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Extracellular signal-regulated kinase activation by parathyroid hormone in distal tubule cells

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Sneddon WB, Yang Y, Ba J, Harinstein LM, Friedman PA. Extracellular signal-regulated kinase activation by parathyroid hormone in distal tubule cells. *Am J Physiol Renal Physiol* 292: F1028–F1034, 2007. First published November 14, 2006; doi:10.1152/ajprenal.00288.2006.—The PTH receptor (PTH1R) activates multiple signaling pathways, including extracellular signal-regulated kinases 1 and 2 (ERK1/2). The role of epidermal growth factor receptor (EGFR) transactivation in ERK1/2 activation by PTH in distal kidney cells, a primary site of PTH action, was characterized. ERK1/2 phosphorylation was stimulated by PTH and blocked by the EGFR inhibitor, AG1478. Upon PTH stimulation, metalloprotease cleavage of membrane-bound heparin-binding fragment (HB-EGF) induced EGFR transactivation of ERK. Conditioned media from PTH-treated distal kidney cells activated ERK in HEK-293 cells. AG1478 added to HEK-293 cells ablated transactivation by conditioned media. HB-EGF directly activated ERK1/2 in HEK-293 cells. Pretreatment of distal kidney cells with the metalloprotease inhibitor GM-6001 abolished transactivation of ERK1/2 by PTH. The role of the PTH1R COOH terminus in PTX-sensitive ERK1/2 activation was characterized in HEK-293 cells transfected with wild-type PTH1R, with a PTH1R mutated at its COOH terminus, or with PTH1R truncated at position 480. PTH stimulated ERK by wild-type, mutated and truncated PTH1Rs 21-, 27- and 57-fold, respectively. Thus, the PTH1R COOH terminus exerts an inhibitory effect on ERK activation. EBP50, a scaffolding protein that binds to the PDZ recognition domain of the PTH1R, impaired PTH but not isoproterenol or calcitonin-induced ERK activation. Pertussis toxin inhibited PTH-stimulated ERK1/2 by mutated and truncated PTH1Rs and abolished ERK1/2 activation by wild-type PTH1R. We conclude that ERK phosphorylation in distal kidney cells by PTH requires PTH1R activation of G_i, which leads to stimulation of metalloprotease-mediated cleavage of HB-EGF and transactivation of the EGFR and is regulated by EBP50.

PTH receptor signaling; receptor internalization; epidermal growth factor transactivation; matrix metalloproteinase; EBP50/NHERF1

EXTRACELLULAR SIGNAL-RELATED kinases 1 and 2 (ERK1/2) are stimulated by several distinct pathways that may employ canonical activation of tyrosine kinase or G protein-coupled receptors (GPCR), GPCR endocytosis (19); scaffolding proteins or molecules such as β-arrestins acting in this capacity (12, 27); and transactivation of epidermal growth factor receptor (EGFR) (40). Some but not all G protein-coupled receptors stimulate ERK1/2 by an endocytic pathway. Internalization of β_2-adrenergic receptors (6, 20), but not of α_{2B}-adrenergic receptors (25), is necessary for ERK activation.

The type 1 PTH receptor (PTH1R) activates multiple signaling pathways in bone and kidney, including ERK (4, 5, 13, 30). In previous work, we showed that PTH stimulated ERK1/2 in distal kidney tubule cells (30). ERK activation is functionally linked to PTH actions. Blockade of ERK1/2 inhibited PTH-stimulated calcium transport in distal kidney cells. PTH stimulation of ERK1/2 in proximal tubule cells appears to be involved in regulating the Na^+-K^+-ATPase (11) and type IIa Na^+/Pi cotransporter (3, 14).

PTH internalizes its cognate receptor in target cells in kidney and bone (31, 35). In separate work, we showed that receptor endocytosis directly contributes to ERK1/2 stimulation in HEK-293 cells heterologously expressing the PTH1R (33). Limited information is available on PTH1R stimulation of ERK in kidney and the relations between PTH1R internalization and ERK activation in this setting (14). The first aim of the present investigation was to fill this gap and test the hypothesis that PTH1R activation and internalization are required for ERK stimulation. PTH receptor activation and internalization can be dissociated in a ligand-dependent manner (28, 31). PTH(1-34) activates and internalizes the PTH1R; PTH(1-31) activates but does not internalize the receptor; while PTH(7-34) does not activate the receptor but efficiently internalizes it. Generally similar observations have recently been reported for PTH1R expressed in HEK293 cells (8).

Transactivation of the EGFR is required for ERK activation by PTH in bone cells (1). Therefore, we also sought to define the requirement for EGFR transactivation in mediating the stimulatory action of PTH on its target cells in the distal nephron. In the course of this analysis, we assessed ERK activation by PTH1Rs truncated or mutated in key regions that affect the binding of regulatory proteins such as the β-arrestins and the 50-kDa ezrin-binding protein, EBP50, (sodium/proton exchanger regulatory factor 1; NHERF1) that are involved in PTH1R internalization. The results show that in defined PTH target cells of the kidney ERK activation involves EGFR transactivation and, within the limit of experimental error, PTH1R endocytosis does not contribute to ERK activation. These findings support the view that the preferred pathway of ERK stimulation is determined in a cell-specific manner and by the immediate cellular demands and biological functions of the cell.

MATERIALS AND METHODS

Cell culture and transfection. The preparation, subcloning, characterization, and culture conditions of distal kidney cells have been described (31). Briefly, cells were maintained in DMEM/F-12 (Mediatech Cellgro 10-090, Herndon, VA) supplemented with 5% fetal

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bovine serum and 10% penicillin/streptomycin. Distal kidney cells that stably express 10^5 PTH1R/cell (D1 cells) were created and maintained as described (31). PTH1R levels were measured using radioligand binding as outlined previously (31). Cells were serum starved overnight in HEPES and glutamine-free DMEM (Cellgro 15–017). Where described, transient transfection was accomplished with FuGene 6 (Roche Diagnostics), according to the manufacturer's recommended protocols.

Reagents. Human [$_{Nle}r^{8,18}$,Tyr^{34}]PTH(1-34) (hPTH[1-34]), hPTH(1-31), and [$dTrp^{12}$,Tyr^{34}]-bovine PTH(7-34) were purchased from Bachem (Torrance, CA). Isoproterenol and salmon calcitonin, AG1478, and bisindolylmaleimide were purchased from Sigma.

ERK1/2 phosphorylation. Confluent D1 cells on six-well plates were serum starved overnight using DMEM (15–017, Mediatech). Cells were incubated with inhibitors, as indicated, followed by treatment with PTH peptides at 37°C for 10 min. The plate was placed on ice and the culture medium was removed. Cells were lysed in 250 μl/well of 0.5% NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40; Sigma) supplemented with a protease inhibitor cocktail consisting of 0.5 mM AEBSF, 150 nM aprotinin, 1 μM E-64, 0.5 mM EDTA, and 1 μM leupeptin (Calbiochem, San Diego, CA) and incubated for 15 min on ice. Cells were scraped and transferred to a 1.5-ml microcentrifuge tube on ice. The cell lysate was then drawn through a 21-gauge needle attached to a 1-ml syringe four times and then placed back on ice for an additional 15 min. Lysates were microcentrifuged at 14,000 rpm at 4°C and the supernatants were transferred to a fresh 1.5-ml microcentrifuge tube on ice. Fifty microliters of lysate were added to 50 μl of 2× Laemmli SDS-PAGE loading buffer (Bio-Rad) containing 5% 2-mercaptoethanol and incubated at 95°C for 2 min. Forty microliters of the lysate was run on duplicate 15% SDS-PAGE gels and then transferred to Immobilon P membranes (Millipore) using the Semi-Dry Method (Bio-Rad). Activated ERK1/2 was measured by phospho-specific immunoblotting of phosphorylated ERK1/2 with a polyclonal antibody (9101) targeted to residues surrounding Thr202/Tyr204 of human p44 MAP kinase (Cell Signaling Technology, Beverly, MA). Total endogenous nonphosphorylated ERK1/2 was measured in parallel (Cell Signaling Technology, 9102). Blots were quantified by densitometric analysis. Results were normalized to total ERK2 and expressed as the fold change of pERK2 under unstimulated control conditions.

Transactivation of ERK1/2. A coculture system was used for analyzing PTH-stimulated ERK activation by EGFR transactivation (21). Distal kidney cells were passaged onto 10-cm dishes and grown to confluence. After serum starving the distal kidney cells overnight in DMEM, the cells were treated with the indicated inhibitors and 10^{-7} M PTH(1-34) for 10 min at 37°C. The conditioned medium was collected and concentrated using a 10,000 molecular weight cutoff Amicon centrifugal (Millipore, Bedford, MA) by centrifugation at 4,000 rpm for 8 min (IEC MP4R). The conditioned media were concentrated from 10 ml to 1–2 ml. The conditioned medium was then applied for 10 min to confluent, serum-starved (DMEM 15-017, Mediatech) HEK-293 cells on six-well plates. HEK-293 cell lysates were prepared and ERK1/2 phosphorylation measured as described above.

Assessment of ERK1/2 activation and the role of G_i by truncated or mutant PTH1Rs. Generation of mutant ETVA-PTH1R, where Ala replaces the COOH-terminal Met of the PTH1R, and truncated 480stop-PTH1R have been described (31). HEK-293 cells were grown on six-well plates and transiently transfected with wild-type PTH1R, ETVA-PTH1R, or 480stop-PTH1R, as indicated. Cells were serum-starved overnight in DMEM with or without 10 ng/ml pertussis toxin. After treatment of PTH(1-34) for 10 min, cell lysates were prepared and ERK1/2 phosphorylation was measured as described.

Second messenger analysis. Adenylyl cyclase activity was measured by cAMP accumulation. Cells were seeded in 24-well plates at 4×10^4 cells/well in DMEM/F12 containing 5% FBS. Cells were washed twice with Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution and incubated with 0.5 μCi [^{3}H]adenine medium at 37°C for 2 h. The cells are treated with 1 mM 3 isobutyl-1-methylxanthine (IBMX) in fresh medium containing 0.1% BSA for 15 min and incubated in the presence of the indicated peptide for 15 min. The reaction was terminated by the addition of 1.2 M TCA, followed by neutralization with 4 N KOH. cAMP was isolated by two-column chromatography (24). Radioactivity was measured by beta scintillation spectrometry.

Protein kinase C activity was determined by ligand-induced phosphotransferase to a specific PKC substrate peptide, QKRPSQRSKYL. Distal kidney cells were passaged onto six-well plates and cultured for 48 h. After overnight incubation in serum-free media, the cells were pretreated for 15 min with 10 μM AG1478, 10 μM bisindolylmaleimide I, or vehicle followed by a 10-min treatment with 100 nM PTH(1-34). Cell lysates were prepared in 0.5% NP-40 lysis buffer, as described above for analysis of ERK phosphorylation. PKC activity was measured with [γ-^{32}P]ATP using a PKC Assay Kit (Upstate Cell Signaling Solutions, cat. no. 17-139; Lake Placid, NY).

Statistics. Data are presented as means ± SE, where *n* indicates the number of independent experiments. Effects of experimental treatments were assessed by paired comparisons within experiments and reported as means ± SE of *n* independent experiments. Paired results were compared by ANOVA with posttest repeated measures analyzed by the Bonferroni procedure (Prism; GraphPad). Differences greater than $P \leq 0.05$ were assumed to be significant.

RESULTS

PTH(1-34) stimulated ERK2 in a time-dependent manner in distal kidney D1 cells (31). Maximal activation was observed at 10 min (Fig. 1, *A* and *B*) and returned to baseline by 30–60 min. As noted previously, PTH(1-34) did not consistently alter

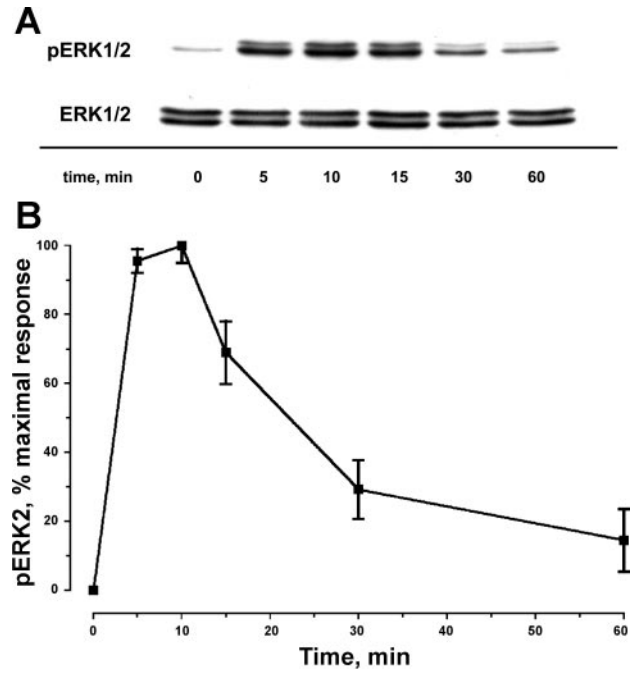

Fig. 1. PTH stimulates ERK2 phosphorylation in a time-dependent manner. Confluent distal kidney cells were serum-starved overnight and then treated for the indicated times with 10^{-7} M PTH(1-34). ERK phosphorylation was measured as detailed in MATERIALS AND METHODS. *A*: representative experiment showing time course of PTH-induced ERK1/2 phosphorylation. *B*: average time course of pERK2 phosphorylation. Results were normalized to the expression of total ERK2. Results are shown as means ± SE of 3 independent, paired experiments.

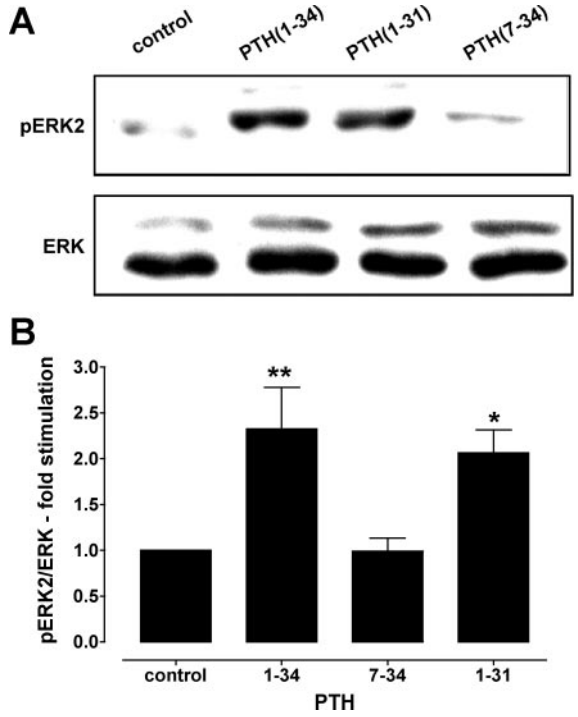

Fig. 2. PTH ligand-selective stimulation of ERK2 phosphorylation. Distal kidney cells were serum-starved overnight and then treated for 10 min with 10^{-7} M PTH(1-34), PTH(1-31), or PTH(7-34) as indicated. ERK phosphorylation was measured as outlined. *A*: representative experiment showing effects of indicated peptide on ERK2 and total ERK. *B*: average results depicting means ± SE of 3 independent experiments. pERK2 phosphorylation was normalized to the expression of total ERK2. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control.

ERK1 levels (30). Therefore, we focused on the effect of PTH peptide fragments on ERK2 using a 10-min stimulation.

PTH(1-34), which activates and internalizes the PTH1R, caused a 2.3-fold increase of ERK2 phosphorylation (Fig. 2, *A* and *B*). PTH(1-31), which stimulates adenylyl cyclase and phospholipase C but does not internalize the PTH1R (28), was nearly as efficacious in activating ERK2. PTH(7-34) activates neither adenylyl cyclase nor phospholipase C but promotes efficient PTH1R internalization. PTH(7-34) failed to exert a significant effect on ERK2 phosphorylation. It is likely, therefore, that PTH1R internalization does not contribute importantly to ERK activation in these defined epithelial cells. We also examined the effects on ERK2 of the two principal circulating forms of PTH, PTH(1-84) and PTH(7-84). PTH(1-84) potently stimulated ERK phosphorylation to the same extent as PTH(1-34). PTH(7-84), like PTH(7-34), failed to affect ERK (data not shown).

ERK activation by several GPCRs involves transactivation of the EGF receptor (19). Transactivation may involve direct association with a receptor tyrosine kinase or may proceed by metalloprotease cleavage of the heparin-binding fragment of epidermal growth factor (HB-EGF), a potent EGFR agonist. HB-EGF is released into the extracellular space and acts in an autocrine or paracrine manner to activate the EGFR, leading to ERK phosphorylation (1, 9).

Determination that EGFR transactivation induced by PTH involves release of HB-EGF was accomplished by challenging distal kidney cells with PTH and applying the conditioned media harvested from these cells to HEK-293 cells. Distal kidney cells were treated for 10-min with PTH(1-34). Conditioned media were collected, concentrated, and applied to wild-type HEK-293 cells, which do not express the PTH1R. Addition of conditioned media from distal kidney cells treated with PTH to HEK-293 cells provoked ERK2 phosphorylation by HEK-293 cells (Fig. 3). Pretreatment of distal kidney cells with the metalloprotease inhibitor GM6001 blocked the ability of the conditioned media from PTH-treated distal kidney cells to activate ERK2 phosphorylation in HEK-293 cells. Direct application of PTH(1-34) to HEK-293 cells did not activate ERK2. These findings establish that ERK activation did not arise from an action of PTH on HEK-293 cells. As expected, direct application of exogenous HB-EGF stimulated ERK2 phosphorylation in distal kidney cells (data not shown). These findings are consistent with the idea that ERK activation in distal kidney cells is mediated by transactivation of the EGFR by the PTH1R. The EGFR inhibitor, AG1478, blocked PTH(1-34)- and PTH(1-31)-stimulated ERK2 activation (Fig. 4*A*) by distal kidney cells, further implicating EGFR transactivation in ERK stimulation by the PTH1R. AG1295, an inhibitor of the platelet-derived growth factor receptor (PDGFR), had no effect (data not shown). Thus the inhibitory effects of AG1478 were specific to ERK signaling insofar as it had no effect on PTH-stimulated adenylyl cyclase or protein kinase C (Fig. 4*B*), two signaling pathways normally activated by PTH in distal kidney cells (7). Thus transactivation proceeds specifically through the EGFR. According to this model, PTH activates metalloprotease-induced cleavage of HB-EGF from distal kidney cells, which is released into the culture media, and in turn, stimulates the EGFR on HEK-293 cells.

The COOH terminus of the PTH1R contains a PDZ-recognition motif (ETVM) that interacts with the 50 kDa ezrin-binding protein (EBP50), a cytoplasmic scaffolding protein (16). We examined the role of the PTH1R COOH terminus on ERK2 activation. These experiments were performed on HEK-293 cells because, unlike the distal kidney cells, they do not express endogenous PTH1R. PTH(1-34) robustly activated ERK2 phosphorylation in HEK-293 cells transfected with wild-type PTH1R (ETVM, Fig. 5). Mutation of the PTH1R PDZ-recognition domain by substituting A for the COOH-terminal M (M593A) (ETVA-PTH1R) abrogates interaction of

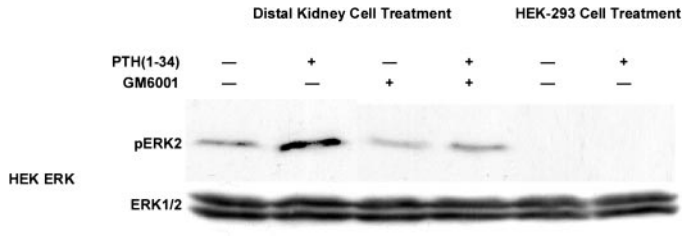

Fig. 3. Transactivation of ERK by PTH. Conditioned media were collected from distal kidney cells that had (+) or had not (−) been treated with PTH(1-34), concentrated, and applied to wild-type HEK-293 cells. Activated, phosphorylated ERK and total ERK were measured as outlined. Media collected from distal kidney cells exposed to PTH(1-34) stimulated ERK2 phosphorylation. Pretreatment of distal kidney cells for 1 h with 10^{-6} M GM6001, a matrix metalloproteinase inhibitor, followed by a 10-min challenge with PTH(1-34), and then applying the concentrated media to HEK-293 cells substantially suppressed ERK transactivation. Direct application of PTH to HEK-293 cells had no effect. Comparable results were obtained in 3 independent experiments.

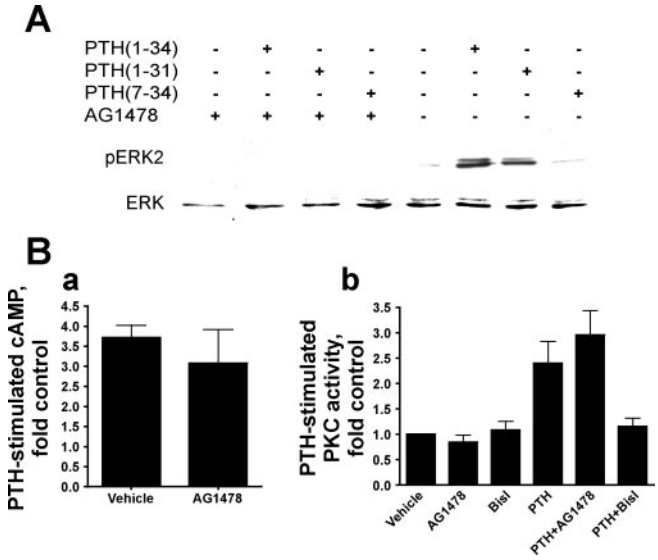

Fig. 4. *A*: inhibition of PTH-induced ERK transactivation by an EGFR blocker. Distal kidney cells were serum-starved overnight and treated for 10 min with vehicle or 1 μM AG1478, an EGFR inhibitor, followed by a 10-min treatment with 10^{-7} M PTH(1-34), PTH(1-31), or PTH(7-34), as indicated. PTH-stimulated ERK phosphorylation was measured as described in MATERIALS AND METHODS. A representative experiment is shown. Similar results were obtained in 2 additional independent experiments. *B*: EGFR inhibition does not block PTH-stimulated cAMP formation or PKC activation. Mouse distal kidney cells were preincubated with 1 μM AG1478 or the PKC inhibitor 10 μM Bisindolylmaleimide I or vehicle for 10 min, followed by a 10-min treatment with 10^{-7} M PTH(1-34). cAMP formation and PKC activity was assayed as described in MATERIALS AND METHODS. Data represent means ± SE of 3 independent experiments.

the receptor with EBP50 (31). PTH(1-34) stimulated ERK2 phosphorylation in HEK-293 cells transiently transfected with ETVA-PTH1R. The magnitude of ERK2 activation was comparable to that of the wild-type PTH1R. In contrast, truncation of the COOH terminus of the PTH1R at position 480 (480stop) resulted in a paradoxical twofold augmentation of PTH(1-34)-stimulated ERK2 phosphorylation compared with the wild-type PTH1R. These findings suggest that the COOH terminus of the PTH1R plays a negative modulatory role in PTH-stimulated ERK activation. Complementary experiments, wherein EBP50 was overexpressed in distal kidney cells revealed a profound inhibitory effect of the presence of EBP50 on PTH-stimulated ERK (Figs. 6 and 7) but not on ERK phosphorylation elicited by isoproterenol or by salmon calcitonin (Fig. 7).

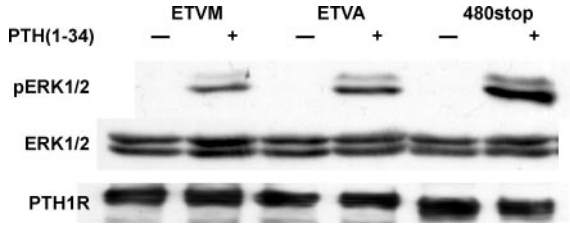

Fig. 5. Structural determinants of PTH1R activation of ERK. HEK-293 cells were transiently transfected with wild-type PTH1R (ETVM), a mutant PTH1R, where the COOH-terminal Met was replaced by Ala (ETVA), or with a truncated PTH1R lacking virtually all of its COOH terminus (480stop). Empty pEGFP-N2 vector served as the negative control. Thirty six hours after transfection, cells were serum-starved overnight and then treated for 10 min with 10^{-7} M PTH(1-34), as indicated. PTH-stimulated ERK phosphorylation was measured as outlined in MATERIALS AND METHODS. Total ERK and PTH1R expression were assessed by immunoblot with antibodies to ERK1/2 and GFP, respectively. A representative experiment is shown. Similar results were obtained in 3 separate experiments.

Binding of the COOH terminus of the PTH1R with the PDZ protein EBP50 affects receptor signaling and internalization (16, 31). It was therefore of interest to determine the effect of EBP50 on activation of ERK by PTH in distal kidney cells. As shown in Fig. 6, PTH(1-34) provoked concentration-dependent phosphorylation of ERK that was markedly attenuated in cells transiently transfected with EBP50. The inhibitory effect of EBP50 on ERK stimulation was specific for PTH in as much as EBP50 failed to suppress ERK activation by either isoproterenol (ISO) or calcitonin (CT) (Fig. 7). These findings fortify the conclusion that the PTH1R COOH terminus contains important regulatory domains involved in ERK activation.

In addition to the well-conserved signaling pathway responsible for activation of ERK by receptor tyrosine kinases, ERK activation may be mediated by pertussis toxin (PTX)-sensitive or -insensitive G proteins and has been shown to be either Ras or PKC dependent (38). The PTH1R couples facultatively with G_i (16, 26). Accordingly, we examined the role of G_i in PTH-stimulated ERK activation. We first analyzed the effects

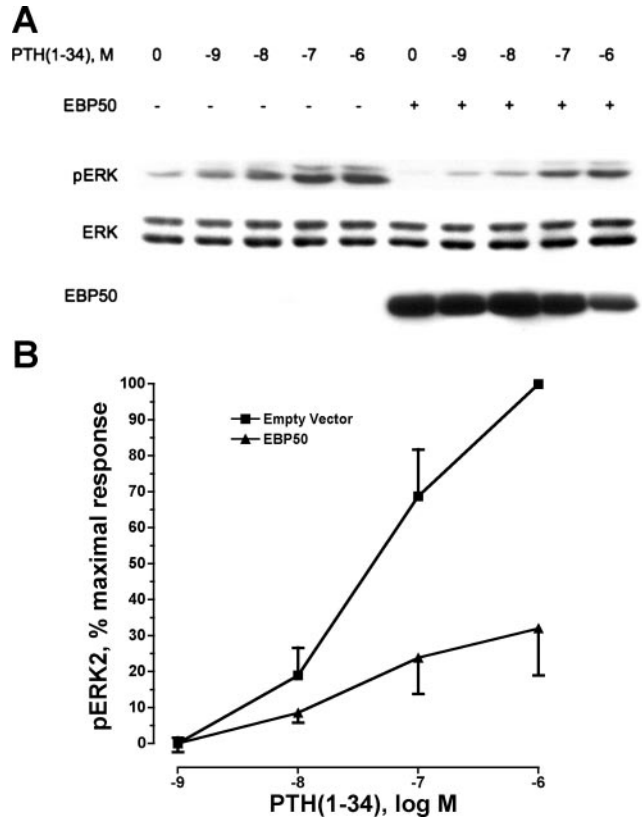

Fig. 6. Involvement of the COOH-terminal PTH1R PDZ recognition domain in PTH-stimulated ERK activation. Distal kidney cells were transiently transfected with HA-EBP50 or empty vector. Forty-eight hours after transfection, cells were serum-starved overnight and treated with the indicated concentration of PTH(1-34) for 10 min. Cell lysates were prepared as outlined in MATERIALS AND METHODS. *A*: results of a sample experiment. *B*: average results of 6 independent experiments performed in parallel with empty vector or EBP50. ERK2 phosphorylation was measured by quantitative densitometry, normalized for total ERK2 expression, and analyzed by 2-way ANOVA. EBP50 significantly inhibited PTH-induced ERK phosphorylation ($P < 0.01$).

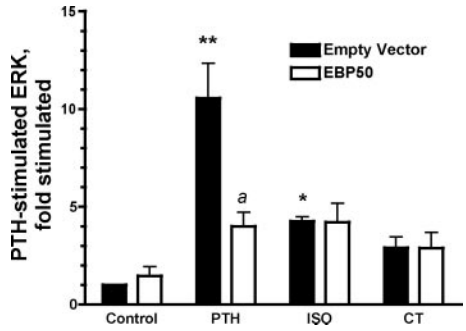

Fig. 7. Specificity of EBP50 inhibition of PTH-stimulated ERK. Distal kidney cells were transiently transfected with HA-EBP50 or empty vector. Forty-eight hours later, the cells were serum-starved overnight and then challenged with 100 nM PTH(1-34), 10 μM isoproterenol (ISO), or 10 μM calcitonin (CT). Results are average ± SE of 5 independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control; $^{a}P < 0.05$ vs. PTH.

of G_i in distal kidney cells. Overnight treatment with PTX caused profound inhibition of PTH-stimulated ERK phosphorylation (Fig. 8*A*). To gain further insight into the structural determinants of PTH receptor that regulate G_i and ERK activation, we transfected HEK-293 cells with the full-length (ETVM) PTH1R, the PTH1R harboring a mutation in the PDZ-recognition domain, M593A (ETVA), and the truncated 480stop-PTH1Rs. Despite different magnitudes of ERK2 phosphorylation, PTX inhibited PTH-stimulated ERK2 activation by all three PTH1Rs (Fig. 8*B*). These results demonstrate that the PTH1R determinants for G_i activation are located upstream of the receptor COOH terminus.

DISCUSSION

A variety of cellular signaling mechanisms mediate or contribute to ERK activation by GPCRs. These include hetero- and monomeric G proteins, EGFR transactivation, and receptor internalization. PTH stimulates ERK in target cells of bone and kidney, where protein kinase C mediates activation (30, 32). Recent findings disclosed that PTH1R activation and internalization of the cognate PTH1R could be dissociated (28). Thus PTH(1-34) activates and internalizes the PTH1R in distal kidney cells, whereas PTH(7-34) promotes receptor endocytosis without activating classical adenylyl cyclase or phospholipase C signaling pathways. PTH(1-31), by contrast, activated the PTH1R without concomitant receptor internalization. In the present study, we took advantage of the ability to dissociate PTH1R activation and internalization using these ligands to determine the requirement for receptor endocytosis on ERK activation by PTH. We further explored the role of EGFR transactivation and the involvement of the heterotrimeric inhibitory G protein, G_i, on PTH-induced ERK stimulation.

Using activation-selective (PTH[1-31]) or internalization-selective (PTH[7-34]) peptides, the present results show clearly that receptor activation, but not internalization, is required for stimulation of ERK in distal kidney cells. Thus PTH(1-31) activates ERK2 but does not internalize the PTH1R (Fig. 2) (28), whereas PTH(7-34), which does not significantly activate ERK (Fig. 2), robustly and rapidly internalizes the PTH1R in distal kidney cells (31). These findings differ from those where the PTH1R is heterologously expressed. In HEK-293 cells receptor endocytosis induced by PTH(7-34) was sufficient to promote ERK activation (33). Moreover, interrupting receptor internalization with the clathrin-binding domain of β-arrestin 1 or applying dominant-negative dynamin inhibited ERK activation by PTH(1-34) without interfering with receptor-induced signaling. These results imply that PTH1R trafficking is involved in the activation of MAP kinases by PTH in cells where the PTH1R is heterologously expressed. The divergence of these observations with the present findings underscores the cell-specific nature of ERK stimulation, even by common GPCRs.

Stable β-arrestin binding to the angiotensin AT1a receptor is required for efficient activation of ERK phosphorylation by angiotensin in COS7 cells (36, 37). Under these circumstances, arrestin forms a signaling scaffold by interacting both with the GPCR and components of the ERK1/2 cascade (18, 37). Where receptor internalization is arrestin dependent, arrestin colocalizes with ERK2 in cytosolic vesicles (37). These observations imply that the role of arrestins in mediating receptor internalization has a direct effect on their ability to promote ERK activation. In distal kidney cells, however, PTH1R internalization does not require the participation of β-arrestins and proceeds independently of ERK activation (29).

Transactivation of the EGFR is another mechanism by which GPCRs stimulate ERK activation (for a review, see Ref. 15). GPCR activation leads to release of preformed EGF receptor ligands in a process known as ectodomain shedding. HB-EGF is synthesized in the cell as a transmembrane precursor that is proteolyzed by a metalloprotease to form a soluble growth factor that is a potent EGFR ligand (23). HB-EGF

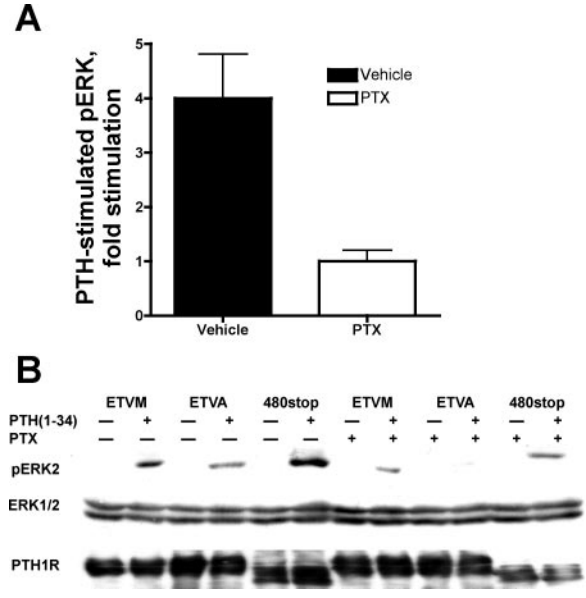

Fig. 8. Pertussis toxin sensitivity of PTH-induced ERK2 activation. *A*: D1 cells were serum-starved overnight in the presence or absence of 10 ng/ml PTX. PTH-stimulated ERK2 phosphorylation was measured as outlined in MATERIALS AND METHODS. *B*: HEK-293 cells were transiently transfected with wild-type PTH1R (ETVM), a mutant PTH1R (ETVA), a truncated PTH1R (480stop) as described in the legend to Fig. 5. Thirty-six hours after transfection, cells were serum-starved overnight in the presence or absence of 10 ng/ml pertussis toxin (PTX) and then treated for 10 min with 10^{-7} M PTH(1-34), where indicated. PTH-stimulated ERK phosphorylation was measured as outlined. Total ERK and PTH1R expression were analyzed by immunoblot with antibodies to ERK1/2 and GFP, respectively. The results are for a representative experiment; similar results were obtained in 3 independent experiments.

directly activates ERK phosphorylation in distal kidney cells and in HEK-293 cells (data not shown). Furthermore, EGFR inhibition with tyrphostin AG1478 blocked activation of PTH-dependent ERK in both distal kidney cells (Fig. 4) and HEK-293 cells (data not shown) without affecting signaling through adenylyl cyclase or protein kinase C (Fig. 4*B*). Using a coculture system, the present results demonstrate that a soluble factor is released from distal kidney cells upon stimulation with PTH and that this factor is able to activate ERK signaling when applied to HEK-293 cells (Fig. 6). Direct application of PTH to HEK-293 cells failed to activate ERK. The metalloprotease inhibitor, GM6001, inhibited release of this factor and abrogated ERK activation by conditioned media. Based on these considerations, this factor is likely to be HB-EGF because its ability to stimulate ERK activity in HEK-293 cells was inhibited by AG1478 (data not shown). EGFR transactivation is also involved in PTH1R activation of ERK in osteoblasts (1), HEK-293 cells (33), and opossum kidney cells (5).

The present findings additionally demonstrate that the COOH terminus of the PTH1R plays a modulatory role in ERK activation. An inhibitory role for the PTH1R COOH terminus has been described for PTH-stimulated cyclic AMP formation (10). Here, a PTH1R truncated at position 480 that lacks most of the COOH terminus stimulated ERK activation to a greater extent than did the full-length receptor (Figs. 5 and 7). EBP50, which binds to the COOH-terminal PDZ recognition motif (16, 31), substantially suppressed PTH-stimulated ERK (Fig. 7). A PTH1R containing a mutation of the PDZ recognition motif (ETVA) was not appreciably affected by EBP50. These findings emphasize that the COOH terminus of the PTH1R plays a negative modulatory role in PTH-stimulated ERK activation. Absence of the PTH1R COOH terminus may expose sequence-specific components that promote ERK activation. Alternatively, it may remove steric hindrance of signaling elements in the third intracellular loop, or reflect impaired desensitization of this mutant receptor, which lacks agonist-induced phosphorylation sites (17, 34). The experiments with mutant receptors were conducted on HEK-293 that do not express endogenous PTH1R. HEK-293 cells were transiently transfected with different PTH receptor constructs so that they could be studied in the absence of a background of wild-type receptor, which would confound the results were the experiments performed in distal kidney cells. In all cases, the wild-type receptor was used as a control and we show that the inhibitor effects of AG1478 and PTX are equivalent in distal kidney and HEK-293 cells.

Pertussis toxin inhibited PTH-stimulated ERK phosphorylation by the full-length and truncated PTH1Rs, indicating that activation of G_i is required (Fig. 7). Pertussis toxin has no effect on 480stop-PTH1R-stimulated cyclic AMP stimulation in COS7 cells (10). The divergence of effects may reflect a direct or indirect interaction of the COOH terminus of the PTH1R with G_i to modulate cyclic AMP formation, but an indirect action with respect to ERK activation. When PTX-sensitive G_i is activated by GPCR ligands, it leads to release of the $\beta\gamma$ subunit that, in turn, activates a matrix metalloprotease that cleaves HB-EGF (22) and leads to EGFR transactivation. This process appears to account for the results in distal kidney cells, where PTX treatment inhibited the ability of conditioned media from PTH-stimulated distal kidney cells to activate ERK in HEK-293 cells. The role of G_i in ERK activation may extend beyond $\beta\gamma$-mediated activation of the metalloprotease because direct application of PTX to HEK-293 cells inhibited ERK phosphorylation by conditioned media from both PTH-treated and -untreated distal kidney cells. This may reflect the contribution of G_i activation to baseline levels of ERK activation in HEK-293 cells because, despite lower overall levels of stimulation, conditioned media from PTH-stimulated distal kidney cells augmented ERK phosphorylation to a greater degree than did conditioned media from untreated cells. The role of EGFR transactivation in ERK1/2 stimulation by PTH is cell specific and does not occur in all cells. Chinese hamster ovary (CHO) cells do not express the EGFR (2). PTH robustly activates ERK1/2 in CHO cells that are stably transfected with the human PTH1R (39). As expected, ERK1/2 activation by PTH in these cells is not inhibited by GM6001 or AG1478, indicating that transactivation of a growth factor receptor likely does not contribute to this process (data not shown).

In summary, PTH activates ERK1/2 phosphorylation in distal kidney cells in a PTX-sensitive manner. The metalloprotease inhibitor GM6001 and the EGFR inhibitor AG1478 also inhibit PTH stimulation of ERK1/2. We, therefore, conclude that ERK1/2 activation in distal kidney cells by PTH(1-34) requires PTH1R activation of G_i that leads to stimulation of metalloprotease-mediated cleavage of HB-EGF and transactivation of the EGFR.

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REFERENCES

1. **Ahmed I, Gesty-Palmer D, Drezner MK, Luttrell LM.** Transactivation of the epidermal growth factor receptor mediates parathyroid hormone and prostaglandin F2α-stimulated mitogen-activated protein kinase activation in cultured transgenic murine osteoblasts. *Mol Endocrinol* 17: 1607–1621, 2003.
2. **Antonelli V, Bernasconi F, Wong YH, Vallar L.** Activation of B-Raf and regulation of the mitogen-activated protein kinase pathway by the $G_o\alpha$ chain. *Mol Biol Cell* 11: 1129–1142, 2000.
3. **Bacic D, Schulz N, Biber J, Kaissling B, Murer H, Wagner CA.** Involvement of the MAPK-kinase pathway in the PTH-mediated regulation of the proximal tubule type IIa Na^+/P_i cotransporter in mouse kidney. *Pflügers Arch* 446: 52–60, 2003.
4. **Chaudhary LR, Avioli LV.** Identification and activation of mitogen-activated protein (MAP) kinase in normal human osteoblastic and bone marrow stromal cells: attenuation of MAP kinase activation by cAMP, parathyroid hormone and forskolin. *Mol Cell Biochem* 178: 59–68, 1998.
5. **Cole JA.** Parathyroid hormone activates mitogen-activated protein kinase in opossum kidney cells. *Endocrinology* 140: 5771–5779, 1999.
6. **Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SSG, Caron MG, Lefkowitz RJ.** Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* 273: 685–688, 1998.
7. **Friedman PA, Coutermarsh BA, Kennedy SM, Gesek FA.** Parathyroid hormone stimulation of calcium transport is mediated by dual signaling mechanisms involving protein kinase A and protein kinase C. *Endocrinology* 137: 13–20, 1996.
8. **Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Loomis CR, Spurney RF, Luttrell LM, Lefkowitz RJ.** Distinct β-arrestin and G protein dependent pathways for parathyroid hormone receptor stimulated ERK1/2 activation. *J Biol Chem* 281: 10856–10864, 2006.
9. **Higashiyama S.** Metalloproteinase-mediated shedding of heparin-binding EGF-like growth factor and its pathophysiological roles. *Protein Pept Lett* 11: 443–450, 2004.

10. **Iida-Klein A, Guo J, Xie LY, Jüppner H, Potts JT Jr, Kronenberg HM, Bringhurst FR, Abou-Samra AB, Segre GV.** Truncation of the carboxyl-terminal region of the rat parathyroid hormone (PTH)/PTH-related peptide receptor enhances PTH stimulation of adenylyl cyclase but not phospholipase C. *J Biol Chem* 270: 8458–8465, 1995.
11. **Khundmiri SJ, Dean WL, McLeish KR, Lederer ED.** Parathyroid hormone-mediated regulation of Na^+-K^+-ATPase requires ERK-dependent translocation of protein kinase Ca. *J Biol Chem* 280: 8705–8713, 2005.
12. **Kolch W.** Coordinating ERK/MAPK signalling through scaffolds and inhibitors. *Nat Rev Mol Cell Biol* 6: 827–837, 2005.
13. **Lederer ED, McLeish KR.** PTH stimulates a biphasic extracellular signal-regulated kinase response in opossum kidney cells through independent signal transduction pathways. *J Am Soc Nephrol* 8: 576A, 1997.
14. **Lederer ED, Sohi SS, McLeish KR.** Parathyroid hormone stimulates extracellular signal-regulated kinase (ERK) activity through two independent signal transduction pathways: role of ERK in sodium-phosphate cotransport. *J Am Soc Nephrol* 11: 222–231, 2000.
15. **Luttrell LM.** "Location, location, location": activation and targeting of MAP kinases by G protein-coupled receptors. *J Mol Endocrinol* 30: 117–126, 2003.
16. **Mahon MJ, Donowitz M, Yun CC, Segre GV.** Na^+/H^+ exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. *Nature* 417: 858–861, 2002.
17. **Malecz N, Bambino T, Bencsik M, Nissenson RA.** Identification of phosphorylation sites in the G protein-coupled receptor for parathyroid hormone. Receptor phosphorylation is not required for agonist-induced internalization Mol. *Endocrinology* 12: 1846–1856, 1998.
18. **McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ.** β-Arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* 290: 1574–1577, 2000.
19. **Pierce KL, Luttrell LM, Lefkowitz RJ.** New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. *Oncogene* 20: 1532–1539, 2001.
20. **Pierce KL, Maudsley S, Daaka Y, Luttrell LM, Lefkowitz RJ.** Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. *Proc Natl Acad Sci USA* 97: 1489–1494, 2000.
21. **Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ.** Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem* 276: 23155–23160, 2001.
22. **Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A.** EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 402: 884–888, 1999.
23. **Riese DJ II, Komurasaki T, Plowman GD, Stern DF.** Activation of ErbB4 by the bifunctional epidermal growth factor family hormone epiregulin is regulated by ErbB2. *J Biol Chem* 273: 11288–11294, 1998.
24. **Salomon Y, Londos C, Rodbell M.** A highly sensitive adenylate cyclase assay. *Anal Biochem* 58: 541–548, 1974.
25. **Schramm NL, Limbird LE.** Stimulation of mitogen-activated protein kinase by G protein-coupled α_2-adrenergic receptors does not require agonist-elicited endocytosis. *J Biol Chem* 274: 24935–24940, 1999.
26. **Schwindinger WF, Fredericks J, Watkins L, Robinson H, Bathon JM, Pines M, Suva LJ, Levine MA.** Coupling of the PTH/PTHrP receptor to multiple G-proteins. Direct demonstration of receptor activation of G_s, $G_{q/11}$, and $G_{i(1)}$ by [α-^{32}P]GTP-γ-azidoanilide photoaffinity labeling. *Endocrinology* 8: 201–209, 1998.
27. **Shenoy SK, Lefkowitz RJ.** Multifaceted roles of β-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochem J* 375: 503–515, 2003.
28. **Sneddon WB, Bisello A, Magyar CE, Willick GE, Syme CA, Galbiati F, Friedman PA.** Ligand-selective dissociation of activation and internalization of the parathyroid hormone receptor. Conditional efficacy of PTH peptide fragments. *Endocrinology* 145: 2815–2823, 2004.
29. **Sneddon WB, Friedman PA.** Arrestin mobilization is facultative for PTH receptor endocytosis. *J Bone Miner Res* 19, *Suppl* 1: S192, 2004.
30. **Sneddon WB, Gesek FA, Friedman PA.** Obligate MAP kinase activation in parathyroid hormone stimulation of calcium transport but not calcium signaling. *Endocrinology* 141: 4185–4193, 2000.
31. **Sneddon WB, Syme CA, Bisello A, Magyar CE, Weinman EJ, Rochdi MD, Parent JL, Abou-Samra AB, Friedman PA.** Activation-independent parathyroid hormone receptor internalization is regulated by NHERF1 (EBP50). *J Biol Chem* 278: 43787–43796, 2003.
32. **Swarthout JT, Doggett TA, Lemker JL, Partridge NC.** Stimulation of extracellular signal-regulated kinases and proliferation in rat osteoblastic cells by parathyroid hormone is protein kinase C-dependent. *J Biol Chem* 276: 7586–7592, 2001.
33. **Syme CA, Friedman PA, Bisello A.** Parathyroid hormone receptor trafficking contributes to the activation of extracellular signal-regulated kinases but is not required for regulation of cAMP signaling. *J Biol Chem* 280: 11281–11288, 2005.
34. **Tawfeek HA, Qian F, Abou-Samra AB.** Phosphorylation of the receptor for PTH and PTHrP is required for internalization and regulates receptor signaling. *Mol Endocrinol* 16: 1–13, 2002.
35. **Teitelbaum AP, Silve CM, Nyiredy KO, Arnaud CD.** Downregulation of parathyroid hormone (PTH) receptors in cultured bone cells is associated with agonist-specific intracellular processing of PTH-receptor complexes. *Endocrinology* 118: 595–602, 1986.
36. **Tohgo A, Choy EW, Gesty-Palmer D, Pierce KL, Laporte S, Oakley RH, Caron MG, Lefkowitz RJ, Luttrell LM.** The stability of the G protein-coupled receptor-β-arrestin interaction determines the mechanism and functional consequence of ERK activation. *J Biol Chem* 278: 6258–6267, 2003.
37. **Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM.** β-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J Biol Chem* 277: 9429–9436, 2002.
38. **Van Biesen T, Luttrell LM, Hawes BE, Lefkowitz RJ.** Mitogenic signaling via G protein-coupled receptors. *Endocr Rev* 17: 698–714, 1996.
39. **Wang B, Sneddon WB, Yang Y, Friedman PA.** NHERF1 switches extracellular signal-regulated kinase signaling by the PTH receptor. *J Bone Miner Res* 20: S41, 2005.
40. **Waters C, Pyne S, Pyne NJ.** The role of G-protein coupled receptors and associated proteins in receptor tyrosine kinase signal transduction. *Semin Cell Devel Biol* 15: 309–323, 2004.