

The Ca^{2+} /calmodulin binding domain of the Ca^{2+} -ATPase linked to the Na^+ , K^+ -ATPase alters transport stoichiometry

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Abstract Using *Xenopus* oocytes as an expression system, we have investigated ion-transport and ouabain-binding properties of a chimeric ATPase (α_1 -CBD; Ishii and Takeyasu (1995) *EMBO J.* 14, 58–67) formed by the α_1 -subunit of chicken Na^+ , K^+ -ATPase (α_1) and the calmodulin binding domain (CBD) of the rat plasma membrane Ca^{2+} -ATPase. α_1 -CBD can be expressed and transported to the oocyte plasma membrane without the β -subunit, and shows ouabain binding. In contrast to ouabain binding, this chimera requires the β -subunit for its cation (Na^+ and K^+) transport activity. α_1 -CBD exhibits an altered stoichiometry of Na^+ - K^+ exchange. A detailed analysis of $^{22}\text{Na}^+$ efflux, $^{86}\text{Rb}^+$ uptake, pump current and ouabain binding suggests that the chimeric molecule can operate in an electrically silent 2Na^+ - 2K^+ exchange mode and, with much lower probability, in its normal 3Na^+ - 2K^+ exchange mode.

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1. Introduction

The Na^+ , K^+ -ATPase and the plasma membrane Ca^{2+} -ATPase belong to P-type ATPases. A common characteristic of these cation transporting ATPases is that they become transiently phosphorylated at an aspartic acid residue. During the transport cycle, the chemical energy of hydrolysis of ATP is converted into gradients of the electrochemical potential of the transported ions. The Ca^{2+} -ATPase is formed by a single polypeptide of about 130 kDa, differs from other P-type ATPases by having an extra C-terminal domain of about 150 amino acid residues. A sequence corresponding to the calmodulin binding site has been identified within this domain [1]. It was shown that the calmodulin binding domain (CBD) interacts with other regions of the pump molecule. Without calmodulin bound this interaction leads to self-inhibition of the pump. Two polypeptide segments have been suggested as possible candidates for the site of interaction with the CBD [2,3].

In contrast to the Ca^{2+} -ATPase, the Na^+ , K^+ -ATPase is a

heterodimer composed of an about 110 kDa α -subunit and a glycosylated β -subunit. Though the α -subunit carries all functional sites, the β -subunit is essential for stabilising the conformation of the α -subunit and for proper insertion of the enzyme complex into the plasma membrane [4–7]. The enzyme can exist in two conformations, an E_1 conformation with the cation binding sites accessible from the cytoplasm, and an E_2 conformation with binding sites oriented to the external medium. During the normal transport cycle, three cytoplasmic Na^+ ions bind to the E_1 -ATP form. This promotes phosphorylation of the enzyme, and leads to occlusion of the Na^+ ions. After a subsequent conformational change to E_2 , the Na^+ ions will be released to the external medium. Thereafter, two external K^+ ions become bound instead of the Na^+ ions. This induces dephosphorylation, and the K^+ ions become occluded. After a conformational change to E_1 , stimulated by ATP binding, the K^+ ions will be released internally, and the transport cycle is completed.

Chimeric molecules (α_1 -CBD) formed by the α_1 -subunit of chicken Na^+ , K^+ -ATPase and the C-terminal fragment of 165-amino-acid residues of the rat plasma membrane Ca^{2+} -ATPase II have been shown to exhibit ATPase activity [8]. These authors used for this chimeric subunit the symbol NNN-CBS. The ATPase activity is Na^+ - and K^+ -dependent, and can be detected only in the presence of Ca^{2+} /calmodulin. This was taken as an additional evidence that the C-terminus interacts with regions that are conserved in the Ca^{2+} - and Na^+ , K^+ -ATPases. Based on comparison of amino-acid sequence, the authors conclude that several regions of the α -subunit form the binding site for the CBD. Interaction with these regions blocks ATPase activity also of the chimera, and block is released in the presence of Ca^{2+} /calmodulin. The activity of the ATPase is, nevertheless, inhibited by ouabain. Also the affinity to the β -subunit of α_1 -CBD is reduced, which opens the question whether the calmodulin-binding domain can substitute for the β -subunit and whether α_1 -CBD exhibits an ATPase activity.

Here we show that the chimeric molecule can be expressed in *Xenopus* oocytes both with and without the β -subunit of the Na^+ pump of *Torpedo* electroplax; the presence of β -subunit is not necessary for ouabain binding but is necessary for transport activity. We also demonstrate that the chimeric ATPase transports Na^+ and K^+ across the cell membrane, and the modified C-terminus leads to an altered Na^+ , K^+ stoichiometry. Part of the results has been published previously [9,10].

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2. Materials and methods

2.1. Oocytes

Females of the clawed toad *Xenopus laevis* were anaesthetised with *m*-aminobenzoic acid ethylester methane sulfonate (MS222, Sandoz, Basel, Switzerland; 1 g/l). Parts of the ovary were removed and treated with collagenase (15 mg/ml) at 19°C for 10 h. Full-grown prophase-arrested oocytes were selected for experiments. For expression of pumps, oocytes were injected with cRNA for the chimeric α -subunits α_1 -CBD (10–15 ng/oocyte) either without or together with cRNA for the β -subunit of the Na^+, K^+ -ATPase of *Torpedo* electroplax (5–10 ng/oocyte). These cells and non-injected control oocytes were stored at 19°C in oocyte Ringer's solution (ORI, see Section 2.5) containing antibiotics (in mg/l: 70 gentamycin, or 30 streptomycin plus 30 penicillin). Experiments were performed after 3–5 days of incubation. Before measurements, oocytes were loaded with Na^+ by incubating the cells in K^+ -free solution ('loading solution') for 40 min. Under these conditions ouabain-binding is accelerated [11] and transport activity at 5 mM external K^+ is maximised.

2.2. Measurements of ouabain binding and $^{86}\text{Rb}^+$ uptake

The number of ouabain binding sites, which is equal to the number of pump molecules per oocytes, was determined by incubating Na^+ -loaded oocytes in K^+ -free solution containing 2.5 μM cold ouabain and 2.5 μM [^3H]ouabain (see [12]). For determination of maximum pump activity by measurements of $^{86}\text{Rb}^+$ uptake, Na^+ -loaded oocytes were incubated in Na^+ -free test solution containing 5 mM Rb^+ (925 kBq/ml) and inhibitors of $\text{K}^+(\text{Rb}^+)$ -selective channels (see Section 2.5) for 12 min. Under the Na^+ -free, 5 mM $\text{K}^+(\text{Rb}^+)$ conditions, pump activity is nearly voltage-independent [13–15], eliminating possible effects of differences or changes in membrane potential on transport activity.

2.3. Voltage-clamp experiments

During the normal transport cycle of the Na^+, K^+ -ATPase, three Na^+ ions are transported out of the cell and two K^+ ions into the cell. As a result of the $3\text{Na}^+-2\text{K}^+$ stoichiometry, the pump generates a current that can be determined in voltage-clamp experiments. Membrane currents were measured at a holding potential of $V_H = -60$ mV. Pump-specific current could be isolated from total membrane current as the current component activated by external K^+ under the condition that all other K^+ -sensitive currents are blocked, or as the current component blocked by specific inhibitors of the Na^+, K^+ -ATPase, like the cardiac glycoside ouabain. Over a wide range of potentials, the currents determined by these two assays are identical [13].

2.4. Na^+ -efflux under voltage clamp

To estimate the rate of Na^+ efflux mediated by the Na^+, K^+ -ATPase, oocytes were microinjected with 20–50 nl of $^{22}\text{NaCl}$ (≈ 100 MBq/ml). An oocyte is then placed in a perfusion chamber mounted on a Geiger-Müller tube and continuously superfused with radioactivity-free solution (Fig. 2). Loss of radioactivity due to efflux of the ^{22}Na was monitored under voltage clamp [16] at $V_H = -60$ mV. The efflux of radioactivity from an oocyte with chimeric pumps obeyed under constant conditions Eq. 1, where y_0 and y are the radioactivity in the oocyte at $t=0$ and t , respectively:

$$y = y_0 e^{-kt} \quad (1)$$

From the rate constant of efflux (k), the number of Na^+ ions transported per second across the cell membrane (I_{Na} , in nC/s) was calculated according to Eq. 2:

$$I_{\text{Na}} = F k [\text{Na}_i] v \quad (2)$$

where $[\text{Na}_i]$ represents the intracellular Na^+ concentration. The volume of the oocyte, v , was assumed to be 0.5 μl , the volume of the aqueous phase of the cytoplasm. During an experiment lasting about 1.5 h $[\text{Na}_i]$ decreases from a value of about 100 mM by 10–20% (compare [15]). The final concentration is still sufficient for maximum activation of the pump by internal Na^+ . The pump-generated flux component was determined as the difference of efflux rates obtained in solutions with and without extracellular ouabain (1 mM) or K^+ (5 mM).

2.5. Solutions

The solutions had the following compositions (in mM):

<i>ORI</i>	90 NaCl, 2 KCl, 2 CaCl_2 , 5 MOPS (adjusted to pH 7.2)
<i>loading solution</i>	110 NaCl, 2.5 sodium citrate, 5 MOPS (adjusted to pH 7.6)
<i>test solution</i>	100 tetramethylammonium (TMA^+) chloride, zero or 5 KCl, 5 MOPS (adjusted to pH 7.8), 20 tetraethylammonium (TEA^+) chloride, 10 BaCl_2

All experiments were performed in Na^+ -free solution. In the nominally Na^+ - and K^+ -free solutions, their concentrations were below 5 μM as determined by flame photometry. TMA^+ was used for iso-osmolar substitution of Na^+ . For activation of the pump 5 mM KCl (or RbCl) were added. To block other K^+ -sensitive pathways, solutions contained Ba^{2+} and TEA^+ . To reduce background currents mediated by Ca^{2+} -activated channels (Cl^- channels), no Ca^{2+} was added to the test solutions.

3. Results

To further evaluate the effect of the CBD on the chimeric pump molecule, we performed measurements of [^3H]ouabain binding, which characterises the extent of incorporation of pump molecules into the plasma membrane, and measured pump-mediated ^{86}Rb uptake, ^{22}Na efflux and current, which characterise transport.

3.1. Measurements of ouabain binding

For detection of ouabain binding, oocytes were incubated for 20 min in K^+ -free solution in the presence of 5 μM ouabain (2.5 μM [^3H]ouabain). Fig. 1A shows the results for non-injected control oocytes, and for oocytes injected with cRNA for only the chimeric α -subunit and for the chimeric α -subunit together with the β -subunit ($\text{T}\beta$) of *Torpedo*. The overall average of ouabain-binding sites of non-injected control oocytes was 3.6×10^9 molecules per oocyte. The presented data were normalised to the average control values for each batch of cells. The normalised number of ouabain-binding molecules in injected oocytes is by a factor of about 3.6 higher than in non-injected oocytes (α_1 -CBD: 3.71 ± 0.52 ; α_1 -CBD/ $\text{T}\beta$: 3.54 ± 0.36). For comparison, oocytes from the same batches were also injected with cRNA for wild-type α - and β -subunit of the Na^+, K^+ pump of *Torpedo*, which increased the number of ouabain binding sites by a factor of 3.53 ± 0.28 ($n=16$). Thus, regardless of the presence of β -subunit, ouabain-binding chimeric molecules are inserted into the oocyte membrane to the same extent as wild-type *Torpedo* pumps.

3.2. Measurements of ^{86}Rb uptake and pump current

To test whether functional ATPases are expressed, measurements of pump-mediated ^{86}Rb uptake and of electrogenic current were performed on the same batches of oocytes that were used for ouabain binding (Fig. 1B,C). The data were normalised to the average control values for each batch of oocytes. Fig. 1B shows for illustration also unspecific $^{86}\text{Rb}^+$ uptake measured in presence of 100 μM ouabain (downwardly directed, filled bar). The results demonstrate that the chimeric α_1 -CBD can accomplish Rb^+ uptake only when cRNA for the β -subunit was co-injected. The Rb^+ uptake for α_1 -CBD/ $\text{T}\beta$ is by a factor of 3.54 ± 0.42 larger than in non-injected oocytes. This factor is identical to that obtained for ouabain binding, and suggests that the Rb^+ uptake per pump molecule is the same as for the endogenous *Xenopus* pump. The measure-

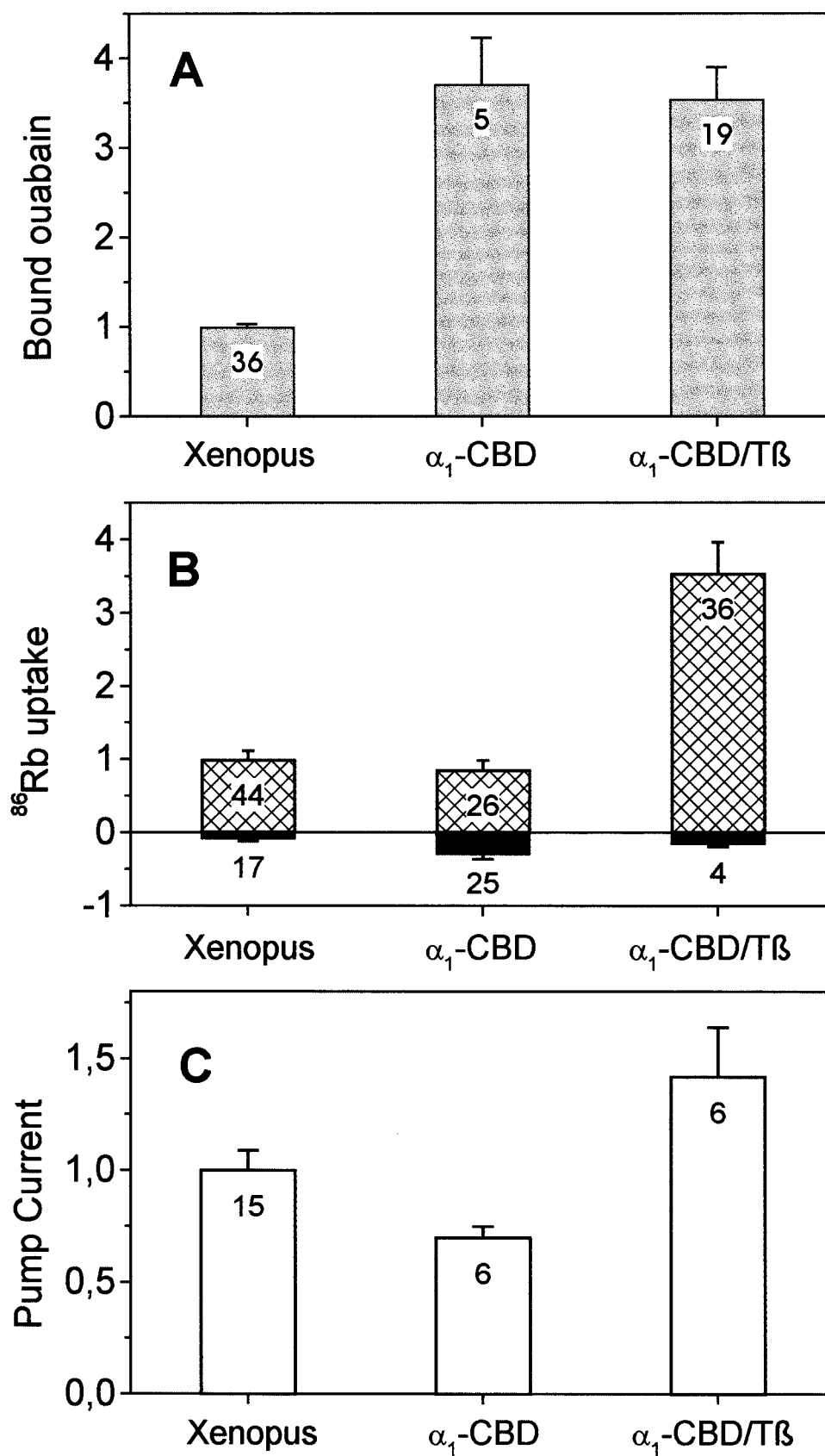


Fig. 1. Ouabain binding (A), and pump-mediated $^{86}\text{Rb}^+$ uptake (B) and current (C) for non-injected control oocytes (*Xenopus*), for oocytes injected with cRNA for the chimeric α -subunit without (α_1 -CBD) and with (α_1 CBD/T β) the β -subunit of *Torpedo* electroplax. In B, the hatched bars represent total uptake of $^{86}\text{Rb}^+$; filled downward-directed bars unspecific uptake determined in the presence of 100 μM ouabain. All data ($\pm$ SEM) were normalised to those of the control oocytes (275.6 ± 9.6 dpm, 8341 ± 473 dpm, and 43 ± 4 nA for bound ouabain, $^{86}\text{Rb}^+$ uptake, and pump current, respectively). Numbers within or below bars give numbers of oocytes.

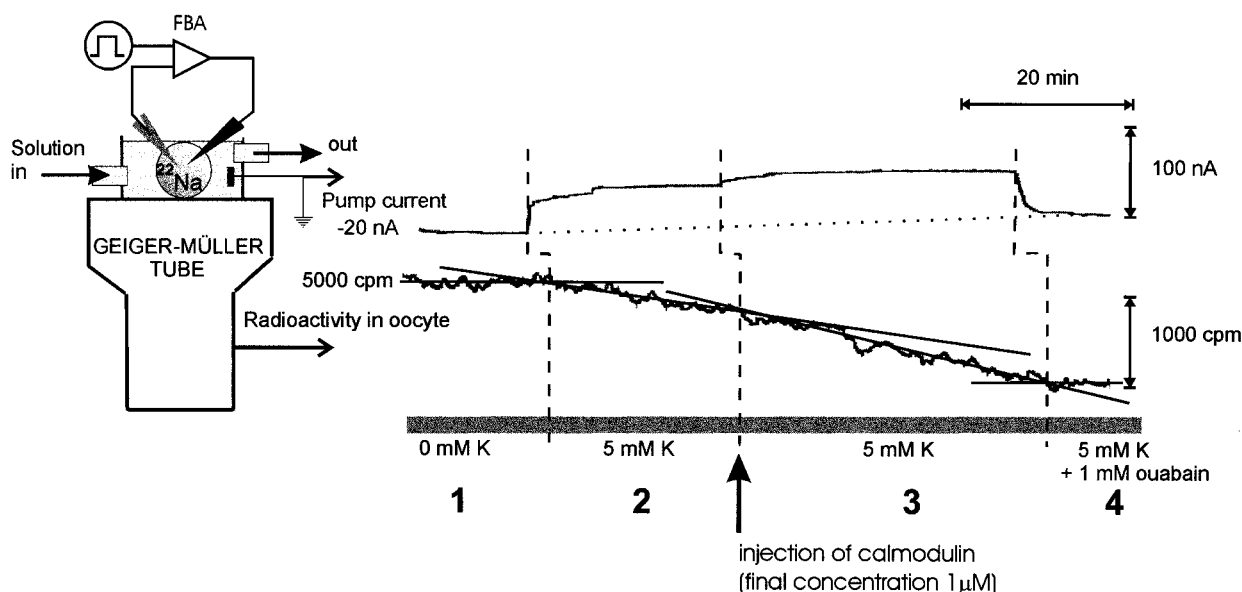


Fig. 2. Simultaneous measurements of $^{22}\text{Na}^+$ efflux and pump current. An oocyte injected with $^{22}\text{Na}^+$ is placed into a chamber, the bottom of which is formed by the mica window of a Geiger-Müller tube (right part). The decline of radioactivity in the oocyte was recorded (left part, lower trace) and fitted piece-wise to the sections indicated by numbers 1–4 by Eq. 1. The fitted k values are proportional to the efflux, the respective rates are (in 10^{-6} s^{-1}) $k_1 \approx k_4 \approx 0$, $k_2 = 60$, $k_3 = 92$. Simultaneously the respective membrane current (upper trace) was recorded at a fixed holding potential ($V_H = -60 \text{ mV}$). Average values of this type of experiments are listed in Table 1.

ments of pump current (Fig. 1C) also illustrate that extra electrogenic current exceeding the endogenous contribution is only produced if cRNA for α_1 -CBD and T β is injected; moreover, injection of cRNA for only the α_1 -CBD subunit even leads to a slight reduction in both $^{86}\text{Rb}^+$ uptake (to 0.86 ± 0.13) and pump current (to 0.70 ± 0.05). Interestingly, when cRNA for the β -subunit was co-injected, the pump current increased only by a factor of 1.42 ± 0.22 .

3.3. Measurements of $^{22}\text{Na}^+$ release and pump current

The discrepancy between the increase factor of 1.5 for pump current and of 3.5 for ouabain binding and Rb^+ uptake was further investigated by measurements of release of $^{22}\text{Na}^+$ from the oocyte monitored under voltage clamp. An example of this kind of experiments is depicted in Fig. 2. The pen recording illustrates that 5 mM external K^+ stimulates release of $^{22}\text{Na}^+$ and generation of pump current. Both signals are completely blocked by ouabain. Microinjection of calmodulin (final concentration 1 μM) amplifies rate of efflux and pump current, illustrating calmodulin sensitivity of the pumps. The fact that transport can be detected even without extra injection of calmodulin suggests that the oocytes contain endogenous calmodulin, though at a non-saturating concentration. In oocytes not injected with RNA, application of 5 mM K^+ produced a current signal of about 25 nA, but the corresponding loss of $^{22}\text{Na}^+$ was too small to be detectable within the time intervals employed (data not shown, but see [16]). There-

fore, the detected outward transport of $^{22}\text{Na}^+$ is predominantly mediated by the newly expressed chimeric pumps. Table 1 summarises the result of the efflux experiments. After subtraction of the contribution of the endogenous pumps, an electrogenic signal of about 20 nA remains that can be attributed to the chimeric pump and can be increased by calmodulin injection to about 25 nA. Injection of calmodulin into control oocytes did not produce a change in pump current (not illustrated). From these data, the number of Na^+ ions and the number of net charges transported by the chimeric pump out of the cell can be calculated; the values for the ratio of transported ions (I_{Na}) to chimera-mediated pump current ($I_{\text{p}}^{\text{chim}} = I_{\text{p}} - I_{\text{p}}^{\text{control}}$) is about 15 (15.6 and 14.1 for calmodulin-injected and non-injected oocytes, respectively).

4. Discussion

The experiments described in Section 3 were performed to determine whether the β -subunit is as essential for the chimeric α_1 -CBD subunit as it is for wild-type α -subunits of Na^+ , K^+ -ATPases, and to look for possible effects of the calmodulin-binding domain (CBD) on transport activity.

It has been demonstrated previously [8] that α_1 -CBD associates with the β -subunit, though with lower affinity than the wild-type, to form an active Na^+ - and K^+ -dependent ATPase, and that its activity can be detected only in the presence of Ca^{2+} and calmodulin. Our data on ouabain binding show that

Table 1
Comparison of rate of $^{22}\text{Na}^+$ efflux (k) and pump current I_{p}

Type of oocyte	k (10^{-6} s^{-1})	I_{Na} (nA)	I_{p} (nA)
Control	not detectable	—	25 ± 4
α_1 -CBD/T β -injected without calmodulin	53 ± 18	254	43 ± 3
α_1 -CBD/T β -injected with calmodulin	106 ± 25	407	51 ± 4

I_{Na} was calculated to Eq. 2.

α_1 -CBD is expressed in the oocytes both with and without β -subunit. Since ouabain is applied to the external medium, it can be concluded that in both cases α_1 -CBD molecules are incorporated into the plasma membrane in a conformation that allows ouabain to bind. Obviously, the calmodulin binding domain can at least in part mimic functions of the β -subunit, i.e. stabilise the α -subunit in a conformation that allows transfer to the plasma membrane and ouabain binding. This is a surprising finding since it is believed that an extracellular domain of the β -subunit is essential for the assembly with the α -subunit and that cytoplasmic or transmembrane regions of the β -subunit play a minor role (see e.g. [17]). CBD, on the other hand, was believed to interact with cytoplasmic regions of the α -subunit of the Na^+, K^+ -ATPase [8]. The chimeric molecules with the cytoplasmic C-terminal CBD but without β -subunit, nevertheless, bind ouabain and are inserted into the oocyte membrane with about the same efficiency as wild-type Na^+, K^+ -ATPase. Noteworthy, sequence comparison between the attached domain and the β -subunit yields no similarities.

The question then arises whether the β -subunit is needed at all for the chimeric ATPase. The measurements of $^{86}\text{Rb}^+$ uptake demonstrate that a functional transporting ATPase is only formed when cRNA for the β -subunit is co-injected. Without β -subunit, Rb^+ uptake even seems to be slightly reduced compared to non-injected oocytes. The increase in Rb^+ uptake in oocytes with α_1 -CBD/T β is identical to the increase in the number of pump molecules; since wild-type Na^+, K^+ -ATPases exhibit a fixed $3\text{Na}^+/2\text{K}^+$ stoichiometry under a large variety of ionic conditions [14,16,18,19], this parallel increase can be taken as evidence that the chimeric α_1 -CBD/T β pump transports, like wild-type pumps, $2\text{K}^+(\text{Rb}^+)$ ions into the cell per pump cycle.

Since it was demonstrated that assembly of an α -subunit with different β -subunits results in different transport characteristics [20–24], one may also expect that the C-terminal CBD of the chimera influences the ATPase. Whereas Rb^+ uptake increased by a factor of 3.5 compared to control oocytes, the pump current increased by a factor of only 1.5, suggesting that the $3\text{Na}^+/2\text{K}^+$ stoichiometry is not maintained and that the modified C-terminus leads to a reduction in the number of extruded Na^+ ions. For further evaluation, simultaneous measurements of Na^+ efflux and pump current were performed. In previous experiments we demonstrated that the ratio of the number of outwardly moved Na^+ ions to outwardly moved net charges ($I_{\text{Na}}/I_{\text{p}}$) is 2.9 for the endogenous *Xenopus* Na^+, K^+ -ATPase (calculated from data of Schwarz and Gu [16]) and 2.7 for the *Torpedo* Na^+, K^+ -ATPase [14], in complete accordance with the $3\text{Na}^+/2\text{K}^+$ stoichiometry. By contrast, chimeric α_1 -CBD/T β exhibits a value of about 15. The simplest explanation for this finding would be that the pump can turnover when two Na^+ ion are bound to the sites accessible from the cytoplasm. In this case, the pump would operate in an electrically silent $2\text{Na}^+/2\text{K}^+$ exchange mode. If 3Na^+ ions can also bind with low probability, the extra Na^+ ion could give a small electrogenic contribution to the overall activity. To account for an ion/charge ratio of 15, the pump

would have to operate with a probability of 0.14 in this $3\text{Na}^+/2\text{K}^+$ exchange mode.

In conclusion, CBD linked to the α -subunit of the Na^+, K^+ -ATPase not only confers Ca^{2+} /calmodulin dependence to the ATPase but also mimics part of the stabilising function of the β -subunit and dramatically alters the interaction with Na^+ . Interestingly, this alteration in cation interaction occurs without any other mutation within the α -subunit.

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