

Permeability of Plasma Membrane Vesicles to Ouabain and Mg^{2+} as a Factor Determining Rate of Binding of Ouabain to Na^+ and K^+ Dependent ATPase

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Na^+ , K^+ -ATPase of the plasma membrane isolated from sheep kidney medulla exhibits functional asymmetry for the cardiac glycoside ouabain. In this vesicular membrane preparation the rate of binding of ouabain was slow (time constant > 60 min) when the vesicles were incubated in the presence of isotonic sucrose. Upon treatment of the preparation with hypoosmotic shock or phospholipase A the initial rate of ouabain binding was enhanced at least 3 fold. In equilibrium a concentration of the ouabain-enzyme-complex was obtained which was about twofold that of the untreated vesicles. This result suggests two types of ouabain binding sites with an approximate stoichiometry of 1 to 1. The stoichiometry seems to be maintained at high concentrations of ouabain where binding curves show a biphasic time course. Additional information about heterogeneity of binding sites comes through experiments in which the vesicles were treated with Mg^{2+} prior to the addition of ouabain. A minor fraction of the binding sites were occupied by ouabain only after longtime incubation with Mg^{2+} .

Introduction

Cardiac glycosides evoke positive inotropic effect on the heart through inhibition of the Na^+ , K^+ -pump. This inhibitory effect is accompanied by binding of these drugs to the Na^+ , K^+ -ATPase. Binding of ouabain the most commonly used cardiac glycoside, has been demonstrated in the presence of Mg^{2+} and inorganic phosphate or in the presence of ATP and Na^+ [1–4]. Since both pathways lead the formation of a phosphoenzyme [5, 6] it was suggested that both are functionally related to the inhibitory action of ouabain on ATP-splitting [5–7]. In this paper Mg^{2+} and P_i supported ouabain binding was studied in a preparation of plasma membrane vesicles isolated from sheep kidney medulla. These vesicles supposedly are tight for ligands of the Na^+ , K^+ -ATPase such as Na^+ or ATP. This assumption is based on the observation that leak producing agents were able to activate ATP-splitting and phosphorylation [8]. Activation of the phosphorylation by Na^+ was slow (time constant of several minutes) although ATP was allowed to react with the enzyme for 5 sec only [9] as if Na^+ had to penetrate to the interior of possibly existing inside-out vesicles to exert its acti-

vation effect. In order to give proof of tight vesicles by a method independent from ATPase- and phosphorylation measurements the effect of tightness was tested on the rate of ouabain binding. The information about the sidespecific reactivity of ouabain or Mg^{2+} is discussed with the known sidedness of the inhibitory binding site in Na^+ , K^+ -ATPase. In experiments with intact cells it has been shown that ouabain interacts with the enzyme from one side only. In squid axon ouabain was inhibitory when applied to the extracellular medium [10]. In analogy ghosts reconstituted from lysed red cells seem to bind ouabain from the outside [11]. In agreement with these experiments a model is presented (Fig. 8) which assumes that ouabain acts on both sides of the membrane: on the outside in right-side-out vesicles and on the inside in inside-out ones. On the basis of this model results known from the literature are discussed which give evidence for heterogeneity of binding sites [12–17].

Materials and Methods

Materials

Plasma membrane vesicles were isolated from sheep kidney medulla by a gradient centrifugation procedure described earlier [8, 9] adapted for the use of zonal rotor gradient centrifugation. Vesicles were stored in isotonic sucrose and generally used within

Abbreviations: MOPS, morpholinopropane sulfonic acid; P_i , inorganic phosphate; EDTA, ethylenediamine tetraacetic acid; TCA, trichloroacetic acid.

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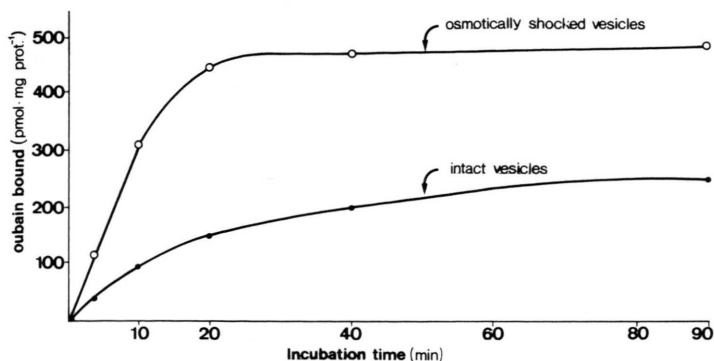


Fig. 1. Effect of hypotonic treatment of plasma membrane vesicles on ouabain binding. Vesicles were incubated with 1 mM MgCl_2 , 0.1 mM Tris EDTA, 100 mM (Tris)₃phosphate and imidazol-MOPS pH 7.0 in the presence and in the absence of 0.7 M sucrose (osmotic shock) for 1 min at standard conditions (methods). Binding was started by the addition of 5 μM [^3H]ouabain and stopped after the time indicated on the abscissa.

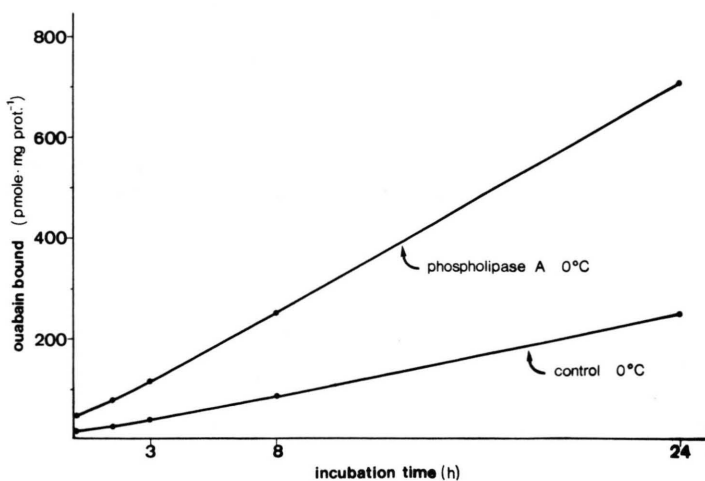


Fig. 2. Effect of phospholipase A on ouabain binding measured at 0 °C. Vesicles were incubated at 0 °C with 0.5 μg phospholipase A (*Naja naja*) in the presence of 1.0 mM CaCl_2 , 0.7 M sucrose in a total volume of 25 μl . Digestion of the vesicles was stopped after 15 min by the addition of 1.1 mM EDTA and the sample diluted with 500 μl of incubation mixture thermostated to 0 °C, which contained 1 μM [^3H]ouabain, 1 mM EDTA and 0.8 mM MgCl_2 . Ouabain binding was stopped after the time indicated on the abscissa.

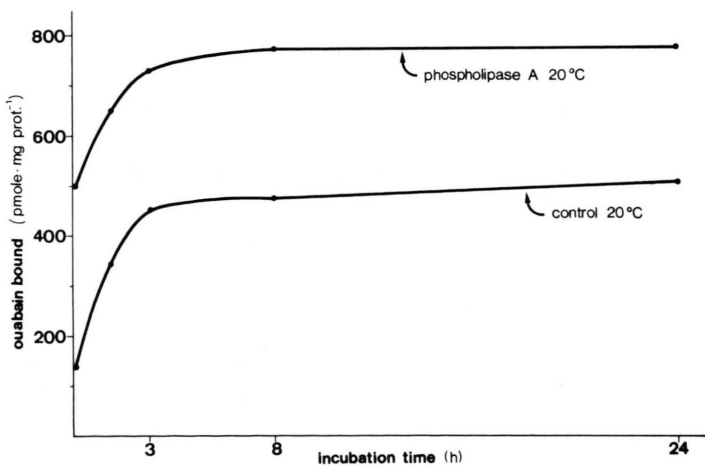


Fig. 3. Effect of phospholipase A on ouabain binding measured at 20 °C. Conditions as described in Fig. 3 except that the incubation mixture was kept at 20 °C.

two weeks. Ouabain and digitoxin p. a. were from Merck, Darmstadt, [^3H]ouabain and [^3H]digitoxin from Amersham, Buchler, Braunschweig. Phospholipase A (Naja naja) was from Koch Light (Bucks, England). Microfilters (0.6 μ , BA 90) were from Schleicher und Schüll, Dassel.

Methods

Ouabain binding experiments were carried out at 0 °C in a total volume of 500 μl containing 0.1 mg protein, 100 mM $(\text{Tris})_3\text{PO}_4$, 10 mM imidazol-MOPS pH 7.0 as stated in the legends of the figures. Unless otherwise described binding was started by the addition of [^3H]ouabain and terminated by 20 fold excess of unlabeled ouabain over the final concentration of the labeled compound in the incubation mixture. Samples were filtered and washed with 20 ml of solution containing 0.5 mM unlabeled ouabain at 0 °C three times on microfilters and counted in a liquid scintillation counter. In contrast to the incubation medium the washing solution was free of isotonic sucrose. This treatment bases on the assumption that in the absence of sucrose vesicles were rather permeable to ouabain so that intravesicular ouabain not bound to the receptor was removed. If not otherwise described unspecific binding was determined by incubation of an aliquot of the samples with ouabain of a specific activity identical to that obtained after dilution with the stopping solution. The further treatment was identical to that described for the specific ouabain binding. Unspecific labeling was generally less than 10% of total ouabain binding.

Results

The effect of membrane tightness on the rate of binding of ouabain in plasma membrane vesicles

In order to detect possibly existing intravesicular ouabain binding sites the effect of leak producing agents on ouabain binding kinetics was examined. Thereby it was assumed that the drug added to the extravesicular space could not attack binding sites located on the inside in tight vesicles.

Fig. 1 shows ($\text{P}_i + \text{Mg}^{2+}$)-supported ouabain binding to plasma membrane vesicles incubated in the presence (upper curve) and in the absence of isotonic sucrose (lower curve). In the absence of sucrose binding saturates after 20 min at a level of 500 pmol ouabain \cdot mg prot. $^{-1}$. This value is enzymatically sig-

nificant because it is in the range of the concentration of the phosphoenzyme measured after similar osmotic treatment of vesicles before the addition of [^{32}P]ATP (300 pmol \pm 80 ($n = 6$) pmol $^{32}\text{P} \cdot$ mg prot. $^{-1}$). In isotonic sucrose however, the rate of initial binding of ouabain is about three times slower. The concentration of the ouabain-enzyme-complex does not approach the value obtained by osmotic shock even after 90 min incubation with ouabain. From these results it was concluded that hypotonic treatment activated both the rate and the extent of binding of ouabain. Furthermore it was inferred that increased permeability of the vesicles for one of the ligands of the Na^+, K^+ -ATPase was contributing to increase in the rate of binding of ouabain, namely that of Mg^{2+} , P_i or of ouabain itself. This assumption is based on the observation that sucrose diminishes passive fluxes of small molecules in membranes [18].

In order to substantiate this interpretation and exclude a possible unspecific effect of sucrose on ouabain binding, vesicles were treated with phospholipase A in the presence of isotonic sucrose. Under these conditions plasma membranes should undergo lipolysis of nonessential phospholipids but Na^+, K^+ -ATPase should not be impaired. Consistent with this interpretation is the fact that under similar conditions acceleration of Na^+ -activation of phosphorylation from ATP is achieved (unpublished results). In the ouabain binding experiments vesicles were first incubated with phospholipase A at 0 °C. Then [^3H]ouabain was added together with Mg^{2+} and P_i to initiate binding of ouabain. Incubation with Mg^{2+} and P_i was performed both at 0 °C (Fig. 2) and 20 °C (Fig. 3). As shown in Fig. 2 phospholipase A activates ouabain binding at 0 °C after 10 sec already to 40 pmol from 20 pmol in the nondigested control. After this rapid initial phase a linear increase of [^3H]ouabain incorporation follows, which is almost 4 times as high as the untreated control (increase from 70 to 275 pmol ouabain \cdot mg prot. $^{-1}$). At 20 °C (Fig. 3) however, the differential increment between 10 sec and 8 h was not significantly increased compared to the control. At this temperature phospholipase A essentially activates the rapid initial component from about 170 to 500 pmol.

From the effect of hypoosmotic treatment and phospholipase A it was concluded that permeability of the vesicles for one of the ligands involved in the binding reaction may have determined rate and extent of ouabain binding. The assumption that lipids

obstruct the access of substrates to the intravesicular face of the membrane is supported by the observation that 0.5% albumin influences level of binding. When albumin was added to the incubation mixture together with phospholipase A (compare ref. [19]) the starting value of binding measured at 20 °C is reduced from about 500 pmol to 350 ± 50 ($n = 2$). Albumin is known to bind lysophosphatides and fatty acids produced by phospholipase A, which normally would make the membrane leaky.

The effect of Mg^{2+} concentration on the rate of ouabain binding

For the design of further experiments it was considered that in a fraction of the tight vesicles access of Mg^{2+} to intravesicular sites could have contributed to the inhibition of ouabain binding. In this view the increase in the rate of ouabain binding obtained by treatment with phospholipase A or osmotic shock might have reflected influx of Mg^{2+} into vesicles. In a test for vesicles with low permeability to Mg^{2+} the preparation was first incubated with Mg^{2+} and thereafter binding was started with [3H]ouabain. As shown in Fig. 4 the initial ouabain binding value increased about twofold compared to the control after 24 h preincubation with Mg^{2+} . This level seems to be not significantly increased when Mg^{2+} was present for 46 h before the addition of ouabain. In contrast binding measured after 24 h still was dependent on Mg^{2+} , as if Mg^{2+} -incubation determined the yield of ouabain-enzyme-complex. Since this effect was so slow it was assumed that it took several days for

Mg^{2+} to reach an equilibrium on both sides of the membrane. Obviously vesicles were tight for Mg^{2+} .

Evidence for heterogeneity of binding sites

In order to saturate the enzyme with Mg^{2+} and improve the reactivity of possible existing vesicles tight for Mg^{2+} , the Mg^{2+} -concentration was raised to 10 mM and binding of cardiac glycosides measured at low concentrations of ouabain. Fig. 5 shows that ouabain binding was considerably accelerated compared to Fig. 4, since an equilibrium was obtained after 1 h incubation with 5.0 μM ouabain. Apparently there were enough empty ouabain binding sites remaining since a second incubation with 50 μM ouabain led to a further increase in ouabain binding level.

Effect of the concentration of ouabain on the rate of formation of the ouabain-enzyme-complex

In a further experiment the effect of high concentrations of ouabain was tested on the initial rate of ouabain binding. As shown in Fig. 6 rate of binding of ouabain was roughly proportional to the ouabain concentration in the range tested (up to 100 μM). Since the rate of ouabain binding was influenced by concentrations more than 100 fold above the dissociation constant [15], the possibility was considered that interaction of the enzyme with ouabain was obstructed in vesicles oriented with the ouabain binding site to the intravesicular space.

This assumption implies that binding kinetics were influenced by the diffusion of ouabain into the vesicles.

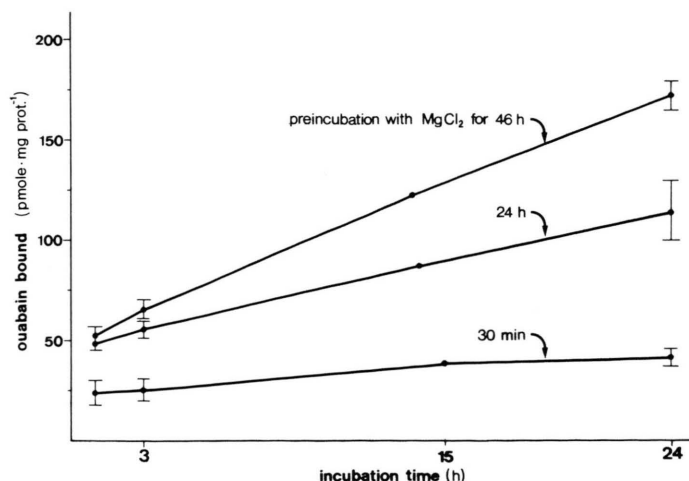


Fig. 4. Effect of Mg^{2+} -preincubation on the rate of ouabain binding. Vesicles were incubated with 1 mM $MgCl_2$ and 0.5 mM EDTA at 0 °C under standard conditions for the time indicated in the figure, then binding was started by the addition of 5 μM ouabain (0 °C).

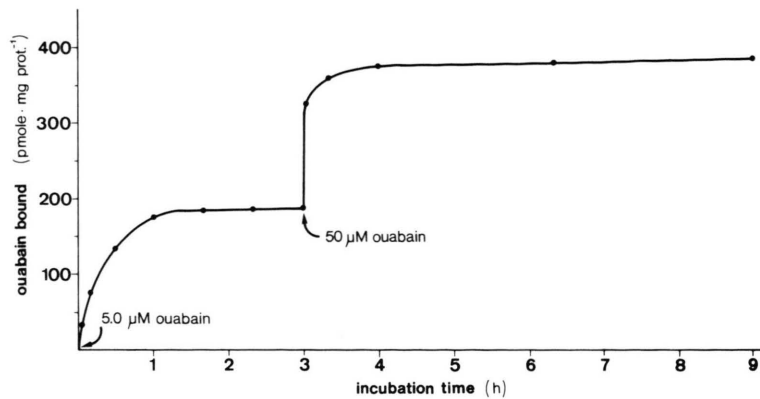


Fig. 5. Binding of ouabain at low and high concentrations of ouabain. Vesicles were incubated with 10 mM MgCl_2 and 1 mM EDTA. After 60 min preincubation at 0°C binding started by the addition of $50\text{ }\mu\text{M}$ ouabain, after another 3 h $50\text{ }\mu\text{M}$ ouabain was added, which had a specific activity identical to the first portion. Stopping of the binding reaction and washing of the controls was performed as described in "methods".

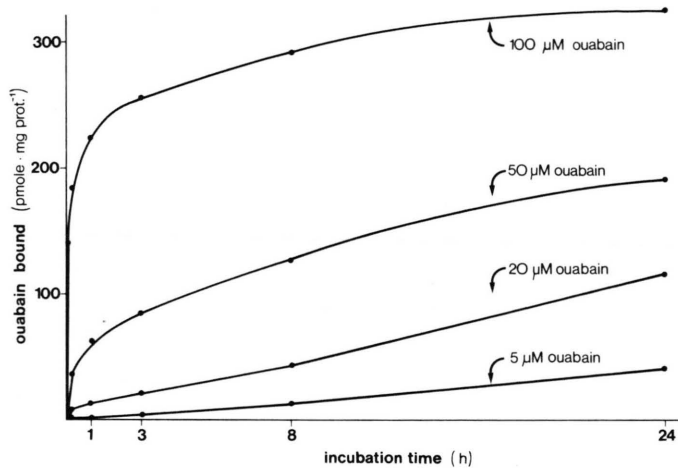


Fig. 6. Dependence of the rate of formation of ouabain-enzyme-complex on concentration of ouabain. Vesicles were incubated with 0.8 mM MgCl_2 , 1 mM Tris EDTA, 0.1 mM $(\text{Tris})_3\text{phosphate}$ and 10 mM imidazol-MOPS pH 7.5 at 0°C . After 5 min ouabain was added in the concentration stated in the figure and stopped as usual after the time indicated on the abscissa.

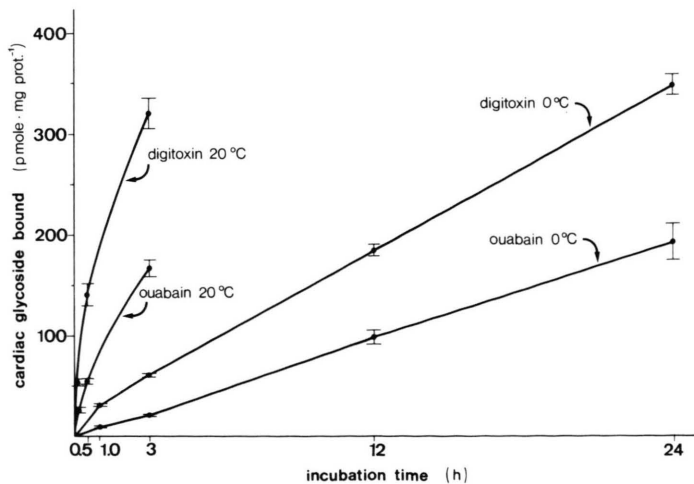


Fig. 7. Comparison of the rate of initial binding of ouabain and digitoxin. Vesicles were incubated in a medium as described in Fig. 5. Binding was started by the addition of $1\text{ }\mu\text{M}$ $[^3\text{H}]$ ouabain or $[^3\text{H}]$ digitoxin thermostated to 0°C or 20°C and binding terminated by the addition of ice cold stopping solution as described in "methods".

Comparison of rate of binding of ouabain with that of digitoxin

In a further experiment the cardiac glycoside ouabain was replaced by its congener digitoxin and rate of initial binding measured at 0 °C and 20 °C. As shown in Fig. 7 rate binding of digitoxin was 2.3 fold more rapid than in the control with ouabain. This increase also is maintained at 20 °C. Since digitoxin is more lipophylic than ouabain, the possibility was considered that higher permeability of the membrane for digitoxin was responsible for the increase in the rate of binding of the cardiac glycosides. An activating effect due to the difference in binding constants was also considered.

Discussion

In this paper osmotic shock and treatment with phospholipase A were used to check the influence of membrane integrity on ouabain binding in a vesicular ATPase preparation. Both procedures make the membrane permeable to small molecules. The effect of detergents on ouabain binding was not tested in order to avoid possible side effects on the Na⁺, K⁺-ATPase as seen from the progressive inhibition of the Na⁺, K⁺-ATPase activity at high detergent concentrations [8]. Binding of ouabain was studied in the presence of Mg²⁺ and P_i. Under these conditions permeability of Mg²⁺, P_i and ouabain has to be taken into account. As shown in the erythrocyte, cell membranes seem to be rather permeable to inorganic phosphate [4].

Mg²⁺-permeability

In order to lower the passive permeability of the plasma membrane vesicles to these ligands of the Na⁺, K⁺-ATPase and detect possible tightness effects on the initial rate of ouabain binding, experiments were performed at 0 °C. Phosphorylation experiments gave evidence that vesicles are tight for sodium at this temperature [9]. Because of the low permeability to Na⁺, it is unlikely that Mg²⁺ added to the extravesicular volume could initiate phosphorylation from P_i on the inside in right-side-out vesicles. An effect of Mg²⁺ on ouabain binding was detected with a time constant of days. Although a slow conformational change cannot be excluded it seems to be probable that this divalent cation had to penetrate the interior of the vesicles and bind to the intracellular side of the membrane. The sidedness for

Mg²⁺ is difficult to demonstrate in the erythrocyte since it seems to be impossible to reseal erythrocyte ghosts in the presence of Mg²⁺ [4]. The initial level of the ouabain-enzyme-complex seen in Fig. 4 could be related to the portion of vesicles leaky to Mg²⁺. As shown in Fig. 3 phospholipase A changes the number of binding sites at equilibrium as if vesicles were made permeable to Mg²⁺. In this respect the effect of phospholipase seems to correspond that of hypotonic treatment.

Since temperature also did alter the amount of ouabain bound immediately after the addition of ouabain (compare Figs 2 and 3), it was considered that the permeability of vesicles to Mg²⁺ was dependent on the temperature. As shown in Fig. 5 the addition of 10 mM Mg²⁺, a concentration far above that to saturate the enzyme, was able to accelerate the rate with which an equilibrium was obtained. Once the level was reached only a slow increase of binding was observed (Figs 1 and 3). Could this mean that vesicles are heterogeneous with respect to permeability to Mg²⁺?

Ouabain permeability

Although the above mentioned factors could complicate binding of ouabain in right-side-out vesicles, especially in experiments, in which both Mg²⁺ and ouabain were present in high concentration, it is difficult to explain the effect of high concentrations of ouabain alone on the basis of Mg²⁺ permeability (Fig. 6).

As shown in Fig. 7 rate of binding was dependent on ouabain at concentrations more than 100 fold higher than the association constant. If not the association constant could account for slow binding it could have been slow passive influx into possibly existing inside-out vesicles. However, this does not mean, that the values measured, was ouabain which was entrapped in vesicles. Free ouabain supposedly is removed from the vesicles by washing with hypotonic buffer. The hypothesis that vesicles were attacked by intravesicular ouabain implies that Mg²⁺ and P_i acts from the outside. Indeed this component of binding was visible at low concentrations of Mg²⁺ where the cation probably cannot act from the intravesicular face of the membrane. The rapid component observed in Fig. 1 also could be dependent on ouabain that passively entered the vesicles. Hypotonic treatment did influence rate of binding more than the level of the ouabain-enzyme-complex in the

equilibrium. Phospholipase A similarly activates the initial rate of binding at 0 °C, but not at 20 °C. At elevated temperatures vesicles supposedly are leaky anyway, so that the effect of phospholipase A is not visible. The hypothesis of intravesicular binding sites appears to be related to several observations known from the literature. In earlier experiments Triton X-100 was shown to enhance ouabain-caused inhibition of the phosphorylation, as if this drug penetrated more rapidly into inside-out vesicles made leaky by Triton X-100 [8]. Treatments with detergents such as Triton X-100 or dodecylsulfate [21] with NaClO₄ [13], lysoytic enzymes [16, 20] or sonication of the enzyme [12] seem to be equivalent in the sense that these procedure remove lipids from the vesicles and make them leaky to ligands of the Na⁺,K⁺-ATPase like ouabain. Increased permeability for ouabain would be responsible for the observation that aged Na⁺,K⁺-ATPase preparation were more rapidly inhibited by ouabain than fresh ones [7]. This is consistent with the experience that the rate of the initial phase of ouabain binding varies with the age of the preparation as if tightness for ouabain decreases with storage of the vesicles (see Fig. 4). It is known whether tightness of the cell membrane to cardiac glycosides has any physiological significance. Experiments with Hela cell [22] and erythrocytes [24] have shown that the binding component saturable at low concentration is responsible for the inhibition of the pump whereas the "nonsaturable component" was uptake into the cell. In Na⁺,K⁺-ATPase preparations isolated in the presence of detergents the permeability and orientation of the membrane certainly do not influence kinetical properties of enzyme. There are results however published in the literature that suggest the existence of vesicles in some preparations [12, 13, 16, 23]. The tightness of other preparations is not known entirely. This is also true for these experiments on which the sequential model of the Na⁺,K⁺-ATPase bases [5].

This paper wants to make clear that it is important to take into account tightness and orientation in ouabain binding experiments with isolated plasma membrane vesicles. The tightness tests for ouabain and Mg²⁺ base on the asymmetric reactivity of the Na⁺,K⁺-ATPase for these ligands in the intact cell. If indeed ouabain is accepted only from the side of the membrane which is opposite to where P_i and Mg²⁺ or ATP is bound two vesicles exist in the preparation (Fig. 8): Inside-out vesicles, which bind oua-

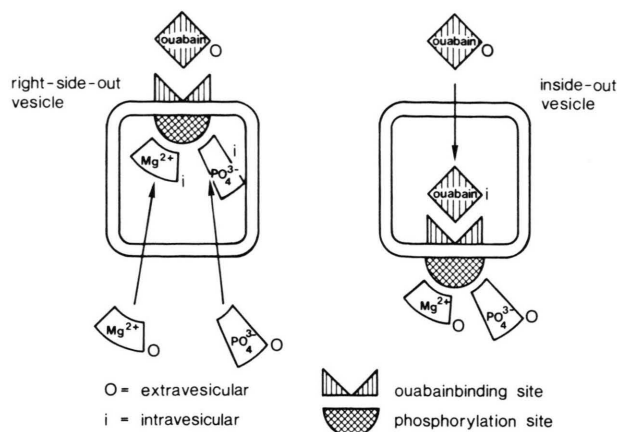


Fig. 8. Reactivity of ouabain binding sites with known sidedness as expected in tight inside-out and right-side-out vesicles.

bain on the inside and right-side-out ones, with the binding sites facing the outside. The results presented here however, suggest heterogeneity of ouabain binding sites within the inside and the outside oriented fractions. There is a model that could provide an explanation for some of the observations. Namely the flip-flop dimeric model proposed by Grosse *et al.* [25] and Schoner [17]. It assumes two sets of sites one with low and one with high affinity for ouabain. In this view slow and fast reacting sites would be equivalent to high and low affinity ones. This assumption is attractive especially through the possible 1 to 1 stoichiometry seen in Figs 1, 2, and 5. This means that initial rates would not only be determined by diffusion of ouabain or Mg²⁺ but also by the affinity constants for these ligands.

However, on the basis of this model it is difficult to explain the effects of phospholipase A or osmotic shock.

If not the dimeric model could explain the observed heterogeneity it could be the presence of sites with unknown functionality. The question that arises is whether ouabain is bound in inside-out vesicles exclusively from the inside or whether there is an extravesicular ouabain binding site whose function is not known. Until further experiments are made in which the effect of ouabain is studied on the phosphorylation it is not possible to reconcile the flip-flop model with the orientation hypothesis.

Acknowledgements

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