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# TIME COURSE OF PUMP INHIBITION BY OUABAIN IN AMPHIBIAN EPITHELIA

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## Summary

Inhibition by ouabain of rheogenic $Na^+$ transport across the basolateral membranes of frog skin is found to be manifest within 3—4 min. This rate of pump inhibition is not different from the rate of diffusion through extracellular tissue layers between the serosal bath and the actual site of action, i.e., the epithelial cell layers. It is concluded that the well-known slow time course of decrease in transepithelial current flow is due to ionic redistribution and conductance changes of the epithelial membranes secondary to pump inhibition.

Complete inhibition of active transepithelial $Na^+$ transport by ouabain in isolated amphibian epithelia requires incubation for more than 30 min even in the presence of comparatively high concentrations ($1 \cdot 10^{-4}$ M) of the glycoside [1]. This is much longer than would be anticipated from the kinetics of ouabain binding to ($Na^+$ + $K^+$)-activated ATPase extracted from other tissues [2], ouabain binding to the isolated frog skin epithelium [3] or to the inhibitory effect of ouabain on disaggregated frog skin cells [4]. The slower time course of ouabain effects on transepithelial $Na^+$ transport and their rather low reversibility could be a consequence of diffusion delay through connective tissue and intercellular spaces [3]. On the other hand, inhibition of the Na pump could be as fast as anticipated while secondary changes at mucosal and/or basolateral membranes resulting from the inhibition are responsible for the observed behaviour of the transcellular current flow. Strong evidence supporting the latter possibility will be provided in the present report.

The experiments were performed on isolated frog skin of *Rana temporaria* mounted in a lucite chamber and impaled with microelectrodes as described recently [5]. The skins were kept short-circuited throughout (i.e.,

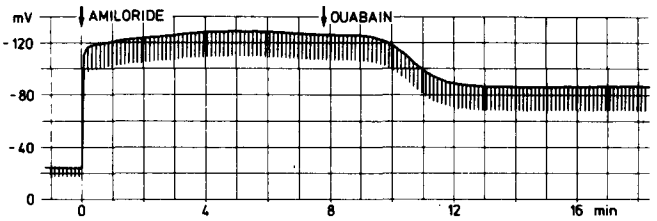


**Fig. 1. Record of the intracellular potential of short-circuited frog skin epithelium (with reference to the mucosal bathing solution; reproduced from the original record). The thin vertical lines reflect voltage changes across the apical border ($\Delta V_o$) upon voltage clamping the transepithelial potential to $V_t$ = 20 mV. $\Delta V_o/\Delta V_t$ represents the fractional resistance of the apical border relative to the transcellular resistance ($R_o/R_c$). The time scale was arbitrarily set '0' when amiloride (final concentration: $1 \cdot 10^{-4}$ M) was added to the mucosal bathing solution. Note the resulting change of $\Delta V_o$ which was equivalent to increase of $R_o/R_c$ from 0.31 to 0.98. Ouabain was added to the serosal side at a final concentration of $2 \cdot 10^{-4}$ M.**

transepithelial potential $V_t$ = 0 mV) and the short circuit current ($I_{sc}$) was recorded on one channel of a strip chart recorder along with the intracellular potential ($V_{sc}$) and the ratio of mucosal border to transcellular resistance $F(R_o)$ as measured from the voltage change across the mucosal border on voltage clamping of the transepithelial potential, $V_t$, to 20 mV for 150 ms every 5—8 s. Both sides of the skin were continuously perfused with Ringers' solution (110 mM NaCl, 2.5 mM $KHCO_3$, 1 mM $CaCl_2$) at a rate which permitted exchange of the chamber fluid within 15 s. Amiloride-containing solution ($1 \cdot 10^{-4}$ M) was applied from the mucosal side, ouabain was added to the serosal perfusion fluid to give a final concentration of $2 \cdot 10^{-4}$ M. Elevation of serosal potassium concentration was made by equimolar substitution of KCl for NaCl in Ringers' solution.

Fig. 1 shows the result from a typical experiment which was started after $I_{sc}$ had stabilized at a value of 78 $\mu A/cm^2$ and a cell was impaled. Addition of amiloride ($1 \cdot 10^{-4}$ M), which has been demonstrated to block $Na^+$ uptake across the mucosal border [6,7], hyperpolarized rapidly $V_{sc}$ from a control value of —24 mV to a maximal value of —129 mV concomitant with a decrease of the short circuit current to less than 2 $\mu A/cm^2$. Simultaneously the fractional resistance of the mucosal border increased to 98% of the transcellular resistance. This is equivalent to a ratio of 50 : 1 for the relation of mucosal to basolateral border resistance and indicates effective blockage of the mucosal membrane $Na^+$ conductance. Under these conditions, when there is virtually no transcellular current flow, equilibrium of the individual ion fluxes must exist across the basolateral border and, accordingly, the measured intracellular potential, $V_{sc}$, represents a direct estimate of the effective e.m.f. across this border ($E_i$) [8]. Perhaps the existing contribution of the apical border e.m.f. may be neglected in view of the high resistance of this border compared to that of the basolateral membranes (i.e., 50 : 1).

A detailed analysis of $E_i$ is of particular interest for resolving the components that contribute to the effective e.m.f. across the basolateral border. From osmotic considerations it can be calculated that a large fraction of $E_i$ is accounted for by the Nernst potential of $K^+$ ($E_K$). If $K^+$ were the only intracellular cation, a maximal value of about —100 mV could be calculated for $E_K$. The hyperpolarization above this value (about 30 mV in the above experiment) after the administration of amiloride has been suggested to be due to

rheogenic $Na^+$ transport* across the basolateral membranes [9]. This component disappears spontaneously within 15—25 min, reflecting presumably the depletion of $Na^+$ from the cytoplasmic tissue pool and concomitant decrease of the pump rate to very low values. In Fig. 1, the onset of this slow spontaneous decay of the hyperpolarization is detectable between 5 and 9 min after the administration of amiloride. Thereupon, ouabain was added to the serosal bathing solution. After a delay of 1 min, $E_i$ decreased rapidly until a steady-state value of −88 mV was reached within 2.5 min. Note that this value of $E_i$ is clearly less than the above mentioned maximal value of $E_K$ and would agree with an entirely passive origin of the basolateral membrane potential.

In order to provide a quantitative estimate of the velocity of the ouabain-induced depolarization, the change in $E_i$ was plotted on a semilogarithmic scale vs. time as shown in Fig. 2. It is evident that more than 80% of the decay is fairly well fitted by a single exponential term with a time constant of 0.70 min and a delay of 2.3 min relative to the actual addition of ouabain. The mean time constant of the ouabain-induced decay of $E_i$ as estimated from this and three other similar experiments was 0.67 ± 0.03 min (± S.E.) which is considerably faster than the rate of spontaneous disappearance of the hyperpolarization with $\tau$ = 4.0 ± 0.6 min [9]. Alterations of the intracellular potassium concentration to explain either one of the time constants are highly unlikely. The intracellular potassium content has been reported to remain constant for 1 h after ouabain incubation or even longer in the presence of mucosal amiloride [10]. Accordingly, the observed ouabain-induced depolarization of $E_i$ would feasibly be associated with inhibition of the rheorgenic Na pump.

It is interesting to compare the velocity of the change in $E_i$ after ouabain addition with the rate of depolarization obtained upon increasing the serosal $[K^+]$. In six experiments the rate of depolarization of $E_i$ upon increasing the serosal $[K^+]$ to values between 28 and 110 mM was found to be described by

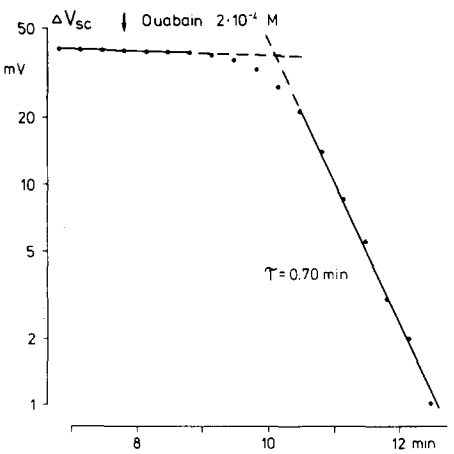


**Fig. 2. Semilogarithmic plot of change in $E_i$ ($E_i$ minus $E_i$ after approach to the steady value after ouabain addition) observed on addition of ouabain to the serosal perfusion solution (arrow). Data are from experiment shown in Fig. 1.**

*The term 'rheogenic' refers to pump operation in a constant current mode, i.e. with quasi-infinite internal resistance of the pump pathway. In contrast, 'electrogenic' would indicate a pump pathway with internal resistance magnitude similar to that of parallel diffusional pathways.

a single exponential with $\tau = 0.75 \pm 0.08$ min (± S.E.). Thus, it would appear that the ouabain-induced depolarization is just as fast, or even faster, than the depolarization by serosal $K^+$, although the diffusion coefficient in free solution of ouabain ($M_r$ 584.7) is, presumably, considerably less than that of potassium. This unexpected rapid ouabain-induced decline of $E_i$ might result from the fact that ouabain is bound to the ($Na^+ + K^+$)-ATPase from concentrations far below $2 \cdot 10^{-4}$ M and binding to the enzyme thus precedes equilibration within the intercellular space.

At face value, the present results appear to be inconsistent with those of a previous study by Cala et al. [3], who reported a time constant of about 30 min for saturation binding of ouabain to ($Na^+ + K^+$)-ATPase of isolated frog skin epithelium. For experimental reasons, they used ouabain at concentrations as low as $1 \cdot 10^{-6}$ M. Unlike their study, $2 \cdot 10^{-4}$ M ouabain was used in the present study in order to obtain a maximal rate of ouabain binding. It may be expected that a saturation binding occurs within times that are 1 to 2 orders of magnitude smaller when the concentration is $2 \cdot 10^{-4}$ M than at concentrations of $1 \cdot 10^{-6}$ M. Thus, the inconsistency between the present data and previous results [3] is only apparent.

A slower time course for the ouabain-induced depolarization of $E_i$ has been observed by Helman et al. [11]. Time constants of greater than 3 min can be derived from these data. On the other hand, Fisher and Helman [12] reported that the time constant for depolarization by serosally added potassium was about 4 min. Both of these studies were performed on skins of *Rana pipiens berliendieri* which apparently differ from *R. temporaria* in that diffusion through connective and intercellular tissue layers is much more restricted. Nevertheless, it is interesting to note that the rate of depolarization after ouabain incubation is, in this species, also faster than that after exposure to high [$K^+$], suggesting similar rapidity of pump inhibition.

The above data demonstrate that the rate of inhibition of ($Na^+ + Ka^+$)-ATPase (at least the fraction connected with the rheogenic Na pump) by ouabain is very rapid and presumably not different from that in separated frog skin epithelium [3], isolated epithelial cells of frog skin [4] or other simpler tissues [2]. Consequently, the reason(s) for the slow time course of the decrease in transepithelial $Na^+$ transport after incubation with ouabain should be reconsidered. It has been shown [11] that profound changes of the ionic conductance of the two epithelial membranes result from the pump inhibition, which follow a time course identical to that of the decrease in transepithelial $Na^+$ transport. Irrespective of the origin of these, presumably secondary changes, which are still unclear and requires further investigation, the problem remains to be solved as to what source of energy accounts for the persisting transepithelial net current flow during the period of 30—40 min after ouabain addition, i.e., when the ($Na^+ + K^+$)-ATPase is, according to the present electrophysiological observations, already blocked.

This obvious deviation from basic rules of thermodynamics could be explained if ouabain would rapidly inhibit only the rheogenic component of the $Na^+$—$K^+$ exhange through the pump pathway, which is, indeed, only detectable by the present method. Presumed, neutral (i.e., 1 : 1) exchange of $Na^+$—$K^+$ would be less sensitive to ouabain, this could result in persistence of

transport until eventually the entire ATPase is blocked. In view of previous results regarding the function of the ($Na^+ + K^+$)-ATPase (cf. Ref. 13), this explanation appears highly unlikely. Evidence for the existence of other, ouabain-insensitive transport mechanisms for Na as suggested for renal tubular cells [14] has not been obtained for frog skin epithelium.

One source of transepithelial current flow after blockage of active transport is given by the fact that $Na^+$ is accumulated in the intracellular space almost entirely due to uptake from the mucosal side [10] whereas $K^+$ is mainly lost to the basolateral side. This will generate a net current flow during the non-steady-state period of approach to the new ionic composition of high $Na^+$/low $K^+$ after ouabain addition. It is not clear whether the amount of $K^+$ lost in exchange for accumulated $Na^+$ can be responsible for the entire charge transfer across the epithelium during the period after ouabain addition. From tracer wash-out analysis, accumulation of some 300 nequiv. $Na^+/cm^2$ after ouabain addition has been reported [15] which would explain persistence of transepithelial current flow for rather extended periods of time. Assuming an initial $I_{sc}$ value of 40 $\mu A/cm^2$ which is an average value for frog skin, a time constant of about 11 min is calculated for the decrease in $I_{sc}$ after ouabain addition which is about in the range of experimental observations [1]. Accordingly, the main fraction of persisting current flow would be accounted for by a secondary change, i.e., the redistribution of intracellular cations after inhibition of the active cation transport while the active transport step, per se, is inhibited already at the very beginning of the incubation period with ouabain. It appears possible that the rate of change in cellular electrolyte concentration, the rate of decrease of the cellular conductance and the time course of change in $I_{sc}$ are causatively interrelated. This, however, requires future experimental work with more appropriate techniques. From the present study it can be concluded that ouabain binding to ($Na^+ + K^+$)-ATPase of intact tight epithelia and the actual onset of the effect are not different in time course from that of other simple systems, i.e., they occur at comparatively high velocity.

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