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INHIBITION OF (Na^+ + K^+)-ATPase BY OUABAIN: INVOLVEMENT OF CALCIUM AND MEMBRANE PROTEINS

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Summary

Treatment of plasma membrane isolated from murine plasmocytoma MOPC 173 with an EDTA-containing buffer resulted in a 300-fold increase in sensitivity of (Na^+ + K^+)-stimulated Mg^{2+}-ATPase to ouabain. This phenomenon was associated with the solubilization by EDTA of phospholipid free proteins (approx. 30 000—34 000 daltons) from the cytoplasmic face of the plasma membrane and with removal of about 90% of the membrane bound Ca^{2+}. The recovery of the original resistance to ouabain required specifically Ca^{2+} and was associated with a binding of the solubilized proteins to the membrane.

Introduction

The (Na^+ + K^+)-stimulated Mg^{2+}-ATPase (Na^+ + K^+-ATPase: EC 3.6.1.3), which is responsible for the coupled active transport of Na^+ and K^+, reacts with and is specifically inhibited by ouabain (for a review, see Ref. 1). This cardiac glycoside binds to the large polypeptide of the enzyme [2] at the external face of the plasma membrane [3]. Sensitivity to ouabain greatly varies between different species [4—9]. As a general rule, enzyme from rodents is least sensitive to ouabain. Enzyme from man, beef and dog is sensitive to the drug whereas the enzymes from guinea pig and rabbit exhibit an intermediate sensitivity. Ouabain-sensitive K^+ influx in Hela cells and rodent firboblasts is completely inhibited by 10^{-6} M and $3 \cdot 10^{-4}$ M respectively [10]. These differences in sensitivity to ouabain have been shown to result from differences in the affinity of the enzyme to ouabain [5,8,9,11,12]. In murine plasmocytoma

cells, it was previously shown that the sensitivity of the membrane-bound ($Na^+ + K^+$)-ATPase to ouabain depended upon the plasma membrane isolation procedure since EDTA, which increased dramatically the sensitivity of the enzyme to ouabain, removed constituents from the inner face of membranes [13,14]. The purpose of this study is to further investigate the nature of these constituents and their relationship to the murine plasma membranes.

Materials and Methods

Plasma membrane isolation. Plasma membranes from murine plasmocytoma MOPC 173 ascitic cells (MF_2S) were purified by isopycnic centrifugation [15]. Inside-out and right side-out membrane vesicles were isolated by concanavalin A affinity chromatography and characterized as previously described [14,16].

Plasma membrane treatment with EDTA. Plasma membranes (0.5 mg/ml protein) were incubated for 45 min with magnetic stirring at 0°C in a pH 6.8 buffer containing 250 mM sucrose and 30 mM imidazole-chloride (imidazole sucrose buffer), and centrifuged 30 min at a speed which yielded 31 000 $\times g$ at the bottom and 24 000 $\times g$ at the top of the sample. The pellet was suspended (0.5 mg protein/ml) in imidazole sucrose buffer and submitted to the same treatment. The second pellet was resuspended at the same protein concentration in imidazole sucrose buffer (pH 6.8) containing 1 mM EDTA (imidazole salt) and incubated for 90 min at 0°C with magnetic stirring. After centrifugation the pellet was resuspended in 60% glycerol and stored at —20°C. No ($Na^+ + K^+$)-ATPase activity was found in the supernatants.

Partial purification of constituents removed by EDTA. Supernatant from the EDTA treatment was extensively dialyzed against EDTA-free buffer and concentrated on an UM 10 Amicon filter, then filtered through an XM 50 Amicon filter. Two fractions were obtained and stored at 4°C; fraction 1 which passed through the XM 50 filter and fraction 2 which did not.

Ouabain sensitivity of ($Na^+ + K^+$)-ATPase activity. The ($Na^+ + K^+$)-ATPase activity was assayed at 37°C according to Ottolenghi [17] using 30 μg plasma membrane protein/ml of incubation medium. The ($Na^+ + K^+$)-ATPase activities of different preparations were in the range of 12—20 μmol P_i/h per mg of protein which was 60% of the total ATPase activity. The sensitivity of ($Na^+ + K^+$)-ATPase was checked with 10^{-7}—10^{-3} M ouabain (Calbiochem) (prepared daily). The mixture was preincubated for 10 min at 37°C in the assay medium containing 4 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 40 mM imidazole-HCl, pH 7.4. The reaction was initiated with the addition of prewarmed ATP (final concentration 2 mM) and carried out over six time points to assess linearity. Every 2 min aliquots were taken and the assay was terminated by transferring them to test tubes containing sulfuric acid and molybdate where the liberated P_i was measured [18]. The activities in the presence of varying ouabain concentrations are expressed as the percentage of ($Na^+ + K^+$)-ATPase activity measured in the absence of ouabain. All results are corrected for ATPase activity measured in the absence of K^+ (ionic strength compensated with choline chloride) which was equal to the activity not inhibited by 10^{-3} M ouabain.

Reconstitution. Reconstitution experiments were carried out as follows: In 1 ml imidazole sucrose buffer, 150 μg of EDTA treated pellet plus $CaCl_2$ (2 ·

10^{-6}—10^{-4} M) were preincubated for 30 min at 4°C with concentrated supernatants. Then, aliquots (about 12 μg of proteins) were taken and the enzymic assay was initiated by transferring these aliquots to incubation tubes for measurement of sensitivity to ouabain. Because other buffers contain ligands that either complex with calcium or increase free calcium concentration in the assays, imidazole sucrose buffer was used in these experiments.

Gel electrophoresis. Sodium dodecyl sulphate-10% polyacrylamide slab gels were prepared according to Laemmli [19]. Gels were stained with 0.05% Coomassie Blue R in 10% acetic acid, 30% methanol and destained in the same solvent. Gels were scanned at 570 nm from the top to the dye position with a Pye-Unicam spectrophotometer.

Determination of lipid-phosphate in EDTA supernatants. 5 μg (0.1 ml) of fractions 1 or 2 from the Amicon filtration were mixed with 0.5 ml 65% $HClO_4$ and 0.1 ml of 30% H_2O_2 and treated for 30 min at 180 to 200°C. The dried material was resuspended in water and aliquots taken for determinations of inorganic phosphate.

Calcium analysis

Calcium determinations were carried out with a Jobin Yvon J.Y. 38 inductively coupled plasma spectrophotometer. Calcium emission was recorded at 393.4 nm. Calcium contamination of the buffers and assay mixtures were measured. Standard solutions were then prepared in deionized water.

Membrane samples were treated as follows: 0.5 ml of membrane suspension was mixed with 4.5 ml of a 1% Triton X 100 solution. This yielded a clear sample which could be directly assayed for calcium content. Blank and standard solutions were prepared in 1% Triton. Calcium content of the blank was 10-fold less than that of the EDTA treated membrane samples.

Results

In native right side-out and inside-out vesicles [14], the $(Na^+ + K^+)$-ATPase was found to be 50% inhibited by 120 ± 20 μM ouabain (symbolized as $E_{1/2}$). After EDTA treatment plasma membrane vesicles retained 90 ± 8% of their $(Na^+ + K^+)$-ATPase activity [13,14]. In EDTA treated right side-out vesicles, $E_{1/2}$ remained unchanged whereas in treated inside-out vesicles, $E_{1/2}$ decreased to 0.4 ± 0.1 μM. Consistent with the above, unfractionated membranes treated with EDTA exhibited a two-step dose response curve to ouabain (Fig. 1). The first step reflects inhibition of $(Na^+ + K^+)$-ATPase of inside-out vesicles whereas the second step reflects the inhibition of right side-out vesicles. Native membranes and EDTA treated membranes contained 1.88 ± 0.11 and 0.17 ± 0.03 μg Ca^{2+}/mg protein respectively (mean of 22 measurements). To demonstrate that a long lasting incubation of membranes with ouabain does not increase the sensitivity to the drug, the $(Na^+ + K^+)$-ATPase activity in native and reconstituted membranes (see below) was measured over a 70 min assay in the presence of varying amounts of ouabain. The amount of proteins in the assay was 5 μg in 0.4 ml assay medium and 40 μl aliquots were taken every 8 min. The kinetics were linear over 70 min whatever the ouabain concentration and the sensitivity to ouabain was the same as membranes incubated only 10 min.

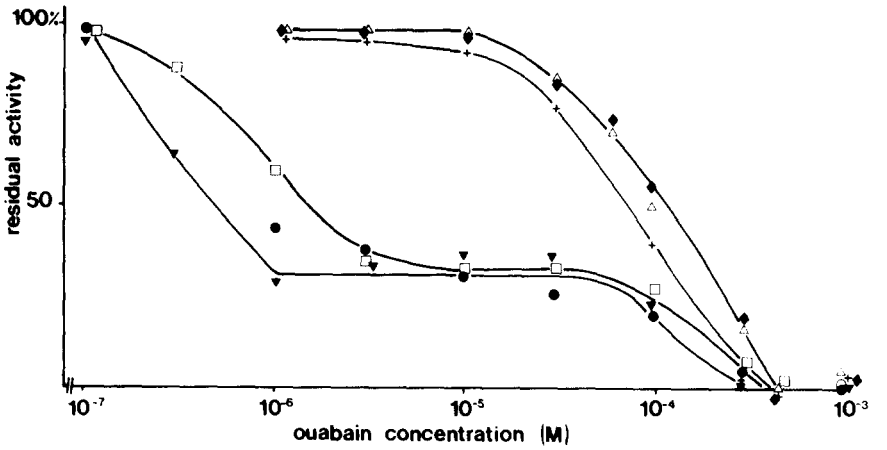

Fig. 1. $(Na^+ + K^+)$-ATPase activity versus ouabain concentration (logarithmic scale) in treated and reconstituted plasma membranes. The ouabain sensitivity of $(Na^+ + K^+)$-ATPase activity was tested in native membranes (△) and in the EDTA treated membranes (▾). Reconstitution experiments were carried out as follows: in 1 ml imidazole sucrose buffer, 150 μg of EDTA-treated membrane pellet plus 10^{-4} M $CaCl_2$ were preincubated for 30 min at 4°C with either 30 μg of fraction 1 (♦) or 150 μg of fraction 2 (□) or 30 μg of fraction 1 pretreated with proteases (●). This pretreatment of fraction 1 was carried out for 3 h at 37°C with 30 μg of this fraction, 7 μg of trypsin and chymotrypsin (Calbiochem) at a protein concentration of 0.2 mg/ml in a 0.1 M Tris-chloride buffer at pH 8.0. Evidence for protein binding to the membrane: $(Na^+ + K^+)$-ATPase sensitivity to ouabain in 31 000 × *g*/30 min pellet of membranes reconstituted with 30 μg fraction 1 and 10^{-4} M $CaCl_2$ (+). Results depicted in this figure are individual experiments which are representative of three to seven independent experiments.

Centrifugation of EDTA-treated membranes was necessary to alter the sensitivity to ouabain. Plasma membranes treated by 1 mM EDTA for 60 min at 4°C without subsequent centrifugation exhibited the same $(Na^+ + K^+)$-ATPase sensitivity to ouabain as native membranes.

The proteins solubilized by EDTA can be removed from the membranes by centrifugation. The effects of repetitive treatments on the shift of sensitivity to ouabain was therefore examined. As shown in Table I, the time of incubation before centrifugation did not affect the increase in sensitivity very much. The number of treatments, however, greatly affected the increase in sensitivity. Note that two successive 30 min treatments with EDTA followed by centrifugations increased the sensitivity to ouabain much more than a single 60 min incubation followed by a centrifugation ($E_{1/2}$: 0.6 ± 0.1 μM and 8 ± 2 μM, respectively).

Another method of altering the equilibrium between free and bound effectors is by dilution. Native plasma membranes were incubated (0.5 mg protein/ml) for 30 min with magnetic stirring at 4°C in a imidazole sucrose buffer and then diluted 100-fold in the same buffer. No alteration in sensitivity was noted. If, however, EDTA containing buffer was used for the dilution, a 5-fold increase in sensitivity was noted (Table I, part B). Further, if native membranes were incubated for 30 min at 4°C in EDTA containing buffer and then centrifuged, the $E_{1/2}$ measured in the pellet was 12 μM. If the pellet was diluted 100-fold (5 μg/ml) in EDTA containing buffer, the $E_{1/2}$ was 3 μM. Centrifuged EDTA treated membranes are still sensitive to dilution. The sensitivity of the diluted enzyme to ouabain was determined over a 40 min time period after adjust-

TABLE I

EVIDENCE FOR AN EQUILIBRIUM BETWEEN MEMBRANE-BOUND AND FREE FORMS OF PLASMA MEMBRANE INNER FACE PROTEINS

$E_{1/2}$ is the ouabain concentration required to inhibit 50% of the $(Na^+ + K^+)$-ATPase activity.

Preincubation periods (min)	EDTA [a,d] containing buffer	Number of centrifugations	100-fold dilution for 10 min before assay	$E_{1/2}$ (μM)
(A)				
30	yes	0	no	120 ± 20
30	yes	1	no	12 ± 2
60	yes	1	no	8 ± 2
90 [c]	yes	1	no	6 ± 2
30 + 30	yes	2	no	0.6 ± 0.1
30 + 30 + 30 [c]	yes	3	no	0.10 ± 0.02
(B)				
30	no [b]	0	yes	120 ± 20
30	yes	0	yes	25 ± 5
30	yes	1	no	12 ± 2
30	yes	1	yes	3 ± 1
30	no	1	no	65 ± 5

[a] 30 mM imidazole chloride, 250 mM sucrose, 1 mM EDTA (as imidazole salt) buffer pH 6.8 at 4°C.

[b] 30 mM imidazole chloride, 250 mM sucrose, buffer pH 6.8 at 4°C.

[c] The time of incubation up to 150 min at 4°C in the presence of 1 mM EDTA containing imidazole sucrose buffer does not affect the specific activity of $(Na^+ + K^+)$-ATPase.

[d] The presence of 0.6 mM imidazole-EDTA (final concentration) in the assay medium did not affect the specific activity of $(Na^+ + K^+)$-ATPase preparation.

ment of the assay medium to the same ionic composition as described under Materials and Methods.

The addition of the non-concentrated and non-dialyzed supernatants from EDTA-treated membranes to the 31 000 × *g*/30 min pellet did not restore the resistance to ouabain even with Ca^{2+}. Concentrated and dialyzed material removed by EDTA from inside-out vesicles, in contrast with that released from right side-out vesicles, was able to restore the original resistance when added back to EDTA treated vesicles with Ca^{2+} [14]. This property was used to characterize the solubilized material during purification. The supernatant from centrifugation for 60 min at 100 000 × *g* was able to restore resistance showing that the material was water soluble. The capacity to restore resistance was retained after filtration and concentration (see Materials and Methods) and was associated only with fraction 1 (Fig. 1 and Table II). The capacity to restore resistance was lost after treatment of fraction 1 with a mixture of trypsin and chymotrypsin (Fig. 1). Under the conditions of pH and temperature and at the protein dilution used here the presence of proteases during preincubation and enzyme assay did not significantly affect $(Na^+ + K^+)$-ATPase activity. An aliquot from fraction 1 incubated without proteases under the same conditions did not significantly affect the restoration capacity. The restoration capacity of fraction 1 was lost by freezing but was found to be stable for at least 4 months at 4°C in aqueous solution. A remarkable feature of this phenomenon was the failure of fraction 1 plus Ca^{2+} to restore resistance if added to the incubation medium at 37°C after initiation of the enzymatic assay. The capacity of frac-

TABLE II

CAPACITY OF FRACTIONS 1 AND 2 TO RESTORE RESISTANCE

Ion concentrations and amounts of fractions 1 and 2 added in the preincubation medium to 150 μg of treated membranes (see Reconstitution in Materials and Methods). Fractions 1 and 2 are dialyzed fractions of EDTA supernatant filtered through a XM 50 Amicon filter.

Ions (M)		Supernatant fractions (μg of protein)		$E_{1/2}$ (μM)
$CaCl_2$	$MgCl_2$	Fraction 1	Fraction 2	
0	0	0	0	0.4 ± 0.1
10^{-4}	$2 \cdot 10^{-3}$	30—150		120 ± 20
10^{-4}	$2 \cdot 10^{-3}$		30—150	0.85 ± 0.15

tion 1 to restore the resistance was also lost if preincubation was carried out at 20°C instead of 4°C (data not shown).

Fraction 2 contained 600 ± 5 nmol phosphorus of phospholipid origin per mg of protein, whereas the amount of phosphorus in fraction 1 was below the limit of detection of the method and must be less than 20 nmol per mg of protein.

Gel electrophoresis in sodium dodecyl sulphate showed that the EDTA supernatants contained major bands at 39 000 and 34 000 daltons and minor

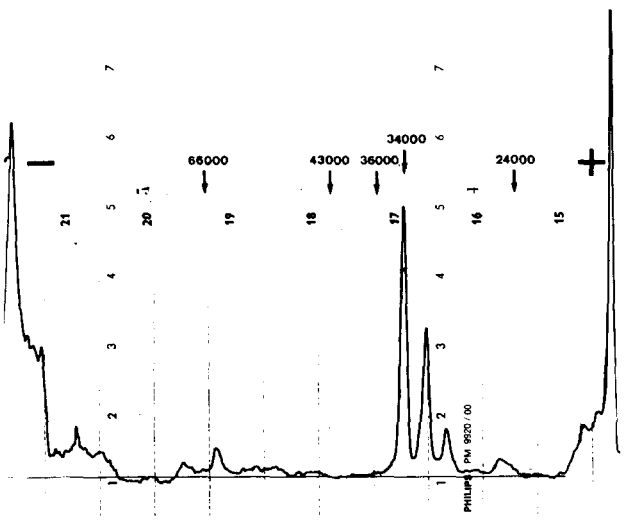

Fig. 2. Gel electrophoresis of the EDTA supernatant. Scan of a Coomassie blue-stained sodium dodecyl sulphate 10% polyacrylamide slab gel (according to Laemmli [19]) of supernatant from EDTA treatment of inside-out vesicles. Gel was scanned at 570 nm from the top of the gel to the dye position in a Pye Unicam 1800 spectrophotometer. Molecular weight standards were: 66 000 bovine serum albumin, 43 000 actin from white muscle of rabbit, 36 000 and 34 000 β- and α-tropomyosin from rabbit white muscle, 24 000 chymotrypsinogen.

TABLE III

IONIC CONDITIONS FOR THE RESTORATION OF THE ORIGINAL RESISTANCE OF $(Na^+ + K^+)$-ATPase TO OUABAIN IN EDTA-TREATED MEMBRANES

The Ca^{2+} concentration in the assay medium was adjusted to $2 \cdot 10^{-5}$ M whatever the Ca^{2+} concentration in the preincubation medium.

Ions (M)		Fraction 1 (μg of protein)	$E_{1/2}$ (μM)
$CaCl_2$	$MgCl_2$		
10^{-4}	0	30—150	120 ± 20
$1.1 \cdot 10^{-5}$ [a]	0	30	120 ± 20
$2.4 \cdot 10^{-6}$ [a,b]	0	30	14 ± 3
0	$2 \cdot 10^{-3}$	30—150	0.4 ± 0.1
10^{-4}	$2 \cdot 10^{-3}$	30	120 ± 20

[a] The Ca^{2+} concentration in the imidazole sucrose buffer was taken into account in these final concentrations.

[b] The amount of Ca^{2+} in the preincubation medium was 4-fold higher than the amount of Ca^{2+} bound to the 150 μg of treated membranes (170 ng Ca^{2+}/mg proteins or 0.64 μM Ca^{2+}).

bands at 30 000 and 60 000 daltons (Fig. 2). Fraction 1 prepared from inside-out vesicles contained approx. 4% of the total membrane proteins. Although varying with different preparations, the amount of protein in the three major bands (30 000 to 34 000 daltons) represented about 50% of the total amount of proteins in fraction 1.

The effect of Mg^{2+} and Ca^{2+} on the restoration capacity was examined (Table III and Fig. 3). When added back to 150 μg of EDTA treated membranes, calcium alone (10^{-4} M), magnesium alone ($2 \cdot 10^{-3}$ M) and fraction 1

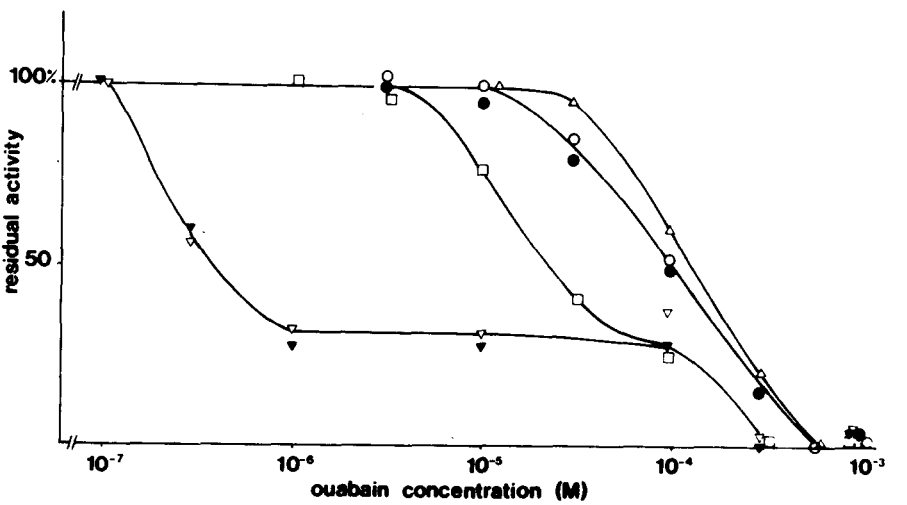

Fig. 3. Effects of varying Ca^{2+} concentrations on the restoration of the original resistance to ouabain. The $(Na^+ + K^+)$-ATPase sensitivity to ouabain was assayed simultaneously in EDTA-treated membrane pellet alone (▼) and EDTA-treated membrane pellet plus 10^{-4} M Ca^{2+} (▽), fraction 1 plus 10^{-4} M Ca^{2+} (●), fraction 1 plus $1.1 \cdot 10^{-5}$ M Ca^{2+} (○) or fraction 1 plus $2.4 \cdot 10^{-6}$ M Ca^{2+} (□). Control: native plasma membranes (△). Results presented are the average of duplicate determinations on three different plasma membrane preparations.

alone (30—150 μg) were ineffective. Fraction 1 (30 μg) plus 10^{-5} M or 10^{-4} M $CaCl_2$ fully restored the original resistance, but 10^{-6} M $CaCl_2$ led only to a partial restoration (Fig. 3 and Table III). These concentrations of calcium did not inhibit the $(Na^+ + K^+)$-ATPase under our assay conditions. Fraction 1 plus $MgCl_2$ ($2 \cdot 10^{-3}$ M) was ineffective and Mg^{2+} alone did not inhibit the restoration induced by Ca^{2+} plus fraction 1 (Table III). Amounts of fraction 1 below 15 μg were generally insufficient for complete restoration.

To determine whether or not recovery of the original sensitivity was associated with a protein binding to the membrane, the following procedure was used. Plasma membranes treated with EDTA were preincubated for 30 min with 30 μg fraction 1 and 10^{-4} M $CaCl_2$. They were then centrifuged for 30 min at 31 000 × *g* and the $(Na^+ + K^+)$-ATPase in the pellet was assayed for its sensitivity to ouabain. There was no loss of total ATPase activity in the pellet. There was no detectable $(Na^+ + K^+)$-ATPase activity in the supernatant. The ouabain sensitivity of the pellet-bound ATPase was the same as in native membranes (Fig. 1). Only the sensitivity of the inside-out vesicle-bound $(Na^+ + K^+)$-ATPase activity was affected.

Discussion

In this paper we have reported that phospholipid-free proteins, bound directly or indirectly via Ca^{2+} to the inner face of membranes can affect the sensitivity of the $(Na^+ + K^+)$-ATPase to ouabain.

The shift of the sensitivity of the enzyme to ouabain seemed not to be due to drastic differences in the rate of drug binding to native and treated membranes since either a 10-min or a 70-min assay in the presence of varying doses of the drug led to the same inhibition of the enzymatic activity. Furthermore, the amount of P_i liberated over these assays was linear with respect to time suggesting that enzyme-ouabain equilibrium was reached in less than two min (the shortest time used) and that ouabain forms a reversible complex with $(Na^+ + K^+)$-ATPase as shown by Allen and Schwartz [5] with preparations from rat heart (insensitive species). The sensitivity of a particular enzyme to ouabain is determined by the affinity of the enzyme for ouabain. To explain the increase in affinity seen in EDTA-treated membranes one must postulate either a decrease in the rate of dissociation and/or an increase in the rate of association. Since the binding of ouabain to plasmocytoma membranes reaches equilibrium in less than two min even in the presence of 20 mM KCl [9,20], we cannot distinguish between these two possibilities. Note that rapid inhibition has also been reported for enzyme prepared from rat hearts [9]. The kinetics of hydrolysis in inhibited inside-out vesicles are linear and the sensitivity to ouabain of native right side-out and inside-out vesicles is the same [14] suggesting the membrane before any EDTA treatment, is freely permeable to ouabain. Therefore, it seems unlikely that the shift in sensitivity is due to a shift in permeability of vesicles to ouabain.

Since the molar ratio of phospholipid to protein in active fraction 1 was less than one and since the capacity to restore was destroyed by treatment of this material with proteases, it seems likely that the active components consist of phospholipid-free proteins. This material is located at the inner face of plasma

membranes since only inside out vesicles are affected by the EDTA treatment and only proteins extracted from these vesicles are able to restore original resistance when added back with Ca^{2+} to treated vesicles. The requirement for calcium in the preincubation appeared to be specific since magnesium (10^{-4}—$2 \cdot 10^{-3}$ M) failed to restore the sensitivity. The effect of Ca^{2+} might be due to Ca^{2+} bound at either the outer or the inner face of the membrane. Asymmetrical effect of Ca^{2+} on membrane-bound enzyme has been reported by Rega et al. [21] who found that Ca^{2+} from the inner face can activate externally located K^+-dependent phosphatase of the erythrocyte membrane.

The shift of sensitivity to ouabain induced by EDTA treatment followed by centrifugation or by dilution in an EDTA containing buffer suggests that a ternary complex of membrane components-Ca^{2+}-protein exists at the inner face of the native plasma membrane. Calcium could serve either as a link between proteins and membranes or as a conformational modifier of membrane binding sites for the proteins. Dilution of native membranes in EDTA containing buffer greatly lowers the concentration of free calcium causing calcium to dissociate from the ternary complex. Treatment of native membranes with EDTA containing buffer reduced the calcium content of the membranes by 90%. Treatment with EDTA followed by centrifugation also causes calcium to dissociate. Furthermore, centrifugation physically separates certain proteins from the membrane-bound ($Na^+ + K^+$)-ATPase. It might be surprising that dilution in imidazole sucrose buffer did not affect sensitivity to ouabain (Table 1b) as imidazole is known to be a weak chelator. In fact, these dilution experiments were done with 5 μg plasma membrane proteins per ml (9.4 ng Ca^{2+}) and imidazole sucrose buffer which contained 64 ng Ca^{2+} per ml (1.4 μM Ca^{2+}). This 6—7-fold higher amount of Ca^{2+} in the medium seems to be sufficient to prevent any shift of sensitivity to ouabain. Under all conditions centrifugation was more effective than dilution in shifting the sensitivity of the membranes to ouabain (Table I). However, a single EDTA treatment and centrifugation did not remove all the proteins involved since sensitivity to ouabain could be affected by subsequent dilution or centrifugation.

The extent and precise location of the binding of the fraction 1 proteins remain unknown. Phospholipids might be involved since it is known (a) that the majority of calcium binding to membranes is to phospholipid polar head groups [22] and (b) interaction of ouabain with purified ($Na^+ + K^+$)-ATPase altered calcium binding to the phospholipids associated with the enzyme [23]. Further, if the native membranes are treated with EDTA plus local anesthetics, which are known to insert into phospholipids, the amount of protein removed by EDTA increases (unpublished observations).

It is unlikely that all the proteins in fraction 1 are specifically related to the effect on ($Na^+ + K^+$)-ATPase. A specific activity of 20 μmoles P_i/mg protein per hour would mean that approximately 1% of the membrane proteins is ($Na^+ + K^+$)-ATPase. Under the conditions of restoration of the original sensitivity (15 μg fraction 1 protein and 150 μg pellet protein) and assuming a molecular weight of 34 000 * and 280 000 daltons, respectively for fraction 1 protein and

* Although the 30 000—36 000 dalton proteins are the major proteins in fraction 1, we cannot exclude the involvement of band(s) weakly stained with Coomassie in the phenomenon described here.

($Na^+ + K^+$)-ATPase, a fraction 1 protein to ($Na^+ + K^+$)-ATPase molar ratio of 100 is implied.

Although recovery of ($Na^+ + K^+$)-ATPase sensitivity to ouabain was complete, we are not suggesting that the reconstituted membranes are identical to the native membranes. In plasmocytoma cells, plasma membrane-bound 5′-nucleotidase activity [15] was lost by EDTA treatment and could not be restored after addition of divalent cations and solubilized proteins (data not shown).

The proteins might be elements of cytoplasmic structure which have been shown to be involved in the modulation of lymphocyte surface receptor mobility [24,25]. The modulation of the external ouabain site by inner face-bound proteins in the presence of calcium might be a similar phenomenon.

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