J. Membrane Biol. 104, 211–221 (1988)

The Journal of
Membrane Biology

Different Sensitivity of ATP + Mg + Na (I) and P_i + Mg (II) Dependent Types of Ouabain Binding to Phospholipase A_2

Petr Svoboda, Evzen Amler, and Jan Teisinger
Institute of Physiology, Czechoslovak Academy of Sciences, Videnska 1083, Praha 4, Czechoslovakia

Summary. The effect of phospholipase A_2 and of related agents on ouabain binding and Na,K-ATPase activity were studied in intact and detergent-treated membrane preparations of rat brain cortex and pig kidney medulla. It was found that phospholipase A_2 (PLA_2) may distinguish or dissociate ouabain binding complexes I (ATP + Mg + Na) and II (P_i + Mg), stimulating the former and inhibiting the latter. Procedures which break the permeability barriers of vesicular membrane preparations, such as repeated freezing-thawing, sonication or hypoosmotic shock failed to mimic the effect of PLA_2, indicating that it was not acting primarily by opening the inside-out oriented vesicles. The detergent digitonin exhibited similar effects on ouabain binding in both ATP + Mg + Na and P_i + Mg media. Other detergents were ineffective.

The ability of PLA_2 to distinguish between ouabain binding type I and II can be manifested even in SDS-treated, purified preparations of Na,K-ATPase. The number of ATP + Mg + Na-dependent sites is unchanged, while the P_i + Mg-dependent sites are decreased in number in a manner similar to that seen in original membranes. This inhibition is completely lost in the reconstituted Na,K-ATPase system, where the ATP- as well as P_i-oriented ouabain sites are inhibited by PLA_2.

Key Words ouabain binding · phospholipase A_2 · Na,K-ATPase

Introduction

Plasma membrane Na,K-ATPase pumps Na out of the cell and K into the cell against concentration gradients using energy derived from ATP hydrolysis (Cantley, 1980; Stahl, 1986). The enzyme is an integral membrane protein with a catalytic ATP site at the interior surface and ouabain binding site at the external surface.

Sweadner (1979, 1985) demonstrated two forms of Na,K-ATPase catalytical subunits, designated as alpha and alpha$^+$. The two isoenzymes differ most strikingly in the inhibition of the enzyme activity by ouabain. The Na,K-ATPase from rat brain axolemma (alpha$^+$) has a high affinity for ouabain, $IC_{50} = 1 \times 10^{-7}$ M, while that from rat kidney medulla (alpha) has a low affinity, $IC_{50} = 3 \times 10^{-5}$ M. Besides this "intertissue" difference, an equally large "interspecies" difference exists—the IC_{50} values for ouabain inhibition of cardiac muscle Na,K-ATPase are 0.3 μM (bovine and dog), 1 μM (rabbit and guinea pig) and 100 μM (rat and mouse *see* Matsuda & Iiwata, 1986). More recently, three distinct forms of Na,K-ATPase alpha-subunits were demonstrated in the rat brain (Schull, Schwartz & Lindgrel, 1986).

An open question at present is whether such differences are based on inherent differences in protein structure of alpha and alpha$^+$ isoenzymes, or whether phospholipids and/or protein-lipid interactions play a decisive role in the ouabain sensitivity of the enzyme. Numerous studies indicate that the Na,K-ATPase activity and ouabain binding are strongly dependent on integrity and physical properties of plasma membrane lipids (Goldman & Albers, 1973; Roelofsen & Van Deenen, 1973; Taniguchi & Iida, 1973; Kimelberg & Papahadjopoulas, 1974; Goodman & Wheeler, 1978; Roelofsen, 1981; Abeywardena & Chernock, 1983; Abeywardena et al., 1984).

We have tested the effects of various lipid modifying agents such as phospholipases A_2, C and D and of detergents on ouabain-binding characteristics, activity and ouabain sensitivity of Na,K-ATPase from various sources. These effects were compared in intact and detergent-treated enzyme preparations from rat brain and pig kidney medulla.

Materials and Methods

Preparations of Microsomal Membranes from Rat Brain

Microsomes from rat brain cortex were prepared as described before (Svoboda & Mosinger, 1981; DeRobertis, et al., 1962).

White rats of the Wistar strain were killed by decapitation, the cerebral cortex was removed and the white matter separated and discarded. The remaining tissue was homogenized in 10 vol of 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, in a tightly fitting Elvehjem-Potter homogenizer. The homogenate was centrifuged first for 20 min at 10,000 × *g*, and the resulting supernatant was centrifuged for 1 hr at 100,000 × *g*. The final pellet was suspended with rehomogenization in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, or in redistilled water and stored at −30°C at 0.5–1.7 mg protein per ml.

Na,K-ATPase Measurement

The Na,K-ATPase activities were determined in two reaction media: (i) 100 mM NaCl, 200 mM KCl, 100 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$, and (ii) 120 mM NaCl, 100 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 2×10^{-4} M ouabain.

The enzyme was preincubated for 5–7 min at 37°C before starting the reaction by addition of 2.5 mM ATP. After 5–10 min at 37°C, the enzyme reaction was terminated with $HClO_4$, the precipitated protein was separated by centrifugation, and the clear supernatant was assayed for inorganic phosphate according to Taussky and Shorr (1951). To verify the pseudo-first order kinetics of the enzyme reaction, i.e., linearity of ATP splitting, in some experiments the ATPase activities were also measured by means of a spectrofluorometric assay in which hydrolysis of ATP was coupled to the oxidation of NADH and monitored continuously on the chart recorder (Pullman et al., 1960).

All chemicals tested were present in incubation media during the assay of ATPase activities. The Na,K-ATPase was expressed as inorganic phosphate released per hour per mg of membrane protein in Na + K + Mg medium minus that in Na + Mg + ouabain medium. Vanadium free (Sigma A 5394 or 2383) was used in all experiments.

Isolation of Na,K-ATPase from Rat Brain and Pig Kidney Medulla

The Na,K-ATPase from rat brain and pig kidney medulla was isolated by the sodium dodecyl sulfate extraction procedure described by Jorgensen (1974) using 0.6 mg SDS/mg protein and a two-step sucrose density gradient. The Na,K-ATPase was recovered from the pellet after centrifugation at 100,000 × *g*. The brain enzyme was isolated according to Sweadner (1974).

Measurement of Ouabain Binding

Brain or kidney medulla microsomal fractions (50–100 μg protein per sample) were incubated with [^{3}H] ouabain in a total volume of 0.4 ml of 2.5 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris-HCl, pH 7.4 (ATP + Mg + Na medium) or in 5 mM NaH_2PO_4, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4 (P_i + Mg medium). In some experiments the ATP + Mg + Na medium without sodium and P_i+ Mg medium with 100 mM NaCl were also used.

After a 5–7 min preincubation, the binding reaction was initiated by [^{3}H] ouabain and continued for 15, 60 or 90 min at 37°C (specified in the figure legends). In "one-point assays" 5–10 nM concentrations of [^{3}H] ouabain were used. In Scatchard plot analyses, the microsomes were incubated with increasing concentrations of [^{3}H] ouabain (2×10^{-10}–4×10^{-7} M). Incubation was terminated by dilution of the reaction mixture with 5 ml of ice-cold buffer, i.e., with ATP + Mg + Na or P_i + Mg medium and the solution was immediately filtered through Whatman GFC filters under very low vacuum (flow-rate 40–60 ml/min). The filters were washed twice with 5 ml of cold buffer and dried overnight at laboratory temperature. The radioactivity was determined in the Beckman L5 spectrometer using a toluene based scintillation cocktail. The nonspecific binding was determined in the presence of excess of unlabeled ouabain 10^{-6}, 10^{-5} or 10^{-4} M. The nonspecific binding accounted for less than 7, 5 and 3% of total binding, respectively. The specific binding was defined as the difference between the total and nonspecific binding.

When using Whatman GFC filters, the recovery of microsomal membrane protein was between 50 and 70%. Therefore, in the case of purified, SDS-treated Na,K-ATPase membrane fragments, the separation of bound and free radioactivity was also carried out by filtration through Schleicher-Schuell (S-S) filters (0.45 μm). The S-S filters were washed with 20 ml of water before use and the results were corrected for the nonspecific binding to filters alone. This type of binding was determined in the presence as well as in the absence of nonradioactive 10^{-4} M ouabain and subtracted from the total and nonspecific binding measured on the filters plus membranes. The S-S filters retained less of SDS-treated Na,K-ATPase than the GFC filters and therefore, the GFC filters were preferentially used. [^{3}H] ouabain (20 or 42 Ci/mmol) was obtained from NEN.

Results

In the ATP + Mg + Na medium, phospholipase A_2 markedly increased [^{3}H] ouabain binding to microsomal membranes beginning at a concentration of 0.001 U per ml (IC_{50} = 0.06 U/ml) (Fig. 1*A*). The maximum effect, 300% over the control level, was reached at 0.2–1 U/ml and a further increase in PLA2 decreased the binding below the peak values. The stimulation of [^{3}H] ouabain binding capacity was found in the rat brain as well as in pig kidney medulla microsomes.

In contrast, in P_i + Mg medium (Fig. 1*B*), PLA_2 inhibited the [^{3}H] ouabain binding at the lowest concentrations (0.001 U/ml). Half-maximum inhibition was observed at 0.06 U/ml and at about 10 U/ml, the level of binding was close to zero. Neither phospholipase C nor D altered the [^{3}H] ouabain binding at 0.01–1 U/ml in the ATP + Mg + Na and P_i + Mg media. Some minor inhibition was observed at 10 U/ml of phospholipase C; phospholipase D was without effect even at this concentration (*data not shown*).

The specificity of phospholipase A_2 action was also demonstrated when the overall Na,K-ATPase was measured as inorganic phosphate production in ATP + Mg + Na + K medium (Fig. 1*C*). In brain microsomes, half-maximum inhibition of the enzyme activity was observed at 0.08 U/ml of PLA_2 (Fig. 1*C*), while PLC and PLD did not effect the enzyme at concentrations up to 1 U per ml.

The sensitivity of the rat brain microsomal Na,K-ATPase to ouabain (IC_{50} = 0.9 μM) was similar to that of SDS-treated enzyme from pig kidney medulla (IC_{50} = 0.7 μM) and was unchanged by PLA_2 (*data not shown*).

The PLA_2 effects on [^{3}H] ouabain binding measured in both ATP + Mg + Na and P_i + Mg media (*see*Fig. 1*A* and *B*) were blocked by EGTA or quinacrine; EDTA was less effective than EGTA and the addition of exogenous calcium at 0.001–1 mM was without effect on the control and PLA_2-stimulated levels of [^{3}H] ouabain binding (*not shown*).

To investigate the time-course of the PLA_2 action in the ATP + Mg + Na medium, phospholipase A_2 was added 8 or 20 min after initiation of the binding reaction by radioactive ouabain (Fig. 2). The addition of PLA_2 produced a rapid increase of binding which was already evident after 2 min. A new steady-state level was reached after 90 min incubation with PLA_2 at 37°C. This finding indicated that PLA_2 increased the rate of association.

Phospholipase A_2 also increased the rate of dissociation of [^{3}H] ouabain (Fig. 2, insert) from the ATP + Mg + Na-oriented sites. In this experiment, dissociation was initiated by addition of unlabeled ouabain to final concentration of 10^{-5} M after a 60-min incubation with the radiolabeled ligand (10 nM) in the presence or absence of PLA_2.

This relatively rapid stimulation of the turn-on and off rates in the presence of ATP + Mg + Na may reflect a change in the number of glycoside receptors and/or an increase in affinity of the binding reaction. This was answered by the Scatchard plot analysis (Fig. 3). In this experiment, the ouabain binding to brain microsomes was measured as a function of increasing total concentrations of the radioligand and the data were analyzed according to Scatchard (1949). The inhibitory effect of PLA_2 on ouabain binding in the P_i+ Mg medium was also analyzed in this way (Fig. 4). In this part of our work, the microsomal membranes were suspended in redistilled water with 2 mM Tris-HCl, pH 7.4, while in former experiments (Figs. 1 and 2) the suspension in isotonic sucrose was used (*see* Materials and Methods).

The incubation of microsomal membranes with increasing concentrations of [^{3}H] ouabain, 2 × 10^{-10}–4 × 10^{-7} M for 90 min at 37°C showed that the specific binding is a saturable process. Plateau values were reached at about 0.1–0.2 μM [^{3}H] ouabain. The ratio between specific and nonspecific sites was unchanged by PLA_2.

The Scatchard plots (*see* Figs. 3 and 4), which were analyzed by a unweighted linear robust regression method of Cressie and Keightley (1981), were linear with correlation coefficients very close to one

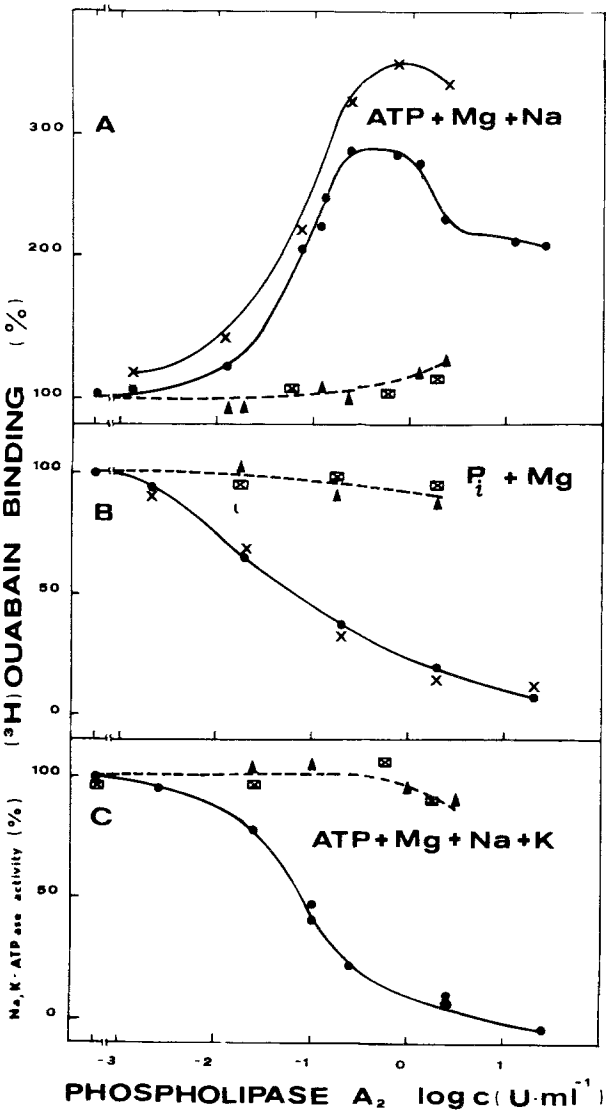


Fig. 1. The dose-response curves of the phospholipase A_2 effect on ouabain binding and Na,K-ATPase activity in brain and kidney microsomes. (*A,B*) The microsomal membranes (50–100 μg protein) prepared from rat brain (●——●) or pig kidney medulla (×——×) were preincubated for 5 min at 37°C with increasing concentrations of PLA_2 (0.0025–25 U per ml) in medium A (2.5 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris-HCl, pH 7.4) or in medium B (5 mM NaH_2PO_4, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4). The binding reaction was initiated by addition of 10 nM [^{3}H] ouabain, continued for 15 min at 37°C and terminated by filtration through Whatman GFC filters. The radioactivity remaining on the filters was determined as described in Materials and Methods. The inhibitors of phospholipase enzyme activity, EGTA (▲------▲) and mepacrine (☒------☒), were used at 10^{-4} M concentrations and were present during both pre- and incubation periods. The results shown are the means of five independent experiments carried out in duplicate. 100% represent the control levels of [^{3}H] ouabain binding without phospholipase A_2 (ATP + Mg + Na = 1.8 pmol/mg protein, P_i + Mg = 3.0 pmol/mg protein). (*C*) The Na,K-ATPase activity was measured as described in Materials and Methods using a 7-min preincubation period in the presence of increasing concentrations of PLA_2 0.0025–25 U/ml. 100% represent the control level of enzyme activity without PLA_2 = 15.1 μmol P_i/hr/mg protein

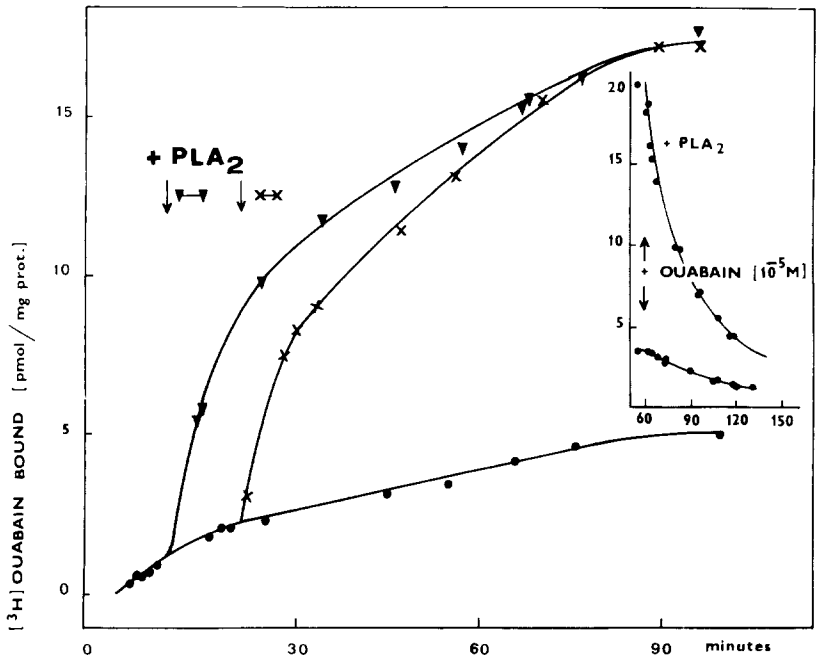


Fig. 2. The time-course of phospholipase A_2 effect on [^{3}H] ouabain binding reaction to the brain microsomes. Brain microsomes suspended in isotonic sucrose (frozen once, 140 μg protein per ml) were incubated at 37°C in ATP + Mg + Na medium (*see* Materials and Methods and legend to Fig. 1) with 10 nM [^{3}H] ouabain. After 8 or 20 min of incubation, 1 U/ml of phospholipase A_2 was added and the time-course of the binding reaction was followed by taking 0.4 ml aliquots from the reaction mixture. A parallel sample without PLA_2 served as control. *Insert:* Brain microsomes were incubated in the presence or absence of PLA_2 as above. After a 60-min incubation, the dissociation of [^{3}H] ouabain was initiated by addition of 10^{-5} M unlabeled ouabain. The time-course of the dissociation was followed by a decrease of [^{3}H] ouabain binding determined in 0.4-ml aliquots of the reaction mixture. The results shown are the means of two experiments carried out in quadruplicates

(0.97–0.99). This indicated a single type of ouabain binding site. As evidenced by the linearity of the Scatchard plot, there is no indication of cooperative interactions or multiple types of glycoside receptors in the case of both ATP + Mg + Na and P_i + Mg-oriented sites. Therefore, in the brain microsomes studied in this work, only one type of ouabain binding site was detected. This linearity also shows that after 90 min the binding reaction reaches equilibrium and, therefore, the PLA_2 effect is unrelated to the nonequilibrium phenomena.

It is also shown in Fig. 3 that in the ATP + Mg + Na medium, the PLA_2 treatment increased the maximum binding capacity (B_{max}) two-fold from 67.6 ± 3.6 pmol/mg to 138.2 ± 14 pmol/mg, while the dissociation constant (K_d) of the binding reaction was unchanged, 15.5 ± 1.3 nM *vs.* 18.6 ± 1.5 nM in control. In the case of P_i + Mg-supported sites (Fig. 4), both the B_{max} and the affinity of the binding reaction were markedly reduced. The B_{max} values decreased from 121.0 ± 5.8 to 36.6 ± 2.4 pmol/mg and the K_d increased from 29.5 ± 2.1 nM to 145.1 ± 9.7 nM. In the absence of PLA_2, both types of binding sites exhibited similar K_d and B_{max} values. After the PLA_2 treatment, however, vastly different numbers are obtained.

Thus, phospholipase A_2 is able to dissociate or distinguish the ATP-oriented (type I) from P_i-oriented (type II) ouabain sites. This indicates that the properties the cardiac glycoside receptor of Na,K-ATPase are modified by interference with the phospholipid environment of the enzyme.

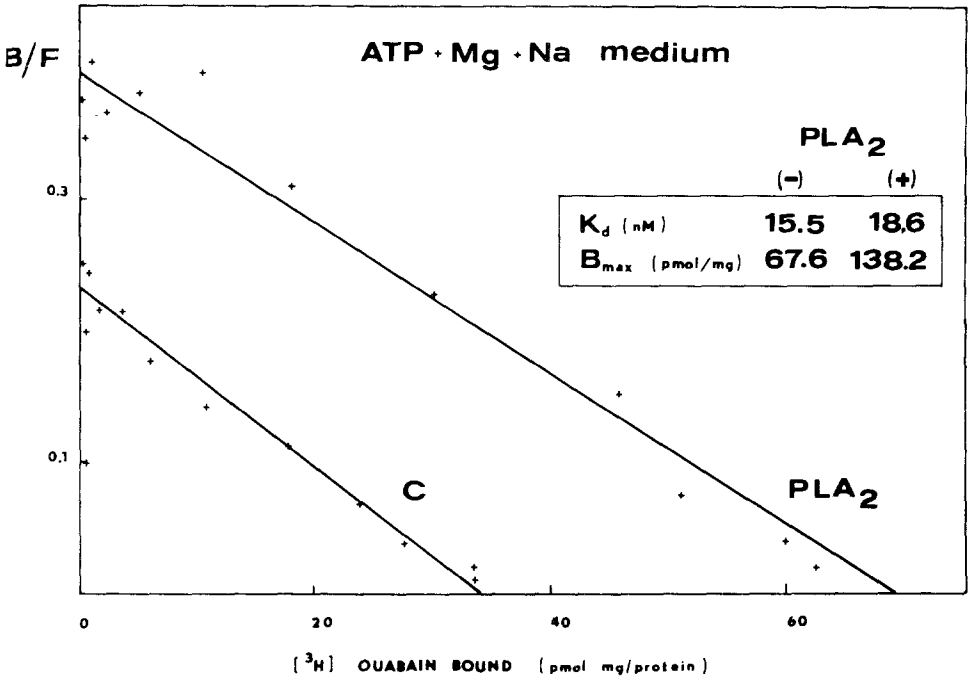
The relative increase of 200% in B_{max} shown in Fig. 3 is less than that demonstrated in the "one-point" experiment in Fig. 1*A* using 10 nM [^{3}H] ouabain. As the increase of binding by PLA_2 is not a function of the total ouabain concentration, this difference is most probably due to the exchange of isotonic sucrose for distilled water in suspending the microsomal membranes. This difference is demonstrated in Fig. 5 where both types of membranes were compared simultaneously.

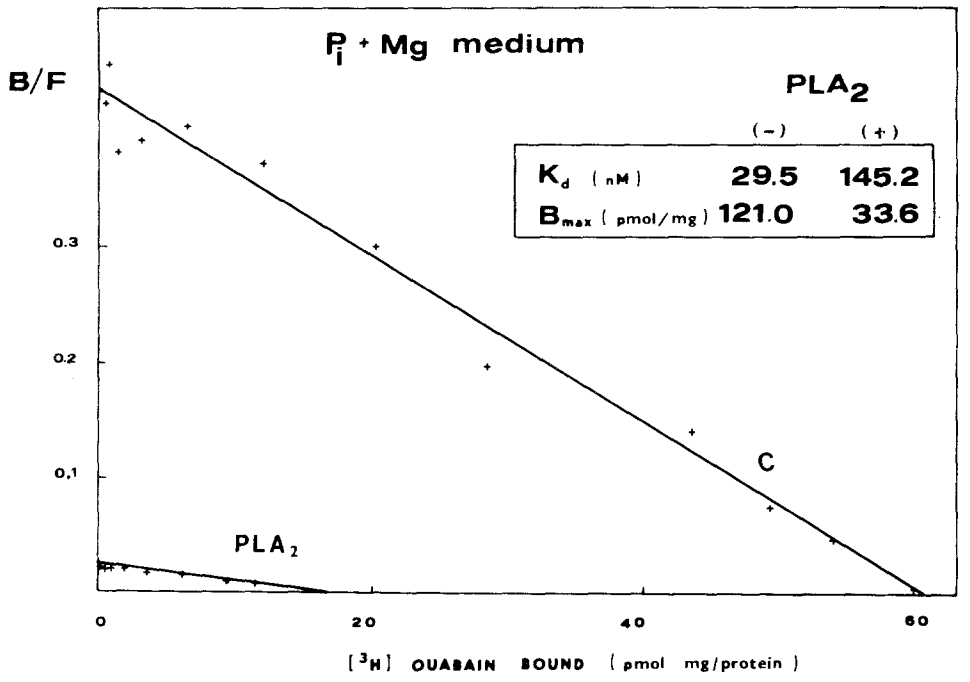
Microsomes suspended in distilled water exhibited higher control levels of binding than those suspended in isotonic sucrose. Therefore, the relative increase after PLA_2 is lower in the former membrane preparation. It is also shown in Fig. 5 that PLA_2 stimulation depends on Na cations. Without sodium, in the ATP + Mg medium, the PLA_2 stimulation is very low, particularly in hypotonically lysed membranes (distilled water).

On the other hand, the inhibition of P_i + Mg-supported ouabain binding by PLA_2 is not dependent on sodium. It can be seen in the presence, as well as in the absence, of Na cations. Sodium cations substantially inhibited the control levels of P_i-supported binding, while the ATP-oriented sites were not affected under control conditions (Fig. 5).

In the hypotonically lysed microsomes there was no evidence for any vesicular forms of membrane fragments (electron microscopy control). Therefore, the PLA_2 effect in "distilled water" microsomes cannot be interpreted as vesicle opening.

We attempted to determine to what extent the





Figs. 3 and 4. Scatchard plot of [^{3}H] ouabain binding to the brain microsomes. Effect of phospholipase A_2. Brain microsomes (suspended in distilled water and frozen once, 50–100 μg protein per sample) were incubated with increasing concentration of [^{3}H] ouabain 2×10^{-10}–4×10^{-4} M in ATP + Mg + Na (Fig. 3) or P_i + Mg (Fig. 4). The incubation was carried out for 90 min at 37°C. When indicated, 1 U/ml of phospholipase A_2 was included in the binding mixture. The separation of the bound and free radioactivity was achieved as described in Materials and Methods using Whatman GFC filters. The binding data were analyzed according to Scatchard (1949), the unweighted linear regression method of Cressie and Keightley (1981) was used

PLA_2 stimulation of [^{3}H] ouabain binding in the ATP + Mg + Na medium could reflect the increase in passive permeability into the resealed, closed vesicles. PLA_2-stimulated ouabain binding could also be demonstrated in permeabilized forms of microsomal membrane preparations (Table 1). Membrane disruptive procedures, such as repeated freezing-thawing, sonication or hypoosmotic shock,

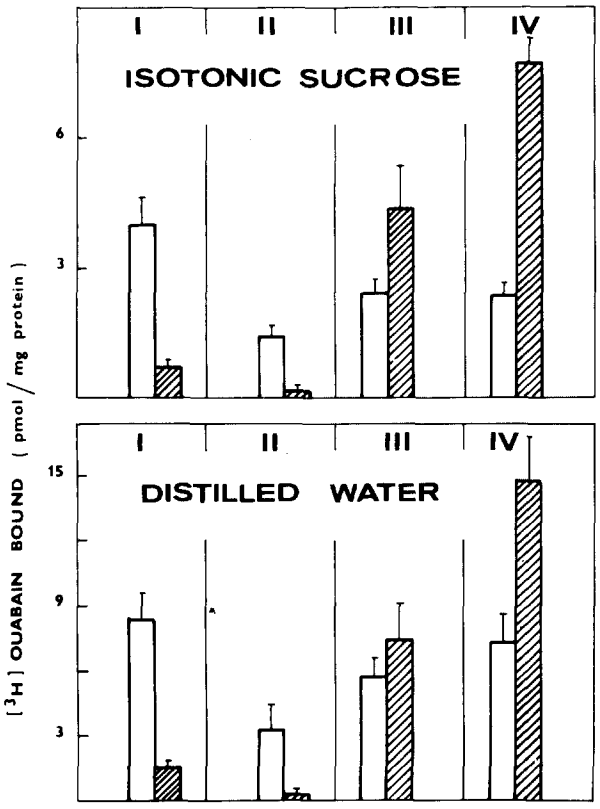


Fig. 5. Comparison of [^{3}H] ouabain binding levels in brain microsomes suspended under iso- and hypoosmotic conditions. The effect of phospholipase A_2. Brain microsomes were prepared as described in Materials and Methods and suspended with rehomogenization in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, or in distilled water at 1.5 mg protein/ml. After a storage for 1–3 weeks at −30°C, the membranes were thawed once and diluted into the incubation media I, II, III and IV. After the preincubation for 7 min at 37°C, the binding reaction was initiated by addition of 6 nM [^{3}H] ouabain and continued for 60 min at 37°C. □ control level of binding, ▨ phospholipase A_2, 1 U/ml. (*I*) 5 mM NaH_2PO_4, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4. (*II*) 5 mM NaH_2PO_4, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl. (*III*) 2.5 mM ATP, 5 mM ATP, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4. (*IV*) 2.5 mM ATP, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl

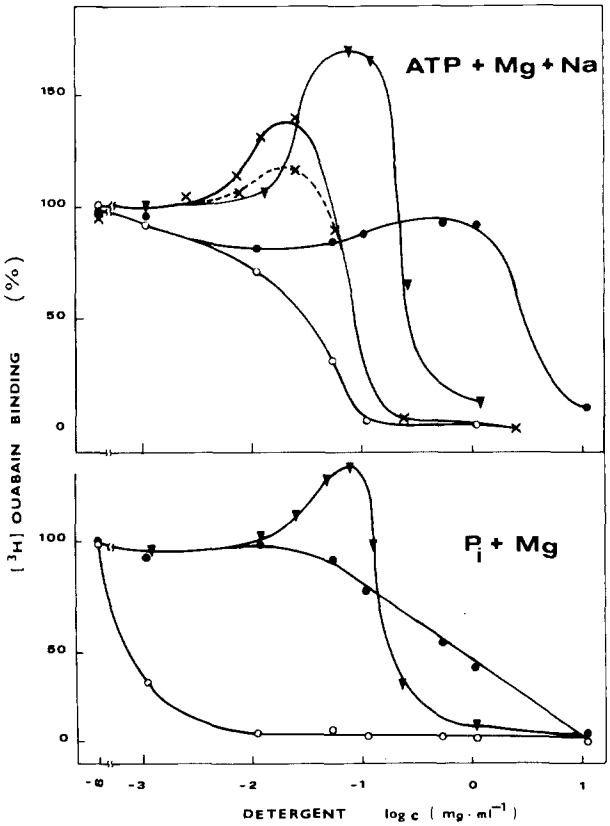


Fig. 6. The effect of detergents on ATP+ Mg + Na and P_i + Mg dependent ouabain binding to the brain microsomes. Brain microsomes were stored in isotonic sucrose or distilled water (*see* Materials and Methods) at −30°C, thawed once and diluted into ATP + Mg + Na or P_i + mg incubation media. After a 7-min preincubation with increasing concentrations of detergents, the binding reaction was initiated by addition of 6 nM [^{3}H] ouabain and continued for 60 min at 37°C. ▼------▼, digitonin-microsomes in distilled water; ×——×, Triton X100-microsomes in sucrose; ×------×, Triton X100-microsomes in distilled water; ●——●, CHAPS-microsomes in sucrose; ○——○, Nonidet P40-microsomes in sucrose. The data are expressed as relative change in % as compared with the control sample, 100% (ATP + Mg + Na) = 3.6 pmol/mg, 100% (P_i + Mg) = 3.1 pmol/mg

increased the basal levels of binding. However, the level of phospholipase A_2 stimulated ouabain binding also increased.

Using these procedures, the Na,K-ATPase activity increased approximately in parallel with the enhancement of ouabain binding. This is in contrast to the PLA_2 effect on the enzyme activity, which was always inhibitory, under a wide variety of experimental conditions tested (Table 1). The P_i + Mg-dependent binding was also increased by freezing-thawing or by osmotic shock.

We questioned whether the PLA_2 action could be mimicked by detergents. The effects of detergents were rather complicated depending on the type used (Fig. 6). Nonidet P40 inhibited at the lowest concentrations, whereas Chaps was ineffective up to 1 mg/ml. Higher Chaps concentrations were also inhibitory. The pattern of inhibition by these detergents was similar in ATP + Mg + Na and P_i + Mg media.

The effect of digitonin and Triton X100 was biphasic. At low concentrations, the binding levels gradually increased to the maximum at about 0.1 mg/ml. The magnitude of this stimulation was different with the two detergents. Digitonin stimulated ouabain binding up to 170–180% in frozen, hypotonically lysed membranes. Ouabain binding was increased by 140% when microsomes were suspended in isotonic sucrose and treated with Triton X100. In microsomes suspended in distilled water, the bind-

Table 1. The effect of membrane disruptive procedures on Na,K-ATPase activity and ouabain binding in brain microsomes

			Na,K-ATPase activity	+ PLA_2	Ouabain binding (ATP + Mg + Na)	+ PLA_2
Microsomes in isotonic sucrose	A	fresh	8.1 (1.00)	2.7	1.45 (1.00)	5.17
	B	frozen once	14.5 (1.79)	1.8	2.32 (1.60)	7.61
	C	frozen 3 times	16.8 (2.07)	1.3	2.71 (1.87)	7.72
	D	sonicated	10.0 (1.23)	2.1	1.75 (1.21)	4.38
Hypothonic medium						
	E	15 min	12.5 (1.54)	1.9	N.M.	N.M.
	F	60 min	9.1 (1.13)	0.8	N.M.	N.M.
Microsomes in distilled water	G	fresh	17.2 (2.13)	1.6	3.65 (2.52)	7.80
	H	frozen once	23.8 (2.94)	2.7	6.20 (4.28)	13.28

Fresh brain microsomes (A) were prepared as described in Materials and Methods and suspended in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, at 0.8–1.2 mg protein per ml. After storage at −30°C for 2–4 weeks, the membranes were thawed once (B) or freezed-thawed 3 times in dry ice plus acetone (C). The frozen and thawed membranes (B) were also sonicated for 30 sec at 0°C (D), or after dilution 10 times with hypothonic 20 mM Tris-HCl pH 7.4 incubated for 15 min (E) or 60 min (F) at 37°. The membranes prepared according to protocol G and H were suspended in redistilled water buffered with Tris, pH 7.4, and measured fresh or frozen once. Na,K-ATPase activity and ouabain binding were assayed as described in methods using a 7-min preincubation with or without phospholipase A_2 (1 U/ml). The ouabain binding reaction was started by addition of 5 nM [^{3}H]ouabain and continued for 60 min at 37°C in ATP + Mg + Na incubation medium. Na,K-ATPase activity = μmol P_i/hr/mg protein, ouabain binding= pmol/mg protein.

The numbers in brackets represent the relative change of Na,K-ATPase activity or of [^{3}H]ouabain binding as compared with fresh microsomes without phosphoilipase A_2. The results shown are means of two experiments carried out in duplicate.

Table 2. Modification of the phospholipase A_2 effect in ATP + Mg + Na medium by Triton × 100

Phospholipase A_2 1 U/ml	Triton × 100 (mg/ml)						
	0.00	0.0012	0.0024	0.012	0.024	0.24	2.4
Absent	100.0	95.9	105.1	134.5	141.2	2.7	1.8
Present	205.4	233.6	239.5	225.4	138.2	1.6	1.8

Brain microsomal membranes in isotonic sucrose were incubated with phospholipase A_2 (1 U/ml) and increasing concentrations of Triton × 100. The binding assay was initiated after a 7-min preincubation by addition of 5 nM [^{3}H]ouabain and continued for 60 min at 37°C. The data are expressed as % of the [^{3}H]ouabain binding to the control microsomal membranes in the absence of PLA_2 and TX 100. The 100% values correspond to 7.2 pmol/mg protein.

ing increased by only 110–120%. The effect of Triton X100 is comparable to that of repeated freezing-thawing or hypoosmotic shock, whereas digitonin seems to interact with the hydrophobic membrane interior to an extent similar to PLA_2.

The stimulation of ouabain binding by digitonin can be demonstrated in the ATP + Mg + Na as well as in P_i + Mg medium, but it is much higher in the former case. This finding indicates that sodium cations play an important role in the detergent and phospholipase A_2 effects on the cardiac glycoside receptor.

Increasing Triton X100 or digitonin concentrations above a critical range of 0.05–0.1 mg/ml completely inhibited ouabain binding. This sudden decrease (Fig. 6) occurred in parallel in the ATP + Mg + Na and P_i + Mg media, and it was detected in a very narrow concentration range. It should be distinguished from the PLA_2 inhibition of type I sites (*see* Fig. 1*B*) which proceeds over a wide range of PLA_2 concentrations.

In the presence of both the detergent and phospholipase A_2, results differing from those with the detergent or phospholipase alone were obtained. Using Triton X100 at a critical concentration of 0.0024 mg/ml (Table 2), the ability of PLA_2 to stimu-

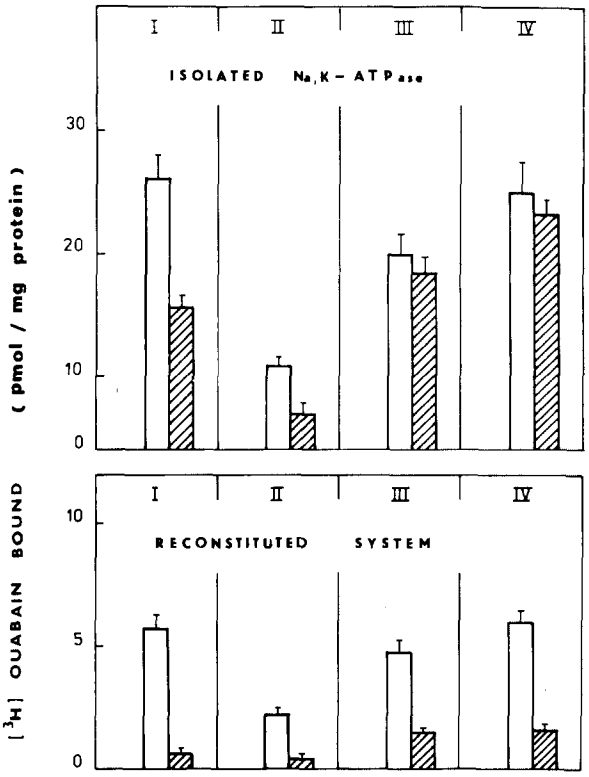


Fig. 7. The effect of phospholipase A_2 on ouabain binding to isolated and reconstituted Na,K-ATPase from pig kidney medulla. The Na,K-ATPase from pig kidney medulla was isolated according to Jorgensen (1974) using the SDS-extraction procedure. In the study of the reconstituted system, Na,K-ATPase was incorporated by sonication and dialysis into the artificial phosphatidylcholine vesicles as described by Karlish and Pick (1981). The ouabain binding assay was as described in Materials and Methods using 6 nM [^{3}H] ouabain, a 60-min incubation period at 37°C and the same amount of protein (30–50 μg) in the purified and reconstituted Na,K-ATPase preparation. Incubation media: (*I*) 5 mM NaH_2PO_4, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4; (*II*) 5 mM NaH_2PO_4, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl; (*III*) 2.5 mM ATP, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4; (*IV*) 2.5 mM ATP, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl. □, Control binding; ▨, phospholipase A_2, 1 U/ml

late [^{3}H] ouabain binding was lost in spite of the fact that the detergent alone increased the binding to 140%.

The ability of detergents to interfere with the PLA_2 effect on ouabain binding was further analyzed in SDS-treated, purified preparations of Na,K-ATPase. The Na,K-ATPase was purified from rat brain and pig kidney medulla according to Jorgensen (1974) and Sweadner (1978). Using SDS, the nonspecific membrane constituents were selectively solubilized and the Na,K-ATPase remained in the membrane. Therefore, these purified preparations represent the membrane bound enzyme, which had been extensively treated with a strong anionic detergent.

The effect of PLA_2 on SDS-treated Na,K-ATPase from rat brain (Fig. 7) was also tested, but the results were different from those obtained with microsomes. The stimulation of ouabain binding was not detected. In the P_i + Mg medium (columns *I* and *II*, Fig. 7) phospholipase A_2 caused a marked inhibition of [^{3}H] ouabain binding. Sodium cations decreased levels of ouabain binding for the PLA_2-treated as well as the control enzyme. In ATP + Mg medium (columns *III* and *IV*), PLA_2 induced a minor inhibition which was at the level of statistical significance. The same result was obtained with or without sodium.

Comparison of the data presented in Figs. 5 and 7 clearly shows that the ability of phospholipase A2 to differentiate between type I and II of ouabain binding sites also occurred in the SDS-treated, purified Na,K-ATPase. The stimulation of ouabain binding by phospholipase A2 was abolished for type I binding, but type II binding was still strongly inhibited.

This ability of PLA_2 was completely lost in a reconstituted system, when the purified Na,K-ATPase from pig kidney medulla was incorporated into the artificial phospholipid vesicles by dialysis and sonication according to Karlish and Pick (1981). Under these conditions (*see* Fig. 7, lower half), phospholipase A_2 inhibited the [^{3}H] ouabain binding regardless of the binding media used. The absolute levels of ouabain binding were much lower than in an identical amount of the nonreconstituted enzyme. This can be easily explained if we assume that a significant portion of binding sites are latent, due to a steric hindrance by abundant phospholipids or to an inside-out orientation of phospholipid vesicles.

Therefore, the results obtained in the reconstituted system provide the final evidence in favor of our hypothesis, that the stimulatory effect of PLA_2 on the intact brain and kidney microsomes cannot be solely interpreted as vesicle opening. If such effects existed, they should have also been demonstrated in the reconstituted system, where more than 80% of the total ouabain binding sites are latent (Figs. 5 and 7).

Discussion

Treatment of Na,K-ATPase with massive doses of phospholipases induced inhibition of the enzyme activity and of ouabain binding (Goldman & Albers, 1973; Roelofsen & Van Deenen, 1973; Goodman & Wheeler, 1978; Lane et al., 1978; Lin-Shiau & Chen, 1982). However, diverse and even controversial results have been described: inhibition (Roelofsen & Van Deenen, 1973; Goldman & Albers, 1973;

Goodman & Wheeler, 1978; Lane et al., 1978; Lin-Shiau & Chen, 1982), no change (Erdman & Schoner, 1973; Taniguchi & Iida, 1973) as well as stimulation (Charnock, Simonson & Almeida, 1977; Walter, 1975, 1979; Schmalzing & Kutschera, 1982).

The reason for such discrepancies may be explained by the data presented in this work, namely by the specific ability of PLA_2 to differentiate between ATP + Mg + Na (type I) and P_i + Mg (type II) ouabain binding sites. In intact microsomal preparations of Na,K-ATPase, phospholipase A_2 stimulates the former and inhibits the later type of binding. The total number of ATP + Mg + Na-dependent sites increased twofold, while the dissociation constant remained unchanged. In the case of P_i + Mg-oriented sites, both B_{max} and K_d were drastically reduced.

The ability of phospholipase A_2 to differentiate between type I and II sites is also observed in SDS-treated, purified preparations of Na,K-ATPase, where the P_i + Mg-dependent binding is inhibited. Finally, in Na,K-ATPase reconstituted in an artificial phospholipid system, the differentiation between type I and II sites is completely lost and the ATP + Mg + Na as well as Pi + Mg-oriented binding is inhibited by phospholipase A_2.

Scatchard plots of [^{3}H] ouabain binding in ATP + Mg + Na and P_i + Mg media indicate a single type of binding site under both ligand conditions. Thus, in our experiments with the rat brain Na,K-ATPase, there is no evidence for the presence of multiple types of ouabain binding (Sweadner, 1979, 1985; Stahl, 1986) or negative cooperativity. This finding is surprising in the view of the present data showing the presence of high (alpha$^+$) and low-affinity (alpha) isoenzymes of Na,K-ATPase towards ouabain (Stahl, 1986). It is also possible that the highest [^{3}H] ouabain concentrations used in our experiments (4×10^{-7} M) is not sufficient for a fractional occupation of the low-affinity sites having K_d in the range of 10^{-5} M.

We now consider to what extent our data can be interpreted in the terms of opening of the tightly sealed membrane vesicles or whether some other mechanisms are involved.

Inhibition of the enzyme activity is usually accompanied by decreased ouabain binding (Goldman & Albers, 1973; Roelofsen & Van Deenen, 1973; Lane et al., 1978; Lin-Shiau & Chen, 1982). In some reports, however, the stimulation of ouabain binding by PLA_2 was described. In such cases, the increase of ouabain binding was accompanied by an increased Na,K-ATPase activity (Charnock et al., 1977; Walter, 1975, 1979).

According to Walter (1975, 1979), the stimulation of [^{3}H] ouabain binding to kidney plasma membrane fragments detected after PLA_2 addition is due to an increased permeability of the resealed, inside-out oriented vesicles. This interpretation does not apply to our data, because the experimental protocol of Walter (1979) differs substantially from ours. Walter isolated the inside-out oriented impermeable vesicles in hypertonic sucrose density gradients, and 0.7 M sucrose was also included in the ouabain binding medium during the assay. The effect of PLA_2 was eliminated when 0.7 M sucrose was omitted from the medium or if the incubation was carried out at 37°C instead of at 0°C.

Charnock et al. (1977) showed increased ouabain binding after the PLA_2 treatment in the detergent-treated, partially purified Na,K-ATPase preparation from the rat brain. This increase was proportional to the stimulation of Na,K-ATPase activity.

Similar data were obtained in early studies on the purification of the enzyme, where detergent activation of the crude membrane preparations was observed (Jorgensen & Skou, 1971; Jorgensen, 1975). The stimulation of ouabain binding was accompanied by stimulation of the enzyme activity and such results were interpreted as opening of the sealed vesicles (Jones, Maddock & Besh, 1980; Forbush, 1982). Therefore, the critical question, i.e., to what extent the PLA_2 effect on the brain microsomes may or may not be explained by vesicle opening, is discussed together with the effect of detergents, which was also tested in this work.

The hypothesis of opening of the right-side-out oriented vesicles after the detergent treatment, which was carefully tested by Forbush (1982) in kidney microsomes, cannot explain our data. In our case, the Na,K-ATPase activity was never stimulated by PLA_2. Beginning with the lowest concentrations and under a wide variety of experimental conditions (*see* Fig. 1 and Table 1), phospholipase A_2 was always inhibitory for Na,K-ATPase activity. Therefore, the intact brain microsomes must be either freely permeable or sealed and inside-out oriented.

If these vesicles are opened, the ouabain binding to ATP-oriented sites will be increased, but why does the P_i-supported ouabain binding decrease? The ouabain binding site, as such, should be oriented towards the inner compartment of the vesicle regardless of the presence of ATP or P_i in the medium, and ATP, Mg and P_i should be freely available for their respective sites at the external surface. It is unlikely that phospholipase A_2 will open the vesicles in the presence of ATP + Mg + Na and close them in P_i + Mg medium. Neither can it be concluded that the permeability for ouabain will increase in the presence of ATP + Mg + Na and decrease in the presence of P_i + Mg.

The first possible explanation of our data combines the opening of the inside-out vesicles with the role of Na^+. Sodium cations were shown to stimulate the ouabain binding in the presence of ATP + Mg and in the absence of K^+ (Lindenmayer & Schwartz, 1973). Therefore, in the ATP + Mg + Na medium, phospholipase A_2 could induce a substantial increase of ouabain binding by enhancing the permeability for both external ouabain and sodium. The effect of 100 mM Na^+ should be oriented to low-affinity Na sites located at the cytoplasmic side of the membrane (Garrahan, Horestein & Rega, 1977).

In the P_i+ Mg medium, sodium strongly inhibits ouabain binding (Albers, Koval & Siegel, 1968). According to Bodeman and Hoffman (1976), this effect of Na^+ also takes place on the cytoplasmic side of the membrane. Therefore the PLA_2 effect mediated via sodium could result in the marked inhibition of the type II ouabain binding (P_i + Mg). However, the data presented in Figs. 1, 5 and 7 clearly show that the PLA_2 inhibits the P_i + Mg-supported binding in the absence of sodium.

The main arguments against opening of inside-out oriented vesicles may be formulated as follows: (i) the PLA_2-induced stimulation of [^{3}H] ouabain binding in microsomes may be demonstrated in permeabilized membranes suspended in distilled water, sonicated or repeatedly frozen (Table 1, Fig. 5), (ii) in the reconstituted system, where at least a fraction of Na,K-ATPase molecules should be in an inside-out orientation (Fig. 7), and permeability problems should be similar to those of the original microsomes, the ATP+ Mg + Na-dependent sites are inhibited by PLA_2 equally well as the P_i+ Mg-oriented sites.

Therefore, it is suggested that the opening of the vesicles represents only the first, minor or partial event in the phospholipase A_2 effect on the membrane. Subsequently or simultaneously with opening of the vesicles, phospholipase A_2 begins to interact with the hydrophobic membrane interior.

The question as to what extent the ability of PLA_2 to differentiate between type I and II of ouabain binding can be interpreted in the terms of E_1–E_2 transition is difficult to answer. Both optimum conditions of ouabain binding are usually related to the E_2 conformation. The fact that there was no difference in fluorescence parameters of anthroylouabain bound in the presence of ATP + Mg + Na or P_i + Mg favors this single intermediate hypothesis (Fortes, 1977; Moczydlowski & Fortes, 1980). However, the ouabain binding complex formed in the presence of P_i + Mg is more stable than in the presence of ATP + Mg + Na (Forbush, 1983). This result seems incompatible with the Post-Albers scheme since both conditions should stabilize E_2 (Cantley, 1980).

Lee and Fortes (1985) studied the anthroylouabain binding to Na,K-ATPase as a function of Na^+ concentrations in the presence of ATP+ Mg and showed that it binds to E_1-P, although with a lower affinity than to E_2-P. They also suggest the existence of a third phosphoenzyme E_x-P, intermediate between E_1-P and E_2-P. In the presence of Na^+ cations the E_1-P, which contains three occluded Na^+ ions can bind cardiac glycosides at a rate comparable to that of E_2-P. In our experiments, the sodium cations were essential for the demonstration of the stimulatory effect of PLA_2 on type I of ouabain binding. It is possible that the balance between E_1-P, E_x-P and E_2-P intermediates responsible for the overall ouabain binding is changed by the physical state of the membrane and thus also by treatment with phospholipase A_2 and detergents.

References

Abeywardena, M.Y., Charnock, J.S. 1983. Modulation of cardiac glycoside inhibition of Na,K-ATPase by membranes: Difference between species. *Biochim. Biophys. Acta* **729:**75–84

Abeywardena, M.Y., McMurichie, E.J., Russel, G.R., Charnock, J.S. 1984. Species variation in the ouabain sensitivity of cardiac Na,K-ATPase: A possible role for membrane lipids. *Biochem. Pharmacol.* **33:**3649–3654

Albers, R.W., Koval, G.J., Siegel, G.J. 1968. Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium activated adenosine triphosphatase. *Mol. Pharmacol.* **4:**324–336

Bodemann, H.H., Hoffman, J.F. 1976*a*. Side-dependent effects of internal versus external Na and K on ouabain binding to reconstituted human red blood cells ghosts. *J. Gen. Physiol.* **67:**497–525

Bodemann, H.H., Hoffman, J.F. 1976*b*. Comparison of the side-dependent effects of Na and K on orthophosphate-, UTP- and ATP-promoted ouabain binding to reconstituted human red blood cell ghosts. *J. Gen. Physiol.* **67:**527–545

Cantley, L.C. 1980. Structure and mechanism of the Na,K-ATPase. *Curr. Topics Bioenerg.* **11:**201–237

Charnock, J.S., Simonson, L.P., Almeida, A.F. 1977. Variation in sensitivity of the cardiac glycoside receptor characteristics of Na,K-ATPase to lipolysis and temperature. *Biochim. Biophys. Acta* **465:**77–92

Cressie, L.C., Keightley, O.D. 1981. Analysing data from hormone-receptor assays. *Biometrics* **37:**235–249

De Robertis, E., DeIraldi, A.P., DeLorez, G.R., Salganicoff, A.L. 1962. Cholinergic and noncholinergic nerve endings in the rat brain. *J. Neurochem.* **9:**23–35

Erdman, E., Schoner, W. 1973. Ouabain-receptor interactions in Na,K-ATPase preparations: III. On the stability of the ouabain receptor against physical treatment, hydrolases and SH reagents. *Biochim. Biophys. Acta* **330:**316–324

Forbush, B., III. 1982. Characterisation of right-side-out membrane vesicles rich in Na,K-ATPase and isolated from dog kidney medulla. *J. Biol. Chem.* **257:**12678–12684

Fortes, P.A.G. 1977. Anthroylouabain A: A specific fluorescent probe for the cardiac glycoside receptor on Na,K-ATPase. *Biochemistry* **16:**531–540

Garrahan, P.J., Horestein, A., Rega, A.F. 1977. The interaction of ligands with the Na,K-ATPase during Na,K-ATPase activity. *In:* Na,K-ATPase: Structure and kinetics. J. C. Skou and J. G. Norby, editors. pp. 261–274. Academic, London

Goldmann, S.S., Albers, R.W. 1973. Sodium-potassium activated adenosine triphosphatase: IX. The role of phospholipids. *J. Biol. Chem.* **248:**867–874

Goodman, S.L., Wheeler, K.P. 1978. Ouabain binding to phospholipid dependent adenosine triphosphatase. *Biochem. J.* **169:**313–320

Jones, L.R., Maddock, S.W., Besh, H.R. 1980. Unmasking effect of alamethicin on beta-adrenergic receptor-coupled adenylate cyclase and cAMP-dependent protein kinase activities of cardiac sarcolemmal vesicles. *J. Biol. Chem.* **255:**9971–9980

Jorgensen, P.L. 1975. Isolation and characterization of the components of the sodium pump. *Q. Rev. Biophys.* **7:**239–274

Jorgensen, P.L. 1974. Purification and characterization of Na,K-ATPase: III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by SDS. *Biochim. Biophys. Acta* **356:**36–52

Jorgensen, P.L., Skou, J.C. 1971. Purification and characterization of Na,K-ATPase: I. Influence of detergents on the activity of Na,K-ATPase in preparations from the outer medulla of rabbit kidney. *Biochim. Biophys. Acta* **233:**366–380

Karlish, S.J.D., Pick, U. 1981. Sidedness of the effects of sodium and potassium ions on the conformational state of the sodium-potassium pump. *J. Physiol. (London)* **312:**505–529

Kimmelberg, H.K., Papahadjopoulos, D. 1974. Effects of phospholipid acyl chain fluidity, phase transitions and cholesterol on Na,K-stimulated triphosphatase. *J. Biol. Chem.* **249:**1071–1080

Lane, L.K., Anner, B.M., Wallick, E.T., Ray, M.V., Schwartz, A. 1978. Effect of phospholipase treatment on the partial reactions of and ouabain binding to a purified sodium and potassium activated adenosine triphosphatase. *Biochem. Pharmacol.* **27:**225–231

Lee, J.A., Fortes, P.A.G. 1985. Anthroylouabain binding to different forms of Na,K-ATPase. *In:* The Sodium Pump. I. M. Glynn and J. C. Ellory, editors. pp. 277–282. Company of Biologists, Cambridge, England

Lindenmayer, G.E., Schwartz, A. 1973. Nature of the transport adenosine triphosphatase digitalis complex: IV. Evidence that sodium-potassium competition modulates the rate of ouabain interaction with Na,K-adenosine-triphosphatase during enzyme catalysis. *J. Biol. Chem.* **248:**1291–1300

Lin-Shiau, S.Y., Chen, C.C. 1982. Effects of beta-bungarotoxin and phospholipase A_2 from Naja Naja snake venom on ATPase activities of synaptic membranes from rat cerebral cortex. *Toxicon* **20:**409–418

Matsuda, T., Iwata, H. 1986. Phospholipid composition of cardiac Na,K-ATPase from various species. *Experientia* **42:**405–407

Moczydlowski, E.G., Fortes, P.A.G. 1980. Kinetics of cardiac glycoside binding to Na,K-adenosine triphosphatase studied with a fluorescence derivative of ouabain. *Biochemistry* **19:**969–977

Pullman, M.E., Penefsky, H.S., Datta, A., Racker, E. 1960. Partial resolution of the enzymes catalyzing oxidative phosphorylation: I. Purification and properties of soluble dinitrophenol stimulated adenosine triphosphatase. *J. Biol. Chem.* **235:**3322–3329

Roelofsen, B. 1981. The (non)specificity in the lipid-requirement of calcium and sodium plus potassium-transporting adenosine triphosphatases. *Life Sci.* **29:**2235–2247

Roelofsen, B., Van Deenen, L.L.M. 1973. Lipid requirement of membrane bound ATPase: Studies on human erythrocyte ghosts. *Eur. J. Biochem.* **40:**245–257

Scatchard, G. 1949. The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51:**660–672

Schmalzing, G., Kutschera, P. 1982. Modulation of ATPase activities of human erythrocyte membranes by free fatty acids or phospholipase A_2. *J. Membrane Biol.* **69:**65–76

Schull, G.E., Schwartz, A., Lindgrel, J.B. 1985. Amino acid sequence of the catalytical subunit of the Na,K-ATPase deduced from the complementary DNA. *Nature (London)* **316:**691–695

Stahl, W.L. 1986. The Na,K-ATPase of the nervous tissue. *Neurochem. Int.* **8:**449–476

Svoboda, P., Mosinger, B. 1981. Catecholamines and the rat brain microsomal Na,K-ATPase: I. Protection against lipoperoxidative damage. *Biochem. Pharmacol.* **30:**427–432

Sweadner, K.J. 1978. Purification from rat brain of an intrisic membrane protein fraction enriched in Na,K-ATPase. *Biochim. Biophys. Acta* **508:**486–499

Sweadner, K.J. 1979. Two molecular forms of Na,K-stimulated ATPase in brain. Separation and difference in affinity for strophantidin. *J. Biol. Chem.* **254:**6060–6067

Sweadner, K.J. 1985. Enzymatic properties of separated isoenzymes of the Na,K-ATPase: Substrate affinities, kinetic cooperativity and ion transport stoichimetry. *J. Biol. Chem.* **260:**11508–11513

Taniguchi, K., Iida, S. 1973. The role of phospholipids in the binding of ouabain to sodium and potassium dependent adenosine triphosphatase. *Mol. Pharmacol.* **9:**350–359

Taussky, H.H., Shorr, E. 1953. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* **202:**675–685

Walter, H. 1975. Tightness and orientation of vesicles from guinea-pig kidney estimated from reaction of adenosine triphosphatase dependent on sodium and potassium ions. *Eur. J. Biochem.* **58:**595–601

Walter, H. 1979. Permeability of plasma membrane vesicles to ouabain and Mg^{2+} as a factor determining the rate of binding of ouabain to Na,K-ATPase. *Z. Naturforsch.* **34:**1224–1231

Received 12 August 1987; revised 7 March 1988