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Functional activity of oligosaccharide-deficient (Na,K)ATPase expressed in *Xenopus* oocytes

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(Na,K)ATPase from *Torpedo californica* was expressed in *Xenopus laevis* oocytes in the presence of tunicamycin by injecting mRNAs for the α- and β-subunits derived from the cloned cDNAs into the oocytes. The oligosaccharide-deficient ATPase thus synthesized was transported to the oocyte plasma membrane, where it exhibited virtually the same ATPase activity, ouabain-binding capacity and $^{86}Rb^+$ transport activity as the fully glycosylated enzyme. We conclude that the oligosaccharide chains on the β-subunit has no effect on the catalytic activities of (Na,K)ATPase.

$(Na^+ + K^+)$ATPase; Oligosaccharide chain; Tunicamycin; (*Xenopus* oocyte)

1. INTRODUCTION

(Na,K)ATPase is a membrane-bound enzyme catalyzing ATP-driven active transport of Na^+ and K^+. It consists of two non-covalently linked subunits, i.e. a catalytic α-subunit of about 100 kDa and a glycosylated β-subunit whose protein moiety has a molecular mass of about 35 kDa [1–4]. The β-subunit has three potential glycosylation sites in its primary structure [5–11]. While the α-subunit has been shown to contain ATP- and ouabain-binding sites as well as a site that is transiently phosphorylated during the catalytic cycle [12], no catalytic functions have been attributed to the β-subunit. However, Noguchi et al. [13] have recently reported that the expression of functionally active (Na,K)ATPase in *Xenopus* oocytes can be achieved only when mRNAs for both α- and β-subunits have been injected, indicating the functional importance of the β-subunit. On the other hand, Tamkun and Fambrough [14] have shown that inhibition of glycosylation of the β-subunit in cultured chick sensory neurons does not affect the subunit assembly, intracellular transport, and in vivo degradation rate of the ATPase. This study was designed to study whether the oligosaccharide chains of the β-subunit are required for ATP hydrolysis and ion transport activities of the enzyme. For this purpose, mRNAs for the two subunits of *Torpedo californica* (Na,K)ATPase were expressed in *Xenopus laevis* oocytes in the presence and absence of tunicamycin, a glycosylation inhibitor. Here we report that the oligosaccharide-deficient ATPase synthesized in the presence of the inhibitor is transported to the cell surface and exhibits virtually the same catalytic activities as the fully glycosylated enzyme.

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Abbreviations: (Na,K)ATPase, sodium- and potassium-dependent adenosine triphosphatase; PMSF, phenylmethanesulfonyl fluoride

2. MATERIALS AND METHODS

Recombinant plasmids used for the synthesis of mRNAs for the subunits of *Torpedo californica* (Na,K)ATPase were constructed as described in [13]. The mRNAs were synthesized in vitro with the aid of SP6 RNA polymerase [15,16]. mRNAs for both α- and β-subunits (0.5 μg/μl each) were injected into *Xenopus laevis* oocytes (about 20 nl per oocyte) and the oocytes were incubated at 19°C for 3 days. When tunicamycin was applied, the mRNAs were injected together with tunicamycin

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(40 μg/ml) into oocytes, which had been incubated at 19°C for 3 days in the presence of the inhibitor (2 μg/ml) [17]. The oocytes were then incubated at 19°C for 3 more days in the presence of tunicamycin (2 μg/ml) prior to examination.

For preparation of oocyte 'microsomes' (plasma membrane fraction), about 300 oocytes were held in 50 mM imidazole/HCl buffer (pH 7.5) containing 250 mM sucrose, 1 mM EDTA and 1 mM PMSF (buffer A) using watchmaker's forceps and a slit was made with the tip of a syringe needle. The oocytes were then gently squeezed to extrude cytoplasmic materials. The crude plasma membrane fraction thus obtained was suspended in buffer A. After homogenization, the microsomes were sedimented by centrifugation as described in [13]. The pellet was suspended in 25 mM imidazole/HCl buffer (pH 7.5) containing 1 M NaSCN, 125 mM sucrose, 0.5 mM EDTA and 0.5 mM PMSF, and the suspension was incubated at 20°C for 1 h. The microsomes were then washed twice with buffer A and finally suspended in 0.4 ml of buffer A.

To label the ATPase subunits, 8–10 oocytes that had been injected with mRNAs for the two subunits were incubated in the presence of L-[U-^{14}C]leucine (spec. act. 340 mCi/mmol; final concentration 0.15 mCi/ml) at 19°C for 3 days. The labeled oocytes were then mixed with 100 non-labeled, non-injected oocytes, which served as a source of carrier membranes. The mixture was treated as the above-mentioned preparation of microsomes. The microsomes were subjected to immunoprecipitation with a mixture of anti-α and anti-β subunit sera as described in [13]. Surface proteins of oocytes, deprived of follicular cells, were iodinated with Na^{125}I by the method of Hubbard and Cohn [18].

ATPase activity of microsomes as well as [21,22-^{3}H]ouabain-binding capacity and $^{86}Rb^{+}$ transport activity of oocytes, deprived of follicular cells, were assayed as described in [13]. Protein was determined by the method of Lowry et al. [19]. Anti-α and anti-β subunit sera were prepared as described in [13]. Tunicamycin was purchased from Sigma and radioactively labeled compounds were from Amersham and NEN. Other chemicals and biochemicals were obtained from Nakarai Chemical Co. and were of reagent or higher grade.

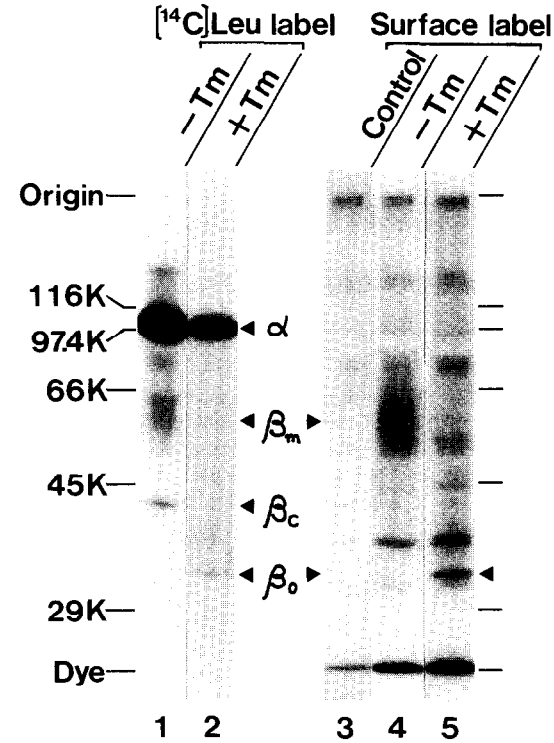

Fig.1. Effect of tunicamycin on the expression of *Torpedo californica* (Na,K)ATPase in *Xenopus* oocytes. Microsomes from mRNA-injected oocytes labeled with [^{14}C]leucine in the absence (lane 1) or presence (lane 2) of tunicamycin (2 μg/ml) were subjected to immunoprecipitation with a mixture of anti-α and anti-β subunit sera. For lanes 3–5, non-injected oocytes (3) or mRNA-injected oocytes incubated without (4) or with (5) tunicamycin (2 μg/ml) were labeled with Na^{125}I from the extracellular surface of the oocytes and subjected to immunoprecipitation as above.

3. RESULTS AND DISCUSSION

Xenopus oocytes, to which mRNAs for both α- and β-subunits of (Na,K)ATPase had been injected, were incubated for 3 days in the presence of L-[^{14}C]leucine and then microsomes (plasma membrane fraction) were prepared therefrom. The microsomes were subjected to immunoprecipitation with a mixture of anti-α and anti-β subunit sera and the immunoprecipitate was examined by SDS-polyacrylamide gel electrophoresis and subsequent fluorography. As reported previously [13], three major radioactive polypeptides were detected (fig.1, lane 1), i.e. the α-subunit of (Na,K)ATPase (about 100 kDa) and two forms of the β-subunit termed β_m and β_c having molecular masses of about 60 and 41 kDa, respectively. It has been shown that β_m and β_c correspond to the fully glycosylated and core glycosylated forms of the β-subunit, respectively [13]. In the oocytes that had been incubated with tunicamycin, the amounts of β_m and β_c were drastically reduced and a band having a molecular mass of 35 kDa (β_o) appeared (fig.1, lane 2), though the mobility of the α-subunit remained unaffected. Anti-β subunit serum alone could precipitate β_m, β_c and β_o (not shown). As reported previously [13,20], digestion of β_m and β_c with endoglycosidase F and endoglycosidase H, respectively, resulted in the formation of a band having the same mobility as β_o. We, therefore, concluded that β_o was the β-subunit devoid of oligosaccharide chains.

To study if these subunits synthesized in oocytes could be transported to the cell surface, constituents of the surface of oocytes (incubated in the absence of [^{14}C]leucine) were iodinated with Na^{125}I and the iodinated proteins were examined by immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in fig.1, both β_m synthesized in the absence of tunicamycin (lane 4) and β_o synthesized in the presence of the inhibitor (lane 5) were iodinated. The origin of other radioactive bands is unknown, but they seem to be non-specific artefacts in view of the fact that they can also be detected in the control oocytes that had not been injected with the subunit mRNAs (lane 3). These results indicated that not only β_m but also the nonglycosylated β-subunit (β_o) could be transported to the oocyte surface. The failure of β_c to be iodinated (lane 4) is consistent with the finding reported by Tamkun and Fambrough [14] that β_c possessing only core sugar chains of high mannose type remained intracellularly and could not be transported to the plasma membrane unless fully glycosylated. The extremely poor iodination of the α-subunit synthesized both in the absence and presence of tunicamycin (lanes 4 and 5) can be accounted for by assuming that iodinatable tyrosine residues in this subunit of the *Torpedo californica* enzyme are rarely exposed to the outer cell surface (cf. [21]). It is evident that the α-subunit was actually transported to the cell surface, because, as shown below, the ouabain-binding capacity and $^{86}Rb^+$ transport activity of the oocytes injected with mRNAs for the two ATPase subunits were much higher than those of control oocytes.

We next examined ouabain-sensitive ATPase activity, [^{3}H]ouabain-binding capacity and $^{86}Rb^+$ transport activity of oocytes that had been injected with the mRNAs and incubated in the presence of tunicamycin. Oocytes that had not received the mRNAs were used as a control to assess the contribution of (Na,K)ATPase inherent to the oocytes. As shown in table 1, the ATPase activity of microsomes from oocytes injected with the mRNAs was definitely higher than that of control microsomes, and no significant difference in ATPase activity was seen between microsomes from tunicamycin-treated and untreated oocytes. The capacity of oocytes to bind [^{3}H]ouabain to their surface was again practically the same between tunicamycin-treated and untreated oocytes, both being about 6-fold higher than that of control oocytes. Furthermore, $^{86}Rb^+$ transport activities of the treated and untreated oocytes were also nearly the same and clearly higher than that of control

Table 1

Effect of tunicamycin on the activities of (Na,K)ATPase expressed in *Xenopus* oocytes

	(Na,K)ATPase activity (μmol/mg per h)	[^{3}H]Ouabain binding activity (fmol/oocyte)	$^{86}Rb^+$ transporting activity (pmol/min per oocyte)
mRNAs non-injected	2.9 (n = 2)	19 (n = 4)	49 (n = 4)
mRNAs injected			
− Tunicamycin	7.3 (n = 3)	119 (n = 4)	172 (n = 4)
+ Tunicamycin	7.4 (n = 3)	114 (n = 4)	122 (n = 2)

Xenopus oocytes were injected with mRNAs for the α- and the β-subunits and incubated in the presence or absence of tunicamycin for 3 days at 19°C. The microsome preparations, which had been treated with 1 M NaSCN, were assayed for ATPase activity in the presence and absence of 1 mM ouabain, and the ouabain-sensitive activity was taken as (Na,K)ATPase activity. The oocytes deprived of follicular cells were assayed for [^{3}H]ouabain-binding and $^{86}Rb^+$ transporting activities

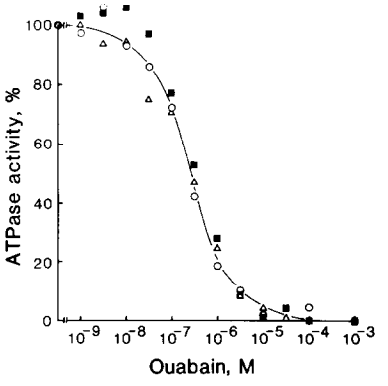

Fig.2. Ouabain sensitivity of the (Na,K)ATPase expressed in *Xenopus* oocytes. Microsomes from non-injected oocytes (○) and from oocytes injected with mRNAs and incubated in the absence (△) or presence (■) of tunicamycin (2 μg/ml) were assayed for ATPase activity in the presence of varied concentrations of ouabain. The microsomes were preincubated in 50 mM imidazole/HCl buffer (pH 7.5) containing 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and indicated concentrations of ouabain at 37°C for 15 min. The reaction was started by the addition of 1 mM ATP.

oocytes. These results indicated that *Torpedo californica* (Na,K)ATPase synthesized in *Xenopus* oocytes and transported to the cell surface in the presence of tunicamycin had virtually the same catalytic activities as that synthesized in the absence of tunicamycin. Hence, we conclude that the oligosaccharide chains of the β-subunit have no significant effect on the functions of the ATPase in the plasma membrane.

In (Na,K)ATPase the ouabain-binding site is generally thought to reside on the α-subunit [22,23]. It is, therefore, held that the difference in ouabain sensitivity between kidney and brain (Na,K)ATPase is due to the difference in the α-subunit [24]. It has, however, been reported that the β-subunit oligosaccharide chains in the kidney enzyme are different from those of the brain enzyme [20], even though the β-subunits from the two sources are identical [9]. The possibility, therefore, arises that oligosaccharide chains of the β-subunit may, at least in part, be responsible for the affinity of (Na,K)ATPase for ouabain. To test this possibility we examined the ouabain sensitivity of the oligosaccharide-deficient ATPase. As shown in fig.2, no significant difference in ouabain sensitivity was detected between the enzymes expressed in oocytes in the presence and absence of tunicamycin. Moreover, the ouabain sensitivity of the ATPase inherent to *Xenopus* oocytes was also identical with those of the expressed enzymes. It can, therefore, be concluded that the absence of oligosaccharide chains on the β-subunit does not affect the ouabain sensitivity of (Na,K)ATPase.

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