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[^{3}H] Ouabain binding to a hydrophobic protein from electroplax membranes

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SUMMARY

After specific binding of [^{3}H] ouabain to electroplax membranes and extraction with chloroform–methanol the ligand was found in the organic phase. After column chromatography in Sephadex LH20 a small fraction of hydrophobic protein carried the bound [^{3}H] ouabain. The maximum specific activity achieved was 4.31 nmoles per mg protein; the specificity of this binding was confirmed by protection experiments. The protein is eluted slightly beyond the cholinergic receptor protein and probably represents a small fragment of the original (Na^+–K^+)-ATPase present in the membrane.

The electric organ of *Electrophorus* is one of the richest sources of (Na^+–K^+)-ATPase, an enzyme which is intimately bound to the electroplax membrane. The cardiac glycoside ouabain is a specific inhibitor of (Na^+–K^+)-ATPase which binds to the enzyme in a rather slow and irreversible manner, each functional molecule being inhibited by 1 molecule of this ligand[1]. In spite of its tightness this binding does not involve the formation of covalent linkages since the ligand may be extracted by acid denaturation or by methanol[2]. Several properties of this binding have led to the suggestion that the interaction may involve nonpolar regions of the peptide chain of the enzyme and the structural characteristics of this cardio-active steroid also suggest that it may develop hydrophobic bonds with a complementary protein surface[3]. However, the presence of hydrogen bonding and stronger polar interactions has also been postulated[1,2].

Previous work from this laboratory, using chloroform–methanol extraction and column chromatography with Sephadex LH20, has shown that several fractions of

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hydrophobic protein (*i.e.* proteolipids) may be isolated from total electroplax of *Electrophorus*[4] or from electroplax membranes[5]. One of these protein fractions was found to bind cholinergic ligands and was considered to represent the cholinergic receptor of the electroplax[6]. Furthermore, some preliminary experiments, in which [^{3}H]ouabain was added to the extract, showed that the radioactivity was eluted in a certain relationship with this cholinergic protein. This finding led us to investigate the possible participation of hydrophobic proteins in the specific binding of the cardiac glycoside. It will be shown here that a protein fraction eluted slightly beyond the cholinergic protein fraction is responsible for the binding of ouabain.

After dissection of 20 g of electric tissue from *Electrophorus* the material was homogenized in the cold with 50 mM Tris–HCl buffer, pH 7.4. A tightly fitted Potter homogenizer, driven by hand, was used until a fine suspension of the tissue was obtained. This was filtered through a double cheese cloth and centrifuged for 1 h at 50 000 × *g* at 0 °C. The supernatant was discarded and the sediment was washed with the buffer and centrifuged twice as above. From the 20 g of original tissue approximately 115 mg of protein were recovered. This amount was resuspended in 4 ml of Tris–HCl buffer, pH 7.9, and divided into two tubes, one for the experiment of specific binding and the other for the control. For the binding experiments with [^{3}H]ouabain (spec. act., 12 Ci/mM, Amersham) the incubation medium contained: 0.1 mM Tris–ethyleneglycol-bis-(β-amino-ethyl ether)-*N*,*N*′-tetraacetic acid, 50 mM Tris–HCl, pH 7.9, 3 mM $MgCl_2$ and 60 mM NaCl, in a total volume of 2.5 ml. In the control experiment 2.08 nmoles of [^{3}H]ouabain were added to the incubation medium (final concentration of ligand, $8.3\ 10^{-7}$ M). In the experiment of specific binding[2] the incubation solution contained 2 mM Na-ATP in addition to the 2.08 nmoles [^{3}H]ouabain. After 3 min of incubation at 37 °C and 10 min at 0 °C, the binding was stopped by the addition of 8 vol. of 50 mM Tris–HCl, pH 7.9, in the cold; this was followed by centrifugation for 30 min at 40 000 × *g*. The membranes were washed twice by centrifugation and were resuspended in 2 ml of buffer. Aliquots were taken to determine protein content and radioactivity (liquid scintillation counting); what remained was lyophilyzed and extracted with chloroform–methanol (2:1, v/v). The lipid extract was concentrated and passed through a Sephadex LH20 column (2.5 cm × 24 cm) as described by La Torre *et al.*[4].

In the experiments of specific binding most of the radioactivity added to the incubation mixture appeared to be bound to the membrane. In the control, without Na-ATP, the radioactivity found in the sediment was only 0.7% of that of the binding experiment. After extraction of the lyophilized material in chloroform–methanol (2:1, v/v) more than 99% of the radioactivity appeared in the organic phase.

As previously described by De Robertis and Fiszer de Plazas[5], in electroplax membranes the hydrophobic protein appears in two main peaks in the chloroform and smaller peaks in the chloroform–methanol mixtures (Fig. 1). The lipid phosphorus (not represented in Fig. 1) is eluted mainly with the second large peak of protein and ends at the point in which radioactivity appears. This is slightly displaced towards the right of the large protein peak and coincides with a small amount of protein detectable by the

TABLE I

DIFFERENT EXPERIMENTS OF [^{3}H] OUABAIN BINDING TO A HYDROPHOBIC PROTEIN FROM ELECTROPLAX MEMBRANES

See description of the various experiments in the text. a, specific binding with addition of 2.08 nmoles [^{3}H] ouabain; b, addition of 0.83 nmole [^{3}H] ouabain to the membrane sediment; c, addition of 0.42 nmole [^{3}H] ouabain to the lyophilized tissue; d, same as c but ligand was added to the extract; e, same as a but with hydration of the lyophilized membranes.

Expt	*Total [^{3}H] ouabain bound (nmole)*	*[^{3}H] Ouabain bound (nmoles/mg protein)*	*Total [^{3}H] ouabain free (nmole)*	*[^{3}H] Ouabain bound / [^{3}H] Ouabain free*
1a	0.649	2.810	0.207	3.130
2a	0.626	4.310	0.682	0.920
3b	0.152	1.024	0.050	3.040
4c	0.002	0.026	0.333	0.007
5d	0.004	0.045	0.232	0.017
6e	0.003	0.060	0.221	0.015

method of Hess and Lewin[7]. In another experiment, not illustrated here, a better fit of the radioactivity with a small absorbance peak at 280 nm was observed. In this experiment the specific activity was 4.31 nmoles of [^{3}H] ouabain per mg of protein (Table I). This figure is about 6.6 times greater than the maximum concentration of ligand found by Albers *et al.*[2]. This suggests that in our case considerable concentration of the binding sites has occurred. It is interesting to remark that in all experiments the protein binding the [^{3}H] ouabain is found immediately after the fraction that binds the cholinergic ligands[4]. The small amount of radioactivity eluted in chloroform–methanol (4:1, v/v) corresponds to free ligand (Fig. 1). In an experiment in which $1 \cdot 10^{-7}$ M ouabain in 5.8 ml of chloroform–methanol (2:1, v/v) was placed on the column most of the radioactivity was eluted with chloroform–methanol (4:1, v/v). A minor amount (about 10%) was eluted with chloroform between 60 and 110 ml, *i.e.* beyond the volume at which the bound ligand is eluted (Fig. 1).

In Table I, Expts 1 and 2 represent the specific binding of [^{3}H] ouabain in the presence of Na-ATP. Expt 3 represents one case in which the [^{3}H] ouabain was added directly to the sedimented membranes. Under these conditions, the same type of result was obtained as in Expts 1 and 2. This finding provoked serious doubts as to the interpretation of the experiments of specific binding. It is possible that in the extract the ligand could detach from its site of specific binding to rebind unspecifically to the protein eluted in the chloroform. To overcome this difficulty several experiments were done in which the [^{3}H] ouabain was added at different stages during the procedure. Those that gave a clear cut answer were the experiments of protection indicated as Expts 4 and 5 in Table I. In these experiments the binding was achieved using unlabelled ouabain in the presence of Na-ATP (specific binding); later [^{3}H] ouabain was added directly to the membranes or to the lipid extract. It may be observed that in both cases there was practically no binding of

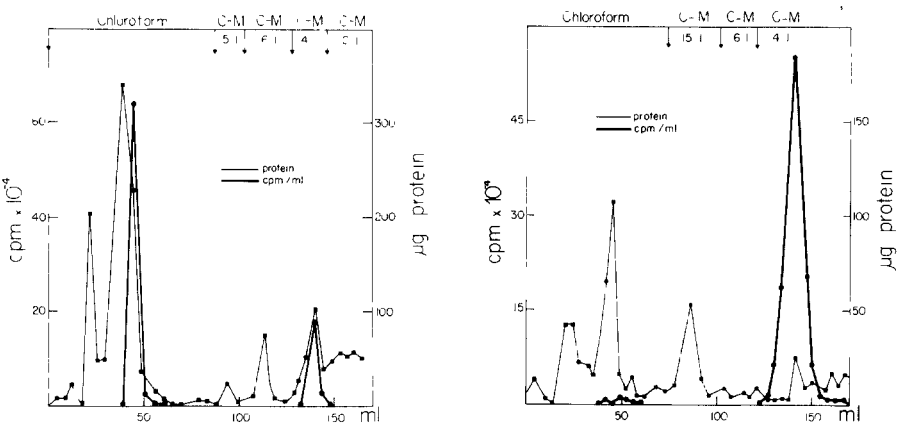

Fig. 1. Chromatogram in Sephadex LH20 of the chloroform–methanol extract corresponding to Expt 1 (Table I). The radioactivity is eluted in a major bound peak at about 45 ml chloroform and a smaller (free) peak in chloroform–methanol (4:1, v/v) (see description in the text).

Fig. 2. Chromatogram in Sephadex LH20 of the chloroform–methanol extract corresponding to Expt 5 (Table I). The protection with unlabelled ouabain in the presence of Na-ATP has resulted in a large peak of free radioactivity (*cf.* Fig 1 and see the description in the text).

[^{3}H] ouabain and the radioactivity appeared free in the chloroform–methanol (4:1, v/v). The ^{3}H-bound/^{3}H-free ratio is 2 orders of magnitude lower than in Expts 1 and 2. One of the protection experiments is represented in Fig. 2. It is evident that in Fig. 1 most of the [^{3}H] ouabain is bound while in Fig. 2 most of the radioactivity is free.

The results presented here suggest that a hydrophobic protein (*i.e.* proteolipid) may be responsible for the binding of ouabain to (Na^+–K^+)-ATPase. A small protein fraction eluted slightly beyond the cholinergic peak[5] appears to contain the sites of binding for the cardiac glycoside. The binding to this fraction may be obtained either on the membranes, in the presence of Na-ATP, or directly by adding the [^{3}H] ouabain to the organic extract (Table I). This would indicate that once the protein fraction is separated by the solvent the sites of binding become available to the ligand. In favor of this interpretation are also the protection experiments which demonstrate that once the binding sites are specifically blocked by the ligand they are no longer available to the [^{3}H] ouabain added to the extract.

It was mentioned earlier that ouabain probably binds to the enzyme by a series of hydrophobic and hydrophilic interactions[1,2]. The fact that ouabain remains bound to a hydrophobic protein in the extract and as a peak in the chloroform may be explained by taking into account that all types of hydrophilic interactions (*i.e.* hydrogen bond, ion–ion and dipolar interactions) are considerably strengthened in hydrophobic media (see ref. 8). In fact, we have experimentally shown that the presence of a small amount of water in a chloroform–methanol medium is able to break the specific binding of ouabain. This is what happened in Expt 6 (Table I) in which the lyophilized tissue was hydrated prior to

its extraction with chloroform–methanol. The presence of a small amount of water could also explain the lower ^{3}H-bound/^{3}H-free ratio in Expt 2 as compared to Expts 1 and 3 (Table I).

Throughout this discussion we have assumed that [^{3}H]ouabain binds to a protein moiety. In the region in which the ligand is bound no lipid phosphorus is detectable; however, the possibility that other lipids could be associated with this fraction is not discarded at present and is being investigated in our laboratory.

In the case of the cholinergic receptor protein we have calculated a molecular weight of about 40 000 (ref. 9). Since the ouabain-binding protein appears nearby in the chromatogram we could assume that it is similar in size or even smaller. The molecular weight of (Na^+–K^+)-ATPase from different sources has been calculated using different methods; since the lowest estimate by ouabain binding for the enzyme from electroplax is 500 000 (ref. 2) we have to conclude that we are probably dealing with a small fragment of the enzyme.

Since (Na^+–K^+)-ATPase may also be specifically labelled with [^{32}P]ATP or with ^{32}P under certain incubation conditions[2] we have carried out preliminary experiments to determine the site of binding of ^{32}P. Results to be published elsewhere suggest that the phosphorylated intermediate of (Na^+–K^+)-ATPase is also a hydrophobic protein but different from the fragment that binds [^{3}H]ouabain.

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