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Feedback inhibition of epithelial Na^+ channels in *Xenopus* oocytes does not require G_0 or G_{i2} proteins

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Received 18 August 1999

Abstract **Regulation of amiloride-sensitive epithelial Na^+ channels (ENaC) is a prerequisite for coordination of electrolyte transport in epithelia. Downregulation of Na^+ conductance occurs when the intracellular Na^+ concentration is increased during reabsorption of electrolytes, known as feedback inhibition. Recent studies have demonstrated the involvement of αG_0 and αG_{i2} proteins in the feedback control of ENaC in mouse salivary duct cells. In this report, we demonstrate that Na^+ feedback inhibition is also present in *Xenopus* oocytes after expression of rat α,β,γ-ENaC. Interfering with intracellular αG_0 or αG_{i2} signaling by coexpression of either constitutively active $\alpha G_0/\alpha G_{i2}$ or dominant negative $\alpha G_0/\alpha G_{i2}$ and by coinjecting sense or antisense oligonucleotides for αG_0 had no impact on Na^+ feedback. Moreover, no evidence for involvement of the intracellular G protein cascade was found in experiments in which a regulator of G protein signaling (RGS3) or β-adrenergic receptor kinase (βARK) was coexpressed together with α,β,γ-ENaC. Although some experiments suggest the presence of an intracellular Na^+ receptor, we may conclude that Na^+ feedback in *Xenopus* oocytes is different from that described for salivary duct cells in that it does not require G protein signaling.**

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Key words: Na^+ feedback; Epithelial Na^+ channel; *Xenopus* oocyte; G protein; G_{i2}; G_0

1. Introduction

Reabsorption of NaCl in epithelial tissues largely depends on the activity of apical amiloride-sensitive Na^+ channels (ENaC). Activity of these channels is tightly controlled in order to adjust cellular uptake of Na^+ to the extrusion capacity of the basolateral Na^+,K^+-ATPase. This feedback inhibition of ENaC has been examined in detail in tight epithelia such as renal collecting duct, colonic mucosa and salivary duct [8,26]. Several mechanisms for apical Na^+ channel inhibition have been suggested that include binding to either extracellular or intracellular modifier sites and participation of other intracellular mediators like H^+, free Ca^{2+} or Cl^- [9,12,26]. More recent experiments clearly favor intracellular Na^+ and Cl^- concentrations as the essential signals for feedback inhibition of ENaC [8]. According to these studies, which were performed on mouse salivary duct cells, an increase in intracellular Na^+ leads to activation of the ubiquitin ligase Nedd4. After activation, Nedd4 mediates ubiquitination and endocytosis of ENaC which occurs in a dynamin-dependent fashion paralleled eventually by a change in single channel open probability [7]. This regulatory limb has been demonstrated to be essential for proper ENaC function and it was shown to be defective in Liddle's disease [2,14]. Recent studies uncovered the signaling pathway for feedback inhibition proximal to Nedd4 in mouse mandibular duct cells [15,16]. According to these patch clamp studies, intracellular Na^+ binds to a putative Na^+ receptor that can be blocked by compounds like amiloride, benzimide-azolylguanidinium (BIG) and 5-*N*-dimethyl-amiloride (DMA). Once activated by intracellular Na^+, the receptor will initiate the Nedd4-dependent cascade. Trimeric G proteins essentially contribute to this process. It was found that pertussis toxin-sensitive G_0 proteins are activated during an increase of intracellular Na^+ [15]. Interestingly, feedback inhibition by intracellular Cl^- does require activation of G_{i2} proteins but seems to be independent of the Nedd4/ubiquitin pathway.

Feedback inhibition of ENaC was also observed in *Xenopus* oocytes [1]. It was demonstrated that it strictly follows the intracellular Na^+ concentration. Moreover, experiments with oocytes that had been cut open suggested the presence of a cytosolic factor that is required for the inhibition of ENaC. Nedd4-dependent regulation of ENaC has also been shown to be present in oocytes [2]. Moreover, mutations causing Liddle syndrome abolish Nedd4-dependent regulation of ENaC and reduce sodium-dependent downregulation of the epithelial sodium channel in the *Xenopus* oocytes [10,14]. However, it remains unknown to what extent G_0 or G_{i2} protein metabolism is involved in the feedback inhibition observed in *Xenopus* oocytes. The results of the present study suggest that feedback inhibition in *Xenopus* oocytes is different from that observed in mandibular duct cells since it does not require G protein function.

2. Materials and methods

2.1. cRNAs for rat epithelial Na^+ channel (rENaC) subunits, G proteins, β-adrenergic receptor kinase (βARK) and regulator of G protein signaling (RGS3)

The three (α,β,γ) subunits of the rat amiloride-inhibitable Na^+ channel (ENaC, kindly provided by Prof. Dr. B. Rossier, Pharmacological Institute of Lausanne, Switzerland) were subcloned into pBluescript, linearized with *Not*I and in vitro transcribed using T7 promoter and polymerase. cDNAs encoding the dominant negative (dn) and the constitutively active (ca) form of the α subunit of G_0 were obtained by site-directed mutagenesis using standard methods (R179C for dn-αG_0; Q205L for ca-αG_0) [18,20]. Analogous mutations were introduced into α subunits of G_{i2} proteins (G204A for dn-αG_{i2}; Q206L for ca-αG_{i2}) and were kindly provided by Dr. J.S. Gutkind (NIDCR, NIH, Bethesda, MD, USA). cDNAs for the βARK [6] and the

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PII: S0014-5793(99)01291-0

RGS3 [11] were generous gifts from Dr. J.S. Gutkind and Prof. Dr. G. Walz (Department of Internal Medicine, University of Freiburg, Germany). cDNAs were linearized using either *Not*I or *Sal*I and cRNA was in vitro transcribed using T7 polymerase and a 5′ cap (mCAP mRNA capping kit, Stratagene). For some experimental protocols oocytes were injected with 50 ng of phosphorothioated sense or antisense oligonucleotides for *Xenopus*-αG_0: 5′-GCTGCTCTTT-CCTCCGCGCTCAGTGTGCAGCCCAT-3′ (as); 5′-ATGGGCTG-CACACTGAGCGCGGAGGAAAGAGCAGC-3′ (s) [22,24]

2.2. Preparation of oocytes and microinjection of cRNA

Isolation and microinjection of oocytes have been described in a previous report [4]. In brief, after isolation from adult *Xenopus laevis* female frogs, oocytes were dispersed and defolliculated by a 30 min treatment with collagenase (type A, Boehringer, Germany). Subsequently oocytes were rinsed and kept in ND96 buffer (in mmol/l): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, pH 7.55, supplemented with theophylline (0.5 mmol/l) and gentamicin (5 mg/l) at 18°C. Oocytes of identical batches were injected with cRNA (each subunit 10 ng) of α-, β-, γ-rENaC and G proteins, βARK or RGS3, respectively, after dissolving cRNAs in about 50 nl double-distilled water (PV830 pneumatic pico pump, WPI, Germany). Oocytes injected with 50 nl double-distilled water served as controls. For some experimental protocols oocytes were coinjected with 50 ng of either sense or antisense oligonucleotides of αG_0.

2.3. Electrophysiological analysis of Xenopus oocytes

Two days after injection, oocytes were impaled with two electrodes (Clark instruments) which had a resistance of 1 MΩ when filled with 2.7 mol/l KCl. A flowing (2.7 mol/l) KCl electrode served as bath reference in order to minimize junction potentials, which were close to zero when bath Cl^- was replaced by gluconate. Membrane currents were measured by voltage clamping of the oocytes (OOC-1 amplifier, WPI, Germany) in intervals from −100 to +20 mV in steps of 20 mV, each 1000 ms. Current data were filtered at 400 Hz (OOC-1 amplifier). Between intervals, oocytes were voltage clamped to −100 mV for 20 s. Data were collected continuously on a computer hard disk at a sample frequency of 1000 Hz and displayed on a computer screen (MacLab, AD Instruments). Data were analyzed using the programs chart and scope (McLab, AD Instruments, Macintosh). Conductances were calculated for the voltage clamp range of −100 to +20 mV according to Ohm's law. Typically current values were measured at the time point 250 ms during the voltage step. During the whole experiment the bath was continuously perfused at a rate of 5–10 ml/min.

2.4. Materials

All used compounds were of the highest available grade of purity. Amiloride and DMA were from RBI (Natick, MA, USA). BIG was from Aldrich Li^+ and $NMDG^+$ were obtained from Merck (Darmstadt, Germany). Pertussis toxin was from Sigma (Deisenhofen, Germany). Statistical analysis was performed according to Student's *t*-test. A *P* value < 0.05 was accepted to indicate statistical significance.

3. Results

After coexpression of the three subunits for rENaC large amiloride-sensitive Na^+ currents were detected (Fig. 1). These ENaC currents were inhibited reversibly by 10 μM amiloride. Oocytes were voltage clamped to −100 mV and in intervals clamp voltage was gradually changed to +20 mV in steps of 20 mV. Over an observation period of 25 min, the negative current gradually decreased and the amount of amiloride-sensitive current was reduced (Fig. 1). In $n = 29$ experiments, the amiloride-sensitive Na^+ conductance was reduced from 22.2 ± 2 to 12.1 ± 1 μS within 25 min. This is also shown in Fig. 2A for the normalized amiloride-sensitive conductance of the initial ENaC conductance. No significant feedback inhibition of ENaC was observed when oocytes were kept at their depolarized membrane voltage (data not shown). When extracellular Na^+ was replaced by Li^+, similar inhibition of ENaC was observed (Fig. 2B). However, when extracellular

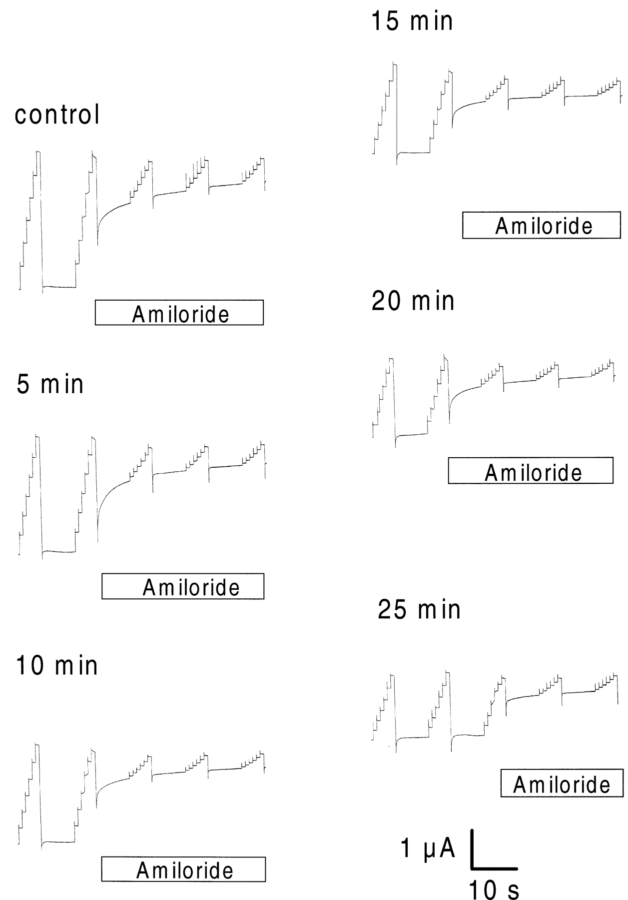

Fig. 1. Feedback inhibition of ENaC expressed in *Xenopus* oocytes. Representative examples of whole cell currents observed in an oocyte expressing α,β,γ-rENaC. Oocytes were voltage clamped to −100 mV and in intervals the clamp voltage was changed in steps of 20 mV (for 1 s) from −100 mV to +20 mV. The effect of amiloride (10 μM) was examined every 5 min. Note the decrease on whole cell conductance with time.

Na^+ was removed and was replaced by $NMDG^+$ feedback inhibition was no longer observed, indicating that influx of either extracellular Na^+ or Li^+ is essential for downregulation of ENaC (Fig. 2B).

We examined to what degree G proteins affect feedback inhibition in *Xenopus* oocytes. To that end, several constructs encoding G proteins were coexpressed with α,β,γ-rENaC subunits. Fig. 3 shows normalized conductances in the presence of extracellular Na^+ and after applying the above described voltage clamp protocol for 25 min. Initial amiloride-sensitive whole cell conductance in oocytes coexpressing various G protein subunits or coinjected with sense and antisense oligonucleotides were not significantly different from those expressing only α,β,γ-rENaC (data not shown). Moreover, injection of only oligonucleotides or sole expression of α subunits of G proteins did not change membrane conductances in *Xenopus* oocytes (data not shown). Neither constitutively active nor dominant negative αG_0 (ca-αG_0, dn-αG_0) had any significant effect on feedback inhibition when compared to controls (α,β,γ-rENaC only). Moreover, injection of antisense oligonucleotides for *Xenopus* αG_0 did not inhibit Na^+ feedback in *Xenopus* oocytes and the results for both sense and antisense were not significantly different (Fig. 3A).

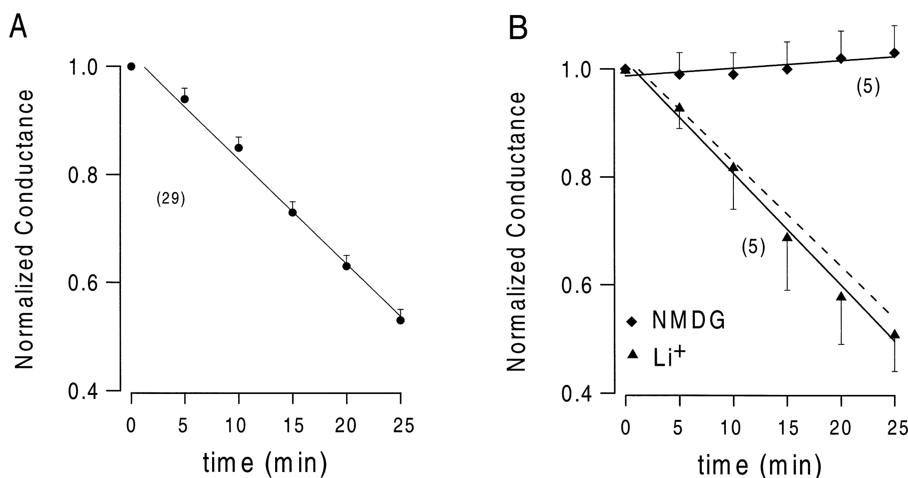

Fig. 2. Feedback inhibition of ENaC expressed in *Xenopus* oocytes. A: Time course of the normalized amiloride-sensitive whole cell conductance (amiloride-sensitive whole cell conductance measured at a given time divided by amiloride-sensitive whole cell conductance under control conditions). B: Time course of the normalized amiloride-sensitive whole cell conductance when extracelluar Na^+ (dashed line, data in A) was replaced by either $NMDG^+$ or Li^+. Means ± S.E.M. (number of experiments).

Because we were unable to detect any contribution of αG_0 to the feedback regulation of ENaC we examined whether another class of G proteins, αG_{i2}, does participate in feedback inhibition of ENaC. To that end, similar experiments were performed by coexpressing ca-αG_{i2} and dn-αG_{i2} together with α,β,γ-rENaC. Again, initial amiloride-sensitive Na^+ conductances were similar to those of controls (data not shown) and no difference in feedback inhibition of ENaC could be detected when compared to the feedback under control conditions (Fig. 3B). This indicates that αG_{i2} is not essential for the control of ENaC activity in *Xenopus* oocytes. We also checked for the contribution of G proteins by coexpressing proteins that interfere in a more general way with intracellular G protein signaling. βARK is known to bind β,γ subunits of trimeric G proteins which then will be no longer available for signal transduction [25,27]. The regulator of G protein signaling (RGS3) acts as a GTPase on several G proteins such as $\alpha G_{q/11}$, αG_s and αG_i and thereby limits G protein-mediated intracellular signal transduction [11]. However, when coexpressed with α,β,γ-rENaC in *Xenopus* oocytes, feedback inhibition of ENaC was not different from that in control cells (Fig. 3B). Additional experiments were performed using pertussis toxin (PTX). In 11 experiments in which oocytes were incubated for 1 h in 0.1–1 μg/ml PTX prior to impalement, the sodium feedback was largely reduced (Fig. 4A, inset). These results, however, are obscured by the fact that in another series of 11 experiments, acute exposure of the oocytes to PTX for only 10 min significantly inhibited amiloride-sensitive Na^+ conductance (Fig. 4A). Taken together, these experiments strongly suggest that in contrast to mouse mandibular duct cells, G protein signaling is not involved in the feedback regulation of ENaC in *Xenopus* oocytes.

Previous studies suggested that G protein signaling activated by an increase of intracellular Na^+ causes feedback

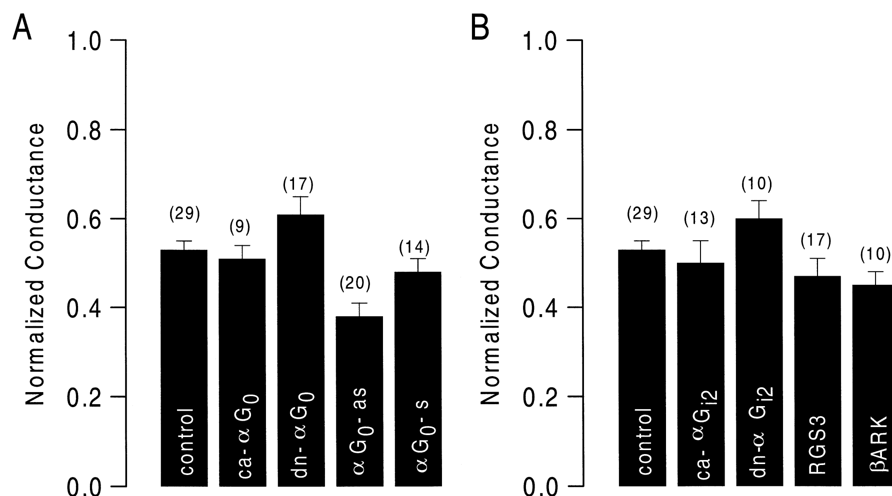

Fig. 3. Summary of the normalized whole cell conductances measured after 25 min of applying the voltage clamp protocol. A: dn-αG_0, ca-αG_0: oocytes coinjected with dominant negative and constitutively active forms of the α subunit of αG_0. αG_0-as, αG_0-s: oocytes coinjected with antisense and sense oligonucleotides for αG_0. B: dn-αG_{i2}, ca-αG_{i2}: oocytes coinjected with dominant negative and constitutively active forms of the alpha subunit of αG_{i2}. RGS3, βARK: oocytes coexpressing regulator of G protein signaling type 3 and β-adrenergic receptor kinase, respectively. Means ± S.E.M. (number of experiments).

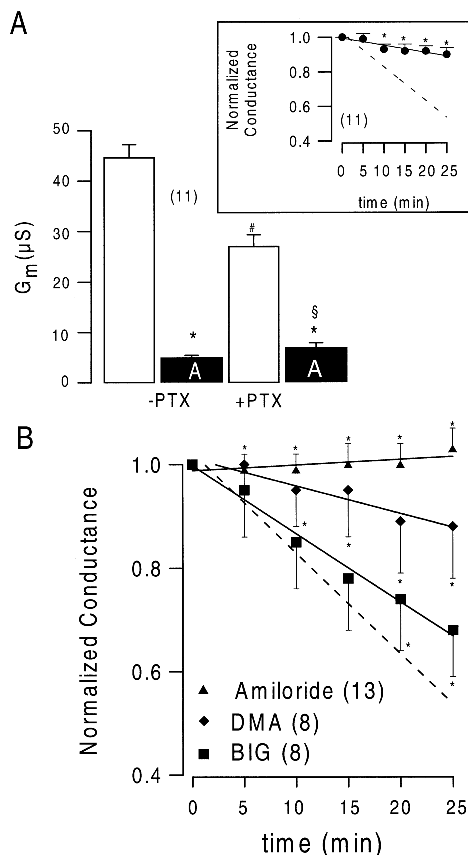

Fig. 4. Feedback inhibition of ENaC expressed in *Xenopus* oocytes. A: Acute inhibition of whole cell conductance and effects of amiloride (A) on ENaC after 10 min perfusion of the oocytes with PTX (0.1–1 µg/ml). Inset: Feedback inhibition of ENaC after 1 h pre-incubation of the oocytes with PTX (0.1–1 µg/ml). B: Feedback inhibition was measured under control conditions (dashed line, data in Fig. 2A) or after incubation overnight in the presence of amiloride (1 µM), or following injection of DMA or BIG (both 200 µM). Means ± S.E.M. (number of experiments). Asterisks indicate significant difference from control; # indicates significant inhibition of the whole cell conductance Gm. § indicates significant inhibition in amiloride-sensitive Na^+ conductance.

inhibition of ENaC that is triggered by binding of Na^+ to a putative intracellular Na^+ receptor [16,19]. This Na^+ receptor was shown to be blocked by inhibitors such as amiloride, BIG or DMA. These blockers may therefore interrupt the Na^+ feedback mechanism. In fact, feedback inhibition of ENaC was significantly reduced when oocytes were kept overnight in amiloride (1 µM) containing ND96 (Fig. 4B). In addition Na^+ feedback was partially blocked when oocytes were injected with either 100 µM DMA or BIG prior to double electrode voltage clamp experiments. These experiments suggest the presence of an intracellular Na^+ receptor that might act as a sensor for intracellular Na^+ concentration. There is, however, no indication that activation of this receptor in *Xenopus* oocytes does couple to G proteins in order to confer the signal on to ENaC channels.

4. Discussion

Na^+ feedback inhibition was observed in *Xenopus* oocytes after expression of exogenous α,β,γ-rENaC. We asked whether the underlying signal transduction is similar to that in mouse mandibular duct cells which required the presence of αG_0 proteins [7,15]. We detected a feedback inhibition similar to what was described in previous reports [1,14]. Feedback inhibition, however, occurs at a much slower rate presumably because of the large oocyte volume and the extended time that is required to enhance the intracellular Na^+ concentration. It has been shown recently that an intracellular Na^+ concentration of around 50 mM is required for inhibition of ENaC [1]. The time that is required to enhance the intracellular Na^+ concentration depends on the magnitude of Na^+ currents expressed in the oocytes. In that respect, it is interesting to note that in the present study about 50% of the amiloride-sensitive Na^+ current was inactivated after 25 min. This is only slightly less than the inhibition observed in previous reports, although in the present study oocytes expressed lower ENaC currents and were kept in high Na^+ Ringer [14]. Moreover, and in contrast to [14], we did not detect larger ENaC currents in oocytes that had been kept in low extracellular Na^+ ($NMDG^+$). In addition, feedback inhibition was similar in both oocytes kept in either low or high extracellular Na^+ during expression (data not shown). This result might be due to the fact that the level of ENaC expression in our experiments was considerably lower than in the previous study. It should be stated that for the experimental series described in this study, comparable levels of ENaC expression was obtained.

The present experiments do not reveal any contribution of G protein signaling to feedback inhibition of ENaC in oocytes. This is based on independent experiments using αG_0 antisense, constitutively active and dominant negative αG_0 and αG_{i2} as well as RGS3 and βARK. The αG_0 antisense was used successfully as a tool to block αG_0-dependent signal transduction in *Xenopus* oocytes in previous studies [22,24]. Moreover, the antisense used here inhibited the ATP-mediated increase in Cl^- conductance in *Xenopus* oocytes overexpressing P_2Y_2 receptors (data not shown). Depending on the cell type, P_2Y_2 receptors couple to $G_{q/11}$, G_i or G_0 proteins [21]. Dominant negative and constitutively active α subunits of G proteins have been successfully used in parallel experiments in our laboratory to block exocytosis of the cystic fibrosis transmembrane conductance regulator (CFTR) in *Xenopus* oocytes (unpublished data). RGS3 is expressed predominantly in renal tubular cells and is known to accelerate the intrinsic GTPase activity of $\alpha G_{q/11}$, αG_s and αG_i. In addition, it might increase the availability of β and γ subunits of trimeric G proteins [5,11]. RGS3 blocked αG_{i2}-mediated inhibition of CFTR in *Xenopus* oocytes in other experiments (unpublished data). βARK can be used as a scavenger for β,γ subunits of trimeric proteins which will then no longer be available for signal transduction [25]. Additional experiments in order to find out whether feedback inhibition of ENaC in *Xenopus* oocytes is sensitive towards PTX were not conclusive, because PTX per se inhibited ENaC. Regarding regulation of ENaC by PTX-sensitive G proteins, results from previous studies have been contradictory: purified epithelial Na^+ channels were activated by αG_{i3}, while PTX activated ENaC in A6 cells [3,23]. Our data would be in agreement with the idea that PTX-sensitive proteins activate ENaC in *Xenopus* oocytes. However, it remains obscure whether Na^+ feedback inhibition of ENaC does involve the action of a PTX-sensitive protein. Nevertheless, none of the present experimental series gave any

hint of a role for G proteins in feedback regulation of ENaC in *Xenopus* oocytes.

A previous report suggested the presence of one or several cytosolic factors that are essential for the Na^+ feedback in oocytes [1]. This unknown cytosolic factor could be the putative intracellular Na^+ receptor suggested in previous reports and supported by data of the present experiments. According to these results, the receptor also accepts Li^+ instead of Na^+. Other studies have shown that an intact C-terminal PY motif is required for feedback inhibition. It has been demonstrated that mutation of the C-terminal PY motif such as occurs in Liddle's disease does interrupt Nedd4-mediated endocytosis and also interferes with feedback inhibition of ENaC [1,2]. Very similar results were obtained in our laboratory (unpublished data). However, other regulatory limbs of ENaC are not affected by these mutations, such as inhibition of ENaC by CFTR [13,17]. Thus, regulation of ENaC by CFTR is probably independent of intracellular Na^+. Moreover, the present results suggest that the mechanisms by which increased intracellular Na^+ concentration is transmitted to Nedd4-dependent inhibition of ENaC depends on the cell type in which feedback inhibition takes place.

Acknowledgements: We gratefully acknowledge the expert technical assistance of Mrs. P. Kindle and Mrs. H. Schauer. Supported by DFG Ku756/2-3, Zentrum klinische Forschung 1 (ZKF1) and Mukoviszidose e.V. DIC is a Fellow of the Medical Foundation of the University of Sydney.

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