

Modulation of the Na,K-Pump Function by β Subunit Isoforms

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ABSTRACT To study the role of the Na,K-ATPase β subunit in the ion transport activity, we have coexpressed the *Bufo* $\alpha 1$ subunit ($\alpha 1$) with three different isoforms of β subunits, the *Bufo* Na,K-ATPase $\beta 1$ ($\beta 1\text{NaK}$) or $\beta 3$ ($\beta 3\text{NaK}$) subunit or the β subunit of the rabbit gastric H,K-ATPase (βHK), by cRNA injection in *Xenopus* oocyte. We studied the K^+ activation kinetics by measuring the Na,K-pump current induced by external K^+ under voltage clamp conditions. The endogenous oocyte Na,K-ATPase was selectively inhibited, taking advantage of the large difference in ouabain sensitivity between *Xenopus* and *Bufo* Na,K pumps. The K^+ half-activation constant ($K_{1/2}$) was higher in the $\alpha 1\beta 3\text{NaK}$ than in the $\alpha 1\beta 1\text{NaK}$ groups in the presence of external Na^+ , but there was no significant difference in the absence of external Na^+ . Association of $\alpha 1$ and βHK subunits produced active Na,K pumps with a much lower apparent affinity for K^+ both in the presence and in the absence of external Na^+ . The voltage dependence of the $K_{1/2}$ for external K^+ was similar with the three β subunits. Our results indicate that the β subunit has a significant influence on the ion transport activity of the Na,K pump. The small structural differences between the $\beta 1\text{NaK}$ and $\beta 3\text{NaK}$ subunits results in a difference of the apparent affinity for K^+ that is measurable only in the presence of external Na^+ , and thus appears not to be directly related to the K^+ binding site. In contrast, association of an $\alpha 1$ subunit with a βHK subunit results in a Na,K pump in which the K^+ binding or translocating mechanisms are altered since the apparent affinity for external K^+ is affected even in the absence of external Na^+ .

INTRODUCTION

The Na,K pump is composed of an $\alpha\beta$ heterodimer. The two subunits are assembled soon after synthesis in the endoplasmic reticulum (Geering, 1990). Although neither hydrolysis of adenosine triphosphate (ATP) nor ion transport function by an isolated subunit has ever been demonstrated, the α subunit has been called the catalytic subunit because it includes the binding site for ATP (Pedemonte and Kaplan, 1990) and the catalytic phosphorylation site (Ohtsubo, Noguchi, Takeda, Morohashi, and Kawamura, 1990). In addition, several lines of evidence (site-directed mutagenesis, covalent binding of ouabain analogues) clearly indicate that the binding site of

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ouabain, the Na,K pump-specific inhibitor, is primarily located on the α subunit; for review see Horisberger, Lemas, Kraehenbühl, and Rossier (1991c). The β subunit has a major role in the maturation and the translocation of the Na,K-pump protein from the endoplasmic reticulum (ER) to the plasma membrane; for review see Geering (1991). However, the role of this β subunit, once the Na,K pump is in its mature and active form at the plasma membrane, is poorly understood.

The structure of the α subunit (with 8–10 putative transmembrane segments) and the existence of other ion-motive P-ATPases that do not include a β subunit make it probable that the α subunit forms the binding sites and the pathway for the transported cations. However, the possibility of a contribution of the β subunit (that includes only one transmembrane segment) is still open. A few recent reports point to this possible role (Eakle, Kim, Kabalin, and Farley, 1992; Schmalzing, Kröner, Schachner, and Gloor, 1992; Jaisser, Canessa, Horisberger, and Rossier, 1992a; Jaisser, Horisberger, and Rossier, 1992b; Lutsenko and Kaplan, 1992). In addition, selective chemical modifications of the β subunit of the gastric H,K-ATPase have been shown to entail loss of function of the holoenzyme (Chow, Browning, and Forte, 1992).

We have reported that the *Xenopus* β 3NaK subunit, as well as the β 1NaK isoform, could assemble with the α 1 subunit to form a functional Na,K pump at the plasma membrane of *Xenopus* oocytes but we were not able to detect any significant physiological differences between the α 1 β 1NaK and α 1 β 3NaK complexes (Horisberger, Jaunin, Good, Rossier, and Geering, 1991a). However, this approach was not powerful enough to detect small physiological differences between Na,K-pump isoforms expressed by cRNA injection, because the noise of the endogenous oocyte Na,K pump was added to the signal of the exogenous Na,K pump. More recently, using the *Xenopus* and *Bufo* α and β subunits, we could show a consistent difference in the apparent affinity for external K^+ between Na,K pumps including a β subunit of the β 1 and β 3 isotype. In the present paper, we have extended this work by studying the kinetics of the activation by external K^+ of Na,K pumps composed of an α subunit and either one of the two known amphibian isoforms of the β subunit, β 1NaK or β 3NaK (Verrey, Kairouz, Schaerer, Fuentes, Geering, Rossier, and Kraehenbühl, 1989; Good, Richter, and Dawid, 1990; Jaisser et al., 1992a), or the β subunit of the most closely related P-ATPase, the stomach H-K-ATPase; for review see Wallmark, Lorentzon, and Sachs (1990). In addition, the effects of the membrane potential and of the presence or absence of external Na^+ on apparent K^+ affinity of all these isoforms was studied. The α subunit of the *Bufo marinus* Na-K-ATPase was chosen because it is well expressed in *Xenopus* oocytes and it confers a relative resistance to ouabain, allowing the study of the function of the artificially expressed *Bufo* Na,K pump after selective inhibition of the endogenous *Xenopus* Na,K pump (Jaisser et al., 1992a,b).

METHODS

Expression of α and β Subunits cRNAs in Xenopus Oocytes

Synthetic cRNAs coding for the α 1 subunit and β 1NaK and β 3NaK subunits of the Na,K-ATPase of *Bufo marinus* (Jaisser et al., 1992a), were prepared from cDNAs subcloned in pSD

vectors containing a 110-bp poly-A tail (Good, Welch, Barkan, Somasekhar, and Mertz, 1988). Similarly cRNA coding for the β subunit of the rabbit gastric H,K-ATPase (Reuben, Lasater, and Sachs, 1990) were prepared. Capped cRNAs were synthesized using SP6 RNA polymerase (Promega Corp., Madison, WI) as described earlier (Melton, Krieg, Rebagliati, Maniatis, Zinn, and Green, 1984; Horisberger et al., 1991a).

Stage V–VI *Xenopus* oocytes were obtained as previously described (Horisberger et al., 1991a) and were injected with 10 ng of Na,K-ATPase α subunit cRNA and 1 ng of Na,K-ATPase β subunit, or 4 ng of H,K-ATPase β subunit cRNA in a total volume of 50 nl. We have shown previously that co-injection of α and β 1NaK or β 3NaK cRNA of *Bufo* Na,K-ATPase induces a large increase in the activity of Na,K pumps at the surface of *Xenopus* oocytes, when compared to oocytes injected with water, α subunit alone or β subunit alone (Jaisser et al., 1992a). Similarly, co-injection of *Xenopus* Na,K-ATPase α and rat gastric H,K-ATPase β subunit cRNAs was shown to result in the expression of functional Na,K-pump (Horisberger, Jaunin, Reuben, Lasater, Chow, Forte, Sachs, and Rossier, 1991b).

Electrophysiological Measurements of Na,K-Pump Activity

Na,K-pump activity was measured in Na⁺-loaded oocytes as the outward current activated by addition of K⁺, in the presence of K⁺ channel blockers, as described earlier (Horisberger et al., 1991a). Briefly, 3–5 d after cRNA injection, the oocytes were first loaded with Na⁺ by a 2-h exposure to a K⁺-free and Ca⁺⁺-free solution. They were kept thereafter in a K⁺ free solution containing 0.4 mM Ca⁺⁺ until the measurements were performed. Whole-cell currents were measured using the two-electrode voltage clamp technique. Current and voltage were recorded under voltage clamp conditions with a Dagan TEV-200 clamp instrument (Dagan Corp., Minneapolis, MN). The TL-1 DMA interface and Pclamp data acquisition program (Axon Instruments, Inc., Foster City, CA) were used to drive the voltage clamp and record voltage and current signals at a sampling rate of 1 KHz. The current signal was low-pass filtered at 25 Hz. Whole-cell current–voltage (*I*-*V*) curves were obtained by recording the current while, starting from a holding membrane potential of –50 mV, rectangular voltage pulses (125 ms) of varying amplitude (from +80 to –80 mV) were applied every 1.5 s and the steady state current was measured at 100 ms after the start of the voltage step.

Specific Measurement of the Exogenously Expressed Na,K Pump

To measure specifically the activity of the exogenous *Bufo* Na,K pumps we took advantage of the relative resistance to ouabain of the *Bufo* Na,K pump ($K_i \sim 50 \mu\text{M}$) and its fast dissociation rate constant (Jaisser et al., 1992a) compared to the *Xenopus* Na,K pump ($K_i < 0.1 \mu\text{M}$) (Canessa, Horisberger, Louvard, and Rossier, 1992). As illustrated in Fig. 1, the activity of the endogenous *Xenopus* Na,K pump was inhibited by exposure for 1 min to 10 μM ouabain. We have shown that this manoeuvre completely inhibits the *Xenopus* Na,K pump for a period of at least 15 min (Canessa et al., 1992; Jaisser et al., 1992a). Ouabain was then removed and a 4–8-min period was allowed for the recovery of the small part of the *Bufo* Na,K-pump activity that could have been transiently inhibited by the 10 μM ouabain (see Fig. 1).

Activation of the Na,K Pump by External Potassium

The activation of the Na,K-pump current by external K⁺ was studied in two separate sets of experiments, in the presence and in the nominal absence of external Na⁺. In the Na⁺ containing solutions, the K⁺-induced current was measured after a stepwise increase of the K⁺ concentration from 0.0 to 0.3, 1.0, 3.0, and 10.0 mM. In the Na⁺-free solutions the K⁺ concentrations were 0.0, 0.02, 0.1, 0.5, and 5.0 mM. The various K⁺ concentrations were obtained by addition of adequate amounts of K-gluconate to the corresponding K⁺-free

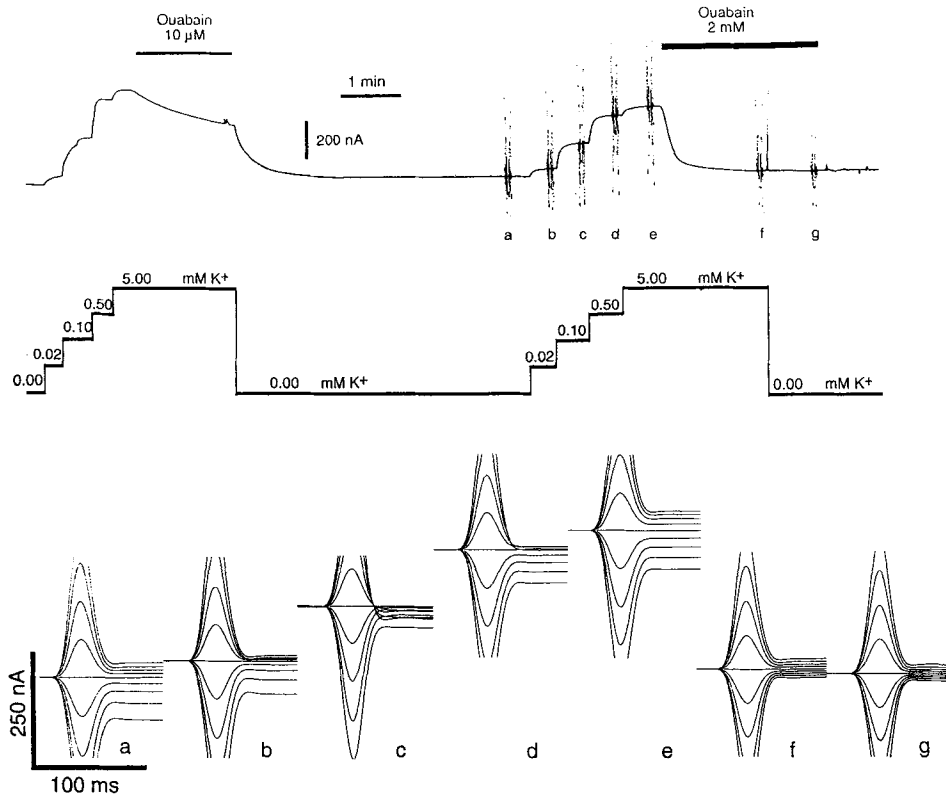


FIGURE 1. (Top) Original tracing of current recording in an oocyte injected with α 1 and β 1NaK cRNA of the *Bufo* Na,K pump, in a sodium-free solution. The holding potential was maintained at -50 mV, except for the series of short voltage steps (a–g). In this example, the K⁺ concentration was first increased in steps from 0 to 5.0 mM. K⁺ activated a large outward current. Then 10 μ M ouabain was added in the presence of 5.0 mM K⁺. The outward current decreases by ~ 100 nA, which corresponds to the ouabain-sensitive endogenous *Xenopus* Na,K pump. The small part of the *Bufo* pump that might also have been inhibited was allowed to recover during the 4–8 min after removal of ouabain. The K⁺ concentration was then increased as indicated and series of voltage steps were obtained (a–e). Thereafter, 2 mM ouabain was added and *I-V* curves were recorded in the presence (5 mM, f) and absence of K⁺ (g). (Bottom) Whole oocyte current recordings during the series of voltage steps at the points a–g indicated in the top panel. Only the first 150 ms of each recording is shown. The current signal was low-pass filtered at 25 Hz. The large current spikes are due to the large uncompensated oocyte membrane capacitance. For further analysis, the current values were read at a point corresponding to the end of the part of the tracings shown in the figure, i.e., 100 ms after the start of the voltage step.

solutions. *I-V* curves were recorded after stabilization of the current at each K⁺ concentration (see examples in Fig. 1, a–e). Then 2 mM ouabain was added and *I-V* curves were recorded in the highest K⁺ concentration (Fig. 1f) and in the K⁺-free solution (Fig. 1g) to estimate the size of K⁺-induced currents not due to the Na,K pump. The series of *I-V* curves were started only after the baseline current was stable and the series of *I-V* curves (a–g) were obtained during a period of < 10 min (see Fig. 1, a–g). No corrections were made for drift with time.

The current induced by K^+ at each potential was obtained by subtracting the current measured in the K^+ -free solution from the current measured in the presence of K^+ . The parameters of the Hill equation:

$$I = I_{\max}/[1 + (K_{1/2}/CK)^{n_H}] \quad (1)$$

were fitted to the data of the current (I) induced by various concentration of K^+ (CK) and yielded least square estimates of the maximal current (I_{\max}), the half-activation constant ($K_{1/2}$), and the Hill coefficient (n_H). The voltage dependence of $K_{1/2}$ was obtained by fitting the parameters of the exponential function:

$$K_{1/2} = K_{1/2}(0) \cdot \exp(kFV/RT) \quad (2)$$

to the $K_{1/2}$ vs. V_m data to obtain least square estimates of the $K_{1/2}$ at 0 mV [$K_{1/2}(0)$] and k , an exponential steepness factor. A nonlinear fit program based on the simplex method (Nelder and Mead, 1965) was used for fitting equations to the data.

All experiments were performed at room temperature (24–26°C). Na,K-pump current measurements were restricted to oocytes showing a membrane resistance $> 0.25 \text{ M}\Omega$. As no differences were observed in water-injected (50 nl) or noninjected oocytes, oocytes of these two groups were pooled to form the "control" group.

Solutions and Drugs

The composition of the solutions used for the electrophysiological measurements were as follows: Na^+ -containing solution (mM) Na^+ 87, Ca^{++} 0.41, Mg^{++} 0.82, Ba^{++} 5, TEA⁺ 10, gluconate 90, Cl^- 22.5, HCO_3^- 2.4, MOPS 10, pH 7.4; Na^+ -free solution (mM) Ca^{++} 0.41, Mg^{++} 0.82, Ba^{++} 5, TEA⁺ 10, Cl^- 22.5, sucrose 140. Ouabain (Sigma Chemical Co., St. Louis, MO) was added from a 0.2 M solution in dimethylsulfoxide for the low concentration (10 μM) solutions, and was directly dissolved in the final solution for the 2 mM concentration. Results are expressed as mean \pm SE (n = number of observations). The statistical significance of differences between means was estimated using Student's t test for unpaired data. $P < 0.05$ was chosen as the level of statistical significance.

RESULTS

Expression of Exogenous Na,K Pumps

Coinjection of α and β subunit cRNAs resulted in the expression of a large exogenous Na,K-pump activity in all groups. Fig. 2 shows that the current due to exogenous Na,K pumps (i.e., the current measured after inhibition of the endogenous Na,K pump) was four to six times higher in the $\alpha 1\beta 1\text{NaK}$ and $\alpha 1\beta 3\text{NaK}$ groups, and about twice higher in the $\alpha 1\beta \text{HK}$ group than the current due to the endogenous Na,K pump, i.e., the current measured in noninjected oocytes before exposure to ouabain. In noninjected oocytes no ouabain sensitive current could be detected after exposure to 10 μM ouabain for 1 min. All the means values of the groups of cRNA-injected oocyte are significantly different from 0 ($P < 0.005$ in each case). In earlier experiments in which ouabain binding was measured in parallel with the activity, we have shown that the Na,K-pump current measured at -50 mV in the presence of 10 mM K^+ was highly correlated with the number of ouabain binding sites and thus is a reliable estimate of the number of Na,K-pump expressed at the surface of the oocyte (Jaunin, Horisberger, Richter, Good, Rossier, and Geering,

1992). A similar relationship between the number of ouabain binding sites and Na,K-pump current (or ouabain-sensitive rubidium uptake) was observed when $\alpha 1\text{NaK}$ alone, $\alpha 1\beta 1\text{NaK}$ and $\alpha 1\beta \text{HK}$ were compared (Horisberger et al., 1991b). Therefore, the lower activity in the $\alpha 1\beta \text{HK}$ group can most probably be attributed to the lower level of expression, possibly due to a less efficient assembly between the amphibian Na,K-ATPase $\alpha 1$ subunit and the mammalian H,K-ATPase β subunit (Horisberger et al., 1991b).

Ouabain-sensitive and Potassium-induced Currents

Fig. 3 shows the results of experiments performed in the presence of external sodium. The main source of variation in the current values was related to the level of expression of active Na,K pump. Therefore all current values were normalized to the ouabain-sensitive current measured in the presence of 10 mM K^+ at -50 mV (mean values indicated in Fig. 2). The upper panel shows the current sensitive to 2 mM

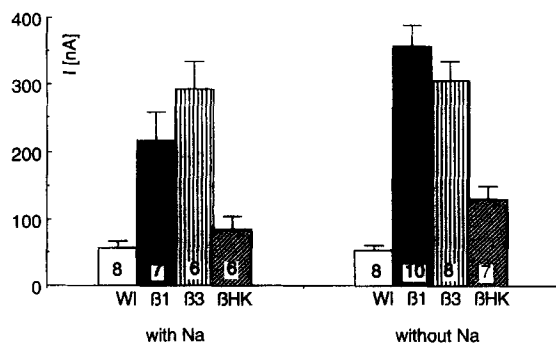


FIGURE 2. Ouabain-sensitive currents at -50 mV. The effect of 2 mM ouabain was recorded in a Na^+ containing solution (with Na) in the presence of 10 mM K^+ , and in Na^+ -free solution (without Na) in the presence of 5 mM K^+ . The number of noninjected (NI) or water-injected oocytes (WI), or of oocytes injected with $\alpha 1\beta 1\text{NaK}$ ($\beta 1$), $\alpha 1\beta 3\text{NaK}$ ($\beta 3$), or $\alpha 1\beta \text{HK}$

(βHK) cRNA is indicated in the columns. The NI/WI oocytes had not been previously exposed to ouabain, and the values indicated by the white columns represent the endogenous *Xenopus* oocyte Na,K-pump current. In the three other groups this endogenous component had been removed by previous exposure to 10 μM ouabain, and the values represent essentially the activity of the exogenous *Bufo* Na,K pump.

ouabain in the presence of 10 mM K^+ (corresponding to the subtraction of the $I-V$ curves e minus f of Fig. 1). In all groups the voltage dependence of the Na,K-pump current was marked at negative potentials ($\sim 50\%$ decrease of the ouabain-sensitive current between -50 and -130 mV) similarly to what has been described in earlier reports in oocyte (Rakowski and Paxson, 1988; Schweigert, Lafaire, and Schwarz, 1988; Wu and Civan, 1991) or other cell types (Horisberger and Giebisch, 1989; Rakowski, Gadsby, and De Weer, 1989; Gadsby and Nakao, 1989; Stimers, Shigeto, and Lieberman, 1990). The voltage dependence tended to be smaller at depolarized membrane potentials, especially for the endogenous pump and for the $\alpha 1\beta \text{HK}$ group. This can be explained by the lower apparent affinity of K^+ for these α/β complexes in the depolarized potential range (see below). K^+ concentrations higher than 10 mM could not be used because significant ouabain-resistant K^+ -induced currents appeared at K^+ concentrations > 10 mM. The middle panel shows that currents induced by 10 mM K^+ were of similar magnitude and had a similar voltage

dependence as the ouabain-sensitive current. The lower panel shows the current induced by 10 mM K^+ in the presence of 2 mM ouabain (corresponding to the subtraction of the I - V curves f minus g of Fig. 1). The size of this current amounted to a few percent of the ouabain-sensitive current in the presence of 10 mM K^+ . For the cRNA-injected oocytes a small residual Na,K-pump current was expected because of the high K_i of ouabain for the *Bufo* Na,K pump (Jaisser et al., 1992a). Assuming a K_i of 50 μ M and simple one-site inhibition kinetics, 2 mM ouabain should inhibit 97.6% of the total current. From these results we conclude that the K^+ -induced currents were essentially due to activation of the Na,K-pump.

Fig. 4 shows the results of similar measurements performed in the absence of external Na^+ . The ouabain-sensitive current (*upper panel*) was slightly voltage sensitive in the negative potential range ($\sim 20\%$ decrease of the ouabain-sensitive current between -50 and -130 mV). Although smaller than that observed in the presence of external Na^+ , this voltage dependence was statistically significant and similar in all groups. Gadsby, Rakowski, and De Weer (1993) have shown that the external Na^+ binding step is the main voltage-dependent step of the sodium translocating part of the pump cycle, and Rakowski, Vasilets, LaTona, and Schwarz (1991) have shown that the apparent affinity of K^+ was also voltage dependent. The presence of a voltage dependence in the absence of external Na^+ and at saturating K^+ concentration suggests that there is another voltage-dependent step in the cycle. Except for the $\alpha 1\beta 1NaK$ group for which the K^+ -induced current was voltage independent, there was a small increase of the K^+ -induced current in the high negative potential range. As the effect of K^+ in the presence of ouabain was negligible (*lower panel*), the discrepancy between the ouabain-sensitive current (*upper panel*) and the current activated by 5 mM K^+ (*middle panel*), at negative membrane potentials, correspond to the presence of a small ouabain-sensitive inward current in the absence of external Na^+ and K^+ . A similar ouabain-sensitive current has been observed by Rakowski et al. (1991) and has been investigated in more detail by Efthymiadis, Rettinger, and Schwarz (1993). The nature of this current is unknown.

*Potassium Activation of the Na,K-Pump Current in the Absence
and the Presence of External Sodium*

The voltage dependence of the current activated by different concentrations of K^+ (corresponding to the subtraction of the I - V curves b - d minus curve a of Fig. 1) in the presence and in the absence of external Na^+ are shown in Fig. 5. Current values were normalized to the ouabain-sensitive current measured in the presence of the highest K^+ concentration at -50 mV.

Potassium activation kinetics were obtained for each membrane potential value by fitting the parameters of the Hill equation (I_{max} , $K_{1/2}$, Hill coefficient) to the K^+ -induced current vs K^+ concentration data for each oocyte (see examples in Fig. 6). The Hill coefficients (n_H) were in the range of 1.5–2.0 for the experiments performed in the presence of Na^+ , and in the range of 0.9–1.3 for the experiments in the absence of Na^+ . There was no obvious voltage dependence of n_H obtained by parameter fitting in either case. Using fixed values of 1.6 and 1.0 for n_H in experiments with Na^+ containing and Na^+ -free solutions, respectively, and fitting the

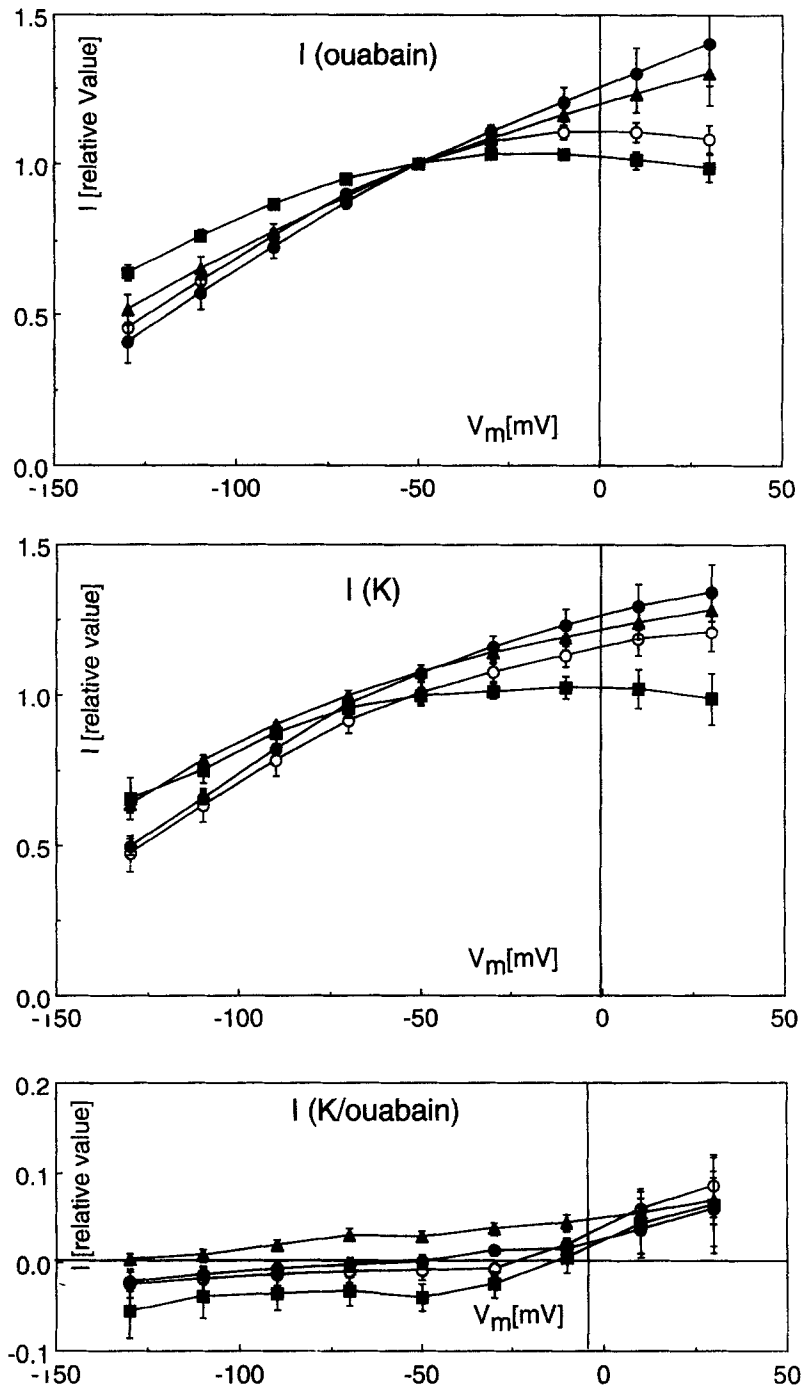


FIGURE 3. Ouabain-sensitive and potassium-activated steady-state current-voltage relationship recorded in the presence of external Na^+ (87 mM). In all three panels the current values (I) normalized to the ouabain-sensitive current at -50 mV membrane potential in the presence

two remaining parameters (I_{\max} and $K_{1/2}$) yielded essentially similar results concerning the $K_{1/2}$ estimates.

The voltage dependence of the $K_{1/2}$ of the activation of the Na,K-pump current by external K^+ is shown in Figs. 7 and 8. In the absence of external Na^+ (Fig. 7) the apparent K^+ affinity was increasing monotonically with the membrane potential in all groups. In the noninjected oocyte group, the voltage dependence of the $K_{1/2}$ was similar to that observed under similar conditions by Rakowski et al. (1991), with an exponential steepness factor of 0.36 ± 0.05 ($n = 6$). In the $\alpha 1\beta 1NaK$ and $\alpha 1\beta 3NaK$ groups the steepness factor of the voltage dependence of $K_{1/2}$ was 0.21 ± 0.02 ($n = 8$) and 0.22 ± 0.02 ($n = 8$), respectively (no significant difference between these two groups). Both these values were significantly smaller than in the noninjected group ($P < 0.005$). Although the $K_{1/2}$ values were similar at high negative membrane potentials, the $K_{1/2}$ at +10 mV was much higher in the noninjected group ($632 \pm 62 \mu M$) than in the $\alpha 1\beta 1NaK$ ($241 \pm 19 \mu M$) and $\alpha 1\beta 3NaK$ groups ($234 \pm 15 \mu M$) ($P < 0.001$). The $K_{1/2}$ values were similar in the $\beta 1NaK$ and in the $\beta 3NaK$ groups. No significant difference could be established at any potential value. The $\alpha 1\beta HK$ group had a much higher $K_{1/2}$ over the whole potential range, with a voltage dependence, k of 0.27 ± 0.06 ($n = 7$), a value not significantly different from those of the $\alpha 1\beta 1NaK$ and $\alpha 1\beta 3NaK$ groups.

In the presence of Na^+ (Fig. 8), the activation by external K^+ presented a different type of voltage dependence. In all groups, both hyperpolarization and depolarization tended to decrease the apparent affinity of K^+ for activation of the pump current, with a maximal apparent affinity around -50 to -30 mV. This indicates that the voltage dependence of the apparent affinity of K^+ results not only from a voltage-dependent step in the binding of K^+ , but also from another step with a reverse voltage dependence, presumably the binding/release of external Na^+ (Gadsby et al., 1993). Again the $\alpha 1\beta HK$ group had a higher $K_{1/2}$ than the other groups. The $\alpha 1\beta 1NaK$ group had a significantly lower affinity for K^+ than the $\alpha 1\beta 3NaK$, ($P < 0.02$ or smaller for each potential value) and this difference was larger in the high negative potential range.

DISCUSSION

In this paper we have extended the previous finding by us (Jaisser et al., 1992a,b) and others (Schmalzing et al., 1992; Eakle et al., 1992) that the structure of the β subunit has an influence on the function of the Na,K pump present at the plasma membrane, and more specifically on the apparent affinity of potassium for its external binding site. The Na,K pump is a transport system that undergoes a complex cycle with at least two conformations, one of which (the E2 conformation)

of 10 mM K^+ are plotted against the membrane potential (V_m). (*Top*) Current sensitive to 2 mM ouabain in the presence of 10 mM K^+ (corresponding to the subtraction of current recordings f minus e of Fig. 1). (*Middle*) Current activated by 10 mM K^+ , (e minus a of Fig. 1). (*Bottom*) Current activated by 10 mM K^+ in the presence of 2 mM ouabain (g minus f of Fig. 1). Note the expanded scale. The values are the mean \pm SE of eight, seven, six, and six measurements in the noninjected or water injected (NI/WI, \circ) or $\alpha 1\beta 1NaK$ ($\beta 1$, \blacktriangle), $\alpha 1\beta 3NaK$ ($\beta 3$, \bullet), and $\alpha 1\beta HK$ (βHK , \blacksquare) cRNA-injected oocytes, respectively.

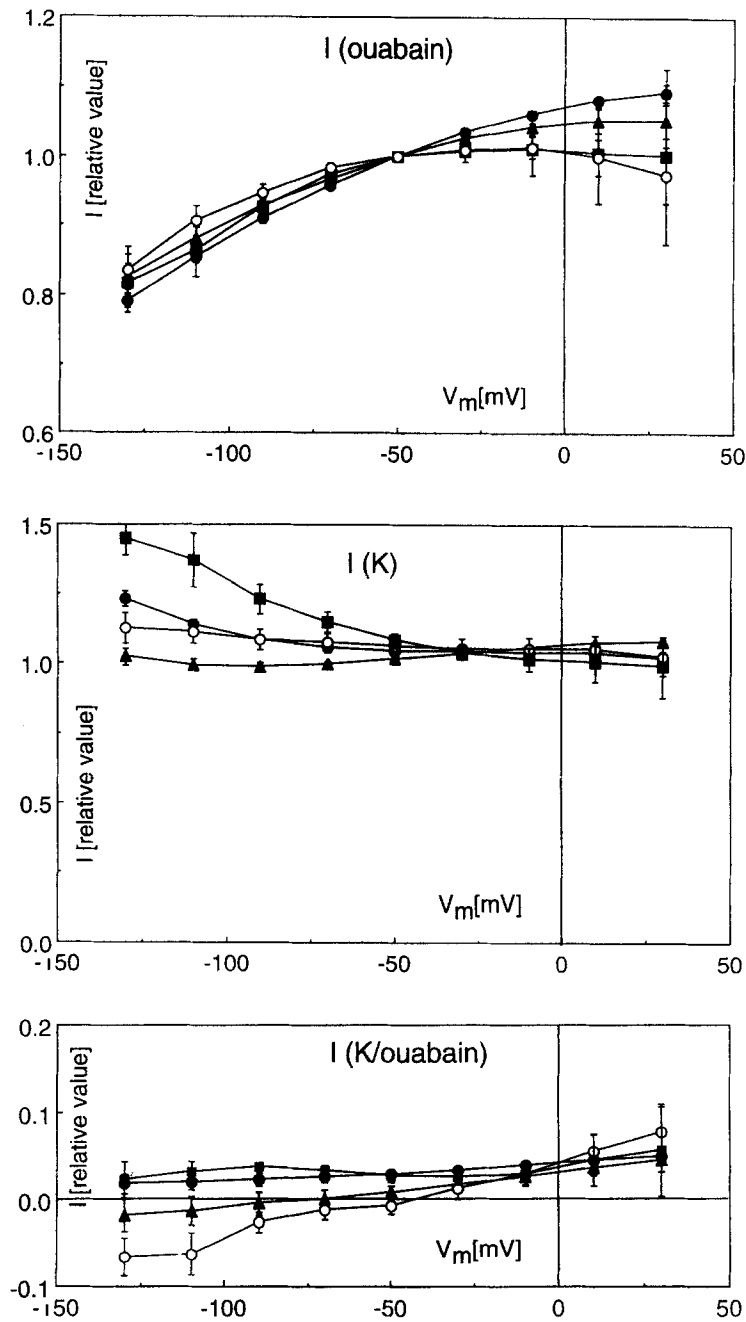


FIGURE 4. Ouabain-sensitive and potassium-activated steady-state current-voltage relationship recorded in the nominal absence of external Na^+ . In all three panels the current values (I) normalized to the ouabain-sensitive current at -50 mV membrane potential in the presence of 5 mM K^+ are plotted against the membrane potential (V_m). (Top) Current sensitive to 2 mM ouabain in the presence of 5 mM K^+ (corresponding to the subtraction of current recordings f

has a high affinity for external K^+ ions. The apparent affinity of K^+ , which we measure as the $K_{1/2}$, will generally be different from the intrinsic affinity (K_m) of the E2 conformation (Läuger, 1991). In principle, modifications of the $K_{1/2}$ could result from a change of the K_m as well as from other alterations in the kinetics of the pump cycle. To investigate the role of the β subunit in the Na,K-pump function, we have examined the potassium activation kinetics of Na,K-pump heterodimers including different β subunits under two conditions, in the presence and in the absence of external Na^+ , and over a wide range of membrane potential.

The most striking difference between pumps including $\beta 1NaK$ and $\beta 3NaK$ isoforms was the apparent affinity for K^+ measured in the presence of external Na^+ . No difference could be detected in the absence of external Na^+ over the whole potential range. In contrast, a much lower apparent affinity for K^+ was observed with the $\alpha 1\beta HK$ complex both in the presence and in the absence of external Na^+ .

To analyze the difference of the apparent affinity for K^+ and its relation to the intrinsic affinity, we have used a simple three-state kinetic model of the Na,K-pump cycle that is described in the Appendix. In this model the apparent affinity, $K_{1/2}$ is related to the intrinsic K_m by the following relation (Eq. A8 in the Appendix):

$$K_{1/2} = K_m \frac{f1 + b1}{f1 + f2} \quad (3)$$

The large difference of $K_{1/2}$ observed between the $\alpha 1\beta 1NaK$ and the $\alpha 1\beta HK$ groups was of roughly similar magnitude in the presence and in the absence of external Na^+ , and at all tested membrane potentials. The most simple explanation for this uniform difference under various conditions is an alteration of the intrinsic K_m , which appears as a factor in the right side of Eq. 3.

The absence of detectable difference between the $\alpha 1\beta 1NaK$ and the $\alpha 1\beta 3NaK$ groups in the absence of external Na^+ makes it very unlikely that the intrinsic K_m was altered. If the change of the apparent affinity observed in the presence of Na^+ has to be attributed to the modification of a single rate constant, an increase of the rate constant $b1$ (i.e., the backward Na^+ translocating step) in the $\alpha 1\beta 3NaK$ group could explain both the higher $K_{1/2}$ in the presence of external Na^+ and the absence of difference in Na^+ -free external solutions. This hypothesis is supported by the observation that the difference of $K_{1/2}$ increases at negative membrane potential. Indeed, as the rate of the backward Na^+ translocating step ($b1$) increases at negative membrane potentials, owing to the voltage dependence of external Na^+ binding (Gadsby et al., 1993), the influence of $b1$ on the $f1 + b1$ term increases. It is, however, obvious that more complex modifications, concerning several rate constants could also produce these results.

minus e of Fig. 1). Note that the vertical scale of this panel was expanded to allow for better visualization of the voltage dependence of the ouabain-sensitive current. *Middle panel:* Current activated by 5 mM K^+ , (e minus a of Fig. 1). (*Bottom*) Current activated by 5 mM K^+ in the presence of 2 mM ouabain (g minus f of Fig. 1); note the expanded vertical scale. The values are the mean \pm SE of 8, 10, 8, and 7 measurements in the noninjected or water injected (NI/WI, \circ) or $\alpha 1\beta 1NaK$ ($\beta 1$, \blacktriangle), $\alpha 1\beta 3NaK$ ($\beta 3$, \bullet), and $\alpha 1\beta HK$ (βHK , \blacksquare) cRNA-injected oocytes, respectively.

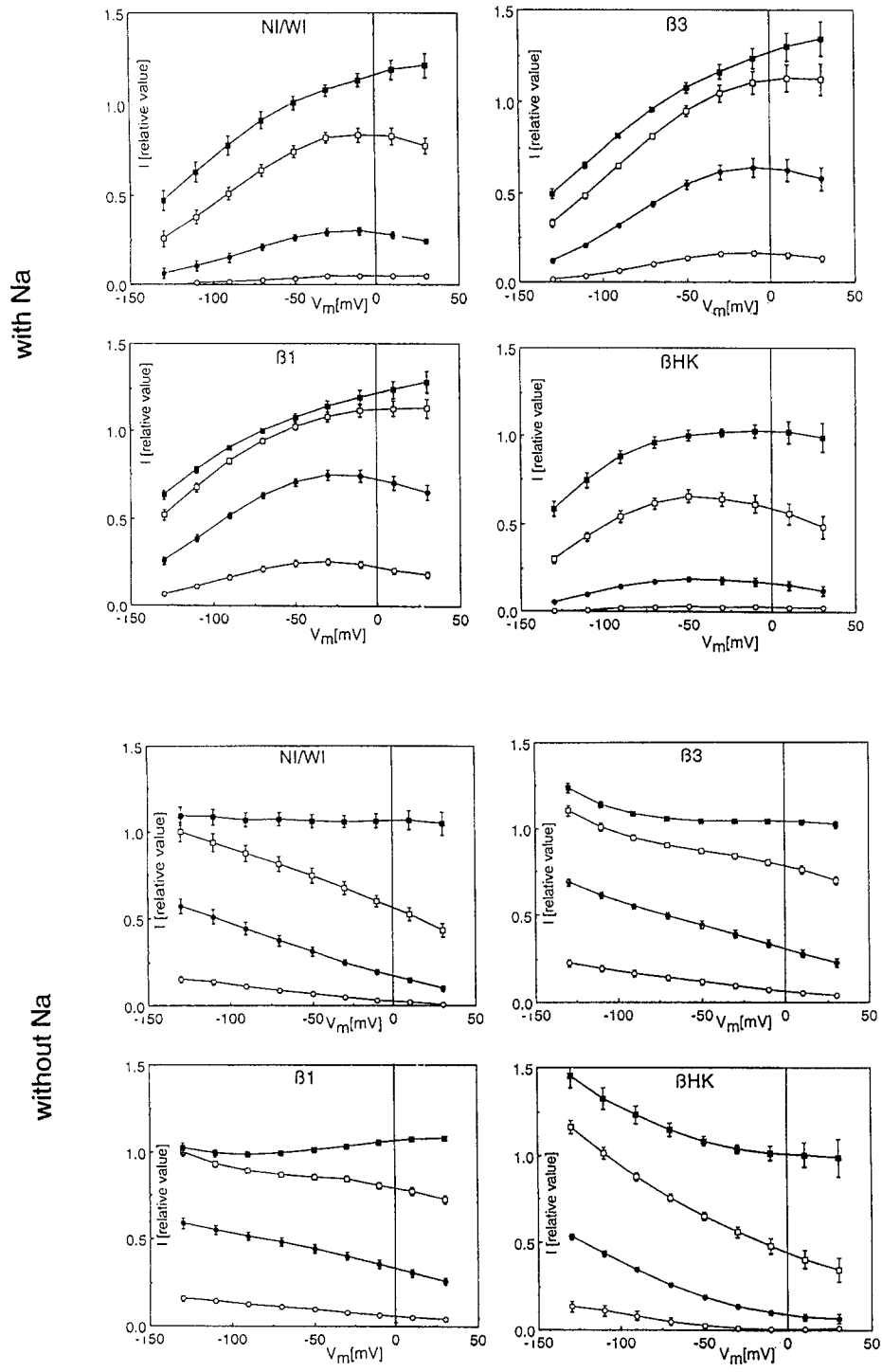


FIGURE 5.

The β subunit might be involved in the function of the Na,K pump because the transmembrane segment of the β peptide chain participates directly in the structure of cation binding or occlusion sites, as suggested by Capasso, Hoving, Tal, Goldshleger, and Karlish (1992). Alternatively, the β subunit, by its close interaction with the α subunit, might modify the structure of the α protein and/or the equilibrium between different conformational states of this subunit. Comparison of the primary sequence of a large number of β subunit isoforms (Horisberger et al., 1991c) indicate that the overall structure is well conserved: a short intracellular amino-terminal sequence is followed by one transmembrane segment and a large extracytoplasmic carboxy-terminal domain. A striking difference is the presence in the $\beta 1$ isoform of a stretch of 15–20 amino acids (corresponding to exon 5) that is not present in the other β isoforms. The differences between β HK and the other β isoforms are more substantial (about 30% identity) and widespread throughout the whole sequence. Studies using chimeric β subunits formed from different isoforms may allow us to determine more precisely which parts of the β protein are involved in the functional differences that we have observed, and help to delineate functional domains of the β subunit.

Although the presence of α and $\beta 3$ NaK subunits has been shown in *Xenopus* oocytes (Jaunin et al., 1992), the exact isoform composition of the protein forming active Na,K pumps at the plasma membrane of this cell type has not yet been determined. Our results obtained with noninjected oocytes show that the function of the endogenous Na,K pump is clearly different from either that of the $\alpha 1\beta 1$ NaK or the $\alpha 1\beta 3$ NaK complexes. In particular, the voltage dependence of the apparent affinity for K^+ is nearly twice as steep for the endogenous than for the $\alpha 1\beta 1$ NaK or $\alpha 1\beta 3$ NaK pumps. The reasons for this difference are not clear. Species differences do not seem to be the explanation since *Xenopus* $\alpha 1\beta 1$ NaK or $\alpha 1\beta 3$ NaK complexes expressed in oocytes by cRNA injection also show a higher apparent affinity for K^+ when compared to endogenous Na,K-pumps studied in non injected oocytes (unpublished results). The existence of an oocyte specific α subunit isoform might explain the difference in K^+ affinity. Deletions of the amino-terminal part of the α subunit have been shown to alter the apparent affinity for K^+ (Bürgener-Kairuz, Horisberger, Geering, and Rossier, 1991), and more specifically the voltage dependence of the apparent K^+ affinity (Vasilets, Omay, Ohta, Noguchi, Kawamura, and Schwarz, 1991). The importance of the amino terminus is further suggested by the presence of a distinct transcript of the $\alpha 1$ isoform in the oocyte and during early development (Bürgener-Kairuz, personal communication).

In conclusion, our results point to an important role of the β subunit in the

FIGURE 5. (opposite) Steady-state current–voltage relationship of the potassium-activated current in the presence of external Na^+ (top four panels, with Na), and in the absence of external Na^+ (bottom four panels, without Na). The current values (I) normalized to the ouabain-sensitive current at -50 mV membrane potential in the presence of 10 mM K^+ (with Na) or 5 mM K^+ (without Na) are plotted against the membrane potential (V_m). The concentrations of K^+ (in mM) are indicated by the symbols as follows: 0.3 (○), 1.0 (●), 3.0 (□), and 10.0 (■) for the top panels (with Na); and 0.02 (○), 0.10 (●), 0.50 (□), and 5.0 (■) for the bottom panels (without Na). The number of experiments are the same as in Figs. 3 and 4.

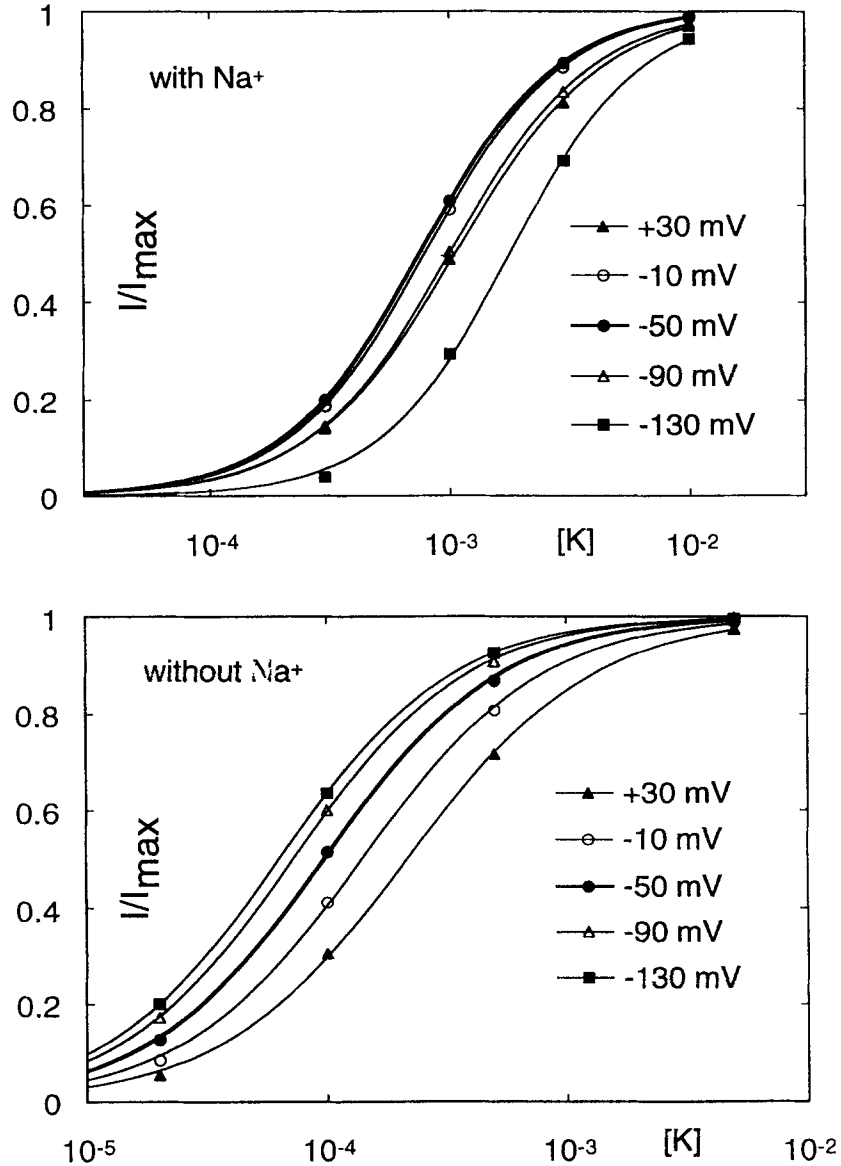


FIGURE 6. Concentration dependence of the K^+ induced current at membrane potentials ranging from +30 to -130 mV, in representative experiments with an $\alpha 1\beta 1NaK$ cRNA-injected oocyte. The K^+ concentration $[K]$ is given in molar. (Top) In the presence of external Na^+ . (Bottom) In the absence of external Na^+ . The continuous lines are the best fitting curve obtained with the Hill equation as described in Methods. Current values have been normalized to the I_{max} obtained by the parameter fitting. For clarity of the figure, the values at only five membrane potentials are presented.

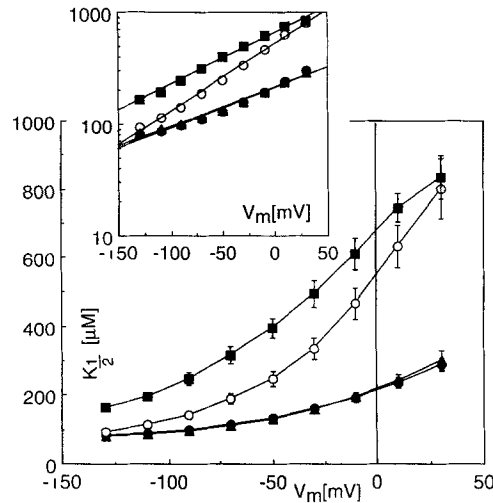


FIGURE 7. Voltage dependence of the half-activation constant ($K_{1/2}$) by external K^+ , in the nominal absence of Na^+ . The $K_{1/2}$ was determined for each measurement by fitting the parameters of the Hill equation to the current versus concentration data, as described in Methods. The mean Hill coefficients at 50 mV were 1.06 ± 0.02 , 1.18 ± 0.03 , 1.12 ± 0.05 , and 1.13 ± 0.06 , in the NI/WI, $\alpha 1\beta 1NaK$, $\alpha 1\beta 3NaK$, and $\alpha 1\beta HK$ groups, respectively, and did not show any consistent voltage dependence. *Inset*: Semilogarithmic plot of the same data. The values are the mean \pm SE of 8, 10, 8, and 7 measurements in the noninjected or water injected (NI/WI, \circ) or $\alpha 1\beta 1NaK$ (\blacktriangle), $\alpha 1\beta 3NaK$ (\bullet), and $\alpha 1\beta HK$ (\blacksquare) cRNA-injected oocytes, respectively.

transport function of the Na,K pump, confirming evidence obtained by other techniques (Eakle et al., 1992; Schmalzing et al., 1992; Lutsenko and Kaplan, 1992). Analysis of the difference of potassium activation kinetics between Na,K-pump dimers including different β subunits suggests that the presence of the gastric H,K-ATPase β subunit modifies the properties of the external binding site of K^+ to the Na,K pump. When compared to the $\beta 1$ subunit, the presence of the Na,K-

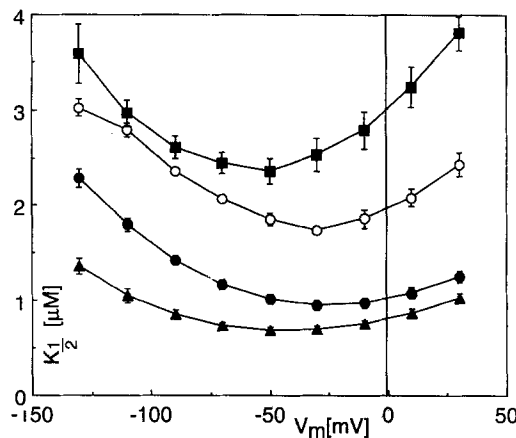


FIGURE 8. Voltage dependence of the half-activation constant ($K_{1/2}$) of external K^+ , in the presence of Na^+ (87 mM). The $K_{1/2}$ was determined for each measurement by fitting the parameters of the Hill equation to the current versus concentration data, as described in the Methods section. The mean Hill coefficients at 50 mV were 1.91 ± 0.06 , 1.65 ± 0.03 , 1.70 ± 0.03 , and 1.83 ± 0.03 , in the NI/WI, $\alpha 1\beta 1NaK$, $\alpha 1\beta 3NaK$, and $\alpha 1\beta HK$ groups, respectively, and did not show any obvious voltage dependence. The values are the mean \pm SE of eight, seven, six, and six measurements in the noninjected or water-injected (NI/WI, \circ) or $\alpha 1\beta 1NaK$ (\blacksquare), $\alpha 1\beta 3NaK$ (\bullet), and $\alpha 1\beta HK$ (\blacktriangle) cRNA-injected oocytes, respectively.

measurements in the noninjected or water-injected (NI/WI, \circ) or $\alpha 1\beta 1NaK$ (\blacksquare), $\alpha 1\beta 3NaK$ (\bullet), and $\alpha 1\beta HK$ (\blacktriangle) cRNA-injected oocytes, respectively.

ATPase $\beta 3$ subunit induces a decrease of the apparent affinity that is most likely due to alterations of the Na^+ translocation kinetics rather than to a direct alteration of the K^+ binding site.

APPENDIX

To analyze the relation between the intrinsic affinity (K_m) and the apparent activation constant ($K_{1/2}$), we used a simple steady-state kinetic model of the Na,K-pump cycle. As illustrated in Fig. 9, this model includes three states: E1, E2, and E2K. An equilibrium binding kinetic with an intrinsic affinity K_m links the E2 and E2K states. The pseudomonomolecular rate constants $f1$ and $b1$ represent the step during which, according to the Post-Albers model, Na^+ ions are released (forward: $f1$) or bound (backward: $b1$). The $f2$ rate constant summarizes all the other steps of the cycle between the E2K and the E1 state. The backward rate constant of the E2K to E1 step is assumed to be slow compared to the other rate constants and is set to 0. The $b1$ rate constant is equal to 0 in the absence of external Na^+ and has a finite value in the presence of Na^+ . From published data (Rakowski et al., 1991; Gadsby et al., 1993), both $b1$ and K_m are expected to be voltage dependent. The observed voltage dependence of the ouabain-sensitive current in the absence of external Na^+ (Fig. 3, top) suggests that either $f1$ or $f2$ are also voltage dependent. It should be noticed that the E1 and E2 states do not have exactly the same meaning as they do in the Post-Albers model.

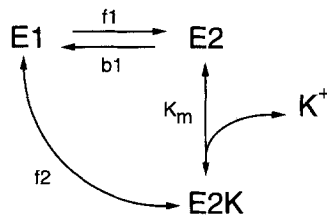


FIGURE 9. Three-state kinetic model of the Na,K-pump cycle.

If we denote by $[E]$ the mole fraction of the state E and by $[K]$ the K^+ concentration, we can write

$$[E1] + [E2] + [E2K] = 1 \quad (\text{A1})$$

$$[E2] \cdot [K] / [E2K] = K_m \quad (\text{A2})$$

$$d[E1]/dt = [E2] \cdot b1 + [E2K] \cdot f2 - [E1] \cdot f1 = 0 \quad (\text{A3})$$

under steady-state conditions.

This system of three equations can be solved for $[E2K]$ as follows

$$[E2K] = f1 / (f1 + f2 + (f1 + b1) \cdot (K_m / [K])) \quad (\text{A4})$$

The $E2K$ mole fraction at the maximal turnover rate, at saturating $[K]$, ($E2K_{\max}$) is given by

$$E2K_{\max} = f1 / (f1 + f2) \quad (\text{A5})$$

The turnover rate (V) can be defined as

$$V = E2K * f2 \quad (\text{A6})$$

and from Eqs. A5 and A6

$$V_{\max} = (f1 \cdot f2) / (f1 + f2) \quad (\text{A7})$$

The K^+ concentration producing a turnover rate (V) equal to $V_{\max}/2$, $K_{1/2}$, can be calculated from A4, A5, and A6 as

$$K_{1/2} = K_m \frac{f1 + b1}{f1 + f2} \quad (\text{A8})$$

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