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Effects of Aminoperimidine on Electrolyte Transport across Amphibian Skin

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Abstract

The effect of aminoperimidine (AP) on transepithelial Na⁺ transport and Cl⁻ conductance (G^{Cl}) of isolated amphibian skin (*Bufo viridis* and *Rana esculenta*) was analyzed using transepithelial and intracellular electrophysiological techniques. AP, applied at concentrations between 30 and 100 μM from the mucosal side, stimulated Na⁺ transport rapidly and reversibly by more than 30% of the control value due to an increase in apical membrane Na⁺ permeability. Influence of AP on basolateral membrane conductance and effective driving force for Na⁺ were negligible. Voltage-activated G^{Cl} of toad skin, but not the resting, deactivated conductance, as well as spontaneously high G^{Cl} in frog skin was rapidly inhibited by AP in a concentration-dependent manner. The half-maximal inhibitory concentration of 20 μM is the highest hitherto reported inhibitory power for G^{Cl} in amphibian skin. The effect of AP on G^{Cl} was slowly and incompletely reversible even after brief exposure to the agent. Serosal application of AP had similar, albeit delayed effects on both Na⁺ and Cl⁻ transport. AP did not interfere with the Cl⁻ pathway after it was opened by 100–300 μM CPT-cAMP, a membrane-permeable, nonhydrolyzed analogue of cAMP. Inhibition of the voltage-activated G^{Cl} by AP was attenuated or missing when AP was applied during voltage perturbation to serosa-positive potentials. Since AP is positively charged at physiological pH, it suggests that the affected site is located inside the Cl⁻ pathway at a certain distance from the external surface. AP affects then the Na⁺ and Cl⁻ transport pathways independent of each other. The nature of chemical interference with AP, which is responsible for the influence on the transport of Na⁺ and Cl⁻, remains to be elucidated.

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Key Words

Cl⁻ conductance
Na⁺ transport
Intracellular electrical
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Benzimidazol-guanidine
cAMP

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Introduction

Transepithelial NaCl transport across amphibian skin epithelium is divided as regards to mechanisms and morphological routes into the transport of Na⁺, which is active and carried out through the principal cells, and the movement of Cl⁻, which is predominantly passive and located to anion-selective pathways separate from the principal cells [for reviews, see 7, 11]). Much of the information on these transport mechanisms has been obtained with the aid of inhibitors and agonists, which are considered more or less specific for individual steps of the transepithelial passage. During the years it turned out that a certain number of agents as MK-196 [16], furosemide [1], procaine [3], or NO₃ [19] affect the transport of both Na⁺ and Cl⁻ pathways. Although it is questionable whether the molecular sites responsive for either effect are the same, comparison of these molecules might provide better insights into the mode of action. In the course of our studies, it turned out that aminoperimidine (AP), which contains a guanidine group and shares some chemical similarities with amiloride, did not inhibit, but rather stimulated Na⁺ transport across frog and toad skin. This response pattern is comparable to that reported previously for the also chemically related benzimidazolguanidine (BIG) [27]. In contrast to BIG, which has no influence on Cl⁻ conductance [9], AP appeared as a potent inhibitor of Cl⁻ conductance, particularly the voltage-activated component across toad skin. This combination of opposite influences could be valuable for the analysis of the transport pathways for the two ions across the skin epithelia of both frog and toad.

Materials and Methods

The experiments were done on toads (*Bufo viridis*) collected in Israel and frogs (*Rana esculenta*) originating from Turkey and Hungary. Abdominal skins were carefully dissected from doubly pithed animals. For transepithelial experiments they were mounted in a modified Ussing chamber (0.4 ml hemichamber volume; 0.5 cm² exposed tissue area); edge damage was prevented by soft rubber gaskets and heavy silicone grease on the mucosal side. The chambers were perfused at >2 ml/min on both sides with Ringer solutions of the following composition (in mM): Na⁺ 115, K⁺ 2.5, Ca²⁺ 1, Cl⁻ 117, Hepes 3.5; pH 7.6. For the analysis of Cl⁻ conductance, Na⁺ transport was eliminated by addition of amiloride (10⁻⁵ M) to the mucosal fluid. Transepithelial electrical parameters were obtained with a voltage-clamp system which determined, in addition to transepithelial potential (V_t) and clamping current (I_t), tissue conductance (G_t) from brief small perturbations of V_t (5 mV, 150 ms every 5 s) using appropriately triggered sample-and-hold amplifiers [17]. Toad skin was kept either short-circuited or depolarized to -30 mV (referred to the mucosal side) to deactivate the Cl⁻ conductance (G^{Cl}). G^{Cl} was activated by intermittent hyperpolarization of V_t to 80 mV (serosal-positive). Where appropriate, G^{Cl} is reported as the difference between G_t at -30 mV and +80 mV. The dependence of G^{Cl} on V_t was determined by sequentially perturbing V_t to several values between 0 and 150 mV returning to the hold value of -30 mV after each voltage step. Individual values of G_t (V_t) were approached with time course up to 5 min; the result must thus be considered as a steady-state G/V relationship. In the present study, approach to steady values of G^{Cl} was notably slower than reported in previous studies [11]; the difference could indicate a species difference between *B. viridis* and *Bufo bufo*. Frog skin was analyzed at short circuit or voltage-clamped to +80 mV. Before and after addition of AP, V_t was briefly (30–60 s) inverted to -30 mV.

Techniques for the determination of intracellular potentials from isolated amphibian skins have been reported previously [18]. In brief, microelectrodes were pulled from borosilicate glass on a Brown-Flaming P80C puller. After filling with 1M KCl, their input resistance was 40–70 MΩ. The skin was impaled perpendicular to the surface from the mucosal side with the aid of a stepping motor micromanipulator (Fa. Frankenberger, Germering, Germany). Transepithelial electrophysiological data were obtained as described above. Skins were kept short-circuited except

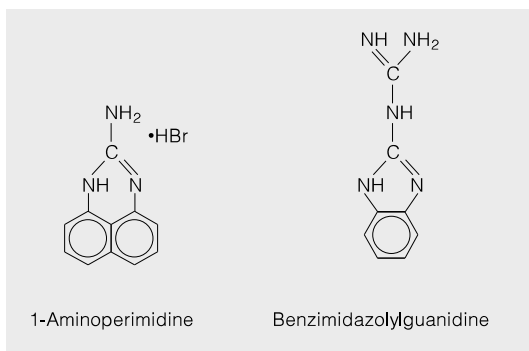


Fig. 1. Structure formula of AP. It should be noticed that the three nitrogen atoms around the carbon atom compose the guanidine moiety, which is fused through two of the nitrogens to the hydrophobic naphthalene group. The pK of AP is 9.6 ± 0.2 (determined in 100 mM NaCl). Accordingly, the molecule is positively charged at the pH used in the present study, and the charge is distributed over the nitrogen atoms. For comparison, the structure formula of BIG is shown, which contains also a guanidine moiety, but is lacking the hydrophobic ring structure.

for brief perturbation of V_t (10 mV, 200 ms every 2 s). The changes of the intracellular potential from the value at short circuit (V_{sc}) during perturbation of V_t were used for estimation of the fractional resistance of the apical membrane, $fR_a = R_a / (R_a + b)$ by similar sample-and-hold circuitry as above [17]. Based on the treatment of an equivalent circuit with two parallel conductive limbs across the cellular and paracellular route [14], specific apical and basolateral membrane conductances, g_a and g_b , were calculated from the changes in G_t induced by amiloride (indicated by $'$), which yields the magnitude of the transcellular conductance g_c ($g_c = G_t - G_t'$). Using the values of fR_a before and during amiloride (fR_a'), specific membrane conductances are obtained according to the following formalism:

$$g_a = g_c / (fR_a \cdot fR_a'), \quad g_b = g_c / (fR_a - fR_a')$$

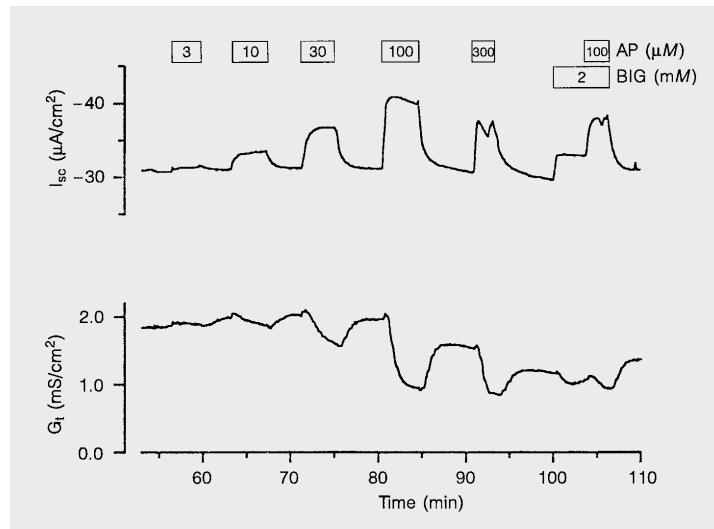
In this notation, fR_a' corrects for remaining apical membrane conductance during amiloride, which may be due to incomplete blockage of Na^+ channels by amiloride or other unspecified apical conductance. fR_a' below 0.96 was considered as the limiting value for valid cellular impalement. Since frog skin used for microelectrode analysis had negligible G^{Cl} , correction for paracellular current recirculation [18] was not required.

All chemicals were of reagent grade. 2-Aminoperimidine hydrobromide (AP), benzimidazolylguanidine (BIG) and 2-chlorophenylthiocyclic 3',5'-adenosine monophosphate (CPT-cAMP) were from Aldrich and Sigma, respectively. Structure formulas of AP and BIG are shown in figure 1. Mean \pm SEM values are reported.

Results

The effect of AP on transepithelial Na^+ transport across toad skin was analyzed in 14 experiments. Figure 2 shows a typical example. Addition of AP at concentrations between 3 and 300 μM led to dose-dependent, immediate increase of the I_{sc} up to 30% at concentration of 100 μM . Stimulation was rapidly reversible after washout of AP at all concentrations. In the presence of 300 μM AP, I_{sc} started to decrease after an initial gain, whereupon the drug was quickly removed; this response, which was consistently observed in 3 other experiments, might indicate toxic side effects and was not further investigated. For comparison, BIG was applied at the usual concentration (2 mM). The increase in I_{sc} induced by 2 mM BIG was clearly smaller than that observed after AP exceeding 30 μM . In the presence of BIG, application of 100 μM AP led to further stimulation of the I_{sc} to about the same level as observed with AP alone. It should be noted that the tissue conductance decreased at all effective concentrations of AP. These patterns are opposite to the expected response of cellular conductance and point to simultaneous inhibitory influence of AP on shunt conductance (see below). On the average of 13 experiments, mucosal addition of 100 μM AP increased the I_{sc} within 3 min by $36.8 \pm 1.2\%$ from a control value of $32.6 \pm 1.6 \mu A/cm^2$. The effect of 2 mM BIG was also tested in 6 of these experiments; the increase of I_{sc} was clearly less than with 100 μM AP ($19.9 \pm 2.5\%$ elevation from

Fig. 2. Effect of increasing concentrations of AP and of BIG in the mucosal perfusion solution on I_{sc} and G_t of toad skin (*B. viridis*) under short-circuited conditions.

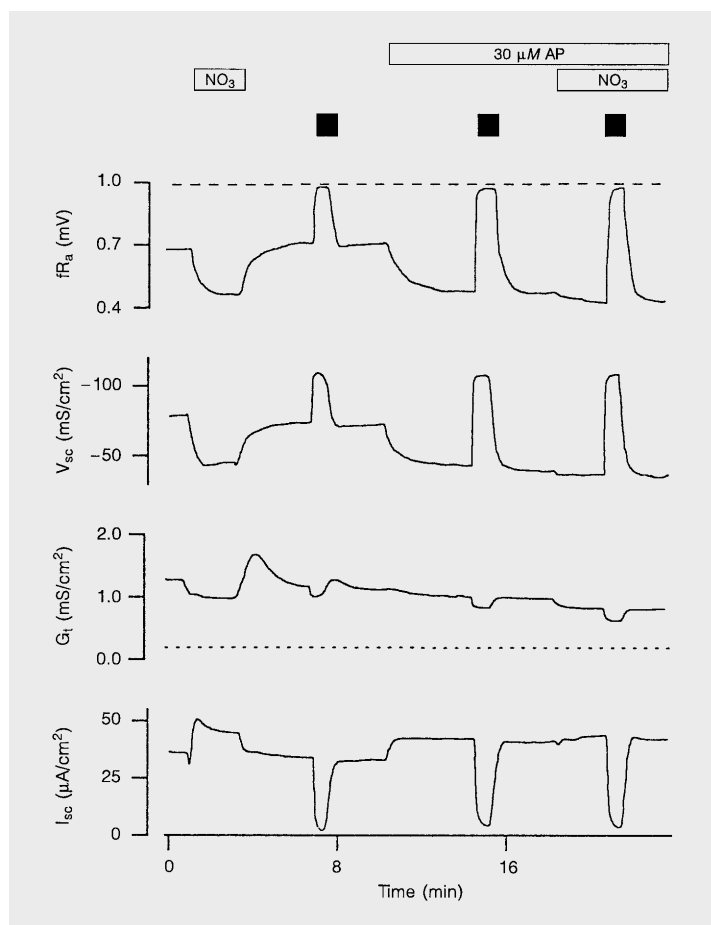


a control value of $26.5 \pm 2.7 \mu A/cm^2$. Tissue conductance never decreased after BIG.

Frog skin responded basically similar. The application of AP was about as effective as in toad skins in blocking G^{Cl} in skins from *R. esculenta* with spontaneously large values of G^{Cl} under short-circuit conditions (see below). Other tissues from *R. esculenta* contained very little Cl^- conductance and could not be used for the study of the Cl^- pathway. These tissues were, on the other hand, favorably suited for microelectrode studies, since they allowed analysis of the sodium pathway independent of possible artifacts, which may arise from change in G^{Cl} [14]. Results from a typical experiment are depicted in figure 3. It shows that application of 30 μM AP increased I_{sc} by approximately 25%, which was associated with considerable depolarization of V_{sc} and decrease in fR_a . Tissue conductance, G_t , was almost unchanged. The changes induced by AP are similar to those observed during a preceding replacement of mucosal Cl^- by NO_3^- , which has been shown to stimulate apical membrane Na^+ conductance [19]. NO_3^- had only very little additional effect in the

presence of AP. Amiloride was repeatedly added for brief periods from the mucosal side, to verify the viability of the impaled cell and to permit calculation of specific membrane conductances from the amiloride-induced changes in G_t and fR_a . It can be derived from the record in figure 3 that apical membrane conductance increased by more than 50% after AP, whereas the basolateral membrane conductance was reduced to approximately 60%. Similar results were obtained in 5 other experiments. On the average, AP increased the I_{sc} by almost 70% from a control value of $17.5 \pm 4.8 \mu A/cm^2$. This was accompanied by depolarization of the epithelial cells from -81.5 ± 2.6 to -52.5 ± 5.7 mV and a decrease of the fractional resistance of the apical membrane by 30% (from 0.81 ± 0.03 to 0.57 ± 0.07). No difference was observed in V_{sc} after blockage of apical sodium entry by amiloride (-106 ± 4 mV in control versus -104 ± 5 mV after AP). Apical membrane conductance, g_a , more than doubled from 0.17 ± 0.03 to 0.38 ± 0.07 mS/cm^2 , whereas the basolateral membrane conductance, g_b , decreased to 66% (from 0.88 ± 0.23 to $0.62 \pm$

Fig. 3. Microelectrode determination of the effect of mucosal AP on principal cells' membrane conductances of frog (*R. esculenta*) skin. Intracellular membrane potential (V_{sc}) and apical membrane fractional resistance (fR_a) are shown in the upper panels. Trans-epithelial current (I_{sc}) and conductance (G_t) are shown in the lower two panels. Dark bars at the top indicate periods where amiloride ($10^{-5} M$) was applied to the mucosal side. Mucosal perfusion with NO_3^- -Ringer is included before and during AP, as indicated by the blocks.



0.21 mS/cm²). This latter behavior is readily explained by the response of inward rectifying basolateral potassium channels upon the decrease of the membrane potential [14].

To further elucidate the effects of AP on cellular and shunt conductance, experiments as depicted in figure 4 were performed on toad skin. The tissues were kept without amiloride and repeatedly probed for voltage-activated Cl⁻ conductance by perturbing V_t from 0 to +80 mV. I_{sc} increased on mucosal application of 1 mM BIG or 100 μM AP as usual (fig. 4, upper panel). Voltage-activated G^{Cl} , which was about 1.6 mS/cm² in the control

period, was essentially unaffected by BIG, but was almost completely dissipated by AP. The decrease in G^{Cl} was not reversible after application of 100 μM AP in the time frame of the present experiments (<1 h). Similar observations were made in 8 other experiments. On the average, G_t at short circuit decreased by $17 \pm 7\%$ after AP from the control value of 1.12 ± 0.16 mS/cm². The voltage-induced gain in G_t , i.e., the value of G^{Cl} , was 1.45 ± 0.19 mS/cm² in the control and decreased to $11.8 \pm 4.1\%$ of this value after the addition of 100 μM AP to the mucosal bath. Figure 4 further shows that the inhibition of the voltage-

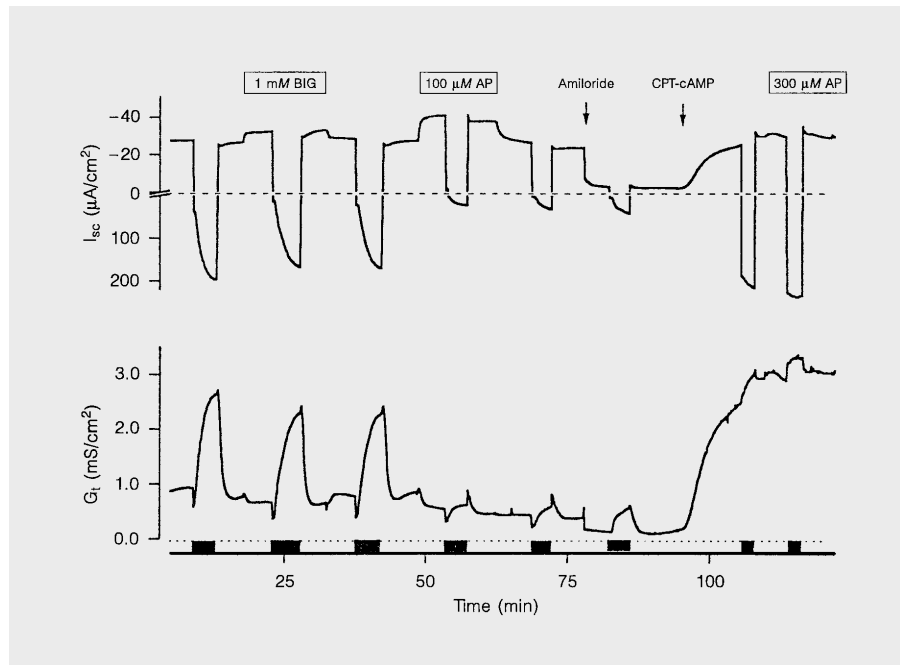


Fig. 4. Effect of mucosal application of BIG or AP on I_{sc} , G_t and voltage-activated Cl^- conductance in short-circuited toad skin (*B. viridis*). At the first arrow, amiloride (10^{-5}) was added to the mucosal perfusing solution, and at the second arrow CPT-cAMP ($300 \mu M$) was added to the serosal perfusing solution. Dark bars at the bottom indicate periods of voltage perturbation from short circuit to $+80$ mV (serosa-positive). Note that different scales of current (I_t) are used above and below the midline in the upper panel.

activated G^{Cl} by AP was completely relieved upon application of $300 \mu M$ of the membrane-permeable, nonhydrolyzed analogue of cAMP, CPT-cAMP to the serosal perfusion fluid in the presence of $10^{-5} M$ amiloride in the mucosal bath. The increase of I_{sc} following CPT-cAMP under these conditions may be explained by the stimulation of glandular Cl^- secretion [24]. The stimulated G^{Cl} at short circuit, which averaged 4.98 ± 1.6 mS/cm² in the presence of CPT-cAMP ($100\text{--}300 \mu M$), was unaltered (4.94 ± 1.5 mS/cm²) upon mucosal addition of $100 \mu M$ AP ($n = 7$). Since CPT-cAMP conductance is voltage-insensitive [8], the effect of voltage activation was not assessed under these conditions.

Serosal application of AP at concentrations $>100 \mu M$ led to a slowly developing stimulation of the I_{sc} , which was in the steady state similar as after mucosal addition of AP. Tissue conductance was comparably affected by serosal and mucosal AP; the effect appeared in the former case merely much more delayed.

The effect of AP on the voltage-activated G^{Cl} was tested in the nontransporting ($10^{-5} M$ mucosal amiloride) condition. Figure 5 shows the typical result when $30 \mu M$ AP was added to the mucosal solution while the tissue was in the inactivated state, i.e., voltage-clamped to -30 mV. Voltage perturbation to $+80$ mV was made 3 min after the addition of AP, and it is

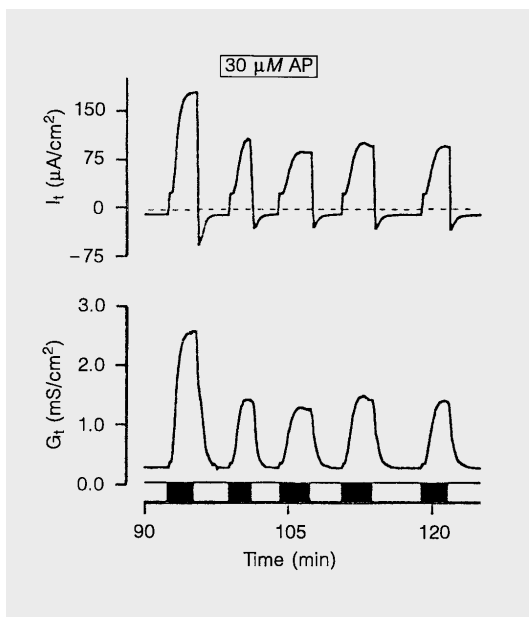


Fig. 5. Inhibition by mucosal AP of voltage-activated Cl^- conductance in toad skin under nontransporting conditions (10^{-5} M amiloride). The dark bars in the lower panel indicate periods of voltage perturbation from -30 to $+80$ mV.

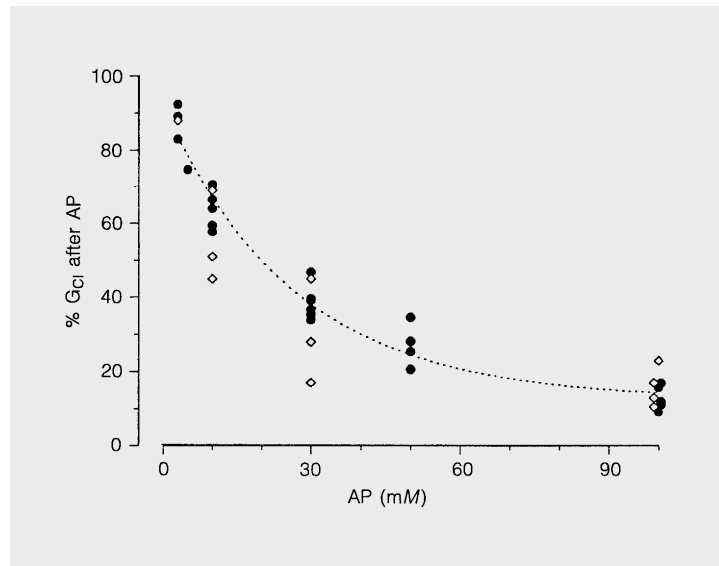
evident that the steady-state inhibitory effect on G_t was exerted at this time already. A second activation test in the continuous presence of the drug did not show larger inhibition. Reversibility of the inhibition was generally incomplete at AP concentrations above $30 \mu\text{M}$ and incubation times of >5 min. G_t recovered slowly if lower concentrations of AP were applied for brief periods of time. The data from 21 experiments with different concentrations of AP are combined in the dose-response relation shown in figure 6. From the regression line of these data, an apparent half-maximal inhibitory concentration of $20 \mu\text{M}$ AP was obtained. Numerical analysis for the inhibitory effect of AP on skins from *R. esculenta* was more difficult, because G_t was either high but intensive to voltage or voltage

activation was so delayed that repeated tests could not be made. At short circuit as well as at the clamp potential of $+80$ mV, inhibition of G_t was slow and apparently not complete at all concentrations of AP (see below). Rapid onset of the action was observed, however, if the tissue was briefly voltage-clamped to -30 mV (<1 min) in the presence of AP and then returned to short circuit or positive clamp potentials. Data from 11 such experiments using 5 frogs, which had G_t between 1.8 and 3.5 mS/cm 2 , are included in figure 6; they indicate that the half-maximal inhibitory concentration of AP in frog skin is comparable to that for toad skin.

Since AP was applied as the Br^- salt, control experiments were performed to test the effect of Br^- in the mucosal solution. In agreement with previous observations [6], NaBr at concentrations of 1 – 2 mM did not affect the voltage-activated G_t (not shown), assuring that the inhibitory effects on G_t are due to the AP moiety of the substance.

In initial experiments, the effect of AP on G_t was tested while the toad skins were voltage-clamped to 80 mV, positive inside. Using this protocol, AP induced a rather minuscule and slowly developing decrease in G_t at all concentrations up to $300 \mu\text{M}$. If the tissue was thereafter briefly returned to the inactivated state, G_t was found inhibited as characteristic for the respective concentration of AP in the next activation period. As it thus appeared that voltage clamping the tissue could prevent the inhibition of G_t by AP, this possibility was analyzed in additional experiments. A typical experiment is shown in figure 7. During voltage perturbation to $+120$ mV, which led to slightly larger activation of G_t than the preceding perturbation to $+80$ mV, neither 30 nor $100 \mu\text{M}$ caused a notable decrease of current or conductance. After brief intermittent inactivation of G_t by voltage clamping to -30 mV, however, the full inhibitory effect

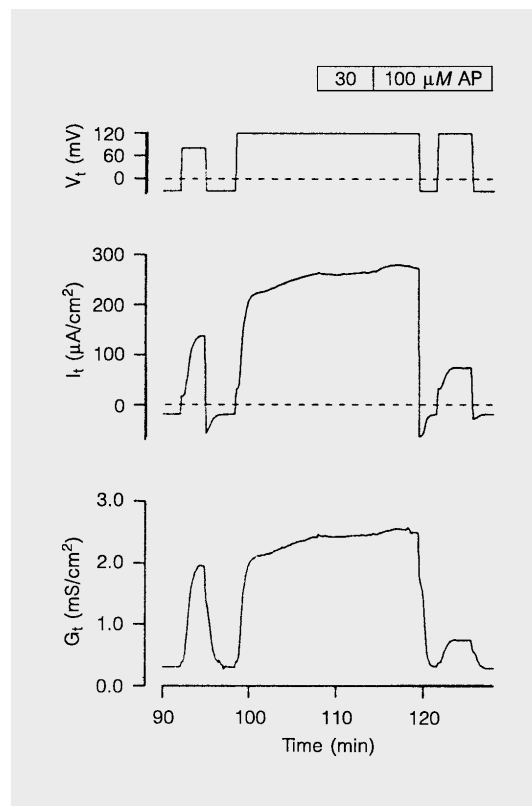
Fig. 6. Dose-response curve of mucosal addition of AP on voltage-activated G_{Cl} in toad skin (filled dots). The data are normalized with respect to the magnitude of G_{Cl} in the activation period preceding addition of AP. The dotted line represents the least square exponential regression line. Depicted, albeit not included in the regression analysis, are also results from frog skins (open diamonds).



occurred. Opposite patterns are detectable at lower clamp potential ($<+60$ mV). In this condition, AP was inhibitory as usual, except for notably slower onset of the effect (not shown). Similar observations were made in all other experiments, including frog skin at short circuit and during voltage perturbation. The degree of 'protective' effect of voltage and the magnitude of required voltage were different between tissues. In general, clamp potentials smaller than $+50$ mV did not prevent the inhibition by AP in any concentration, whereas essentially full protection for more than 10 min was observed upon voltage perturbation to more than $+100$ mV.

Steady-state G/V relationships were performed on 7 pieces of skin before and after partial inhibition of G_{Cl} by submaximal doses

Fig. 7. Effect of voltage perturbation on the inhibition of G_{Cl} by mucosal AP in toad skin. Note apparently missing response on 30 and 100 μM AP in toad skin, voltage-clamped to $+120$ mV. Full effect of AP was observed after brief intermittent change to -30 mV.



of AP (10–30 μM), which decreased the absolute magnitude of G^{Cl} to 37% of the control value. The sigmoidal shape of the conductance/voltage relation, similar as shown previously [8], was not notably different after AP and under control conditions, but the clamp potential resulting in half-maximal activation of G^{Cl} was shifted to a slightly larger value (from 59 mV in the control to 71 mV during AP). This indicates that AP reduces the voltage sensitivity of the chloride pathway.

Discussion

Uptake of Na^+ and Cl^- across amphibian skin epithelium is carried out separately and can be influenced independently by the same and separate inhibitors or agonists. Although the fundamental mechanisms of transport are similar in toad and frog skin, the expression of certain functional states can be different between the genera as well as among different species. This is particularly evident for G^{Cl} . Whereas considerable voltage-activated G^{Cl} was generally observed in skins from *B. viridis*, skins from *R. esculenta* were either lacking notable Cl^- conductance or contained spontaneously high G^{Cl} at short circuit, which was in most cases insensitive to voltage or developed over extended periods of time (5–10 min). Other species of frogs display the same variability [10]. Nevertheless, if G^{Cl} is present, differences in response patterns are no more pronounced among species than between the two genera. The same applies to the effectiveness of modulators of Na^+ transport in different species of frogs and toads [for references see 4]. Accordingly, results from either tissue cannot, a priori, be generalized except for conditions of coincidence.

Our experiments show that AP inhibits voltage-activated G^{Cl} of amphibian skin. At the same time, it stimulates Na^+ transport

across the skin, which is unquestionably accomplished by the principal cells of the epithelia cell layer [for references, see 7]. The microelectrode data indicate that AP increases apical membrane conductance of these cells. A similar effect on Na^+ transport is exerted by a number of other substances which bear fundamentally different chemical structures. Among them are the amiloride-related pyrazine derivate BIG, the 'loop' diuretics furosemide and MK-196, the SH-group reactive agent *p*-chloromercuribenzenesulfonic acid (PCMBS), the local anesthetic procaine, anions as NO_3^- and heavy metal ions [1, 3, 9, 12, 16, 19, 21]. The mode of action by these agents is different from the stimulation caused by mineralocorticoids or by increased level of cellular cAMP induced through different means (ADH, PGE_2 , forskolin [for references, see 2]). All substances noted above are effective after application from the mucosal side. As regards the stimulation of I_{sc} by BIG and PCMBS, it was suggested by Zeiske and Lindemann [27] in 1974, that these agents affect structures responsible for self-inhibition of the Na^+ channels, thereby removing the inhibitory influence of Na^+ on its own pathway. A similar mode of action was generally implied for the other agents. Despite the enormous gain in the understanding of the Na^+ channel in the last years, no radically different explanation has been proposed, except for the verification that the number of open Na^+ channels is increased without alteration of the single channel conductance [for reviews, see 4, 5, 25]. In view of the dissimilar chemical structure of these substances, a specific action on particular structures of the Na^+ channel seems improbable. Rather, it suggests effects at multiple sites or of more general nature, such as displacement of surface charge distribution at the entry site or in the neighboring vicinity, which facilitates binding of Na^+ to the gating structures of the Na^+ chan-

nel. AP, which is amphiphilic and positively charged at physiological pH, could partition itself in the apical membrane and exert its stimulatory effect on the Na⁺ pathway by altering the surface potential in the vicinity of charge-sensitive molecules.

Direct influence of AP on basolateral membrane properties of principal cells is negligible. Since AP is membrane permeable, the slower response on application from the serosal side seems to be due to diffusion delay before arrival at its apical site of action. The microelectrode data show that the basolateral membrane conductance decreased after AP; this response appears to be secondary and can be explained by the depolarization of the cell subsequent to increased Na⁺ entry. Parallel decrease in basolateral membrane potential and conductance has previously been reported for other conditions [13] and reflects response pattern of inwardly rectifying basolateral K⁺ channels [14]. Since the intracellular potential in the presence of amiloride was unchanged by AP in concentrations below 100 μM, direct effects of AP on the Na⁺/K⁺-ATPase can be ruled out.

Half-maximal inhibition (K_{1/2}) of the voltage-activated G^{Cl} by mucosal application of AP occurred at a concentration of 20 μM whereas the resting, inactivated conductance was unaffected. On a molecular basis, AP is thus one of the most effective inhibitors of the voltage-activated Cl⁻ conductance across amphibian skin. Interestingly, comparable K_{1/2} of 24 μM was obtained for the inhibition of Cl⁻ channels activity in rat colon by NPPB [23]. The mode of action of these substances should be considered in the light of the present knowledge and a recent working model for the voltage-activated Cl⁻ conductance of amphibian skin [20]. At least two functional components must be involved in the pathway, i.e., a regulatory site, which is opened by serosa-positive electrical potential, followed by an anion-con-

ductive channel. The problem of the intraepithelial location of the route for conductive Cl⁻ movement is still unsettled [11, 15] and has not been attempted in the present study; yet, the pathway has already proved to be highly complex and influenced through transepithelial potential, cAMP and a number of agonists such as theophylline or procaine [7]. Substances that inhibit voltage-activated G^{Cl} could either interfere with the sensor which opens the gate through voltage activation, or they could obliterate the already inserted conductive path. Hitherto, no means exists to distinguish between these alternatives. It is interesting to note that opposite effect on G^{Cl} in amphibian skin is exerted by another amphiphilic substance, the local anesthetic procaine, which was supposed to act due to partitioning in the lipid phase of the membrane in uncharged form [3]. AP has a pK of 9.6 [pers. unpubl. determination] and is positively charged at physiological pH. This difference between procaine and AP could account for the opposite interference with Cl⁻ pathways.

AP inhibited G^{Cl} effectively, when applied to the external side of the tissue at short circuit (frog skin) or serosa-negative clamp potentials (frog and toad skin). Although the chemical nature of the proteinic group(s), which is responsible for the voltage activation is still obscure, our experiments reveal a certain important property regarding the interaction of AP and the voltage-activated G^{Cl}. AP contains a guanidine group, which can be protonated with pK of 9.6. Accordingly, the molecule bears a positive charge at physiologically tolerated pH values (6–8.5). Due to this charging of AP, it was anticipated that serosa-positive clamp potentials prevent the access to its binding site in the affected pathway. This was indeed observed. Accordingly, it can be concluded that access of the ionic form of AP, rather than the lipophilic moiety, to some sensitive site in the Cl⁻ pathway is required for

inhibition of activated G^{Cl} . The 'protective' function was gradually reduced at less positive clamp potentials ($<+60$ mV), where the inhibitory effect of AP could be almost completely elicited after extended duration of application. The delayed effect of AP was particularly evident in frog skin, where inhibition of G^{Cl} at short circuit was incomplete. In all cases, however, brief reversal of the potential to serosa-negative values established the full inhibition. This suggests that access of AP to the site of action is strongly accelerated by the electrical gradient. Since the binding of AP is not readily reversible, we cannot estimate the location of the binding site in the pathway as has been done from the voltage dependence of amiloride binding [22, 26]. It is also impossible to decide whether interaction occurs from the solution side or includes partitioning of AP in the lipid phase with consecutive movement to a position more inside the membrane.

The slight shift of the conductance/voltage relationship to higher clamp potentials could be a consequence of such voltage-dependent entrance of AP to its site of action with partial rejection at higher clamp potentials. It has to be found out whether inhibition occurs at the voltage sensor or whether properties of the ion-selective path are altered.

In conclusion, AP was found to be a sensitive and selective inhibitor of amphibian skin G^{Cl} , whereas it stimulated Na^+ transport. The inhibitory effect depends on the transepithelial potential, but the precise site of action remains to be elucidated.

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