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Rapid report

Hypertonicity enhances expression of functional $Na^+/K^+/2Cl^-$ cotransporters in Ehrlich ascites tumour cells

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Abstract

Ehrlich cells exposed to a hypertonic medium for five hours respond by an increased expression of $Na^+/K^+/2Cl^-$ cotransport proteins as estimated from immunoprecipitations using polyclonal anti-cotransporter antibodies. The 3.4-fold increase in cotransport expression is followed by a concomitant 2.6-fold increase in the maximal bumetanide-sensitive K^+ influx during regulatory volume increase, indicating a 2.6-fold increase in the number of functional cotransporters in the plasma membrane. © 1997 Elsevier Science B.V.

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$Na^+/K^+/2Cl^-$ cotransport is present in most animal cells, and participates in transepithelial transport of salt and water (see [1,2]). In symmetrical cells, activation of $Na^+/K^+/2Cl^-$ cotransport is one of the principal cellular responses to shrinkage, and it promotes the uptake of KCl and water to restore cell volume upon cell shrinkage (regulatory volume increase: RVI). Although the major role of the cotransporter in single cells is to maintain and regulate cell volume, activation of the $Na^+/K^+/2Cl^-$ cotransporter has been reported to be one of the first mitogenic signals [3–6], providing another important function of this transporter in symmetrical cells.

Activation by specific agonists of the $Na^+/K^+/2Cl^-$ cotransporter depends on the tissue of origin, and a variety of signal transduction pathways have been suggested to be involved in this activation of the cotransporter (see [1,2,7,8]). Short-term regulation by agonists is mediated by phosphorylation and dephosphorylation of the cotransport protein itself (e.g. [9–14]).

Prolonged cell shrinkage has been shown to result in an increased expression of Na^+-coupled osmolyte cotransporter, such as the Na^+/Cl^--dependent taurine transporter [15], and the Na^+-dependent inositol transporter [16]. The increased expression of these transporters is due primarily to an increased transcription rate [17]. In contrast, the effect of prolonged exposure to hypertonicity on $Na^+/K^+/2Cl^-$ cotransport expression and activity remains to be investigated.

In Ehrlich cells, polyclonal antibodies raised against proteins of the $Na^+/K^+/2Cl^-$ cotransporter,

Abbreviations: RVI, regulatory volume increase

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recognised proteins of ~80 and ~40 kDa in Western blots of cell membranes, and inhibited function of the cotransporter [18]. The inhibition of cotransport function was measured by two independent methods, i.e. inhibition of regulatory cell volume increase after cell shrinkage, and inhibition of unidirectional bumetanide-sensitive Cl^- influx after cell shrinkage. In addition, Fab fragments from these antibodies were equipotent in inhibiting cell volume regulation after cell shrinkage. Thus the antibodies were concluded to bind to an extracellular domain of the cotransporter [18]. In mouse kidney and in *Xenopus laevis* oocytes, proteins of 140 kDa and 175 kDa, respectively, were recognised by these anti-cotransport antibodies [19,20]. We have now employed the antibodies to investigate the effect of prolonged exposure to a hypertonic medium on the expression of the $Na^+/K^+/2Cl^-$ cotransporter in Ehrlich cells: expression of cotransporters is increased 3.4-fold, and this increase is followed by a concomitant 2.6-fold increase in the maximal cotransport activity as measured during a RVI response upon extensive cell shrinkage.

Materials and methods. Immunoprecipitation of $Na^+/K^+/2Cl^-$ cotransporters. Immunoprecipitation of ^{35}S-labelled $Na^+/K^+/2Cl^-$ cotransporters were conducted according to Harlow and Lane [21], after a five hour exposure of Ehrlich cells to a labelling mix of 70% [^{35}S]methionine, 30% [^{35}S]cysteine (0.2 MBq/ml) (Promix™, Amersham Int., UK) in RPMI 1640 isotonic medium (control) (Biological Industries, Israel) or in RPMI 1640 hypertonic medium (60 mOsm sucrose added).

$Na^+/K^+/2Cl^-$ cotransport activity. $Na^+/K^+/2Cl^-$ cotransport activity during RVI was measured as the unidirectional, bumetanide-sensitive K^+ influx using $^{86}Rb^+$ (Risø, Denmark) as a tracer for K^+ (see [22]).

Results and discussion. Metabolic labelling of Ehrlich cells with [^{35}S]methionine/[^{35}S]cysteine results in a rapid labelling of a wide range of proteins. Using polyclonal antibodies against the $Na^+/K^+/2Cl^-$ cotransporter [18], we have immunoprecipitated cotransport proteins of Ehrlich cells. Fig. 1 shows a fluorograph of ^{35}S-labelled immunoprecipitated (Fig. 1, lane 2) or non-precipitated proteins (Fig. 1, lane 1) from Ehrlich cells. The polyclonal antibodies specifically precipitates four bands which on Fig. 2, lane 1, can be recognised as ~40 kDa, 76 kDa, 80 kDa and 205 kDa (Fig. 1, lane 2; Fig. 2, lane 1). From Fig. 1, lane 2, it is seen that the 205 kDa band is completely removed by immunoprecipitation. The ~40 kDa and 76–80 kDa proteins correspond well with the previously purified bumetanide-binding proteins [23] as well as with the proteins recognised in Western blots [18]. The 205 kDa protein immunoprecipitated here (Fig. 1), has not previously been shown to be detected in Western blots of Ehrlich cell membranes. A similar protein was, however, found during the purification of the bumetanide-binding proteins from Ehrlich cells, when these proteins were separated using gel electrophoresis under non-denaturing conditions [23]. In crude extracts of mouse kidney, the antibodies detected a protein of 150 kDa ([19]), and in *Xenopus laevis* oocytes, a 175 kDa protein is the target of these polyclonal antibodies [20]. Recent cloning of a variety of $Na^+/K^+/2Cl^-$ cotransporters have revealed a molecular size of the primary translation products of 140–160 kDa [24], which increases posttranslationally to 160–200 kDa due to glycosylation of the protein. The molecular weights of the glycosylated cotransporters correspond well with the immunoprecipitated 205 kDa protein (Figs. 1 and 2). Further, the antibodies has previously been found to inhibit both volume regulation after cell shrinkage [18] as well as the bumetanide-sensitive Cl^- and K^+ influxes during the RVI response following cell shrinkage [18,22]. Thus, the 205 kDa, the ~80 kDa, and/or the ~40 kDa proteins precicipated by the antibodies are likely to be constituents of the Ehrlich cell $Na^+/K^+/2Cl^-$ cotransporter.

The effect of prolonged exposure to a hypertonic medium on the expression of the $Na^+/K^+/2Cl^-$ cotransporter has not yet been described. We therefore estimated the level of expression by immunoprecipitation of newly synthesised cotransporters. Ehrlich cells were metabolically labelled with [^{35}S]methionine/[^{35}S]cysteine during incubation in either RPMI 1640 medium (calculated final osmolarity: 300 mOsm) or RPMI 1640/sucrose medium (calculated final osmolarity: 360 mOsm). An incubation period of 5 h was chosen to ensure significant expression of cotransporters. Fig. 2 shows immunoprecipitated material from cells incubated for 5 h in hypertonic medium (lane 1), or in isotonic medium (lane 2). Samples containing equal amounts of la-

belled total cellular protein (newly synthesised) are subjected to immunoprecipitation in the two cases. It is evident that the amount of labelled precipitated proteins in cells exposed to hypertonic stress is larger than in control cells. All three bands at 41, 76–80 and 205 kDa are increased in two experiments, while in gels from three other experiments a significant increase was observed only in the 205 kDa band as compared to the control. Densitometric analysis of five gels like the one shown in Fig. 2, revealed that the total amount of labelled immunoprecipitated protein (total peak area) was increased, and further that the integrated density of the 205 kDa band is increased 3.4-fold in hypertonically stressed cells as compared to unchallenged cells (Table 1). An additional 50 kDa band, not found in isotonic medium, was seen in all the immunoprecipitates from cells in hypertonic medium. Since the same amount (equal number of cpm) of newly synthesised total cellular protein was used for each immunoprecipitation from either control or hypertonically stressed cells, the increased radioactivity of the bands is due to an increased expression of the $Na^+/K^+/2Cl^-$ cotransporter relative to the majority of cellular proteins. The increased expression of cotransporters is not necessarily an increase of the protein in the plasma membrane. Whether the increase in the expression of cotransporters corresponded to an increase in functional carriers in the membrane, was tested by measurement of the bumetanide-sensitive K^+ influx ($Na^+/K^+/2Cl^-$ cotransport activity) during RVI. The cotransport activity was measured after addition of a hypertonic experimental medium where the external concentrations of Na^+, K^+ and Cl^- were well above the apparent K_m values (see [25]). This ensures that the inward driving force for the $Na^+/K^+/2Cl^-$ cotransporter is high, and that the rate of transport is maximal (equal to V_{max}). Fig. 3 shows the cotransport activity during RVI in Ehrlich cells after prolonged isotonic or hypotonic preincuba-

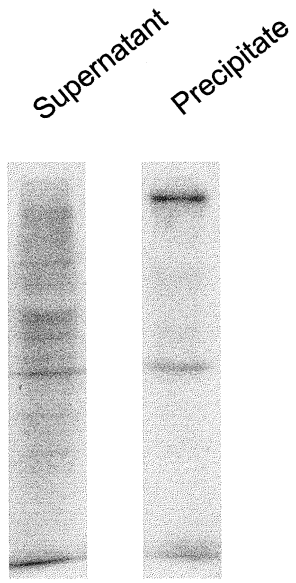

Fig. 1. Immunoprecipitation of $Na^+/K^+/2Cl^-$ cotransporters from Ehrlich cells. Immunoprecipitation of proteins was performed essentially as described by Harlow and Lane [21]. Polyclonal antibodies against bumetanide-binding proteins of Ehrlich cells were used [18]. Ehrlich ascites tumour cells (hyper diploid strain) were harvested as described previously [22], and kept at 37°C in a waterbath with gentle shaking. Cells were incubated at cytocrit 0.5% with ^{35}S-labelled methionine/cysteine mix (0.1–0.2 MBq/ml) in RPMI 1640 medium. The period of incubation was 5 h. After the incubation, cells were washed twice in RPMI 1640 (centrifuged for 45 s, $700\times g$) and lysed in lysis buffer: 150 mM NaCl, 1.0% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0) at 4°C. The cell lysate was incubated for 30 min on ice, and then centrifuged for 10 min, $20\,000\times g$, 4°C. The supernatant containing solubilized proteins was transferred to a new tube containing 50 μl pre-immune serum from the rabbit used for antigen production preabsorbed to protein A-Sepharose beads (Pharmacia, Sweden). The solubilized proteins were incubated rotating for one hour at 4°C, and the mixture was spun for 1 min, $10\,000\times g$, 4°C to collect the proteins binding antibodies from normal rabbit serum. The supernatant was transferred to a new tube containing specific anti-serum preabsorbed to protein A-Sepharose, and incubated overnight at 4°C with slow rotation. The protein A-Sepharose/antibody/cellular protein complex were collected by centrifugation (1 min, $10\,000\times g$, 4°C). A 10 μl sample of the supernatant (non-immunoprecipitated proteins; lane B) was mixed with 50 μl Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM β-mercaptoethanol, 60 mM Tris (pH 6.8), 0.01% Bromphenol blue). The protein A-Sepharose antibody complex was washed once in lysis buffer followed by washes in 80% (v/v) lysis buffer (diluted with water), 60% lysis buffer, 40% lysis buffer and 20% lysis buffer. After the final wash, 50 μl of Laemmli sample buffer was added (lane A). Samples (non-immunoprecipitated and immunoprecipitated proteins) were incubated at 95°C for 10 min, and separated by SDS-PAGE (9%). The gels were stained with Coomassie blue, treated with 1 M sodium salicylate, and subsequently dried under vacuum. The ^{35}S-labelled proteins were subsequently visualized by fluorography. Similar results were obtained in three other experiments.

Table 1
Effect of prolonged hypertonicity on $Na^+/K^+/2Cl^-$ cotransport expression and activity

	Control	5 h hypertonic pretreatment	P
$Na^+/K^+/2Cl^-$ cotransport activity during RVI (relative to control)	1 (3)	2.6 ± 0.51 (3)	≤ 0.006
Amount of $Na^+/K^+/2Cl^-$ cotransporters (relative to control)	1 (4)	3.4 ± 1.40 (4)	≤ 0.02

$Na^+/K^+/2Cl^-$ cotransport activity during RVI was measured in paired experiments as described in Fig. 3: the cotransport activity was measured at 12 ± 6.0 μmol (g dry wt.)$^{-1}$ min^{-1} (Control) and 30×11.8 (5 h hypertonic pretreatment) using 630 mOsm saline solution (in mM: 300 Na^+, 300 Cl^-, 10 K^+, 2 Ca^{2+}, 2 Mg^{2+}.2 SO_4^{2-}, 2 HPO_4^{2-}, 3.3 Mops, 3.3 Tes, 5 Hepes, 630 mOsm calculated, pH 7.4). The amount of cotransporters were measured by densitometric analysis of gels. Briefly, fluorographs were transferred to a Compaq Desktop computer using CREAM™ software (Kem-En-Tec, Denmark) equipped with a Panasonic CCTV camera (model WV-BL200). Gels were scanned, and arbitrary densitometric values obtained using the CREAM™ software. In each experiment, the 205 kDa band of controls was taken as a measure for the amount of $Na^+/K^+/2Cl^-$ cotransporters. It was set to 1 and the densitometric values were calculated relative to the control. Mean $\pm$ S.D. is shown and the number of independent experiments are given in parenthesis.

tion. During RVI, the cotransport activity in control cells is 12 ± 6.0 μmol (g dry wt.)$^{-1}$ min^{-1} (mean $\pm$ S.D., $n = 3$). This is less than the cotransport activity normally found during RVI, but still much higher than the activity found at isotonic steady state (1 ± 0.8 μmol (g dry wt.)$^{-1}$ min^{-1}, mean $\pm$ S.D., $n = 4$; see [22]). In cells exposed to a hypertonic medium for five hours, the cotransport activity during RVI is increased to 30 ± 11.8 μmol (g dry wt.)$^{-1}$ min^{-1} (mean $\pm$ S.D., $n = 3$), which is significantly higher than in control cells ($P \leq 0.006$ in a paired Student's t-test). Thus, the increased expression of $Na^+/K^+/2Cl^-$ cotransport protein, demonstrated by immunoprecipitation (Fig. 1, Table 1), is accompa-

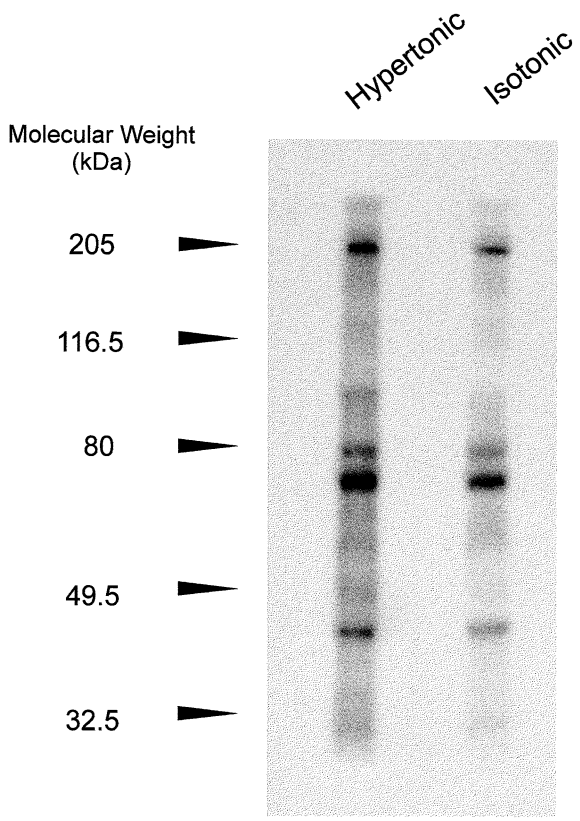

Fig. 2. Effect of hypertonic stress on the amount of immunoprecipitated $Na^+/K^+/2Cl^-$ cotransporters from Ehrlich cells. Immunoprecipitation of proteins was performed essentially as described in Fig. 1. Ehrlich cells were incubated at cytocrit 0.5% with ^{35}S-labelled methionine/cysteine mix (0.1–0.2 MBq/ml) in RPMI 1640 medium (control) or in RPMI 1640 medium with 60 mosM sucrose (hypertonic). The period of incubation was 5 h. After the incubation cells were washed twice in RPMI 1640 (centrifuged for 45 s, $700 \times g$) and lysed in lysis buffer: 150 mM NaCl, 1.0% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0) at 4°C. Upon preclearing with normal rabbit serum, the lysate was spun for 1 min, $10\,000 \times g$, 4°C and an aliquot of the supernatant was counted using liquid scintillation counting. Equal amounts of cpm from the control and hypertonic tubes, corresponding to equal amounts of newly synthesized protein (within five hours), were transferred to new tubes containing specific anti-serum preabsorbed to protein A-Sepharose. (No significant difference in cpm/mg dry wt. between isotonic and hypertonic treated cells was observed in the four experiments). After the immunoprecipitation and subsequent washes (as in Fig. 1), 50 μl of Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM β-mercaptoethanol, 60 mM Tris (pH 6.8), 0.01% Bromphenol blue) was added, and the immunoprecipitated proteins were incubated at 95°C for 10 min, and separated on a 9% cel using SDS-PAGE. The gels were stained using Coomassie blue, treated with 1 M sodium salicylate, and subsequently dried under vacuum. The precipitated proteins were subsequently visualized by fluorography. Similar results were obtained in four other experiments.

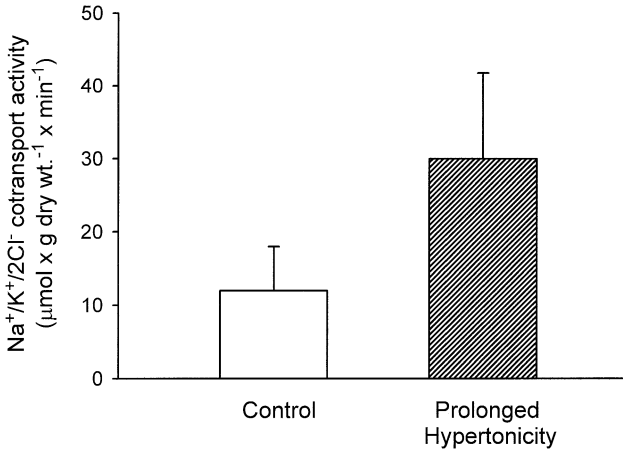

Fig. 3. Effect of prolonged exposure to a hypertonic medium on the activity of the $Na^+/K^+/2Cl^-$ cotransporter during regulatory volume increase. The $Na^+/K^+/2Cl^-$ cotransporter activity during a RVI response upon cell shrinkage was measured as the bumetanide-sensitive K^+ influx using $^{86}Rb^+$ as a tracer for K^+. Ehrlich ascites tumour cells (hyper diploid strain) were harvested as described previously [22]. The cells were kept at 37°C in a waterbath with vigorous shaking. The cytocrit was adjusted to 0.5%, and cells were incubated for 5 h in RPMI medium (Control) or in RPMI 1640 supplemented with 60 mOsm sucrose (Hypertonic stress). After the incubation, cells were centrifuged for 30 s, 700 × *g*, and resuspended in hypertonic standard medium (in mM: 300 Na^+, 300 Cl^-, 10 K^+, 2 Ca^{2+}, 2 Mg^{2+}, 2 SO_4^{2-}, 2 HPO_4^{2-}, 3.3 Mops, 3.3 Tes, 5 Hepes, 630 mOsm calculated, pH 7.4) containing ^{86}Rb (50000 Bq/ml). The influx experiments were conducted as described previously [22]. Samples were taken at intervals for removal of extracellular ^{86}Rb by cation exchange chromatography. In half of the experiments, bumetanide (final concentration: 30 μM) was present in the fluxmedium. The unidirectional K^+ influx, presented in μmol (g dry wt.)$^{-1}$ min^{-1}, was calculated by linear regression from the radioactivity of the cell lysates, the specific activity of the medium and the dry weight of the cell suspension. All correlation coefficients were ≥ 0.98. The bumetanide-sensitive, unidirectional K^+ influx taken to represent the $Na^+/K^+/2Cl^-$ cotransporter, was calculated as the difference between the unidirectional K^+ influx of the cells exposed to bumetanide and the unidirectional K^+ influx of cells in the absence of bumetanide. The results are presented as mean ± S.D., with the number of independent experiments in brackets.

nied by a concomitant increase in maximal cotransport activity (Fig. 3, Table 1), suggesting that a hypertonic stimulus results in an increase in functionally expressed cotransporters in the plasma membrane. $Na^+/K^+/2Cl^-$ cotransport activity is measured at V_{max}, and therefore a stoichiometric relationship between transport and the amount of protein of 1:1 might be expected. Upon prolonged exposure to hypertonicity, the maximal rate of transport is increased 2.6 ± 0.51 fold, which is not significantly different from the increase in the amount of transporters (3.4 ± 1.40 fold).

When hypertonicity is maintained for 24 h or more, i.e. longer than the incubation time applied is this study (5 h), cells slowly replace the excess of KCl taken up immediately after cell shrinkage by accumulating non-perturbing (or compatible) osmolytes (see [17,26]). In e.g. Madin–Darby canine kidney (MDCK) cells cultured in hypertonic medium for days, accumulation of the compatible osmolytes betaine and inositol result from an increased transcription of the mRNAs encoding the Na^+-coupled cotransporter for each osmolyte [16,27]. It appears that Ehrlich cells during the first few hours after onset of hypertonicity attempt to recover cell volume by an increased expression of $Na^+/K^+/2Cl^-$ cotransporters, and a concomitant increase in maximal transport rate. After this initial period, a switch in cellular osmolyte content probably takes place from a high $[KCl]_i$, the electrolytes responsible for most of the intracellular osmotic activity in the initial period, to a gradual increase in the amount of compatible osmolytes resulting from increased expression of the Na^+-coupled organic osmolyte cotransporters. This is important to the cells since high $[K^+]_i$ has a perturbing effect on many processes whereas the organic osmolytes are compatible. Whether the increased expression of $Na^+/K^+/2Cl^-$ cotransporters is a result of an increased transcription rate as in the case of the Na^+-coupled osmolyte transporters (see [26]), awaits structural information on the DNA level of the Ehrlich cell $Na^+/K^+/2Cl^-$ cotransporter in order to measure mRNA abundance by northern hybridization.

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References

[1] M. Haas, Am. J. Physiol. 267 (1994) C869–C885.
[2] E.K. Hoffmann, P.B. Dunham, Int. Rev. Cytol. 161 (1995) 173–262.

[3] J.P. Anger, L. Vernhet, A. Hichami, A. Corlu, E. Lepalabe, A. Troussard, A. Legrand, Arch. Int. Pharmacodyn. Ther. 329 (1995) 307–318.
[4] E. Berman, I. Sharon, H. Atlan, Biochim. Biophys. Acta 1239 (1995) 177–185.
[5] R. Panet, M. Markus, H. Atlan, J. Cell. Physiol. 158 (1994) 121–127.
[6] O. Bussolati, J. Uggeri, S. Belletti, V. Dall'Asta, G.C. Gazzola, FASEB J. 10 (1996) 920–926.
[7] M. Haas, Annu. Rev. Physiol. 51 (1989) 443–457.
[8] H.C. Palfrey, M.E. O'Donnell, Cell Physiol. Biochem. 2 (1992) 293–307.
[9] M. Haas, D.G. McBrayer, C. Lytle, J. Biol. Chem. 270 (1995) 28955–28961.
[10] J.D. Klein, W.C. O'Neill, Am. J. Physiol. 269 (1995) C1524–C1531.
[11] J.D. Klein, P. Perry, W.C. O'Neill, J. Membr. Biol. 132 (1993) 243–252.
[12] M.E. O'Donnell, A. Martinez, D. Sun, Am. J. Physiol. 269 (1995) C1513–C1523.
[13] E.B. Pewitt, R.S. Hedge, M. Haas, H.C. Palfrey, J. Biol. Chem. 265 (1990) 20747–20756.
[14] J. Torchia, Q. Yi, A.K. Sen, J. Biol. Chem. 269 (1994) 29778–29784.
[15] S. Uchida, H.M. Kwon, A. Yamauchi, A.S. Preston, F. Marumo, J.S. Handler, Proc. Natl. Acad. Sci. USA 90 (1993) 7424–7429.
[16] H.M. Kwon, A. Yamauchi, S. Uchida, A.S. Preston, A. Garcia-Perez, B. Burg, J.S. Handler, J. Biol. Chem. 267 (1992) 6297–6301.
[17] H.M. Kwon, In: K. Strange (Ed.), Cellular and Molecular Physiology of Cell Volume Regulation, CRC Press, Ann Arbor, 1994, pp. 383–394.
[18] P.B. Dunham, F. Jessen, E.K. Hoffmann, Proc. Natl. Acad. Sci. USA 87 (1990) 6828–6832.
[19] M. Haas, P.B. Dunham, B. Forbush III, Am. J. Physiol. 260 (1991) C791–C804.
[20] W. Suvitayavat, H.C. Palfrey, M. Haas, P.B. Dunham, F. Kalmar, M.C. Rao, Am. J. Physiol. 266 (1994) C284–C292.
[21] E. Harlow, D. Lane, Antibodies. A Laboratory Manual, Cold Harbor Spring Laboratory, Cold Spring Harbor, 1988, pp. 1–711.
[22] B.S. Jensen, F. Jessen, E.K. Hoffmann, J. Membr. Biol. 131 (1993) 161–178.
[23] P.W. Feit, E.K. Hoffmann, F. Jessen, M. Schirdt, P. Kristensen, P.B. Dunham, J. Membr. Biol. 103 (1988) 135–147.
[24] J.A. Payne, B. Forbush III, Curr. Opin. Cell Biol. 7 (1995) 493–503.
[25] S.M. O'Grady, H.C. Palfrey, M. Field, Am. J. Physiol. 253 (1987) C177–C192.
[26] H.M. Kwon, J.S. Handler, Curr. Opin. Cell Biol. 7 (1995) 465–471.
[27] A. Yamauchi, S. Uchida, H.M. Kwon, A.S. Preston, R.B. Robey, A. Garcia-Perez, M.B. Burg, J.S. Handler, J. Biol. Chem. 267 (1992) 649–652.