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κ Opioid Receptor Interacts with Na^+/H^+-exchanger Regulatory Factor-1/Ezrin-Radixin-Moesin-binding Phosphoprotein-50 (NHERF-1/EBP50) to Stimulate Na^+/H^+ Exchange Independent of G_i/G_o Proteins*

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We previously showed that Na^+/H^+-exchanger regulatory factor-1/Ezrin-radixin-moesin-binding phosphoprotein-50 (NHERF-1/EBP50) co-immunoprecipitated with the human κ opioid receptor (hKOR) and that its overexpression blocked the κ agonist U50,488H-induced hKOR down-regulation by enhancing recycling. Here, we show that glutathione *S*-transferase (GST)-hKOR C-tail interacted with purified NHERF-1/EBP50, whereas GST or GST-C-tails of μ or δ opioid receptors did not. GST-hKOR C-tail, but not GST, bound HA-NHERF-1/EBP50 transfected into Chinese hamster ovary cells and endogenous NHERF-1/EBP50 in opossum kidney proximal tubule epithelial cells (OK cells). The PDZ domain I, but not II, of NHERF-1/EBP50 was involved in the interaction. Association of NHERF-1/EBP50 with hKOR C-tail enhanced oligomerization of NHERF-1/EBP50. NHERF-1/EBP50 was previously shown to regulate Na^+/H^+-exchanger 3 (NHE3) activities in OK cells. We found stimulation of OK cells with U50,488H significantly enhanced Na^+/H^+ exchange, which was blocked by naloxone but not by pertussis toxin pretreatment, indicating it is mediated by KORs but independent of G_i/G_o proteins. In OKH cells, a subclone of OK cells expressing a much lower level of NHERF-1/EBP50, U50,488H had no effect on Na^+/H^+ exchange, although it enhanced p44/42 mitogen-activated protein kinase phosphorylation via G_i/G_o proteins similar to that in OK cells. Stable transfection of NHERF-1/EBP50 into OKH cells restored the stimulatory effect of U50,488H upon Na^+/H^+ exchange. Thus, NHERF-1/EBP50 binds directly to KOR, and this association plays an important role in accelerating Na^+/H^+ exchange. We hypothesize that binding of the KOR to NHERF-1/EBP50 facilitates oligomerization of NHERF-1/EBP50, leading to stimulation of NHE3. This study provides the first direct evidence that a G protein-coupled receptor through association with NHERF-1/EBP-50 stimulates NHE3.

The κ opioid receptor (KOR)[1] is one of the multiple types (at least μ, δ, and κ) of opioid receptors that mediate pharmacological effects of opioid drugs and physiological actions of endogenous peptides. Activation of κ opioid receptors *in vivo* results in many effects, including analgesia (1–3), dysphoria (2–4), and water diuresis (1, 2). At cellular levels, stimulation of κ opioid receptors activates G_i/G_o proteins resulting in downstream changes, such as inhibition of adenylyl cyclase, activation of p42/p44 mitogen-activated protein (MAP) kinase, increase in K^+ conductance, and decrease in Ca^{2+} conductance (for a review, see Ref. 5). κ opioid receptors of the human, rat, mouse, and guinea pig have been cloned (for reviews, see Refs. 6 and 7), and the receptors belong to the rhodopsin subfamily of G protein-coupled receptors (GPCRs) (8).

PDZ domains were originally identified in the proteins postsynaptic density-95 (PSD-95), discs-large, and zona occludens-1 (ZO-1) as repeats of about 90 amino acids containing the conserved motif Gly-Leu-Gly-Phe. These domains mediate protein-protein interactions by interacting with the last few C-terminal residues of their target proteins (9, 10). Many GPCRs have been shown to interact with PDZ domain-containing proteins, and such interactions play important roles in signal transduction and receptor regulation (for a review, see Ref. 11).

Na^+/H^+ exchanger regulatory factor-1 (NHERF-1)/ezrin-radixin-moesin (ERM)-binding phosphoprotein-50 (EBP50) is a PDZ domain-containing protein. Rabbit NHERF-1 was first purified and cloned (12), and the human homolog was subsequently cloned, termed EBP50 (13) or hNHERF (14). The term

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[1] The abbreviations used are: KOR, κ opioid receptor; hKOR, human κ opioid receptor; β_2-AR, β_2-adrenergic receptor; BCECF-AM, acetoxymethyl ester of 2′,7′bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; CHO cells, Chinese hamster ovary cells; DI and DII, NHERF-1/EBP50 PDZ domains I and II; FL, full-length NHERF-1/EBP50; GPCR, G protein-coupled receptor; GST, glutathione *S*-transferase; HA, hemagglutinin epitope (YPYDVPDYA); hdor, human δ opioid receptor; hmor, human μ opioid receptor; HRP, horseradish peroxidase; MAP, mitogen-activated protein; NHE3, Na^+/H^+ exchanger 3; NHERF-1/EBP50, Na^+/H^+ exchanger regulatory factor-1/ezrin-radixin-moesin (ERM)-binding phosphoprotein-50; nor-BNI, norbionaltorphimine; OK cells, opossum kidney proximal tubule epithelial cells; PDZ, PSD-95/Discs-large/ZO-1 homology; PTX, pertussis toxin; PSD-95, post-synaptic density protein of 95 kDa; TMA, tetramethylammonium; U50,488H, (−)(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)cyclohexyl]benzeneacetamide; OKH, the clonal subline "H" of OK cells; PVDF, polyvinylidene difluoride; MOPS, 4-morpholinepropanesulfonic acid; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); PKA, protein kinase A; PKC, protein kinase C.

NHERF-1/EBP50 is used throughout this report. NHERF-1/EBP50 is widely distributed in tissues and is particularly enriched in those containing polarized epithelia. NHERF-1/EBP50 is a cofactor essential for cAMP-mediated inhibition of the activity of Na^+/H^+ exchanger 3 (NHE3), a subtype of the Na^+/H^+ exchangers found in renal apical membranes. Through the ERM-binding domain, NHERF-1/EBP50 binds to the ezrin-radixin-moesin family of actin-binding proteins and thus joins NHE3 with the actin cytoskeleton. This complex facilitates NHE3 phosphorylation and hormonal regulation of Na^+/H^+ exchange (for reviews, see Refs. 15 and 16). In renal proximal tubules, NHE3 secrets H^+ into the lumen that, in turn, leads to the production of HCO_3^-, which re-enters the blood. Such NHE3-initiated ion transport mediates the re-absorption of NaCl and $NaHCO_3$.

Hall *et al.* (17) have shown that NHERF-1/EBP50 is associated with the β_2-AR, and this interaction plays an important role in β_2-AR-mediated regulation of the NHE3 activity independent of G proteins. This finding resolves the paradox, that activation of β_2-AR enhances intracellular cAMP but does not inhibit NHE3 activity. The observation revealed a mechanism by which GPCRs, such as β_2-AR, through their associated protein(s), influence cellular signaling events independent of G proteins.

We have recently demonstrated that NHERF-1/EBP50 co-immunoprecipitates with the human κ opioid receptor (hKOR) via its first PDZ domain (18). This association appears to serve as a signal for internalized hKOR to be sorted to the recycling pathway and thus blocks U50,488H-induced down-regulation of the hKOR, without affecting its internalization (18). The C-terminal sequence of the hKOR, NKPV, is distinctly different from the sequence, D(S/T)*X*L, the optimal C-terminal motif in the β_2-AR for NHERF-1/EBP50 binding (19).

Although NHERF-1/EBP50 co-immunoprecipitates with the hKOR and it appears to be a PDZ domain-mediated interaction (18), it is not clear whether NHERF-1/EBP50 binds directly to the C-terminal domain of the hKOR. In this study, we employed the pull-down techniques using the fusion protein of glutathione *S*-transferase (GST) and the C-terminal domain of the hKOR (GST-hKOR C-tail) to examine its interaction with NHERF-1/EBP50, purified from a bacterial expression system, expressed in CHO cells and endogenously expressed in OK cells, a cell line derived from opossum kidney proximal tubules. As in OK cells, NHERF-1/EBP50 is known to be a regulator of NHE3 (16, 28), and κ opioid binding sites have been shown to be present (20); thus, we examined in these cells whether activation of the κ opioid receptor regulates Na^+/H^+ exchange, and, if so, whether NHERF-1/EBP50 plays a role.

EXPERIMENTAL PROCEDURES

Materials—OK cells and minimum essential medium were purchased from ATCC (Manassas, VA), OKH cells were kindly provided by Dr. Judith A. Cole of the University of Memphis, and OKH-N1 cells were a gift from Dr. Eleanor D. Lederer of the University of Louisville. (−)U50,488H was purchased from Tocris (Ellisville, MO), naloxone and norbionaltorphimine (nor-BNI) were obtained from NIDA. pGEX-4T-1 bacterial expression system, Glutathione Sepharose 4B and Bulk GST Purification Module were purchased from Amersham Biosciences. pET-30a(+) bacterial expression vector, S-protein HRP conjugate and BugBuster protein extraction reagent were obtained from Novagen Co. (Madison, WI). ProBond™ nickel-chelating resin and ProBond™ purification system, Superscript II reverse transcriptase, hygromycin B, LipofectAMINE, Dulbecco's modified Eagle's medium/Ham's F-12, and fetal calf serum were purchased from Invitrogen. The following reagents were purchased from the indicated companies: Acetoxymethyl ester of 2′,7′bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM), Molecular Probes (Eugene, OR); *Escherichia coli* BL21-CodonPlus (DE3)-RP, Stratagene (La Jolla, CA); Triton X-100 and protease inhibitor mixture tablets, Roche Applied Science; enhanced chemiluminescence (ECL) Western blotting detection reagents and BCA protein assay kit, Pierce; Immobilon™-P PVDF membrane and Microcon centrifugal filter devices, Millipore (Bedford, MA); monoclonal anti-HA antibody (HA.11), Covance Co. (Berkeley, CA); monoclonal anti-actin(beta) antibody, Abcam, Inc. (Cambridge, MA); rabbit polyclonal anti-$G\alpha_{i\text{-}3}$, anti-G_{12}, and anti-G_{13} antibodies, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); PhosphoPlus p44/42 MAP kinase (Thr^{202}/Tyr^{204}) antibody kit and p44/42 MAP kinase antibody kit, Cell Signaling (Beverly, MA); and Advantage cDNA Polymerase Mix and NucleoTrap mRNA Mini kit, Clontech (Palo Alto, CA). Restriction enzymes and other chemicals used in the molecular biology experiments were purchased from Promega (Madison, WI), Roche Applied Science, and Qiagen Co. (Valencia, CA). Pertussis toxin (PTX), nigericin, and commonly used chemicals were purchased from Sigma-Aldrich.

cDNA Construction and Purification of GST Fusion Proteins—Six GST fusion proteins were used in the studies: GST-hKOR C-tail, GST-hmor C-tail, GST-hdor C-tail, GST-FL, GST-DI, and GST-DII. The C-terminal domain of the hKOR-(334–380), hmor-(336–394) or hdor-(322–372), NHERF-1/EBP50 (full-length, FL), NHERF-1/EBP50-(1–151) (PDZ domain I, DI), or NHERF-1/EBP50-(152–358) (PDZ domain II, DII) was cloned into the pGEX-4T-1 bacterial expression system, which contains the GST sequence 5′ to the inserted sequence, and transformed into *E. coli* BL21-CodonPlus (DE3)-RP to express. The *E. coli* cells overexpressing GST fusion proteins or GST were solubilized in BugBuster protein extraction reagent at room temperature for 30 min according to the Novagen manual, and then adsorbed onto glutathione-Sepharose 4B beads (15 min at room temperature) and washed three times with phosphate-buffered saline. The beads were ready for pull-down analysis, and the amounts of loaded proteins were semiquantified by use of Coomassie Blue staining (SDS-gels) and Ponceau S staining (PVDF membranes), respectively. In addition, those proteins used for overlay assays were further eluted from the beads with 10 mM glutathione/50 mM Tris-HCl (pH 8.0) buffer, and glutathione was removed through multiple rounds of concentration and dilution using Microcon™ centrifugal filter devices with fresh buffer of 25 mM Tris/0.8% NaCl/0.1% Tween 20 (pH 7.6) (TBS-T buffer). The concentrations of the purified proteins were determined by using a BCA protein assay kit.

cDNA Construction and Purification of His_6/S Tag-NHERF-1/EBP50—NHERF-1/EBP50 cDNA was cloned into the pET-30a(+) bacterial expression vector, which carries N-terminal His_6 tag and S tag, and transformed into *E. coli* BL21(DE3). The protein was purified from medium by use of ProBond™ nickel-chelating resin according to the manufacturer's instructions. The His_6 tag fusion proteins were eluted from the beads with 0.5 M imidazole/500 mM NaCl/50 mM sodium phosphate buffer, pH 8.0, at room temperature for 20 min. Imidazole was removed in a similar way so as to remove glutathione as above, and the protein concentration was detected by using a BCA protein assay kit.

Cell Culture—CHO cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37 °C. Opossum kidney proximal tubule epithelium cells (OK, OKH, and OKH-N1 cells) were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in minimum essential medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). OKH-N1 cells, transfected stably with full-length rabbit NHERF-1/EBP50 in the pcDNA3.1(+)-Hygro vector, were maintained in the presence of 0.25 mg/ml hygromycin B.

Assessment of Direct Protein-Protein Interactions by Pull-down Techniques—This assessment was performed as described by Einarson and Orlinick (21).

Interaction of hKOR-C-tail with Purified NHERF-1/EBP50—GST (control), GST-hKOR C-tail, GST-hmor C-tail, or GST-hdor C-tail bound to glutathione-Sepharose 4B beads at ~10 μg of each protein/20 μl of resin, with less loading of GST-hKOR C-tail due to its poor binding capability. The beads (20 μl each) were incubated with purified His_6/S-tag-NHERF-1/EBP50 (10 μg), respectively, in 1 ml of TBS-T buffer containing 3% bovine serum albumin at 4 °C with end-over-end rotation for 1 h. The beads were washed twice with ice-cold 3% bovine serum albumin in TBS-T buffer and three times with TBS-T buffer to remove nonspecific binding. The Sepharose 4B beads were incubated with 40 μl of 2× SDS-PAGE sample buffer at 100 °C for 5 min to dissociate proteins from beads and centrifuged to remove beads. The supernatants (20 μl of each) were subjected to 15% SDS-PAGE, and the gel was transferred to Immobilon™-P PVDF membrane, which was rinsed three times with TBS-T buffer and blocked with 2% nonfat dry milk in TBS-T buffer. The His_6/S tag-NHERF-1/EBP50 fusion protein was detected by blotting with an S-protein HRP conjugate (1:5000), followed

by enhanced chemiluminescence reagents. The membranes was also stained with 0.1% Ponceau S in 5% acetic acid to reveal the relative loading amounts of the GST (26 kDa), GST-hKOR C-tail (34 kDa), GST-hmor C-tail (37 kDa), and GST-hdor C-tail (35 kDa).

Interaction of hKOR-C-tail with HA-NHERF-1/EBP50 or Its Fragment Expressed in CHO Cells—CHO cells were transiently transfected with 8 μg/100-mm dish of HA-NHERF-1/EBP50 in pcDNA3, HA-NHERF-1/EBP50-(1–151) (PDZ domain I (DI)) in pBK/CMV, or HA-NHERF-1/EBP50-(152–358) (PDZ domain II (DII)) in pBK/CMV by use of LipofectAMINE (50 μl) following the manufacturer's instructions. 2–3 days after transfection, cells were solubilized in lysis buffer (50 mM Tris HCl/150 mM NaCl/1 mM EDTA/1% (v/v) Triton X-100), and centrifuged at 16,000 × *g* for 30 min. Supernatants were incubated with glutathione-Sepharose 4B beads pre-loaded with either GST or GST-hKOR-C-tail as described as above in the 1% Triton X-100/1 mM EDTA/TBS buffer (25 mM Tris/150 mM NaCl, pH 7.6) at 4 °C with end-over-end rotation overnight. The beads were washed three times with ice-cold TBS-T buffer. The proteins were eluted from the beads with 2× SDS-PAGE sample buffer, resolved with 10% SDS-PAGE and transferred onto Immobilon™-P PVDF membranes. HA fusion proteins were detected with immunoblotting using monoclonal anti-HA antibody (1:4000) followed by goat anti-mouse IgG-HRP conjugate and enhanced chemiluminescence reagents.

Interaction of hKOR-C-tail with Endogenous NHERF-1/EBP50 in OK Cells—OK cells (~1×10^7) were lysed and solubilized with 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) in the presence of Roche Applied Science protease inhibitors mixture (1 tablet/10 ml) for 1 h at 4 °C and centrifuged (16,000 × *g*, 30 min). The supernatants were used for pull-down experiments using beads loaded with either GST-hKOR C-tail or GST as described as above, except that NHERF-1/EBP50 was detected by immunoblotting with a polyclonal anti-rabbit NHERF-1/EBP50 antibody (1:500) followed by goat anti-rabbit IgG-HRP conjugate and enhanced chemiluminescence reagents.

Detection of NHERF-1/EBP50 Oligomerization by Overlay Assays—This was performed according the method of Lau and Hall (22). Purified GST and GST fusion proteins (GST-FL, GST-DI, and GST-DII) (2 μg/lane) were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were incubated with purified His_6-S.tag-NHERF-1/EBP50 (10 μg/ml) alone or in the presence of GST or GST-hKOR C-tail at concentration of 10 μg/ml in TBS-T containing 5% nonfat milk overnight at 4 °C. Membranes were washed three times with 10 ml of TBS-T buffer (10 min each time) and then incubated for 1 h at room temperature with S-protein horseradish peroxidase conjugate at dilution of 1:4000 in the TBS-T buffer for detection of overlaid His_6-S.tag-NHERF-1/EBP50 proteins. Finally, membrane were washed as above and visualized via enhanced chemiluminescence using an ECL kit.

p42/p44 MAP Kinase Phosphorylation—OK and OKH cells were transferred to 12-well plates, grown overnight, and changed to serum-free medium for 3 h approximately overnight to reduce basal MAP kinase phosphorylation. Cells were treated with or without U50,488H (1 μM), naloxone (10 μM), or nor-BNI (1 μM) at 37 °C for 5 min and then lysed with 2× SDS-PAGE sample buffer. For PTX-treated cells, cells were pre-treated with PTX (100 ng/ml, 12–16 h) before incubation with any drugs. Aliquots of the lysates were separated on SDS-PAGE and transferred to Immobilon™-P PVDF membranes. Phosphorylated MAP kinase was detected by Western blot using a PhosphoPlus p44/42 MAP kinase (Thr^{202}/Tyr^{204}) antibody kit according to the manufacturer's instructions. Membranes were then stripped by incubating at 50 °C for 30 min with 0.1 mM 2-mecaptoethanol and 2% SDS in 62.5 mM Tris buffer (pH 6.8) and rinsed twice with TBS-T buffer at room temp. Total p44/42 MAP kinase on the membranes was detected by Western blot using p44/42 MAP kinase antibody. Images were captured with a FUJIFILM LAS-1000 imaging system and analyzed with ImageGauge software.

Determination of Intracellular pH (pH_i) in OK, OKH, or OKH-N1 cells—pH_i of cells was determined by microphotometry of the fluorescence emission of the pH-sensitive dye, acetoxymethyl ester of 2′,7′bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM), using dual wavelength excitation according to the method of Weinman *et al.* (23). Cells were cultured in serum-free medium for overnight on 40-mm glass coverslips to full confluence and loaded with 6.5 μM BCECF-AM in 20 mM HEPES buffer, pH 7.4, containing 35 mM NH_4Cl, 90 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM tetramethylammonium (TMA)-PO_4, and 25 mM glucose for 20 min at room temperature. Coverslips were transferred to an FSC2 chamber (Bioptechs) and rapidly perfused at a constant flow rate with solutions warmed to 37 °C. Cells were acidified for 5 min with 20 mM HEPES, pH 7.5, 130 mM TMA-Cl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM TMA-PO_4, and 25 mM glucose. Na^+/H^+ exchange transport was initiated by substituting 130 mM NaCl for 130 mM TMA-Cl in the perfusion solution. BCECF fluorescence was measured at excitation wavelengths of 500 and 440 nm and an emission wavelength of 550 nm. The NH_4Cl pulse was used to achieve an initial pH_i of about 6.0, and only cells with initial pH_i values between 5.8 and 6.0 were included for analyses. Na^+/H^+ exchange, expressed as change in pH_i (ΔpH_i)/10 s, represented the initial slope of transport activity measured between 5 and 20 s of sodium-dependent pH_i recovery. Over this brief time period, the relationship between pH_i and time was essentially linear. To examine the effects of U50,488H and other opioid drugs on Na^+/H^+ exchanger activity, cells were pretreated with the drug(s) during the final 15 min of dye loading and continuously throughout the perfusion process. For PTX-treated cells, cells were pretreated with PTX (100 ng/ml, 12–16 h) before U50,488H treatment. At the end of each experiment, the cells were equilibrated in pH clamp media containing 20 mM HEPES, 20 mM MOPS, 115 mM KCl, 14 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM TMA-PO_4, 25 mM glucose, and 10 μM nigericin at pH 6.2 and 7.3 for calibration of fluorescence intensity with pH. On any given coverslip, Na^+/H^+ exchange was measured in several fields, and the determinations were averaged to constitute a single observation. All measurements were made on cells at the same passage on the same day.

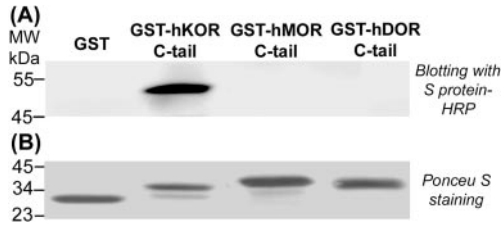

FIG. 1. **Direct interaction between the hKOR C-tail and NHERF-1/EBP50.** Purified His_6-S.tag-NHERF-1/EBP50 was incubated with glutathione-Sepharose 4B beads pre-loaded with GST, GST-hKOR C-tail, GST-hmor C-tail, or GST-hdor C-tail for 1 h at 4 °C. The beads were washed extensively, and the bound proteins were eluted from the beads, resolved by 10% SDS-PAGE, and transferred onto an Immobilon™-P PVDF membrane. *A*, the His_6-S.tag-NHERF-1/EBP50 fusion protein was detected by blotting with S-protein HRP conjugate followed by enhanced chemiluminescence reagents. *B*, the same membrane was stained with 0.1% Ponceau S in 5% acetic, which shows the molecular weights and amounts of the GST and GST fusion proteins loaded. Each figure represents one of the two (GST-hmor C-tail and GST-hdor C-tail) or six (GST-hKOR C-tail and GST) experiments with similar results.

RESULTS

Direct Interaction between the C-terminal Domain of the Human κ Opioid Receptor (hKOR) and NHERF-1/EBP50—We have previously shown that NHERF-1/EBP50 is associated with the hKOR in CHO cells and overexpression of NHERF-1/EBP50 blocks agonist-induced down-regulation of the hKOR (18). Here we determined whether there was a direct interaction between NHERF-1/EBP50 and C-terminal domain of hKOR by GST fusion protein pull-down techniques. As shown in Fig. 1, GST-hKOR C-tail bound His_6-S.tag-NHERF-1/EBP50, but GST did not, indicating that NHERF-1/EBP50 binds directly to the C-terminal domain of the hKOR. In contrast, the C-tails of human μ and δ opioid receptors did not bind NHERF-1/EBP50, demonstrating the selectivity of the interaction of the hKOR C-tail with NHERF-1/EBP50.

We next determined whether the C-terminal domain of hKOR was associated with NHERF-1/EBP50 expressed in CHO cells. HA-tagged full-length NHERF-1/EBP50 and its two fragments containing PDZ domains I and II were expressed in CHO cells and Triton X-100-solubilized fractions were used for pull-down experiments. Fig. 2*A* showed that GST-hKOR C-tail strongly bound NHERF-1/EBP50 and its PDZ domain I-containing fragment but not its domain II-containing fragment, in cell lysates, whereas GST did not bind significantly to any of the constructs. These results

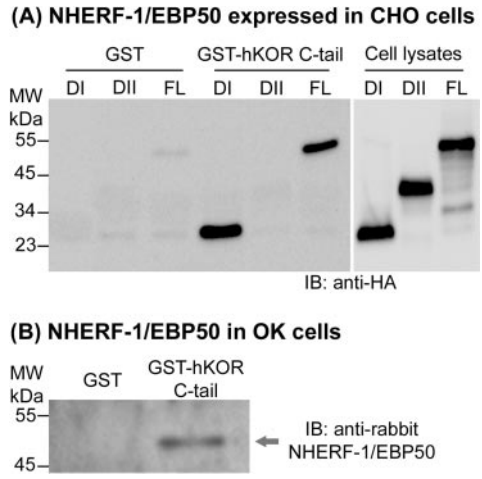

FIG. 2. **Association of the hKOR C-tail with NHERF-1/EBP50 expressed in CHO cells or present endogenously in OK cells.** *A*, HA-NHERF-1/EBP50 expressed in CHO cells. CHO cells were transfected with the cDNA construct of HA-NHERF-1/EBP50 (full-length, *FL*) or the 1–151 fragment containing the PDZ domain I (*DI*) or the 152–358 fragment containing the PDZ domain II (*DII*). 2–3 days later, similar numbers of transfected cells were lysed and solubilized with 1% Triton X-100 and centrifuged. Pull-down experiments were performed with the supernatants, and glutathione-Sepharose beads pre-bound with GST-hKOR C-tail or GST. Bound proteins were resolved on SDS-PAGE, blotted onto membranes, and HA-NHERF-1/EBP50 and its PDZ DI and PDZ DII domains were detected by immunoblotting with monoclonal antibody against HA (HA.11) (*left panel*). In the *right panel*, a fraction of the same batches of transfected cells were subjected to SDS-PAGE, transferred, and immunoblotted with the same anti-HA antibody to reveal the expression levels of HA-NHERF-1/EBP50, its DI and DII fragments. *B*, NHERF-1/EBP50 present endogenously in OK cells. OK cells were lysed and solubilized with 1% Triton X-100 and centrifuged. Supernatants were used for pull-down experiments, and NHERF-1/EBP50 was detected by immunoblotting with a polyclonal anti-rabbit NHERF-1/EBP50 antibody. The data shown in each panel of this figure are representative of three independent experiments with similar results.

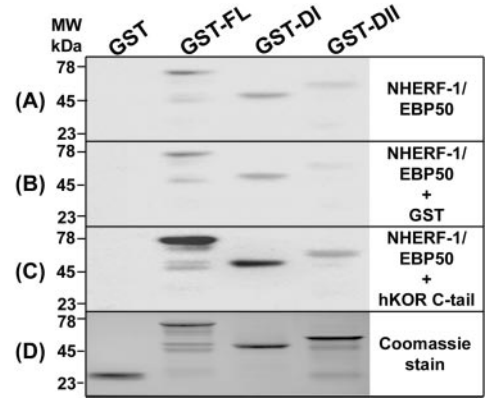

FIG. 3. **Oligomerization of NHERF-1/EBP50 is facilitated by the hKOR C-tail.** *A*, overlay of His_6-S.tag-NHERF-1/EBP50 onto GST-full-length NHERF-1/EBP50 (*GST-FL*), GST-NHERF-1/EBP50 PDZ domain I (*GST-DI*), and GST-NHERF-1/EBP50 PDZ domain II (*GST-DII*). Purified GST fusion proteins (2 μg each) were resolved on SDS-PAGE, transferred onto PVDF membranes, and overlaid with the His_6-S.tag-NHERF-1/EBP50 (10 μg/ml), which was probed with S protein-HRP conjugate followed by enhanced chemiluminescence. *B*, overlay as in *A* in the presence of GST (10 μg/ml). *C*, overlay as in *A* in the presence of GST-hKOR C-tail (10 μg/ml). *D*, Coomassie Blue staining shows the relative sizes and loading amounts of the GST fusion proteins. The data shown in each panel of this figure are representative of three independent experiments with similar results.

indicate that NHERF-1/EBP50 expressed in CHO cells interacts with the C-terminal domain of the hKOR and the PDZ domain I is responsible for the interaction.

Opossum kidney proximal tubule cells (OK cells) have been shown to express NHERF-1/EBP50 (28) and κ opioid binding sites endogenously (20). To test whether the hKOR C-tail interacted with NHERF-1/EBP50 in OK cells, GST pull-down experiments were performed using 1% Triton X-100-soluble fraction of OK cells. As shown in Fig. 2*B*, GST-hKOR C-tail, but not GST, was associated with a protein band of ~50 kDa, which was recognized by a polyclonal anti-rabbit NHERF-1/EBP50 antibody. These results indicate that the C-terminal domain of the κ opioid receptor binds endogenous NHERF-1/EBP50 in OK cells.

hKOR C-tail Enhanced Oligomerization of NHERF-1/EBP50—It has been reported that the NHERF-1/EBP50 oligomerizes via PDZ domains (22, 24–26), and binding of the C termini of the β_2-adrenergic receptor or the platelet-derived growth factor receptor facilitated its oligomerization (22, 25). Here we examined whether the binding of hKOR C-tail had any effect on NHERF-1/EBP50 oligomerization. GST fusion proteins were subjected to SDS-PAGE and transferred onto membranes, and overlay was performed with His_6-S.tag-NHERF-1/EBP50. As shown in Fig. 3, His_6-S.tag-NHERF-1/EBP50 bound strongly to GST-NHERF-1/EBP50 and GST-NHERF-1/EBP50 PDZ domain I (Fig. 3*A*), but it bound weakly to GST-NHERF-1/EBP50 PDZ domain II and did not bind GST. The results suggest that NHERF-1/EBP50 oligomerization is primarily via its PDZ domain I, consistent with the report of Shenolikar *et al.* (24, 27). Binding of His_6-S.tag-NHERF-1/EBP50 to GST-NHERF-1/EBP50 and GST-NHERF-1/EBP50 PDZ domain I was significantly enhanced by the presence of GST-hKOR C-tail (Fig. 3*C*), compared with that in the presence of GST (Fig. 3*B*) or without GST or any GST fusion protein (Fig. 3*A*). The results indicate that the interaction of NHERF-1/EBP50 and hKOR-tail facilitates the PDZ domain I-mediated NHERF-1/EBP50 oligomerization.

Presence of κ Opioid Receptors in OK Cells: U50,488H Enhanced p44/42 MAP Kinase Phosphorylation in a Naloxone- and PTX-sensitive Manner—To determine whether OK cells express functional κ opioid receptors, we examined whether administration of U50,488H, a selective κ opioid agonist affected p44/42 MAP kinase phosphorylation. U50,488H enhanced p44/42 MAP kinase phosphorylation by 1.9- to 2.3-fold, compared with the control (Fig. 4). The U50,488H effect was blocked by either nor-BNI, a selective κ opioid antagonist, or naloxone, a nonselective opioid antagonist, indicating that the effect of U50,488H is mediated by κ opioid receptors. In OK cells pretreated with PTX, U50,488H failed to affect p44/42 MAP kinase phosphorylation (Fig. 4), demonstrating that κ opioid receptor stimulation of p44/42 MAP kinase phosphorylation is dependent on activation of the G_i/G_o proteins in OK cells.

U50,488H Stimulated NHE3 Activity in OK Cells via κ Opioid Receptor in a PTX-insensitive Manner—Whether activation of κ opioid receptors regulated Na^+/H^+ exchange was investigated in OK cells, which express only NHE3 among the subtypes of Na^+/H^+ exchangers (28). Following intracellular acidification, control OK cells had an initial $\Delta pH_i/10$ s (a measure of Na^+/H^+ exchange rate) of 0.030 ± 0.002 ($n = 9$) (Fig. 5, *A* and *C*). In the presence of U50,488H, the $\Delta pH_i/10$ s was increased to 0.043 ± 0.003 ($n = 7$) (Fig. 5, *A* and *C*), which was significantly different from the control ($p < 0.01$). Naloxone, which by itself had no effect ($\Delta pH_i/10$ s = 0.032 ± 0.002, $n = 4$), blocked U50,488H-induced enhancement ($\Delta pH_i/10$ s = 0.028 ± 0.001, $n = 4$) (Fig. 5, *A* and *C*), indicating that the effect is mediated by the κ opioid receptor. The μ opioid agonist [D-Ala^2,*N*-methyl-Phe^4,Gly^5-ol]enkephalin (DAMGO) and the δ opioid agonist [D-Pen^{2,5}]enkephalin (DPDPE) had no effect on Na^+/H^+ exchange in OK cells (data not shown). Interestingly, in OK cells

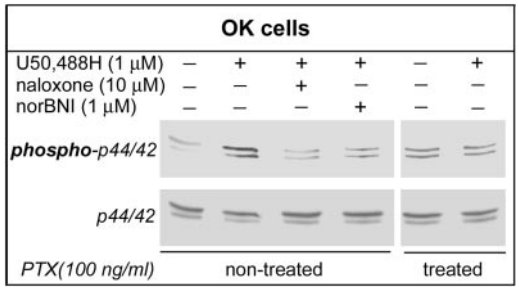

FIG. 4. **Presence of κ opioid receptors in OK cells: U50,488H stimulated p42/p44 MAP kinase phosphorylation via κ opioid receptors.** OK cells were cultured in serum-free medium for 3 h approximately overnight to reduce basal MAP kinase phosphorylation. Cells were treated with or without U50,488H (1 μM), naloxone (10 μM), or nor-BNI (1 μM) as indicated at 37 °C for 5 min. For PTX-treated cells, cells were pre-treated with PTX (100 ng/ml, 12–16 h) before incubation with any drugs. Aliquots of the cell lysates were separated on SDS-PAGE, transferred onto membranes, and phosphorylated p44/42 MAP kinase was detected by immunoblotting with specific antibody against phospho-p44/42 MAP kinase. Subsequently, membranes were stripped, and total p44/42 MAP kinase was detected by blotting with p44/42 MAP kinase antibody. The data shown in each panel of this figure are representative of three independent experiments with similar results.

pretreated with PTX, the ΔpH_i/10 s of control cells and U50,488H-treated cells were 0.031 ± 0.002 (n = 4) and 0.044 ± 0.002 (n = 4) (Fig. 5, *B* and *C*), respectively, which were similar to corresponding data in cells not treated with PTX. The PTX treatment paradigm was shown previously to completely abolish agonist-induced increase in [^{35}S]GTPγS binding mediated by κ opioid receptors (29). These results indicate that stimulation of NHE3 activity by κ opioid receptor activation is not mediated by G_i/G_o proteins.

U50,488H Failed to Stimulate NHE3 Activity in OKH Cells, Which Express a Much Lower Level of NHERF-1/EBP50—Because the stimulatory effect of κ opioid receptor on NHE3 activity in OK cells is independent of G_i/G_o proteins, we investigated whether the effect was mediated by the association of the κ opioid receptor with NHERF-1/EBP50. The OKH cell line is a subclone of OK cells, which were shown to express a much lower level of NHERF-1/EBP50 compared with OK cells (30). The lower expression of NHERF-1/EBP50 in OKH cells was confirmed by immunoblotting (Fig. 6*A*, the *top panel*), whereas the expression levels of $G\alpha_i$ (the *middle panel*) were similar. Incubation of OKH cells with U50,488H did not have any effect on NHE3 activity (Fig. 7*A*). Control and U50,488H-treated OKH cells had initial rates of Na^+/H^+ exchange (ΔpH_i/10 s) of 0.025 ± 0.003 (n = 4) and 0.023 ± 0.002 (n = 4). In OKH cells U50,488H enhanced p44/42 MAP kinase phosphorylation by ~2-fold, similar to that in OK cells, which was blocked by naloxone and PTX pretreatment (Fig. 6*B*), indicating that κ opioid receptor-G_i/G_o proteins signaling pathway is still intact. These results suggest that acceleration of Na^+/H^+ exchange following κ opioid receptor activation is independent of G_i/G_o proteins and mediated by NHERF-1/EBP50 in OK cells.

Transfection of NHERF-1/EBP50 into OKH Cells Restored the Ability of U50,488H to Stimulate NHE3 Activity—We used OKH-N1 cells to further explore the role of NHERF-1/EBP50 in U50,488H-induced increase in Na^+/H^+ exchange. OKH-N1 cells are OKH cells stably transfected with the full-length rabbit NHERF-1/EBP50 at an expression level comparable to that in OK cells (Fig. 6*A*, *top panel*). These cells expressed a similar level of $G\alpha_i$, compared with either OK or OKH cells (Fig. 6*A*, the *middle panel*). Following acidification of OKH-N1 cells, U50,488H significantly increased the ΔpH_i/10 s, compared with the untreated control ($p < 0.05$, n = 6) (Fig. 7*B*). These results further support the notion that U50,488H stimulated Na^+/H^+ exchange is mediated by NHERF-1/EBP50.

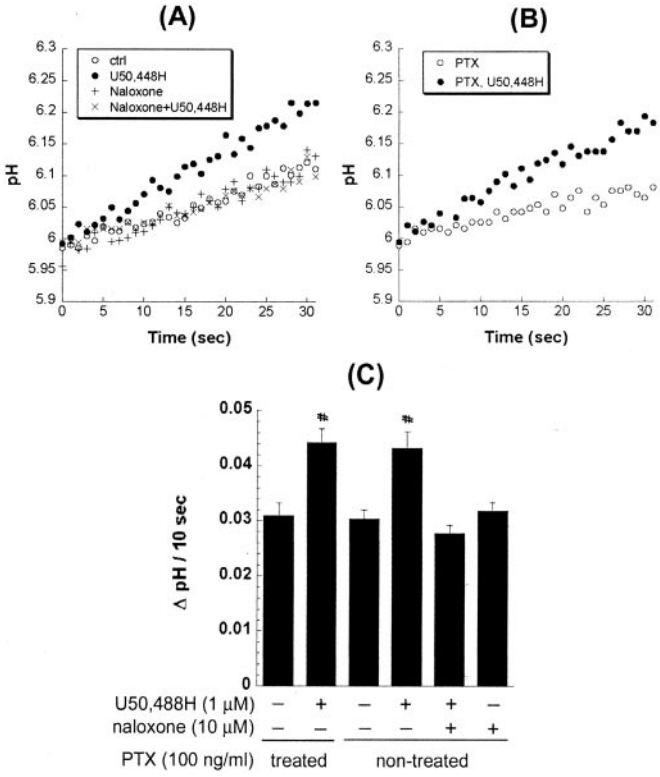

FIG. 5. **U50,488H stimulated Na^+/H^+ exchange in OK cells independent of PTX-sensitive G proteins.** *A*, effects of U50,488H and/or naloxone on Na^+-dependent pH_i change. *B*, effects of PTX pretreatment on the U50,488H action. OK cells grown on coverslips were loaded with BCECF-AM, acidified, and intracellular pH (pH_i) was determined after switching to Na^+-containing buffer (at time 0) as described under "Experimental Procedures." *A*, cells were treated with 1 μM U50,488H, 10 μM naloxone, or both during the final 15 min of dye loading and continuously throughout the perfusion process. *B*, cells were pre-treated with PTX (100 ng/ml, 12–16 h) before incubation with 1 μM U50,488H as in *A*. *A* and *B* show representative data of Na^+-dependent pH_i changes in the first 30 s. *C*, initial rates of pH_i changes (ΔpH_i/10 s) are calculated based on pH_i changes between 5 and 20 s of sodium-dependent pH_i recovery as described under "Experimental Procedures." On any given coverslip, Na^+/H^+ exchange was measured in several fields, and the determinations were averaged to constitute a single observation. Each value represents the mean ± S.E. of at least four independent experiments with duplicate coverslips. #, $p < 0.01$, compared with cells without opioid drug treatment by one-way analysis of variance followed by Dunnett's multiple comparison test.

DISCUSSION

NHERF-1/EBP50 Binds to the C-tail of the κ Opioid Receptor via the First PDZ Domain—We have previously shown that the hKOR co-immunoprecipitated with NHERF-1/EBP50, and a PDZ domain-mediated interaction has been proposed to occur between the C-tail of the hKOR and NHERF-1/EBP50 (18). In this study, using pull-down techniques, we show that the hKOR C-tail interacted directly with NHERF-1/EBP50, purified from bacterial expression system or expressed in CHO cells, via its PDZ domain I and bound endogenous NHERF-1/EBP50 in OK cells. Notably the interaction of the hKOR C-tail with NHERF-1/EBP50 expressed in CHO cells was much stronger than its association with NHERF-1/EBP50 purified from bacterial expression system, which is probably due to lack of post-translational modifications of proteins in bacteria. The binding of NHERF-1/EBP50 to the C-tail of the hKOR is much weaker than its binding to the C-tail of the β_2-adrenergic receptor.[2] This finding is consistent with the observation that the C-terminal sequence of the hKOR, NKPV, is distinctly

[2] P. Huang and L.-Y. Liu-Chen, unpublished observations.

different from the sequence, D(S/T)*X*L, the optimal C-terminal motif in the β_2-AR for EBP50/NHERF binding (19). Although the opossum κ opioid receptor has not been cloned, it is very likely that the four amino acids of the C-terminal domain are identical to those in the hKOR as they are conserved in the human, guinea pig, rat, and mouse KORs (31–34).

Activation of κ Opioid Receptors Enhanced NHE3 Activity via NHERF-1/EBP50—In OK cells, the selective κ agonist U50,488H enhanced Na^+/H^+ exchange in a naloxone-sensitive manner. Because the NHE3 is the only subtype of NHEs in OK cells (28), these results indicate that U50,488H stimulated NHE3 activity via κ opioid receptors. Interestingly, the stimulatory effect is PTX-resistant, indicating that κ opioid receptor regulates the activity of NHE3 by a mechanism independent of activation of G_i/G_o proteins, which are the predominant G proteins coupled to the receptor (5). In OKH cells that express a much lower NHERF-1/EBP50 level, κ opioid receptor activation did not affect NHE3 activity; although it enhanced p42/p44 MAP kinase phosphorylation similar to that in OK cells. Moreover, stably transfecting NHERF-1/EBP50 into OKH cells restored the stimulatory effect of κ opioid receptors on NHE3 activity as shown in OKH-N1 cells. Thus, κ opioid receptor regulation of NHE3 activity is NHERF-1/EBP50-dependent.

It should be noted that OK, OKH, and OKH-N1 cells express similar levels of total $G\alpha_i$ proteins, thus excluding their role in regulation of Na^+/H^+ exchange. The κ opioid receptors have been shown to couple to G_o proteins in addition to G_i proteins. However, by use of rabbit polyclonal anti-rat $G\alpha_o$ antibody (Calbiochem), we were not able to detect specific bands in the three cell lines. One possibility is that OK cells have low or no $G\alpha_o$, because G_o proteins have been reported to be predominantly expressed in neuronal cells. Another likely scenario is that the anti-rat $G\alpha_o$ antibody may not recognize opossum $G\alpha_o$ proteins. The κ opioid receptor, like many other G_i/G_o-coupled receptors, has been demonstrated also to interact with PTX-insensitive G proteins, such as G_z (35), G_{16} (36), and G_s (37). However, these G proteins are not likely to be involved in the observed stimulation of NHE3 by κ opioid receptor activation, because G_z is predominantly expressed in neuronal tissues (38, 39) and G_{16} is expressed only in hemopoietic cells (40). In addition, stimulation of G_s proteins has been shown to inhibit NHE3 activity (41). G_q proteins have not been reported to be coupled to opioid receptors (5). Thus, this NHERF-1/EBP50-dependent mechanism is unlikely to be mediated by these heterotrimeric G proteins. Lin *et al.* (42) reported that G_{12} and G_{13} proteins stimulated NHE3 activity. By immunoblotting, we found that the three cell lines expressed $G\alpha_{12}$ and $G\alpha_{13}$, and OKH and OKH-N1 cells have higher expression levels of both G proteins than OK cells (data not shown). To the best of our knowledge, opioid receptors have not been shown to couple to G_{12} and G_{13} proteins. However, the possibility cannot be ruled out that G_{12} and G_{13} may play a role in κ opioid receptor-mediated stimulation of NHE3 activity.

The current study provides the first direct evidence that, through association with NHERF-1/EBP-50, a GPCR enhances NHE3 activity, although stimulation of NHE3 by the β_2-AR acting via NHERF-1/EBP-50 is implicit in the findings of Hall *et al.* (17). Interaction of NHERF-1/EBP-50 with NHE3 has been demonstrated to be critical for cAMP-mediated inhibition of NHE3 activity (27). However, activation of the β_2-AR does not result in inhibition of NHE3 activity, unlike that of other G_s-coupled receptors such as the parathyroid hormone receptor (41). NHERF-1/EBP-50 binds to the C terminus of the β_2-AR via PDZ domain-mediated interaction (17). When the interaction is disrupted by mutation in the β_2-AR, activation of the mutant receptor, which enhanced cAMP formation similar to

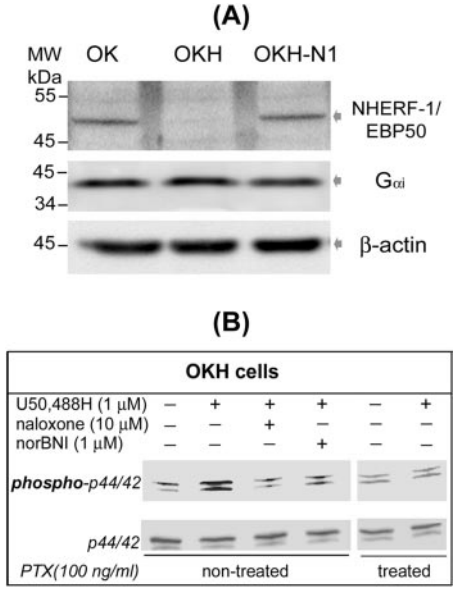

FIG. 6. *A*, OKH cells express a lower level of NHERF-1/EBP-50 than OK cells. Similar numbers of OK, OKH, and OKH-N1 cells were lysed and lysates were separated with SDS-PAGE and protein bands were transferred onto membranes. Immunoblotting was performed as described under "Experimental Procedures" first with polyclonal anti-rabbit NHERF-1/EBP50 antibody (1/1000 dilution) (the *top panel*). After stripping, the membranes were probed with rabbit polyclonal anti-$G\alpha_{i\text{-}3}$ antibody at 1/200 dilution (the *middle panel*). Following the second stripping, the membranes were blotted with monoclonal anti-actin(beta) antibody at 1/50,000 dilution (the *bottom panel*). Anti-$G\alpha_{i\text{-}3}$ antibody recognizes all $G\alpha_i$ proteins, but not other $G\alpha$ subunit proteins. *B*, in OKH cells U50,488H stimulated p44/p42 MAP kinase phosphorylation via κ opioid receptors as in OK cells. Experiments were performed in a similar manner as in Fig. 4. The data shown in each panel of this figure are representative of three independent experiments with similar results.

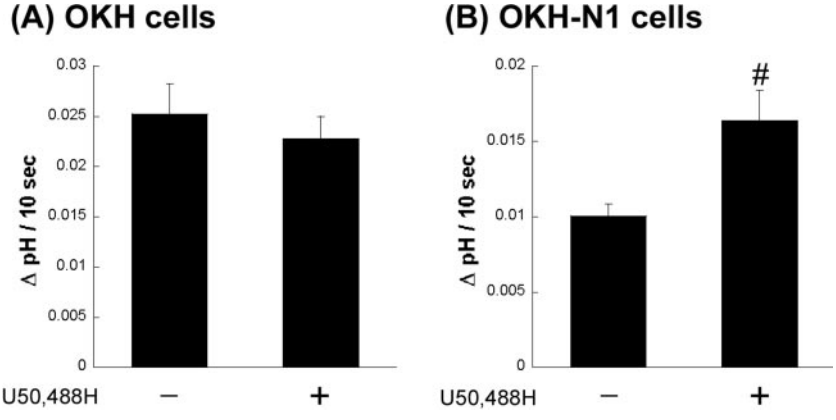

FIG. 7. **U50,488H did not affect Na^+/H^+ exchange in OKH cells but significantly stimulated Na^+/H^+ exchange in OKH-N1 cells.** Experiments were conducted in a similar manner as in Fig. 5. The data of the Na^+-dependent pH_i recovery from acid loading in the absence or presence of U50,488H were collected, and the initial rates of pH_i changes are shown for OKH cells (*A*) and OKH-N1 cells (*B*), respectively. Each value represents the mean ± S.E. of at least four independent experiments. #, $p < 0.05$, compared with control cells without U50,488H treatment by paired *t* test.

the wild-type, leads to inhibition of the NHE3 activity. These results suggest that the lack of inhibition following activation of the β_2-AR is likely to be due to two opposing effects on NHE3: cAMP-mediated inhibition via G_s proteins and stimulation via interaction with NHERF-1/EBP50 independent of G_s.

Activation of κ opioid receptors in rat cardiac myocytes increased pH_i by enhancing Na^+/H^+ exchange, which is dependent on PKC activation (43, 44). This PKC-related mechanism is likely to be mediated by PTX-sensitive G proteins through the $\beta\gamma$ subunits, because κ receptor activation in the heart causing another effect, arrhythmia, involves PTX-sensitive G proteins, phospholipase C, and PKC pathway (45). However, NHERF-1/EBP50 may not be involved, because NHE1 is the main Na^+/H^+ exchanger present in cardiomyocytes (46, 47).

Mechanisms Underlying Stimulation of NHE3 Activity following Activation of κ Opioid Receptors—How κ opioid receptor interacts with NHERF-1/EBP50 to stimulate NHE3 in OK cells remains to be investigated. NHERF-1/EBP50 is associated with NHE3 and ezrin, which was proposed to bind to cAMP-dependent protein kinase (PKA) (27). NHERF-1/EBP50 thus brings the ezrin-PKA complex into proximity of the tail of NHE3. This juxtaposition is believed to facilitate NHE3 phosphorylation by PKA, resulting in reduction of NHE3 activity (27).

Hall *et al.* (17) and Weinman *et al.* (24, 27) have proposed that when β_2-AR is activated by an agonist, the receptor binds NHERF-1/EBP50 with such a high affinity that it displaces NHE3 binding, resulting in disinhibition of NHE3 function. Although it remains possible that the κ opioid receptor competes with NHE3 for NHERF-1/EBP50 as the β_2-AR, the affinity of the κ opioid receptor for NHERF-1/EBP50 is considerably less. Accordingly, we favor an alterative hypothesis whereby the κ opioid receptor promotes the oligomerization of NHERF-1. If a basal level of PKA activity is present in OK cells to maintain NHERF-1/EBP50-mediated inhibition of NHE3, activation of the κ opioid receptor enhances its association with NHERF-1/EBP50 binding and causes oligomerization of NHERF-1/EBP50, which, in turn, eliminates the inhibition of NHE3 by NHERF-1/EBP50 to unmask the stimulatory effect. Our previous observation, that pretreatment of CHO cells expressing the hKOR and NHERF-1/EBP50 with U50,488H increased the amount of NHERF-1/EBP50 co-immunoprecipitated with the hKOR (18), supports this hypothesis. NHERF-1/EBP50 oligomers may represent a pool of inactive NHERF-1/EBP50, which is incapable of inhibiting NHE3 (24, 27). Here we showed that the hKOR C-tail promoted oligomerization of NHERF-1/EBP50, similar to the C-terminal domain of the platelet-derived growth factor receptor or the β_2-AR (22, 25). The oligomerization of NHERF-1/EBP50 may render it inactive in inhibiting NHE3. The κ opioid receptor stimulatory effect on NHE3 in OK cells is thus revealed.

We recognize that the association between the κ opioid receptor, NHERF-1/EBP-50, and NHE3 may also involve other signaling pathways. NHERF-1/EBP50 has been shown to associate with a variety of signaling protein(s) that might affect NHE3 function directly or indirectly, including phospholipase C β1 and β2 isoforms (48, 49), a receptor for activated C kinase (50), EPI64 (51), nadrin (51), and ERM proteins (52). ERM proteins, as conformationally regulated binding sites for NHERF-1/EBP50, have been implicated in regulating the Rho and Rac signaling pathways (52, 53). One or several of these NHERF-1/EBP50-associated proteins may affect NHE3 function by yet unidentified mechanisms, which may be regulated by the κ opioid receptor activation in an NHERF-1/EBP50-dependent mechanism.

Use of OK Cells in the Study—Whether activation of κ opioid receptors regulated Na^+/H^+ exchange was investigated in OK cells, because these cells have been shown to express NHERF-1/EBP50, NHE3, and κ opioid binding sites endogenously (20, 28). However, using OK cells has its limitations. The expression level of κ opioid receptors in the cell line is very low, barely detectable with [^{3}H]diprenorphine binding. Such a low level of κ opioid receptors does not yield detectable agonist-induced [^{35}S]GTPγS binding. Even though p42/p44 MAP kinase phosphorylation is a sensitive assay, U50,488H caused only a modest increase in p42/p44 MAP kinase phosphorylation.

CONCLUSION

In conclusion, we have shown that NHERF-1/EBP50 binds directly to the C-tail of the κ opioid receptor through its first PDZ domain and that this interaction plays an important role in stimulation of NHE3 activity mediated by the κ opioid receptor independent of PTX-sensitive G proteins. We have observed that κ opioid receptor mRNA is present in the rat kidney as determined by RT-PCR,[3] and it is therefore plausible that κ opioid agonists may stimulate NHE3 in renal proximal tubules via a PDZ domain-mediated interaction between the κ opioid receptor and NHERF-1/EBP50. Although the physiological significance of our finding is presently unknown, the NHERF-1/EBP50-dependent regulation of Na^+/H^+ exchange by κ opioids may potentially play a role in the regulation of proximal tubule sodium and hydrogen ion transport.

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