J. Membrane Biol. 148, 51–56 (1995)

The Journal of
Membrane
Biology

The Expression of Na^+/K^+-ATPase β-Subunit cRNA Injected into *Xenopus* Oocytes is Affected by Coinjection with α-Subunit cRNA

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Received: 20 September 1994/Revised: 27 June 1995

Abstract. The cRNA for *Torpedo californica* Na^+/K^+-ATPase β-subunit (cRNAβ) was injected into *Xenopus* oocytes alone or with the cRNA for the Na^+/K^+-ATPase α-subunit (cRNAα). When cRNAβ was injected alone, the amount of the β-subunit that accumulated in oocytes increased with increasing amounts of injected cRNAβ. When cRNAβ and cRNAα were injected simultaneously, less β-subunit accumulated than when cRNAβ was injected alone, whereas the Na^+/K^+-ATPase activity increased markedly. The decrease in the accumulation of the β-subunit was dose-dependent upon the cRNAα. The mutant β-subunit unable to assemble with the α-subunit accumulated in oocytes independently of cRNAα, suggesting that post-translational control mechanisms may serve to reduce the accumulation of the β-subunit.

Key words: Na^+/K^+-ATPase — cDNA expression — Subunit assembly — *Xenopus* oocyte

Introduction

Na^+/K^+-ATPase mediates the active transport of Na^+ and K^+ across the plasma membrane of animal cells (Jørgensen & Andersen, 1988; Geering, 1990; Lingrel et al., 1990; Horisberger et al., 1991; Vasilets & Schwarz, 1993). The minimum functional unit of the enzyme is a protomer composed of a catalytic α- and a glycosylated β-subunit. The α-subunit contains sites for ATP hydrolysis, ion binding and the specific inhibitor, ouabain. On the other hand, the role(s) of the β-subunit in the catalytic function remains unknown. There is increasing experimental evidence that the β-subunit plays an important role in the biogenesis of Na^+/K^+-ATPase. The β-subunit assembles with, and thereby stabilizes, the newly synthesized α-subunit (Tamkun & Fambrough, 1986; Noguchi et al., 1987; Geering et al., 1989; Horowitz et al., 1990). Only the α-subunit assembled with the β-subunit can be targeted to the plasma membrane, where Na^+/K^+-ATPase mediates active transport (Takeyasu et al., 1988; Zamofing, Rossier & Geering, 1988; Ackermann & Geering, 1990; Noguchi, Higashi & Kawamura, 1990*b*). Therefore, the β-subunit is indispensable for the α-subunit to be correctly inserted into the membrane and to exhibit its functional properties on the plasma membrane. While investigating αβ assembly using *Xenopus* oocytes into which cRNAs for each of the subunits were injected (Noguchi, Higashi & Kawamura, 1990*b*), we found that the expression of the β-subunit was reduced by coinjection with the α-subunit. In this study, the effect of the α-subunit on the expression of the β-subunit was investigated by injecting cRNAβ alone or with cRNAα into *Xenopus* oocytes. The results indicated that the β-subunit was downregulated when injected with cRNAα, whereas the α-subunit was rather stabilized by coinjection with the β-subunit.

Materials and Methods

Construction of Deletion Mutants

The plasmids pSPTα and pSPTβ, containing the cDNA encoding the α- and β-subunit, respectively, of Na^+/K^+-ATPase of *Torpedo californica* electroplax were as described (Noguchi et al., 1987). We constructed the deletion mutants pSPTβΔ1 and pSPTβΔ2 lacking the coding region of the β-subunit from Tyr244 to the C-terminus and from Asp165 to Lys290, respectively, as follows. A 1901-bp *Hae*III (–35)/

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*Pvu*II fragment was excised from pNKAβ1 (Noguchi et al., 1986) and purified. For pSPTβΔ1, the 1901-bp fragment was further digested with *Dra*I and *Sca*I, the resulting 765-bp *Hae*III (−35)/*Sca*I (731) and 146-bp *Dra*I (1721)/*Pvu*II fragments were purified and ligated with *Sma*I-cleaved pSP65. For pSPTβΔ2, the 1901-bp fragment was further digested with *Sau*3AI. The resulting 157-bp *Hae*III (−35)/*Sau*3AI (123), 996-bp *Sau*3AI (871)/*Pvu*II fragments were purified and inserted into the *Sma*I site of pSP65. The construction of these plasmids was verified by restriction endonuclease analysis. Each cRNA was synthesized *in vitro* from the respective plasmid which had been linearized by digestion with *Sal*I using SP6 RNA polymerase.

Expression in *Xenopus* Oocytes

The synthesized cRNAs were injected into *Xenopus laevis* oocytes (stages IV and V). Unless otherwise specified, the concentration of each cRNA was 0.5 μg/μl and about 20 nl was injected per oocyte (Noguchi et al., 1987). The oocytes were incubated at 19°C for 3 days in modified Barth's medium containing 100 μg/ml each of ampicillin, streptomycin and cefmenoxime and 15 μg/ml nystatin. Translation products were labeled with either L-[U-^{14}C]leucine (310 mCi/mmol; final concentration, 0.15 mCi/ml) or L-[4,5-^{3}H]leucine (142 Ci/mmol; final concentration, 0.3 mCi/ml). The former was used when the translation products were quantified by using Fujix BAS 2000 (Fuji film). Immunoprecipitation and fluorography were performed as according to standard procedures (Noguchi, Higashi & Kawamura, 1990*b*).

Membrane Preparation and Assay of ATPase Activity

About 400 oocytes for each preparation were homogenized in 6 ml of 250 mM sucrose, containing 50 mM imidazole-HCl buffer (pH 7.5) and 1 mM EDTA (solution A). The homogenate was centrifuged at 7,000 × *g* for 10 min on a 50% sucrose cushion (1 ml); then the supernatant was recovered and further centrifuged at 160,000 × *g* for 30 min. The resulting pellet was suspended and incubated at 20°C for 1 hr 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 the ouabain-insensitive ATPase activity (Noguchi et al., 1987). The suspension was then centrifuged at 160,000 × *g* for 30 min. The pellet was washed twice with solution A and suspended in 0.4 ml solution A. The ATPase activity was then assayed at 37°C in a reaction mixture (0.2 ml) containing (in mM): 50 imidazole-HCl buffer (pH 7.5), 140 NaCl, 14 KCl, 5 $MgCl_2$, 1 ATP and microsomes (20–80 μg protein) in the presence or absence of 1 ouabain. Na^+/K^+-ATPase activity was obtained by subtracting the ATPase activity measured in the presence of ouabain from that measured in its absence.

Materials

Enzymes used for the construction of mutants and *in vitro* transcription were purchased from TaKaRa, Nippon Gene and Toyobo. Antisera raised against the α- and β-subunit of *T. californica* Na^+/K^+-ATPase have been described (Noguchi et al., 1987). Other chemicals were obtained from Nacalai Tesque and were of reagent or higher grade.

Results

Xenopus oocytes, into which varying amounts of cRNA for the β-subunit (cRNAβ) of *T. californica* Na^+/K^+-ATPase alone had been injected, were incubated for 3 days in the presence of [^{14}C]leucine, and Triton extracts were immunoprecipitated with anti-β-subunit antiserum. The fluorograms are shown in Fig. 1*A*. Two major radioactive polypeptides were detected: fully (βm) and core-glycosylated (βc) forms of the β-subunit having molecular masses of about 60 and 41 kDa, respectively. The amounts of both β-subunits were quantified using a Fujix BAS 2000. The expression of both subunits increased with increasing amounts of injected cRNAβ (Fig. 1*B*).

As reported (Noguchi, Higashi & Kawamura, 1990*b*) and shown in Fig. 4*A* (lane 1) when the cRNA for the α-subunit (cRNAα) was injected alone, the α-subunit was unstable and the low levels of α-subunit accumulated in the oocytes. However, when cRNAα and cRNAβ were injected simultaneously, the expression level of the α-subunit was markedly increased (Fig. 4*A*, lane 2). As Noguchi et al. (1990*a*) have shown, the improvement is due to the β-subunit that assembles with and thereby stabilizes the newly synthesized α-subunit. Otherwise the α-subunit is degraded soon after the synthesis.

On the contrary, the expression level of the β-subunit in oocytes decreased after the coinjection of cRNAα with cRNAβ. As shown in Fig. 2, when cRNAα was injected into oocytes together with cRNAβ at a molar ratio of 1:1, the accumulation of the β-subunit decreased by about 50% from that in the absence of cRNAα at both concentrations of cRNAβ tested (6.0 and 30 ng/oocyte). However, the Na^+/K^+-ATPase activity notably increased after coinjection of cRNAβ with cRNAα. (The Na^+/K^+-ATPase activity found in oocytes injected with cRNAβ alone could be due to exogeneous β-subunit forming complexes with endogenous α-subunit originally stored in oocytes (Verrey et al., 1989).)

The decrease in β-subunit accumulation was studied by injecting varying amounts of cRNAα at a fixed amount of injected cRNAβ. The results are shown in Fig. 3. The levels of the β-subunits, both βc and βm, decreased with increasing amounts of injected cRNAα, whereas the accumulation of the α-subunit and Na^+/K^+-ATPase activity increased.

One possible explanation for the decreased expression of the β-subunit in oocytes after coinjection with cRNAα was that cRNAα competed with cRNAβ for translation machinery such as the ribosomes of *Xenopus* oocytes. If the affinity of cRNAα for the machinery is higher than that of cRNAβ, the synthesis of the β-subunit in oocytes could be reduced by coinjection with cRNAα, leading to the decreased expression of the β-subunit. To exclude this possibility, we constructed deletion mutants in the COOH-terminal domain of the β-subunit. The COOH-terminal domain of the β-subunit is involved in the assembly with the α-subunit and hence a structural disturbance in the COOH-terminal domain results in a

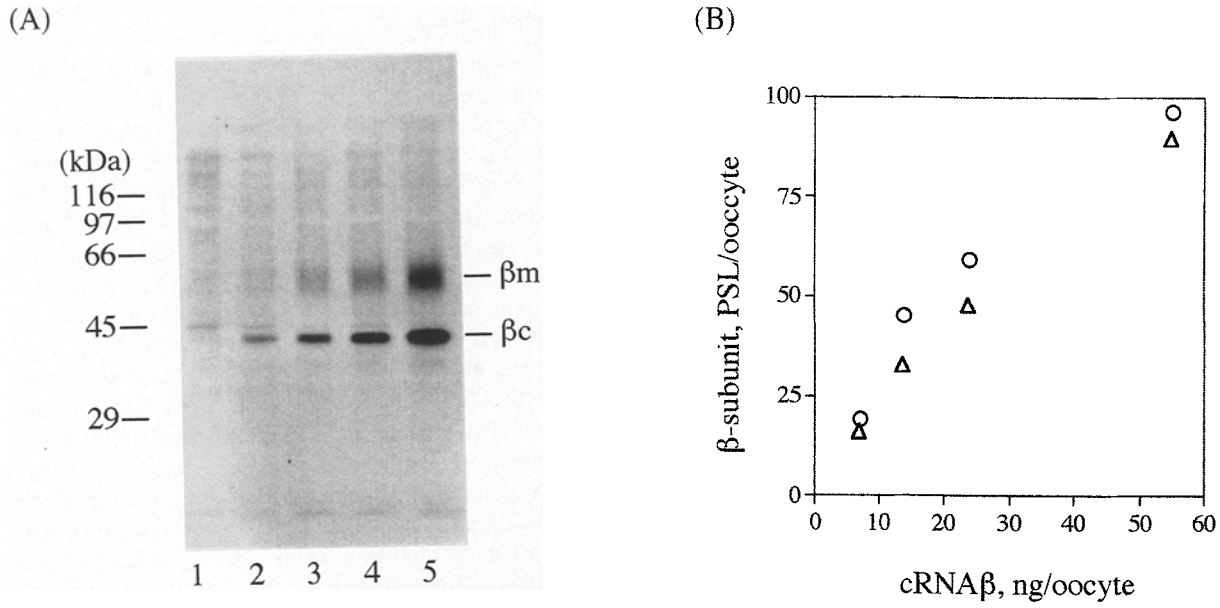

Fig. 1. The β-subunit accumulated in *Xenopus* oocytes injected with varying amounts of cRNAβ. Oocytes were injected with cRNAβ (lane 1, none; lanes 2–5, 7.0, 14, 24 and 55 ng/oocyte) and incubated at 19°C for 3 days in the presence of [^{14}C]leucine. The oocytes were homogenized in the presence of 1% Triton X-100, and the translation products were immunoprecipitated with anti-β-subunit antiserum. The immunoprecipitates were resolved by SDS-gel electrophoresis and visualized by fluorography. In (*A*), a fluorogram is shown where βm and βc represent fully glycosylated (mature) and core-glycosylated β-subunits, respectively. In (*B*), the amounts of βm (○) and βc (△) of the injected oocytes were quantified using Fujix BAS 2000. PSL is a unit defined by the manufacture of the analyzing system and is proportional to the level of radioactivity.

molecule that does not assemble with the α-subunit (Beggah et al., 1993; Noguchi, Mutoh & Kawamura, 1994). We speculated that the expression of the mutant cRNAβ would not be affected by cRNAα, if post-translational processes such as αβ assembly were responsible for the decreased level of the β-subunit.

A scheme of the mutants that we constructed are shown in Fig. 4*B*. Plasmid pSPTβΔ1 lacked the sequence encoding the COOH-terminal 62 amino acid residues in addition to a large part of the 3′ noncoding sequence. Plasmid pSPTβΔ2 lacked the sequence within the coding region corresponding to Asp165-Lys290.

The cRNAs for the mutants were injected into oocytes together with cRNAα. Triton extracts from the cRNA-injected oocytes incubated in the presence of [^{3}H]leucine, were immunoprecipitated with a mixture of anti-*Torpedo* α- and anti-*Torpedo* β-subunit antisera, and resolved by electrophoresis and visualized by fluorography. The fluorograms are shown in Fig. 4*A*. The major translation products of pSPTβΔ1 and pSPTβΔ2 were assigned core-glycosylated forms (βΔ1c and βΔ2c, respectively) from their molecular masses. The bands around 60 kDa on lanes 4 and 5 are probably due to βΔ1c dimer. The band of the α-subunit in oocytes injected with cRNAα plus cRNAβΔ1 (lane 4) or cRNAβΔ2 (lane 6) was much more faint than that in oocytes injected with cRNAα plus wild-type cRNAβ (lane 2) and showed similar intensity to that of oocytes injected with cRNAα alone (lane 1). Moreover, the α-subunit was not copre-

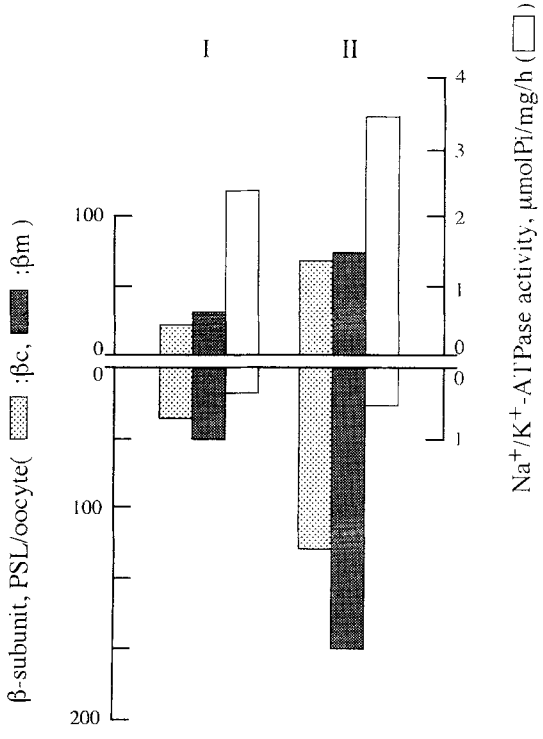

Fig. 2. Expression of the β-subunit decreased upon coinjection with cRNAα Alone (lower panel), or in combination with cRNAα (upper panel), cRNAβ was injected into oocytes. The amounts of injected cRNAβ were 6.0 (I) and 30 ng/oocyte (II) at a fixed molar ratio of cRNAα:cRNAβ = 1:1 when cRNAα was injected.

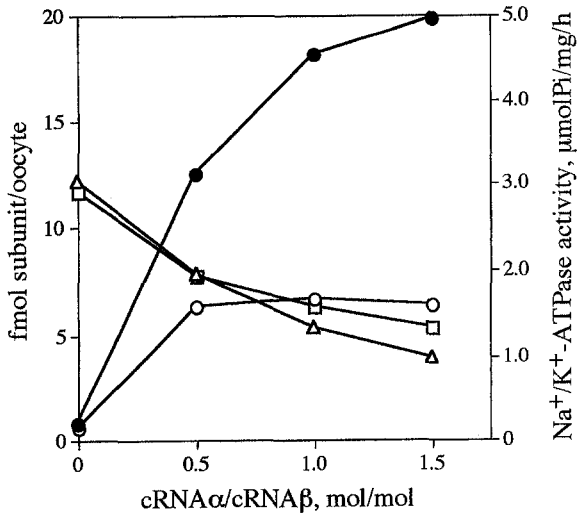

Fig. 3. Effect of cRNAα on the expression of the β-subunit. Varying amounts of cRNAα were injected into oocytes together with cRNAβ the concentration of which was kept constant (10 ng/oocyte). The amounts of the α-(○) and β-subunits (△: βc, □: βm) were quantified as described in the legend to Fig. 1 and expressed in fmol/oocyte. The Na^+/K^+-ATPase activity of microsomes (●) was assayed under standard condition.

cipitated with βΔ1 or βΔ2 using antiserum against the β-subunit (*data not shown*). These results confirmed that the mutants of the β-subunit with deletions in the COOH-terminal domain including βΔ1 and βΔ2, could not assemble with the α-subunit.

As shown in Fig. 4*A*, when these mutant β-subunit cRNAs were injected into oocytes, cRNAβΔ1 and cRNAβΔ2 were expressed independent of coinjected cRNAα and more efficiently than the wild-type β-subunit (lanes 4–7). An equal expression of all β-subunits was attempted by injecting reduced amounts of mutant cRNAs. The results are shown in lanes 8–11 and were essentially the same as those in lanes 4–7. From these results, we concluded that the expression of the mutant cRNAβ was not influenced by coinjection with cRNAα.

Discussion

The role of the β-subunit in the catalytic cycle of Na^+/K^+-ATPase is unclear, although the β-subunit must be coexpressed and assembled with the α-subunit to form a functional enzyme (Noguchi et al., 1987). The β-subunit stabilizes the nascent α-subunit leading to the formation of an active αβ assembly, otherwise the α-subunit is degraded soon after the synthesis (Noguchi, Higashi & Kawamura, 1990*a*). We examined whether or not the expression of the β-subunit was affected by the α-subunit in the biogenesis of the enzyme. We injected cRNAβ alone or with cRNAα into *Xenopus* oocytes and assayed the levels of the translation products. When cRNAβ was injected alone, the levels of not only βc, but also βm, increased linearly with increasing amount of cRNAβ. Since the βm is the fully glycosylated population of the β-subunit that has passed through Golgi apparatus to plasma membranes, we concluded that the β-subunit can be transported to plasma membranes independently of the α-subunit. This is in contrast to the conclusions of Ackermann and Geering (1990). They have claimed that the α- and β-subunits depend on each other for transport out of the endoplasmic reticulum based upon the results of pulse-chase labeling experiments using the *Xenopus* oocyte expression system. This discrepancy may be due to the difference in species from which cRNAβ is derived: ours was from *Torpedo* and theirs from *Xenopus laevis*. Indeed, Schmalzing, Kröner and Gloor (1993) have recently found in the *Xenopus* oocyte system, that the apparent transport incompetence of the β-subunit is a peculiarity of the homologous (*Xenopus*) β-subunit and that heterologous β-subunits such as that from *Torpedo* is efficiently routed to plasma membranes without the α-subunit.

The accumulation of the β-subunit in oocytes decreased upon coinjection with cRNAα as shown in Figs. 2 and 3. The mutant β-subunit unable to assemble with the α-subunit was accumulated independently of the α-subunit (Fig. 4). The stability of cRNAβ was not significantly affected by coinjection with cRNAα (*data not shown*). These results suggest that the decrease in the accumulation of the β-subunit occurs post-translationally. One possible explanation is that the overexpression of functional αβ assembly in plasma membranes might downregulate the formation of the assembly by degrading the stabilizer of the α-subunit, that is, the β-subunit. If so, the accumulation of the β-subunit in oocytes could be decreased by coinjection with cRNAα. The β-subunit may regulate, through αβ assembly, the number of pump molecules transported to plasma membranes. The β-subunit may upregulate and downregulate the number of pump molecules by stabilizing the nascent α-subunit and by being destroyed, respectively. The other possibility is that the direct interactions of nascent or newly synthesized α-subunit with nascent β-subunit regulate translation of the β-subunit so that both subunits may be synthesized stoichiometrically (Fambrough et al., 1994), otherwise β-subunit is overexpressed. As shown in Fig. 3, synthesis of βc and βm decreased gradually as increasing amounts of cRNAα coinjected with cRNAβ, and sum of βc and βm approached to the level of the α-subunit. (When immunoprecipitated with antiserum to the α-subunit, nonspecific bands were frequently found within a broad band of βm. The amount of βm in Fig. 3 was, therefore, overestimated.) Through this stoichiometric synthesis, the α- and β-subunits may be up- and downregulated, respectively. The mutant β-subunits that lack the ability to interact with the α-subunit support this possibility.

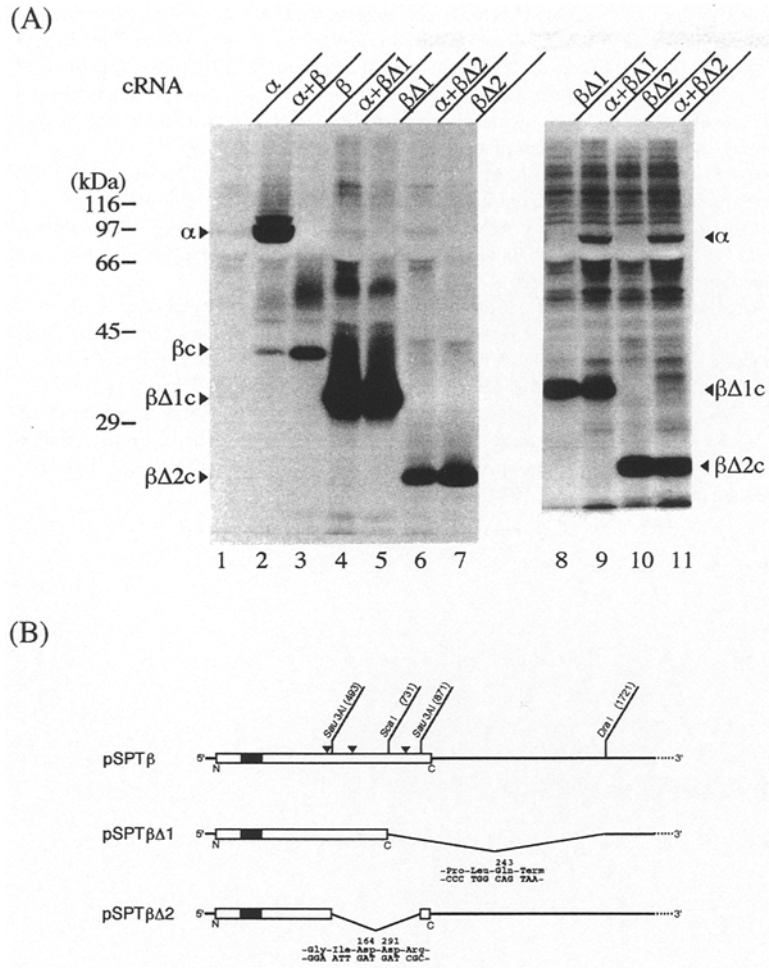

Fig. 4. Translation products in *Xenopus* oocytes injected with cRNAα and mutant cRNAβ. In (*A*), oocytes were injected with wild-type cRNAβ (10 ng/oocyte, lanes 2 and 3), a deletion mutant of βΔ1 (10 ng/oocyte, lanes 4 and 5), and βΔ2 (10 ng/oocyte, lanes 6 and 7) with or without cRNAα (10 ng/oocyte) and incubated at 19°C for 3 days. As a control, cRNAα alone was injected (lane 1). In order to see an equal expression of all β-subunits, reduced amounts of mutant cRNAβ were injected. The cRNAβΔ1 (2.0 ng/oocyte, lanes 8 and 9) and βΔ2 (7.0 ng/oocyte, lanes 10 and 11) were injected with or without cRNAα (10 ng/oocyte). Triton extracts were immunoprecipitated with a mixture of antisera against *Torpedo* α- and β-subunits. In (*B*), construction of the deletion mutants, pSPTβΔ1 and pSPTβΔ2, from the *Torpedo* Na^+/K^+-ATPase β-subunit gene in the plasmid pSPTβ are shown. (▼) and (■) represent potential glycosylation sites and a transmembrane segment, respectively.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 05259226, No. 06454149).

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