Thyroid hormone potentiates glucocorticoid-evoked airway Na^+ transport without affecting α-ENaC transcription

K. Richard, S.J. Ramminger, L. Forsyth, A. Burchell, S.M. Wilson*

Lung Membrane Transport Group, Division of Maternal and Child Health Sciences, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, UK

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Abstract Glucocorticoid and thyroid hormones (T_3) are important for the development of the lungs' Na^+ absorbing phenotype, which is essential for the integrated functioning of the respiratory tract. Electrometric studies of H441 airway epithelial cells confirmed that dexamethasone increases apical Na^+ conductance (G_{Na}) and demonstrated that T_3 facilitates this control over G_{Na}. Assays of transcriptional activity showed that dexamethasone caused concentration-dependent activation of the human α-ENaC promoter ($EC_{50} \sim 5$ nM) but, despite its clear effect on G_{Na}, T_3 had no effect upon the transcriptional response to dexamethasone. The facilitation of Na^+ transport may thus reflect control over events downstream to transcription.
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1. Introduction

During fetal life, fluid is secreted into the lumen of the developing lung establishing a distending pressure that is crucial to lung morphogenesis [1]. However, this liquid must be removed from the lung by the time of birth, since the retention of liquid in the alveolar space can impede oxygenation of the blood. Indeed, such retention of fetal lung liquid is a component of respiratory distress syndrome (RDS), the commonest cause of death amongst premature infants in the developed world (see, e.g., [2]). Studies on fetal lambs showed that liquid is normally absorbed from the lungs during labor and birth and established that this absorption is driven by active Na^+ transport [3–5]. Moreover, this process is dependent upon epithelial Na^+ channels [6], which are composed of three subunits (α-, β- and γ-ENaC) encoded by separate genes [7–9]. It is clear that α-ENaC is vital for lung function as artificial deletion of this gene abolishes lung liquid clearance and thus precipitates death from respiratory distress within 48 h of birth [6], whereas deletion of β- or γ-ENaC does not cause overt pulmonary pathology (see review [10]).

Glucocorticoid hormones play an important role in pulmonary physiology by controlling the development of the lungs' Na^+ absorbing phenotype (see review [5]) and controlled expression of the α-ENaC gene appears to be an important part of this process [11–14]. However, administering glucocorticoids to immature fetuses does not cause precocious development of an absorptive phenotype unless given in conjunction with thyroid hormone (T_3) and so both hormone classes are important to the development of pulmonary Na^+ absorption [15,16]. Although the mechanisms by which T_3 can contribute to the control of pulmonary Na^+ transport are virtually unknown, it has been suggested that this hormone can augment the glucocorticoid-evoked activation of the α-ENaC promoter [14] and so, in the present study, we explore the effects of T_3 upon glucocorticoid-evoked Na^+ transport [17,18] and α-ENaC transcription in an epithelial cell line (H441) derived from the human airway.

2. Methods

2.1. Electrometric studies

H441 cells were grown for 7 days on permeable culture membranes (Costar Snapwells, 10^6 cells cm^{-2}) in a fully defined medium prepared using fetal bovine serum (8.5%) that had been extensively dialyzed to remove hormones/growth factors (see [17]). This medium was supplemented with dexamethasone (0.3–300 nM) and/or 10 nM T_3 as detailed in the text; 10 nM T_3 was used because previous studies of isolated rat distal epithelial cells had revealed clear physiological effects at this concentration [19]. H441 cells can form polarized epithelial sheets when grown on permeable supports (e.g., [13,17]) and previous work has shown that these cultures respond to dexamethasone, a synthetic glucocorticoid, with an increase in the rate of transepithelial Na^+ transport that reflects increased expression of a Na^+ conductance identical to that associated with heterologous co-expression of α-, β- and γ-ENaC [13,17,18]. In the present study, H441 cells in monolayer culture were, therefore, mounted in Ussing chambers so that amiloride-sensitive (10 μM) short circuit current (I_{Amil}, a measure of transepithelial Na^+ transport) and apical Na^+ conductance (G^+_{Na}) could be measured using previously described methods [17].

2.2. Assay of α-ENaC transcriptional activity

The 5′ end of the human α-ENaC gene (GenBank Accession No. U81961) includes two transcription start sites that allow the synthesis of two distinct α-ENaC transcripts (α-ENaC-1 and α-ENaC-2) [20–22]. In the present study, a 2.2 Kb sequence corresponding to nucleotides −1388 to +830 (relative to the start site for the α-ENaC-1 transcript) was amplified (PCR) from H441 cell genomic DNA using primers (sense CACGGTACCACCCAGCACCCAGAGAGCAGACGAA, antisense: ATACTCGAGCTGACCTCGAGCTGTGTCCTGATTC) that included *Kpn*I and *Xho*I sites in their 5′ ends (underlined). This fragment (designated KR1) which includes exon 1A, intron 1 and the start site for the α-ENaC-2 transcript [22], was then cloned into the *Kpn*I/*Xho*I site of the *p*GL3-basic vector. This lies upstream to a *Photinus pyralis* (firefly) luciferase gene so that activation of the KR1

* Corresponding author. Fax: +44-1382-632-597.
E-mail address: s.m.wilson@dundee.ac.uk (S.M. Wilson).

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sequence would evoke synthesis of this fluorescent protein. The hormonal regulation of this construct was explored by transfecting (4 μg well^{-1}, Ca_2PO_4 co-precipitation) H441 cells on 12 well plates with the basic *p*GL3 plasmid or the *p*GL3 plasmid containing the KR-1 sequence (*p*GL3-KR1). In each experiment, cells were also transfected with a second plasmid (*p*RL-TK, 0.12 μg) incorporating a constitutively active promoter upstream to a coelenterate (*Renilla reniformis*) luciferase gene. The transfected cells were incubated for 18 h before being glycerol shocked (15% v/v glycerol, 2 min at room temperature) and, after a 4 h recovery period, stimulated with dexamethasone and/or T_3. The cells were then harvested, lysed and the amounts of *Photinus* and *Renilla* luciferase isoforms produced were measured (Wallac 1420 Victor plate reading luminometer/Promega dual luciferase assay system). We are aware that some authors have reported that glucocorticoids can activate the *p*RL-TK plasmid [23] but, in the present study, the highest dexamethasone concentration used (200 nM) had no discernible effect upon *Renilla* luciferase formation and so this signal was used to control for variations in transfection efficiency. Transcriptional activities are normalized to the background activity determined in cells expressing the empty *p*GL3 vector.

3. Results and discussion

3.1. Effects of T_3 and dexamethasone upon the spontaneous I_{SC}

Electrometric studies ($n = 5$) of cells maintained (7 days) in medium supplemented with 10, 30 or 100 nM dexamethasone confirmed (e.g., [17]) that H441 cells become incorporated into electrically resistive cell sheets in the presence of this hormone; the transepithelial resistances (R_t of these cultured epithelia were thus 334 ± 76, 491 ± 70, and $440 \pm 47\ \Omega\,cm^2$, respectively. Moreover, as anticipated by earlier work [13,14,17] such cultures generated a spontaneous short circuit current that was almost completely (>90%) inhibited by adding 10 μM amiloride to the solution bathing the apical side of the cell layer. Analysis of these data showed that I_{Amil} was larger in cells that had been exposed to higher concentrations of dexamethasone (10 nM: 2.7 ± 0.7 μA cm^{-2}, 30 nM: 6.8 ± 1.6 μA cm^{-2}, and 100 nM: 12.9 ± 2.0 μA cm^{-2}). Parallel studies of age-matched cells that were exposed to 10 nM T_3 as well as dexamethasone showed that T_3 did not affect R_t (10 nM dexamethasone: $474 \pm 56\ \Omega\,cm^2$; 30 nM dexamethasone: $543 \pm 112\ \Omega\,cm^2$; and 100 nM dexamethasone: $427 \pm 65\ \Omega\,cm^2$), demonstrating that this hormone does not influence the formation of a coherent epithelial layer. However, T_3 clearly augmented I_{Amil} in cells exposed to 10 nM (5.6 ± 0.6 μA cm^{-2}, $P < 0.001$ compared with control data presented above) and 30 nM (11.0 ± 2.2 μA cm^{-2}, $P < 0.05$) dexamethasone. Although T_3 also seemed to enhance the currents generated by cells treated with 100 nM dexamethasone (18.1 ± 3.7 μA cm^{-2}), this effect was not statistically significant ($P < 0.1$). As H441 cells do not transport significant amounts of Na^+ if maintained in glucocorticoid-free medium [17,18], these data confirm [13,14,17] that dexamethasone evokes a concentration-dependent stimulation of Na^+ transport in these cells and show that T_3 can augment the responses evoked by submaximal concentrations of dexamethasone.

3.2. The control of G_{Na}

Transepithelial Na^+ transport is normally restricted by the rate at which Na^+ can cross the apical membrane and our earlier studies thus showed that the glucocorticoid-induced Na^+ transport seen in H441 cells reflects an increase in G_{Na} [17,18]. As the data presented above suggest that T_3 may also influence the conductive properties of this membrane, we explored the effects of this hormone upon the glucocorticoid-evoked increase in G_{Na} [17]. As anticipated [17], these experiments showed that dexamethasone caused a concentration-dependent increase in G_{Na} but also established that this response was almost doubled by 10 nM T_3 (Fig. 1). Whilst these data are highly suggestive of a potentiating interaction between the two hormones, this hypothesis cannot be formally accepted in the absence of data that define the effects of T_3 upon G_{Na} in the absence of dexamethasone. However, undertaking such experiments was not straightforward, since R_t must be $>200\ \Omega\,cm^2$ to allow G_{Na} to be measured and, in our hands [17], H441 cells do not become incorporated into such resistive cell sheets in the absence of glucocorticoids. It was therefore not possible to explore the effects of T_3 upon G_{Na} directly and so we used an alternative approach in which cells were maintained in dexamethasone-containing (200 nM) medium for 6 days in order to ensure the formation of resistive cell layers. The cells ($n = 3$) were then divided into three groups that were respectively maintained in control (i.e., hormone free), dexamethasone-containing (200 nM) or T_3-containing (10 nM) medium for a further 48 h before G_{Na} was measured; this time interval was chosen because pilot studies had shown that R_t fell below $200\ \Omega\,cm^2$ if cells were deprived of dexamethasone for 72 or 96 h. These experiments showed that in control cells ($R_t = 333 \pm 5\ \Omega\,cm^2$), G_{Na} was 83 ± 16 μS cm^{-2} and this value was ~20% of that measured in dexamethasone-treated cells (376 ± 46 μS cm^{-2}, $P < 0.05$, $R_t = 408 \pm 42\ \Omega\,cm^2$). Withdrawal of dexamethasone thus causes a fall in G_{Na} an observation that accords with previous data which have shown a rapid reduction in basal Na^+ transport under such conditions (e.g., [13]). Studies of cells maintained in T_3-containing medium ($R_t = 284 \pm 44\ \Omega\,cm^2$) showed that G_{Na} was 145 ± 30 μS cm^{-2}, a value that did not differ significantly from control and which was ~38% of that measured in dexamethasone-treated cells ($P < 0.01$). Whilst these data suggest strongly that T_3 cannot sustain the dexamethasone-induced increase in G_{Na} the mean value of G_{Na} measured in T_3-treated cells was greater than control and, although this was not statistically significant, we were concerned that this small

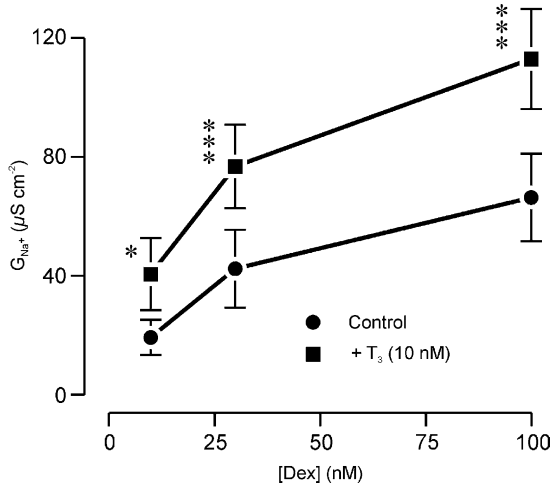

Fig. 1. T_3-evoked control over G_{Na}. Apical Na^+ conductance (G_{Na}) was measured in H441 cells, grown to confluence on Costar Snapwell membranes, by monitoring the amiloride sensitive (10 μM) currents evoked by imposing inwardly directed Na^+ gradients upon cultures that had been basolaterally permeabilized by adding nystatin (75 μM) to the solution bathing the apical side of the cell layer. Data are means ± S.E.M., $n = 5$, asterisks denote statistically significant effects of T_3 (*$P < 0.05$, ***$P < 0.005$).

difference may indicate a direct action of T_3. However, even if this possibility is accepted, then analysis of these data shows that, at best, T_3 can sustain ~20% of the dexamethasone-induced increase in G_{Na} and this small effect cannot account for the large increases in G_{Na} shown in Fig. 1. The present data thus suggest strongly that the large T_3-evoked increase in G_{Na} seen when cells are simultaneously exposed to both hormones (Fig. 1) must reflect a potentiating interaction between the two hormone classes.

Whilst the present data, in common with previous in vivo studies [15,16], suggest that T_3 augments the response to dexamethasone, in the present study dexamethasone could still increase G_{Na} if administered without T_3 see also [13,17,18]. The situation in H441 cells thus differs from that described in the fetal lamb, where exogenous glucocorticoids cannot cause precocious maturation of lung liquid absorption unless administered with T_3 [15]. This discrepancy may imply that in vitro studies can detect increases in G_{Na} that are not large enough to cause detectable liquid absorption in vivo. It is, however, also worth noting that naturally occurring glucocorticoids were used in the in vivo studies [15], whilst dexamethasone was used in the present experiments. It is possible that this synthetic glucocorticoid may act with greater efficacy, which may allow T_3-independent events to be detected.

3.3. Assay of transcriptional activity

It is now well accepted that glucocorticoids control transcription of the α-ENaC gene by activating a glucocorticoid response element (GRE) in this gene's promoter region and this is thought to be an important part of the mechanism that allows such hormones to control epithelial Na^+ transport [12–14,24]. Studies of rat α-ENaC promoter showed that although T_3 could not evoke transcription per se, it did enhance glucocorticoid-evoked transcription [12], which suggests strongly that the T_3-evoked enhancement of G_{Na}, which we now report, may be due to increased α-ENaC transcription. We therefore explored the effects of dexamethasone and T_3 on the transcriptional activity of a 2.2 Kb gene fragment that includes ~1.4 Kb of the upstream, promoter region of the human α-ENaC gene (see Section 2).

The first such experiments (Fig. 2A) showed that the basal activity of this construct was only ~6-fold greater than the background activity measured using the empty *p*GL3 vector. Such low intrinsic activity has been reported in earlier studies of both the rat and human α-ENaC promoters [13,14,21]. However, exposing the cells to a concentration of dexamethasone (200 nM, 24 h) that substantially increases G_{Na} (present study, [17]) increased transcriptional activity ~10-fold (Fig. 2A). More detailed studies of this response showed that this dexamethasone-induced transcription first became evident after 2–4 h and reached a clear peak at 16 h (Fig. 2B). Fig. 2C shows the results of experiments in which cells were exposed to a range of dexamethasone concentrations (0.3–200 nM) for this length of time. Analysis of these data showed that, under control conditions, the concentration needed for half maximal activation (EC_{50}) was 4.7 ± 0.3 nM and that maximal responses occurred at concentrations above 30 nM; we are unaware of any previous work that has studied the concentrations of dexamethasone needed to activate this promoter. Fig. 2C also includes the results of experiments in which H441 cells were exposed to 10 nM T_3 in addition to dexamethasone and analysis of these data showed that this hormone had no significant effect upon the

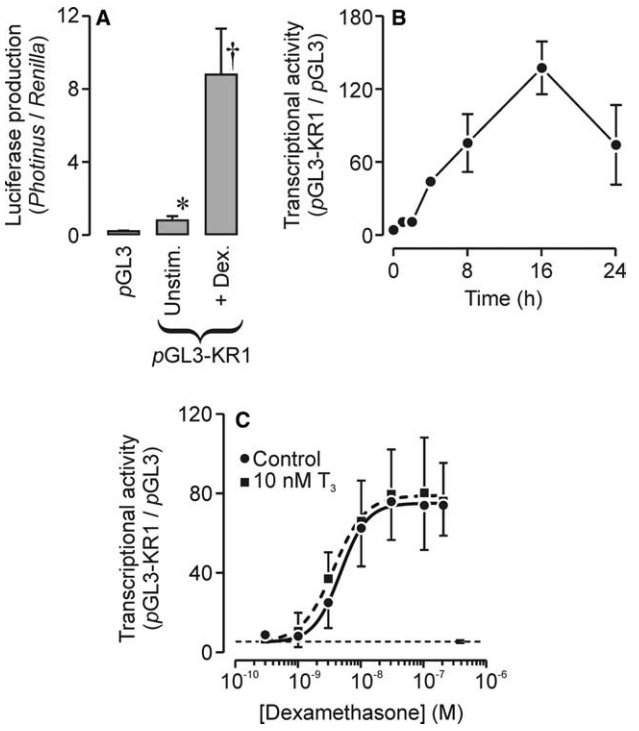

Fig. 2. Dexamethasone-induced activation of *p*GL3-KR1. (A) Luciferase formation (24 h) in H441 cells ($n = 5$) transfected with the empty *p*GL3 vector or with *p*GL3-KR1. Cells expressing *p*GL3-KR1 were either maintained under basal (i.e., unstimulated) conditions or exposed to dexamethasone (200 nM); * statistically significant ($P < 0.05$) difference between cells expressing *p*GL3-KR1 and *p*GL3; † statistically significant ($P < 0.001$) effect of dexamethasone upon *p*GL3-KR1-expressing cells. (B) Time course for dexamethasone-induced (200 nM) activation of *p*GL3-KR1 ($n = 3$). Data are normalized to the background expression level measured in parallel studies of cells expressing *p*GL3. (C) Responses to a range of concentrations of dexamethasone (3–200 nM, 16 h, $n > 4$ for each point) quantified under control conditions and in the presence of 10 nM T_3. The sigmoid curves were fitted to the experimental data using a least squares regression procedure (*GraFit 5*, Erithacus Software), whilst the dashed line shows the activity of the KR-1 construct in unstimulated cells. All data are means ± S.E.M.

EC_{50} for dexamethasone (3.7 ± 0.3 nM) or upon the magnitude of the response to maximally effective concentrations of this hormone (Fig. 2C).

3.4. Significance of present findings

Our earlier studies of H441 cells [17,18] identified a glucocorticoid-dependent, apical Na^+ conductance and it is now clear that the induction of this conductance is augmented by T_3. This accords well with data from fetal lambs, which showed that the development of the lungs' Na^+ absorbing phenotype is blocked by fetal thyroidectomy [15,16]. However, whilst this effect was reversed by exogenous T_3, administering T_3 to immature fetuses did not cause precocious maturation of an absorptive phenotype unless given in conjunction with a glucocorticoid. It is therefore clear that both hormone classes are important to the maturation of pulmonary Na^+ absorption and this raises the possibility that poor maturation of Na^+ absorption, as well as impaired lung growth and surfactant synthesis, may contribute to increased incidence of respiratory illness seen amongst congenitally hypothyroid infants [25]. Moreover, studies of hypothyroid rats have revealed an

abnormally low rate of epithelial Na^+ transport in another absorptive tissue, the distal colon, and this deficit, which can be corrected by exogenous T_3, provides evidence of a more general role for this hormone in the control of epithelial Na^+ transport [26]. However, despite its potential importance, the mechanisms underlying this phenomenon are almost totally unknown.

The present assays of transcriptional activity confirmed that dexamethasone activates the α-ENaC promoter and extended upon this earlier work by establishing the relationship between transcriptional activity and the concentration of dexamethasone used. However, the most important result to emerge from these experiments was that T_3, at a concentration that doubles the dexamethasone-induced increase in G_{Na} cannot sustain the dexamethasone-induced increase in G_{Na} and also has no effect upon the corresponding activation of the human α-ENaC promoter (Fig. 2). This failure to augment dexamethasone induced transcription was surprising as structural analysis of the rat [12] and the human [20] promoters has shown that each contains at least 2 binding sites with the potential to allow T_3 to regulate transcription and because earlier work had provided strong evidence of a potentiating interaction between these hormones [12]. However, the present data refer to the human gene, whereas the previous study [12] was undertaken using the rat α-ENaC promoter and there are surprisingly large differences between these sequences [12,21,22]. It is therefore possible that the apparent discrepancy between these studies (present study, [12]) may reflect species-specific differences. Furthermore, the previously reported effect of T_3 was documented in COS7 cells co-expressing heterologous glucocorticoid and thyroid hormone receptors [12], whilst the present study reports effects mediated by the endogenous receptors expressed by H441 cells. It is, therefore, possible that potentiating interactions may only be seen in cells overexpressing these nuclear receptors.

The KR-1 promoter fragment used in the present study starts at a point that is ~1.4 Kb upstream to the α-ENaC-1 start site and thus includes the whole of intron 1 and the GRE that has previously been shown to regulate transcription [13,20]. The present data thus show that T_3 cannot modulate responses mediated via this regulatory element. It is, however, still possible that (i) T_3-sensitive elements lying further upstream may control transcription in vivo or (ii) that inhibitory elements in intron-1 may override any stimulatory effects. However, it is important to remember that increased α-ENaC transcription is only one of the mechanisms by which glucocorticoids can control G_{Na} as PY domains in each of the ENaC subunits allow a number of proteins, of which NEDD-4/2 is the archetypical example, to bind to the channel complex and target it for internalization and degradation. As well as evoking α-ENaC transcription, glucocorticoids interact with this pathway by controlling the expression of serum and glucocorticoid regulated kinases (SGK-1, SGK-2, and SGK-3), enzymes that can phosphorylate and thus inactivate NEDD-4/2 [27]. This has the effect of increasing G^+_{Na} by inhibiting the internalization/degradation of ENaC [14,28]. A growing body of evidence now shows that this is a centrally important mechanism that allows epithelial Na^+ transport to be controlled by hormones/growth factors [27]. Moreover, it is clear that this pathway does contribute to the control of pulmonary Na^+ transport (see, e.g., [14,29]).

T_3-evoked augmentation of glucocorticoid-induced pulmonary Na^+ transport has now been documented in rat [30], sheep [15,16] and human (present study) cells, but the data presented here suggest strongly that this can occur with no increase in α-ENaC transcription. The potentiating interaction between glucocorticoid and thyroid hormones may thus involve a signaling pathway downstream to transcription (e.g., SGK-1, NEDD-4/2) or control over the expression of other ENaC subunits.

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References

[1] Harding, R. and Hooper, S.B. (1996) J. Appl. Physiol. 81, 209–224.
[2] O'Brodovich, H. (1996) Proc. Assoc. Am. Physicians 108, 345–355.
[3] Walters, D.V. and Olver, R.E. (1978) Pediat. Res. 12, 239–242.
[4] Olver, R.E., Ramsden, C.A., Strang, L.B. and Walters, D.V. (1986) J. Physiol. Lond. 376, 321–340.
[5] Olver, R.E., Walters, D.V. and Wilson, S.M. (2004) Annu. Rev. Physiol., 66, 77–101.
[6] Hummler, E., Baker, P., Gatzy, J., Berrmann, F., Verdumo, C., Schmidt, A., Boucher, R. and Rossier, R.C. (1996) Nature Genetics 12, 325–328.
[7] Canessa, C.M., Horisberger, J.D. and Rossier, B.C. (1993) Nature 361, 467–470.
[8] Canessa, C.M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J.D. and Rossier, B.C. (1994) Nature 367, 463–466.
[9] Ishikawa, T., Marunaka, Y. and Rotin, D. (1998) J. Gen. Physiol. 111, 825–846.
[10] Barker, P.M. and Olver, R.E. (2002) J. Appl. Physiol. 93, 1542–1548.
[11] Schultz-Baldes, A. et al. (2001) Pflügers Arch. 443, 297–305.
[12] Otulakowski, G., Raffii, B., Bremner, H.R. and O'Brodovich, H. (1999) Am. J. Respir. Cell. Mol. Biol. 20, 1028–1040.
[13] Sayegh, R., Auerbach, S.D., Li, X., Loftus, R.W., Husted, R.F., Stokes, J.B. and Thomas, C.P. (1999) J. Biol. Chem. 274, 12431–12437.
[14] Itani, O.A., Auerbach, S.D., Husted, R.F., Volk, K.A., Ageloff, S., Knepper, M.A., Stokes, J.B. and Thomas, C.P. (2002) Am. J. Physiol. Lung Cell. Mol. Physiol. 282, L631–L641.
[15] Barker, P.M., Brown, M.J., Ramsden, C.A., Strang, L.B. and Walters, D.V. (1990) Pediat. Res. 27, 588–591.
[16] Barker, P.M., Strang, L.B. and Walters, D.V. (1990) J. Physiol. Lond. 424, 473–485.
[17] Ramminger, S.J., Richard, K., Inglis, S.K., Land, S.C., Olver, R.E. and Wilson, S.M. (2004) Am. J. Physiol. Lung Cell. Mol. Physiol. 287, L411–L419.
[18] Clunes, M.T., Butt, A.G. and Wilson, S.M. (2004) J. Physiol. Lond. 557, 809–819.
[19] Ramminger, S.J., Inglis, S.K., Olver, R.E. and Wilson, S.M. (2002) J. Physiol. Lond. 544, 567–577.
[20] Mick, V.E., Itani, O.A., Loftus, R.W., Husted, R.F., Schmidt, T.J. and Thomas, C.P. (2001) Mol. Endocrinol. 15, 575–588.
[21] Baines, D.L., Janes, M., Newman, D.J. and Best, O.G. (2002) Biochem. J. 364, 537–545.
[22] Thomas, C.P., Auerbach, A., Stokes, J.B. and Volk, K.A. (1998) Am. J. Physiol. 274, C1312–C1323.
[23] Ibrahim, N.M., Marinovic, A.C., Price, S.R., Young, L.G. and Fröhlich, O. (2000) BioTechniques 29, 782–784.
[24] Wang, H.-C., Zentner, M.D., Deng, H.-T., Kim, K.-J., Wu, R., Yang, P.-C. and Ann, D.K. (2000) J. Biol. Chem. 275, 8600–8609.
[25] Cuestas, R.A., Lindall, A. and Engel, R.R. (1976) N. Engl. J. Med. 295, 297–302.
[26] Pachá, J., Pohlová, I. and Zemanová, Z. (1996) Gastroenterology 111, 1551–1557.
[27] Debonneville, C. et al. (2001) EMBO J. 20, 7052–7059.
[28] Friedrich, B. et al. (2003) Pflügers Arch. 445, 693–696.
[29] Snyder, P.M., Steines, J.C. and Olson, D.R. (2004) J. Biol. Chem. 279, 5042–5046.
[30] Folkesson, H.G., Norlin, A., Wang, Y., Abedinpour, P. and Matthay, M.A. (2000) J. Appl. Physiol. 88, 416–424.