Endocrine, *vol. 30, no. 3, 343–352, December 2006* 0969–711X/06/30:343–352/$30.00
ENDO (Online) ISSN 1559-0100

Mutation of Phenylalanine-34 of Parathyroid Hormone Disrupts NHERF1 Regulation of PTH Type I Receptor Signaling

David Wheeler and W. Bruce Sneddon

Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Internalization of the PTH type I receptor (PTH1R) is regulated in a cell- and ligand-specific manner. We previously demonstrated that the sodium/proton exchanger regulatory factor type 1 (NHERF1; EBP50) is pivotal in determining the range of peptides that internalize the PTH1R. Antagonist PTH fragments can internalize the PTH1R in some kidney and bone cell models. PTH(7-34), which binds to, but does not activate, the PTH1R, internalizes the PTH1R in kidney distal tubule (DT) cells, where NHERF1 is not expressed. The effect of antagonist PTHrP peptides has not, to this point, been assessed. PTH1R internalization was measured by real-time confocal fluorescence microscopy of DT cells stably expressing 10^5 EGFP-tagged PTH1R/cell *(1)*. PTHrP(7-34) internalized the PTH1R in a manner indistinguishable from PTH(7-34). Introduction of NHERF1 into DT cells, however, blocked PTH(7-34)–, but not PTHrP(7-34)–, induced PTH1R internalization. To delineate the sequences within PTHrP that determine whether PTH1R internalization is affected by NHERF1, chimeric PTH/PTHrP fragments were tested for their ability to induce PTH1R internalization. PTH(7-21)/PTHrP (22-34), PTH(7-32)/PTHrP(33-34), and PTH(7-33)/PTHrP(34) at 1 μ*M* each internalized the PTH1R 50–70% in a NHERF1-independent manner. When the C terminus of PTHrP was replaced with homologous amino acids from PTH, NHERF1 inhibited PTH1R internalization. It was determined that simply mutating F34 to A in PTH induced PTH1R internalization in a NHERF1-independent manner. None of the chimeric peptides activated the PTH1R but all effectively competed for 1 n*M* PTH(1-34) in cyclic AMP assays. In addition, all chimeric peptides competed for radiolabeled PTH(1-34) in binding assays in DT cells. PTH(1-34) and PTHrP(7-34), but not PTH(7-34), efficiently recruited β-arrestin1 to plasma membrane PTH1Rs. We, therefore, conclude that PTH(1-34) and PTHrP(7-34) induce a conformational change in the PTH1R that promotes arrestin binding and dissociates NHERF1 from PTH1R internalization.

Key Words: Hormones and receptors; PTH/PTHrP; signaling.

Introduction

Parathyroid hormone (PTH) is an 84-amino-acid peptide hormone that is secreted by the parathyroid glands in response to low serum calcium levels. PTH binds to and activates the PTH/PTHrP receptor, also known as the type I PTH receptor (PTH1R). The first 34 amino acids of PTH are sufficient to bind with high affinity and activate the PTH1R. Positions 1–6 are important for adenylyl cyclase stimulation by the PTH1R, the first two positions being required for full activity *(2–5)*. Positions 1 and 29–32 are critical for stimulation of phosphoinositide turnover and activation of protein kinase C *(6,7)*. Sequences distal to position 15 are thought to be important for binding of PTH to the N terminal extracellular ligand-binding domain of the PTH1R. The PTH-related protein (PTHrP) is synthesized and released by many normal and malignant cell types. It acts typically in an autocrine or paracrine fashion, in contrast to the endocrine actions of PTH. In bone development, PTHrP plays a pivotal role as a growth factor in bone development, delaying chrondrocyte differentiation and promoting bone growth *(8)*. PTHrP is a 141-amino-acid hormone that has high sequence identity to PTH at its N terminus, sharing 8 of 13 amino acids. PTHrP also binds to and activates the PTH1R. The first 34 amino acids of PTHrP, like PTH, can fully activate the PTH1R. There are, however, differences in the interactions between PTH and PTHrP with the PTH1R, even in regions of high identity between these two peptides *(4)*.

The PTH1R belongs to Class B (also called Class II) of the seven-transmembrane family of guanine nucleotide binding protein (G protein)–coupled receptors (GPCRs) *(9)*. Receptors in this family bear some structural similarity and exhibit a defining characteristic in that they activate both adenylyl cyclase and phospholipase C (PLC) *(10–12)*. The responses of the PTH1R to agonists are regulated by

Received November 16, 2006; Revised December 11, 2006; Accepted December 21, 2006.

Author to whom all correspondence and reprint requests should be addressed: W. Bruce Sneddon, PhD, University of Pittsburgh School of Medicine, Department of Pharmacology, E-1103 Biomedical Science Tower, Pittsburgh, PA. E-mail: wbs2@pitt.edu

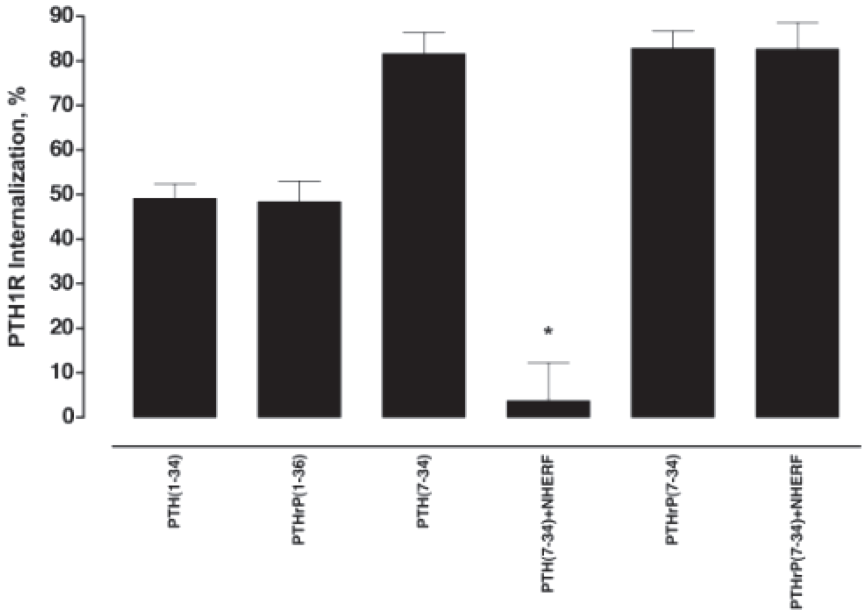

Fig. 1. PTH(7-34) and PTHrP(7-34) both induce PTH1R endocytosis. The effect of 10^{-6} *M* of the indicated peptides on PTH1R internalization in kidney distal tubule cells expressing 100,000 PTH1R/EGFP per cell (D1 cells). Receptor endocytosis was measured by real-time quantitative confocal microscopy in D1 cells that had been transfected with empty pcDNA3 vector or NHERF1. The extent of PTH1R internalization after a 15 min peptide exposure is presented. Results are the means ± SEM of triplicate determinations in three independent experiments. $*p < 0.01$ for PTH(7-34) in the presence of NHERF1 vs the absence of NHERF1 as determined by a one-way ANOVA with a post-hoc Bonferroni test for statistical significance.

multiple mechanisms. Upon activation, downstream second messengers can feed back and attempt to shut off the signal from the PTH1R. Typically, G protein–coupled receptor kinase 2 (GRK2) phosphorylates the agonist-activated receptor *(13,14)*. This promotes arrestin recruitment *(15–17)* and uncoupling of the receptor from its cognate G proteins, G_s and G_q. Following this desensitization, the PTH1R is endocytosed into intracellular compartments after which it can be recycled back to the plasma membrane (resensitization) *(18)*, or targeted for degradation, leading to receptor downregulation *(19,20)*. We have demonstrated that both the synthetic PTH(7-34) and the secreted form of the peptide PTH(7-84), which bind to but do not activate the PTH1R, internalize the PTH1R very efficiently in kidney distal tubule cells and rat osteosarcoma ROS 17/2.8 cells *(1)*. In contrast, neither PTH(7-34) nor PTH(7-84) internalizes the PTH1R in kidney proximal tubule cells or the human SaOS-2 osteosarcoma cell line. The cell-specific effects of PTH(7-34) on PTH1R internalization depend of the expression of NHERF1 *(1)*. NHERF1 is expressed in kidney proximal tubule cells and SaOS cells but is absent in kidney distal tubule and ROS17/2.8 cells *(1)*. Expression of NHERF1 inhibits PTH(7-34) induces PTH1R internalization in kidney distal tubule cells *(1)*. In contrast, expression of a dominant negative NHERF1 promotes PTH(7-34)-induced PTH1R internalization in proximal tubule cells where NHERF1 is expressed at high levels. The synthetic peptide PTHrP(7-34), like PTH(7-34), is a PTH1R antagonist. To this point, it has not been compared with PTH for its ability to induce PTH1R endocytosis.

Results

In previous studies, we demonstrated that PTH(7-34) internalizes the PTH1R and that this internalization is regulated by NHERF1. Because PTH and PTHrP both bind to and activate the PTH1R, we sought to determine if PTHrP-stimulated PTH1R internalization was similarly regulated. PTH(1-34) and PTHrP(1-36), both PTH1R agonists, share 8 of 13 N terminal amino acids. PTH(7-34) and PTHrP(7-34), by comparison, share four amino acids between positions 7 and 13. We, therefore, inquired whether these sequence disparities translated into differentially regulated PTH1R internalization. PTH(1-34) and PTHrP(1-36) at 1 μ*M* each internalized the PTH1R 50% after 15 min (Fig. 1) in mouse kidney distal tubule cells that stably express 10^5 PTH1R/cell (D1 cells). These cells do not express NHERF1 *(1)*. Expression of NHERF1 does not inhibit PTH1R internalization by these agonists *(1)* (data not shown). PTH(7-34) and PTHrP(7-34) at 1 μ*M* internalized the PTH1R 80% after 15 min in D1 cells (Fig. 1). PTH(7-34)–induced PTH1R internalization was blocked by expression of NHERF1 (Fig. 1) *(1)*, PTHrP(7 34)–stimulated PTH1R internalization was not inhibited by NHERF1 (Fig. 1).

In order to determine the sequences within PTHrP that promote PTH1R internalization by a NHERF1-independent mechanism, a series of chimeric PTH/PTHrP peptides were

Table 1
PTH and PTHrP(7-34) and Chimeric Peptides were Designed to Identify Sequences Within PTH That Confer NHERF1-Sensitivity to PTH1R Internalization

PTH(7-34)	**L**M**H**N	L**GK**HLNSME**R**	VEW**L**RKKLQD	V**H**NF
PTHrP(7-34)	**L**L**H**D	K**GK**SIQDLR**R**	RFF**L**HHLIAE	I**H**TA
PTH(7-21)PTHrP(22-34)	**L**M**H**N	L**GK**HLNSME**R**	VFF**L**HHLIAE	I**H**TA
PTHrP(7-21)PTH(22-34)	**L**L**H**D	K**GK**SIQDLR**R**	REW**L**RKKLQD	V**H**NF
PTH(7-32)PTHrP(33-34)	**L**M**H**N	L**GK**HLNSME**R**	VEW**L**RKKLQD	V**H**TA
PTHrP(7-32)PTH(33-34)	**L**L**H**D	K**GK**SIQDLR**R**	RFF**L**HHLIAE	I**H**NF
PTH(7-33)PTHrP(34)	**L**M**H**N	L**GK**HLNSME**R**	VEW**L**RKKLQD	V**H**NA
PTHrP(7-33)PTH(34)	**L**L**H**D	K**GK**SIQDLR**R**	RFF**L**HHLIAE	I**H**TF

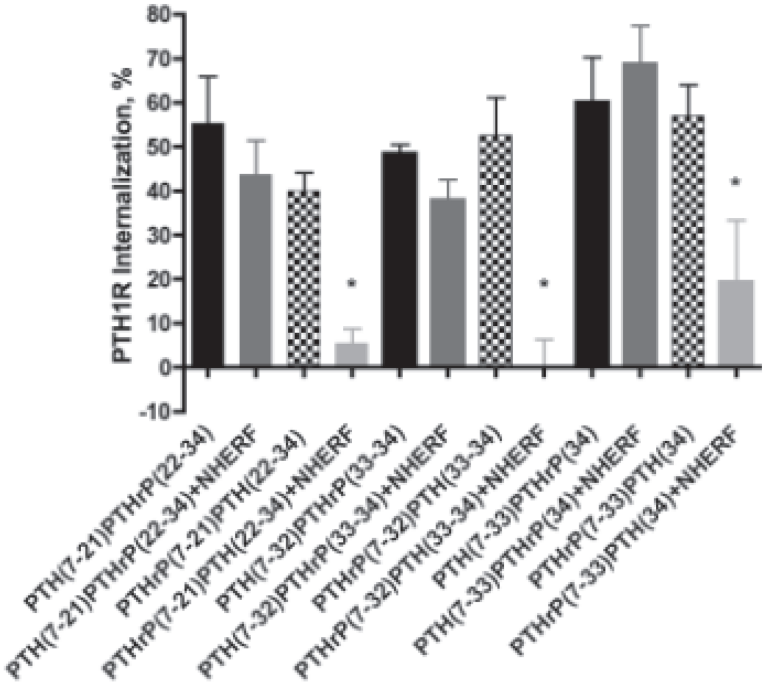

Fig. 2. NHERF1 inhibition of PTH1R internalization is dependent on sequences in the C terminus of PTH(7-34). The effect of 10^{-6} *M* of the indicated peptides on PTH1R internalization in D1 cells. Receptor endocytosis was measured as outlined in Fig. 1. The extent of PTH1R internalization after a 15 min peptide exposure is presented. Results are the means ± SEM of triplicate determinations in four independent experiments. *$p < 0.01$ in the presence of NHERF1 vs the absence of NHERF1 as determined by a one-way ANOVA with a post-hoc Bonferroni test for statistical significance.

designed (Table 1). Previous work of Gardella indicated, that the C terminus of PTH is incompatible with the N terminus of PTHrP with respect to receptor binding *(28)*. Positions 5, 19, and 21 were found to be critical to maintaining binding activity of chimeric peptides. We, therefore, decided that the dividing point for initial chimeric peptides would be at position 21. PTH(7-21)PTHrP(22-34) internalized the PTH1R 55% after 15 min in D1 cells (Fig. 2). This is somewhat less than PTH(7-34) and may reflect a reduced efficiency of the chimeric peptides to induce a conformational change in the PTH1R that promotes receptor internalization. Expression of NHERF1 had a minimal effect on PTH(7-21)PTHrP(22-34)–stimulated PTH1R endocytosis. The reverse chimera, PTHrP(7-21)PTH(22-34), internalized the PTH1R 40% after 15 min (Fig. 2) and expression of NHERF1 completely blocked this effect. These data suggest that replacement of PTH sequences between positions 22 and 34 with homologous amino acids from PTHrP dissociate NHERF1 and PTH1R internalization.

PTH(8-34) and PTH(9-34) internalized the PTH1R 45% and 40%, respectively, and this endocytosis was blocked by expression of NHERF1 (Fig. 3). In contrast, neither PTH (10-34) nor PTH(7-31) internalized the PTH1R in D1 cells (Fig. 3). This indicated that positions 7-9 and 32-34 might be important for promoting PTH1R internalization by PTH. We have also demonstrated that PTH(7-34)–induced PTH1R internalization is PKC dependent *(22)*. Positions 29–32 are critical for activation of PKC by PTH (*6,7*). Taken together, this suggested that positions 32–34 of PTH might be important amino acids for PTH1R endocytosis. Both PTH and

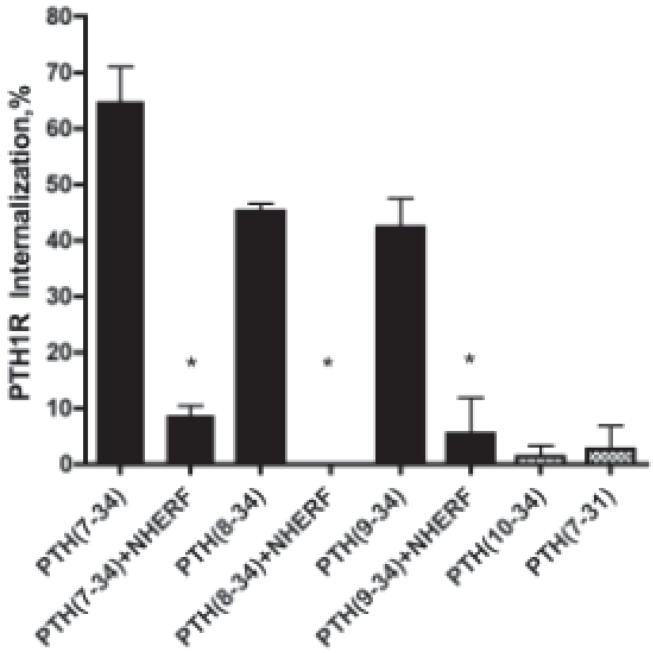

Fig. 3. Induction of PTH1R internalization requires positions 7–9 and 32–34 of PTH(7-34). The effect of 10^{-6} *M* of the indicated peptides on PTH1R internalization in D1 cells is presented. Receptor endocytosis was measured by real-time quantitative confocal microscopy in D1 cells that had been transfected with empty pcDNA3 vector or NHERF1. The extent of PTH1R internalization after a 15 min peptide exposure is presented. Results are the means ± SEM of triplicate determinations in four independent experiments. *$p < 0.01$ in the presence of NHERF1 vs the absence of NHERF1 as determined by a one-way ANOVA with a post-hoc Bonferroni test for statistical significance.

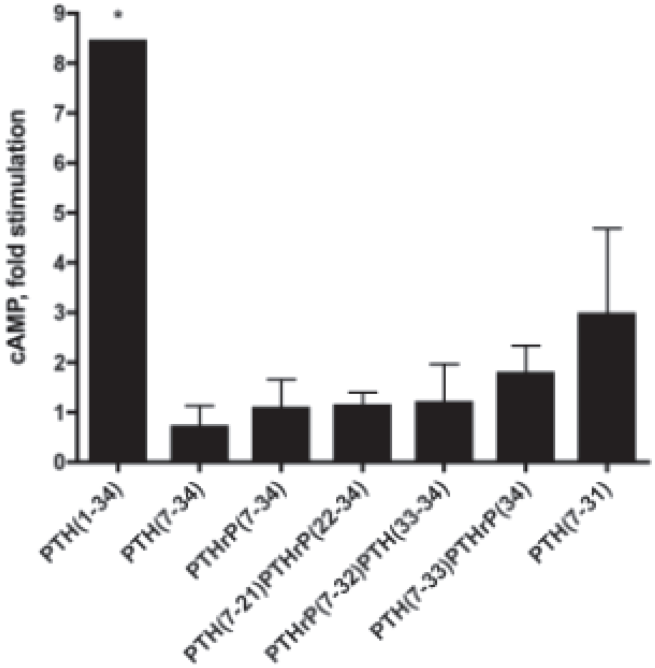

Fig. 4. Stimulation of adenylyl cyclase by chimeric PTH/PTHrP peptide fragments. D1 cells were exposed to 10^{-6} *M* of the indicated peptide for 15 min at 37°C and cAMP accumulation was measured. Data are the mean ± SEM normalized to unstimulated controls ($n = 3$). *$p < 0.01$ vs control.

PTHrP share a histidine at position 32. Therefore, chimeric PTH/PTHrP peptides were designed where positions 33 and 34 were substituted. PTH(7-32)PTHrP(33-34) internalized the PTH1R 50% in D1 cells and this process, as with PTHrP (7-34), was not inhibited by NHERF1 expression (Fig. 2). PTHrP(7-32)PTH(33-34) internalized the PTH1R 53% and, like PTH(7-34), this action was completely inhibited by NHERF1 expression (Fig. 2). These data clearly demonstrate that mutation of N33 and F34 in PTH promotes NHERF1-insensitive PTH1R internalization by PTH(7-34).

PTH and PTHrP have the amino acids NF and TA in positions 33–34, respectively. Asparagine and threonine are polar amino acids that constitute a relatively conservative substitution at position 33. In contrast, phenylalanine 34 of PTH has a large aromatic sidechain and is very different in size from alanine 34 of PTHrP. We, therefore, focused our attention on position 34. PTH(7-33)PTHrP(34) internalized the PTH1R in a NHERF-insensitive manner, whereas PTHrP(7-33)PTH(34) stimulated PTH1R internalization with the same magnitude but in a NHERF-sensitive manner (Fig. 2). A single mutation of F34 in PTH therefore alters the pathway of PTH1R trafficking by PTH(7-34).

Peptide concentrations of 1 μ*M* were employed for all of the chimeric PTH/PTHrP PTH1R internalization experiments. All of the peptides induced PTH1R internalization at this concentration and we wanted to test their respective antagonist properties in D1 cells. Therefore, we determined the ability of each of these peptides to stimulate cyclic AMP formation on their own in D1 cells and to inhibit cyclic AMP formation by 1 n*M* PTH(1-34). As shown in Fig. 4, only PTH(1-34) stimulated cyclic AMP formation in D1 cells (8.3-fold of control). Neither PTH(7-34), PTH(7-31), nor any of the chimeric PTH/PTHrP peptides tested affected cyclic AMP. In contrast, all of the peptides tested, with one exception, inhibited cyclic AMP formation by 1 n*M* PTH(1-34) with EC_{50}s in the 200–2000 n*M* range and these values were not affected by NHERF1 expression (Table 2). The notable exception to this was PTHrP(7-21)PTH(22-34), which inhibited PTH(1-34)–stimulated cyclic AMP formation with an EC_{50} of 0.2 n*M* in D1 cells. Expression of NHERF1 in D1 cells shifted the EC_{50} for this peptide to 200 n*M*.

We also performed binding studies using [^{125}I]PTH(1-34) as a tracer in D1 cells in the presence and absence of NHERF1 (Table 3). Both PTH(7-34) and PTHrP(7-34) inhibited radiolabeled PTHrP binding with IC_{50} values of 50 n*M*. Most of the chimeric peptides inhibited tracer binding with similar affinity. There were exceptions to this, however. Similar to its ability to inhibit cyclic AMP formation by PTH(1-34), PTHrP(7-21)PTH(22-34) robustly inhibited tracer binding in D1 cells with an IC_{50} of 3 p*M*. To our knowl-

Table 2
EC_{50} Values ± SEM for Inhibition by Chimeric PTH/PTHrP Peptides of 1 n*M* PTH(1-34)–Stimulated Cyclic AMP Formation in D1 Cells[a]

Peptide	EC_{50} (−NHERF), n*M*	EC_{50} (+NHERF), n*M*
PTH(7-21)PTHrP(22-34)	500 ± 10	700 ± 15
PTHrP(7-21)PTH(22-34)	0.2 ± 0.05	200 ± 10
PTH(7-32)PTHrP(33-34)	800 ± 10	1300 ± 10
PTHrP(7-32)PTH(33-34)	200 ± 5	300 ± 10
PTH(7-33)PTHrP(34)	200 ± 15	200 ± 10
PTHrP(7-33)PTH(34)	2000 ± 10	1500 ± 15

[a]Data represent $n = 3$ performed in triplicate.

Table 3
IC_{50} Values ± SEM for Inhibition of Radiolabeled PTH(1-34) Binding in D1 Cells[a]

Peptide	IC_{50} (-NHERF), n*M*	IC_{50} (+NHERF), n*M*
PTH(7-34)	50 ± 5	300 ± 10
PTHrP(7-34)	50 ± 5	20 ± 0.5
PTH(7-21)PTHrP(22-34)	50 ± 0.5	5 ± 0.5
PTHrP(7-21)PTH(22-34)	0.005 ± 0.001	0.4 ± 0.05
PTH(7-32)PTHrP(33-34)	4 ± 0.05	0.2 ± 0.05
PTHrP(7-32)PTH(33-34)	90 ± 5	400 ± 15
PTH(7-33)PTHrP(34)	6 ± 0.5	60 ± 15
PTHrP(7-33)PTH(34)	300 ± 15	40 ± 5

[a]Data represent $n = 3$ performed in triplicate.

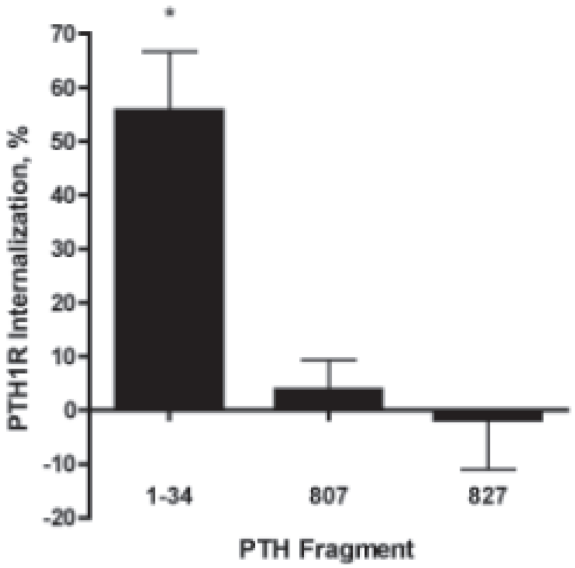

Fig. 5. J domain antagonists do not internalize the PTH1R . The effect of 10^{-6} *M* of the indicated peptides on PTH1R internalization in D1 cells. Receptor endocytosis was measured by real-time quantitative confocal microscopy in D1 cells that had been transfected with empty pcDNA3 vector or NHERF1. The extent of PTH1R internalization after a 15 min peptide exposure is presented. Results are the means ± SEM of triplicate determinations in three independent experiments. *$p < 0.01$ vs control as determined by a one-way ANOVA with a post-hoc Bonferroni test for statistical significance.

edge, this is the highest affinity PTH1R ligand described to date. This is a very exciting finding and is the focus of ongoing investigations in our laboratory. The presence of NHERF1 shifted the IC_{50} for this peptide to 400 p*M*. PTH (7-32)PTHrP(33-34) and PTH(7-33)PTHrP(34) also inhibited tracer binding with greater affinity than PTH(7-34), with IC_{50}s of 4 n*M* and 6 n*M*, respectively. The data indicate that all of the chimeric PTH/PTHrP peptides tested in the PTH1R internalization should be bound to the PTH1R at maximal levels at a concentration of 1 μ*M* and NHERF1 expression has a modest effect on the binding.

Based on photoaffinity crosslinking studies, PTH1R antagonists such as PTH(7-34) are thought to primarily interact with the extracellular N terminus of the PTH1R (the N domain), with minimal contacts to the transmembrane helix bundle and extracellular loops (the J domain) *(2,29, 30)*. Recently, a new class of synthetic PTH1R antagonists was characterized that bind to the J domain *(31)*. We tested two of these peptides for their ability to stimulate PTH1R internalization in D1 cells. Neither peptide 807 ([$Deg^{1,3}$, Bpa^{2},Nle^{8},Q^{10},A^{12},W^{14},Y^{15}]rPTH(1-15)NH2) nor peptide 827 ([$Ac5c^{1}$,Bpa^{2},Aib^{3},Nle^{8},Q^{10},A^{12},W^{14},Y^{15}]rPTH(1-15) NH2) internalized the PTH1R (Fig. 5) as compared with 55% internalization by PTH(1-34). Both peptides were used at 1 μ*M*, well above the IC_{50} for competition with radiolabeled PTH(1-15) of 100 n*M* *(31)*. These data provide a clear distinction between the classical N domain PTH1R antagonists that can internalize the receptor in the absence of NHERF1 and the J domain antagonists that lack this ability.

There are conflicting reports on the importance of receptor phosphorylation in the induction of PTH1R internalization. We previously reported that both PTH(1-34) and (7-34) induce PTH1R phosphorylation *(22)*. We therefore tested PTH(7-34), PTHrP(7-34), and the chimeric peptides for their respective abilities to stimulate PTH1R phosphorylation in D1 cells in the presence or absence of NHERF1 (Fig. 6) using in vivo receptor phosphorylation as described previously *(22)*. All of the peptides induced PTH1R phosphorylation at 1 μ*M*. There may have been a modest inhibitory effect of NHERF1 on PTH1R phosphorylation by PTH(7-32)PTHrP(33,34) and PTHrP(7 32)PTH(33-34). This is not likely related to PTH1R internalization because, despite inducing similar levels of receptor phosphorylation, only PTH(7-32)PTHrP(33,34) is able to internalize the receptor in the presence of NHERF1. While all of the peptides tested reproducibly induced PTH1R phosphorylation, the apparent enhanced ability of PTH(7-21)PTHrP (22-34), PTH(7-32)PTHrP(33,34), and PTHrP(7-32)PTH (33-34) to induce PTH1R was not reproducible.

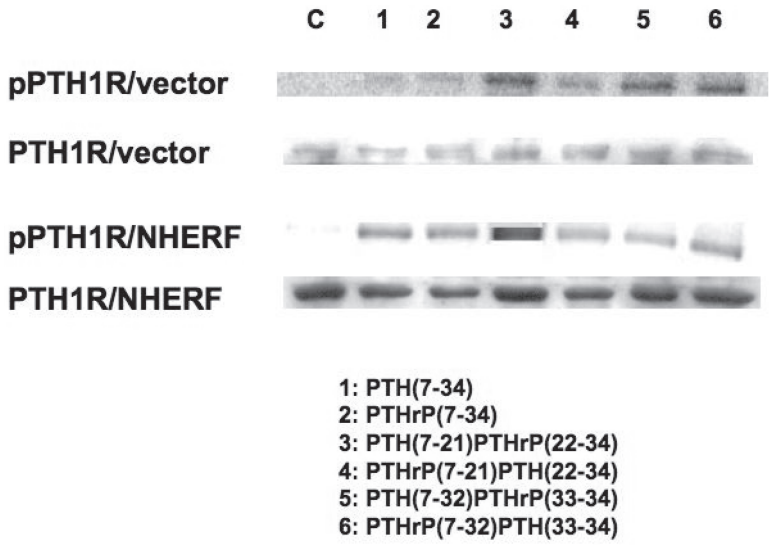

Fig. 6. Chimeric PTH/PTHrP peptides all induce PTH1R phosphorylation. PTH1R phosphorylation was assessed in D1 cells in the presence or absence of NHERF1 as described in Methods. pPTH1R represents the phosphorylated PTH1R. PTH1R represents the level of PTH1R/EGFP as measured by Western blots using a polyclonal anti-EGFP antibody. D1 cells were exposed to 10^{-6} *M* of chimeric peptides 1–6 or vehicle (C) as outlined in Methods. The data were reproduced three times. A representative experiment is presented.

The β-arrestins play a key role in the internalization of GPCRs. We sought to determine if NHERF1 regulates PTH1R mobilization of β-arrestins or arrestin-dependent receptor internalization. We hypothesized that differences therein underlie the ability of some PTH1R ligands to dissociate PTH1R internalization from NHERF1. The binding of β-arrestin1 to PTH1R residing on the plasma membrane of D1 cells was measured using image cross-correlation spectroscopy (ICCS). The fraction of binding was measured in cells with and without NHERF1 (Fig. 7). Binding of arrestin to PTH1R was negligible before the addition of ligand, independent of NHERF1 expression. Addition of PTH(1-34) promoted a dramatic increase in the binding of β-arrestin1 to PTH1R. This increase was modestly but significantly reduced by the expression of NHERF1 ($p = 0.004$). Addition of PTH(7-34) did not induce any increase in β-arrestin-1–bound receptor, independently of the expression of NHERF1. In contrast, 100 n*M* PTHrP(7-34) caused a robust increase in the binding of β-arrestin1 to PTH1R, which was modestly but significantly ($p = 0.04$) decreased by NHERF-1 expression.

Discussion

In our earlier work, we demonstrated that both the synthetic PTH(7-34) and the secreted form of the peptide PTH (7-84), which bind to but do not activate the PTH1R, internalize the PTH1R very efficiently in kidney distal tubule cells and rat osteosarcoma ROS 17/2.8 cells *(1)*. In fact, PTH(7-34) is more efficacious than PTH(1-34) at inducing PTH1R internalization in these cells. The mechanistic details that account for this difference are, at present, unknown. In contrast, neither PTH(7-34) nor PTH(7-84) internalizes the PTH1R in kidney proximal tubule cells or the human

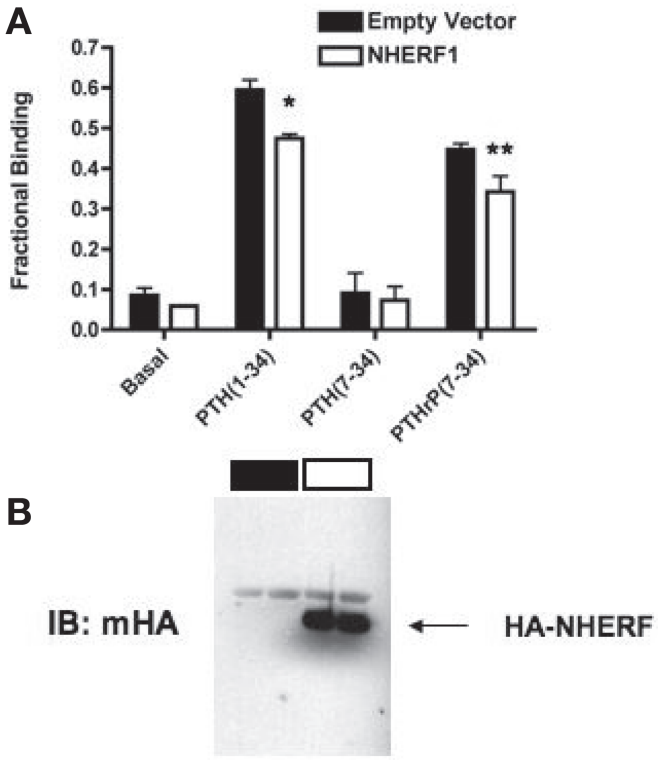

Fig. 7. (**A**) The binding of β-arrestin1 to PTH1R residing on the plasma membrane of D1 cells was measured using image cross-correlation spectroscopy. The fraction of binding was measured in cells with and without NHERF1. The following ligands were used to promote arrestin binding: PTH(1-34) (100 n*M*), PTH(7-34) (100 n*M*), and PTHrP(7-34) (100 n*M*). Data represent mean ± SEM of four independent measurements. *$p < 0.005$, **$p < 0.05$ vs empty vector as determined by a one-way ANOVA with a post-hoc Bonferroni test for statistical significance. (**B**) D1 cells were concurrently transfected as in **A**, lysates prepared, and HA–NHERF1 expression was confirmed in duplicate by immunoblot using a monoclonal anti-HA antibody.

SaOS-2 osteosarcoma cell line. The cell-specific effects of PTH(7-34) on PTH1R internalization depend of the expression of NHERF1 *(1)*. Expression of NHERF1 inhibits PTH(7-34)-induced PTH1R internalization. In this article, we demonstrate that PTHrP(7-34) also internalizes the PTH1R but in a NHERF1-independent manner (Fig. 1). We determined that mutation of phenylalanine 34 of PTH induces PTH1R internalization by a NHERF1-independent mechanism (Fig. 2). Conversely, F34–PTHrP(7-34) (A34 replaced by the homologous amino acid from PTH) fails to alter NHERF1-regulation of PTH1R internalization. Photoaffinity crosslinking studies using photoreactive L-*p*-benzoylphenylalanine (Bpa)–substituted PTH(1-34) and PTHrP(1-34) peptides have identified interactions between the C terminal of the peptide ligands and the extracellular N terminal of the PTH1R. Alanine scanning mutagenesis and crosslinking experiments identified position 23 in the C terminal of PTHrP as having important contacts with the ligand-binding domain of the PTH1R. Bpa^{23}–PTHrP(1-36) binds to threonine-33 and glutamine-37 in the N terminal extracellular domain *(32)*. This interaction is important for binding of the agonist, PTH(1-34), as well as the antagonist, PTH(7-34). Furthermore, Bpa^{27}–PTH(1-34) has been shown to crosslink to leucine-261 in the first extracellular loop *(33)*. The distal portion of PTH(7-34) is critical for induction of receptor internalization. Deletion of positions 32–34 eliminates PTH1R sequestration (Fig. 3) and also results in a reduction of the ability of N-terminally truncated PTH peptides to bind the PTH1R (data not shown). It is tempting to speculate that the large aromatic sidechain of F34 in PTH and the small sidechain of A34 in PTHrP have very different interactions with the ligand binding domain of the PTH1R and that these interactions underlie conformational changes in the PTH1R that could dissociate NHERF1 from the PTH1R.

The interactions of antagonists that bind to the N terminal extracellular domain of the PTH1R are clearly different from antagonists that interact with the transmembrane bundle and the extracellular loops. We demonstrated that the "J domain antagonists," in contrast to the "N domain antagonists" that we are focusing on, are unable to internalize the PTH1R in the presence or absence of NHERF1 (Fig. 5). This is consistent with a role for peptide binding to the N terminus of the PTH1R transducing a signal and altering the receptor conformation that leads to internalization in some cells. The J domain antagonists used in this study were highly modified PTH(1-15) peptides that were optimized for their ability to bind the PTH1R with high affinity *(31)*. In contrast, the native PTH(1-15) sequence only efficiently binds and initiates conformational changes in the receptor in the presence of additional sequences that promote peptide binding to the extracellular N terminus *(34)*.

Receptor phosphorylation plays an important role in the classical mechanism of GPCR internalization. Typically, the agonist-activated receptor is phosphorylated by GRK2 *(13,14)*. This promotes arrestin recruitment (*15–17*) and uncoupling of the receptor from its cognate G proteins, G_s and G_q. Following this desensitization, the PTH1R is endocytosed into intracellular compartments after which it can be recycled back to the plasma membrane (resensitization) *(18)*, or targeted for degradation, leading to receptor downregulation *(19,20)*. The C terminal of the PTH1R contains multiple sites for serine phosphorylation, but there are conflicting data from different cell lines concerning the role that phosphorylation plays in PTH1R internalization. We presented data in this article (Fig. 6) that demonstrate that PTH(7-34), PTHrP(7-34), and chimeric PTH/PTHrP peptides of the same length all induce PTH1R phosphorylation in D1 cells in the presence or absence of NHERF1. These data can be interpreted in a couple of different ways. First, PTH1R phosphorylation by N terminally truncated PTH/PTHrP peptides may be an epiphenomenon that is not related to PTH1R internalization because it occurs even in the presence of NHERF1-induced blockade of receptor endocytosis. Alternatively, antagonist-induced PTH1R phosphorylation may be required for receptor internalization but, in this instance, NHERF1-induced inhibition of PTH1R internalization occurs downstream of PTH1R phosphorylation. This will have to be tested in future studies. PTH1R phosphorylation and internalization has been examined in other cell lines. In LLC–PK1 cells, mutation of seven C-terminal serine residues to alanine markedly reduced agonist induced PTH1R internalization *(35)*. A murine model with a genetic knock-in of this phosphorylation-deficient receptor exhibited exaggerated cAMP and calcemic responses to administered PTH, likely as a result of a reduction in PTH1R desensitization and internalization *(36)*. A similarly substituted, phosphorylation-deficient PTH1R, when expressed at high levels in HEK 293 cells, was internalized normally in response to agonist binding *(24)*. In contrast, a similarly substituted phosphorylation-deficient opossum PTH1R exhibited a 30% reduction in agonist-induced receptor internalization in HEK293 cells *(37)*. This underscores the cell-specific nature of the regulation of PTH1R internalization.

We demonstrated that PTH(7-34) does not recruit β-arrestin1 (Fig. 7) but is, nonetheless, able to robustly internalize the PTH1R *(1)*. This is different from the findings of the Lefkowitz laboratory *(38)*, who determined that PTH(7-34) promoted an interaction between the β-arrestins and the PTH1R in transfected HEK293 cells. The discrepancy likely reflects differences in methodology. The Lefkowitz study examined co-immunoprecipitation of β-arrestins with the PTH1R in whole cell lysates, whereas we were using ICCS to examine only the PTH1R at the plasma membrane. We determined that NHERF1 completely inhibits this β-arrestin–independent PTH1R internalization induced by PTH(7-34). In contrast, both PTH(1-34) and PTHrP(7-34) recruit β-arrestin1 to the PTH1R and receptor internalization is not inhibited by NHERF1. Romero and colleagues,

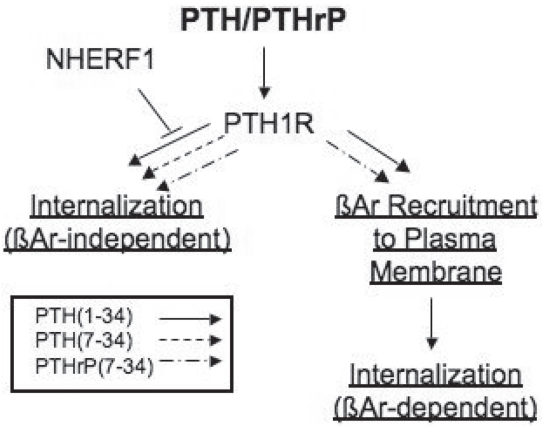

Fig. 8. A working model of NHERF1 regulation of PTH1R internalization. PTH(1-34) and PTHrP(7-34) each recruit β-arrestins to the PTH1R and internalize the PTH1R in a NHERF1-independent manner. PTH(7-34) does not recruit β-arrestins and internalizes the PTH1R in a NHERF1-inhibitable manner. NHERF1 inhibits β-arrestin-independent PTH1R internalization while only modestly affecting β-arrestin binding to the PTH1R.

using total internal reflection and confocal microscopy, have determined that NHERF1 tethers the PTH1R to actin filaments (G. G. Romero, personal communication). This slows down receptor movement in and out of the membrane and facilitates formation of complexes with other cytoskeletal proteins. The PTH1R is concentrated in actin stress fibers in the presence of NHERF. This does not prevent internalization of the PTH1R by ligands that promote β-arrestin recruitment, but it does slow down this process. Tethering by NHERF, however, prevents arrestin-independent internalization of the PTH1R by the antagonist PTH(7-34). In the absence of NHERF1, the PTH1R moves much more freely in the membrane and is more diffusely distributed within the cell and the plasma membrane. We propose that this freedom of movement permits the PTH1R to be internalized without antecedent β-arrestin binding. It is unclear why changing F34 to A in PTH(7-34) promotes PTH1R internalization in the absence of NHERF1. Presumably this alters the receptor conformation induced by ligand binding such that β-arrestins are recruited and NHERF1 is no longer able to tether the PTH1R to actin and it internalizes on binding to A34-PTH(7-34) or PTHrP(7-34). We propose a model in Fig. 8 to summarize these findings. We conclude that NHERF1 blocks arrestin-independent but not arrestin-dependent PTH1R internalization. Furthermore, the differential ability to recruit β-arrestins between PTH(7-34) and PTHrP(7-34) underlies their inability or ability, respectively, to dissociate PTH1R internalization from NHERF1 regulation.

The ability of the synthetic PTHrP(7-34) fragment to induce PTH1R internalization, while not physiological, is informative in terms of how PTH1R levels can be exogenously regulated. Under pathophysiological circumstances such as renal failure, N-terminally truncated PTH peptides are secreted by the parathyroid glands, the most predominant of these being PTH(7-84) *(39)*. Levels of these peptides are elevated and may contribute to PTH resistance by selectively downregulating the PTH1R in specific tissues such as renal distal tubules, where NHERF1 is absent. If a less selective downregulation of the PTH1R is desirable, synthetic peptides could be designed that break the NHERF1-dependence of this process and promote PTH1R internalization in a broader range of cells and tissues. Replacement of F34 in PTH with alanine would likely accomplish this goal. Very little is known about the role of PTH1R conformational changes that occur on binding N-terminally truncated PTH and PTHrP peptides and how this affects arrestin recruitment. It is interesting that the two fragments, PTH(1-34) and PTHrP(7-34), promote very different signaling cascades (the former robustly stimulates cAMP formation, while the latter does not) and yet they both efficiently recruit β-arrestin1 to the PTH1R. Future studies in our laboratory will focus on characterizing PTH1R receptor conformations that promote arrestin binding and how this dissociates the PTH1R from NHERF1.

Methods

Peptides

The chimeric peptides used in the study are described in Table 1. The University of Pittsburgh School of Medicine, Department of Molecular Genetics and Biochemistry Peptide Core Facility synthesized these peptides, and hPTH(7-31), as free acid amides. Each lyophilized peptide was reconstituted in 10 m*M* sodium acetate at a concentration of 100 m*M* and stored at –80°C. In addition, hPTH(8-34), (9-34), and (10-34) were obtained as a gift from Harald Juppner at Massachusetts General Hospital in Boston, MA.

Cell Culture and DNA Transfection

The preparation, subcloning, characterization, and culture conditions of mouse kidney distal tubule cells have been described *(21)*. Cells were grown in a 50:50 mix of DMEM/F12 (10–092-CV; Mediatech, Inc.), which was supplemented with 5% heat-inactivated FCS (Invitrogen) and 1% PS (5 mg penicillin, 5 mg streptomycin/mL; Invitrogen Life Technologies) in a humidified atmosphere of 95% air–5% CO_2 at 37°C. The immortalized mouse DT cells were stably transfected with human (hPTH1R)/enhanced green fluorescent protein (EGFP) as described previously *(22)*. The EGFP moiety lies in frame at the C terminus of the PTH1R. We have previously demonstrated that this does not interfere with PTH1R signaling nor does it impair the interaction between NHERF1 and the PTH1R *(1)*. These D1 cells were maintained in DMEM/F12 supplemented with 5% FCS, 1% PS, and 1% G418 (50 mg/mL solution, Invitrogen). D1 cells express 10^5 receptors/cell as determined

by Scatchard analysis of radioreceptor binding assays using [^{125}I]hPTH(1-34) as the radioligand *(23)*. D1 cells plated on 25 mm glass coverslips, where indicated, were transiently transfected with 1 μg mNHERF1 (a gift from Edward Weinman at the University of Maryland School of Medicine) or empty pcDNA3.1 vector (Invitrogen), using Fugene 6 (Roche Applied Science). Fluorescent PTH1R internalization studies were performed 48 hr post-transfection.

Quantitative, Real-Time Fluorescence Measurement of PTH1R Internalization

PTH1R internalization was examined and quantified in D1 cells as described previously *(1)*. Receptor internalization is measured as a loss of plasma membrane fluorescence after addition of PTH fragments and results are consistent with internalization of radiolabeled PTH(1-34) *(1)*. Control experiments confirm that no photobleaching of EGFP occurs when D1 cells are treated with vehicle.

Cyclic AMP Assays

Ligand-stimulated accumulation of cAMP was determined in the presence of 1 m*M* 3-isobutyl-1-methylxanthine. cAMP was measured chromatographically as previously described *(5)*. Where activation properties of the chimeric PTH/PTHrP peptides were being assessed, cAMP was measured in cells treated for 15 min with 1 μ*M* of the indicated peptide. In competition experiments, cells were incubated with increasing concentrations of chimeric peptide concurrently with 1 n*M* hPTH(1-34). Competition curves were fit and EC_{50} values calculated using a four-point logistic algorithm (Prism, GraphPad Software).

Radioligand Binding

Confluent D1 cells on 24 well plates (100,000–200,000) were incubated on ice for 2 h with 100,000 cpm of high-pressure liquid chromatography–purified [^{125}I][Nle^{8,18}Tyr^{34}] hPTH(1-34)NH2 in 250 μL of Dulbecco's modified Eagle's medium/F-12 medium containing 5% fetal bovine serum, essentially as described *(23,24)*. In brief, cells grown to confluence in 24-well plates were incubated for 2 h on ice to prevent internalization of the radioligand and to achieve equilibrium binding. Under these conditions, the concentration of radioligand was 0.1 n*M*. Where indicated, the radioligand was incubated concurrently with various concentrations of competitor peptides. Following incubation, the cells were washed twice with ice-cold phosphate-buffered saline and collected in 0.5 mL of 0.1 *N* NaOH, and bound [^{125}I]PTH was assessed using a Wallac Wizard 1470 Automatic Gamma Counter. Specific binding competition curves were fit and IC_{50} values calculated using a four-point logistic algorithm (Prism).

In Vivo Receptor Phosphorylation

PTH1R phosphorylation by chimeric PTH/PTHrP peptides in D1 cells was performed exactly as described previously *(22)*. As a control, PTH1R levels were assessed using Western blots as described *(22)* using a rabbit polyclonal anti-GFP antibody (Invitrogen).

Image Cross-Correlation Spectroscopy (ICCS)

These experiments were based on the experimental approach described by Wiseman et al. and Bacia and Schwille *(25,26)*. ICCS involves rapid (approx 60 ms/frame) two-channel scanning of a small section of the plasma membrane of cells containing two different protein-conjugated dyes. Correlations between the fluctuations in the fluorescence intensity of the two channels are used to generate a cross-correlation function, which can then be used to calculate the fractional binding of one of the fluorescent species to the other *(27)*. For these experiments, D1 cells were cultured in Mattek dishes and co-transfected with PTH1R/EGFP and mRed (a monomeric variant of dsRed)–β-arrestin-1. Only cells that expressed comparable amounts of both fluorescent proteins, as determined by confocal microscopy, were used for these studies. All confocal microscopy studies were conducted on an Olympus Fluoview 1000 equipped with a SIM scanner using the photon counting option to ensure accurate quantitation of the fluorescence fluctuations. The 488 nm line of an argon laser and the 543 nm line of a HeNe laser were used to excite eGFP and mRed, respectively. Emission bandpasses were optimized to eliminate bleedthrough using the spectral detection system of the instrument. Images were collected at 50–60 ms intervals. Up to 300 images per experiment were collected. The cells were kept at 37°C using an open perfusion incubator (Harvard Apparatus, Inc). The data collected were exported to ImageJ and analyzed using a plug-in specifically written to calculate image autocorrelation and image cross-correlation. This plug-in was based on code originally developed by Tully (Compix, Inc). The fractional degree of binding of PTH1R to arrestin was calculated from the ratio of the amplitudes of the cross-correlation function and the PTH1R and β-arrestin1 autocorrelation functions, as described by Kim et al. *(27)*.

Statistical Analyses

Where indicated, a one way analysis of variance (ANOVA) was performed, followed by post-hoc Bonferroni analysis using Prism software.

Acknowledgments

This study was supported by the NIH, NIDDK R21DK 068066. The author acknowledges Peter A. Friedman, PhD of the University of Pittsburgh School of Medicine, Pittsburgh, PA, Guillermo G. Romero, PhD of the University of Pittsburgh School of Medicine, Alessandro Bisello, PhD of the University of Pittsburgh School of Medicine, Pittsburgh, PA, and Thomas A Gardella, PhD of Massachusetts General Hospital, Boston, MA, for technical assistance and

helpful discussions before, during, and after the completion of these studies. I also thank Simon Watkins, PhD and the University of Pittsburgh Center for Biologic Imaging, where all of the microscopy studies were performed.

References

1. Sneddon, W. B., Syme, C. A., Bisello, A., et al. (2003). *J. Biol. Chem.* **278,** 43787–43796.
2. Adams, A. E., Bisello, A., Chorev, M., Rosenblatt, M., and Suva, L. J. (1998). *Mol. Endocrinol.* **12,** 1673–1683.
3. Maeda, S., Wu, S. X., Green, J., et al. (1998). *J. Am. Soc. Nephrol.* **9,** 175–181.
4. Behar, V., Bisello, A., Bitan, G., Rosenblatt, M., and Chorev, M. (2000). *J. Biol. Chem.* **275,** 9–17.
5. Bisello, A., Chorev, M., Rosenblatt, M., Monticelli, L., Mierke, D. F., and Ferrari, S. L. (2002). *J. Biol. Chem.* **277,** 38524–38530.
6. Takasu, H., Guo, J., and Bringhurst, F. R. (1999). *J. Bone Mineral Res.* **14,** 11–20.
7. Jouishomme, H., Whitfield, J. F., Gagnon, L., et al. (1994). *J. Bone Mineral Res.* **9,** 943–949.
8. Chung, U. I., Lanske, B., Lee, K., Li, E., and Kronenberg, H. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 13030–13035.
9. Horn, F., Vriend, G., and Cohen, F. E. (2001). *Nucleic Acids Res.* **29,** 346–349.
10. Abou-Samra, A. B., Freeman, M., Juppner, H., Uneno, S., and Segre, G. V. (1990). *J. Biol. Chem.* **265,** 58–62.
11. Bringhurst, F. R., Juppner, H., Guo, J., et al. (1993). *Endocrinology* **132,** 2090–2098.
12. Guo, J., Iida-Klein, A., Huang, X. W., Abou-Samra, A. B., Segre, G. V., and Bringhurst, F. R. (1995). *Endocrinology* **136,** 3884–3891.
13. Dicker, F., Quitterer, U., Winstel, R., Honold, K., and Lohse, M. J. (1999). *Proc. Natl. Acad. Sci. USA* **96,** 5476–5481.
14. Flannery, P. J. and Spurney, R. F. (2001). *Biochem. Pharmacol.* **62,** 1047–1058.
15. Ferrari, S. L., Behar, V., Chorev, M., Rosenblatt, M., and Bisello, A. (1999). *J. Biol. Chem.* **274,** 29968–29975.
16. Tawfeek, H. A. and Abou-Samra, A. B. (1999). *J. Bone Mineral Res.* **14,** S542.
17. Vilardaga, J. P., Frank, M., Krasel, C., Dees, C., Nissenson, R. A., and Lohse, M. J. (2001). *J. Biol. Chem.* **276,** 33435–33443.
18. Chauvin, S., Bencsik, M., Bambino, T., and Nissenson, R. A. (2002). *Mol. Endocrinol.* **16,** 2720–2732.
19. Tian, J., Smogorzewski, M., Kedes, L., and Massry, S. G. (1994). *Am. J. Nephrol.* **14,** 41–46.
20. Ureña, P., Kubrusly, M., Mannstadt, M., et al. (1994). *Kidney Internatl.* **45,** 605–611.
21. Friedman, P. A., Gesek, F. A., Morley, P., Whitfield, J. F., and Willick, G. E. (1999). *Endocrinology* **140,** 301–309.
22. Sneddon, W. B., Magyar, C. E., Willick, G. E., et al. (2004). *Endocrinology* **145,** 2815–2823.
23. Ferrari, S. L. and Bisello, A. (2001). *Mol. Endocrinol.* **15,** 149–163.
24. Malecz, N., Bambino, T., Bencsik, M., and Nissenson, R. A. (1998). *Mol. Endocrinol.* **12,** 1846–1856.
25. Wiseman, P. W., Squier, J. A., Ellisman, M. H., and Wilson, K. R. (2000). *J. Microsc.* **200,** 14–25.
26. Bacia, K. and Schwille, P. (2003). *Methods* **29,** 74–85.
27. Kim, S. A., Heinze, K. G., Bacia, K., Waxham, M. N., and Schwille, P. (2005). *Biophys. J.* **88,** 4319–4336.
28. Gardella, T. J., Luck, M. D., Wilson, A. K., et al. (1995). *J. Biol. Chem.* **270,** 6584–6588.
29. Lee, C., Luck, M. D., Juppner, H., Potts, J. T. Jr., Kronenberg, H. M., and Gardella, T. J. (1995). *Mol. Endocrinol.* **9,** 1269–1278.
30. Carter, P. H., Shimizu, M., Luck, M. D., and Gardella, T. J. (1999). *J. Biol. Chem.* **274,** 31955–31960.
31. Shimizu, N., Dean, T., Tsang, J. C., Khatri, A., Potts, J. T. Jr., and Gardella, T. J. (2005). *J. Biol. Chem.* **280,** 1797–1807.
32. Mannstadt, M., Luck, M. D., Gardella, T. J., and Juppner, H. (1998). *J. Biol. Chem.* **273,** 16890–16896.
33. Greenberg, Z., Bisello, A., Mierke, D. F., Rosenblatt, M., and Chorev, M. (2000). *Biochemistry* **39,** 8142–8152.
34. Bergwitz, C., Gardella, T. J., Flannery, M. R., et al. (1996). *J. Biol. Chem.* **271,** 26469–26472.
35. Qian, F., Leung, A., and Abou-Samra, A. (1998). *Biochemistry* **37,** 6240–6246.
36. Bounoutas, G. S., Tawfeek, H., Frohlich, L. F., Chung, U. I., and Abou-Samra, A. B. (2006). *Endocrinology* **147,** 4674–4679.
37. Vilardaga, J. P., Krasel, C., Chauvin, S., Bambino, T., Lohse, M. J., and Nissenson, R. A. (2002). *J. Biol. Chem.* **277,** 8121–8129.
38. Gesty-Palmer, D., Chen, M., Reiter, E., et al. (2006). *J. Biol. Chem.* **281,** 10856–10864.
39. D'Amour, P., Brossard, J. H., Rousseau, L., et al. (2005). *Kidney Int.* **68,** 998–1007.