STUDIES ON ION PERMEABILITY: IV. THE MECHANISM OF OUABAIN ACTION ON THE Na⁺-ION EFFLUX IN FROG MUSCLES

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SUMMARY

Ouabain at concentrations above that needed to produce a maximal effect on the equilibrium distribution of K^+ and Na^+ ion in frog muscle (10^{-6} M), when introduced into the washing solution produces an increase of the half time of exchange, t_1, of the Na^+-ion efflux only when the original Na^+-ion efflux was fast. Pre-incubation with ouabain and Na^{22}, produces greater and more predictable effects on the t_1 which increases with the length of pre-incubation up to about 30 hours at $25°C$. Incubation in a moist chamber of muscles briefly exposed to ouabain and then washed in a ouabain-free solution to remove ouabain in the **extracellular** space, cause continued increase of t_1 up to 24 hours.

INTRODUCTION

Ouabain and other cardiac glycosides decrease intracellular K^+ and increase intracellular Na^+ ion. Two mechanisms for these actions have been considered: according to the membrain theory, ouabain acts by interfering with a postulated Na-K pump;[1,2] according to the association-induction hypothesis, ouabain acts as a cardinal adsorbent, shifting the intrinsic equilibrium constant, $K^{oo}_{Na \to K}$, toward a diminished K^+-ion preference over Na^+ ion in their adsorption on cooperatively linked protein sites.[3,4]

In preceding papers, Ling and **Bohr** have reported the results of studies on the steady level distributions of alkali-metal ions: (a) Equilibrium K^+ and Na^+-ion distribution in the presence of ouabain from 8 x 10^{-9} to 3.27 x 10^{-7} M, follows the prediction of a two (or multiple) cardinal site model derived from the association-induction hypothesis.' (b) Normal frog muscle cells selectively accumulate alkali-metal ions in the rank order of $Rb^+ > K^+ > Cs^+ > Li^+ > Na^+$. Contrary to the anticipation of the membrane-pump model, ouabain does not produce an equalization of the distribution ratios of the five ions; instead, the equilibrium distribution assumes a new rankorder: $K^+ > Li^+ > Rb^+ > Cs^+ > Na^+$.[6]

Both of these rank orders were those predicted theoretically.[3]

In the present paper we shall report our studies on the effect of variation in the duration of exposure to ouabain on the kinetics of Na^+-ion efflux of frog sartorius muscles.

MATERIALS AND METHODS

All experiments were performed on the isolated sartorius muscles of northern leopard frogs (*Rana pipiens pipiens*, Schreber) from Wisconsin or Vermont. The basic Ringer phosphate solution used throughout the experiment has the same composition described in a preceding paper.[7] Ouabain was from Sigma Chemical Co., St. Louis, Mo. Carrier-free Na^{Z2} was obtained from Nuclear Science and Engineering Corp., Pittsburg, Pa.

The method of studying the efflux of labeled Na^+ ion, using a well-type γ-scintillation counter was also fully described.[7] To load with Na^{22}, we isolated the muscles sterilely and incubated them at 25°C in a small volume (3 to 5 ml) of Ringer-GIB medium containing streptomycin and penicillin, each at a concentration of 125 µg/ml for at least 18 hours but usually longer.'

RESULTS

Effect of Ouabain Added to the Washing Solution

Though little emphasized, it has been known for some time that the rate of Na^+-ion efflux from normal frog muscle shows considerable variations. Inclusion in the washing solution of ouabain at a concentration considerably above that producing a near maximal effect on the equilibrium distribution of K^+ and Na^+ ion (3.27 x 10^{-7} M),[5] may or may not produce a discernible effect on the rate of Na^+-ion efflux, depending on the time constant of Na^+-ion efflux prior to the application of the drug. Thus, at a concentration of 10^{-6} M, no effect was observed in response to ouabain after an exposure of 100 minutes (Fig. 1) when the original rate of efflux was slow [the half time of exchange (t,) of the "main" portion of the curve was about 60 minutes]. Yet, at the same concentration of ouabain, a noticeable effect was seen after an exposure of 40 minutes when the original efflux rate was high $(t_{1/2} = 30$ min) (Fig. 2). Similarly, Figure 3 shows that at a concentration of 10^{-7} M, ouabain did not produce any observable effect on the Na^+-ion efflux after an exposure of 240 minutes, when the original efflux rate was 50 minutes.

Effect of Re-Incubation with Ouabain .

Whereas 10^{-7} M (10^{-6} M) ouabain added into the washing solution produced no or relatively small effects on the rate of Na^+-ion efflux, a pronounced effect was observed if the same concentration of ouabain was introduced into the pre-incubation solution con-
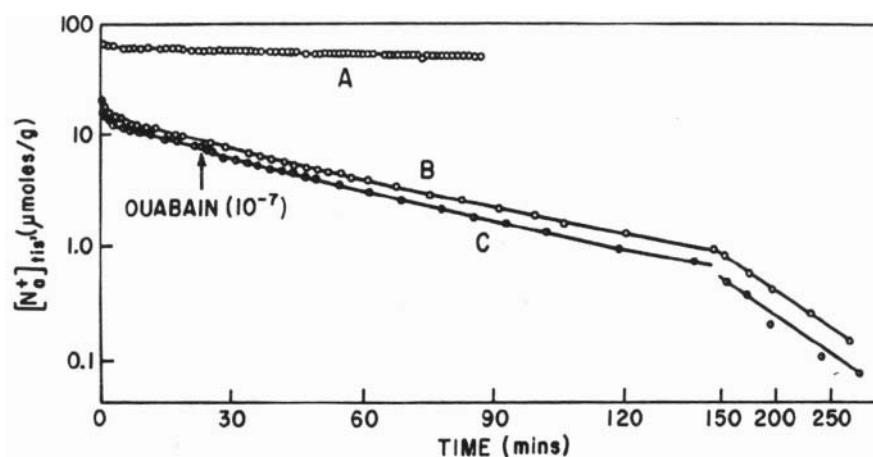
Figure 3. Effect of ouabain on the Na$^+$-ion efflux of frog sartorius muscle.
B and C were the Na$^+$-ion efflux of a pair of sartorius muscles from the same animal and incubated in Na^{22}-labeled normal Ringer phosphate (NRP) for the same length of time (18 hours at 25°C). Whereas both B and C were washed in NRP during the first 23 minutes, only C was switched to a Ringer solution containing 10^{-7} M ouabain. Note change of scale of abscissa after 150 minutes. A was incubated in a similar Na^{22}-containing NRP for the same length of time at the same temperature, except this pre-incubation solution contained 10^{-7} M ouabain. The washing solution for A was NRP containing no ouabain.

taining labeled Na$^+$ ion even though the washing solution used in the efflux study contained no ouabain at all. The top curve of Figure 3 shows that, in this case, $t_{1/2}$ of the bulk of the intracellular Na$^+$ ion has increased from 30 to 290 minutes.

Since, in these experiments, the muscles were exposed to ouabain for a much longer period of time (18 hours) than in those experiments where the ouabain was introduced in the washing solution (2 to 4 hours), we raised the question whether the length of exposure time could account for the difference.

Effect of the Duration of Pre-Incubation in Ouabain on Subsequent Na^c-Ion Efflux

Figure 4 shows the effect of a variation of the duration of pre-incubation of sartorius muscles in a solution containing both 10^{-6} M ouabain and Na^{22}, on the subsequent rate of labeled Na$^+$-ion efflux. In each case, the washing solution used in the efflux study was a Ringer phosphate solution containing 10^{-6} M ouabain. Estimated from the efflux curve, the value of $t_{1/2}$ rises sharply after a two-hour period of exposure to 10^{-6} M ouabain and then increases steadily, but less rapidly, with further exposure to ouabain until the value of t,, levels off after the exposure time has exceeded 30 hours.

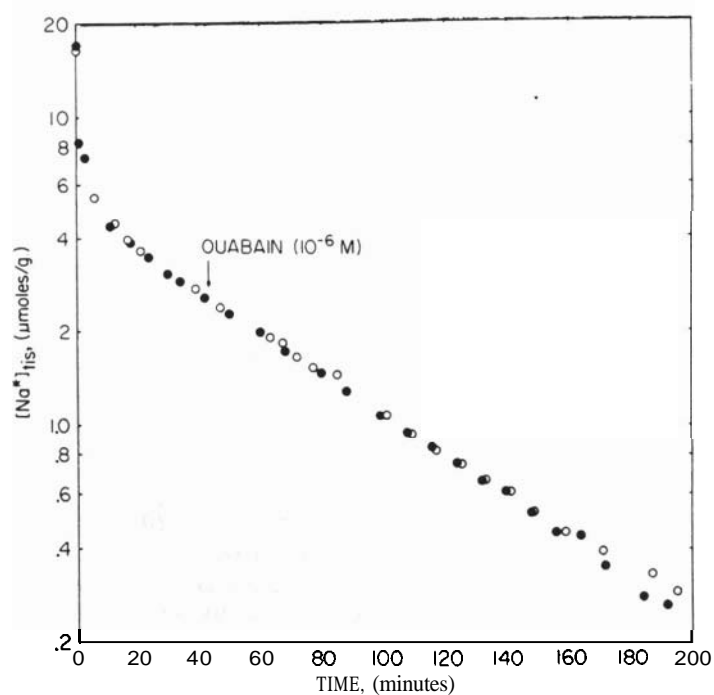This data shows that the duration of exposure to ouabain does indeed play a major

Figure 1. Effect of ouabain on the **Na⁺-ion** efflux of the frog sartorius muscle.
The **Na⁺-ion** efflux of a pair of sartorius muscles from the same frog and incubated in **Na²²-labeled** normal Ringer phosphate for the same length of time **(24** hours at **25°C).** The filled circles represent a muscle washed throughout in NRP. The open circles represent a muscle washed for **42** minutes in NRP, at which time the washing solution was switched to a Ringer containing 10^{-6} M ouabain.
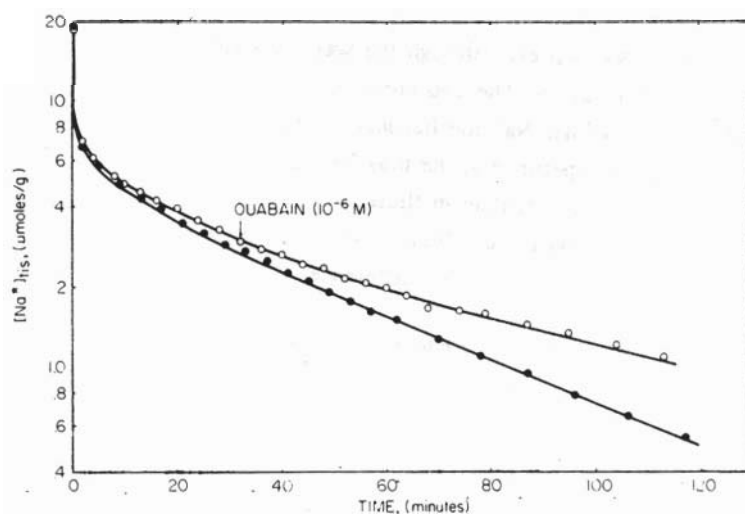

Figure 2. Effect of ouabain on the **Na⁺-ion** efflux of the frog sartorius muscle.
The experiment was identical to that shown in Figure 1, except that the washing solution was changed to a Ringer solution containing 10^{-6} M ouabain after **32** minutes.
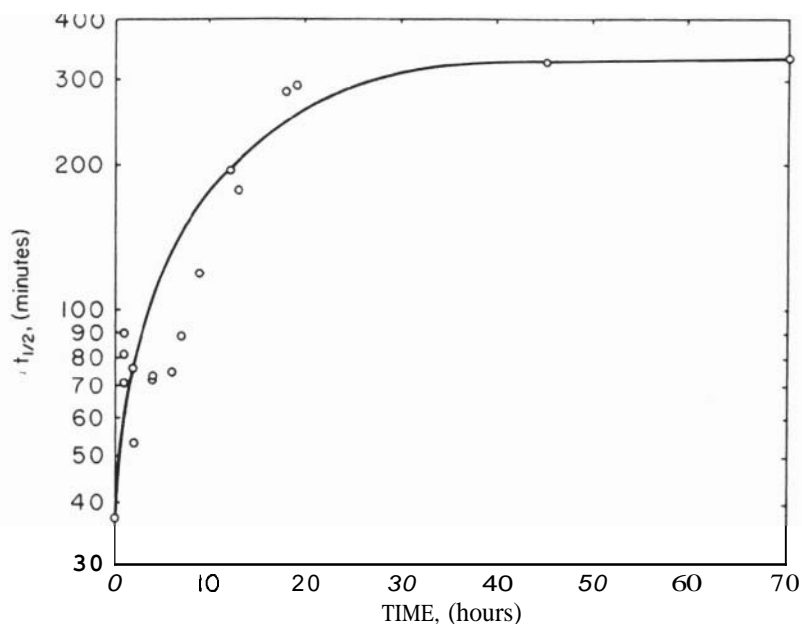
Figure 4. Effect of pre-incubation with ouabain on the Na^+-ion efflux of the frog sartorius muscle. The ordinate shows the length of time of incubation in Na^{22} Ringer solution containing 10^{-6} M ouabain. All muscles were incubated at least 18 hours in Na^{22} Ringer phosphate solution, exposure to ouabain being in the final hours of incubation. The Na^{22} was washed out for one hour or more with Ringer phosphate solution containing 10^{-6} M ouabain. The counts per minute remaining in the tissue were plotted on a semi-log scale versus time and the half time for exchange $(t_{1/2})$ was measured by drawing a tangent to the efflux curve at 30 minutes. Each point represents one experiment.

role in determining $t_.$ It also shows that ouabain added in the pre-incubation solution containing Na^{22} is, in general, more effective than when added to the washing solution. We next attempt to answer the question whether or not the slow completion of ouabain action is due to slow entry of ouabain into the muscle cells.

Effect of Duration of Pre-Incubation in Muscle Exposed Briefly to Ouabain

After isolated sartorius muscles had been equilibrated for 18 hours at $25°C$ in an Na^{22}-containing normal Ringer-GIB medium, enough 10^{-3} M ouabain was added to the bathing solution to give a final ouabain concentration of 10^{-4} M. After shaking at 25°C for **30** minutes, the muscles were removed, blotted and introduced into a vigorously shaken flask containing one liter of cold Ringer phosphate ($0°C$) for 20 minutes. This length of time was shown to be long enough to remove virtually all ouabain in the extracellular space (extending the washing period to one hour did not alter the results) (Table 1).

Table 1. Effect of Incubation in a Moist Chamber on the $t_{1/2}$ of Na-Ion Efflux.
All muscles were fust incubated at 25°C in a normal Ringer phosphate (NRP) solution containing 10^{-4} ouabain for 30 minutes followed by a vigorous washing in 1 Liter of cold NRP. After blotting, the muscles were incubated in a moist chamber for different lengths of time indicated in the first column. Data are the half time of exchange of the "main" portion of the Na-ion efflux and are in minutes. Washouts carried out at 25°C.

Duration of Incubation	Half Time of Exchange $(t_{1/2})$ (minutes)								
	12/3/1968	12/5/1968	8/13/1969	9/2/1969	9/3/1969	9/8/1969	9/12/1969	3/10/1972*	3/12/1972†
1 min	17;25							55;65	25;34;22
5-10 min	68		68;54		72	65			
30-60 min		77;82		119	125		84		
2-3 hrs	58		140;114			71		98;151	
4-6 hrs	106		130		139		105	103;140	24;31;21
18-24 hrs	153;127;124	133;160;136	124	136;180		123;135;172		99;202;117;235	
28-48 hrs	160;150		151	198	180	130	128;135		

* One hour of cold wash.

† No exposure to ouabain.

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After blotting again, the muscles were placed in moist chambers kept at $25°C$. After different time intervals, muscles were then taken out and their Na^+-ion effluxes in normal Ringer phosphate solution studied. Table 1 shows the results of one typical series of these experiments. The time duration label refers to the time of incubation in the moist chamber only.

In these experiments, all muscles had been exposed to the same concentration of ouabain for the same length of time (1 hour pre-incubation and 20 minute washing at $0°C$). Since the extracellular space had been already freed of ouabain, it could not serve as an additional source of ouabain to the cell. Yet, incubation continued to increase the values of t,, for hours. Incubation in moist chambers of control muscles not treated with ouabain, showed no change of the time constant (see Table 1).

DISCUSSION

In a preceding paper we have shown that there is no unusual diffusion barrier in the extracellular space of frog sartorius muscle and that diffusion of solutes in this space follows well-known laws.' We have no data on the diffusion coefficient of ouabain. From its molecular weight (548) one can roughly estimate that it would take approximately 20 minutes for ouabain to reach a concentration 90% of that in the external solution.[10,11] Therefore, the observed slow progress of ouabain action in increasing $t_{1/2}$ over a period of many hours, has little to do with the time for ouabain to reach the cell surface.

Indeed, the data presented in Table 1 show quite clearly that this slow progress of ouabain action occurs mainly after the ouabain has entered into the cells. This is difficult to reconcile with the idea that ouabain produces its effect by poisoning a postualted pump located in the cell membrane, a layer located at the outermost boundary of the cell.

The membrane theory can predict the existence of only one rate constant for the Na^+-ion efflux. In this case, it would be extremely difficult to see why treatment for the same length of time with the same concentration of ouabain should produce different effects depending on the time of subsequent incubation.

On the other hand, these observations are in harmony with the association-induction hypothesis. In this theory, the bulk of intracellular K^+ ion and a fraction of intracellular Na^+ ion in normal resting cells is adsorbed on the β- and γ-carboxyl groups of cell proteins as a result of a more favorable free energy of adsorption for K^+ ion.[3,4] Thus, our previous work has shown that $K^{oo}_{Na \to K}$ is around 130 in normal frog muscle cells and ouabain reduces it by a factor of 10.[4,5] The increase of relative Na^+-ion preference leads to the displacement of adsorbed K^+ ion by adsorbed Na^+ ion.

In preceding publications, it has been shown that the Na^+-ion efflux can be resolved

into at least two fractions[7] and that the slow fraction, conventionally regarded as rate-limited by membrane permeability, was shown to be actually rate-limited by desorption. Thus, the effect of ouabain is to produce a steady increase of more strongly adsorbed Na^+ ion. This stronger adsorption, in turn, increases the activation energy barrier for the exchange desorption, hence, a reduced rate of the Na^+-ion efflux seen in Figures 2 and 4 and Table 1.

The ouabain-induced transition from the cooperative K^+ state to the Na^+ state is, like many other cooperative phenomena, slow in onset, hence the delayed completion of ouabain effect after the drug has already entered into the cells.

The association-induction model also offers a reasonable explanation of the greater effectiveness of ouabain added to the pre-incubation solution than ouabain added to the washing solution. Conversion of K^+ ion adsorbing sites to Na^+ ion adsorbing sites should be similar in either case, but only when the free Na^+ ion in the environment is labeled, as is the case in the pre-incubation, can we "see" the newly adsorbed Na^+ ion which, exchanging slowly, creates a slowing down of the Na^+-ion efflux.

When ouabain is added to the washing solution—usually some time after washing has begun—much of the labeled free Na^+ ions in and out of the cells originally introduced during pre-incubation with Na^{22} have already been washed away and replaced by non-labeled Na^+ ion. Conversion of free Na^+ ions to adsorbed Na^+ ions under this condition will involve only unlabeled, hence, invisible Na^+ ions. This, then, explains why ouabain added to the pre-incubation solution always brings about a visible slowing down of $t_{1/2}$ (Fig. 4), while the same concentration of ouabain added to the washing solution for the same length of time only does so sometimes, i.e., when the muscle had originally a faster rate of Na^+-ion exchange.

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