NHERF-1 and the Cytoskeleton Regulate the Traffic and Membrane Dynamics of G Protein-coupled Receptors*

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The sodium-hydrogen exchange regulatory factor 1 (NHERF-1/EBP50) interacts with the C terminus of several G protein-coupled receptors (GPCRs). We examined the role of NHERF-1 and the cytoskeleton on the distribution, dynamics, and trafficking of the β2-adrenergic receptor (β2AR; a type A receptor), the parathyroid hormone receptor (PTH1R; type B), and the calcium-sensing receptor (CaSR; type C) using fluorescence recovery after photobleaching, total internal reflection fluorescence, and image correlation spectroscopy. β2AR bundles were observed only in cells that expressed NHERF-1, whereas the PTH1R was localized to bundles that parallel stress fibers independently of NHERF-1. The CaSR was never observed in bundles. NHERF-1 reduced the diffusion of the β2AR and the PTH1R. The addition of ligand increased the diffusion coefficient and the mobile fraction of the PTH1R. Isoproterenol decreased the immobile fraction but did not affect the diffusion coefficient of the β2AR. The diffusion of the CaSR was unaffected by NHERF-1 or the addition of calcium. NHERF-1 reduced the rate of ligand-induced internalization of the PTH1R. This phenomenon was accompanied by a reduction of the rate of arrestin binding to PTH1R in ligand-exposed cells. We conclude that some GPCRs, such as the β2AR, are attached to the cytoskeleton primarily via the binding of NHERF-1. Others, such as the PTH1R, bind the cytoskeleton via several interacting proteins, one of which is NHERF-1. Finally, receptors such as the CaSR do not interact with the cytoskeleton in any significant manner. These interactions, or the lack thereof, govern the dynamics and trafficking of the receptor.

Cell membranes are highly heterogeneous structures consisting of an ensemble of fluctuating microdomains with distinct lipid and protein compositions. These microdomains play important functions in signal transduction processes by increasing the rate and efficiency of coupling of key intermolecular interactions involved in specific signaling processes (1–3). The cytoskeleton has also been implicated in the regulation of signal transduction processes by serving as a substrate for the anchoring of specific proteins (4), regulating traffic (5), and partitioning the cell membrane into microdomains through the formation of effective barriers to the diffusion of lipids and proteins present in the bulk of the plasma membrane (6, 7). However, this general model of the cytoskeleton’s role in the regulation of signaling processes depicts a somewhat passive picture. Most studies of the relationships among cytoskeletal structures and signaling processes focused on the effects of extracellular signals on cytoskeletal reorganization rather than on the effects of the cytoskeleton upon signaling pathways.

Here, we examined the effects of the expression of NHERF-1/EBP50 in the dynamics of three GPCRs: the β2-adrenergic receptor (β2AR), the parathyroid hormone receptor (PTH1R), and the calcium-sensing receptor (CaSR). The β2AR and the PTH1R are type A and B receptors, respectively, and were chosen because of their documented interactions with NHERF-1 (10, 12, 14–18). The CaSR is a type C receptor that does not interact with NHERF-1, thus serving as an important control. The data show that the β2AR and the PTH1R are closely associated to actin stress fibers by a mechanism that is modulated by their interactions with NHERF-1. NHERF-1 expression was not required for the cytoskeletal association of the PTH1R; however, the β2AR was found in bundles only in cells expressing NHERF-1. The CaSR was not associated with the cytoskeleton independently of NHERF-1 expression. The

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2 The abbreviations used are: PDZ, postsynaptic density protein (PSD95)/Drosophila disc large tumor suppressor (DlgA)/Zo-1 protein (PDZ)2 domains (which interact with a variety of signaling molecules) and a C-terminal Ezrin-Radixin-Moesin (ERM)-binding domain (that enables these proteins to interact with cytoskeletal structures) (8–10). Two of the members of this family, the Na+/H+ exchange regulatory factors 1 and 2 (NHERF-1 and -2), interact with and modulate the function of several G protein-coupled receptors (10–13). However, although the data show unequivocally that NHERF-1 and NHERF-2 modulate the function of GPCR, a unified hypothesis to explain the multiple roles of these scaffolding molecules is lacking.
diffusion of the PTH1R and the \( \beta_2 \)AR was strongly influenced by the expression of NHERF-1. Finally, we show that the effects of NHERF-1 on PTH1R traffic are consistent with a novel model in which NHERF-1 interferes with the binding of arrestin to the activated PTH1R.

**EXPERIMENTAL PROCEDURES**

**Materials and Constructs**—Anti-NHERF-1 antibody was purchased from Upstate Biotechnology, Inc. Anti-FLAG (M1) was from Sigma, and anti-hemagglutinin (HA11) was purchased from Covance. Anti-rabbit IgG antibody conjugated to TRITC was obtained from Jackson ImmunoResearch. All other materials were purchased from Sigma unless otherwise noted. A pEGFP-N2 plasmid encoding a full-length human PTH1R carboxyl-terminal eGFP fusion protein (PTH1R-eGFP) was kindly provided by C. Silve (INSERM, Paris, France). Similar plasmids coding for the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)-AR-eGFP) and the calcium-sensing receptor (CaSR-eGFP) were kindly supplied by Dr. Jeffrey Benovic (Thomas Jefferson University) and Dr. Gerda Breitwieser (Geisinger Medical Center), respectively.

**Cell Culture**—Chinese hamster ovary N10 cells (CHO-N10) were cultured in Ham’s F-12 medium (Mediatech, Inc.) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin solution at 37 °C/5% CO\(_2\). All live cell imaging experiments were done in Mattek dishes in complete medium. Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche Applied Science) were used for transient transfections.

**Confocal Microscopy**—An Olympus Fluoview 1000 confocal microscope equipped with a SIM scanner was used for all experiments unless otherwise indicated. For live cell imaging experiments, the cells were kept at 37 °C using an open perfusion microincubator (Harvard Apparatus Inc.).

**Immunocytochemistry**—Cells were cultured on glass coverslips, transfected with the desired plasmids, and allowed to grow until 80% confluent. The coverslips were washed in phosphate-buffered saline and fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline at 4 °C. Cells were permeabilized with 5% nonfat milk, 0.1% Triton X-100 for 1 h at 4 °C and then stained with either phalloidin-TRITC (3 nm) or anti-NHERF-1 antibody (1 \( \mu \)g/ml) overnight at 4 °C. Phalloidin-stained cells were washed four times with phosphate-buffered saline and mounted using gelvatol. Anti-NHERF-1-stained cells were further treated with anti-rabbit IgG conjugated with TRITC (1:1000) in 5% nonfat milk for an additional 2 h at room temperature. The cells were then washed four times with phosphate-buffered saline, mounted with gelvatol, and examined by confocal microscopy.
Fluorescence Recovery after Photobleaching (FRAP)—All FRAP measurements were done focusing the microscope onto the plasma membrane adjacent to the coverslip. This region of the cell was chosen for two main reasons: 1) it provides a large surface, and 2) many supplemental studies were done using total internal reflection fluorescence (TIRF), a technique that is limited to the observation of the cell membranes adjacent to the coverslip. The plasma membrane was located using a Z-stack scanning procedure (20). Circular regions of interest (between 90 and 200 μm²) were selected and bleached with a 1-s pulse from a 405-nm laser line using the Fluoview 1000 SIM scanner, whereas recovery data were acquired using the instrument’s main scanner and the 488-nm line of an argon gas laser. This short pulse was selected to ensure a Gaussian bleaching spot. To maximize reproducibility of the experimental conditions, all data were acquired in the photon-counting mode of the instrument. Sixty images were then collected at intervals of 1–1.6 s. The images were exported to MetaMorph (Universal Imaging, Inc.), and the average fluorescence intensities of the bleached regions and control regions in other cells or far removed regions of the same cell were obtained. The data were fitted to a single exponential decay and plotted using GraphPad Prism. The diffusion coefficient was calculated using the Stokes-Einstein equation.

TABLE 1
Effects of NHERF-1 expression on receptor distribution

|                  | NHERF-1(−) | NHERF-1(+)|                  | NHERF-1(−) | NHERF-1(+) |
|------------------|------------|-----------|-------------------|------------|------------|
|                   | Percentage fluorescence in bundles | Fraction of cells with visible bundles | Percentage fluorescence in bundles | Fraction of cells with visible bundles |
| β₂AR              | 1.2 ± 0.6 (n = 38) | 1/38 | 19.4 ± 2.7 (n = 27)* | 21/27 |
| PTH1R             | 34.4 ± 3.0 (n = 22) | 21/22 | 57.4 ± 2.8 (n = 34)*,* | 34/34 |
| M593A-PTH1R       | 17.4 ± 2.9 (n = 16) | 12/16 | 17.69 ± 3.0 (n = 12) | 9/12 |
| PTH1R + ΔERM-NHERF-1 | 14.5 ± 1.3 (n = 15) | 8/15 | 12.1 ± 1.4 (n = 39) | 12/39 |
| CaSR              | ND         | 0/22 | ND                | 0/19 |

*a Different from NHERF-1(−) cells (p < 0.001).
*b Different from cells expressing M593A-PTH1R or ΔERM-NHERF-1 (p < 0.001).

ND, none detected.
for two-dimensional diffusion ($D = r^2/(4\tau_d)$, where $r$ is the radius of the bleached spot, and $\tau_d$ is the half-life of fluorescence recovery).

**Image Correlation Spectroscopy (ICS) and Image Cross-correlation Spectroscopy (ICCS)**—These studies were based on the experimental techniques described by Wiseman et al. (21–23) and recently reviewed by Bacia and Schwille (24) and Kim et al. (25). The technique measures the correlation of an image with itself after a defined latency time $\tau$. This correlation is a function of the mobility of the fluorescent molecules. The experimental design is, in principle, identical to that used for fluorescence correlation spectroscopy, except that in ICS a larger region of the cell is imaged. This allows the acquisition of spatial information that is not available in typical fluorescence correlation spectroscopy experiments. Two different experimental designs were used for these studies. In some cases, the data were collected with a Fluoview 1000 confocal microscope focused onto the plasma membrane of the cell adjacent to the coverslip. In others, the data were obtained using an Olympus-based TIRF system (see below). For confocal microscopy experiments, a small section of the plasma membrane ($<50 \mu m^2$) was rapidly scanned (50–60 ms/frame) under low laser power, and up to 300 images were collected. For the TIRF experiments, up to 200 images were obtained consecutively using a 500-ms exposure time. With either experimental set-up, fluorescence loss due to photobleaching of the sample was almost negligible. The image data were exported to ImageJ and analyzed using a plug-in specifically written to calculate the autocorrelation function of the data. This plug-in was based on code developed by A. Tully and E. Levitan (University of Pittsburgh). The resulting autocorrelation data were exported to GraphPad Prism and fit to a single species two-dimensional diffusion model ($G(\tau) = K(1 + \tau/\tau_d)^{-1} + G_0$), where $\tau_d$ is the charac-
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characteristic time constant, $K$ is a proportionality factor, and $G_C$ is a term that accounts for spatial autocorrelation) as described by Hebert et al. (21). The data were also fit to a two-state diffusion model without any significant improvement in the quality of the fit. Therefore, all analyses reported in this paper were done using the single-species diffusion model. For calibration purposes, the diffusion of free intracellular GFP was determined using ICS and FRAP. The values for the diffusion coefficient of GFP obtained by both methods were comparable. ICCS experiments were conducted essentially using the same design, except that images were collected from both green (eGFP) and red (monomeric dsRed) channels. The cross-correlation calculation was performed using an ICCS plug-in for ImageJ developed for this purpose. Fractional binding was calculated from the ratio of the amplitude of the cross-correlation function to the amplitude of the autocorrelation function for PTH1R. These calculations are based on the method described by Kim et al. (25).

**Total Internal Reflection Microscopy—TIRF microscopy** studies were conducted using an Olympus 1X71 equipped with a 150-milliwatt argon laser, an Olympus 60 TIRF objective, a Princeton CCD camera, and Sutter filter wheels. The imaging work station was controlled with SimplePCI software (Compix, Inc.). Typical exposure times of 500 ms were used for most experiments. For ICS measurements, up to 200 images were collected continuously. For endocytosis experiments, images were taken once every 30 s for up to 20 min. The data collected were exported to ImageJ and analyzed using the appropriate plug-ins.

**Endocytosis Studies**—These experiments were done using TIRF microscopy. Cells with or without NHERF-1 were imaged at 37 °C at 30-s intervals for 1–2 min before the addition of the ligand. The microscope was refocused after stimulation of the cells, and image acquisition was continued for an additional 20 min at 30-s intervals. Fluorescence intensity data of the whole cell or of specific regions of interest were exported to GraphPad Prism and analyzed by fitting to a single exponential decay.

**Statistical Analysis**—All multiple comparisons were done using ANOVA followed by Tukey’s post-test pairwise comparisons using the analysis routines built in GraphPad Prism. Plates from a minimum of five independent experiments were used for every procedure. Most of the experiments described here were replicated 12 times or more. ICS and ICCS studies were done using ImageJ plug-ins developed in house. These are available upon request.

**RESULTS**

**Expression of NHERF-1 in CHO-N10 Cells**—These studies were done using a new cell line, derived from Chinese hamster ovary cells. These cells, termed CHO-N10, express NHERF-1 in a tetracycline-inducible manner. We chose CHO cells as a model system for these studies, because they do not express detectable levels of NHERF-1 (Fig. 1). Fig. 1 also shows the induction of NHERF-1 expression 24 h after the addition of various doses of tetracycline. As shown, the expression of NHERF-1 by CHO-N10 cells is exquisitely sensitive to tetracycline. Therefore, CHO-N10 cells appear to be an ideal system to examine the effects of NHERF-1 expression on the dynamics and biochemistry of its interacting proteins. All experiments reported below were done inducing NHERF-1 expression with 50 ng/ml tetracycline for 24 h.

**GPCRs That Contain C-terminal PDZ Binding Motifs Are Tethered to Actin Stress Fibers**—Since NHERF-1 binds GPCR and the cytoskeleton, we first examined the effects of NHERF-1/EBP50 on the distribution of GPCR in live cells transfected with β2AR-eGFP, PTH1R-eGFP, or CaSR-eGFP. TIRF microscopy revealed that the β2AR-eGFP construct aggregated on puncta and bundle-like structures that closely resembled cytoskeletal stress fibers only in cells expressing NHERF-1/EBP50 (Fig. 2, A and B). In contrast, PTH1R-eGFP accumulated in bundles even in the absence of NHERF-1 expression (Figs. 2, C and D, and 3A). The CaSR chimeras were never observed in bundles (Fig. 2, D and E). Several lines of evidence suggested that these bundle structures were linked to the cytoskeleton. For instance, prolonged serum starvation of the cells reduced significantly the prevalence of receptor bundles. Furthermore, the addition of latrunculin A to disrupt the cytoskeleton rapidly dissipated most of the PTH1R bundles (see, for instance, Fig. 3B), such that 15 min after the addition of latrunculin, no bundles were visible. The relative distribution of the receptors in bundles was determined by morphometric analyses. The results of these measurements are summarized in Table 1.

**Effect of NHERF-1 Expression on the Diffusion of the β2AR and the CaSR**

![Graph showing the effect of NHERF-1 expression on the diffusion of β2AR and CaSR](image)

**FIGURE 5. Effects of NHERF-1 expression on the diffusion of β2AR and CaSR in the plasma membrane of CHO-N10 cells.** CHO-N10 cells were transfected with β2AR-eGFP or CaSR-eGFP, and NHERF-1 expression was induced with 50 ng/ml tetracycline for 24 h. The diffusion of the fluorescent receptors was examined by TIRF-ICS. One hundred images were collected per set at a rate of 3 images/s. The measured total intensities were corrected for photobleaching using a normalization plug-in for ImageJ. The corrected intensities were fit to a single-component two-dimensional diffusion model to determine the diffusion coefficient and the immobile fraction of the receptor. Statistically significant differences ($p < 0.01$) between basal and tetracycline-induced, NHERF-1-expressing cells are denoted by the asterisks ($n = 6$ for all experiments).
These results illustrate the significant effects of the expression of NHERF-1 on the subcellular distribution of the β₂AR. The effects of NHERF-1 on the distribution of the PTH₁R were much less dramatic although significant. In contrast, NHERF-1 expression had no detectable effects on the distribution of the CaSR. Finally, to demonstrate that these distributions were not peculiar to CHO cells, the distribution of the PTH₁R was examined in HEK293, rat osteosarcoma (ROS), and human osteosarcoma (SaOS₂) cells. In all cases, a significant fraction of the PTH₁R was found in bundle-like structures (data not shown).

To determine the relationship between the receptor bundles and the cytoskeleton, CHO-N10 cells expressing PTH₁R-eGFP were fixed and stained with TRITC-conjugated phalloidin. Fig. 3C shows clearly that the bundles parallel actin fibers. As expected, NHERF-1 co-localized with these fibers.

The data shown in Fig. 1 clearly suggested a major difference in the subcellular distributions of the CaSR on one side and the β₂AR and the PTH₁R on the other. We hypothesized that the differences in the distribution of these receptors were a consequence of the fact that the β₂AR and the PTH₁R contain a PDZ binding motif at their C terminus, whereas the CaSR does not. Since the PDZ binding motifs of the β₂AR and the PTH₁R interact with NHERF-1, we proceeded to examine the relevance of these interactions for the stability of the receptor bundles using the PTH₁R as a model. CHO-N10 cells were transfected with 1) M593A-PTH₁R-eGFP (a PTH₁R-eGFP in which the C-terminal methionine has been mutated to alanine, which results in a dramatic reduction of the affinity of the receptor for NHERF-1 (26)) or 2) wt-PTH₁R-eGFP co-transfected with a deletion mutant of NHERF-1 that does not contain the ERM-binding domain (∆ERM-NHERF-1, which has intact PDZ domains and acts as a dominant negative regarding most of the effects of NHERF-1, primarily because it does not interact with the cytoskeleton (18)). In both cases, the PTH₁R bundles were still present, albeit much less so than in the cells that expressed wt-PTH₁R-eGFP alone (Table 1 and Fig. 3, D and E).

Thus, the binding of the PTH₁R to the cytoskeletal fibers is mediated to a significant extent by the interaction of the C terminus of the receptor with specific PDZ domain-containing proteins.

**NHERF-1 Expression Modulates Receptor Diffusion**—We next studied the effects of the cytoskeleton and NHERF-1 expression on PTH₁R mobility. Diffusion coefficients were measured using a confocal microscope focused at the plasma membrane closest to the coverslip using FRAP and ICS techniques (Fig. 4). Fig. 4A shows a representative FRAP experiment carried out with cells that do not express NHERF-1 mobility. Diffusion coefficients were calculated for several cells from 5–16 plates for each condition. These results are summarized in Fig. 4B. The diffusion of the PTH₁R was significantly slower, and the fraction of immobile receptors was...
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significantly greater in cells that expressed NHERF-1. The results obtained with FRAP and ICS were internally consistent; both methods demonstrated that NHERF-1 significantly decreased the diffusion coefficient and mobile fraction of PTH1R (Fig. 4B). It should be noted that the numbers obtained with ICS and FRAP were not identical. These differences are not unusual and have been attributed to the existence of confinement zones that have a much greater relative influence in the data collected by ICS, because the region imaged is significantly smaller (27).

To demonstrate that the effects of NHERF-1 on receptor mobility were primarily due to the interaction of PTH1R with NHERF-1, we examined the diffusion of M593A-PTH1R. M593A-PTH1R diffused very rapidly in comparison with wt-PTH1R in the presence of NHERF-1 (Fig. 4C). Likewise, wt-PTH1R diffused very rapidly when co-expressed with NHERF-1 mutants that do not bind the receptor (such as S1S2, in which the core binding sequences of the PDZ domains have been scrambled) or that fail to interact with the cytoskeleton (such as ΔERM-NHERF-1) (Fig. 4C).

Finally, we examined the role of the cytoskeleton in the lateral mobility of PTH1R (Fig. 4D). Blocking actin polymerization with latrunculin A increased dramatically the diffusion coefficient while decreasing the immobile fraction of wt-PTH1R-eGFP. Interestingly, NHERF-1 decreased the diffusion coefficient of PTH1R in latrunculin A-treated cells, suggesting the presence of a macromolecular complex that includes NHERF-1, PTH1R, and other proteins that may interact with the second PDZ domain or with the ERM binding motif of NHERF-1. These observations demonstrate that the restricted mobility of the PTH1R observed in cells that express NHERF-1 is a consequence of two concomitant phenomena: 1) the interactions of the receptor with NHERF-1 and 2) the interactions of NHERF-1 with the cytoskeleton.

Since NHERF-1 also interacts with the β2AR with high affinity, we predicted that NHERF-1 expression should have similar effects on β2AR diffusion. The results are shown in Fig. 5. NHERF-1 dramatically reduced the mobility of the β2AR. Importantly, NHERF-1 had no discernible effects on the diffusion of the CaSR, which was included as a negative control. Therefore, the effects of NHERF-1 are specific and not due to generalized changes in the structure of the cytoskeleton in the vicinity of the plasma membrane. Interestingly, the effects of NHERF-1 on β2AR mobility were independent of the presence of ligand, thus suggesting that the interactions between the β2AR and NHERF-1 do not require ligand binding.

**Local Effects of NHERF-1 on Receptor Lateral Diffusion**—Because the distribution of the PTH1R on the cell surface was not homogeneous, we examined the dynamics of the receptor in different subregions of the cell using TIRF microscopy. “Bundle” and “bulk” regions within each cell were identified based on the concentration of observable bundles within the region of interest (ROI) (Fig. 6A). Small (<2.5 μm in diameter) circular ROI were selected within these regions, and the diffusion coefficient of the receptor was measured by ICS. As shown in Fig. 6B, the autocorrelation functions of the PTH1R within “bulk” and “bundle” regions were very different. Within the bundles, in the absence of NHERF-1, about 60% of the receptor molecules diffused with a coefficient of 0.1 μm²/s (Fig. 6C). In contrast, the receptor diffused very slowly (D = 0.019 μm²/s) in the bulk region of the cell. This finding strongly suggests that the receptor diffuses along the bundles. However, this diffusion was still very slow when compared with that observed in the presence of latrunculin A; thus, we conclude that the motion of the receptors along the bundles is limited by the binding of the receptor to the cytoskeleton. NHERF-1 expression drastically reduced the diffusion coefficient of the fast moving component within the bundles without exerting a statistically significant effect on the diffusion coefficient of PTH1R molecules within the bulk membrane (Fig. 6C). Conversely, the expression of NHERF-1 increased significantly the immobile fraction of the PTH1R within the bundles. Because the data show that PTH1R bundles coincide with actin stress fibers (Fig. 3C), these results suggest that the effects of NHERF-1 on the lateral diffusion of the PTH1R are effectively limited to the population of receptors located in very close proximity to actin fibers.

**Perturbation of the Effects of NHERF-1 Expression by the Addition of Ligand**—The diffusion data shown in Figs. 4 and 5 suggest that NHERF-1 effectively tethers PTH1R and β2AR to the cytoskeleton. Inasmuch as the interactions of some GPCR with NHERF-1 appear to be ligand-dependent (28), we hypoth-

![Effect of Ligand Addition on the Membrane Dynamics of the PTH1R, the β2AR and the CaSR](image-url)
addition of isoproterenol did not alter the diffusion coefficient of the β2AR; however, the immobile fraction of the receptor was reduced substantially. These results suggest that ligand induces the dissociation of the β2AR-NHERF-1 complexes from the cytoskeleton without affecting the binding of NHERF-1 to the receptor.

**NHERF-1 Expression Modulates PTH1R Internalization**—To investigate the effects of NHERF-1 on receptor traffic, we examined the effects of the addition of ligand on the surface distribution of the PTH1R using TIRF microscopy. In these experiments, images were collected at 30-s intervals after the addition of ligand. PTH-(1–34), a well characterized PTH agonist (18, 26), induced the disappearance of these receptor bundles (Fig. 8A). Fig. 8B shows a kymograph of the xy projections of the cells shown in Fig. 8A. Interestingly, the PTH1R internalized significantly more slowly at the edges of the cell. Collected intensity data were fit to a single exponential to estimate an apparent first order rate of internalization. The plateau values from these fits were used to calculate the fraction of internalized receptor. In the presence of PTH-(1–34), 70–80% of the receptors internalized. Strikingly, NHERF-1 expression reduced the rate of internalization of the receptor by about 50% without affecting significantly the fraction of internalized receptors. In contrast, the antagonist PTH-(7–34) induced slower receptor internalization, which was abrogated by the expression of NHERF-1. We conclude that NHERF-1 expression affected differentially receptor internalization, depending on the nature of the ligand used to induce endocytosis.

**Effects of NHERF-1 Expression on the Binding of β-Arrestin to the PTH1R**—To gain some insight into the mechanisms by which NHERF-1 modulates PTH1R dynamics and internalization, CHO-N10 cells were transfected with PTH1R-eGFP and β-arrestin-1 fused to mRFP (a monomeric variant of the Discosoma protein dsRed). These cells were then studied using ICCS analysis, a technique developed to study protein-protein interactions in live cells. This method is based on a time-dependent analysis of the cross-correlation between the fluorescence intensities of two targets labeled with distinct fluorophores (24): proteins that are associated with a common macromolec-

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**FIGURE 8. Effects of the expression of NHERF-1 on the internalization of PTH1R.** A, CHO-N10 cells treated with vehicle (NHERF−) or tetracycline (NHERF+) were examined by TIRF microscopy after the addition of 100 nM PTH-(1–34). Images were collected at 30-s intervals. The figure shows representative images obtained at the indicated times. B, kymograph of the cells shown in A. The image data were processed as follows. After correction for photobleaching, a y axis maximal projection of the cell was obtained. A pseudocolor look up table was applied to the projection. As shown, the intensity decayed significantly more rapidly in the NHERF− cell shown in A. Furthermore, receptor internalization was somewhat slower at the edges of both cells. C, summary of the PTH1R endocytosis data. The data show the summary of 12–18 cells (12 separate coverslips) per condition. The internalization rate constant was calculated fitting the intensity data to a single exponential, whereas the fraction of surface receptors internalized was calculated from the span of the decay function obtained from these fits. ANOVA analyses followed by Tukey’s post-test comparisons revealed that NHERF-1 expression had a significant effect on the rate of endocytosis of the PTH1R, independently of the ligand used to induce internalization. Moreover, PTH-(7–34)-induced internalization of the receptor was almost completely blocked by NHERF-1 (p < 0.001, n = 12).
ular complex diffuse together, whereas those that are not diffuse in random directions with respect to one another. This approach has been previously used to measure dynamic protein-protein association (24, 25).

In order to eliminate experimental artifacts due to signal saturation, we selected cells in which the fluorescence intensities of PTH1R-eGFP and mRFP-β-arrestin-1 were comparable. In all cases, we calculated the cross-correlation function of the same cells, immediately before and at various times after the addition of the ligand. The fractional degree of binding was experimentally determined by measuring the ratio of the amplitudes of the PTH1R autocorrelation curve and the PTH1R-β-Arrestin cross-correlation curve, as described by Bacia et al. (24). The addition of PTH-(1–34) increased substantially the binding of PTH1R to arrestin in a time-dependent manner from a low basal value to almost 100% (Fig. 9A), indicative of the formation of a complex that included arrestin and the receptor. NHERF-1 reduced the rate of formation of this complex 3-fold, suggesting that NHERF-1 binding interferes with arrestin recruitment to the receptor. This observation also indicates that NHERF-1 dissociation probably precedes arrestin binding.

Since PTH1R internalization induced by PTH-(7–34) was significantly slower, we examined the interactions of the receptor with β-arrestin-1 after the addition of this ligand. PTH-(7–34) did not increase the cross-correlation between PTH1R and β-arrestin-1 (Fig. 9B). To confirm that the ICCS data reported in Fig. 9B reflected PTH1R-arrestin interactions, CHO-N10 cells were co-transfected with FLAG-tagged β-arrestin-1 and hemagglutinin-tagged PTH1R. The cells were treated with PTH-(1–34) or PTH-(7–34) for 15 min, and the binding of PTH1R to arrestin was determined by co-immunoprecipitation methods (Fig. 9C). As shown, PTH-(1–34) induced the formation of complexes with β-arrestin, whereas PTH-(7–34) did not. This suggests that PTH-(7–34)-induced internalization is arrestin-independent. Because NHERF-1 expression completely blocked PTH-(7–34)-induced PTH1R internalization, we also conclude that NHERF-1 affects PTH1R endocytosis by a dual mechanism: 1) reducing the rate of arrestin binding to the receptor, and 2) blocking arrestin-independent endocytic processes.

DISCUSSION

Direct, functional interactions of GPCRs with the cytoskeleton were initially suggested several years ago (29). We describe here three different modes of interaction of GPCR with the cytoskeleton: 1) some GPCR, such as the β2AR, are linked to the cytoskeleton via their interactions with NHERF-1; 2) some GPCRs, such as the PTH1R, interact with the cytoskeleton through their interactions with other proteins in addition to NHERF-1; and 3) some GPCRs, such as the CaSR, do not appear to interact with the cytoskeleton at all.
The interactions of the β₂AR with the cytoskeleton appeared to be mediated almost exclusively by NHERF-1, as there was no evidence of cytoskeletal localization of the β₂AR in cells that did not express NHERF-1. However, surprisingly, the association of PTH1R to cytoskeletal structures was not strictly dependent on the expression of NHERF-1, suggesting that NHERF-1 is only one of several cellular components mediating the interactions of PTH1R with the actin cytoskeleton. Consistent with this, at least two other cytoskeleton-related proteins have been reported to interact with PTH1R: Tctex-1, a dynein light chain (30), reportedly linked to the actin cytoskeleton (31), and 4.1G, a protein directly associated with actin (32). The binding sites for these two proteins are located near the C terminus of PTH1R, upstream of the PDZ domain binding motif (ETVM), which appears to be excluded (30, 32). These interactions may be sufficient to promote partial binding of PTH1R to the cytoskeleton. However, interference with the C-terminal PDZ domain binding motif significantly decreased the accumulation of PTH1R in bundles, suggesting that other PDZ domain-containing proteins may participate in the linkage of the PTH1R to the cytoskeleton. Nevertheless, our data strongly suggest strong interactions between the PTH1R and NHERF-1 in live cells; the PTH1R becomes effectively immobilized by its interactions with NHERF-1 and the underlying cytoskeletal fibers. The functional consequences of these interactions are probably multiple. The activation of phospholipase C-dependent pathways by PTH1R appears to be regulated by binding to NHERF and the cytoskeleton (16, 33).

Much less is known about the functional consequences of tethering the β₂AR to NHERF-1 and the cytoskeleton, and most of what we know about these is limited to the effects of NHERF-1 on β₂AR traffic. It is clear that β₂AR recycling is tightly regulated by NHERF-1 expression, since disruption of the cytoskeleton impairs β₂AR recycling and increases its targets by linking them to the cytoskeleton.

Our diffusion studies also shed important light on the influence of ligand binding on interactions of GPCR with NHERF-1. We hypothesized that ligands that reduce the affinity for NHERF-1 should increase the lateral mobility of the receptor. That seems to be the case for the PTH1R; ligand binding induced a significant increase of the diffusion coefficient and the mobile fraction of PTH1R in cells that expressed NHERF-1, whereas it was without significant effects in cells that did not. This indicates that, after ligand binding, 1) the interactions of the PTH1R with NHERF-1 and other partners have been altered, and 2) the association of PTH1R with the cytoskeleton has been impaired by either releasing NHERF-1 from the cytoskeleton or by direct disruption of the cytoskeleton. However, the case of the β₂AR is somewhat different. NHERF-1 expression immobilizes non-stimulated β₂AR, strongly suggesting that β₂AR-NHERF-1 complexes exist in the absence of ligand. Five minutes after the addition of ligand, the mobile fraction of the β₂AR was significantly increased without changing its diffusion coefficient. This strongly suggests that the binding of the β₂AR to NHERF-1 is not significantly altered by the addition of ligand, although the association of β₂AR-NHERF-1 complexes to the cytoskeleton probably is. The permanence of β₂AR-NHERF-1 complexes after the addition of ligand is consistent with previously published data. In fact, it was suggested that the interactions of the β₂AR and NHERF-1 were induced by ligand (28). However, more recent work by Cao et al. (34) established that GRK5-dependent phosphorylation of Ser⁴¹¹ impaired NHERF-1 binding to the β₂AR, suggesting that ligand binding might negatively influence the binding of NHERF-1 to the β₂AR. Thus, the binding of NHERF-1 to the β₂AR appears to be temporally and dynamically regulated by signaling cascades downstream the activation of the receptor. Our results suggest that, at least within 5 min after ligand addition, the β₂AR remains associated to NHERF-1, but the receptor degradation (34). More recently, it has been reported that the ability of isoproterenol to activate cAMP production is increased by disruption of the cytoskeleton (35), but this observation has not been linked to NHERF-1 expression or function.

The diffusion of the β₂AR and the PTH1R but not the CaSR was strongly influenced by NHERF-1. The expression of NHERF-1 reduced the diffusion coefficient of the β₂AR and the PTH1R while increasing significantly the immobile fraction of these receptors. These results are consistent with the report of Bates et al. (27), who showed that the diffusion of the CFTR was also strongly influenced by NHERF-1 binding. These results prove that NHERF-1 immobilizes
interactions of NHERF-1 with the cytoskeleton or the integrity of the cytoskeleton itself are altered as a consequence of the treatment. It should be noted that several effects of cAMP and protein kinase A on the disruption of the cytoskeleton have been documented (36–39).

The reduced lateral mobility of membrane proteins induced by NHERF-1 is likely to result in the accumulation of receptors in NHERF-1-enriched regions. Furthermore, the reduced mobility of the receptors should have significant modulatory effects on the traffic of these receptors. It was recently shown that, in HEK293 cells (which express NHERF-1 at very high levels), the interactions of PDZ domain-containing proteins with the C terminus of GPCRs regulate the dynamics of the endocytosis of these receptors by delaying the recruitment of dynamin, inducing abortive events that reduce the efficiency of endocytosis, or selecting subpopulations of clathrin-coated pits (40). Here we show that the interactions with NHERF-1 govern the overall dynamics of receptors that contain C-terminal PDZ binding motifs. We propose that, since endocytic events are initiated randomly on the cell surface and have a finite duration (19), a significant reduction of the lateral mobility of the cargo molecules will probably reduce the probability of accumulation of cargo in clathrin-coated pits, thus reducing the net rate of endocytosis. Furthermore, at least in the case of the PTH1R, the interaction of the receptor with NHERF-1 delays the binding of β-arrestin. Delayed β-arrestin recruitment implies delayed recruitment of AP-2 and clathrin and, as a consequence, delayed recruitment of dynamin to complete the endocytic process. Thus, our findings provide a simple, self-consistent explanation for the reduced rate of endocytosis of GPCR containing C-terminal PDZ binding motifs observed in the presence of NHERF-1. Furthermore, we show that different ligands can induce selective, alternative pathways of GPCR internalization via mechanisms that may or may not involve arrestin and that are tightly regulated by NHERF-1. The general model we propose is shown in Fig. 10. GPCRs that contain a C-terminal PDZ binding motif are tethered to the cytoskeleton via direct interactions with NHERF-1. Some of these, such as the PTH1R, may bind the cytoskeleton via multiple additional interactions that may include other PDZ domain-containing targets. In general, NHERF-1 binding plays a major role in anchoring these receptors to the cytoskeleton, reducing receptor mobility, and dynamically interfering with the binding of arrestin after ligand stimulation. Furthermore, the binding of NHERF-1 to some of these receptors blocks completely arrestin-independent receptor internalization processes, such as PTH(7–34)-induced PTH1R endocytosis. These data, in conjunction with those of Puthenveedu and von Zastrow (40), suggest that this may be a very general mechanism for the regulation of GPCR trafficking.

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