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THE MEASUREMENT OF OUABAIN BINDING AND SOME RELATED PROPERTIES OF DIGITONIN-TREATED (Na^+, K^+)-ATPase

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Summary

1. Digitonin-treated membrane preparations purified from dog kidney lose their (Na^+,K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity, but the K^+-phosphatase and Na^+-dependent ADP-ATP exchange activities survive and remain ouabain-sensitive. Because the enzyme preparations consist largely of pure (Na^+,K^+)-ATPase, these effects of digitonin must be intrinsic to the Na^+ pump.

2. Concomitant with these enzymatic changes, digitonin treatment alters the sensitivity of the phosphatase and exchange activities to ouabain.

3. Attempts to measure ouabain binding by the usual centrifugation or filtration methods proved unsuccessful. A filtration method involving a double 0.01 μm filter and omitting water washes is necessary to demonstrate ouabain binding. Under these conditions, ouabain binding capacity appears to be unchanged in the presence of digitonin, but the apparent dissociation constant is doubled.

4. Ouabain binding is rendered more reversible by digitonin treatment, since washing filters with water removes a large fraction of bound ouabain without affecting the retention of exchange activity.

5. The double filter method traps essentially all of the ADP-ATP exchange activity on the filter. However, a large and somewhat variable proportion of the K^+-phosphatase activity passes through the filter. Sodium dodecyl sulfate polyacrylamide gel analysis of the filtrate shows that a small amount of fil-

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Abbreviations: (Na^+,K^+)-ATPase, the membrane adenosine 5′-triphosphate phosphohydrolase (EC 3.6.1.3) sensitive to cardiac glycosides and requiring simultaneously Na^+, K^+ and Mg^{2+} for full activity; K^+-phosphatase, the membrane acyl phosphate phosphohydrolase (EC 3.6.1.7) sensitive to ouabain and requiring both K^+ and Mg^{2+} for full activity (and catalyzed by the (Na^+,K^+)-ATPase); EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; SDS, sodium dodecyl sulfate.

trable protein catalyzes this phosphatase activity at greatly increased turnover rates. Both subunits of the (Na^+,K^+)-ATPase are present in this latter protein fraction.

Introduction

A considerable body of evidence now establishes that the (Na^+,K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity in most mammalian tissues is a function of the Na^+ pump. This conclusion is particularly strengthened by recent experiments inserting the (Na^+,K^+)-ATPase into the membrane of liposomal vesicles, where its pumping function can be directly observed [1—4]. Purification of this pump in a number of laboratories has established its qualitative subunit composition and the fact that ouabain binding, K^+-phosphatase and ADP-ATP exchange activities are indeed carried out by the pump itself (see, for example, ref. 5). However, the details of pump architecture and how the active subunits interact with one another to produce cation pumping remain unclear, partly because functional pump subunits have never been isolated.

Previous efforts in our laboratory suggested that digitonin treatment of (Na^+, K^+)-ATPase preparations might lead to functional subunit preparations [6,7]. These studies showed that digitonin could disrupt (Na^+,K^+)-ATPase function without significant effect on its Na^+-dependent ADP-ATP exchange or K^+-dependent acetyl phosphatase activities. The enzyme preparations used were insufficiently active however to effectively attempt subunit isolation. Furthermore, our previous investigation of the effects of digitonin on enzyme properties was rather limited in scope. With the advent of a rapid, reliable means for obtaining relatively pure, high activity (Na^+,K^+)-ATPase preparations [8], we can now show that the previously reported digitonin effects are indeed intrinsic to the pump. An additional feature of the digitonin-treated pump not previously investigated in detail is its interaction with cardiac glycosides. Preliminary evidence with impure preparations suggested that sensitivity to ouabain is altered by digitonin [6]. We now present the results of experiments delineating digitonin's effect on ouabain binding. These experiments also provide additional insight into some physical properties of the digitonin-disrupted membrane enzyme.

Methods

Preparation of purified (Na^+,K^+)-ATPase

Freshly dissected, whole dog kidneys were frozen and stored at −20° C until used. 6—12 frozen kidneys were thawed overnight in the refrigerator by immersing in 0.25 M sucrose/0.001 M EDTA, pH 7.4. The dark red outer medulla was excised and enzyme prepared in the cold essentially as described by Jorgensen [8]. The minced tissue was homogenised in a Waring blendor in 9 volumes of the above medium, then further homogenized by hand in a loose-fitting Potter-Elvehjem homogenizer. After filtering through 2 layers of cheesecloth, the suspension was centrifuged at 10 000 × g for 30 min. The superna-

tant fluid was decanted and centrifuged at 30 000 × *g* for 35 min. The resulting pellets were suspended in 0.7 times the original volume of cold distilled water, combined and analysed for protein content by the method of Lowry et al. [9]. These microsomes were refrigerated overnight or until used (usually within 3 days).

The microsomal suspension was treated with dodecyl sulfate in the following manner: Microsomal protein was added (to a final concentration of 3.6 mg/ml) to a solution containing 25 mM Tris buffer, pH 7.6 at 25° C, 2 mM EDTA and 3 mM ATP (Na^+ salt). A stock solution of dodecyl sulfate (6 mg/ml) was added drop by drop with rapid stirring to give a final concentration of 1.2 mg/ml. (Either sodium dodecyl sulfate or the Tris salt yield similar results). After vigorous stirring at 20° C for 45 min, the somewhat clarified suspension was layered on a column of 50% (w/w) glycerol and centrifuged in the Beckman Ti60 head for 90 min at 60 000 rev./min. We have found that the use of a column of 50% glycerol is more convenient than the discontinuous sucrose gradient described by Jorgensen [8], and yields comparable results. The supernatant fluid was aspirated and discarded and the resulting pellets suspended by hand in cold distilled water. The resulting enzyme has a specific activity of 1200—1500 μmol phosphate released per mg protein per h. It is stable in the refrigerator for 1—2 weeks.

The purified enzyme shows one prominent band on SDS polyacrylamide gel electrophoresis at a position characteristic of the larger subunit of the (Na^+,K^+)-ATPase. In addition, a lightly staining band is seen at the position expected for the glycoprotein subunit. Heavy overloading of the gels reveals traces of other bands, but these do not exceed 5—10% of the staining material. These results are similar to those reported by Jorgensen for enzyme prepared from the kidneys of other species [8].

Measurement of enzyme activities

(Na^+,K^+)-ATPase activity was determined at 37° C in 25 mM Tris, pH 7.4/5 mM ATP/6 mM $MgCl_2$/100 mM NaCl/15 mM KCl/1 mM dithiothreitol and 10—50 μg enzyme protein per ml. Tubes containing 1 mM ouabain in addition served as controls; the ouabain-insensitive hydrolysis of ATP was always negligible in the purified enzyme preparations. The incubation was initiated with ATP and stopped by adding an equal volume of 15% trichloroacetic acid. The orthophosphate released was estimated by Gomori's method [10]. The rate of hydrolysis of ATP was linear with enzyme concentration and constant with time under the conditions stated. When digitonin is present, it interferes with the Gomori procedure. The method of Berkowitz, described by Uesugi and coworkers [11], or the method of Canessa et al. [12] avoided this difficulty.

ADP-ATP exchange activity was measured by the method of Glaze and Wadkins using [^{3}H]ADP [13]. The concentrations of ADP and ATP were each approximately 6 mM, and did not change significantly during the course of the reaction. The Mg^{2+} concentration was 1.2 mM, EGTA was 1 mM, Na^+ was 20 mM and K^+ was omitted; otherwise the incubation conditions were those described for the (Na^+,K^+)-ATPase assay. A ouabain-containing sample provided the control, as described above, and showed negligible exchange activity (see Table IV).

K^+-dependent acetyl phosphatase activity was estimated by measuring the rate of disappearance of acetyl phosphate using the Lipmann-Tuttle method [14]. The incubation medium was as for the (Na^+,K^+)-ATPase assay, except that the Mg^{2+} concentration was 10 mM, Na^+ was omitted, and 6 mM acetyl phosphate substituted for ATP. A ouabain-containing blank corrected for the minimal non-enzymic hydrolysis. Digitonin does not interfere with this colorimetric procedure. Because the disappearance of acetyl phosphate is non-linear with time, inital rates were estimated by the "exact" numerical method of Cornish-Bowden [15].

Measurement of [^{3}H]ouabain binding

[^{3}H]ouabain diluted with various concentrations of unlabelled ouabain was presented to (Na^+,K^+)-ATPase preparations in the following medium: 25 mM Tris, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, 100 mM NaCl, 1 mM dithiothreitol and 100—300 μg enzyme protein per ml. After incubation at 37° C, the reaction mixture was submitted to ultracentrifugation or membrane filtration as described under Results. The incubation time was sufficient for the binding reaction to reach equilibrium. Non-specific trapping of ouabain was estimated in the presence of 1 mM unlabeled ouabain. Centrifugal separation of enzyme-bound ouabain followed the procedure of Lindenmayer and Schwartz [16]. Filter separation of enzyme-bound ouabain was accomplished using a Millipore 3025 Sampling Manifold and various types of filters as described in the text. The protein-containing filter was dissolved in 10 ml Bray's solution [17] for

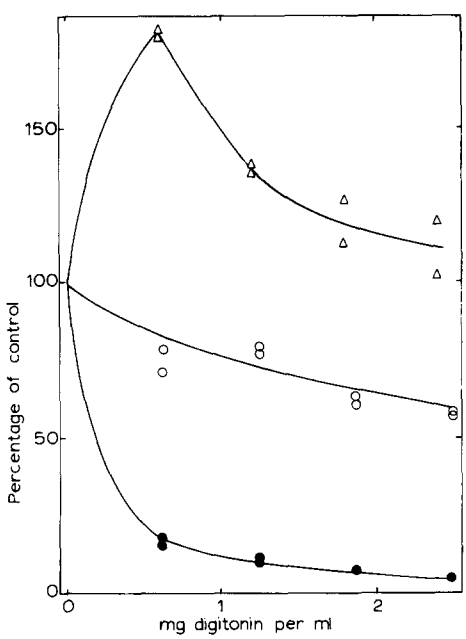

Fig. 1. Effect of increasing concentrations of digitonin on ouabain-sensitive (Na^+,K^+)-ATPase, K^+-phosphatase and ADP-ATP exchange activities of the purified enzyme. Each sample was exposed to digitonin in the presence of 1 mM EGTA in the standard assay medium for measuring that activity. Open circles, K^+-phosphatase; filled circles, (Na^+,K^+)-ATPase; open triangles, ADP-ATP exchange activity.

counting. A 0.1-ml aliquot of filtrate was counted in similar fashion and quench-corrected with the aid of an internal standard-calibrated external standard.

Treatment with digitonin

Commercial samples of digitonin were dissolved by heating in distilled water, then cooling to form a supersaturated stock solution. Addition of the stock 24 mg/ml digitonin solution to protein-containing tubes to various final concentrations prevented reprecipitation of the digitonin.

Materials

[8-^{3}H]ADP was purchased from Schwarz/Mann as a 50% ethanolic solution and dried on a rotary evaporator or in a vacuum desiccator over P_2O_5. The labeled ADP and unlabeled ADP, ATP and acetyl phosphate were converted to the Tris form by passage through a column of Dowex-50/Tris.

[^{3}H]ouabain was obtained from New England Nuclear Corporation as an ethanol/benzene solution. The solvent was removed with a stream of N_2 just prior to use, since aqueous solutions were found to be unstable on prolonged storage. Radiopurity of the labeled ouabain was periodically determined by thin-layer chromatography and by exhaustive binding to (Na^+,K^+)-ATPase as described by Hansen and Skou [18].

Digitonin was obtained from Sigma Chemical Company or Matheson, Coleman and Bell. Other chemicals were reagent grade.

Results

The results in Fig. 1 show that the effects of digitonin on the purified (Na^+, K^+)-ATPase and its associated acetyl phosphatase and ADP-ATP exchange reactions are similar to those reported with less enriched preparations [7]. A somewhat gratifying observation is that K^+-phosphatase activity loss is less marked with the purified enzyme than previously reported. Control experiments (not illustrated) show that activity changes occur immediately on exposure to digitonin and the resulting activity does not change over the course of an hour or so. Alkali metal cation composition of the medium does not appear to influence the activity changes observed.

TABLE I

TYPICAL CONCENTRATIONS OF OUABAIN REQUIRED TO INHIBIT HALF-MAXIMALLY SOME ACTIVITIES OF THE Na^+ PUMP

Exposure to ouabain took place in each case in the standard assay medium for measuring that activity (see Methods). Values are expressed as $I_{0.5}$, that concentration required to inhibit half-maximally the activity in question. Digitonin concentration, 2.4 mg/ml; this concentration is used in all subsequent experiments because it inhibits (Na^+,K^+)-ATPase activity nearly completely (Fig. 1).

	$I_{0.5}$ (μM)	
	without digitonin	with digitonin
(Na^+, K^+)-ATPase	3.2	—
K^+-phosphatase	11	4.2
ADP-ATP exchange	0.23	0.41

Table I shows that digitonin treatment affects the interaction of the enzyme with ouabain. Other investigators have previously noted the lower sensitivity of the untreated K^+-phosphatase activity to ouabain [19]. The $I_{0.5}$ values for (Na^+,K^+)-ATPase and K^+-phosphatase activities are higher than those for the exchange activity, and undoubtedly reflect the well-known effect of K^+ on the rate of ouabain binding [20]. Nevertheless, it is apparent that digitonin treatment renders the K^+-phosphatase more sensitive to ouabain (as reported previously, ref. 6), while the ADP-ATP exchange activity is slightly less sensitive.

To more quantitatively define ouabain interaction with the digitonin-treated (Na^+,K^+)-ATPase, we attempted to measure the extent of [^{3}H]ouabain binding to the enzyme by centrifuging the complex and analyzing the pellet. Specific [^{3}H]ouabain binding to the pellet in control enzyme preparations was linear with protein concentration and with specific activity of the enzyme. The total capacity of untreated enzyme by this method was 136 pmol ouabain bound/μmol phosphate per min, a value somewhat higher than that reported previously for rabbit kidney (Na^+,K^+)-ATPase [21]. Preliminary experiments revealed, however, that digitonin-treated enzyme pellets contained considerably less bound ouabain. These pellets, moreover, appeared smaller than those of control preparations, suggesting that some of the enzyme did not sediment when digitonin was present. Accurate measurement of protein content of the supernatant fluid was not possible because of interference by constituents of the reaction medium, especially dithiothreitol. Increasing the sedimentation time to 4 h did not suffice to quantitatively precipitate the enzyme-bound ouabain, but did demonstrate that binding occurs in the presence of digitonin. Subsequent experiments showed however, that the percentage recovery of bound ouabain in the pellet varied considerably from one experiment to the next. For example, in two experiments centrifuged for 5 h, the binding capacities in the presence of digitonin were 39% and 23% of the controls. For this reason, we attempted to use filtration to separate enzyme-bound radioactivity.

Initial studies with untreated enzyme preparations were carried out by the method of Van Winkle et al. [22] using 0.45 μ filters. Assay of filtrates for unretained K^+-phosphatase activity showed no significant amount of enzyme activity. After filtration each sample was washed three times with 2-ml aliquots of distilled water to reduce the filter blank. In such experiments, ouabain-binding capacity averaged 97 pmol/μmol phosphate per min, a value again comparable to that of Jorgensen [21]. When digitonin-treated enzyme preparations were tested under these conditions, however, no ouabain binding was observed, suggesting that the filter did not retain the protein. Indeed, analysis of the filtrate for K^+-phosphatase activity showed that at least 83% of the enzyme activity passed through the filter. Reduction of filter pore size to 0.05 μm did not improve filter retention of bound ouabain.

The lack of ouabain binding to filters contrasted sharply with the partial binding observed using the centrifugation procedure, and suggested that the washing procedure was removing bound ouabain. We therefore ran some preliminary tests to determine whether binding could quantitated when the washing procedure was omitted. These experiments showed that the unwashed filter blank at 10^{-8} M ouabain represented 10% of the total radioactivity in control samples and about 5% of the total in the presence of digitonin, thereby

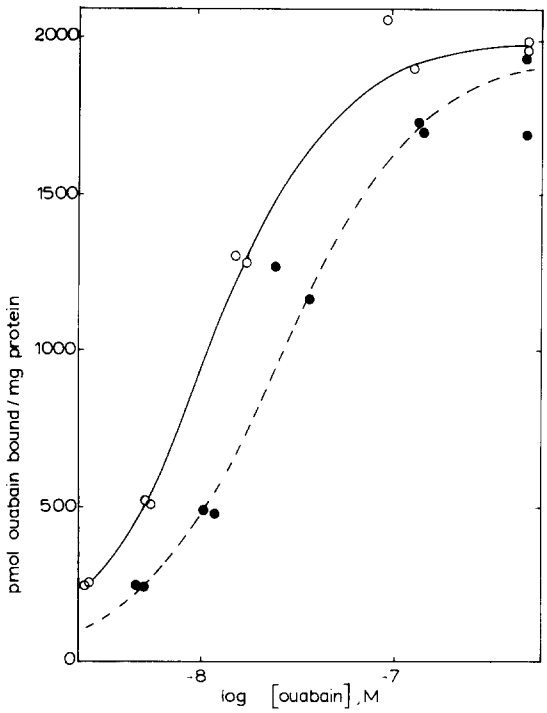

Fig. 2. Retention of protein-bound [^{3}H]ouabain by double 0.01-μm Schleicher and Schuell filters. Enzyme was treated with 2.4 mg digitonin/ml and ouabain binding measured as described under Methods. Open circles, untreated enzyme; filled circles, digitonin-treated enzyme. Enzyme-bound ouabain was separated using two Schleicher and Schuell 0.01-μm filters in series and omitting the water wash.

permitting reasonably accurate measurements of binding. Under these conditions, digitonin-treated enzyme preparations showed some ouabain binding, but the observed capacity was well below that of the untreated enzyme. (Schleicher and Schuell filters with 0.01 μm nominal pore size were used in

TABLE II

EFFECT OF DIGITONIN ON SOME PARAMETERS OF OUABAIN BINDING BY (Na^+,K^+)-ATPase

Ouabain binding was determined as described under Methods using double 0.01-μm filters and no washing. Results were analyzed by linear regression of (ouabain)/(pmol ouabain bound/mg protein) upon (ouabain). The correlation coefficient for the regression line from each experiment exceeded 0.99. The specific binding by control enzyme averaged 105 ± 4 pmol (μmol/min)$^{-1}$. Digitonin concentration, 2.4 mg/ml.

Expt.	Binding Capacity (pmol/mg)		Percentage of control	Apparent K_d (nM)	
	Control	Digitonin		Control	Digitonin
1	2930	2560	87.4	11.5	15.5
2	1861	1939	104.2	11.0	21.1
3	2249	2140	95.1	16.4	33.2
4	2137	2317	108.4	15.5	27.9
5	2168	—	—	10.5	—
Average	2269	2239	98.3	11.7	21.4
S.D.	398	264	8.2	4.0	9.5

these experiments in the hopes of increasing retention further). Concommittant measurements of K^+-phosphatase activity in the filtrate revealed that a large proportion of that activity was still passing through the filter. The amount of this filtrable activity also seemed to vary widely from one experiment to the next, suggesting that perhaps the effective particle size of the digitonin-treated enzyme was close to the pore size of the filter. For this reason we then tried two 0.01 μm filters in series to determine whether retention could be improved. To ensure that we could properly evaluate the results, we measured [^{3}H]-ouabain binding, ADP-ATP exchange activity and K^+-phosphatase activity. An example of the ouabain-binding results is shown in Fig. 2. While maximal binding capacity after digitonin treatment now differed little from the control, a reproducible increase in apparent dissociation constant for ouabain was observed. Table II presents the results of a series of such experiments comparing the binding curves of control and digitonin-treated enzyme preparations.

Based on these results, one might conclude that retention of the digitonin-treated protein on the double filter was essentially complete. Table III shows, however, that while such filtrates contain only small amounts of ouabain-sensitive ADP-ATP exchange activity, there is a large and variable percentage of the K^+-phosphatase activity that passes through the 0.01 μm double filter. This dichotomy between the filtration properties of ouabain-binding and exchange activities, on the one hand, and K^+-phosphatase activity on the other suggests that different enzyme species resulting from digitonin action are catalyzing the two classes of activity.

One possible explanation of these results is that the filtration process "inhibits" the exchange activity but not the phosphatase activity in the filtrate. Such selective inhibition might result from shearing forces applied to the sample during its passage through the extremely small pores of the filter. Evidence

TABLE III

ANALYSIS OF OUABAIN-SENSITIVE K^+-PHOSPHATASE AND ADP-ATP EXCHANGE ACTIVITIES IN FILTRATES

Samples containing 100 μg of enzyme protein in 25 mM Tris, pH 7.4, 1 mM dithiothreitol, 1 mM EGTA and 5 mM Mg^{2+} were treated with 2.4 mg digitonin per ml and then passed through two 0.01-μm filters placed in series. The filtrates were assayed for K^+-phosphatase activity as described under Methods. Identical samples were assayed for ADP-ATP exchange activity, except that the Mg^{2+} concentration during filtration was 1.4 mM. K^+-phosphatase activity is expressed as μmol hydrolyzed/min per mg protein in the unfiltered control. ADP-ATP exchange activity is expressed as μmol ATP exchanged/hr/mg protein in the unfiltered control. Values are mean ± standard deviation with number of determinations in parentheses.

Activity	Expt.	Unfiltered	Filtered	Percentage in filtrate
K^+-phosphatase	1	8.38 ± 0.49 (2)	4.90 ± 1.04 (4)	58.5
	2	8.75 ± 0.42 (2)	3.40 ± 1.94 (8)	38.9
	3	18.1 ± 4.00 (2)	6.71 ± 0.23 (2)	37.2
	4	12.1 ± 0.09 (2)	7.90 ± 0.19 (2)	65.5
ADP-ATP Exchange	5	47.9 ± 1.6 (3)	−0.1 ± 3.7 (10) *	0
	6	46.9 ± 0.5 (2)	1.0 ± 2.0 (4) *	4.1

* The ouabain-containing controls averaged slightly more activity than those without ouabain in some cases (see Table IV). Obviously, very low exchange activities in the filtrate are difficult to measure.

against this view comes from experiments in which different filter sizes and combinations were used to trap the enzyme. When a single 0.05 μm Millipore filter was used, an average of 31% of the exchange activity was found in the filtrate, with a range between 14 and 59%. Using two 0.05 μm filters in series reduces this value to 1.3% in the filtrate. With 0.01 μm Schleicher and Schuell filters, a single filter allowed 33% of the exchange activity to pass, while the double filters gave the results shown in Table III. Taken together, these results

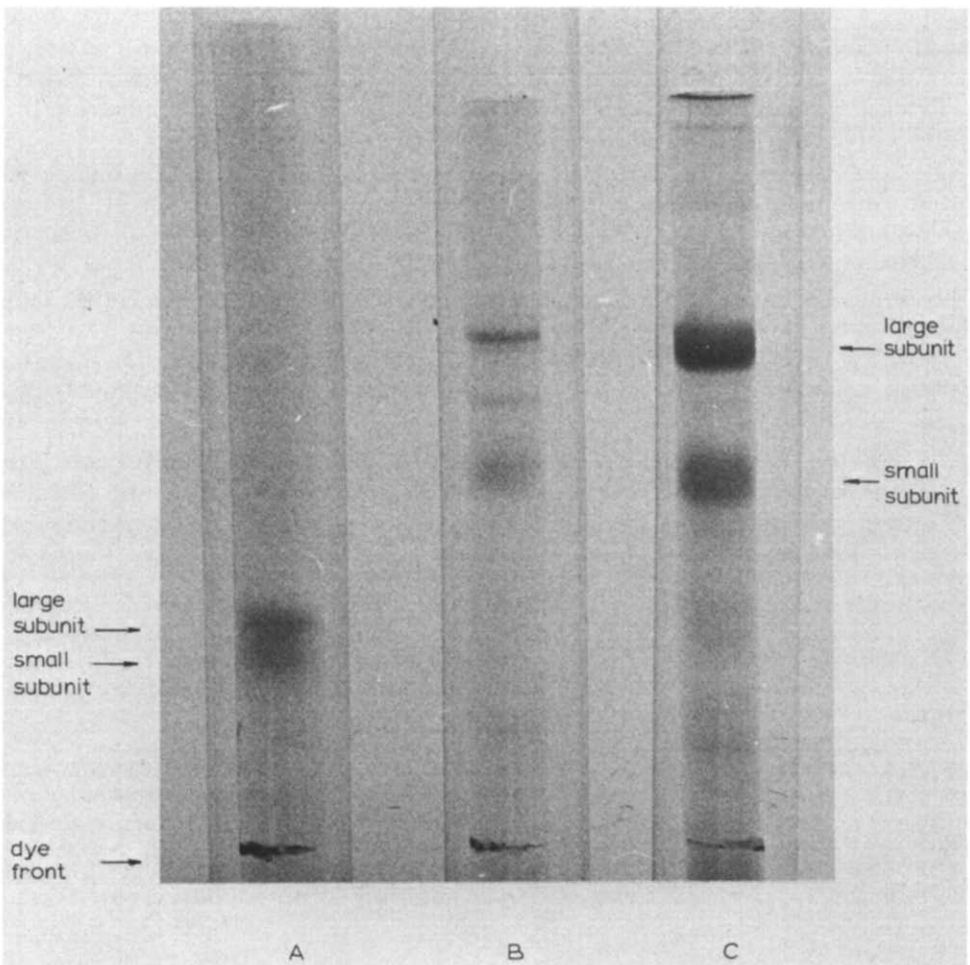

Fig. 3. SDS polyacrylamide gel electrophoresis of native enzyme and filtrates obtained after digitonin treatment. Enzyme was treated as described in Table III and a number of such filtrates combined and concentrated using Amicon B-15 Mini-con concentrators. The concentration factor ultimately obtained was 375-fold and 13% of the volume of the concentrated sample was then taken for electrophoresis. This amount of protein was applied to 3.3 and 7% SDS polyacrylamide gels and electrophoresis carried out in Tris/acetate buffer, pH 7.4, containing 0.2% SDS and 1% β-mercaptoethanol [26]. Staining was with Coomassie Blue. A, Filtrate electrophoresed on 3.3% acrylamide. B, Filtrate on 7% acrylamide. C, Untreated enzyme (50 μg) on 7% acrylamide. The amount of protein electrophoresed in A and B derived in each case from 1040 μg of unfiltered enzyme. While the amount is unknown, it is obviously less than 5% of the original unfiltered protein.

suggest that filtration per se does not inactivate the ADP-ATP exchange activity.

We made several attempts to measure ADP-ATP exchange activity directly on filter-trapped enzyme, with only partial success. In these studies, between 6 and 14% of the original activity could be detected when the filters were cut in small pieces and immersed in exchange assay medium. This low recovery was undoubtedly due to two factors: (1) poor physical mixing of the assay system with the filters present in the tube and (2) more important, the enzyme protein is undoubtedly sequestered and layered deep in the filter pores, thereby restricting the access of substrate to the catalytic sites. Nevertheless, enough activity was detected on the filter to suggest that exchange activity is truly retained by the filter, not simply inactivated.

The K^+-phosphatase activity remaining in the filtrate after passage through two 0.01 μm filters in series is catalyzed by a small amount of the original protein. This conclusion is based on the results in Fig. 3, which show that the filtrate must be concentrated some 375-fold in order to visualize the protein on SDS polyacrylamide gels. Both subunit types are present in the filtrate in a proportion similar to that seen in the native enzyme. It is unlikely that these subunits are arranged in the normal fashion, however, since sulfhydryl cross-linking studies reveal a large change in their disposition in the membrane after digitonin treatment [25].

The lack of ouabain binding to water-washed filters might be explicable in two ways: (1) ouabain may bind to the enzyme reversibly when digitonin is present (this is not the case for untreated controls [23,24]); (2) ouabain may be bound irreversibly to the digitonin-treated enzyme, but water washes the enzyme through the filter because of its smaller particle size. To discriminate

TABLE IV

EFFECT OF WATER WASHES ON RETENTION OF ENZYME-BOUND OUABAIN AND ADP-ATP EXCHANGE ACTIVITY BY DOUBLE FILTERS

Ouabain binding to digitonin-treated protein was measured using double 0.01-μm filters as described under Methods. Samples washed twice with 1.0-ml aliquots of distilled water were compared to unwashed controls (after correcting for non-specific binding as described in Methods). ADP-ATP exchange activity is expressed as μmol/h per mg protein in sample prior to filtration. Values with asterisks showed no significant difference ($P > 0.2$) between samples with or without ouabain.

Measurement	Unwashed	Washed	Percentage in filtrate after washing
Ouabain bound on filter:			
Capacity (pmol/mg protein)	2161	390	82
Apparent K_d, nM	31	149	
ADP-ATP exchange in filtrate:			
—ouabain	0.668 ± 0.868 (6) *	3.95 ± 4.25 (6) *	
+ouabain	1.28 ± 2.05 (6) *	1.94 ± 2.55 (6) *	
Δ	—0.612	2.01	4.2
ADP-ATP exchange of unfiltered control:			
—ouabain	49.7 ± 2.33 (2)		
+ouabain	2.28 ± 1.81 (2)		
Δ	47.4		

between these two possibilities, ouabain binding and ADP-ATP exchange activities were monitored in separate experiments comparing washed with unwashed filtrations. The results appear in Table IV. Washing reduced total ouabain binding to 18% of that of an unwashed control. Accompanying this loss of binding activity was a 5-fold increase in apparent dissociation constant (compare Fig. 2), suggesting that either the rate of ouabain association is decreased or that of dissociation increased. It is unlikely that much enzyme is washed through the filter, however, since only 4% of the original exchange activity is found in the filtrate after washing (even this amount is too small to measure, as shown by the standard deviations). Therefore, the most likely conclusion is that ouabain binding is rendered reversible by digitonin treatment.

Discussion

Since the purified (Na^+,K^+)-ATPase responds to digitonin treatment in a fashion similar to that reported previously for less pure preparations, it is highly likely that these effects are intrinsic to the pump itself, and do not reflect secondary actions of digitonin-disrupted membrane components not associated with the pump. Because the "partial" reactions observed remain sensitive to high concentrations (1 mM) of ouabain, we felt that measurement of ouabain binding provided an additional probe of the effects of digitonin on enzyme structure and function. The results in Table I provide an indirect indication that ouabain binding as well as its effects on enzyme function are altered by digitonin treatment. The phenomena involved are obviously complex, since the Na^+-dependent exchange activity is rendered less sensitive to ouabain inhibition, while the K^+-dependent phosphatase activity is more readily inhibited. One possible interpretation of these results is that K^+ antagonism of ouabain binding [20] is somewhat reduced by digitonin treatment. This view is only partly tenable, however, since a 10-fold difference in $I_{0,5}$ values remains when the effects of ouabain on Na^+-dependent exchange and K^+-dependent phosphatase are compared.

Attempts to measure ouabain binding by the centrifugation method employed so successfully by others [16,21] were frustrated by the change in physical properties of the enzyme caused by digitonin. Untreated enzyme behaved as predicted from the results of other workers. Sufficient sedimentation of digitonin-treated enzyme did occur, however, to indicate that some ouabain binding takes place. Prolongation of the centrifugation time increased the amount of bound ouabain in the pellet, but not enough to provide reliable estimates of binding capacity, especially since the extent of sedimentation appeared to vary widely from one preparation to another.

Adequate retention of enzyme-bound ouabain in the presence of digitonin by filtration through Millipore or Schleicher and Schuell filters also proved to be a problem. This problem has two aspects:

(1) Washing the filter with water to lower the filter blank, a standard practice in ouabain-binding studies, reduces the binding to essentially zero. One possible explanation of this finding is that ouabain binding to the enzyme is rendered reversible by digitonin treatment. Alternatively, water washing may exacerbate the second aspect of the problem.

(2) Apparently the digitonin treatment reduces the membrane particle size of the enzyme preparation to such an extent that it will in part pass through a filter of 0.01 μm nominal pore size. While accurate calibration of pore sizes in such filters is problematic, one possible interpretation of the results is that digitonin treatment reduces the membrane enzyme preparation to a size approaching that expected for single pump units (and perhaps single subunits). Whether this is indeed so will obviously require further experimentation. A somewhat satisfactory resolution of this problem was to use a double layer of 0.01-μm filters and attempt to determine how well the enzyme is retained by the filter by quantitating ouabain binding, ADP-ATP exchange and K^+-phosphatase activities.

When ouabain binding was measured in this manner, retention of digitonin-treated enzyme by the filter appeared to be essentially complete (Fig. 2), on the assumption that total binding capacity is insensitive to digitonin treatment. The apparent dissociation constant is approximately doubled in the presence of digitonin (Table II), a fact consistent with the results obtained for the ouabain-sensitivity of the ADP-ATP exchange activity (Table I). The assumption that binding capacity is unaltered by digitonin treatment is supported by the fact that essentially all of the ADP-ATP exchange activity is retained by the filter (Table III). These observations taken together are consistent with the view that essentially all the membrane protein is trapped by a double 0.01-μm filter system.

Contrasting sharply with the consistency just mentioned is the appearance of a large fraction of the K^+-phosphatase activity in the filtrate. This phenomenon is even more startling when one considers that it remains ouabain-sensitive. How can one account for the fact that apparently all of the ouabain-binding and exchange activity is trapped by the filter, yet much of the ouabain-sensitive K^+-phosphatase activity is not. There are at least two possible explanations of these results:

(1) Only a small amount of enzyme protein passes through the filter; this fraction catalyzes a disproportionate amount of K^+-phosphatase activity but little exchange activity. Such a hypothesis requires an increased phosphatase turnover number for this fraction.

(2) Alternatively, digitonin might "freeze" the enzyme in two forms, one which catalyzes exchange and the other K^+-phosphatase activity. Each might represent a sizable fraction of membrane protein, but show different filtration properties. If this were true, then we must conclude that even our double filter system does not retain all the protein and, additionally, that total ouabain-binding capacity is increased after digitonin treatment. The results in Fig. 3 show unequivocably that only a very tiny fraction of the enzyme protein passes through the double 0.01-μm filters. This fraction is responsible for the disproportionate K^+-phosphatase activity of the filtrate, yet does not catalyze any unusual amount of exchange activity. It is not clear whether the small amount of filtrable enzyme with these unusual properties is created by digitonin action or simply pre-exists in the membrane as a distinct entity prior to treatment. The different filtration properties of the exchange and phosphatase activities do not, however, arise from conformational differences induced by alterations in medium composition since the media used for filtration in both cases were vir-

tually identical. We therefore conclude that both ouabain-binding and exchange studies present a true picture of protein retention by the filter membrane. This conclusion is supported by the SDS polyacrylamide gel results.

The 7% SDS gels show both subunits present in the filtrate, as well as a third band with a mobility intermediate between those of the enzyme units. The source of this band is unknown, but traces of it are sometimes seen in the purified ATPase preparation (as shown in Fig. 3). This band is apparently enriched by the filtration process, relative to other minor contaminants of the preparation. Alternatively, it may arise from the native enzyme by proteolysis or intramolecular disulfide exchange, but our results do not bear on this question. Assuming that it is enzymatically inactive, however, this leaves the two types of ATPase subunits to account for the K^+-phosphatase activity in the filtrate. Whether both types are involved in the enzyme activity or not remains to be proven.

It is apparent from the results of Table IV that ouabain no longer binds "irreversibly" in the presence of digitonin, but behaves more like an aglycone. Yoda has shown that the rate of dissociation of cardiac glycosides is largely controlled by the sugar portion of the glycoside [23,24]. It is tempting to speculate that digitonin, itself a glycoside, interferes with "irreversible" ouabain binding by interaction at the sugar binding site. Further studies with aglycones may resolve this point. Nevertheless, an unfortunate effect of the reversibility observed is that it renders kinetic studies more difficult. Flow rates with double 0.01-μm filters are too slow to obtain reliable binding rates at short time intervals.

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