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Phosducin, β-arrestin and opioid receptor migration

Rüdiger Schulz [a,*], Andrea Wehmeyer [a], John Murphy [b], Karin Schulz [c]

[a] *Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Königinstr. 16, D-80539 Munich, Germany*
[b] *Max-Planck-Institute for Biochemistry, Department of Cell Biology, Am Klopferspitz 18, D-82152 Martinsried, Germany*
[c] *Gene Center, University of Munich, Feodor-Lynen-Str. 25, D-81377 Munich, Germany*

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Abstract

Internalization of G protein-coupled opioid receptors depends on multiple criteria, including the affinity of drugs to their receptors and the state of the receptor–G protein interaction. Most recent studies reveal that cytosolic components like phosducin and arrestin interfere with receptor internalization, that is phosducin impairs receptor phosphorylation and arrestin enhances endocytosis by uncoupling the receptor from its G protein. This study was designed to examine the mutual effect phosducin and arrestin exert on receptor endocytosis. Neuronal NG 108-15 hybrid cells transiently expressing the μ-opioid receptor, which has been fused to green fluorescence protein, were employed to study internalization of the fluorescent μ-opioid receptor construct in living cells by means of confocal laser scanning microscopy. Fluorescent μ-opioid receptors were detected in drug-naive cells both at the cell membrane and at cell surface protrusions, most likely filopodia, microspikes and retraction fibres. The opioid receptors present in the cell membrane internalize upon etorphine (1 nM) exposure, a process clearly blocked in cells overexpressing phosducin. However, coexpression of both phosducin and β-arrestin 1 reverses this blockade. In contrast to etorphine, morphine fails to internalize μ-receptors expressed in NG 108-15 cells. When arrestin is overexpressed in these cells, morphine gains the ability to induce endocytosis, and this process is left unaffected by phosducin. The findings suggest that endocytosis of activated μ-opioid receptors primarily depends on arrestin-triggered uncoupling of the receptor from its G protein complex. Drug-induced receptor phosphorylation appears of subordinate significance for receptor internalization.

Keywords: Endocytosis; μ-Opioid receptor; Phosducin; Arrestin; NG 108-15 cell; Green fluorescence protein

1. Introduction

Activation of G protein-coupled receptors, including the opioid receptors, brings about desensitization and possibly receptor internalization (Lefkowitz, 1998). The underlying biochemical processes have been reported to depend on multiple mechanisms, such as the affinity of ligands and their intrinsic activity (Kovoor et al., 1998), the phosphorylation of receptors (Zhang et al., 1998), the activity of arrestins (Krupnick and Benovic, 1998), and the formation of clathrin-coated pits (Lin et al., 1997). On the other hand, there is evidence to demonstrate that sequestration of receptors does not require each of the criteria mentioned, e.g., receptor phosphorylation (Murray et al., 1998), and no strict relation exists between receptor affinity of drugs and their ability to cause receptor internalization (Gaudriault et al., 1997). The present study was designed to more closely investigate opioid receptor endocytosis under the influence of the cytosolic components phosducin (Lee et al., 1987) and β-arrestin 1 (Krupnick and Benovic, 1998).

The phosphoprotein phosducin (Lee et al., 1992) has been reported to represent an ubiquitous cytosolic component (Danner and Lohse, 1996), which upon receptor stimulation translocates towards the cell membrane (Schulz et al., 1998a). Phosducin binds to Gβγ liberated from the G protein trimer by receptor activation (Blüml et al., 1997), thereby preventing G protein-coupled receptor kinases (GRKs) to bind to the occupied G protein dimer. Gβγ serves cytosolic GRKs as membrane anchor (Hawes et al., 1994), a prerequisite to phosphorylate agonist-occupied receptors. Phosphorylation of receptors, including the opioid receptors, is required to induce desensitization (Hawes et al., 1994; Pitcher et al., 1995; Schulz et al., 1998b). Moreover, binding of phosducin to Gβγ hinders reassociation of G protein subunits to form a trimer (Lee et

* Corresponding author. Tel.: +49-89-2180-2662; Fax: +49-89-34-23-16; E-mail: schulz@pharmtox.vetmed.uni-muenchen.de

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al., 1992), which eventually results in an increased GTPase activity of Gα subunits (Bauer et al., 1992). These functions of phosducin, specifically those relating to neutralization of Gβγ, may contribute to inhibition of receptor-mediated endocytosis (Lin et al., 1997), a mechanism most recently documented for opioid receptors in NG 108-15 neuronal hybrid cells stably overexpressing phosducin (Schulz et al., 1999).

Further cytosolic components most critical for the process of receptor endocytosis are the arrestins (Lefkowitz, 1998). These proteins redistribute following receptor activation and tightly bind to the C-terminal of phosphorylated receptors, thereby uncoupling the receptor from its G protein (Ferguson et al., 1996). As consequence of the interruption of signal transmission endocytosis of receptors will be initiated (Krupnick and Benovic, 1998). These findings seem to attribute also to μ-opioid receptors, as β-arrestin represents a strong cellular constituent accounting for rapid receptor endocytosis in cooperation with G protein-coupled receptor kinases (Kovoor et al., 1997).

The studies reported here examine the effect of phosducin and β-arrestin on internalization of μ-opioid receptor activated by etorphine and morphine, respectively. These ligands were selected since etorphine is well known to trigger rapid endocytosis of μ-receptors, while morphine has been communicated to lack the ability to bring about receptor internalization (Keith et al., 1996). Employing these opioids we tested the mutual effect of phosducin, which is known to prevent internalization (Schulz et al., 1999), and β-arrestin, which stimulates internalization (Kovoor et al., 1997). These cellular processes were made visible in living NG 108-15 neuronal hybrid cells, using confocal laser scanning microscopy to follow the migration of μ-opioid receptors fused to green fluorescence protein (Schulz et al., 1999).

2. Materials and methods

2.1. Materials

2.1.1. Cell culture

Neuroblastoma × glioma hybrid NG 108-15 cells were raised in DMEM medium supplied with 10% fetal calf serum (Schulz et al., 1998a) and penicillin (100 IU/ml)/streptomycin (100 μg/ml). If not otherwise mentioned, experiments were conducted with cells at 70% confluency.

2.1.2. Compounds

Morphine–HCl was purchased from Merck (Mannheim, Germany), etorphine–HCl was provided by National Institute of Drug Abuse (USA). Phalloidin and all further compounds were obtained from Sigma (Taufkirchen, Germany).

2.1.3. Antibodies

The β-arrestin antibody was purchased from Dianova (Heidelberg, Germany), using a dilution of 1:250, the peroxydase-conjugated second antibody was employed at a concentration of 1:3000.

2.2. Treatment of cells

Cells were raised on glass coverslips and exposed to the opioid under investigation at 37°C (cell incubator) for time periods indicated under Section 3. For examination of fluorescence the coverslip carrying the adherent living cells were submitted to confocal microscopy at 23°C. Optical sections of the cells were taken at 1 μm intervals moving from bottom to top. The cells were kept during microscopy at the same drug concentration they were exposed during the incubation period at 37°C.

2.3. Gel electrophoresis and Western blotting

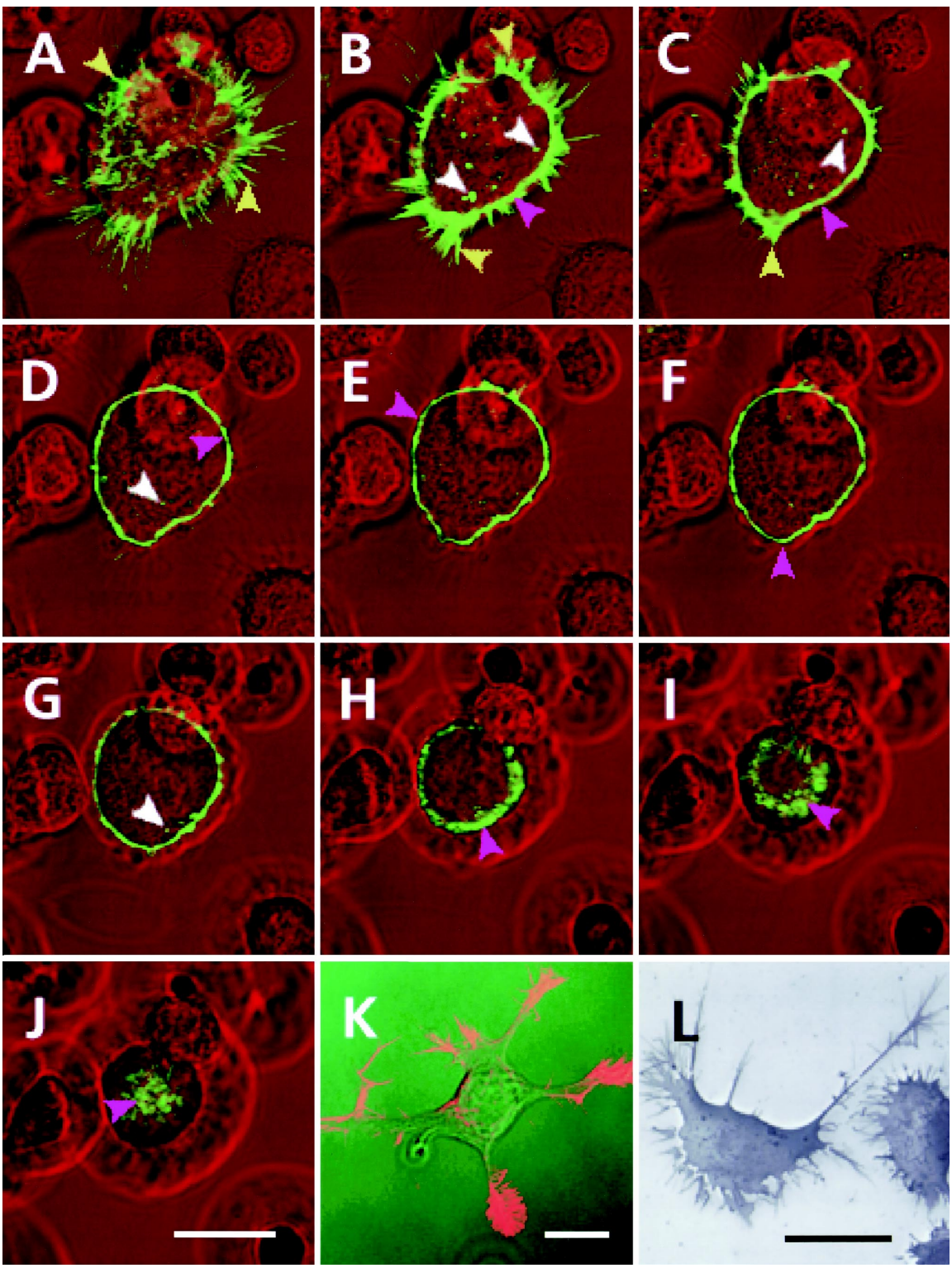
Gel electrophoresis was conducted as described by Schulz et al. (1998a). Cells were taken up in equal volume of phosphate buffered saline/ethylenediaminetetraacetic acid, sonicated, and centrifuged ($2 \times 10^4 g$, 15 min). The clear supernatant was termed 'cytosol' and applied to electrophoresis. After separation of proteins they were transferred onto polyvinyldifluoride membranes (Immobilon P, Millipore, Bedford, UK) and the immunoreactive bands were detected by enhanced chemoluminescence (ECL®, Amersham, Braunschweig, Germany). Quantification of bands was conducted by densitometry (Enhanced Analysis System, Herolab, Wiesloch, Germany).

Fig. 1. Confocal imaging of a single living neuronal NG 108-15 cell (images A to J), expressing the μ-opioid receptor fused to EGFP. Optical sections were monitored successively in 1 μm intervals. Image A exhibits the optical section closest to the surface of the coverslip (1 μm from the bottom). Ten optical sections (images A to J) out of 16 taken are displayed. The most distant section given (image J) was monitored 10 μm from the bottom. μ-Opioid receptors (green fluorescence) were found in association with the cell membrane (D to J, pink arrows), and with membrane protrusions resembling microspikes or lamellipodia (A to C, yellow arrows). Few fluorescent endosomes (white arrows) were detected close to the bottom sections (images B, C), while the remaining frames exhibit strongly reduced cytosolic fluorescence (images D to G). Specimen K displays a confocal image of a highly differentiated NG 108-15 cell stained with phalloidin, revealing the distribution of actin filaments. These morphological structures are associated with the fluorescent μ-opioid-receptor/EGFP construct. Frame L reflects a scanning electron microscopic photograph of NG 108-15 cells, exhibiting cell membrane protrusions (ectoplasm composed pseudopodia; see arrows), resembling fluorescent structures displayed in image B. Scale bars, 10 μm.

2.4. Construction of expression vectors

2.4.1. μ-Opioid receptor / enhanced green fluorescence protein (EGFP) construct

The fusion of cDNA encoding the EGFP to the μ-opioid receptor DNA is detailed by Schulz et al. (1999). Briefly, the rat μ-opioid receptor (pRc/CMC-μ-receptor; Chen et al., 1993) was amplified by PCR. This amplified fragment was cleaved with *Sac*I (μ-receptor F) or *Bsp*120I (μ-receptor R), and cloned into the *Sac*I/*Bsp*120I multiple cloning site of pEGFP-C3 (Clontech, Palo Alto, CA, USA). In frame cloning of the μ-opioid receptor was verified by sequencing (MediGene, Martinsried, Germany).



2.4.2. β-Arrestin 1

The cDNA encoding bovine β-arrestin 1 was removed (*Not*I and *Apa*I) from pBC-β-arrestin (Lohse et al., 1990) and inserted into pBC-SK-dhfr, resulting in pBC-β-arrestin-dhfr.

2.5. DNA transfection

Cell monolayers were transiently transfected with cDNA encoding the μ-receptor/EGFP employing the *N*-1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP)-method as described by the manufacturer (Boehringer, Mannheim, Germany). Transient cotransfection of cells (10 cm petri dish) with cDNAs encoding the fluorescent μ-opioid receptor (10 μg DNA) and β-arrestin 1 (10 μg DNA), respectively, followed the same DOTAP-method. Cells were examined 48 h after transfection.

2.6. NG 108-15 cells stably expressing phosducin

Wehmeyer and Schulz (1998) detail construction of the plasmid encoding phosducin as well as the generation of stably phosducin expressing NG 108-15 cells. This publication also describes their pharmacological characteristics, including the development of supersensitivity to prostaglandin E_1. Cells carrying the empty vector were termed 'NGvec' and those overexpressing phosducin were designated 'NG 8' (clone 8).

2.7. Confocal microscopy

The Zeiss LSM-410 Instrument was used, employing a 40×1.3 oil-immersion objective. The 488 nm laser line induced excitation, and the fluorescent signals were collected by means of a 510 nm long pass-filter. Phalloidin staining of cells was conducted according to the manufacturer's instructions (confocal images were taken by means of TRITC-filter). The digitized images were processed using Adobe Photoshop 4.0 (Mountain View, CA, USA).

2.8. Scanning electron microscopy

Cells were prepared as described by Claviez et al. (1986). Briefly, the specimens were fixed on glass coverslips (mixture of 2% glutaraldehyde and 0.02% OsO_4 in phosphate buffer, pH 6, for 15 min at 4°C in the dark), and after dehydration (ethanol) the preparations were coated with gold, and examined by a JOEL scanning electron microscope.

3. Results

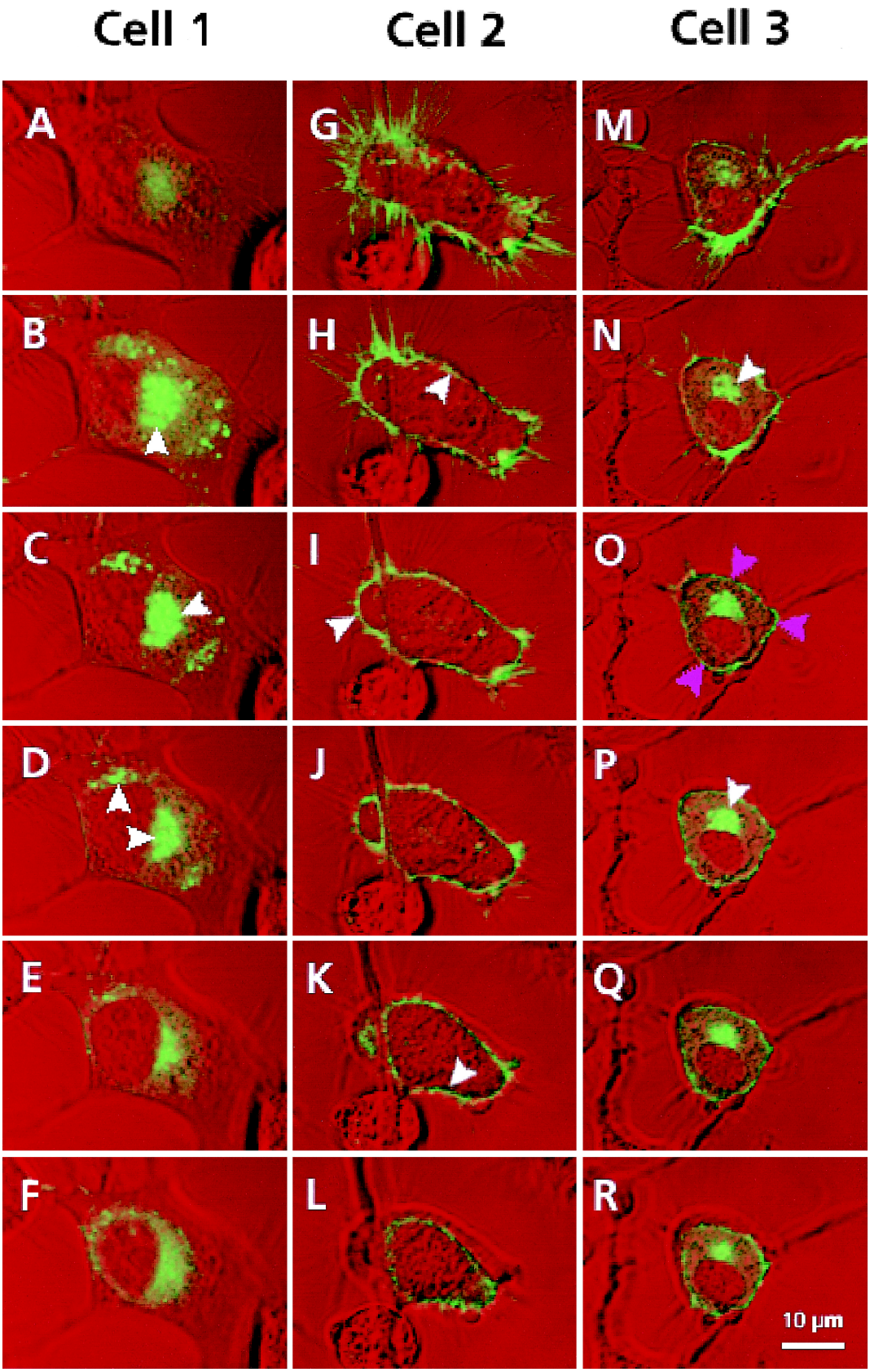
3.1. Distribution of μ-opioid receptors (fluorescence) in NG 108-15 cells

Confocal microscopy of a single optical section from NG 108-15 cells expressing the μ-opioid receptor/EGFP fusion protein revealed fluorescence almost exclusively associated with the cell membrane (Schulz et al., 1999). This study examines the location of fluorescence (EGFP-tagged μ-receptors) throughout cells by monitoring confocal sections at 1 μm intervals from bottom to top. Fig. 1 (images A to J) displays the distribution of fluorescence of a representative NG 108-15 cell transfected to exhibit the μ-opioid receptors/EGFP fusion proteins. The bottom optical section (image A, 1 μm above the surface of the coverslip) exhibits fluorescent structures resembling membrane protrusions characterized as microspikes, lamellipodia or filopodia (yellow arrows). These membrane structures contain dense meshwork of actin filaments, which form stress fibers or even longer extensions (filopodia). These surface-associated, distinct structures progressively disappear (images B, C) as the optical sections were taken more distant the coverslip surface (3 to 7 μm from the bottom). These confocal sections exhibit fluorescence almost exclusively associated with the plasma membrane (images D to G, pink arrows), although unevenly distributed. Noteworthy, the cytoplasm contains only traces of fluorescence, while the nucleus is free of detectable staining (see also Figs. 2 and 4). Optical sections close to the top of the cell (images H to J) display increasingly diffuse fluorescence (pink arrows), indicating larger membrane areas covered by each section of confocal microscopy. These findings caused us to apply the same technique of monitoring cell sections when examining the effect of certain drugs on endocytosis.

Specimen K of Fig. 1 was imaged by confocal microscopy of a phalloidin-stained NG 108-15 cell, focusing

Fig. 2. The effect of etorphine on NG 108-15 cells transfected to express the μ-opioid receptor/EGFP construct (cell 1), the μ-opioid receptor/EGFP construct in NG 8 cells overexpressing phosducin (cell 2), and the μ-opioid receptor/EGFP construct plus phosducin (NG 8) plus β-arrestin 1 (cell 3). Images were taken by confocal microscopy of living cells. Cell 1 (frames A to F) was exposed to 1 nM etorphine. Successive images were taken in 1 μm interval, starting 1 μm from the bottom (A) up to the top of the cell (image F, 9 μm above cover slip). Six optical sections out of 10 monitored are presented. Note the strong internalization of μ-receptors (fluorescence, white arrows) with almost no fluorescence detected in the membrane. Frames G (bottom) to L (top) display images taken in 1 μm intervals of cell 2, which expresses the μ-opioid receptor/EGFP construct in NG 108-15 cells stably overexpressing phosducin (NG 8). The cell was exposed to 1 μM etorphine for 45 min at 37°C. Note the association of fluorescence with the membrane (white arrows) even at a saturating etorphine concentration. Cell 3 (images M, bottom, to R, top) represents frames of a phosducin expressing NG 8 cell transiently cotransfected to express the μ-opioid receptor/EGFP construct and β-arrestin. The cell was challenged with etorphine (1 nM) for 15 min. Note the internalization of fluorescence (white arrows) even in the presence of phosducin. However, receptor-associated fluorescence remains in the membrane (pink arrows). Each cell displayed stands for at least four further cells examined. Scale bar, 10 μm.

at an optical section 4 μm above the surface (center of the cell) of the coverslip. The cell displays characteristic phalloidin-stained actin filaments, cellular structures resembling the morphology of fibres displayed in images A to C.



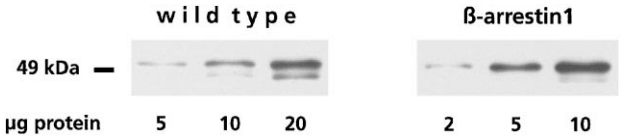


Fig. 3. Western blot analysis of 'cytosol' from NG 108-15 cells. Cytosol of wild type cells and cells (NGS) transiently transfected to express β-arrestin 1 (2 days) was submitted to electrophoresis, and arrestin immunoreactivity was visualized by ECL. The two bands detected (e.g., 10 μg protein of wt cells) appear to represent β-arrestin 1 (55 kDa) and β-arrestin 2 (45 kDa). Densitometry revealed an at least 30-fold higher expression of β-arrestin 1 in transfected cells as compared to wt NG 108-15 cells.

The raster electronic micrograph given (image L) demonstrates the presence of filopodia associated with the cell membrane.

3.2. The effect of etorphine on opioid receptor distribution

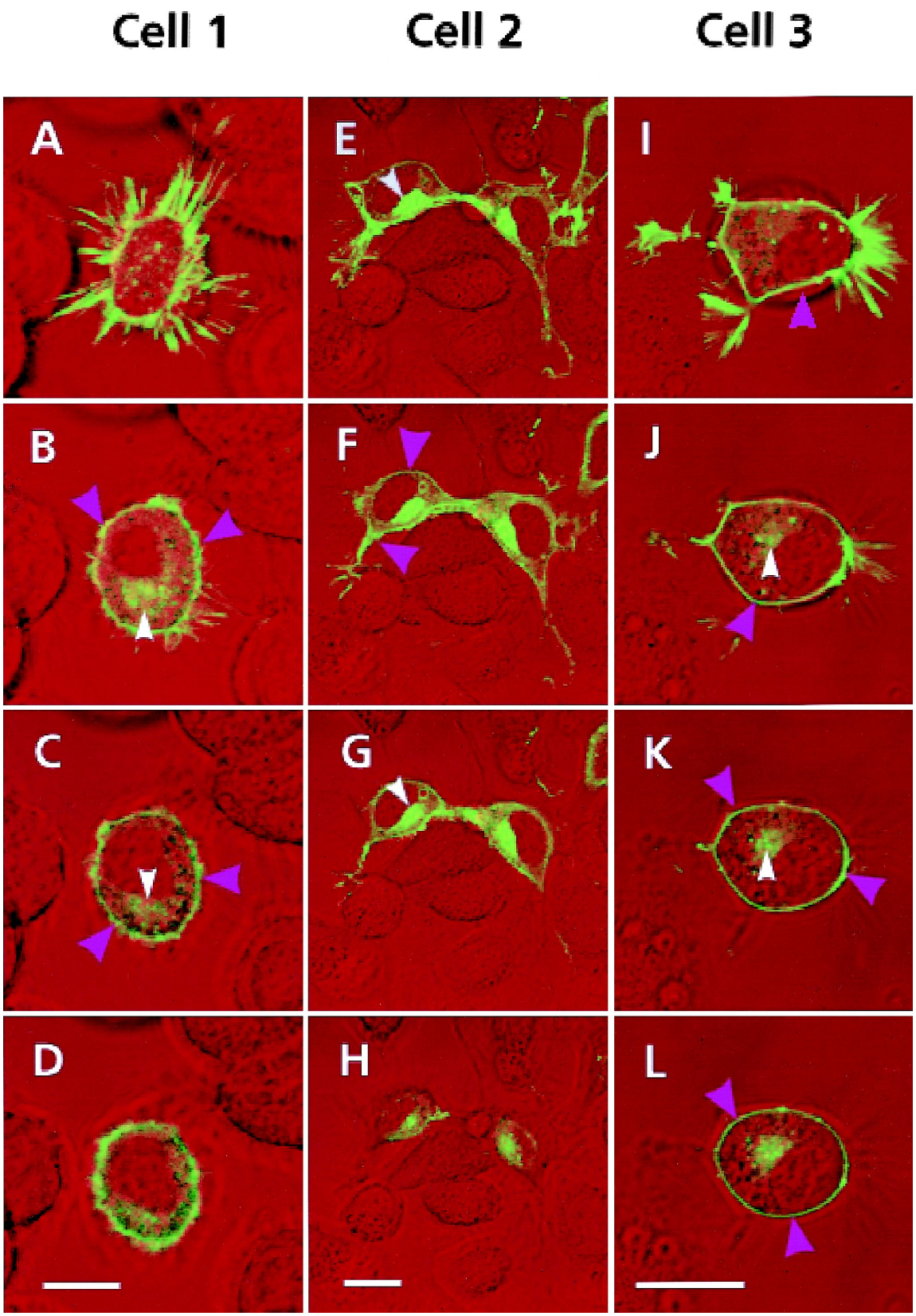
Fig. 2 exhibits the opiate response of NG 108-15 cells transfected to express (i) the μ-opioid receptor/EGFP construct, (ii) the μ-opioid receptor/EGFP construct in NG 8 cells overexpressing phosducin, and (iii) the μ-opioid receptor/EGFP construct, phosducin (NG 8) and β-arrestin 1. Fig. 2 (A to F) displays the response of a NG 108-15 cell expressing the μ-opioid receptor/EGFP and challenged with 1 nM etorphine for 15 min at 37°C. The images (A, cell bottom, to F, top) reveal that the opiate causes redistribution of the fluorescent material to the cytosol, leaving the cell membrane with traces of fluorescence (optical sections D and E, white arrows). Fluorescence is also associated with stress fibers and pseudopodia, indicating a failure of etorphine to affect the localization of opioid receptor/EGFP constructs associated with these structures. Cells challenged with 0.1 nM etorphine exhibit only a partial internalization of the μ-opioid receptor construct (data not given). Phosducin overexpressing NG 8 cells expressing the μ-receptor/EGFP construct failed to respond with internalization when exposed to 1 nM etorphine (15 min, 37°C). Using 1 μM of the opiate under these experimental conditions, few early endosomes were detected as possible signs of internalization. The run through images of a single phosducin overexpressing cell presented in Fig. 2 (G, bottom, to L, top) display the distribution of fluorescence exposed to 1 μM etorphine (representative for further six cells examined). These cells fail to internalize activated receptors, as the fluorescence is associated with the cell membrane (white arrows). This finding is contrasted with NG 8 cells expressing Phd cotransfected to express in addition the μ-receptor/EGFP construct and β-arrestin. Although no information can be given for the absolute concentration of β-arrestin within a single Phd-overexpressing NG 8 cell, Western blotting of a transfected NG 8 cell population reveals an at least 30-fold increase above that found in wt NG 108-15 cells (Fig. 3). Apparently, the increased level of β-arrestin overcomes the inhibition of μ-receptor internalization by the phosphoprotein phosducin (Fig. 2M to R). Even 1 nM of etorphine triggers a rapid and efficient endocytosis of μ-receptors (central endosomes, white arrows) despite the presence phosducin. However, while most of the fluorescence appears in the cytosol, traces of fluorescence are still detectable in the cell membrane (image O, pink arrows).

3.3. The effect of morphine on receptor internalization

Morphine and etorphine have in common to inhibit the activity of the adenylyl cyclase system, but they exhibit distinct differences with respect to their ability to internalize opioid receptors. We observed here that NG 108-15 cells transiently transfected to express the μ-opioid receptor/EGFP construct failed to induce opioid receptor internalization upon stimulation with morphine (1 μM, 30 min, 37°C). Fig. 4 represents optical sections (Cell 1, A to D, bottom to top) of a cell exposed to 1 μM morphine. The opiate largely fails to bring about internalization even at concentrations saturating the opioid receptor population. It appears that the cytosol displays minor amounts of diffuse fluorescence with only weak indications of an accumulation of fluorescence (images B and C, white arrows). In contrast, morphine at a very low concentration (1 nM) brings about sequestration of receptors (fluorescence, Cell 2, images E to H) when NG 108-15 cells were cotransfected with β-arrestin. However, the endocytosis of fluorescence is incomplete as compared to the effect of 1 nM etorphine (see Fig. 1), since fluorescence is still visible in the cell membrane (Cell 2, F, pink arrows). Moreover, transient coexpression of the μ-receptor/EGFP construct and β-arrestin in NG 8 cells overexpressing phosducin also exhibits receptor endocytosis, when stimulated with 1 nM

Fig. 4. The effect of morphine on internalization of the μ-opioid receptor/EGFP construct expressed in NG 108-15 cells. Confocal images of living cells were taken in 1 μm intervals from bottom to top (see Figs. 1 and 2). Four out of 11 optical sections monitored are presented for each cell. Cell 1 (images A to D; bottom to top) displays optical sections after exposure to 1 μM morphine at 37°C for 30 min. Under these experimental conditions, the cell displays fluorescence (μ-opioid receptors) mainly associated with the cell membrane (pink arrows). Some diffuse fluorescent material is detected in the cytoplasm, displaying moderate central vesicles (white arrows). 1 nanomolar morphine completely failed to affect the distribution of fluorescence. Cell 2 (images E to H) was transfected to express the fluorescent μ-opioid receptor construct and β-arrestin 1. Challenge of the cell with 1 nM morphine (37°C, 15 min), a concentration expected to occupy 10 to 20% of μ-receptors, caused receptor endocytosis (white arrows), although fluorescence was still detectable in the cell membrane (pink arrows). Cell 3(images I to L) expressed the fluorescent μ-opioid receptor and β-arrestin 1 in NG 8 cells overexpressing phosducin. After exposure to morphine (1 nM, 37°C, 15 min) fluorescence was detected in cytoplasm (white arrows) as well as in association with the cell membrane(pink arrows). Scale bars, 10 μm.

morphine (Cell 3, images I to L), indicating that internalization of opioid receptors is induced despite the presence of phosducin. Again, internalization is incomplete at 1 nM morphine, as the membrane retains fluorescence (pink



arrows). Exposure to 1 μM morphine (37°C, 30 min) brought about a strong opioid receptor internalization.

4. Discussion

μ-Opioid receptors fused to EGFP are incorporated into the membrane of NG 108-15 cells, they retain their function and will be sequestered upon exposure to the appropriate opioid (Schulz et al., 1999). Here we report that fluorescence (μ-opioid/EGFP constructs) associated with the cell membrane exhibits a characteristic distribution. That is, confocal microscopy of optical sections close to the bottom of the cell reveals morphological structures resembling pseudopodia or nerve growth cones (Evans et al., 1997). Scanning electron microscopy supports this interpretation, and phalloidin staining let us assume the formation of stress fibers (Theriot and Mitchison, 1991). These morphological structures originate from cell membranes, they carry different receptors (Tsui et al., 1985) and, thus, are likely to host residual μ-receptor/EGFP constructs. These receptors appear to have lost their function as the fluorescent material fails to internalize when cells are exposed to etorphine (see below). In marked contrast, fluorescence monitored more distant to the bottom is almost exclusively located in the cell membrane, and these fluorescent receptors appear highly functional as judged by their ability to internalize upon activation. As expected, images close to the top of the cell increasingly display diffuse fluorescence, which should be distinguished from mechanisms associated with internalization of fluorescent receptors. In general, confocal sections reveal that the receptor construct is associated with the plasma membrane, and, after etorphine challenge, with the cytosol, while it spares the cell nucleus.

The process of opioid-receptor internalization is controlled by phosducin and β-arrestin (Krupnick and Benovic, 1998), respectively. The cytosolic phosducin has already been demonstrated to inhibit etorphine-induced endocytosis in NG 108-15 cells (Schulz et al., 1999), a mechanism proposed to relate to the competition of phosducin and G protein-coupled receptor kinases for Gβγ, serving as anchor for the kinases to bring about phosphorylation of activated receptors. wt NG 108-15 cells appear to be devoid of phosducin (Wehmeyer and Schulz, 1998) but they express receptor kinases (Schulz et al., 1998a,b) and β-arrestin (this study). Those cells transiently transfected to express the μ-opioid receptor/EGFP construct are highly sensitive to etorphine, as 1 nM of the alkaloid almost completely internalizes the fluorescent receptors. In principle, this result is in line with reports regarding endocytosis of G protein-coupled receptors (Cao et al., 1998). Our findings strongly suggest an interference of the phosducin with μ-opioid receptor phosphorylation at the C-terminus, and turns the attention to another cytosolic protein, that is arrestin. The phosphorylated receptor strongly increases its affinity to β-arrestin (Lefkowitz, 1998), which functions to uncouple the activated receptor from the G protein, and to trigger the process of receptor sequestration (Krupnick and Benovic, 1998). Our data clearly indicate that arrestin expressed in sufficiently high concentrations overcomes the action of phosducin to impair internalization. Thus, β-arrestin is supposed to bind to the activated, unphosphorylated receptor, thereby triggering the process of internalization. The literature provides ample evidence for this notion, indicating binding of arrestin to agonist-occupied, non-phosphorylated receptors, although with reduced affinity (Gurevich et al., 1995, 1997). The data suggest that overexpression of arrestin is a sufficient condition to internalize unphosphorylated, etorphine-occupied opioid receptors despite the presence of phosducin.

Apparently, uncoupling of the opioid receptor from its G protein is superposed the process of receptor phosphorylation to cause internalization. This notion is highly suggested by our experiments examining the response to morphine, as it largely fails to induce sequestration of fluorescence (μ-opioid/EGFP) even at saturating morphine concentration. It is likely that this failure is due to an insufficient phosphorylation of activated receptors by G protein-coupled receptor kinase 3 (Kovoor et al., 1998; Zhang et al., 1998), resembling a situation which explains the attenuation of receptor internalization in the presence of phosducin. However, as the concentration of β-arrestin increases, uncoupling of the activated receptor is believed to occur, and this in turn triggers internalization of morphine-activated opioid receptors. Our data may indicate a less efficient sequestration of opioid receptors as compared to those images taken from etorphine-exposed NG 108-15 cells. Regardless of the degree of receptor phosphorylation caused by the individual opioids, internalization of activated receptors takes place upon interruption of intracellular signalizing, a mechanism brought about by arrestin. Although arrestin was detected in wt NG 108-15 cells, overexpression of this cytosolic protein was required to trigger receptor endocytosis. It remains to be seen whether internalization of opioid receptors is paralleled by functional parameters, e.g., the control of cAMP generation.

References

Bauer, H.B., Müller, S., Puzicha, M., Pippig, S., Obermaier, B., Helmreich, E.J.M., Lohse, M., 1992. Phosducin is a protein kinase A-regulated G-protein regulator. Nature 358, 73–76.

Blüml, K., Schnepp, W., Schröder, S., Beyermann, M., Macias, M., Oschkinat, H., Lohse, M.J., 1997. A small region in phosducin inhibits G-protein beta gamma-subunit function. EMBO J. 16, 4908–4915.

Cao, T.T., Mays, R.W., von Zastrow, M., 1998. Regulated endocytosis of G-protein-coupled receptors by a biochemically and functionally dis-

tinct subpopulation of clathrin-coated pits. J. Biol. Chem. 38, 24592–24602.

Chen, Y., Mestek, A., Liu, J., Hurley, J.A., Yu, L., 1993. Molecular cloning and functional expression of a mu-opioid receptor from rat brain. Mol. Pharmacol. 44, 8–12.

Claviez, M., Brink, M., Gerisch, G., 1986. Cytoskeletons from a mutant of dictyostelium discoideum with flattened cells. J. Cell Sci. 86, 69–82.

Danner, S., Lohse, M.J., 1996. Phosducin is an ubiquitous G-protein regulator. Proc. Natl. Acad. Sci. 93, 10145–10150.

Evans, L.L., Hammer, J., Bridgman, P.C., 1997. Subcellular localization of myosin V in nerve growth cones and outgrowth from dilute-lethal neurons. J. Cell Sci. 110, 439–449.

Ferguson, S.S.G., Downey, W.E. III, Colapietro, A.M., Barak, L.S., Ménard, L., Caron, M.G., 1996. Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 271, 363–366.

Gaudriault, G., Nouel, D., Farra, C.D., Beaudet, A., Vincent, J.-P., 1997. Receptor-induced Internalization of selective peptidic mu and delta opioid ligands. J. Biol. Chem. 272, 2880–2888.

Gurevich, V.V., Dion, S.B., Onorato, J.J., Ptasienski, J., Kim, C.M., Sterne-Marr, R., Hosey, M.M., Benovic, J.L., 1995. Arrestin interactions with G-protein-coupled receptors: direct binding studies of wild type and mutant arrestins with rhodopsin, β2-adrenergic, and m2 muscarinic cholinergic receptors. J. Biol. Chem. 270, 720–731.

Gurevich, V.V., Pals-Rylaarsdam, R., Benovic, J.L., Hosey, M.M., Onorato, J.J., 1997. Agonist–receptor–arrestin, an alternative ternary complex with high affinity. J. Biol. Chem. 272, 28849–28852.

Hawes, B.E., Touhara, K., Kurose, H., Lefkowitz, R.J., Inglese, J., 1994. Determination of the G beta gamma-binding domain of phosducin—a regulatable modulator of G beta gamma signaling. J. Biol. Chem. 269, 29825–29830.

Keith, D.E., Murray, S.R., Zaki, P.A., Chu, P.C., Lissin, D.V., Kang, L., Evans, C.J., von Zastrow, M., 1996. Morphine activates opioid receptors without causing their rapid internalization. J. Biol. Chem. 271, 19021–19024.

Kovoor, A., Nappey, V., Kieffer, B.L., Chavkin, C., 1997. Mu and delta opioid receptors are differently desensitized by the coexpression of β-adrenergic receptor kinase 2 and β-arrestin 2 in *Xenopus* oocytes. J. Biol. Chem. 272, 27605–27611.

Kovoor, A., Celver, J.P., Wu, A., Chavkin, C., 1998. Agonist induced homologous desensitization of μ-opioid receptors mediated by G protein-coupled receptor kinases is dependent on agonist efficacy. Eur. J. Pharmacol. 5, 704–711.

Krupnick, J.G., Benovic, J.L., 1998. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu. Rev. Toxicol. 38, 289–319.

Lee, R.H., Lieberman, B.S., Lolley, R.N., 1987. A novel complex from bovine visual cells of a 33000-dalton phosphoprotein with beta- and gamma-transducin: purification and subunit structure. Biochemistry 26, 3983–3990.

Lee, R.H., Ting, T.D., Lieberman, B.S., Tobias, D.E., Lolley, R.N., Ho, Y.K., 1992. Regulation of retinal cGMP cascade by phosducin in bovine rod photoreceptor cells—interaction of phosducin and transducin. J. Biol. Chem. 267, 25104–25112.

Lefkowitz, R.J., 1998. G protein-coupled receptors: III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. J. Biol. Chem. 273, 18677–18680.

Lin, F.-T., Krueger, K.M., Humphrey, E.K., Daaka, Y., Fredericks, Z.L., Pitcher, J.A., Lefkowitz, R.J., 1997. Clathrin-mediated endocytosis of the β-adrenergic receptor is regulated by phosphorylation/dephosphorylation of β-arrestin 1. J. Biol. Chem. 272, 31051–31057.

Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G., Lefkowitz, R.J., 1990. β-Arrestin: a protein that regulates β-adrenergic receptor function. Science 248, 1547–1550.

Murray, S.R., Evans, C.J., von Zastrow, M., 1998. Phosphorylation is not required for dynamin-dependent endocytosis of a truncated mutant opioid receptor. J. Biol. Chem. 273, 24987–24991.

Pitcher, J.A., Touhara, K., Payne, E.S., Lefkowitz, R.J., 1995. Pleckstrin homology domain-mediated membrane association and activation of the β-adrenergic receptor kinase requires coordinate interaction with the Gβγ subunit and lipid. J. Biol. Chem. 270, 11707–11710.

Schulz, R., Schulz, K., Wehmeyer, A., Murphy, J., 1998a. Translocation of phosducin in living neuroblastoma×glioma hybrid cells (NG 108-15) monitored by red-shifted green fluorescent protein. Brain Res. 790, 347–356.

Schulz, K., Müller, S., Belke-Louis, G., Schulz, R., 1998b. Rat beta-adrenergic receptor kinases 1 and 2 in mouse neuroblastoma×rat glioma NG 108-15 hybrid cells. Biochem. Pharmacol. 55, 65–70.

Schulz, R., Wehmeyer, A., Schulz, K., Murphy, J., 1999. The effect of phosducin on opioid receptor function. J. Pharmacol. Exp. Ther. 289, 599–606.

Theriot, J.A., Mitchison, T.J., 1991. Actin microfilament dynamics. Nature 352, 126–131.

Tsui, H.C., Lankford, K.L., Klein, W.L., 1985. Differentiation of neuronal growth cones: specialization of filopodial tips for adhesive interactions. Proc. Natl. Acad. Sci. U.S.A. 82, 8256–8260.

Wehmeyer, A., Schulz, R., 1998. Phosducin expression in NG 108-15 hybrid cells enhances prostaglandin E1 stimulated adenylate cyclase activity. Life Sci. 62, PL127–PL134.

Zhang, J., Ferguson, S.S., Barak, L.S., Bodduluri, S.R., Laporte, S.A., Law, P.Y., Caron, M.G., 1998. Role of G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. Proc. Natl. Acad. Sci. U.S.A. 95, 7157–7162.