Heterologous expression of a mammalian epithelial sodium channel in yeast

Soma Sen Gupta, Cecilia M. Canessa*

Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06510, USA

Received 25 July 2000; accepted 11 August 2000

Edited by Maurice Montal

Abstract The α and β subunits of the amiloride-sensitive rat epithelial sodium channel (αβENaC) were expressed in the yeast *Saccharomyces cerevisiae*. We used a combination of yeast strains, including a mutant in the secretory pathway (*sec6*), and Western blotting techniques, to show that αβENaC was synthesized and targeted through the secretory system to the plasma membrane. Yeasts expressing αβENaC were more sensitive to salt than the parent strain. In addition, amiloride, a specific blocker of ENaC, was found to suppress salt sensitivity in the yeast strain expressing αβENaC.

Key words: ENaC; Amiloride; *ENA1* (*PMR2*); Degenerin

1. Introduction

ENaC is an amiloride-sensitive epithelial channel highly selective for Na^+ over K^+ ($P_{Na^+}/P_{K^+} = 100$) which mediates sodium reabsorption in the distal part of the renal tubule, the lung, the distal colon and ducts in several exocrine glands [1,2]. ENaC belongs to a large family of cation channels known as the ENaC/degenerin that also includes several mammalian acid-activated channels expressed in the nervous system (ASICs), the degenerins from *Caenorhabditis elegans* involved in the transduction of mechanosensitive stimuli, and the neuropeptide-activated channel from the ganglion of *Helix aspersa* (FaNaCh) [3]. ENaC channels are made up of three homologous subunits designated as α, β and γ. Each subunit contains two transmembrane domains and the amino- and carboxy-termini in the cytoplasmic side. Various combinations of subunits: αβ, αγ or α alone, form channels with distinct functional properties [4,5].

Structural studies of the ENaC/degenerin family of ion channels have been hampered so far by the difficulty in obtaining sufficient quantities of these proteins. The requirement for both a large yield and an inexpensive source of recombinant protein for structure–function studies has called for the development of non-mammalian expression systems. Expression in eukaryotic microorganisms such as the yeast *Saccharomyces cerevisiae* can provide an invaluable source of large and functional quantities of a mammalian protein. However, it is important to determine whether a protein expressed in a heterologous system is functional. In addition, it is important to verify that membrane proteins are not sequestered in the endoplasmic reticulum (ER). The nicotinic acetylcholine receptor from *Torpedo californica* and the human β_2-adrenergic receptor are examples of heterologous membrane proteins that have been produced successfully in yeast [6].

To establish whether ENaC could be expressed in yeast, the cDNAs encoding the α and β subunits of ENaC were subcloned into a yeast–mammalian shuttle vector (pSVA14) which harbors a heat-shock element that makes it possible to turn on synthesis of ENaC by heat-shock. Both the wild-type and a yeast strain carrying a temperature-sensitive *sec6-4* mutation [7] were used in these studies, to examine whether ENaC was delivered through the secretory pathway to the plasma membrane. In addition, a simple functional assay was used to assess the functional competence of ENaC expressed in yeast.

2. Materials and methods

2.1. Yeast strains

S. cerevisiae strains TF23 (*ura3-52, his3Δ200, leu2-3,112, GAL2*) and SY1 (*Matα, ura3-52, leu2-3,112, his4-619, sec6-4*^{ts}, *GAL2*) were used in these studies. TF23 was a gift from T. Ferreira. SY1 has been described in detail by Nakamoto et al. [8], and the *sec6-4*^{ts} mutation by Schekman and Novick [7].

2.2. Plasmid construction and yeast transformation

A construct containing the coding sequences of the α and β subunits of ENaC in a single polypeptide was made as previously described [3]. The αβ dimer was subcloned into the expression vector pSVA14 [9] in the sites *Hin*dIII and *Bam*HI, under the control of a heat-shock-activated promoter. pSVA14 ± αβENaC were transformed into yeast cells by the method of Ito et al. [10]. Plasmid loss was checked by growing the transformed yeast cells aerobically at 30°C under non-selective conditions for 48 h and then plating a suitable dilution on selective and non-selective media.

2.3. Drop tests

TF23 cells transformed with either pSVA14 or pSVA14-αβENaC were grown at 30°C on synthetic medium lacking leucine (CSM−leu medium from BIO101, Vista, CA, USA) and containing 2% (w/v) glucose. The cells were then diluted to ∼1 A_{600}/ml in sterile water and 2.5 μl droplets from each strain were placed on YPD medium containing 2% (w/v) bactopeptone, 1% (w/v) yeast extract (both from Difco, MI, USA), 2% (w/v) glucose and supplemented with 0, 0.5 or 1.0 M NaCl. The plates were incubated at 37°C and photographed after 24 h.

2.4. Growth curves

TF23 cells or TF23 transformed with pSVA14-αβENaC were grown aerobically at 30°C for 14 h on 25 ml of synthetic medium lacking leucine and containing 2% (w/v) glucose. After 14 h, the yeast strains were diluted to ∼0.2 A_{600}/ml into 25 ml fresh CSM−leu medium containing 2% (w/v) glucose, with or without 1 M NaCl. These cells were allowed to double at 30°C, such that the A_{600}/ml was 0.4. The strains were then shifted to 39°C for 2 h to turn on the heat-shock promoter, and subsequently returned to 30°C. The A_{600}/ml was measured every hour for a period of 24 h.

*Corresponding author. Fax: (1)-203-785 4951.
E-mail: cecilia.canessa@yale.edu

0014-5793/00/$20.00
PII: S0014-5793(00)01977-3

2.5. Amiloride sensitivity test

TF23 transformed with pSVA14-αβENaC and TF23 with pSVA14 (i.e. strain transformed with empty vector) were grown aerobically in CSM−leu medium, until an $OD_{600} \sim 0.5$. 0.5 ml of each culture was then subjected to the following conditions: 1 M NaCl, 1 M NaCl plus 2.5 μM amiloride, 1 M NaCl plus 5.0 μM amiloride, 1 M NaCl plus 10.0 μM amiloride. The controls neither contained NaCl nor amiloride. Each condition was carried out in duplicate. The strains were grown at 39°C for 24 h, and the A_{600}/ml values of the stationary phase cultures were recorded.

2.6. Isolation of yeast plasma membrane

Plasma membranes were prepared essentially as described by McCusker et al. [11]. Briefly, TF23 or SY1 cells ± pSVA14-ENaC and ± 1 M NaCl were grown to an A_{600}/ml ∼ 2 in CSM−leu containing 2% (w/v) glucose. The cells were then heat-shocked for 2 h at 39°C to turn on the heat-shock element of pSVA14. After heat-shock, the SY1 cultures were then returned to 23°C for a further 3 h, to permit the secretory vesicles to fuse with the plasma membrane. All the subsequent steps were carried out at 4°C. The cells were harvested by centrifugation, and resuspended in 6 ml Perlin lysis buffer (50 mM Tris–HCl, pH 7.75, 0.3 M sucrose, 5 mM EDTA, 1 mM EGTA, and protease inhibitors including 1 mM DFP), and lysed by passage through a French Pressure Cell (SLM-Aminco, Urbana, IL, USA) at 20 000 psi. The lysate was adjusted to pH 7.5 with Tris base. Following low speed ($3500 \times g$ for 5 min) and high speed ($14\,000 \times g$ for 10 min) centrifugations, the supernatant was centrifuged at $200\,000 \times g$ for 45 min, and the membrane pellet was washed with 1 mM EGTA/Tris buffer (pH 7.5) with protease inhibitors and centrifuged at $200\,000 \times g$ for 35 min, and resuspended in a small volume of the same buffer.

2.7. Isolation of yeast secretory vesicles

SY1 cells were grown to mid-exponential 1 A_{600}/ml at 23°C in minimal medium supplemented with 2% (w/v) galactose. The cells were harvested and resuspended in minimal medium supplemented with 2% (w/v) glucose for 3 h; and transferred to 39°C for two additional hours. The cells were harvested, washed and lysed, and secretory vesicles were isolated by sucrose density gradient centrifugation as described by Ambesi et al. [12].

2.8. Protein assay

Protein concentrations were measured by the method of Lowry et al. [13].

2.9. Western blotting

To visualize αβENaC in either the secretory vesicles or plasma membranes, protein samples containing 20–60 μg were subjected to 10% SDS–PAGE followed by immunoblotting on to an Immobilon P membrane [8] and probed with polyclonal antiserum raised in rabbits against the amino-terminus of the α subunit of ENaC [14]. The membranes were washed and treated with 1 μCi of [^{125}I]protein A (ICN, Irvine, CA, USA), and exposed to a PhosphorImager screen for 24 h. The bands were then visualized with a PhosphorImager programmed with ImageQuant Software Version 5 (Molecular Dynamics, Sunnyvale, CA, USA).

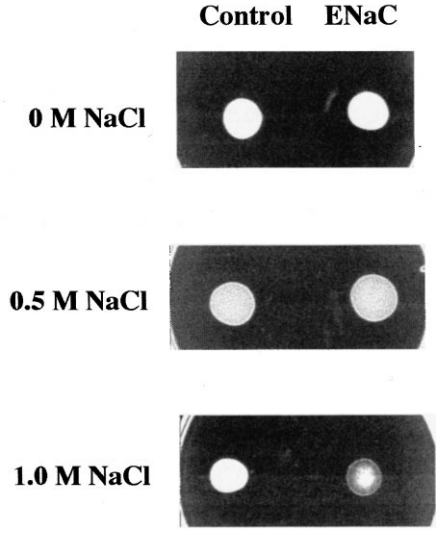

Fig. 1. Drop tests of TF23 transformed with pSVA14 empty vector (control) or pSVA14-αβENaC (ENaC) on YPD medium supplemented with 0, 0.5 or 1.0 M NaCl. The plates were incubated at 37°C for 24 h.

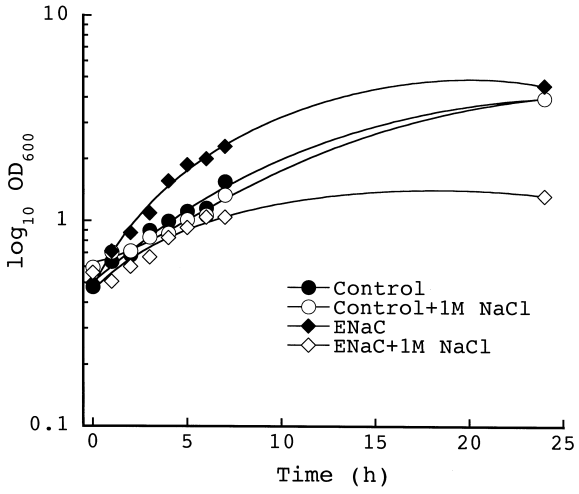

Fig. 2. Growth curves of parental TF23 (control) ± 1 M NaCl and TF23 transformed with pSVA14-αβENaC (ENaC) ± 1 M NaCl at 30°C. Cells were subjected to an initial heat-shock at 39°C for 2 h to turn on the synthesis of αβENaC, and then returned to 30°C for 24 h, and the A_{600}/ml was recorded at regular intervals ($n = 4$, S.E.M. ∼ 5%).

3. Results

3.1. pSVA14-αβENaC confers salt sensitivity to TF23 yeast

When αβ channels are expressed in *Xenopus* oocytes, they are constitutively open and exhibit high selectivity to sodium ions [4]. To examine whether αβENaC is functionally active when expressed in yeast, we tested the effect of high sodium concentration added to the medium on the rate of growth of TF23 transformed with pSVA14 empty vector and TF23 transformed with pSVA14-αβENaC.

TF23 transformants were 'seeded' on YPD plates containing 0, 0.5 or 1.0 M NaCl. The heat-shock element in the pSVA14 vector was activated by incubating the plates at 37°C for 24 h. All TF23 strains grew equally well in the control (0 M NaCl) and in 0.5 M NaCl. However, when the concentration of NaCl was increased to 1.0 M, the strain transformed with αβENaC grew poorly in comparison to the strain transformed with empty vector (Fig. 1).

We also examined the effect of NaCl on the growth of TF23 ± pSVA14-αβENaC in liquid media. The heat-shock element in the vector pSVA14 was turned on by incubating the strains at 39°C for 2 h. After the 2 h heat-shock, the strains were incubated at 30°C for 24 h. Growth curves were constructed by measuring the absorbance at 600 nm every 2 h (Fig. 2). The parent TF23 strain grew well in 0 and 1.0 M NaCl. The strain expressing αβENaC seemed to grow slightly better than TF23 at 30°C in the absence of NaCl, although the difference was not statistically significant. However, in the presence of 1.0 M NaCl, the αβENaC strain exhibited a 70% reduction in growth after 24 h. In addition, when the strain

lost the plasmid (pSVA14-αβENaC), by growing the cells under non-selective conditions, it reverted to the wild-type phenotype being able to grow well in high salt medium.

The results from the drop test and the growth curves indicate that αβENaC confers salt sensitivity to the parent TF23 yeast strain, suggesting that functional sodium channels are expressed in the plasma membrane of transformed TF23 cells.

3.2. Effect of amiloride on salt sensitivity of TF23

Amiloride is a specific blocker of ENaC with a half-inhibition constant of 1 μM for αβ channels [3,4]. If salt sensitivity is a property conferred by functional channels, the presence of amiloride in the medium should at least partially block the activity of αβ expressed in TF23 cells and allow them to grow in high salt medium. We tested this hypothesis by growing TF23 transformed with either pSVA14 or pSVA14-αβENaC in 1 M NaCl with increasing concentrations of amiloride. Fig. 3 shows that in high salt medium, cells expressing αβENaC have a 40% reduction in growth which returns to approximately normal levels in the presence of 2.5 μM amiloride. In contrast, the control is not affected by either high salt or amiloride.

3.3. ENaC is targeted to the plasma membrane

The previous functional assays strongly suggested that αβENaC channels are expressed at the cell surface where they mediate the entrance of sodium into the cell. To determine the location(s) of the αβ proteins in the transformed yeast strains, we performed Western blot analysis of various cellular membrane fractions. To see whether αβENaC was targeted through the secretory pathway to the plasma membrane, the strain SY1 expressing pSVA14-αβENaC was used to isolate secretory vesicles and plasma membranes.

All the functional assays were carried out with TF23 because it is neither *sec6* nor temperature-sensitive. To see whether the channel was expressed in TF23 in either the presence or absence of 1 M NaCl, plasma membranes were prepared from this strain ± pSVA14-αβENaC. Western blotting was carried out using a well characterized anti-α antibody [14].

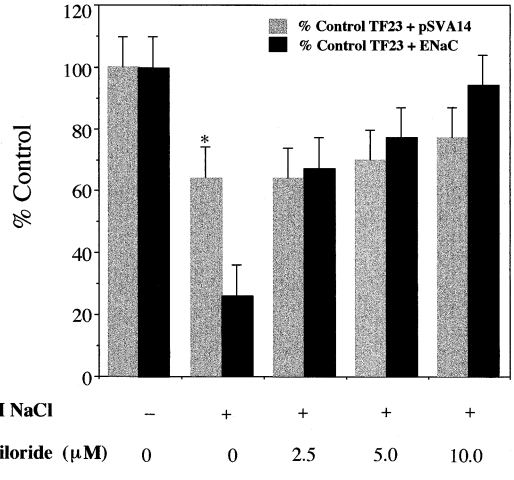

Fig. 3. Effect of amiloride on growth at 39°C in the presence of 1.0 M NaCl. A_{600}/ml values were recorded after 24 h for TF23 transformed with pSVA14 (TF23+pSVA14) and TF23 transformed with pSVA14-αβENaC (TF23+ENaC), all values were expressed as a % of the control ($n=2$, S.E.M. ~ 10%, $^*P=0.02$).

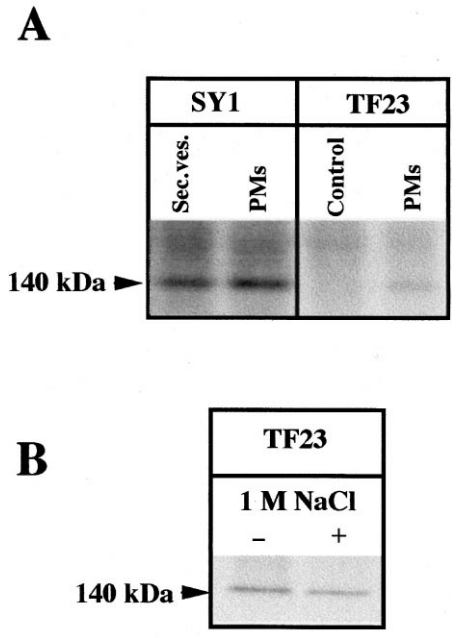

Fig. 4. A: Western blotting of 20 μg secretory vesicles (Sec. ves.) and 20 μg plasma membranes (PMs) of SY1 transformed with pSVA14-αβENaC. TF23 plasma membrane preparations of control (empty vector), and TF23 transformed with pSVA14-αβENaC (PMs). B: Western blotting of 60 μg TF23 PMs in the presence or absence of 1 M NaCl.

Fig. 4A shows that αβENaC is expressed in both the secretory vesicles and plasma membrane preparations of SY1, hence αβENaC is targeted through the secretory system to the plasma membrane in yeast. Fig. 4A,B shows that αβENaC is also expressed in the plasma membrane of TF23 in the presence or absence of salt. However, the relative levels of αβENaC getting to the plasma membrane in SY1 and TF23 are different.

4. Discussion

Most yeasts can sustain normal growth in the presence of high external sodium concentrations in spite of the very large electrochemical gradient favoring the influx of sodium into the cytoplasm (resting membrane potential of approximately −300 mV). Typically, salt tolerance in yeast is both strain- and pH-dependent [15]. Yeast can withstand NaCl concentrations within the range of 1–2 M. Sodium tolerance results from a combination of very low sodium permeability of the plasma membrane and the expression of a family of genes that efficiently extrude sodium, for example *ENA1* (*PMR2*) [16].

Expression of this mammalian sodium selective epithelial sodium channel made yeast more sensitive to salt. This result was anticipated if some functional αβ channels were expressed at the cell surface.

In contrast to the voltage-dependent sodium channels that require depolarization of the plasma membrane for activation, αβ channels are constitutively open with a very high open probability ($P_o \sim 1$). A large influx of sodium in transformed TF23 cells was the most likely cause for toxicity manifested by a decrease in the rate of growth when sodium was present in the medium. The effect was specific because transformation with the empty plasmid did not alter the phenotype. Most

convincing was the demonstration that amiloride reverted the salt-sensitive phenotype. Amiloride inhibition of other proteins was not expected with the doses employed in our experiments (<10 μM). Moreover, the action of amiloride was mainly on plasma membrane proteins because at pH lower than 7.0 almost all the drug is positively charged making it impermeant to the plasma membrane.

In the experiments that addressed the traffic of αβENaC in several membrane compartments, we showed that αβ is expressed in both secretory vesicles and plasma membranes isolated from SY1. This result suggests that the αβ channels are going through the secretory pathway to the plasma membrane, as opposed to being sequestered in the ER where they will eventually be degraded. The data indicate that αβENaC is correctly folded and targeted to the plasma membrane in spite of that the yeast genome does not have any homologues to the ENaC/degenerin family of proteins.

Acknowledgements: We thank Professor Carolyn W. Slayman for her generosity in providing laboratory space, equipment, and reagents. We thank Ken Allen for his invaluable technical assistance and stimulating discussions, and Dr. T. Ferreira for providing the *S. cerevisiae* strain TF23. This work was supported by NIH Grant HL56163.

References

[1] Canessa, C.M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Gautschi, J.-D., Horisberger and Rossier, B.C. (1994) Nature (Lond.) 367, 463–467.
[2] Canessa, C.M., Merillat, A.-M. and Rossier, B.C. (1994) Am. J. Physiol. 267, 1682–1690.
[3] McNicholas, C.M. and Canessa, C.M. (1997) J. Gen. Physiol. 109, 681–692.
[4] Fyfe, G.K. and Canessa, C.M. (1998) J. Gen. Physiol. 112, 423–432.
[5] Garty, H. (1997) Physiol. Rev. 77, 359–396.
[6] Romanos, M.A., Scorer, C.A. and Clare, J.J. (1992) Yeast 8, 423–488.
[7] Schekman, R. and Novick, P.J. (1982) in: The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression (Strathern, J.N., Jones, E.W. and Broach, J.R., Eds.), pp. 361–398, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[8] Nakamoto, R.K., Rao, R. and Slayman, C.W. (1991) J. Biol. Chem. 266, 7940–7949.
[9] Verjovski-Almeida, S., Kurtenbach, E. and Slayman, C.W. (1994) Subcellular localization of rabbit calcium-ATPase expressed in yeast, Annals of the Annual Meeting of the 'Sociedade Brasileira de Bioquimica e Biologia Molecular', p. 65, Caxambu.
[10] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[11] McCusker, J.H., Perlin, D.S. and Haber, J.E. (1987) Mol. Cell Biol. 7, 4082–4088.
[12] Ambesi, A., Allen, K.E. and Slayman, C.W. (1997) Anal. Biochem. 251, 127–129.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Duc, C., Farman, N. and Canessa, C.M. et al. (1994) J. Cell. Biol. 127, 1907–1921.
[15] Sychrova, H., Ramirez, J. and Pena, A. (1999) FEMS Microbiol. Lett. 171, 167–172.
[16] Rodriguez-Navarro, A., Quintero, F.J. and Garciadeblas, B. (1994) Biochim. Biophys. Acta 1187, 203–205.