The Role of Membrane Trafficking in G Protein-Coupled Receptor Regulation

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To all those sacrificed for the sake of my curiosity...

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ABBREVIATIONS

A1R	Adenosine receptor 1
ACSF	Artificial cerebrospinal fluid
ADA	Adenosine deaminase
AP-2	Adaptor protein 2
AR	Adrenergic receptor
Arf6	G protein ADP-ribosylation factor 6
ARNO	Guanine nucleotide ADP-ribosylation factor nucleotide-binding site opener
AT1A	Angiotensin receptor 1A
B1R	Bradykinin B1 receptor
B2R	Bradykinin B2 receptor
BK	Bradykinin
BPA	Biotinylation protection assay
CAM	Constitutively activated mutant
CCKR	Cholecystokinin receptor
CCP	Clathrin-coated pit
CB1	Cannabinoid receptor 1
CCR5	Chemokine receptor 5
cGASP	carboxyterminal domain of GASP
CLASP	Clathrin-associated sorting protein
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
COP	Coatomer protein
D1R	Dopamine D1 receptor
D2R	Dopamine D2 receptor
DADLE	[D-Ala2, D-Leu5] enkephalin
DAMGO	[D-Ala2, N-Me Phe4, Gly-ol5]-enkephalin
DOR	Delta opiate receptor
DRM	Detergent-resistant microdomain
DUB	deubiquitinylation protein
E/ANTH	Epsin/AP180 N-Terminal Homology domain
EE	Early endosome
EEA1	Early endosomal antigen 1
eEF	Eukaryotic elongation factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial-specific nitric oxide synthase
EFA6	Exchange factor for Arf-6
EGFP	Enhanced green fluorescent protein
ENTH	Epsin N-Terminal Homology domain
Eps15	Epidermal growth factor receptor pathway substrate 15
ER	Endoplasmatic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ERK	Extracellular signal regulated protein kinase
ESCRT	Endosomal sorting complex required for transport
ETA	Endothelin receptor A
ETB	Endothelin receptor B
FBS	Fetal bovine serum
FERM	4.1 (Four.one), Ezrin, Radixin and Moesin
FYVE	Fab1, YOTB, Vac1, EEA1
GABABR	Gamma aminobutyric acid B receptor
GAP	GTPase activating protein
GASP	GPCR Associated Sorting Protein
GEF	Guanine nucleotide exchange factor
GDI	Guanine nucleotide dissociation inhibitor
GDS	Guanine nucleotide dissociation stimulator
GIT1	G protein-coupled receptor kinase-interactor 1
GLP-2R	Glucagon-like peptide 2 receptor
GnRHR	Gonadotropin releasing hormone receptor
GPCR	G Protein-Coupled Receptors
G protein	Guanine nucleotide regulatory protein
GRAFS	Glutamate, rhodopsin, Adhesion, frizzled and secretin GPCR family
GRK	GPCR regulated protein kinase
GST	Glutathione-S-transferase
H1	Histamine 1 receptor
HEK	Human embryonic kidney
HRP	horseraddish peroxidase
Hrs	Hepatocyte growth factor regulated tyrosine kinase
IL-1	Interleukin 1
KD	Kallidin
KHRB	Krebs-Ringer HEPES buffer
KOR	Kappa opiate receptor
LBPA	Lysobisphosphatidic acid
LDLR	Low density lipoprotein receptor
LE	Late endosome
M2MR	Muscarinic acetylcholine receptor M2
MAPK	Mitogen-activated protein kinase
MCD	Methyl-b-cyclodextrin
MDC	Monodansylcadaverin
Mdm2	Transformed mouse 3T3 cell double minute 2
MEM	Minimum Essential Medium
mGluR	Metabotropic glutamate receptor
MHC	Major histocompatibility antigen
MOR	Mu opiate receptor
MVB	Multivesicular body
mVPS4	Mammalian vacuolar protein sorting 4
NK1	Neurokinin 1 receptor
NMDA	N-methyl D aspartate
nNOS	Neuronal-specific nitric oxide synthase
NO	Nitric oxide
NSF	N-ethylmaleimide-sensitive fusion protein
PAR1	Protease-activated receptor 1
PBS	Phosphate-buffered saline
PDZ	Post-synaptic-density-protein 95 Drosophila disc-large tumor-suppressor (DlgA) Zo-1 protein
PH domain	Pleckstrin homology domain
PI3K	Phosphatidylinositol 3-kinase
PI5K	Phosphatidylinositol 5-kinase
PIP	Phosphatidylinositol phosphate
PKA	cAMP-dependent protein kinase
PKC	Calcium- and phospholipid-dependent protein kinase or protein kinase C
PLD	Phospholipase D
PLC	Phospholipase C
PLA	Phospholipase A
PNGase F	Protein N-glycosidase F
PX domain	Phox homology domain
rab5	Ras-like in rat brain 5
RAMP	Receptor activity modifying protein
RE	Recycling endosome
RGS	Regulator of G protein signaling
SDS	Sodium dodecylsulfate
SEE	Static early endosome
SHR	Spontaneously hypertensive rats
SMAP1	Small Acidic Protein 1
SNAP25	Synaptosomal-associated protein 25
SNARE	SNAP Receptor
SNX	Sorting nexin
TBS	Tris-buffered saline
TfnR	Transferrin receptor
TGN	Trans-Golgi network
TM	Transmembrane
TNFα	Tumor necrosis factor alpha
TOM1	Temperature dependent Organization in Mitotic nucleus
UIM motif	Ubiquitin interacting motif
V1A	Vasopressin receptor 1A
VAMP2	Vesicle-associated membrane protein 2
VIP1R	Vasoactive intestinal peptide 1 receptor
VTA	Ventral tegmental area

SUMMARY

G protein-coupled receptors (GPCR) constitute the largest and most diverse family of cell surface receptors in eukaryotic cells. This group of receptors is represented in all eukaryotic species, present in all mammalian cells and tissues, and influence aspects of all physiological events known. As such, this family has been, and continues to be the major therapeutic target in most diseases, may they either be of exogenous or endogenous origin.

Highly complex molecular machineries regulate every aspect of GPCR production, function, and destruction. Enormous efforts over the past four decades have brought us from a theoretical indication of their existence, via the cloning of their genes and the elucidation of their activity states and interaction partners, all the way to the minute details of the movements within the receptors, which result in major physiological effects. However, we are still only beginning to appreciate these processes, how they may be reconstituted in their physiological setting, and how they may be further exploited for drug development.

One mode of regulation of GPCR is the movement of these receptors within the membrane compartments of the cellular environment where they are operating. Although originally seriously questioned, early findings in the 1980-s of agonist-promoted movements of GPCR are now considered highly important regulatory mechanisms that are cruical for proper receptor function. These mechanisms, now generally called GPCR membrane trafficking, are the topics of this thesis.

I have used three separate rhodopsin family receptor systems, the opiate, the dopamine, and the bradykinin systems to investigate the impact of endocytosis and postendocytic trafficking on receptor integrity and tissue responsiveness to specific receptor agonists. These receptor systems regulate a multitude of physiological events including 1) autonomic functions such as breathing, heart rate, blood pressure, and bowel movements; 2) pathogen-protective tasks such as immune cell proliferation and migration, vascular permeability, and induction of proinflammatory mediators; 3) sensory perception via positive and negative modulation of nociceptive transmission; 4) higher cognitive, motivational, and goal-oriented functions in the central nervous system. Albeit major differences exist between these groups of receptors in both effector systems utilized and physiological effects elicited, they overlap in their distribution and share modulatory control over some common circuits such as nociceptive signaling.

Elucidating the machinery regulating the postendocytic sorting of the Delta Opioid Receptor (DOR) is of great importance. The reason is the potential clinical use of DOR agonists as analgesic agents, which has, so far, been limited by the short halflife of this receptor following agonist stimulation. In study I, a novel protein was found that interacts with the DOR carboxyterminal domain and acts as a mediator of sorting of the internalized receptor to the degrading lysosomal pathway. This protein was named GPCR associated sorting protein or GASP. Further, it was established that overexpression of a truncated c-terminal form of GASP, cGASP, acts as a dominant-negative modulator on DOR targeting to lysosomes. Indeed, perturbation of this interaction leads to recycling of fully functional receptors to the plasma membrane, and thus extend DOR halflife.

Considering the imbalance and excessive activity of the dopamine system that is suggested to occur in a number of pathological conditions such as attention disorder/ADHD, schizophrenia, and Tourette's syndrome, and the protective role of dopamine D2 receptors

(D2R) against the development of addiction, a better understanding of D2R regulation is crucial for future drug development. In study II, dopamine D1 (D1R) and D2R were shown to differ in their postendocytic choice; i.e. D1R recycles to the plasma membrane, whereas D2R degrades via lysosomal targeting. Further, D2R sorting to lysosomes was shown to be GASP-dependent, and abrogating the D2R-GASP interaction resulted in recycling of the D2R to the plasma membrane. Evaluation of the physiological value of these findings was carried out by using an ex vivo rat brain slice system. Abrogation of D2R-GASP interaction in this physiological model led to resensitization of D2R.

One of the major roles of the B1 (B1R) and B2 (B2R) bradykinin receptors is to activate acute inflammatory processes and facilitate both acute and chronic nociceptive signaling. This makes these receptors ideal targets for immunomodulatory and analgesic treatments. Little is known about the membrane trafficking of these receptors, which is particularly true for the B1R, therapeutically the most interesting receptor since it is induced by injury and thus expressed primarily in the pathological state. Study III was designed to address the initial steps of receptor internalization and postendocytic choices after rapid internalization of the human bradykinin B1R and B2R in a human cell model system (HEK293). By doing so, the endocytic pathways utilized by these two receptors were mapped. The studies revealed that B2R is rapidly endocytosed and recycles to the plasma membrane. In contrast, B1R is constitutively endocytosed and sorted for destruction via lysosomal targeting. Further, it was established that B1R, in contrast to most GPCR, is stabilized on the plasma membrane by the cognate ligand des-Arg10-KD. This is therapeutically interesting since a clinically useful antagonist at this receptor should not only block the agonist response but also promote endocytosis.

Considering that plasma membrane stabilization of B1R appears to be important for agonist signaling of this receptor, Study IV was set up to investigate various means of stabilizing the receptor and determine their effect on signaling. I found that blockers of clathrin-mediated endocytosis and coexpression with B2R resulted in stabilization of B1R on the plasma membrane in the absence of agonist. In contrast to agonist-promoted stablization, agonist-induced B1R signaling was severly attenuated in both of these cases. Further, I found that B1R lacks carboxyterminal palmitoylation common to most rhodopsin family GPCR including B2R. Together with the established knowledge that B1R does not get phosphorylated either in response to agonist activation or in the absence of agonist, we now conclude that any interacting regulatory machinery must by necessity interact with the unmodified receptor. This greatly simplifies the future search for B1R interaction partners.

In conclusion, the investigation of the three receptor systems described has revealed that GPCR are subject to rapid membrane trafficking and that this is a receptor-specific regulatory mechanism, which dramatically impacts GPCR activity. Further, our finding of GASP as an interaction partner for DOR, D2R and B1R and part of their postendocytic targeting to lysosomes is a novel finding of a noncovalent interaction supporting membrane trafficking of GPCR. Our ex vivo findings from the D2R model system indicate that receptor activity can be regulated by the targeted alteration of a receptor trafficking phenotype. Lastly, the finding that agonist can act as a membrane stabilizing agent for the active form of the B1R is a novel regulatory mechanism of GPCR trafficking. This effect raises the issue of what parameters should be addressed when new drugs are screened for receptor activity. Potentially, a future analgetic drug targeting B1R should be an agonist on endocytosis, and at the same time an antagonist on signaling – one type of biased ligand.

INTRODUCTION

The Superfamily of G Protein-Coupled Receptors

Classes and Evolution of G Protein-Coupled Receptors

G protein-coupled receptors (GPCR) constitute a heterogenous group of receptors representing with more than a 1000 genes in the human genome. This superfamily of receptors share low sequence homology. Instead, they are recognized by their common structural topography of seven transmembrane-spanning regions interconnected by three intracellular (i1, i2 and i3) and three extracellular (e1, e2 and e3) loops (Graul and Sadee 2001; Schioth and Fredriksson 2005). These receptors also contain highly variable N- (extracellular) and C- (intracellular) terminal domains. GPCR respond to numerous endogenous as well as exogenous stimuli including mechanical stretch, hormones, neurotransmitters, proteases, nutritional lipids, light and odours.

The family of GPCR is ancient being represented in most phyla of eukaryots with characterized members in fungi, plants, nematoda, insects, and vertebrates (Graul and Sadee 2001; Schioth and Fredriksson 2005). The recent finding in bacteria of a light-sensing rhodopsin-like protein (rhodopsin being the first GPCR to be cloned, from bovine retina) has raised the possibility that GPCR exist even in prokaryots, although the structure of this bacterial protein has to be characterized further before this question can be resolved (Graul and Sadee 2001; Schioth and Fredriksson 2005). Several types of viruses also carry functional GPCR that are utilized after infection of their host to hijack the eukaryotic cellular machinery and direct cellular functions such as adhesion, extravasation, differentiation, and proliferation to name a few (Polson, Wang et al. 2002; Paulsen, Rosenkilde et al. 2005; Vischer, Vink et al. 2006).

The fact that close orthologs of most human GPCR can be found in most mammals indicates that little redundancy exist between receptors of this superfamily. Further, the ability to express and correctly regulate GPCR from the human genome in yeast, two organism lineages with more than a billion years of separate development (Schioth and Fredriksson 2005), indicate the importance of this group of proteins throughout eukaryotic evolution. Also, recent genomic studies trace GPCR regulatory proteins back to very early common eukaryotic ancestors although specialization and expansion of groups of proteins have occurred fairly recently in evolution. Notably, humans do not always display the most complex setup of GPCR and GPCR regulatory proteins as exemplified by the greater number of both odor, and, more surprisingly, non-odor sensing GPCR in mus musculus (Vassilatis, Hohmann et al. 2003; Bjarnadottir, Gloriam et al. 2006).

Early attempts to categorize GPCR have grouped the receptors in systems of A through F or 1 through 5 (Attwood and Findlay 1994; Kolakowski 1994; Horn, Weare et al. 1998; Bockaert and Pin 1999). The latest attempt utilizes five groups with the acronym GRAFS for Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin (Fredriksson, Lagerstrom et al. 2003; Schioth and Fredriksson 2005). Of these groups, rhodopsin is the one containing the most members in higher vertebrates. The rhodopsin group is further subdivided into α, β, γ, and δ which are further subdivided into subgroups depending on the type of ligand with which they interact (Schioth and Fredriksson 2005). The GPCR model systems utilized in the studies presented in this dissertation are all part of the α and γ subgroups of the rhodopsin group.

How to Apply Lessons Learned From Evolution and Genomal Organization on GPCR Investigations
Great receptor integrity and low redundancy may have consequences on the applicability of findings in one receptor system on the next. In fact, accumulating evidence indicate that there are exceptions to most of the established rules of how GPCR act, or are acted upon by regulatory mechanisms, making it difficult to predict the outcome of studies in new receptor systems. Indeed Qanbar and Bouvier state that:"…each GPCR is a unique protein with unique structural and functional details"; not a comforting thought in a protein family the size of the GPCR superfamily. However, as the literature grows on GPCR interacting proteins, and lipid species in many different model system – so does the available tools for assessing novel GPCR, GPCR activities, and GPCR regulators. It is also comforting to see the growing number of receptor systems and species models being utilized for the study of GPCR activity and regulation.

Another concern in GPCR studies is the ability to translate results from basic cell models to physiological systems. Do in vitro systems reflect in vivo situations? In the past this has been adressed using several different cell models in parallell to exclude cell-specific phenomena. Lately the demand on basic GPCR studies being anchored and confirmed in vivo has grown considerably. Whereas we all benefit from increased demands on the quality of scientific work, the implications from the evolutionary studies mentioned above, and the multiple uncontrollable variables that exist in animal models, make it imperative that early experiments delineating novel GPCR activities are carried out in the simplest models imaginable. Cell biological and biochemical investigations are costly and tedious endevours that are hard to combine with work in animal models. They do however allow for the elimination of many erroneous hypotheses, and lead to more pointed and relevant questions for future animal model investigations. Ethical and practical issues clearly favour in vivo studies built on in vitro results. A vast amount of information, on the conditions necessary for proper GPCR function, is now available are now available which can aid in the choice of suitable cell models.

GPCR Activation, Effector Coupling, and Conformational Changes

Receptor-G protein Coupling
According to the classic model of GPCR activity, the agonist favors an active receptor conformation that allows for a tight association of the heterotrimeric guanine nucleotide regulatory protein (G-protein) to the cytosolic face of the GPCR, thus forming the so called ternary complex (Fig. 1) (De Lean, Stadel et al. 1980). The inactive G-protein α-subunit carries a GDP molecule, which keeps this subunit in a high affinity state for the $\beta\gamma$ subunits. The tight interaction between the active receptor and the G-protein results in the release of the GDP molecule, which, under physiological conditions leads to the immediate binding of GTP. GTP induces a new conformation of the G-protein α subunit that has a low affinity for the $\beta\gamma$ subunits and which in turn leads to the dissociation of the G-protein into individual α and $\beta\gamma$ subunits. These subunits are subsequently free to interact with and modulate downstream effectors. Inactivation of the G-protein subunits is dependent on the α subunit intrinsic GTPase activity; hydrolysis of GTP closes the activation cycle by favoring the formation of the inactive $\alpha\beta\gamma$ complex.

The specificity of receptor signaling depends on G protein-specific interactions with downstream effectors. There are currently 16 known genes coding for distinct α subunits with an additional 12 gene products generated through differential splicing. Further, 5 β and

12 γ subunit coding genes have been identified. There are limitations to the combination of subunits, four families G proteins can be identified: Gs (Gs and Golf), Gi (Gtr, Gtc, Gg, Gi1-3, Go and Gz), Gq (Gq, G11, G14 and G15/16), and G12 (G12 and G13). Examples of effector systems influenced by these G-proteins are shown in Fig. 1, and are excellently reviewed elsewhere (Offermanns 2003; McCudden, Hains et al. 2005).

Receptor Conformation and Activity

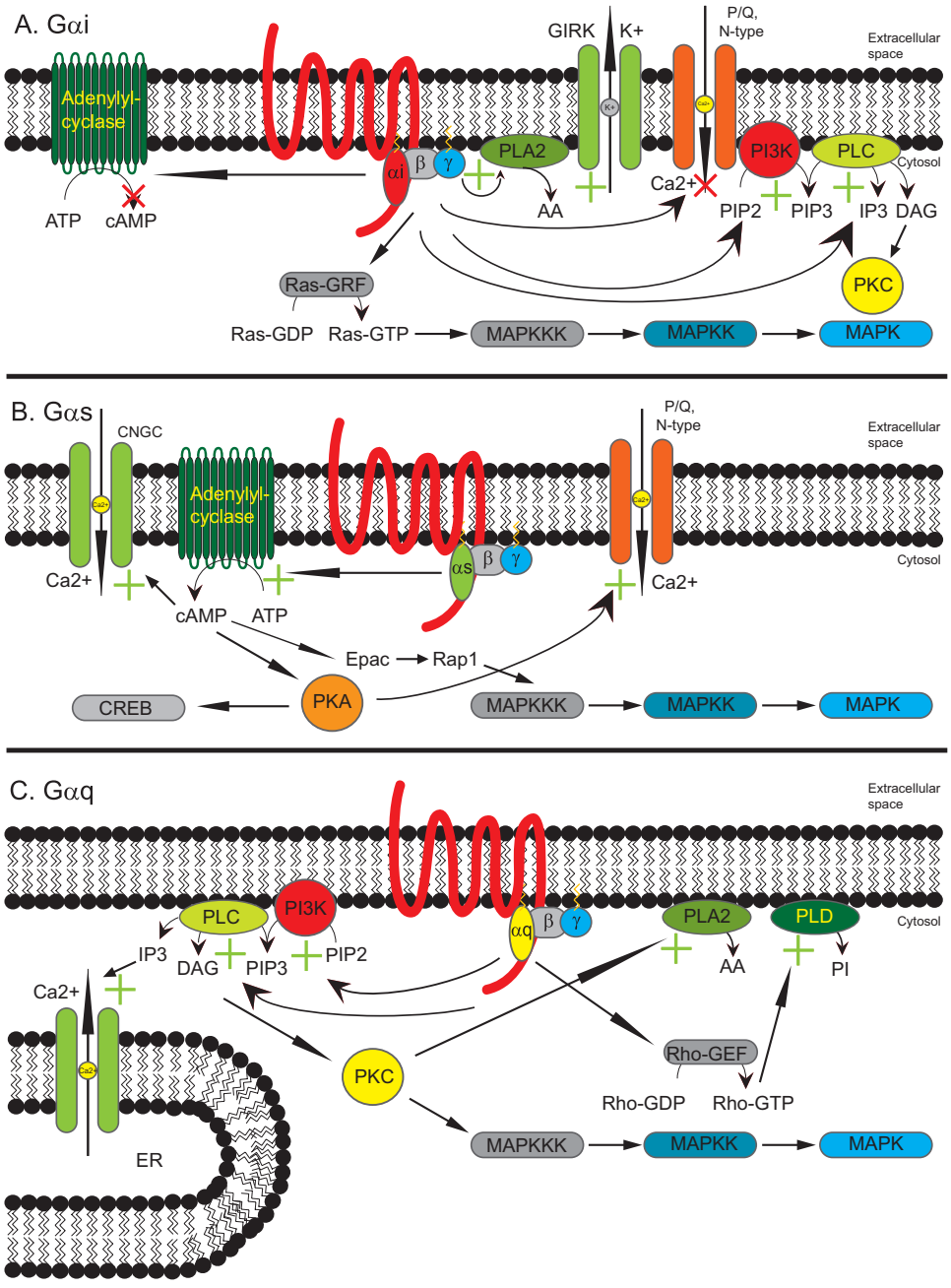
The notion that agonist binding to a receptor is the only way to induce the active receptor conformation was challenged in the early 1990-s. It was then shown that constitutively activated mutants (CAM) of β2-AR (exchange of ic3 between β2-AR and α1-AR) showed an increased propensity to shift into an active conformation in the absence of agonist, with an increased affinity for the agonist that correlated with intrinsic agonist activity, increased potency of agonists, and increased intrinsic activity of partial agonists (Samama, Cotecchia et al. 1993).

Based on these conclusions, a model named the revised ternary complex model was inferred. This model holds that the receptor is in an equilibrium between an inactive R state and an active R* state, the latter which couples to the G-protein. In this model, the agonist shifts the equilibrium towards the active R* state by stabilizing this conformation. This theory has been used to explain the spontaneous activity of receptors, i.e. activity in the absence of ligand or so called constitutive activity. In other words the propensity for constitutive activity relates to the intrinsic spontaneous rate of receptor protein isomerization from the inactive R to the active R* state. This model also accommodates the finding that certain drugs, called inverse agonists, act in the absence of agonist to dampen basal receptor signaling. In this model, an inverse agonist stabilize the inactive R state of the receptor thus shifting the equilibrium toward this state and silencing constitutive signaling. On the other hand, a neutral antagonist would then be a ligand that binds equally well to both the R and R* states and hence does not change the equilibrium between their states and the intrinsic receptor activity.

Indications from several studies have since been interpreted to mean that the receptor can adopt more than one active R* state (Mathis and Leeb-Lundberg 1991; Spengler, Waeber et al. 1993; Robb, Cheek et al. 1994; Perez, Hwa et al. 1996; Whistler and von Zastrow 1998). Several possible explanations to these phenomena have been raised, and some of the answers may lie in the specific requirements necessary for G protein activation.

Fig. 1 – Schematic representation of G protein-mediated receptor signaling pathway. A, Gai-mediated pathways. B, Gas-mediated pathways. C, Gaq-mediated pathways. PLA2 - phospholipase A2; PLD - phospholipase D; PLC - phospholipase C; PKA - protein kinase A; PKC - protein kinase C; PI3K - phosphoinositide 3 kinase; Ras-GRF - Ras guanine nucleotide release factor; PIP2 - phosphoinositide-4,5-diphosphate; IP3 - inositol-triphosphate; DAG - diacylglycerol; AA - aracidonic acid; GIRK - gated inwardly rectified potassium channel; P/Q N-type - voltage sensitive calcium channel; MAPK, MAPKK, MAPKKK - mitogen activated kinases; CREB - cAMP response element binding protein; Epac - exchange protein directly activated by cAMP; Rap1 - rhoptry-associated proetin 1; Rho-GEF - Rho guanine nucleotide exchange factor.

Fig. 1


A. Gαi
Adenylyl-cyclase
ATP
cAMP
αi
β
γ
PLA2
AA
GIRK
K+
P/Q, N-type
Ca2+
PI3K
PLC
Cytosol
Extracellular space
PIP2
PIP3
IP3
DAG
PKC
Ras-GRF
Ras-GDP
Ras-GTP
MAPKKK
MAPKK
MAPK
B. Gαs
CNGC
Adenylyl-cyclase
αs
Ca2+
cAMP
ATP
Epac
Rap1
PKA
CREB
C. Gαq
PLC
PI3K
αq
PLA2
PLD
IP3
DAG
PIP3
PIP2
AA
PI
Rho-GEF
Rho-GDP
Rho-GTP
ER

Structural Basis of Receptor G Protein Function
Although receptor conformation is known to be key in the signal transduction process, very little is actually known about how GPCR activates the G-protein. That the activation process does involve movements within the protein is best illustrated by studies on the visual GPCR rhodopsin.

Rhodopsin contains covalently bound 11-cis-retinal as a ligand and exists in a conformation in which 11-cis-retinal acts as an inverse agonist. Photon absorption by 11-cis-retinal converts the molecule into all-trans-retinal, an agonist for rhodopsin. The ends of 11-cis-retinal are tethered to transmembranal regions 3 (TM3) and 6 (TM6), and light-promoted transformation of retinal from cis to trans introduces differences in binding of retinal to these domains and alterations in the distance between the two TM segments. The relative movements of TM3 and TM6 are of crucial importance for activation of transducin, the rhodopsin specific G-protein (Rao and Oprian 1996). The changes necessary to accommodate the transformation from inactive to active receptor progresses through several intermediate steps, which correspond to the chemical changes of amino acid residue side chains and sterical changes of amino acid residues that convert each involved residue from inactive to active conformation. This is called the binary transition principle (Perez and Karnik 2005). It is these steps that may correspond to the additional active receptor conformations mentioned above. Thus, binary transition of residues and groups would implicate that the number of possible conformations is limited by the transitional states and the number of involved groups/residues (2n , where 2 connotes active and inactive and n the number of groups/residues involved).

Several mutations of human rhodopsin that alter the activity status of the receptor, by interfering with conformationally crucial residues, have been implicated in human disease. Retinal degeneration is caused by a point mutation (K296E) that renders the receptor constitutively active in the absence of retinal (Rao and Oprian 1996). A milder form of this disease is caused by another point mutation (G90D) that results in slightly increased constitutive activity and leads to congenital night blindness (Rao and Oprian 1996). Both mentioned diseases are thus caused by a decreased tendency to adopt the inactive conformation. At the other end of the activity spectrum, autosomal retinitis pigmentosa is caused by a misfolding due to an abnormal disulfide bridge that renders the receptor silent.

The need for separation of TM3 and TM6 in rhodopsin has since been confirmed as a general mechanism for GPCR avtivation, although exceptions may exist (Ward, Hamdan et al. 2002). Further, extensive mutational analysis has shown that motifs in TM3, in the third intracellular loop i3, the last few residues of TM7 (the NPXXYX5F motif; see below) and the carboxyterminal are of importance both for the activation of the G-protein and the GPCR G-protein binding specificity as reviewed recently by Kristiansen (Kristiansen 2004).

Ligand-Induced Differential Signaling – Functional Selectivity
A spectral analysis of purified β2-AR, with a covalently attached fluorochrome at TM6, showed the stabilization of two separate receptor-conformational states in β2-AR in response to two separate ligands, a full agonist and a partial agonist (Ghanouni, Gryczynski et al. 2001). Further, a number of endogenous ligands for the β2-AR has been shown to differentially induce specific receptor conformations that, in turn, correspond to specific cellular functions of the receptor (Swaminath, Xiang et al. 2004).

The above examples represent a growing number of studies that highlight a discrepancy between the intrinsic efficacy model inferred in the 1960-s and the actual measured signaling via several ligand-receptor pairs. Intrinsic efficacy is defined as the stimulus elicited per receptor by a ligand. This measure only allows for differences in quantity, not quality of the stimulus such that, for example an antagonist is expected to antagonize all signaling pathways affected via that receptor equally well, whereas a full agonist is expected to activate all pathways affected via the receptor equally well. Likewise once the intrinsic efficacy of a ligand is known from one model system this is expected to apply to all other situations involving that ligand-receptor couple. In fact, what has been shown indicate that signaling via GPCR is far more finely tuned than mere “volume” control.

For example, the cellular context a GPCR is expressed in, or functional modifications of a GPCR in a controlled cellular context, can result in that one and the same ligand may act as either an agonist or an inverse agonist. A ligand that can act as both agonist or inverse agonist is called a Protean agonist, after the greek god Proteus that could change shape at will. One example of this is the bradykinin B2 receptor (B2R) ligand Icatibant, which apparently acts as a partial agonist when B2R display a low spontaneous activity, and as an inverse agonist when B2R display high spontaneous activity (Leeb-Lundberg, Mathis et al. 1994; Fathy, Leeb et al. 1999; Quitterer, Zaki et al. 1999). The spontaneous activity of a receptor has been reported to be modulated through several mechanisms including levels of GTP and G-protein.

Receptors have also been reported to be stabilized in an inactive, but G protein coupled state, so called precoupling, which is exemplified by the delta opioid receptor (DOR) with antagonists and inverse agonists (Alves, Salamon et al. 2003),and the histamine H1 and H2 receptor with inverse agonists (Monczor, Fernandez et al. 2003; Fitzsimons, Monczor et al. 2004). Further, dopamine D2 receptors (D2R) were reported to couple differentially with specific G proteins depending on the ligand with which the receptor had been stimulated (Gazi, Nickolls et al. 2003).

Such preferential signaling by a selective stabilization of specific conformational states of a receptor has been given several different names including ”agonist-directed trafficking of receptor stimulus”, ”biased agonism”, ”differential engagement”, and ”stimulus trafficking, among others. This phenomenon has lately recieved a growing amount of attention because of the potential opportunity to fine-tune the effect elicited via a specific ligand-receptor interaction and constitutes a new avenue in modern pharmacology. This selective conformational stabilization has implications on several levels of GPCR regulation as will be clarified in the next section.

GPCR-Interacting Proteins Affect Signaling

GPCR may exist in preformed complexes on the plasma membrane with other proteins which may modulate both activational status and signaling pathway utilized. For example dopamine D1 receptor (D1R) can form complexes with inactive NMDA receptors which block lateral movement and allow D1R concentration in spines where NMDA receptors are localized thus enhancing local D1R signaling (Scott, Zelenin et al. 2006). Last but not least a growing number of studies imply that oligomerization of receptors, as homo-oligomers, hetero-oligomers or poly-oligomers of homogenous or heterogenous character, may play a more or less prominent role in GPCR signaling. Metabotropic GABA receptors for example are dependent on the on heterodimerization of GABABR1 and GABABR2 for 4

the extracellular space or on the plasma membrane (e.g. kallikreins, acetylcholine esterase, and surface-bound peptidases such as endopeptidase 24.11) (Davis and Konings 1993; Roques, Noble et al. 1993; Blakely and Bauman 2000) (Fig. 2).

Several proteins such as sorting nexin 13 (SNX13), SNX14 and SNX25 have been shown to serve as regulators of G-protein signaling (RGS) and can both aid in receptor activation by catalyzing the activation of the G-protein, and attract effector systems, or aid in GTP hydrolysis, and hence catalyse the silencing of the GTP protein (GAP function, abbreviaton for GTPase activating proteins) (Fig. 2). SNX13 was the first of these proteins to be shown to have a modulatory role on GPCR signaling, more specifically a role as a GAP on β2-AR signaling via Gαs (Zheng, Ma et al. 2001). A whole group of proteins is now called regulators of G protein signaling (RGS), and is composed of a diverse set of proteins divided by function and structural similarities into four subfamilies B/R4, A/RZ, C/R7, and D/R12. These proteins usually have a negative modulatory effect on GPCR signaling via a GAP activity. For example, RGS9 of the C/R7 subfamily has been implied in negative modulation of MOR, a mouse knock-out model of RGS9 show a sharp increase in MOR agonist effect (Zachariou, Georgescu et al. 2003). GAIP and RGS4 together can antagonize Gαq activation of phospholipase C (Hepler, Berman et al. 1997). But RGS proteins may also serve as signaling nodes that aids in preassembly of the signaling complex to the GPCR (Tinker 2006).

Phosphorylation of the receptor protein may also impede receptor function directly and thus make up part of the desensitization process. These direct modifications may involve only the activated the receptor – called homologous desensitization, or may be caused by compensatory adjustments to a chronic stimuli or the activity of other receptors, called heterologous desensitization. Heterologous desensitization is usually carried out by effector kinases such as PKA and PKC, whereas the initial face of homologous desensitization is carried out by a class of specific serine/threonine G-protein receptor kinases (GRKs). Activity of either kinase system does not result in an easily recognizable phosphorylation pattern of consensus motifs. Rather each kinase/receptor pair result in an individual phosphorylation pattern, a fact that complicates considerably the prediction and investigation of GPCR phosphorylations. Further, exceptions to the mentioned phosphorylation rule do exist such as the purine receptor P2Y1, a receptor that is phosphorylated by PKC in an agonist-dependent fashion.

Modification of a GPCR via phosphorylation may serve other purposes in addition to desensitization. For example, PKA mediated phosphorylation of the β2-AR has been shown to mediate a switch in signaling from Gαs to Gαi (Daaka, Luttrell et al. 1997).

The GRK family of proteins includes seven members, GRK1 – GRK7, of which GRK1 and GRK7 are only expressed in rod and cone cells of the retina, respectively. GRK4 has a very limited expression pattern in cerebellum, testis and kidneys, whereas GRK2, GRK3, GRK5 and GRK6 are expressed in high abundance throughout the body. Further GRK2 and GRK3 are recruited to the plasma membrane through the interaction with the βγ-subunit of the active G-protein and by a pleckstrin homology domain (PH-domain) with phospholipids, phosphoinositides (Fig. 3), at the inner face of the plasma membrane (see below for lipid alterations during endocytosis). GRK5, GRK1/7 and GRK4/6 on the other hand are constitutively associated to the plasma membrane through direct interaction with phospholipid head-groups, farnesylation, and palmitoylation, respectively (see below for discussion on lipid modifications of proteins). The receptor specificity of each GRK is not

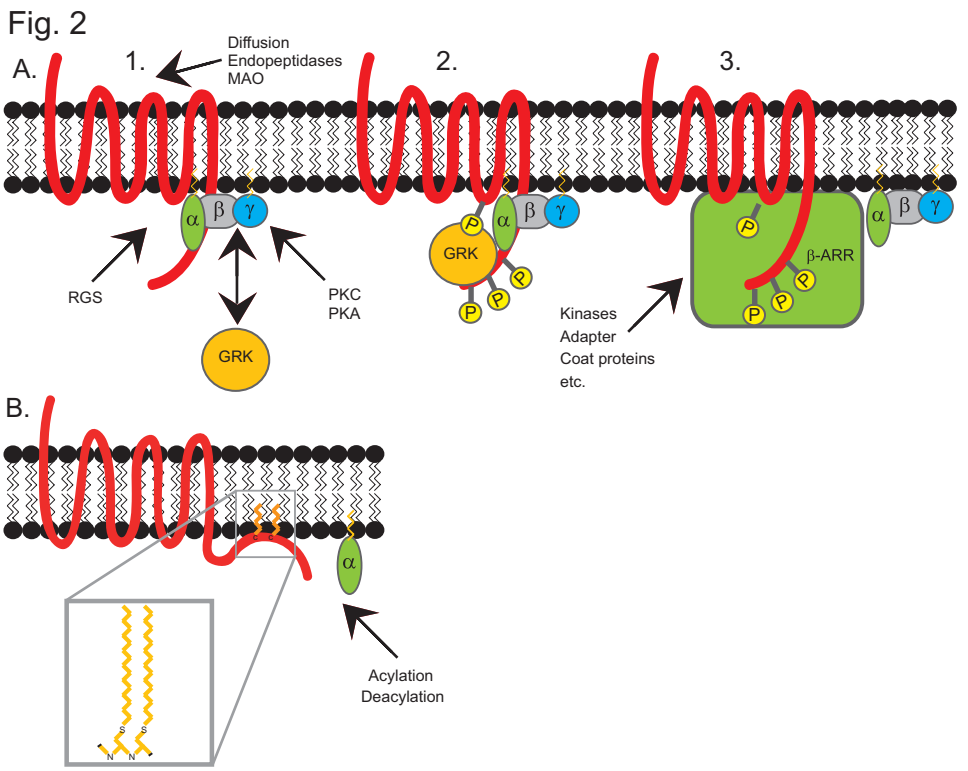


Fig. 2 – Schematic representation of covalent modifications and protein-protein interactions involved in receptor desensitization. A, steps (1-3) in receptor desensitization. B, receptor-effector acylation. RGS - regulator of G protein signaling; β-ARR - beta-arrestin.

absolutely established although it seems like most GPCR are phosphorylated by GRK2 or GRK3 in response to agonist activation. There is no consensus sequence phosphorylated by the GRKs in response to agonist activation. Both serine and/or threonine residues bordered by acidic residues in the carboxy-terminal tail and/or in the i3 loop may be phosphorylated in response to agonist. Also enzymatic activity of the GRK is determined by interaction with the active conformation of the receptor, with or without ligand bound, limiting phosphorylation to receptors in that state.

Phosphorylation by GRKs does not block signaling through the receptor, but it does increase the receptor affinity for a cytosolic family of proteins, arrestins, that ultimately sterically hinder further G-protein activation. Arrestin 1 and 4 are expressed in rods and cones of the retina, respectively, whereas arrestin 2 and 3 are expressed ubiquitiously. Arrestin 2 and 3 are, for historical reasons, known as β-arrestin1 and 2, respectively, (ar-

restins of β adrenergic receptor) and will be refered to as such in the text. Arrestins are recruited to the active receptors through the increased affinity for the receptor in the active conformation, through the increase in affinity for the phosphorylated receptor, and through the recognition of the same kind of altered phospholipids mentioned for the GRKs above. The human lutropin receptor is an exception to the rule in that it does desensitize in a β-arrestin-1-dependent manner but it does so independently of receptor phosphorylation (Min, Galet et al. 2002).

Both GRKs and arrestins have additional roles in GPCR actvity regulation. For example, GRKs contain an RGS domain that can catalyze the G-protein GTPase activity. There are examples of GPCR such as the endothelin receptors ETA and ETB that inactivate through a phosphorylation-independent GRK-dependent mechanism (Freedman, Ament et al. 1997). Arrestins on the other hand function as scaffolds for numerous effectors including phosphodiesterases, enzymes that metabolize cAMP and thus are able to counteract the Gαs dependent signaling (Perry, Baillie et al. 2002).

Another covalent modification of the receptor protein besides phosphorylation, that can be altered by agonist activation is palmitoylation (James and Olson 1989; Mumby 1997). Acylation of receptor proteins is achieved through the formation of a labile, easily modifiable thioester bond between cysteine residues in the carboxy-terminal of the receptor and palmitate (Fig. 2). Studies have shown that deacylation leads to desensitization of the β2-adrenergic receptor (β2AR) and the adenosine A3 receptor through a PKA-mediated phosphorylation of a site normally hindered in the acylated carboxy-terminus (Moffett, Adam et al. 1996; Palmer and Stiles 2000). On the other hand, phosphorylation of the vasopressin receptor V1a both in the presence and absence of agonist is dependent on palmityolation, indicating the variable outcomes of this modification reported from different receptor model systems (Hawtin, Tobin et al. 2001). Similarily, acylation of the Gαs subunit leads to plasma membrane targeting, whereas activation by the binding of GTP leads to deacylation (Iiri, Backlund et al. 1996). This could possibly result in a negative effect on signaling through clearance of Gαs from the plasma membrane .

Ligand-Induced Differential Desensitization

Rapid agonist induced uncoupling and desensitization is, as activation and signaling, dependent on a specific receptor conformation. It has been shown, for example for the B2R, that the spontaneously attained active state can be desensitized (Fathy, Leeb et al. 1999). This implies that ligand binding is not a prerequisite to desensitization. Several ligands have also been shown to result in a signaling activation of a specific receptor, but fail to, or act poorly to trigger the inactivating events resulting in receptor desensitization (Whistler and von Zastrow 1998; Velazquez, Garrad et al. 2000). Desensitization via phosphorylation of the angiotensin receptor AT1A , on the other hand, can occur without activation of the normal Gαq pathway, further supporting that these are two states achieved through separate receptor conformations (Thomas, Qian et al. 2000). Also, CCR7 has two naturally occuring ligands, CCL19 and CCL21, of which only CCL19 promotes receptor phosphorylation and β-arrestin recruitment (Kohout, Nicholas et al. 2004).

Thus, just as activation, phosphorylation and desensitization can be select targets, for specific ligands that result in modulated receptor activity. This also has implications for the further regulation of GPCR, as will become from the discussion of membrane trafficking below.

G Protein-independent Signaling – Direct Receptor-Effector coupling
GPCR can signal via direct interaction with enzymes or channels, or via scaffolding proteins, in addition to G protein-mediated signaling. For example, the β2-AR interacts directly with the Na+/H+ exchange protein NHERF/EBP50, via a PDZ ligand domain in the carboxy-terminal of the receptor (Hall, Premont et al. 1998). This interaction allows for a direct influence of the receptor on cell acidity. Another example is that of the B2R that, when inactive bind and inhibit the activity of eNOS and nNOS, and, when activated by the cognate ligand bradykinin, release these two enzymes in an active form (Ju, Venema et al. 1998; Golser, Gorren et al. 2000). Also, metabotropic glutamate receptors 1 and 5 (mGluR1 and mGluR5) form a complex with Ral and phospholipase D (PLD) through which they modulate several intracellular cascades including their own activity (Bhattacharya, Babwah et al. 2004).

A number of proteins involved in the desensitization process such as arrestins and dynamin act as platforms for the assembly of enormous protein signaling complexes – so called receptorsomes. An example of this is the β2-AR, which through recruited β-arrestin 2 is able to activate c-Src, ERK, Rho A, and E3 ubiquitin ligase with various results on cytoskeletal, chemotactic, and apoptotic processes (Luttrell, Ferguson et al. 1999; DeFea, Vaughn et al. 2000; Shenoy, McDonald et al. 2001).

The activation of additional layers of signaling through scaffolding seems even more complex in light of the varied effect that different ligands have on which proteins to be recruited to the activated receptor. For example, ERK activation via CCR7 only occur in response to CCL19 and not in response to CCL21, since only CCL19 results in β-arrestin recruitment (Kohout, Nicholas et al. 2004).

Further, the tissue-specific levels of both scaffolding proteins and proteins involved in the intracellular transduction cascade initiated by GPCR may very well play a crucial role in determining the specific activity caused by the ligand binding to the receptor. How desensitization of non-G protein-coupled signaling of GPCR is regulated via desensitization will most likely vary depending on receptor and type of interaction. However, there are extra levels of desensitization discussed below that may limit the extent of such signal transduction.

Addtional Layers of GPCR Activity Regulation; GPCR Membrane Trafficking

Internalization of Desensitized Receptors
Most GPCR are cleared from the plasma membrane within minutes after uncoupling and phosphorylation through the process of receptor-mediated endocytosis (FIG. 3). This clearance and the ensuing intracellular sorting of the receptors is generally called membrane trafficking of GPCR. This process may be too slow to contribute to the initial rapid desensitization of the GPCR, at least for receptors that internalize only in response to ligand binding. Instead, trafficking may affect the timing, steady state receptor levels on the plasma membrane, and/or the long term sensitivity of the receptor system through resensitization or downregulation. These processes are key in the subsequent response of the cell to the receptor ligand.

The importance of endocytic regulation of GPCR is underscored by a number of pathological conditions caused either by genetic receptor defects or drugs that elicit an im-

proper receptor trafficking phenotype. An example of the former is the naturally occuring mutations in the DRY/H motif of the V2 vasopressin receptor. The arginine in this motif, when mutated into a cysteine or leucine, results in a constitutively active receptor that only desensitizes in the presence of ligand and leads to the disease ”Nephrogenic Syndrome of Inappropriate Antidiuresis” (NSIAD) (Feldman, Rosenthal et al. 2005). If, on the other hand, the same arginine is mutated into a histidine, the altered receptor constitutively recruits β-arrestin and internalizes resulting in clearance of the receptor, and this manifests itself clinically as the disease ”Nephrogenic Diabetes Insipidus” (NDI) (Rosenthal, Antaramian et al. 1993). An example of the latter is morphine, an opioid drug used since the days of ancient Greece (first documented use by Galen) as a highly potent analgesic. Morphine exerts its positive analgesic effects through the μ opioid receptor (MOR). This drug also promotes severe side effects such as tolerance and dependence. Recent research has shown that morphine fails in several respects to elicit a normal trafficking pattern of the receptor, i.e. phosphorylation and β-arrestin-mediated desensitization and internalization (Whistler and von Zastrow 1998; Whistler, Chuang et al. 1999). Furthermore, rescue of the trafficking through receptor mutation or combinatorial drug treatment results in diminished tolerance and dependence, and enhanced analgesia (Finn and Whistler 2001; He, Fong et al. 2002; He and Whistler 2005).

Steps Involved in GPCR Internalization

The process of receptor-mediated endocytosis is not unique to GPCR but a ubiquitous cellular process involved in several basic mechanisms such as the uptake of nutrients, lipid membrane turnover, i.e. to accommodate cellular growth or shrinkage, antigen processing, and transcytosis of material from one side to the other in a polarized cell. Many of the details involved in GPCR endocytosis have been extrapolated from earlier cell biological studies of endocytic model systems such as the internalization of the epidermal growth factor receptor (EGFR), the transferrin receptor (TfnR; receptor involved in nutrient, iron uptake), and the low density lipoprotein receptor (LDLR; uptake of nutritional lipids). Many of the classes of proteins that are involved in these endocytic systems are also active in GPCR endocytosis, but additional classes of proteins specialized in the task of handling signaling receptors exist, and numerous more are being discovered each year (see Fig. 3).

Albeit numerous differences exist between non-GPCR and GPCR, and even between different GPCR, some features are common for all endocytic processes. First, endocytosis is energy- and temperature-dependent, involve ATP or GTP expenditure to accommodate the necessary modifications in several enzymatic steps, and require physiological temperature, osmotic pressure, and pH to function. Second, the process is selective for the cargo included. Little passive inclusion of bystanders can be detected, and the endocytic process of one type of activated GPCR may exclude other types of activated GPCR. Third, the lipids surrounding the GPCR are not inert but are in fact directly implicated in the recruitment of the necessary effectors for endocytosis. Lipids may also be modified by enzymatic intervention to change their shape in order to accommodate the altered structural requirements forced upon the membrane during the formation of a vesicle from a flat surface. Indeed, the plasma membrane is not a homogenous sea of lipids but parted into regions of differing lipid and protein composition, each with its own properties. Fourth, all the information required for the correct postendocytic targeting of the formed vesicle within the cell is included in the vesicle.

Fig. 3

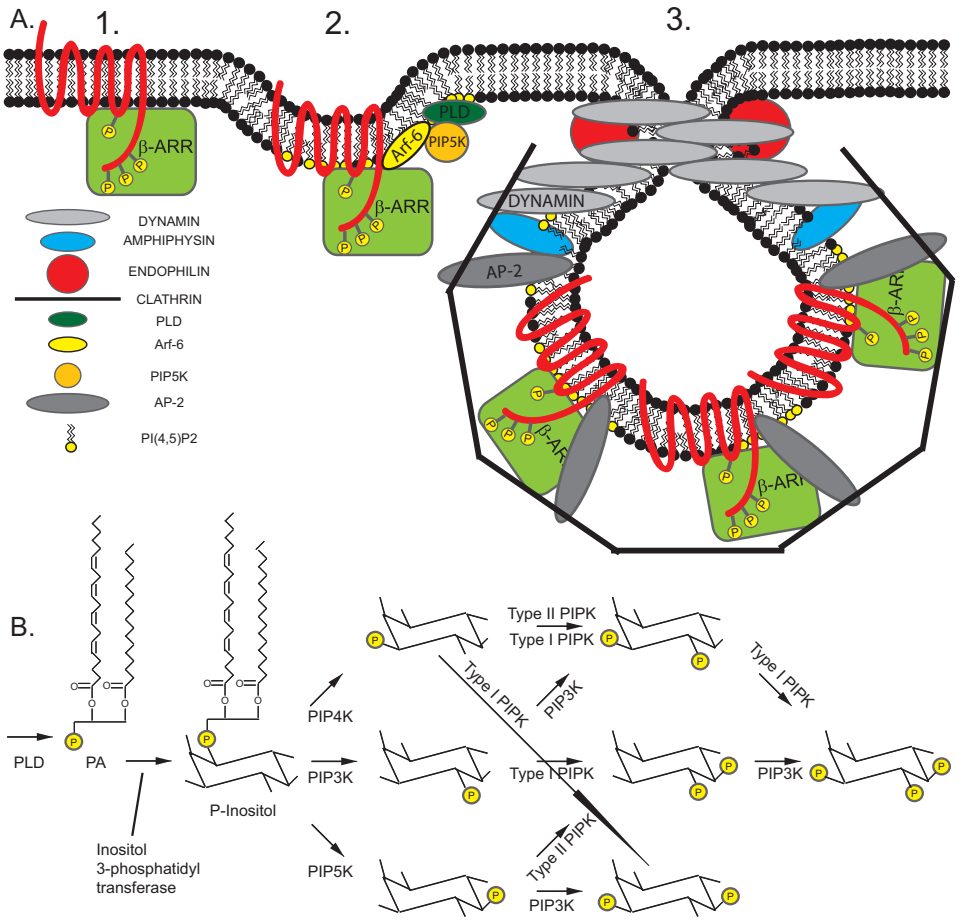


Fig. 3 – Schematic representation of covalent modifications, protein-protein interactions, and phosphoinositide metabolism involved in clathrin-mediated receptor endocytosis. A, steps (1-3) in receptor endocytosis. β-ARR - beta-arrestin; Arf-6 - ADP ribosylation factor 6; PLD - phospholipase D;AP-2 - adaptor protein 2 ; PIPK - phosphatidylinositol kinase; PA - phosphatidic acid; P-inositol - phosphatidylinositol; PIP - phosphatidylinositolphosphate.

Internalization
The first step of receptor internalization is the selection of the cargo to be included in the assembling plasma membrane pit that is to become the endocytic vesicle. This process is followed by a cascade of events where a major machinery assembles around the deactivated GPCR which is necessary for membrane reshaping into a vesicle and subsequent fission from the plasma membrane. It is still not known whether the concentration of the GPCR in the pit formations on the plasma membrane is due to receptor movements to the ”hot spot” of endocytosis, or the recruitment of the machinery to the spot where the receptors are inactivated. The emerging view of large congregates of GPCR multimers, and the fact that measurements of speed of receptor movement in the membrane fail to record an increase in movement in response to agonist, favor the latter alternative.

Even though most GPCR are affected by members of the endocytic machinery outlined below, no consensus machinery exist that is needed for the internalization of all GPCR. Instead a plethora of interacting partners is emerging, and the importance of each of these partners for the endocytosis of individual receptors will have to be addressed separately in future studies.

Selectivity of cargo inclusion in an endocytic pit can be achieved through two separate mechanisms: 1) the formation of plasma membrane subdomains based on protein and lipid composition – e.g. lipid rafts; 2) cargo-specific adaptors that recognize both the cargo and the downstream machinery necessary for endocytosis. It is now clear that internalization of different types of GPCR utilize either one or both of these mechanisms for recognition of cargo. The pathways utilized are usually divided into three groups based on key proteins involved in the process: 1) clathrin-dependent pathway, 2) lipid raft pathway with or without the aid of caveolin, and 3) macropinocytosis.

Clathrin, which is part of a family of proteins called coat proteins, is a multimeric protein consisting of three heavy and three light chains (see fig 4A) aggregated into a triskelion (Harrison and Kirchhausen 1983; Kirchhausen, Harrison et al. 1987). A number of clathrin multimers can form a lattice on the intracellular face of the plasma membrane or on membranes of intracellular compartments such as the trans golgi network and endosomes. The locale for formation of clathrin lattices is dependent on the adaptor protein that links the suitable coat to the cargo (Prasad and Lippoldt 1989; Gallusser and Kirchhausen 1993). Clathrin formation and cargo accumulation results in the formation of a clathrin-coated pit that invaginates, and pinches off from the plasma membrane, to form a clathrin-coated vesicle.

Lipid rafts constitute another group of membrane microdomains that may or may not be coated with membrane caveolin. As the name implies, these structures have a specific lipid composition with the outer leaflet highly enriched in glycosphingolipids and cholesterol (Brown and London 1998). The classification of these structures is based on the finding that not all membranes can be dissolved in all solvents, which has resulted in the additional name detergent-resistant microdomains (DRM) (London and Brown 2000). Lipid rafts devoid of caveolin are relatively small (<50nm diameter), whereas caveolin-containing lipid rafts are slightly bigger (≤200nm diameter) (Simons and Ikonen 1997). Refined methods that would allow their study in a living cellular context, especially the non-coated version, are hot research topics. Most people studying lipid rafts consider them as transiently forming short-lived structures with the exceptions of the caveolae, that seem to be extraordinarily stable (van Deurs, Holm et al. 1993; Thomsen, Roepstorff et al. 2002). Non-caveolin

DRM are flat or invaginated (rare, or extremely transient structures that are mostly found in cells with a blocked lipid raft endocytic pathway), whereas caveolin-enriched lipid rafts form flask-shaped invaginations of plasma membrane coated by large numbers of polymerized caveolin molecules. Targeting of GPCR to DRM is thought to be achieved through either protein-protein interaction with caveolin via a ϕXϕXXXXϕ or ϕXXXXϕXXϕ motif on the cytosolic face of the protein, through fatty acylation such as palmitoylation (Fig. 2) of the receptor, or alterations of the active receptor protein conformation that favor localization to the DRM because of the local lipid composition (Couet, Li et al. 1997; Gimpl, Burger et al. 1997; Lamb, Zhang et al. 2002; Eroglu, Brugger et al. 2003; Alves, Salamon et al. 2005).

A third endocytic pathway that has recently been implied in the regulation of the epidermal growth factor receptor is macropinocytosis (Orth, Krueger et al. 2006). This process starts as large ruffles at the plasma membranes and continues by the engulfment of the entire ruffle including macrmolecules in the extracellular space. Certain markers for rafts are included in micropinocytic bodies. Macropinocytosis has been implicated in regulation of the EGFR but has yet to be proven important in GPCR membrane trafficking.

The Clathrin-Dependent Machinery

AP-2 is a heterotetrameric plasma membrane-specific adaptor protein that recognizes the cytosolic face of transmembrane proteins through a number of different motifs (see Fig. 3) (Gallusser and Kirchhausen 1993). The AP-2 protein consists of four subunits α, β2, δ2, and μ2. The α-subunit is involved in targeting of AP-2 to the plasma mebrane, and also aids in the interaction with several other proteins, such as Eps15, which are necessary for subsequent steps (DPW or DPF motif-containing endocytic proteins) (Mousavi, Malerod et al. 2004). β2 contains a clathrin box motif (LLNLD), a common motif in clathrin interacting proteins, which is recognized by, and bind to the N-terminal β-propeller of the clathrin protein (ter Haar, Harrison et al. 2000). μ2 binds to phospholipids in the plasma membrane and is involved in cargo sequence motif recognition (Aguilar, Ohno et al. 1997; Rohde, Wenzel et al. 2002). The role, and the sequence of these endocytic cargo motifs depend on whether they are part of a constitutively endocytosing transmembranal proteins, or of a signaling receptors (Mousavi, Malerod et al. 2004). AP-2 is aided in catalysing formation of clathrin coat assembly by several proteins. Eps15 can form multimers via a coiled coil central domain, and acts as a polymerizer for AP-2, AP-3 (AP180), and Epsin, among other proteins (Cupers, ter Haar et al. 1997; Salcini, Chen et al. 1999). Epsin and AP-3 aid AP-2 in catalysing clathrin coat formation (Hao, Luo et al. 1999; Kalthoff, Alves et al. 2002). Amphiphysin is another hub protein that binds to AP-2, clathrin, and the plasma mebrane via one terminal and the central portion of the protein, and to endophilin, dynamin, and synaptojanin at the other end of the protein (Wigge and McMahon 1998; Zhang and Zelhof 2002). This kind of multiple scaffolding, and strengthening of interaction via these scaffolds is a common theme for the internalization machinery.

β-Arrestin is an additional adaptor for GPCR, which, in addition to the receptor, binds to both AP-2 (C-terminal β-arrestin interacts with β2-subunit of AP-2), and clathrin (clathrin binding motif is close to AP-2 binding motif in the β-arrestin protein) (Goodman, Krupnick et al. 1996; Laporte, Oakley et al. 1999; Laporte, Oakley et al. 2000; Claing, Laporte et al. 2002). In this respect, β-arrestin acts as a clathrin-associated sorting protein (CLASP), proteins that diversify the number of proteins recognized by AP-2 and the clathrin machinery (Edeling, Mishra et al. 2006). CLASP proteins contain a DEnX1-2FXXFLXXXR motif that binds to the β2-subunit of AP-2, a motif also seen in epsin (Edeling, Mishra et al. 2006).

β-Arrestin activity in internalization is modified by phosphorylation of the protein. Both β-arrestin 1 and 2 are unable to bind to clathrin when phosphorylated (Lin, Krueger et al. 1997; Claing, Perry et al. 2000). Activation of the GPCR leads to dephosphorylation of β-arrestins through yet unknown phosphatases. The form of β-arrestin that activates Erk depends on the receptor system. In the case of β2-AR, β-arrestin 1 activates Erk. This mechanism seems to serve as a negative feedback on β-arrestin 1 through Erk phosphorylation. On the other hand, β-arrestin 2 is phosphorylated, and thus hindered from interacting with clathrin by casein kinase II phosphorylation (Lin, Miller et al. 1999; Lin, Chen et al. 2002; Shenoy and Lefkowitz 2003).

β-Arrestin interaction with the kinase Src, and colocalization to desensitized, uncoupled receptors is important for the phosphorylation of proteins involved in regulating internalization (Ahn, Maudsley et al. 1999). One of the substrates for Src is dynamin, and a block of Src induced dynamin phosphorylation leads to a perturbed β2-AR internalization (Ahn, Maudsley et al. 1999; Miller, Maudsley et al. 2000).

β-Arrestin, AP-2, and clathrin all bind to modified phosphorylated lipids in the inner leaflet of the plasma membrane via pleckstrin homology domains (PH domains) (Gaidarov, Krupnick et al. 1999; Rohde, Wenzel et al. 2002). In response to β2-AR signaling, Grk2, which initiates receptor desensitization by receptor phosphorylation, also interacts with and activate phosphatidylionsitol 3-kinase (PI3K), a protein that converts phosphatidylinositol diphosphate, PIP2 into PIP3 (Naga Prasad, Barak et al. 2001; Naga Prasad, Laporte et al. 2002). Inhibitors of PI3K (e.g. the mushroom poison wortmannin) lead to a block of β2AR internalization, whereas disruption of the Grk2/PI3K augments β2-AR signaling (Naga Prasad, Barak et al. 2001; Naga Prasad, Laporte et al. 2002; Perrino, Naga Prasad et al. 2005). Phosphorylation of PIP2 is just one of a number of intricate steps that regulate lipid metabolism near the activated GPCR (see Fig. 3B and 4 for an overview). Breaking this lipid turnover process at any one level leads to severe impairments of GPCR turnover, signaling, and/or cell survival, see recent review (Gruenberg 2003). The cascade of events set in motion by GPCR activation and deactivation leads to a transient increase in the level of these modified lipids, which in turn results in enhanced recruitment of proteins with lipid binding domains.

In addition to the role of PI3K in lipid metabolism, this protein also modulates the cortical cytoskeleton by phosphorylating tropomyosin and promoting actin bundling, which is of importance for the transport of GPCR to the forming clathrin coated pits on the plasma membrane. This process may also lend strength to the pinching off and movement of formed vesicles (Gaidarov, Krupnick et al. 1999; Naga Prasad, Jayatilleke et al. 2005).

β-Arrestin has multiple additional roles beyond its function as signaling silencer and adaptor for coats. This protein also serves to anchor numerous additional proteins to the desensitized receptor. Three of these proteins directly involved in internalization are N-ethylmaleimide-sensitive fusion protein (NSF), G protein ADP-ribosylation factor 6 (Arf-6), and the corresponding guanine nucleotide ADP-ribosylation factor nucleotide-binding site opener (ARNO) (McDonald, Cote et al. 1999; Claing, Chen et al. 2001).

NSF acts later in the endocytic process to regulate the activity of receptors on the vesicle and on the target membrane, so called SNAREs (Sollner, Bennett et al. 1993). This protein also has a direct catalyzing effect on receptor internalization even though the mechanism for this is unknown (McDonald, Cote et al. 1999).

Arf-6 activity is controlled by the GEF ARNO (Chardin, Paris et al. 1996). Arf-6 activity in turn controls phosphoinositide 5-kinase (PI5K) both through direct interactions and indirectly through activation of phospholipase D (PLD) (Brown, Gutowski et al. 1993). This lipase produces phosphatidic acid, a substrate that among other things function as a cofactor for PI5K activity. One type of PLD, PLD2, has been shown to be crucial for the internalization of AT2R and MOR (Koch, Brandenburg et al. 2003; Du, Huang et al. 2004). In fact, the activation of PLD2 by MOR is dependent on which agonist is used, and agonists that do not lead to PLD2 activation do not promote receptor internalization (Koch, Brandenburg et al. 2004). Therefore, this system presents an example of biased agonism on endocytosis. Activation of PI5K by Arf-6 results in the accumultaion of PI(4,5)P2, a lipid recognized by several proteins via PH, FERM, PX, ENTH, FYVE, and tubby domains (Honda, Nogami et al. 1999). Arf-6 activity also modulates cytoskeletal alterations leading to reshaping of the actin cortical cytoskeleton possibly to lend strength to plasma membrane shape-changes and vesicle fission (Donaldson 2003). Further, Arf-6 interacts with both AP-2 and SMAP1, and SMAP1 in turn interacts with clathrin, leading to enhanced plasma membrane recruitment of adaptors and coat proteins (Krauss, Kinuta et al. 2003; Paleotti, Macia et al. 2005; Tanabe, Torii et al. 2005). Arf-6 activity is positively modulated by GEFs other than ARNO such as GRP1, and EFA6, and negaitevely by GAPs such as SMAP1, GIT1 and 2, and possibly several others, as recently reviewed (D'Souza-Schorey and Chavrier 2006). The latter two are also implicated in GRK activity regulation and thus act as negative modulators of internalization at an early step in the pathway (Premont, Claing et al. 1998). Several GPCR such as β2-AR, AT1R, ETBR, V2R, and muscarinic M2MR show direct dependence on Arf-6 for their internalization (Houndolo, Boulay et al. 2005). Notably Arf-6 depletion affects all known internalization pathways for GPCR, indicating the importance of this protein in signaling receptor internalization.

Besides the numerous activities of β-arrestins, these proteins can be modified further to accommodate new interactions. One such modification found to be essential for the internalization of a number of receptors is ubiquitination. Polyubiquitination was initially postulated to be the signal for targeting to and destruction by the proteasome. Direct monoubiquitination of receptor proteins or indirectly of adaptor β-arrestin has been shown to act as targets for internalization from the plasma membrane through interactions with proteins via ubiquitin binding domains. For example, β-arrestin interacts with and is ubiquitinated by the ubiquitin ligase Mdm2, and disruption of this interaction and/or the sites for ubiquitination results in blocked endocytosis of the β-AR (Shenoy, McDonald et al. 2001).

There are examples of GPCR that show β-arrestin 1- and 2-independent clathrin-dependent internalization. These include the protease-activated receptor 1 (PAR1) and the metabotropic glutamate receptor 5 (mGluR5) (Paing, Stutts et al. 2002; Bhattacharya, Babwah et al. 2004). How these receptors endocytose is still under debate. PAR1 requires phosphorylation and is dynamin- and clathrin-dependent but can internalize fully in β-arrestin 1 and 2 knock-out mouse embryonal fibroblasts. β2-AR internalization was blocked in the same cell line. It is possible that PAR1 interacts directly with the AP-2 protein but that there also exist other possible adaptors involved in clathrin-coated pit and vesicle formation such as the E/ANTH proteins. Members of this family such as epsin AP180 and CALM have been found to bind directly and selectively to cargo in the clathrin-coated pits. It is clear that the cytosolic carboxyterminal tail of the PAR1, and especially a putative AP-2 binding motif (YSIL) in the tail, is a crucial domain for internalization of this receptor (Paing, Temple et al. 2004). mGluR5, on the other hand, seems to create an intracellular scaffold consisting of the proteins Ral, RalGDS, and PLD2 (Bhattacharya, Babwah et al.

2004). The activity of this scaffold of proteins leads to the formation of phosphatidic acid, which in turn directly and indirectly through activation of PI5K and formation of PIP2 attracts AP-2 and clathrin. An interesting feature of the mGluR5 receptor is that an inverse agonist, 2-methyl-6-(phenylethynyl)pyridine, blocks basal receptor activity but does not block receptor endocytosis indicating that the scaffold formation and internalization are independent of receptor activity (Fourgeaud, Bessis et al. 2003). Constitutive internalization is a phenomenon reported for several GPCR but is most often connected to receptor activity. The number of alternative scaffolding molecules and routes utilized for the internalization of GPCR will most likely grow as the study of more receptor model systems progresses.

There has been considerable debate over the role of clathrin in the formation of clathrin-coated vesicles. One role suggested for clathrin is that of deforming the membrane during pit and vesicle formation. However, several other proteins seem involved in this process. Indeed, and membranes actually seem able to form vesicles in the absence of a coat. It is possible that the main function for clathrin is to hold the correct machinery together during vesicle formation (Ford, Mills et al. 2002). One example of a protein that is part of this machinery is amphiphysin, which localizes to clathrin-coated pits via binding to lipids and clathrin, and can in turn attract dynamin, and synaptojanin to the edge of the forming vesicle (McMahon, Wigge et al. 1997; Wigge and McMahon 1998; Zhang and Zelhof 2002). Dynamin is a GTPase that forms a multimeric complex around the growing clathrin-coated pit, and later the neck of the vesicle, and this protein has been implicated in the bending of the membrane, and fission of the vesicle from the membrane. This effect is achieved either mechanically through a wringing mechanism, or indirectly by attracting effectors, as reviewed (Mousavi, Malerod et al. 2004). Dynamin also tethers endophilin to the clathrin-coated pit, and may also regulate endophilin activity (Schmid, McNiven et al. 1998). Endophilin is a lysophosphatidic acyl transferase that adds acyl groups to lysophosphatidic acid, which alters the lipid shape from an inverted cone to a cone shape that may enhance curvature of the membrane (Schmidt, Wolde et al. 1999). Other proteins involved in altering the lipid structure are epsin and synaptophysin. Epsin can insert itself into the membrane via a hydrophobic helical domain and thus separate lipid head groups, and lowering the energy required to bend the membrane (Ford, Mills et al. 2002). Synaptophysin is closely related to caveolin in function, and polymerizes and alters the shape of membranes by interacting with cholesterol (Thiele, Hannah et al. 2000). Dab2, which is also recruited to the forming pit via AP-2, receptor motifs, and lipid interactions seems to act as a link to actin motor proteins such as myosin VI, indicating that the cortical cytoskeleton may have a role in vesicle formation (Morris, Arden et al. 2002). Another dynamin-interacting protein is synaptojanin, a phosphatase of PI(4,5)P2 that is implicated in fission, uncoating of the vesicle after fission, and tethering of the formed vesicle to the cytoskeleton. These functions are shared by other proteins such as Hsc70 (Harris, Hartwieg et al. 2000; Newmyer, Christensen et al. 2003). Cytoskeletal contacts are thought to regulate endosomal vesicle movements through direct polymerization of actin or through the action of motor proteins like myosins.

The rab family of proteins is yet another group of proteins of importance for the budding, fission, and subsequent trafficking. These small G-proteins are organelle membrane-specific and go through the same cycle of activation and deactivation as described for other G-proteins. The rab5 protein is specific for the plasma membrane and early endosomal membranes. This protein is not only important for both the budding/fission process of vesicles containing GPCR but also for subsequent steps of membrane fusion between

formed vesicles and between vesicles and larger organelles (McLauchlan, Newell et al. 1998; Seachrist, Laporte et al. 2002). There is a connection between vesicle receptors (SNAREs) and rab proteins, which in the case of rab5 seems to involve a direct interaction (Armstrong 2000). Rab5 is most likely both involved in the selection of the proper SNAREs for the endocytic vesicle and a regulator of their activity (Seachrist and Ferguson 2003). EEA1 (early endosomal antigen 1) is one rab5 effector characterized for the endocytic vesicle, which causes homotypic fusion of early endosomes (Simonsen, Lippe et al. 1998; Christoforidis, McBride et al. 1999). Rab5 has been reported to interact directly with GPCR and is essential for internalization of several differentially trafficking GPCR (Seachrist and Ferguson 2003).

Alternative Routes and the Non-Clathrin Dependent Machinery for Internalization
The cellular machinery necessary for internalization may vary as indicated above. One such variation that has recieved growing attention is the internalization through non-clathrin dependent structures. The two main pathways mentioned are the lipid raft and the caveolae pathway. Several groups are now accepting to fuse these two pathways into one based on the their many shared mechanisms unraveled so far, (see for example (Nabi and Le 2003)). Others are less categorical and instead divide the lipid raft-dependent internalization pathways depending on the machinery necessary for each pathway, (see for example (Kirkham and Parton 2005)). Further, the caveolae, a structure categorized by the inclusion of the protein caveolin, seems to be a relatively stable surface structure with a very limited ability to pinch off from the plasma membrane (Thomsen, Roepstorff et al. 2002). Non-caveolar lipid rafts, on the other hand, seem to be able to undergo very rapid internalization in the absence of any detectable coat (Deckert, Ticchioni et al. 1996; Kirkham, Fujita et al. 2005). Raft structures can also be seen in early endosomes and throughout the entire biosynthetic pathway (Mukherjee and Maxfield 2000; Sharma, Choudhury et al. 2003; Mogelsvang, Marsh et al. 2004). On the other hand, the role of caveolae in signaling is undisputed. The altered ability of some receptors to endocytose in the absence of caveolin may therefore be due to the inability of these receptors to activate the chain of events necessary for receptor internalization, rather than a block of an actual caveolin-dependent internalization pathway.

Most studies of the lipid raft/caveolae involvement in GPCR internalization are based on methods that deplete the plasma membrane of cholesterol through agents such as filipin, nystatin, and methyl-β-cyclodextrin, through dominant-negative versions of proteins involved, or through knock-down of the expression of caveolin or other proteins involved. Lipid raft pathways lack sensitivity to blockers of the clathrin pathway such as monodansylcadaverine (MDC), acidity (e.g. acetic acid), potassium depletion, or hypertonic media (high sucrose or NaCl). It has in fact been found through these studies that a few proteins such as dynamin, actin, epsin, and Arf-6 are shared between the clathrin and non-clathrin pathways. Several phosphorylated lipids involved in clathrin-dependent internalization are also active in the non-clathrin pathway. The extent of overlap between coated and non-coated internalization pathways will not be solved until more receptors have been extensively characterized.

A few examples of receptors that utilize the non-clathrin-dependent pathway are the vasoactive intestinal peptide 1 receptor (VIP1R), the muscarinic acetylcholine receptor 2 (M2MR), the endothelin-1 receptors A and B (ETAR, ETBR), the adenosine receptor (A1R), the cholecystokinin receptor (CCKR), some of the adrenergic receptors (α2AR, β1AR), chemokine receptor 5 (CCR5), the glucagon-like peptide 2 receptor (GLP-2R),

and the gonadotropin relase hormone receptor (GnRHR) The internalization mechanisms of these receptors are briefly outlined below to clarify the diversity through which the non-clathrin-dependent pathways operate.

Agonist-stimulated internalization of the VIP1R was recently shown to be arrestin- and Arf-6-independent but was almost completely blocked by a dominant-negative version of dynamin (K44E) and by MCD pretreatment (Houndolo, Boulay et al. 2005). These features seem to be typical of a clathrin-independent receptor internalization. The M2MR is similarly not blocked by clathrin blockers and does not require arrestins for internalization (Delaney, Murph et al. 2002). This receptor does however require Arf-6, but is insensitive to GIT-1, and the dynamin K44E mutant (Delaney, Murph et al. 2002; Houndolo, Boulay et al. 2005). Two other dynamin mutants, one that lacks the GTP binding site (A1-272) and another that is unable to be stimulated by PIP2 (K535M), both block M2MR internalization (Delaney, Murph et al. 2002). Delaney et. al. also showed that M2MR quickly enters transferrin-positive early endosomes after internalization, indicating that clathrin-dependent and clathrin-independent pathways merge after internalization. M2MR is a good example of the difficulties that exist when trying to interpret the effect of dominant-negative models on internalization. Dynamin is, just as β-arrestin, a large protein that acts as a scaffold for several types of effectors, and the particular effectors recruited via dynamin will most likely vary with the receptor type.

This above reasoning might be why GLP-2R, yet another receptor where internalization is blocked by cholesterol depletion, is reported to be independent of dynamin for internalization (Estall, Yusta et al. 2004). GLP-2R has only been tested for K44E sensitivity. Estall et. al. also reported that GLP-2R colocalize transiently with caveolin on intracellular vesicles, but that receptor and caveolin are separated within 20-60 minutes after internalization. The ETBR is yet another Arf-6-dependent receptor that directly interacts with caveolin and for which internalization is largely blocked by cholesterol depletion and by dynamin (K44E) (Claing, Perry et al. 2000; Houndolo, Boulay et al. 2005). The ETBR has also been reported to be blocked by hypertonic sucrose media, a classical clathrin-disrupting agent, and by mutant forms of β-arrestin (Paasche, Attramadal et al. 2001; Gregan, Jurgensen et al. 2004). Thus ETBR apparently has the ability to internalize through both clathrin-dependent and clathrin-independent pathways. This is in fact not an all to uncommon feature since it is shared by CCKR and β1-AR (Roettger, Rentsch et al. 1995; Rapacciuolo, Suvarna et al. 2003).

Chosing which pathway to utilize for internalization might be influenced by differential phosphorylation. In the case of β1-AR, a low agonist concentration results in GRK-mediated clathrin-dependent internalization, whereas a high agonist load causes additional PKA-mediated phosphorylation and clathrin-independent internalization (Rapacciuolo, Suvarna et al. 2003). CCR5, on the other hand, showed almost total and equal dependence on both clathrin (sucrose inhibition, recruited arrestin in response to agonist), and lipid rafts (nystatin and filipin inhibition) for internalization, as well as a high dependence of the actin cytoskeleton (pretreatment with an actin depolymerizing agent cytochalasin D) (Mueller, Kelly et al. 2002; Mueller and Strange 2004). If this means that lipid rafts can enter a cell in a clathrin-dependent manner is yet to be determined. The sensitivity of CCR5 to particular blockers varied slightly with the ligand utilized, again emphasizing the differential effect that various ligands can have on receptor conformation and activity (Mueller, Kelly et al. 2002; Mueller and Strange 2004).

Finally, the avian GnRH-R internalization is completely insensitive to clathrin blockers such as MDC and sucrose, but is blocked by dynamin K44E, filipin, and cyclodextrin as well as by a dominant-negative version of caveolin-1 (caveolin-1(Δ1-81)) (Pawson, Maudsley et al. 2003). This could reflect an inability to activate the receptor in the absence of functional caveolin, or a perturbed recruitment of the correct internalization machinery for the receptor, and therefore does not have to imply that the receptor is internalized with caveolin. Interestingly, GnRH-R internalization could be enhanced by the overexpression of β-arrestin indicating that cell-type specific levels of interacting molecular species may influence the pathway chosen by a GPCR (Pawson, Maudsley et al. 2003).

In summary, several different levels of regulation exist within the clathrin-independent pathway(s) of internalization. In addition, there is evidence for overlap between clathrin-indepedent and clathrin-dependent mechanisms of internalization. The great variability in sensitivity to the array of endocytic perturbations available emphasize the importance of a tight control of terminology in order to avoid misleading epithets. In the end, it seems necessary to investigate each GPCR for interaction partners and utilized pathways under more than one set of conditions to fully understand the process of internalization for that receptor.

The Postendocytic Choices

Once internalized, GPCR face at least three alternative paths; 1) recycling to the plasma membrane for reinsertion and utilization; 2) sorting along a degradative pathway to the lysosome or the proteasome leading to proteolysis; 3) intracellular storage. An additional role of internalization is for transport of receptors to other cellular loactions, e.g. along an axon to a nerve terminal, but this process does not involve receptor activation. Also, it seems that intracellular pools of receptors may form that can be recruited to the plasma membrane when needed. It is currently unknown if agonist activation can lead to storage of internalized receptors.

The alternate routes taken by internalized receptors traverses both transiently formed and more permanent endosomal organelles in the cytosol (Fig. 4). I will start out by briefly surveying the organelles involved, and then expand on the mechanisms that regulate the transport of GPCR through these organelles.

Membrane compartments in the endocytic, degradative, and recycling pathway

The uncoated primary vesicles go through cycles of homotypic fusion plus fusion to pre-formed early endosomes carrying several additional proteins. The size of the vesicles thus grow over time and may include many different kinds of endocytosed GPCR and other protein species such as transferrin receptors and EGFR (Cao, Mays et al. 1998). Due to a vacuolar proton pump, the pH drops to roughly pH6 in the early endosome and continues to drop along the endosomal structures towards the late endosome and lysosome, where the pH is 4 (Yamashiro and Maxfield 1984; Maxfield and Yamashiro 1987; Van Dyke 1996). Initially, this serves to facilitate dissociation of the ligand from the receptor and receptor dephosphorylation thus resulting in recycling and resensitization of recycling GPCR (Geuze, Slot et al. 1983). Later, the declining pH serves to optimize the conditions for specialized hydrolases within the limiting membrane of the lysosome. The emerging early endosome takes the shape of an octopus with a spheric center with protruding tubular membranal structures (Gruenberg 2001). The vesicular part retains GPCR destined for the degrading pathway, whereas the protruding tubules fill up with recycling GPCR (Dunn, McGraw et al. 1989; Mayor, Presley et al. 1993). Effectors necessary for the consecutive

steps are brought in both by the action of Rab proteins and by the combined attraction of the modified lipids and the surfaces of the GPCR facing the cytosolic side of the vesicle. For some receptors, the same interacting proteins that initiated internalization, such as β-arrestin, remain tethered as address tags throughout the intracellular sorting steps.

The tubular structures either rapidly recycle back to the plasma membrane with its cargo or are transported to the perinuclear space for a more slow sorting (Yamashiro, Tycko et al. 1984). Meanwhile, the vesicular remnant starts to endocytose material to the intraluminal space forming a vesicle, called a multivesicular body (MVB), which is filled with smaller vesicles (Gruenberg and Stenmark 2004). The MVB is also called a carrier vesicle because of its ability to transport the cargo along microtubules while maturing into a late endosome.

Later steps involve the fusion to a late endosome or lysosome enriched in hydrolases that help digest the cargo. It is important to realize that what seem like an end station in one endocytic pathway may just be an intermediate checkpoint in another. MHC class II molecules are for example loaded with cargo in lysosomes, from which they bud off, and recycle to the plasma membrane (Chow, Toomre et al. 2002). Thus intracellular membrane-bound pathways feed into each other. Mannose-6-phosphate receptors readily recycles between the early endosome and golgi network, or late endosome and golgi network, carrying enzymes to the endosomal pathway.

Lipids in intracellular sorting

The lipid composition of each endosomal organelle is quite complex and reflect both their position in the cell and their function (Gruenberg 2003) (see Fig. 4). For example, the phosphoinositides play as crucial a role in the intracellular compartments operating as catalyzers and protein docking partners as in the plasma membrane. Each level of sorting involves a different set of phosphoinositides thus restricting the activity of each lipid species. PI(4,5)P2 is enriched in budding and newly formed vesicles, PI3P is highly enriched in the early endosome and multivesicular bodies/late endosomes, and PI(3,5)P2 may be enriched in late endosomes and lysosomes (Gillooly, Morrow et al. 2000). A block of the activity of kinases involved in the production of these phosphoinositides leads to a block of endocytic transport through the affected pathway. An example of this is the effect of wortmannin seen on MVB maturation, where bloated early endosomes amass without further endosomal maturation.

Lysobisphosphatidic acid is enriched in the inner vesicles of multi vesicular bodies, where the inherent head group cone-shape of this lipid helps to induce negative curvature and aid in fission of these inner vesicles (Matsuo, Chevallier et al. 2004).

Cholesterol is enriched in several structures such as the recycling tubules, late endosomes, lysosomes, and the trans golgi network (Gagescu, Demaurex et al. 2000; Mobius, van Donselaar et al. 2003). This lipid too seem important for the correct localization of certain proteins involved in sorting, such as Rab9, and thus indirectly control protein and lipid sorting within the cell (Ganley and Pfeffer 2006).

Lipid rafts are formed in the endoplasmic reticulum where they support protein folding (Sarnataro, Campana et al. 2004; Campana, Sarnataro et al. 2006). Further lipid rafts are found in the golgi apparatus and have a role in targeting of proteins to correct cellular membranes (Simons and van Meer 1988; Brown and Rose 1992; Simons and Ikonen

Fig 4.

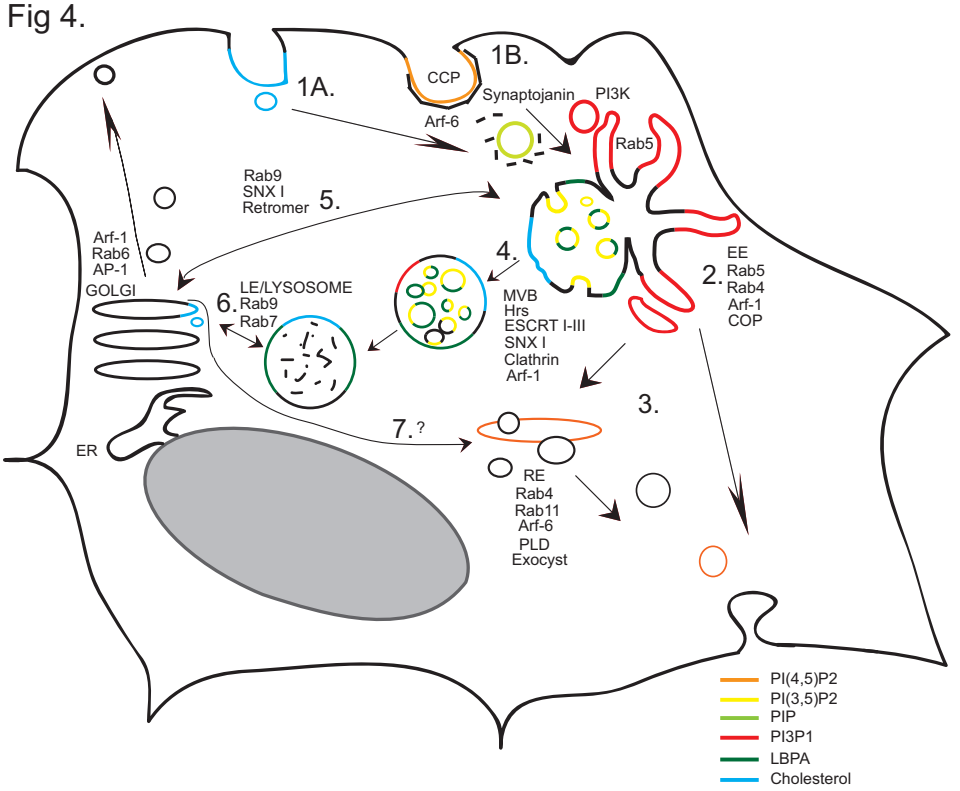


Fig. 4 – Schematic representation of endosomal organelles, proteins, and lipids involved in receptor endocytosis and post-endocytic sorting. 1, internalization via lipid rafts (A) and clathrin-coated pits (CCP) (B); 2, Early endosome (EE) sorting; 3, Fast and slow endosome recycling; 4, Sorting via multivesicular bodies (MVB); 5, Retromer sorting; 6, Golgi retrieval; 7, Alternative maturational route.

1997; Cheong, Zacchetti et al. 1999). Lipid rafts internalized via endocytosis remain intact and traverse through the endosomal structures either to the inner vesicles of multivesicular bodies, or back to the trans golgi network (Fivaz, Vilbois et al. 2002). Most integral membrane proteins that are endocytosed in lipid raft structures only briefly remain in these structures after internalization, whereas others such as cholera toxin, caveolin, and glycosylphosphatidylinositol-anchored or acylated proteins seem to adhere more firmly to the intracellular trafficking of the raft (Fivaz, Vilbois et al. 2002; Estall, Yusta et al. 2004).

Connection between the endocytic and biosynthetic pathways

The endocytic pathway is at several steps interconnected with the biosynthetic pathway, such that material can be transported between the golgi network, most often the TGN, and the early or the late endosome (Ghosh, Dahms et al. 2003). Both lipids and proteins shuttle between the two pathways, and disruption of a pathway often has dire consequences for the integrity of the entire cell (Ghosh, Dahms et al. 2003; Gruenberg 2003). Clinically

manifested storage disorders, for example, may affect the shuttling of cholesterol between the degradative and biosynthetic pathways ultimately leading to both plasma membrane depletion of the lipid, and compromised protein sorting in the degradative pathway (Mukherjee and Maxfield 2004; Ganley and Pfeffer 2006). Importantly, newly synthesized proteins can traverse endosomal structures (Rodriguez-Boulan and Musch 2005). Newly synthesized asialoglycoprotein receptor H1, E-cadherin, and transferrin receptors traverse a tubular compartment after they have left the trans golgi network (Leitinger, Hille-Rehfeld et al. 1995; Laird and Spiess 2000; Lock and Stow 2005). This tubular structure later fuses to Rab11- and transferrin-positive recycling endosomes, after which the newly synthesized proteins reach the plasma membrane (Laird and Spiess 2000; Lock and Stow 2005). These interconnections may confuse investigations of intracelllular trafficking, and underscores the necessity to scrutinize between internalized and biosynthetic pathways. A mere fractionation or whole cell staining approach would not be able to discriminate between receptors in the endocytic and biosynthetic pathways.

In conclusion, exocytosis in the biosynthetic pathway is by no means a default process, and the intricate designs available to sort lipids and proteins to the right cellular localization, utilizes the same endosomal structures traversed by endocytic material.

Proteins involved in postendocytic organelle sorting, movement, and maintenance
Most knowledge of the intracellular protein sorting machineries stem from studies of yeast, and of epidermal growth factor receptor trafficking in mammalian cells. Almost all proteins involved in intracellular protein sorting in yeast have homologues in mammals. However, the complexity is far greater in the higher eukaryots, with higher number of members of each family of proteins, and additional sets of proteins not present in yeast. This reflects the growing need of specific protein regulation in higher eukaryots.

To review all proteins known to act in the intracellular sorting of the endocytic pathways is of course beyond the scope of this text. However, most proteins belong to a few well-defined groups that I will outline below in the general order in which they appear in the endocytic pathway. First, there is the Ras family of GTPases (Rab and Arf proteins) and their respective effectors including phosphoinositide kinases, tethering factors, vesicle fusion factors, motor proteins, and cargo recognition proteins among others. Second, there are the proteins involved in specific recognition of cargo destined for degradation based on recognition of ubiquitination. I will also include various other effector systems controlled by the members of this group. The third and last group is examples of scantily characterized proteins involved in sorting and pathway-connecting machineries such as the retromer complex.

Rab proteins
The Rab proteins constitute the largest subfamily of the Ras GTPase superfamily. These proteins have a significant role in establishing the function of intracellular endosomal organelles. They act as effector coordinators in cascades that quickly, and with high precision, guide vesicles and cargo to the right destination within the cell. There are more than sixty Rab family members found to date in mammals, 11 in yeast, most of them linked with highly specified membrane areas, and often with little or no overlap with other members of the family.

Rab activity, as all GTPase protein activity, is dictated by the actions of GAPs and GEFs. A Rab cascade is organized so that the effector of one Rab is the GEF of the next Rab in a specific endocytic pathway (Walch-Solimena, Collins et al. 1997; Ortiz, Medkova et al.

2002). Thus, a directed progression occurs through the pathway.

Rab proteins are modified posttranslationally by the addition of two geranylgeranyl lipid moieties to the protein, which are used for membrane association (Kinsella and Maltese 1991; Kinsella and Maltese 1992). In the inactive GDP-bound form, Rab proteins are bound by a GDP dissociation inhibitor (GDI), that blocks insertion of the lipid moieties into membranes, and thus retains the inactive Rab in the cytosol (Garrett, Kabcenell et al. 1993; Garrett, Zahner et al. 1994; Shapiro and Pfeffer 1995). A GDI dissociation factor (GDF) activates the Rab, allowing it to associate, by means of recruitment via GEFs, effectors, and modified lipids to the correct membrane (Pfeffer and Aivazian 2004; Aivazian, Serrano et al. 2006).

Rab proteins are active, via effectors, in altering membrane lipid composition, lend force to endosomal movement, connect cargo to the right vesicle, tethering vesicles to target membranes, and induce membrane fusion. For example, Rab5 attracts and activates PI3K to the membrane, resulting in lipid modifications (Christoforidis, Miaczynska et al. 1999). This is of importance for further effector recruitment, such as early endosomal antigen 1 (EEA1), to Rab5, but also for other machineries discussed below (Stenmark, Aasland et al. 1996; Christoforidis, McBride et al. 1999).

Rab proteins can also bind directly or indirectly to motor proteins such as myosins that run along actin cytoskeletal tracks, or dynein and kinesin, that run in opposite directions respectively, on microtubules (reviewed in (Mallik and Gross 2004)).

Rab9 acts as as a cargo selector by binding to the protein tail-interacting protein of 47kDa, which, in turn, acts as an adaptor for the mannose-6-phosphate receptor (Carroll, Hanna et al. 2001). This allows the recycling of the mannose-6-phosphate receptor between the trans golgi network and late endosomes.

Several Rab proteins utilize tethering factors such as EEA1, Golgin, and exocyst as effectors. These effectors act, as the name implies, as guides in tethering the vesicle and target membranes in close proximity to each other. For example, Rab5 attracts EEA1, when aided by rabex-5 and N-ethylmaleimide-sensitive factor (NSF), to membranes of early endosomes, leading to homotypic tethering of these maturing vesicles (Christoforidis, McBride et al. 1999; McBride, Rybin et al. 1999). This allows for the activity of vesicular, and target membrane N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), to form a trans-SNARE complex, that can overcome the energy barrier necessary for membrane fusion to take place (Chen and Scheller 2001). The SNARE for Rab5- and EEA1-dependent homotypic fusion of early endosomes is syntaxin 13 (Christoforidis, McBride et al. 1999; McBride, Rybin et al. 1999).

Rab5 is located to budding, and uncoated vesicles, and early endosomes, whereas Rab4 overlap with Rab5 in early endosomes, and with Rab11 in recycling endosomes (Sonnichsen, De Renzis et al. 2000). Rab9, and Rab7 can be found in the MVB, late endosomes, and lysosomes, whereas Rab6 is mainly found in Golgi (Barbero, Bittova et al. 2002; Mallard, Tang et al. 2002) (Fig. 4).

ARF proteins
Another subfamily of Ras GTPase proteins includes the ADP-ribosylation factors (ARF). There are six mammalian ARF proteins divided up in three classes, ARF1, -2, and –3 make up class I, ARF4, and –5 make up class II, whereas ARF-6 makes up class III. Class I ARF proteins regulate coat assembly and lipid modifications in the exocytic pathway (Bonifacino and Glick 2004). Also, ARF1 regulate coat formation on endosomal membranes by recruiting various adaptor proteins (AP-1, -3, -4, and GGA proteins) (Kirchhausen 2000; Bonifacino 2004). Class II proteins have no known function as of yet. ARF-6 on the other hand is very important in a number of tasks concerning internalization, and postendocytic sorting (D'Souza-Schorey, Li et al. 1995; Peters, Hsu et al. 1995).

ARF proteins are just as other GTPases, regulated by GAP, and GEF proteins, (see review for examples (D'Souza-Schorey and Chavrier 2006)). ARF proteins go through a conformational change of their Switch regions when activated, just as Rab proteins, allowing the recruitment of effectors to the surface formed (Pasqualato, Menetrey et al. 2001). Structural motifs in the proteins, plus localization in the cell determine their specified cellular tasks (Peters, Hsu et al. 1995). Brefeldin, a fungal metabolite, specifically blocks the activity of ARF1-3 GEF proteins active in in golgi, thus providing a valuable tool to assess endocytic membrane trafficking while blocking the biosynthetic pathway (D'Souza-Schorey and Chavrier 2006).

As already discussed, ARF-6 controls the activity of internalization by activating PI5-kinase and phospholipase D. Activity of phospholipase D results in formation of phosphatidic acid, a precursor to the phosphorylated inositides necessary for recruitment necessary for a number of proteins to membranes. Further, phosphatidic acid catalyzes the activity of several enzymes involved in lipid modification and internalization, including PI5-kinase. Direct interaction of ARF-6 with Rac1 and partner of Rac1, and formed PI4,5P2 lead to remodeling of the actin cytoskeleton, necessary for endosomal movement and structural changes of membranes (Radhakrishna, Klausner et al. 1996; D'Souza-Schorey, Boshans et al. 1997; Schafer, D'Souza-Schorey et al. 2000; Santy, Ravichandran et al. 2005).

ARF-6 is further involved in targeting of internalized lipids and vesicular cargo to the recycling pathway, possibly by utilizing some of the same effectors as Rab11 (Aikawa and Martin 2003; Prigent, Dubois et al. 2003). ARF-6 can, for example, control the exocyst complex, and thereby tether recycling vesicles to the plasma membrane (Prigent, Dubois et al. 2003). The recycling pathway regulated by ARF-6 is dependent on phospholipase D activity (Jovanovic, Brown et al. 2006; Padron, Tall et al. 2006).

Unbiased screens for Arf-6 interacting proteins should, considering the noted importance of Arf-6 in GPCR internalization, and the numerous effects of this protein, especially in the recycling pathway, significantly aid in the elucidation of intracellular protein sorting machineries. Arf-6 is definitely an important node in the intracellular paths of proteins internalized through all known pathways.

Hepatocyte growth factor regulated tyrosine kinase (Hrs), the endosomal sorting complex required for transport (ESCRT), and MVB biogenesis
Hrs is a multidomain adaptorprotein that is active in protein sorting in the early endosome and multivesicular body. The yeast homologue of Hrs is called vps27p for vacuolar protein sorting 27 protein. As the name implies, vps proteins are involved in sorting of cargo to the vacuole, which is the yeast endosome corresponding to the mammalian lysosome. There

are more than 50 vps proteins known to date. Of those, 17 are essential for the sorting of material to and from the MVB in yeast (Katzmann, Odorizzi et al. 2002). Several lines of evidence show that monoubiquitination has a crucial role in targeting of membrane proteins to the MVB/vacuolar pathway. Hrs is one of a number of proteins that can recognize and interact with mono-ubiquitin conjugated proteins via the ubiquitin interacting motifs (UIM) (Raiborg and Stenmark 2002; Shih, Katzmann et al. 2002).

Hrs is localized to clathrin coated surfaces of the early endosome (Raiborg, Bache et al. 2002; Myromslien, Grovdal et al. 2006) (Fig. 4). At this location Hrs interacts with ubiquitinated cargo, PI3P via a FYVE (Fab1, YOTB, Vac1, EEA1) domain, and TSG101 (Raiborg, Bremnes et al. 2001; Bache, Brech et al. 2003; Katzmann, Stefan et al. 2003). TSG101 is part of the mammalian ESCRT complex I, a complex consisting of three subunits (Myromslien, Grovdal et al. 2006). This complex attracts two more ESCRT complexes, ESCRT complex II and –III (Katzmann, Odorizzi et al. 2002). Together, the ESCRT complexes can initiate the invagination of the early endosomal membrane, leading to the formation of intralumenal vesicles destined for lysosomal targeting and destruction (Katzmann, Odorizzi et al. 2002). The final step of the invagination process is dependent on the mVPS4 protein, that assembles into a multimere, and allow the dissasembly, and reutilization of the ESCRT complexes (Babst, Sato et al. 1997; Babst, Wendland et al. 1998). mVPS4 is also implied in ubiquitin –independent sorting to the MVB and lysosome (Reggiori and Pelham 2001). There are reports of additional proteins that aid in recognition of ubiquitinated cargo, and target this cargo to Hrs. An example is the TOM1 and TOM1 L1/L2 proteins, that bind to ubiquitinated internalized IL-1R, and aid in targeting this receptor to Hrs/TSG101 for lysosomal targeting (Brissoni, Agostini et al. 2006).

Hrs has numerous extra tasks in the endocytic pathway. For example, Hrs competes off VAMP2 from the early endosomal homotypic fusion complex (VAMP2-SNAP25-Syntaxin13) by binding to SNAP25, thus limiting the growth of the early endosome (Sun, Yan et al. 2003). Further, Hrs interacts with several other proteins implicated in membrane trafficking, such as eps15 (which also contain UIM domains), and sorting nexin-1 (SNX-1) ((Sun, Yan et al. 2003) and references therein). Hrs can, via SNX-1 interact with the retromer complex (see below), and the cytoskeleton associated recycling or transport complex, both of which support cargo recycling (Myromslien, Grovdal et al. 2006). Hrs acts independently of the ESCRT complexes when cooperating in conjunction with these two systems. SNX-1 has also been implied in EGFR degradation, possibly aided by Hrs (Seaman 2005).

A recurring theme in most of these proteins is their ability to interact with modified, phosphorylated lipids via FYVE and Phox homology (PX) domains. There are more than 70 known proteins with these kinds of domains today, including the family of sortin nexins, and several members of the ESCRT complex proteins (Gruenberg and Stenmark 2004). Many of these proteins are involved in membrane trafficking regulation, and not least in sorting into the intraluminal vesicles of the MVB. Rab5 recruits pI3-kinase to the early endosome; see above (and Fig. 4). Block of PI3-kinase activity by inhibitors like wortmannin, or block of modified lipids (PI3P) by overexpression of tandem repeated FYVE domains, block sorting into the MVB, and further sorting to the lysosome of for example the ubiquitinated EGFR (Fernandez-Borja, Wubbolts et al. 1999; Futter, Collinson et al. 2001; Petiot, Faure et al. 2003).

Production of intralumenal vesicles also seem coupled to formations of coats, both cloth-

rin, and non-clathrin, possibly coatomer protein coats, on the early endosome (Whitney, Gomez et al. 1995; Aniento, Gu et al. 1996; Daro, Sheff et al. 1997; Gu, Aniento et al. 1997) (Fig. 4). Block of coatomer proteins and ARF-1 alter the structure and block the function of early endosomes, indicating the importance of these coats in endosomal integrity (Gu and Gruenberg 2000).

A recently discovered step in the biogenesis of the MVB is the formation of a cholesterol platform through the action of the cholesterol-interacting protein Annexin II (Emans, Gorvel et al. 1993; Harder, Kellner et al. 1997; Mayran, Parton et al. 2003). Several Annexin II are thought to form a form of coat that can interact with the actin cytoskeleton (Gruenberg and Stenmark 2004). Block of Annexin II results in block of MVB formation, indicating that Annexin II is a key ingredient in the formation of this endosomal structure (Mayran, Parton et al. 2003). Annexin II has also been implicated in the regulation of recycling endosome location and structure (Zobiack, Rescher et al. 2003).

Numerous additional interaction partners for the Hrs, Annexin proteins, and the components of the ESCRT complexes, that aid in their function, or specify cargo that utilize these proteins for sorting, are likely to be found in future studies.

The Retromer complex and unclassified sorting proteins
The retromer complex is a pentameric protein complex active in retrieving proteins, most often involved in transport of enzymes to the degradative pathway, from the early endosome, or the maturing MVB, to the golgi network. The complex consists of mammalian VPS35p and mVPS35p that is induced by mVPS29p to bind to cargo on the EE/MVB membrane. MVPS29p binding to mVPS35p links mVPS35p to a sortin nexin dimer, probably homodimeric SNX1 or heterodimeric SNX1/SNX2. Establishing the link between mVPS29p and mVPS35p is facilitated by mVPS26p (Haft, de la Luz Sierra et al. 2000). The SNX proteins are thought to deform the membrane via their Bin/Amphiphysin/Rvs (BAR) domain, forming transport tubules that can recycle material from the forming late endosome (Zhong, Lazar et al. 2002). MVPS26p aid in membrane localization of the complex by promoting mVPS35p binding to the membrane (Seaman, McCaffery et al. 1998; Reddy and Seaman 2001).

Sorting nexins are also involved in several additional membrane trafficking feats, such as endosomal movements on microtubules (SNX23). SNX13 acts as an RGS to Gαs, and Gαs, in turn, can stimulate SNX13 directed downregulation of EGFR. SNX9 associate with the plasma membrane and catalyzes clathrin-mediated internalization. These and other functions are reviewed excellently in (Worby and Dixon 2002; Carlton, Bujny et al. 2005).

A number of PDZ domain proteins, with various functions, may also be involved in intracellular trafficking of membranebound cargo. GIPC/synectin acts as a PDZ-mediated link between cargo, in uncoated internalized vesicles, and the myosin VI motor protein, thus linking the endosome to transport on the actin cytoskeleton (Naccache, Hasson et al. 2006).

Summary of proteins in trafficking
A growing number of proteins that govern the intracellular sorting of internalized cargo are being discovered. Several of these classes of proteins are most likely to take part in intracellular sorting of GPCR. Future studies ought to take advantage of the solid knowledge acquired from other sorting models, when trying to elucidate the machinery responsible for GPCR trafficking.

Predestined intracellular fate of cargo
It was noted relatively early that cargo may enter cells through more than one type of clathrin-coated pit. β2-AR, for example, can enter the cell both through clathrin-coated pits (ccp, Fig. 3 and 4) that contain Tfn, and those that do not (Cao, Mays et al. 1998). The separation of cargo prior to internalization was very recently explored for a few compunds involved in cell metabolism and growth (Tfn, EGF and LDL). The result was the identification of two separate early endosomal (EE, Fig. 4) populations (Lakadamyali, Rust et al. 2006). The most frequently occuring EE (65%) of the early endosomes (EE) is called static EE (SEE) since it shows very little movement and a slow maturation towards forming an MVB. The second population is called a dynamic EE (DEE), which, as the name implies, is a rapidly moving organelle that quickly (<1min) matures into a late endosome. The DEE formation and movement is dependent on microtubules making the pathway nocodazole sensitive. Transferrin receptors that endocytose through an AP-2-mediated clathrin-dependent mechanism target both populations indiscriminately, and is also able to recycle through both. Cargo destined for degradation such as EGF and LDL, on the other hand, tend to favor the DEE over the SEE to a great extent thus facilitating the rapid sorting of these cargos to the degrading compartments.

Interestingly, EGF and LDL are endocytosed through mechanisms with alternative adaptor proteins containing E/ANTH domains. These domains interact with tubulin and the formation and movement of the primary uncoated vesicle and the DEE is nocodazole sensitive.

Another interesting feature of the clathrin-coated pit populations is that the loading of cargo in the specific classes of pits is saturable. The only way to exceed the maximum cargo load is to lower the temperature during loading and then raise temperature back to physiological levels. This seems to result in a conversion of ccps destined for SEE to be converted into ccps destined for DEE.

Proteolysis by proteasomal targeting
Polyubiquitinylation, meaning the conjugation of more than four units of 78 amino acid units of ubiquitin to a lysine residue in a protein, is a crucial step in targeting a protein for destruction by proteasomal degradation (see review (Roos-Mattjus and Sistonen 2004)). Ubiquitin is conjugated to proteins via the action of three types of enzymes, E1 (one type that can form two splice variants in mammals), E2 (more than 25 types in mammals), and E3 (a very diverse set of genes divided in two groups, RING and HECT, based on structure and function).

E1 activates ubiquitin in an ATP-consuming reaction, and transfers the activated ubiquitin to E2, which is the conjugation enzyme. E3 is the, so called, ligase enzyme that recognizes both E2 loaded with ubiquitin, and the target protein. The exact target motif recognized, in the protein, is in most cases not known. The fact that the E3 enzyme family is so diverse most likely reflects the diversity of these targets. A number of deubiquitinylation proteins also exist (DUBs, also called isopeptidases) that are able to cleave off conjugated ubiquitin, via cysteine protease activity, at the amide bond after the last amino acid in ubiquitin. The 26S proteasome is a protein megacomplex present in the nucleus and cytoplasm, consisting of a dimer of at least 32 separate subunits each, and a staggering molecular weight of about 2.5MDa. Importantly, transmembranal proteins can, during their passage through the endoplasmic reticulum, be targeted to the proteasome. This process is carried out by a poorly understood machinery called ERAD (Endoplasmic Reticulum Associated Degradation), and can recognize misfolded proteins, unthread them from the

membrane, into the cytosol. In the cytosol, ERAD catalyzes the polyubiquitinylation of the unthreaded proteins, thus targeting them for proteasomal destruction. Whether this process can occur at other cellular membranes is not known.

GPCR Sorting Through the Endosomal Compartments
The length of agonist stimulation is a very important factor when dissecting the intracellular sorting of endocytosed GPCR. Most GPCR are downregulated, by proteolysis, in response to the agonist if the agonist is delivered at a sufficient concentration during a sufficiently long period. This fact not only hints to the important role of cellular adaptation in receptor signaling and regulation but also indicate that most receptors can adopt more than one intracellular fate. Theoretically, such dual fates could occur in response to short stimuli given the right conditions. The many receptor modifications that take place in response to ligand binding have the potential to drastically alter the repetoir of proteins engaged by the receptor and result in several alternative sorting routes.

As mentioned briefly before some receptors internalize quite rapidly in the absence of ligand binding. The role of constitutive internalization is still a matter of debate. It has been assumed in the past that constitutive endocytosis leads to rapid recycling of the receptor to the surface. However, this has recently been challenged, not least by our own studies on the bradykinin B1 receptor (see Study III). Therefore, constitutive endocytosis must be viewed as an individual property of each receptor rather than a default process.

GPCR recycling has thus far been reported to depend on dephosphorylation of the internalized receptor, receptor motifs like type I or II PDZ ligand sequences or internal PDZ ligand motifs, and interaction with proteins such as NHERF/EBP50, NSF, and the elongation factors eEF1A1 and eEF1A2. Degradation, on the other hand, has been attributed to ubiquitination (of receptor or an adaptor such as β-arrestin), tyrosine-based motifs (e.g. YXXϕ), and interaction with a number of proteins such as GPCR associated sorting protein (GASP, see Study I), SNX1, Hrs, and Vps4 (reviewed in (Trejo 2005)).

In order to simplfy the presentation of alternative intracellular GPCR sorting routes, I will next examplify a few receptor model systems.

Postendocytic Sorting of the Adrenergic Receptors
β2-AR is as mentioned internalized through a GRK, β-arrestin 2, Arf-6, NSF, AP-2, and clathrin-dependent mechanism in response to agonist activation. After internalization this receptor is rapidly delivered to vesicles that display markers of early endosomes and comigrates with recycling markers such as transferrin back to the plasma membrane (von Zastrow and Kobilka 1992). Recycling of the receptor is sensitive to monensin and is dependent on the interaction with a carboxyterminal tail binding protein named NHERF/EBP50 through a PDZ binding domain and a PDZ type I ligand in the receptor tail (Cao, Deacon et al. 1999; Liang, Curran et al. 2004). Ubiquitination of the receptor or phosphorylation within the SLL PDZ ligand motif results in a rerouting of the receptor to the degradative pathway, thus allowing the receptor a dual postendocytic fate (Cao, Deacon et al. 1999). In support of this view, it has been shown that the receptor, in addition to interacting with recycling mediating proteins such as NHERF/EBP50 and NSF, also interact with proteins that determine postendocytic degradation such as GASP and SNX1 (Whistler, Enquist et al. 2002; Heydorn, Sondergaard et al. 2004).

β1-AR is internalized via a β-arrestin- and clathrin-dependent pathway when phosphory-

lated by GRK and through lipid rafts when phosphorylated by PKA. β1-AR is not as readily internalized as the β2-AR, and ends up in morphologically distinct vesicles close to the plasma membrane (Liang, Curran et al. 2004). This receptor recycles through a monensin-insensitive pathway back to the plasma membrane (Liang, Curran et al. 2004).

α2B-AR is a clathrin-dependent internalizing receptor in response to agonist whereas the α2A-AR endocytose through both lipid raft and clathrin-dependent mechanisms (Olli-Lahdesmaki, Scheinin et al. 2003). α2B-AR recycling is in contrast to α2A-AR sensitive to brefeldin A (Olli-Lahdesmaki, Scheinin et al. 2003). Brefeldin A is an antibiotic that terminates transport of receptors from the trans golgi network to the plasma membrane via perturbation of ARF function, indicating that this organelle is involved in postendocytic sorting and recycling of the α2B-AR (D'Souza-Schorey and Chavrier 2006). The α2B-AR halflife has been reported to decline sharply after agonist exposure, indicating that this receptor is targeted to lysosomes to a large degree after agonist induced internalization (Heck and Bylund 1997). The degree of internalization of the α2A-AR varies radically depending on animal species, type of cell, and type of ligand. For example, the drug dexmedetomidine radically increases phosphorylation and in turn internalization of the α2A-AR resulting in a more rapid and robust resensitization (Olli-Lahdesmaki, Tiger et al. 2004). On the other hand, in murine cells or in cells with low arrestin expression, internalization is considerably lower, which results in decreased resensitization (Olli-Lahdesmaki, Tiger et al. 2004).

Postendocytic Sorting of the PAR-1 Receptor is SNX1 Dependent
The thrombin receptor or protease-activated receptor 1 is irreversibly activated by the proteolytic cleavage of its N-terminal region by the protease thrombin (Ramachandran, Klufas et al. 1997). The fact that the receptor is irreversibly activated makes it imperative to maintain rapid desensitization, internalization and intracellular trafficking. β-arrestin seems to be involved in the rapid desensitization but not the internalization of PAR-1 (Paing, Stutts et al. 2002). On the other hand, phosphorylation of a classical tyrosine-based motif in the carboxy-terminal receptor tail seems crucial for internalization (Paing, Temple et al. 2004). Once internalized, PAR-1 is targeted for degradation in lysosomes (Wang, Zhou et al. 2002; Gullapalli, Wolfe et al. 2006). This degradative fate can be blocked by overexpression of a dominant-negative version of SNX1 indicating that this protein, or possibly the heterodimeric partner of SNX1/SNX2, is crucial for the sorting of this receptor (Wang, Zhou et al. 2002; Gullapalli, Wolfe et al. 2006). It has also been shown that PAR-1 is able to interact with the GASP protein (Heydorn, Sondergaard et al. 2004).

Postendocytic Sorting of the Adenosine Receptor
The adenosine A1R requires the adenosine deaminase (ADA) surface protein both for activation and internalization (Escriche, Burgueno et al. 2003). When activated in the presence of ADA, the A1R is internalized through caveolae, as assessed by morphology, co-immuno-precipitation of A1R with caveolin 1, and transmission electron microscopy colocalization with caveolin 1 (Escriche, Burgueno et al. 2003). A1R and ADA are separated after internalization and recycle back to the plasma membrane independently of each other (Escriche, Burgueno et al. 2003). The intracellular organelles that A1R are morphologically classified as a caveosome, an endosome for caveolae . The recycling of the receptor is sensitive to NEM, an alkylating agent that inhibits the fusion of vesicles to target membranes (Escriche, Burgueno et al. 2003).

Dual Fate Postendocytic Sorting of the CCK Receptor Along two Separate Paths
Agonist-stimulated CCKR internalizes both through clathrin-dependent and -indepen-

dent pathways. Clathrin-mediated endocytosis results in a translocation of the receptor to a cluster of vesicles in the perinuclear space from which it does not recycle (Roettger, Rentsch et al. 1995). In contrast, the smooth uncoated vesicles formed in addition to the clathrin dependent ones remain in close proximity to the plasma membrane and allow rapid recycling of the CCKR (Roettger, Rentsch et al. 1995).

Slow Recycling of the GLP-2R
The glucagon-like peptide 2 receptor GLP-2R is internalized through a clathrin-independent mechanism and colocalizes transiently with caveolin-1 during internalization (Estall, Yusta et al. 2004). The postendocytic separation of GLP-2R and caveolin-1 is complete within one hour of internalization (Estall, Yusta et al. 2004). Internalized receptors are then routed to the perinuclear space from which it is recycled but at a very slow rate (Estall, Yusta et al. 2004). The rate of recycling varies between cell lines and ranges from two to four hours. A block of GLP-2R internalization with filipin enhances desensitization, and block of recycling of the receptor by monensin blocks resensitization and enhances desensitization (Estall, Yusta et al. 2004). These effects underscore the role of receptor membrane trafficking in receptor activity control.

Recycling of the muscarinic acetylcholine M4 receptor
It was, surprisingly enough, recently shown that the muscarinic acetylcholine receptor M4, (mACh4R), which is rapidly internalized in response to agonist, recycling, is modulated by elongation factors, eEF1A1 and eEF1A2 (McClatchy, Fang et al. 2006). Overexpression of eEF1A1 resulted in a significantly slowed recycling of the receptor that in turn lowered the steady state number of receptor on the plasma membrane after agonist-induced internalization of the receptor. The same study showed that recycling of the mACh4R was dependent on myosin Vb, indicating a role for actin in movements of recycling vesicles.

Implications of the Current Knowledge of GPCR postendocytic Sorting
The lack of cohesive rules for how receptors are sorted after internalization, and the low number of interaction partners found so far that regulate these postendocytic movements, have implications for the future research of this huge family of receptors. First, generalizations from a few receptor model systems cannot explain the intricate regulation of each individual receptor. Second, the results from investigating a receptor may vary between different ligands, species, and even between different cells within the same species making it imperative to allow the characterization of several ligands and models. Third, ethical reasons argue for studies initially in heterologous systems in various cellular background to minimize the number of animals used. Fourth, the system of intracellular compartments handling the sorting of the receptor is highly dynamic and can only be reliably studied if treated as such – calling for methods with high temporal resolution. Fifth, receptor activity needs to be assessed since surface receptor numbers per se is not a measurement of proper receptor activity.

The complex regulatory machinery operating in concert with each receptor is most likely best studied through a combination of approaches. Notably, unbiased approaches to elucidate interaction partners are still called for. Historically, this has been approached by yeast two- or three-hybrid screens. These are extremely tedious investigations, whereas the great improvement in masspectrometry and maldi-tof in combination with GST-fusion proteins of receptor domains is a very attractive alternative.

Receptor Model Systems – Pharmacology and Physiology

Three Families of GPCR

I have been working on elucidating the role of membrane trafficking in GPCR regulation in three separate receptor models. All three model systems are rhodopsin family receptors with little sequence homology but many structural similarities. My initial work was carried out on the opioid receptors MOR and DOR and was executed in an in vitro heterologous HEK293 model system or in vitro cell-free experimental setups. The second project was carried out using the dopamine D1 and D2 receptors. This project was carried out through a combination of in vitro and ex vivo experiments. The third project presented in my third manuscript and the added supplementary data was based on the bradykinin B1 and B2 receptors. This project was carried out in an in vitro heterologous cell model, in an in vitro cell model with endogenous receptor expression, and in a primary ex vivo culture of endogenous receptor expressing cells. Below is a short introduction to each receptor model system.

Opioid Receptors

There are four receptors in the opioid receptor family: mu receptor (MOR), delta receptor (DOR), kappa receptor (KOR) and nociceptin receptor (Waldhoer, Bartlett et al. 2004). Each of these receptors except the nociceptin receptor have been reported to have splice variants. Further, several reports now show modulatory effects of opioid receptor hetero- and homo-oligomerization giving rise to a plethora of pharmacological species (Jordan and Devi 1999; Gomes, Jordan et al. 2000; Pascal and Milligan 2005; Waldhoer, Fong et al. 2005; Gupta, Decaillot et al. 2006).

These receptors are expressed both in peripheral tissues, immune cells and the central nervous system (Mansour, Fox et al. 1995; Quock, Burkey et al. 1999; Sharp 2006). They all preferentially signal through the Gi pathway but are known to switch coupling to Gs and Gz under special circumstances. Endogenous opioid ligands are formed through metabolism of precursor proteins. Proenkephalin, proopiomelanocortin, and prodynorphin form enkephalin, beta-endorphine, and dynorphin, respectively (Raynor, Kong et al. 1994). Enkephalin and beta-endorphines stimulate MOR and DOR whereas dynorphin activates KOR.

MOR, DOR, and KOR are found in high abundance in several locations in the brain, including striatum, hypothalamus, periaqueductal grey, locus ceruleus, and the ventral tegmental area (Margolis, Hjelmstad et al. 2005). This area recieves input from several other brain regions and in turn elicit a response through dopaminergic projection neurons to ventral striatum and medial prefrontal cortex (Margolis, Hjelmstad et al. 2005). These in turn are areas involved in reward-induced behavior and attention/working memory, respectively (Taha and Fields 2005; German and Fields 2006; Taha and Fields 2006). MOR activity leads to euphoria and reinforced behavior, whereas KOR activity results in dysphoria and termination of conditioned behavior or in aversive effects (German and Fields 2006). At least some of the opposing effects of these two receptors are due to their opposing influnce on the VTA circuits (Margolis, Hjelmstad et al. 2003; Margolis, Hjelmstad et al. 2005).

MOR and DOR are known to have a negative modulatory role in nociceptive signaling, whereas the nociceptin and KOR receptors contribute both to nociceptive signals and analgesic effects depending on site of action. The analgesic effect of opioid receptors

is exerted via several separate direct and indirect systems that modulate nociceptive signaling. MOR, DOR, and KOR are present on peripheral free nerve endings, dorsal root ganglion cell bodies, presynaptic dorsal horn nociceptive afferents, and postsynaptic projection neurons on the spinal cord level (Trafton, Abbadie et al. 1999; Stein, Machelska et al. 2001). MOR and DOR are also present on GABA-ergic neurons regulating the signaling tonus of the periaqueductal gray and on the locus ceruleus noradrenergic projection neurons, whereas KOR can be found on neurons in the rostro-ventral medulla (Tershner, Mitchell et al. 2000; Meng, Johansen et al. 2005). When activated, the periaqueductal gray in turn activates serotonergic projection neurons of the raphe nuclei in the brain stem. Locus ceruleus and Raphe nuclei projection neurons in turn activate enkephalic interneurons at the level of the dorsal horn in the spinal cord leading to release of enkephalin and activation of pre- and postsynaptic opioid receptors that in turn attenuates signaling through the nociceptive tracts. Activation of KOR in rostro-ventral medulla blocks analgesia elicited by morphine when injected into the periaqueductal grey (Meng, Johansen et al. 2005). There are indications that opiate receptor activity can elicit different physiological effects depending on gender. For example, rostro-ventral medullary activity of KOR in females support morphine analgesia rather than block it (Tershner, Mitchell et al. 2000), and male pain threshhold and responsiveness to morphine is higher than in females (Mitrovic, Margeta-Mitrovic et al. 2003). In the periphery, stress can induce release of opiate ligands from white blood cells inducing a peripheral analgesic effect through the opiate receptors on free nerve endings. Peripheral release of opioids can also reduce inflammation (Stein, Machelska et al. 2001).

Much like the bradykinin B1R, DOR has been reported not to be expressed on the plasma membrane under naïve conditions. Already starting at DOR maturation, Petaja-Repo and collegues has reported that most DOR do not reach full maturity in a HEK293 model system (as assessed by complexity of glycosylation and plasma membrane levels compared to total cell level of DOR) and that the vast majority of newly synthesized DOR are instead targeted for proteasomal destruction directly from the ER via the ERAD system (Petaja-Repo, Hogue et al. 2001). This is very much in agreement with the maturation profile of the B1R (Fortin, Dziadulewicz et al. 2006). Further, stimulation of PC12 cells with nerve growth factor leading to differentiation of the cell line results in a redistribution of DOR from the plasma membrane to an intracellular receptor reserve pool (Kim and von Zastrow 2003). Numerous reports place the DOR in intracellular structures such as neuropeptide granules in sensory fiber nerve endings in naïve tissues whereas several stimuli such as nociceptive signaling, morphine treatment, inflammation and bradykinin signaling results in redistribution of the DOR to the plasma membrane thus enhancing tissue responsiveness to DOR elicited analgesia (Cheng, Liu-Chen et al. 1997; Cahill, Morinville et al. 2001; Wang and Pickel 2001; Cahill, Morinville et al. 2003; Morinville, Cahill et al. 2003; Morinville, Cahill et al. 2004; Guan, Xu et al. 2005; Julius and Basbaum 2005; Patwardhan, Berg et al. 2005). Heterologous expression systems that lack necessary trafficking machinery for intracellular storage will miss this added layer of complexity in receptor trafficking. Considering the similarities in maturational problems reported by us and others for the B1R and DOR and the similar distribution and time of expression seen for these two receptors (see below for B1R induction of expression), we find it pertinent that future studies address the localization and maturation of the B1R in neuronal tissue.

Both DOR and MOR uncoupling and desensitization after short agonist activation proceed via the classical Grk phosphorylation and β-arrestin recruitment (Zhang, Ferguson et al. 1998; Zhang, Ferguson et al. 1999; Lowe, Celver et al. 2002), whereas desensitization

Effector	DOR	MOR
G protein	$G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{z}$, $G\alpha_{15/16}$	$G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{z}$, $G\alpha_{11}$, $G\alpha_{15/16}$
Adenylate cyclase	AC A	C
Phospholipase	PLCβ, PLA_2 P	LCβ, PLA_2, PLD
Protein kinase	PKC, PI3K, Erk	PKC, PI3K, Erk
Ionchannel K	IR, GIRK, L-type Ca^{2+}	KIR, GIRK, L-type Ca^{2+}
Transciption factor	CREB, Sap, c-Myc, TALI, RNA pol II, Stat, Elk-1, ER	CREB, Sap, c-Myc, TALI, RNA pol II, Stat, Elk-1, ER
Other enzymes	rsk1,2, S6 kinase, TH, PP2c	rsk1,2, S6 kinase, TH, PP2c

after chronic stimuli may include several other forms of phosphorylation. However, the two receptors differ in their ability to rapidly desensitize in two aspects. First, MOR uncoupling has been suggested to be dependent on phosphorylation of a threonine residue in the second intracellular loop whereas DOR uncoupling depends on phosphorylation of several residues in both the second and fourth intracellular domains (Celver, Lowe et al. 2001; Lowe, Celver et al. 2002). Second, MOR has a weaker ability two recruit β-arrestin after activation and phosphorylation of the receptor, and this seem to be caused by a lower ability of MOR to activate β-arrestin (Lowe, Celver et al. 2002). Uncoupling and desensitization, at least of DOR, can be avoided by activation of ERK possibly by a block of arrestin recruitment (Eisinger, Ammer et al. 2002). KOR and the nociceptin receptor are less well characterized than MOR and DOR but seem to be Grk phosphorylated and recruit β-arrestin in response to acute agonist activation.

Rapid internalization of both DOR and MOR has been reported to proceed via a Grk- and β-arrestin-dependent mechanism (Whistler and von Zastrow 1998; Whistler, Chuang et al. 1999; Whistler, Tsao et al. 2001). Internalization seems to occur even after a brief pulse of agonist exposure and seems to be independent of signaling (Remmers, Clark et al. 1998; Zaki, Keith et al. 2001). DOR has been suggested to have multiple modes of internalization both with and without agonist. One study shows that mouse DOR lacking its entire carboxy-terminal domain is able to internalize when stimulated with agonist (Whistler, Tsao et al. 2001). Further, alanine substitutions of all carboxy-terminal phosphorylation sites rendered the receptor unable to internalize in response to agonist, and this loss of function could be rescued by mutating the alanines into aspartates thereby mimicking constant phosphorylation. The phosphorylation-deficient carboxy-terminal domain was, unlike the normal receptor tail, unable to recruit β-arrestin 2 in response to agonist. Together, this implies that the DOR tail contains regulatory elements that act as an internalization-brake until inactivated through phosphorylation and recruitment of β-arrestin 2. This point is strengthened by several studies reporting that the constitutive endocytosis of both DOR

and MOR is enhanced when the receptors are expressed in truncated forms lacking the carboxyterminal tail (Trapaidze, Keith et al. 1996; Murray, Evans et al. 1998).

Recently, these data have been disputed in a study that claims that mutant phosphorylation-deficient mouse DOR can recruit both β-arrestin 1 and 2 and conclude that arrestin binding, but not Grk2 phosphorylation, is required for DOR internalization (Zhang, Wang et al. 2005). However, these authors neither eliminate all phosphorylation sites in the carboxyterminal of the DOR nor prove that the mutant receptor does not get phosphorylated. Thus, it is possible that the retained internalization and arrestin recruitment by the mutant receptor is due to a reduced but not abolished phosphorylation of the carboxyterminal domain. Nevertheless its interesting to learn that DOR can recruit both forms of arrestin and that the receptor-arrestin interaction is crucial for receptor internalization.

MOR internalization has been reported to be dependent on Grk phosphorylation of a couple of carboxyterminal threonines (Celver, Xu et al. 2004). Further, these authors report that an acidic cluster proximal to these threonines is necessary for the phosphorylation to occur. A variant of MOR with all carboxyterminal threonines and serines replaced by alanines has also been shown to be unable to internalize (Finn and Whistler 2001).

Further, morphine and etorphine, two opioid receptor ligands that are structurally very similar differ in that morphine unlike etorphine is unable to promote both adequate phosphorylation and β-arrestin recruitment for both DOR and MOR (Keith, Murray et al. 1996; Keith, Anton et al. 1998; Whistler and von Zastrow 1998; Zhang, Ferguson et al. 1999). This phenotype can be rescued for the MOR but not the DOR by overexpression of Grk 2 and/or β-arrestin (Whistler and von Zastrow 1998; Zhang, Ferguson et al. 1999). Thus, small differences in agonist structure can have profound effects on desensitization and internalization of these two receptors because of selective recruitment and activation of receptor regulating machineries. This is further supported by the finding that PLD2 is associated with MOR, and that MOR activity in response to agonist leads to increased PLD2 activity and enhanced MOR internalization (Koch, Brandenburg et al. 2004; Koch, Wu et al. 2006). This effect of PLD2 was further seen for both DOR and cannabinoid receptor 1 (CB1) (Koch, Wu et al. 2006). However, morphine stimulation of MOR failed to stimulate PLD2 activity and MOR internalization (Koch, Wu et al. 2006). Yet another protein that has been implicated in MOR internalization is synaptophysin, a protein involved in dynamin recruitment (Liang, Wu et al. 2006). Overexpression of a truncated form of synaptophysin lacking the domain responsible for the recruitment of dynamin to the activated receptor resulted in an attenuated MOR internalization indicative of the importance of both these proteins in MOR internalization.

A growing number of reports indicate that GPCR when activated transiently associate with lipid rafts, this has also been shown in vitro for the DOR (Lamb, Zhang et al. 2002; Alves, Salamon et al. 2005; Xu, Yoon et al. 2006). The authors of this report show that this transient localization only occurs in response to agonist activation and they therefore argue that the altered receptor conformation achieved favor the partitioning of the receptor into the lipid raft fraction of the membrane. As described earlier, lipid rafts are relatively bulkier and tend to attract proteins involved in GPCR signal transduction. It is therefore attractive to speculate that a transient shift into lipid rafts might support GPCR signaling, and this might in turn explain why so many GPCR have been shown to associate with these structures. At the same time, this might explain the confusion regarding the mechanism of internalization of activated GPCR. Simply put, the great number of GPCR that transiently

pass through the lipid raft membrane compartment in order to signal properly, followed by internalization via the clathrin route, could easily be mistaken for receptors that do internalize via non-clathrin pathways. Several studies both in vitro and in vivo clearly show that DOR, regardless of the mode of phosphorylation, internalise via the clathrin-dependent pathway (Keith, Murray et al. 1996; Xiang, Yu et al. 2001; Lee, Cahill et al. 2002; Marie, Lecoq et al. 2003).

The postendocytic trafficking routes of the opioid receptors are as well studied as the internalization aspects of these receptors. The least well-studied receptors are again the nociceptin and KOR. Our own investigations indicate that KOR is poorly internalized in heterologous systems (Jennifer Whistler unpublished data). However, it has been reported that KOR recycling in CHO cells is facilitated by EBP50/NHERF, and that the interaction is governed by a PDZ ligand domain in the most distal part of the carboxyterminal tail of the KOR (Li, Chen et al. 2002). Several studies, including our study I shows that DOR and MOR are differentially sorted after agonist activation leading to targeting to lysosomes for destruction and recycling to the plasma membrane, respectively (Dingledine, Valentino et al. 1983; Tsao and von Zastrow 2000; Finn and Whistler 2001; Whistler, Tsao et al. 2001; Marie, Lecoq et al. 2003). It has also been established that this postendocytic degrading fate of DOR occurs even after only one brief stimulation with agonist and thus does not correspond to the general downregulation seen with many receptors in response to chronic stimulation (Tsao and von Zastrow 2000). Some of the human homologues of the yeast vacuolar sorting proteins discussed earlier may be involved in DOR postendocytic although ubiquitination is unnecessary for this sorting to occur (Tanowitz and Von Zastrow 2002; Hislop, Marley et al. 2004). Also, a di-leucine motif in the second intracellular loop of DOR may influence the rate of sorting along the degrading pathway (Wang, Loh et al. 2003). Further, recycling of MOR has recently been shown to be actively regulated via part of the carboxyterminal domain rather than an unregulated process driven by membrane bulk flow (Tanowitz and von Zastrow 2003). This regulated recycling of MOR may be executed by Hrs (Hanyaloglu, McCullagh et al. 2005).

Functionally, the postendocytic fate of a receptor will, as discussed earlier, determine the future responsiveness of the cellular circuit to subsequent rounds of agonist challenge. This was elegantly shown to be true for MOR both in vitro by cAMP measurements in a HEK293 cell model system, and ex vivo by patch clamp in rat locus ceruleus neurons (Finn and Whistler 2001; Alvarez, Arttamangkul et al. 2002). In contrast, DOR fail to resensitize after agonist activation, and this failure can be transplanted to the MOR via carboxyterminal transplantation (Finn and Whistler 2001).

Elucidating the machinery regulating the postendocytic sorting of the DOR is of great importance because of the potential clinical use of DOR agonists as analgetic agents, and the limited halflife of the receptor after agonist stimulation reported. Based on our knowledge at the time, we therefore designed study I in order to elucidate the role of the DOR carboxyterminal tail in shunting the internalized receptor to the degrading, lysosomal pathway.

Dopamine Receptors

The dopamine receptors are part of the catcholamine receptor group. They are usually divided up in two groups, the D1R-like (D1R and D5R), and the D2R-like (D2R, D3R and D4R) receptors. D1R-like receptors lack introns in their genes, and code for GPCR that have a short third intracellular loop and a long carboxyterminal domain. In contrast, D2R-like receptors all have introns and code for GPCR that have a very long third intracellular loop and a short carboxyterminal domain. In catecholamine receptors, this latter receptor structure is a telltale sign of Gi coupling. Additional splice variants exist of the D2R and the D3R that either include (D2Rlong, D3Rlong) or exclude (D2Rshort, D3Rshort) a long third intracellular loop. D4R has considerable polymorphism in humans based again on a variation in the length of the third intracellular loop. This polymorphism has some effects on the pharmacological profile of D4R, but the physiological effects of these variations are not understood. It has been proposed that the length of the third intracellular loop affects coupling efficiency. D5R also have a number of pseudogenes in different locations in the genome but only one of the copies is functional. D1R-like receptors couple to Gs and stimulate adenylate cyclase, while D2R and D4R have been shown to decrease levels of cAMP via Gi while the main D3R signaling pathway is still unclear. D2R-like receptors further affect the MAP kinase cascade, Ca2+ channels (D2R and D3R), and stimulate arachidonic acid release.

Pharmacologically, all members of the family can be stimulated by dopamine, but several ligands specific for each subgroup, and in some cases even for specific receptors, do exist. For example, benzazepines (SCH23390 and SKF83566) selectively antagonises D1R-like receptors, whereas butyrophenones (eg haloperidol) and substituted benzamides (eg sulpiride) antagonizes D2R-like receptors. Further raclopride and clozapine show selective antagonism at D2R/D3R and D4R, respectively. Clozapine, when administered to handle psychosis in schizophrenia, is used at a subsaturating concentration for the D2R but saturating for the D4R, which has led some to believe that this receptor is central in the ethiology of this disease.

All dopamine receptors can be found in the central nervous system, whereas members of each group, but so far not all individual receptors, can be found in peripheral tissues such as retina, kidney, and heart. In the CNS, substantia nigra, nucleus ruber, and the ventral tegmental area are the main areas for dopaminergic projection neurons. These areas project rostrally to thalamus, striatum, amygdala, pyriform cortex, hippocampus, anterior cingulate cortex, and prefrontal cortex, all of which have expression of dopaminergic receptors. They also project caudally to the lateral parabrachial nucleus and to locus ceruleus. The receptors modulate a vast number of physiological processes such as movement via regulation of the basal ganglia, mood via effects on the limbic system and prefrontal cortex, cognition via several areas of the cortex, reward via activities in the mesolimbic projections, and interestingly also endogenous analgesic effects via influence on neuronal activity in the locus ceruleus and Raphe nuclei of the reticular formation. The exact expression patterns of each specific receptor are not completely known. Several factors such as species differences, overlapping pharmacological properties, and discrepancies between mRNA and detectable receptor protein levels complicate the picture. Speculations on what expression patterns most closely image reality are beyond the scope of this text. A few general statements about the receptors of choice, D1R and D2R are however in place. The D2R has in several studies been indicated to function as an autoreceptors; i.e. localize to dopaminergic neurons and modulate levels of activity in these neurons. Further, D2R localize to both pre- and postsynaptic membranes, whereas D1R more often

Effector	D1R	D2R
G protein	$G\alpha_s$	$G\alpha_{i/o}$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_z$
Adenylate cyclase	AC	AC
Phospholipase	PLC, PLA_2	PLCβ, PLA_2, PLD
Protein kinase	PKA, PKC	PKC, PI3K, Erk
Ionchannel	GIRK, K^+ efflux	GIRK, L-type Ca^{2+}
Other enzymes	Na^+/H^+ antiport, Na^+/K^+ ATPase	Na^+/H^+ antiport, Na^+/K^+ ATPase

localize to postsynaptic membranes. Several reports have by different experimental approaches also been able to show a colocalization of both receptors in certain neurons of for example the striatum. The two receptors therefore have the opportunity to influence each other's activity and trafficking patterns, respectively, via hetero-dimerization.

Several pathological conditions have been linked to alterations of dopamine signaling in the central nervous system. Dopamine is for example postulated to play a central role in reinforcing drug seeking behaviour and is thus postulated to play a central role in addiction (Volkow, Fowler et al. 1999; Volkow, Wang et al. 2006). Further, the classical symptoms in Parkinson's disease are caused by selective cell death of neurons in the dopaminergic projection areas. It has also been suggested based on various animal models and clinical findings that several less defined syndromes such as schizophrenia, hyperactive disorder ADHD, and Tourette's syndrome to name a few may stem at least in part from malfunctioning hyperactive dopaminergic circuits (Jones, Gainetdinov et al. 1999; Zhuang, Oosting et al. 2001). Lack of dopamine as in Parkinson's disease can be remedied through replacement therapy, but the other syndromes are much harder to address. It has been suggested that dopamine D2 autoreceptor may serve a protective role in all of the above mentioned syndromes (Jones, Gainetdinov et al. 1999; Volkow, Fowler et al. 1999; Zhuang, Oosting et al. 2001; Volkow, Wang et al. 2006) and thus make this receptor and the regulation of its activity very interesting targets for future drug development.

Both D1R and D2R have been reported to internalize in a GRK2/3, β-arrestin, and clathrin-dependent manner (Tiberi, Nash et al. 1996; Kim, Valenzano et al. 2001; Macey, Liu et al. 2005), although they do not colocalize in endosomal structures (Vickery and von Zastrow 1999). Studies of postendocytic sorting of D1R and D2R have clearly shown a recycling phenotype for the D1R both in vitro (Vickery and von Zastrow 1999), and in vivo (Ariano, Sortwell et al. 1997), whereas the results from D2R are more ambigous. Several in vivo reports suggest that chronically elevated dopamine levels result in D2R downregulation (Jones, Gainetdinov et al. 1999; Volkow, Fowler et al. 1999) whereas an in vitro (HEK293) study indicate that D2R recycles albeit slower than D1R receptor (Vickery and von Zastrow 1999). Another report that utitilizes both in vitro (HEK293) and ex vivo models

show that D2R and D3R sorting can be affected by the GIPC protein resulting in a rerouting from the lysosomal pathway (Jeanneteau, Diaz et al. 2004).

The conflicting data may stem from the difficulty of studying the D2R in vitro. We found in our Study II the D2R internalization is much more limited than D1R internalization in response to dopamine stimulation. This may obscure the readout of less sensitive assays.

Interestingly, D1R recycling has been attributed to a sorting motif located in its proximal carboxyterminal domain (Vargas and Von Zastrow 2004), which challenges the notion of a default recycling pathway for internalized GPCR. In this study, the authors transplanted the recycling phenotype from D1R to DOR by addition of a 20 amino acid stretch to the carboxyterminal domain of the DOR. Unfortunately, the study does not convincingly show how transplantation of this putative domain alters the interaction of DOR with GASP. Recycling of the DOR/D1R chimera might thus occur due to a block of DOR/GASP interaction rather than a specific interaction with a recycling machinery. However, the truncated D1R does not recycle nor degrade indicating that this proximal carboxyterminal domain is of importance for D1R recycling. This is reminiscent of our results from the bradykinin receptors (see Study III) and together with a growing amount of studies on facilitated recycling of GPCR prompts further investigation in the future.

Considering the imbalance and excessive activity of the dopamine system suggested in the pathological conditions mentioned above, and the protective role of D2R against the development of addiction, a better understanding of D2R regulation is crucial for future drug development, and led us to conduct Study II.

Bradykinin Receptors

There are two bradykinin receptor family members B1R and B2R. Both receptors are coded for by three exons in two separate genes in close proximity to each other on chromosome 14q32. The entire coding sequence of B1R is within the third exon whereas a short stretch of B2R is coded for by the second exon of the B2R gene. An alternative exon 2 may exist called exon 2b. No other splice variants have as of yet been discovered. A third putative bradykinin receptor GPR100, also known as GPCR142 or relaxin-3 receptor-2, responds to bradykinin and is antagonized by icatibant, a B2R specific antagonist. However GPR100 normally respond to the ligand relaxin-3 via $G\alpha i$ and only show similar response to bradykinin as B2R when coexpressed with the promiscous $G\alpha$ protein $G\alpha 16$. Further, this receptor is unable to make B2R redundant as shown by B2R knock-out animal models. Human and rabbit bradykinin receptors are more closely related in an evolutionary pespective than human and mouse or human and rat for both B1R and B2R. This is reflected in the B1R protein where most of the carboxyterminal tail is missing in mouse and rat (15aa after the NPXXY motif) as compared to human and rabbit (41aa after the NPXXY motif). In contrast, B2R from all four species have the same length of the tail (59aa after the NPXXY motif). Considering the many functions ascribed to this domain, this species difference may have profound effects on the membrane trafficking of the receptor in these separate species and therefore also on the choice of cell and animal models in the study of the B1R.

Pharmacologically, the bradykinin receptors respond to peptide agonists formed through enzymatic cleavage of polypeptide precursors. High-molecular kininogen is thus processesed into Bradykinin (RPPGFSPFR) a B2R agonist, which can be further metabolized to

Effector	B2R	B1R
G protein	$G\alpha_q$, $G\alpha_i$, $G\alpha_{12/13}$	$G\alpha_q$, $G\alpha_i$
Phospholipase	PLCβ, PLA_2, PLD	PLCβ, PLA_2
Protein kinase	PKC, PI3K, Akt, FAK, Erk, JAK	Erk
GTP exchange factor (GEF)	RhoA	
Transciption factor	Stat, NF-kB, AP-1 NF-IL-6, Elk-1	
Other enzymes	eNOS	eNOS

des-Arg9-Bradykinin (RPPGFSPFR), a B1R agonist and the preferred endogenous B1R agonist in mouse and rat. Further, low-molecular weight kininogen can be processed to form Lys-Bradykinin (also called Kallidin, LRPPGFSPFR), which is a less selective B1 and B2 agonist, and can in turn be metabolized further to Lys-des-Arg10-Bradykinin (also called des-Arg10-Kallidin, LRPPGFSPF), which is a highly specific B1 agonist in for example human and rabbit. B2-specific agonists are primarily formed through the action of plasma and tissue kallikreins, whereas B1 specific agonists are formed by the additional action of arginine carboxypeptidases. Numerous synthetic agonists and antagonists, both peptide and nonpeptide, have been synthesized and a few compounds are now in clinical use. When active, the receptors both preferentially signal via Gαq or Gαi but B2R is able to signal via several other G proteins such as Gαs, Gα12/13. B2R stimulate phospholipase Cβ via Gαq, translocation and activation of PKC, stimulation of phospholipase A2, phospholipase D, NO release via direct activation of eNOS, promotes activation of the MAP kinase cascade via phosphorylation, activates the JAK/STAT pathway, and regulates the activity of focal adhesion kinase and other focal adhesion-associated proteins and cytoskeletal regulators via phosphorylation. Besides the acute signaling effects that involve effects on cAMP and mobilization of intracellular and extracellular calcium, these various signaling pathways modulate the activity of a host of transcription factors that in turn regulate the levels of a diverse range of proinflammatory cytokines. One of these cytokines is IL-1β that in turn is involved in B1R upregulation. B1R is also reported to act via Gαq and Gαi, leading to similar effects as B2R. Although the two receptors share most effector systems they are not functionally redundant nor do they result in similar intracellular signaling patterns. One reason for this might be the high spontaneous rate of activity in the B1 receptor relative B2R, but several other aspects of receptor regulation differ between the two as will be discussed below. A number of rigorous pharmacological studies have already mapped crucial domains within each of the two receptors involved in ligand binding, phosphorylation, and activity. It is possible through these studies to deduce the central role played by the carboxyterminal domain, also called the fourth intracellular domain, in the pharmacology of these receptors. This prompted us to address the role of this domain in the design of Study III.

Both receptors are expressed in numerous tissues albeit most of these tissues express B1R only after induction through TNFα, IL-1β, LPS, intact pathogens like Burkholderia cenocepacia , Staphylococcus aureus, and via the B1-specific agonist des-Arg10-kallidin (Marceau, Larrivee et al. 1999; Sardi, Daray et al. 1999; Ma, Hill et al. 2000; Ma and Heavens 2001; Phagoo, Reddi et al. 2001; Phagoo, Reddi et al. 2005; Bengtson, Phagoo et al. 2006). Both receptors have been confirmed in endothelial cells, smooth muscle cells (both vascular and nonvascular), fibroblasts, epithelial cells, leukocytes, and both peripheral and central nervous tissue (Bhoola, Ramsaroop et al. 1997; Mahabeer, Naidoo et al. 2000; Ricupero, Romero et al. 2000; Wotherspoon and Winter 2000; Valdes, Germain et al. 2001; Prat, Biernacki et al. 2005). The B2R tissue distribution is of much less controversy than B1R. A basic problem is that tissue damage such as the one induced by preparation of tissue for immunodetection or in situ hybridization may induce the expression of the B1R. This and the fact that methods, antibodies, and probes used differ between studies are likely reasons behind the discrepancies reported. Further, B1R levels are relatively low even after induction, which indicates that methodological sensitivity is crucial for detection. Our own unpublished data using our N-terminal anti-B1R antibody indicates that B1R is expressed in fare abundance in several areas of rat brain located to the dendritic neuronal surface. Further, the same antibody when applied to transmission electron microscopy of rat dorsal horn show a lamina II expression of B1R and B2R in or near synapses (Johan Enquist, unpublished data). Also, indicative of their function, both receptors are upregulated in several tissues in different pathological conditions. For example, spontaneously hypertensive rats (SHR) show an initial higher B2R abundance over Wistar Kyoto rats (WKR), whereas B1R in the SHR are upregulated compared to WKR at later stages in this disease model (Qadri, Hauser et al. 2002). Further, streptozotocin-induced diabetes, paw formalin injection, and severing of the sciatic nerve in experimental models of epileptic seizures, diabetic pain, chronic inflammation, and neuropathic pain all lead to B1R upregulation in spinal cord gray matter and on dorsal root ganglion cells (Cloutier, de Sousa Buck et al. 2002; Ongali, Campos et al. 2003; Vianna, Ongali et al. 2003; Ongali, Campos et al. 2004). Similar results of upregulation of receptors have been shown in post-mortem studies on human diabetic or hypertensive donors relative control (de Sousa Buck, Ongali et al. 2002). Upregulation or the detection of the two receptors have also been confirmed in certain forms of human cancer, notably prostate cancer, lymphoma, sarcoma, and squamous cell carcinoma (Wu, Akaike et al. 2002; Taub, Guo et al. 2003).

Notably, most tissues that express either receptor also express the enzymes necessary for the production of the agonists, see for example Mahabeer et. al. work from 2001 for distribution of receptors and tissue kallikrein in human brain regions (Mahabeer, Naidoo et al. 2000; Valdes, Germain et al. 2001).

Physiologically, deletion of either or both of these two receptors is not lethal, which could either indicate redundancy or that they are activated only under certain physiological conditions such as vascular shear, tissue injury, inflammation, and dietary stress – conditions under which several studies show an increased effect of bradykinin receptor agonists or antagonists. In support of this, several studies show that stress-induced conditions in the knock-out animals lead to a flawed physiological response. For example, neutrophil extravasation is blocked in B1R-knock outs, a physiological response that normally might be achieved through an axonal reflex since the same result is achieved through peripheral nerve destruction via capsaicin or block of other important receptors on the peripheral nerve such as NK1 or H1 receptors (Pesquero, Araujo et al. 2000).

Similarily, no effect on the vascular system can be seen in the B2R knock-out animal model, except a mild cardiomyopathy and slight insulin resistance, unless the animals are presented with a high dietary salt regime in which case they develop malignant hypertension (Alfie, Sigmon et al. 1997; Cervenka, Harrison-Bernard et al. 1999; Duka, Kintsurashvili et al. 2001; Maestri, Milia et al. 2003). Knock-out of either receptor also leads to a loss of central wind-up and reduced central sensitization in response to nociceptive stimulation (Pesquero, Araujo et al. 2000; Wang, Kohno et al. 2005).

Double B1R and B2R knock-out animals also lack the shock response to systemically administered LPS, a result indicative of the important role of these two receptors in the acute inflammatory response (Cayla oral presentation Hypertension 2002, 40:391-92).

A seemingly endless number of studies approach the physiological role of bradykinin receptors by direct activation of receptors in different tissues. Intravenous application of either B1R (after B1R induction) or B2R agonists result in bradykardia and hypotension via peripheral loss of resistance, whereas the same agonists when injected extravascularly or intrathecaly induce hypertension and tachykardia (Beauchamp, Lemieux et al. 1991; Green, Janig et al. 1997; Marceau and Bachvarov 1998; Cloutier and Couture 2000; deBlois and Horlick 2001; Cloutier, de Sousa Buck et al. 2002; Qadri, Hauser et al. 2002). The former has been attributed to the receptor-stimulated production of NO, production of prostacyclin via PLA2 that result in increase in cAMP in smooth muscles, effects on non NO-sensitive calcium channels in smooth muscle cells, the breakdown of intercellular junctions leading to the formation of edema, and by increasing diuresis (Furchgott 1983; Cockcroft, Chowienczyk et al. 1994; deBlois and Horlick 2001; Wojciak-Stothard and Ridley 2002; Batenburg, Garrelds et al. 2004; Hebert, Regoli et al. 2005). The latter effect is most likely due to reflex activation of the sympathetic autonomic system via peripheral nerve activation or, in the case of intrathecal administration possibly also to activation of central cardiovascular control centers such as the hypothalamus (Beauchamp, Lemieux et al. 1991; Cloutier and Couture 2000; Cloutier, de Sousa Buck et al. 2002).

Further, studies have clearly established the effect of initiation, potentiation and inhibition of bradykinin receptor activity on nociceptive signaling. B1R and B2R agonist injection in peripheral tissue leads to hyperalgesia, whereas bradykinin release in the spinal cord potentiates NMDA signaling in the nociceptive pathway (Pesquero, Araujo et al. 2000; Fox, Wotherspoon et al. 2003; Wang, Kohno et al. 2005). In contrast bradykinin release in trigeminal ganglion cells induce the upregulation of DOR leading to an enhanced analgesia thus acting as a negative feedback to bradykinin induced hyperalgesia (Patwardhan, Berg et al. 2005).

Finally, a direct injection of bradykinin into the lateral or fourth brain ventricles results in analgesia (Couto, Correa et al. 1998; Couto, Moroni et al. 2006). The latter effects together with our unpublished data on the wide distribution of the receptors in the brain indicate that much is still to be learned of the central effects of bradykinin receptor activity. It is interesting to note the substantial tissue distribution overlap of these receptors with the opiate receptors in particular, but also with the dopamine receptors. Further, the functions of these different receptor systems seem to either completely oppose each other or regulate the temporal induction and responsiveness of the opposing receptor system.

Regulation of bradykinin receptor activity is, as indicated, directed to all levels of receptor

protein handling including synthesis, maturation, cellular localization, clearance, as well as through the restricted formation of ligands, that are in turn further destabilized by the same set of endopeptidases that dispose of extracellular opioid ligands (Norman, Lew et al. 2003). B2R has a limited induction in most tissues, whereas B1R is strongly induced as discussed above. Further, when induced, several studies of cell models, including our Study III, show a slow maturation of B1R with a majority of receptors apparently degraded via proteasomal activity prior to incorporation into the plasma membrane (Fortin, Dziadulewicz et al. 2006). On the other hand, B2R follows the archetypal GPCR scheme of rapid agonist-induced phosphorylation, followed by uncoupling mediated through β-arrestin 2 recruitment, sequestration, dephosphorylation and recycling (Munoz, Cotecchia et al. 1993; de Weerd and Leeb-Lundberg 1997; Haasemann, Cartaud et al. 1998; Pizard, Blaukat et al. 1999; Houle, Larrivee et al. 2000; Bachvarov, Houle et al. 2001; Lamb, De Weerd et al. 2001; Simaan, Bedard-Goulet et al. 2005).

B1R, on the other hand, shows no signs of phosphorylation, with or without agonist (Blaukat, Herzer et al. 1999; Faussner, Bauer et al. 2005) (Blaukat unpublished observations, Leeb-Lundberg unpublished observations). Further, B1R does not show any significant desensitization in response to agonist activation (Mathis, Criscimagna et al. 1996; Faussner, Proud et al. 1998; Faussner, Bathon et al. 1999). Instead, several reports indicate that intracellular messengers plateau, or in the case of calcium can be induced to oscillate over time, in response to agonist activation. Also, several studies indicate that the numbers of B1R surface receptors actually increase over time, to some degree rapidly enough to exclude de novo synthesis (Faussner, Bathon et al. 1999; Phagoo, Reddi et al. 2001). Some studies do indicate that there is a slight receptor-specific internalization of B1R agonist but significantly less than the amount of bradykinin internalized with the B2R (Lamb, De Weerd et al. 2001). Block of protein synthesis has further leads to a relatively rapid clearance of B1R from the cell surface (Audet, Petitclerc et al. 1994; Sardi, Daray et al. 1999; Fortin, Bouthillier et al. 2003).

The literature is quite contradictory in describing the machinery behind the internalization of these two receptors. One publication claim to have shown internalization of B2R via caveolae based on a morphological study, whereas several studies show association of B2R and B2R-associated G proteins with detergent-resistant membrane components (de Weerd and Leeb-Lundberg 1997; Haasemann, Cartaud et al. 1998; Lamb, De Weerd et al. 2001). Here, the words sequestration, meaning hidden, and internalized might add to the confusion since they need not mean the same thing. In the future, it is important to elucidate both sequestration mechanisms and the mechanisms by which actual removal of receptor protein from the plasma membrane occur. No one has as of yet seriously addressed the phenomenon through for example targeted disruption of specific coat proteins, or adaptors for that matter. B2R has been shown to internalize in a β-arrestin 2- and dynamin-independent manner in HEK293 cells (Lamb, De Weerd et al. 2001). However, there are also claims that release of β-arrestin 2 from the B2R is necessary for the receptor to recycle, indicating that this protein may have a prominent role in B2R trafficking (Simaan, Bedard-Goulet et al. 2005). Further, B1R internalization has been claimed to be both arrestin- and dynamin- dependent (Lamb, De Weerd et al. 2001) but also that agonist-occupied B1R target caveolae (Sabourin, Bastien et al. 2002). This latter claim has been disputed in another study from our lab (Lamb, Zhang et al. 2002). Several studies have been based on carboxy-terminally EGFP-tagged receptor constructs but recent findings indicate that at least some aspects of B2R trafficking is disturbed by this form of receptor modification (Kalatskaya, Schussler et al. 2006), indicating that this form of modi-

fication is less than optimal for membrane trafficking studies.

There is an absolute lack of prior studies that attempts to clarify the postendocytic choices made by the B1R, nor has anyone investigated what molecular interactions dictate postendocytic choices of either of the bradykinin receptors.

Obviously two receptors distributed in so many tissues and, that show species-specific differences in domains crucial to membrane trafficking, need to be studied under very controlled conditions. Preferably, these studies should be pursued in cells from various tissues, and by means of direct investigation of the receptor proteins in order for us to draw any conclusions on the impact of membrane trafficking on B1R and B2R regulation.

Thus we designed study III to be as controlled but minimally modified as possible by using human bradykinin receptors minimally modified by N-terminal synthetic tags and expressed in a human fibroblast cell line (HEK293). Our aim with the study was to characterize the initial steps of receptor internalization and postendocytic choices after rapid internalization within this model system. Even so, we knew already from the start that several factors including high receptor expression (Faussner, Bauer et al. 2003), constitutive expression of B1R, which is normally only expressed after induction, and the restricted amount of proteins involved in membrane trafficking regulation of GPCR expressed in HEK293 are all cause for concern and are all good reasons to interpret data with great care. However, we found this to be a necessary first step towards an improved understanding of the physiology of these receptors.

AIMS

1. **Study the mechanism underlying postendocytic sorting of DOR to the degrading lysosomal pathway after rapid agonist-stimulated internalization in a heterologous model system.**

2. **To investigate the postendocytic trafficking of the dopamine D1 and D2 receptor in response to rapid agonist induced internalization in a heterologous model system.**

3. **To investigate the influence of GASP on postendocytic sorting and ex vivo signaling of the dopamine D2 receptor.**

4. **To investigate the rapid accumulation of B1R on the plasma membrane after agonist activation.**

5. **To investigate internalization mechanisms and postendocytic fate of B1R and B2R in a heterologous model system.**

6. **To investigate plasma membrane stabilization, activation, and uncoupling mechanisms of the B1R receptor.**

7. **To find interaction partners involved in the B1R regulation.**

Aim 1 was addressed in study I.
Aim 2 and 3 were investigated in study II.
Aim 4 and 5 were addressed in study III.
Aim 6 and 7 were addressed in study IV – supplementary data.

METHODS

Study I

Radioligand binding assay
Agonist-induced down-regulation of receptors was assayed in intact cells using a previously described method (Law, Hom et al. 1982). Briefly, monolayers of cells expressing FLAG-tagged MOR, DOR or D-MOR were incubated for 3 h at 37°C in the absence or presence of 10 μM DAMGO or 10 μM DADLE respectively. To ensure a saturating concentration of peptide agonist over the incubation period, monolayers incubated were supplemented with fresh peptide every hour during the incubation. At the end of the incubation, cells were lifted with PBS supplemented with EDTA and washed four times by centrifugation with 10 ml of warm (37°C) PBS. Then cells were washed once by centrifugation in 10 ml of Krebs-Ringer HEPES buffer (KHRB: 110 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, pH 7.3). Radioligand binding was carried out in 120 μl of KHRB containing equal amounts of washed cells (50-100 μg of protein) and ligand concentrations as above. Incubations were carried out for 30 min at room temperature, and cells were harvested and washed using vacuum filtration on glass fiber filters as above. For all determinations, bound radioligand represented <= 10% of total radioligand present in the incubation, and nonspecific binding (defined as above) was <= 10% of counts isolated on glass fiber filters.

Biotin protection-degradation assay
Stably transfected cells expressing FLAG-tagged receptors were grown to 80% confluency washed 2 times with cold phosphate buffered saline (PBS) then incubated in 3 mg/ml disulfide cleavable (sulfo-NHS S-S biotin, Pierce) in PBS at 4°C for 30 minutes with gentle agitation. Cells were washed 2 times with Tris buffered saline and placed back into medium for treatment. Cells labeled 100% biotinylated were left on ice in PBS. Cells labeled 100% stripped were also left on ice in PBS then stripped as described below. Cells were treated with 5 μM agonist for 30 minutes or 3 hours, washed 2 times with cold PBS and the remaining cell surface biotinylated receptors were stripped in 50 mM glutathione, 0.3 M NaCl, 75 mM NaOH, 1% fetal bovine serum at 4°C for 30 minutes. Glutathione was quenched with a 20 minute wash of PBS with 50 mM iodoacetamide, 1% bovine serum albumin. Cells were extracted in 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris-HCl pH 7.4 containing 1 μM leupeptin, 1 μM pepstatin A, 1 μM aprotinin, 2.5 μM Pefabloc SC, and cell debris was removed by centrifugation at 10,000 x g for 10 minutes at 4°C. Receptors were immunoprecipitated using M2 mouse anti-FLAG antibody (Sigma), rabbit anti-mouse linker (Jackson Immunoresearch) and protein A Sepharose (Pharmacia) overnight. Precipitates were extensively washed, and deglycosylated for 2 hours at 37°C with PNGase F (New England Biolabs). Proteins were denatured in SDS sample buffer with no reducing agent and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and the membrane blocked in Tris buffered saline containing 0.1% Tween and 5% nonfat milk for 1 hour. Biotinylated proteins were visualized by incubating with the Vectastain ABC immunoperoxidase reagent (Vector Laboratories), followed by development with ECL reagents (Amersham).

Fluorescence microscopy
Cells were grown on glass coverslips and incubated in media containing 3.5 g/ml M1 mouse anti-FLAG monoclonal antibody (Sigma) to label surface receptors. Cells were then treated as described with 5 μM of the alkaloid agonist etorphine for the indicated time periods. For recycling experiments cells were incubated for 30 minutes with etorphine followed by a wash and incubation for an additional 30 minutes with 10 μM of the opiate antagonist naloxone. Cells were fixed using 4% formaldehyde in phosphate-buffered saline and permeabilized using 0.1% Triton X100. For visualization of receptor and GFP tagged GASP, fixed specimens were then incubated with Texas Red conjugated donkey anti-mouse antibody (Jackson Immunoresearch) to detect M1 antibodies bound to tagged

receptors. For visualization of FLAG-tagged receptors relative to (amino-terminally) HA-tagged GASP, a mouse anti-HA IgG1 (HA.11, clone 16B12, Covance) was used. Selective detection of anti-FLAG (IgG2b) and anti-HA (IgG1) was accomplished using subsequent incubation of specimens with a rabbit anti-mouse IgG2b linker antibody (Zymed), then with FITC-conjugated goat anti-mouse IgG1 (Boeringer Mannheim) to visualize HA and Texas Red-conjugated donkey anti-rabbit serum (Jackson Immunoresearch) to visualize FLAG. For co-localization of FLAG-tagged receptors with LAMP1 and LAMP2, H4A3 and H4B4 mouse IgG1 reagents (Developmental Studies Hybridoma Data Bank) were used with the same series of detection steps. Epifluorescence microscopy was performed using an inverted Nikon microscope fitted with a Nikon 60XNA1.4 objective, standard filter sets (Omega Optical) and a cooled CCD camera (Princeton Instruments). Confocal fluorescence microscopy was performed using a Zeiss LSM510 microscope fitted with a Zeiss 63XNA1.4 objective in single photon mode with standard filter sets and standard (1 Airy disc) pinhole.

Membrane Cyclic AMP Assay
Membrane Preparations: Cells were grown to 80% confluency and then pretreated with 5 μM DAMGO or DADLE for 10 min, treated with 5 μM DAMGO or DADLE for 3 hours followed by washout and antagonist chase at 37°C for 30 min, or left untreated. Cells were lifted in PBS 0.04% EDTA, washed four times in 15 ml of cold PBS, and then resuspended in 1 ml of cold buffer of 25 mM MgCl2, 75 mM Tris•HCl , and 2 mM EDTA, pH 7.5 and pelleted at 2,000 x g for 5 min. The pellet was resuspended with a glass potter in the same buffer and assayed immediately. Adenylyl Cyclase Assay: Membranes were incubated in 30 mM Tris•HCl , 1 mM EDTA, 50 μM GTP, 0.1 mM cAMP, 40 μM ATP, 10 mM creatine phosphate, 200 units/ml creatine phosphokinase, 1 μCi [32P]-ATP, and 10 μM forskolin with or without 10 μM DAMGO or DADLE at 37°C for 30 min. Reactions were stopped by addition of HCl to 1 μM and applied to acidic alumina spin-columns (Pierce). Columns were washed and eluted according to the manufacturer's instructions and eluate counted in a scintillation counter.

Yeast Two-Hybrid Screen
The COOH-terminal tail of murine DOR-1 (residues 337 to 391) was used to identify interacting clones, and the interacting clones were isolated from a 293 cell-derived cDNA library (Clontech, Palo Alto, CA) using the Gal4-based MATCHMAKER system (Clontech). A total of 2.5 X 106 recombinants were screened.

Affinity Chromatography Assay
DOR, MOR, β-2AR, α2b-AR, D4R, and V2R receptor carboxy-terminal tails starting after the NPXXY motif, and cGASP were cloned into the pGEX4t1 vector and carboxy-terminal tail-GST fusion proteins were produced and attached to glutathione-Sepharose (Sigma). DOR carboxyterminal MBP fusion protein was constructed corresponding to GST fusion. Full length GASP was in vitro-translated using the TNT Quick Coupled Transcription/Translation Systems (Promega) with 35S-labeled methionine. Fusion proteins and radiolabeled probe were mixed in a wash solution containing 0.1% Triton X-100 and incubated for 1 hr at room temperature. Glutathione resin was washed several times followed by denaturation by boiling in SDS sample buffer and proteins fractionated by SDS-PAGE. Proteinconcentrations of receptor tails and controls were estimated by Coomassie staining of the gel and pulled down probe was visualized by autoradiography. Competition of FL- and c-GASP for DOR-MBP was carried out essentially as affinity chromatography for DOR-GST, but with incremental added concentrations of GST-cGASP.

Co-immunoprecipitation
Cells were grown to confluency, washed 2 times with PBS and lysed in 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris-HCl pH 7.4 containing 1 μM leupeptin, 1 μM pepstatin A, 1 μM aprotinin, 2.5 μM Pefabloc SC, and cell debris was removed by centrifugation at 10,000 x g for 10

minutes at 4°C. An aliquot of lysate was removed for GASP control blot. Lysate was incubated with M2 anti-FLAG affinity resin (Sigma) overnight. Pellets were extensively washed and deglycosylated with PNGase F (New England Biolabs) for 2 hours at 37°C and then eluted in SDS sample buffer. For co-immunoprecipitation with endogenous GASP, one fourth of eluate was separated by SDS-PAGE on a 12% gel (receptor blot), three quarters on a 7% gel (GASP blot). For co- immunoprecipitation with cGASP eluate was run on a 12 % gel that was later cut and blotted separately for cGASP and receptor. Proteins were transferred to nitrocellulose. GASP blots were incubated for 2 hours with rabbit anti-GASP (1:4000). cGASP blots were incubated with rabbit anti-GFP antibodies for 2 hours (1:200) (Clontech). Both were followed by 1 hour with HRP-conjugated anti-rabbit antibody (NEB) (1:3000), and visualized with ECL plus (Amersham). Receptor blots were incubated with biotinylated M2 (1:250) (Sigma) for two hours, followed by visualization with Vectastain ABC reagents (Vector) and ECL plus. HA-11 (Covance) was used for blotting at 1:1000 for 2 hours for HA-GASP blots.

Study II

Animals.
Male Sprague-Dawley rats, 22-28 days of age were used. Animal care was in accordance with Ernest Gallo Clinic and Research Center Animal Care and Use Committee.

Drugs and Reagents.
Quinpirole, SCH23390 dopamine, raclopride, baclofen, haloperidol, and FLAG antibodies were purchased from Sigma. Hemagglutinin (HA) antibodies were purchased from Covance (Princeton, New Jersey), and both donkey anti-rabbit Cy-3 and anti-sheep FITC were purchased from Jackson ImmunoResearch. The polyclonal dopamine D2 receptor (D2R) antibody was purchased from Chemicon (D2R 5084). The horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies were purchased from NEB. To generate the anti-GASP antibodies, the C-terminal domain of GASP (amino acids 943-1395) was cloned into pRSETa from Invitrogen, and His6-cGASP was expressed in Escherichia coli BL21 RIL and purified by using Ni2+-chelating Sepharose. Rabbit antisera to the purified His6-cGASP was produced at Zymed.

Slice Preparation and Electrophysiology.
Horizontal slices (230 µm) containing the VTA were prepared as described (9). Artificial cerebrospinal fluid (ACSF) contained 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 18 mM NaHCO3, 11 mM glucose (pH 7.2-7.4 and milliosmolarity 301-305) and was bubbled with 4°C carbogen. After cutting, slices recovered for at least 45 min at 32°C in carbogen-bubbled ACSF. During patch-clamp experiments, slices were submerged and continuously perfused (using a peristaltic pump, 2ml/min) with ACSF warmed to 31-32°C, and supplemented with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 µM), picrotoxin, and sodium metabisulfite (50 µM), as described (10). Cells were visualized with an upright microscope using infrared differential interference contrast illumination. Whole-cell voltage clamp recordings were made with 2.5-3.5 M electrodes using a Multiclamp700A amplifier (Axon Instruments) in current clamp mode. The potassium methanesulfonate-based internal solution consisted of 0.95% (vol/vol) KOH, 0.38% (vol/vol) methanesulfonic acid, 20 mM Hepes, 0.2 mM EGTA, 2.8 mM NaCl, 2.5 mg/ml MgATP, 0.25 mg/ml GTP (pH 7.2-7.4, 275-285 milliosmolarity). Data were acquired by passing 2-KHz DC current through an amplifier. Cells were set to -60 mV 10-15 min before each quinpirole application and the membrane potential determined every 2 s (single traces) or 30 s (grouped data).

Cell Culture and Immunocytochemistry
Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were grown in DMEM

(Gibco BRL) supplemented with 10% fetal bovine serum (HyClone). N-terminal FLAG- and/or HA-tagged D1R or D2R constructs (Vickery and von Zastrow 1999) were stably expressed in HEK293 cells. GFP-cGASP (see study I) constructs were either stably or transiently expressed. For generation of clonal stable cell lines, single colonies were chosen and propagated in the presence of selection-containing media. The antibody-feeding immunocytochemistry and recycling experiments were essentially as described (Finn and Whistler 2001), except dopamine (10 μM, 60 min), haloperidol (20 μM), or SCH23390(20 μM) were used. Briefly, cells stably expressing FLAG-tagged D1R or D2R were grown on coverslips to 50% confluency. Live cells were fed M1 antibody (Sigma) directed against the FLAG tag (1:1,000, 30 min). Cells were then treated with agonist (10 μM dopamine, 60 min) or left untreated. Untreated cells were then fixed with 4% formaldehyde in PBS. Residual surface receptors (those not internalized by agonist) in cells treated with agonist were stripped of antibody by washing in PBS without calcium (the M1 interaction is calcium sensitive). Cells were then either fixed as above or first treated with antagonist (20 μM haloperidol for D2R, or 20 μM SCH23390 for D1R) for 30 min to assess recycling and then fixed as above. After fixation, cells were permeabilized in blotto with 0.1% Triton X-100 and stained with fluorescently conjugated secondary antibody (1:500, Molecular Probes).

Biotin Protection Degradation Assay (BPA).
HEK293 cells stably expressing N-terminal FLAG- or HA-tagged D1R or D2Rs either alone or together with GFP-cGASP were grown to 80% confluency in 10-cm plates and subjected to the BPA protocol as described in ref. 12 except the D1R and D2R agonist dopamine (10 μM), the D2R antagonist haloperidol (20 μM), or the D1R antagonist SCH23390 (20 μM) were used. Briefly, cells were treated with 0.3 mg/ml disulfide-cleavable biotin (Pierce) for 30 min at 4°C. Cells were then washed in PBS and placed in prewarmed media for 15 min before treatment with ligand (or no treatment) for the specified period. Concurrent with ligand treatment, 100% and strip plates remained at 4°C. After ligand treatment, plates were washed in PBS, and remaining cell surface biotinylated receptors were stripped in 50 mM glutathione/0.3 M NaCl/75 mM NaOH/1% FBS at 4°C for 30 min. Cells were quenched with Tris buffer, then lysed in 0.1% Triton X-100/150 mM NaCl/25 mM KCl/10 mM Tris•HCl, pH 7.4, with protease inhibitors (Sigma). Cleared lysates were immunoprecipitated with anti-FLAG or HA antibodies, treated with PNGase (New England Biolabs) for 2 h and resolved by SDS/PAGE and visualized with streptavidin overlay (Vectastain ABC immunoperoxidase reagent, Vector Laboratories). For quantification, at least three blots for each condition were quantified by using Scion IMAGE software package. Agonist treatment for 60 min was designated 100% for each agonist/condition tested.

Coimmunoprecipitation from HEK293 Cells.
HEK293 cells stably expressing D1R, D2R, or no heterologous receptor were grown to confluency and washed two times with PBS, and lysates were prepared as described in study I, in 0.1% Triton X-100/150 mM NaCl/25 mM KCl/10 mM Tris•HCl, pH 7.4, with protease inhibitors (Sigma). Cleared lysate was incubated with M2 anti-FLAG affinity resin (Sigma) for 1 h at 4°C, washed extensively, and deglycosylated with PNGase (New England Biolabs) for 2 h. Precipitates were resolved on a 4-20% gradient Tris•HCl precast gel (Bio-Rad) and transferred to nitrocellulose, and the blots were cut below the 75-kDa marker band to separately immunoblot for either receptor (lower blot) or GASP (upper blot). GASP blots were incubated for 2 h with rabbit anti-GASP (1:1,000) and for 1 h with HRP-conjugated anti-rabbit antibody (NEB) (1:4,000, 1 h at room temperature), then visualized with ECL plus (Amersham Pharmacia). Receptor blots were incubated for 2 h with biotinylated M2 (1:250) (Sigma), then visualized with streptavidin overlay (Vectastain ABC reagents, Vector Laboratories) and ECL plus.

GST Competition
The last 14 amino acids of the D2R were fused to the C terminus of GST, and the fusion protein was generated as described in study I. Affinity between in vitro-translated GASP protein and GST-D2R was tested in the presence or absence of rabbit anti-GASP antibodies (250 ng/ml) or control rabbit IgG (250 ng/ml) antibodies.

GST Pull-Down from Rat Brain
Rat brain synaptosomal membranes were prepared from adult rats as described (Hunter, Burstein et al. 1986), and lysates were prepared in 0.1% Triton X-100/150 mM NaCl/25 mM KCl/10 mM Tris•HCl, pH 7.4, with protease inhibitors (Sigma) and Complete (Roche Diagnostics). Cleared lysate was incubated with empty glutathione resin (B, beads), resin bound to empty GST (G), resin bound to a GST-D1R fusion protein (D1), or resin bound to a GST-D2R fusion protein (D2) as described above, for 2 h at 4°C, then washed extensively. Equivalent protein levels of GST, GST-D1R, and GST-D2R were determined before the GST pull-down experiment and confirmed by Coomassie stain of the gel. Precipitates were resolved on a 4-20% gradient Tris•HCl gel (Bio-Rad), transferred to nitrocellulose, and immunoblotted for GASP by using rabbit anti-GASP (1:1,000) antibody, and incubated for 1 h with HRP-conjugated anti-rabbit antibody (NEB) (1:4,000), then visualized with ECL plus (Amersham Pharmacia).

VTA Slice Preparation and Immunohistochemistry
Adult rats were deeply anesthetized with halothane and fixed with 4% paraformaldehyde (Sigma) by using a standard perfusion procedure (Kharazia, Jacobs et al. 2003). Double immunofluorescence immunohistochemistry of coronal sections (35 µM) containing the VTA were performed by using rabbit anti-GASP (1:1,000) or rabbit anti-D2R (1:500 Chemicon) and sheep anti-tyrosine hydroxylase (TH), a marker for dopamine production (1:500, Chemicon). Secondary fluorescent antibodies were donkey anti-rabbit Cy-3 and donkey anti-sheep FITC (1:250). Mounted sections were examined by using LSM 510 laser confocal microscope (Zeiss), or Nikon Eclipse E600 microscope equipped with Spot-2 color CCD camera (Technical Instruments, San Francisco, CA). Preincubation of the anti-GASP antibody with the protein used for the immunization, resulted in a loss of immunostaining.

Coimmunoprecipitation from Rat Brain
Rat brain synaptosomes were prepared from adult rats as described (Hunter, Burstein et al. 1986) and lysates prepared in 0.1% Triton X-100/150 mM NaCl/25 mM KCl/10 mM Tris•HCl , pH 7.4, with protease inhibitors (Sigma) and Complete (Roche Diagnostics). Cleared lysate was incubated with either anti-mouse dopamine D2 receptor D2R antibody-coated (2.5, 5, and 10 µg/ml, Santa Cruz Biotechnology no. SC5303) protein G-agarose beads (Invitrogen) or 1 mg/ml BSA-coated (Sigma) protein G-agarose beads for 2 h at 4°C, washed extensively with 1% Triton X-100/150 mM NaCl/25 mM KCl/10 mM Tris•HCl, pH 7.4, with protease inhibitors (Sigma and Roche Diagnostics), and then deglycosylated with PNGaseF (New England Biolabs) for 2 h. Sample buffer was added, and the samples were boiled for 5 min at 95°C. Precipitates were resolved on a 4-20% gradient Tris•HCl precast gel (Bio-Rad), transferred to nitrocellulose, and the blots cut below the 75-kDa marker band to separately immunoblot for either D2 receptor (lower blot, monoclonal anti-D2R, Santa Cruz Biotechnology, 1:250 overnight at 4°C) or GASP (upper blot, 1:1000, overnight at 4°C). Immunoreactive bands were detected by using either HRP-conjugated anti-rabbit (GASP) or HRP-conjugated anti-mouse antibody (D2R) (NEB) (1:4,000), then visualized with ECL plus (Amersham Pharmacia).

Study III

Cell Culture and DNA Constructs

IMR90 human embryonic lung fibroblast cells (American Type Culture Collection) were grown in Minimum Essential Medium containing 200 U/ml penicillin, 200 μg/ml streptomycin, 2 mM L-glutamine (Invitrogen) and 10% fetal bovine serum (FBS) (HyClone) in 5% CO2 at 37°C. HEK293 cells (American Type Culture Collection) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL) supplemented with 10% FBS in 10% CO2 at 37°C. The human B2R and B1R cDNA were subcloned into a pcDNA3.1 vector containing a zeosin selection marker. An N-terminal artificial signal sequence, as previously described (Whistler et al., 2002), and the FLAG sequence tag were added in series to make the B2R and B1R constructs named SFB2 and SFB1, respectively. Receptor constructs with exchanged C-terminal tails starting from the first amino acid after the NPXXY motif (B1CB2 and B2CB1) and B1R truncated after residue Phe319 (B1Stop320), which is located 7 residues beyond the NPXXY motif, were created by PCR. GFP-β-arrestin 2 was kindly provided by Dr. Marc Caron, Duke University Medical Center, Durham, N.C., USA (Barak et al., 1997). The cells were transfected using the calcium phosphate precipitate method. Single colonies were then chosen and propagated in the presence of selection-containing media to generate clonal stable cell lines.

Radioligand Binding Assay

Assays were performed on particulate preparations and on intact cells as previously described (Leeb, Mathis et al. 1997; Phagoo, Poole et al. 1999). Pretreatment of intact cells with agonist at 37ºC was followed by a 6-min wash with ice-cold 50 mM glycine-HCl, pH3, and two washes in ice-cold phosphate-buffered saline (PBS) to remove the agonist.

Phosphoinositide Hydrolysis

Confluent cells were labeled with 0.4 μCi/well [3H]myoinositol for 16-20 hrs in inositol-free DMEM containing 0.5% BSA in 48-well dishes, washed in Leibovitz's L-15 medium (pH 7.4) containing 10 mM LiCl, and then stimulated with increasing concentrations of agonist for 30 min at 37°C. The cells were then lysed with 100 mM formic acid, and the lysate was mixed with anion-exchange resin in a 48-well plate with a small hole in the bottom of each well. The resin was then washed sequentially with water and 60 mM ammonium formate by aspiration. Inositol phosphates were eluted from the resin with 1 M ammonium formate and counted for radioactivity in a Beckman LS6000 scintillation counter.

FACS Analysis

Confluent cells in 100-mm dishes were treated with and without agonist in DMEM, 10% FBS. The cells were then trypsinized and washed with ice-cold PBS plus Ca2+/Mg2+. Cells were then resuspended in 100 μl ice-cold PBS plus Ca2+/Mg2+ with 50% FBS, 0.4 μg M1 anti-FLAG antibody conjugated with APC using a commercial kit (Prozyme), 1 μg mouse IgG1 kappa (MOPC 21), incubated for 20 min at 4°C, which was followed by 2 washes in PBS plus Ca2+/Mg2+. The cell pellet was finally resuspended in 1 ml PBS plus Ca2+/Mg2+ with 50% FBS. Cells (~20,000) were counted by FACS, and each receptor-positive cell line was gated against untransfected HEK293 cells to reduce background staining. Mean fluorescence was calculated for the gated signal-positive population of untreated and agonist-treated cells.

Biotinylation Protection Assay

Confluent cells were washed twice with ice-cold PBS and then incubated with 0.3 mg/ml disulfide-cleavable sulfo-NHS-S-S-biotin (Pierce) in PBS for 30min at 4°C with gentle agitation. The cells were then washed twice with Tris-buffered saline (TBS) to quench the biotinylation reaction and returned to DMEM, 10% FBS for treatments. Cells were allowed to reequilibrate to growth conditions for 30 min at 37ºC prior to further experimentation. Cells labeled Total and strip in the figures were left on ice in TBS. At 37ºC, cells were treated without (NT) or with 1 μM desArg10kallidin (DAKD) or 1 μM brady-

kinin (BK) (AG) for the indicated intervals and washed twice with ice-cold TBS. The remaining cell surface-biotinylated receptors on the cells together with those cells designated strip were stripped with 50 mM glutathione, 0.3 M NaCl, 75 mM NaOH, 1% FBS for 30 min at 4°C. The glutathione was then quenched using a 20-min wash with PBS containing 50 mM iodoacetamide, 1% bovine serum albumin. Cells were then extracted in 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 25 mM KCl containing a complete protease inhibitor cocktail (Roche), and cell debris was removed by centrifugation at 10,000 x g for 10 min at 4°C. Receptors were immunoprecipitated in the extraction buffer by incubating in anti-FLAG M2 affinity resin (Sigma) overnight at 4°C. The precipitates were washed extensively and sequentially in the extraction buffer and in 10 mM Tris-HCl, pH 7.4. The receptors were then deglycosylated for 2hr at 37°C with PNGase F (New England Biolabs). Proteins were denatured in sodiumdodecylsulfate (SDS) sample buffer without reducing agent, fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and the membrane was blocked in TBS containing 0.1% Tween-20 and 10% nonfat milk for at least 1 hr. Biotinylated proteins were visualized by incubating with the Vectastain ABC immunoperoxidase reagent (Vector Laboratories) followed by development with ECL reagents according to the manufacturers instructions (Amersham).

Biotinylation Degradation Assay

Confluent cells were washed twice with ice-cold PBS and then incubated with 0.3 mg/ml disulfide-cleavable sulfo-NHS-S-S-biotin in PBS for 30 min at 4°C with gentle agitation. The cells were then washed twice with TBS to quench the biotinylation reaction and returned to DMEM and 10% FBS. Cells were then left on ice (Total) or returned to 37°C for 30 min after which they were incubated without (NT) or with 1 μM DAKD or BK (AG) for 120 min at 37°C. The receptors were then extracted, immunoprecipitated, and detected as described above under “Biotinylation protection assay.”

Fluorescence Microscopy

Cells were propagated in growth media on glass coverslips to 50% confluency and then treated in one of three ways. For whole cell receptor distribution experiments, cells were incubated without and with agonist or antagonist for 30 min at 37°C. For cell surface receptor redistribution experiments, cells were incubated in media containing 3.5 μg/ml primary mouse anti-FLAG M1 monoclonal antibody (Sigma) for 30 min at 37°C to label surface receptors. The cells were then incubated without agonist or antagonist or a combination of the two for an additional 30 min at 37°C. For receptor recycling experiments, cells were incubated as described immediately above followed by stripping of remaining cell surface M1 antibody by depleting the media of Ca2+ with PBS containing 0.1% ethylenediaminotetraacetic acid (EDTA) at room temperature. The cells were then further incubated without and with agonist or antagonist for an additional 30 min at 37°C. In all experiments, cells were then fixed using 3.5% formaldehyde in PBS and permeabilized using 0.1% Triton X-100. For visualization of receptors, fixed specimens were incubated with Alexa488-labeled anti-mouse IgG2b antibody (Molecular Probes) to detect FLAG M1 antibodies bound to the tagged receptors. For colocalization of FLAG-tagged receptors with LAMP 1 and LAMP 2, primary H4A3 and H4B4 mouse IgG1 (Developmental Studies Hybridoma Data Bank) antibodies were incubated with secondary Alexa568-labeled anti-mouse IgG1 antibody (Molecular Probes), to detect LAMP 1 and LAMP 2, along with secondary Alexa488-labeled anti-mouse IgG2b, to detect FLAG M1 antibodies bound to receptors. Images were collected by confocal microscopy using a Nikon Eclipse confocal microscope. Quantification of colocalized fluorescence was done using the Imaris software (Bitplane AG).

β-Arrestin Recruitment

Cells stably expressing GFP-β-arrestin 2 were transiently transfected with SFB1, SFB2, SFB1CB2, or SFB2CB1. Transfected cells were plated on polylysine-treated glass coverslips in 6-well dishes for 24 h before assay. The cells were then incubated in growth media without and with agonist for 5 min

at 37°C. Cells were then fixed in PBS containing 3.5% formaldehyde for 30 min followed by primary staining with M1 anti-FLAG antibodies and secondary staining with Alexa568-labeled anti-mouse IgG1 antibody. Images were then collected by confocal microscopy using a Nikon Eclipse confocal microscope.

In Vitro Affinity Assay of GST Fusion Proteins and GASP.
B1R and B2R receptor Cterminal tails starting after the NPXXY motif were cloned into the pGEX4t1 vector and Cterminal tail-GST fusion proteins were produced and attached to glutathione-Sepharose (Sigma). Full length GASP was in vitro-translated using the TNT Quick Coupled Transcription/Translation Systems (Promega) with 35S-labeled methionine. Fusion proteins and radiolabeled probe were mixed in a wash solution containing 0.1% Triton X-100 and incubated for 1 hr at room temperature. Glutathione resin was washed several times followed by denaturation by boiling in SDS sample buffer and proteins fractionated by SDS-PAGE. Proteinconcentrations of receptor tails and controls were estimated by Coomassie staining of the gel and pulled down probe was visualized by autoradiography.

Co-Immunoprecipitation of Receptor and GASP.
Confluent cells were washed twice with ice-cold PBS and lysed in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM Na2HPO4) with complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 14,000 xg for 15 min at 4ºC. An aliquot of the cleared lysates was withdrawn from each sample tocompare the GASP concentration by immunoblotting as described below. The rest of the cleared lysate was immunoprecipitated using 15 μl M2 anti-FLAG affinity resin (Sigma) for 1 hr at 4°C, washed extensively with 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 25 mM KCl containing a complete protease inhibitor cocktail (Roche), and deglycosylated with PNGaseF (New England Biolabs) for 2 hr. Precipitates were resolved on a 4-20% gradient Tris-HCl precast gel (Bio-Rad) and transferred to a PVDF membrane. The blots were cut below the 75-kDa marker band to separately immunoblot for either receptor (lower blot) or GASP (upper blot). GASP blots were incubated for 2 hr at room temperature with rabbit anti-GASP (1:1000) and for 1 hr at room temperature with HRP-conjugated anti-rabbit antibody (New England Biolabs) (1:4000), then visualized with ECL Plus (Amersham Pharmacia). Receptor blots were incubated for 2 hr with biotinylated FLAG M2 antibody (1:250) (Sigma) and then visualizedwith streptavidin overlay (Vectastain ABC reagents, Vector Laboratories) and ECL Plus.

Data Analysis.
Data was analyzed by the Student's t-test as indicated.

Study IV

Transmission Electron Microscopy
Postembedment staining of lowicryl-embedded specimens. Rat spinal cord dorsal horn, cervical level were stained with anti-B1R and -B2R antibodies at 1:1000 concentration according to Larsson and Broman, personal communication.

RESULTS AND COMMENTS

I. Modulation of Postendocytic Sorting of G Protein-Coupled Receptors

The opioid receptors μ and δ have previously been shown to rapidly undergo endocytosis via a phosphorylation-, β-arrestin 2-, and clathrin-dependent pathway in response to agonist activation. It has further been shown that these two receptors differ in their postendocytic where MOR rapidly recycles and resensitizes, whereas DOR is rapidly downregulated. Earlier studies also show that the distal carboxyterminal domain is crucial for the recycling and resensitization of the MOR. The aim of this study was to characterize the differences between MOR and DOR in postendocytic sorting after agonist-induced internalization.

Alterations of MOR by introducing the carboxyterminal region of DOR led to desensitization, lack of resensitization, and downregulation of the receptor protein in response to agonist activation. Similarily DOR was substantially degraded within 3 hr of agonist stimulation, but this degradation phenotype was abated in a DOR/MOR chimeric receptor consisting of the DOR with a MOR carboxyterminal domain. Confocal microscopy of these two receptors indicated that the difference in postendocytic sorting was due to the specific targeting of DOR to late endosomes and lysosomes.

We next sought to identify DOR carboxyterminal interacting protein species by using the yeast two-hybrid technique. Several of the clones identified coded for parts of the carboxyterminal of a large protein expressed in many tissues and enriched in the central nervous system. The gene had previously been cloned by the Kazusa DNA Research Institute in Japan and coded for a 1395 predicted protein, clone KIAA0443. We raised an antisera for the protein using the carboxyterminal part of the protein. Staining of HEK293 cells showed a diffuse cytosolic localization of GASP. In vitro affinity and co-immuno precipitation of receptor and target protein from a heterologous cell model confirmed the preferential affinity of the protein for DOR over MOR. We suggested that the protein should be named G Protein-Coupled Receptor Associated Sorting Protein, acronym GASP.

Further, we showed that, when expressed alone, the carboxyterminal domain of GASP (cGASP) could displace the binding of full length GASP to DOR both in vitro and in the heterologous expression system. We therefore studied the effect of overexpression of cGASP on DOR postendocytic sorting. Confocal microscopy showed that DOR, when coexpressed with cGASP, was able to recycle to the plasma membrane after agonist-stimulated internalization, which indicated an altered receptor postendocytic sorting fate. This was further supported by the findings that endocytosed DOR no longer colocalized with late endosomal and lysosomal markers. The biotinylation protection assay confirmed normal agonist promoted receptor internalization, but an increased DOR protein stability in the presence of agonist over 3 hours. Further, radioligand binding showed a significantly increased number of surface DOR after agonist exposure when DOR was coexpressed with the truncated form of GASP. Normal degradation rates of the EGFR in cGASP-expressing cells indicated that the degradative pathway per se was not adversely affected in these cells, but instead that the effects were due to a specific interference with the sorting machinery of the DOR. Thus, cGASP acts as a dominant-negative regulator of postendocytic GPCR lysosomal targeting.

The in vitro affinities of the carboxy-terminal domains of the V2R, β2-AR, α2b-AR, and dopamine D4R were also determined in order to establish whether the GASP protein could influence the postendocytic sorting of other GPCR. α2b-AR carboxyterminal showed strong interaction with GASP whereas the V2R carboxyterminal showed essentially no interaction, both results which are in line with earlier reports of the postendocytic fate of these two receptors. The postendocytic fate of D4R is unknown, but this result may indicate that this receptor has a degrading phenotype. Surprisingly, the normally recycling β2-AR (see text above for mechanism) showed a strong interaction with GASP. A degrading mutant form of β2-AR, β2-AR-Ala, exhibited affinity for cGASP in a heterologous model system, and overexpression of cGASP was able to decrease the rate of β2-AR-Ala degradation. It has been shown in a previous study that ubiquitination of the β2-AR leads to degradation of the receptor by decreasing the receptor affinity for a protein necessary for recycling and thereby possibly allowing a receptor GASP interaction. However, since ubiquitination of DOR has been reported not to be necessary for lysosomal targeting, and since we show here that GASP has a high affinity for unmodified receptor carboxy-terminals in vitro, ubiquitination of the receptor is not an absolute prerequisite for GASP binding.

Significance

This study shows that, in addition to covalent receptor modifications, a direct protein-protein interaction between a GPCR and the sorting protein GASP can lead to the targeting of the receptor to a degrading pathway. This raises the possibility that modulation of receptor protein stability through the targeted disruption of a receptor-GASP interaction may alter tissue receptor responsiveness.

II. Dopamine Responsiveness is Regulated by Targeted Sorting of D2 Receptors

The dopamine receptors D1R and D2R differ in their response to prolonged agonist exposure in that D1R surface numbers are barely affected, whereas D2R show a significant decrease in surface receptor density. The aim of this study was to elucidate postendocytic trafficking of the D2R and investigate whether GASP influence the postendocytic fate of receptor.

We first established stable cell lines expressing D2R, D1R or double stable cell lines expressing both D2R and D1R. We then investigated the postendocytic fate of the two receptors in these cells. Confocal microscopy and the biotinylation protection degradation assay showed that D2R is unable to recycle after agonist promoted internalization, and that the receptor is degraded to a significant degree after 3 hours of agonist exposure. In contrast, the D1R readily recycled back to the plasma membrane and showed no significant degradation after 3 hours of agonist exposure. The two receptors retained their respective postendocytic fate when coexpressed in the same cells indicating that proximity of the two receptors does not confer postendocytic sorting – an important point since coexpression of these two receptors in neuronal tissue has been reported.

Due to the similarities between the D2R and DOR postendocytic sorting we next examined the GASP affinity for D2R and D1R. GASP showed a considerably greater affinity for the D2R in our model system. Further, we were able to establish that the dominant-nega-

tive model for GASP, cGASP, was able to both facilitate recycling of the D2R and delay agonist-promoted degradation of the receptor in our model cell line.

We next found evidence for the in vivo interaction between D2R and GASP by coimmunoprecipitation of the D2R and GASP from rat brain. We were also able to establish a higher affinity for D2R over D1R carboxyterminal tails in rat brain lysates. Further, we established in vivo proximity between D2R and GASP by showing that GASP and tyrosine hydroxylase colocalized in rat ventral tegmental area (VTA) neurons.

Earlier studies have shown that recycling receptors resensitize, and that this resensitization can be measured as receptor activity after rechallenge using the patch-clamp technique. Patch-clamp whole cell analysis of rat VTA neurons showed that D2R responsiveness to agonist is desensitized after a single round of agonist exposure, and that the receptor is unable to resensitize over a period of 35 minutes. We next tried to find ways to deliver the dominant-negative mutant cGASP to the ex vivo brain slice neurons in order to test if we could alter receptor sorting and thus activity. However, we failed to establish a functional protocol for this response. Instead, we discovered that a high dose of GASP antibodies, raised against the receptor binding carboxyterminal part of the protein, were able to displace GASP from the D2R carboxyterminal tail using an in vitro affinity protocol. We also showed that inclusion of the antibody in the pipette buffer allowed the antibody to enter the rat VTA tyrosine hydroxylase positive neurons during whole cell patch. Dendritic spines, the presumed neuronal localization of D2R, was clearly shown to be filled with antibodies by this technique. Inclusion of the antibody in the patch-clamp protocol caused a degree of hyperpolarization of the neuron in response to the secondary stimulation. This response was further blocked by a D2R specific antagonist thus establishing that the inclusion of the antibody manifests as a rescue phenotype on D2R responsiveness to a second round of agonist stimulation.

Sginificance

This study shows that the GASP protein consistently interact with certain GPCR and support the sorting of these receptors to the degrading pathway. Further, this study shows that a GASP-receptor interaction can take place in vivo. Also, this in vivo interaction leads to a functional desensitization after agonist exposure and that responsiveness can be rescued by abbrogation of the D2R/GASP interaction. The study also indicates that data obtained from the HEK293 cell model system may be directly translatable to in vivo phenotypes of GPCR.

III. Bradykinin Receptor Regulation in Response to Agonist Activation

Earlier studies on the membrane trafficking of the bradykinin receptors B1R and B2R have generally employed highly modified receptors. Also, the use of several different model systems and the tendency to rely on indirect approaches such as radioligand binding and/or internalization have created a rather complex and sometimes contradictory picture of bradykinin receptor trafficking. The aim of this study was therefore to establish a model of heterologously expressed minimally modified receptors in HEK293 cells, and utilize this model to characterize bradykinin receptor trafficking.

An initial finding was that a 30-minute period of agonist stimulation of endogenously expressed receptors in a lung fibroblast cell line, IMR90, resulted in an increase and a decrease in the surface density of the B1R and B2R, respectively, as assessed by ligand binding. This phenotype was confirmed in our heterologous model system, ruling out up-regulation of gene expression as an explanation for the increased surface density of B1R.

Earlier studies had established that B2R is rapidly phosphorylated on several serine and tyrosine residues in the cytosolic carboxyterminal domain, whereas the B1R fails to undergo such modification. Because of the important role that the carboxyterminal plays in facilitating internalization, we decided to create receptors with exchanged carboxyterminal domains. These chimeric receptors showed an altered internalization phenotype in that B1CB2 acquired the ability to endocytose in response to agonist, whereas B2CB1 lost endocytic responsiveness to agonist. Further, we established that B2R, but not B1R, is able to recruit β-arrestin in response to agonist activation, and that these respective abilities were completely transplantable through the carboxyterminal domain exchanges.

We also showed that B1R, unlike B2R, is unstable on the plasma membrane in the absence of agonist, and is constitutively endocytosed. Again, this phenotype was fully reversed in the chimeric B1CB2 and B2CB1 receptor chimeras. Interestingly, the rate of internalization of the B1R was significantly decreased by the agonist. We also showed that regardless of the route or mode of entry, receptor internalization for all constructs could be blocked by disrupting clathrin coat formation, whereas lipid raft disassembly had no effect on the internalization process.

The postendocytic fate of the bradykinin receptors was investigated using confocal microscopy, biotinylation protection, and biotinylation degradation assays, and these assays clearly showed that B1R is targeted for degradation through the late endosomal and lysosomal pathway. B2R, on the other hand, was able to recycle back to the plasma membrane after agonist induced internalization. Again these postendocytic fates were fully transplantable through the carboxyterminal domain.

Given the similarity of the postendocytic fate of the B1R to that of D2R and DOR and the obvious reliance on the carboxyterminal domain for the proper postendocytic sorting, we tested the affinity of GASP for the two receptors. Both in vitro affinity and coimmunoprecipitation showed that the B1R, and in particular the carboxyterminal domain of the B1R, showed a greater affinity for GASP than the B2R, and that this affinity was at least in part transplantable via the carboxyterminal tail. We next tried to establish cells that coexpressed B1R and the dominant negative form of GASP, cGASP, but we were unable to attain any viable clones. Nevertheless, studies of the stability of the receptors starting on the plasma membrane clearly showed that the carboxyterminal domain of the B1R determines the rate of receptor degradation. Further, alterations that lower the receptor affinity for GASP reduce the rate of receptor degradation and vice versa.

Significance

This study shows that an endogenous cognate agonist can act as an inverse agonist on receptor endocytosis. This implies that future drug target investigations of B1R ought to address the effect of the candidate drug on the B1R plasma membrane density and stability. Again, the data obtained from our heterologous HEK293 cell model system matched those of an endogenously expressing receptor model

– lung fibroblast IMR90 cells, adding weight to the choice of HEK293 cells as a sensible choice of model system to assess GPCR function. Further, this study showed that the agonist-promoted internalization of B2R proceeds through a β-arrestin 2 and clathrin-dependent pathway, whereas constitutive and agonist-promoted internalization of the B1R proceeds via a β-arrestin-2-independent but clathrin-dependent pathway. Both the mode of internalization and the postendocytic fate are determined by the carboxyterminal domain of these two receptors.

IV. Regulation of the Bradykinin B1 Cell Surface Stability

Very little is known about bradykinin receptor expression in vivo. I investigated B1R and B2R expression and distribution in rat spinal cord using two antisera produced in our lab. Transmission electron microscopy indicated that both receptors are expressed in lamina II of the dorsal horn, in postsynaptic densites (Fig 5A, below). Further immunohistological investigation of naïve rat brain sections indicated that B1R is expressed in several areas where B2R has been reported to be expressed (data not shown). The finding that B1R and B2R are co-localized in naïve neuronal tissue led us to investigate the effect, if any, of receptor co-expression on B1R and B2R localization and activity.

To this end, differentially tagged receptors (Flag-B1R and HA-B2R) were co-expressed in HEK293 cells. The expression, trafficking, and activity profiles of the two receptors were then evaluated. Each receptor expressed to the same degree whether it was present alone and in combination with the other. Data from confocal microscopy and the biotinylation protection assay indicated that spontaneous B1R internalization is reduced by co-expression with B2R. Further, stimulation with the B2R- specific ligand bradykinin resulted in internalization of both receptors (Fig. 5B). B2R agonist-stimulated PI hydrolysis was significantly increased by receptor co-expression, whereas B1R agonist-stimulated PI hydrolysis was almost completely abolished (Fig. 5C and D). At least two possible explanations exist for the reduced B1R activity. These are 1) a direct inhibitory effect of an interaction with B2R, and 2) an inhibitory effect by the stabilizing B1R in the plasma membrane. We addressed these two possibilities by determining the effect of stabilizing B1R on the cell surface using hypertonic media as described in study III. Doing so significantly reduced the ability of B1R to mediated agonist stimulation of PI hydrolysis. On the other hand, B2R was completely unaffected by this treatment (Fig 5E).

Based on these findings, I propose that when present in the plasma membrane in the absence of agonist B1R spontaneously and irreversibly desensitize over time.

In contrast to B1R, hypertonic media did not affect the agonist-stimulated activity of B1CB2, in which the B2R carboxy-terminal tail had been substituted in B1R. It has been reported that the carboxy-terminal domain of B1R is not phosphorylated in response to agonist stimulation. I found here that this B1R domain is also not palmitoylated (data not shown). This is in sharp contrast to the B2R carboxy-terminal domain, which is both phosphorylated and palmitoylated. Thus, the carboxy-terminal domain of B1R lacks all the typical GPCR covalent modifications. Nevertheless, the B1R domain still seems to be crucial in B1R desensitization.

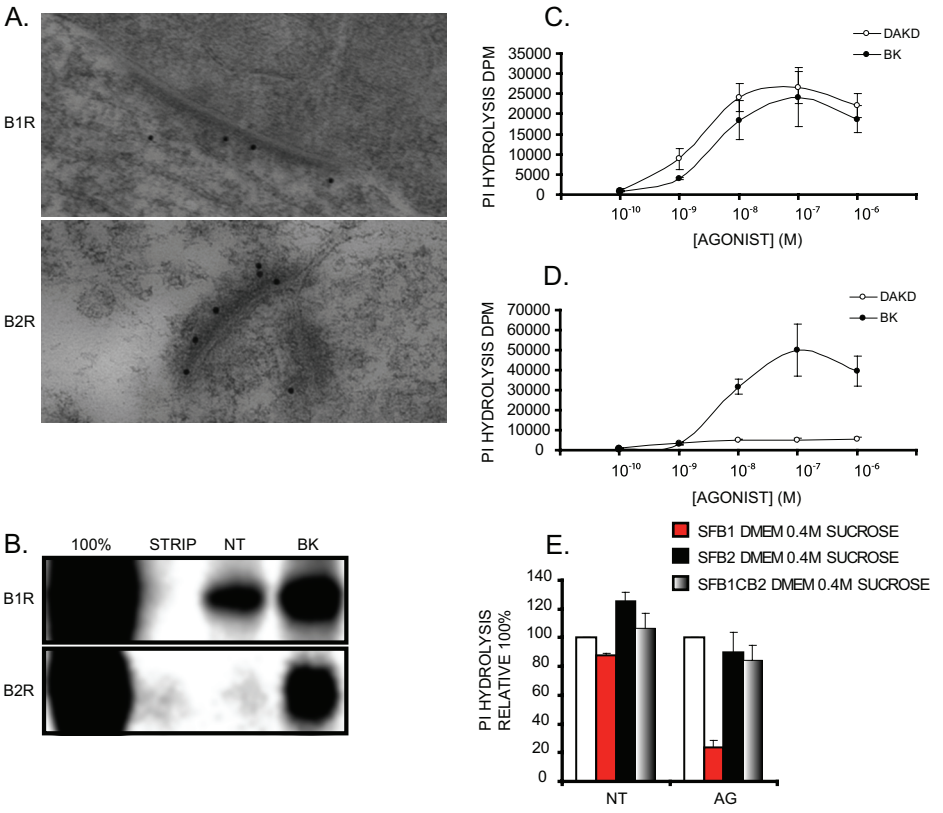
To begin to search for partners with which the B1R carboxy-terminal domain interacts, GST-chromatography combined with mass-spectrometry was performed. Two putative

B1R interacting proteins were found in HEK293 lysates including heat-shock protein of 70 kDa (Hsp70) and elongation factor 1α (eEAAF1α). Hsp70 has been reported to aid in the selection of unfolded proteins in the endoplasmic reticulum for the ERAD system, leading to proteasomal targeting. Hsp70 has also been implicated in the uncoating of endocytic vesicles. On the other hand, eEAAF1α has been shown to interact with the muscarinic receptor M4MR (see section on postendocytic choices) and to attenuate recycling of this receptor to the plasma membrane.

Significance

This study shows that B1R and B2R are coexpressed in vivo, and that coexpression in vitro leads to stabilization of B1R on the cell surface. Further, B1R activity is spontaneously attenuated when the receptor is stabilized on the plasma membrane. This stabilization is not due to covalent modifications of the carboxy-terminal domain. Hsp70 and eEAAF1α are two putative proteins that may influence receptor activity and/or trafficking by interacting with the B1R carboxy-terminal domain.

Fig.5



CONCLUSIONS AND FUTURE PERSPECTIVES

In these studies, I have shown that the ability of a GPCR to signal in response to activation in a tissue can be modulated by manipulations of the receptor membrane trafficking phenotype. Further, manipulation of receptor membrane trafficking can either be achieved via biased agonism or by targeted disruption of the interaction between a receptor and the receptor trafficking machinery. Thus, this work is indicative of the significant value of expanding our knowledge of specific mechanisms governing the membrane trafficking of GPCR. To compile and utilize the great amount of data that continues to be collected on GPCR trafficking is a formidable future task.

The identification and characterization of the protein GASP raises the possibility that influencing this protein can modulate the responsiveness of a cell to a drug. Indeed, the targeted disruption of the interaction between GASP and a specific GPCR could potentially increase the therapeutic window for a drug by reducing receptor degradation and increasing receptor recycling. Further, a biased agonist that increases or decreases the affinity of a GPCR for an interacting protein such as GASP would similarily have the potential to modulate the efficiency and longevity of agonist signaling through that receptor. These are intriguing opportunities in terms of DOR and D2R since both of these receptors are important clinical targets in several pathological conditions, but are both known to rapidly downregulate in response to agonist activation, presumably in part via GASP.

GASP has now been shown to be part of a larger family of proteins. Thus, the GASP protein investigated in the three studies presented here is now connoted GASP-1. It would be of great general interest to further investigate the role(s) of the other members of this family on GPCR trafficking. Knock-out animal models of GASP-1 and the close homolog GASP-2 are currently under construction in our laboratories.

B1R is currently one of the most interesting therapeutic targets for inflammatory pain. In sharp contrast to DOR and D2R, B1R fails to desensitize and is stabilized on the plasma membrane in response to the cognate agonist des-Arg10-KD. Enhanced B1R signaling, which is induced under pathological conditions, would thus be expected to continue to proceed until the agonist is cleared. The effect of other ligands on B1R stability is currently unknown. The failure in the past decade to produce an efficient inverse agonist may indeed reflect the fact that current ligands stabilize the receptor on the cell surface. We propose that screening for ligands that enhance B1R endocytosis is a possible effective alternative approach to develop a receptor antagonist.

We interpret my data on B1R constitutive desensitization and internalization to mean that B1R ability to signal is dependent on both the time spent on the plasma membrane, and in which conformation B1R is on the plasma membrane. Further we believe that the full length receptor contains carboxyterminal domains necessary for the stabilizing effect of des-Arg10-KD. Thus we hypothesize that prolonged periods of time spent on the surface in an agonist bound state results in decreased desensitization whereas receptors stabilized in a non-agonist bound state tend to desensitize. Since B1stop320 desensitizes over time but is less proned for internalization than the B1R one might speculate that desensitization and internalization are events determined by separate domains in the B1R. Further, we and others have failed to detect any modifications of the B1R carboxyterminal domain regardless of ligand occupancy. This greatly simplifies the search for putative interaction partners with this B1R domain.

Finally recycling of GPCR has recently come into focus, and the view that GPCR recycle via a default bulk membrane flow has been challenged. If B2 recycling is regulated by a receptor specific machinery, this machinery may be a good future drug target for analgesic intervention to acute pain. We have already shown that the B1CB2 has an altered lowered affinity for GASP and show a robust recycling phenotype. Further the carboxyterminal domain of the B2R is isolated in this chimera allowing us to specifically address the role of this domain. We also have an eminent tool for finding B2R tail interacting proteins using our constructed GST-carboyterminal tail fusion protein construct. A future approach in determining the mode of B2R recycling could therefore be to 1) construct and investigate postendocytic trafficking of the B1stopNPXXY; 2) use the B2R-gst fusion protein in the masspectrometry setup to look for interaction partners; 3) find the motifs necessary for the interaction with these proteins via mutational analysis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

På alla nivåer av cellbaserat liv är effektiv kommunikation med omgivningen ett krav. Cellens alla livsviktiga funktioner kräver dock ett skydd i form av ett dubbelt lipidlager, det så kallade cellmembranet. Samtidigt som ett intakt cellmembran är en förutsättning för cellens överlevnad utgör det ett problem då det skiljer cellen från signaler från dess omvärld. Signalöverföring via proteiner som sträcker sig över båda lipidlagren är lösningen för att kunna ta in information, bearbeta den och reagera korrekt på de miljömässiga krav som ställs.

Den största enskilda gruppen av signalerande cellmembranproteiner heter G protein-kopplade receptorer. De har blivit döpta baserade på en bland dem allmän signaleringsprincip – genom aktivering av ett så kallat G protein på insidan av cellmembranet. Gruppen utgörs av drygt 1000 kodande regioner i den mänskliga arvsmassan – eller cirka 3% av alla de sekvenser som kodar för proteiner. G protein-kopplade receptorer har väldigt varierad aminosyrasekvens men är istället lika i deras fysiska utformning. De består alla av sju stycken regioner, aminosyrasekvenser, som sträcker sig över membranet, med sammanhållande intra- och extracellulära bryggor samt en extracellulär så kallad N-terminal och en intracellulär C-terminal. Strukturen kan liknas vid en orm som har flätat sig upp och ner genom membranet och det reflekteras i ett av de engelska namnen på dessa receptorer – serpentine receptors. De yttre regionerna kan reagera på de substanser, så kallade ligandrer som kan interagera med receptorn. De inre regionerna har som uppgift att förmedla signalen på insidan av cellen samt utgöra en yta av receptorn genom vilken cellen kan reglera dess funktion.

De olika varianterna av G protein-kopplade receptorer reagerar på snart sagt alla typer av ligandrer inklusive ljus, lukt, smak, ämnen frisatta på grund av vävnadsskada, hormoner och nervsignalsubstanser, för att nämna några. Dessa receptorer är mer eller mindre involverade i alla fysiologiska förlopp i alla celler och vävnader i hela kroppen. Detta faktum avspeglas i att nästan 40% av alla läkemedel, och över 90% av alla läkemedel verksamma i vår hjärna och ryggmärg, riktar sig mot dessa receptorer.

När en ligand binder till en G protein-kopplad receptor överförs signalen via rörelser i receptorproteinet till G proteinet på insidan av cellen. Dessa rörelser uppstår på grund av strukturförändringar som sker då liganden binder till proteinet och ger i sin tur upphov till mätbara elektriska signaler. Överföringen startar en kaskad av signaler vars effekt kan vara allt från aktivering av gener, produktion av proteiner, cellförflyttning, nyproduktion av DNA och celldelning, till frisättning av fria radikaler och fortledning av elektriska signaler längs meterlånga neuron.

För att åstadkomma rätt effekt vid exakt rätt tillfälle krävs att cellen har förmåga att noga kontrollera receptorns aktivitet. En mängd regleringsmekanismer finns därför tillgängliga som begränsar receptorns signalering. De snabbaste sätten att reglera sker genom direkta förändringar av receptorproteinet, så kallad fosforylering och avkoppling av G proteinet. Det finns även en uppsättning långsammare men mera vittgående regleringsmekanismer som innefattar förflyttningar av hela delar av cellmembranet tillsammans med de receptorer som sitter trädda genom detsamma in i cellen. Detta sker genom bildandet av små lipidbubblor - vesikler. Denna senare typ av reglering kallas membrantrafik. De snabba kontrollmekanismerna är nu ganska väl förstådda, däremot är kunskapen om de långsammare mekanismerna fortfarande mycket begränsade. När receptorer befinner sig i vesikler

inuti cellen finns två möjliga val – antingen kan de snabbt återvända till ytan, eller så kan de brytas ner. Det första valet leder till att cellen snabbt kan återanvända receptorn, det vill säga cellen kan åter igen svara på liganden. Det senare valet leder till att cellen inte kan svara på ny stimulering med den typen av ligand innan cellen har producerat en ny uppsättning av receptorer, ett betydligt långsammare förlopp.

Hittills och kanske av tradition har farmakologisk forskning inriktat sig på att undersöka hur läkemedlen reagerar med den yttre delen av receptorn. Undersökningarna som utförts har baserats på vilken effekt som kunnat mätas inuti cellen som respons på inbindningen av liganden till ytan. Däremot har nästan ingen hänsyn tagits till de långsammare regleringsmekanismerna, membrantrafik har mest setts som en obskyr sidoeffekt. Detta är givetvis ett förhållningssätt som på flera plan ger en skev bild av de fysiologiska förloppen och möjligheterna att påverka desamma. Primärt riskerar man att ge upphov till bieffekter genom att inte tillgodose alla regleringsmekanismer. Dessutom förloras möjligheten att finjustera aktiviteten som utförs av den receptor som man undersöker.

Målet med mina doktorandstudier är att åskådliggöra effekten av membrantrafik på regleringen av tre grupper av inbördes närbesläktade G protein-kopplade receptorer. Alla tre grupperna: opiatreceptorer, dopaminreceptorer och bradykininreceptorer, är involverade i en rad sjukdomsförlopp. Opiatreceptorer och dopaminreceptorer, som båda hämmar smärta, anses vara centrala i utvecklandet av missbruk, de ligger bakom motivationen till att använda droger. Dopaminreceptorer anses också vara viktiga faktorer vid utvecklingen av schizofreni och vid koncentrationsstörningar som ADHD. Bradykininreceptorerna är involverade i utvecklandet av tumörer samt reglering av inflammation, blodtryck och smärtsignalering. Kort sagt, en ökad förståelse för den fysiologiska regleringen av dessa receptorer skulle potentiellt vara till mycket stor klinisk nytta.

Under mina studier har jag gjort två stycken huvudsakliga fynd och ett viktigt metodologiskt framsteg som kommer att underlätta framtida studier.

För opiatreceptorn delta och dopaminreceptorn 2l, har jag tillsammans med min forskargrupp funnit och karakteriserat ett protein som vi har döpt till GASP (engelska för GPCR Associated Sorting Protein). Detta protein deltar i sorteringen av dessa båda receptorer inuti cellen och medför degradering av receptorerna. Jag har visat att blockering av detta proteins funktion gör att receptorerna kan återvända till ytan där de kan återanvändas. Denna återanvändning ger därmed potentiellt en ökad effekt av läkemedel riktade mot dessa båda receptorer.

För bradykininreceptorn B1 har jag visat att en kroppsegen ligand kan stabilisera receptorn på ytan och öka dess signaleringsförmåga. Eftersom B1 receptorn är involverad i signalering av smärta är konsekvensen av denna slutsats att den kroppsegna liganden ökar smärtsignaleringen. Jag har också visat att de receptorer som tas in i cellen bryts ner och att detta möjligen regleras av GASP. Slutsatsen blir att en ligander som ökar membrantrafiken av B1 receptorn har potentialen att verka som ett smärtstillande medel.

Min karakterisering av B1 receptorn har vidare givit vid handen att den så kallade C-terminalen av denna receptor är, till skillnad från andra receptorer, omodifierad. Detta är en del av G protein-kopplade receptorer som brukar modifieras kemiskt, genom addering av molekyler, som del av de snabba regleringsmekanismerna. Denna typ av reglering verkar saknas i B1 receptorn vilket radikalt ökar möjligheterna att söka efter andra proteiner som

interagerar med B1 receptorn via C-terminalen. Baserat på dessa fynd har jag ställt samman en försöksuppställning som syftar till att snabbt isolera och karakterisera proteiner som interagerar med denna domän i B1 receptorn oavsett vilken cellvävnad receptorn uttrycks i. Med hjälp av denna metod är det dessutom enkelt att med stor noggranhet avgöra exakt vilka aminosyror i C-terminalen som styr de potentiella interaktioner man upptäcker. Mina preliminära resultat indikerar att B1 via sin C-terminal interagerar med proteiner som styr både utmognad av proteinet samt tidigare nämnd membrantrafik. Dessa fynd kan nu på ett relativt enkelt vis valideras i de cellmodeller jag har skapat.

Mina studier av membrantrafiken av receptorerna i de tre ovan nämnda receptorgrupperna har styrkt vikten av studier av dessa reglermekanismer och indikerat potentiella mål för framtida läkemedelsutveckling.

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