Critical role of protein kinase C α and calcium in growth factor induced activation of the Na^+/H^+ exchanger NHE1

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Abstract The ubiquitously expressed Na^+/H^+ exchanger (NHE1) plays an important role in the regulation of the intracellular pH. Induction of NHE activity by phorbol esters and inhibition of growth factor-mediated stimulation of the NHE by protein kinase C (PKC) inhibitors suggest an implication of PKCs in the regulation of the NHE. Expression of PKC isotype-specific dominant negative and constitutively active mutants or downregulation of PKC by isotype-specific antisense oligonucleotides revealed that stimulation by epidermal growth factor (EGF) or phorbol ester of the NHE in NIH3T3 cells is a PKCα-specific effect. Elevation of cytoplasmic calcium by a Ca^{2+} ionophore or thapsigargin causes a growth factor-independent stimulation of the NHE predominantly mediated by calcium/calmodulin kinase II. It is concluded that in NIH3T3 cells overexpressing the EGF receptor (EGFR6 cells), EGF requires cPKCα for the activation of the NHE, while calcium/calmodulin-dependent kinases are essential in thapsigargin induced stimulation of the NHE. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Na^+/H^+ exchanger; NHE1; Protein kinase C isotype; Calcium; Epidermal growth factor; NIH3T3

1. Introduction

Na^+/H^+ exchangers (NHE) are important regulators of cytoplasmic pH. Up to now, six isoforms (NHE1–NHE6) have been identified [1,2]. The NHE1 isoform [3] is ubiquitously expressed in mammalians and is characterized by high amiloride sensitivity, 12 transmembrane domains and the cytoplasmic carboxy-terminus for interaction with several intracellular regulators such as calcium, calcium/calmodulin or protein kinases. Activation of the NHE by extracellular signalling is associated with phosphorylation of serine residues suggesting a kinase-dependent regulation. Although it has been demonstrated that phorbol esters (TPA) increase the phosphorylation of the NHE1 in fibroblasts [4], mutation of a protein kinase C (PKC) consensus phosphorylation site [5] did not attenuate the TPA-induced activation of the NHE1 arguing against a direct PKC-mediated phosphorylation. Numerous observations, however, emphasize a crucial role of PKCs in regulation of the NHE. Inhibitors of PKC like staurosporine or PKC depletion by long-term treatment with phorbol esters prevent the activation of the NHE [6,7]. Induction of PKC activity by diacylglycerol or short-term exposure to TPA leads to activation of the NHE [8]. These results indicate an implication of classical or novel PKC isoforms in the regulation of the NHE. Direct evidence for this suggestion and characterization of the corresponding PKC isozyme, however, have not been presented.

In epidermal growth factor receptor-6 (EGFR6) cells it has been demonstrated that stimulation of the EGF receptor by its ligand leads to an activation of the mitogen-activated protein (MAP) kinase pathway [9,10]. The Ras-Raf1-MEK signalling pathway was shown to be crucial for the activation of the growth factor- and Ras-dependent stimulation of the NHE1 in CCL39 fibroblasts [11,12]. These findings strongly suggest that an implication of this pathway in regulation of the NHE in EGFR6 cells has to be considered.

Increase in cytoplasmic calcium concentration has been demonstrated to stimulate sodium influx by activation of the NHE [13]. Application of calcium ionophores immediately activates the NHE1 [5]. Calcium could lead to an activation of calcium-dependent kinases like PKC and calcium/calmodulin-dependent protein kinases or promote the binding of calmodulin to high-affinity binding sites of the NHE [14]. The activation of classical PKCα is known to be calcium-dependent [15]. It is conceivable, therefore, that a member of the classical PKC subfamily acts as a putative regulator of NHE. EGFR6 cells express the PKC isotypes α, ε, λ and ζ.

In order to address the question which PKC isotypes are involved in the regulation of the NHE, representatives from each of the three major sub-families of PKC, i.e. classical (cPKC), novel (nPKC) and atypical (aPKC) were investigated. Dominant negative and constitutively active mutants of each of these PKC isotypes were employed to elucidate the implication of the PKC by transient transfection of EGFR6 cells. In addition, PKC antisense oligonucleotides (PKCα and PKCε) were used to downregulate the expression of these proteins. Embryonic fibroblasts derived from a PKCα−/− 'knock-out' mouse were employed to substantiate the results obtained by the transfection assays. The role of calcium in the regulation of the NHE was studied either by reducing the cytoplasmic free calcium by the calcium chelators BAPTA and EGTA or by increasing the cytoplasmic calcium concentration by A23187 and thapsigargin. The role of calcium/calmodulin-dependent protein kinases was studied by application

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of the inhibitor KN-93. The possible implication of the MAP kinase pathway in regulation of the NHE was investigated by employing the MAP kinase inhibitor PD-98059 and transient transfection of a constitutively active MEK.

The studies revealed that in EGFR6 cells stimulation by EGF leads to a cPKCα-dependent activation of the NHE and that the calcium-dependent activation of cPKCα is sufficient for the stimulation of the NHE. Neither novel nPKCε and atypical PKCλ nor Erk1/2 are implicated in the EGF-mediated activation of the NHE in this cell system while calcium/calmodulin-dependent kinases are critical in thapsigargin-induced stimulation of the NHE.

2. Materials and methods

2.1. Materials

EGF, ATP, TPA, EGTA, A23187, EIPA, thapsigargin, nigericin, aprotinin and leupeptin were obtained from Sigma (Vienna, Austria) and BCECF-AM and BAPTA-AM from Molecular Probes Europe (Leiden, The Netherlands). Cell culture media were purchased from Bender (Vienna, Austria), Opti-Mem I medium from Life Technologies (Vienna, Austria) and the TransFast™ transfection reagent from Promega (Mannheim, Germany). Monoclonal anti-NHE1 antibody was obtained from Chemicon (Biomedica, Vienna, Austria) and polyclonal rabbit anti-PKCα, PKCε, PKCλ and PKCζ antibodies from Santa Cruz Biotechnology (Heidelberg, Germany). Tachisorb was purchased from Calbiochem (Lucerne, Switzerland).

2.2. Cell culture

NIH3T3 fibroblasts overexpressing EGFR (EGFR6), [9] were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine at 37°C in a humidified atmosphere (95% air, 5% CO_2).

2.3. Production of PKCα knock-outs and controls

Production of PKCα-deficient (PKCα−/−) mice and genotypic determination were performed as will be described elsewhere (manuscript in preparation). In brief, PKCα knock-outs were generated using the standard techniques of the gene targeting approach. To generate a PKCα−/− mouse line the 129/SV strain was used for the backcross. F2 homozygotes and 129/SV wild type (wt) were used for the generation of PKCα−/− embryonic fibroblast cells (EMFI) using standard protocols [18].

2.4. Plasmids, oligonucleotides and transfection protocols

The generation of dominant negative (DN) PKC mutants ($\alpha_{K/R}$, $\varepsilon_{K/R}$, $\zeta_{K/R}$, $\lambda_{K/W}$) and constitutively active (CA) PKC mutants ($\alpha_{A/E}$, $\varepsilon_{A/E}$, $\zeta_{A/E}$, $\lambda_{A/E}$) has been described previously [16,17]. PKCα sense (ATGGCTGACGTTTACCCG), PKCα antisense (CGGGTAAACGTCAGCCAT), PKCε sense (AGGTCACATCAGCCCTCATG) and PKCε antisense (CATGAGGGCTGATGTGACCT) oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). NIH3T3 cells (EGFR6) were seeded in 10 cm wells containing circular glass coverslips. Transfection of subconfluent monolayers with the indicated expression vectors (all subcloned into the expression vector pEF1-neo) or oligonucleotides was carried out with Transfast™ as described by the manufacturer.

2.5. Western blot analysis

Western blot analysis of NHE1 and PKC isotypes was performed as described previously [16].

2.6. NHE activity and pH_i

The cells were grown on round glass coverslips (22 mm). One day after plating the cells were kept in low serum medium (0.5% FCS) for 36–48 h for serum starvation. At this time point average cell count per coverslip was approximately 0.5×10^5 cells. pH_i (intracellular pH) was determined by fluorescence spectrophotometry employing BCECF (2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein). Cells attached to coverslips were washed in HCO_3-free HEPES-buffered saline (HBS) (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM glucose, 20 mM HEPES/NaOH, pH 7.4). The cells were loaded with BCECF by incubation in HBS containing 1 μM BCECF acetoxy methyl ester for 10 min at 37°C and subsequent washing (two times) with HBS. Fluorescence (excitation, 502 or 440 nm; emission, 530 nm) was measured with a single cell imaging system (Applied Imaging, Sunderland, UK). The cytoplasmic pH_i values were calculated from the ratio of the fluorescence intensities I_{502}/I_{440}. The ratio of the fluorescence intensities I_{502}/I_{440} was calibrated to pH_i using a nigericin calibration procedure as described [6]. The NHE activity was determined by measuring the cytoplasmic pH_i after acidification by a NH_4Cl preloading technique [11]. The recovery of pH_i from acid load ($\Delta pH_i/\Delta t$) was determined from the slope of the pH_i tracing.

3. Results

3.1. Expression patterns of NHE1 and PKC isozymes in EGFR6 and EMFI cells

The expression levels of NHE1 were analyzed in EGFR6 cells [10] and in EMFI cells (wt or PKCα−/−) by Western blot analysis (Fig. 1a). As shown in Fig. 1b, EGFR6 cells express the PKC isozymes cPKCα, nPKCε and aPKCλ/ζ. In vivo expression and biological activities of DN (K/R and K/W) or CA mutants (A/E) of the various PKC isoforms have been described previously [16]. According to expectations and in contrast to the corresponding wt EMFIs, cPKCα was not detectable in the PKCα−/− cell line (Fig. 1c).

3.2. Activation of the NHE1

The NHE activity was determined by measuring the cytoplasmic pH_i after acidification by NH_4Cl employing a single cell imaging technique. The basal activity of the NHE after 2 days of serum starvation and a defined acid load procedure by applying the NH_4Cl preloading technique was comparable in all cell lines ($1.06\pm0.15\times10^{-3}$ ΔpH_i/s). The NHE activity could be increased by stimulation of the EGFR6 cells with either EGF or ATP, the latter acting via R7G receptors as shown in Fig. 2. Typically for the NHE1 isoform, the activity was completely inhibited by preincubation with 100 μM of the

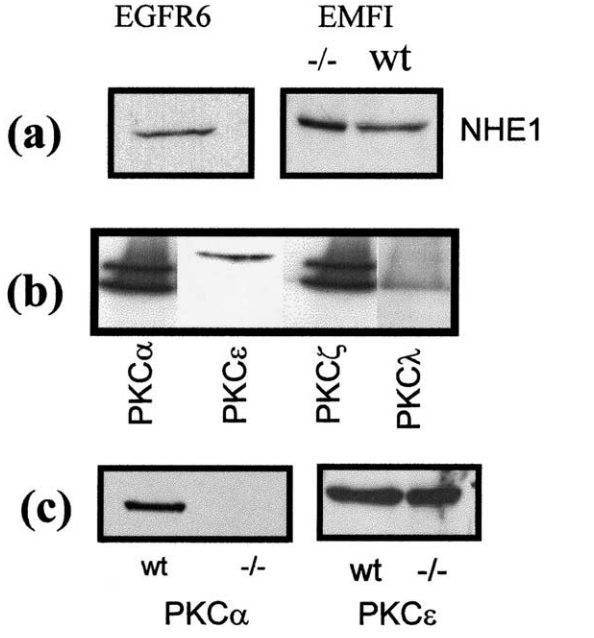

Fig. 1. Expression patterns of NHE1 and PKC isozymes in NIH3T3 (EGFR6) and EMFI cells. Western blot analysis of (a) the NHE subtype NHE1 in EGFR6 and EMFI (wt and PKCα−/−), (b) the PKC isotypes in EGFR6 cells and (c) the PKC isotypes α and ε in EMFI.

amiloride analogue EIPA. The activation of the NHE by stimulation of PKC with TPA in this cell system is demonstrated. The EGF- and TPA-induced activation of the NHE was suppressed by the PKC inhibitor GF109303X or depletion of PKC after long-term treatment (24 h) with phorbol ester. In contrast to EGFR6, in PKCα−/− EMFI cells, the NHE could be activated neither by TPA nor by ATP. This result points to a critical role of the PKC isoforms PKCα or/and PKCε in the regulation of the NHE.

As described previously [9,10] stimulation of EGFR6 cells by EGF causes an increase in inositol phosphate and diacylglycerol formation followed by an increase of the cytoplasmic calcium concentration, possibly leading to an activation of PKC. Taken together these results indicate an implication of PKCs in the EGF-mediated pathway of the NHE activation. Therefore, the role of classical, novel and atypical PKC isozymes in regulation of the NHE1 was investigated in more detail.

3.3. Role of different PKC isozymes in activation of the NHE

Transient transfection of EGFR6 cells with $PKC\alpha_{K/R}$ and $nPKC\varepsilon_{K/R}$ caused an almost complete inhibition of the EGF-induced activation of the NHE (Fig. 3) and a significant suppression of the TPA-mediated stimulation. The activation of the NHE, however, was not affected by expression of $PKC\zeta_{K/W}$. This result strongly suggests an implication of cPKCα and nPKCε in the activation of the NHE. These suppositions were checked by antisense constructs. Transfection of EGFR6 cells with PKCα antisense oligonucleotides reduced the expression of cPKCα in a dose-dependent manner (Fig. 4a). The same result was obtained by PKCε antisense oligonucleotides depleting PKCε in this cell system (Fig. 4b). PKCα antisense oligonucleotides at a concentration of 3 μM were able to inhibit the TPA- and EGF-induced activation of the NHE, comparable to $PKC\alpha_{K/R}$ (Fig. 4c). In contrast to $nPKC\varepsilon_{K/R}$, the PKCε antisense construct did not affect the EGF-induced activation of the NHE (Fig. 4d). $PKC\alpha_{A/E}$ but not $PKC\varepsilon_{A/E}$ provoked a weak but significant activation of the NHE (Fig. 4e).

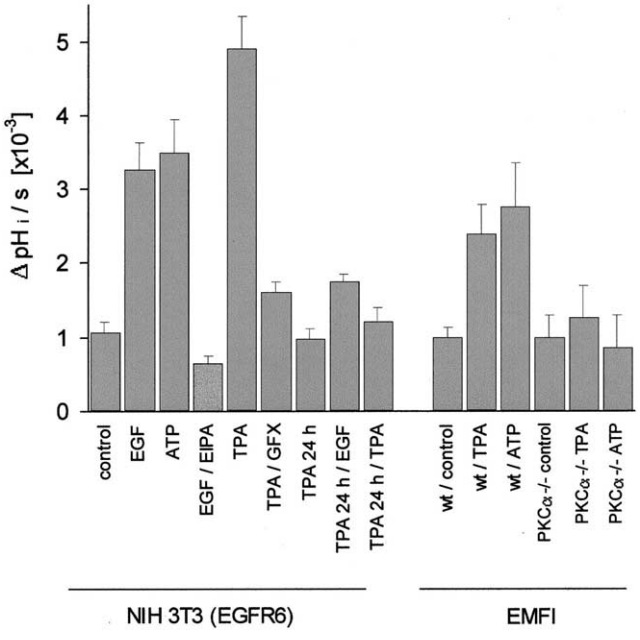

Fig. 2. Activation of the NHE. The NHE activity of EGFR6 cells was determined by measuring the time-dependent recovery of the cytoplasmic pH_i after acidification by NH_4Cl employing a single cell imaging technique as described in Section 2. After 2 days of serum starvation induction by 0.1 μM EGF and 1 μM ATP (5 min) revealed a threefold increase of the activity which could be blocked by the NHE inhibitor EIPA. The phorbol ester (0.1 μM TPA, 5 min) and EGF-induced activation of the NHE is depressed by the PKC inhibitor GF109303X and after depletion of PKC by long-term treatment with TPA (24 h). Bars represent mean ± S.E.M. ($n \geq 10$). In wt EMFI cells the NHE is activated by 0.1 μM TPA and 1 μM ATP. EMFI cells derived from a corresponding PKCα−/− knock-out mouse revealed no significant stimulation of the NHE by TPA or ATP. Bars represent mean ± S.E.M. ($n \geq 6$).

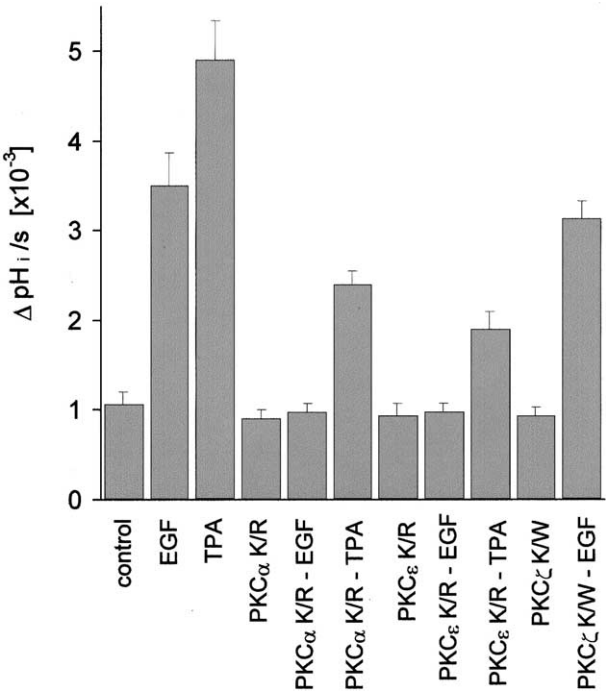

Fig. 3. Role of representatives from each of the three major subfamilies of the PKC isotypes in EGF- and TPA-induced activation of the NHE. EGFR6 cells were transfected with kinase-defective $PKC\alpha_{K/R}$, $nPKC\varepsilon_{K/R}$ or vector control pEF-neo (mock) together with the reporter plasmid GFP as a selection marker. Inhibition of the EGF-induced NHE activity was seen after expression of both DN isotypes α and ε. Essentially the same result was obtained after stimulation of the NHE by TPA. Expression of the DN isoform of aPKCζ failed to inhibit the EGF-mediated activation of the NHE. Bars represent mean ± S.E.M. ($n \geq 10$).

3.4. Calcium-dependent activation of the NHE

Stimulation of EGFR6 cells by EGF causes an increase of the cytoplasmic calcium concentration by release of calcium from intracellular stores followed by a store depletion-activated calcium influx as demonstrated previously [9]. Binding of intracellular free calcium by BAPTA and simultaneous removal of the extracellular calcium by EGTA inhibits the EGF- and TPA-induced activation of the NHE (Fig. 5a). Elevation of cytoplasmic calcium by thapsigargin or the calcium ionophore A23187 leads to a growth factor-independent activation of the NHE (Fig. 5b). To test whether the calcium-induced stimulation of the NHE is cPKC-dependent, thapsigargin- and A23187-mediated activation of the NHE was carried out in EGFR6 cells expressing $PKC\alpha_{K/R}$. The kinase-defective $PKC\alpha_{K/R}$ partially reduced the calcium-induced ac-

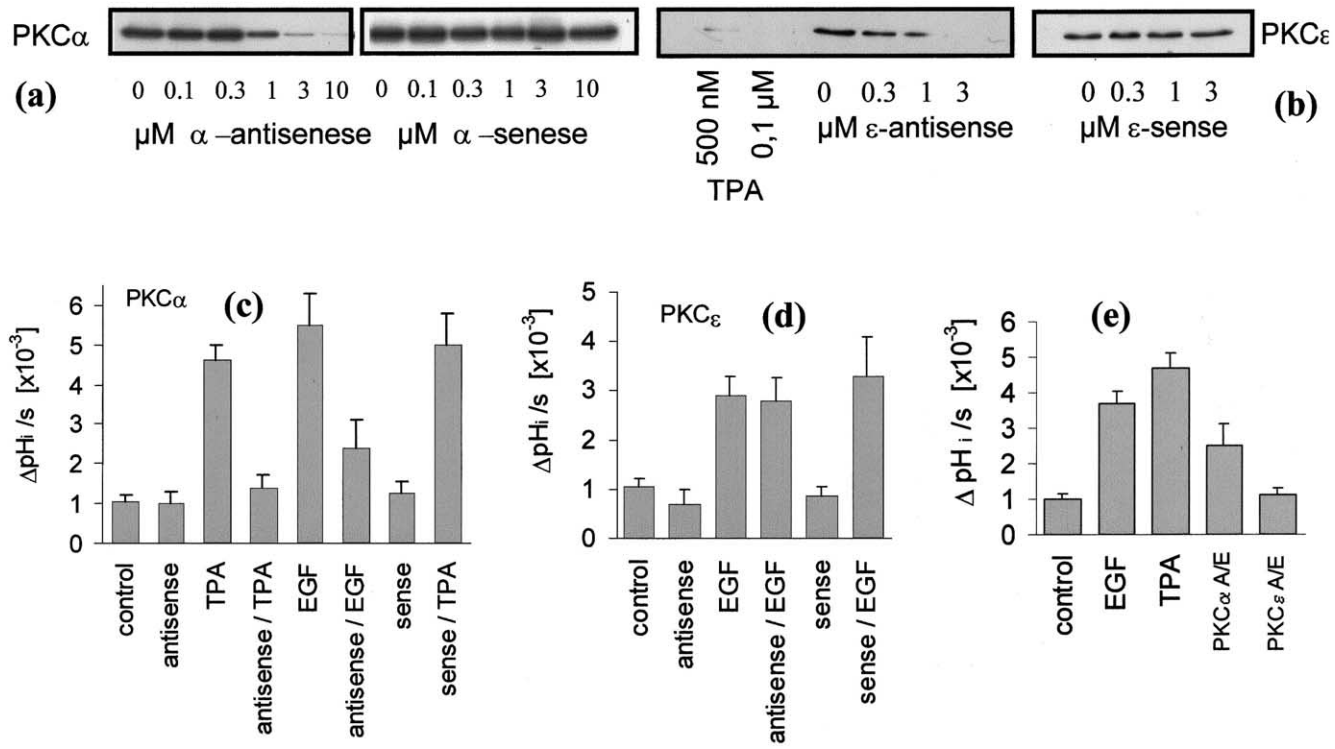

Fig. 4. Depletion of PKCα and PKCε by antisense oligonucleotides. EGFR6 cells were incubated with increasing concentrations of antisense oligonucleotides as indicated. The concentration-dependent depletion of PKCα (a) and PKCε (b) by the antisense oligonucleotides but not by the corresponding sense oligonucleotides is demonstrated. The EGF- and TPA-induced activation of the NHE is inhibited by depletion of PKCα (c) but unaffected after depletion of PKCε (d). The basal activity of the NHE is increased by overexpression of $PKC\alpha_{A/E}$ but not of $PKC\varepsilon_{A/E}$ (e). Bars represent mean ± S.E.M. ($n \geq 10$).

tivation of the NHE. The thapsigargin-induced activation of the NHE, however, was almost completely inhibited by the calcium/calmodulin II kinase inhibitor KN-93 (Fig. 5c). It is concluded, that the stimulation of the NHE by calcium results from activation of calcium-dependent kinases like cPKCα and calcium/calmodulin kinases.

3.5. Erk-independent activation of the NHE

In EGFR6 cells it has been demonstrated that stimulation of the EGF receptor by EGF leads to an activation of the MAP kinase pathway [9,10]. The Ras-Raf1-MEK signalling pathway was shown to be essential for the activation of the Ras-dependent stimulation of the NHE1 in CCL39 fibroblasts [11]. Moreover, in this cell system, the growth factor-induced activation of the MAP kinase cascade plays a predominant role in NHE1 stimulation [12]. It was, therefore, decided to investigate whether stimulation of the MAP kinase pathway by EGF leads to an activation of the NHE in EGFR6 cells.

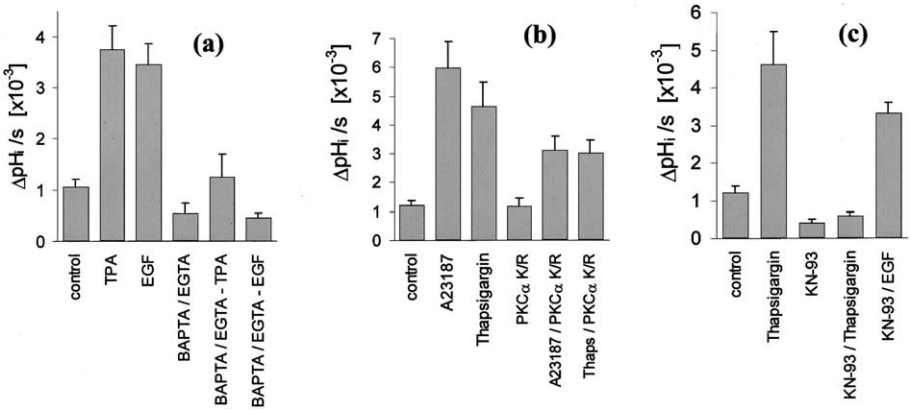

Fig. 5. Calcium-dependent activation of the NHE. Removing of cytoplasmic free calcium by BAPTA/EGTA reduces the basic activity of the NHE and inhibits the EGF- and TPA-induced stimulation of the NHE (a). Increase of cytoplasmic calcium by addition of 1 μg/ml A23187 and 1 μM thapsigargin leads to a growth factor-independent activation of the NHE and transfection of $PKC\alpha_{K/R}$ attenuates the A23187- and thapsigargin-induced activation of the NHE (b). Inhibition of calcium/calmodulin kinase II by KN-93 (10 μM for 18 h) represses the basal and the thapsigargin-induced activation of the NHE, while the EGF-mediated stimulation of the NHE remains unaffected (c). Bars represent mean ± S.E.M. ($n \geq 10$).

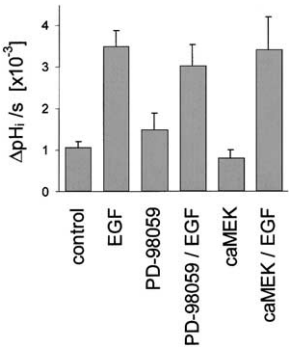

Fig. 6. The EGF-stimulated activation of the NHE is not affected by the MAP kinase pathway. Preincubation of the cells with the MAP kinase inhibitor PD-98059 (50 μM) for 18 h shows no significant effect on the EGF-stimulated activation of the NHE and overexpression of a constitutively active form of MAP kinase (caMEK) is ineffective to stimulate the NHE in the absence of EGF. Bars represent mean ± S.E.M. ($n \geq 8$).

Employing the MEK inhibitor PD-98059, however, revealed no significant attenuation of the EGF-induced activation of the NHE. Furthermore, overexpression of a constitutively active MEK failed to activate the NHE in the absence of EGF (Fig. 6). These data indicate that the MEK > ERK cascade is not involved in the EGF-mediated activation of the NHE.

4. Discussion

NIH3T3 cells overexpressing the EGF receptor (EGFR6 cells) contain the PKC isotypes cPKCα, nPKCε, aPKCλ and aPKCζ. The data presented here demonstrate that the growth factor-induced activation of the ubiquitously expressed isoform of the NHE, NHE1, selectively requires cPKCα. This conclusion is based on the observations that (i) downregulation of PKC by long-term exposure to TPA, (ii) expression of kinase-defective $PKC\alpha_{K/R}$, and (iii) depletion of cPKCα by isotype-specific antisense oligonucleotides lead to an almost complete inhibition of the phorbol ester- and the EGF-induced stimulation of the NHE. (iv) Final proof of this concept came from experiments employing PKCα−/− embryonic fibroblasts derived from the PKCα−/− knock-out mouse. Compared to the corresponding wt cell line, these cells expressed about the same level of NHE1 and nPKCε, but no detectable amount of cPKCα as shown in Fig. 1d. Whereas in the wt cell line the stimulation by TPA and ATP induced a significant activation of the NHE similar to EGFR6 cells, this was not the case in the corresponding PKCα−/− cells (Fig. 2).

Both isotypes of PKC, cPKCα and nPKCε, are activated by diacylglycerol or the widely used more active surrogate TPA. Stimulation of the EGFR6 cells by EGF leads to an activation of PLCγ [10] and thereby to formation of diacylglycerol and inositol 4,5-bisphosphate which in turn cause an increase in cytoplasmic calcium [9]. Consequently, this signalling cascade induced by EGF could activate cPKCα as well as nPKCε. If PKCε were implicated in the regulation of the NHE, it would be expected that expression of $nPKC\varepsilon_{K/R}$ causes an inhibition of the EGF- or TPA-induced activation of the NHE. As demonstrated in Fig. 3, this was indeed the case. Depletion of nPKCε, however, failed to inhibit the EGF-induced activation of the NHE (Fig. 4b,d). Thus, an expression of DN PKCε somehow interferes with endogenous PKCα, perhaps by competition with a joint cofactor like diacylglycerol. Expression of DN, kinase-deficient mutants of PKCα and PKCε is, therefore, not sufficient to unambiguously define a PKC dependence in this system. The failure of PKCε-depleted cells to inhibit the EGF-induced activation of the NHE clearly indicates the operation of a PKCε-independent pathway. This conclusion is furthermore supported by the finding that removal of cytoplasmic free calcium by BAPTA blocked the EGF-induced stimulation of the NHE (Fig. 5a), although the activation of nPKCε is known to be calcium-independent, due to the lack of the calcium binding site C2 of the regulatory domain. Furthermore, in PKCα−/− cells which express a normal level of nPKCε (Fig. 1c) no significant activation of the NHE by TPA and ATP could be observed (Fig. 2). Collectively these findings indicate that nPKCε is not implicated in the PKC-dependent regulation of the NHE.

The MAP kinase pathway is considered essential in growth factor-stimulated activation of the NHE1 in CCL39 cells [12]. Members of aPKCs have been described previously as crucial downstream targets and regulators in the Ras-MAP kinase pathway in epithelial cells [16]. Therefore, it was attractive to study the implication of the MAP kinase cascade in regulation of the NHE. However, in contrast to CCL39 cells, blockade of this pathway by a MAP kinase inhibitor failed to attenuate the EGF-induced activation of the NHE in EGFR6 cells and overexpression of constitutively active MEK was insufficient to activate the NHE growth factor independently (Fig. 6). Finally, DN isotypes of PKCλ and PKCζ did not affect the EGF-mediated stimulation of the NHE.

In addition to PKCα, the concentration of cytoplasmic free calcium is critical for the regulation of the NHE in EGFR6 cells. Removing the cytoplasmic calcium by BAPTA/EGTA leads to a reduction of the basal activity of the NHE (Fig. 5a) and the EGF-induced pathway is completely inhibited under these conditions. Elevation of cytoplasmic calcium by thapsigargin and A23187 was able to mimic a growth factor-independent stimulation of the NHE which could only in part be inhibited by DN $PKC\alpha_{K/R}$ (Fig. 5b). These findings support a model in which the thapsigargin- and A23187-induced stimulation of the NHE is mediated in part by a calcium-mediated activation of the calcium-dependent PKCα, but to a greater extent by another, PKC-independent mechanism [14]. Indeed, a strong inhibition of the thapsigargin-induced activation of the NHE was observed by application of the calcium/calmodulin kinase II inhibitor KN-93 (Fig. 5c), indicating a predominant role of the calmodulin kinase II in the calcium-induced stimulation of the NHE. In view of the poor inhibition by KN-93 of the EGF-induced activation of the NHE it must be concluded that the EGF-induced stimulation of the NHE is predominantly mediated by PKCα and not by a KN-93-sensitive kinase.

In summary, the data presented here clearly demonstrate that among the PKC isotypes expressed in our cell systems, only PKCα is implicated in the EGF- and TPA-induced activation of the NHE in EGFR6 cells. Surprisingly, no contribution to the activation of the NHE by the MAP kinase cascade, which has been shown to be important in CCL39 cells [12], could be demonstrated in this cell system. High levels of cytosolic free calcium are also sufficient to stimulate the NHE via calcium/calmodulin kinase II. Full activation of

the NHE by EGF, however, depends on the combined effects of active PKCα and calcium.

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