EFFECTS OF MILLIMOLAR CONCENTRATIONS OF GLUTARALDEHYDE ON THE ELECTRICAL PROPERTIES OF FROG SKIN

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SUMMARY

1. The effects of millimolar concentrations of glutaraldehyde on the electro-physiological properties of the epithelium of frog skin (*Rana temporaria*) were investigated. We recorded short-circuit current (I_{sc}), transepithelial conductance (G_t) and impedance (Z_t), fractional resistance (f_{R_o}) and the potential difference across the apical membrane (V_0). We used either Na^+ or K^+ as major mucosal cations to compare the effects on transepithelial Na^+ and K^+ currents (I_{Na} and I_K) and thus on the apical Na^+ and K^+ permeabilities.

2. At concentrations above 0·005 % (w/v) or 0·5 mM, glutaraldehyde irreversibly and completely inhibits both I_{Na} and I_K within 2–3 h. The initial time courses of the inhibition of transepithelial currents following serosal and mucosal applications of the compound markedly differ.

3. Glutaraldehyde decreased G_t in sulphate Ringer solutions while it augmented G_t severalfold in chloride Ringer solution.

4. Measurements of the transepithelial impedance of tissues incubated with sulphate solutions showed that glutaraldehyde increased the resistances of both apical and basolateral membranes significantly. The capacitance of the apical membrane was augmented, while the basolateral membrane capacitance was drastically decreased.

5. Microelectrode impalements of the granulosum cells showed that glutaraldehyde decreased V_0 by more than 40 mV and increased f_{R_o}, which reached values around 90%.

6. The role of free amino groups in ion-transporting proteins and the potential non-fixative uses of protein cross-linkers in epithelia are discussed.

INTRODUCTION

The pentanedialdehyde, usually named glutaraldehyde (GA), is the protein cross-linking reagent (Han, Richard & Delacourte, 1984) with the widest use as histological fixative (Hopwood, 1973) and coupling agent in immunochemical studies (Reichlin, 1980). In epithelial physiology, GA was used as a fixative (at concentrations higher than 2% w/v) for preserving water and urea channels induced by antidiuretic

hormone in urinary bladders (Eggena, 1983), or by hypertonic outer solutions in the amphibian skin (Aboulafia & Lacaz-Vieira, 1985).

As most cross-linkers, GA has no specificity towards a given class of proteins. Because of this lack of selectivity among macromolecules, little attention was paid to its 'mild' effects on various cellular processes. However, it is undisputed that GA is reasonably specific in the simple chemical sense for it cross-links mainly the free ϵ-amino groups of lysine (Korn, Feairheller & Filachione, 1972). This makes GA a potentially informative tool for probing the participation of free amino groups and of lysine residues in various cellular processes. On a priori grounds, the occurrence of some clear-cut functional effects of GA would justify concluding that the proteins involved in those functions do contain such residues in some key positions. The effects of GA on nerve membranes (Horn, Brodwick & Eaton, 1980; Mărgineanu, Katona & Popa, 1981) led to this conclusion before the amino acid sequence of the axonal sodium channels became known. Recently, the finding by Kondo & Imai (1987) that, in the hamster, millimolar concentrations of GA inhibit Cl^- transport in the thin ascending limb of Henle's loop was interpreted in a similar way.

A practical reason for investigating the effects of low concentrations of GA on the skin is the prospect that the mild GA treatment, which is widely used in red blood cell studies (Corry & Meiselman, 1978), might also become a useful procedure for modulating the physico-chemical properties of epithelial tissues.

In the present study, we report the effects of millimolar concentrations of GA on the frog skin. This epithelial tissue was chosen because it is a useful model of tight epithelia in general, it can be easily isolated with minimal damage and is stable during in vitro electrophysiological experiments. We found fairly reproducible patterns in the inhibition of I_{sc}, together with rather impressive impedance changes, resulting from the modification of resistive as well as capacitive properties. The impedance measurements were performed in ionic conditions which allowed the discrimination of the characteristics of the apical and basal membranes and the study was complemented with microelectrode recordings of the apical membrane potential and fractional resistance.

Our results show that GA largely increases the (mainly paracellular) chloride conductivity of the skin. In chloride-free solutions, it raises the resistances of both apical and basolateral membranes. This compound also increases the apical capacitance, but produces a large decrease in the basolateral capacitance. Some of the results were communicated at the summer meeting of the Belgian Society of Physiology (Mărgineanu & Van Driessche, 1989).

METHODS

Frogs (*Rana temporaria*), weighing around 25 g, were kept at 17 °C with free access to tap water. The abdominal skin of doubly pithed animals was dissected and mounted in an Ussing-type lucite chamber, previously described in detail by De Wolf & Van Driessche (1986). The chamber ensured negligible edge damage and the continuous perfusion with fresh solutions of both the mucosal (M) and serosal (S) sides of the epithelium.

Paired experiments were done with intact pieces of skin and the isolated epithelium. The removal of the corium was achieved after 1–2 h exposure to 60 U/ml collagenase (Worthington, Freehold, NJ, USA) (Fisher, Erlij & Helman, 1980).

The transepithelial potential was clamped to zero with a low-noise voltage clamp (Van Driessche & Lindemann, 1978) and the short-circuit currents (I_{sc}) were recorded on a standard X–T recorder.

The transepithelial conductance (G_t) was calculated from the current changes caused by 1 s voltage pulses of 10 mV amplitude. These measurements appear on the I_{sc} tracings (Fig. 1 and Fig. 3) as vertical bars, with lengths proportional to G_t.

Solutions. NaCl Ringer solution contained (in mM): 115 NaCl; 2·5 KHCO$_3$; 1 CaCl$_2$. Na$_2$SO$_4$ Ringer solution consisted of (in mM): 57·5 Na$_2$SO$_4$; 2·5 KHCO$_3$; 1 CaSO$_4$. Cs$_2$SO$_4$ Ringer solutions (in mM): 57·5 Cs$_2$SO$_4$; 2·5 KHCO$_3$; 1 CaSO$_4$. K$_2$SO$_4$ Ringer solution (in mM): 57·5 K$_2$SO$_4$; 2·5 KHCO$_3$; 1 CaSO$_4$. The pH of all solutions was adjusted to 8·0.

The GA solutions were prepared immediately before use by dissolving into the appropriate Ringer solution the suitable quantity from a 25 % (w/v) aqueous solution (Fluka, Buchs, Switzerland). The UV spectrum of the purchased reagent presented a single absorption maximum at 280 nm and the absorbance ratio at 235 *versus* 280 nm was 0·136, which indicates a good purity (Korn *et al.* 1972). A 1 % (w/v) GA solution would correspond to 0·1 mM for the monomeric form of GA, but it is generally preferred to give the concentration of aqueous GA solutions in % (w/v) because of the uncertainty about the polymerization state.

Sodium currents (I_{Na}) were blocked with 60 μM mucosal amiloride (a gift from Merck, Sharp and Dohme, West Point, PA, USA). I_{sc} was stimulated with 0·1 U/ml oxytocin (Sigma, St Louis, MO, USA) added to the serosal compartment.

Impedance measurements. The transepithelial electrical impedance was recorded with a computerized sine-wave method while the mean voltage across the epithelium was clamped to zero. A voltage signal composed of fifty-three sine waves was imposed on the tissue via the command input of the voltage clamp. The transepithelial current (I_t) and voltage (V_t) signals were recorded consecutively. This procedure allowed us to use the same 48 dB/octave anti-aliasing filter for I_t and V_t. Reed relays were used to select I_t or V_t as input for the anti-aliasing filter and analog-to-digital converter. A synchronization pulse from the signal generator was used to initiate the sampling of a sweep of data points. The impedance (Z) was calculated as the ratio of the Fourier-transformed voltage and current signals. The calculations were done with data collected from a single sweep of sine waves. To avoid interferences from transient behaviour of the membrane and equipment, the sweep for data collection was preceded by three sweeps of the command signal. Two impedance curves each consisting of fifty-three frequency lines were recorded consecutively. The fundamental frequencies of the two curves were 0·05 and 3·2 Hz. The two curves were merged and in this paper plotted as impedance loci (Nyquist plots).

A two-membrane model was assumed for calculations of the apical and basolateral equivalent resistances (R_a and R_b) and capacitances (C_a and C_b). Each membrane was described by a simple RC network. Moreover, we assumed a complex dielectric constant ($\epsilon' - j\epsilon''$) which is generally found in biological membranes and results in the depression of the centre of the semicircle into the region of the positive reactance. With this assumption the impedance of one cell membrane can be described by the Cole–Cole equation (Cole & Cole, 1941):

$$Z(f) = \frac{R_m}{1 + (j\omega R_m C_m)^\alpha}, \tag{1}$$

where $j = \sqrt{-1}$, and R_m and C_m represent the membrane resistance and capacitance respectively. $\omega = 2\pi f$ is the circular frequency and $\alpha = 1 - \theta$. θ is the angle in radians equal to $2\phi/\pi$, where ϕ is the angle (in degrees) between the real axis and the line that connects the centre of the semicircle with its intersections with the real axis. Using eqn (1) to describe the impedances of apical and basolateral membranes and assuming an ohmic resistance for the impedance of the paracellular shunt path (R_p), we calculated the transepithelial impedance as:

$$Z_t = R_s + \frac{As^\alpha + B}{Cs^{2\alpha} + Ds^\alpha + 1}, \tag{2}$$

where R_s represents the resistance between the voltage electrodes in series with the epithelium and $s = j\omega$. A, B, C and D are related to the resistance and capacitance of the apical and basolateral membrane:

$$A = R_p[R_a(R_b C_b)^\alpha + R_b(R_a C_a)^\alpha]/N, \tag{3}$$

$$B = R_p(R_a + R_b)/N, \tag{4}$$

$$C = R_p(R_a C_a R_b C_b)^\alpha/N \tag{5}$$

and $\quad D = \{R_b(R_a C_a)^\alpha + R_a(R_b C_b)^\alpha + R_p[(R_a C_a)^\alpha + (R_b C_b)^\alpha]\}/N, \tag{6}$

with $N = R_a + R_b + R_p$. A, B, C and D were determined by the non-linear curve fitting of eqn (2) to the impedance data. The real as well as imaginary parts were utilized in the fitting procedure. Weight factors, equal to the absolute value of the recorded data, were attributed to the data points. In this way all impedance data points delivered a comparable contribution to the sum of squares of differences. R_a, R_b, C_a and C_b were calculated by solving eqns (3)–(6). R_p was independently determined by blocking the transcellular ion movements. We used 50 μM-amiloride to block I_{Na} and Cs_2SO_4 Ringer solution to inhibit I_K.

Impedance curves were usually recorded at 10 min intervals, each record taking about 150 s. Except for these interruptions, I_{sc} was continuously recorded and the 10 mV/1 s pulses were applied at intervals of several minutes.

Microelectrode experiments were done with the technique of Nagel (1978). The skin was mounted horizontally in a modified Ussing-type chamber. The tissue was continuously perfused on both sides of the 0·4 cm² of exposed area. The transepithelial potential difference was clamped to zero, except for the 200 ms periods during which the transepithelial potential difference was clamped to 20 mV for measurements of fractional resistance (f_{R_0}) and G_t. These voltage pulses were repeated at 5 s intervals. The impalements were made from the mucosal side with a stepping-motor micro-manipulator. Before shifting to GA solutions on the serosal side, the intracellular location of the microelectrode was checked by short exposure to either Cs_2SO_4 Ringer solution or to chloride Ringer solution in which sodium was replaced by the impermeant cation N-methyl-D-glucamine (NMDG). Both procedures are expected to make the fractional resistance of the apical membrane approach 100 % and to cause its hyperpolarization together with characteristic changes in I_{sc} and G_t. The fractional resistance, f_{R_0}, is the ratio between the apical and the whole transcellular resistances, expressed as a percentage. A Flaming–Brown P-87 puller was used to pull the micropipettes from fibre capillaries (Hilgenberg, Malsfeld, FRG). The input resistance of the microelectrodes, filled with 3 M-KCl solution, ranged between 40 and 60 MΩ. The apical membrane potential (V_0) is measured between the microelectrode, intracellularly located in the principal epithelial cells and the mucosal voltage electrode. The parameters f_{R_0}, V_0, G_t, I_{sc} and the resistance of the microelectrode were simultaneously recorded with a multichannel strip-chart recorder and with a data collection program into a computer system. The data collected by the digital data acquisition system were replotted as illustred in Fig. 7.

The experiments were done in April and May, at room temperature (around 25 °C), with less than 1·5 °C variation throughout the protocols.

RESULTS

Effects on I_{sc} and G_t

Preliminary experiments, aimed to establish the experimental protocols, indicated that in the submillimolar range (below 0·005 % or 0·5 mM), GA produces an initial increase of I_{sc}. The stimulatory phase lasted for 10–20 min and was followed by a slow inhibition. The variability among preparations was large in this concentration range, while above 0·005 % (w/v), GA consistently inhibited I_{sc}. The main features of GA-induced inhibition of I_{sc} were the same at increasing GA concentrations but, as expected, the changes proceeded at a higher rate. As we were interested in the effects of mild GA treatment, the highest GA concentration we used was 0·1 % (w/v). The inhibitory effect could not be reversed upon washing out the drug. In view of this, throughout the experiments reported here, the tissues remained in contact with GA, once it was applied. In the experiments with sulphate Ringer solutions, we worked at the median GA concentration of 0·025 % (w/v).

The effect of GA on skins incubated with NaCl solutions is illustrated in Fig. 1. Glutaraldehyde was either added to the mucosal (M, panel *A*) or to the serosal (S, panel *B*) side. Independent of the side of application, long exposure (2 h) to GA caused a reduction of I_{Na} to values close to zero. In experiments with 0·025 % (w/v)

GA on the serosal side, I_{Na} dropped in 60 min from $24.6 \pm 1.7 \,\mu A/cm^2$ to $5.4 \pm 2.1 \,\mu A/cm^2$ ($n = 4$) and in four experiments with mucosal 0.025% (w/v) GA, I_{Na} decreased from $20.5 \pm 3.8 \,\mu A/cm^2$ to $4.2 \pm 0.2 \,\mu A/cm^2$. A marked difference between the effects of mucosal and serosal application of GA was observed in the initial phase

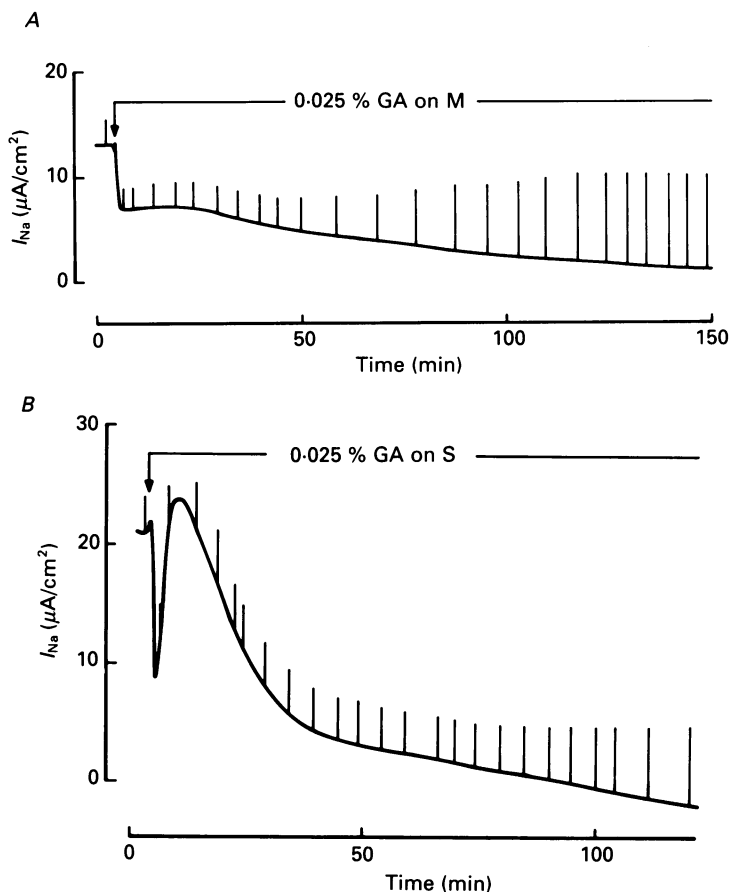Fig. 1. Time course of the effect of glutaraldehyde on the short-circuit current across isolated frog skins. The tissues were incubated with NaCl Ringer solution on both sides. The transepithelial current is indicated on the ordinate as I_{Na} because it is mainly carried by Na^+ ions, which cross the apical membrane down their electrochemical gradients and are actively pumped out of the epithelial cells across the basolateral membrane. Glutaraldehyde (GA; 0.025% w/v) was added to the mucosal (M, panel A), or the serosal compartment (S, panel B). The vertical bars are the current responses caused by 10 mV pulses of 1 s duration.

after application of the compound. From M side (Fig. 1A), GA caused a rapid drop followed by a plateau and a quasi-exponential fall. On the other hand, serosal GA (Fig. 1B) caused an initial drop in I_{Na} followed by a recovery phase, where I_{Na} sometimes surpassed the control values, as in the example shown in Fig. 1B. This stimulatory phase was then followed by a progressive fall of I_{Na}. The differences in

initial time courses of I_{Na} inhibition from mucosal and serosal sides were consistently observed in a series of twenty-four experiments with skins immersed in NaCl Ringer solution.

Immediately following GA application the transepithelial conductance was sometimes reduced, but in all of the twenty-four experiments with skins bathed in
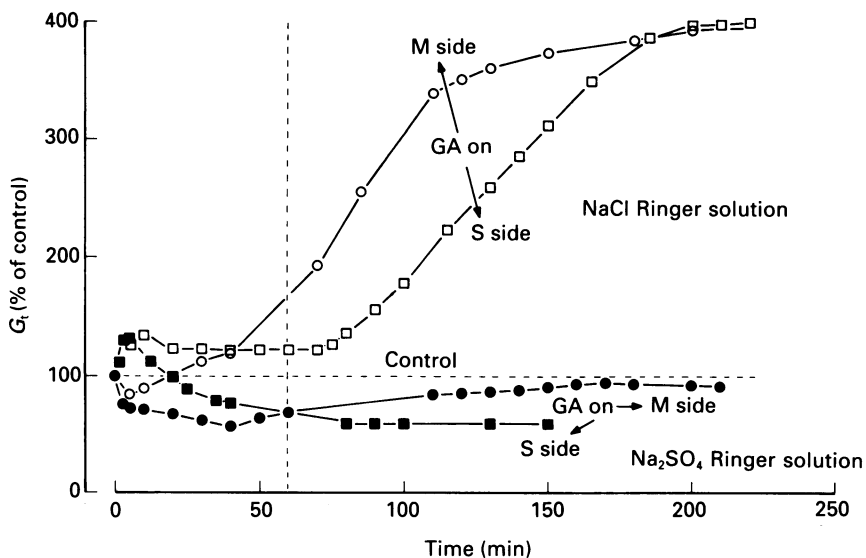

Fig. 2. Time course of the transepithelial conductance (G_t) after GA application. The skins were bathed on both sides with either NaCl Ringer solution (open symbols), or with Na_2SO_4 Ringer solution (filled symbols) and exposed to 0·025% (w/v) GA on either mucosal (M) side (circles) or serosal (S) side (squares). The figure shows data from single experiments, but is representative for twelve experiments (four conditions, three experiments in each case). The G_t values were normalized with respect to control. The control conductance, pooled for the six skins immersed in NaCl Ringer solution, was $0·35 \pm 0·05$ mS/cm^2 and for the six skins immersed in Na_2SO_4 Ringer solution it was $0·26 \pm 0·02$ mS/cm^2 (mean $\pm$ s.e.m.).

NaCl Ringer solution, G_t markedly increased after 60 min of GA treatment (Fig. 2). The events described above occurred in a similar way in experiments with the isolated epithelium.

Similar experiments to those shown in Fig. 1 were performed with skins incubated with Na_2SO_4 Ringer solutions. They showed an essentially similar time course of I_{Na} after GA administration, but we did not observe any increase in G_t after long exposure. Figure 2 compares the time courses of G_t after mucosal and serosal GA application in skins incubated with either NaCl or Na_2SO_4 Ringer solutions. These results demonstrate that long exposure to GA opens a conductive pathway which depends on Cl$^-$ in the incubation media. Moreover, as we were unable to depress the GA-induced G_t with amiloride, we are dealing with an increase of Cl$^-$ conductance. It is likely that this pathway resides in the paracellular route for ion movements or perhaps in a specialized Cl$^-$ conductive cell type (Hviid Larsen & Kristensen, 1978). In order to investigate the effects of GA on the cation movements across the principal

epithelial cells, we used sulphate solutions as incubation media. So far we demonstrated that GA inhibits I_{Na}. It remains unclear from these data whether GA inhibits the apical Na^+ uptake directly by blocking the Na^+-selective channels, or by diminishing the driving force (intracellular potential difference) by inhibition of the
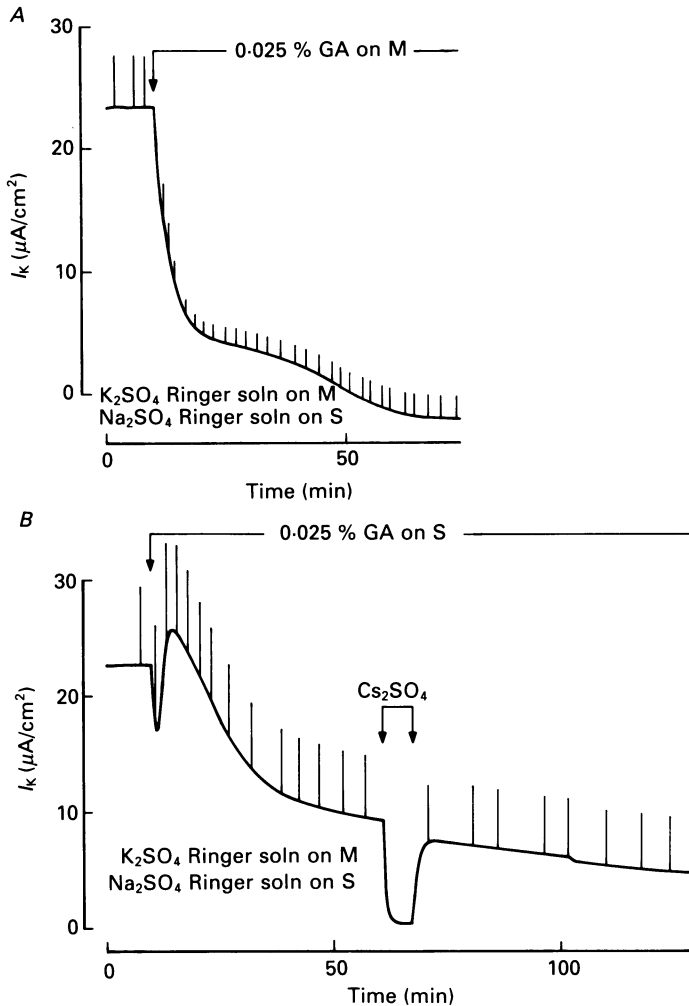

Fig. 3. Effect of GA on the short-circuit current in skins incubated with sulphate Ringer solutions. The serosal solution was Na_2SO_4 Ringer solution, while the mucosal side was exposed to K_2SO_4 Ringer solution. Since in these conditions the transepithelial current is chiefly carried by K^+ ions, which flow down their electrochemical gradient, the current is indicated as I_K on the ordinate. 0·025 % (w/v) GA was added to either mucosal (M, panel *A*), or serosal (S, panel *B*) compartments.

basolateral K^+ conductance or reduction of the active transport and thus the electrogenicity. Further experiments described in this study were designed to localize the site of action of GA. As the apical membrane of the skin of *Rana temporaria* contains K^+ channels which reside in parallel with the Na^+ channels in the

granulosum cells (Van Driessche, 1984), we also tested whether the currents carried by these channels are inhibited by GA. Figure 3 illustrates such experiments in which K^+ currents were recorded in the presence of a M–S-oriented K^+ gradient: in the mucosal compartment there was K_2SO_4 Ringer solution and the serosal one
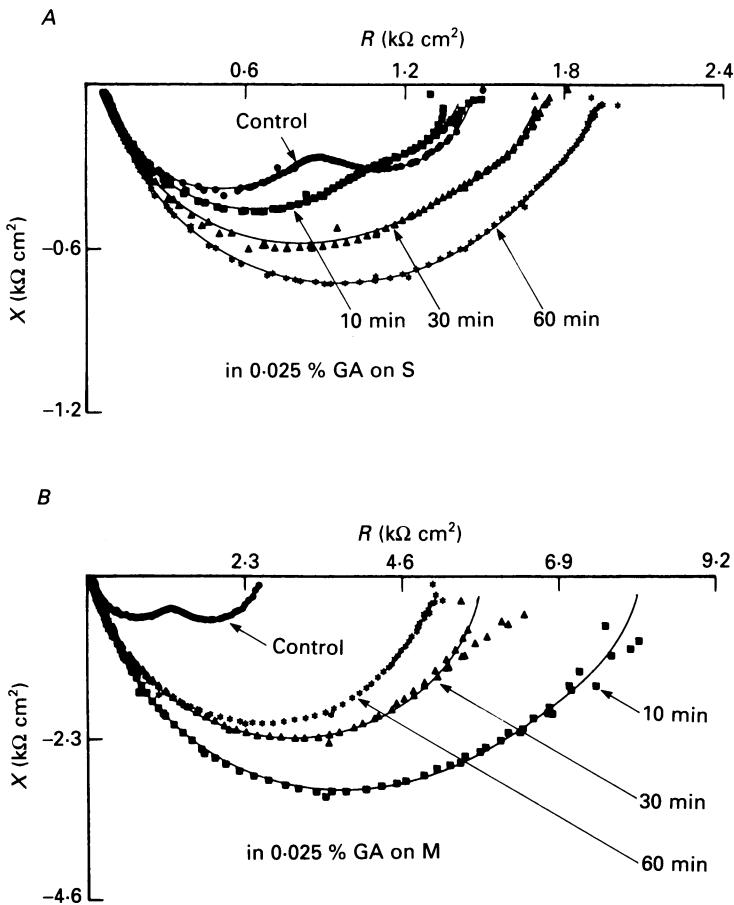

Fig. 4. Effect of GA on transepithelial impedance of frog skins. Nyquist plots of the impedance of frog skins before (control) and after different periods of exposure to 0·025 % (w/v) GA on either serosal (S, panel *A*) or mucosal (M, panel *B*) side. The skins were bathed on both sides with Na_2SO_4 Ringer solution, which is hypotonic, and treated with oxytocin (0·1 U/ml) on the serosal side. The impedance loci (or Nyquist plots) consist of the ensemble of points defined by the values of resistance (*R*) and capacitive reactance (*X*) of the skin, each corresponding to a sine wave of a given frequency. The high-frequency points are to the left.

contained Na_2SO_4 Ringer solution. Surprisingly enough, the time courses of I_K following serosal as well as mucosal application of GA strongly resembled those discussed above for I_{Na}. Mucosal GA elicited a biphasic inhibition of I_K, whereas serosal administration displayed rapid inhibition followed by a stimulatory action and a subsequent quasi-exponential fall. Thus, it appears either that the effects shown in Figs 1 and 3 are the result of GA attack on one protein at the apical

membrane and one at the basolateral membrane regulating both I_{Na} and I_K, or that different proteins are affected by GA with similar time courses. On the basis of these data alone, we cannot rule out any of the two possibilities, but the first one seems simpler. In none of the experimental situations described so far was any recovery observed with GA on M side, as exemplified in Figs 1*A* and 3*A*.
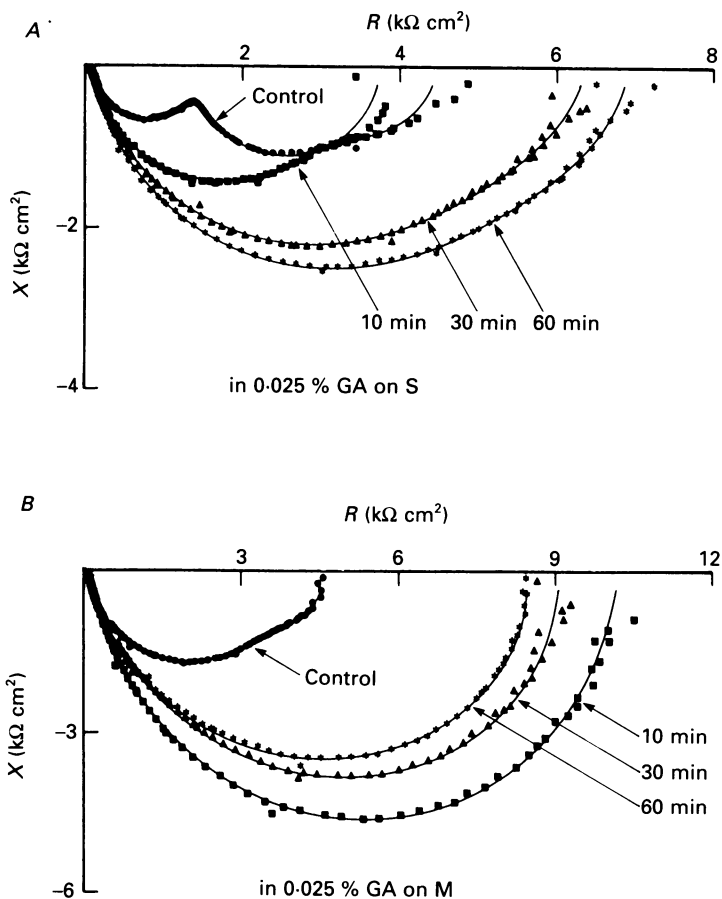

Fig. 5. Same kind of impedance loci as in Fig. 4 for frog skins bathed with K_2SO_4 Ringer solution on M side and with Na_2SO_4 Ringer solution on S side.

The above presented indications that GA inhibits the apical channels for both Na^+ and K^+ and that it opens a paracellular way for Cl^- suggest the occurrence of major resistive changes within the epithelial cell membranes. In order to locate and to quantify these changes, we performed impedance measurements.

Impedance measurements

In NaCl Ringer solution, the transepithelial impedance is determined over the entire frequency range by the characteristics of only the apical membrane (Van Driessche, 1984), which makes the Nyquist plot appear simply semicircular. In order to reliably obtain R_a, C_a, R_b and C_b and to observe the effect of GA treatment on each

of them, the two semicircles corresponding to high and low frequencies have to be distinct. To obtain this separation, we performed the impedance measurements on skins bathed with hypotonic sulphate solutions (Smith, 1971) and treated with oxytocin (0·1 U/ml in the serosal compartment).
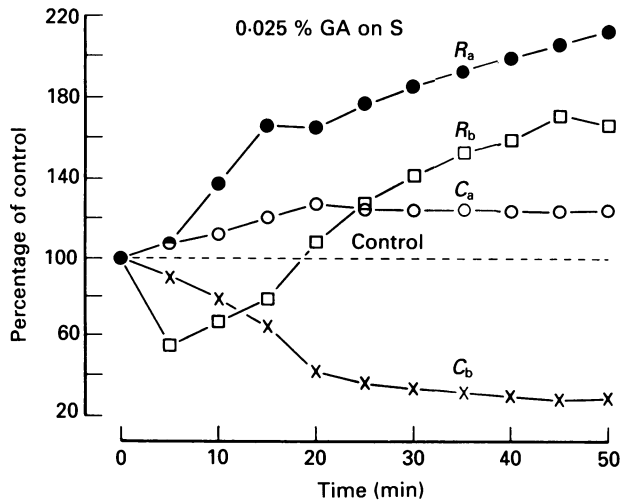

Fig. 6. Time courses of the resistances (R_a and R_b) and capacitances (C_a and C_b) of the apical and basolateral membranes of a frog skin bathed with Na_2SO_4 Ringer solution on S side and with K_2SO_4 Ringer solution on M side and exposed since zero time to 0·025 % (w/v) GA on S side. All the parameters are normalized with respect to the values before GA (control). The graph shows data from a single experiment, but which are typical for a series of five experiments in which the control values were as listed in Table 1.

As illustrated in Fig. 4, the application of GA in either compartment produces conspicuous effects on the impedance of the skin, when in bilateral Na_2SO_4 Ringer solution.

Qualitatively, two features consistently appear on the impedance records: (i) GA causes, from both M and S sides, a progressive merging of the two semicircles, such that, after less than 60 min, the characteristics of the two membranes cannot be distinguished; (ii) when GA is in compartment S, the changes in impedance are progressive, while from the M side, it produces the largest impedance change within the first 10 min. Figure 5 shows that the same features (i) and (ii) appear in the impedance records of the skins bathed with K_2SO_4 solution on M side and with Na_2SO_4 Ringer solution on S side.

The characteristic time course of the changes in apical and basolateral resistances and capacitances is shown in Fig. 6 for a typical experiment, in which the impedance recordings were made at 5 min intervals and it was possible to have reliable double fits of them up to 50 min. This is not always possible, especially when GA is in the M compartment (because of the above-mentioned feature (ii)). The absolute values of the apical and basal parameters for the series of experiments done with GA in the S compartment, before and after 30 min exposure to the drug, are given in Table 1.
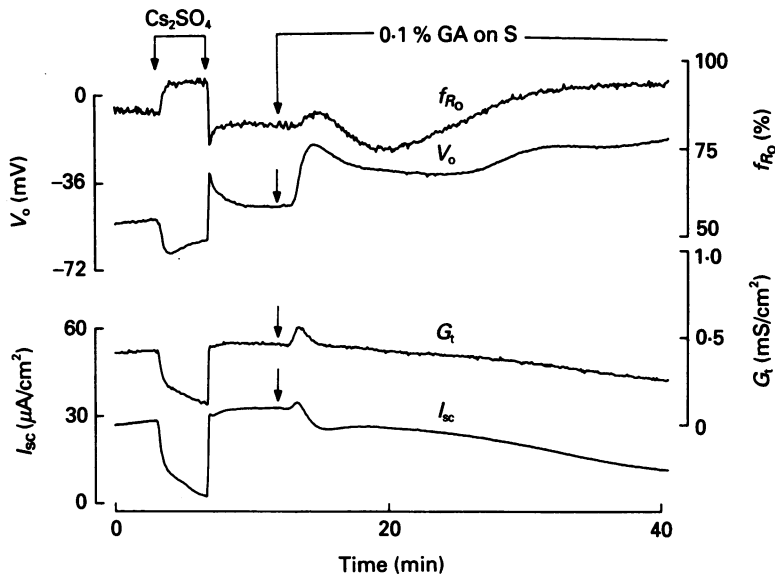
Fig. 7. Microelectrode recording showing the changes in the fractional resistance (f_{R_o}) and the transmembrane potential (V_o) of the apical membrane of a frog skin. 0·1 % (w/v) GA was applied on S side at the moment indicated by the arrows. The transepithelial conductance (G_t) and the short-circuit current (I_{sc}) were simultaneously recorded. The skin was bathed on both sides with Na_2SO_4 Ringer solution, except for the brief period when Cs_2SO_4 Ringer solution was applied on M side.

TABLE 1. Values of the apical (subscript a) and basolateral (subscript b) resistances (R) and capacitances (C) of frog skins bathed with sulphate Ringer solutions, before (control) and after 30 min exposure to 0·025 % (w/v) glutaraldehyde (GA) in the S compartment. Mean ± S.E.M., $n = 5$

	R_a (kΩ cm^2)	R_b (kΩ cm^2)	C_a (μF/cm^2)	C_b (μF/cm^2)
Control	2·76 ± 0·62	4·22 ± 1·06	1·58 ± 0·12	43·54 ± 7·03
30 min in GA	5·19 ± 1·17	6·00 ± 1·25	2·58 ± 0·36	12·27 ± 1·06

The percentage ratios of R_a, R_b, C_a and C_b values after 30 min exposure to GA, against the corresponding control values, are: 188, 142, 163 and 28, respectively. These ratios, calculated with the data in Table 1, confirm that Fig. 6 is representative for the ensemble.

Microelectrode recordings

In order to ensure that the effects of GA will appear within the usual duration of successful punctures, in the microelectrode experiments we used more concentrated (0·1 % w/v) GA solutions.

Figure 7 shows examples of recordings made with skins immersed in Na_2SO_4 Ringer solutions. I_{sc} and G_t, which were recorded with the conventional macroelectrodes, convey essentially the same information as obtained with the chamber used in impedance measurements. However, because of different perfusion rates, chamber geometry and differences in GA concentration, the time courses of the

changes appear to be different. The initial fall of I_{sc} is not as steep as on the recordings in Figs 1 and 3, but is still clearly present. The recovery is less pronounced and the subsequent slower decrease is equally well seen. G_t traces show a tendency to decrease, in accord with data in Fig. 2.

The evolution of the two parameters specific for microelectrode records (f_{R_0} and V_0) is rather similar in NaCl (not shown) and in Na_2SO_4 Ringer solutions. In both cases there was a rapid rise and fall of f_{R_0}, followed by its slower increase towards a steady value. Before GA treatment, f_{R_0} was $64.6 \pm 8.2\%$ and it reached saturation at $89.7 \pm 2.1\%$ (means $\pm$ S.E.M., nine experiments), which indicated a more pronounced increase of R_a values compared with R_b.

An effect of GA, revealed by microelectrode experiments, is the large depolarization of the apical membrane of epithelial cells. In both chloride and sulphate Ringer solutions, the decrease in the absolute values of V_0 had an initial rapid phase and a subsequent slower one, the overall depolarizations produced in 30 min being 42.5 ± 4.3 mV (mean $\pm$ S.E.M., nine experiments).

The major change in V_0 occurred rapidly, within 5 min after GA administration. Such depolarizations of the intracellular potential can be caused by either an elevation of the apical membrane conductance, a reduction of the basolateral membrane conductance, or by a decrease of the electrogenicity of the active transport mechanism. However, during the phase where V_0 depolarized rapidly, minor changes in f_{R_0} occurred. Therefore it is most likely that the initial depolarization is not related to alterations of the membrane conductances, but originates from an inhibition of the electrogenic active transport. On the other hand, during the following long exposure to GA, only minor changes in V_0 occurred, whereas the major effect was the change in f_{R_0}. Therefore long time effects of GA seem to originate from changes in the conductance of apical as well as basolateral membrane. This agrees with the results of impedance measurements presented above.

DISCUSSION

A previous report that millimolar concentrations of GA inhibit I_{sc} in frog skins bathed in NaCl Ringer solution (Mărgineanu, Rucăreanu, Flonta & Finichiu, 1984), attributed the effect to the inhibition of Na^+–K^+ pumps. In that study, none of the other parameters reported here could be followed up and equipment limitation led to skipping the first drop in I_{sc}. The values of I_{sc} are a composite measure of the function of ionic channels – whose population is multifarious on both apical and basolateral membranes (see review by Van Driessche & Zeiske, 1985) – and of the basolateral Na^+–K^+ pumps. Together with the fact that GA cross-links the lysinyl residues on whichever protein they are, this makes any simple explanation of the inhibition by GA of I_{sc} appear unrealistic.

The matching of GA effects on I_{sc} and G_t in intact and in split skins allows the elimination of the possibility that they might be due to cross-linking by GA of the collagenous extra-epithelial structures. The concentration limits of what one could consider as 'mild' effects of GA on the electrophysiological properties of frog skin are 0.005% (w/v), for the appearance of I_{sc} inhibition, and 0.1% (w/v), for the complete blocking of I_{sc} within 1 h. The investigation reported in a brief abstract by

Fallenstein & Lindemann (1979), concerning the effect of GA on the apical Na^+ permeability of frog skin, was done at the above-mentioned upper limit of GA concentration (0·1 % w/v).

Probably the most straightforward inference from our data is that prolonged exposure to millimolar concentrations of GA results in a large increase in the chloride permeability of the frog skin. This evidence, illustrated by Fig. 2, together with the fact that the transitory recovery is observed only with GA on S side, prompted us to tentatively ascribe that recovery to the activation by GA of an outwardly directed Cl^- current. However, until acquiring more direct data to support the explanations, we refrain from speculating on it.

The increases in R_a and R_b, revealed by impedance analysis (Table 1), indicate that GA blocks both apical and basolateral ionic channels. Within a simplified view of epithelial organization (Kristensen & Ussing, 1985), one would consider that GA blocks the apical sodium channels and the basolateral potassium channels. However, the similarity of GA effects when I_{sc} is carried by either Na^+ (Fig. 1) or K^+ (Fig. 3), indicates that the apical K^+ channels (Van Driessche, 1984) are also blocked, or that the two ionic pathways depend on the same protein. It was already noted that the larger increase of R_a with respect to R_b is confirmed by the rise in f_{R_0} (Fig. 7) and it should be noted that it also agrees with the larger drop in I_{sc} values when GA is applied on M side (Fig. 3*A*), as compared with serosal applications (Fig. 3*B*).

Summing up the above discussion, it appears that the apical sodium channels and the apical and basolateral potassium channels all possess free amino groups, probably belonging to lysine residues, in such locations that their cross-linking by GA results in the promotion of closed conformations. In contrast, in the proteins transporting Cl^-, the cross-linking of the free amino groups promotes the conducting conformation.

The quasi-exponential fall in I_{sc}, equally well seen both on the conventional records (Figs 1 and 3) and on those from the microelectrode chamber (Fig. 7), might be attributed to the GA-induced increase of both R_a and R_b (Table 1) while, as suggested by microelectrode experiments, the first drop in I_{sc} is to be attributed to the rapid block by GA of the $Na^+–K^+$ pumps.

A noteworthy effect of GA treatment of the frog skin is the very large decrease in basolateral capacitance. This might be, in principle, ascribed to decreases in either or both surface and dielectric constants of the basolateral membrane as a result of protein cross-linking. However, if the dielectric constant would actually be changed, a similar effect on the apical membrane could be expected. Because we observed the opposite, we consider that the large GA-induced decrease in C_b is due mainly to the reduction of the basolateral membrane area. In any event, the very occurrence of capacity changes of such an extent casts doubts on the validity of the assumption – implicit in electron microscopical studies – that GA fixation preserves the geometric properties of the cells.

In conclusion, the present work provides information on the manner in which the electrical properties of the isolated amphibian skin and the transepithelial active transport of sodium are influenced by the protein cross-linker glutaraldehyde, at concentrations which are up to three orders of magnitude less than those used in fixation procedures. The quite pronounced effects reported here appear as a strong

indication that lysine residues are present in some key positions of the proteins regulating transepithelial ion transport. A practical sequel of this study is that if GA is to be included as a stabilizing ingredient in Ringer solutions and in storage fluids of the epithelial grafts, its concentration should not exceed 0·005 % (w/v).

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