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Biochimica et Biophysica Acta 1370 (1998) 169–174

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Cu^{2+} reveals different binding sites of amiloride and CDPC on the apical Na channel of frog skin

M.L. Flonta [b,*], J.De Beir-Simaels [a], D. Mesotten [a], W. Van Driessche [a]

[a] *Laboratorium voor Fysiologie, K.U.Leuven, Campus Gasthuisberg, B- 3000 Leuven, Belgium*
[b] *Dept. Physiology and Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independentei 91–95, Bucharest 76201, Romania*

Received 8 August 1997; revised 4 November 1997; accepted 4 November 1997

Abstract

The effect of Cu^{2+} ions, present in the mucosal bathing solution, on the transepithelial short-circuit current (I_{sc}) and conductance (G_t) and on the blocker-induced noise of apical Na channels, was studied on the isolated ventral skin of the frog *Rana temporaria*. Cu^{2+} effects were concentration-dependent, the full effect being reached at 50 μmol/l. Cu^{2+} increased I_{sc} and G_t; this effect was eliminated by high concentrations of amiloride (30 μmol/l) and of CDPC (150 μmol/l). Cu^{2+} markedly reduced the corner frequency (f_c) of the Na channel noise, while having virtually no effect on the f_c of CDPC-induced noise. Cu^{2+} reduces the association rate constant of amiloride to the Na channel to one third; this effect is interpreted as indicating competition between Cu^{2+} and amiloride for the same (negatively charged) binding site on the channel, while CDPC appears to bind on a different site. © 1998 Elsevier Science B.V.

Keywords: Frog skin; Copper; Na channel; Blocker-induced noise; Amiloride; CDPC

1. Introduction

The apical Na channel of frog skin is a member of a newly identified ion-channel family, which is characterized by sensitivity to amiloride.

The primary structure of an epithelial Na channel (ENaC) was first elucidated in 1994 [1]. The channel is thought to be composed of three homologous but nonidentical subunits, named α, β and γ [2]. The α-subunit alone can form fully functional amiloride-sensitive Na channels, whereas the β- and γ-subunits cannot [3]. In turn, the presence of β- or γ-subunits increases the amiloride-sensitive current ~10 fold. In spite of much new molecular information on the ENaC, a number of questions need further investigation. Specifically, what regions of the α-, β-, or γ-subunits contain the conduction pore, what regions are involved in channel gating, and what regions interact with regulatory effectors or known blockers? A way to obtain information which can be used to predict some structural features of the channel is to investigate the binding affinities of the effective blockers and how these are influenced by other interacting agents. With this idea in mind we have studied the effect of Cu^{2+} on the apical Na channel of amphibian skin. This tissue is a good model of tight epithelia in general, it can be easily isolated with

* Corresponding author. Fax: +40-1-2233933; E-mail: florita@bio.bio.unibuc.ro

PII S0005-2736(97)00261-7

minimal damage and it is stable for many hours in vitro.

Copper is a trace element essential for life [4], whose deficiency or excess entail serious pathological consequences [5]. Some years ago, Fereira [6–8] showed that Cu^{2+} stimulates the short-circuit current (I_{sc}) through amphibian epithelia. However, I_{sc} is a composite measure of the function of ionic channels – whose population is multifarious on both apical and basolateral membranes [9] – and of the basolateral Na^+/K^+ pumps, so that the effect of Cu^{2+} on I_{sc} has to be dissected into its elementary components.

Using a noninvasive method, Na channel characteristics were derived from the analysis of blocker-induced lorentzian noise, produced by two pyrazine derivatives, amiloride and CDPC (6-chloro-3,5-diaminopyrazine-2-carboxamide). Here we report data showing that Cu^{2+} competes with amiloride for the same binding site on the apical Na channel, while it has only minor affinity for the CDPC-binding site. This latter site thus appears to be distinct from the amiloride-binding site.

2. Methods

Frogs (*Rana temporaria*), weighing around 25 g, were kept at 17°C with free access to tap water. The abdominal skin of doubly pithed animals was dissected and mounted in an Ussing-type lucite chamber, as previously described [10]. The chamber ensured negligible edge damage and allowed the continuous perfusion with fresh solutions of both the mucosal ($_m$) and serosal ($_s$) sides of the epithelium, at a rate of 5 ml/min. The tissue area in contact with bathing media was 1 cm^2.

The transepithelial potential was clamped to zero with a low-noise voltage clamp [11] and the short-circuit current (I_{sc}) was recorded on a standard X-T recorder. The transepithelial conductance (G_t) was calculated from the current changes caused by 256 ms voltage pulses of 5 mV amplitude, applied every 14 s.

2.1. Solutions

All solutions were freshly prepared with highly purified water (Milli-Q Water Purification System, Millipore). In order to eliminate the contribution of Cl^- to the measured I_{sc} and G_t, Cl-free Ringer solutions, having SO_4^{2-} as the major anion, were used. Na_2SO_4 Ringer solution, bathing both sides of non-depolarized skins, consisted of (in mmol/l): 115 Na^+, 58.5 SO_4^{2-}, 2.5 K^+, 2.5 HCO_3^-, 1 Ca^{2+}. K_2SO_4 Ringer solution, used to depolarize the serosal side of the epithelium, contained (in mmol/l): 97.5 K^+, 20 Na^+, 58.5 SO_4^{2-}, 2.5 HCO_3^- and 1 Ca^{2+}. The pH of all solutions was adjusted to 8.0. The concentrations in the mucosal compartment of amiloride hydrochloride, Sigma) and of CDPC (6-chloro-3,5-diaminopyrazine-2-carboxamide, Aldrich) respectively ranged from 2 to 40 μmol/l ($[AMI]_m$) and from 10 to 150 μmol/l ($[CDPC]_m$) [12]. Copper was added as $CuSO_4$; free Cu^{2+} concentrations are estimated by preliminary conductance measurements, to be 30% lower than the values given in the case of both amiloride and CDPC.

2.2. Noise analysis

The fluctuation in current was recorded and analyzed in the frequency domain, as previously described [13]. The technique exploits the fact that the average macroscopic Na^+ current (μA) consists of the random flickering of millions of independent channels (pA) which gives rise to fluctuations (nA) around the mean current. These data can be resolved into their frequency components using the Fourier transform. The current signal was high-pass filtered with a 24 dB/octave Butterworth type filter with a cut-off frequency of 0.3 Hz. Furthermore, to prevent aliasing, the signal was low-pass filtered with a 48 dB/octave Butterworth filter (cut-off frequency = 850 Hz). Power density spectra (PDS) were calculated from Fourier transformed records of 4096 points collected over a 2 s period, yielding a fundamental frequency of 0.5 Hz. Averaged PDS of 2048 frequencies were obtained from 50 records. Frequencies above 760 Hz were discarded in further analysis. Spectra containing relaxation noise were fitted with the sum of a lorentzian component and a background noise term (A/f^{α}) according to the following formula:

$$S(f) = \frac{S_o}{1 + (f/f_c)^2} + \frac{A}{f^{\alpha}}$$

The plateau value (S_o) and the corner frequency (f_c) of the lorentzian function, determined by nonlinear regression analysis of the PDS, were used for the calculation of single channel current and channel density, according to a two-state model for channel opening and closing [9,14]. A represents the power density at 1 Hz and α the slope of the $1/f$ noise component.

The model predicts a linear dependence of f_c on the concentration of the fluctuation-inducing blocker [B]:

$$2\pi f_c = k_{01}[\mathrm{B}] + k_{10} \quad (1)$$

where k_{01} and k_{10} respectively are the ON- (association) and the OFF- (dissociation) rate constants. It also allows calculation of the single channel current (i_{Na}) and the number of open channels (N_o) in the presence of the blocker, from the plateau value (S_o) and the macroscopic Na^+ current (I_{Na}):

$$i_{Na} = \frac{S_o(2\pi f_c)^2}{4 I_{Na} k_{01}[B]} \quad (2)$$

$$N_o = I_{Na}/i_{Na} \quad (3)$$

3. Results

The data in Fig. 1 shows the stimulatory effect of Cu^{2+} on the transepithelial current and conductance. The onset of dose-dependent inhibition of I_{sc} and G_t by amiloride was slowed down after the addition of Cu^{2+} on the apical side of the skin (Fig. 1(A)), the time constant of the exponential fall rising from about 1 s to more than 3.5 s after Cu^{2+} addition. The slowing by Cu of the onset of CDPC block of I_{sc} was less obvious (Fig. 1(B)).

In all experiments, $[Cu^{2+}]_m$ was 50 μmol/l, because a preliminary investigation of the effect of $[Cu^{2+}]_m$ on blocker-induced noise of apical Na channels indicated this as the lowest concentration giving a maximal f_c change, 10 min after Cu^{2+} addition (Fig. 2).

The effect of Cu^{2+} on the spectrum of amiloride-induced noise of the apical Na-channel is illustrated in Fig. 3, showing the decrease of the corner frequency and the increase of the plateau values of the

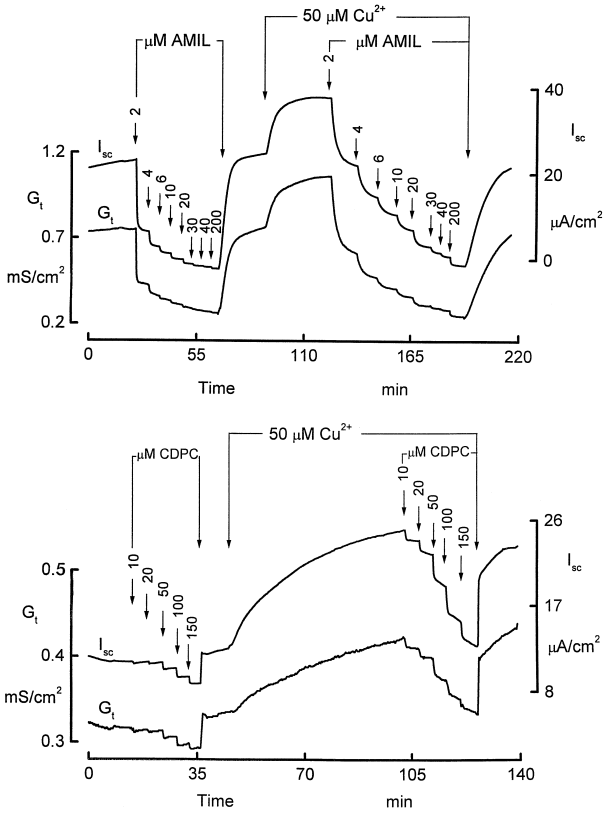

Fig. 1. Effect of Cu^{2+} (50 μmol/l, in the mucosal compartment) on the short-circuit current and transepithelial conductance of frog skin, in the presence of increasing mucosal concentrations of amiloride (AMI) (A) and of CDPC (B). $[AMI]_m$ was successively: 2, 4, 6, 10, 20 and 30 μmol/l and $[CDPC]_m$: 10, 20, 50, 100 and 150 μmol/l. The records are representative for (A, above) 5 and (B, below) 7 experiments.

recorded lorentzian spectra. Fig. 3 is representative for 6 experiments in identical conditions and for the general appearance of the crude noise data. In accord

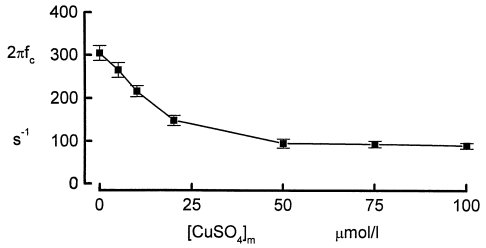

Fig. 2. Effect of mucosal concentration of Cu^{2+} on the corner frequency of the amiloride-induced noise of apical Na channels. f_c was recorded 10 min after increasing $[CuSO_4]_m$, in the presence of 20 μmol/l $[AMI]_m$.

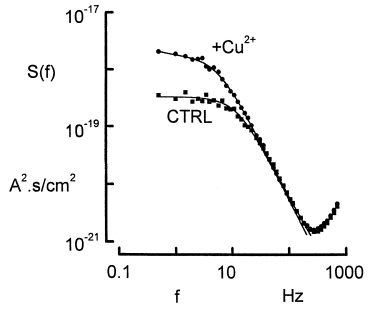

Fig. 3. Effect of 50 μmol/l $[Cu^{2+}]_m$ on the Na^+ current power density spectrum, recorded in the presence of 6 μmol/l $[AMI]_m$.

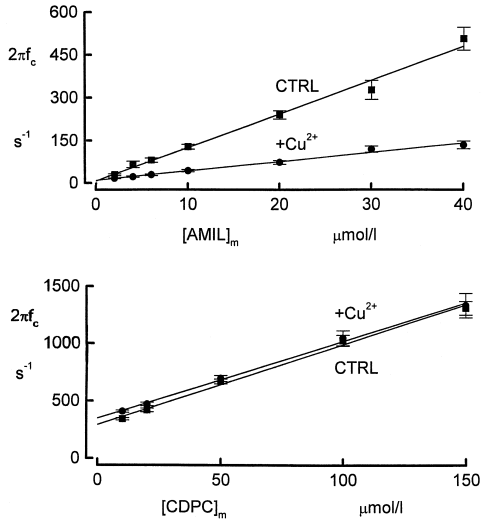

Fig. 4. Effect of 50 μmol/l $[Cu^{2+}]_m$ on the dependence of noise corner frequency on blocker concentration, in the case of 6 amiloride-treated frog skins (A, above) and of 7 CDPC-treated frog skins (B, below).

with Eq. (1) above, the dependence of f_c on mucosal blocker concentration was approximately linear, in the case of both amiloride and CDPC (Fig. 4). A clear-cut effect of Cu^{2+}, namely to reduce the slope of the f_c - $[blocker]_m$ dependence, was observed when the blocker was amiloride (Fig. 4(A)), but not when the blocker was CDPC (Fig. 4(B)), while the intercepts on the ordinate are less influenced by Cu^{2+} in either case.

In order to have an isolated apical membrane subjected to Cu^{2+} effects, the basolateral membrane was depolarized with high K^+ Ringer solution in the serosal compartment. The dependence of f_c on $[amiloride]_m$ was then studied in control conditions and in the presence of 50 μmol Cu^{2+}/l at the mucosal side. The ensemble of blocker kinetic constants (k_{01} and k_{10}) and of apical Na channel characteristics (i_{Na} and N_0, calculated with Eqs. (2) and (3) above) are given in Table 1, for non-depolarized frog skins treated with amiloride and CDPC, and for depolarized frog skins treated with amiloride, both without and in the presence of Cu^{2+}.

In order to further characterize the binding of Cu^{2+} on the Na channel, the influence of transepithelial voltage on the f_c of the noise induced by 20 μmol/l amiloride was studied, in control sulphate Ringer and in the presence of 50 μmol/l Cu^{2+} (Fig. 5). In control f_c increases at positive or negative

Table 1
Effect of 50 μmol/l $[Cu^{2+}]_m$ on blocker ON/OFF rate constants of amiloride and CDPC, and on the parameters of frog skin apical Na channel, as revealed by blocker-induced noise. k_{01} - association (ON) constant, k_{10} - dissociation (OFF) constant, i_{Na} - single channel current and N_0 - open channel density. Values are given as mean ± S.E.M

Conditions		k_{01} (μmol^{-1}s^{-1})	k_{10} (s^{-1})	i_{Na} (pA)	$N_0 \times 10^6$ (cm^{-2})
Amiloride-treated					
Non-depolarized ($N = 6$)	Control	11.77 ± 0.56	8.03 ± 11.77	0.40 ± 0.11	106.8 ± 35.8
	Cu^{2+}	3.30 ± 0.17	9.91 ± 3.52	0.70 ± 0.16	83.6 ± 20.0
K-depolarized ($N = 5$)	Control	10.63 ± 0.30	13.10 ± 6.29	0.36 ± 0.07	98.8 ± 23.3
	Cu^{2+}	3.28 ± 0.01	15.74 ± 0.32	0.35 ± 0.08	126.3 ± 33.0
CDPC-treated ($N = 7$)					
	Control	6.96 ± 0.29	295.3 ± 24.8	0.67 ± 0.11	28.03 ± 5.08
	Cu^{2+}	6.68 ± 0.20	351.2 ± 16.5	0.57 ± 0.06	40.45 ± 8.23

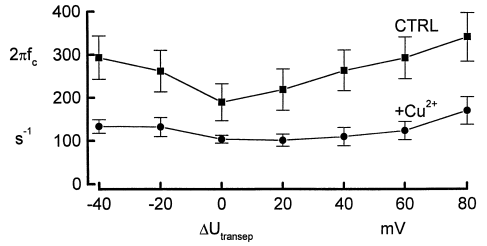

Fig. 5. Effect of 50 μmol/l $[Cu^{2+}]_m$ on the dependence of noise corner frequency on transepithelial voltage, in the case of 5 frog skins. The concentration of amiloride in the mucosal compartment was 20 μmol/l.

transepithelial potentials; this effect is reduced by copper.

4. Disscussion

The large increases in transepithelial (short-circuit) current and conductance, produced by Cu^{2+}, are associated with an increased entrance of Na^+ through the apical membrane, as indicated by the records in Fig. 1, which show that blockers of the Na channel abolished the Cu-induced increase. Thus, in the presence of the highest dose of amiloride (30 μmol/l), I_{sc} had virtually the same value with or without Cu^{2+} (Fig. 1(A)) and even the less potent blocker CDPC completely eliminated the Cu-induced increase above the control value. The concentration-dependent increase of the transepithelial current by Cu^{2+} is reflected in the quasi-exponential fall of f_c of the noise induced by a fixed concentration of blocker (Fig. 2), as well as in the increased plateau values, illustrated in Fig. 3.

A larger transepithelial ionic current, as produced by Cu^{2+} could, in principle, be attributed to increases in either the current passing through each channel (i_{Na}), or the number of open channels (N_0), or both. The characteristics of the Na channel, revealed by the analysis of amiloride-induced current fluctuations (Table 1) shows that, in non-depolarized frog skin, Cu^{2+} significantly increased i_{Na}, while it tended to decrease N_0. However, when the basolateral membrane was depolarized with an excess of potassium, i_{Na} was not influenced by the addition of Cu^{2+}, while N_0 tended to increase, indicating the possible existence of a group of voltage dependent Na channels. This could be suggested also by the influence of transepithelial voltage on the copper induced decrease of f_c values at higher positive or negative transepithelial potentials (Fig. 5).

The most conspicuous effect of Cu^{2+} was the reduction to one third of the association constant k_{01} between amiloride and the apical Na channel, in both non-depolarized and depolarized frog skins (Table 1). Amiloride is thought to stabilize the closed conformation of the Na channel via the interaction of its positively charged guanidinum group with a carboxyl group critically placed at the entrance of the channel [15],possibly on the H_2 hairpin structure[16]. The strong decrease of k_{01} by Cu^{2+} could arise from the electrostatic shield by this cation of the net negative charge present at the binding site for amiloride, on the Na channel protein. Our data suggest that Cu^{2+} binding, which hampers the attachment of amiloride, does not prevent (and even favours) the passage of Na^+ through the channel, as shown by the increased I_{sc}. The idea of competition between Cu^{2+} and amiloride for the same binding site is further supported by the fact that in the presence of high concentrations of amiloride, the stimulatory effect of Cu^{2+} on I_{sc} vanishes (Fig. 1(A)). In this case the conditions are very far from equilibrium between amiloride and copper.

As one might expect within the context of such a model, the basolateral depolarization did not significantly influence either the value of k_{01} in control mucosal solution, or the effect of Cu^{2+} on it. On the other hand, the dissociation rate constant k_{10}, on which Cu^{2+} alone had virtually no influence, tended to be increased in depolarized skins, the difference in k_{10} between non-depolarized and depolarized skins approaching significance in the presence of Cu^{2+}. The basolateral depolarization, which makes the intracellular compartment less negative, might facilitate the dissociation of the charged blocker from its binding site, particularly in the presence of the competitor Cu^{2+}, as a result of reduced transapical field. The same combination of electrostatic conditions appears to promote the tendency towards an increased number of open channels.

The association rate (k_{01}) of the amiloride analogue CDPC with apical Na channels, i.e. the slope of the curves in Fig. 4(B), was only negligibly influenced by Cu^{2+}, strikingly different from amiloride

(Fig. 4(A)). This indicates that the CDPC molecule, which is neutral at physiological pH, blocks the channel upon binding at a different site from amiloride. The dissociation rate constant of the CDPC–Na channel complex, k_{10}, was more than ten times higher than for the amiloride–Na channel complex (Table 1). This is in agreement with Helman and Baxendale [17] and with previous results from this laboratory, which indicated a lifetime of the blocker-channel complex much shorter for CDPC (4 ms), than for amiloride (75 ms) [18]. Cu^{2+} still increased k_{10}, in agreement with its tendency (above discussed) to promote the open configuration of the channel.

In summary, this study indicates a strong competition between Cu^{2+} and the charged pyrazine derivative amiloride, for binding on the apical Na channel of frog skin, and the lack of such an antagonism between Cu^{2+} and the neutral pyrazine derivative CDPC, thus pointing to the two blockers having different binding sites on the Na channel.

Molecular techniques have revealed additional epithelial Na channel family members. If the δ-ENaC is coexpressed with the β- and γ-subunits, instead of the α-subunit, the channel is 30-fold less sensitive to amiloride and has a unit conductance twice as high [19]. This suggests that the amiloride binding site could be located on the α-subunits, and it would be interesting to determine the CDPC sensitivity of this novel channel.

Acknowledgements

M.L.F. acknowledges support from the European Community (S & T Cooperation with Central and Eastern European Countries -PECO Grant 1993). She also expresses her gratitude to Dr. Gordon Reid for improving the English.

References

[1] C.M. Canessa, L. Schild, G. Buell, B. Thoreus, I. Gantschl, J.-D. Horisberger, B.C. Rossier, Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits, Nature 367 (1994) 463–467.

[2] D.J. Benos, C.M. Fuller, V.G. Shlyonsky, B.K. Berdiev, I.I. Ismailov, Amiloride-sensitive Na^+ channels: Insights and Outlooks, New Physiol.Sci. 12 (1997) 55–61.

[3] I.I. Ismailov, M.S. Awayda, B.K. Berdiev, J.K. Bubien, J.E. Lucas, C.M. Fuller, D.J. Benos, Triple-barrel organisation of ENaC, a clonned epithelial Na^+ channel, J. Biol. Chem. 271 (1996) 807–816.

[4] W. Mertz, The essential trace elements, Science 213 (1981) 1332–1338.

[5] D.D. Perrin, R.P. Agarwal, Multimetal–multiligand equilibria: a model for biological systems, in: H. Siegel (Ed.), Metal Ions in Biological Systems, vol. 2, Mixed-ligand complexes, Marcel Dekker, NY, 1973, pp. 167–206.

[6] K.T.G. Ferreira, The effect of Cu on isolated frog skin, Biochim. Biophys. Acta 203 (1970) 555–567.

[7] K.T.G. Ferreira, The effect of cooper on frog skin. The role of sulfhydryl groups, Biochim. Biophys. Acta 510 (1978) 298–304.

[8] K.T.G. Ferreira, The mechanism of action of Cu^{2+} on the frog skin, Biochim. Biophys. Acta 552 (1979) 341–345.

[9] W. Van Driessche, W. Zeiske, Ionic channels in epithelial cell membranes, Physiol. Rev. 65 (1985) 833–903.

[10] I. De Wolf, W. Van Driessche, Voltage dependent Ba^{2+} block of K^+ channels in apical membrane of frog skin, Am. J. Physiol. 251 (1986) C696–C706.

[11] W. Van Driessche, B. Lindemann, Voltage dependence of the blocking rate constants of amiloride at apical Na channels, Rev. Sci. Instrum. 49 (1978) 52–57.

[12] S.I. Helman, N.I. Kizer, Apical sodium ion channels of tight epithelia as viewed from the perspective of noise analysis, Current Topics in Membranes and Transport, vol. 37, Academic Press. Inc., 1990, pp. 117–155.

[13] W. Van Driessche, W. Zeiske, Spontaneous fluctuations of potassium channels in the apical membrane of frog skin, J. Physiol. 299 (1980) 101–116.

[14] W. Van Driessche, Noise and Impedance Analysis, in: J.A. Schafer, G. Giebisch, P. Kristensen, H.H. Ussing (Eds.), Methods in Membrane and Transporter Research, R.G. Landes Company, 1994, pp. 27–80.

[15] J. Warncke, B. Lindeman, Voltage dependence of the blocking rate constants of amiloride at apical Na channels, Pflugers Arch. 405 (1985) 89–94.

[16] C.M. Canessa, What is new about the structure of the epithelial Na^+ channel?, New Physiol. Sci. 11 (1996) 195–201.

[17] S.I. Helman, L.M. Baxendale, Blocker-related changes of channel density. Analysis of a three-state model for apical Na channels of frog skin, J. Gen. Physiol. 95 (1990) 647–678.

[18] D.G. Margineanu, W. Van Driessche, Comparison of amiloride and CDPC effects on the frog skin electrophysiology, Pflugers Arch. 415 S1 (1990) R33.

[19] R. Waldman, G. Champigny, F. Bassilana, N. Voilley, M. Lazdunski, Molecular cloning and functional expression of a novel amiloride-sensitive Na^+ channel, J. Biol. Chem. 270 (1995) 27411–27414.