The mechanisms involved in the regulation of the epithelial sodium channel (ENaC) via the cAMP pathway are not yet completely understood. The aim of the present study was to investigate cAMP-mediated ENaC regulation in Xenopus laevis oocytes heterologously expressing the three subunits (αβγ) of rat ENaC and to determine the ENaC regions important for mediating the stimulatory effect of cAMP. In oocytes treated for about 24 h with 1 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 μM forskolin (FSK) so as to increase intracellular cAMP, the amiloride-sensitive whole cell current (∆I_{amil}) was on average 10-fold larger than ∆I_{amil} in matched control oocytes. This effect on ∆I_{amil} was paralleled by an increase in ENaC surface expression caused by a reduced rate of ENaC retrieval. In addition, IBMX/FSK also enhanced ENaC open probability from about 0.2 to 0.5. The stimulatory effect of IBMX/FSK was dependent on the presence of intact PY motifs in the C termini of the channel. Mutagenesis of putative protein kinase A and CK-2 consensus motifs in the cytosolic domains of the channel did not reveal critical sites involved in mediating the stimulatory effect of IBMX/FSK. In contrast, site-directed mutagenesis of two putative ERK-consensus motifs (T613A in βENaC and T623A in γENaC) largely reduced the stimulatory effect of IBMX/FSK. Phosphorylation of these ERK sites has previously been reported to enhance the interaction of ENaC and Nedd4 (Shi, H., Asher, C., Chigaev, A., Yung, Y., Reuveny, E., Seger, R., and Garty, H. (2002) J. Biol. Chem. 277, 13539–13547). Using co-expression experiments we demonstrated that mutating the two ERK sites attenuates the inhibitory effect of Nedd4-2 on ENaC currents. We conclude that an increase in intracellular CAMP favors the dephosphorylation of the two ERK sites, which reduces channel retrieval and increases P_{o} by modulating ENaC/Nedd4 interaction. This defines a novel regulatory pathway likely to be relevant for cAMP-induced stimulation of ENaC in vivo.

The apically localized amiloride-sensitive epithelial sodium channel (ENaC)\(^2\) plays a critical role in fluid and electrolyte homeostasis and is widely expressed in absorptive epithelia such as the aldosterone-sensitive distal nephron, the distal colon, respiratory epithelia, sweat and salivary ducts (1, 2). ENaC is composed of three homologous subunits (α, β, and γ) that contain two transmembrane domains, a large extracellular loop, and short intracellular amino and carboxyl termini. The functional importance of a highly conserved PPXY sequence (the PY motif) in the carboxyl terminus of each subunit was recognized in Liddle syndrome (3). The PY motifs mediate ENaC binding to WW domains of the ubiquitin ligase Nedd4/Nedd4-2. This binding of Nedd4/Nedd4-2 to ENaC leads to ubiquitination of lysine residues in the N terminus of the channel and to channel internalization and proteasomal degradation (4, 5). In Liddle syndrome, mutations and/or deletions of the PY motif in β or γ ENaC reduce the Nedd4/Nedd4-2-mediated endocytic retrieval of ENaC from the membrane. This results in an increase in the number of ENaC channels in the membrane, which in turn causes enhanced sodium absorption and hypertension in patients with Liddle syndrome (6, 7).

ENaC phosphorylation by kinases and dephosphorylation by phosphatases has long been thought to contribute to ENaC regulation (1). In cultured renal epithelial cells, aldosterone, the main hormonal regulator of ENaC activity, and insulin have been shown to increase the phosphorylation of the C termini of the α-, β-, and γ-subunits of ENaC (8, 9). Moreover, the C termini of ENaC subunits expressed as glutathione S-transferase fusion proteins were found to be phosphorylated by cytosolic fractions derived from rat colon (10). This phosphorylation is thought to involve at least three different types of kinases, including the extracellular-regulated kinase (ERK) and casein kinase 2 (CK-2) (11).

There is a growing body of evidence that the serum and glucocorticoid-inducible kinase isoform 1 (SGK1) is an important contributing factor in the signal transduction cascade of aldosterone action on epithelial sodium transport (12). However, ENaC activation by SGK1 is rather complex and not yet completely understood (13–16). Similarly, the molecular mechanisms involved in ENaC stimulation by the cAMP-activated protein kinase A (PKA) pathway are not yet fully understood. It is well established that the antidiuretic hormone (vasopressin) enhances sodium absorption in isolated cortical collecting tubules and that the effect of antidiuretic hormone is synergistic to that of aldosterone (17). Vasopressin functions by binding to V2 receptors and activating adenylyl cyclase. Its effect on sodium transport is mimicked by membrane permeable cAMP analogues, phosphodiesterase inhibition with IBMX or adenylyl cyclase activation with forskolin (FSK). Recent evidence suggests that V2 receptor stimulation reduces sodium excretion in healthy humans. This indicates that the cAMP pathway of ENaC regulation is indeed relevant for sodium homeostasis in humans (18).
ENaC Activation by cAMP Involves ERK Phosphorylation Sites

It is generally assumed that the response to antidiuretic hormone is mediated by the activation of PKA and subsequent phosphorylation events. Whereas it has been proposed that PKA may directly phosphorylate ENaC (8), definitive proof for this has not yet emerged and as far as we know a functionally relevant PKA site has not yet been identified in any of the three ENaC subunits. Alternatively, PKA may modify other not yet identified proteins involved in ENaC regulation. Moreover, cAMP may affect ENaC via the recently identified EPaC pathway (19).

Considerable efforts have been made to address the question whether cAMP primarily increases the open probability of ENaC or its surface expression. Whereas there is convincing experimental evidence for both mechanisms, the relative importance of the two mechanisms remains a matter of debate and may vary under different experimental conditions and from tissue to tissue. There is little doubt that cAMP can have major effects on ENaC surface expression. Indeed, data from a quantitative approach, using a combination of short circuit current ($I_{SC}$) measurements and antibody detection of epitope-tagged ENaC expressed at the cell surface, indicate that in Madin-Darby canine kidney (MDCK) cells, the increase in $I_{SC}$ produced by cAMP, can be accounted for entirely by a proportional increase in the surface density of ENaC (20). Enhanced surface expression may be caused by stimulation of channel insertion or by inhibition of channel retrieval, and there is evidence that both mechanisms contribute to a cAMP-mediated increase in ENaC surface expression. It has been reported that acute ENaC stimulation by cAMP is mediated by exocytotic insertion from a recycling channel pool (21) and that a cAMP-mediated stimulation of ENaC translocation to the cell surface is dependent on the C-terminal PY motifs (22). As a mechanism for the inhibition of ENaC retrieval by cAMP, it has been proposed that PKA phosphorylates Nedd4-2 in a similar manner as SGK (13, 14), thereby inhibiting Nedd4-2-mediated channel ubiquitination, retrieval, and subsequent proteasomal degradation (23).

Interestingly, several groups including our own (24) have previously failed to demonstrate ENaC stimulation by increasing intracellular cAMP in Xenopus laevis oocytes heterologously expressing ENaC. These findings suggested that activation of the cAMP/PKA pathway may not be sufficient for ENaC activation in the oocyte system and that, in the native tissue additional factors may be involved that are missing in the oocytes. However, a 2–3-fold stimulation of ENaC currents has recently been reported in oocytes heterologously expressing rat ENaC that had been exposed to a mixture containing the membrane permeant cAMP analogue chlorophenylthio-cAMP and IBMX for 1 h (25). This finding led us to hypothesize that a prolonged increase in intracellular cAMP may be necessary to obtain a robust stimulatory effect on ENaC in the oocyte expression system. Exposure times to drugs, to increase intracellular cAMP, may well have been too short in previous studies and a potential stimulatory effect of cAMP may have been obscured by concomitant channel “run down” (24, 26). Indeed, in the present study we demonstrate that a prolonged exposure (about 24 h) of oocytes to 1 mM IBMX and 1 μM FSK resulted in an average on a 10-fold increase in ENaC currents. This was associated with a concomitant increase in channel surface expression, caused by an inhibition of channel retrieval. In addition, an increase in channel open probability contributed to the stimulatory effect of IBMX/FSK. Using site-directed mutagenesis, we identified ENaC regions that are functionally relevant for the stimulatory effect of cAMP. We found no evidence for a role of putative PKA or CK-2 sites in mediating the stimulation. Instead, our results indicate that two putative ERK consensus motifs in the C termini of β and γ ENaC are critically involved in the regulatory pathway, by which cAMP activates the channel.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—The full-length cDNAs encoding the three subunits of wild-type (WT) rat ENaC (α, β, and γ ENaC) (27) were in pGEM-HE. Those encoding the truncated rENaC subunits αP61stop, βR56stop, and γR60stop were in pSD5. FLAG-tagged ENaC clones were those described by Firsov et al. (6). cRNA that encodes a mutant β-ENaC subunit with a cysteine introduced to the degenerin site of ENaC (βS518C) was in pSD5 (28). All these clones were kindly provided by Bernard C. Rossier and Laurent Schild (Lausanne, Switzerland). Mouse kidney Nedd4-2 cRNA cloned in pcDNA3 was a gift of Sharad Kumar (Adelaide, Australia). Linearized plasmids were used as templates for cRNA synthesis (mMessage mMachine RNA Transcription Kit, Ambion, Austin, TX) using either T7 (WT-ENaC, truncated γ-ENaC, and Nedd4-2) or SP6 (truncated α-, β-ENaC, and βS518C) as promoters. The αS621A mutation used has been described previously (16). Mutations were introduced using site-directed mutagenesis extension overlap PCR or the QuikChange II Site-directed Mutagenesis Kit (Stratagene, The Netherlands). Mutations were confirmed by sequence analysis. The following mutant channel subunits were generated (sequences of the forward and reverse primers are given in parentheses): αS617A-ENaC (5′-CTACGGCCGATCCTGCTGACCTAG-3′; 5′-TGGGACACAGTCCGCGGACCCGGAACGGCTGAG-3′), αS617A/αS621A-ENaC (5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′), αS617A/αS621A/αS631-ENaC (5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′), αS617A/αS621A/αS623A-ENaC (5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′), αS617A/αS621A/αS623A/S631A-ENaC (5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′), αS617A/αS621A/αS623A/S631A/S633A-ENaC (5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′/5′-CTACGGCCGATCCTGCTGACCTAG-3′).
oocytes were kept in "high sodium" (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.3 Ca(NO₃)₂, 0.4 CaCl₂, 0.8 MgSO₄, 10 HEPE, adjusted to pH 7.4 with NaOH) or in "low sodium" (in mM: 1 NaCl, 60 N-methyl-D-glucamine, 40 KCl, 0.3 Ca(NO₃)₂, 0.4 CaCl₂, 0.8 MgSO₄, 10 HEPE, adjusted to pH 7.4 with HCl) modified Barth's saline supplemented with 100 units/ml penicillin (sodium salt) and 100 µg/ml streptomycin. Unless stated otherwise, oocytes were studied 2 days after mRNA injection. Oocytes were routinely clamped at a holding potential at −60 mV. The amiloride-sensitive current (ΔIAm) was determined by subtracting the corresponding current value measured in the presence of 2 µM amiloride from that measured prior to the absence of amiloride in a NaCl solution (in mM: 95 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPE, adjust to pH 7.4 with NaOH). Data are given as mean ± S.E.; n indicates the number of oocytes; N indicates the number of different batches of oocytes used; significance was evaluated by using the Mann-Whitney test or the Wilcoxon signed-rank test for not normally distributed data.

Surface Labeling of Oocytes—Experiments were essentially performed as described previously (30–32) using mouse anti-FLAG M2 as primary antibody (Sigma). Peroxidase-conjugated affinity sheep anti-mouse IgG (Chemicon, Boronia Victoria, Australia) was used as secondary antibody. Chemiluminescence of individual oocytes placed in 50 µl of Power Signal ELISA solution (Pierce) was quantified in a Turner TD-20/20 luminometer (Sunnyvale, CA) by integrating the signal over a period of 15 s. Results are given in relative light units (RLU).

Western Blotting—Oocytes were homogenized in ice-cold buffer solution (containing in mM: 100 β-glycerophosphate, 1.5 MgCl₂, 5 EGTA, 1 dithiothreitol, 20 HEPE (pH 7.5) supplemented with 1 phenylmethylsulfonyl fluoride, 0.2 sodium orthovanadate, 0.5 NaF prior to use) by pipetting them up and down several times and passing them five times through a 27-gauge needle. Subsequently, samples were centrifuged at 1,500 × g for 20 min at 4 °C. The proteins in the supernatant were separated on a 10% SDS-PAGE gel, followed by electrophoretic transfer to a polyvinylidene difluoride membrane in a semi-dry blotting chamber. The blot was processed according to the protocol recommended by the manufacturer of the rabbit anti-MAPK (Upstate, Lake Placid, NY) and phospho-p44/42 MAPK antibody (Cell Signaling Technology, Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Rockland, Gilbertsville, PA) was used as secondary antibody. Bound antibody was detected by chemiluminescence (ECL Plus™ Western blotting Detection Reagent, Amersham Biosciences).

Solutions and Chemicals—3-Isobutyl-1-methylxanthine (IBMX), FSK, brefeldin A (BFA), PD 98059, SB 203580, roscovitine, SP 600125, progesterone, and amiloride hydrochloride were purchased from Sigma. To prepare stock solutions, BFA and progesterone were dissolved in ethanol, whereas amiloride was dissolved in water. All other substances were dissolved in Me₂SO. Stock solutions were stored as recommended by the manufacturer and were diluted in aqueous solutions immediately prior to use in the experiments. Me₂SO and ethanol were added to control solutions as appropriate. The sulphydryl reagent MTSET (12-trimethylammonium)ethylmethanethiosulfonate bromide) was obtained from Toronto Research Chemicals (Toronto, Canada).

RESULTS

Increasing Intracellular cAMP by Prolonged Exposure to IBMX/FSK Stimulates ENaC Currents in X. laevis Oocytes—One day after cRNA injection, oocytes heterologously expressing the three subunits (αβγ) of rat ENaC were exposed to a combination of 1 mM IBMX and 1 µM forskolin (IBMX/FSK) to increase intracellular cAMP. ENaC activity was assessed by measuring the amiloride-sensitive whole cell current (ΔIAm). Fig. 1A summarizes data from a representative set of experiments performed in one batch of oocytes. One group of oocytes was exposed to IBMX/FSK for 24 h, the other group served as a time-matched control. Exposure to IBMX/FSK largely increased ΔIAm as illustrated by the average I/V plots shown in Fig. 1A. The I/V plots also demonstrate that in both groups of oocytes the reversal potential of ΔIAm was close to zero. This is consistent with the well known fact that ENaC-expressing oocytes, kept in the presence of high extracellular Na⁺, will become severely Na⁺ loaded with an intracellular Na⁺ concentration approaching that of the extracellular solution (27, 32). A significant stimulatory effect of IBMX/FSK on ENaC currents was observed in all batches of oocytes tested. On average a 24-h exposure to IBMX/FSK increased ΔIAm by a factor of 9.7 ± 0.8 (n = 88, Fig. 1B). A time course of the stimulatory effect of IBMX/FSK is shown in Fig. 1C. The stimulatory effect of IBMX/FSK persisted up to 30 h and became increasingly prominent with prolonged exposure. Fig. 1C also demonstrates that the effect of IBMX/FSK was reversible after washout.

IBMX/FSK Stimulates ENaC Currents by Increasing the Surface Expression of the Channel—The stimulation of ΔIAm in oocytes exposed to IBMX/FSK may be caused by an increase in channel open probability (Pₒ) of ENaC, by an increase in the number of ENaC channels expressed at the cell surface, or by a combination of both effects. To investigate this question we assessed ΔIAm and surface expression of extracellular FLAG-tagged ENaC (ENaC-FLAG) using a chemiluminescence assay (30, 33). The results shown in Fig. 1D demonstrate that surface expression and ΔIAm were concomitantly increased in oocytes treated with IBMX/FSK. These findings indicate that an increase in the number of ENaC channels expressed at the cell surface is an important mechanism underlying the stimulatory effect of IBMX/FSK on ΔIAm.

IBMX/FSK Also Increase the Open Probability of ENaC—To address the question whether IBMX/FSK also alters channel open probability, we performed experiments using the S518C mutant of the β-subunit of
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FIGURE 2. cAMP increases channel P_o as demonstrated by experiments using the property of the JSS518C mutant ENaC to be converted by MTSET to a channel with a P_o of about 1. Representative whole cell current recordings are shown in control (A) and IBMX/FSK (24 h)-treated (B) oocytes expressing the JSS518C mutant ENaC consisting of wild-type α- and γ-subunits (αWT, γWT) and a β-subunit in which Ser-518 is replaced by a cysteine (βJSS18C). To increase the open probability of the mutant channel to about 1, the sulfhydryl reagent MTSET (1 mM) was added to the bath solution as indicated by the hatched bars. Amiloride (2 μM) was present as indicated by the filled bars. The whole cell current was continuously recorded at a holding potential of −60 mV interrupted by brief voltage step protocols (see legend of Fig. 1), which were performed prior to each solution exchange. C, summary of data from similar experiments as shown in A and B performed in different batches of oocytes (n = 2). Δ_{IAM} values were normalized to the mean value of Δ_{IAM} of the corresponding control group from the same batch of oocytes before MTSET treatment and without IBMX/FSK stimulation. MTSET caused a significant increase of Δ_{IAM} in both control oocytes (***, p < 0.001) and in oocytes pretreated with IBMX/FSK (*, p < 0.05).

drat ENaC (βSS518C). This mutant channel can be converted from a channel with a normal open probability to a channel with an open probability of nearly one by exposing the channel to the sulfhydryl reagent MTSET, which destabilizes the closed state of the channel (28). As illustrated by two representative whole cell current traces in Fig. 2, A and B, application of MTSET caused a substantial increase in Δ_{IAM} in both control and IBMX/FSK-treated oocytes. On average, MTSET increased Δ_{IAM} about 6-fold in control oocytes and about 2-fold in IBMX/FSK-treated oocytes (Fig. 2C). If MTSET converted the open probability of all active ion channels present at the cell surface to one, these findings indicate that the average open probability of the channels prior to the application of MTSET must have been about 0.17 and 0.5 in control and IBMX/FSK-treated oocytes, respectively. This finding indicates that in addition to stimulating the surface expression of ENaC, treatment with IBMX/FSK also increases the average channel open probability by about 3-fold. However, a 3-fold increase in open probability is not sufficient to account for the 7-fold increase in Δ_{IAM} observed in IBMX/FSK-treated oocytes expressing the βSS518C mutant channel (Fig. 2C), which is of similar magnitude to the stimulatory effect of IBMX/FSK on Δ_{IAM} in oocytes expressing wild-type ENaC (see above). Indeed, the finding that after stimulation by IBMX/FSK the application of MTSET causes a further 2.4-fold increase in Δ_{IAM} indicates that the open probability of the channel is only moderately increased by IBMX/FSK. Thus, our MTSET data confirm that an increase in surface expression plays a major role in mediating the stimulatory effect of IBMX/FSK on Δ_{IAM}.

FIGURE 3. cAMP inhibits channel retrieval. One day after cRNA injection, oocytes were incubated in the presence (squares) or absence (circles) of IBMX for 24 h followed by incubation with (filled symbols) or without 18 μM brefeldin A (BFA) (open symbols) for up to 24 h. At the times indicated Δ_{IAM} was measured in 10–12 oocytes from each experimental group. Δ_{IAM} values measured at −60 mV were normalized to the corresponding mean value of Δ_{IAM} at time 0 h. The figure summarizes normalized results from three similar sets of experiments performed in three different batches of oocytes.

The IBMX/FSK-induced Increase in ENaC Surface Expression Is Due to an Inhibition of Channel Retrieval—Channel surface expression reflects the balance between channel insertion and channel retrieval. Thus, IBMX/FSK may increase the surface expression of ENaC by enhancing its delivery to the cell surface, by inhibiting ENaC retrieval, or by a combination of both processes. To assess the rate of ENaC retrieval, we inhibited delivery of new channels to the plasma membrane by adding 18 μM BFA to oocytes 2 days after injection with ENaC cRNA. BFA is a fungal metabolite that inhibits the secretory pathway of newly synthesized proteins, without affecting clathrin-mediated endocytosis (34). Because it has been reported that high concentrations of FSK can inhibit and reverse the effect of BFA on Golgi morphology by a CAMP-independent mechanism (35), we did not use FSK in the BFA experiments but only used IBMX to increase intracellular cAMP. Fig. 3 illustrates the effect of BFA on Δ_{IAM} in control oocytes and in oocytes pretreated with IBMX for 24 h and maintained in the continuous presence of IBMX throughout the experiment. In control oocytes, Δ_{IAM} was decreased by 88% within 6 h after addition of BFA, which is in good agreement with data reported previously (7, 32). In non-treated control oocytes, Δ_{IAM} remained more or less stable, whereas in IBMX-treated cells, Δ_{IAM} continued to increase throughout the 24-h period examined. This suggests that in the IBMX-treated oocytes, the channel insertion rate exceeded channel retrieval rate during this period. Importantly, in IBMX-treated oocytes, BFA had essentially no inhibitory effect on Δ_{IAM} during the first 6 h of exposure to BFA. This is in sharp contrast to the dramatic inhibitory effect of BFA in control oocytes. Even after 24 h of BFA exposure a substantial fraction of Δ_{IAM} was preserved in the IBMX-treated oocytes (Fig. 3). These findings indicate that the increase in intracellular cAMP by IBMX enhances Δ_{IAM} by reducing the net retrieval rate of ENaC, thereby stabilizing ENaC expression in the membrane. In the presence of continued channel insertion this explains the large increase in Δ_{IAM} and enhanced surface expression of ENaC observed in oocytes exposed to IBMX or IBMX/FSK for a prolonged period of time.

Deletion of the C Termini or Mutation of the PY Motifs of All Three ENaC Subunits Largely Abolishes the Stimulatory Effect of cAMP—The stimulatory effect of IBMX/FSK on ENaC currents and surface expression has some similarity to the effect of channel mutations that cause Liddle syndrome. Moreover, the reduced effect of BFA on ENaC currents in oocytes treated with IBMX is reminiscent of the finding that BFA has little effect on the activity of ENaC with Liddle syndrome mutation because the retrieval of the mutated channel by clathrin-mediated endocytosis is defective (36). The analysis of Liddle syndrome mutations has revealed the essential role of the C termini and their PY motifs for channel retrieval. Therefore, we tested the effect of IBMX/FSK on ENaC channels with C-terminal truncations and mutated PY motifs (Fig. 4). The sites of the C-terminal truncations were analogous...
MECHANISM THAT MEDIATES THE STIMULATORY EFFECT OF cAMP.

The C Terminus and PY Motif of Each Subunit Contributes to the Stimulatory Effect of cAMP. To determine the relative importance of the C termini and PY motifs of each ENaC subunit for the stimulatory effect of IBMX/FSK, the individual effects of the C-terminal truncation or PY mutation of: (A) the α-subunit (αY673A; αT); (B) the β-subunit (βY673A; βT); and (C) the γ-subunit (γY673A; γT) were tested in similar experiments as shown in Fig. 4 (**, p < 0.01; ***, p < 0.001).

The C Terminus and PY Motif of Each Subunit Contributes to the Mechanism That Mediates the Stimulatory Effect of cAMP—To determine the relative importance of the C termini and PY motifs of three ENaC subunits, we performed an additional series of experiments in which we tested the effect of C-terminal truncation or PY mutation of each individual ENaC subunit. As shown in Fig. 5, it was sufficient to delete the C terminus of the α-, β-, or γ-subunits to achieve a large inhibition of cAMP-mediated ENaC stimulation. Similarly, mutating the PY motif of the α-, β-, or γ-subunits resulted in a substantial reduction of the stimulatory effect of cAMP on ENaC currents (Fig. 5). Interestingly, the mutation of the PY motif of the β-subunit had the most pronounced inhibitory effect. This suggests that an intact PY motif of the β-subunit is particularly important for mediating the stimulatory effect of cAMP (Fig. 5B).

Mutagenesis of Putative PKA Consensus Motifs in the Cytosolic Domains of αβγENaC Does Not Reveal a Critical Site Involved in Mediating the Stimulatory Effect of IBMX/FSK—PKA efficiently phosphorylates proteins containing the amino acid consensus sequence RXRS. However, this motif represents only one-half of all physiological PKA phosphorylation sites (38). Therefore, we used three less restrictive consensus sequences (RXX[S/T], RX[S/T], or KXX[S/T]) to search for putative PKA consensus motifs in the cytosolic domains of ENaC. The identified PKA consensus motifs in the α- and γ-subunits are highly conserved across species, with the exception of the motif at Thr-579 in γENaC, which is present in rat and mouse but not in human. The serine and threonine residues of the putative PKA motifs were mutated to alanine and the mutant channels were functionally expressed in oocytes to test the effect of the mutations on the stimulatory response to IBMX/FSK. As shown in Table 1 none of the mutants abolished the stimulation by IBMX/FSK. Indeed, the stimulatory effect of IBMX/FSK was fully preserved in the αT67A and γT579A mutants, whereas more than 50% of the stimulatory effect was preserved in the other mutants tested. In addition to being part of a putative PKA consensus site, the residue Thr-67 in αENaC is also part of a putative CK-2 consensus motif. Nevertheless, mutating this site had no effect on the stimulatory effect of IBMX/FSK. The residue Ser-621 in αENaC has recently been shown to be essential for ENaC activation in outside-out patches by recombinant SGK1 (16). However, neither Ser-621 nor the adjacent Ser-617 residue seemed to be essential for the stimulation by IBMX/FSK. The partial inhibitory effects of several individual mutations may indicate that the mutated sites are relevant for channel function. However, these mutations reduced the stimulatory effect of IBMX/FSK by less than 50%. Therefore, they are unlikely to affect essential PKA sites. This interpretation is also supported by the finding that there was no additive inhibitory effect of the double mutant S617A/S621A of the α-subunit. Taken together, these findings suggest that the effect of IBMX/FSK is not dependent on a direct phosphorylation of ENaC at the PKA sites tested.

FIGURE 4. Deletion of the C terminus or mutation of the PY motifs of all three ENaC subunits largely abolishes the stimulatory effect of cAMP. A, oocytes expressing wild-type αβγENaC (WT) or C terminally truncated α-, β-, and γ-ENaC subunits (αT, βT, γT) were incubated with (filled bars) or without (open bars) IBMX/FSK for 24 h followed by the assessment of ΔIAm at −60 mV. ΔIAm values measured in the corresponding non-treated oocytes were normalized to the mean value of ΔIAm measured in the IBMX/FSK-stimulated oocytes (N = 6). B, in similar experiments (N = 4) the effect of IBMX/FSK was tested in oocytes expressing ENaC with all three PY motifs mutated (αY673AβY673AγY672A). The IBMX/FSK-stimulated ΔIAm was significantly reduced in oocytes expressing mutant channels compared with the IBMX/FSK-stimulated ΔIAm in matched WT-ENaC expressing oocytes (***, p < 0.001).

FIGURE 5. The C terminus and PY motif of each subunit contributes to the stimulatory effect of cAMP. To determine the relative importance of the C termini and PY motifs of each ENaC subunit for the stimulatory effect of IBMX/FSK, the individual effects of the C-terminal truncation or PY mutation of: (A) the α-subunit (αY673A; αT); (B) the β-subunit (βY673A; βT); and (C) the γ-subunit (γY673A; γT) were tested in similar experiments as shown in Fig. 4 (**, p < 0.01; ***, p < 0.001).
Mutagenesis of Putative CK-2 Consensus Motifs Does Not Affect the Stimulatory Effect of IBMX/FSK—Because CK-2 has been reported to be involved in ENaC regulation (11), we also investigated the role of putative CK-2 sites characterized by an [S/T]XX(E/D) motif (39). Putative CK-2 consensus motifs are present in all three subunits (Table 1). They are highly conserved across species with the exception of the motif at Thr-599 in γENaC, which is only found in mouse and rat. Both the Ser-631 residue of βENaC and the Thr-599 residue of γENaC have been reported to be phosphorylated by CK-2 (11). As mentioned before, the Thr-67 residue in αENaC contributes to a CK-2, as well as a PKA consensus motif. The Ser-633 residue in βENaC has recently been reported to be phosphorylated by GRK2 (G protein-coupled receptor kinase) rather than by CK-2. In contrast, the phosphorylation is thought to render the channel insensitive to inhibition by Nedd4-2 (40). As reported in Table 1, mutating the Ser or Thr residues of the putative CK-2 consensus sites to alanine did not inhibit the stimulatory effect of IBMX/FSK on ENaC currents. These findings indicate that these putative CK-2 sites are not involved in mediating the effect of IBMX/FSK.

Mutation of Two ERK Consensus Motifs Largely Reduces the Stimulatory Effect of IBMX/FSK on ENaC—Recently, the Thr-613 residue of βENaC and the Thr-623 residue of γENaC have been identified as ERK phosphorylation sites by in vitro assays (41). As shown in Fig. 6A these ERK sites are highly conserved in different species. We investigated the effect of mutating these ERK sites on the IBMX/FSK-induced stimulation of ENaC. Mutating Thr-613 of βENaC to alanine reduced the stimulatory effect of IBMX/FSK to 31% of its control value. Similarly, mutating Thr-623 of γENaC to alanine reduced the stimulatory effect to 48%. Importantly, when both sites were mutated simultaneously, the stimulatory effect of IBMX/FSK was reduced even further to 12% of its control value (Fig. 6B). These findings indicate that both ERK sites are important for mediating the stimulatory effect of IBMX/FSK on ENaC.

Mutation of the Two ERK Consensus Motifs (βT613A, γT623A) Increases Δ\text{IA} \text{ami} by Reducing the Rate of ENaC Retrieval—Phosphorylation of the two ERK sites of ENaC has been reported to facilitate the interaction of β- and γENaC with Nedd4, thereby favoring ENaC retrieval from the plasma membrane (41). Thus, mutating Thr-613 and γThr-623 should enhance macroscopic ENaC currents by preventing the phosphorylation of these sites. Indeed, we could show that Δ\text{IA} \text{ami} was 6.3-fold higher in oocytes expressing the double mutant βT613A/γT623A than in oocytes expressing wild-type ENaC (Fig. 7A). This is in agreement with previously reported findings (41, 42). To further investigate the functional relevance of the ERK consensus sites, we performed experiments using BFA as a tool to assess the channel retrieval rate in oocytes expressing wild-type ENaC or ENaC with the double mutation βT613A/γT623A. As shown before, application of BFA resulted in a rapid decline of ENaC currents in oocytes expressing wild-type ENaC. In contrast, in oocytes expressing ENaC with the double mutation βT613A/γT623A, the BFA-induced decline of Δ\text{IA} \text{ami} was significantly reduced (Fig. 7B). These findings confirm the hypothesis that ENaC retrieval is inhibited by mutating the ERK sites in the C termini of β- and γENaC.

Mutation of the Two ERK Consensus Motifs (βT613A, γT623A) Reduces the Inhibitory Effect of Co-expressed Nedd4-2 on Δ\text{IA} \text{ami}—Because phosphorylation of the two ERK sites has been reported to promote the interaction of ENaC and Nedd4 (41), we hypothesized that mutating these sites should reduce Nedd4-mediated channel retrieval.

**FIGURE 6.** The stimulatory effect of cAMP on ENaC is largely reduced by mutating two putative ERK consensus sites. A, amino acid sequence alignment illustrates the highly conserved ERK phosphorylation sites in ENaC (Thr613 in rat and γENaC [Thr623 in rat] in different species. The ERK consensus motifs [S/T]XX(E/D) are followed by the PY motif [T/PXXY] (bold letters), B, the effect of IBMX/FSK was tested on ENaC with a mutated ERK site in the β-subunit (βT613A) or in the γ-subunit (γT623A) or on a channel in which both ERK sites were mutated (βT613A, γT623A). Experiments were essentially performed as those summarized in Table 1 (***, p < 0.001).

**TABLE 1**

Mutating putative PKA or CK-2 consensus motifs does not have a major effect on ENaC stimulation by cAMP

The mutations listed affect putative PKA and/or CK-2 consensus sites as indicated. Experiments were essentially performed as described in Fig. 1. To summarize data from different batches (N) of oocytes (n), the stimulatory effect of IBMX/FSK on mutant channels was normalized (%) to the average effect of IBMX/FSK on WT-ENaC in the same batch of oocytes. The stimulatory effect of IBMX/FSK was largely preserved in all mutants tested. Some mutants partially reduced the stimulatory effect of IBMX/FSK compared to WT-ENaC control.

| Mutation       | Consensus motif | Normalized stimulatory effect of IBMX/FSK (%) | N  | n |
|----------------|----------------|---------------------------------------------|----|---|
| α-ENaC         | T67A           | PKA/CK-2                                   | 111 ± 14 | NS* | 3  | 45 |
|                | T109A          | PKA                                        | 64 ± 15 | *** | 3  | 44 |
|                | S617A          | PKA                                        | 65 ± 8  | *** | 4  | 60 |
|                | S621A          | PKA                                        | 60 ± 6  | *** | 4  | 60 |
|                | S617A/S621A    | PKA                                        | 56 ± 9  | *** | 3  | 45 |
|                | S631A          | CK-2                                       | 84 ± 9  | *** | 3  | 43 |
|                | S633A          | CK-2                                       | 112 ± 18| NS  | 3  | 45 |
| β-ENaC         | T75A           | CK-2                                       | 85 ± 13 | NS  | 3  | 43 |
|                | T599A          | PKA                                        | 115 ± 14| NS  | 3  | 44 |
|                | T630A          | PKA                                        | 91 ± 11 | *** | 3  | 44 |
|                | S638A          | PKA                                        | 66 ± 8  | *** | 3  | 40 |
| γ-ENaC         | S633A          | CK-2                                       | 58 ± 6  | *** | 3  | 45 |

* NS, not significant.
** p < 0.001.
*** p < 0.05.
**** p < 0.01.

g-ENaC contributes to a CK-2, as well as a PKA consensus motif. The Ser-633 residue in βENaC has recently been reported to be phosphorylated by GRK2 (G protein-coupled receptor kinase) rather than by CK-2. This phosphorylation is thought to render the channel insensitive to inhibition by Nedd4-2 (40). As reported in Table 1, mutating the Ser or Thr residues of the putative CK-2 consensus sites to alanine did not inhibit the stimulatory effect of IBMX/FSK on ENaC currents. These findings indicate that these putative CK-2 sites are not involved in mediating the effect of IBMX/FSK.
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FIGURE 7. Mutating the ERK sites stimulates ENaC currents by reducing Nedd4-2-mediated channel retrieval. A, comparison of baseline $\Delta I_{\text{amp}}$ in oocytes expressing wild-type (WT) ENaC (open column) or mutant ENaC ($\beta T613A$, $\gamma T623A$) with both ERK sites mutated (filled column). To summarize data from different batches of oocytes ($N = 13$), individual $\Delta I_{\text{amp}}$ values were normalized to the mean $\Delta I_{\text{amp}}$ value of WT-ENaC expressing oocytes from the corresponding batch. B, effect of BFA on $\Delta I_{\text{amp}}$ in oocytes expressing mutant ENaC (triangles) and wild-type ENaC (circles). One day after injection of cRNA one group of oocytes (filled symbols) was exposed to BFA (18 μM) starting at time 0, whereas another group of non-treated oocytes served as control (open symbols). At the times indicated $\Delta I_{\text{amp}}$ was measured in 10–12 oocytes from each experimental group. $\Delta I_{\text{amp}}$ values measured at −60 mV were normalized to the corresponding mean value of $\Delta I_{\text{amp}}$ at time 0. The figure summarizes normalized results from three similar sets of experiments performed in three different batches of oocytes. C, mutating the two ERK sites largely reduces the inhibitory effect of Nedd4-2. Oocytes were injected with wild-type or mutant ENaC cRNA (0.1 ng/subunit) alone (open columns) or in combination with 1 ng of Nedd4-2 cRNA (filled columns) and maintained in low sodium Barth’s solution to reduce sodium loading. Two days after cRNA injection $\Delta I_{\text{amp}}$ was measured at −60 mV. To summarize data from different batches of oocytes individual $\Delta I_{\text{amp}}$ values were normalized to the mean $\Delta I_{\text{amp}}$ value of the corresponding control group without Nedd4-2. Normalized $\Delta I_{\text{amp}}$ was significantly larger in oocytes co-expressing mutant channel and Nedd4-2 than in oocytes co-expressing WT-ENaC and Nedd4-2 ($**$, $p < 0.001$).

To test this hypothesis, we coexpressed mouse kidney Nedd4-2 with either wild-type ENaC or with ENaC carrying the $\beta T613A$ and $\gamma T623A$ mutations. For these experiments, oocytes were maintained in modified low sodium Barth solution to minimize sodium feedback inhibition and retrieval of ENaC by endogenous X. laevis Nedd4 (7, 26, 32). We have previously demonstrated that under these conditions the relative inhibitory effect of heterologously expressed Nedd4-2 is even larger than in sodium-loaded oocytes (43). As shown in Fig. 7C, co-expression of mouse Nedd4-2 reduced $\Delta I_{\text{amp}}$ by about 70% in oocytes expressing wild-type ENaC. In contrast, in oocytes expressing the $\beta T613A/\gamma T623A$ mutant, the inhibitory effect of Nedd4-2 co-expression was substantially reduced. These findings confirm the functional importance of the two ERK sites for modifying ENaC/Nedd4-2 interaction.

No Evidence for ERK Inhibition by IBMX/FSK—Because PKA has been reported to inhibit ERK activation in X. laevis oocytes (44, 45), we investigated the possibility that the stimulatory effect of IBMX/FSK was due to an inhibition of ERK. Inhibition of ERK by IBMX/FSK would reduce the phosphorylation of the two ERK sites of ENaC, thereby reducing ENaC/Nedd4 interaction resulting in a stabilization of the channel in the membrane. ERK is known to require phosphorylation for its activation (46). Using phosphospecific antibodies and Western blot analysis, we could demonstrate that the level of phosphorylated active ERK was very low in control oocytes expressing ENaC and did not further decrease in IBMX/FSK-treated oocytes (Fig. 8). Our findings are in agreement with previous studies reporting minimal amounts of active ERK in G$_2$-arrested Xenopus oocytes (44, 45, 47). Thus, in the oocyte expression system, it is unlikely that the stimulatory effect of IBMX/FSK on ENaC is mediated by an inhibition of endogenous ERK. This conclusion is further confirmed by our finding that exposure of ENaC expressing oocytes to PD 98059 (30 μM) or to U0126 (10 μM), two known inhibitors of the MAPK cascade (48), had no consistent stimulatory effect on $\Delta I_{\text{amp}}$ (data not shown). We also tested the P38 inhibitor SB 203580 (10 μM) (49) and the relatively nonspecific c-Jun N-terminal kinase inhibitor SP 600125 (10 μM) (49). Neither of the two inhibitors significantly increased $\Delta I_{\text{amp}}$ (data not shown). Thus, it is unlikely that an inhibition of MAP kinases plays a major role in mediating the stimulatory effect of IBMX/FSK.

Mutating ERK Sites Partially Prevents the Stimulatory Effect of Roscovitine—Because the ENaC residues $\beta T613A$ and $\gamma T623A$ are each followed by a proline, these threonines may also be phosphorylated by cyclin-dependent kinases (CDKs) (50). Therefore, we tested the effect of roscovitine, a known inhibitor of CDKs (51). As shown in Fig. 9, exposure of wild-type ENaC expressing oocytes to roscovitine (80 μM) resulted in a 3.9-fold increase in $\Delta I_{\text{amp}}$. This effect of roscovitine is in agreement with a recent report from another laboratory (52). Importantly, the stimulatory effects of roscovitine and IBMX/FSK on $\Delta I_{\text{amp}}$ were not additive, indicating that the effects may be mediated by the
same pathway. This conclusion is further supported by the finding that the stimulatory effect of roscovitine was substantially reduced in oocytes expressing ENaC with the βT613A/γT623A mutations (Fig. 9), which is reminiscent of the finding that these mutations also reduce the stimulatory effect of IBMX/FSK (see above). Collectively, these data suggest that endogenous CDKs in the oocytes may contribute to tonic ENaC inhibition by keeping the two ERK sites (βT613/γT623) of ENaC phosphorylated. This tonic inhibitory effect of CDKs is revealed by the stimulatory effect of roscovitine and may be prevented or reversed by exposure of the oocytes to IBMX/FSK resulting in channel activation.

**DISCUSSION**

We have demonstrated that in X. laevis oocytes heterologously expressing ENaC, an increase in intracellular cAMP by prolonged exposure of the cells to IBMX/FSK increases ENaC currents by about 10-fold. This is partly due to an increase in ENaC surface expression that results from an inhibition of channel retrieval, leading to stabilization of the channel in the membrane. In addition, we present evidence that an increase in channel open probability also contributes to the stimulatory effect of IBMX/FSK. Thus, the stimulatory effect of IBMX/FSK on ENaC is reminiscent of the effect of channel mutations causing Liddle syndrome. These mutations affect highly conserved C-terminal PY motifs of ENaC that are critical for Nedd4-2-mediated channel retrieval and have been shown to increase both channel surface expression and channel open probability (6). Interestingly, the stimulatory effect of IBMX/FSK was largely reduced in oocytes expressing ENaC channels with Liddle syndrome mutations or C-terminal truncations. This indicates that the C termini and PY motifs of ENaC are critically involved in mediating the stimulatory effect of IBMX/FSK. Our findings in the oocyte expression system are in agreement with observations previously made in Fisher rat thyroid epithelial cells heterologously expressing ENaC. In this system, Liddle syndrome mutations affecting the PY motif of the β-subunit also disrupted the stimulatory effect of cAMP on ENaC (22).

Using site-directed mutagenesis, we demonstrated that the effect of IBMX/FSK was not dependent on the presence of putative PKA or CK-2 sites in the cytoplasmic termini of ENaC. Thus, whereas CK-2 has been reported to phosphorylate C-tail fusion proteins of ENaC (11), CK-2-mediated phosphorylation of ENaC does not seem to be involved in the stimulatory effect of IBMX/FSK. Similarly, putative PKA sites are unlikely to contribute to the effect of IBMX/FSK, which is in agreement with the absence of convincing evidence in the literature for direct PKA-mediated ENaC phosphorylation.

A major finding of the present study was the fact that the Thr-613 residue in βENaC and the Thr-623 residue in γENaC are essential for mediating the stimulatory effect of IBMX/FSK. Recently, these two residues located immediately before the PY motifs have been identified as ERK phosphorylation sites by in vitro assays (41, 42). The threonine in this position is one of only three cytoplasmic serine/threonine residues fully conserved in all β- and γ-subunits cloned so far. Multiple proline residues near the phosphorylation site suggest that these sites can be phosphorylated by proline-directed kinases, e.g. by members of the MAPK family such as ERK, p38, or c-Jun amino-terminal kinase. Using plasmam resonance studies, it was shown that peptides having phosphothreonine at positions β-613 or γ-623 bind WW domains of Nedd4 two to three times better than the non-phosphorylated analogues. Moreover, mutating these threonine residues resulted in increased ENaC currents in the X. laevis oocyte expression system (41). This is in agreement with the findings of our present study. Because mutated ERK sites cannot be phosphorylated, the ability of the mutated channel to interact with endogenous Xenopus Nedd4 or overexpressed Nedd4-2 can be expected to be reduced, resulting in a stabilization of the channel in the plasma membrane. Indeed, in the present study we could demonstrate that mutating the two ERK sites reduced the rate of channel retrieval and reduced the inhibitory effect of co-expressed Nedd4-2. This is in agreement with a recent report (53) that the inhibitory effect of Nedd4-2 on mouse ENaC heterologously expressed in CHO cells was reduced by a T628A mutation in γ-mENaC. This mutation corresponds to the T623A mutation in γ-rENaC used in the present study. Importantly, we also found that mutating the two ERK sites largely reduced the stimulatory effect of IBMX/FSK. These findings demonstrate the functional importance of these sites.

There are several recent studies suggesting that ERK activation may play a role in ENaC inhibition and may serve as a negative regulator of ENaC-mediated sodium absorption (54–56). Whereas the long-term effect of ERK is thought to involve an inhibition of ENaC transcription (55, 56), the short-term inhibition is likely to be mediated by phosphorylation of the two ERK sites identified in the C termini of the β- and γ-subunits of the channel (41). Indeed, recent evidence has emerged that in the oocyte expression system activation of the ERK pathway by progesterone results in ENaC inhibition (42, 47, 52). The inhibitory effect of progesterone was partially reduced by mutating the serine residues of the ERK consensus sites βThr-613 and γThr-623 to alanine, and it was demonstrated that the βT613A mutation abolished both basal and progesterone-induced phosphorylation of βENaC (42). Taken together, these findings suggest that ERK-mediated phosphorylation of these sites does indeed inhibit ENaC, most likely by facilitating its interaction with Nedd4/Nedd4-2 (41).

Our findings suggest a novel regulatory role of the ERK sites because we demonstrate that cAMP-mediated stimulation of ENaC critically depends on their presence. The phosphorylation state of these residues will depend on the balance of activities of kinases and phosphatases in a given cell. Thus, stimulation of ERK, e.g. by progesterone, is likely to shift the balance toward phosphorylation of these sites causing channel inhibition (42). In contrast, an increase in intracellular cAMP may reduce phosphorylation of the sites, thereby reducing a tonic inhibitory effect of ERK. The concept that ENaC activity may be tonically inhibited by a baseline phosphorylation of its ERK sites is supported by the recently reported finding that inhibition of ERK stimulates transepithelial sodium transport in cultured renal epithelial cells (57).

Because it has been demonstrated that PKA prevents progesterone-induced activation of ERK in Xenopus oocytes (44, 45) we initially hypothesized that the stimulatory effect of cAMP on the ENaC current may be due to an inhibition of ERK activation, resulting in a reduced phosphorylation of βThr-613 and γThr-623. However, using Western blot analysis we were unable to demonstrate a decrease in the proportion of active phosphorylated ERK in oocytes treated with IBMX/FSK. Interestingly, the level of active phosphorylated ERK was very low in untreated control oocytes and remained low in IBMX/FSK-treated oocytes. Our findings are in good agreement with reports from other laboratories that the level of active phosphorylated ERK is low in resting oocytes and only increases during oocyte maturation, which can be stimulated by progesterone (44, 45, 47). Moreover, we failed to demonstrate a stimulatory effect of ERK inhibitors and inhibitors of other MAP kinases on ENaC currents. In a previous study, the p38 MAP kinase inhibitor SB 203580 was even found to have an inhibitory effect on ENaC currents in Xenopus oocytes (58), which also argues against an involvement of MAP kinases in the tonic phosphorylation of the ERK sites. Collectively, these findings indicate that in the resting oocyte ERK or other MAP kinases do not play a major role in the tonic phosphorylation.
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The complex interplay of the mechanisms involved. blocks retrieval of ENaC from the membrane may mask the effect of any treatment that affects ENaC/Nedd4 interaction by phosphorylation or dephosphorylation of the ERK sites not only affects Nedd4-mediated channel retrieval but also has an important effect on channel gating, i.e. \( P_O \).

In summary, our experiments indicate that an increase in channel open probability combined with an increase in channel surface expression are the two mechanisms by which CAMP leads to enhanced ENaC currents. We have identified the critical importance of two ERK sites, \( \beta \)-Thr-613 and \( \gamma \)-Thr-623, in mediating this regulation and propose that dephosphorylation of these two sites reduces channel retrieval and increases \( P_O \) by modulating ENaC/Nedd4 interaction. We conclude that the two ERK sites are important modulators of ENaC activity and that modification of their phosphorylation state by hormonal signaling may contribute to the dynamic responsiveness of ENaC function, e.g. to changes in intracellular cAMP levels.

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