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Functional expression of N-terminal truncated α-subunits of Na,K-ATPase in *Xenopus laevis* oocytes

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N-terminal deletion mutants of Na,K-ATPase α_1 isoforms initiating translation at Met^{34} (α_1T_1) or at Met^{43} (α_1T_2) were expressed in *X. laevis* oocytes. Compared to β_3 cRNA injected controls, the co-expression of $\alpha_1$wt, α_1T_1, α_1T_2 with β_3 subunits results in a 2- to 3-fold increase of ouabain binding sites, parallelled by a concomitant increase in Na,K-pump current. The apparent $K_{½}$ for potassium activation of the α_1T_2/β_3 Na,K-pumps is significantly higher than that of the α_1wt/β_3 or α_1T_1/β_3 Na,K-pumps expressed at the cell surface. Total deletion of the lysine-rich N-terminal domain thus allows the expression of active Na,K-pump but with distinct cation transport properties.

Na,K-pump; Ouabain binding; Potassium activation; α_1 Isoform; β_3 Isoform

1. INTRODUCTION

Na,K-ATPase is an α/β heterodimeric plasma membrane protein, responsible for the maintenance of the high K^+, low Na^+ concentrations of the intracellular milieu. All the functional sites (Na, K, ATP binding sites) appear to be located on the α subunit (for review, see [1]). The primary sequence of the α subunit and its three isoforms (α_1, α_2, α_3) has been deduced from cDNAs isolated from invertebrates and vertebrates (review in [2]). The N-terminal domain faces the cytoplasm; it diverges most among the three types of α isoforms. Interestingly, the comparison of several α_1 isoforms (human, sheep, rat, pig, chicken, frog, toad) indicates the existence of three methionines as possible translation-initiation sites characterized by a conserved Kozak consensus sequence [3]. In the purified kidney enzyme, the first methionine (Met^1) is predominantly used; the first 5 amino acids are removed posttranslationally, leaving Gly^5 as the first amino acid to be sequenced in the native enzyme [4]. A striking characteristic of the N terminus of α_1 isoform is a lysine-rich domain with an excess of positively charged amino acids. The Lys^{39} residue, according to the *X. laevis* sequence [9] represents a highly conserved tryptic site which is found in all α isoforms (for review, see [5]). It has been proposed that the N-terminal domain (Met^1 to Lys^{39}) represents a cation-selective gate, or is perhaps implicated in ion transport by the formation of a salt bridge (for review, see [5]).

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Recently, we have obtained evidence by primer extension and S1 mapping for the existence of two distinct pools of α_1 mRNA isoforms during the early development of *Xenopus laevis* embryos (Burgener-Kairuz et al., submitted). The predicted size for the full-length α_1 isoform is 3.4 kb, with a transcription initiation site at −55 bp from the ATG coding for Met^1. By contrast, the short transcript has an expected size of 3.26 kb with a transcription-initiation site located +87 bp downstream from the first ATG coding for Met^1. As shown in Fig. 1, the short transcript could initiate its translation at Met^{34}, which displays a good Kozak consensus sequence motif [3]. In order to test the possible functional significance of this observation, we have prepared deletion mutants of the α_1 N-terminal domain. The first mutant, α_1T_1 should initiate at Met^{34} and leaves the tryptic site intact. The second mutant α_1T_2 can only initiate at Met^{43}, removing the conserved tryptic site at Lys^{39}.

We measured the number of ouabain binding sites and Na,K-pump currents in *Xenopus laevis* oocytes. Our results indicate that both mutants are able to support the expression of Na,K-pumps at the cell surface. In addition, the α_1T_2 mutant co-expressed with β-subunits shows a significantly lower apparent affinity for potassium than either α_1T_1 or $\alpha_1$wt.

2. MATERIALS AND METHODS

2.1. *Deletion mutant at the N terminus of the α_1 isoform*

The near full-length α_1 cDNA (−45 to +3349 bp = $\alpha_1$wt) from *Xenopus laevis* previously cloned from an A6 kidney cell library [9] and a β_3 cDNA cloned from a *Xenopus neurula* library [7,8] were inserted into the pSD5 vector [6]. We have previously shown that both the β_1

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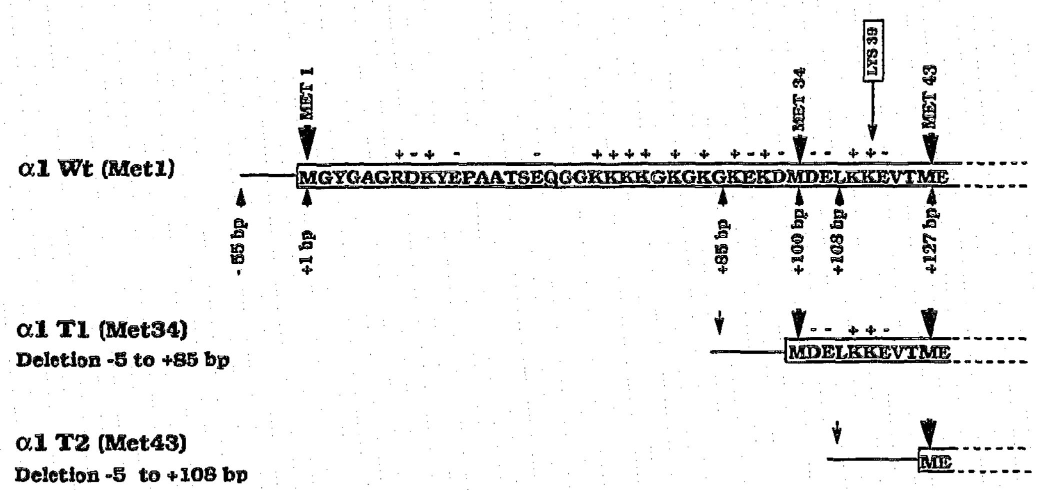

Fig. 1. N-terminal end of Na,K-ATPase α_1 isoform of *X. laevis*. The amino acid sequence deduced from cDNA cloning [9] is shown. According to Kozak's consensus sequence, translation initiates at Met[1]. There are 12 positively charged and 8 negatively charged amino acids. Lys[39] is a highly conserved tryptic site described by Jørgensen [4,5]. The α_1T_1 mutant was engineered (see section 2) so that Met[34] becomes the preferred translation-initiation site. The α_1T_2 mutant can only initiate at Met[43], according to Kozak's consensus sequence. The only other methionine before the first transmembrane domain (Met[60]) has no Kozak's consensus sequence and therefore cannot initiate translation efficiently.

and the β_3 isoforms can support the expression at the surface of an active Na,K-pump with similar functional properties [12].

Oligonucleotide primers were synthesized and purified using an Applied Biosystems DNA synthesizer, according to the methods supplied by the manufacturer. Primers were based on the sequences of the *Xenopus laevis* α_1 subunit cDNA. The primers required for PCR deletion consisted of:

(A_1) A forward deletion oligonucleotide with a 89 nucleotides deletion (−22 to +99 bp α_1T_1):

5'-CTACCACAGAAGCACCG/GGGAAGGAGAAAGAC-3'
−22 −6/+85 +99

(A_2) A forward deletion oligonucleotide (−22 to +123 bp α_1T_2), with a 112 nt deletion:

5'-CTACCACAGAAGCACCG/GCTAAAGAAGGAAGTG-3'
−22 −6/+108 +123

(B) A reverse hybrid primer downstream of a *Sma*I restriction site (+246 to +232 bp), composed of a 3' 15 nucleotide sequence complementary to the cDNA inverse strand, and a 5' 15 nucleotide unique sequence (underlined):

5'-<u>GATACTGGGCTATCC</u>GAGGGCATTGGGTCC-3'
+246 +232

(C) A forward primer upstream of the primer A and complementary to part of the SP6 promoter and the *Eco*RI restriction site in the pSD5 polylinker vector:

5'-GACACTATAGAATACACGGAATTCGAGCTCG-3'

(D) A reverse primer of identical sequence to the 5' 15 nucleotide, half of primer B. 5'-<u>GATACTGGGCTATCC</u>-3'

Mutants were obtained according to a published procedure [10]. PCR steps were carried out with Gene Amp kits (Perkin-Elmer/Cetus) in 100 μl volumes using 2.5 U of Taq polymerase for each reaction. The PCR cycles were 1 min at 94°C, 1 min at 40°C and 1 min at 72°C. The step 1 reaction contained 50 ng each of primers A1 or A2 and B and 10 ng of *Nae*I-linearized pSD5 α_1 and was cycled 20 times. The expected 194 bp deleted product of A1 step 1 and the 171 bp deleted product of A2 step 1 was used as a primer for step 2 reaction. The step 2 reaction contained 2 μl of the step 1 reaction and 10 ng of the *Nae*I-linearized pSD5 α_1 and was run three cycles of 1 min at 94°C, 15 min at 40°C and 4 min at 72°C. Primers C and D were then added (500 ng each), and 25 additional PCR cycles completed consisting of 1 min at 94°C, 1 min at 55°C and 45 sec at 72°C. The final 230-bp fragment for α_1T_1 and 207-bp for α_1T_2 were phenol-extracted, ethanol-precipitated and digested with *Eco*RI and *Sma*I. The resulting fragments were extracted from a 1% low-melt agarose gel following electrophoresis and cloned into PSD5 α_1 in place of the corresponding wild-type *Eco*RI–*Sma*I fragment. The deletions were checked by sequencing of both DNA strands.

2.2. *Oocyte injection with* Xenopus *α and β cRNA*

Stage V–VI oocytes were obtained from *Xenopus* females (African *Xenopus* Facility Noordhoek, Republic of South Africa) by removal of ovary segments from an anesthetized frog. Oocytes were defoliculated with 0.25% collagenase (type 1A, Sigma) in modified Barth's saline (MBS) without Ca^{2+}. After overnight incubation in complete MBS, oocytes were injected with either 7 ng β_3, or 10 ng $\alpha_1$wt + 7 ng β_3, or 10 ng α_1T_1 + 7 ng β_3 or 10 ng α_1T_2 + 7 ng β_3 cRNAs (in a total volume of 50 nl). cRNAs were obtained by in vitro transcription of linearized templates.

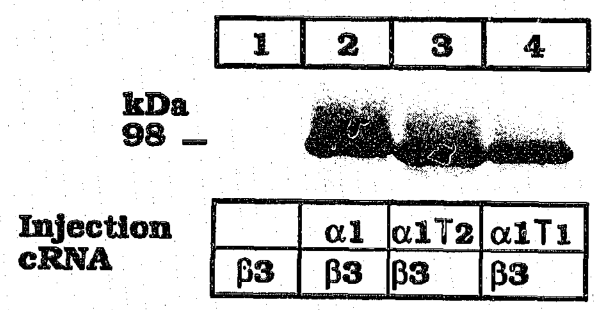

Fig. 2. Translation of $\alpha_1$wt, α_1T_1 and α_1T_2 in oocytes injected with the corresponding cRNAs and β_3 cRNA. Oocytes were labeled with [^{35}S]methionine (pulse 4 h, chase 24 h) and immunoprecipitates were analyzed on SDS-PAGE. Lane 1, β_3 cRNA alone; lane 2, $\alpha_1$wt + β_3 cRNA; lane 3, α_1T_2 + β_3 cRNA; lane 4, α_1T_1 + β_3 cRNA.

As shown in Fig. 2, the cRNAs coding for wild-type (lane 2), T_1 (lane 4), and T_2 (lane 3) mutants were equally well translated in the *Xenopus laevis* oocyte metabolically labeled by [^{35}S]methionine (4 h pulse, 48 h chase), followed by immunoprecipitation, according to a published protocol [11]. Within the resolution of SDS-PAGE, small down-shifts in apparent molecular mass are observed between $\alpha_1$wt (98 kDa), T_2 and T_1 mutants.

2.3. *Na,K-pump current measurements*

Electrophysiological measurements were performed 3 days after cRNA injection, as described previously [12]. Na,K-pump-generated currents were estimated by measuring the outward current produced by adding 10 mM K^+ to a K^+-free solution (97 mM Na^+, 0.82 mM Mg^{2+}, 0.41 mM Ca^{2+}, 90 mM gluconate, 22 mM Cl^-, 10 mM MOPS) while the membrane potential was set at −50 mV and in the presence of 5 mM barium to block currents flowing through K^+ channels. Na,K-pump current measurements were restricted to oocytes showing a total membrane conductance smaller than 5 μS, measured in the K^+-free solution.

Results are expressed as mean ± SE, and the Student's *t*-test was used to evaluate the statistical significance of differences between means.

2.4. *Ouabain binding*

Ouabain binding to oocytes was measured following the procedure of Jaunin et al. (submitted). Briefly, oocytes were loaded with Na^+ for 1 h at room temperature with a K^+-free solution 1 (110 mM NaCl, 10 mM Tris-HCl, pH 7.4) followed by a 20-min incubation with 0.28 μM [^{3}H]ouabain (Amersham, sp. act. 45 Ci/mmol) in a solution 2 containing 90 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4. Oocytes were extensively washed after incubation, individually transferred to Eppendorf tubes and solubilized with 100 μl of 5% SDS. Individual solubilized oocytes were counted after the addition of 10 ml Scintillator 299 (Packard). Non-specific ouabain binding was determined in parallel experiments by including a 1000-fold excess of cold ouabain in the reaction mixture. Non-specific binding amoun... to 3–7% of the total binding. All experimental data shown represent total ouabain binding.

3. RESULTS AND DISCUSSION

3.1. *N-terminal deletion α_1 mutants can express functional sodium pumps in* Xenopus *oocytes*

Four groups of oocytes were injected with either β_3 cRNA alone or together with $\alpha_1$wt, α_1T_1, α_1T_2 cRNAs. The mean values of K^+-induced outward current in the 4 experimental groups are shown in Fig. 3 (panel A). As shown previously [12], (Jaunin et al., unpublished observations), co-injection of $\alpha_1$wt and β_3 cRNA resulted in a 2-fold increase in the Na,K-pump current compared to the β_3 control. The first deletion mutant α_1T_1, co-injected with β_3 cRNA led to an even larger Na,K-pump current, compared to the $\alpha_1$wt β_3. The second deletion mutant α_1T_2 led to a nearly 2-fold increase in the Na,K- pump current, not significantly different from the level of expression reached by the $\alpha_1$wt. As shown in Fig. 3 (panel B), there was a parallel increase in the number of ouabain sites expressed at the cell surface, demonstrating a good relationship between induced Na,K- pump currents and ouabain binding sites. This suggests that the observed variation in Na,K-pump current expressed at the cell membrane is not due to an intrinsic change in the function of the pump but is related to variations of the expression of Na,K-ATPase at the cell surface.

3.2. *Deletion of the first 42 amino acids of α_1 leads to the expression of Na,K-pumps with a lower affinity for potassium*

Since the removal of positively and/or negatively charged amino acids could affect the transport properties of the mutated α_1 isoforms, we have measured the half activation constant ($K_{(1/2)}$) for external K^+, and compared the values obtained between the $\alpha_1$wt/β_3 to that of α_1T_1/β_3 and α_1T_2/β_3. As shown in Fig. 4, the $K_{1/2}$ for the wild-type was close to 1.7 mM, as reported previously [12]. The $K_{1/2}$ for K^+ activation of the α_1T_1 mutant was unchanged (i.e. 1.7 mM), while the $K_{1/2}$ for K^+ activation of the α_1T_2 mutant was larger (2.3 mM), a difference which was highly significant with respect to the $\alpha_1$wt and the α_1T_1 mutant. The Hill coefficients were similar in the three groups, averaging 1.58, a value in the range of those published by other investigators [13].

A change of the apparent $K_{1/2}$ could be due to any modification of the cation binding site(s). However, as

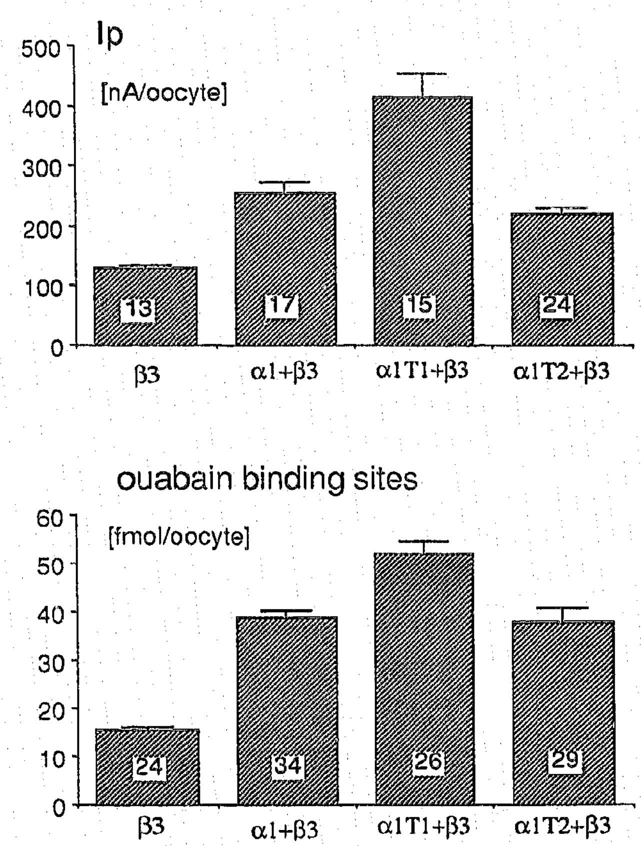

Fig. 3. Na,K-pump current (I_p, upper panel) measured as the outward current activated by increasing the K^+ concentration from 0 to 10 mM, and number of ouabain binding sites (lower panel) in four experimental groups (see text) of sodium-loaded oocytes. Co-injection of each of the 3 forms of α_1 subunit with the β_3 subunit mRNA induced a significant increase of the Na,K-pump current and the number of ouabain binding sites. The number of observations is indicated in each column. Values are means ± SE.

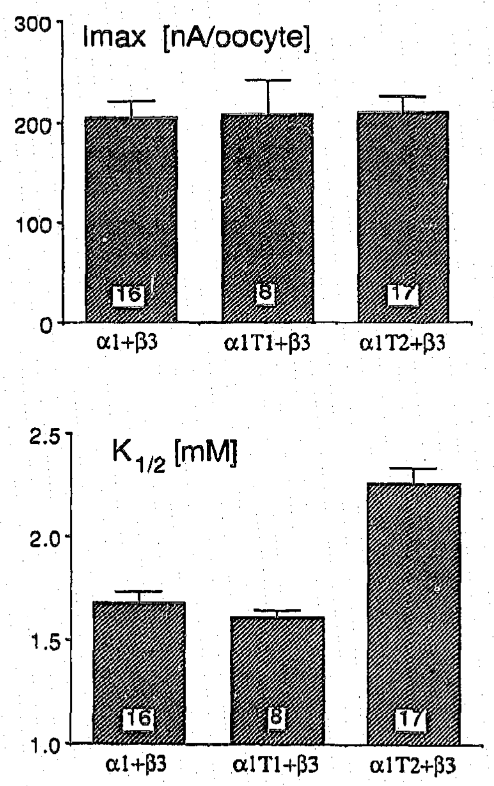

Fig. 4. Maximal Na,K-pump current (I_{max}, upper panel) and half activation constant ($K_{1/2}$, lower panel) in 3 experimental groups of oocytes. These values were obtained by fitting the observed K^+-activated currents to the Hill equation. In these experiments, I_{max} was similar in the 3 groups. However, the $K_{1/2}$ was significantly higher in the group injected with the largest truncation (α_1T_2) than in the wild-type (α_1) and the shorter truncation (α_1T_1). The Hill coefficients were similar in the 3 groups (α_1, 1.58 ± 0.02; α_1T_1, 1.54 ± 0.03; and α_1T_2, 1.61 ± 0.04 mM). The number of observations is indicated in each column. Values are means ± SE.

Rakowski et al. [14] have proposed that the K^+ binding site is positioned in the membrane's electrical field, a modification of the location of this binding site in the membrane's electrical field could also result in a change of the apparent $K_{1/2}$. Alternatively, because K^+ ions bind specifically to the E2 conformation of the enzyme, any change of the kinetics resulting in an alteration of the ratio of the E1/E2 conformations could result in a change of the apparent affinity of external K^+ ions. More work is needed to determine the precise cause of the observed modification of the $K_{1/2}$.

3.3. *Possible implication of the N terminus of the α_1 isoform in the function and the expression of Na,K-ATPase*

In the present study, we have been able to test directly the hypothesis that the highly positively charged N terminus of α_1 isoforms could modulate the function of Na,K-ATPase. Our results indicate that the deletion of this end of the molecule (up to 42 amino acids) does not prevent the assembly and the expression of functional pumps at the surface of oocytes. Interestingly, when all lysine residues were removed, a highly significant change in the $K_{1/2}$ for K^+ activation was observed. Our data do not provide any clue about the molecular mechanisms by which the changes are induced. Since the α_1T_1 mutant does not differ significantly from the $\alpha_1$wt in the functional properties tested thus far, one can tentatively conclude that Lys^{38}, Lys^{39} and possibly Asp^{35} and Glu^{36} are of special importance in determining the wild-type phenotype. This can now be directly tested by site-directed mutagenesis. The physiological relevance of our findings has yet to be established in intact cells and in vivo. However, the fact that short transcripts of α_1 isoforms (initiating at +87 bp from ATG (see Fig. 1)) have been observed during early development (Burgener-Kairuz et al., submitted) and in various tissues of the adult animal, strongly suggests that the truncated α_1 isoform could represent a Na,K-ATPase with novel functional properties in vivo. From this point of view, the regulation of gene expression of this novel α_1 isoform could also be quite distinct from that of the full-length α_1 isoform so far described, in that the α_1 gene could be under the control of at least 2 distinct promoters.

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