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Anthracene-9-Carboxylic Acid Inhibits an Apical Membrane Chloride Conductance in Canine Tracheal Epithelium

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Summary. Canine tracheal epithelium secretes Cl from the submucosal to the mucosal surface via an electrogenic transport process that appears to apply to a wide variety of secretory epithelia. Cl exit across the apical membrane is thought to be a passive, electrically conductive process. To examine the cellular mechanism of Cl secretion we studied the effect of anthracene-9-carboxylic acid (9-AC), an agent known to inhibit the Cl conductance of muscle membrane. When added to the mucosal solution, 9-AC rapidly and reversibly decreases short-circuit current and transepithelial conductance, reflecting a reduction in electrogenic Cl secretion. The inhibition is concentration-dependent and 9-AC does not appear to compete with Cl for the transport process. The decrease in current and conductance results from a decrease in the net and both unidirectional transepithelial Cl fluxes without substantial alterations of Na fluxes. Furthermore, 9-AC specifically inhibits a Cl conductance: tissues bathed in Cl-free solutions showed no response to 9-AC. Likewise, when the rate of secretion and Cl conductance were minimized with indomethacin, addition of 9-AC did not alter transepithelial conductance. In contrast, neither removal of Na from the media nor blockade of the apical Na conductance with amiloride prevented a 9-AC-induced decrease in transepithelial conductance. We also found that the effect of 9-AC is independent of transepithelial transport: 9-AC decreases transepithelial conductance despite inhibition of Cl secretion with ouabain or furosemide. Intracellular electrophysiologic techniques were used to localize the effect of 9-AC to a reduction of the electrical conductance of the apical cell membrane: 9-AC hyperpolarizes the electrical potential difference across the apical membrane and decreases its relative conductance. 9-AC also prevents the characteristic changes in the cellular electrical potential profile, transepithelial conductance, and the ratio of membrane conductances produced by a reduction in mucosal bathing solution Cl concentration. These results indicate that 9-AC inhibits Cl secretion in tracheal epithelium by blocking an electrically conductive Cl exit step in the apical cell membrane. Thus, they support a cellular model of Cl secretion in which Cl leaves the cell across a Cl permeable apical membrane driven by its electrochemical gradient.

Key Words tracheal epithelium · Cl secretion · anthracene-9-carboxylic acid · Cl permeability · electrophysiology

Introduction

In the investigation of epithelial transport, inhibitors of ion transport have provided powerful probes for understanding the way that ions move across cell membranes. For example, amiloride has been an invaluable tool for understanding electrogenic Na transport. Considerable insight into the mechanism of epithelial ion transport has also been provided by the use of furosemide to inhibit Cl-cotransport processes, substituted stilbenes to inhibit electrically neutral Cl movement, and barium to inhibit K conductance. Unfortunately, there is no widely used agent that inhibits the cellular Cl conductance in either Cl-secreting or non-Cl-secreting epithelia.

However, Palade and Barchi (1977) and Bryant and Morales-Aguilera (1971) have inhibited the Cl conductance of muscle membranes with carboxylic acids. Anthracene-9-carboxylic acid (9-AC) proved to be the most potent inhibitor of the Cl conductance in rat diaphragm muscle. They showed that 9-AC reversibly inhibited Cl conductance in a concentration-dependent manner. The structure of 9-AC is shown in Fig. 1.

Canine tracheal epithelium secretes Cl from the submucosal or basolateral surface to the mucosal or apical surface (Olver, Davis, Marin & Nadel, 1975; Al-Bazzaz & Al-Awqati, 1979; Widdicombe, Ueki, Bruderman & Nadel, 1979). Chloride exit from the cell across the apical membrane appears to be an electrically conductive process with Cl

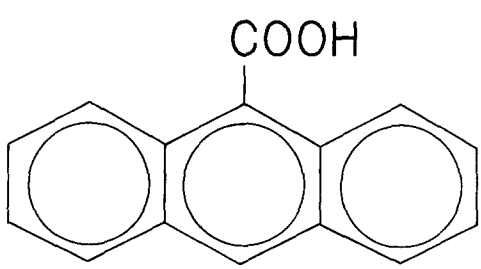

Fig. 1. Chemical structure of anthracene-9-carboxylic acid, 9-AC

moving passively down a favorable electrochemical gradient (Welsh, Smith & Frizzell, 1982, 1983; Welsh, 1983*c*; Shorofsky, Field & Fozzard, 1983). In this study, we examined the ability of 9-AC to inhibit Cl secretion and block the Cl conductance of canine tracheal epithelium.

List of Symbols

Ψ_t, Ψ_a, Ψ_b	The electrical potential difference across the epithelium, the apical membrane and the basolateral membrane, respectively, in millivolts.
I_{sc}	The short-circuit current, i.e., the current required to clamp Ψ_t to zero, in $\mu A \cdot cm^{-2}$.
G	Electrical slope conductance, in $mS \cdot cm^{-2}$.
R	Electrical slope resistance, in $\Omega \cdot cm^2$.
E	Electromotive force, in millivolts.
a, b, p, t	As subscripts, refer to apical, basolateral, paracellular and transepithelial, respectively.
Cl, Na, K	As superscripts refer to the respective ions.

Materials and Methods

Tracheal epithelium was prepared as previously described (Welsh & Widdicombe, 1980). Mongrel dogs (20 to 40 kg) of either sex were anesthetized with pentobarbital (25 mg/kg intravenously) and the trachea was removed. After stripping off the muscular layer, we used the posterior membranous portion of the trachea. The bathing solution contained (in mM): 118.9 NaCl, 20.4 $NaHCO_3$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$ and 10 glucose. The solution was bubbled with 95% O_2 – 5% CO_2 (pH 7.4 at 37 °C).

For measurement of transepithelial electrical properties and radioisotope fluxes, tissues were mounted in Ussing chambers (1.5 cm^2 surface area). The transepithelial electrical potential difference (Ψ_t) (referenced to the mucosal solution) was automatically clamped to zero (the short-circuit condition) by automatic voltage-current clamps (University of Iowa, Bioengineering). Transepithelial slope conductance (G_t) was calculated from the change in current required to clamp Ψ_t to ± 10 mV (pulses delivered by a pulse generator built into the voltage-current clamp; duration 0.5 or 1 sec; period 60 sec). Na and Cl transport rates were determined from the unidirectional and calculated net transepithelial fluxes of ^{22}Na and ^{36}Cl measured in paired tissues from the same dog. ^{22}Na (5 μCi) and ^{36}Cl (7 μCi) were added to the appropriate side of the tissue (the volume of fluid on each side of the tissue was 10 ml). Forty-five min were allowed for fluxes of ^{22}Na and ^{36}Cl to reach a steady state, and then 3 to 4 samples of both bathing solutions were taken at 10- to 15-min intervals during each control and experimental period. For ^{14}C mannitol fluxes 5 μCi of the isotope was added to the bathing solution, 2 hr were allowed for equilibration, and then samples were taken at 15-min intervals.

For measurement of intracellular electrical properties the epithelium was mounted, mucosal surface up, as a flat sheet between two halves of a Lucite® micropuncture chamber (0.125 cm^2 aperture). Both the mucosal and submucosal surface of the epithelium were continuously perfused by gravity flow from reservoirs above the chamber at a rate of $2 ml \cdot min^{-1}$. The perfusion reservoir was water-jacketed so that the solution bathing the tissue was maintained at 37 °C.

The techniques for measuring transepithelial and intracellular electrical properties, construction of microelectrodes, and performance of cellular impalements were the same as those previously described (Welsh et al., 1982). We measured the electrical potential difference across the apical cell membrane (Ψ_a) and the membrane conductance ratio (G_a/G_b):

$$\frac{G_a}{G_b} = \frac{\Delta\Psi_t}{\Delta\Psi_a} - 1 \quad (1)$$

where G_a and G_b refer to the conductances of the apical and basolateral membranes and $\Delta\Psi_t$ and $\Delta\Psi_a$ refer to the changes in Ψ_t and Ψ_a induced by transepithelial current pulses.

Epinephrine (10^{-6} M, submucosal solution) (Elkin-Sinn) was used to stimulate Cl secretion (Smith, Welsh, Stoff & Frizzell, 1982) and unless otherwise noted, was present throughout. Furosemide was a generous gift of Hoechst Pharmaceuticals, Somerville, N.J. Amiloride was a generous gift of Merck, Sharp and Dohme Research Laboratores, West Point, Pa. Indomethacin, ouabain, and Anthracene-9-carboxylic acid were obtained from Sigma Chemical (St. Louis, Mo.).

Values are presented as means ± SEM. Statistical significance was evaluated using a paired or unpaired *t*-test as indicated; $P < 0.05$ was considered statistically significant.

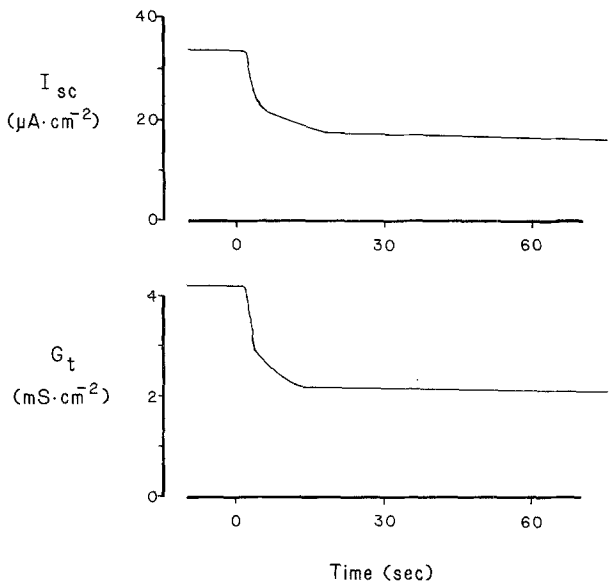

Fig. 2. Time course of the electrical response to 9-AC. Tracing from one representative tissue in which secretion was stimulated with epinephrine. 9-AC (10^{-2} M) was added to the mucosal solution at time zero

Results

Mucosal 9-AC Decreases Short-Circuit Current and Transepithelial Conductance

To determine if 9-AC had an effect on ion transport in tracheal epithelium, we added 9-AC to the mucosal bathing solution of stimulated tissues (epinephrine, 10^{-6} M, submucosal solution). The time course of a typical response is shown in Fig. 2. 9-AC (10^{-2} M) rapidly decreased both the short-circuit current (I_{sc}) and G_t.

Next, we asked which surface of the epithelium

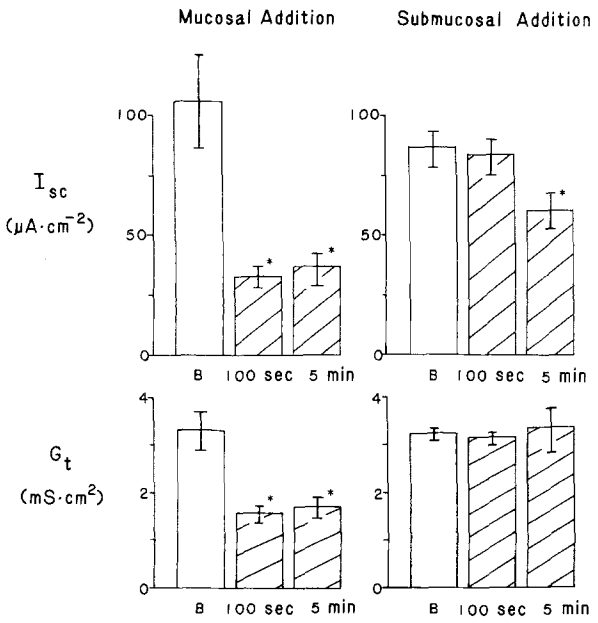

Fig. 3. Sidedness of the effect of 9-AC in secreting tissues. 9-AC (10^{-2} M) was added to either the mucosal or submucosal solution and I_{sc} and G_t were measured during a baseline period "*B*" and 100 sec and 5 min following additon of 9-AC. Epinephrine was present throughout to maximize the rate of Cl secretion. * Value different from the baseline value, $P < 0.01$

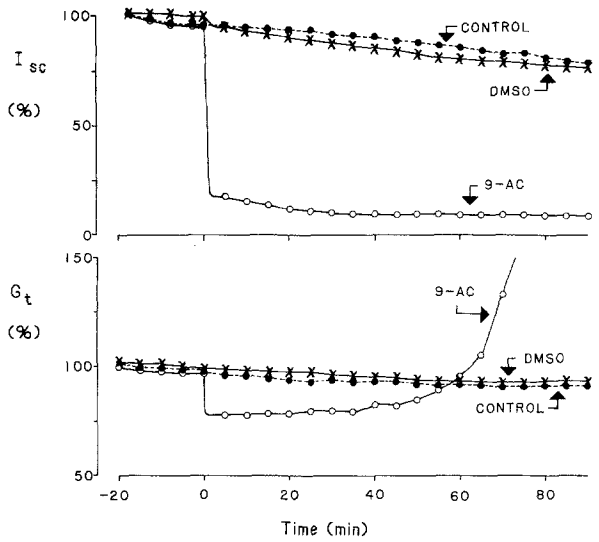

Fig. 4. Effect of 9-AC and DMSO on I_{sc} and G_t. Values are from three epinephrine-treated tissues from the same animal. One received no intervention, "Control"; one received 9-AC (10^{-2} M, mucosal solution); one received a volume of DMSO equal to that given with 9-AC. 9-AC and DMSO were added at time zero. Initial values of I_{sc} for "Control," "9-AC," and "DMSO" tissues were 163, 99 and 144 $\mu A \cdot cm^{-2}$, respectively. Initial values for G_t were 6.1, 5.7 and 8.0 $mS \cdot cm^{-2}$, respectively

was sensitive to 9-AC. The drug was added to either the mucosal or submucosal surface and I_{sc} and G_t were measured 100 sec and 5 min later. Figure 3 shows that mucosal addition rapidly decreased I_{sc} and G_t. In contrast, submucosal addition had no acute effect; 5 min after addition there was a slight decrease in I_{sc}, but no change in G_t. These results suggest that the primary effect of 9-AC is at the apical membrane. It is not clear why submucosal 9-AC produced a small delayed decrease in I_{sc}, but it may have resulted from the movement of a small amount of 9-AC across the epithelium to the mucosal surface. For the remainder of the study 9-AC was added only to the mucosal bathing solution.

9-AC was added to the bathing solution as an aliquot of a 0.5 M stock solution in dimethyl sulfoxide (DMSO). To determine if DMSO produced effects similar to those seen with 9-AC we added DMSO alone. A representative experiment is shown in Fig. 4. DMSO alone did not produce the acute changes in I_{sc} and G_t observed with 9-AC nor did it produce the delayed increase in G_t observed with 9-AC. The late increase in G_t produced by 9-AC is discussed below.

The inhibition of Cl secretion and decrease in G_t produced by 9-AC was largely reversible. Figure 5 shows the I_{sc} and G_t in eight stimulated tissues before addition of 9-AC, 10 to 15 min following addition of 9-AC, and then, during a recovery period, 60 min following replacement of the mucosal solution with Ringer's that did not contain 9-AC.

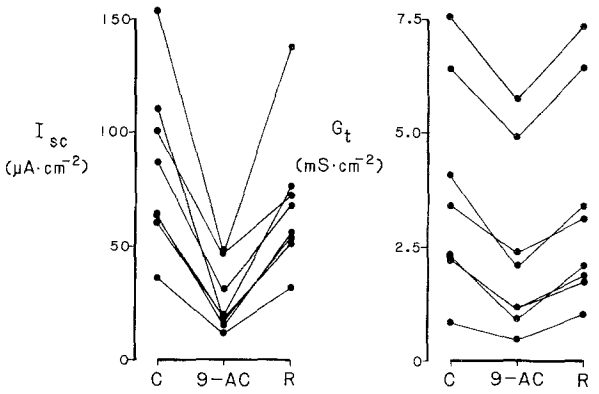

Fig. 5. Reversibility of the effect of mucosal 9-AC. Short-circuit current and G_t were measured during a control period "*C*," 10 to 15 min following addition of 9-AC (10^{-2} M) to the mucosal bathing solution, and during a recovery period "*R*," 50 to 70 min following replacement of the mucosal bathing solution. Each point represents values obtained in one tissue

9-AC Concentration-Response Relation

Figure 6 shows the effect of adding progressively increasing concentrations of 9-AC to secreting tissues. Both I_{sc} and G_t decreased as the concentra-

tion of mucosal 9-AC increased. To avoid the possibility of any time-dependent effects, a cumulative dose-response relation was also performed with increasing concentrations of 9-AC added at 40-sec intervals. However the results were identical to those obtained when increasing concentrations of 9-AC were added at 5-min intervals (Fig. 6). I examined the dose-response relation of 9-AC and I_{sc} and G_t for Michaelis-Menten kinetics with linear transformations such as the Lineweaver and Burk plot and Hill plot, but they failed to yield a straight line. While this may suggest that 9-AC does not interact with a single membrane site, the inability to fit the data to commonly used enzyme kinetic formalisms may also indicate that such analyses are too simplistic for a process as complicated as transepithelial transport.

In an attempt to determine whether 9-AC competes with Cl for the conductive transport process, we examined the effect of increasing concentrations of 9-AC in tissues bathed in solutions containing varying Cl concentrations. Gluconate was substituted for Cl symmetrically for 1 hr before addition of 9-AC. Figure 7 shows that I_{sc} decreased with decreasing bathing solution Cl concentrations and, as a result, the magnitude of the response to 9-AC was less at low Cl concentrations. However, when the response to 9-AC is replotted as % initial I_{sc} (lower half of Fig. 7) the response in different Cl concentrations is indistinguishable. These results suggest that 9-AC does not compete with Cl, but rather inhibits the transport process independent of the Cl concentration.

Chloride Specificity of 9-AC's Effect

The results presented so far suggest that 9-AC inhibits a Cl conductive process, thereby inhibiting secretion. To further examine the Cl specificity of the effect, we performed several experiments. First, the effect of 9-AC was examined in tissues in which the rate of Cl secretion was minimized by the mucosal addition of indomethacin (10^{-6} M, for 90 min) (epinephrine was not present). Indomethacin decreases the endogenous rate of protaglandin production, decreases intracellular cAMP, minimizes the apical membrane Cl conductance, and thus decreases the rate of Cl secretion (Al-Bazzaz, Yadava & Westenfelder, 1981; Smith et al., 1982; Welsh et al., 1982). In indomethacin-treated tissues, 9-AC (10^{-2} M, mucosal solution) did not significantly change G_t: G_t was 1.3 ± 0.1 mS · cm^{-2} before, and 1.2 ± 0.1 mS · cm^{-2} following the addition of 9-AC ($n = 4$). However, there was a decrease

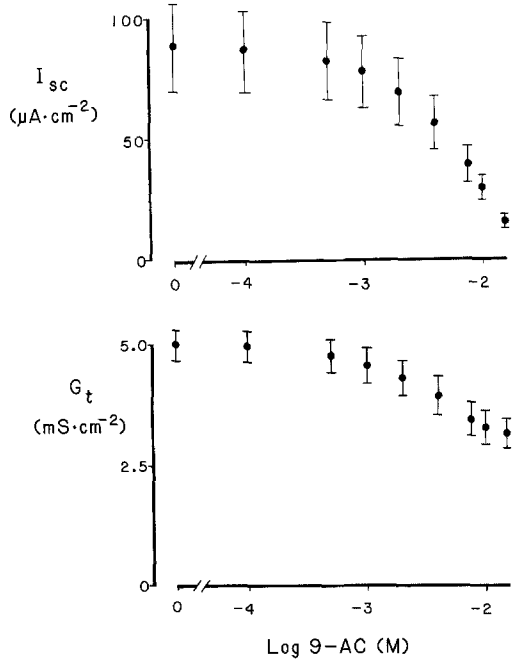

Fig. 6. Effect of mucosal 9-AC concentration on I_{sc} and G_t. Each point represents the mean ± SEM of four determinations. I_{sc} and G_t were measured 5 min after addition of each concentration

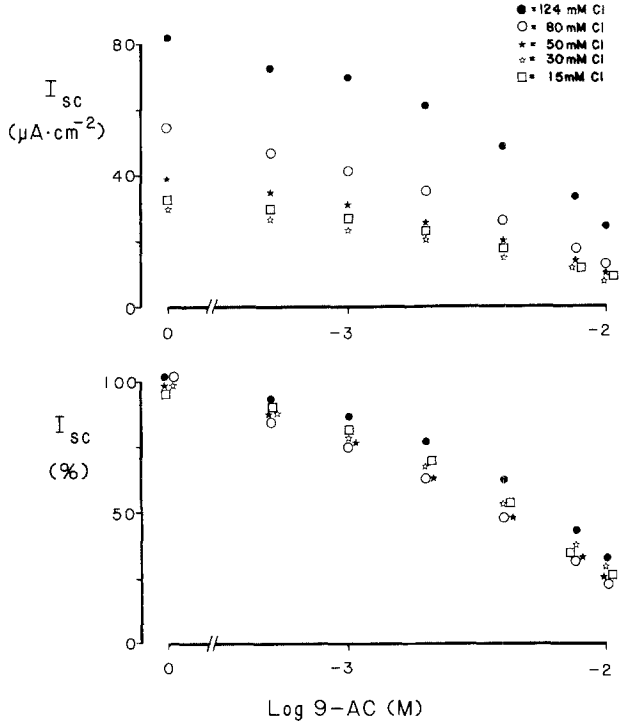

Fig. 7. Effect of mucosal 9-AC concentration on I_{sc} in five tissues bathed in varying Cl concentrations. Each point represents the mean of five determinations. Values were obtained 5 min after addition of each concentration. In the lower portion of the Figure, data are replotted as percent initial I_{sc}

in I_{sc} from 40 ± 11 to 26 ± 8 $\mu A \cdot cm^{-2}$ ($P < 0.05$). It is possible that this decrease in current may be due to a Low rate of Cl secretion that persists despite treatment with indomethacin (Al-Bazzaz et al., 1981; Welsh et al., 1983). However, the main point is that when there is little or no apical Cl conductance, the effect of 9-AC is minimal.

Further evidence that 9-AC inhibits a Cl conductance was obtained by examining the effect of 9-AC on G_t in tissues bathed in NaCl Ringer's, ($I_{sc} = 92 \pm 27$ $\mu A \cdot cm^{-2}$), Cl-free Ringer's (gluconate substitution) ($I_{sc} = 30 \pm 14$ $\mu A \cdot cm^{-2}$), and Na-free Ringer's (choline substitution) ($I_{sc} = 6 \pm 2$ $\mu A \cdot cm^{-2}$). Figure 8 shows that 9-AC significantly decreased G_t when Cl was present in the bathing solution (NaCl Ringer's and Na-free Ringer's) but not when Cl-free media bathed the tissues. In tissues bathed in NaCl I_{sc} decreased to 29 ± 8 $\mu A \cdot cm^{-2}$, in Cl-free Ringer's I_{sc} decreased to 18 ± 7 $\mu A \cdot cm^{-2}$, and in Na-free Ringer's I_{sc} decreased to 3 $\mu A \cdot cm^{-2}$. These results suggest that the effect of 9-AC is dependent upon the presence of Cl.

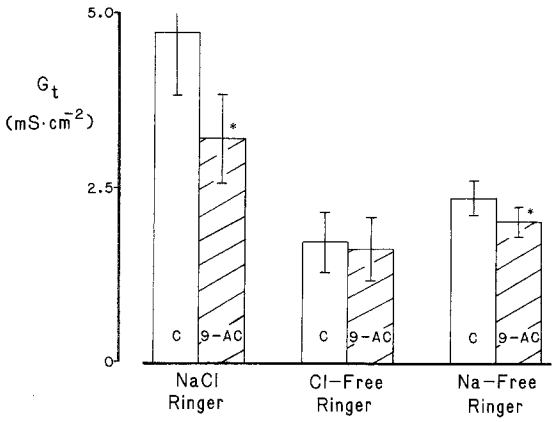

Fig. 8. Effect of ion substitutions on the response to 9-AC. Transepithelial conductance (G_t) was measured in seven tissues bathed in NaCl Ringer's, seven tissues bathed with Cl-free gluconate Ringer's, and seven tissues bathed in Na-free choline Ringer's. Tissues were incubated in the respective solution for at least 1 hr before addition of 9-AC. Conductance was measured during a control period "*C*," immediately before, and 100 sec following addition of 9-AC (10^{-2} M) to the mucosal bathing solution. Epinephrine (10^{-6} M, submucosal solution) was present throughout. $^{*}P < 0.01$

To directly examine the effect of 9-AC on transepithelial Cl movement, simultaneous transepithelial fluxes of Na and Cl were measured in seven pairs of tissues; the results are shown in Table 1. We measured fluxes during 3 periods: first, when the rate of secretion was minimized by the addition of indomethacin (10^{-6} M, mucosal solution, for 60 min before the first flux period); second, when Cl secretion was maximized by the addition of epinephrine (10^{-6} M, submucosal solution); and third, following the addition of 9-AC (10^{-2} M, mucosal solution) (epinephrine remained in the submucosal solution during the third period). Following the addition of 9-AC, I_{sc} and G_t decreased to values not different from those measured under baseline conditions when the rate of secretion was minimal. This suggests that 9-AC may, at least partially, block the epinephrine-induced changes in membrane conductance. This point is further illustrated by an examination of the Cl fluxes. The changes in unidirectional and net Cl fluxes parallel the changes in I_{sc} and G_t: epinephrine increases the fluxes, while the subsequent addition of 9-AC returns the fluxes to values similar to those found under baseline conditions. In contrast to the changes in electrical properties and Cl fluxes, the changes in Na fluxes following addition of 9-AC were trivial. These results provide further evidence that the effect of 9-AC is specific for Cl.

Table 1. Effect of 9-AC on transepithelial electrical properties and ion fluxes[a]

	I_{sc} ($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)	G_t ($mS \cdot cm^{-2}$)	J^{Na}_{ms} ($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)	J^{Na}_{sm}	J^{Na}_{Net}	J^{Cl}_{ms} ($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)	J^{Cl}_{sm}	J^{Cl}_{Net}
Baseline	0.73 ±0.10	1.22 ±0.12	1.08 ±0.07	0.57 ±0.12	0.51 ±0.10	1.04 ±0.18	1.27 ±0.17	−0.23 ±0.10
Epinephrine	2.18[b] ±0.35	2.46[b] ±0.11	1.05 ±0.09	0.66[b] ±0.11	0.39 ±0.07	1.80[b] ±0.23	3.48[b] ±0.51	−1.68[b] ±0.39[b]
9-AC and epinephrine	0.82[b] ±0.10	1.41[b] ±0.10	1.10 ±0.08	0.89[b] ±0.15	0.27 ±0.09	0.80[b] ±0.15	1.18[b] ±0.14	−0.38[b] ±0.07

[a] Seven tissue pairs were studied during three consecutive periods: during a "baseline period," following addition of epinephrine (10^{-6} M, submucosal solution), and following addition of 9-AC to epinephrine-treated tissues. Indomethacin (10^{-6} M, mucosal solution) was present during the baseline period to minimize the spontaneous rate of Cl secretion and remained in the mucosal solution during the subsequent two periods. A negative number indicates a net flux from submucosa to mucosa, in accord with the direction of current flow.

[b] $P < 0.02$ compared to the preceding period.

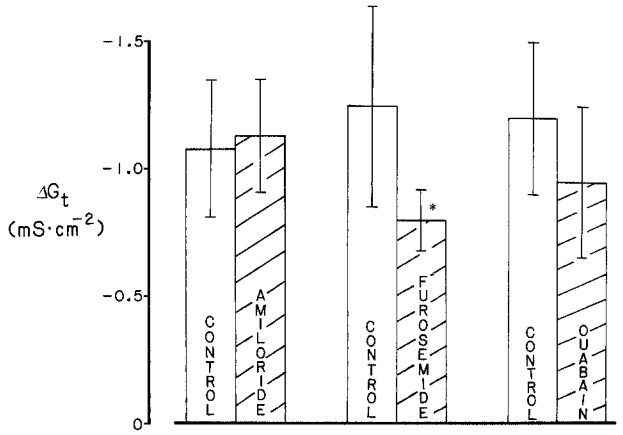

Fig. 9. Change in transepithelial conductance (ΔG_t) produced by 9-AC in tissues in which Na and/or Cl transport was inhibited. Five tissue pairs were studied for each condition. In each pair, one tissue served as a control, and one tissue was treated with either amiloride (10^{-4} M, mucosal solution for 10 min before adding 9-AC), furosemide (10^{-3} M, submucosal solution for 30 min before adding 9-AC) or ouabain (10^{-4} M, submucosal solution, for 45 min before adding 9-AC). The change in transepithelial conductance was measured 100 sec following addition of 9-AC (10^{-2} M) to the mucosal bathing solution. *Value significantly different from matched control, $P<0.05$

The Effect of 9-AC Is Independent of Active Transport

The results presented so far suggest that 9-AC inhibits Cl secretion by blocking a Cl conductive process. However, it is also possible that 9-AC might inhibit Cl transport indirectly, perhaps by inhibiting the Na pump or a process involved in transcellular Cl transport other than an apical Cl conductance. To investigate this possibility, we examined the effect of 9-AC when either Na transport was inhibited by amiloride, Cl transport was inhibited by furosemide, or both were inhibited by ouabain. Five pairs of tissues were examined for each of the conditions; one tissue of the pair served as a control and one received the drug of interest. When I_{sc} and G_t had stabilized following addition of amiloride, furosemide, or ouabain, we measured the change in G_t produced by 9-AC.

The results are shown in Fig. 9. First, amiloride inhibits Na absorption in tracheal epithelium by inhibiting an apical Na conductance (Widdicombe & Welsh, 1980; Welsh et al., 1983). Although amiloride decreased I_{sc} from 70 ± 17 to 57 ± 12 $\mu A \cdot cm^{-2}$ ($n=5$, $P<0.05$), 9-AC decreased G_t to the same extent as we observed in tissues that did not receive amiloride. This suggests that the effect of 9-AC is not dependent upon active Na absorption, nor an apical Na conductance. Second, furosemide inhibits Cl secretion by inhibiting electrically neutral, Na-dependent Cl entry at the basolateral membrane, thereby decreasing intracellular Cl activity (Welsh, 1983*b*, *c*). In addition, furosemide slightly decreases the apical membrane conductance, a decrease which may partially result from the decrease in intracellular Cl activity. When furosemide was added to the submucosal solution, I_{sc} decreased from 70 ± 6 to 27 ± 7 $\mu A \cdot cm^{-2}$ ($n=5$, $P<0.01$). Despite the inhibition of Cl secretion, 9-AC still decreased G_t. The decrease in G_t in furosemide-treated tissues was slightly less than that in control tissues, a finding most likely resulting from the small decrease in apical conductance produced by furosemide (Welsh, 1983*b*). These data suggest that the effect of 9-AC is not dependent on active secretion, but is consistent with an inhibition of an apical Cl conductance. Third, ouabain decreases both Na and Cl transport in tracheal epithelium by inhibiting the Na-K-ATPase that provides the energy for transport (Al-Bazzaz & Al-Al-Awqati, 1979; Widdicombe et al., 1979). Submucosal ouabain decreased I_{sc} from 43 ± 8 to 2 ± 2 $\mu A \cdot cm^{-2}$ ($n=5$, $P<0.01$) but the decrease in G_t produced by 9-AC remained intact. These results provide convincing evidence that 9-AC has an effect even in the absence of transepithelial transport, a finding most consistent with the conclusion that 9-AC inhibits an electrically conductive, passive transport process.

Delayed Effect of 9-AC

Although the effect of 9-AC was reversible in the short term, with prolonged exposure 9-AC progressively and irreversibly increased G_t (Fig. 4). To determine if the increase in G_t might partially result from an increase in paracellular conductance, we examined the effect of prolonged exposure to 9-AC on the transepithelial flux of mannitol, which provides a marker of paracellular pathway permeability in tracheal epithelium (Welsh & Widdicombe, 1980). Figure 10 shows that with prolonged exposure, 9-AC increased both the unidirectional mannitol flux and G_t. In addition, these changes were accompanied by a decrease in I_{sc}. These findings suggest that during prolonged exposure 9-AC may be toxic to the epithelium, further inhibiting transport, and increasing the permeability of the paracellular pathway.

Intracellular Electrophysiologic Studies of 9-AC's Effect

To localize the effect of 9-AC with greater certainty, we turned to intracellular electrophysiologic techniques. Figure 11 shows the time course of the

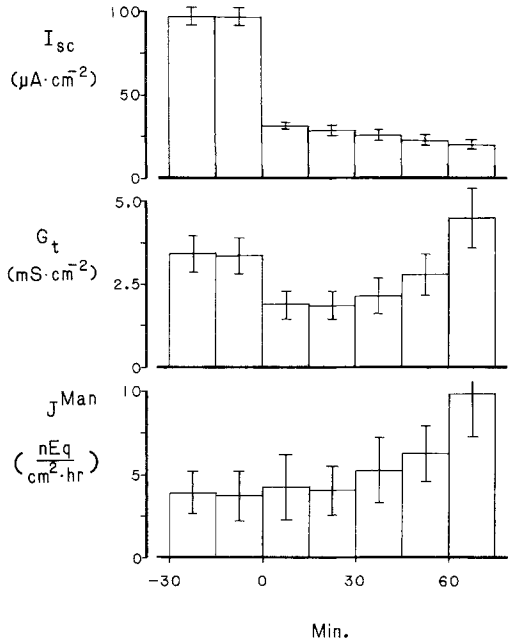

Fig. 10. Long-term time course of the effect of 9-AC on electrical properties and transepithelial mannitol flux. 9-AC (10^{-2} M) was added to the mucosal bathing solution at time zero

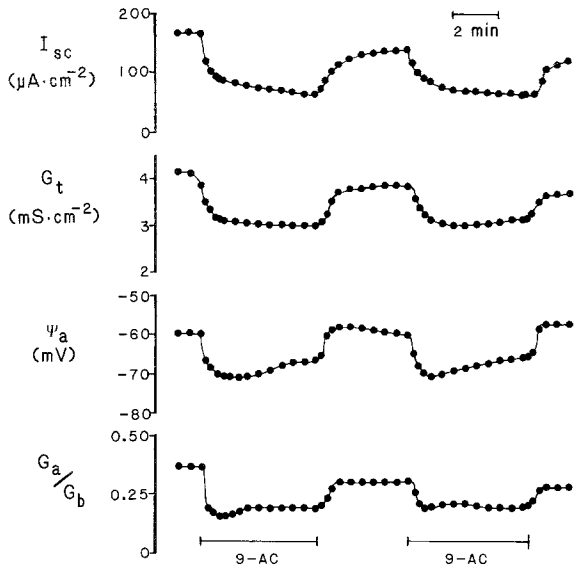

Fig. 11. Time course of the effect of 9-AC on transepithelial and intracellular electrical properties under short-circuit conditions in one representative tissue. 9-AC (10^{-2} M) was present in the mucosal solution during the periods indicated. Epinephrine (10^{-6} M) was present throughout to stimulate Cl secretion

changes in transepithelial and intracellular electrical properties obtained in one representative, secreting tissue.

Mucosal 9-AC reversibly decreased both I_{sc} and G_t. Following addition of 9-AC, Ψ_a (and Ψ_b, under short-circuit conditions $\Psi_a = -\Psi_b$ so that $\Psi_t = 0$) followed a slightly biphasic pattern, first hyperpolarizing and then depolarizing slightly. This biphasic pattern of Ψ_a was frequently, but not always observed. For the tissue represented in Fig. 11, G_a/G_b also followed a slightly biphasic pattern, decreasing abruptly with addition of 9-AC and then increasing slightly. The biphasic pattern of G_a/G_b occurred in a few tissues, but was not consistently found. Finally, Fig. 11 shows that the decrease in G/G_b was complete before the decrease in G_t had reached a steady state. Again, this time dissociation of the decrease in G_a/G_b and G_t was not always found. The significance of these time-dependent changes will be discussed subsequently.

Table 2. Effect of 9-AC on transepithelial and intracellular electrical properties[a]

	I_{sc} ($\mu A \cdot cm^{-2}$)	G_t ($mS \cdot cm^{-2}$)	Ψ_a (mV)	G_a/G_b
Baseline	139 ± 8	5.0 ± 0.4	-50 ± 2	0.68 ± 0.13
9-AC	$88^b \pm 6$	$4.3^b \pm 0.4$	$-60^b \pm 2$	$0.27^b \pm 0.04$

[a] Values are mean ± SEM of sixteen impalements in six tissues. Values were obtained during a "baseline" period and then 20 to 60 sec following addition of 9-AC (10^{-2} M, mucosal solution). Epinephrine (10^{-6} M, submucosal solution) was present throughout to stimulate Cl secretion.

[b] $P < 0.001$ compared to the baseline value.

Table 2 shows the effect of 9-AC on the transepithelial and intracellular electrical properties obtained in 16 cellular impalements in six tissues. Tissues were stimulated with epinephrine and the electrical properties were measured just before and 20 to 60 sec (at the peak of the response) following addition of 9-AC (10^{-2} M, mucosal solution).

The decrease in G_a/G_b combined with a decrease in G_t clearly indicates that 9-AC decreases G_a. There is no evidence to suggest an alternative to this interpretation, that 9-AC rapidly and simultaneously decreases the conductance of the paracellular pathway while increasing basolateral conductance. Certainly 9-AC did not initially alter transepithelial Na (Table 1) or mannitol fluxes (Fig. 10); if 9-AC decreased the conductance of the paracellular pathway the transepithelial flux of Na and/or mannitol might be expected to decrease. Table 2 and Fig. 11 also show that 9-AC hyperpolarizes Ψ_a. The hyperpolarization of Ψ_a can be readily explained by a decrease in G_a; the voltage drop due to current flowing across a membrane with a decreased conductance may be larger following addition of 9-AC. An analogous, but reversed, situation is observed on stimulation of secretion; G_a increases and Ψ_a depolarizes.

Table 3. Effect of 9-AC on the change in electrical properties produced by mucosal Cl substitution[a]

	G_t (mS · cm^{-2})	G_a/G_b	Ψ_t (mV)	Ψ_a (mV)	Ψ_b (mV)
Control					
124 mM Cl	6.2 ±1.1	1.04 ±0.10	30 ±7	−31 ±5	62 ±6
10 mM Cl	3.4 ±0.6	0.52 ±0.07	46 ±6	−16 ±5	62 ±6
Δ	−2.8[b] ±0.5	−0.52[c] ±0.20	+16[b] ±3	+16[b] ±2	−1 ±3
9-AC					
124 mM Cl	3.9 ±0.8	0.39 ±0.05	19 ±5	−41 ±5	60 ±6
10 mM Cl	2.9 ±0.6	0.29 ±0.04	21 ±5	−27 ±4	48 ±5
Δ	−1.1[c, d] ±0.3	−0.10 ±0.09	+2[d] ±2	+15[c] ±5	−13[b] ±4

[a] Results represent mean ± SEM of values obtained in six tissues when the mucosal solution contained either 124 or 10 mM Cl. Values were not obtained for the first 2 min following a change in mucosal Cl concentration. Tissues were studied both in the absence and presence of mucosal 9-AC (10^{-2} M). Epinephrine (10^{-6} M, submucosal solution) was present throught to stimulate Cl secretion. Δ represents the difference between values at 124 and 10 mM mucosal Cl.

[b] Value different from zero, $P < 0.005$.

[c] Value different from zero, $P < 0.05$.

[d] Δ-9-AC different from Δ-Control, $P < 0.005$.

We also examined the effect of 9-AC on the change in membrane conductance ratio and cellular electrical potential profile produced by mucosal Cl substitution; the results are shown in Table 3. The results represent the mean ± SEM of measurements obtained in six tissues; two to six cellular impalements were performed in each tissue under each experimental condition. Measurements were made when both surfaces of the epithelium were bathed with the normal Ringer's (123.7 mM Cl) and when the Cl concentration of the mucosal solution was decreased to 10 mM by gluconate substitution. The tissues were then returned to the normal Ringer's solution and 9-AC was added to the mucosal solution. Measurements were then repeated with both 124 mM Cl and 10 mM Cl Ringer's on the mucosal surface. Epinephrine was present in the submucosal solution throughout to stimulate Cl secretion. Tissues that showed a time-dependent deterioration before completion of the study (*see* Fig. 10) were discarded.

First, let us examine the change in G_t and G_a/G_b. Decreasing mucosal Cl concentration from 124 to 10 mM decreases G_t and G_a/G_b, changes consistent with an apical Cl conductance, and similar to those previously reported (Welsh et al., 1982; Shorofsky et al., 1983). 9-AC not only decreased both G_t and G_a/G_b, but also minimized the fall in G_t and prevented the decrease in G_a/G_b that normally result from a decrease in mucosal Cl concentration. While some decrease in G_t is to be expected following a decrease in mucosal Cl concentration [due to the decrease in conductance of the paracellular pathway (Welsh & Widdicombe, 1980)], the fact that 9-AC minimized the Cl-dependent decrease in G_t and G_a/G_b substantiates the conclusion that 9-AC blocks an apical Cl conductance.

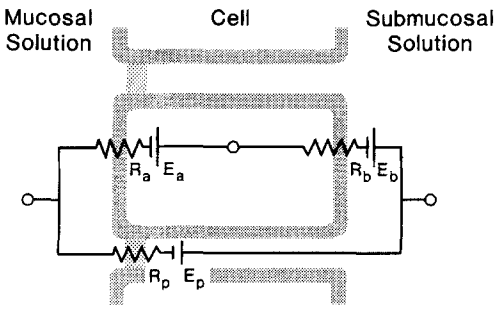

Fig. 12. Equivalent electrical circuit model of the epithelium. *See* text and list of symbols for details

Next, consider the changes in voltage. Although the voltage changes are impossible to interpret quantitatively, a qualitative consideration can be made with the equivalent circuit model shown in Fig. 12. A decrease in mucosal Cl concentration will generate a mucosal solution negative emf (or voltage) across the apical membrane and the paracellular pathway. Depending upon the relative conductance of these two paths, circular current flow through the cell and paracellular pathway may or may not result. These changes will hyperpolarize Ψ_t and depolarize Ψ_a. These are the changes shown in Table 3 and previously reported (Welsh et al., 1982; Shorofsky et al., 1983).

Next consider the effect of a decrease in mucosal Cl concentration when the only change is the elimination of the apical Cl conductance. (Note, however, that the entire conductance of the apical membrane would not be abolished.) Under these conditions, decreasing mucosal Cl concentration would generate a mucosal solution negative emf across the paracellular pathway but not the apical membrane. The mucosal solution negative paracellular emf would induce circular current flow from the mucosal solution through the paracellular path and back through the cellular pathway. Cellular current flow in this direction would depolarize Ψ_a (as shown in Table 3) and would be expected to

hyperpolarize Ψ_b. However, Ψ_b depolarized (Table 3). A possible explanation for this discrepancy would be a decrease in G_b. In light of the evidence that 9-AC secondarily decreases G_b (*see below*) this is the most likely explanation for the findings. Another possibility that cannot be ruled out is that G_b decreased as a result of the decrease in mucosal Cl concentration. Other effects of 9-AC are also possible, but seem less likely considering the previous results and the observation that Ψ_b was unchanged after 9-AC (Table 3). In any event, the voltage changes are certainly consistent with the previous results that indicate that 9-AC blocks an apical Cl conductance and secondarily increases basolateral conductance.

Discussion

Anthracene-9-carboxylic acid (9-AC) inhibits Cl secretion by canine tracheal epithelium. We have localized this effect to an inhibition of an electrically conductive Cl exit step at the apical cell membrane.

In previous studies of muscle membrane, Palade and Barchi (1977) found that a variety of aromatic carboxylic acids which are analogs of benzoic acid reduced the membrane Cl conductance (G^{Cl}) of rat diaphragm. 9-AC, which was the most effective analog studied, specifically inhibited G^{Cl}. The inhibition was initially rapid, reaching a stable value by 10 min. Inhibition was reversible, concentration-dependent, and voltage-independent. Their structure-activity analysis led them to conclude that 9-AC partitions into the lipid phase of the muscle membrane and produces its effect by interacting with a single transport process. The conclusion that 9-AC blocks G^{Cl} was also made by Bryant and Morales-Aguilera (1971) from electrophysiologic studies of goat intercostal muscles and by Hayward and Barchi (1980) from measurement of ^{36}Cl efflux from rat skeletal muscle. In addition, Levitan and Barker (1972) observed that salicylate and other aromatic monocarboxylic acids decrease the Cl conductance of molluscan neurons. However they did not test 9-AC.

Our findings are consistent with the observations noted above. The results that suggest that 9-AC does not compete with Cl for a membrane transport site (Fig. 7), also agree with the conclusion of Palade and Barchi (1977), that 9-AC does not block G^{Cl} via stearic hindrance but rather binds to a lipophilic membrane site and alters the anion channel. One difference in the effect of 9-AC on tracheal epithelium and muscle is that in trachea 10^{-3} to 10^{-2} M 9-AC is required to inhibit transport and decrease G_t, while in muscle 10^{-5} to 10^{-4} M 9-AC is sufficient to decrease membrane conductance. The reason for this difference is not apparent since, as discussed below, the drug appears to block a Cl conductance in both preparations. One possible explanation is a difference in the membrane lipids of muscle and trachea that influences the partitioning of 9-AC into the membrane.

These findings support a cellular model of Cl secretion in which Cl leaves the cell passively, across a Cl-conductive apical membrane. Previous evidence for conductive Cl exit in tracheal epithelium is as follows. First, ion substitution studies indicate that the electrical potential difference across the apical membrane (Ψ_a) and the apical conductance (G_a) are dependent on the Cl concentration in the mucosal bathing solution (Welsh et al., 1982; Shorofsky et al., 1983). Second, there is a direct relation between G_a and the secretory rate (Welsh et al., 1982, 1983; Shorofsky et al., 1983). Third, the electromotive force across the apical membrane is primarily determined by the Cl activity gradient across the apical membrane (Welsh, 1983*b*, *c*; Welsh et al., 1983). Fourth, Cl is accumulated in the cell at an activity greater than predicted for a passive distribution, thus providing an electrochemical gradient that favors passive Cl exit (Welsh, 1983*c*).

In tracheal epithelium changes in apical membrane Cl conductance are temporally related to changes in basolateral K conductance (Welsh et al., 1982; Welsh et al., 1983*a*; Shorofsky et al., 1983). Following addition of secretagogue, G_a^{Cl} increases and then G_b^K increases. The coupling of G_b^K and G_a^{Cl} is an important regulatory mechanism that serves to maintain a negative intracellular voltage and prevents large changes in intracellular ion concentrations (Smith & Frizzell, 1982; Welsh, 1983*d*; Welsh et al., 1983).

In light of the relation between G_a^{Cl} and G_b^K it is appropriate to ask whether analogous changes occur following inhibition of G_a^{Cl}. That is, when G_a^{Cl} is reduced by 9-AC, is there a secondary decrease in G_b? Evidence to suggest that this may be the case can be obtained from an examination of the acute electrical response to addition of 9-AC shown in Fig. 11. The major points are: (1) following addition of 9-AC, I_{sc} and G_t decreased monotonically; (2) G_a/G_b decreased in a biphasic manner so that G_a/G_b fell to its lowest value approximately 40 to 50 sec following the onset of the response and subsequently increased slightly. When G_a/G_b had decreased maximally, G_t had not achieved its

lowest value; (3) Ψ_a (and Ψ_b) changed in a biphasic manner, hyperpolarizing maximally and then depolarizing slightly, but remaining more negative than before addition of 9-AC.

Although not as dramatic, this pattern is qualitatively the reversal of that which occurs following addition of epinephrine (*see* Figs. 7 and 8 of Welsh et al., 1982, and Figs. 2 and 3 of Welsh et al., 1983). The sequence of events following addition of epinephrine is first, an increase in G_a causing G_a/G_b to increase, G_t to increase, and Ψ_a to depolarize. Then G_b increases causing a decrease in G_a/G_b, further increase in G_t, and partial repolarization of Ψ_a. Thus, the biphasic changes following addition of 9-AC may represent a primary effect of 9-AC on G_a^{Cl} followed by a secondary decrease in G_b.

Further evidence to suggest that the decrease in G_a produced by 9-AC is accompanied by a decrease in G_b can be obtained from consideration of an equivalent electrical circuit model shown in Fig. 12. If I assume that 9-AC only affects G_a, and not G_b or G_p, I can calculate values for G_b and G_p based on the values of G_t and G_a/G_b obtained before and after addition of 9-AC (Welsh et al., 1983). When this calculation was made for the individual data that contributed to Table 2, I frequently obtained negative values for either G_b or G_p. This indicates that the assumption that 9-AC only affects G_a is wrong. When taken together with the biphasic changes shown in Fig. 11, and the effect of mucosal Cl substitutions on the cellular electrical potential of 9-AC-treated tissues (Table 3), these results are most consistent with a secondary decrease in G_b that accompanies the fall in G_a. The decrease in G_b most likely represents a fall in G_b^K since the basolateral membrane is primarily K permeable.

Although a 9-AC-induced decrease in G_p certainly cannot, by itself, explain the results, I cannot rule out the possibility that 9-AC may have produced a small decrease in G_p. However, several points suggest that any effect of 9-AC on G_p would be small compared to the effect on G_a. First, 9-AC did not significantly alter G_t in nonsecreting, indomethacin-treated tissues, i.e., when G_a^{Cl} was quite small or absent. Second, 9-AC did not alter G_t in tissues bathed in Cl-free solutions, i.e., when both G_a^{Cl} and G_p^{Cl} are low or absent. Third, 9-AC did not significantly decrease the unidirectional fluxes of Na nor mannitol, both of which would have reflected a decrease in paracellular permeability.

In addition to its effect on G_a^{Cl} in tracheal epithelium it is possible that 9-AC may prove to be a useful agent in the study of the Cl permeability of other epithelia. However, one problem with its use is that it appears to be toxic following prolonged exposure. Palade and Barchi (1977) also observed that "with longer exposure times" it may be cytotoxic and irreversibly decrease muscle resting potential. The mechanism of these long-term changes are unknown but our results (Fig. 10) suggest both a cellular deterioration (I_{sc} decreased) and an increase in paracellular permeability (G_t and mannitol fluxes increased in parallel). Thus, studies utilizing the drug must be sufficiently short to minimize cytotoxic effects.

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