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Expression of functional ($Na^+ + K^+$)-ATPase from cloned cDNAs

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Functional ($Na^+ + K^+$)-ATPase is formed in *Xenopus* oocytes injected with α- and β-subunit-specific mRNAs derived from cloned *Torpedo californica* cDNAs. Both the mRNAs are required for the expression of functional ($Na^+ + K^+$)-ATPase.

($Na^+ + K^+$)-ATPase; cDNA expression; Ouabain binding; $^{86}Rb^+$ transport; (*Xenopus* oocyte)

1. INTRODUCTION

Sodium- and potassium-dependent adenosine triphosphatase [($Na^+ + K^+$)-ATPase], which mediates the active transport of Na^+ and K^+, is widely distributed among animal cell membranes and consists of two types of subunits, α and β [1–4]. The larger α-subunit is thought to have the catalytic role, whereas no definite role has been assigned to the smaller β-subunit. The primary structures of both the subunits from various sources have been deduced by cloning and sequence analysis of the cDNAs [5–14]. The present investigation shows that functional ($Na^+ + K^+$)-ATPase is formed in *Xenopus* oocytes by microinjection of α- and β-subunit-specific mRNAs synthesized by transcription in vitro of cloned *Torpedo californica* cDNAs. The subunit requirement for the expression of functional ($Na^+ + K^+$)-ATPase has also been studied.

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Abbreviation: ($Na^+ + K^+$)-ATPase, sodium- and potassium-dependent adenosine triphosphatase

2. MATERIALS AND METHODS

The recombinant plasmids used for the synthesis of ($Na^+ + K^+$)-ATPase subunit-specific mRNAs were constructed as follows. The ~2.1-kilobase-pair (kb) *Hpa*II fragment from clone pNKAα87 [5] was treated with T_4 DNA polymerase in the presence of four deoxyribonucleoside triphosphates and cleaved with *Nde*I. The resulting ~0.8-kb fragment was ligated with the *Aha*III (−38)/*Nde*I (2557) fragment from pNKAα87 and *Sma*I-cleaved pSP65 [15] (numbers in parentheses indicate the 5′-terminal nucleotide generated by cleavage). The plasmid pSPNKAα thus obtained, carrying the entire coding sequence for the *T. californica* ($Na^+ + K^+$)-ATPase α-subunit in the same orientation as the SP6 promoter, was identified by restriction endonuclease analysis. The ~1.9-kb *Hae*III (−35)/*Pvu*II (on vector) fragment from clone pNKAβ1 [7] was inserted into the *Sma*I site of pSP65 in the same orientation as the SP6 promoter to yield pSPNKAβ.

($Na^+ + K^+$)-ATPase subunit-specific mRNAs were synthesized in vitro as in [15,16], using *Sal*I-cleaved pSPNKAα and pSPNKAβ as templates. The sizes of the resulting α- and β-subunit-specific mRNAs estimated by electrophoresis on 1.5% agarose gels agreed with those expected (~3.4 and

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~1.9 kilobases, respectively), except that the β-subunit-specific mRNA preparations contained an additional RNA species of ~1.7 kilobases. The α- and the β-subunit-specific mRNA, together (mRNA concentration, 0.1 or 0.5 μg/μl each) or separately (0.5 or 3 μg/μl), were injected into *X. laevis* oocytes (average volume injected per oocyte, ~20 nl). The oocytes were incubated at 19°C for 3 days as in [17] before being tested.

For labelling translation products, oocytes were incubated in the presence of L-[U-^{14}C]leucine (spec. act. 340 mCi/mmol; final concentration, 0.15 mCi/ml). Extracts were prepared from 8–10 oocytes as in [17], except that aprotinin was omitted. The cell extracts (corresponding to 3 oocytes each) were subjected to immunoprecipitation using rabbit antisera to *T. californica* ($Na^+ + K^+$)-ATPase subunits as specified and protein A-Sepharose. The resulting immunoprecipitates were analysed by electrophoresis on 0.1% SDS/10% polyacrylamide gels [18], followed by fluorography [19]. ($Na^+ + K^+$)-ATPase from *T. californica* electric organ and its subunits were purified as in [5,7].

Unless otherwise specified, microsomes from oocytes were prepared at 0–4°C as follows. About 440 oocytes were homogenized in 6 ml of a solution containing 250 mM sucrose, 50 mM imidazole-HCl buffer (pH 7.5) and 1 mM EDTA (solution A). The homogenate was centrifuged at 7000 × *g* for 10 min on a 50% sucrose cushion (1 ml), and the supernatant was further centrifuged at 160000 × *g* for 30 min. The resulting pellet was suspended and incubated at 20°C for 1 h in 4 ml of a solution containing 1 M NaSCN, 125 mM sucrose, 25 mM imidazole-HCl buffer (pH 7.5) and 0.5 mM EDTA to reduce ouabain-insensitive ATPase activity [20]. The suspension was then centrifuged at 160000 × *g* for 30 min, and the pellet was washed twice by homogenization in 4 ml of solution A followed by centrifugation, suspended in 0.4 ml of solution A and used as a microsome preparation.

ATPase activity was assayed at 37°C in a reaction mixture (total volume, 0.2 ml) containing 50 mM imidazole-HCl buffer (pH 7.5), 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 1 mM ATP and microsomes (20–80 μg protein) in the presence and absence of 1 mM ouabain as in [21], except that the P_i released was determined according to [22]. Ouabain-sensitive ATPase activity was obtained by subtracting the ATPase activity measured in the presence of ouabain (ouabain-insensitive activity) from that measured in its absence (total activity). The ouabain-insensitive activity was 6–17 and 16–27% of the total activity for oocytes injected with both the α- and β-subunit-specific mRNAs at concentrations of 0.5 and 0.1 μg/μl each, respectively, and 34–64% of the total activity for noninjected oocytes and oocytes injected with either the α- or the β-subunit-specific mRNA (3 μg/μl) alone. Protein was determined as in [23] with bovine serum albumin as a standard.

For the assay of [^{3}H]ouabain-binding activity on the cell surface, 40 oocytes were treated with 1 mg/ml collagenase as in [24] to be deprived of follicular cells and were incubated at 19°C for 6 h with 0.1 μM [21,22-^{3}H]ouabain (4.1 Ci/mmol) in modified Barth's medium [25] from which KCl was omitted. The amount of [^{3}H]ouabain binding measured in the presence of 1 mM unlabelled ouabain [5–7% of the total binding for oocytes injected with both the α- and β-subunit-specific mRNAs (0.5 μg/μl each) and 31–50% of the total binding for noninjected oocytes and oocytes injected with either the α- or the β-subunit-specific mRNA (3 μg/μl) alone] was subtracted to eliminate nonspecific binding.

$^{86}Rb^+$ uptake by oocytes was measured as follows. After removal of follicular cells [24] and incubation at 4°C for 2 h in modified Barth's medium [25] supplemented with 20 μM monensin [26] and with NaCl to give a final concentration of 140 mM, 50–60 oocytes were incubated at 19°C in 1 ml of a solution containing 5 mM ^{86}RbCl (1.0 mCi/mmol), 140 mM NaCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM Hepes-NaOH buffer (pH 7.6) and 20 μM monensin (solution B) in the presence and absence of 1 mM ouabain. At intervals of 3 min, 10 oocytes each were withdrawn, washed with ice-cold solution B containing unlabelled 5 mM RbCl instead of ^{86}RbCl, centrifuged through dinonyl phthalate/silicone oil (1:1, v/v) [27] and counted for $^{86}Rb^+$.

3. RESULTS AND DISCUSSION

mRNAs specific for the α- and β-subunits of *T.*

californica ($Na^+ + K^+$)-ATPase were synthesized by transcription in vitro of the respective cDNAs and were injected together or separately into *Xenopus* oocytes. The resulting translation products were identified by labelling the polypeptides with [^{14}C]leucine and isolating them by immunoprecipitation, followed by SDS-polyacrylamide gel electrophoresis. As shown in fig.1, two major translation products with estimated relative molecular masses of 100 and 41 kDa were found in oocytes injected with both the α- and β-subunit-specific mRNAs (mRNA concentration, 0.5 μg/μl each for lanes 3,4 or 0.1 μg/μl each for lanes 5,6), whereas no labelled polypeptide was immunoprecipitated from the extract of noninjected, control oocytes (lanes 1,2). The polypeptide of 100 kDa was indistinguishable in mobility from the native α-subunit and corresponded to the major translation product formed by injection of the α-subunit-specific mRNA (3 μg/μl) alone, which was immunoprecipitated specifically with antiserum to the α-subunit (lanes 7–9). On the other hand, the polypeptide of 41 kDa migrated faster than the native β-subunit (~60 kDa) and corresponded to the major translation product formed by injection of the β-subunit-specific mRNA (3 μg/μl) alone, which was immunoprecipitated specifically with antiserum to the β-subunit (lanes 10–12). Injection of the β-subunit-specific mRNA (3 μg/μl) alone manifested an additional diffuse band corresponding to about 60 kDa, and this species also reacted specifically with antiserum to the β-subunit (lanes 10–12).

The amount of the α-subunit found in oocytes injected with the α-subunit-specific mRNA alone at a concentration of 0.5 μg/μl (not shown) was much smaller than that present in oocytes injected with both the α- and β-subunit-specific mRNAs at the same concentration (0.5 μg/μl each). When the α-subunit-specific mRNA alone was injected at a higher concentration (3 μg/μl), the amount of the α-subunit found was comparable to that found when both the mRNAs were injected at a lower concentration (0.1 μg/μl each) (fig.1, lanes 5,7). In contrast, the amount of the β-subunit found in oocytes increased with increasing concentrations (0.1, 0.5 and 3 μg/μl) of the β-subunit-specific mRNA injected, irrespective of simultaneous injection of the α-subunit-specific mRNA (fig.1, lanes 3,5,10).

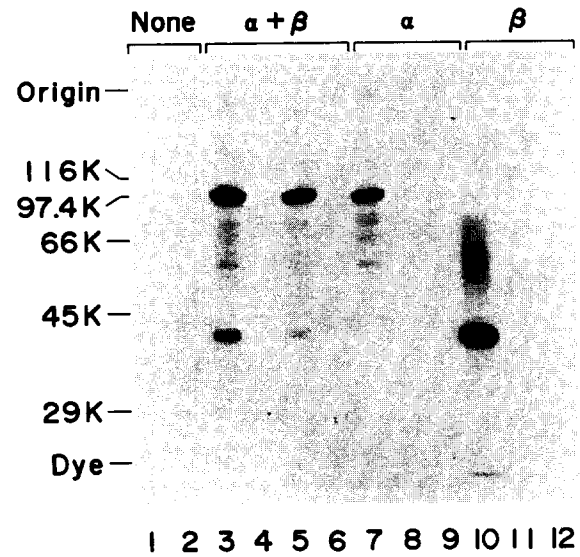

Fig.1. Fluorogram of SDS-polyacrylamide gel electrophoresis of the polypeptides resulting from translation of ($Na^+ + K^+$)-ATPase subunit-specific mRNAs in *Xenopus* oocytes. Cell extracts from noninjected oocytes (lanes 1,2) and from oocytes injected with both the α- and β-subunit-specific mRNAs (mRNA concentration, 0.5 μg/μl each for lanes 3,4 or 0.1 μg/μl each for lanes 5,6) or with the α- (3 μg/μl) (lanes 7–9) or the β-subunit-specific mRNA (3 μg/μl) alone (lanes 10–12) were subjected to immunoprecipitation with a mixture of anti-α- and anti-β-subunit sera (lanes 1–6) or with anti-α- (lanes 7,8,12) or anti-β-subunit serum alone (lanes 9–11); lanes 2,4,6,8 and 11 represent controls for lanes 1,3,5,7 and 10, respectively, i.e. immunoprecipitates obtained in the presence of a large excess of the purified native α- and β-subunits (lanes 2,4,6), α-subunit (lane 8) or β-subunit (lane 11). The fluorogram was exposed at −80°C for 24 h. The size markers used were *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa) and bovine erythrocyte carbonic anhydrase (29 kDa) (Sigma).

Fig.2 shows experiments designed to elucidate the observed difference in size between the major β-subunit polypeptide synthesized in oocytes and the native β-subunit. Microsome preparations from oocytes injected with the β-subunit-specific mRNA (3 μg/μl) alone (lanes 2,5) or together with the α-subunit-specific mRNA (0.5 μg/μl each) (lanes 3,6), as well as purified native ($Na^+ + K^+$)-ATPase (lanes 1,4), were treated with en-

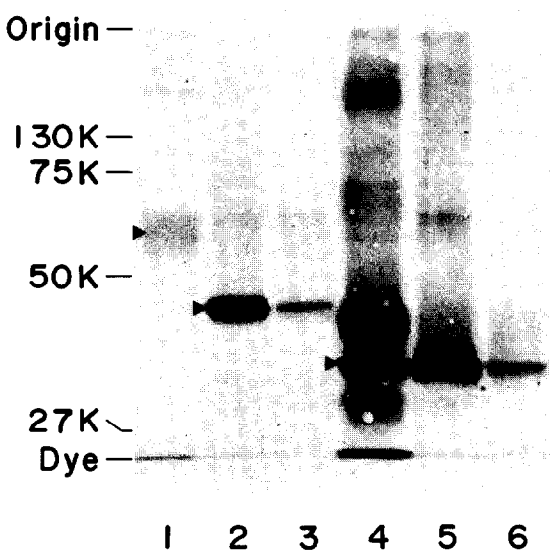

Fig.2. Immunoblotting analysis of $(Na^+ + K^+)$-ATPase β-subunit polypeptides before and after endoglycosidase F treatment. Purified native $(Na^+ + K^+)$-ATPase (lanes 1,4) and the enzyme in microsomes from *Xenopus* oocytes injected with the β-subunit-specific mRNA (mRNA concentration, 3 $\mu g/\mu l$) alone (lanes 2,5) or with both the α- and β-subunit-specific mRNAs (0.5 $\mu g/\mu l$ each) (lanes 3,6) were electrophoresed on a 0.1% SDS/10% polyacrylamide gel before (lanes 1–3) and after endoglycosidase F treatment [28,29] (lanes 4–6) and were then blotted onto a nitrocellulose sheet [30]. The blot was incubated with rabbit anti-β-subunit serum, and the bound antibody was detected by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G [31]. Untreated β-subunit polypeptides as well as the smallest major species of treated β-subunit polypeptides are indicated by arrowheads. The size markers used (pre-stained) were rabbit muscle phosphorylase *b* (130 kDa), bovine serum albumin (75 kDa), chicken ovalbumin (50 kDa) and soybean trypsin inhibitor (27 kDa) (Bio-Rad).

doglycosidase F, which removes *N*-linked carbohydrate side chains [28,29]. This treatment resulted in an increase in the electrophoretic mobility of the β-subunit polypeptides (probed with β-subunit-specific antiserum) in all three preparations. The smallest major species observed after endoglycosidase F treatment had an estimated molecular mass of 35 kDa, which agrees well with that of the protein moiety of the β-subunit calculated from its primary structure (34671 Da) [7]). These results suggest that the difference in molecular mass between the major β-subunit polypeptide produced in oocytes and the native β-subunit is attributable to a difference in glycosylation.

Oocytes injected with both the α- and β-subunit-specific mRNAs (0.5 $\mu g/\mu l$ each) were examined for different functional parameters of $(Na^+ + K^+)$-ATPase; noninjected oocytes were used as controls to assess endogenous $(Na^+ + K^+)$-ATPase. First, the ouabain-sensitive ATPase activity in microsomes from the injected oocytes (109 ± 46 nmol/min per mg protein, mean ± SD, $n = 8$) was 6-fold higher than that in microsomes from noninjected oocytes (18 ± 8 nmol/min per mg protein, $n = 8$); a typical experiment is shown in fig.3. Secondly, the [^{3}H]ouabain-binding activity on the surface of the injected oocytes (193 ± 51 fmol/oocyte, $n = 3$) was 7-fold higher than that of noninjected oocytes (28 ± 6 fmol/oocyte, $n = 3$). Finally, the $^{86}Rb^+$ uptake by the injected oocytes was much greater than that by noninjected oocytes, as exemplified in fig.4. This difference was due to an increase in ouabain-sensitive $^{86}Rb^+$ transport activity in the injected oocytes because, in the presence of 1 mM ouabain, no significant difference in $^{86}Rb^+$ uptake was observed between the two groups of oocytes (fig.4). These results indicate that the α- and β-subunit-specific mRNAs derived from the cloned cDNAs can direct the formation of functional $(Na^+ + K^+)$-ATPase in the plasma membrane of *Xenopus* oocytes.

Using this expression system, we next examined whether both the α- and β-subunit-specific mRNA are required for the expression of functional $(Na^+ + K^+)$-ATPase. The [^{3}H]ouabain-binding activity on the surface of oocytes injected with either the α- or the β-subunit-specific mRNA (3 $\mu g/\mu l$) alone [20 ± 4 fmol/oocyte ($n = 3$) and 21 fmol/oocyte (mean of 18 and 24 fmol/oocyte), respectively] was no more than that of noninjected oocytes (see above). Likewise, the ouabain-sensitive ATPase activity in microsomes from oocytes injected with either the α- or the β-subunit-specific mRNA (3 $\mu g/\mu l$) alone [18 ± 6 nmol/min per mg protein ($n = 4$) and 24 ± 4 nmol/min per mg protein ($n = 3$), respectively] was as low as that in microsomes from noninjected oocytes (see above), as exemplified in fig.3. It should be noted that the amount of the α-subunit found in oocytes injected with the α-subunit-specific mRNA

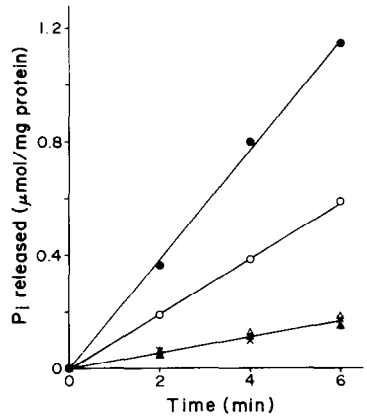

Fig.3. Ouabain-sensitive ATPase activities in microsomes from *Xenopus* oocytes injected with $(Na^+ + K^+)$-ATPase subunit-specific mRNAs. Assays were carried out with microsomes from noninjected oocytes (×) and from oocytes injected with both the α- and β-subunit-specific mRNAs [mRNA concentration, 0.1 μg/μl each (○) or 0.5 μg/μl each (●)] or with the α- (3 μg/μl) (▲) or β-subunit-specific mRNA (3 μg/μl) alone (△).

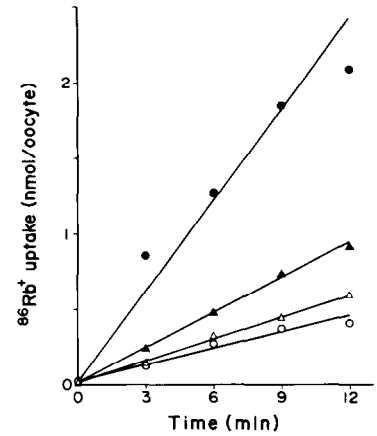

Fig.4. $^{86}Rb^+$ uptake by *Xenopus* oocytes injected with $(Na^+ + K^+)$-ATPase subunit-specific mRNAs. $^{86}Rb^+$ uptake was measured for oocytes injected with both the α- and β-subunit-specific mRNAs (mRNA concentration, 0.5 μg/μl each) (circles) and for noninjected oocytes (triangles) in the presence (open symbols) and absence (closed symbols) of 1 mM ouabain.

(3 μg/μl) alone was comparable to that present in oocytes injected with a low concentration (0.1 μg/μl each) of both the α- and β-subunit-specific mRNAs (see above) and that the latter oocytes exhibited an ouabain-sensitive ATPase activity in microsomes much higher than that of noninjected oocytes (fig.3).

Our results show that the α-subunit-specific mRNA alone is unable to direct the formation of functional $(Na^+ + K^+)$-ATPase, despite the fact that a sufficient amount of the α-subunit is produced. On the basis of previous studies, it seems unlikely that the β-subunit is directly involved in catalytic activity [4]. One possibility is that the coexistence of the β-subunit is required for the correct conformation of the α-subunit necessary for $(Na^+ + K^+)$-ATPase activity. A significant interaction between the two subunits is suggested by the observations that the susceptibility of the β-subunit to trypsin depends on combinations of ligands that bind to the α-subunit [32] and that reduction of a disulphide bond located in the β-subunit results in inactivation of the enzyme, both the reduction and the inactivation being protected by K^+ and Na^+ [33,34]. An alternative possibility is that the β-subunit is required for localizing the α-subunit into the membrane. Consistent with this notion is the observation that the assembly in vivo of the α- and β-subunits occurs concurrently with or immediately after polypeptide synthesis [35]. Studies with cell-free translation systems have yielded variable results as to the dependence of membrane insertion of the α-subunit on the presence of the β-subunit [36,37]. Our finding that the amount of the α-subunit in the cell extract is markedly reduced in the absence of the β-subunit is compatible with the view that the α-subunit is stabilized by the coexistence of the β-subunit.

Recently, it has been reported that mouse Ltk^- cells transfected with chicken $(Na^+ + K^+)$-ATPase β-subunit cDNA produce the immunologically measurable chicken β-subunit on the cell surface [14]. Another recent study shows that mouse $(Na^+ + K^+)$-ATPase α-subunit cDNA, introduced into monkey CV-1 cells, confers ouabain resistance [38].

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