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Visualizing Preference of G Protein-Coupled Receptor Kinase 3 for the Process of κ-Opioid Receptor Sequestration

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ABSTRACT

G protein-coupled receptor kinases (GRKs) phosphorylate opioid receptors, which eventually results in receptor sequestration. With respect to κ-opioid receptors, it is known that internalization occurs in a species-specific manner. That is, the agonist-occupied human κ-receptors will sequester whereas murine receptors fail to do so. This investigation concentrates on the internalization of κ-opioid receptors, employing laser scanning microscopy as a major technique to examine receptor internalization in living cells. For this reason, we fused green fluorescence protein to κ-receptors, and DsRed-fluorescent protein to GRK2 and GRK3. All fusion proteins retained their biologic activities. Permanent cell lines (HEK 293, NG 108-15) were transfected to express either green fluorescent κ-receptors or to coexpress the tagged receptor and a specific GRK-DsRed construct. The localization of fluorescent receptors and GRKs was monitored by confocal microscopy before and after opioid exposure of transfected cells. Activation of the murine κ-receptors triggers rapid translocation of tagged GRKs toward the cell membrane, but receptor internalization was not observed. The agonist-occupied human κ-receptor also causes translocation of GRK2- and GRK3-DsRed, which was followed by the formation of vesicles carrying the green fluorescent κ-receptors. Moreover, the green fluorescent vesicles consistently harbour red fluorescent GRK2 and GRK3, respectively. The phenomenon of κ-receptor internalization as well as cointernalization of GRKs is blocked by phosducin, indicating a critical role of G protein-βγ subunits for κ-receptor sequestration. Comparing the effect of over-expressed GRK2 and GRK3 on sequestration of κ-receptors, we conclude that GRK3 more strongly induces κ-receptor internalization than GRK2.

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G protein-coupled receptor kinases (GRKs) phosphorylate G protein-coupled receptors (GPCRs), including opioid receptors (Lefkowitz, 1998; Chavkin et al., 2001). This process facilitates binding of cytosolic β-arrestin to GPCRs, which is followed by cointernalization of receptors and arrestin (Gurevich and Benovic, 1995; Evans et al., 2001). The functional interaction of GRKs with their substrate, the opioid receptors, critically depends on factors such as the receptor type (Carman and Benovic, 1998), the ligand activating the receptor (Kovoor et al., 1998), the composition of Gβγ subunits (Müller et al., 1997), or the cellular concentration of the receptor kinase (Roettger et al., 1997). However, the ability of GRKs to trigger receptor internalization depends on phosphorylation of amino acid motifs, e.g., threonine/serine, located at the C terminus of the receptor (Guo et al., 2000; Celver et al., 2001). This finding is important because the murine and human κ-opioid receptors exhibit species variations with respect to these domains (Simonin et al., 1995), and these differences have been suggested to account for species specific κ-receptor internalization (Blake et al., 1997; Li et al., 1999).

This investigation aims to assess the contribution of distinct GRKs to the process of κ-receptor internalization. Since receptor kinases phosphorylate GPCRs with different preferences (Carmann and Benovic, 1998), and Gβγ subunits display different affinities to GRKs (Wu et al., 1998), we decided to examine more closely the effect of GRK2 and GRK3 on activated mouse and human κ-opioid receptors. Both GRKs anchor at Gβγ subunits attached to the cell membrane to bring about receptor phosphorylation (Müller et al., 1997). For the present investigation, we employed laser scanning microscopy (LSM) to analyze in real time the internalization of opioid receptors, and simultaneously followed the fate of GRKs. For identification of these molecules, we fused the κ-receptors to enhanced green fluorescence protein (EGFP; Heim et al., 1995) and the GRKs to red fluorescence protein (DsRed; Wall et al., 2000). The fusion proteins were expressed in HEK 293 cells and in NG 108-15 cells. The results

ABBREVIATIONS: GRK, G protein-coupled receptor kinase; GPCR, G protein-coupled receptor; LSM, laser scanning microscopy; EGFP, enhanced green fluorescence protein; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; U-50488, *trans*(±)-3,4-dichloro-*N*-methyl-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; U-69592, (5*a*,7*a*,8*b*)-(+)*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide; GTPγS, guanosine-5′-*O*-[γ-thio]-triphosphate; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

demonstrate distinct features for κ-receptors and the receptor kinases 2 and 3. First, activation of mouse and human κ-receptors triggers translocation of cytosolic GRK2 and GRK3 toward the cell membrane, but only human κ-receptors will internalize. Second, κ-receptors cointernalize with GRK2 and GRK3. Third, GRK3 more efficiently promotes internalization of κ-receptors compared with GRK2.

Materials and Methods

Chemicals

The reagents used were of analytical grade and purchased from Sigma (Taufkirchen, Germany). Opioid receptor ligands U-50488 [*trans*(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide], U-69593 [(5*a*,7*a*,8*b*)-(+)*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide], and guanosine-5′-*O*-[γ-thio]-triphosphate (GTPγS) were purchased from Sigma; DAMGO ([D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin) was obtained from Bachem (Heidelberg, Germany) and naloxone from Dupont (Geneva, Switzerland). The enzyme inhibitor Complete was from Roche Applied Science (Mannheim, Germany). The restriction enzymes used for the construction of expression vectors were purchased from MBI Fermentas (St. Leon-Rot, Germany).

Radiolabeled Tracers

^{125}I-cAMP (2000Ci/mmol) was obtained from Amersham Biosciences (Braunschweig, Germany), and [^{3}H]diprenorphine (36 Ci/mmol) was purchased from PerkinElmer Life Sciences (Dreieich, Germany).

Cell Culture Reagents

Reagents were purchased from Invitrogen (Karlsruhe, Germany), fetal calf serum from PAN (Nürnberg, Germany), and cell culture material from NUNC GmbH & Co. KG (Wiesbaden, Germany).

Antibodies

The anti-cAMP antiserum was obtained from Bio-Yeda (Rehovot, Israel), the anti-DsRed antibody from BD Biosciences Clontech (Heidelberg, Germany), the polyclonal anti-GRK2 and -3 antibodies from Santa Cruz Biotechnology (Heidelberg, Germany), and the monoclonal anti-GRK2/3 antibody from Upstate Biotechnology (Lake Placid, NY).

Cell Culture

HEK 293 cells and neuroblastoma × glioma hybrid 108-15 cells were grown for confocal microscopy on glass coverslips (2.4 × 3.2 cm), maintained in DMEM, and supplemented with 10% fetal calf serum as described (Ammer and Schulz, 1993). Experiments were conducted with cells at 60% confluency.

DNA Transfection

Cells were transfected with Effectene Reagent (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol and used for experiments 48 h after transient transfection. Stable transfections of HEK cells to express κ-opioid receptor-EGFP were conducted according to standard procedures, using G418 selection.

Receptor Binding Studies

Opioid receptor binding studies (see Vachon et al., 1987) were conducted with freshly prepared membranes from drug-exposed cells. Membranes (100 μg of protein, 10^6 cells) were incubated for 30 min at 30°C with the ligand (tritiated tracer) in the absence and presence of 10 μM cold opioid to define nonspecific binding.

Western Blots

Proteins were resolved as described (Ammer and Schulz, 1993). Samples were electrophoresed on polyacrylamide gels under reducing conditions (10% gel, 200 mM dithiothreitol). Samples were boiled or left unboiled (see text) for SDS-PAGE. Markers provided size calibration, and proteins were electroblotted to nitrocellulose membrane at 4°C. Membranes were washed with 0.2% Tween-20 in Tris-buffered saline and incubated with the primary antibody overnight at 4°C. After appropriate secondary antibody steps (1 h, 25°C, 1:20,000) and rinsing, the blots were developed with enhanced chemiluminescence detection.

cAMP Assay

A slightly modified method (Ammer and Schulz, 1997) was employed. Briefly, cells were seeded onto 96-well plates (4.8×10^4 cells/well), allowed to settle for 2 h at 37°C in supplemented DMEM. Adenylyl cyclase was stimulated by 10 μM forskolin in the presence of 0.25 mM 3-isobutyl-1-methylxanthine over 15 min at 37°C. Assays were conducted in duplicate.

Construction of Expression Vectors

Human κ-Opioid Receptor-EGFP. The plasmid pcDNA1 AMP encoding the human κ-opioid receptor (Simonin et al., 1995) was provided by Dr. Kieffer. We amplified the insert by PCR (Taq-DNA polymerase; New England Biolabs, Schwalbach, Germany), using the following primers: F 5′-GCC GCG AGC TGC AGC GCT CAC-3′; R 5′-GCG TAC GGT ACC ATA CTG GTT TAT TCA TC-3′ (stop codon eliminated, construction of *Kpn*I cleavage site). The PCR fragment was cleaved with *Pst*I (F) and *Kpn*I (R) and inserted into the *Pst*I/*Kpn*I multiple cloning site of pEGFP-N3 (Clontech).

Mouse κ-Opioid Receptor-EGFP. The plasmid pBacPAK8 encoding the mouse κ-opioid receptor was provided by Dr. Bell (Yasuda et al., 1993). The κ-receptor insert was amplified by PCR, using the following primers: F 5′-GGA TCC CTG CAG CGC TCA CCA TGG-3′; R 5′-GCT CTT GGG CCC CAT ACT GGC TTA TTC-3′ (stop codon eliminated). The PCR fragment was cleaved with *Pst*I (F) and *Apa*I (R) and inserted into the *Pst*I/*Apa*I multiple cloning site of pEGFP-N3 (Clontech).

μ-Opioid Receptor-EGFP. The construction of the expression vector encoding the μ-receptor-EGFP sequence is detailed by Schulz et al. (1999b).

GRK2-DsRed. The vector pBC encoding the bovine GRK2 sequence was provided by Dr. Benovic (Benovic et al., 1989). Amplification of GRK2 was achieved by PCR, using the primers F 5′-GTC GAT AAG CTT ATG GCG GAC CTG GGA GGC GG-3′ (*Hin*dIII introduced) and R 5′-GAC TTA GTC GAC CAG AGG CCG TTG GCA CTG C-3′ (stop codon eliminated, *Sal*I introduced). The obtained PCR fragment was cut with *Hin*dIII (F) and *Sal*I (R) and cloned into the *Hin*dIII/*Sal*I sites of pDsRed-N1 (Clontech).

GRK3-DsRed. The plasmid pCDNA3 encoding the bovine GRK3 sequence was provided by Dr. Benovic (Benovic et al., 1991). The GRK3 insert was amplified by PCR, using the primer F 5′-CAG TGT GCT GGA ATT CGG CGT CCG-3′ and the primer R 5′-CAG TGT GCT GGA ATT CGG CGT CCG-3′ (stop codon eliminated). The PCR fragment was cleaved with *Eco*RI (F) and *Sac*II (R) and cloned in-frame into the *Eco*R1/*Sac*2 sites of the multiple cloning site of pDsRed1-N1.

Phosducin-EGFP. The construction of the phosducin-EGFP fusion protein has been reported (Schulz et al., 1998b). Each fusion protein (DNA sequence) was sequenced to control in-frame cloning and correctness of inserts.

κ-Opioid Receptor. The receptor was cloned into pcDNA3.1.

Confocal Microscopy

For LSM studies, cells were transfected and grown on coverslips (2.4 × 3.2 cm). After 2 days, they were placed in a device (PeCon, Zeiss, Germany) and maintained at 37°C, pH 7.4 (continuous CO_2

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flow) in a volume of 3 ml of DMEM. The drug under investigation was left in contact with cells until the end of the experiment. The transfected cells displayed a spectrum of fluorescence ranging from low to high. Studies were conducted preferentially with clones emitting medium fluorescence as judged by eye. Laser scanning confocal images were collected with an inverted Zeiss LSM 510 microscope (63 × 1.3 oil immersion Plan-Neofluar objective). For excitation the 488-nm argon-ion laser was used and the HeNe laser for 543 nm. The emission was collected with band pass filter 505 to 530 and long pass 560-nm filter, which allowed simultaneous monitoring of EGFP-tagged receptors and DsRed-tagged GRKs with no bleed through between channels. Images from EGFP and DsRed fluorescence patterns were processed as one-color images or merged as two-color overlays. The digitized images were prepared as graphics using Adobe Photoshop software.

Quantification of Receptor Internalization

Receptor internalization was judged by the presence of clearly detectable and definable green fluorescent vesicles monitored by confocal microscopy. The observation period (time after drug exposure) was limited to 15 min; thereafter vesicles will accumulate and form clusters. The confocal images were taken at medium level of the cell body. For identification of vesicles colocalizing EGFP-tagged κ-receptors and GRK-DsRed, the corresponding red and green fluorescent images were merged to quantify yellow (red and green fluorescence), green, and red fluorescent vesicles. The numbers of internalized vesicles are expressed as means ± S.E.M.

Results

The studies were designed to investigate mechanisms of κ-opioid receptor internalization. For this reason, κ-receptors as well as GRKs were fused to fluorescent proteins to follow their fate in living cells by LSM. Since the fluorescent molecules consist of about 30 kDa, we first examined the function of opioid receptors and GRKs when fused to fluorescent proteins.

Functionality of Fluorescent κ-Opioid Receptors and GRKs

κ-Receptors of mouse and man were fused to EGFP; the constructs were expressed in HEK cells and tested for their affinity to the κ-receptor ligand U-50488 in the absence and presence of GTPγS (10 μM). Figure 1 demonstrates the potency of U-50488 to displace [^{3}H]diprenorphine at the human κ-receptor and κ-receptor-EGFP construct, respectively. Evidently, GTPγS shifts the concentration-response curve to the right, confirming G protein control of GPCRs (Lefkowitz, 1998), including the κ-receptor-EGFP construct. In addition, the tagged κ-receptor displays identical binding characteristics compared with the wild-type receptor. No binding differences were observed between human κ-receptors and those of the mouse (data not given).

Tests for functionality of GRKs fused to DsRed rest on findings by Zhang et al. (1998) that over-expression of GRK2 attenuates μ-opioid receptor activity to inhibit adenylyl cyclase activity. This technique was used to examine the function of GRK2 and GRK3 fused to DsRed. HEK cells permanently expressing μ-opioid receptors, EGFP-tagged μ-opioid receptors, human κ-receptors, and EGFP-tagged κ-receptors, respectively, were transfected to express the individual GRK-fusion protein. Figure 2A displays the results obtained with μ-receptors and GRK2. Adenylyl cyclase was stimulated (forskolin, 10 μM), and cAMP accumulation was assayed in the presence of increasing DAMGO concentrations. Coexpression of GRK2 and GRK2-DsRed, respectively, in these cells attenuated the opioid activity to a similar degree, regardless whether wild-type receptor kinase or tagged kinase was over-expressed. Identical results were obtained with GRK3 and GRK3-DsRed. Analogous experiments were conducted with HEK cells expressing the EGFP-tagged human κ-opioid receptor. The results given in Fig. 2B report the response to U-50488, a κ-selective ligand, of cells over-expressing the fused κ-receptors only or fused κ-receptors plus distinct GRKs. The opioid brings about an inhibition of forskolin-stimulated cAMP accumulation up to 40%. Over-expression of GRK2, GRK3, and GRK3-DsRed, respectively, fails to attenuate the potency of U-50488 to inhibit adenylyl cyclase. Similar results were seen with cells over-expressing GRK2-DsRed (data not given). To rule out that a strongly reduced over-expression of exogenous GRKs in κ-receptor carrying HEK cells accounts for the observed failure of GRKs, we examined, by using Western blotting, the degree of expression of GRK2- and GRK3-DsRed in HEK cells permanently expressing EGFP-tagged μ- or κ-opioid receptors (Fig. 3). Cytosol of transfected cells was electrophoresed, and expression of the different GRKs fused to DsRed was detected by the anti-DsRed antibody. Densitometry of the immunostained bands (110 kDa) revealed very similar expression levels for GRK2/3-DsRed in cells coexpressing the tagged μ- and κ-receptors. The results do not suggest that different expression levels of the GRK fusion proteins account for the different responses (cAMP accumulation) of the transfected HEK cells (Fig. 2, A and B).

κ-Opioid Receptor Sequestration

Translocation of GRKs and κ-Receptors: Confocal Microscopy. HEK cells were transfected to stably express either the EGFP-tagged mouse κ-receptor or the tagged human receptor. Using LSM, the receptors (green fluorescence) were localized almost exclusively in the cell membrane. When challenged with U-50488 (10 nM) an estimation by eye

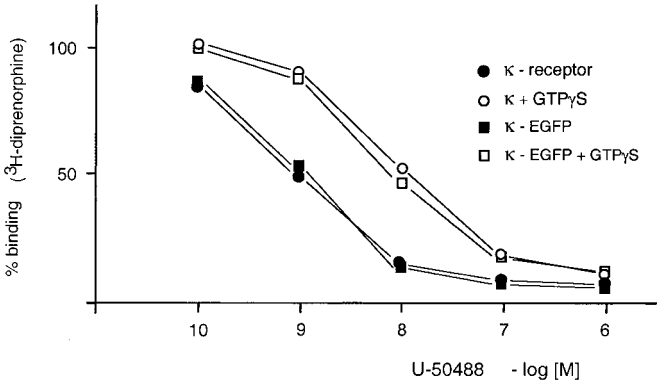

Fig. 1. HEK cells expressing κ-opioid receptors and EGFP-tagged κ-opioid receptors, respectively, were employed for receptor binding studies. Cell membranes were incubated with [^{3}H]diprenorphine in the absence (solid symbols) and presence (open symbols) of GTPγS (10 μM) for 30 min at 30°C. Membranes were exposed to increasing concentrations of the κ-receptor ligand U-50488. Displacement of the tritiated opioid antagonist is expressed as percentage of binding in the absence of the κ-agonist. Unspecific binding was assayed with 10 μM U-50488. The data given are the averaged results of three experiments with the S.E.M. for each data point less than 10%. Abscissa, molar concentrations of U-50488; ordinate, percentage of total [^{3}H]diprenorphine receptor binding in the presence of increasing concentrations of U-50488.

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failed to detect internalization (fluorescent vesicles) of mouse κ- or human κ-receptors within 30 min (data not given). Identical results were obtained when the EGFP-tagged κ-receptors were transiently expressed in NG 108-15 cells.

NG 108-15 cells were cotransfected to transiently express GRK2-DsRed as well as the mouse and the human κ-receptor-EGFP, respectively. Figure 4 displays confocal images of two cells. One cell expresses both the murine κ-receptor-EGFP (images A–C) and GRK2-DsRed (images D–F), and the second cell expresses the human κ-receptor-EGFP (images G–I) and GRK2-DsRed (images J–L). Both cells were challenged with U-50488 (5 nM). Activation of the mouse receptor results in translocation of the tagged kinase (red fluorescence) towards the cell membrane in less than 1 min (image E), and accumulation of GRK2-DsRed remains visible at the cell membrane for 3 min (images not shown) and vanishes thereafter (image F). However, no internalization of mouse κ-receptors (generation of green fluorescent vesicles) was observed during the course of the experiment (15 min, images A–C). Like the mouse receptor, activation of human κ-receptors also triggers an immediate translocation of the kinase to the cell membrane (image K). In contrast to the mouse receptor, the activated human receptor does internalize (observation period 15 min, image I). Most interestingly, the image displaying the "red channel" fluorescence clearly demonstrates the appearance of vesicles, exhibiting red fluorescence (GRK2-DsRed, image L). Superimposing images I and L reveals strict colocalization of κ-receptors and GRK2 (not shown), indicating cointernalization of κ-receptors and GRK2.

Identical experiments conducted with HEK cells inconsistently uncovered translocation of GRK2-DsRed. Noteworthy, expression of GRK3-DsRed in NG 108-15 cells resulted in the formation of patchy red fluorescent material unevenly distributed in the cytosol. In general, this phenomenon was not observed with HEK cells. It is assumed that oligomerization of DsRed-constructs (Baird et al., 2000) accounts for this observation.

Difference between GRK2 and GRK3 to Promote Internalization of the Human κ-Opioid Receptor. Confocal microscopy failed to clearly demonstrate internalization of the κ-receptor-EGFP expressed in HEK cells (observation periods up to 1 h). When the cells coexpressed tagged κ-receptors with GRK2-DsRed and GRK3-DsRed, respectively, receptor internalization is observed in response to U-50488 and U-69593 challenge. Figure 5 displays two cells, each permanently expressing κ-receptors tagged with EGFP and GRK2-DsRed (images A–I) or GRK3-DsRed (images J–R). The confocal images given cover the distribution of fluorescence before (0 min) and after (1 and 15 min), to challenge with the κ-ligand U-50488 (10 nM). Image A (0 min) presents several cells, exhibiting green fluorescence (κ-receptors) primarily associated with cell membranes (A and B), but only two cells coexpress major amounts of GRK2-DsRed (A and C). After 1 min of exposure to U-50488, translocation of GRK2-

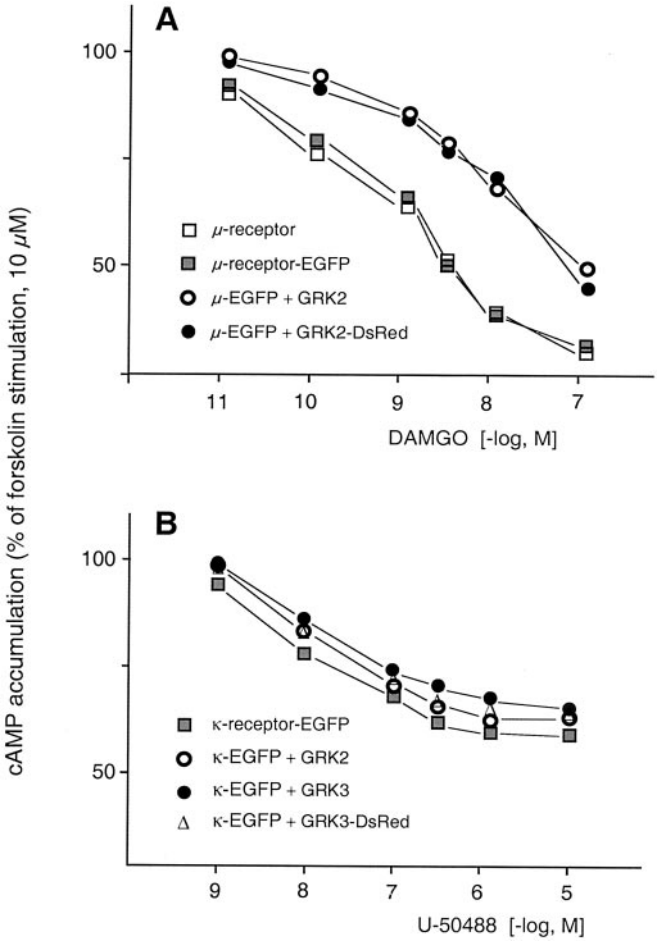

Fig. 2. Effect of GRK over-expression on opioid activities to inhibit forskolin-stimulated cAMP generation of HEK cells stably expressing wild-type μ-opioid receptors and EGFP-tagged μ-receptors, respectively (A), or the κ-opioid receptor-EGFP (B). A, HEK cells were transfected to express μ-receptors (squares, open and closed) and in addition GRK2 (circles) or GRK2-DsRed (closed circles). Cells were stimulated with forskolin (10 μM) and simultaneously exposed to increasing concentrations of the μ-receptor ligand DAMGO. Basal cAMP levels of controls amount to 11 ± 4 fmol/well (1.2 times 10^4 cells, 15 min). Forskolin-stimulated levels amount to 520 ± 60 fmol/well. Data represent averaged cAMP concentrations of three independent experiments with S.E.M of less than 12%. Ordinate, percentage of cAMP concentration of forskolin-stimulation in the absence of DAMGO; abscissa, molar DAMGO concentration. B, HEK cells stably expressing κ-receptors-EGFP (squares) and transiently GRK2 (circles), GRK3 (closed circles), and GRK3-DsRed (triangles), respectively, were stimulated with forskolin (10 μM). cAMP assays in the presence of increasing concentrations of U-50488 were conducted as described under A.

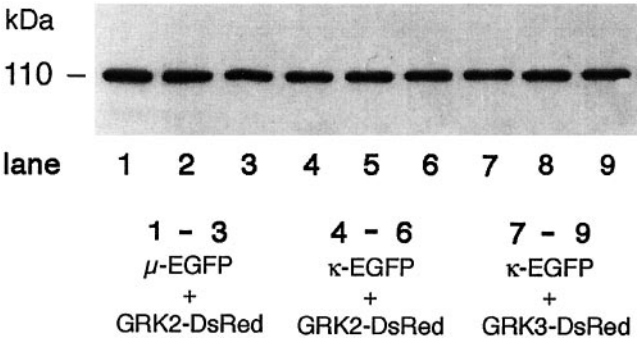

Fig. 3. Western blotting of cytosol (10 μg of protein each lane) from HEK cells permanently expressing EGFP-tagged μ- and κ-receptors, respectively. Cells have been cotransfected with constant amounts of cDNA coding for GRK-DsRed. The following cotransfections were conducted: μ-opioid receptor-EGFP (μ-EGFP) and GRK2-DsRed; κ-opioid receptor-EGFP (κ-EGFP) and GRK2-DsRed; κ-EGFP and GRK3-DsRed. Immunoreactive GRK2/3-DsRed (110 kDa) was revealed by anti-DsRed antibodies. Each lane represents an independent cotransfection of a newly raised HEK cells. The experiment shown stands for two series of experiments (18 independent cotransfections).

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DsRed toward the cell membrane is observed, although hardly visualized on reproduced image F. After 15 min of receptor activation, green fluorescent vesicles (κ-receptors) are accumulating in the cytosol, mainly in those two cells clearly expressing GRK2-DsRed (image H). Cells lacking visible red fluorescence (GRK2-DsRed) largely failed to exhibit convincing receptor internalization. However, the internalized vesicles carrying EGFP-tagged κ-receptors also exhibit

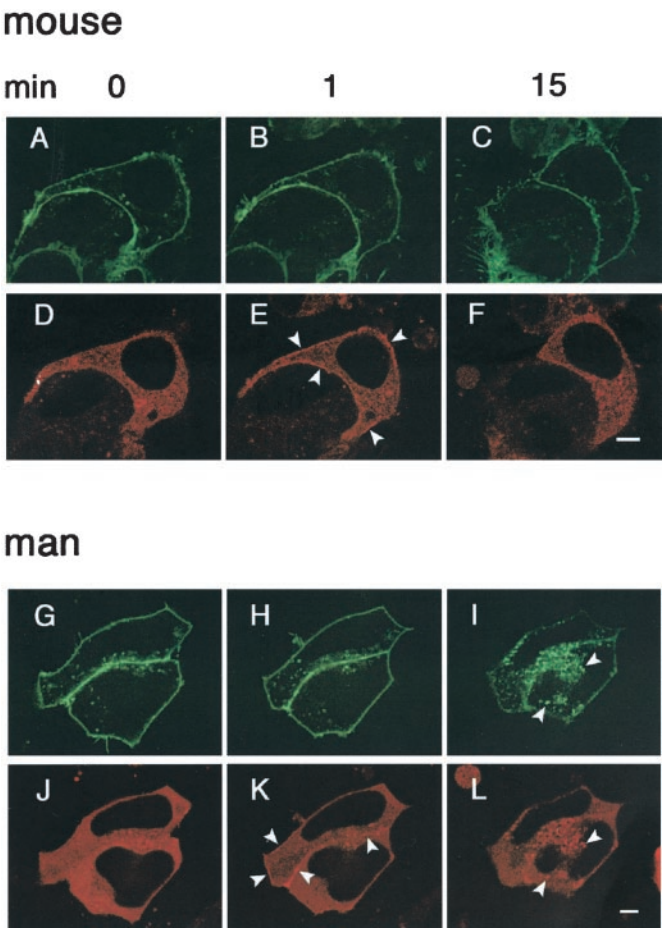

Fig. 4. Fluorescence imaging of NG 108-15 cells coexpressing κ-receptors fused to EGFP and GRK2 fused to DsRed. Images of the tagged mouse κ-opioid receptor (A, B, C) are given before (0 min, image A) and after (1 and 15 min, images B, C) to U-50488 (5 nM) exposure of cells. Coexpression of GRK2-DsRed of this cell is given (images D, E, F). Analogous confocal images are given for the human κ-opioid receptor-EGFP and GRK2-DsRed (G to L). The NG 108-15 cell expressing mouse κ-receptors (green fluorescence, images A to C) and GRK2-DsRed (red fluorescence, D, E, F) reveals translocation of cytosolic GRK2-DsRed toward the cell membrane within 1 min after U-50488 exposure (image E, arrowheads point to translocated GRK2-DsRed). However, the mouse receptor fails to internalize upon opioid challenge, as no formation of green vesicles migrating off the cell membrane was monitored. Stimulation of human κ-receptor by U-50488 also triggers translocation of GRK2-DsRed toward the membrane (image K, arrows). In contrast to the mouse receptor, the activated human κ-receptor sequesters as indicated by the accumulation of green vesicles in the cytosol (15 min after drug exposure, image I). Confocal microscopy uncovers cointernalization of receptors and GRK2-DsRed as revealed by the formation of red vesicles (image L). The arrowheads of images I and L point out colocalization of κ-receptor-EGFP and GRK2-DsRed. Almost each green vesicle of image I is accompanied by red fluorescence (image L). Arrowheads in I and L highlight two coincident green and red vesicles. The response of κ-receptors in the cells shown is representative for more than 15 specimens from independent experiments. However, for better visibility of human κ-receptor internalization, we selected a HEK cell exhibiting a strong internalization. Calibration bars indicate 2 μm.

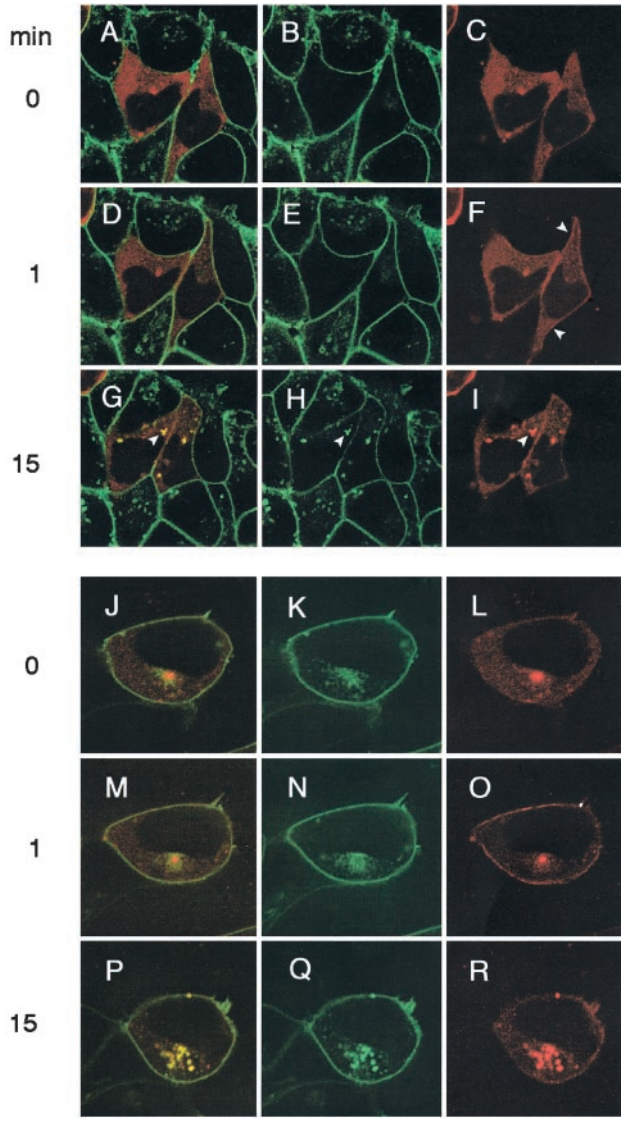

Fig. 5. Response of HEK cells stably expressing human κ-opioid receptor and transiently GRK2-DsRed (images A–I) or GRK3-DsRed (images J–R). Optical sections A and B reflect membrane located EGFP-tagged κ-receptors and cytosolic GRK2-DsRed (A and C) before drug treatment (0 min). Although all cells carry EGFP-tagged receptors, only two cells express high levels of GRK2-DsRed. Exposure of cells to U-50488 (10 nM) triggers translocation of red-GRK toward the cell membrane (F, 1 min, arrowheads point to enrichment). After 15 min, the cells expressing GRK2-DsRed exhibit internalization of opioid receptors (image H) and appearance of red vesicles (GRK2-DsRed, image I). When images H and I were superimposed, colocalization of vesicles is apparent (G). Arrows of H and I indicate colocalization of green and red vesicles (green receptors and red GRK2). Merging images H and I reveals the emission of yellow fluorescence (G, arrow), indicating a close spatial proximity (below optical resolution) of green- and red-tagged proteins. J, K, and L reflect confocal images of a HEK cell displaying green fluorescent human κ-receptor-EGFP (J, K) and GRK3-DsRed fluorescence (J, L) before U-50488 (10 nM, 0 min) treatment. After drug challenge (1 min), a strong translocation of cytosolic GRK3-DsRed toward the cell membrane is monitored (O), while no change in distribution of κ-receptors was observed. After 15 min, a strong internalization of receptors (green vesicles, image Q) is seen. Simultaneous monitoring of GRK3-DsRed distribution uncovers the formation of red vesicles (R). When images Q and R are merged, very close colocalization of receptors and GRK3 is documented (yellow fluorescence, P). The cells displayed here expressing GRK2-DsRed and GRK3-DsRed are representative of more than 10 cells each. The GRK3-DsRed-transfected cells consistently exhibited much stronger translocation of GRK3-DsRed and subsequent κ-receptor internalization compared with cells expressing GRK2-DsRed. Scale bars: A, 7 μm (images A to I); J, 4 μm (images J to R).

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red fluorescence, indicating cointernalization of κ-receptors and GRK2-DsRed (image I). This phenomenon becomes obvious when merging images H and I. The superimposed "red" and "green" vesicles suggest a very close colocalization of fluorescent entities below optical resolution as the emitted fluorescence turns yellow.

The cell overexpressing GRK3-DsRed (images J–R) was also exposed to U-50488 (10 nM), and confocal images were taken up to 15 min thereafter. Before drug exposure, green fluorescent opioid receptors are localized in the cell membrane (Fig. 5, images J and K), and the red fluorescent kinase is homogeneously distributed in the cytoplasma (J, L). Within 1 min after drug challenge, an intense translocation of GRK3-DsRed toward the the cell membrane takes place (image O), where it remains accumulated for several minutes (not shown). Thereafter we observed a strong internalization of vesicles carrying green fluorescence (κ-receptor, 15 min, image Q). Display of the red channel reveals vesicles exhibiting red fluorescence (GRK3-DsRed, image R). Superimposition of images Q and R discloses colocalization of red and green fluorescent material, strongly indicating cointernalization of κ-receptors and GRK3. Again, the occurrence of yellow fluorescence confirms a very close location of fluorescent receptors and kinases.

Sequestration of Human κ-Opioid Receptors and GRKs: Quantification. The results obtained by LSM technique suggested to us an increased rate of κ-receptor internalization in presence of exogenous GRK3 compared with GRK2. Figure 6 presents the mean number of LSM-detectable vesicles generated within 15 min after challenge of cells with U-50488 (20 nM). Under these conditions a moderate internalization of EGFP-tagged κ-receptors is noted ($p < 0.01$) in the absence of exogenous GRK2/3-DsRed. In the presence of GRK2-DsRed, internalization is enhanced. A separate documentation of receptor-related fluorescence and GRK-related fluorescence reveals that almost all vesicles transport both κ-receptors and GRK2. Analogous experiments with GRK3-DsRed demonstrate the highest internalization rate of κ-receptor. A mean number of 11.5 internalized green vesicles indicates a significant enhancement compared with cells expressing GRK2-DsRed (6.3 vesicles). Again, green and red fluorescence was colocalized in about 90% vesicles analyzed.

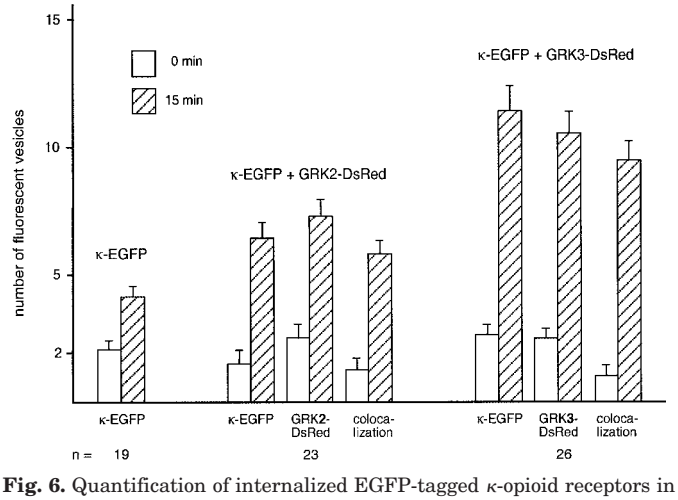

Fig. 6. Quantification of internalized EGFP-tagged κ-opioid receptors in HEK cells. Vesicles carrying green fluorescence were monitored by LSM, and their number before challenge with U-50488 (20 nM) as well as 15 min after was registered. Columns represent the number of vesicles in the absence of exogenous GRK and the presence of GRK2-DsRed and GRK3-DsRed, respectively. Confocal images were analyzed for green fluorescent κ-receptors (κ-EGFP), for red fluorescent GRKs (GRK-DsRed), and for colocalization of red and green fluorescence after merging the corresponding green and red fluorescent images. Degree of significance of differences between the number of internalized vesicles carrying κ-receptors: absence and presence of GRK2-DsRed $p < 0.05$; absence and presence of GRK3-DsRed $p < 0.01$; n, number of cells analyzed, each cell represents an independent transfection.

Internalization of Human κ-Opioid Receptors: Receptor Binding Studies. The LSM technique proved suitable to analyze receptor sequestration in single cells. On the other hand, receptor binding studies provide an alternative technique to evaluate receptor internalization, requiring about 10^6 cells per assay. Figure 7 demonstrates κ-receptor binding studies conducted with membranes of HEK cells exposed to U-50488. In HEK cells permanently expressing EGFP-tagged κ-receptors, binding studies failed to detect receptor internalization upon prolonged U-50488 exposure, using experimental conditions employed for LSM studies (20 nM, 30 min, 37°C). When these cells were transiently transfected to coexpress GRK2 the identical treatment of cells triggered a moderate disappearance (5%, $p < 0.06$) of κ-receptors from the cell membrane. However, over-expression of GRK3 reduced κ-receptors for 17% ($p < 0.02$).

The Effect of Phosducin on κ-Receptor Internalization. Phosducin competes with GRK2/3 for binding to Gβγ subunits, thereby preventing phosphorylation of GPCRs. Since phosducin attenuates μ- and δ-opioid receptor internalization (Schulz et al., 1999a,b), we transfected HEK cells permanently expressing the human κ-receptor-EGFP with cDNAs coding for phosducin-EGFP and for GRK3-DsRed. Cells were challenged with U-50488 (10 nM), and those expressing the three different fusion proteins were selected for analysis by LSM. The results clearly demonstrate that internalization of the human κ-opioid receptor is strongly attenuated in the presence of phosducin, because almost no newly generated vesicles were observed up to 30 min after drug application.

Expression of Endogenous and Exogenous GRKs in HEK Cells. Cytosol of wild-type HEK cells as well as of cells

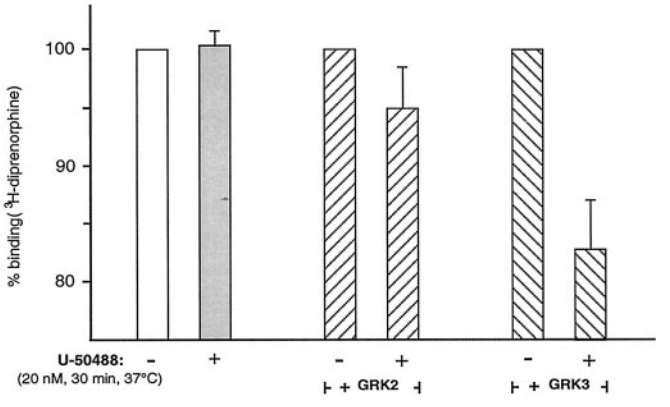

Fig. 7. Effect of prolonged exposure (30 min, 37°C) of HEK cells expressing EGFP-tagged κ-receptors to U-50488 (20 nM) in the absence of exogenous GRKs, and in the presence of GRK2 and GRK3, respectively. κ-Receptor capacity of cell membranes was estimated analogous to the technique given for Fig. 1. The decline of binding sites in the presence of GRK2 reached a level of significance of $p < 0.06$, in the presence of GRK3 $p < 0.02$. $n = 3$ for each experimental design.

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over-expressing GRKs was submitted to Western blot analysis (Fig. 8). Noteworthy, the monoclonal antibody employed for detection of GRKs recognizes both GRK2 and GRK3 (manufacturer's instructions). The immunoreactive bands migrating at 79 kDa (marker) represent endogenous (lanes 1 and 4) as well as over-expressed GRK2 (lane 2), and GRK3 (lane 5). Moreover, HEK cells clearly express GRK2-DsRed (lane 3, 110 kDa) and GRK3-DsRed (lane 6).

We further examined whether DsRed constructs in HEK cells exist as tetramers or even octamers as has been suggested (Baird et al., 2000; Gross et al., 2000). These authors report the detection of DsRed oligomers (120–200 kDa immunoreactive material) when submitted unboiled to electrophoresis. However, the high molecular mass DsRed material disappeared when the samples were boiled. We applied this experimental approach to HEK cells expressing DsRed and GRK3-DsRed and submitted boiled and unboiled cytoplasma samples (10 μg of protein) to gel electrophoresis. Western blot analysis (anti-DsRed antibodies) revealed a single immunoreactive band (30 kDa) for cells expressing DsRed and a single band at 110 kDa for cells expressing GRK3-DsRed. Boiled and unboiled samples displayed identical Western blot results (data not shown). We found no support for the notion that DsRed or DsRed constructs form oligomers, or that the DsRed constructs tested here undergo proteolysis to form DsRed and GRK3.

Discussion

Opioid receptors couple to G proteins and may internalize when activated (Carmann and Benovic, 1998). However, the κ-opioid receptor displays an unusual feature as sequestration occurs in a species-specific manner. That is, the human κ-receptor internalizes whereas the murine receptor fails (Li et al., 1999). We report here that GRK2 over-expression in HEK cells facilitates sequestration of human κ-receptors, which is even more pronounced in cells expressing exogenous GRK3. Moreover, the activated human κ-opioid receptor consistently cointernalizes with GRK2- and GRK3-DsRed, respectively.

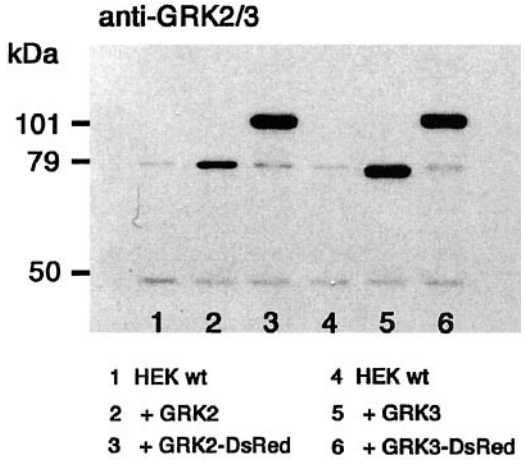

Fig. 8. Western blot analysis of cytosolic proteins (5 μg) from HEK cells. Samples were from HEK wild-type cells (lanes 1 and 4), from cells over-expressing GRK2 (lane 2), GRK2-DsRed (lane 3), GRK3 (lane 5), and GRK3-DsRed (lane 6). The monoclonal antibody employed to develop immunoreactive bands does not differentiate between GRK2 and GRK3, and detects equally well GRK2-DsRed and GRK3-DsRed. The blot is representative of two independent experiments.

In this study, the confocal microscopy has been employed as a major technique to uncover the fate of activated κ-opioid receptors and GRKs in living HEK cells. However, the detection of specific proteins by LSM requires their labeling with fluorescent compounds such as EGFP or DsRed. Although the generation and expression of relevant fusion proteins was achieved, the size of the attached fluorescent labels (EGFP, DsRed) of about 30 kDa (Heim et al., 1995; Baird et al., 2000) raises concern about the conservation of function of tagged receptors and GRKs. The test conducted for κ-opioid receptor constructs examined their binding affinities in the presence and absence of GTPγS. The results clearly document that wild-type κ-receptors and κ-receptors fused to EGFP displayed identical IC_{50} values for U-50488 to displace tritiated diprenorphine, resembling already published reports (Simonin et al., 1995). In addition, an intact functional coupling between tagged and non-tagged receptors and G proteins was proven as the affinity for the κ-ligand declined in the presence of GTPγS. These results suggest that the fluorescent label does not at all disturb the function of κ-receptors.

We further examined the function of GRKs fused to DsRed, employing the finding by Zhang et al. (1998) that over-expression of GRK2 attenuates the μ-opioid receptor-induced inhibition of cAMP accumulation. Both exogenous GRKs and the corresponding tagged receptor kinases bring about an identical inhibition of μ-opioid receptors-triggered control on adenylyl cyclase activity. In principle, the same experimental outcome was observed in HEK cells expressing δ-opioid receptors (unpublished observation). Thus, the studies clearly document that DsRed fused to GRK2/3 does not disturb the function of these constructs. However, conflicting results were obtained with cells expressing human κ-opioid receptors, as coexpression of GRKs as well as of tagged GRKs failed to attenuate the κ-ligand-induced inhibitory action on cAMP generation. This failure does not relate to a reduced expression of exogenous kinases in HEK cells expressing κ-receptors, since Western blotting of cytosol separated from μ- and κ-opioid receptor carrying cells reveals very similar GRK-DsRed expression levels. On the other hand, the tagged GRKs exert function as they facilitate the process of μ-, δ-, and of κ-opioid receptor sequestration, as do the wild-type GRKs. It remains to be understood how the over-expressed GRKs differently effect the control of adenylyl cyclase activity via μ- and δ-receptors and via κ-receptors, while the process of μ- and κ-receptor sequestration is facilitated by these exogenous kinases.

Specific isoforms of GRKs phosphorylate their substrates, the GPCRs, with different preferences (Carman et al., 1999). The major outcome of the experimental approaches employed here suggests that differences exist between GRK2 and GRK3 to promote internalization of the human κ-opioid receptor. This conclusion rests in particular on the LSM technique conducted with living cells transfected to express fluorescent opioid receptors and GRKs. We noticed that cells over-expressing GRK3-DsRed responded to U-50488 more strongly to internalize vesicles carrying κ-receptors compared with cells expressing exogenous GRK2-DsRed. Quantification of vesicles ascertainable by confocal microscopy in GRK3-DsRed expressing cells revealed that about twice the number was present in the cytosol within 15 min after drug

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challenge compared with that in the presence of exogenous GRK2-DsRed. Opioid receptor binding studies largely confirmed that more human κ-receptors disappeared from the cell surface in cells expressing GRK3-DsRed compared with the tagged GRK2. It should be pointed out that LSM studies with living cells were selected by eye and investigated, whereas each receptor binding study represents the mean of 10^6 cells. All these cells carry κ-receptors but transient cotransfection with GRK-DsRed is achieved only for an estimated 20 to 30% cells per batch with variable expression levels per cell. We confirmed findings (Li et al., 1999), that the endogenous GRK2 level of HEK cells and even over expressed GRK2 proved poorly effective to internalize κ-receptors, although internalization of δ- and μ-receptors is strong in these cells (Schulz et al., 2002). Elevating the kinase activity by over-expression of GRK2-DsRed enhances κ-receptor internalization but the effect was of a moderate degree. In contrast to GRK2, GRK3 was not detected by Western blotting in HEK cells. When GRK3-DsRed was expressed, we observed the strongest receptor internalization by LSM at rather low kinase concentrations as judged by the intensity of red fluorescence (GRK3-DsRed) in the cytosol, and an analogous result was obtained with receptor binding studies. These observations led us to propose a preferred phosphorylation of human κ-receptors by GRK3 as a prerequisite of an effective receptor sequestration.

The interpretation of our findings with GRK-DsRed constructs should consider the tendency that DsRed forms oligomers under specific conditions (Baird et al., 2000; Gross et al., 2000). We analyzed the expressed DsRed as well as GRK3-DsRed in HEK cells by means of Western blot technique and found absolutely no hint for the formation of DsRed oligomers or of GRK3-DsRed tetramers in cytosol samples not boiled prior to electrophoresis (heating destroys DsRed oligomers; Baird et al., 2000). In addition, there was no indication that GRK3-DsRed breaks down intracellularly, generating DsRed and GRK3. There is sufficient reason to assume that GRK3-DsRed accounts for the observed internalization of κ-opioid receptors. Moreover, DsRed is known to exist both in the cytosol and the nucleus (Schulz et al., 2002), but confocal images of cells expressing GRK3-DsRed revealed the absence of red fluorescence in the nucleus. Thus, the findings reported here strongly support the view that DsRed and DsRed constructs exist as monomers in HEK cells.

This study also provides evidence for living cells by means of the LSM technique that activation of κ-opioid receptors triggers accumulation of GRKs at the cell membrane. Although translocation of cytosolic kinases subsequent to GPCRs activation has been described (Ferguson et al., 1996; Pitcher et al., 1998), our observation with respect to the κ-receptors deserves attention. Activation of both the mouse and the human κ-receptor causes translocation of GRKs, but only the human κ-receptor undergoes sequestration. Thus, GRK translocation is triggered by receptor activation regardless of the receptor's ability to undergo sequestration.

The LSM technique further revealed that κ-opioid receptors and GRKs cointernalize. The process is initiated by κ-receptor activation, causing translocation and binding of GRKs to Gβγ subunits attached to the interior of the cell membrane (Hekmann et al., 1994). We assume GRKs strongly bind to the Gβγ subunits preventing their immediate dissociation and release back to the cytosol. The critical function of Gβγ for the process of internalization becomes clear when phosducin-EGFP is included. Phosducin is known to compete with GRK2/3 for Gβγ, thereby protecting the receptor from phosphorylation (Müller et al., 1997; Schulz et al., 1998a) and internalization (Schulz et al., 1999a,b). The very close location of fluorescent receptors and GRKs in microdomains of the membrane may account for the observed cointernalization. In fact, the yellow fluorescent vesicles documented in our study strongly support the notion that κ-receptors and GRKs cointernalize. The observation reported here is not unique to the κ-receptor as a recent report by Ruiz-Gomez and Mayor (1997) suggested that G protein-coupled excitatory acting β-adrenergic receptors cointernalize with GRK2. We speculate that our finding may contribute to an understanding of process regulating signal transmission. Receptor internalization not only removes receptive sites from the cell surface but also diminishes available cytosolic GRKs. Thus, vesicles carrying κ-opioid receptors and in addition GRKs may function as scavengers for these kinases, which in turn may contribute to receptor-mediated signal transmission. Our findings may gain interest in light of the report that levels of GRK2 and GRK3 alter during the state of opioid tolerance and dependence (Hurle, 2001).

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