The Amiloride-sensitive Epithelial Sodium Channel α-Subunit Is Transcriptionally Down-regulated in Rat Parotid Cells by the Extracellular Signal-regulated Protein Kinase Pathway*

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Previous studies have shown that an inducible Raf-1 kinase protein, ΔRaf-1:ER, activates the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK)-signaling pathway, which is required for the transformation of the rat salivary epithelial cell line, Pa-4. Differential display polymerase chain reaction was employed to search for mRNAs repressed by ΔRaf-1:ER activation. Through this approach, the gene encoding the α-subunit of the amiloride-sensitive epithelial sodium channel (α-ENaC) was identified as a target of activated Raf-1 kinases. α-ENaC down-regulation could also be seen in cells treated with 12-O-tetradecanoyl-1-phorbol-13-acetate (TPA), indicating that the repression of steady-state α-ENaC mRNA level was dependent upon the activity of protein kinase C, the target of TPA, as well. Pretreatment of cells with a specific inhibitor of the ERK kinase pathway, PD 98059, markedly abolished the down-regulation of α-ENaC expression, consistent with the hypothesis that the ERK kinase-signaling pathway is involved in TPA-mediated repression. Moreover, through the use of transient transfection assays with α-ENaC-reporter and activated Raf expression construct(s), we provide the first evidence that activation of the ERK pathway down-regulates α-ENaC expression at the transcriptional level. Elucidating the molecular programming that modulates the expression of the α-subunit may provide new insights into the modulation of sodium reabsorption across epithelia.

The expression of the amiloride-sensitive epithelial sodium channel (ENaC), a member of the DEG/ENaC ion channel superfamily (1), on the apical surface of select epithelia regulates the rate of sodium reabsorption. Its sodium channel activity has been identified in distal segments of kidney tubule, airway epithelium, skin, bladder, colon, sweat, and salivary glands. By using functional expression assays in a Xenopus oocyte system, three ENaC subunits, denoted α-, β-, and γ-ENaC, have recently been identified, cloned, and characterized (2-4). These three subunits share considerable amino acid identity (34-37%) and a similar topological structure (5). Although the α-subunit is sufficient for channel activity, expression of the β- or γ-subunit(s) of ENaC alone or concurrently does not reconstitute an amiloride-sensitive conductance in Xenopus oocytes, but either subunit augments the channel activity of α-ENaC by three- to five-fold. When both β- and γ-ENaC are co-injected with α-ENaC, greater than 100-fold potentiation of the amiloride-sensitive current is observed (3). Thus, optimal activity of the amiloride-sensitive epithelial sodium channel requires a heteromultimeric complex consisting of α-, β-, and γ-subunits (6, 7). ENaC plays a major role in the control of fluid and electrolyte homeostasis, as demonstrated by the discovery that mutations in ENaC subunits are associated with hypertensive or hypotensive genetic diseases such as Liddle’s syndrome (8) or pseudohypoaldosteronism type 1 (9). Though the exact subunit composition of ENaC is still being resolved, the expression of a functionally active sodium channel is dependent on the presence of α-ENaC. Indeed, mouse α-ENaC(-/-) neonates, generated by homologous gene targeting, develop respiratory distress and die within 40 h from an inability to clear the lung liquid (10).

The expression of ENaC is up-regulated by glucocorticoids in the human fetal lung, where mRNAs of all three subunits increase in response to dexamethasone. In contrast, the induction of ENaC genes by aldosterone appears to be subunit-specific, since β- and γ-ENaC, but not α-ENaC mRNAs, are increased in rat distal colon (11, 12), whereas in the kidney, vasopressin is reported to up-regulate all three subunits (13). However, the molecular components that constitute the complete signaling pathway(s) of these events are far from clear.

In most eukaryotic cells, gene expression is regulated by several distinct signaling modalities. Mitogen-activated protein (MAP) kinases, characterized as proline-directed serine/threonine kinases (14), are important cellular-signaling components that transduce signals from cell membrane to nucleus in response to a wide variety of stimuli (15-17). Several MAP kinases have been identified to date, including the extracellular signal-regulated protein kinase (ERK) (18, 19), the C-Jun NH2-terminal/stress-activated protein kinase (20, 21), and p38 (also known as Mkp2/CBPSP) (22-24). Each MAP kinase is activated by a distinct kinase module, which consists of MAP kinase kinase, MAP kinase kinase, and MAP kinase. These kinase modules may provide a structural basis for different signaling cascades and thereby relay extracellular stimuli to specific effectors. The MAP kinase kinases in the ERK-signaling module are MAPK/ERK kinase (MEK) 1 and 2 (25), although the MAP kinase kinase kinases includes Raf-1 and

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‡The abbreviations used are: ENaC, epithelial amiloride-sensitive sodium channel; MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; MEK, MAPK/ERK kinase; PKC, protein kinase C; TPA, 12-O-tetradecanoyl-1-phorbol-13-acetate; kb, kilobase pairs; CAT, chloramphenicol acetyltransferase; ER, estrogen receptor.
MEKK1 (26, 27). Biochemical and genetic studies have revealed that the Raf/MEK/ERK signaling pathway is a pivotal cascade that culminates in phosphorylation of downstream cytosolic and nuclear substrates, ultimately leading to phenotypic cellular changes. The mechanisms involved in the activation events for the ERK kinase cascade have been studied in detail. Raf-1 kinase is activated through interaction with membrane-associated Ras-GTP and in part by phosphorylation (28, 29). In addition, there is ample evidence for the protein kinase C (PKC)-dependent pathway of ERK kinase activation via Raf-1 kinase (30). Since PKC is the major effector for phorbol esters, it has been implicated in the activation of the Raf/ERK pathway (31, 32). For instance, treatment with phorbol esters (e.g., TPA) leads to a rapid activation of Ras and/or Raf in most cell types (33–35). More direct evidence for the involvement of PKC in regulating this pathway has come from transfection or microinjection experiments, which report that TPA treatment or expression of PKC-α and PKC-γ can induce Raf/MEK/ERK pathway activation (34, 36, 37).

In our efforts to elucidate the molecular mechanisms of Raf-1-mediated cell differentiation, proliferation, and transformation, we employed the polymerase chain reaction-based differential display method (38) to identify genes that are regulated in response to Raf-1 activation. In this report, we demonstrate that the α-ENaC mRNA level is selectively down-regulated. Because the α-subunit of ENaC is critical for channel activity, we have investigated the signaling pathway that modulates its expression. Recently, the α-ENaC gene has been shown to be organized into 13 exons and spans at least 17 kb of the human genome (39). We have characterized the first 5 kb of the α-ENaC promoter and engineered several α-ENaC/CAT reporter constructs to study the molecular regulatory mechanism(s) for ENaC gene expression. Although much attention has focused on the up-regulation of ENaC, to date very little is known about the molecular mechanisms by which cells down-regulate ENaC expression. This paper proposes one such mechanism in salivary epithelial cells, where MAP kinase/ERK activation leads to down-regulation of α-ENaC by repressing the transcription of the α-ENaC gene.

MATERIALS AND METHODS

Cell Culture and Stable Transfection—The rat parotid epithelial cell line Pa-4, also known as parotid C5 cells (40), was plated out on Primaria culture dishes (Falcon) in Dulbecco’s modified Eagle’s medium (1:1) supplemented with 5% fetal calf serum, insulin (5 μg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (1 μM), glutamate (5 mM), and kanamycin monosulfate (60 μg/ml) and maintained in a humidified atmosphere of 5% CO2 and 95% air at 35 °C. A stably transfected Pa-4RAf-1-ER ELON cell line was established by transfecting Pa-4 cells with the plasmid pLNCXα-ENaC-1-ER (41) by a LipofectAMINE™-mediated method and selected with G418 (G418, 600 μg/ml). Corresponding phage plaques were selected, diluted, replated, and rescreened to obtain single phage clones. One purified positive clone, named ΔENaC-actin probe was also included to ensure that the quality and quantity of RNA between lanes were comparable. All blots were washed in 0.1× SSC (0.15 M NaCl and 0.015 M sodium citrate), 0.5% SDS solution at 60 °C. To improve autoradiographic detection, exposure was extended to 2 weeks at −80 °C between two thin layers of x-ray film. The membranes were also subjected to electronic autoradiography with the InstantImager 228 (Packard Instrument Co.) to quantitate radioactivity in each band for comparative analysis.

Western Blot Analysis—Parental and ΔRAf-1-ER stably transfected Pa-4 cells were washed and pelleted at 4 °C in Earle’s balanced salt solution. Equal amounts of total protein from cell lysates were resolved on 10% SDS-polyacrylamide gel electrophoresis, electroblotted on Immobilon-P Transfer Membrane (Millipore), and incubated with either anti-α-ENaC antibody (courtesy of Dr. Peter Smith, Allegheny University) or anti-eIF-2α antibody (courtesy of Dr. Roger Duncan, University of Southern California). ERK1/2 blots were probed with either Anti-Active™ MAP kinase (Promega, WI) or anti-ERK1 C-16-G (Santa Cruz Biotechnology, Inc., CA) antibodies. Horseradish peroxidase-labeled secondary antibody (Amersham) and the enhanced chemiluminescence (ECL) detection system (Amersham) were used to visualize proteins of interest.

Identification and Characterization of Genomic Clone Encoding Rat α-ENaC Gene 5′-Flanking Region—To study the rat α-ENaC gene promoter/enhancer, a male Sprague-Dawley rat genomic library (in λ DASH II, Stratagene. La Jolla, CA) was screened with the 5′ labeled 5′-most α-ENaC cDNA probe as described previously (43). Four positive plaques were identified from an initial screening of four rat genomic equivalents. Corresponding phage plaques were selected, diluted, replated, and rescreened to obtain single phage clones. One purified positive clone, named ΔENaC-actin probe was also included to ensure that the quality and quantity of RNA between lanes were comparable. All blots were washed in 0.1× SSC (0.15 M NaCl and 0.015 M sodium citrate), 0.5% SDS solution at 60 °C. To improve autoradiographic detection, exposure was extended to 2 weeks at −80 °C between two thin layers of x-ray film. The membranes were also subjected to electronic autoradiography with the InstantImager 228 (Packard Instrument Co.) to quantitate radioactivity in each band for comparative analysis.

RESULTS AND DISCUSSION

We have been using the rodent salivary gland as a model system to study the genomic response(s) leading to cellular hypertrophy and hyperplasia. Recently, we have shown that
changes in the morphology of the parotid salivary gland correlate with the activation of Raf-1 kinase (42). To study the phenotypic consequences of Raf-1 activation in the rat parotid salivary gland, a parotid epithelial cell line (Pa-4) was stably transfected with a gene encoding a human Raf-1 kinase/estrogen receptor fusion protein (ΔRaf-1:ER). ΔRaf-1:ER responds to estradiol in a dose- and time-dependent manner (41, 45), allowing Raf-1 kinase activity to be selectively and conditionally stimulated. Furthermore, activation of ΔRaf-1:ER is known to be independent of the endogenous estrogen receptor (ER) since either ER agonists or antagonists activate the ΔRaf-1:ER protein (41, 45, 46).

Activation of ΔRaf-1:ER resulted in a number of morphological changes in Pa-4 cells (42) reflecting the changes in mRNA expression. We compared these changes in mRNA expression between the parental (Pa-4) and ΔRaf-1:ER-transfected cell line (Pa-4ΔRaf-1:ER) using the polymerase chain reaction-based method, mRNA differential display. Reverse transcription-polymerase chain reaction products from Pa-4 and Pa-4ΔRaf-1:ER cells revealed a ~125-base pair fragment (B4) that was markedly decreased in Pa-4ΔRaf-1:ER cells (data not shown). Sequence analysis indicated that B4 was identical to the 3’ terminus of the rat α-ENaC cDNA. To confirm the decrease in the α-ENaC message in Pa-4ΔRaf-1:ER cells, a Northern analysis was performed on total RNA prepared from Pa-4 and Pa-4ΔRaf-1:ER cells using the B4 fragment as a probe (Fig. 1A). In confirmation of the results from the mRNA differential display, a single band corresponding to the reported size of the α-ENaC message (~3700 bases) (2) was detected in RNA from Pa-4 and Pa-4ΔRaf-1:ER cells (Fig. 1A), whereas a dramatic decrease in signal was observed in ΔRaf-1:ER-transfected cells (Fig. 1A, 3rd and 4th lanes).

Estradiol alone did not decrease the expression of α-ENaC in Pa-4 cells (Fig. 1A, 1st and 2nd lanes), indicating that the down-regulation of α-ENaC mRNA is a consequence of ΔRaf-1:ER activity and not an ER-mediated event via the estrogen response element. Moreover, the basal α-ENaC expression in unstimulated Pa-4ΔRaf-1:ER cells was already repressed in comparison to the α-ENaC level in parental Pa-4 cells. This was not surprising in that the Pa-4ΔRaf-1:ER cell line has already been shown to exhibit a low level of ΔRaf-1:ER kinase activity (42), probably due to an incomplete inhibition of the ΔRaf-1:ER protein in these stably transfected cells (see below). Thus it is possible that this remaining ΔRaf-1:ER activity is high enough to repress α-ENaC expression.

To determine the relative amount of α-ENaC protein, a Western analysis using antibody against α-ENaC was performed. Consistent with the Northern analysis (Fig. 1A), a significant decrease in the amount of α-ENaC protein was detected in Pa-4ΔRaf-1:ER cells (Fig. 1B, upper panel). The amount of the translation factor eIF-2α in Pa-4 and Pa-4ΔRaf-1:ER cell lysates was also determined (Fig. 1B, lower panel) to demonstrate that the decrease in α-ENaC was not due to nonspecific down-regulation or protein degradation. The half-life of α-ENaC protein is reported to be relatively short at about 1 h (47). Although we do not know if ΔRaf-1:ER activation directly affects ENaC function, it is likely that the reduced availability of both α-ENaC message and protein would lead to diminished sodium absorption across the parotid epithelium.

The ΔRaf-1:ER protein has been reported to mimic the action of endogenous Raf-1 kinase (41, 42). However, the low level of ΔRaf-1:ER kinase activity in Pa-4ΔRaf-1:ER cells made it necessary for us to investigate whether activation of the Raf/MEK/ERK-signaling cascade by a pharmacological means would down-regulate α-ENaC in the parental cell. Since PKC activates Raf-1 kinase, we treated cells with a known PKC activator, TPA. A Northern analysis was performed on Pa-4 cells that were treated with TPA at intervals up to 12 h. A marked decrease in α-ENaC mRNA was observed within 6 h post-treatment (Fig. 2A), implicating that the Raf-1 cascade down-regulates the α-ENaC gene. Although PKC activation with TPA induces ERK kinase activity in many cell types (48), we investigated whether TPA could activate ERK1/ERK2 in Pa-4 cells. A Western analysis of total protein from TPA-treated Pa-4 cells was performed with antibody that recognizes activated ERK2 and cross-reacts with activated ERK1. The kinetics of TPA-induced ERK activation were transient, as indicated by an increase in ERK phosphorylation within 5 min that declined to basal levels after 6 h. There was no change in the level of either ERK1 or ERK2 after TPA treatment, as indicated by an immunoblot probed with antibody that recognizes both ERK1 and ERK2. These data correlate well with the Northern analysis (Fig. 2B) in which no further decrease in α-ENaC mRNA is observed between 6 to 12 h. These results suggest that TPA transiently activates the Raf/MEK/ERK-signaling module by demonstrating TPA-mediated transient activation of ERK1/2 in Pa-4 cells.

We also compared the level of ERK activation between untreated ΔRaf-1:ER cells and TPA-treated Pa-4 cells to determine whether the sustained basal elevation of ΔRaf-1:ER kinase was sufficient to down-regulate α-ENaC. Fig. 2C indicates that the basal level of activated ERK was elevated in ΔRaf-1:ER cells to a level comparable with that in TPA-stimulated Pa-4 cells. Although the level of activated ERK is slightly higher in TPA-treated Pa-4 cells, activation is transient. Therefore, it is conceivable that in ΔRaf-1:ER cells, the sustained
Eighteen cells were treated with 25 or 100 ng/ml TPA for indicated time periods. Sed, blotted, and probed with 32P-labeled probes of rat a Northern analysis of a cells through the activation of ERK kinase. The Northern blot was performed and analyzed as described in antibody against active ERK2 (protein was fractionated by size, transferred, and probed with either period. Cell lysates were prepared at intervals, where 12 from Pa-4 cells, which were treated with TPA (100 ng/ml) over a 6-h

m of total RNA from individual samples were electrophore-
m, a Western analysis of cell lysate from either Pa-4 or

C-Jun NH2-terminal kinase activity (51). Thus, Pa-4 cells were

Total RNA was prepared from Pa-4 cells that were treated with or without TPA (100 ng/ml) in the presence of actinomycin D (5 μg/ml) to block transcription for 4, 8, and 12 h. Equal amounts of RNA were used in the Northern analysis to estimate the half-life for a-ENaC mRNA. Based on our results (Fig. 3), the half-life for a-ENaC mRNA in parotid cells was calculated to be approximately 8 h. If the transcription of a-ENaC gene is completely inhibited by TPA treatment, there should be about 35% a-ENaC mRNA in cells after 12 h co-treated with actinomy-
cin D and phorbol ester, as shown in Fig. 3B. Taken to-
gene expression at the post-transcriptional level, Pa-4
cells were treated with the transcription inhibitor, actinomycin D, in the presence or absence of TPA. If TPA-mediated ERK activation increases a-ENaC mRNA turnover by a post-transcriptional mechanism, the addition of TPA to actinomycin D-treated cells should augment a decrease in the a-ENaC message. The concentration of actinomycin D (5 μg/ml) used in these experiments has been shown to completely inhibit the incorpo-
ration of [3H]uridine into the acid-soluble fraction (52). Total RNA was prepared from Pa-4 cells that were treated with or without TPA (100 ng/ml) in the presence of actinomycin D (5 μg/ml) to block transcription for 4, 8, and 12 h. Equal amounts of RNA were used in the Northern analysis to estimate the half-life for a-ENaC mRNA. Based on our results (Fig. 3), the half-life for a-ENaC mRNA in parotid cells was calculated to be approximately 8 h. If the transcription of a-ENaC gene is completely inhibited by TPA treatment, there should be about 35% a-ENaC mRNA in cells after 12 h co-treated with actinomycin D and phorbol ester, as shown in Fig. 3B. Taken to-
tgether, these results indicate that the addition of TPA does not increase the turnover rate of a-ENaC mRNA.

elevation of activated ERK is sufficient to down-regulate a-ENaC mRNA expression.

To further support this model, the MEK inhibitor, PD 98059, was employed to confirm the pathway by which TPA treatment led to a decrease in a-ENaC mRNA. PD 98059 specifically blocks the activation of ERK kinase by inhibiting the kinase activity of the ERK upstream kinase (MEK), thereby preventing the phosphorylation and activation of ERK (49, 50). Its specificity has been tested against at least 18 kinases, including PKC and the highly homologous C-Jun NH2-terminal kinase, where PD 98059 appears to have no effect on PKC or C-Jun NH2-terminal kinase activity (51). Thus, Pa-4 cells were exposed to 20 μM PD 98059 15 min before TPA treatment. A Northern analysis on a-ENaC mRNA levels in Pa-4 cells showed that the presence of PD 98059 inhibited TPA-induced down-regulation of a-ENaC expression (Fig. 2C, lanes 2 and 4). Therefore, we propose that the activation of the ERK signaling is sufficient to down-regulate a-ENaC gene expression.

To investigate whether ERK activation regulates a-ENaC gene expression at the post-transcriptional level, Pa-4 cells were treated with the transcription inhibitor, actinomycin D, in the presence or absence of TPA. If TPA-mediated ERK activation increases a-ENaC mRNA turnover by a post-transcriptional mechanism, the addition of TPA to actinomycin D-treated cells should augment a decrease in the a-ENaC message. The concentration of actinomycin D (5 μg/ml) used in these experiments has been shown to completely inhibit the incorporation of [3H]uridine into the acid-soluble fraction (52). Total RNA was prepared from Pa-4 cells that were treated with or without TPA (100 ng/ml) in the presence of actinomycin D (5 μg/ml) to block transcription for 4, 8, and 12 h. Equal amounts of RNA were used in the Northern analysis to estimate the half-life for a-ENaC mRNA. Based on our results (Fig. 3), the half-life for a-ENaC mRNA in parotid cells was calculated to be approximately 8 h. If the transcription of a-ENaC gene is completely inhibited by TPA treatment, there should be about 35% a-ENaC mRNA in cells after 12 h co-treated with actinomycin D and phorbol ester, as shown in Fig. 3B. Taken to-
tgether, these results indicate that the addition of TPA does not increase the turnover rate of a-ENaC mRNA.

To determine whether the down-regulation of a-ENaC is mediated, at least in part, at the transcriptional level by the ERK kinase pathway, we subcloned a 4.9-kb and a 1.4-kb DNA 5′-flanking fragment of the rat a-ENaC gene into a CAT reporter plasmid (a-ENaC/CATl and a-ENaC/CATs, respectively, Fig. 4A). The promoter/enhancer activities of these constructs were examined by transiently transfecting Pa-4 cells and measuring the CAT activity in cell lysates (Fig. 4). The truncation of the a-ENaC promoter/enhancer from −4.9 kb to −1.4 kb had a modest effect on basal CAT activity (Fig. 4B, lanes 1 and 5). Furthermore, the CAT activities from both a-ENaC/CATl- and a-ENaC/CATs-transfected cells were down-regulated by TPA treatment approximately four- to five-fold (Fig. 4B, lanes 2 and 6). Consistent with ERK-mediated down-regulation of a-ENaC, PD 98059 inhibited the TPA-dependent decrease in a-ENaC/ CAT activity. Moreover, cotransfection of Pa-4 cells with a-ENaC/CATs and a constitutively active form of Raf-1 kinase (RafBXB) (44) showed a dose-dependent decrease in CAT activity, whereas triple transfection with ERK2 (53) had a cooper-
effective (Fig. 4C). Together, these results suggest that the major regulatory region(s) that mediates the basal and/or regu-
ulated a-ENaC expression is located within the 1.4 kb of the proximal 5′-flanking region. In summary, our results provide the first demonstration that activation of the MAP kinase/ERK cascade can specifically down-regulate a-ENaC promoter/enhancer activities.
To further explore the nature of the down-regulation of \( \alpha \)-ENaC expression mediated by ERK signaling, we considered whether concurrent protein synthesis is necessary to down-regulate the \( \alpha \)-ENaC message. Protein synthesis was blocked in Pa-4 cells prior to TPA treatment using the protein synthesis inhibitor emetine (10 \( \mu \)g/ml). A Northern analysis indicated that \( \alpha \)-ENaC expression was unaffected by emetine treatment (6 h) alone and that the TPA-mediated repression (Fig. 5, 2nd lane) was markedly inhibited (60%) by co-treatment with emetine (Fig. 5, 4th lane). The same inhibitory effect on TPA-mediated down-regulation was also observed using another protein synthesis inhibitor, cycloheximide (10 \( \mu \)g/ml, data not shown). Therefore, our results indicate that concurrent protein synthesis is required for ERK-dependent down-regulation of \( \alpha \)-ENaC gene expression. These results taken together suggest that \textit{de novo} protein synthesis is required for transcriptional repression of \( \alpha \)-ENaC through the ERK kinase pathway. Whether or not repression is mediated through a modification of protein-DNA or protein-protein interaction by this newly synthesized factor is unknown at this moment. Detailed deletion mapping of the \( \alpha \)-ENaC promoter could help identify both the \textit{cis} elements and \textit{trans} factors that regulate the \( \alpha \)-ENaC gene.

Previous studies have provided several examples in which activated ERK targets transcription factor phosphorylation, presumably through regulating transcription via various response elements (54–56). One of the best-studied mechanisms through which the Raf/MEK/ERK pathway modulates gene expression involves the phosphorylation of the Ets family of transcription factors, such as Elk-1 and Ets-2 (45, 57). ERK2-mediated phosphorylation of Elk-1 in its carboxyl-terminal transactivation domain or of threonine 72 in the pointed domain of Ets-2 is believed to be essential for their transactivation switch. However, little work has been done to identify and characterize the transcription factor(s) mediating negative regulation in response to activation of the ERK kinase-signaling module. One can envision at least two models to explain the differential transcriptional control of the \( \alpha \)-ENaC gene expression in response to ERK activation. The first model assumes that in parotid cells, \( \alpha \)-ENaC gene transcription is down-regulated by an aforementioned \textit{trans}-acting factor(s), such as Elk-1. The second model postulates that the ERK kinase pathway acts as a positive regulator by inducing a novel \textit{trans}-acting factor, which negatively regulates \( \alpha \)-ENaC gene expression. The latter model is supported by the use of protein synthesis inhibitors, emetine or cycloheximide, both of which inhibit the down-regulation of \( \alpha \)-ENaC mRNA mediated through the ERK kinase cascade. Current efforts are directed toward elucidating the \textit{cis}-acting elements involved in down-
regulating α-ENaC steady-state level by the activation of the ERK kinase pathway.

Data in this report from transient transfection assays of two α-ENaC/CAT reporter genes show that the ERK kinase-mediated transcriptional repression of the α-ENaC promoter/enhancer activity occurs via the cis regulatory element(s) located within the 1.4-kb 5′-flanking region of the α-ENaC gene. The inhibitory effect of PD 98059 on TPA-mediated down-regulation of reporter activity is also consistent with the ability of TPA to activate ERK. Moreover, the cooperative effect between RafBXB and ERK-mediated suppression of CAT (Fig. 4C) underscores this point. Surprisingly, PD 98059 has a negligible effect on the basal level of α-ENaC message (Fig. 2C). This could be explained by results from recent studies, which support the notion that different magnitudes of ERK activation control its intracellular distribution (58–60). It has been proposed that both duration and intensity of ERK activation explain how the same signaling cascade is used by different growth factor receptors or pharmacological agents, where a variety of signals use the same cascade to elicit distinct changes in gene expression, cell proliferation, and differentiation (61). Recently, it has been shown that phosphorylated (active) ERK can form a homodimer with either phosphorylation state (60). Many substrates of ERK are dimers as well, including basic helix-loop-helix and leucine zipper proteins; ERK dimers may activate substrate dimers through a single interaction. Thus, ERK dimers may be composed of phosphorylated and unphosphorylated forms. As a mixture of fully active and hemiactive dimers, ERK may impact the time course, the activity threshold, and the efficacy involved in regulating downstream events (62). Taken together, this information supports our observation that the treatment of PD 98059 alone has a negligible effect on basal α-ENaC expression. However, our results do not exclude that an alternative signaling pathway could exist that also down-regulates α-ENaC expression. The data presented here provides experimental evidence and a rationale for the analysis of other modulators of ERK activation with implications for normal and abnormal cell physiology. This raises the possibility that the negative regulation of α-ENaC gene expression mediated by ERK activation in parotid cells can be viewed in the larger framework of tissue-specific regulation of α-ENaC gene expression. Are putative negative (trans-acting factors) constitutively expressed in tissues lacking α-ENaC expression? Are there cell-type-specific pathways to regulate α-ENaC gene expression? Each of these questions will require further studies. The biological advantage of the ERK-mediated α-ENaC down-regulation is that it provides a more flexible and precise way to regulate gene expression in response to extracellular stimuli or pharmacological agents. The physiological implications for ERK-mediated α-ENaC down-regulation may be extensive and underlie many important aspects of homeostatic regulation for epithelial sodium transport.

The hyperactivity of the epithelial sodium channel has been recently implicated in a number of human diseases. For example, mutations that increase ENaC activity are associated with hypertension. In cystic fibrosis, misregulation of ENaC by defective cystic fibrosis transmembrane conductance regulator has been proposed to cause hyperabsorption of sodium in airway epithelia (63). Under these conditions, down-regulating α-ENaC gene expression would be a way to restore normal rates of sodium absorption in epithelial tissues. The ability to repress α-ENaC gene expression has important implications for relieving symptoms in sodium channel-associated anomalies. Further investigation that focuses on the trans-acting factor(s) involved in regulating ENaC expression may also identify novel therapeutic strategies for medical conditions such as hypertension and perhaps cystic fibrosis as well. Moreover, other members of the DEG/ENaC superfamily, which includes proteins involved in mechanotransduction, proprioception, neurotransmission, as well as fluid and electrolyte homeostasis (64–66) may be regulated by an ERK-dependent pathway. Thus, it is possible that ERK-dependent regulatory pathways may play an important role in modulating various aspects of cellular function controlled by DEG/ENaC superfamily members.

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FIG. 5. Down-regulation by TPA requires de novo protein synthesis. A Northern analysis of α-ENaC mRNA in control and TPA-stimulated Pa-4 cells cultured in the absence (−) or presence (+) of the protein synthesis inhibitor emetine (10 μg/ml) for 6 h is shown. The Northern blot was performed as described in Fig. 2, and the experiments have been repeated for three times; the percent of inhibition on TPA-mediated repression was about 60%.
