Internalization Determinants of the Parathyroid Hormone Receptor Differentially Regulate β-Arrestin/Receptor Association*

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β-Arrestins have been implicated in regulating internalization of the parathyroid hormone receptor (PTHR), but the structural features in the receptor required for this effect are unknown. In the present study performed in HEK-293 cells, we demonstrated that different topological domains of PTHR are implicated in agonist-dependent receptor internalization; truncation of the cytoplasmic tail (PTHR-TR), selective mutations of the cytoplasmic tail to remove the sites of parathyroid hormone (PTH)-stimulated phosphorylation (PTHR-PD), and mutations in the third transmembrane helix (N289A) or in the third cytoplasmic loop (K382A) resulted in a 30–60% reduction in ^{125}I-PTH-related protein internalization. To better define the role of these internalization determinants, we have tested the ability of these mutant PTHRs to associate with β-arrestins by using three different methodological approaches: 1) ability of overexpression of β-arrestins to restore the internalization of ^{125}I-PTH-related protein for the mutant PTHRs; 2) visualization of PTH-mediated trafficking of β-arrestin1 and -2 fused to the green fluorescent protein with receptors by confocal microscopy; 3) quantification of β-arrestin1-green fluorescent protein translocation by Western blot. Our data reveal that the receptor' cytoplasmic tail contains determinants of β-arrestin interaction that are distinct from the phosphorylation sites and are sufficient for transient association of β-arrestin2, but stable association requires receptor phosphorylation. Determinants in the receptor's core (Asn-289 and Lys-382) appear to regulate internalization of the receptor/β-arrestin complex toward early endocytic endosomes during the initial step of endocytosis.

Agonist binding to most G protein-coupled receptors (GPCRs)[1] is quickly followed by the internalization of the agonist-receptor complex into endocytic vesicles. The model developed from studies of the β_2-adrenergic receptor views internalization as a process facilitated by binding of β-arrestin proteins to agonist-activated receptors after phosphorylation of the receptors by G protein-coupled receptor kinases (GRKs) (1, 2). Phosphorylation of GPCRs by GRKs is a prerequisite for the mobilization of cytosolic β-arrestins. Binding of β-arrestins to GRK-phosphorylated receptors results in the physical uncoupling of receptors from their cognate G proteins and terminates agonist-mediated signaling (3, 4). It was shown recently that β-arrestins bind clathrin, a major component of the clathrin-based endocytic machinery, with high affinity and serve as an adaptor that targets activated and phosphorylated receptors to clathrin-coated pits (5–7). In the case of the β_2-adrenergic receptor, receptor internalization is the consequence of the formation of a complex between β-arrestin2, the clathrin adaptor protein AP2, clathrin, and the activated receptor (8). Internalization has at least two outcomes: directing the receptor to a compartment where the phosphates are removed, allowing resensitization, and movement of the receptor to lysosomes for degradation (2, 9, 10).

Little is known about the structural determinants of GPCRs involved in receptor internalization and/or in arrestin interaction. The current model for the understanding of the arrestin-receptor interaction mechanism is based on various studies of visual arrestin interaction with rhodopsin (11–16). Visual arrestin binding with rhodopsin requires GRK-1 phosphorylated residues, and the contact of phosphorylated residues with a cationic region of arrestin switches it into an active conformation, enabling interaction with an exposed binding site on activated rhodopsin.

The receptor for parathyroid hormone (PTH) and PTH-related protein (PTHrP) is involved in the regulation of calcium homeostasis and in bone remodeling (17). Agonist occupancy of the PTH/PTHrP receptor (PTHR) leads to activation of adenylyl cyclase (via G_s), and phosphatidylinositol-specific phospholipase C_β (via G_q). PTH-induced activation of the PTHR results in the internalization of the PTH-receptor complex via the clathrin-coated pit pathway and involves β-arrestin2 (18, 19). Recently, we have demonstrated that neither the internalization nor the mobilization of β-arrestins (*i.e.* β-arrestin1 and β-arrestin2) to PTHR required the receptor activation isoform that is necessary for activation of both G_s and G_q proteins (*i.e.*

[1] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; PTHR, parathyroid hormone/parathyroid hormone-related protein receptor; GFP, green fluorescent protein; PBS, phosphate-buffered saline; DHB, serum-free Dulbecco's modified Eagle's medium containing HEPES and bovine serum albumin; TR, truncation; PD, phosphorylation-deficient.

relative displacement of helix 3 and helix 6 of the receptor; Ref. 20). This indicates that G protein activation is not an absolute prerequisite for receptor internalization. In the present study, we investigated the molecular basis of PTHR internalization by examining the interaction of β-arrestins with a series of internalization-deficient receptors.

EXPERIMENTAL PROCEDURES

Materials—PTH-(1–34) was obtained from Bachem. β-Arrestin2 and β-arrestin1 were fused to the GFP as described (20, 21). The preparation of β-arrestin1 antibody has been described previously (22). PTHR and GFP antibodies were obtained from BAbCO (Richmond, CA) and CLONTECH (Palo Alto, CA), respectively. Expression vectors pCDNA3 and pCEP4 were from Invitrogen (San Diego, CA).

Receptor cDNAs—Site-directed mutagenesis was performed on the opossum PTH/PTHrP receptor cDNA (23). Constructions of PTHR-PD (phosphorylation-deficient receptor, mutation of serine residues at positions 483, 485, 486, 488, 495, and 498 to alanine residues), PTHR-TR (carboxyl terminus-truncated receptor after the residue 474) and K382A (mutation of Lys-382 to Ala, in the third intracellular loop near helix 5) have been described previously (24–26). Construction of N289A (mutation of Asn-289 to Ala, in helix 3) was performed using the transformer site-directed mutagenesis kit (CLONTECH, Palo Alto, CA) based on the method described by Deng and Nickoloff (27). Mutations were verified by sequencing. Receptor cDNAs were cloned into pCDNA3 and pCEP4 for transient and stable expression, respectively. Arrestin cDNAs (β-arrestin1, β-arrestin2, β-arrestin1-GFP, and β-arrestin2-GFP) were cloned into pCDNA3.

Receptor Expression—HEK-293 cells (ATCC, CRL 1573) served as the expression system for the wild type and mutant receptors. Cells were maintained in culture at 37 °C under a humidified atmosphere with 7% CO_2 in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Invitrogen). Cells were transfected using a protocol based on the calcium-phosphate method as described previously (28). Selection of stably transfected cells was initiated 2 days after transfection by the addition of hygromycin (200 μg/ml). Selection was generally complete after 3–4 weeks of hygromycin treatment. Stock cell lines were cultured in the continuous presence of hygromycin, except when subcultured for experiments in which case hygromycin was omitted.

Intact Cell Phosphorylation—To measure phosphorylation of the PTH receptors in intact cells, transiently transfected HEK-293 cells were labeled in six-well plates with 100 μCi/well [^{32}P]orthophosphate in phosphate-free Dulbecco's modified Eagle's medium for 2 h. Labeled cells were stimulated as indicated, solubilized in 0.8 ml of radioimmune precipitation buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA, 50 mM NaF) for 30 min on ice, and the receptors were immunoprecipitated with 10 μg of mouse monoclonal anti-PTHR antibody. Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and PhosphorImager analysis.

Determination of Receptor Internalization Kinetics—Transfected cells were grown for 3 days in six-well dishes. After incubation for 1 h at 37 °C with 1 ml of DHB (serum-free Dulbecco's modified Eagle's medium containing 20 mM HEPES and 0.1% bovine serum albumin), cells were incubated with 0.5 ml of DHB containing ^{125}I-PTHrP-(1–34) (100,000 cpm) at 37 °C for the indicated times. Previous studies have demonstrated that PTH-(1–34) and PTHrP-(1–34) bind equipotently the PTH/PTHrP receptor (23). To stop the incubation, the cells were placed on ice and rapidly washed with 1 ml of ice-cold phosphate-buffered saline (PBS). The cells were incubated for 2 × 5 min in 0.5 ml of acid wash solution (150 mM glycine/50 mM acetic acid, pH = 3) to remove the surface-bound radioligand. The supernatants containing the acid-released radioactivity were collected, and the cells were treated with 1 ml of 0.8 N NaOH to solubilize the acid-resistant radioactivity. The nonspecific binding was measured in parallel experiments using 10^{-6} M PTH-(1–34). The radioactivity was quantified in a γ-counter. The percentage of internalization was calculated after deduction of the respective nonspecific value: % internalization = [(cpm acid-resistant)/ (cpm acid-resistant + cpm acid-released)] × 100. The data were fitted to single-phase exponential curves. The endocytic rate constant k_e was determined as reported previously (20).

Assays for Receptor Function—Ligand binding and cAMP assays were carried out following our previously published procedures (29). In brief, for binding studies, transfected cells were incubated in DHB buffer for 2 h at 0 °C, followed by a 2-h incubation with the same buffer containing 100,000 cpm ^{125}I-PTH-(1–34) with or without unlabeled PTH-(1–34). Cells were washed twice with iced phosphate-buffered saline and extracted with 0.8 N NaOH, and cell-associated ^{125}I-PTH-(1–34) was counted. Competition curves were fitted to a one-site competitive binding curve. For cAMP assays, cells were washed twice with HEPES buffer and incubated with isobutylmethylxanthine (0.5 mM) in the same buffer for 1 h at room temperature. Cells were stimulated with different PTH-(1–34) concentrations at 37 °C for 10 min. Cellular cAMP was extracted and measured by radioimmunoassay (Immunotech).

Assays for β-Arrestin Translocation and Colocalization with the PTHR—β-Arrestin translocation was monitored by two assays: confocal scanning microscopy visualization in intact cells and immunoblot analysis.

β-Arrestin1 or β-arrestin2 translocation assays were performed in transiently transfected HEK-293 cells. The cells were grown on glass coverslips coated with poly-L-lysine and cotransfected with cDNAs of β-arrestin2 fused to GFP (β-arrestin2-GFP) or β-arrestin1-GFP and PTHR. 48 h after transfection, cells were incubated with 100 nM PTH-(1–34) for different times, and fixed for 20 min in 4% paraformaldehyde. Fixed cells were observed with an oil immersion objective (×40) using a Leica (TCS) confocal laser microscope.

Colocalization of β-arrestin2-GFP with rhodamine-labeled wild type or mutant receptors was performed on HEK-293 cells stably expressing the wild type or the mutant PTHR. Cells were grown on glass coverslips and transiently transfected with β-arrestin2-GFP cDNA. After 48 h of transfection, the cells were incubated with 1 μM PTH for different times at 37 °C. Then, the cells were fixed for 20 min in 4% paraformaldehyde and permeabilized using 0.2% Triton. The cells were incubated with mouse OK-1 PTHR antibody at 4 °C overnight. After washing with PBS, the cells were incubated with donkey anti-mouse IgG-rhodamine conjugate secondary antibody at room temperature for 1 h. The cells were mounted on glass slides and examined with a Leica TCS NT/SP confocal microscope.

For immunodetection, transiently transfected HEK-293 cells were detached with a rubber policeman and pelleted by low speed centrifugation; the supernatant was discarded and the cells were lysed in PBS containing protease inhibitors (Calbiochem) by sonication. The lysate was centrifuged at 800–1000 × g for 10 min to remove unbroken cells, and the supernatant was further centrifuged at 20,000 × g for 30 min. The resulting supernatant represents the cytosolic fraction and the pellet the membrane fraction. Membrane proteins solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with a protease inhibitor mixture (Calbiochem) and soluble protein fractions of transfected cells coexpressing PTHR and β-arrestin1-GFP or β-arrestin2-GFP were separated on 7.5% SDS-PAGE. Proteins were transferred onto an Immobilon P transfer membrane (Millipore, Bedford, MA). Membranes were reacted with anti-β-arrestin1 antibody (1:3000) or anti-GFP (1:5000), and anti-rabbit IgG (1:20,000) coupled to peroxidase, and immunoreactive bands were visualized using chemiluminescence (Pierce). The data were calibrated for similar levels of surface receptors.

Data Analysis—Concentration/response, competition and internalization kinetics were analyzed by computer-assisted nonlinear regression (GraphPad, San Diego, CA). If not otherwise indicated, all experiments were replicated at least three times in independent experiments, and the results are expressed as mean ± S.E.

RESULTS

Characterization of Agonist-dependent Internalization of PTHR in HEK-293 Cells—As shown in Fig. 1*A*, the PTHR rapidly internalizes in response to agonist when expressed transiently in HEK-293 cells. As monitored by radioligand binding, the maximal extent of internalization was observed after 20 min and represents 55–60% of the cell-specific associated [^{125}I-PTHrP]. The presence of 0.45 M sucrose (an agent that destabilizes clathrin-coated pits; Ref. 30) reduced the maximal extent of PTHR internalization by 80%. This supports the involvement of clathrin-coated pits in PTH-mediated PTHR internalization (Fig. 1*A*). Overexpression of β-arrestin2 enhanced the maximal extent of PTHR internalization by 15–20%, whereas overexpression of β-arrestin1 did not significantly increase the magnitude of PTHR internalization (Fig. 1*A*). Similar effects were observed after overexpression of functional β-arrestins fused to the green fluorescent protein (*i.e.* β-arrestin1-GFP or β-arrestin2-GFP, data not shown). Overexpression of β-arrestins did not modify the endocytic rate con-
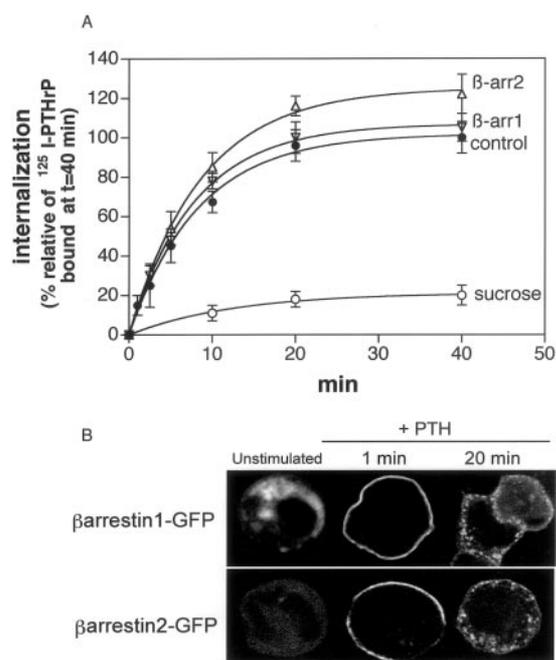
FIG. 1. **Characterization of agonist-dependent internalization of PTHR expressed in HEK-293 cells.** *A*, internalization of wild type PTHRs transiently expressed in HEK-293 cells were measured using internalization of ^{125}I-PTHrP-(1–34) for up to 40 min. Cells were assayed 48 h after transfection with cDNAs for PTHR alone (*control*) or PTHR plus β-arrestin1 (*β-arr1*) or β-arrestin2 (*β-arr2*). The effect of 0.45 M of sucrose on PTHR internalization was also measured (*sucrose*). Acid-resistant and acid-sensitive binding were determined and the specific internalized binding was determined as described under "Experimental Procedures." The maximal extent of ^{125}I-PTHrP-(1–34) internalization was for control (60 ± 5.2%, $n = 3$), β-arrestin1 (63 ± 6.5%, $n = 3$), β-arrestin2 (75 ± 5.0%, $n = 3$), and sucrose (12 ± 2.2%, $n = 3$). The internalization value of the control (PTHR alone) at $t = 40$ min was set as 100%. *B*, β-arrestin-GFP translocation with the activated PTHR. HEK-293 cells transiently coexpressing the PTHR and β-arrestin1-GFP or β-arrestin2-GFP were incubated at 37 °C with or without PTH (100 nM) for the times indicated. Cells were then fixed with 4% paraformaldehyde. Images were visualized on a confocal laser scanning microscope and are representative of an entire population of cells of three independent experiments.

stant of the PTHR ($k_e \approx 0.10$ min^{-1} for both β-arrestin isoforms *versus* $k_e = 0.12 \pm 0.03$ min^{-1} for PTHR).

Confocal scanning microscopy was used to visualize the translocation of β-arrestin1-GFP and β-arrestin2-GFP to the PTHR in response to PTH in transiently transfected HEK-293 cells. Fig. 1*B* shows that β-arrestin1-GFP and β-arrestin2-GFP were homogeneously localized in the cytosol of cells expressing the PTHR at the resting state. Within minutes, upon PTH stimulation (100 nM), β-arrestin1-GFP and β-arrestin2-GFP translocated equally well from the cytosol to the cell membrane (Fig. 1*B*, *1 min*). After longer PTH exposure, β-arrestin1-GFP and β-arrestin2-GFP clusters were present inside the cell (Fig. 1*B*, *20 min*). The punctated pattern of β-arrestin-GFP fluorescence presumably reflected internalization of PTHR with β-arrestins into endocytic vesicles. These data demonstrated that both β-arrestin isoforms translocate equally well to the activated receptor and show, as do recent studies for β-arrestin2 (18, 19), the involvement of β-arrestins in PTH-mediated PTHR internalization.

Different Topological Domains of PTHR Are Implicated in PTH-dependent Receptor Internalization—We found a series of PTHR mutants with a reduced ability to internalize [^{125}I-PTHrP] when transiently expressed in HEK-293 cells (Fig. 2, *A* and *B*). Truncation of the cytoplasmic tail (PTHR-TR) resulted in a severe loss of internalization (50% relative to the wild type
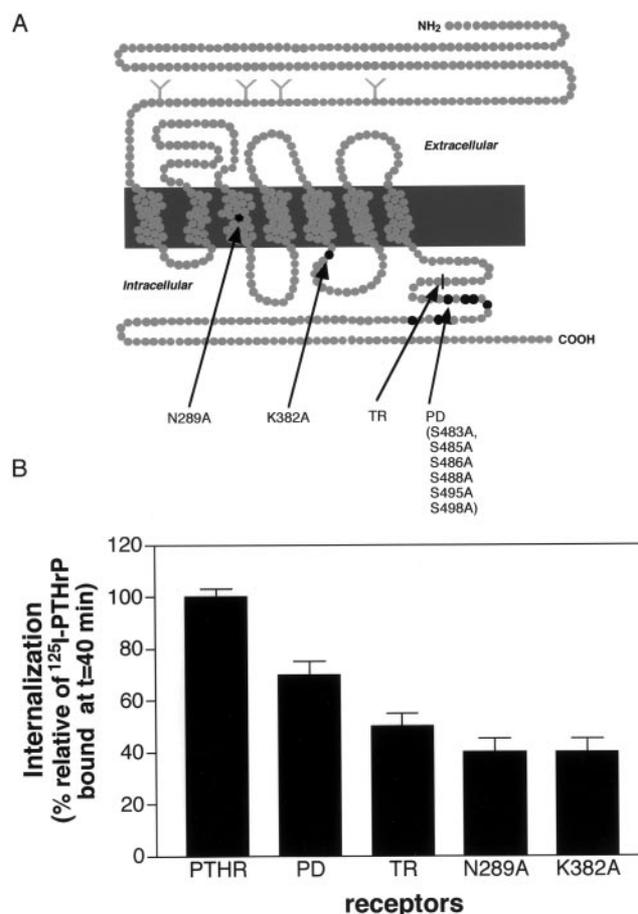
FIG. 2. **Multiple domains of PTHR mediate PTH-induced receptor internalization.** *A*, schematic representation of the PTHR. Mutated residues in PTHR are shown in *black circles*. *B*, HEK-293 cells transiently expressing the wild type or mutant receptors were incubated with ^{125}I-PTHrP-(1–34) at 37 °C for 40 min. Acid-resistant and acid-sensitive binding were determined, and the specific internalized binding was determined as described under "Experimental Procedures." The maximal extent of ^{125}I-PTHrP-(1–34) internalization was for PTHR (60 ± 3.8%, $n = 20$), PTHR-PD (42 ± 0.32%, $n = 12$), PTHR-TR (30 ± 2.3%, $n = 8$), N289A (24 ± 2.5%, $n = 8$), and K382A (22 ± 1.5%, $n = 9$). The internalization of PTHR control was set to 100%, and each *bar* represents the mean ± S.E.

PTHR). Mutation of the cytoplasmic tail to remove the sites of PTH-stimulated phosphorylation (PTHR-PD) produced a 30% reduction (relative to the wild type receptor) in PTHR internalization. Two additional mutations in the body of the PTHR, N289A (in the third transmembrane helix) and mutation K382A (in the third cytoplasmic loop), were found to reduce receptor internalization by 60%. Similar internalization deficiency was also observed in HEK-293 cells stably expressing PTHR-TR, N289A, or K382A (data not shown). In contrast, however, PTHR-PD stably expressed in HEK-293 cells internalizes to levels similar to that observed for PTHR in response to agonist (reported previously in Ref. 25).

The pharmacological properties of PTHR, PTHR-PD, PTHR-TR, and K382A have been described in our previous studies (24–26). The wild type as well as mutant receptors displayed comparable cell surface receptor expression and similar high PTH binding affinity (Table I). N289A displayed a 4-fold decrease in binding affinity and a 2-fold higher EC_{50} value (Table I). K382A displayed a strong reduction of the cAMP (Table I) as well as inositol phosphate responses (data not shown), confirming our previous results (24).

Phosphorylation of Wild Type and Mutant Receptors—Because agonist-mediated receptor phosphorylation might facili-

TABLE I
Binding and signaling characteristics of wild type and mutants of PTHR

HEK-293 cells transiently expressing the receptors were examined for ^{125}I-PTHrP binding competition and PTH-(1–34)-mediated cAMP production. Basal and maximal PTH-(1–34)-mediated cAMP production were 2–3 and 70–80 pmol/30-mm well ($n = 3$), respectively.

	Receptors/cell	K_i	PTH-mediated cAMP	
			% of maximum	EC$_{50}$
	$\times 10^6$	nM		nM
PTHR	0.80 ± 0.02	2.3 ± 0.1	100	0.25 ± 0.05
N289A	0.90 ± 0.08	9.4 ± 1.1	100 ± 17	0.50 ± 0.05
K382A	0.86 ± 0.05	2.9 ± 0.5	5 ± 0.8	0.30 ± 0.03
PTHR-TR	0.69 ± 0.03	1.4 ± 0.2	120 ± 5	0.31 ± 0.16
PTHR-PD	0.61 ± 0.05	1.8 ± 0.3	100 ± 15	0.17 ± 0.08

tate PTH-mediated receptor internalization, we examined the relationship between PTH-dependent internalization and phosphorylation of the receptors. As demonstrated previously (25, 31), PTHR undergoes a PTH-dependent ≈3-fold increase in phosphorylation, and then runs as a broad band at approximately 85 kDa on an autoradiograph (data not shown). Truncation of the tail (PTHR-TR) or mutation of certain Ser residues in the tail (as in PTHR-PD) abolished PTH-promoted receptor phosphorylation. In contrast, the mutant N289A and the signaling-impaired receptor K382A maintained intact phosphorylation characteristics (Fig. 3). These results show that the absence of phosphorylation in PTHR-TR and PTHR-PD may be responsible for the internalization defect. However, our data for N289A and K382A indicate that the decrease of PTH-mediated internalization can occur even when there is no reduction in receptor phosphorylation. Thus, additional mechanisms must regulate PTHR internalization.

Differential Effect of β-Arrestins on the Internalization of PTHR Mutants—Given that β-arrestins are involved in the process of PTHR internalization, we next assessed if the diverse mutations reduce PTHR internalization through a β-arrestin-dependent mechanism. We reasoned that overexpression of β-arrestins with the internalization-deficient receptors might compensate for a possible decrease in receptor affinity for β-arrestins. We coexpressed β-arrestin2 or β-arrestin1 with the different mutants in transiently transfected HEK-293 cells and tested by a radioligand assay the specific agonist-mediated internalization of the wild type and mutant receptors. Fig. 4 shows that overexpression of β-arrestin2 restored the internalization defect of the phosphorylation-deficient and the truncated PTHRs to 80–85% of that seen with the wild type PTHR in presence of overexpressed β-arrestin2. As observed for β-arrestin2, overexpression of β-arrestin1 ameliorated the internalization extent of PTHR-TR (to 75% of the wild type PTHR+β-arrestin1) but was less able to increase the internalization defect of PTHR-PD. In contrast to the cytoplasmic tail mutants, β-arrestin1 and β-arrestin2 were much less effective in promoting internalization of N289A and K382A mutant receptors; β-arrestin2 or β-arrestin1 only weakly rescued the internalization of these receptors (40–45% of wild type PTHR+β-arrestins).

Kinetic analysis of receptor internalization was performed in the absence or presence of exogenous β-arrestin2. Fig. 5 shows that, in the absence of β-arrestin2, the internalization-deficient receptors internalized at a lower rate than wild-type PTHR ($k_e \approx 0.06$–0.09 min^{-1} for the mutants *versus* 0.12 min^{-1} for the wild-type receptor). Coexpression of β-arrestin2 had no effect on the internalization rate of N289A and K382A ($k_e = 0.060 \pm 0.004$ min^{-1} and 0.080 ± 0.004 min^{-1}, respectively), but resulted in a significant increase in the internalization rate constant of PTHR-TR and PTHR-PD (k_e in min^{-1} for PTHR-TR =

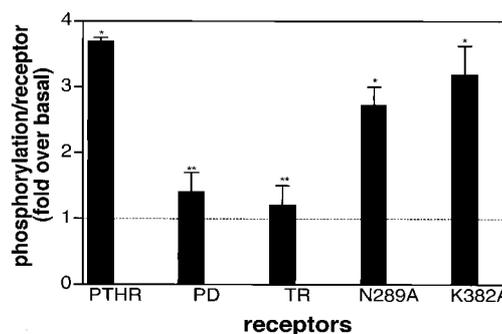FIG. 3. **PTH-mediated phosphorylation of wild type and mutant PTHR in intact cells.** Transfected cells were labeled with ^{32}P and incubated with or without PTH (1 μM) for 10 min. The receptors were immunoprecipitated and resolved by 8% SDS-PAGE, and phosphorylation was quantified by PhosphorImager analysis of the autoradiograph. PTH-mediated receptor phosphorylation (-fold over basal): PTHR = 3.68 ± 0.06 ($n = 7$), N289A = 2.72 ± 0.28 ($n = 4$), K382A = 3.18 ± 0.44 ($n = 4$), PTHR-PD = 1.10 ± 0.27 ($n = 4$), PTHR-TR = 1.05 ± 0.05 ($n = 2$). Bars represent the means ± S.D. of two to seven independent experiments. *, $p < 0.05$ and **, $p < 0.01$ when compared with PTHR.
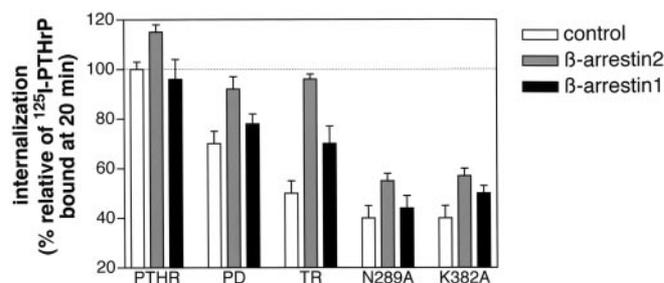

FIG. 4. **Effect of β-arrestins on the internalization of wild type and mutant PTHR.** Internalization of wild type or mutant PTHRs transiently coexpressed with β-arrestin1 or β-arrestin2 in HEK-293 cells were measured using internalization of ^{125}I-PTHrP-(1–34) for 20 min as described under "Experimental Procedures." The mutants designated *PD* and *TR* refer to PTHR-PD and PTHR-TR, respectively. The extent of ^{125}I-PTHrP-(1–34) internalization was for PTHR (control: 60 ± 3.8%, $n = 20$; β-arrestin1: 57.6 ± 4.8%, $n = 10$; β-arrestin2: 70 ± 4%, $n = 17$), PTHR-PD (42 ± 3.2%, $n = 12$; β-arrestin1: 47 ± 3%, $n = 5$; β-arrestin2: 55.2 ± 3.1%, $n = 8$), PTHR-TR (30 ± 2.3%, $n = 8$; β-arrestin1: 42 ± 4%, $n = 5$; β-arrestin2: 57.6 ± 2.0%, $n = 9$), N289A (24 ± 2.5%, $n = 8$; β-arrestin1: 26.4 ± 2.6%, $n = 3$; β-arrestin2: 33.0 ± 2.9%, $n = 3$), and K382A (22 ± 2.5%, $n = 9$; β-arrestin1: 27.5 ± 1.9%, $n = 4$; β-arrestin2: 31.4 ± 2.0% $n = 6$). The internalization of PTHR control was set to 100%, and each *bar* represents the mean ± S.E.

0.17 ± 0.01 and for PTHR-PD = 0.12 ± 0.03). These results demonstrated the existence of two classes of receptor mutants. Class 1 mutants (the cytoplasmic tail mutants: PTHR-TR and PTHR-PD) are those where overexpression of β-arrestins can partially rescue agonist-mediated internalization; class 2 mutants (N289A and K382A) are those where overexpression of β-arrestins was not or poorly effective in the amelioration of PTHR internalization. The absence of phosphorylation in the class 1 mutants could reduce the affinity of the PTHR for β-arrestins, and overexpression of β-arrestins might therefore partially compensate for the decrease in receptor affinity for arrestin. The reduced ability of exogenous β-arrestins to restore internalization of class 2 mutants might be the consequence of a weak (or absent) PTH-induced β-arrestin association. Alternatively, the absence of sensitivity to overexpressed β-arrestins might reflect a defect in the internalization process of N289A and K382A that is independent of β-arrestin.

Confocal Imaging of PTH-mediated Trafficking of β-Arrestin2-GFP with the Wild Type or with the Mutant Receptors—To examine more directly the association of β-arrestin2, wild type, and mutant receptors, we visualized by confocal scanning mi-
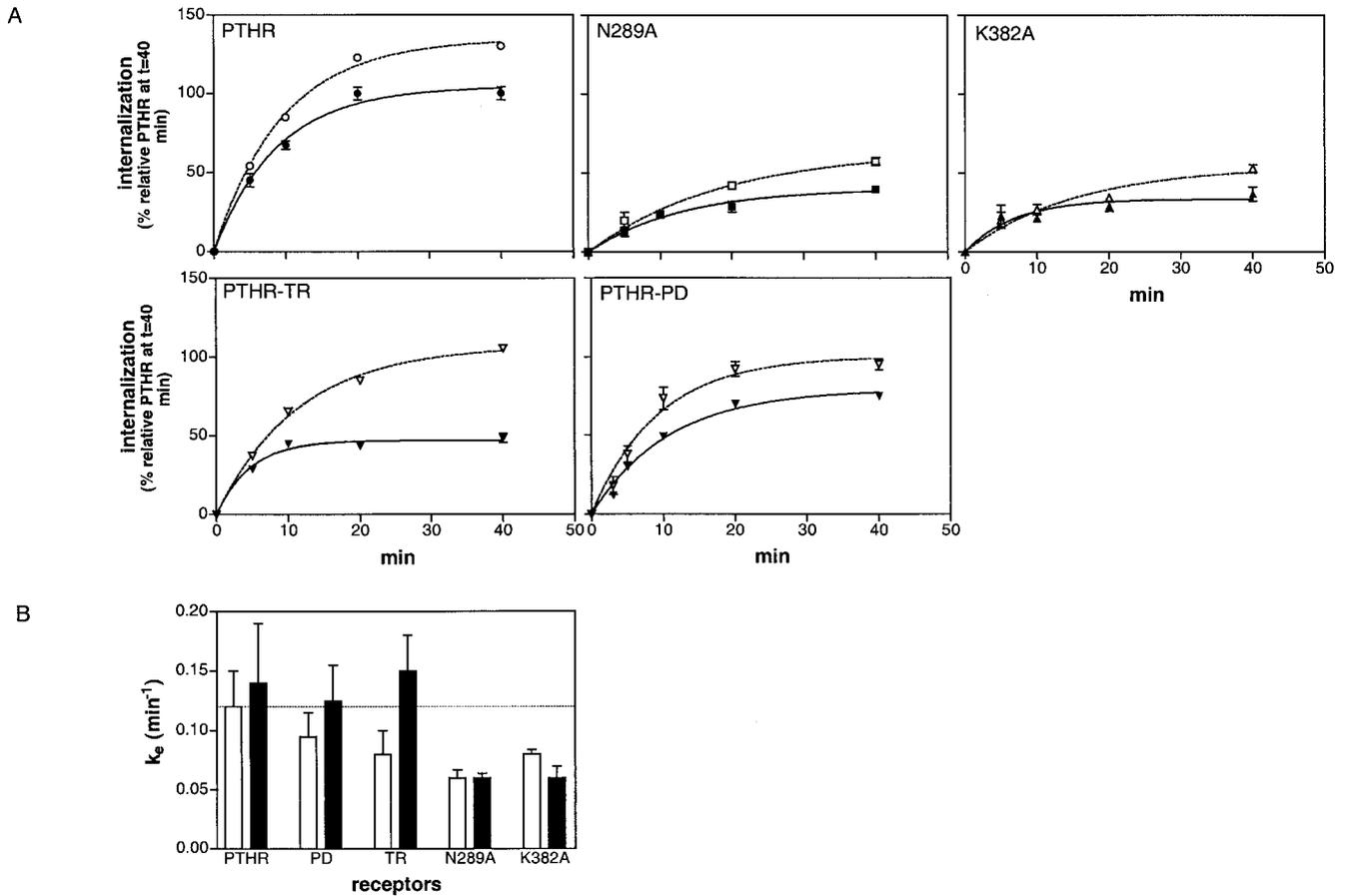
FIG. 5. **Kinetics for agonist-dependent internalization of the wild type and mutant PTHR in the absence or presence of overexpressed β-arrestin2.** *A*, time course of agonist-mediated internalization of wild-type and mutant receptors in the absence (*solid line*) and presence of overexpressed β-arrestin2 (*dotted line*) was performed as described under "Experimental Procedures." The data represent the mean ± S.E. of three or four independent experiments performed in triplicate. *B*, endocytic rate constants (k_e) in the absence (*white bars*) and presence (*black bars*) of overexpressed β-arrestin2 were calculated as described under "Experimental Procedures." Each *bar* represents the mean ± S.D. of three or four independent experiments.

croscopy the trafficking of β-arrestin2-GFP in response to PTH in HEK-293 cells stably expressing the receptors. For these experiments, receptors were labeled with the rhodamine-conjugated anti-PTHR mouse monoclonal antibody and the colocalization between the receptor and the transiently expressed β-arrestin2-GFP was visualized by merging the green and red fluorescence, resulting in a yellow signal.

Fig. 6*A* shows that in the absence of PTH, the PTHR was present at the plasma membrane (red fluorescence) and β-arrestin2-GFP was homogeneously localized throughout the cytosol (green fluorescence). Addition of PTH promoted a colocalization of the PTHR with β-arrestin2-GFP at the plasma membrane (*t* = 2 min). After 5-min exposure with PTH, PTHR colocalized with β-arrestin2-GFP in vesicles close to the plasma membrane presumably reflecting the localization of the receptor/arrestin complex in clathrin-coated pits. Later (*t* = 30 min) the entire population of receptor apparently colocalized with β-arrestin2-GFP in vesicular structures. Thus, agonist-activated PTHR forms a stable complex with β-arrestin2 that is maintained in endocytic vesicles after 30 min (Fig. 6*A*) but also after 1 h (data not shown).

Different results were obtained with the cytoplasmic tail mutant receptors. As observed for cells expressing the wild type PTHR in the absence of PTH, β-arrestin2-GFP was homogeneously localized in the cytosol of cells expressing the mutants PTHR-TR or PTHR-PD (Fig. 6, *B* and *C*). β-Arrestin2-GFP translocation was clearly diminished in cells expressing PTHR-TR (Fig. 6*B* at *t* = 2 and 5 min). Indeed, this receptor

was not able to deplete the pool of cytosolic β-arrestin2-GFP as efficiently as observed for the wild type PTHR. The absence of phosphorylation in PTHR-TR was not responsible for the weak translocation of β-arrestin2, given that PTHR-PD retained the ability to translocate efficiently β-arrestin2-GFP in response to PTH (Fig. 6*C*, *t* = 2 and 5 min). Consistent with the ability of exogenous β-arrestin2 to increase agonist-internalization of PTHR-TR and PTHR-PD, β-arrestin2-GFP/receptor complex showed with time a punctate pattern in the cytosol (Fig. 6, *B* and *C* at *t* = 30 min). However, PTHR-PD differed from the wild type PTHR at longer PTH incubation (*t* = 30 min), in that β-arrestin2-GFP interacted transiently with PTHR-PD whereas it interacted stably with the PTHR (Fig. 6, compare *A* and *C* at *t* = 30 min). These data suggest that the receptor's cytoplasmic tail contains determinants of β-arrestin2 interaction that are distinct from the phosphorylation sites and are sufficient for transient association of β-arrestin2, but stable association requires receptor phosphorylation.

No colocalization between β-arrestin2-GFP and N289A or K382A was observed in absence of PTH (Fig. 6, *D* and *E*, *t* = 0 min). After 2 min of PTH incubation, β-arrestin2-GFP translocated to the plasma membrane of cells expressing N289A or K382A (Fig. 6, *D* and *E*). After a 5-min treatment with PTH, the β-arrestin2-GFP/receptor complex appears uniformly distributed at the periphery of the cell. This contrasts with cells expressing the wild type PTHR, where β-arrestin2-GFP clusters were clearly visible at or near the plasma membrane (Fig. 6*A*, *5 min*). By 30 min of PTH exposure, however, some clusters
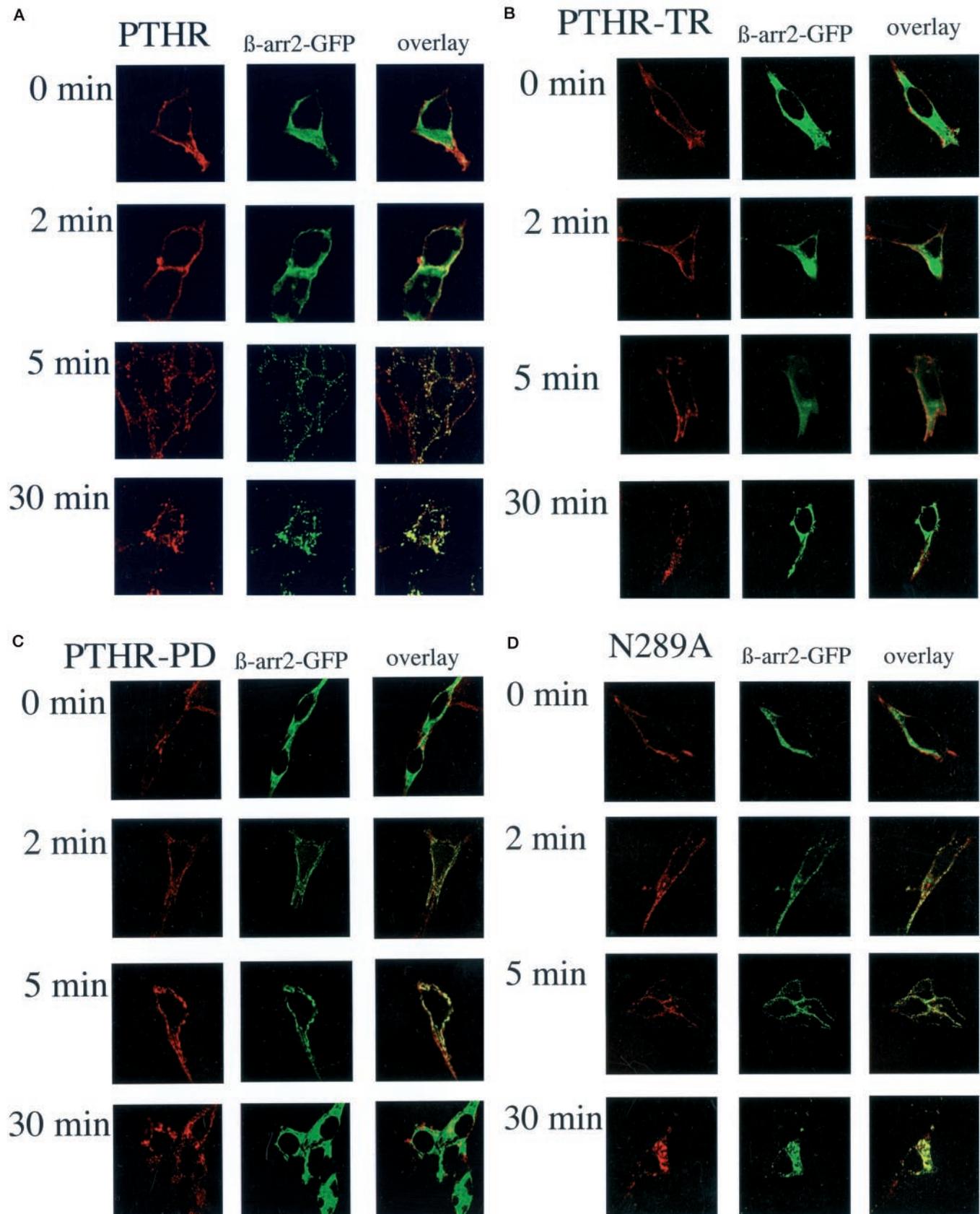
FIG. 6. **Fluorescence confocal microscopy of the colocalization of β-arrestin2-GFP and PTHRs in HEK-293 cells stably expressing wild type or mutant PTHR.** Colocalization of β-arrestin2-GFP with rhodamine-labeled PTH receptor was performed on HEK-293 cells stably expressing the wild type receptor (*A*), PTH-TR (*B*), PTHR-PD (*C*), N289A (*D*), or K382A (*E*). Cells were grown on glass coverslips and transfected with β-arrestin2-GFP cDNA. After 48 h of transfection, the cells were incubated with 1 μM PTH for different times at 37 °C. The cells were then fixed for 20 min in 4% paraformaldehyde, permeabilized using 0.2% Triton, and incubated with mouse OK-1 PTHR antibody at 4 °C overnight. After washing with PBS, the cells were incubated with anti-mouse IgG-rhodamine conjugate antibody at room temperature for 1 h. The cells were mounted on glass slides and visualized by a confocal microscope. The pictures are representative of the entire population of cells and of three independent experiments.
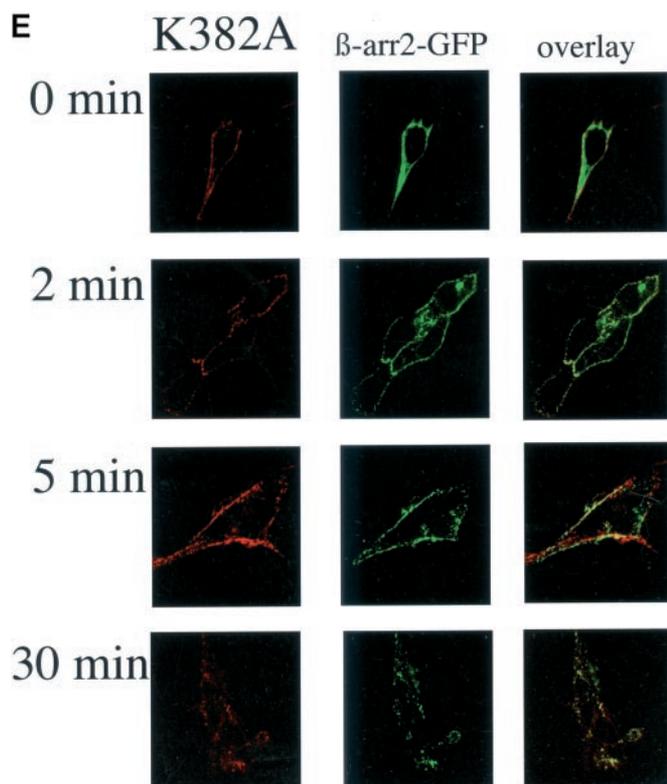
FIG. 6—*continued*

containing N289A/β-arrestin2-GFP (Fig. 6*D*) or K382A/β-arrestin2-GFP (Fig. 6*E*) were also visible in the cell interior. These data indicate that at longer agonist exposure the N289A and K382A also undergo β-arrestin-dependent endocytosis. Thus, the reduced ability of exogenous β-arrestin2 to restore internalization of N289A and K382A is not because of an impaired association or trafficking with β-arrestin2. Despite the ability of N289A and K382A to cointernalize with β-arrestin2, these results indicate a differential capacity of the wild type PTHR and mutants N289A or K382A to generate endocytic vesicles (Fig. 6, compare *A*, *D*, and *E* at *t* = 5 min).

These results were further confirmed in HEK-293 cells transiently expressing the receptors and the two β-arrestin isoforms. The relationship between mutant PTHRs and patterns of β-arrestin2 translocation observed in the stable expression were conserved for both β-arrestin isoforms in HEK-293 cells transiently expressing the receptors. Fig. 7*A* shows the data for the wild type PTHR and the N289A and K382A mutants transiently expressed in HEK-293 cells. In the absence of PTH, β-arrestin1-GFP or β-arrestin2-GFP were uniformly distributed in the cytosol of cells expressing the wild type PTHR or mutant receptors N289A and K382A (data not shown). After 5 min of PTH exposure, β-arrestin1-GFP and β-arrestin2-GFP clusters were present near the plasma membrane but also in the interior of cells expressing the wild type PTHR (Fig. 7*A*). This punctated pattern of β-arrestin(1/2)-GFP fluorescence observed for PTHR contrasts with the homogeneous localization of β-arrestin(1/2)-GFP fluorescence at the plasma membrane of cells expressing the mutant N289A or K382A (Fig. 7*A*). This suggests that the receptor's determinants Asn-289 and Lys-382 might mediate their partial inhibitory effect on receptor internalization by regulating the recruitment of receptor/arrestin complex into early endosomes.

*Immunolocalization of β-Arrestin1-GFP to PTHRs Revealed That Arrestin/N289A and Arrestin/K382A Complexes Do Not Efficiently Dissociate from the Plasma Membrane—*To further explore the interaction between β-arrestins and N289A or K382A, we quantified the stability of the association between β-arrestins and the receptor by analysis of cellular localization of β-arrestin1-GFP or β-arrestin2-GFP by subfractionation and Western blots of HEK-293 cells transiently expressing the receptors and β-arrestin(1/2)-GFP. The difference in size between β-arrestin(1/2) ($\approx$45 kDa) and β-arrestin(1/2)-GFP ($\approx$73 kDa) allows differentiation of the endogenous from the recombinant β-arrestin. The use of anti-GFP antibody did not allow us to establish an accurate quantification of the β-arrestin1/2-GFP translocation because of a high unspecific background. Using anti-β-arrestin1 antibodies circumvented this problem. In unstimulated cells coexpressing β-arrestin1-GFP and PTHR, no β-arrestin1-GFP was detected in the plasma membrane fraction (Fig. 7*B*). After 5 and 10 min of PTH (100 nM) exposure, we observed a considerable β-arrestin1-GFP translocation ($\approx$40% of total expressed β-arrestin1-GFP at 10 min) to the plasma membrane (Fig. 7*B*). β-Arrestin1-GFP could also be coimmunoprecipitated with PTHR from the plasma membrane, indicating a physical association between both proteins (data not shown). No translocation in response to PTH was observed in cells transfected only with β-arrestin1-GFP (data not shown). At later times (20–40 min), the fraction of β-arrestin1-GFP associated with the membrane fraction diminished rapidly.

A different pattern of translocation of β-arrestin1-GFP was seen in cells expressing N289A or K382A (Fig. 7*B*). The fraction of cytoplasmic β-arrestin1-GFP that translocated in the presence of PTH was slightly lower and delayed for N289A ($\approx$33% at 20 min) and for K382A ($\approx$30% at 20 min). However, the most striking difference was the reduced capacity of β-arrestin1-GFP to dissociate from the plasma membrane. Indeed, β-arrestin1-GFP remaining associated with the plasma membrane after 40 min of PTH exposure was $\approx$5% for PTHR *versus* $\approx$25–30% for K382A or N289A. Our data suggest that the disappearance of β-arrestin1-GFP from the plasma membrane expressing PTHR reflects the co-internalization of the complex PTHR/β-arrestin to endocytic vesicles. The reduced ability of the β-arrestins to detach from the plasma membrane expressing N289A or K382A suggests that these receptors have a reduced ability of their receptor/β-arrestin complexes to proceed into endocytic vesicles, thereby reducing the pool of receptor able to internalize.

DISCUSSION

*Multiple Receptor Determinants Regulate Agonist-mediated PTHR Internalization—*Agonist-mediated receptor internalization provides an important mechanism to regulate GPCR functions. Internalization allows: (*a*) decrease of the magnitude of the receptor-mediated G protein signals (desensitization), (*b*) receptor resensitization (via recycling), (*c*) degradation (down-regulation), and (*d*) coupling to other signaling pathways (*e.g.* MAP kinases). The receptor's structural determinants required to mediate internalization are poorly characterized in GPCRs. Although an internalization consensus motif for GPCRs has not been identified, a variety of GPCR internalization motifs that have been characterized so far include 1) clusters of serine/threonine residues that are phosphorylated in the presence of agonist (10), 2) a dileucine-based motif in the carboxyl terminus (32), and 3) tyrosine-based motifs (NP(X)$_{2,3}$Y) found in the end of helix 7 of the class I GPCRs (*i.e.* rhodopsin-like receptors). In addition to these cytoplasmic endocytic motifs in the receptor, receptor mono-ubiquitination appears to provide an alternative signal for GPCR internalization (33, 34). However, these motifs are not consistently involved in GPCR internalization.

Previous studies have indicated that multiple receptor determinants in the cytoplasmic loops and the carboxyl terminus
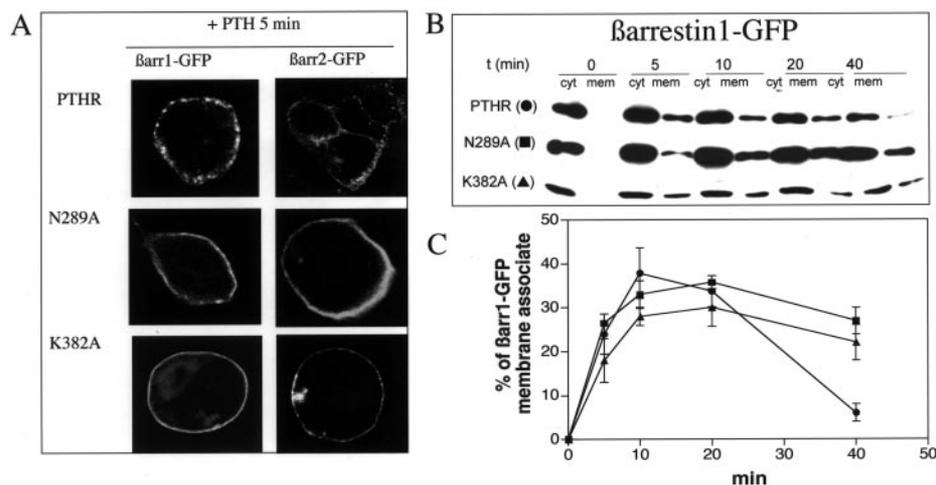
FIG. 7. **Immunoblot analysis of time dependence of β-arrestin1-GFP translocation.** *A*, β-arrestin-GFP translocation with the activated wild type PTHR or mutants N289A or K382A. HEK-293 cells transiently coexpressing the wild type or mutant receptors and β-arrestin1-GFP or β-arrestin2-GFP were incubated at 37 °C with or without PTH (100 nM) for 5 min. Cells were then fixed with 4% paraformaldehyde. Images were visualized on a confocal laser scanning microscope and are representative of an entire population of cells of three independent experiments. *B*, time course of β-arrestin1-GFP translocation in response to 100 nM PTH in HEK-293 cells transiently coexpressing β-arrestin1-GFP and the receptors. Cytosolic proteins (10 μg for K382A and 20 μg for the other receptors) and solubilized membrane proteins (10 μg for K382A and 20 μg for the other receptors) were separated by SDS-PAGE and β-arrestin1-GFP was visualized by immunoblot analysis with a specific β-arrestin1 antibody. *C*, representative time course of β-arrestin1-GFP translocation. The intensity of bands on immunoblots shown in B were quantified by densitometry, and percentage of translocation was calculated as: $100 \times \text{amount}(\beta\text{-arrestin1-GFP})_{\text{membrane}}/\text{amount}(\beta\text{-arrestin1-GFP})_{\text{membrane+cytosol}}$. Data represent the mean ± S.D. ($n = 7$ experiments for PTHR and $n = 3$ experiments for the mutants).

are involved in arrestin binding (35–37). In agreement with these findings, the present study demonstrated that three topological domains of PTHR are required for an efficient PTH-dependent receptor internalization and differently regulate the association with β-arrestins: determinants in the carboxyl terminus (including the phosphorylation sites), Asn-289 in helix 3, and Lys-382 in the third intracellular loop.

The carboxy-tail of the PTHR contains determinants for β-arrestin association because truncation of the carboxy-tail (in PTHR-TR) diminished the ability of the receptor to translocate efficiently β-arrestin2-GFP within minutes of agonist exposure. These determinants are different from the sites for PTH-stimulated phosphorylation, because PTHR-PD showed reduced association with β-arrestin2 during the process of receptor endocytosis but normal β-arrestin translocation during the initial step of endocytosis. Thus, phosphorylation of the serines in the carboxy tail of PTHR stabilizes the interaction with arrestins as indicated by a recent study for class I GPCRs (38).

The endocytic machinery for receptor internalization recognizes internalization signals localized in the cytoplasmic part of the receptor. However, a few studies have reported a role for transmembrane residues in the internalization process of single transmembrane receptors for insulin (39, 40) and interleukin-2 (41). To our knowledge, our results are the first to report a residue within the helix 3 of a GPCR playing a critical role in receptor internalization. How does the Asn-289 residue mediate an effect in the internalization process of PTHR? It seems unlikely that Asn-289 located in the middle of helix 3 (Fig. 2*A*) could interact with the endocytic machinery to promote receptor internalization. According to the model developed by Bywater *et al.* (42) for the GLP-1 receptor (42), the conserved residue Asn-289 appears to be oriented toward other helices rather than to the membrane environment. Therefore, the effect of the N289A mutation on receptor internalization probably reflects a defect in the conformational change of the receptor required for internalization.

The observation that the mutations that have been shown to affect internalization of PTHR do not necessarily affect PTH-mediated signaling suggests that G protein activation is not *per se* coupled to internalization of the receptor. The independence of G protein activation *versus* receptor internalization was recently documented by the finding that PTHR internalization was not affected by blocking receptor conformational changes that drastically block G protein activation (20). Thus, it is improbable that the absence of PTH-mediated signaling in the mutant K382A is the cause for the defect in the receptor internalization.

Our findings suggest that β-arrestin association with the activated PTHR is differently regulated by the receptor's internalization determinants; β-arrestin interaction was not prevented in mutants N289A and K382A, whereas no amelioration was observed in receptor internalization. Conversely, cytoplasmic tail mutants reduced β-arrestin interactions, whereas both isoforms were capable of increasing receptor internalization.

Arrestin/Receptor Interaction and Receptor Internalization—The current model for the understanding of arrestin interaction with the receptor is based on rhodopsin studies and the recent crystal structures of visual arrestin and β-arrestin (12, 13, 43). The model proposes that an initial binding of arrestin to the phosphorylated residues in the carboxyl tail of the receptor disrupt intramolecular charge-charge interactions between highly basic residues in arrestin. This disruption induces a conformational change in arrestin that promotes binding to the activated receptor and stabilizes this interaction by association of arrestin's amphipathic α helix I with the cell membrane as suggested by Han *et al.* (43). Our results indicate that features in the activated PTHR other than phosphorylation of the carboxyl terminus tail are responsible for initial translocation of β-arrestins to the membrane. Phosphorylation of the PTHR stabilizes this interaction, perhaps by promoting the interaction of arrestin's α helix I with the cell membrane. The initial translocation of β-arrestin to the cell membrane appears to depend upon a determinant(s) in the cytoplasmic tail of the PTHR (difference between PTHR-TR and PTHR-PD), but the nature of this determinant is unclear.

Our data further differentiate the capacities of β-arrestins to associate efficiently with activated PTHR from their abilities to promote receptor internalization. Indeed, we have demonstrated that the inability of β-arrestin overexpression to pro-

mote the agonist-mediated internalization of N289A and K382A was not reflected by a reduced capacity of these receptors to translocate β-arrestins to the cell membrane. Rather, the maintenance of the β-arrestin/receptor complex in the plasma membrane might explain the internalization resistance of N289A and K382A. Conversely, β-arrestins were able to increase the internalization of the cytoplasmic tail mutants without displaying an efficient interaction with the receptors.

Thus, we conclude that the cytoplasmic tail and the site for GRKs phosphorylation are necessary for β-arrestin interaction, and could serve as affinity modulator for β-arrestins, whereas Asn-289 and Lys-382 are necessary for the subsequent translocation toward the endocytic endosomes. Our further working hypothesis is that in the receptor/β-arrestin complex the highly conserved residues Asn-289 and Lys-382 in the PTHR might regulate a conformational variation or modification (*e.g.* ubiquitination; Ref. 34) in β-arrestins that enables binding to the endocytic machinery (*e.g.* clathrin, AP-2).

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