The Na^+,K^+-ATPase carrying the carboxy-terminal Ca^{2+}/calmodulin binding domain of the Ca^{2+} pump has $2Na^+,2K^+$ stoichiometry and lost charge movement in Na^+/Na^+ exchange

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Abstract An altered ion-transport stoichiometry from $3Na^+,2K^+$ to $2Na^+,2K^+$ is observed in a chimeric Na^+,K^+-ATPase, which carries the Ca^{2+}/calmodulin binding domain (CBD) of the plasma membrane Ca^{2+}-ATPase at its carboxy-terminus [Zhao et al., FEBS Lett. 408 (1997) 271–275]. The ouabain-resistant mutant of this chimera (ORα1-CBD) was constructed to further investigate the effect of the CBD on ion-transport properties. The ORα1-CBD still shows the $2Na^+,2K^+$ stoichiometry. The loss of electrogenicity is accompanied by the disappearance of transient charge movements in the Na^+/Na^+ exchange mode. We conclude that the binding of the third Na^+ ion, but not of the two others, in $3Na^+,2K^+$ transport mode apparently senses the electric field, and that the voltage-dependent Na^+ binding is likely to be lost in the chimera with CBD.

Key words: Ion transport stoichiometry; Na^+,K^+-ATPase chimera; Ca^{2+}/calmodulin domain

1. Introduction

The Na^+,K^+ATPase and the Ca^{2+}-ATPase of the plasma membrane (PM) belong to the family of P-type ATPases, which become temporarily phosphorylated at an aspartic acid residue in a conserved cytoplasmic domain during the transport cycle (see e.g. [1]). The energy liberated by the hydrolysis of ATP is converted into energy stored in the electrochemical gradients of the ions transported across the cell membrane. In the Na^+,K^+-ATPase, 3 Na^+ are pumped out of the cell and 2 K^+ into the cell for 1 ATP hydrolyzed, whereas in the PM Ca^{2+}-ATPase, 1 Ca^{2+} is pumped out of the cell for 1 ATP hydrolyzed.

The Na^+,K^+-ATPase is composed of two subunits: a large catalytic α subunit of about 110 kDa and a smaller glycosylated β subunit of about 55 kDa. The α subunit hosts all functionally relevant sites including the binding sites for ATP, for the transported cations, for specific inhibitors as well as the catalytic phosphorylation site and other regulatory sites. The β subunit is known to be essential for the enzyme to be completely translocated into the plasma membrane with full enzymatic activity [2–5]. The $3Na^+,2K^+$ stoichiometry of the ion transport means that this pump is electrogenic, generating outward-directed net movement of positive charge. This current can serve as a measure for the transport activity in voltage-clamp experiments. Experiments on *Xenopus* oocytes revealed for the first time that the transport activity of the Na^+,K^+-ATPase is regulated by at least two voltage-dependent steps in the reaction cycle of the pump [6]. These voltage-dependent steps are associated with the extracellular binding of Na^+ and K^+ to the pump molecule, and can be explained in terms of an access channel model [7–11]. Under the conditions with high Na^+ on both sides of the membrane, no extracellular K^+ and with low K^+ intracellularly, the Na^+,K^+ pump can operate electrically silently. In this mode, the pump transports $3Na^+$ ions both in forward and backward direction through the Na^+-translocating half of the pump cycle. Though the net transport is non-electrogenic, a transient pump-mediated current can be detected in response to voltage steps that reflect predominantly the voltage-dependent interaction of extracellular Na^+ with the pump molecule through the access channel (for a review see [12]).

The plasma membrane Ca^{2+}-ATPase is formed by a single peptide of about 130 kDa. A characteristic difference from the Na^+,K^+-ATPase is an additional carboxy-terminal segment of about 160 amino acids. This segment contains a number of domains involved in regulation of enzyme activity (for review see [13]), one of which is a site for the binding of Ca^{2+}/calmodulin [14]. In the absence of Ca^{2+}/calmodulin, this domain interacts with several other cytoplasmic regions, which results in auto-inhibition of ATPase activity [13].

Ishii and Takeyasu [15] reported previously on the properties of a chimeric ATPase that consists of the complete α1 subunit of chicken Na^+,K^+-ATPase and, attached to its carboxy-terminus, the regulatory region with the Ca^{2+}/calmodulin binding domain (CBD) of rat PM Ca^{2+}-ATPase II. This chimera, expressed together with the β subunit, showed Na^+- and K^+-dependent ATPase activity only in the presence of Ca^{2+}/calmodulin. Furthermore, we could show recently by expression in *Xenopus* oocytes [16] that the CBD mimics a part of the function of the β subunit; the chimeric α subunits without the β subunit can be expressed on the cell surface and bind extracellularly applied ouabain, a Na^+,K^+ pump-specific inhibitor. This demonstrates that at least those parts of the α subunit are incorporated into the membrane, which are essential for ouabain binding. Complete function as a Na^+- and K^+-transporting ATPase, however, is obtained only when the β subunit was coexpressed. Interestingly, the chimeric pump can operate in a non-electrogenic mode with stoichiometry that has been changed from $3Na^+,2K^+$ to $2Na^+,2K^+$ [16].

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For further investigation of the effect of CBD on the transport mechanism, we constructed ouabain-resistant mutants of the α1 subunit (ORα1) and the chimeric subunit (ORα1-CBD). In this case, the experiment can be performed in the presence of low concentrations of ouabain that inhibit the endogenous pump but not the OR pumps.

2. Materials and methods

2.1. Construction of ouabain-resistant α subunits of chicken Na^+,K^+-ATPase

The chimeric α subunit (α1-CBD) of Na^+,K^+-ATPase, composed of chicken α1 subunit and the carboxy-terminal 165 amino acids of the Ca^{2+}-ATPase II of rat plasma membrane, was constructed as described previously [15]. For the construction of ouabain-resistant α1 and α1-CBD, Thr^{116} was replaced by Arg, and Asn^{127} by Asp using conventional oligonucleotide-directed mutagenesis. Introducing charged amino acids at these positions has been used previously to convert ouabain-sensitive Na^+,K^+-ATPases to the ouabain-resistant forms [17,18]. Final products were ligated into a vector, pNKS, which has a poly-A signal at the 3′ end of multiple cloning sites. The nucleotide sequences of these constructs were confirmed by the conventional procedure. cRNA was prepared as described in the manufacturer's instruction (Ambion, Austin, TX, USA).

2.2. Oocytes

Fully grown oocytes of *Xenopus laevis* were obtained and treated as described previously (see [16]). For expression, cRNA for the α1 subunits (ORα1 or ORα1CBD) (10–15 ng/oocyte) together with cRNA for the β subunits of Na^+,K^+-ATPase of *Torpedo* electroplax (5–10 ng/oocyte) was injected. These cells and non-injected control oocytes were stored for 3–5 days at 19°C. Before measurements, oocytes were loaded with Na^+ by incubation in Na^+ loading solution for 40 min. Thereafter, cells were kept for at least 30 min in post-loading solution.

2.3. Functional characterization of the pumps

The protocols for measuring $^{86}Rb^+$ uptake and pump-generated steady-state current were identical to those described in our previous report [16]. To block endogenous *Xenopus* pumps, 1 or 10 mM ouabain was added to the external solutions. Pump-mediated Rb^+ uptake was determined by incubation in Rb-flux solution containing 5 mM $^{86}RbCl$. Pump-mediated currents were determined as the component of membrane current activated by 5 mM external K^+ in the presence of 5 mM $BaCl_2$ and 20 mM (TEA)Cl to block K^+-sensitive channels.

In addition to functional characterization of the pumps by Rb^+ uptake and steady-state current measurements, we determined the charges moved in the electrical field during Na^+/Na^+ exchange by measuring the pump-mediated current transients under two-electrode voltage clamp in test solution with 90 mM NaCl. Current was filtered at 3.3 kHz and sampled at 10 kHz. Rectangular voltage pulses of 20 ms duration were applied from a holding potential of −60 mV.

The contribution of Na^+/Na^+ exchange to the current signal was determined as the difference of the current in the presence and in the absence of 90 mM Na^+ in the external bath solution. The amount of moved charges was calculated by integration of the transient component of the current signal.

2.4. Solutions

The composition of the solutions used in the experiments was: Na^+-loading solution: 110 mM NaCl, 2.5 mM sodium citrate, 5 mM MOPS (pH 7.6). Post-loading solution: 90 mM sodium gluconate, 7 mM NaCl, 0.82 mM $MgCl_2$, 2.4 mM $NaHCO_3$, 5 mM $BaCl_2$, 0.41 mM $CaCl_2$, 10 mM MOPS (pH 7.2). Rb-flux solution: 90 mM (TMA)Cl, 20 mM (TEA)Cl, 10 mM $BaCl_2$, 5 mM $NiCl_2$, 5 mM MOPS (pH 7.4). Test solution: 90 mM (TMA)Cl (or 90 mM NaCl), 20 mM (TEA)Cl, 5 mM $BaCl_2$, 2 mM $NiCl_2$, 5 mM MOPS (pH 7.8).

3. Results

To investigate the function of exogenous Na^+,K^+ pumps in *Xenopus* oocytes, it is necessary to subtract or eliminate contributions of the endogenous *Xenopus* pump. This can be done in two ways. (1) One characterizes the endogenous Na^+,K^+ pump in control oocytes that have not been injected with cRNA. In injected oocytes from the same batches, the signals originating from both the endogenous and the exogenous pumps are recorded, from which the contribution determined in the control oocytes is then subtracted. This procedure was used in our previous investigation on the effect of the CBD-containing region [16]. However, this protocol is time-consuming and has some uncertainties including possible remaining contributions from the pumps formed by endogenous α subunits and the *Torpedo* β subunit. (2) One uses cRNA of an ouabain-resistant mutant. The experiments are then performed in the presence of low concentrations of ouabain (1 μM) that block the endogenous pumps but still have no effect on the exogenous ouabain-resistant pumps. The latter protocol is less time-consuming, avoids remaining endogenous contribution, and was used in the experiments described here.

3.1. Ouabain sensitivity of the ouabain-resistant pumps

We have discovered recently that mutations supposed to affect cation translocation may, surprisingly, partially or even completely restore ouabain sensitivity [18]. Therefore, we determined the dependence of $^{86}Rb^+$ uptake of the ORα1 and of the ORα1-CBD pumps on ouabain concentration. Fig. 1 shows that the dependences can be described by the sum of two components. The component with higher sensitivity, with an apparent K_I value of 0.13 μM, represents inhibition of the endogenous pump. This value is compatible with the 0.04 μM obtained from the inhibition of endogenous pump current in the presence of 3 mM K^+ [19]. The components with low affinity represent the inhibition of OR pumps. The calculated apparent K_I values for the different pump species are listed in Table 1. The ORα1 pump exhibits an extremely high apparent K_I value of more than 10 mM while the ORα1-CBD pump shows a value of 0.3 mM. Although addition of CBD obviously increases the sensitivity to ouabain by more than one order of magnitude, the sensitivity is, nevertheless, three orders of magnitude lower than for the endogenous Na^+,K^+ pump. We therefore could use 1 or even 10 μM ouabain to block the endogenous pumps, and measure the pure contribution of the OR pumps.

3.2. Effect of CBD on Rb^+ uptake and electrogenicity

The next question was whether CBD in the ouabain-resistant mutant has the same effects on the ion transport as we

Table 1
Comparison of ouabain sensitivity and transport characteristics

Pump type	K_I^{ouab}	Rb uptake (dpm)	Pump current (at −60 mV)	Q_{max}	z	V_m
Xenopus	0.13 μM	1735 ± 162	48 ± 2 nA	1.0 nC[a]	0.72[a]	−44 mV[a]
ORα1	12.3 mM	5516 ± 416	137 ± 13 nA	5.7 nC	0.64	−145 mV
ORα1-CBD	0.3 mM	3626 ± 391	32 ± 6 nA	1.4 nC	0.50	−133 mV

[a]Values taken from [24].

found for the ouabain-sensitive variant [16]. We had demonstrated that both ouabain-sensitive ATPases were expressed in the oocytes to about the same extent, but electrogenicity was lost to a large extent in the chimeric α1-CBD pump. This was demonstrated by the finding that the increase in the number of pump molecules (determined by the number of ouabain binding sites) is paralleled by the same relative increase in pump-mediated Rb^+ uptake [16]. Both the ouabain-resistant ORα1 and ORα1-CBD pumps are functionally incorporated into the oocyte plasma membrane. Table 1 lists the data for the ouabain-sensitive Rb^+ uptake mediated by the endogenous pumps in non-injected control oocytes, and for the uptake mediated by the exogenous pumps in injected oocytes determined in the presence of 1 or 10 μM ouabain. The uptake mediated by ORα1 and the ORα1-CBD pumps is 3.2 and 2.1 times higher, respectively, than the uptake by the endogenous Na^+,K^+-ATPase. Measurements of the steady-state pump current under two-electrode voltage clamp yielded ORα1-mediated current 2.9 times higher than the endogenous pump current, compatible with the 3.2-fold increase of Rb^+ uptake. The voltage independence of pump current in Na^+-free medium with 5 mM K^+ [7] is maintained in the ORα1 pump (data not shown). In contrast to the ORα1 pumps, the ORα1-CBD pumps generate only a much smaller electrogenic current, which amounts to only 70% of the endogenous pump current (compare Table 1). Since electrogenicity originates from the $3Na^+$,$2K^+$ stoichiometry, we can conclude that also the ORα1-CBD pump operates with high probability in an electrically silent mode with $2Na^+$,$2K^+$ stoichiometry like the ouabain-sensitive chimera.

3.3. Transient current in Na^+/Na^+ exchange

In the wild-type Na^+,K^+ pump, the interaction of the extracellular Na^+ with the pump represents the dominating voltage-dependent step [9–11,20,21]. In the $3Na^+/3Na^+$ exchange mode, the voltage-dependent interaction of external Na^+ with the pump molecule can be detected as transient charge movements in response to voltage steps [11,22,23] (see Fig. 2A). The charges moved during the on and off responses were determined by integrating the transient current signal. The on and off charge movements are nearly identical as has already been demonstrated previously [24]. The voltage dependence of the charge distribution can be described by the Fermi function (Fig. 2C):

$$Q(V) = \frac{Q_{+\infty} + Q_{-\infty}}{1 + \exp(-z(V - V_m)F/RT)} - Q_{-\infty} \quad (1)$$

$Q_{max} = Q_{+\infty} + Q_{-\infty}$ represents the maximum charge that can be moved during the associated reaction steps, and is a measure of the number of pump molecules if $3Na^+/2K^+$ stoichiometry is maintained. z represents the effective valency of the moved charges. The fitted parameters are given in Table 1 together

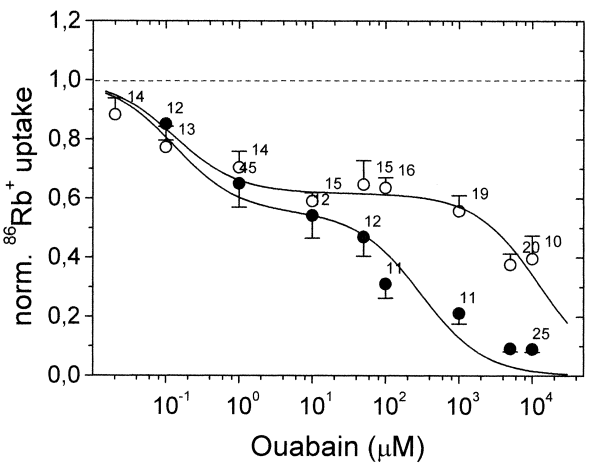

Fig. 1. Dependence of $^{86}Rb^+$ uptake on ouabain concentration. Open symbols are data for the ORα1 pump and are averaged (±S.E.M.) from two to five experiments with 10 oocytes each. Filled symbols are data for the ORα1-CBD pump and are averages (±S.E.M.) from one to five experiment with 10 oocytes each. The solid lines represent fits of:

$$\Phi = \Phi_1 \frac{K_{I1}}{K_{I1} + [\text{ouabain}]} + \Phi_2 \frac{K_{I2}}{K_{I2} + [\text{ouabain}]}$$

to the data. K_{I1} represents the apparent K_I value for inhibition of Rb^+ uptake by the endogenous *Xenopus* pump, K_{I2} the K_I value for inhibition of uptake by the ORα1 and ORα1-CBD pump, respectively. The K_I values are given in Table 1.

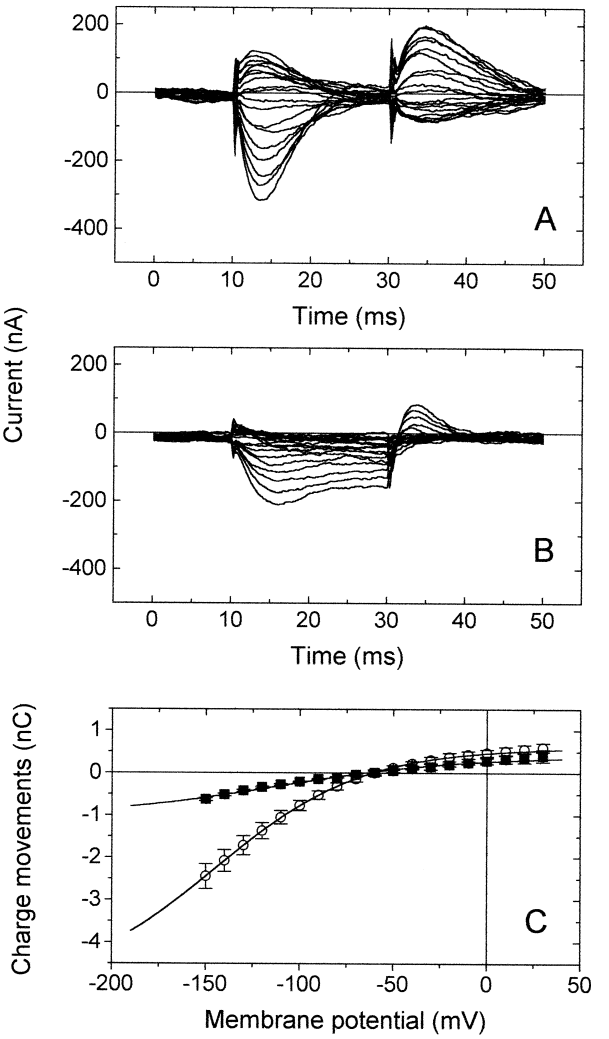

Fig. 2. Transient currents in Na^+,Na^+ exchange mode under TEVC. Transient pump-mediated currents are elicited by rectangular voltage pulses applied from the holding potential for an oocyte with expressed ORα1 pumps (A) and with ORα1-CBD pumps (B). C: Charge movements during the transients. Data are averages from three experiments (±S.E.M.), and were obtained by integration of the transient signals. The solid lines represent fits of Eq. 1 to the data. The fitted parameters are listed in Table 1.

with published data for the endogenous *Xenopus* pump [24]. The Q_{max} for the ORα1 pump but not for ORα1-CBD is clearly elevated compared to that for the *Xenopus* pump. The increase is compatible with the increase of the respective values for Rb^+ uptake and pump current. For the ORα1-CBD pump, compared with the ORα1 pump, the transient signal is drastically reduced (Fig. 2B,C). Obviously, the two transported ions do not sense the electrical field during binding from external medium. The third Na^+ transported in the normal Na^+,K^+ pump but not by the ORα1-CBD pump is the ion that contributes predominately to charge movement and the voltage-dependent interaction.

4. Discussion

4.1. The mutations $Thr^{116}Arg$ and $Asn^{127}Asp$ lead to ouabain insensitivity

As has been demonstrated previously [17,18], mutation of the external amino acids bordering the first two transmembrane segments of the α subunit to charged amino acids results in a reduction of ouabain sensitivity. For the α1 subunit of chicken, these mutations lead to an extraordinarily low sensitivity with an apparent K_I value of 10 mM. Adding the carboxy-terminal regulatory region containing the CBD of the Ca^{2+}-ATPase to the cytoplasmic carboxy-terminus of the Na^+,K^+-ATPase partially restores ouabain sensitivity, reducing the K_I value for inhibition of Rb^+ uptake to 0.3 mM. The K_I value of ORα1-CBD is, nevertheless, still high enough to use the 'low-ouabain' protocol for subtraction of the endogenous contribution to the transport signal. Previously we have shown that mutation of putative sites for cation interaction also modifies ouabain sensitivity [18]. These findings and the present observation demonstrate that modification of only the cytoplasmic carboxy-terminus affects the extracellular ouabain binding, illustrating the high flexibility of the protein. Interesting in this respect are also the previous findings that the interaction of the α subunit with the β subunit is affected by CBD [15,16] though it is believed that the interaction between the α and β subunits occurs in extracellular domains [25].

4.2. The mutation to ouabain resistance does not affect the role of CBD in the ion transport mechanism

As we have shown for the ouabain-sensitive chimeric pump (α1-CBD), also the ORα1-CBD pump can obviously operate with high probability in an electrically silent mode. This is demonstrated by the fact that the ORα1-CBD pumps can mediate Rb^+ uptake 2.1 times higher than for the *Xenopus* pumps, but the electrogenic contribution is even less than that of the endogenous pump (about 70%). As we have discussed for the ouabain-sensitive chimeric pump [16], also the ORα1-CBD pump can most likely operate in the $2Na^+$,$2K^+$ transport cycle; the data in Table 1 yield a probability of more than 60% for this electrically silent mode.

4.3. The transient charge movements in the chimeric pump are reduced to the same extent as the electrogenic steady-state current

The Q_{max} value of the ORα1-CBD pumps is smaller than that of the ORα1 pumps by a factor of 4.2. This is nearly identical to the ratio in electrogenicity of 4.1 represented by the steady-state pump current (Table 1), and suggests that the remaining charge movements reflect the still existing $3Na^+$,$2K^+$ contribution generated by the ORα1-CBD pump. This is supported by the finding that the persisting charge movements can be described by similar effective valency z and midpoint potential V_m as for the ORα1 pump. A straightforward interpretation would be that Na^+,K^+ transport in the chimera is dominated by $2Na^+$,$2K^+$ transport. Partially the pump can still operate in its normal electrogenic $3Na^+$,$2K^+$ mode. In Na^+/Na^+ exchange, the pump could operate in $2Na^+/2Na^+$ and partially in $3Na^+/3Na^+$ exchange, but only the $3Na^+/3Na^+$ mode contributes to the transient current. In conclusion, this means that the movement of the 2 Na^+ ions does not contribute to detectable charge movements in the electrically silent $2Na^+$,$2K^+$ mode.

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References

[1] Schwarz, W. (1994) Cell. Physiol. Biochem. 4, 77–80.
[2] Noguchi, S., Mishina, M., Kawamura, M. and Numa, S. (1987) FEBS Lett. 225, 27–32.
[3] Ackermann, U. and Geering, K. (1990) FEBS Lett. 269, 105–108.
[4] Noguchi, S., Higashi, K. and Kawamura, M. (1990) Biochim. Biophys. Acta 1023, 247–253.
[5] Takeyasu, K., Tamkun, M.M., Renaud, K.J. and Fambrough, D.M. (1988) J. Biol. Chem. 263, 4347–4354.
[6] Lafaire, A.V. and Schwarz, W. (1986) J. Membr. Biol. 91, 43–51.
[7] Rakowski, R.F., Vasilets, L.A., LaTona, J. and Schwarz, W. (1991) J. Membr. Biol. 121, 177–187.
[8] Bielen, F.V., Glitsch, H.G. and Verdonck, F. (1991) J. Physiol. 442, 169–189.
[9] Gadsby, D.C., Rakowski, R.F. and DeWeer, P. (1993) Science 260, 100–103.
[10] Vasilets, L.A., Ohta, T., Noguchi, S., Kawamura, M. and Schwarz, W. (1993) Eur. Biophys. J. 21, 433–443.
[11] Hilgemann, D.W. (1994) Science 263, 1429–1432.
[12] Rakowski, R.F., Gadsby, D.C. and DeWeer, P. (1997) J. Membr. Biol. 155, 105–112.
[13] Carafoli, E. (1994) FASEB J. 8, 993–1002.
[14] James, P., Maeda, M., Fischer, R., Verma, A.K., Krebs, J., Penniston, J.T. and Carafoli, E. (1988) J. Biol. Chem. 263, 2905–2910.
[15] Ishii, T. and Takeyasu, K. (1995) EMBO J. 14, 58–67.
[16] Zhao, J., Vasilets, L.A., Yoshimura, S.H., Gu, Q., Ishii, T., Takeyasu, K. and Schwarz, W. (1997) FEBS Lett. 408, 271–275.
[17] Price, E.M. and Lingrel, J.B. (1988) Biochemistry 27, 8400–8407.
[18] Vasilets, L.A., Takeda, K., Kawamura, M. and Schwarz, W. (1997) Biochim. Biophys. Acta (in press).
[19] Schwarz, W. and Gu, Q.-B. (1988) Biochim. Biophys. Acta 945, 167–174.
[20] Nakao, M. and Gadsby, D.C. (1989) J. Gen. Physiol. 94, 539–565.
[21] Wuddel, I. and Apell, H.J. (1995) Biophys. J. 69, 909–921.
[22] Holmgren, M. and Rakowski, R.F. (1994) Biophys. J. 66, 912–922.
[23] Rettinger, J., Vasilets, L.A., Elsner, S. and Schwarz, W. (1994) in: The Sodium Pump (Bamberg, E. and Schoner, W., Eds.), pp. 553–556, Steinkopff Verlag, Darmstadt.
[24] Rakowski, R.F. (1993) J. Gen. Physiol. 101, 117–144.
[25] Lemas, M.V., Hamrick, M., Takeyasu, K. and Fambrough, D.M. (1994) J. Biol. Chem. 269, 8255–8259.