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Cyanide Inhibition of Chloride Conductance Across Toad Skin

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Abstract. The effect of cyanide (CN^-) on voltage-activated or cAMP-induced passive chloride conductance (G^{Cl}) was analyzed in isolated toad skin. Comparatively low concentrations of CN^- inhibited G^{Cl} almost completely and fully reversibly, regardless of whether it was applied from the mucosal or serosal side. The IC_{50} was 180 ± 12 μM for voltage-activated G^{Cl} and 305 ± 30 μM for the cAMP-inducted conductance. At [CN] <100 μM, the initial inhibition frequently declined partly in the continuous presence of CN^-. Inhibition was independent of the presence of Ca^{2+}. Inhibition was stronger at more alkaline pH, which suggests that dissociated CN^- is the effective inhibitor. The onset of the inhibition of voltage-activated or cAMP-induced G^{Cl} by CN^- occurred with half-times of 34 ± 10 sec, whereas reversibility upon washout was twice as fast (18 ± 7 sec). If [CN^-] <200 μM was applied under inactivating conditions (serosa −30 mV), the reduction of G^{Cl} was stronger upon subsequent voltage-activation than under steady-state activated conditions. This effect was essentially complete less than 30 sec after apical addition of CN^-, but G_t recovered thereafter partially in the continuous presence of CN^-. Dinitrophenol inhibited G^{Cl} similarly, while omission of oxygen did not affect it. These observations, as well as the time course of inhibition and the full reversibility, suggest that interference of CN^- with oxidative phosphorylation and subsequent metabolic depletion is not the reason for the inhibition of G^{Cl}. We propose that the inhibition is directly on G^{Cl}, presumably by competition with Cl^- at a rate-limiting site in the pathway. Location and molecular nature of this site remain to be identified.

Key words: Mitochondria-rich cells — Tight junctions — Electrophysiology — cAMP — Dinitrophenol

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Introduction

A major fraction of transepithelial chloride movement across the abdominal skin of toads occurs through voltage-sensitive, conductive transport sites with hitherto unresolved morphological localization and molecular characteristics (for review *see* 11, 20). The pathway involved, which seems to be regulated in the long-term by the environmental conditions, e.g., chlorinity of the surrounding medium [29], is characterized by the unconditional dependence on the presence of chloride in the apical incubation solution and the slow activation of conductance by serosa-positive transepithelial potentials with time constants in the order of tens of seconds to some minutes. Although the overall pattern of chloride movement has the appearance of inward rectification at steady state, the permeation path itself is not rectifying and has been shown to have a low selectivity for halides [5, 20]. Since direct cellular approaches to this pathway, such as intracellular electrical measurements [33], electron probe microanalysis [26, 30], patch-clamp analysis [32] and quantitative vibrating probe [4, 13, 28] have, as yet, failed to identify the involved site(s) conclusively, we resorted to observing the effect of various agonists and inhibitors on G^{Cl} in the voltage-clamp mode. A number of physiological effectors and pharmacological agents have been tested for their influence on the conductance of this pathway [14, 15], and have led to the suggestion of control by cAMP [34]. Inhibition, on the other hand, was observed with a variety of agents (for reference *see* 11, 17, 20). In preliminary experiments we observed effective and reversible inhibition of voltage-activated G^{Cl} by micromolar CN^-. CN^-, a powerful inhibitor of oxidative metabolism, has been used previously in amphibian skin [1, 7, 19], but effects were variable and apparently unrelated to metabolic inhibition in some cases [1]. The movement of Cl^- is passive and thus independent of metabolic energy supply, except for

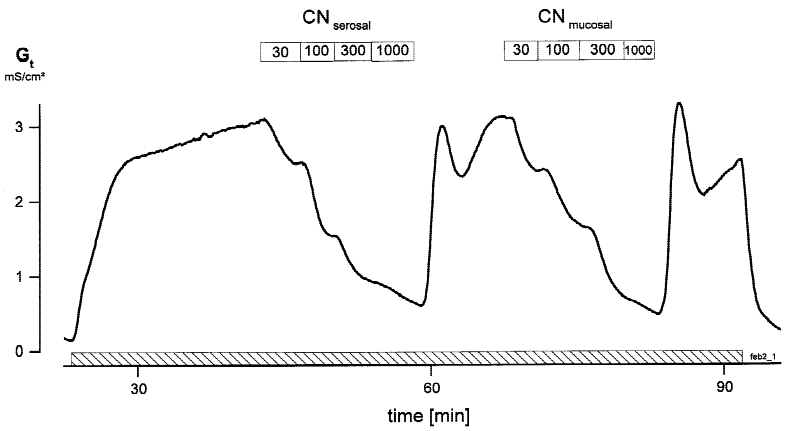

Fig. 1. Response of G_t on application of stepwise increasing [CN^-] from the serosal or mucosal side of isolated toad skin epithelium. The hatched bar above the time scales indicates voltage perturbation from −30 to +80 mV, which leads to activation of G^{Cl} to a steady state level exceeding 3 mS/cm^2. Baseline conductance under inactivated conditions was 0.23 mS/cm^2.

that required to maintain the active state of the pathway. It thus appears possible that CN^- exerts direct effects on the conductive pathway for Cl^-. The aim of the present study was to investigate the influence of CN^- on G^{Cl} systematically.

Materials and Methods

Skins from *Bufo viridis* (collected in Israel), *Bufo regularis* (obtained from Egypt) and *Bufo americanus* (obtained from Carolina Biological Supplies) were used in this study. The animals were kept in the laboratory at room temperature with free access to distilled water and fed weekly with mealworms ad libitum. Techniques for obtaining the intact skin or the isolated epithelium, as well as the experimental procedure for electrophysiological analysis of the voltage-activated chloride conductance (G^{Cl}) have been described recently [12]. Transepithelial potential difference, clamping current and tissue conductance have the notations V_t, I_t, and G_t, where G_t was determined from the current deflection on brief perturbations of V_t (4 mV, 200 msec every 3 sec). The tissues were superfused at >2 ml/min on both sides with Ringer solutions of the following composition (in mM): Na^+ 115, K^+ 2.5, Ca^{2+} 1, Mg^{2+} 1, Cl^- 117, Hepes 3.5; pH 7.6. After elimination of Na^+ transport by the addition of 10 μM amiloride to the mucosal fluid, tissues were depolarized to −30 mV to deactivate G_t. Thereafter, G_t was activated by intermittent hyperpolarization of V_t to 80 mV (serosa positive). Values of G^{Cl} reported in this study reflect the difference between G_t at −30 and +80 mV. CN^- was applied from the apical or basolateral sides under two experimental conditions: (i) CN^- was added cumulatively to the respective side, with new addition being made after G_t had approached a steady state. On a few occasions, CN^- was washed out between application of two successive concentrations. (ii) CN^- was applied in the inactivated state at −30 mV, and V_t was perturbed to +80 mV after a brief period of fluid equilibration (15–40 sec). Usually, CN^- was washed out in these experiments within 3–5 min to permit a second test of the time course during steady-state perturbation. Similar experiments were carried out with SCN^- and 2,4-dinitrophenol (DNP) at appropriate concentrations.

The time to half maximal inhibition and recovery of the effect of CN^-, and for the voltage-activation was calculated from the conductance measurements recorded during the experiments every 2.5 sec. The values were plotted on a semilogarithmic scale *vs.* time, and the time to half maximal effect was determined by linear regression of the linear part of the curve.

Concentration relationships of the effect of CN^- on the voltage-activated G^{Cl} were constructed after normalization of the values of G^{Cl} to the value measured under the appropriate control conditions. Data were fitted to a sigmoidal regression function using software (Origin, Microcal). In the presence of CPT-cAMP, the values of G^{Cl} could not be estimated without knowledge of the Cl^--independent tissue conductance. For these experiments, G_t under control conditions and after application of CN^- is reported and used for the calculation of the concentration-response relationships.

Results

The effect of CN^- on the voltage-activated G^{Cl} was tested in the first series after the approach of G^{Cl} to the steady state level during voltage perturbation to +80 mV. Experiments with apical addition of CN^-, were done on intact skins from all species of toads, whereas for serosal application of CN^- only isolated epithelia from *Bufo viridis* were used, to minimize the diffusion delay in connective tissues layers. Figure 1 shows the typical response of the voltage-activated G^{Cl} in an experiment of the latter type. Sequentially increasing [CN^-] were first applied from the serosal side. At the highest [CN^-] (1 mM) more than 80% of G^{Cl} was blocked. Washout of CN^- rapidly returned G^{Cl} to the prior control level; overshoot, as in the experiment shown, was observed frequently. Subsequent testing of the same [CN^-] from the apical side revealed essentially the same inhibition of G^{Cl} and similar recovery after washout. At each [CN^-] a steady state of inhibition was approached within 2.5 min; the time course of inhibition was not notably different for apical or serosal application. Interestingly, reversibility of the effect upon washout was considerably faster than the inhibition for both test sequences (*see* below). CN^- did neither affect the deactivated G^{Cl} (i.e., G_t at −30 mV) nor G_t in the absence of mucosal Cl^- at activating V_t. These observations indicate that unspecific tissue conductance is insensitive to CN^-

The sensitivity of skins from *Bufo viridis*, *Bufo regularis* and *Bufo americanus* was not notably different and, in the light of the observed variability, the data were

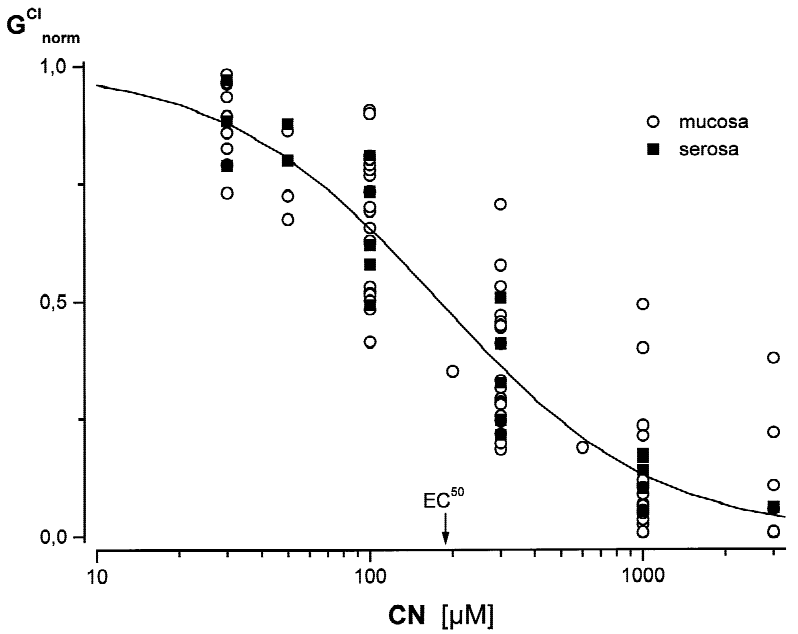

Fig. 2. Concentration-response relationship between G^{Cl} (normalized to control values of the individual experiments) and the [CN^-] in the mucosal or serosal perfusion solution. The calculated regression line is based only on the mucosal data points.

pooled. Results from 21 experiments witih apical and 5 experiments with serosal application of CN^- are included in Fig. 2. The data are normalized for the magnitude of G^{Cl} under control conditions. From the sigmoidal regression curve for the apical data an IC_{50} of 180 ± 12 μM is obtained. The data from serosal application are not included in the regression analysis, but it is evident that they fall in the same range. In other experiments, the effect of prior application of CN^- on the contralateral side was tested, but the concentration-response relation for mucosal application did not differ with the presence or absence of CN^- on the serosal side. For comparison with a recent study from our laboratory [12], the effect of SCN^- on G^{Cl} was tested on skin preparations from the same group of *Bufo viridis*. An IC_{50} of 9.4 + 4.3 mM, indicating that SCN^- is more than 50 times less effective than CN^-, was obtained from concentration-response experiments in 11 skins from 7 animals. In view of the fact that changes in intracellular Ca^{2+} have been invoked frequently in studies on the response on CN^-, we examined for the response to CN^- after serosal incubation with nominally Ca^{2+}-free Ringer with 2 mM EGTA for more than 30 min. The ratio of inhibition in the tissues treated in this way and exposed to 300 μM CN^- was 1.14 ± 0.31 compared with matched tissue pieces from the same animals treated normally ($n = 5$) and was not significantly different from 1.

Due to the high pK (9.3) for the dissociation of the weak acid HCN, the major fraction of cyanide will not be dissociated at the pH (7.6) of the incubation solutions. To examine the relative role of HCN and CN^- in the inhibition of G^{Cl}, we tested the influence of the pH in the apical Ringer solution on the response to 100–200 μM CN^-. Figure 3 shows typical results of these experiments ($n = 5$). After activation of G^{Cl} by perturbation of V_t to +80 mV, 100 μM CN^- was applied in two subsequent periods during which the apical side of the skin was first exposed to a pH of 7.0 and then to pH 9.0. It is evident that the inhibition is notably larger at the more alkaline pH (to 44% of the control G^{Cl}) than at pH = 7.0 (to 78% of the control). On the average, inhibition by identical [CN^-] increased by a factor of 1.9 ± 0.6 at pH = 9 compared with pH = 7. In comparable experiments at apical pH 6.2, the sensitivity of G^{Cl} to CN^- was clearly reduced (to 43 ± 36% of the effect at pH = 7.6; $n = 4$). These observations indicate that inhibition increases with elevated concentrations of dissociated CN^-. Attempts to quantify the responses in terms of free CN^- were unsuccessful, presumably because the pH at the putative site in the Cl^- pathway is not the same as in the bulk solution.

The half-time for the onset of inhibition was 34 ± 7 sec for mucosal ($n = 15$) and 27 ± 10 sec for serosal application of CN^- ($n = 8$). The logarithmically linear components were preceded by delays of 20–45 sec. G^{Cl} recovered after washout of CN^- with a half time of 18 ± 7 sec ($n = 11$), irrespective of the side of application and after a similar delay as the onset of inhibition. Half-times for onset and reversibility of the inhibition by CN^- were not detectably affected by the concentration of CN^-. The half-time for the activation of G^{Cl} by voltage in the present experiments was 25–70 sec. A delay of 15–40 sec was present between voltage perturbation and the analyzed linear time course.

The chloride conductance pathway can be transformed to a permanently open state by incubation with

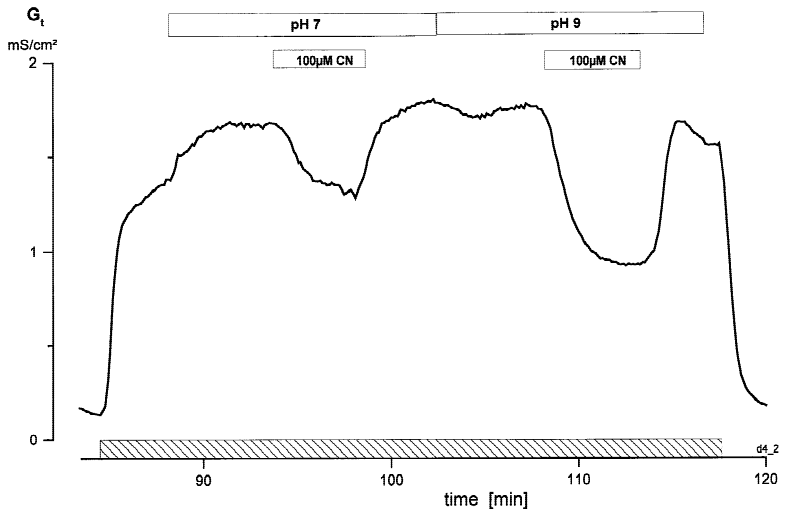

Fig. 3. Effect of pH in the apical solution on the inhibition of G_t by 100 µM CN^-. Activation of voltage-sensitive G^{Cl} was done by voltage perturbation from −30 to +80 mV as indicated by the hatched bar above the time scale.

high [cAMP], as achieved with the membrane-permeable, nonhydrolyzed analogue, CPT-cAMP [12]. The effect of CN^- on G_t in this condition, which is voltage-insensitive, was analyzed under short-circuit conditions. As shown in Fig. 4, CN^- decreased the cAMP-induced G_t promptly, reversibly and dose dependently. It appears that slightly higher concentrations of CN^- are required for the blockage of the cAMP-induced G_t than for inhibition of the voltage-activated G^{Cl}. This is confirmed by the concentration-response relations for the effect of CN^- on the CPT-cAMP induced G_t in 12 tissues (Fig. 5), which yields an apparent IC_{50} of 305 ± 30 µM. This value is almost twice as high as the concentration of CN^- required to block the voltage-activated G^{Cl}, but the significance of the difference is questionable in view of the scatter among different tissues. Reversibility was comparably fast as the onset of the inhibitory effect. Half-times of the onset and the reversibility of inhibition by CN^- during CPT-cAMP were 35–50 sec and thus slightly longer than those of the voltage-activated G^{Cl}. CN^- also effectively and reversibly blocked the conductance remaining after replacement of apical Cl^- by SO_4^{2-} in the cAMP-induced state (Fig. 3). As shown in the figure, this experimental maneuver leads to a reversal of current flow due to efflux of Cl^- (measured as I_{sc}) driven by the serosa to mucosa transepithelial concentration gradient for this ion. Under these conditions, apical CN^- (3 mM) reduced reversibly the transepithelial conductance and current to values comparable to those in the voltage-inactivated state before cAMP.

The patterns of inhibition were notably different when CN^- was added to the apical solution in the inactivated state shortly before the voltage perturbation. The typical finding is shown in Fig. 6. After a control perturbation to +80 mV and return to −30 mV, which displayed the usual time course and magnitude of voltage-activation and inactivation of G^{Cl}, 200 µM CN^- was applied 15 sec prior to a second perturbation to +80 mV. Evidently, the inhibitor had elicited its effect already during this brief period of pre-incubation; activation of G^{Cl} was always almost completely eliminated. As shown in the subsequent part of the record, in which 200 µM CN^- was applied after intermittent washout of the inhibitor, the magnitude of the inhibition by addition of CN^- before activation was notably larger than that exerted by the same concentration of CN^- under steady-state activated conditions. Similar observations were made in more than 10 experiments in which [CN^-] <200 µM was applied. With higher concentrations, inhibition was too strong under both conditions and no difference could be revealed. The inhibition of G^{Cl} by lower concentrations of CN^- was frequently spontaneously reversed in the continuous presence of CN^-. Figure 7 shows an example of this response pattern. Application of 200 µM CN^- for as little as 15 sec prior to voltage perturbation to +80 mV prevented almost completely the activation of G^{Cl} (to 3.7 mS/cm^2 in preceding control periods). This strong inhibition declined spontaneously and partially so that G^{Cl} after 7 min had reached values characteristic of inhibition under steady-state conditions (compare with Fig. 2). The subsequent test application of 200 µM CN^- during steady-state activation of G^{Cl} (after intermittent washout of CN^-) shows similar partial relief after initially stronger inhibition. This kind of transitory steady-state inhibition was observed frequently at lower [CN^-] (<200 µM).

Dinitrophenol (DNP) exerted similarly reversible inhibition of G^{Cl} with a comparable time course, but with considerably greater affinity than CN^-. The data after mucosal (n = 4) or serosal (n = 3) application were very similar and gave an IC_{50} of 56 ± 8 µM. DNP on the mucosal side inhibited the cAMP-induced G^{Cl} (2 experiments) as effectively as the voltage-activated conductance. Azide at 10 mM inhibited G^{Cl} by less than 30%,

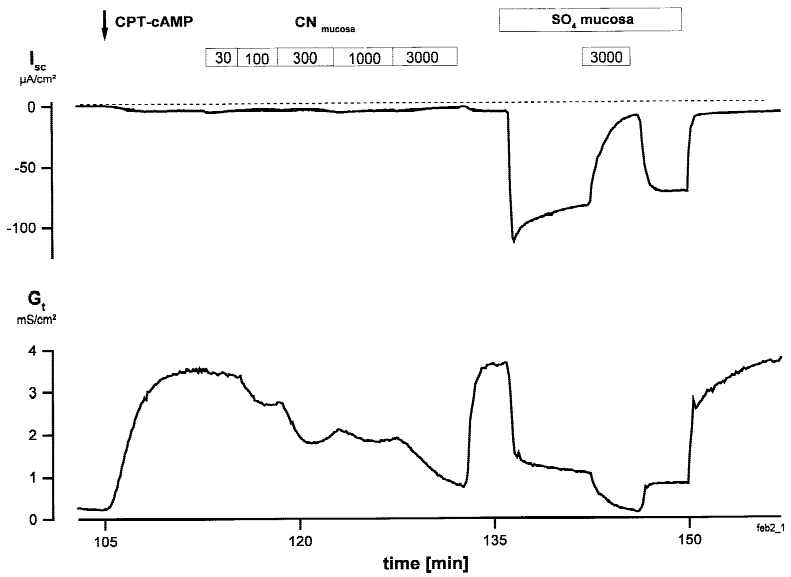

Fig. 4. Effect of mucosal application of CN^- on G_t and I_{sc} under short-circuit conditions. CPT-cAMP (100 µM) was added to the serosal side at the arrow to induce the voltage-insensitive Cl^- conductance. Note the inhibition of I_{sc} by CN^- during Cl^--free perfusion of the mucosal side, which reflects interference with Cl^- current from serosa to mucosa.

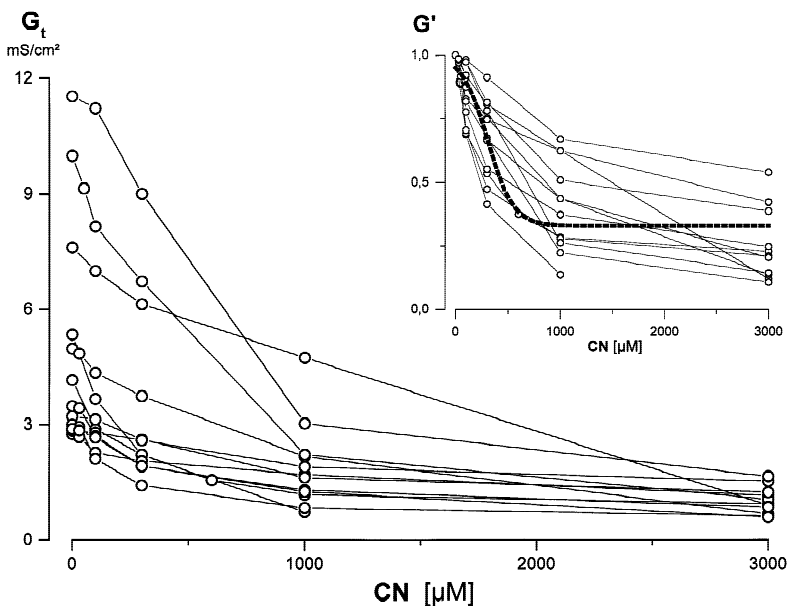

Fig. 5. Concentration-response relationship of the inhibition of CPT-cAMP (100 µM) induced G_t by mucosal CN^-. The inset reports the same data normalized for the magnitude of G_t under the respective control conditions along with the sigmoidal regression line.

whereas CN^- at 1 mM inhibited it in the same tissue more than 80%. Omission of O_2 by perfusion of tissues with solutions vigorously aerated with N_2 did not affect the voltage-activated conductance. The magnitude and the time course of activation of G^{Cl} were hardly affected compared with matched control tissues during observation periods of up to 2 hr in this condition.

Discussion

Unlike the reduction of mucosal $[Cl^-]$ by dilution of the mucosal Ringer solution or by replacement with impermeable anions such as SO_4^{2-} or gluconate—procedures that lead to dissipation of G^{Cl} due to the lack of the permeating Cl^- ion—mucosal application of the anions NO_3^-, I^- or SCN^- inhibits the voltage-activated G^{Cl} of toad skin [12]. Half-maximal inhibitory concentrations of about 25 mM (NO_3^-) and 8 mM (I^- and SCN^-) have been reported and a similar sensitivity was observed for SCN^- in the present study. On a molar basis CN^- turned out to be a remarkably more potent inhibitor. Inhibition of the steady-state, voltage-activated G^{Cl} occurred with an IC_{50} of less than 200 µM after mucosal or serosal application, and was not different when CN^- was present on both sides. The effective form (HCN or CN^-), as well as the actual concentration of the inhibitor at the site of action, are not clear. At a pH of 7.6 in the incubation

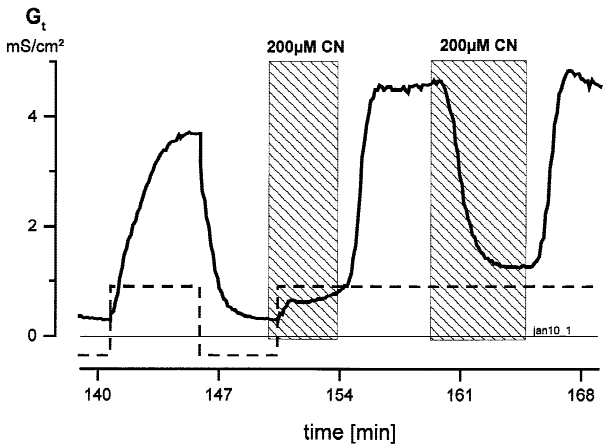

Fig. 6. Response of G_t upon addition of 200 μM CN^- to the mucosal side 15 sec before voltage perturbation from −30 to +80 mV (for voltage-activation of G^{Cl}) or under steady state activated conditions. The dashed line represents V_t and hatched areas indicate the time of presence of CN^- in the mucosal perfusion solution.

solution, the major fraction of the weak acid HCN (pK = 9.3) will be undissociated. Since inhibition was more pronounced at alkaline pH, this suggests that CN^- is the active agent and this would imply for lower effective concentrations. Although CN^- is a known metabolic inhibitor, it is open to question whether the inhibition is due to metabolic deprivation, or rather results from direct interference of CN^- with the activation process or permeation sites in the Cl^- pathway. Since CN^- combines avidly with the mitochondrial cytochrome C oxidase and prevents reduction of the catalytic site at concentrations in the order of 1 μM [35] or less [10], interference with oxidative metabolism could lead to inhibition of G^{Cl}. Indeed, the influence of CN^- has in many situations been associated with deprivation of cellular ATP and resulting activation of K_{ATP} channels (*see* i.e., 9), but these effects were mostly induced at comparatively high [CN^-] (1–5 mM) and additional interception of glycolytic metabolism. The effects of CN^- are not well defined for amphibian skin. The magnitude and time course of changes in ATP after CN^- are unknown, and in any case it must be kept in mind that a notable fraction of energy can be derived from anaerobic metabolism [23]. Previous investigations have reported inhibition of the active Na^+ transport across skins from *Bufo bufo* and *Rana pipiens* [6, 19], but this response was incomplete even at much higher concentrations of CN^- (5 mM) than those which effectively blocked G^{Cl} in the present study. In skins from *Bufo arenarum*, no effect at all was observed with 5 mM CN^- [1]. Interestingly, NaCl uptake across the open-circuited, intact frog skin was inhibited by more than 50% with less than 50 μM CN^- [7]. This response, which was not associated with changes in electrolyte composition and hardly any reduction in oxygen consumption, seems to be unrelated to metabolic arrest. Instead, a decrease in Cl^- permeability, consistent with our observations and actually suggested by those authors on the basis of the observed hyperpolarization of the skins, can likewise explain the decrease of NaCl uptake at open circuit. Preliminary data from our laboratory show that CN^- may have different influences on Na^+ transport, i.e., a fast and essentially reversible inhibition of the I_{sc} at concentrations that effectively inhibit G^{Cl}, followed by irreversible decrease of Na^+ transport with a slow time course, observed at [CN^-] above 500 μM.

The present study was carried out under nontransporting conditions, i.e., after blockade of transepithelial Na^+ transport by the application of amiloride, which eliminates a greater fraction of the metabolic expense. Under these conditions, a number of arguments can be raised against a quantitatively important role of metabolism for the observed effects of CN^- on G^{Cl}: (i) Transepithelial movement of Cl^- is driven by the imposed electrical gradient and thus passive. Accordingly, metabolic energy is not required. (ii) The inhibition of G^{Cl} by CN^- is fast and fully reversible. Half-times for onset of inhibition and reversibility were 25–40 sec and were unrelated to the [CN^-]. This is difficult to explain on a metabolic basis, in particular in view of the reportedly slow inhibition and even slower reversibility of the mitochondrial respiration after exposure to CN^- [35]. (iii) In other systems, influence on ATP-sensitive K^+ channels or changes in intracellular Ca^{2+} could be associated with the effect of CN^- [8]; the latter were eliminated by the removal of Ca^{2+}. K^+ channels have only minimal influence on G^{Cl} [22], and our results show no change in the responsiveness of toad skin G^{Cl} to CN^- after omission of serosal Ca^{2+} and chelation with EGTA. (iv) At lower [CN^-] (<200 μM), a tendency of the inhibited G^{Cl} to recover rather than to continue to decrease was observed with longer periods of exposure. This was particularly evident in those experiments where CN^- was applied at low concentration very briefly before the voltage perturbation. Under these conditions, the maximal effect was exerted immediately at the beginning of the activation period and thereafter declined partially. This behavior is opposite to what would be expected for metabolic depletion, because intracellular stores of ATP should be increasingly depleted the longer the period of exposure. (v) For effective inhibition of metabolic activity, CN^- is generally applied in millimolar concentrations and under elimination of glycolytic pathways. The latter was not done in the present study. (vi) Azide (10 mM), which blocks electron transfer in a manner similar to CN^-, had only little effect, nor did interference with oxidative metabolism by omission of O_2, a maneuver that inhibits the metabolically dependent Na^+ transport by 50–70% [24], influence G^{Cl}. Taken together, these exclude depletion of metabolic energy as the mechanism

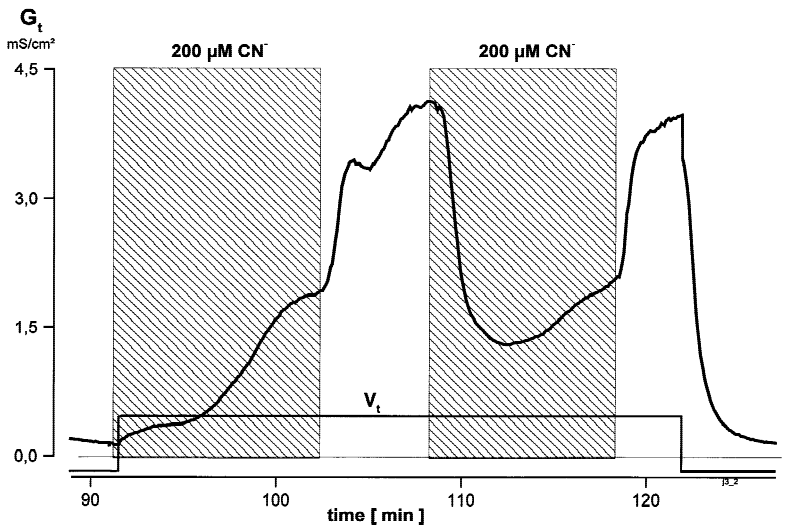

Fig. 7. Partial reversibility of the inhibition of G_t by 200 μM CN^- in the mucosal solution after application of CN^- before voltage perturbation and during steady-state activation of G_t. The dotted line represents V_t, and the hatched areas indicate the presence of CN^-.

inhibiting G^{Cl}, and hence it would appear that CN^- interferes directly with the passage of Cl^- or the activation of this pathway, independent of any influence, real or potential, on metabolic activity. Although it cannot be excluded that other particularly sensitive metabolic pathways might exist to explain the effect of CN^-, it is interesting to note that direct effects of CN^- unrelated to metabolic interference have been reported also for K^+-channels in mouse motor nerve terminals [2].

DNP, an uncoupler of oxidative phosphorylation, was even more potent than CN^- (IC_{50} = 56 μM) and reduced G^{Cl} with a similar time course and reversibility as CN^-. Although metabolic deterioration, cannot be explicitly excluded in this case, we suggest by analogy to the response of G^{Cl} to application of other related phenolic derivatives (*unpublished observations*) that the inhibition by DNP is also due to interference with specific sites in the conductive path for Cl^-. Our results corroborate previous observations on the skin of *Bufo arenarum* [1] in which DNP, albeit at notably a higher concentration (0.8 mM), inhibited the active transport of Na^+ slowly with half-times of 15–40 min, whereas it blocked transepithelial G^{Cl} considerably faster with half times below 10 min. These authors concluded that integrity of oxidative energy metabolism is necessary to sustain the I_{sc}, i.e., active Na^+ transport, but that it is not required for the maintenance of the Cl^--dependent tissue conductance. They suggested that depletion of ATP might be particularly important for cellular reactions involved in the activation of G^{Cl}. If this were so, this could explain the blockade of voltage-activated G^{Cl} by two other inhibitors of mitochondrial energy transfer, rotenone and antimycin (*unpublished observations*). This irreversible inhibition, which developed considerably slower than that after CN^-, may indicate that the activation process, i.e., the conformational change resulting in the increase of G^{Cl}, and/or the maintenance of this state depend on the operation of oxidative metabolism. In contrast to the conclusion of Castillo & Orce [1], we believe it unlikely that these reactions are mediated by changes in the metabolism of cAMP, because CN^-, DNP and the other mitochondrial inhibitors all effectively blocked the cAMP-induced G^{Cl}. Although the magnitude of IC_{50} for the inhibition by CN^- was somewhat higher in the case of the cAMP-induced G^{Cl} (attained after incubation with the nonhydrolyzable membrane permeable analogue CPT-cAMP) than for the voltage-activated G^{Cl}, the inhibitory power of CN^- on both the voltage-activated and cAMP-induced conductance suggests that the main effect of the anions is on the permeation barrier itself and not on the activation site. It may therefore be speculated that inhibition is due to the permeability of CN^-, which is able to enter the Cl^- pathway under both voltage-activated and cAMP-induced conditions, and to bind with high affinity to sites within the pathway. It is interesting to note that I_{sc} and G^{Cl} in the skin of *Bufo arenarum* were insensitive to CN^- [1], and CN^- ineffective on the skin of *Rana ridibunda* (*unpublished observations*). This suggests that the variable effect on CN^- on G^{Cl} in different species is not through metabolic arrest, but different functional properties of the various species, in particular the pharmacological response patterns.

The analysis of the voltage-activated as well as the cAMP-induced Cl^- pathway(s) across amphibian skin is hampered by the lack of concrete knowledge with respect to morphological location. As the apical membrane of principal cells is impermeable to Cl^- (for refs. *see* 11, 20), two other fundamentally different pathways have been proposed for this passive transport, i.e., transcellular movement via mitochondria-rich cells [33] and paracellular passage through the tight junctions between the outermost living epithelial cell layer [25]. Unequivocal experimental proof for either of these possibilities has

not yet been provided. However, recent observations using a video-assisted voltage probe technique [28, 31] demand reconsideration of the substantive arguments favoring the transcellular pathway [20]. Despite the limited value of any transepithelial observations in this context, some of our results for the inhibition of G^{Cl} after CN^- may help to elucidate the problem.

CN^- effectively inhibits G^{Cl} after application from the serosal side with the same half-maximal inhibitory concentration as from the apical side. Furthermore, half-times for the onset of inhibition are the same for apical and basolateral application. If Cl^- movement is through MR cells, the inhibition by CN^- from the serosal side may occur in two ways: (i) Since transcellular current flow requires substantial Cl^- conductances of both apical and basolateral membranes, inhibition at one membrane would already suffice for cessation of transepithelial current flow. Similar sensitivity of the putative Cl^- channels in apical and basolateral membranes to CN^- would explain the observations. Unfortunately, the conductivity of basolateral Cl^- channels in MR-cells is unknown; analyses of the exchange rate of Cl^- for Br^- [3] suggest a rather low Cl^- permeability. (ii) Due to the presence of mainly uncharged HCN in solutions of cyanide, serosal CN^- may readily equilibrate in the intracellular space after permeation across the basolateral membrane. Inhibition of apical Cl^- channels, which must possess similar sensitivity from either face, would then occur from the cytosolic side. Independence of the concentration-response relation and the time course of inhibition by CN^- from the side of application are more easily explained if the Cl^- conductance pathway is localized in the paracellular route, because the actual [CN^-] in the extracellular space comes into contact with either the mucosal or the serosal openings of the tight junction. With HCN as the major source of inhibition, the permeation is not limited to the aqueous phase in the lateral spaces, but may comprise membranes and intracellular spaces. The prevailing presence of the undissociated HCN can also explain the lack of effect of the transepithelial clamping potential on the time course and concentration-response relationships for mucosal *vs.* serosal addition, because diffusion of the uncharged moiety can extend toward the actual reaction sites, where dissociation of HCN liberates the effective agent, unless HCN itself is the inhibitor.

Compared with the time required for diffusion to the apical membrane, the half time of more than 30 sec for the onset of inhibition after the addition of CN^- to the mucosal perfusion solution is rather slow. Using the same experimental setup, blockage of apical membrane Na^+ channels by mucosal amiloride, as well as the dissipation of G^{Cl} upon mucosal substitution of SO_4^{2-} for Cl^- occur with half-times less than 5 sec. It is perhaps not incidental that the time course of inhibition by CN^- is comparable to that of the increase in, or the dissipation of, voltage-activated G^{Cl} after voltage perturbation. The latter, which have been reported to be 10 to more than 60 sec depending on the conditions and animal species [16, 18, 21 and own present data], are certainly much longer than conceivable for the gating of membrane channels. At present, the mechanism of the increase in G^{Cl} by voltage activation or induction by cAMP is essentially unknown, particularly since the pathways involved (membrane channels or tight junctions) have not been identified. Nevertheless, to explain the slowness of these processes a sequence of cellular reactions must be considered, in which voltage and PKA act on a common final step. Furthermore, the apical presence of Cl^- seems to play an essential role in these activation steps (*see also* 18). In this event, competition between CN^- and Cl^- at a specific site in the pathway could result in the inhibition of G^{Cl} and explain the time course of inhibition, as well as the much faster reversal of the effect. Competition between Cl^- and CN^- could also lead to the remarkable difference in apparent affinity of CN^-, depending on the protocol of application. Application of CN^- before activation of the pathway increased the apparent affinity drastically compared with the effect under steady-state activated conditions. If Cl^- and CN^- were to compete for a binding site that must be occupied by Cl^- to open the pathway, the substitution of CN^- for Cl^- already bound to its binding site will occur gradually and approach the final state at a rate determined by the affinities of CN^- and Cl^-. Addition of CN^- before activation, in contrast, presents the binding sites with both Cl^- and CN^-. In the course of activation these sites will load preferentially with CN^-, because it has higher affinity than Cl^-. As a result, the opening of the pathway will be delayed until the equilibrium between Cl^- and CN^- with the same inhibition as during steady-state application is eventually reached. The observation that the inhibition by CN^- at low concentrations was often partly reversible may indicate recruitment of conductive sites by partial inhibition; this would be in agreement with our finding that G^{Cl} showed frequently an overshoot after washout of CN^-. This response needs further investigation.

The effect of CN^- may be envisaged in the frame of a schematic model for the Cl^- conductance pathway that was proposed recently [27] on the basis of the available experimental data. Although the precise location is yet unidentified, it should involve two elements, i.e., a conductive path controlled by a voltage-sensitive regulator. Each of these elements may be influenced by a variety of agents. CN^- (and DNP) seem to compete or block the conductive path with a high affinity, and are similarly effective, regardless of whether the conductance was induced through serosal hyperpolarization or by incubation with cAMP at high concentrations.

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References

1. Castillo, G., Orce, G. 1995. Metabolic inhibition and chloride transport in isolated toad skin. *Arch. Physiol. Biochem.* **103:**149–159
2. Chao, K.F., Liu, S.H., Lin-Shiau, S.Y. 1996. Suppression of potassium currents by cyanide on the mouse motor nerve terminal. *Neuroscience Letters* **203:**105–108
3. Dörge, A., Rick, R., Gehring, K., Thurau, K. 1978. Preparation of freeze-dried cryosections for quantitative X-ray microanalysis for electrolytes in biological soft tissues. *Pfluegers Arch.* **373:**85–97
4. Foskett, J.K., Ussing, H.H. 1986. Localization of chloride conductance to mitochondria-rich cells in frog skin epithelium. *J. Membrane Biol.* **91:**251–258
5. 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
6. Hoshiko, T., Grossman, R.A., Machlup, S. 1988. Effects of basolateral ouabain, amphotericin B, cyanide and potassium on amiloride noise during voltage clamp of *Rana pipiens* skin support sodium-amiloride competition. *Biochim. Biophys. Acta* **942:**186–198
7. Huf, E.G., Doss, N.S., Wills, J.P. 1957. Effects of metabolic inhibitors and drugs on ion transport and oxygen consumption in isolated frog skin. *J. Gen. Physiol.* **41:**397–417
8. Inoue, M., Imanaga, I. 1998. Activation of Ca^{2+}-dependent K^+ channels by cyanide in guinea pig adrenal chromaffin cells. *Am. J. Physiol.* **274:**C105–C111
9. Inoue, M., Sakamoto, Y., Yano, A., Imanaga, I. 1997. Cyanide suppression of inwardly rectifying K^+ channels in guinea pig chromaffin cells involves dephosphorylation. *Am. J. Physiol.* **273:**C137–C147
10. Jones, M.G., Bickar, D., Wilson, M.T., Brunori, M., Colosimo, A., Sarti, P. 1984. A re-examination of the reactions of cyanide with cytochrome c oxidase. *Biochem. J.* **220:**57–66
11. 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. p. 100–121. Surrey Beatty & Sons, Chipping Norton
12. Katz, U., Nagel, W. 1995. Effects of cyclic AMP and theophylline on chloride conductance across toad skin. *J. Physiol.* **489:**105–114
13. Katz, U., Scheffey, C. 1986. The voltage-dependent chloride current conductance of toad skin is localized to mitochondria-rich cells. *Biochim. Biophys. Acta* **861:**480–482
14. Katz, U., Van Driessche, W. 1988. Effect of theophylline on the apical sodium and chloride permeabilities of amphibian skin. *J. Physiol.* **397:**223–236
15. Kristensen, P. 1983. Exchange diffusion, electrodiffusion and rectification in the chloride transport pathway of frog skin. *J. Membrane Biol.* **72:**141–151
16. 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
17. Kristensen, P., Ussing, H.H. 1992. Epithelial Organization. *In:* The Kidney: Physiology and Pathophysiology, Second Edition. D.W. Seldin and G. Giebisch, editors. p. 265–285. Raven Press, New York
18. Lacaz-Vieira, F., Procopio, J. 1988. Comparative roles of voltage and Cl ions upon activation of a Cl conductive pathway in toad skin. *Pfluegers Arch.* **412:**634–640
19. Larsen, E.H. 1973. Effect of amiloride, cyanide and ouabain on the active transport pathway in toad skin. *Munksgaard. Copenhagen* **0:**132–143
20. Larsen, E.H. 1991. Chloride transport by high-resistance heterocellular epithelia. *Physiol. Rev.* **71:**235–283
21. Larsen, E.H., Rasmussen, B.E. 1983. Membrane potential plays a dual role for chloride transport across toad skin. *Biochem. Biophys. Acta* **728:**455–459
22. Larsen, E.H., Ussing, H.H., Spring, K.R. 1987. Ion transport by mitochondria-rich cells in toad skin. *J. Membrane. Biol.* **99:**25–40
23. Leaf, A., Renshaw, A. 1957. The anaerobic active ion transport by isolated frog skin. *Biochem. J.* **65:**90–93
24. Leaf, A., Renshaw, A. 1957. Ion transport and respiration of isolated frog skin. *Biochem. J.* **65:**82–90
25. Nagel, W. 1989. Chloride conductance of amphibian skin. Localization to paracellular pathways. *Miner. Electrol. Metabol.* **15:**163–170
26. Nagel, W., Dörge, A. 1990. Analysis of anion conductance in frog skin. *Pfluegers Arch.* **416:**53–61
27. Nagel, W., Katz, U. 1997. Effects of NEM on voltage-activated chloride conductance in toad skin. *J. Membrane Biol.* **159:**127–135
28. Nagel, W., Somieski, P., Shipley, A.M. 1998. Mitochondria-rich cells and voltage-activated chloride current in toad skin epithelium. *J. Membrane Biol.* **161:**131–140
29. Rajendran, V.M., Kashgarian, M., Binder, H.J. 1989. Aldosterone induction of electrogenic sodium transport in the apical membrane vesicles of rat distal colon. *J. Biol. Chem.* **264:**18638–18644
30. Rick, R. 1994. Short-term bromide uptake in skins of *Rana pipiens*. *J. Membrane Biol.* **138:**171–179
31. Somieski, P., Nagel, W. 1998. Localizing transepithelial conductive pathways using a vibrating voltage probe. *J. Exp. Biol.* **201:**2489–2495
32. Sörensen, J.B., Larsen, E.H. 1996. Heterogeneity of chloride channels in the apical membrane of isolated mitochondria-rich cells from toad skin. *J. Gen. Physiol.* **108:**421–433
33. 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
34. 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
35. Wilson, D.F., Erecinska, M. 1977. Ligands of cytochrome c oxidase. *Methods Enzym.* **53:**191–201