasionally even lacking, despite the rather high concentration of the maleimide. Furthermore and unlike the influence on the voltage-sensor, the inhibitory effect of NEM on the conductive path is reversible. It must be concluded that this effect is not related to the covalent, and thus irreversible binding of NEM to proteinic SH-groups. Similarly, the strong inhibition of G_{Cl} after application of the membrane-impermeable eosin-5-maleimide (EMA), which would at face value suggest some reactive element(s) at the extracellular side of the apical membrane, is evidently not due to reaction of the maleimide-group, since the inhibition is rapidly reversible. Indeed, unpublished own experiments indicate that G_{Cl} is also inhibited by eosin at similar concentrations as with EMA. The origin of these inhibitory effects requires further experimental work.

The precise location of the site which is affected by NEM cannot be verified with any certainty at present, since the membrane permeability of NEM is finite. The ineffectiveness of the membrane-impermeable maleimide, EMA, as well as the weak inhibition of G_{Cl} by the organo-mercurial PCMBS, which has only low membrane permeability, suggest that the sensitive site of the voltage sensor cannot be located at the extracellular face of the apical membrane. Accordingly, modification of intracellular SH-groups involved in regulation of the Cl^- pathway could explain the inhibition by NEM. In view of the rapid onset of the inhibitory effect after mucosal addition of NEM and the quick termination after washout, when low concentrations of NEM were applied for brief periods of time, it appears likely that these sites are located close to, or even inside the apical membrane. This would also agree with the delayed and modest inhibitory action, observed after serosal application of NEM at higher concentrations.

Brief incubation with NEM at concentrations below 100 μM, which rapidly dissipated some 40–60% of the activated G_{Cl}, was usually followed by a slow partial return of the voltage sensitivity of the Cl^- pathway. As it appears very unlikely that binding of NEM to proteins could be reversible within the present time scale, return of the voltage-activation could indicate that new reactive sites (channels or part thereof) are inserted in the Cl^- pathway, or that previously inactive sites, which were inaccessible to the inhibitory action of NEM, are activated. Our observations suggest that the total amount of modifier molecules is limited and can be used up by repeated inhibition of the accessible fraction with NEM. It is interesting to note that—similar as under control conditions—the partly inactivated G_{Cl} is reversibly increased after addition of theophylline. This activation, which keeps the time course and the voltage/conductance relation of G_{Cl} unchanged, is most likely unrelated to possible changes of intracellular cAMP due to inhibition of the phosphodiesterase and depends in magnitude on the activity of G_{Cl}, remaining after NEM. CPT-cAMP, in contrast, is able to fully activate the Cl^- pathway irrespective of the state of the voltage-sensitive control site, which could indicate that the Cl^- pathway is composed of physically separate fractions. Further analysis is necessary to identify these properties.

SH agents, such as NEM are widely used to analyze the chemical basis of a variety of biological activities. The concentrations commonly used are higher than 0.5 mM, and inhibition of metabolic activity due to the influence on mitochondrial ATPases or other nonspecific effects must often be suspected. In our experiments, NEM was effective at fairly low concentrations and within a time frame, which makes notable influence on the supply of metabolic energy unlikely. This notion is supported by the observation that active transepithelial Na^+ transport was little affected at NEM concentrations, which led to complete dissipation of G_{Cl}. Since voltage-activated Cl^- flux is passive, metabolic deficiency is expected to show up even less than for active Na^+ transport. Furthermore, the prompt response of G_{Cl} on additions of CPT-cAMP with essentially control-like increase in Cl^- conductance, indicates that the Cl^- pathway itself remained intact. Accordingly, we suggest that the observed inhibition of voltage-activated G_{Cl} by NEM is the result of a specific interference of the maleimide with proteinic groups of the permeation pathway, notably one of the control sites and may help to identify the molecular entity involved in this transepithelial transport function. Imai et al., (1988) studied similar behavior in the effect of NEM on Cl^- conductance in the thick ascending limb (TAL) of the hamster kidney. NEM, applied at the rather high concentration of 1 mM, irreversibly inhibited the chloride conductance of TAL; after complete development of this inhibition, reapplication of NEM stimulated G_{Cl}. In view of the reversibility, however, this seems to be unrelated to covalent binding to SH-groups, and could be similar, although opposite in direction, to the reversible inhibition of the cAMP-induced G_{Cl}, observed in the present study.

In conclusion, we have found that NEM at low concentrations inhibits *selectively* the voltage-activated Cl^- conductance across toad skin epithelium, at a site separate from the cAMP-sensitive regulator. At higher concentrations, the binding site which might be the voltage-sensitive gate of the pathway or part of it, is fixed by the maleimide in a voltage-insensitive configuration, but is still amenable to the activation by cAMP.

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References

Harck, A.F., Larsen, E.H. 1986. Concentration dependence of halide fluxes and selectivity of the anion pathway in toad skin. *Acta Physiol. Scand.* **128:**289–304

Helman, S.I., Miller, D.A. 1971. In vitro techniques for avoiding edge damage in studies of frog skin. *Science* **173:**146–148

Imai, M., Kondo, Y., Koseki, C., Yoshitomi, K. 1988. Dual effect of N-ethylameimide on Cl- transport across the thin ascending limb of Henles loop. *Pfluegers Arch* **411:**520–528

Imai, M., Yoshitomi, K., Taniguchi, J., Suzuki, M. 1994. Regulation of Cl- conductance in the thin ascending limb of Henle's loop. *Jap. J. Physiol.* **44:**S261–268.

Katz, U, Nagel, W. 1994. Biophysics of ion transport across amphibian skin. *In:* Amphibian Biology. Volume II: The Integument. H. Heatwole and G.T. Barthalmus, editors. pp. 100–121. Surrey Beatty & Sons, Chipping Norton

Katz, U, Nagel, W. 1995. Effects of cyclic AMP and theophylline on chloride conductance across toad skin. *J. Physiol.* **489:**105–114

Katz, U., Van Driessche, W. 1988. Effect of theophylline on the apical sodium and chloride permeabilities of amphibian skin. *J. Physiol.* **397:**223–236

Kristensen, P., Larsen, E.H. 1978. Relationship between chloride exchange diffusion and a conductive chloride pathway across the isolated skin of the toad (*Bufo bufo*). *Acta Physiol. Scand.* **102:**22–34

Larsen, E.H. 1991. Chloride transport by high-resistance heterocellular epithelia. *Physiol. Rev.* **71:**235–283

Larsen, E.H., Kristensen, P. 1978. Properties of a conductive cellular chloride pathway in the skin of the toad (*Bufo bufo*). *Acta Physiol. Scand.* **102:**1–21

Nagel, W., Garcia-Diaz, J.F., Essig, A. 1983. Cellular and paracellular conductance patterns in voltage-clamped frog skin. *In:* Membrane Biophysics II. Physical methods in the study of epithelia. M.A. Dinno, A.B. Callahan, and T.C. Rozzell editors. p. 221–231. Alan R. Liss, New York

Willumsen, N.J., Larsen, E.H. 1986. Membrane potentials and intracellular Cl activity of toad skin epithelium in relation to activation of the transepithelial Cl conductance. *J. Membrane Biol.* **94:**173–190

Willumsen, N.J., Vestergaard, L., Larsen, E.H. 1992. Cyclic AMP- and β-agonist-activated chloride conductance of a toad skin epithelium. *J. Physiol.* **449:**641–653