Coexpression of α_1 with putative β_3 subunits results in functional Na^+/K^+ pumps in *Xenopus* oocytes

(α_1 isoform/ β_1 isoform/ Na^+/K^+ -ATPase/electrogenic transport/voltage dependence)

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ABSTRACT The active Na^+/K^+ pump is composed of an α and a β subunit. Until now, three putative isoforms of the β subunit have been identified that share sequence similarity. We have expressed the β_1 and β_3 isoforms of *Xenopus laevis* Na^+/K^+ -ATPase in *Xenopus* oocytes to compare functional properties of the Na^+/K^+ pump, including either of these two isoforms. Na^+/K^+ pump current, estimated as K^+ -induced outward current in voltage-clamped oocytes, was doubled by coexpression of α_1 subunits with either isoform of the β subunit compared to expression of α_1 subunits alone. The kinetics of activation by external K^+ and the voltage dependence of the electrogenic activity of the Na^+/K^+ pump were similar with both β isoforms, indicating that both β_1 and β_3 isoforms can support expression at the oocyte surface of an active Na^+/K^+ pump with similar functional properties.

The Na^+/K^+ pump (Na^+/K^+ -ATPase) is a ubiquitous transport system in vertebrate plasma membranes, responsible for maintaining the high- K^+ , low- Na^+ concentrations of the intracellular milieu. Although all the functional sites (Na^+ -, K^+ -, and ATP-binding sites) are located on the larger α subunit, the active enzyme always includes a smaller glycosylated β subunit (for review, see ref. 1). One function of this β subunit is to support structural and functional maturation of the α subunit and allow its transport to the cell membrane (for review, see ref. 2). The first identified β isoform, β_1 , has been described in several species and is the β subunit of the Na^+/K^+ -ATPase isolated from kidney as well as other tissues (for review, see ref. 3). In addition, a putative β_2 subunit has been identified by sequence similarity to the β_1 subunit and is preferentially expressed in the nervous system (4). This isoform is nearly identical with the adhesion molecule on glia cloned from mouse brain (5). The β_2 isoform has been copurified with the α_2 subunit of rat brain Na^+/K^+ -ATPase (6). More recently, Good *et al.* (7) have isolated in *Xenopus laevis* a cDNA encoding a protein showing significant homology with both β_1 and β_2 isoforms. This cDNA was taken from a group of transcripts expressed during early development and in the adult nervous system (8). Although no other β isoform similar to rat β_2 or mouse adhesion molecule on glia has been identified in *Xenopus*, this isoform of the β subunit of *Xenopus* Na^+/K^+ -ATPase has been named β_3 because of important size and sequence dissimilarities with known mammalian β_2 isoforms (7). Structure similarities with the β_1 subunit and preliminary evidence showing association with the α subunit (9) suggest that β_3 isoform might also support the Na^+/K^+ -ATPase activity of an α subunit, but a functional role for this protein has not yet been demonstrated.

The purpose of our study was to determine whether this putative β_3 subunit isoform could form, with an α subunit, a competent and functional Na^+/K^+ pump at the plasma membrane. We took advantage of the electrogenic activity of the Na^+/K^+ pump (10) to study the function of Na^+/K^+ pump as a K^+ -activated or strophanthidin-sensitive current in voltage-clamped *Xenopus* oocytes previously injected with cRNAs coding for α and β subunits. A similar approach has already been used to study the functional properties of Na^+/K^+ -ATPase by injecting heterologous cRNA (*Torpedo* cRNA in *Xenopus* oocytes) (11).

MATERIALS AND METHODS

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 removing ovary segments from an anesthetized frog. Oocytes were defolliculated with 0.25% collagenase (type 1A; Sigma) in modified Barth's saline without Ca^{2+} (12). After overnight incubation in complete modified Barth's solution, oocytes were injected with either 10 ng of α_1 , or 10 ng of α_1 plus 7 ng of β_1 , or 10 ng of α_1 plus 7 ng of β_3 cRNA (in a total 50-nl volume). cRNA were obtained by *in vitro* transcription of linearized templates according to Melton *et al.* (13). For templates, cDNAs from α_1 and β_1 subunits cloned from *Xenopus* kidney-derived A6 cells (14) or from β_3 cloned from a *Xenopus* neurula library (8) were recloned into the plasmid pSD5, which allows for synthesis of capped, full-length, poly(A)⁺ RNA (15).

Na^+/K^+ Pump Current Measurements. Electrophysiological measurements were done 2–3 days after cRNA injection. To increase the Na^+/K^+ pump activity and to decrease variation of this activity, the oocytes were first loaded with Na^+ by a 2-hr exposure to K^+ - and Ca^{2+} -free solution (16). Whole-cell currents were then measured with the two-electrode voltage-clamp technique (electrode resistance 1–3 M Ω); a Dagan 8500 clamp instrument (Dagan Instruments, Minneapolis) was used. For determination of current–voltage (*I*–*V*) curves, data were recorded and analyzed with the PClamp system (Axon Instruments, Burlingame, CA). All experiments were done at room temperature (22–25°C).

Na^+/K^+ pump-generated current was estimated by measuring the outward current produced by adding 10 mM K^+ to a K^+ -free solution, while the membrane potential was set at –50 mV and 5 mM barium was present to block currents flowing through K^+ channels. Na^+/K^+ pump current measurements were restricted to oocytes showing a total membrane conductance <5 μS , measured in K^+ -free solution. Voltage dependence of the Na^+/K^+ pump activity was determined by establishing the whole-cell *I*–*V* curve in the

presence of 10 mM K^+ before and after addition of 25 μ M strophanthidin. Starting from a holding membrane potential of -60 mV, rectangular voltage pulses (400 ms) of various amplitude were applied every 2 s, and steady-state current was measured at 300 ms after start of the voltage step.

The kinetics of activation by external K^+ was studied by measuring the current induced by successive addition of 0.3, 1.0, 3.0, and 10 mM K^+ (in replacement of Na^+). The Na^+/K^+ pump current values obtained in each oocyte were fitted to the Hill equation:

$$I = I_{\max} / [1 + (K_{1/2}/K_o)^n],$$

where I is the measured current at each external K^+ concentration (K_o), $I_{\max}$ is the maximal current, $K_{1/2}$ is the half-activation constant, and n is the Hill coefficient. Best-fitting parameters were obtained by a nonlinear method with the Simplex algorithm (17).

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

Solutions and Drugs. All solutions had a low-chloride concentration to prevent swelling of depolarized oocytes and to decrease leak currents. The composition of the control solution was as follows: 97 mM Na^+ , 0.0 mM K^+ , 5 mM Ba^{2+} , 0.82 mM Mg^{2+} , 0.41 mM Ca^{2+} , 90 mM gluconate, 22.5 mM Cl^- , 2.4 mM HCO_3^- , and 10 mM Mops $^-$ /Mops-H. The sodium loading solution was similar, except that no Ba^{2+} and no Ca^{2+} were added, and 0.5 mM EGTA was added. The high- K^+ solution contained 10.0 mM K^+ instead of sodium. The pH of all solutions was set to 7.2. Strophanthidin (Sigma) was added from a 100 mM stock solution in dimethyl sulfoxide.

RESULTS

K^+ -Induced Currents. We first established the validity of using the K^+ -induced current to measure Na^+/K^+ pump-generated current. We used Ba^{2+} , a known blocker of several types of K^+ channels (18), to abolish K^+ currents through conductive pathways. In the presence of 5 mM Ba^{2+} and at a holding potential of -50 mV, increasing extracellular K^+ from 0 to 10 mM induced a large outward current in *Xenopus* oocytes (in contrast to the inward current expected for K^+ conductance). This current could be inhibited by 25 μ M strophanthidin, a specific inhibitor of Na^+/K^+ -ATPase (Fig. 1). Changing K^+ concentration in the presence of 25 μ M strophanthidin only induced negligible current changes: -0.2

FIG. 1. Effect of K^+ and strophanthidin on whole-oocyte current (I), at -50 -mV holding potential. This tracing was recorded with an α_1 and β_1 cRNA-injected oocyte; Ba^{2+} (5 mM) was present throughout the experiment. Starting from a K^+ -free solution, K^+ (10 mM) was added and then removed. There was a large (170 nA) outward K^+ -induced current; this K^+ effect was completely reversible. Then, in the presence of 10 mM K^+ , 25 μ M strophanthidin was added, which abolished the K^+ -induced current. Removing or adding K^+ in the presence of strophanthidin had very little effect. The current signal was filtered at 20 Hz.

± 1.2 nA (range, -4.3 to $+5.2$ nA; $n = 8$). This result indicates that K^+ conductance was efficiently blocked by Ba^{2+} at this holding potential. Thus, K^+ -induced current is a valid measurement of Na^+/K^+ pump current under our experimental conditions.

Next, to look for functional pumps containing the β_3 subunit, we measured K^+ -induced current in *Xenopus* oocytes injected with either α_1 , α_1 plus β_1 , or α_1 plus β_3 cRNAs. In *Xenopus* oocytes, the rate of functional Na^+/K^+ pump synthesis is limited by the availability of newly synthesized β subunits (19). Indeed, injection of β_1 cRNA alone does increase the number of ouabain-binding sites (19). To ensure that the amount of newly synthesized Na^+/K^+ pumps after β cRNA injection would not be limited by the amount of α subunit, we coinjected in this study all oocytes with cRNA coding for the α_1 subunit. In addition, the group injected with the α_1 cRNA alone provided an appropriate control group because expression of α subunits alone does not result in the formation of functional pumps (20, 21).

Fig. 2 shows mean values of K^+ -induced outward current in the three experimental groups. Injection of both β_1 and β_3 cRNAs together with α_1 cRNA induced a large, >2 -fold increase in Na^+/K^+ pump current compared with injection of α_1 cRNA alone. Amplitudes of the current increases induced by β_1 and β_3 isoforms were similar.

The mean membrane conductance, measured in K^+ -free solution, was similar in the three groups: α_1 , 1.1 ± 0.3 ($n = 14$); α_1 plus β_1 , 1.3 ± 0.2 ($n = 15$); and α_1 plus β_3 , 1.5 ± 0.4 μ S ($n = 13$). This result confirmed that the large differences in current induced by changes of K^+ recorded in the three groups cannot be explained by differences in the size of leak conductances.

Voltage Dependence of the Na^+/K^+ Pump Current. In an attempt to detect functional differences between the Na^+/K^+ pump assembled with β_1 or β_3 isoforms, respectively, voltage dependence of the strophanthidin-sensitive current in the α_1 plus β_1 and α_1 plus β_3 groups was studied in oocytes selected for a large Na^+/K^+ pump current and stable electrode impalements with high-input resistance. Fig. 3 shows original recordings of the whole-cell current (in an oocyte from the α_1 plus β_3 group) before and after addition of 25 μ M strophanthidin and the resulting I - V curves. The general shape of the I - V curve of the strophanthidin-sensitive current was similar to that seen under similar conditions in noninjected *Xenopus* oocytes (22) or in other cell types (23, 24)—e.g., the Na^+/K^+ pump current was strongly voltage dependent at high negative membrane potentials, but it saturated around 0 mV. Fig. 4 compares the voltage dependence of the Na^+/K^+ pump current in α_1 plus β_1 and α_1 plus β_3 cRNA-injected oocytes.

FIG. 2. K^+ -induced currents. Mean values ($\pm$ SE) of the change of whole-oocyte current (I), at -50 -mV holding potential, induced by increasing K^+ concentration from 0.0 to 10.0 mM in the presence of 5 mM Ba^{2+} . The three groups of oocytes had been injected with α_1 ($n = 14$), α_1 plus β_1 ($n = 15$), and α_1 plus β_3 ($n = 13$) cRNAs of the Na^+/K^+ -ATPase subunits. $\star$, $P < 0.001$ when compared with α_1 group.

FIG. 3. (Upper) Original recordings of whole-oocyte current measured in a solution containing 10 mM K^+ before (10 K) and 1 min after addition of 25 μM strophanthidin (10 K + S). This oocyte had been injected with α_1 and β_3 cRNA. Current (I) was recorded during a series of voltage-clamp steps (400-ms duration, 2-s interval) to potentials from +20 to -140 mV, starting from a holding potential of -60 mV. The current signal was filtered at 20 Hz but has not been corrected for capacitive transients. Whole-cell current (I) and membrane potential (V_m) were both measured at 300 ms after start of the voltage step to establish I - V curves shown below. (Lower) The curve of strophanthidin-sensitive current ($\bullet$), obtained by subtraction of current before (Δ) and 1 min after ($\square$) strophanthidin addition, shows the voltage dependence of the Na^+/K^+ pump-generated current.

The current values are reported as fraction of the current at 0 mV. There were no obvious differences in the voltage dependence of the Na^+/K^+ pump electrogenic activity between the two groups. The shape of the I - V relation of the Na^+/K^+ pump current in the control group (α_1 cRNA injected) was similar (data not shown).

K^+ Activation. To evaluate another functional property of the Na^+/K^+ pump in oocytes injected with α_1 plus β_1 or α_1 plus β_3 cRNA, we have measured the half-activation constant ($K_{1/2}$) of external K^+ . An original recording of current during exposure to increased K^+ concentrations and the corresponding concentration-response relation are shown in Fig. 5. Mean values of the maximal current $K_{1/2}$ and Hill coefficient obtained by fitting to the Hill equation are shown in Table 1. These variables did not differ significantly between the two groups. The $K_{1/2}$ and Hill coefficient values are in the ranges reported earlier in a variety of tissues (25).

DISCUSSION

We have demonstrated that injection of α_1 and β_3 isoform cRNAs into *Xenopus* oocytes increases the K^+ -induced current, indicative of an increase in Na^+/K^+ pump activity.

FIG. 4. I - V behavior of Na^+/K^+ pump in β_1 and β_3 groups. The strophanthidin-sensitive current (I_p), normalized to the current at 0 mV, is plotted against membrane voltage (V_m). Mean absolute values of the strophanthidin-sensitive current at 0 mV were similar: 199 ± 18 nA ($n = 6$) and 210 ± 7 nA ($n = 5$) for β_1 and β_3 groups, respectively. Vertical bars indicate SE; for several points, SE values are smaller than sizes of the symbols.

Under our experimental conditions, this K^+ -induced current is similar to the strophanthidin-sensitive current (Fig. 1). This increased K^+ -induced current is similar to that seen in oocytes injected with the α_1 and β_1 isoform cRNA. Previously, we have shown (i) that injection of β_1 cRNA alone increases the number of ouabain-binding sites due to association with endogenous α subunits (19) and (ii) that injection of α_1 and β_1 cRNA causes the correct synthesis and post-translational processing of α_1 - β_1 proteins (20). In addition, injection of α_1 and β_3 cRNAs leads to correct synthesis of both subunits, including the proper maturation of the α_1 subunit, a step required for enzyme function (9). Therefore, we suggest that this additional Na^+/K^+ pump activity results

FIG. 5. (A) Original recording of whole-oocyte current (α_1 plus β_1 cRNA-injected oocyte) when it was exposed to 0.0, 0.3, 1.0, 3.0, 10 mM K^+ and 10 mM K^+ plus 20 μM strophanthidin. Membrane potential was set to -50 mV. (B) Logarithm of K^+ concentration-response of K^+ -induced Na^+/K^+ pump current. Solid line is drawn from Hill equation, $I = I_{max}/[1 + (K_{1/2}/[K^+])^n]$, with the best-fitting parameters, in this case: $I_{max} = 189$ nA, $K_{1/2} = 1.72$ mM, and $n = 1.58$.

Table 1. Kinetic parameters of activation of Na⁺/K⁺ pump current by external K⁺

	$I_{\max}$, nA	$K_{1/2}$, mM	Hill coefficient	n
$\alpha_1 + \beta_1$	157 ± 12	1.59 ± 0.05	1.56 ± 0.03	14
$\alpha_1 + \beta_3$	179 ± 21	1.69 ± 0.06	1.63 ± 0.03	15

Maximal current ($I_{\max}$), half-activation constant ($K_{1/2}$), and Hill coefficient were obtained by fitting the current activated by increased K⁺ concentrations to the Hill equation. Differences between the two groups are not statistically significant. n , number of observations.

from the formation of an active α_1 - β_3 complex, synthesized after α_1 and β_3 cRNA injections.

Several additional ideas could explain the increased K⁺-induced current, but they appear improbable. The amplitude and the direction of the K⁺-induced current exclude trivial explanations, such as alterations of passive K⁺ conductive pathways. In principle, the large increase in Na⁺/K⁺ pump rheogenic activity could be either from an increase in the number of active Na⁺/K⁺ pumps at the plasma membrane or from a change of the activity of a constant number of pump units. However, the second hypothesis appears improbable, as current was measured under near-saturating conditions (high intracellular Na⁺ and high extracellular K⁺). Also, injection of β_3 cRNA could stimulate the synthesis of α_1 - β_1 enzyme complexes from endogenous maternal RNA pools. Such an effect seems unlikely because *Xenopus* oocytes and early embryos contain maternal mRNA for both β_1 and β_3 isoforms (7), and we have previously shown that injection of β_3 cRNA causes synthesis of β_3 protein without any detectable increase of synthesis of β_1 protein (9).

No functional difference could be found between voltage dependence of the electrogenic activity of the Na⁺/K⁺ pump containing either the β_1 or the β_3 isoform. Considering the >2-fold increase in amplitude of pump current induced by β cRNA injection, at least half of the strophanthidin-sensitive current was from Na⁺/K⁺ pumps containing the β subunit expressed from the injected cRNA. The similarity of the I - V curves indicated that the voltage dependence of the Na⁺/K⁺ pump activity was similar in α_1 - β_1 and α_1 - β_3 pumps. The Na⁺/K⁺ stoichiometry of these two isoforms of the Na⁺/K⁺ pump is most probably similar, as voltage dependence of an electrogenic ion pump is expected to depend on the number of charges moved across the membrane electrical field during a transport cycle (26). Similarly, we were unable to detect any difference in the kinetics of the activation by outside K⁺ in two groups of oocytes in which a large increase of the Na⁺/K⁺ pump current had been induced by α_1 plus β_1 or α_1 plus β_3 cRNA injection.

The absence of detectable physiological differences between α_1 plus β_1 and α_1 plus β_3 isoforms suggests that the differences between β -subunit isoforms concern domains not directly involved in the ion-translocating activity of the Na⁺/K⁺ pump. However, many other functional properties (Na⁺ and ATP-binding kinetics, for instance) remain to be studied.

In conclusion, we show here that a putative β_3 isoform can support expression of active Na⁺/K⁺ pumps at the plasma membrane. The transport properties of the Na⁺/K⁺ pump containing this β_3 isoform appear similar to that containing the β_1 subunit.

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