Parathyroid hormone receptor internalization is independent of protein kinase A and phospholipase C activation

HESHAM A. W. TAWFEEK, JIAN CHE, FANG QIAN, AND ABDUL B. ABOU-SAMRA
Endocrine Unit, Massachusetts General Hospital and Harvard Medical School,
Boston, Massachusetts 02114

Received 2 March 2001; accepted in final form 18 April 2001

Parathyroid hormone receptor internalization is independent of protein kinase A and phospholipase C activation. Am J Physiol Endocrinol Metab 281: E545–E557, 2001.—Parathyroid hormone (PTH) and PTH-related peptide (PTHrP) binding to their common receptor stimulates second messenger accumulation, receptor phosphorylation, and internalization. LLC-PK1 cells expressing a green fluorescent protein-tagged PTH/PTHrP receptor show time- and dose-dependent receptor internalization. The internalized receptors colocalize with clathrin-coated pits. Internalization is stimulated by PTH analogs that bind to and activate the PTH/PTHrP receptor. Cell lines expressing a mutant protein kinase A regulatory subunit that is resistant to cAMP and/or a mutant receptor (DSEL mutant) that does not activate phospholipase C internalize their receptors normally. In addition, internalization of the wild-type receptor and the DSEL mutant is stimulated by the PTH analog [Gly¹,Arg¹⁹]hPTH-(1–28), which does not stimulate phospholipase C. Forskolin, IBMX, and the active phorbol ester, phorbol-12-myristate-13-acetate, did not promote receptor internalization or increase PTH-induced internalization. These data indicate that ligand-induced internalization of the PTH/PTHrP receptor requires both ligand binding and receptor activation but does not involve stimulation of adenylate cyclase/protein kinase A or phospholipase C/protein kinase C.

Parathyroid hormone/parathyroid hormone-related peptide receptor; G protein-coupled receptor; cytoplasmic loops; extracellular domains; internalization; green fluorescent protein

PARATHYROID HORMONE (PTH) and PTH-related peptide (PTHrP) bind equivalently to a common G protein-coupled receptor, the PTH/PTHrP receptor (5, 38), which mediates the endocrine actions of PTH on mineral ion homeostasis and the multiple actions of PTHrP on different tissues in adult and fetus (39, 45, 51). The PTH/PTHrP receptor is highly expressed in bone and kidney and has a low level of expression in numerous fetal and adult tissues (47, 70).

Activated G proteins stimulate cellular effectors, which increase the intracellular levels of several second messengers, such as cAMP, calcium, diacylglycerol, and inositol trisphosphates (5). Binding of PTH or PTHrP activates the receptor to transduce its signal to different G proteins (54, 61). Both Gs and Gq have been shown to be involved in PTH/PTHrP receptor signaling (54, 61). Receptor activation and stimulation of intracellular second messengers are transient processes that become desensitized in the continuous presence of stimulus. Desensitization may involve several cellular processes, which include receptor phosphorylation by kinases, interaction of β-arrestin(s), uncoupling from G protein(s), and/or receptor internalization (10, 16, 42). Internalization is important for receptor desensitization, and/or initiation of other signaling pathways (10, 16, 42). β-Arrestins act as adaptor molecules that target G protein-coupled receptors to internalize via a classic clathrin-coated pit-mediated pathway (14, 48). Some G protein-coupled receptors, such as bradykinin B2 receptor (22), m2 muscarinic acetylcholine receptor (25), and endothelin receptor (20) associate with caveolae (9, 15, 24, 55). Caveolae are small, flask-shaped invaginations of the plasma membrane, characterized by high levels of cholesterol and glycosphingolipids and by the presence of caveolin, an integral membrane protein. Previous studies showed that hyperosmolar sucrose, which disrupts clathrin-coated pits (35), interferes with PTH/PTHrP receptor internalization (36). This suggested that clathrin-coated pits are involved in PTH/PTHrP receptor internalization. Pharmacological manipulation of the cell milieu with drugs that block or activate protein kinases has also suggested that protein kinase C (PKC) is involved in internalization (26). Such pharmacological studies of the cellular mechanisms of PTH/PTHrP receptor internalization require direct experimental support.

The PTH and PTHrP analogs PTH-(1–34) and PTHrP-(1–36) are full agonists both in vivo and in vitro. The amino terminal-truncated PTH analog PTH-(3–34) binds to the receptor with a high affinity but is a partial agonist. Recently, shortened analogs with point and clustered mutations have been characterized, some of which showed altered signaling properties (63, 65). Such selective signaling agonists provide tools to examine the role of specific regions on receptor function.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of different receptor-activated signals in the process of receptor internalization. The carboxy-terminal-truncated PTH analog [Ala<sup>10</sup>,12, Arg<sup>14</sup>]rPTH-(1–14) has an enhanced signaling activity compared with the native PTH-(1–14) peptide but has a low binding potency in radioreceptor competition assays (62, 63). In contrast, the short PTH peptides [Ala<sup>1</sup>,12, Gln<sup>10</sup>, His<sup>11</sup>, Trp<sup>14</sup>]rPTH-(1–14) and [Ala<sup>1</sup>,10,12, Arg<sup>11</sup>, Trp<sup>14</sup>]rPTH-(1–14) show both enhanced signaling and enhanced binding compared with other PTH-(1–14)-based analogs (62, 63). These peptides provide a tool to study the structural and the functional properties of the PTH analogs required for receptor internalization. The PTH analog [Gly<sup>1</sup>, Arg<sup>19</sup>]hPTH-(1–28) increases cAMP normally but stimulates phospholipase C (PLC) weakly (65). This signal-selective analog is useful to dissect the role of adenylate cyclase (AC) vs. PLC signaling in receptor internalization.

The PTH/PTHrP receptor, which has a mutation in the second cytoplasmic loop, the DSEL mutant receptor, binds PTH and increases cAMP accumulation normally but does not increase PLC activity (37). The DSEL mutant receptor thus provides a means to examine receptor internalization in the absence of PLC signaling.

In this study, we used green fluorescent protein (GFP)-tagged PTH/PTHrP receptors, mutant PTH/PTHrP receptors, and ligands with altered functional properties to examine the cellular mechanisms involved in ligand-induced PTH/PTHrP receptor internalization.

**MATERIALS AND METHODS**

**Materials.** [Nle<sup>8,18</sup>, Tyr<sup>34</sup>]-bPTH-(1–34)-NH<sub>2</sub> (NlePTH), [Nle<sup>8,18</sup>, Tyr<sup>34</sup>]-bPTH-(3–34)-NH<sub>2</sub> [PTH-(3–34)] and [Tyr<sup>36</sup>]hPTHrP-(1–36)-NH<sub>2</sub> (PTHrP) were synthesized by a solid-phase method (Endocrine Unit, Mass. General Hospital, Boston, MA), purified by HPLC, and characterized by amino acid hydrolysis, NH<sub>2</sub> terminal sequencing, and mass spectrometry. All chemicals were of the highest grade available and were obtained from either Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Na<sup>125</sup>I (2,125 Ci/mmol) and [<sup>32</sup>P]orthophosphate (8,500–9,120 Ci/mmol) were purchased from New England Nuclear (Boston, MA); restriction enzymes were purchased from New England BioLabs (Beverly, MA), Promega (Madison, WI), or GIBCO-BRL (Gaithersburg, MD); and lipofectin reagents were obtained from GIBCO-BRL. Tissue culture media were prepared by the Massachusetts General Hospital media facility; fetal bovine serum (FBS) was from Sigma; streptomycin-penicillin was from GIBCO-BRL; and flasks, plates, and other tissue culture supplies were from Corning (Oneonta, NY). Phorbol-12-myristate-13-acetate (PMA) was from Calbiochem (San Diego, CA); IBMX and forskolin were purchased from Sigma; monoclonal antibodies against clathrin and PKC<sub>α</sub> were purchased from Transduction Laboratories (Lexington, KY); and CY3-labeled goat anti-mouse antiserum was purchased from Amersham (Arlington Heights, IL). Vectashield was from Vector Lab (Burlingame, CA), peroxidase-labeled rabbit anti-sheep and goat anti-mouse antisera were from Sigma, Immobilon membranes were from Millipore (Bedford, MA), and the chemiluminescence kit was from New England Nuclear.

**Cell culture.** COS-7 and LLC-PK<sub>1</sub> cells were cultured in DMEM supplemented with 10% FBS. All media contained 1 μg/ml streptomycin and 100 U/ml penicillin. The cells were incubated in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub> at 37°C. Media were replaced every other day.

**Construction of GFP-tagged receptors.** GFP was introduced in the sequence of the rat PTH/PTHrP receptor cDNA cloned in pcDNA1, R158H, by creating unique restriction enzyme sites by means of site-directed mutagenesis and/or polymerase chain reaction. The veracity of the mutant cDNAs was determined by restriction enzyme mapping and sequence analysis. Two GFP-tagged rat PTH/PTHrP receptors were constructed: E2gfp-PTH/PTHrP receptor and Tgfp-PTH/PTHrP receptor. In the E2gfp-PTH/PTHrP receptor, the sequence W69, T70, P71, A72, S74 from exon E2 of the rat PTH/PTHrP receptor was replaced with the 251 amino acids of the E-GFP sequence. The GFP coding region was digested from the E-GFP plasmid (Clonetech) and ligated in frame in the middle of exon E2, an extracellular domain whose deletion or mutation does not disturb ligand binding or signal transduction (46). The location of the E-GFP insertion within exon E2 was selected so that the epitopes recognized by the anti-rat PTH/PTHrP receptor antibody G48 (57) are not interrupted. For the Tgfp-PTH/PTHrP receptor, the GFP coding sequence was inserted in an existing NoI site at the end of the carboxy-terminal tail, which resulted in the deletion of two amino acids from the carboxy-terminal tail, P580 and M591, and addition of 251 amino acids of the GFP.

**Cell transfection.** For transient transfection experiments, COS-7 cells, in 10-cm plates, were transfected with 5 μg of plasmid DNA by use of the DEAE-dextran method. One day after transfection, the cells were trypsinized and replated in 24-well plates for use in radioligand binding, PTH-stimulated cAMP accumulation, and double-antibody binding assays or in 6-well plates, which were used for detection of immunofluorescence.

To study PTH/PTHrP receptor internalization, we developed LLC-PK<sub>1</sub> cell lines stably expressing the GFP-tagged PTH/PTHrP receptor. LLC-PK<sub>1</sub> cells were cotransfected with the GFP-tagged PTH/PTHrP receptor cDNAs and the psv2Neo plasmid by use of lipofectin. The cells were grown in the presence of G418. Cell colonies that survived selection were expanded and examined for expression of GFP under a fluorescent microscope. Twenty cell lines were developed and subcloned. Cell lines that had radioligand binding >10% of the total added radioactivity per well (of a 24-well plate) were expanded and examined for receptor expression by Western blot analysis. Nonspecific binding to untransfected cells was <1% of the total added radioactivity. All of the cell lines expressed one 107-kDa immunoreactive band that corresponded to the expected molecular mass of the fusion protein.

**Phosphorylation of the PTH/PTHrP receptor.** Cells were labeled with [<sup>32</sup>P]orthophosphate (0.5 mCi/3 ml medium for confluent cells in one 6-cm tissue culture dish) for 120 min and challenged with PTH for variable time periods (0–40 min) during the last 0–40 min of the labeling period. The cells were then rinsed (3×) with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer. The cell lysate was immunoprecipitated with the PTH/PTHrP receptor antiserum (G48 antisemum).

**Immunoprecipitation and Western blot.** For immunoprecipitation and Western blots, we used G48 antibody that was raised in sheep against a 20-amino acid synthetic rat PTH/PTHrP receptor peptide (E2D: YPESKENKDVPTGSR-RGRPC), which corresponds to residues 88–108 (57), and was subsequently boosted with eight other synthetic receptor fragments that represent different domains from the extra-cellular loops, the cytoplasmic loops, and the carboxy-terminal tail. The immunoglobulins were precipitated with satu-
rated ammonium sulfate, rinsed (3×), and then coupled to CNBr-activated Sepharose beads. Normal sheep serum was processed in an identical manner to construct normal IgG-Sepharose beads. The cell lysates were incubated with the normal sheep IgG beads for 1 h, and the supernatant was collected and further incubated with the PTH/PTHrP receptor antiserum beads for 1 h at 4°C. The beads were rinsed (3×) with PBS. The receptor was then eluted from the beads with sodium dodecyl sulfate (SDS)-sample buffer, and the lysate was analyzed using 5–20% gradient SDS-PAGE and autoradiographed.

For Western blots, the RIPA buffer cell lysate was mixed (1:1) with 2× SDS-sample buffer and analyzed by 5–20% gradient SDS-PAGE. The proteins were blotted onto Immobilon membranes, and the membranes were blocked with 5% nonfat dry milk and 0.2% Tween-20. The membranes were then incubated with G48 antiserum at a dilution of 1:5,000. A peroxidase-labeled rabbit anti-goat antiserum was added, and the bands were developed using chemiluminescence.

**Subcellular fractionation of PKC.** Cytosolic and membrane fractions containing PKC were separated as described (49). Equal amounts of proteins, measured using a Bio-Rad protein assay kit, were analyzed on a 10% SDS-PAGE. Western blot analysis was performed as described in Immunoprecipitation and Western blot by use of a mouse monoclonal antibody that recognizes PKCα (1:1,000, 5 h at 20°C) and a peroxidase-labeled goat anti-mouse antiserum (1:2,000, for 3 h). The immunoreactive protein bands were detected by chemiluminescence.

**Cell surface receptor quantification.** Cell surface expression of the PTH/PTHrP receptor was assessed using G48 antibody (57). Cells grown in 24-well plates were rinsed (3×) with PBS, pH 7.4, containing 5% heat-inactivated FBS and incubated at room temperature for 2 h in 0.5 ml of PBS with the G48 antibody or nonimmune IgG. The cells were then rinsed (4×) with 5% FBS/PBS, rinsed (4×) with PBS, and incubated at room temperature for an additional 2 h with a rabbit anti-sheep antibody (Sigma) and rinsed with PBS (4×), and a 125I-labeled goat anti-rabbit IgG (200,000 cpm/well) was then added for 2 h. The incubation was terminated by removing the supernatant and rinsing the cells with PBS (3×). The cells were then solubilized in 1 N NaOH, and the radioactivity was counted.

**Radioiodination of NlePTH.** NlePTH was radioiodinated using the chloramine-T method, as previously described (5, 38). Two micrograms of NlePTH and 1.5 mCi of Na125I were reacted in the presence of 10 μg of chloramine-T at room temperature for 20 s in 0.2 M phosphate buffer (pH 7). The reaction was quenched with 20 μg of metabisulfite. Carrier protein, 1 ml of 0.1% bovine serum albumin (BSA) in 0.1% trifluoroacetic acid, was added; the mixture was loaded on a C18 Sep-Pack cartridge, the free iodine and salts were rinsed, and the peptide was then eluted with 50% acetonitrile and 0.1% trifluoroacetic acid. The eluted peptide was diluted 1:1 with 0.1% trifluoroacetic acid to bring the concentration of acetonitrile to 25%. The sample was loaded on a C18 reverse-phase HPLC column that was equilibrated with 20% acetonitrile and 0.1% trifluoroacetic acid. The iodinated peptide was eluted with a 30–50% gradient of acetonitrile in 0.1% trifluoroacetic acid over 30 min at a rate of 2 ml/min. Fractions (0.3 ml) were collected and counted.

**Radioreceptor assay.** Cells grown in 24-well plates were rinsed (2×) with 1 ml of binding buffer (50 mM Tris·HCl, 100 mM NaCl, 5 mM KCl, 2 mM CaCl2, 5% heat-inactivated horse serum and 0.5% FBS, pH 7.7) and incubated with [125I]-labeled NlePTH in binding buffer at 15°C for 4 h in the presence or absence of 10−11−10−6 M nonradioactive NlePTH (5, 38). In some experiments, increasing concentrations of the radioligand (100,000–1,000,000 cpm/well), which correspond to 0.1–10 nM of peptides were applied in the presence and absence of 1 μM NlePTH. Cells were then washed (3×) with cold PBS and solubilized with 1 N NaOH, and the radioactivity was determined by γ-counter (Micromedic System, model 6/400 Plus). The binding data were analyzed using Scatchard plots.

**Measurement of intracellular cAMP accumulation.** Intracellular cAMP accumulation was measured as previously described (4). Cells grown in 24-well plates were preincubated in serum-free DMEM containing 0.1% BSA, 10 mM HEPES (pH 7.42), and 1 mM IBMX at room temperature for 10 min and further incubated at 37°C for an additional 15 min after agonists (PTH, 100 nM) or vehicles were added. The reaction was terminated by aspiration of the medium, washing of the cells with cold PBS, and freezing of the plate on dry ice. cAMP was extracted by thawing the cells in 1 ml of 50 mM HCl, and cAMP concentrations were measured by a specific radioimmunoassay.

**Confocal microscopy and colocalization studies.** Cells seeded on coverslips in 6-well plates were grown as a monolayer until they reached 60–80% confluency. Cells were then rinsed (2×) with PBS and were then challenged with PTH or other analogs for the indicated time period in serum-free DMEM containing 20 mM HEPES buffer and 0.1% BSA at 37°C in a CO2 incubator. The medium was removed, and the cells were rinsed (2×) with cold PBS and fixed with 4% paraformaldehyde (in PBS) for 15 min at room temperature. Fixed cells were then washed (3×) with PBS, each wash lasting 5 min, and then mounted using Vectashield, and the coverslips were sealed on the slides with nail polish.

For colocalization experiments, cells were further permeabilized with 0.1% SDS for 5 min followed by (3×) PBS rinses (5 min each). The permeabilized cells were incubated with the primary antibody (monoclonal anti-clathrin) diluted 1:100 in PBS for 3 h at room temperature. The unbound antiserum was washed (3×) with PBS. A secondary antibody tagged with a red fluorescent dye (Cy3-conjugated goat anti-mouse IgG) was added (1:1,000 in PBS) for 2 h at room temperature, and the cells were then rinsed (3×) with PBS, mounted, and sealed.

The confocal microscopy images were obtained using a Radiance 2000 Laser Scanning system configured with 40×, 63×, and 100× oil objective. Green and red images were captured sequentially at 1024/1024 resolution with 2 optical zoom, iris diaphragm, and a125I-labeled goat anti-rabbit IgG (200,000 cpm/well) was added (1:1,000 in PBS) for 2 h at room temperature, and the cells were then rinsed (3×) with PBS, mounted, and sealed.

**Statistical analysis.** The results are representative of the means ± SD of at least three experiments. Each condition was carried out in triplicate.

**RESULTS**

**Characterization of the GFP-tagged PTH/PTHrP receptor in COS-7 and LLC-PK1 cells.** The functional properties of the E2gfp-PTH/PTHrP receptor (E2gfp-PPR) and the Tgfp-PTH/PTHrP receptor (T2gfp-PPR) and the Tgfp-PTH/PTHrP receptor (T2gfp-PPR)
were examined in transiently transfected COS-7 cells. The apparent dissociation constant ($K_d$), maximal ligand binding, $EC_{50}$, and maximal PTH-stimulated cAMP accumulation of the two GFP-tagged receptors were similar to those of the wild-type PTH/PTHrP receptor (WT-PPR) (Fig. 1, A and B).

To study internalization of the GFP-tagged PTH/PTHrP receptors, we stably transfected LLC-PK$_1$ cells,
a porcine renal tubular cell line, with E2gfp- and Tgfp-PTH/PTHrP receptor cDNAs. Data obtained in several cell lines showed that both E2gfp- and Tgfp-PTH/PTHrP receptors are rapidly internalized after PTH challenge (data not shown). The E2gfp-PTH/PTHrP receptor-expressing cells were selected for subsequent studies.

LLC-PK1 cell lines stably expressing WT-PPR and E2gfp-tagged PTH/PTHrP receptors (GFP-PPR); ~200,000 receptors per cell, were selected and expanded. Receptor expression from these cell lines was compared. Cell surface receptor immunoreactivity of the WT-PPR and the GFP-PPR was in the range of 7–8% of total radioactivity added (Fig. 1C), whereas nonspecific binding to untransfected cells was <0.5% of the total radioactivity added. Western blot analysis revealed one single band (~107 kDa) that represents the expected size of the fusion protein (80 kDa for the PTH/PTHrP receptor + 27 kDa for the GFP backbone = 107 kDa, Fig. 1D) and one band for the WT-PPR with the expected size of 80 Kd (Fig. 1D). The GFP-PPR in the LLC-PK1 stable cell line bound PTH with high affinity (Fig. 1E) and increased cAMP accumulation (Fig. 1G) after PTH challenge; the apparent binding \( K_d \), maximal binding, \( E_{50} \), and maximal cAMP stimulation were similar to those of the wild-type receptors (Fig. 1, E and F).

We have recently shown (57) that PTH causes rapid phosphorylation of the PTH/PTHrP receptor. Therefore, we examined whether the GFP-PPR is normally phosphorylated after challenge with PTH. Both basal and PTH-dependent phosphorylation of the GFP-PPR were similar to those of the WT-PPR (Fig. 1G).

**PTH causes rapid internalization of the GFP-tagged PTH/PTHrP receptors.** Under basal conditions, the GFP-PPRs were mostly localized to the plasma membrane (Fig. 2, *time 0*). Treatment with PTH (100 nM for 40 min) caused internalization of GFP-PPRs, as evidenced by appearance of dense cytoplasmic vesicles observed at different cellular planes (Fig. 2, PTH). The effects were time and dose dependent (Figs. 3 and 4). The earliest effects were observed at 5 min of incubation with 100 nM of PTH. After 20–40 min of PTH treatment, receptor internalization was more dramatic, and most of the receptors became localized to a perinuclear zone (Fig. 3).

**Internalization of GFP-tagged PTH/PTHrP receptors is clathrin dependent.** Clathrin-coated vesicles have been shown to be involved in internalization of several G protein-coupled receptors (18, 31, 41, 53, 58, 66, 76). To study the involvement of clathrin-coated vesicles in PTH/PTHrP receptor internalization, the cells were permeabilized and incubated with a monoclonal anti-clathrin antibody, the major coat protein of clathrin-coated vesicles. Cy3-labeled goat anti-mouse secondary antibody was used to visualize clathrin immunoreactivity. The GFP-PPRs (in green) and the labeled clathrin-coated pits (in red) were scanned simultaneously in the same plane and visualized using the confocal microscope (Fig. 5A). Colocalization of GFP-PPRs and clathrin in the same vesicles is shown (yellow and orange, Fig. 5A). As reported by Huang et al. (36), treatment with 0.5 M sucrose for 40 min, which is known to disrupt clathrin-coated pit formation (35), blocked the effects of PTH (100 nM for 40 min) on PTH/PTHrP receptor internalization (Fig. 5B).

---

**Fig. 2. Internalization of GFP-PPR.** LLC-PK1 cells stably expressing GFP-PPR, plated on coverslips, were treated with NlePTH at 37°C, and were then fixed, mounted, and examined with the confocal microscope. Cells treated with NlePTH (100 nM) for 40 min were examined at the xy planes (H, M, L) and xz plane (V). H is at one-third cell thickness from the apical cell surface, M is at two-thirds cell thickness from the apical cell surface, and L is just above the basal membrane. Plane V is vertically across the middle of the cell.
Biochemical characteristics of ligand requirements for internalization. To study the role of ligand binding and receptor activation in PTH/PTHrP receptor internalization, a series of modified PTH peptides, which exhibited variable binding and signaling properties, were used. PTHrP-(1–36), whose binding and signaling properties are similar to those of PTH, stimulated PTH/PTHrP receptor internalization with a potency that was similar to that of PTH (Fig. 6, A and B). The amino terminal-truncated PTH analog, PTH-(3–34), which binds with high affinity to the receptor but which results in only partial activation, did not stimulate internalization (data not shown). The carboxy terminal-truncated PTH analog, [Ala\(^{3,10,12}\),Arg\(^{11}\)]rPTH-(1–14), which has enhanced signaling activity compared with the native backbone peptide but which has very low binding potency in radioreceptor competition assays (62, 63), did not stimulate internalization of the PTH/PTHrP receptor (Fig. 6A). The lower binding and activation properties of the carboxy terminal-truncated hPTH-(1–28) compared with hPTH(1–34) (65) correlated with its lower stimulation of internalization (Fig. 7, A and B). In contrast, the short PTH peptides [Ala\(^{3,12}\),Gln\(^{10}\),Har\(^{11}\),Trp\(^{14}\)]rPTH-(1–14) and [Ala\(^{3,10,12}\),Arg\(^{11}\),Trp\(^{14}\)]rPTH-(1–14), which showed enhanced binding and activation when compared with other PTH-(1–14) peptides (62, 63), did stimulate internalization (Fig. 6, A and B).

Role of the PLC/PKC pathway in PTH/PTHrP receptor internalization. PTH stimulates multiple signaling pathways including AC/protein kinase A (PKA) and PLC/PKC. To explore the role of the PLC/PKC signaling pathway in internalization, we took advantage of a signal-selective PTH analog, [Gly\(^{1}\),Arg\(^{19}\)]hPTH-(1–28), which increases cAMP normally but stimulates PLC only weakly (65). In addition, we used the cell line...
SD19, an LLC-PK1 cell line that stably expresses the DSEL mutant rat PTH/PTHrP receptor (37), which normally increases cAMP accumulation but is severely defective in PLC stimulation. PTH-(1–34) stimulated internalization of the wild-type and the DSEL mutant receptors (Fig. 8A) as did the AC-selective PTH analog, [Gly1,Arg19]hPTH-(1–28) (Figs. 7, A and B, and 8B). Receptor internalization stimulated by [Gly1,Arg19]hPTH-(1–28) was less than that stimulated by PTH-(1–34) but was higher than that caused by hPTH-(1–28). Taken together, these data suggest that activation of the PLC signaling pathway is not required for PTH/PTHrP receptor internalization.

Although these data strongly rule out a major role for PLC activation in internalization, they do not rule out the potential of PTH to stimulate PKC through other pathways, such as phospholipase D (29, 64) and phospholipase A2 (23). Therefore, involvement of PKC was directly explored with a pharmacological approach using the phorbol ester PMA, which binds to and activates different groups of PKC. The cells were treated with PMA (1 μM) for 20 min prior to and during PTH treatment for 40 min. A long-term treatment with PMA (400 nM) for 16 h was also used to downregulate endogenous PKC. Short- and long-term treatments with PMA induced PKC translocation to the cell membrane and decreased cellular levels, respectively (Fig. 9A). Neither the activation nor the downregulation of PKC resulted in a significant effect on PTH-stimulated internalization (Fig. 9B). The role of PKA was further examined by studying PTH/PTHrP receptor internalization in the SD19AB4 cell line, a subclone of SD19 cells that stably expresses a dominant negative mutant of the type 1 regulatory subunit of PKA that has point mutations in both the A and B cAMP-binding sites. PKA in the SD19AB4 cell line is resistant to cAMP activation (30, 32). Incubation of these cells with different doses of PTH for 40 min caused internalization that was similar to that of the wild-type receptor (Fig. 8A).

**DISCUSSION**

Cellular responses to agonists of G protein-coupled receptors are initiated by a biochemical agonist-receptor interaction that rapidly alters the intracellular environment. Receptor activation initiates several cellular processes that include both stimulation of the signaling pathways and desensitization of the cellular response.

Downregulation and desensitization of the PTH/PTHrP receptor have been documented in several cell line models (3, 6, 7, 19, 28, 30, 34, 52, 56, 75). Inter-
nalization of a $^{125}$I-labeled PTH radioligand has also been demonstrated in cell lines expressing native PTH/PTHrP receptors (11, 68) and in cells transfected with recombinant PTH/PTHrP receptors (32, 36). Recently PTHrP (1, 33, 43, 44) and the PTH/PTHrP receptor (73, 74) have been localized to the nuclear and/or nucleolar regions and have been suggested to have a regulatory role in nuclear events; the process of internalization may play a role in delivering these molecules from the cell surface to the nucleus. In this study, we used cell lines expressing a functional GFP-tagged PTH/PTHrP receptor to examine internalization. Furthermore, cell lines expressing mutant PTH/PTHrP receptors deficient in PLC signaling, PTH analogs with selective AC signaling capability and variable receptor binding properties, and cell lines with defective PKA signaling were used to examine the signaling mechanisms regulating PTH/PTHrP receptor internalization. The data indicate that PTH/PTHrP receptor internalization does not require stimulation of AC/PKA or PLC/PKC; however, it requires ligand binding and receptor activation.

The biochemical characteristics of the ligand receptor interaction required for internalization of the PTH/PTHrP receptor are an area that needs extensive investigation. To achieve this goal, we compared the effects of a series of modified PTH peptides with variable binding and signaling properties on PTH/PTHrP receptor internalization. PTH-(1–34) and PTHrP-(1–36), which bind to the receptor with high affinity and increase its intrinsic activity, were able to stimulate internalization more efficiently than PTH-(1–28), which has a lower affinity and weaker agonist property. These results indicate that both binding and activation may be important for internalization. On the other hand, PTH-(3–34), a partial agonist/antagonist that binds to the receptor with high affinity, did not stimulate internalization; this suggests that binding alone is not sufficient to induce PTH/PTHrP receptor internalization. Similarly, [Ala$^{3,10,12},$Arg$^{11},$Trp$^{14}$]rPTH-(1–14), which has an enhanced receptor activation but does not show a detectable binding to the receptor (63), did not evoke internalization, thus indicating that receptor activation is not sufficient to stimulate internalization. Taken together, these data suggest that receptor internalization may require interaction with PTH analogs that show both stable binding and efficient activation. This notion may be supported by the finding...
that the short PTH peptides, [Ala\(^{3,12}\), Gln\(^{10}\), Har\(^{11}\), Trp\(^{14}\)]-rPTH-(1–14) and [Ala\(^{3,10,12}\), Arg\(^{11}\), Trp\(^{14}\)]rPTH-(1–14), which have enhanced binding and activation properties compared with the parent peptide PTH-(1–14) (62, 63), actually stimulated internalization. Alternatively, because these two peptides displayed better activation than other (1–14) peptides, there may be a threshold of receptor activation required for internalization.

The direct demonstration that both the PTH/PTHrP receptor and clathrin colocalize to the same internalized vesicles suggests that the PTH/PTHrP receptor internalizes through clathrin-coated pits. These data are in agreement with previous studies that indirectly assessed the role of clathrin in PTHrP receptor internalization using hyperosmolar sucrose to block radioligand internalization (36) or by demonstrating that the PTHrP receptor colocalizes with transferrin (26), a marker of clathrin-coated pits. Interestingly, colocalization of the PTHrP receptor with clathrin was detected not only at the cell membrane but in the perinuclear area as well. These data indicate involvement of clathrin-coated pits in both the early steps of internalization from the cell membrane and the late stage of transport to or in the perinuclear structure.

The PTH/PTHrP receptor is known to transmit signal through AC/PKA and PLC/PKC pathways. PTH was also shown to stimulate several other signaling pathways, such as mitogen-activated protein kinase (21, 72), phospholipase D (29, 64), and phospholipase A\(_2\) (23). The interactions of these signals at some point may play a crucial role in regulating receptor internalization. The data presented here suggest that the PLC/PKC signaling pathway does not play a critical role in PTH-induced PTHrP receptor internalization. First, we showed that PTH stimulated internalization of the DSEL mutant PTH/PTHrP receptor, which is deficient in PLC signaling. Second, the PTH analog [Gly\(^{1}\), Arg\(^{19}\)]hPTH(1–28), which increases cAMP accumulation but does not stimulate PLC (65), was a potent stimulator of internalization of both the wild-type and the DSEL mutant PTH/PTHrP receptor. Third, depletion of PKC content with chronic PMA treatment did not block internalization. Finally, the effects of the different PTH analogs on internalization did not correlate with their signaling potency. Taken together these data indicate that internalization of the PTH/PTHrP receptor is not mediated via activation of the PLC/PKC pathways. Recently, it was reported (26, 27) that PTH/PTHrP receptor internalization is blocked by...
staurosporine, an inhibitor of PKC, and that it was promoted by PMA (26, 27). The same investigators also showed that Bpa1-PTHrP-(1–36), which did not increase intracellular calcium concentrations but had normal cAMP stimulation, did not stimulate internalization. These data suggested that PTH/PTHrP receptor internalization is PKC dependent. The discrepancy between these data and ours may arise from the difference in the cell line model (LLC-PK1 vs. HEK293) and/or the receptor species (rat vs. human). Results gained by the use of phorbol esters are to be interpreted carefully, because they bind to non-PKC kinases (60, 71) and nonkinase molecules (2, 8, 17, 40, 50, 59).

Fig. 8. Effects of increasing concentrations of NlePTH or [Gly\(^1\),Arg\(^{19}\)]hPTH-(1–28) on the internalization of WT or DSEL mutant PTH/PTHrP receptors in presence or absence of a dominant negative mutant of the regulatory subunit of PKA. A: LLC-PK\(_1\) cell lines stably expressing WT-PPR, DSEL mutant PTH/PTHrP receptor (SD19), or DSEL mutant PTH/PTHrP receptor together with a mutant regulatory subunit of protein kinase A (PKA) (SD19AB4) were treated with increasing concentrations of NlePTH for 40 min at 37°C. Cell surface receptor immunoreactivity was measured using G48 antiserum. Control cells were treated with vehicle. Data are means ± SD of triplicates. B: LLC-PK\(_1\) cell lines stably expressing WT-PPR or DSEL mutant PTH/PTHrP receptor (SD19) were treated with increasing concentrations of [Gly\(^1\),Arg\(^{19}\)]hPTH-(1–28) for 40 min at 37°C. Cell surface receptor immunoreactivity was measured using G48 antiserum. Control cells were treated with vehicle. Data are means ± SD of triplicates.

Fig. 9. Effects of forskolin, IBMX, or phorbol-12-myristate-13-acetate (PMA) on PTH/PTHrP receptor internalization. A: Western blot analysis of membrane and cytosolic fractions from LLC-PK\(_1\) cells stably expressing the WT-PPR after treatment with PMA for 20 min (1 μM) or 16 h (400 nM) using anti-PKCα antibody. B: effects of forskolin, IBMX, or PMA on WT-PPR internalization. LLC-PK\(_1\) cell line stably expressing WT-PPR was treated with forskolin (30 μM) or IBMX (2 mM) for 10 min or with PMA (1 μM) for 20 min, then NlePTH (100 nM) was added, and the incubation was continued for an additional 40 min (1st 4 sets of columns) or with PMA (400 nM) for 16 h, and then with PTH for 40 min (last set of columns). Cell surface PTH/PTHrP receptor immunoreactivity was measured using G48 antiserum. Control cells were treated with vehicle. Data are means ± SD of triplicates.
Additionally, staurosporine becomes a nonselective kinase inhibitor when used at a micromolar concentration (69).

PTH-induced PTH/PTHrP receptor internalization in a cell line overexpressing a dominant negative PKA regulatory subunit suggests that stimulation of PKA is not required for internalization. These data are further supported by the finding that pharmacological concentrations of forskolin or IBMX did not influence PTH/PTHrP receptor internalization. This is in agreement with our previous report (67) and those of others (6, 7) showing that downregulation of the PTH/PTHrP receptor can occur via cAMP-independent mechanisms. Similarly, desensitization of recombinant PTH/PTHrP receptors expressed in LLC-PK1 cells was not affected by overexpression of a mutant regulatory subunit of PKA (32).

PTH has been shown to cause a rapid and transient phosphorylation of the PTH/PTHrP receptor on its carboxy-terminal tail (12, 13, 57). Agonist-stimulated PTH/PTHrP receptor phosphorylation was not blocked by inhibitors of PKA or PKC. Because phosphorylation is important for PTH/PTHrP receptor internalization (our unpublished data), these data then support the notion that PTH/PTHrP receptor internalization is not dependent on PKC or PKA.

Collectively, the results suggest that the PTH/PTHrP receptor undergoes internalization in response to PTH treatment in a dose- and time-dependent manner. The process of internalization involves clathrin-coated pits. The data also show that AC/PKA and PLC/PKC pathways do not play a major role in receptor internalization. Therefore, internalization may involve a novel signaling pathway and/or a critical receptor conformation that requires a stable agonist binding.

We thank Dr. Thomas Gardella for providing the PTH-(1–14) analogs and Dr. Richard F. Bringhurst for providing the [Gly1,Arg19]hPTH-(1–28) peptide and the SD19 and SD19AB4 LLC-PK1 stable cell lines. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-11794–26 and NRSA, DK-10087–01.

REFERENCES


23. Derrickson BH and Mandel LJ. Parathyroid hormone inhibits Na+-K+-ATPase through Gs/Gt and the calcium-indepen-


40. Kaczanietz MG, Lewin NE, Bruns JD, and Blumberg PM. Characterization of the cysteine-rich region of the Caenorhabditis elegans protein Unc-13 as a high affinity phorbol ester receptor.


55. Qian F, Leung A, and Abou-Samra AB. Agonist-dependent phosphorylation of the parathyroid hormone parathyroid hor-


