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Sodium- and Potassium-Dependent Adenosine Triphosphatase of Electric Organ: Interaction with Ouabain *in Situ*, in a Membrane Fraction, and in the Solubilized Form

LILIANE ZUBLER-FAIVRE AND YVES DUNANT

Département de Pharmacologie, Ecole de Médecine, 1211 Genève 4, Switzerland

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SUMMARY

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$(Na^+ + K^+)$-ATPase (EC 3.6.1.3) of the electric organ of *Torpedo marmorata* has been studied *in situ*, in an enriched membrane-bound fraction, and after solubilization. *In situ*, ouabain did not modify cholinergic transmission of a single impulse but impaired the ability of the tissue to maintain repetitive activity. Pieces of electric organ were incubated in the presence of [^{3}H]ouabain. As much as 0.7-0.9 nmole was bound per gram of tissue, even after prolonged washing. In the membrane fraction, ouabain binding was measured in the presence of ATP and Na^+. There was a good correlation between the amount of bound glycoside and the degree of enzymatic inhibition. Binding in the presence of Mg^{2+} and P_i was more stable and corresponded to 3.13 nmoles of sites per gram of starting tissue. *Torpedo* $(Na^+ + K^+)$-ATPase could be solubilized using the nonionic detergent Lubrol W, and was subjected to gel filtration and sucrose gradient centrifugation. The solubilized enzyme behaved as a homogeneous molecular species having a Stokes radius of 10.7 nm and a sedimentation coefficient of 10.5 S, indicating an apparent molecular weight of 475,000. The activity of the solubilized $(Na^+ + K^+)$-ATPase was the same as that for the enzyme inserted normally in the membrane: about 30 molecules of ATP hydrolyzed per site per second at 37°.

INTRODUCTION

The $(Na^+ + K^+)$-ATPase, or sodium pump, is present in the membranes of most, if not all, living cells. It is, however, more concentrated in certain tissues which need, when functioning, to expel large quantities of sodium ions. This is the case for the electric organs of fishes, where $(Na^+ + K^+)$-ATPase is necessary to restore the membrane potential after an electrical discharge.

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The present work is a study of the $(Na^+ + K^+)$-ATPase of the electric organ of *Torpedo marmorata*. This very homogeneous tissue consists of a great number of prisms, arranged side by side, each built up by about 500 electroplaques. The latter are covered on their ventral surface by nerve terminals; their dorsal faces are not innervated, but show a fine, rich tubular network. When the nerves are activated, their terminals release acetylcholine (1), which acts at receptor sites on the electroplax ventral faces and results in a large number of synchronized postjunctional po-

tentials. Together these generate a strong discharge, positive toward the dorsal face of the fish.

The discharge is thus believed to be due to a very large simultaneous downhill movement of sodium ions through all the innervated electroplax membranes. The recovery of the initial ionic gradient must then depend on the action of the $(Na^+ + K^+)$-ATPase of the cells. It is not surprising that, in this tissue, most of the ATPase activity is Na^+- and K^+-dependent (2).

It is therefore of interest to elucidate in a quantitative fashion the properties of the $(Na^+ + K^+)$-ATPase of *Torpedo marmorata* electric organ. In the present work the activity of this enzyme and its interaction with a specific inhibitor, ouabain, have been studied at three different levels: fragments of tissue, a subcellular membrane fraction, and the solubilized protein. It has been found that solubilization of the *Torpedo* electric organ $(Na^+ + K^+)$-ATPase does not affect the rate of its hydrolytic activity. A short account of these findings has been published (3).

MATERIALS AND METHODS

Animals (*Torpedo marmorata*) were provided by the Station de Biologie Marine, Arcachon, France. Both electric organs were removed under light anesthesia with tricaine, 1:3000 in sea water. [^{3}H]Ouabain (13 Ci/mmole) was purchased from New England Nuclear, and ATP, from Boehringer/Mannheim; Lubrol W was a gift from ICI Pharma. All other chemicals were of reagent grade.

Subcellular Fractionation

The fresh tissue was homogenized in a solution containing 0.5 M sucrose and 0.33 M urea, 9 ml/g of tissue (4). The homogenate was filtered and centrifuged at 500 × *g* for 5 min. The pellet was resuspended in distilled water and centrifuged again at 500 × *g* for 5 min. This pellet, obtained after osmotic shock and enriched in membrane-bound enzyme, is designated $P_{(H_2O)}$. It was resuspended in about 15 volumes of distilled water to test enzymatic activity, or in about 3 volumes for binding tests.

Solubilization of $(Na^+ + K^+)$-ATPase

Fresh organ (50 g) was homogenized in 250 ml of a solution containing 0.32 M sucrose and 1 mM EDTA, pH 8, using a VirTis homogenizer at 22,500 rpm for 1 min. The homogenate was centrifuged at 1300 × *g* for 30 min, the supernatant was discarded, and the pellet was resuspended in distilled water and centrifuged again under the same conditions. A solution of 16% Lubrol W was added to the second pellet to obtain a final concentration of 1.6% (5). The mixture was allowed to stand for 15 min and then centrifuged at 100,000 × *g* for 1 hr. The supernatant was carefully removed and concentrated 4–6-fold under nitrogen pressure (Amicon cell with XM-100 filter). The solution obtained is called "Lubrol W extract," 1 ml of which corresponded to about 10 g of starting tissue.

Determination of Enzymatic Activity

$(Na^+ + K^+)$-ATPase activity was estimated by the rate at which the enzyme liberated inorganic phosphate from ATP. The conditions of incubation were those described by Modolell and Moore (6). The incubation was stopped with an ice-cold aqueous solution of 40% trichloracetic acid, and P_i was determined according to Bonting *et al.* (7). When the samples contained Lubrol W, the incubation was stopped with 25% trichloracetic acid in chloroform; the Lubrol W separated with the organic phase, and colorimetric estimation was performed as usual on the aqueous phase. Enzymatic ATPase activity is expressed as micromoles of P_i liberated during incubation for 1 hr at 37°; 1 μmole of P_i per hour at 37° = 1 unit. The specific activity, expressed in units per milligram of total protein, was found to be linear with the amount of tissue extract.

Binding of Labeled Ouabain to Subcellular Fraction ($P_{(H_2O)}$)

Two different methods were used to measure binding of the radioactive drug to the membrane-bound enzyme.

In the presence of sodium, ATP, and magnesium. First 0.2 ml of the pellet,

$P_{(H_2O)}$, corresponding to about 200 μg of protein, was incubated for 3 min at 37° in the following medium: $MgCl_2$, 1 mM; NaCl, 60 mM; ATP-Na, 1 mM; Tris-HCl, 20 mM, pH 7.5; unlabeled ouabain, various concentrations; and [^{3}H]ouabain, 50 nCi. The reaction was stopped by plunging the incubation tubes into ice; the tubes were then centrifuged at 27,000 × *g* and the supernatants were discarded. The pellets were dissolved in 2.5 N NaOH, and the radioactivity was counted after addition of Bray's scintillation liquid (8).

In the presence of magnesium and inorganic phosphate. The same tissue fraction (0.2 ml) was incubated for 30 min at 37° in the following medium: $MgCl_2$, 4 mM; H_3PO_4, 1 mM; Tris-HCl, 20 mM, pH 7.5; unlabeled ouabain, various concentrations; and [^{3}H]ouabain, 50 nCi. Radioactivity in the pellets was counted as above.

Radioactivity present in the pellets but not specifically bound was measured for both methods in a series of tubes containing the same amounts of tissue fraction and of labeled and unlabeled ouabain, but with an incubation medium composed only of 200 mM NaCl. In this case no specific binding occurred, the radioactivity uptake being directly proportional to ouabain concentration. Consequently these values were subtracted from the above counts in order to obtain specific binding. This method (9) avoids the washing of pellets, in which ouabain is reversibly fixed.

Physiological Effects and Binding of Ouabain in Intact Tissue

One or several prisms were excised carefully and equilibrated in a solution isotonic for elasmobranchs. This contained NaCl, 280 mM; KCl, 5.6 mM; $CaCl_2$, 4.4 mM; $MgCl_2$, 1.2 mM; $NaHCO_3$, 7 mM; NaH_2PO_4, 1.2 mM; glucose, 5.5 mM; sucrose, 100 mM; and urea, 300 mM, pH 7.2 (10). After 1 hr ouabain was added at various concentrations. The incubation lasted for 2 hr at room temperature. The nerves in the tissue were then stimulated by field shocks (10) at 10/sec. The response was recorded, and the responses of ouabain-treated prisms were compared with those of controls.

In separate experiments, fragments of prisms were incubated for 2 hr in the above solution containing, in addition, 2 μM ouabain and [^{3}H]ouabain, 20 nCi/ml. The unbound ouabain was washed out, the tissue fragments were wiped and dissolved in 2.5 N NaOH, and the remaining radioactivity was measured.

Gel Filtration of Solubilized Enzyme

Measurement of activity. Between 2 and 3 ml of Lubrol W extract were applied to a 1.6 × 40 cm column filled with Sepharose 6B (Pharmacia) and eluted at a rate of 10–12 ml/hr with the following buffer: Tris-HCl, 0.1 M, pH 7.0; EDTA, 1 mM; and Lubrol W, 0.1% (v/v). The optical density of the eluate was recorded during elution, and 1-ml fractions were collected and tested for enzymatic activity and for protein content.

Measurement of ouabain binding. A 2-ml portion of the Lubrol W extract was incubated for 30 min at 37° with $MgCl_2$, 4 mM; Tris-HCl, 20 mM, pH 7.5; ouabain, 0.5 μM; and [^{3}H]ouabain, 200 nCi, in a total volume of 3 ml. The elution buffer was the same as above with, in addition, $MgCl_2$, 4 mM, and phosphate, 1 mM, pH 7.0. The mixture was applied to the same column, and fractions were collected in the same way as for enzymatic determination. Radioactivity was measured in each fraction according to the method already described.

Localization of Enzyme on Sucrose Gradients

Measurement of activity. The Lubrol W extract (1 ml) was applied to the top of a linear, continuous, 10–40% sucrose gradient, 35 ml, pH 7.3. The tubes were centrifuged at 26,000 rpm in the Beckman rotor SW 27 of a Sorvall OTD 2 ultracentrifuge. After 19 hr of centrifugation, the tubes were removed and divided into fractions of 2 ml. The enzymatic activity was determined on 0.05-ml samples from each fraction.

Measurement of ouabain binding. The Lubrol W extract (0.3 ml) was incubated for 30 min at 37° with $MgCl_2$, 4 mM; H_3PO_4, 1 mM; Tris-HCl, 20 mM, pH 7.5; ouabain, 0.5 μM; and [^{3}H]ouabain, 50 nCi, in a total

volume of 0.5 ml. The reaction was stopped by plunging the tubes into ice. Then 0.5 ml of the mixture was applied to the top of 4.5 ml of a linear, continuous, 10–40% sucrose gradient formed in the following medium: $MgCl_2$, 4 mM; Na_2HPO_4-NaH_2PO_4, 1 mM; Tris-HCl, 10 mM; EDTA, 1 mM; and Lubrol W, 0.1%, pH 7.0. After 15 hr of centrifugation at 37,000 rpm in the SW 65 Ti rotor of a Spinco centrifuge, the tubes were divided into 19 fractions, and radioactivity was determined directly in each fraction after addition of scintillation liquid.

Proteins

Proteins were measured with the method of Lowry *et al.* (11), using bovine serum albumin as standard.

Acetylcholinesterase activity was determined according to the method of Johnson and Russel, cited by Duguid and Raftery (12). The Lubrol W extract (10 μl) was added to 0.1 ml of the incubation solution containing acetylcholine, 1 mM, and 100 nCi of [*acetyl*-^{3}H]choline in phosphate buffer, 25 mM, pH 7.0. After 20 sec at 21°, the reaction was stopped with 0.1 ml of a solution containing monochloracetic acid, 1 M; NaCl, 2 M; and NaOH, 0.5 M. The [^{3}H]acetate produced by hydrolysis of acetylcholine was extracted and counted by adding a scintillation mixture composed of 10% isoamyl alcohol in toluene with 1.14 g/liter of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 18.95 g/liter of 2,5-diphenyloxazole.

RESULTS

Total ATPase in Homogenate and Membrane Fractions

Total ATPase was first assayed in the sucrose-urea homogenate. The specific activity obtained was 22.05 ± 2.22 units/mg of protein, corresponding to 350.6 ± 35.3 units/g of fresh tissue (means ± SE).

After this homogenate had been centrifuged at 500 × *g* for 5 min, 65% of the ATPase activity was recovered in the pellet and 17% in the supernatant. The specific activity was higher in the pellet and lower in the supernatant than in the homogenate (Table 1). When the pellet was resuspended in distilled water and centrifuged again, nearly all the ATPase activity was recovered in the second pellet, $P_{(H_2O)}$, and the specific activity was approximately 2-fold higher. This result indicates that, during osmotic shock and subsequent centrifugation, an important amount of extraneous protein was eliminated.

The second pellet, $P_{(H_2O)}$, was used to measure ouabain binding to membrane fragments. A similar preparation, after sucrose-EDTA homogenization and osmotic shock, was used as starting material for the solubilization of ATPase. Attempts were made to obtain a membrane fraction of higher specific activity by subjecting the sucrose-urea homogenate or $P_{(H_2O)}$ to sucrose gradient centrifugation, but this did not result in preparations richer than $P_{(H_2O)}$.

Inhibition by Ouabain of (Na^+ + K^+)-ATPase Activity

As cardiac glycosides inhibit the sodium pump specifically, ouabain can be used as a tool to determine the proportion of ATPase activity which is Na^+- and K^+-dependent. In homogenates from *Torpedo* electric organ, 90.2 ± 0.9% of the total activity was inhibited by ouabain. In the absence of sodium or potassium, the activity was reduced to the same slow level as after treatment with ouabain. Consequently 90% of the total ATPase activity was considered to be (Na^+ + K^+)-ATPase. The proportion of ouabain-sensitive activity was even higher (93.8 ± 1.2%) in $P_{(H_2O)}$.

The enriched membrane fraction was incubated in the presence of various ouabain concentrations in order to determine the kinetics of (Na^+ + K^+)-ATPase inhibition by the glycoside. No effects were observed at concentrations lower than 10 nM, and at higher concentrations the enzymatic activity was impaired; the ouabain concentration producing 50% inhibition (K_i) was 0.3 μM (Fig. 1).

Ouabain Binding to Membrane Fraction

As described in MATERIALS AND METHODS, $P_{(H_2O)}$ was incubated in the presence of [^{3}H]ouabain and various concentrations of unlabeled ouabain to measure the affinity

TABLE 1

$(Na^+ + K^+)$-ATPase activity of Torpedo marmorata electric organ

Recovery after subcellular fractionation and solubilization is expressed per gram of original tissue and as a percentage of the value in the homogenate.

Fraction	Protein	Recovery	ATPase activity			Specific $(Na^+ + K^+)$-ATPase activity	[^{3}H]Ouabain sites		Activity per site
			Total	$(Na^+ + K^+)$-ATPase	Recovery				
	mg/g	*% homogenate*	*units/g*	*units/g*	*% homogenate*	*units/mg protein*	*nmoles/g*	*pmoles/mg protein*	*molecules/sec*
Whole tissue							0.7–0.9[a]		
Homogenate	15.9	100	350.6	315.6	100	19.85	3.13[b]	196.8[b]	
First centrifugation									
Supernatant				53.6	17	5.45			
Pellet				211.5					
Second centrifugation: $P_{(H_2O)}$	5.25	34	224.4	205.1	65	42.9	2.03	425	28
Solubilization: 100,000 × *g* supernatant	1.9	12		79	25	41.3			
Lubrol W extract for gel filtration	0.86	5.4		17.5	5.6	20.4			
Gel filtration column peak		3.2		7.7	2.5	15.2		162.8	25

[a] Values obtained after the tissue had been washed (probably underestimated).

[b] Values obtained from those measured in $P_{(H_2O)}$, which contained 65% of the initial activity.

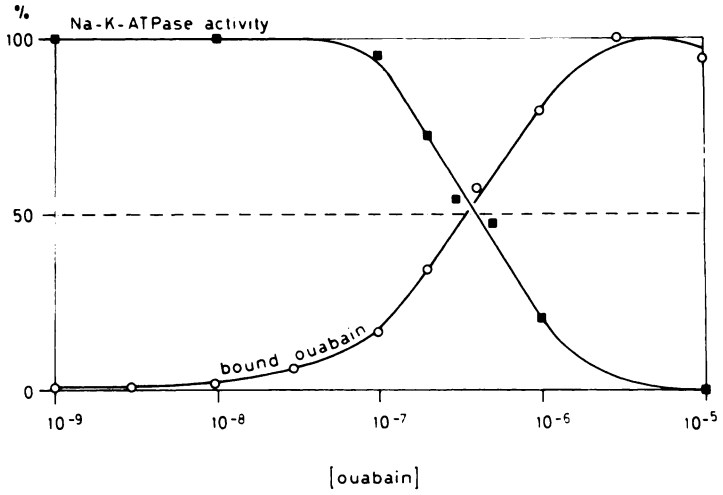

FIG. 1. *Interaction of ouabain with $(Na^+ + K^+)$-ATPase of enriched membrane fraction from Torpedo marmorata electric organ*

■, enzymatic activity expressed as percentage of control tested in the absence of ouabain. Half-inhibition was obtained with 0.3 μM ouabain. ○, [^{3}H]ouabain binding measured on the same membrane fraction in the presence of ATP, Na^+, and Mg^{2+}. The K_{aff} was 0.3 μM.

of the glycoside for the membrane-bound enzyme. Different results were found for the two different incubation media used.

In the presence of sodium, ATP, and magnesium. Binding was maximal after 3 min at 37°. When incubation was prolonged, binding decreased to 50% after 30 min and to 25% after 60 min. Maximum

binding occurred at 3 μM ouabain, and half-maximal binding (affinity coefficient, K_{aff}), at 0.3 μM. This incubation medium was similar to that in which the enzymatic activity was measured. Consequently the inhibition and binding curves may be compared (Fig. 1). It can be seen that they cross at the values of K_i and K_{aff}. There is an evident correlation between the amount of bound glycoside and the degree of enzymatic inhibition.

In the presence of magnesium and inorganic phosphate. Binding was more stable than with Na^+, ATP, and Mg^{2+} and was maintained during further manipulations, such as gel filtration and sucrose gradient centrifugation. In this medium, the K_{aff} was about 0.1 μM and binding was maximal at 0.5 μM ouabain (Fig. 2). Above this concentration binding appeared to decrease. The precision of measurement, however, was low at high ouabain concentrations, since the specific binding became very small compared with the total radioactivity in the pellet. The dependence of specific binding on the ionic composition of this medium was also studied. The binding was maximum at 1.0 mM P_i and 1.0–4.0 mM Mg^{2+}.

Maximum specific binding in the first medium reached 425 ± 56 pmoles of sites per milligram of protein. From this value and the amount of enzymatic activity found in the same fraction, it can be calculated that one site can hydrolyze 1.1×10^5 molecules of ATP per hour, or 28 molecules/sec. Expressed per gram of starting tissue, binding in $P_{(H_2O)}$ was 2.03 nmoles/g. Since this fraction contains 65% of the activity of the homogenate, it is estimated that 1 g of fresh electric organ contains 3.13 nmoles of active sites.

Solubilization of $(Na^+ + K^+)$-ATPase

The sucrose-EDTA-homogenized membrane fraction from *Torpedo* electric organ was treated with a number of agents which, in other tissues, have proved to be useful in solubilizing the $(Na^+ + K^+)$-ATPase: EDTA with $NaHCO_3$, Triton X-100, and deoxycholate. However, even after salting out of the detergent or attempts to reactivate the enzyme with phosphatidylserine, no enzymatic activity was recovered in the soluble phase, i.e., in the supernatant after 100,000 × *g* centrifugation.

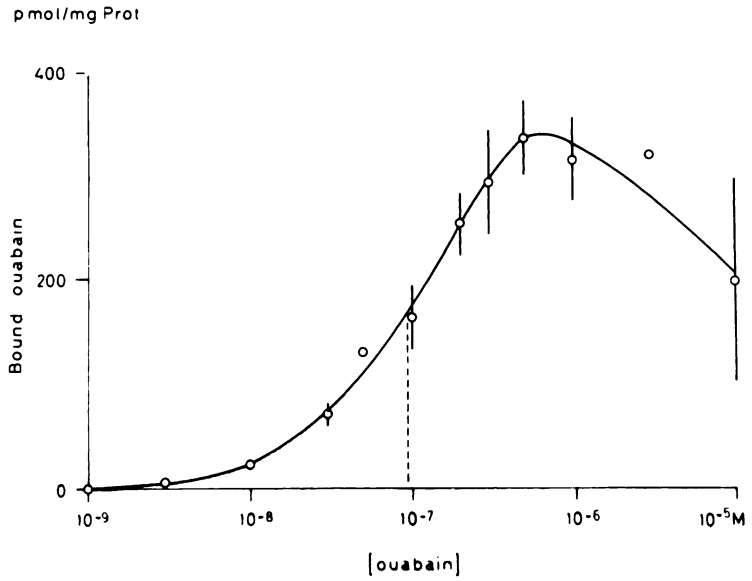

FIG. 2. *[^{3}H]Ouabain binding to $(Na^+ + K^+)$-ATPase of enriched membrane fraction in the presence of 4 mM Mg^{2+} and 1 mM P_i*

The K_{aff} was 0.1 μM. Values are means ± standard errors.

Finally, Lubrol W, a nonionic detergent, was found to be successful in solubilizing the *Torpedo* $(Na^+ + K^+)$-ATPase in an active form. The efficiency of solubilization was not constant from experiment to experiment, but as much as 30–50% of the enzyme initially present could be recovered in the soluble phase. As shown in the following experiments, the solubilized enzyme retained its functional properties and its affinity for ouabain.

Gel Filtration of Solubilized Enzyme

When the Lubrol W extract was run on the Sepharose 6B column, all the enzymatic activity was found in a single peak. The extract was also incubated with [^{3}H]ouabain and applied to the same column. The peak of enzyme-bound ouabain coincided with that of $(Na^+ + K^+)$-ATPase activity, whereas unbound drug was eluted after the total volume of the column (Fig. 3). Thus the solubilized enzyme behaved as a homogeneous molecular species which could be localized both by its hydrolytic activity and by its affinity for the glycoside. At the maximum of the elution peak, the activity per site was 0.93×10^5 molecules of inorganic phosphate produced per hour (or 25 molecules/sec/site). This figure is very close to that obtained when $(Na^+ + K^+)$-ATPase was still located in the membrane.

The specific activity of the $(Na^+ + K^+)$-

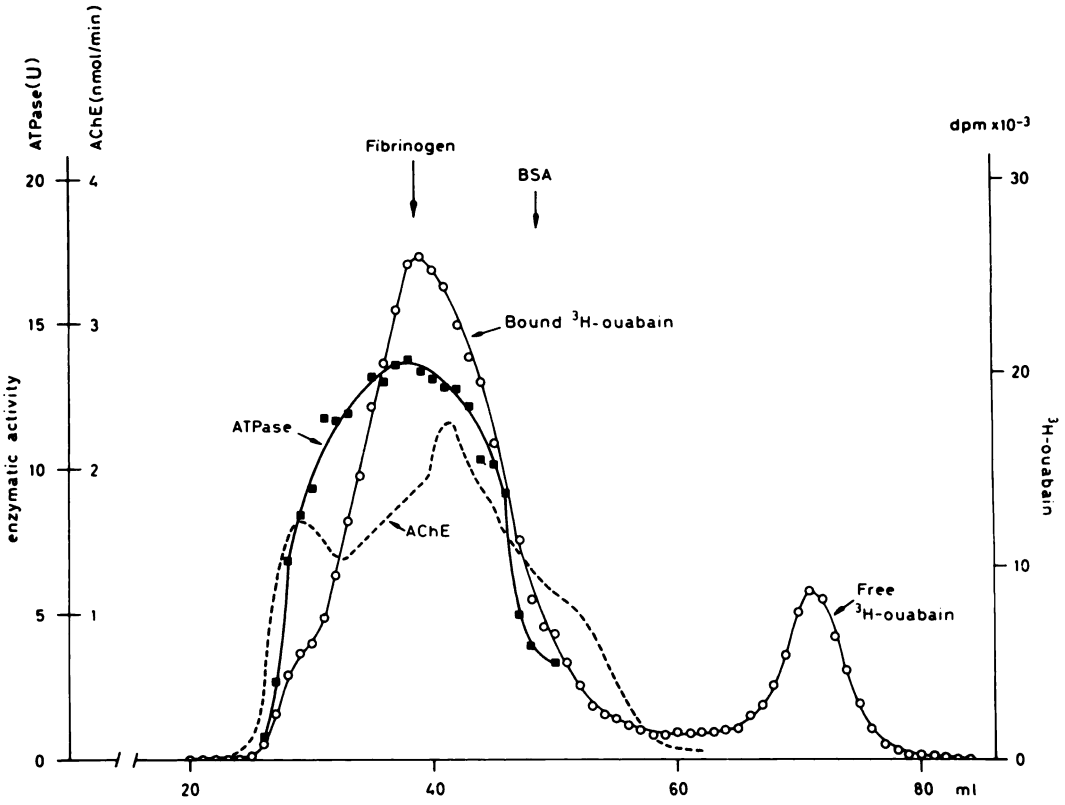

FIG. 3. *Gel filtration of solubilized $(Na^+ + K^+)$-ATPase and acetylcholinesterase (AChE) from electric organ of Torpedo marmorata*

The Lubrol W extract was applied to the top of a Pharmacia K 16/40 column filled with Sepharose 6B and eluted with Tris–EDTA–Lubrol W buffer (total volume, 71 ml; void volume, 27 ml; flow rate, 12 ml/hr; 1 ml/fraction). ■, $(Na^+ + K^+)$-ATPase activity, expressed in units per fraction (1 unit = 1 μmole of P_i liberated from ATP per hour at 37°). - - -, acetylcholinesterase activity measured in the same fractions. ○, disintegrations per minute of [^{3}H]ouabain found in the fractions when the Lubrol W extract had been incubated with the radioactive drug and then run on the same column under the same conditions.

ATPase was no higher for the peak chromatographic fraction than for the starting Lubrol W extract. This unexpected finding can be explained if most of the membrane proteins of the tissue were also concentrated at the level of the ATPase. Indeed, the activity of acetylcholinesterase was measured in the same fractions and was recovered as several peaks in the same region (Fig. 3).

Marker proteins were also run on the column. The peak of the solubilized (Na^+ + K^+)-ATPase was close to that of human fibrinogen, corresponding to a Stokes radius of 10.7 nm [see Bon *et al.* (13)].

Ultracentrifugation on Sucrose Gradients

As in the gel filtration experiments, (Na^+ + K^+)-ATPase applied to a sucrose gradient was recovered in a single peak, which could be located both by its enzymatic activity and by its binding to radioactive ouabain (Fig. 4). In this peak the specific activity was no higher than that of the Lubrol W extract, and again a large amount of acetylcholinesterase was found in these fractions. From comparison with the positions of marker proteins [human albumin, IgG, and IgM (14)], the sedimentation coefficient of the solubilized enzyme is about 10.5 S.

In the binding experiments the free ouabain remained at the top of the tube even in the presence of protein other than (Na^+ + K^+)-ATPase (proteins from human serum). This excluded any nonspecific ouabain-protein binding.

Experiments with Intact Tissue

Fragments of electrogenic tissue were incubated with ouabain (20 μM) for 2–3 hr, a time sufficient to allow complete diffusion of the drug into the lamellar structure of the prisms, whose diameter is 0.3–0.5 cm. The nerves in the fragments were then activated by field shocks and the electrical response of the tissue was recorded. The response to the first stimulus was not affected by ouabain treatment, when compared with control fragments. In contrast, ouabain produced a marked effect on responses to repetitive stimulation. When this tissue is stimulated at a frequency of 10/sec, the amplitude of the electrical response falls off in about 3 min. The decrease is not monotonic but shows one or several plateaus [see Dunant *et al.* (10)]. Ouabain shortened the late phase of this

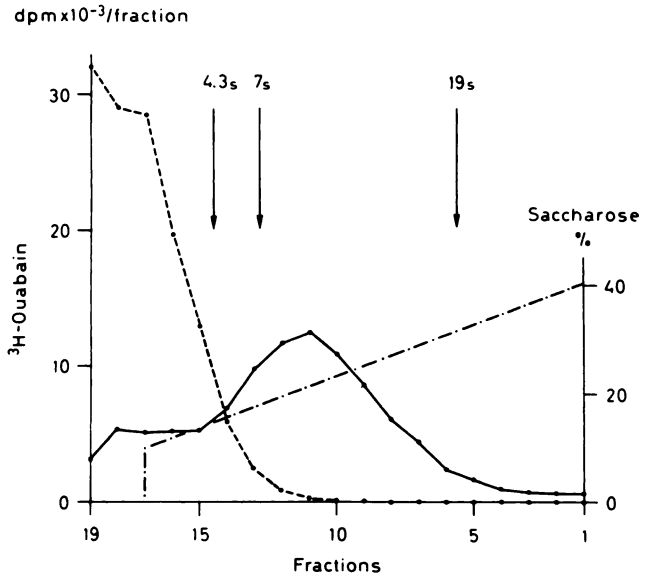

FIG. 4. *Ultracentrifugation of solubilized (Na^+ + K^+)-ATPase on sucrose gradients*
——, Lubrol W extract after incubation with [^{3}H]ouabain; - - -, after incubation of the [^{3}H]ouabain in the presence of proteins of human serum; no binding occurred. Fraction 1 corresponds to the bottom of the tube. 4.3 S, 7 S, and 19 S: positions of albumin, IgG, and IgM from normal human serum.

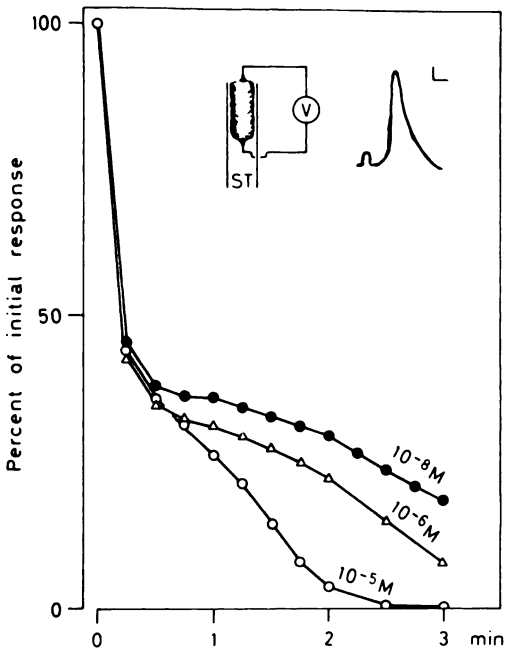

FIG. 5. *Effects of ouabain on electrophysiological response of electrogenic tissue of Torpedo marmorata*

Prisms of electroplax were dissected and incubated with various ouabain concentrations. These were then subjected to field shocks (ST). An example of the response discharge is shown (calibration, 0.2 V and 2 msec). Repetitive stimulation was applied at 10/sec for 3 min. At 1 μM ouabain, the evolution of the response was the same as in the unstimulated controls. With concentrations of 10 μM and above, the response vanished after only 2 min of stimulation. The response to the initial shock was not affected by ouabain (2.3 V at 10 nM, 2.7 V at 1 μM, and 2.6 V at 10 μM; means of three samples).

"fatigue" curve (Fig. 5). This effect was dose-dependent. With ouabain at 10 μM or higher, transmission was exhausted after only 2 min of stimulation. The range of concentrations affecting transmission was higher than that which inhibited (Na^+ + K^+)-ATPase activity in the membrane fraction (Fig. 1).

The binding of [^{3}H]ouabain was also studied with intact tissue. When pieces of tissue were incubated with the glycoside and not washed subsequently, specific binding could hardly be measured. The concentration of drug found in the tissue was nearly proportional to that of the medium. It is probable that in these experiments the specific binding was too small to be detected, owing to the large amount of drug present in the extracellular space. The extracellular space, in this tissue, represents about 77% of the total volume.[1]

In contrast, binding to the intact tissue could be observed after washing out of the drug. The fragments were incubated for 2 hr in the presence of labeled and nonlabeled ouabain (1 μM) and then washed with drug-free physiological medium. During the first 4 hr of washing out, radioactivity decreased in the tissue until it reached a level which remained stable even when the tissue was washed for 20 hr more. The amount of glycoside which remained firmly bound to the fragments under these conditions was 0.7–0.9 nmole/g of wet tissue. This indicates that the binding of ouabain to electrogenic tissue is virtually irreversible.

DISCUSSION

(Na^+ + K^+)-ATPase Activity and Localization

The electrogenic tissue of *Torpedo marmorata* contains 92% water (1) and only 1.59% proteins. Nevertheless, its (Na^+ + K^+)-ATPase is very abundant, and most of it is ouabain sensitive. The number of ouabain binding sites (3.13 nmoles/g) is slightly higher than that of acetylcholine receptors or of active sites of acetylcholinesterase (15).

Most of the *Torpedo* (Na^+ + K^+)-ATPase is located in a heavy fraction which sediments during centrifugation at moderate speed. This result confirms that of Glynn *et al.* (2). As demonstrated by Sheridan *et al.* (4), this fraction contains most of the dorsal (noninnervated) membrane of electroplax, characterized as rather large fragments of the rich network of tubules. It is probable that most of the sodium pumping sites are situated at this membrane and have the role of restoring and maintaining ionic gradients, responsible for the membrane potential, after an electrical discharge. It must be kept in mind, however, that many fragments present in this fraction come from the presynaptic terminals and are also expected to contain (Na^+ + K^+)-ATPase.

[1] Unpublished observations.

In the electric organ of *Electrophorus electricus* it has been possible to separate by centrifugation on discontinuous sucrose gradients the membrane fragments of the noninnervated membrane from those of the postsynaptic membrane. Whereas the former were very rich in $(Na^+ + K^+)$-ATPase activity, little activity was associated with the latter (16). Our attempts to obtain such an ATPase-rich membrane fraction by similar methods failed with *Torpedo marmorata*. The figures published by Duguid and Raftery (12), using *Torpedo californica*, did not show a higher specific activity than in our fraction $P_{(H_2O)}$.

Osmotic shock and further centrifugation increased the specific activity by a factor of 2, probably by eliminating cytoplasmic proteins still present in the first pellet. The $P_{(H_2O)}$ fraction is very convenient for binding experiments, since 94% of its ATPase activity is ouabain-sensitive.

Experiments with [^{3}H]ouabain showed that the glycoside interacts with a unique class of binding sites. When the medium contained Na^+ and ATP, there was an excellent correlation between the number of sites occupied by the drug and the inhibition of enzymatic activity. In the presence of Mg^{2+} and P_i, a medium which does not allow activity, the affinity for ouabain was higher and the binding more stable.

The affinity of the *Torpedo* $(Na^+ + K^+)$-ATPase for ouabain seems remarkably similar to those reported for sodium pump extracted from nervous tissues of different species (17–19). The sensitivity of the heart enzyme, however, is subject to marked species differences (19).

Experiments with Whole Tissue

Ouabain, up to 0.1 mM, did not affect the electrophysiological response to an isolated stimulus. The responses to repetitive stimulation, however, were affected by the glycoside. The "fatigue" of transmission occurred earlier than normally. The effect was dose-dependent, requiring concentrations somewhat higher than those needed to block the enzyme in a homogenate. Possibly the early fatigue of the ouabain-treated tissue is due to disappearance of the ionic electromotive gradient in the course of repetitive activity. Preliminary experiments, using a method which allows measurements of this ionic force (20), have not confirmed this, but rather have indicated that ouabain produces a failure in the conductance change of the postsynaptic membrane during activity.

This physiological effect of ouabain can be compared with that on other synaptic and nonsynaptic preparations. In mammalian nonmyelinated fibers, ouabain has little effect on the action potentials, but decreases the hyperpolarization which follows a salvo of "tetanic" activity (21). At the frog neuromuscular junction, the postsynaptic potential is amplified for a long time before being blocked (22). In mammalian neuromuscular junction (23) and sympathetic ganglion (24), this glycoside causes a large increase in spontaneous transmitter release, most probably because of depolarization of the presynaptic nerve endings.

Ouabain binding in the electric organ of *Torpedo marmorata* was more difficult to obtain with whole tissue than with the homogenate or fractions. However, washing experiments showed that binding in intact tissue was quite stable. Only about one-third of the sites could be labeled. It is quite possible that, under these conditions, a number of sites are not accessible to the drug.

Hydrodynamic Properties of Solubilized Enzyme

Most of the methods used for solubilizing the sodium pump in other tissue failed with the *Torpedo marmorata* electric organ. Only Lubrol W, a nonionic detergent, succeeded in providing $(Na^+ + K^+)$-ATPase activity in soluble form. In this respect the *Torpedo* enzyme, or its membrane environment, behaved quite differently from those of other tissues and species. Lubrol W also proved to be successful for solubilizing the $(Na^+ + K^+)$-ATPase from the rectal gland of *Squalus acanthias*, another elasmobranch (5).

The solubilized $(Na^+ + K^+)$-ATPase from *Torpedo marmorata* electric organ

behaved as a homogeneous molecular species. By comparison with marker proteins, its Stokes radius was estimated to be 10.7 nm, and its sedimentation coefficient to be 10.5 S. The density of the enzyme-detergent complex has not been measured. Nevertheless, an estimate of its molecular weight can be given, since there is a relatively good correlation between molecular weight and the product of the Stokes radius and sedimentation coefficient [see Bon *et al.* (13)]. Thus the solubilized (Na^+ + K^+)-ATPase from *Torpedo* has an apparent molecular weight of about 475,000, and seems to be an elongated and not a globular protein.

The specific activity at the peak of the gel filtration column was no higher than in the Lubrol W extract, probably because of the presence of much acetylcholinesterase in these fractions. Acetylcholinesterase was recovered in several peaks corresponding to the different elongated and globular forms encountered in these tissues (see ref. 13).

Several attempts were made to analyze the solubilized (Na^+ + K^+)-ATPase by acrylamide gel electrophoresis in a solution of sodium dodecyl sulfate or under other conditions. It was not possible in these experiments to make the Lubrol W-treated enzyme migrate in the gel.

The turnover number of the electric organ (Na^+ + K^+)-ATPase (about 30 molecules of ATP per site per second at 37°) is surprisingly close to the values reported for other preparations. For example, that of guinea pig taenia coli is 22 (25), and that of rabbit brain is 33 (26). The rabbit vagus nerve contains 4.6×10^{14} sites/g of fresh tissue (27), and its activity is 120 units/g;[2] consequently its turnover number should be 43.

It is remarkable that after extensive treatment the solubilized (Na^+ + K^+)-ATPase of *Torpedo* electric organ keeps the same turnover number as the enzyme inserted normally in the membrane. Despite the presence of detergent, the sodium pump extracted from the *Torpedo marmorata* electric organ seems to keep intact all its functional properties.

[2] M. Chmouliovsky, personal communication.

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