Human M3 muscarinic acetylcholine receptor protein-protein interactions: roles in receptor signaling and regulation

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by

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… a mis padres Isabel y Oscar,
a Elle, Carmen, et le toujours aimé Pilulo,
a mis hermanos de la diáspora
a todos los que aún no han podido cumplir sus sueños y
siguen luchando por ellos.

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CHAPTER 1

Introduction

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1.1 G PROTEIN COUPLED RECEPTORS

G-protein-coupled receptors (GPCRs) form the largest superfamily of proteins in the human body. About 1% of the human genome encodes for over 1000 GPCRs [1]. They are located at the cell surface and are expressed ubiquitously. GPCRs regulate diverse intracellular responses and are therefore involved in all kinds of diseases, making them interesting therapeutic targets [2]. The name GPCRs originates from the first step in the signal transduction that comprises the direct activation of a hetero-trimeric G protein. However, this is something of a misnomer, as G-proteins are able to couple to other proteins which are not GPCRs, and GPCRs are able to signal without G-proteins [3]. Because of this, GPCRs are more recently referred to by some authors as heptahelical, serpentine or 7-transmembrane (7-TM) receptors. This last name, 7-TM, is derived from the fact that GPCRs are transmembrane proteins, which cross the mebrane seven times. Each individual TM domain is an α-helix which is connected to the next TM domain via alternating intracellular and extracellular loops. These start with an extracellular amino.terminal tail (N-terminal) and end with an intracellular carboxyl-terminal tail (C-terminal). This basic motif has recently been verified by x-ray crystallography on bovine rhodopsin and human β-adrenergic [4,5] (Figure 1.1.1).

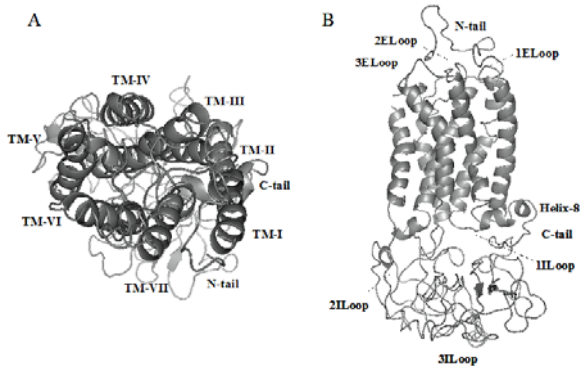

Figure 1.1.1 *Computer model of the human M_3 muscarinic receptor, based on the crystal structure of rhodopsin [4] (PDB code: 1F88), and prepared using Pymol. The structure is viewed from outside of the cell (A) and parallel to the membrane (B). The structure has five key features: 1. seven transmembrane α helices; 2. three external loops (1Eloop-3Eloop); 3. three cytosolic loops (1ILoop-3ILoop); 4. N-terminal tail (N-tail) and a C-terminal tail (C-tail); and 5. cytosolic helix VIII.*

The superfamily of GPCRs can be activated by a plethora of different stimuli. There is a great chemical diversity among the endogenous ligands, including protein hormones, lipids, peptides, biogenic amines, nucleotides and ions [6,7]. Furthermore, the sensation of exogenous stimuli, including light odor and taste, is mediated through GPCRs [6,8]. The bundle of 7-TM domains forms a cavity large enough to fit small ligands, such as biogenic amines. However, larger ligands, such as peptides and hormones, generally interact and bind to the N-terminal tail and/or extracellular loops [9].

GPCRs are involved in wide variety of diseases, including asthma, hypertension, Parkinson´s disease, cancer, and HIV infection [10,11]. The presence of GPCRs at the cell surface makes these receptors very accessible to therapeutic drugs. It is estimated that more than half of all modern drugs are targeted at GPCRs, and several ligands are found among the worldwide top-100-selling pharmaceutical products. Sales for individual drugs reach up to several billions of euros per year [12,13].

Although all GPCRs belong to one superfamily, these receptors are also highly variable and can be subdivided into smaller families on the basis of sequence homology and/or pharmacological characteristics. In general, GPCRs are divided into three major subfamilies: rhodopsin-like (family A), secretin-like (family B) and the metabotropic neurotrasmitter receptor-like (family C). The yeast pheromone (family D), the cAMP receptors (family E) and the frizzled/smoothened family make up three minor subfamilies [6,7,14,15] (Figure 1.1.2).

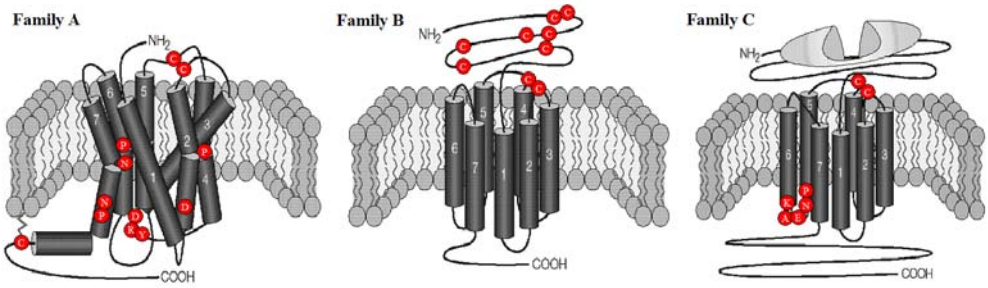

Figure 1.1.2: *GPCRs can be divided into different families on the basis of their structural and genetic characteristics. The figure shows schematic representations of receptor monomers, and illustrates in red circles some key structural aspects of the three main GPCR families (A, B, C). Figure adapted from* [16]

Family A forms the largest of the three major subfamilies, and contains 90% of all GPCRs. Family A includes, among others, aminergic receptors (muscarinergic, dopaminergic, histaminergic, serotonergic and adrenergic receptors), peptides, and rhodopsin, as well as the large subfamily of olfactory receptors. The overall homology among all type A receptors is low; it is restricted to a number of highly conserved key residues. The high degree of conservation among these key residues suggests that they play an essential role in the structural or functional integrity of the receptors. The only residue consistently present among all family A receptors is arginine, found in the Asp- Arg-Tyr (DRY) motif located on the cytoplasmic side of TM-3, which is believed to be involved in G protein activation [17]. However, other fingerprint elements such as the conserved NSxxNPxxY motif are also clearly associated with rhodopsin family. In addition, six different groups of receptors have been further classified within this subfamily of receptors with regards to their phylogeny [18].

Subfamily B consists of approximately 25 GPCRs [19]. A range of neuropeptides and peptide hormones, like glucagons, calcitonin and secretin, recognizes these GPCRs [20]. One of the hallmark features of this family is the large extracellular N-terminal tail containing numerous cysteines, which probably forms a network of disulfide bridges [20]. Unlike family A, subfamily B receptors do not contain the important DRY motif involved in the activation of family A GPCRs.

Hallmark features for family C receptors include a short and highly conserved intracellular loop 3 (3ILoop), a relatively long C-terminal tail, and an exceptionally long N-terminal tail, which is shaped like a Venus-fly trap to facilitate ligand binding (500–600 amino acids). This region shares a low but significant sequence similarity to bacterial periplasmic binding proteins (PBPs) and is considered the glutamate binding site equivalent to the known amino acid binding site of PBPs [21]. This subfamily includes metabotropic glutamate, γ-aminobutyric acid (GABA), calcium, vomeronasal, mammalian pheromone receptors, and recently identified putative taste receptors. As with subfamilies A and B, subamily C receptors contain two putative disulfide-forming cysteines in extracellular loops 1 and 2 (ECL 1 and 2). Otherwise, they do not share any conserved residues with subfamily A or B receptors.

The A-F subclassification of the GPCR superfamily is widely accepted. However, Fredriksson et al. performed the first phylogenetic study of the entire superfamily of GPCRs (comprising about 2% of the genes in the human genome) and proposed a different, more accurate classification [22]. Their analysis demonstrated that there are five main subfamilies of human GPCRs, and that they share a common evolutionary origin within each subfamily. Glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin otherwise known as the GRAFS classification, based on the initials of each of the subfamily names. Three of these subfamilies, the rhodopsin (A), secretin (B), and glutamate (C) families, correspond to the A-F clan system, whereas the two other families, adhesion and frizzled, were not included in the clan system [23]. The rhodopsin receptors make up the largest family, divided into four main groups with 13 distinct branches.
The human genome project has revealed the existence and position of all GPCRs. For several GPCRs, however, their respective cognate ligand is still unknown. These GPCRs are known as orphan GPCRs. Excluding the olfactory GPCRs, approximately 300 full open reading frame (ORFs) have been identified to encode putative GPCRs. Out of these 300, about 100 are considered orphan receptors [19]. Much effort has been invested in attempts to de-orphanize these receptors, as well as to link them to possible diseases. In addition to these orphan receptors, many gene encoding GPCRs can be alternatively spliced, adding to the complexity and magnitude of the superfamily [24].

G PROTEIN COUPLING AND HETEROTRIMERIC G PROTEINS CONCEPTS

The interaction of GPCRs with its proper heterotrimeric G protein is the first molecular step in signal transduction that regulates cellular functions [25]. The G protein consists of three subunits, Gα, Gβ and Gγ. The Gα subunit harbours an intrinsic GTPase activity, which is able to hydrolyze GTP to GDP. The Gβ and Gγ subunits form the dimeric Gβγ-subunit that can either associate to or dissociate from the Gα subunit, depending on whether or not the Gα is bound to GDP or GTP, respectively. In its resting state, the Gα subunit is bound to GDP and is associated to a hetero-dimeric Gβγ-subunit. While bound to the Gα subunit, the Gβγ-subunit inhibits the spontaneous release of GDP from the Gα subunit [26,27].

Upon agonist binding to the receptor, the activated receptor subsequently activates the intracellular heterotrimeric G protein by causing an increase in the rate of GDP-GTP exchange in the Gα subunit. Upon activation, the now GTP-bound Gα subunit dissociates from the Gβγ-subunit, and both the Gα and Gβγ-subunit subsequently activate their respective effectors. Hydrolysis of GTP to GDP by the intrinsic GTPase returns the Gα subunit to its inactive, GDP-bound state, followed by re-association with a Gβγ-subunit [3].

There appear to be more than twenty different α subunits (including splice variants), six β subunits, and fourteen γ subunits [28,29]. However, only four main classes of hetero-trimeric G-proteins can be distinguished: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$ [30]. Gs proteins stimulate adenyl cyclase; $G\alpha_i$ proteins inhibit adenyl cyclase and activate G proteins coupled inwardly rectifying potassium channels. $G\alpha_q$ proteins activate phospholipase Cβ, which leads to the hydrolysis of phosphatidylinositol biphosphate (PIP_2), resulting in the formation of diacyl glycerol (DAG) and inositol triphosphate ($InsP_3$), with corresponding increases in intracellular Ca^{2+}-ions and protein kinase C (PKC) activation [31,32]. $G\alpha_{12}$ proteins activate Rho guanine-nucleotide exchange factors [32] (Figure 1.1.3).

A second classification of G proteins can be made based on their sensitivity to Vibrio cholerae toxin (CTX) or Bordetella pertussis toxin (PTX). CTX prevents the hydrolysis of Gs GTP to its GDP bound state and, as a result, the G protein remains in the active state. PTX prevents the release of bound GDP, locking Gα in the inactive state.

Structures of all the subunits have been discovered by x-ray crystallography in various activation states and in complexes with various effector and regulatory proteins. The α subunit is structurally homologous to other members of the guanine nucleotide binding protein superfamily; the β subunit forms a β-propeller consisting of toroidal arrangement of β sheet; and the α-helical γ subunit is usually found as a complex with the β unit [33-35].

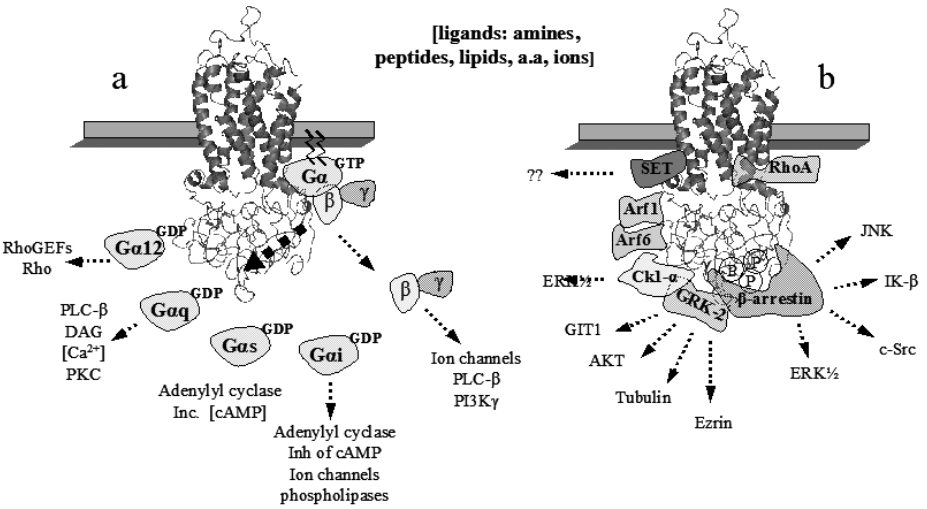

Figure 1.1.3: *Overview of GPCR signalling pathways. a, G-protein coupling and signalling pathways mediated through GPCR activation. GPCRs can be stimulated by a wide assortment of ligands like biogenic amines, ions, lipids and hormones. Activated GPCRs can transfer the extracellular stimulus into intracellular signals through binding to a variety of G proteins ($G\alpha_i$, $G\alpha_q$, $G\alpha_s$, $G\alpha_{12}$), which can activate intracellular and nuclear effectors. b, heterotrimeric G protein independent signalling mediated by GPCRs. Upon agonist stimulation of a GPCRs, β-arrestins, GRK and other scaffold/accessory proteins are recruited and activated. They subsequently activate or inhibit various signalling pathways.*

Originally, receptors were thought to interact with only one type of heterotrimeric G-protein. However, it is now understood that, depending on cellular and external conditions, GPCRs can interact diversely and signal via multiple pathways [36]. In addition, ligands have also been discovered that act as agonist in systems devoid of constitutive activity, but become inverse agonist in constitutively active systems. These ligands are known as protean ligands [37].

GPCRs can also activate small monomeric G-proteins such as Ras [38]. The Gβγ-dimer can separately activate several different signalling pathways, such as adenylyl cyclases, PLC-β, a multitude of kinases, as well as ion channels [32,39].

Moreover, GPCRs can signal independently of G proteins. This signalling is mediated by direct interaction with receptors with scaffold/accessory proteins, such as β-arrestin and G-protein receptor kinases (GRK). This results in the activation of cytoplasmic effectors such as Src, Ras and ERK, which ultimately play a role in chemotaxis and apoptosis [40,41] (Figure 1.1.3b).

GPCR STRUCTURE AND ACTIVATION

The development of therapeutic drugs acting on GPCRs is largely based on two different strategies. For a newly identified target, large libraries are tested in a high throughput screening (HTS) approach. These libraries frequently consist of a selection of known drugs, a large diversity of scaffolds, and a times a selection of naturally occurring compounds. A more rational approach to drug design can be employed when detailed information is known about the target, especially when data is obtained by mutation of the ligand bingind pocket. Rational-based drug design may benefit a great deal from the elucidation of the high-resolution structures of GPCRs. So far, the crystallization of GPCRs other than rhodopsin and the β-adrenergic receptor has not yielded high-resolution structural data [4,5]. However, using the structure of these receptors as a template, homology models of GPCR can be created, which are useful for rational drug design on these GPCRs [42].

Although all GPCRs share the same topology of a 7TM receptor, they do not necessarily share a high sequence homology. However, within the large subfamily of family A GPCRs, there are several amino acid residues which seem to be important for either the structural integrity or signal transduction, and which are conserved among these family A GPCRs [18] (Appendix 1).

Using indirect methods, some progress has been made towards an understanding of the conformational changes that are associated with GPCR activation [43]. Ultimately, however, it may require structures of multiple receptors bound to multiple ligands, including inverse agonists, agonists, and antagonists, for us to flesh out the details of the conformational changes associated with receptor activation. In addition, different agonists may channel individual receptors to different G proteins, and thereby to different second messenger systems, a phenomenon that would require the existence of multiple active conformations and further demonstrate the need for additional high-resolution structures [44].

Despite everything, the accumulated evidence suggests that GPCR activation may involve a change in the relative disposition of H-III and VI [45]. Elegant site-directed spin labelling studies carried out with rhodopsin indicated that rhodopsin activation might involve a rigid bodily movement of the cytoplasmatic end of H-

VI, away from the C terminus of H-III. In agreement with this concept, cross-linking of the cytoplasmatic ends of H-III and H-VI, either via disulfide bonds in rhodopsin or via metal ion bridges in rhodopsin and other GPCRs, prevented receptor activation [46].

Recently, the study of H1 histamine receptor activation revealed that Asn 7.45 (according to Ballesteros-Weinstein numbering [47]) might play a crucial role in transferring the conformational rearrangement of the receptor upon agonist binding, to receptor activation. These residue positions appear to function as link between the ligand binding pocket and the conformational transition of Asn 7.49 (part of the NPxxY motif), which leads to GPCR activation. The amino acids in these proposed mechanisms are conserved throughout the family A GPCRs (~70%), suggesting that the observed mechanisms may also be applicable to other rhodopsin-like GPCRs as well [48].

Several models have been proposed for GPCR activation. The most widely used model, the extended ternary complex, ETC, and its extension the cubic ternary complex, CTC, (Figure 1.1.4; for a review, see [9]), assumes that, in the absence of a ligand, there is equilibrium between two functionally and structurally different states: the inactive (low affinity) (R) and the active (high affinity) (R*). The ability of ligand to induce physiological responses - the efficacy of the ligand - varies: a full agonist binds and stabilizes the active state (R*), which has high coupling efficiency for a specific G protein (R*G). A partial agonist binds and stabilizes the active state less efficiently, and causes only a partial G protein activation. A neutral antagonist recognizes all receptor forms equivalently and has no effect on the equilibrium between (R) and (R*) states. An inverse agonist binds and stabilizes the inactive state (R), thus decreasing the basal, agonist independent, G protein activity. The ETC model can describe these different events. The CTC model includes the possibility that the inactive receptor forms (R and AR) interact with G protein, which is not allowed in the ETC model. Although the CTC model is formally more correct than the ETC model, this correctness comes at a price of carrying too many parameters to allow for useful estimation based on experimental observations. In turn, this can make the model less predictive. Therefore, in practical terms, it is worth considering whether the more complex CTC model is worth applying to experimental data instead of the ETC model. The various binding models do not, however, describe the molecular

basis of ligand binding and G protein activation, because of the lack of empirical GPCR: ligand: G protein complex structures.

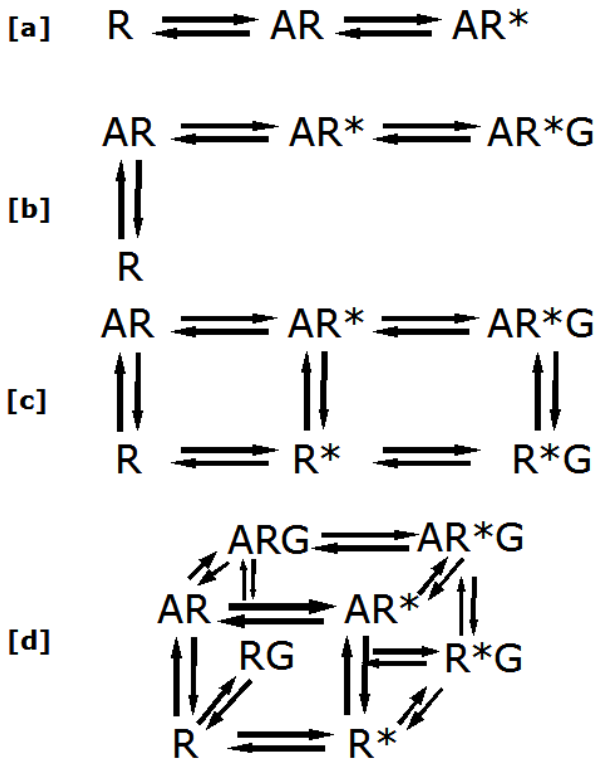

Figure 1.1.4: *Models describing GPCR systems. The traditional model merely describes the binding of agonist (A) to the receptor (R) and subsequent activation of this complex (AR). The ternary complex introduces the G-protein (G) as a third partner, which can interact with the activated agonist-receptor complex (AR*). The ternary complex was extended after the discovery of ligand-independent activation of the receptor, allowing for the formation of R*G. The cubic ternary complex makes up a thermodynamically more complete model. Figure adapted from* [49].

GPCR SIGNALLING AND DESENSITIZATION

In addition to the above, biochemical and biophysical studies with various members from GPCR family A indicate that some GPCRs, undergo spontaneous activation in the absence of agonist [50,51]. G protein signalling is not a constant process; high activation of GPCR reduces the ability to be stimulated in the future (desensitization), whereas low activation increases that ability (sensitization). Agonist-induced desensitization has been classified into agonist-specific (homologous) or agonist-nonspecific (heterologous) desensitization. After agonist

binding, primary desensitization is controlled by the receptor phosphorylation. This occurs via second messenger kinases or by a distinct family of GRKs (for reviews, see [52,53]). Heterologous desensitization involves second messenger kinases, such as protein kinase A (PKA) and protein kinase C (PKC), directly uncoupling GPCRs from their G proteins [54-56]. Second messenger kinases can also mediate agonist-non-specific desensitization, where kinase activation by one type of GPCR causes phosporylation of another GPCR. Homologous desensitization, meanwhile, involves GRKs and the two non-visual arrestins (β-arrestin 2 and 3) [57,58]. In this, the agonist-occupied or actived GPCR is phosphorylated by GRKs. GRK phosphorylation causes binding of β-arrestin to the GPCR, which inhibits binding between GPCR and the G protein. The GRK family of kinases is comprised of seven family members (GRK1-7). GRK1 (or rhodopsin kinase), GRK2 (or βark1, β-adrenergic receptor kinase), GRK3 (or βark2) and GRK7 are cytosolic, whereas GRK4, GRK5 and GRK6 are associated with the plasma membrane by some lipidic modification of their amino acids. Their phosphorylation usually occurs at serine/threonine clusters. Several research groups have shown that mutating the serines and threonines in these clusters to alanines abolishes phosphorylation and inhibits internalization [59,60]. The vast complexity of GPCR signalling has recently been further underlined by results suggesting that GPCRs may not exclusively work via heterotrimeric G proteins [37,38].

There is continuous movement of receptors between the plasma membrane and endosomal vesicles, and between synthesis in the endoplastic reticulum via Golgi apparatus and lysosomal degradation (Figure 1.1.5). Agonist activation of the receptor usually causes the removal of receptors from the plasma membrane to clathrin coated vesicle pits by a process called internalization. After this, internalized receptors are either recycled to the plasma membrane (resensitization) or targeted for degradation in lysosomes (down regulation). Constant stimulus causes receptor loss from the plasma membrane. There are several different internalization pathways reported, in addition to internalization via clathrin, some of which work via caveolae or uncoated vesicles [58]. These processes can occur over time frames ranging from seconds (phosphorylation), to minutes (endocytosis), to hours (downregulation). The extent of receptor desensitization varies from the complete termination of receptor signalling, as

observed in the visual and olfactory systems, to the attenuation of agonist potency and maximal responsiveness, such as observed for the β2-adrenergic receptor [61-63]. However, the extent of receptor desensitization is regulated by a number of factors that include receptor structure and cellular environment.

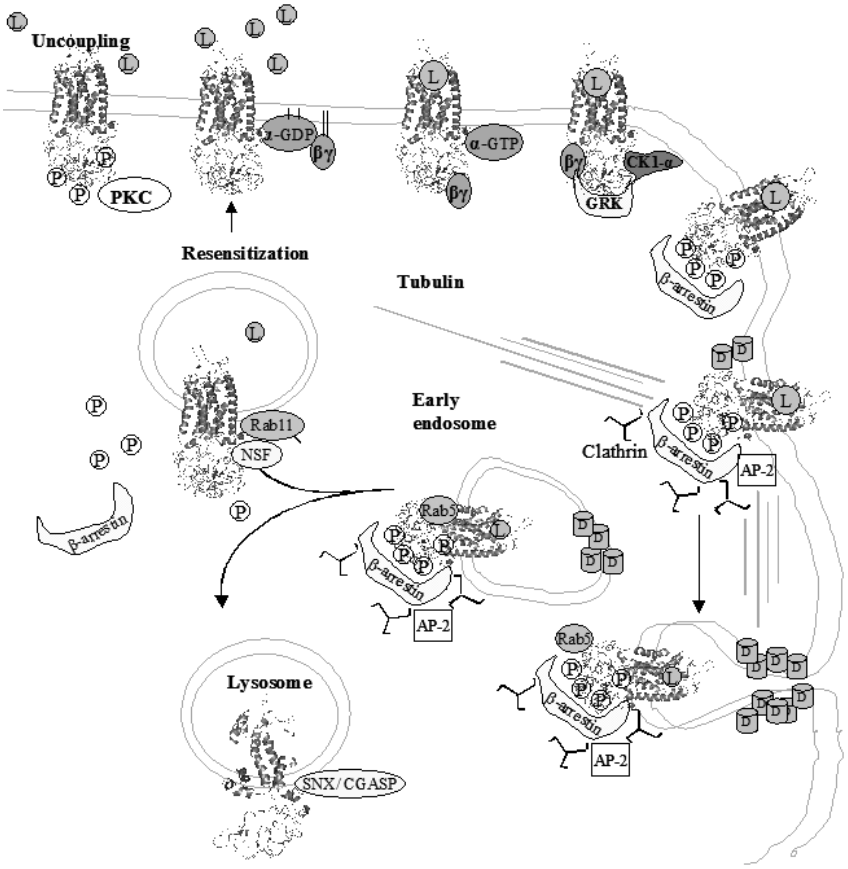

Figure 1.1.5: *Trafficking and homologous desensitization of GPCRs. Activation of a GPCR by an agonist leads to the dissociation of α and βγ subunits. The free βγ dimers recruit G-protein receptor kinases (GRKs) to the receptor, where they specifically phosphorylate agonist-occupied receptors. This, in turn, leads to the recruitment of β-arrestin to the receptor and targets the receptor- β-arrestin complexes to clathrin-coated pits. The receptor is internalised into acidic endosomes and then either dephosphorylated and recycled to the cell surface or degraded. Figure adapted from [64].*

Over the years, numerous reports have addressed the role of receptor sequestration in desensitization and resensitization. While some evidence suggests that this phenomenon is part of the desensitization process, other evidence suggests that internalization is instead one of the means by which receptors are

resensitized. Indeed, trafficking of an uncoupled receptor to endosomal compartments allows dephosphorylation and recycling of the receptor to the cell surface [65]. This is in contrast to receptor "downregulation" observed after prolonged agonist exposure, which leads to targeting of the receptors to degradation pathways [66]. Once internalized, receptors are targeted to recycling or degradative pathways. Some G-protein-coupled receptors, including the β2-adrenergic receptor, can be recycled back to the plasma membrane, as a fully competent receptor within minutes of being internalised [67]. Other receptors, such as the vasopressin type-2 receptor, are detained within the cell for longer time periods before being recycled back to the cell surface while others, like the δ-opioid or thrombin receptors, are degraded [68,69]. GRKs and β-arrestins appear to be key regulatory molecules for receptor internalization since these proteins have been shown to interact with components of the clathrin coated vesicle pathway. In response to GPCR activation, cytosolic β-arrestin proteins translocate to the plasma membrane and then subsequently redistribute to clathrin/coated pits bound to receptors. The clathrin-coated vesicle pathway is the best-characterized endocytic route, and is utilized by constitutively recycling receptors, tyrosine kinase receptors, and numerous G-protein-coupled receptors. Clathrin is a trimeric protein arranged as a triskelion when assembled, and is the major structural protein of the characteristic polygonal lattice of the coated pit. Plasma membrane coated pits also contain the clathrin adaptor protein AP-2, which binds to β-arrestins and to clathrin. Nevertheless, not all GPCRs necessarily internalize in a β-arrestin-/clathrin-dependent manner.

Experimental evidence suggests that GPCRs may also be internalized through alternative endocytic pathways. Some GPCRs have been found in cholesterol rich plasma membrane structures termed caveolae [70,71]. These domains are also known as signalling domains, but appear to contain proteins involved in the formation and budding of vesicles such as the dynamin molecule. The use of biochemical agents to disrupt these structures has been effective in modifying the endocytosis of certain GPCRs [72-74]. Finally, some receptors are suspected to use a third alternative endocytic pathway. No coat or adaptor proteins have been identified for the generation of these vesicles [75]. However, GPCR desensitization and endocytosis can act as molecular switches coupling GPCRs to alternative signal transduction pathways. β-Arrestins not only function in the molecular switch

required for GPCR desensitization and internalization, but also act as scaffolds to transduce and compartmentalize the alternative signals. In fact, β-arrestins have the ability to interact with a variety of endocytic and signalling proteins such as c-Src [76], MAPKs and Raf [77].
Several recent studies suggest that GPCRs can exist as dimers or as part of larger oligomeric complexes. Although GPCR dimerization is not essential for GPCR function, it might contribute to the diversity of signalling in vivo by directly altering ligand binding, desensitization or compartmentalization [78].

GPCR INTERACTING PROTEINS

In cells, multiprotein complexes mediate most of the functions. In neurons these complexes are directly involved in the proper neuronal transmission, which is responsible for phenomena like learning, memory and development. In the recent years, studies based on two-hybrid screens, proteomic, biochemical and cell biology approaches have shown that intracellular domains of GPCR contain sequence motifs that are known to direct protein-protein interactions and, therefore, have the theoretical capacity to interact with a wide range of other proteins [79]. These interactions are the basis of a protein network associated to these receptors, which includes scaffolding proteins containing one or several PDZ (post-synaptic density-95, discs large, zona occludens-1) domains, signalling proteins, and proteins of the cytoskeleton. This determines receptor properties, such as cellular compartmentalization, signalling and functional integration [79,80].
The topology of GPCRs allows several potential regions for these interactions; the extracellular loops are relatively short and, thus, extracellular interactions are likely to be dominated by the longer N-terminal sequences or antibody recognition [81]. On the intracellular face of the receptors, since both the C-terminal tail and the third intracellular loop can be considerable in size, resulting in two regions with the major number of interaction that has been found. The extent of these GPCR-protein interactions varies, ranging from transitory interactions (i.e. signalling purposes) to more stable interactions. However, GPCR-protein assemblies should be considered dynamic complexes that contribute to the intricate process of downstream signalling [82].

A large number of interactions with GPCRs on their intracellular faces have been described apart from those classically involved in signal transduction. A classical example of proteins that interact with GPCRs involves cytoskeletal associations, since several GPCRs interact with cytoskeletal anchoring polypeptides. This is the case of α-filamin and dopamine D2 receptors [83], α-actinin and adenosine A2A receptors [74] and the Shank family of proteins and several GPCRs including type I metabotropic glutamate receptor 1 (mGluR1) or somatostatin receptor type 2 (SSTR2) [84].

In the last few years, interactions between GPCRs and PDZ-domain containing proteins have been reported. PDZ domains are commonly present in a variety of multi-domain proteins and play a role in organizing signalling cascades, anchoring proteins to specific subcellular compartments, and regulating cell signalling by orchestrating assembly/disassembly of macro-molecular complexes. There are three general classes of PDZ domains based on their recognition specificity: class I domains, which interacts with carboxyl-terminal motifs S/T-X-Φ (where Φ indicates a hydrophobic residue and X any aminoacid), Class II domains, which recognize the Φ-X-Φ motif, and class III domains, which preferentially bind to D/E-X-Φ sequences [85]. For example, β2AR contains the DSLL motif at the carboxyl terminus, whereas β1AR displays an ESKV motif. Such differences determine their association to different PDZ domain-containing proteins. Six β1AR-interacting PDZ proteins, including PSD-95, MAGI-2, MAGI-3, CNrasGEF, GPIC and CAL have been identified to date by several approaches (two-hybrid screening, co-immunoprecipitation and fusion protein overlays and pull-down experiments) [86-88]. PSD-95, MAGI-2 and CAL have a dramatic impact on β1AR receptor trafficking, while the other PDZ proteins affect receptor by signal modulation [89]. NHERF (Na+/H+ exchanger regulatory factor) interaction with the β2-adrenergic receptor has been shown to promote both its clustering and endocytosis [90]. The Homer-1b protein interaction with the mGluR1 has been demonstrated to modulate Ca2+ induced mobilization of these receptors [91]. In addition, a direct interaction of calmodulin (CaM) with the 3ILoop of the dopamine D2 receptor [92], M_1 muscarinic receptor and μ-opioid receptors [93] has been described. Calmodulin was found to bind to the human D2 receptor at the AA 208-226 domain in a Ca2+ dependent manner. In line with these results, Ca2+

induced activation of calmodulin inhibits G-protein activation by the D2 receptor [92].

Spinophilin, a protein phosphatase regulatory protein that is enriched in the dentritic spines [94] has been found to interact with both, dopamine D2 receptor and α2-adrenergic receptor through a novel non-PDZ domain-mediated mechanism, allowing it to act as a scaffold that links these GPCRs with cell signal proteins like PP1 (Protein phosphatase 1). 14-3-3ξ is another protein that interacts with the 3ILoop of the β_2-adrenergic receptor in its basal or inactive state, but not upon receptor stimulation and control receptor retention at the basolateral surface [95]. Also, the 3Iloop of some GPCRs, such as β1AR/β3AR and D4R, contains polyproline motifs that bind to SH3 domain-containing proteins. β1AR interacts with endophilins, a protein family that forms complexes with amphiphisyn, synaptojanin and dynamin, all of which are involved in presynaptic vesicle trafficking. Endophilin binding increases agonist-dependent β1AR internalization, probably by acting as an adaptor protein between receptor-containing vesicles and endocytic components such as dynamin, which is required for clathrin-coated vesicle fission [96].

Direct interaction between RGS2 and the 3Iloop of muscarinic, adrenergic, and dopamine receptors has been reported [97,98]. An intriguing aspect of RGS functioning is how specificity is attained in order to regulate different G proteins linked to different receptors, since RGS can non-selectively inhibit several G protein families. In line with this, basal association of RGS2 with the third intracellular loop of the β1aAR, but not the β1AR [99], has been reported. Such interaction allows for the formation of a pre-complex of RGS proteins with defined receptors, so RGS is positioned to rapidly modulate G protein upon agonist challenge. For example, M_1 muscarinic receptors specifically bind RGS2, but not the closely related RGS16 [100].

In addition to direct protein interactions at the intracellular domains of GPCRs, there is increasing evidence that extracellular protein-protein interactions might play roles in modulating the pharmacology of GPCRs or their subcellular localization. The enzyme adenosine deaminase (ADA), a multifunctional protein that is anchored to different proteins on the cell surface, constitutes an example of this type of protein. In fact, evidence has been obtained to show that ADA could form heteromeric complexes with the both adenosine A1 and A2B receptors

[101,102], an interaction which appears to be essential for high affinity agonist binding to adenosine receptors.

Apart from the interactions taking place in the intracellular and extracellular sides of the membrane, GPCRs are able to display protein-protein interactions at the membrane level with other receptors or ion channels [82]. The widely recognized role of dimerization for many cell surface proteins contrasts with the classic models proposed for GPCRs, which were generally believed to function as monomers and to signal through downstream G-proteins in a 1:1 stoichiometric ratio. Until recently, it was believed that distinct sets of intramolecular interactions within the receptor would characterize the active and inactive conformations upon ligands binding. Recent data indicates that, in addition to the specific intramolecular interactions defining the activation states of the receptor, intermolecular receptor interactions might also be important [103].

Both receptor dimerization, as well as receptor interactions with accessory proteins, have been documented and are proposed to participate in GPCR activity [80]. Furthermore, during the last decade, several findings have pointed out that GPCR dimerization is not limited to homodimers, but that they can interact with both closely and distantly related members of other GPCR subfamilies to form heterodimers (hetero-oligomers) [103-106] (Table 1.1.1).

Table 1.1.1: *Overview of homo- and hetero-oligomerization of GPCRs superfamily. Specificy of hetero-oligomers is depicted by listing receptors that have been reported as not being able to form hetero-oligomers with relevant receptors. Abbreviations: CCR5, chemokine receptor; CCKR, cholecystokinin receptor; V_2R, vasopressin receptor; A_1R, angiotensin type 1 receptor.*

Rptor	Homo-	Hetero-oligomerization	Specificity
α_{1a}-AR	[107,108]	A_{1b}-AR [107], δOR [108]	M71 [109], α_{1d}-AR [110]
α_{1b}-AR	[107,111,112]	α_{1a}-AR [107], α_{1d}-AR [110,113], H_1R [111,112], CCR5 [107]	M71 [109], CCR5 [107]
α_{1d}-AR		A_{1b}-AR [110,113], β_2-AR [110]	M71 [109], α_{1a}-AR [114], α_2-AR, β_1-AR, $H_{1\text{-}3}R$, $M_{1\text{-}5}R$, $D_{1\text{-}2}R$
α_{2a}-AR	[115]	MOR [116], β_1-AR [117]	α_{1d}-AR [110], M71 [109]
β_1-AR	[118,118,119]	A_{2a}-AR, β_2-AR [118,120]	α_{1d}-AR [110], M71 [109]

β_2-AR	[118-123]	β_1-AR [118,120], M71 [109], β_3-AR [124], α_{1d}-AR [110], κOR [125]	CCR5 [121], CCKR [126], $MT_{1a}R$ [121], MT2R [123], κOR [127], D2SR [119,127,128], $GABA_BR1$-2 [118,122]
β_3-AR	[124]	B_2-AR [124]	M71 [109]
D_1	[129,130]	$D_{2L}R$ [131-133], NMDA [134], A_1R [135]	α_{1d}-AR [110], α_{2a}-AR [136]
$D_{2L}R$	[129,137-141]	SSTR5 [142], $D_{1\text{-}3}R$ [131-133,139,143], 5-$HT_{1B}R$ [140], α_{2a}-AR [136,144,145]	α_{1d}-AR [110], A_1R [135,146], M_4R [115]
$D_{2S}R$	[139]	$D_{2L}R$ [139]	β_2-AR [147]
D_3R	[129,148,149]	A_{2a}-AR [149], $D_{2L}R$ [143]	
CCR2	[150,151]	CCR5 [151]	
H_1R	[152]	A_{1b}-AR [111,112]	α_{1d}-AR [110], H_4R [153]
5-$HT_{1A}R$	[154]	5-$HT_{1B}R$ [154], 5-$HT_{1D}R$ [154]	
5-$HT_{1B}R$	[140,154-157]	5-$HT_{1A}R$ [154], 5-$HT_{1D}R$ [154,156,157], 5-$HT_{2B}R$ [158]	$D_{2L}R$ [140]
5-$HT_{1D}R$	[129,154,156,157]	5-$HT_{1A}R$ [154], 5-$HT_{1B}R$ [154,156,157]	
5-$HT_{2C}R$	[159,160]	M_4R [160]	CCR5 [161]
5-$HT_{4D}R$	[162]	β_2-AR [162], 5-$HT_{4G}R$ [162], $GABA_BR2$ [154]	
M_1R	[163]	M_2R [163]	α_{1d}-AR [110], Smoothened [163]
M_2R	[164-167]	M_1R [163], M_3R [163,168,169]	Δor [167], κOR [167], μOR [167], α_{1d}-AR [110], Smoothened [163]
M_3R	[163,169-171]	α_{2c}-AR [169,171,172], M_1R [163], M_2R [163,168,169]	M_1R [170], M_2R [170], V_2R [170], α_{1d}-AR [110], Smoothened [163]
M_4R	[115]	5-$HT_{2C}R$ [160]	α_{1d}-AR [110], $D_{2L}R$ [115]
ΔOR	[173,174]	A_{1a}-AR [108], β_2-AR [97], μOR [175]	M_2R [167]
KOR	[173,174]	B_2-AR [127], μOR [176]	M_2R [167], β_2-AR [127]
MOR	[173,174]	A_{2a}-AR, δOR [175], κOR [176]	M_2R [167]
V_2R	[177]	V_1R [105,177], Oxytocin [177]	M_3R [170]
$GABA_BR1$		$GABA_BR2$ [178-180]	β_2-AR [122]
$GABA_BR2$		5-$HT_{1A}R$ [154], $GABA_BR1$ [178-180]	β_2-AR [118]
mGluR1	[173]	$A_{1A}R$ [144,181]	

Homodimerization is defined as the physical association between identical proteins, whereas heteromerization is defined as the association between non-identical proteins. This association can be between two monomers to form dimmers, or between multiple monomers to form receptor oligomers. As available techniques do not allow for a distinction between dimers or higher-order oligomers, the term dimers is often used, being the simplest form of an oligomer's functional unit that can explain the observations.

1.2 MUSCARINIC RECEPTOR FAMILY

Acetylcholine and acetylcholine receptors

Since the beginning of the last century, when Sir Henry Dale (1914) presented his Acetylcholine (ACh) classification and Otto Löwi (1921) revealed for the first time that a chemical transmitter, "Vagusstoff", released from vagal nerve termini decelerated the beating of the heart, ACh, has been described as the naturally occurring neurotransmitter for muscarinic receptors. Ach is synthesized in the cytoplasm of nerve terminals by the enzyme choline acetyltransferase, and is then transported into synaptic vesicle. Depolarization of the nerve terminal causes an influx of calcium into the nerve terminal and evokes the release of Ach into the synaptic cleft. The actions of Ach are determined by the enzymes acetylcholinesterase (AChE) and the plasma butyrylcholinesterase, which hydrolyzes Ach [182]. The actions of Ach are mediated through two classes of receptors: nicotinic and muscarinic receptors. Classification is based in their pharmacological properties and protein structure [183].

Muscarinic Ach receptors (mAChRs) are now known to belong to the members of the superfamily of GPCRs [184,185]. They are abundant in the central nervous system as well as in non-neural tissues that are innervated by the parasympathetic nervous system, such as cardiac and smooth muscle and many exocrine glands. Five genes (m1 - m5) encode muscarinic receptor proteins exhibiting a rhodpsin-like architecture with seven transmembrane domains [186]. They exhibit high sequence homology with each other and with other GPCRs within their seven transmembrane helices, whereas the sequences are more variable at the amino terminal extracellular region, lying at the entrance of ligands, and at the third intracellular loop. In particular, the carboxyl-terminal end of the third intracellular

loop of the receptor has been implicated in the specificity of G protein coupling and shows extensive homology within M_1, M_3, and M_5 receptors and between M_2 and M_4 receptors [184,187,188]. Conserved regions in the second intracellular loop also confer specificity for proper G protein recognition [186,189].

The five muscarinic receptor subtypes have been identified and cloned (M_1 - M_5) [190] (Table 1.2.1). The odd-numbered receptors (M_1, M_3, and M_5) couple to $G_{q/11}$, and thus activate phospholipase C, which initiates the phosphatidylinositol trisphosphate cascade. This leads to the dissociation of phosphatidyl 4,5-bisphosphates (PIP2) into two components, i.e., inositol 1,4,5-trisphosphate (IP 3) and diacylglycerol (DAG). IP3 mediates Ca2+ release from the intracellular pool (endoplasmic reticulum), whereas DAG is responsible for activation of protein kinase C. On the other hand, PIP2 is required for the activation of several membrane proteins, such as the "M current" channel and Na+/Ca2+ exchangers, and muscarinic receptor-dependent depletion of PIP2 inhibits the function of these proteins [191-194]. Depending upon the variety of downstream effectors, different cells exhibit diverse functional muscarinic responses. For example, in vascular smooth muscle cells, muscarinic M_3 receptor activation leads to elevation of intracellular Ca2+ to induce contraction of the cells, whereas the signal from the same receptor (M_3) in vascular endothelial cells activates the endothelial nitric oxide synthase (NOS), leading to NO production and causing the relaxation of adjacent vascular smooth muscle cells [195,196]. In glandular tissues, M_3 receptor-mediated phosphatidylinositol turnover leads to hormone secretion [197,198]. In the brain, activation of postsynaptic M_1 or M_3 receptors leads to inhibition of a potassium channel, called the "M current", via depletion of PIP2, which mediates basal control of neuronal excitability [199,200]. Activation of the post-synaptic muscarinic receptor, that is, inhibition of the M current, does not appear to lead directly to the firing of the action potential, but rather enhances the neuronal response to excitatory input.

The even-numbered receptors (M_2 and M_4) are coupled to the $G_{i/o}$ class of G proteins, and inhibit adenylyl cyclase activity. These receptors also activate G protein-gated potassium channels, which leads to hyperpolarization of the membrane in excitable cells. In the heart, M_2 muscarinic receptor dependent activation of this channel contributes to the deceleration of the beating of the heart [201,202]. In neurons, both M_2 and M_4 muscarinic receptors are present on the axon

terminals and inhibit neuronal excitability, which results in the negative feedback of neurotransmitter release. In smooth muscle cells, the M_2 receptor inhibits adenylyl cyclase and counteracts the adrenergic responses [203]. As shown in Table 1.2.1, there are many agonists and antagonists for the muscarinic receptors. Some of them are selective for certain receptor subtypes and are often used as pharmacological tools for dissecting the function of the individual muscarinic ACh receptors [204,205]. However, their specificity is not absolute and it is usually difficult to explicitly identify the precise physiological roles of individual muscarinic ACh receptors in vivo.

Table 1.2.1: *Muscarinic receptor subtypes and their pharmacology. Table adapted from* [200]

Subtypes	**M_1R**	**M_2R**	**M_3R**	**M_4R**	**M_5R**
G proteins/ Signal transduction	$G\alpha_{q/11}$,PLCβ, IP_3/DAG ↑, Cdc42/RhoA, MAPK, JNK	$G\alpha_{i/0}$, $_C$AMP ↓, Direct coupling to [K+] and [Ca^{2+}] channel	$G\alpha_{q/11}$, PLCβ, IP_3/DAG ↑, PLD↑, RhoA activation	$G\alpha_{i/0}$, $_C$AMP↓, Direct coupling to K+ channel	$G\alpha_{q/11}$, PLCβ, IP_3/DAG↑
Non-selective agonist	Acetylcholine (muscarinic & nicotinic), Carbachol (muscarinic & nicotinic), Muscarine (muscarinic receptor specific), Pilocarpine (muscarinic receptor specific), Oxotremorine-M (muscarinic receptor specific), Metoclopramide (muscarinic receptor specific), Bethanechol (muscarinic receptor specific)				
Non-selective antagonist	Atropine, Scopolamine, QNB (Quinuclidinyl-α-hydroxydiphenylacetate)				
Subtype-selective agonist	McN-A-343 L-689,660 Xanomeline CDD-0097	_	L-689,660	_	_
Subtype-selective antagonist	Pirenzepine Telenzepine MT7	AF-DX 116 AF-DX 384 Methoctramine Himbacine Tripitramine	4-DAMP p-Fluorohexahydrosiladifenidol Darifenacin	TropicamideHimbacine AF-DX 384	_

GENE AND RECEPTOR STRUCTURAL UNDERSTANDING

Muscarinic ACh receptors are among the earliest members of the seven-transmembrane G protein-coupled receptor family to be defined pharmacologically. They are generated from 5 different genes in the human genome (NCBI, Map Viewer at http://www.ncbi.nlm.nih.gov/mapviewer) and diverged from a common ancestor, with this evolutionary relationship reflected in their structures. Similarly to several other members of the GPCR gene family, they are coded by intronless open reading frames formed by a TATA-less promoter, with large intron and initiation consensus elements. So far only few single nucleotide polymorphisms have been identified and could not be associated with either atopic asthma or schizophrenia [206-208].

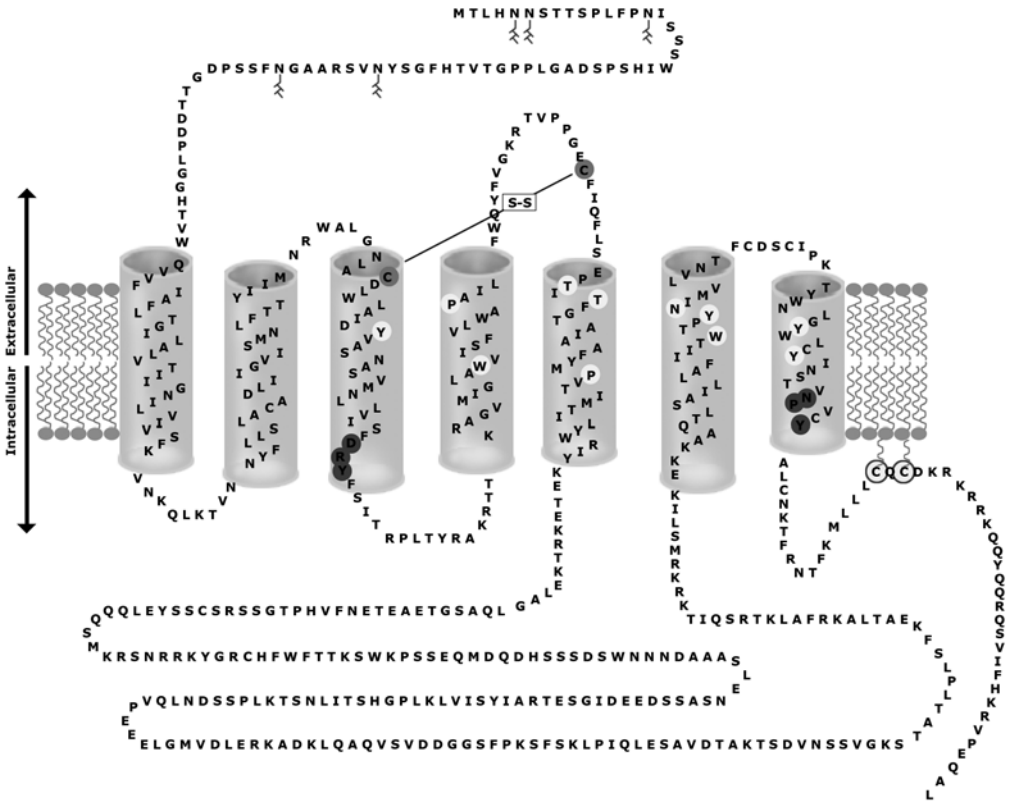

Figure 1.2.1: *Amino acid sequence and secondary structure model of human M_3 muscarinic receptor. The residues crucial for the binding to acetylcholine, shown as filled white circles, are found within in the TM regions. Cysteines residues in the 1Eloop and 2Eloop form a disulphide*

bridge that is shown as a solid line. The most conserved motifs (DRY and NPxxY) are represented by black circles. Predicted N-glycosylation sites are shown as a root in the N-terminal tail. Palmitoylation of two conserved cysteines in the C-terminal tail are also highlight.

Muscarinic receptors share essential homology with rhodopsin, are palmitoylated in the C-terminal tail and potentially N-glycosilated in the N-terminal tail based on the presence of consensus N-glycosilation sites [209] (Figure1.2.1).

Analyses using scanning mutagenesis and homology modelling suggested that similar activation mechanisms exist for several of the cationic amine receptors, including mAChRs, and the neuropeptide receptors [6]. Alanine substitution mutation and scanning mutagenesis have allowed the identification of most of the residues in the transmembrane region that possess the binding site for ACh in the mAChR [45,210,211] (Figure 1.2.1). A negatively charged residue in TM3, $Asp^{3.32}$, is suggested to associate with the polar head of ACh, and is conserved among all the amine receptors, but not among the other peptide receptors [212]. Mutation of $Thr^{5.39}$ and $Thr^{5.42}$ impaired agonist binding and/or activation, and $T^{5.42}C$ was affinity labelled by an agonist derivative [210,213]. Most of these hydrophilic residues appear to be aligned to a plane in the transmembrane regions and comprise the ACh-binding pocket [214]. In addition, in the M_1 receptor, $Ala^{5.46}$ has also been implicated in agonist binding [210]. Many aminergic ligands also form critical interactions with residues in H-V. The critical H-V residues are not as conserved as is $Asp^{3.32}$, but the positions and interactions nonetheless appear to be conserved. These theories have been well confirmed by the homology modelling of the M_1 mAChR structure and docking simulation with Ach [186].

As described above, M_1, M_3 and M_5 receptors (called "Group I") activate $G\alpha_{q/11}$, whereas the M_2 and M_4 receptors ("Group II") activate PTX-sensitive $G\alpha_{i/o}$. Amino acid position and length analysis revealed that amino acid length of the N-terminal regions is highly variable, with from 20 to over 100 amino acid residues, whereas the C-terminal region is more conservative, being 40-45 amino acid residues long. However, frequency distribution revealed high variability for both tails. In contrast to the three extracellular loops and the first and second intracellular loop, with a short length, a large length characterizes 3Iloop (more than 140 residues). The amino acid residues along this loop are well conserved within "Group I" or "Group II", but the homology is relatively low between

"Group I" and "Group II". The specificity for the coupling between the receptor and the G protein α-subunit is considered to be determined within the area of 3ILoop just beneath the membrane, which should be associated with the N- and C-terminal regions of the Gα subunit. The residues in the M_2 receptor, Val, Thr, Ile and Leu, in 3ILoop ~ TM6 (VTIL motif) are shown to be crucial to the interaction with the C-terminus of the PTX-sensitive $G\alpha_{i/o}$ subunit, whereas the residues in the M_3 receptor, Ala, Ala, Leu and Ser in the corresponding region 3ILoop ~ TM6 (AALS motif) are suggested to be important in coupling to the C-terminus of the $G\alpha_q$ subunit [215]. On the other hand, Asp-Arg-Tyr residues in the second intracellular loop (2ILoop) (DRY motif) are well conserved among many GPCRs, including rhodopsin, and the 2ILoop is reported to be crucial for the activation of several G protein α-subunits [216]. 2Iloop, together with the first intracellular loop (1ILoop), is also characterized by the presence of basic residues in the membrane junction, which has been associated with the stable interaction of helix with phospholipids.

REGULATION OF MUSCARINIC RECEPTOR SIGNALLING AND DESENSITIZATION

Prolonged agonist activation of mAChRs decreases their action, which is referred as "receptor desensitization". Desensitization means a weakening of the signal transmission under conditions of a long-lasting stimulation by ligands. Despite the persistent effect of extracellular stimuli, the signal is no longer passed into the cell interior, or only in a weakened form, during desensitizing conditions. Receptor desensitization is achieved by three mechanisms that are different in their time-courses; (1) uncoupling between the receptor and G protein (~ 2~3 minutes), (2) reversible decrease in the number of cell surface receptors by internalization (~10~30 minutes), and (3) down-regulation of the transcription of the mAChR (~ 2~3 hours) [186,217,218]. For mAChRs and most other GPCRs, agonist-induced phosphorylation of the receptor facilitates receptor internalization.

Receptor phosphorylation is mediated by kinases, including G protein receptor kinases (GRKs), casein kinase 1α (CK1α), and diacylglycerol-regulated protein kinase C (PKC) [219,220]. PKC phosphorylated in an agonist-independent and Gβγ-independent manner both in vitro and in vivo alike has been shown for M_1 and M_3

receptors. Particularly, serine and threonine residues in the C-terminus (likely candidates are Ser451, Thr455, and Ser457) and the C-terminal portion of Loop3I (likely candidates are Thr354 and Ser356) of the M_1 receptor have been described. Mammalian M_2 receptors do not appear to serve as substrate for PKC [221-223]. In contrast, GRKs recognize the agonist-bound form of the receptor and mainly phosphorylate serine and threonine residues in the middle of the 3ILoop of mAChRs [53]. Alanine substitution mutagenesis of the putative phosphorylation sites in the 3ILoop of the M_1 (Ser/Thr residues in Ser284MetGluSerLeuThrSerSerGlu292) [224], M_2 (Ser/ Thr residues in Ser286ThrSerValSer290 and Thr307ValSerThr Ser311) [224,225], and M_3 receptors (Ser residues in 349Ser AlaSerSer352) markedly decreases receptor internalization [224].

Once they are phosphorylated by the GRKs, mAChRs are bound to β-arrestin, which can interact with clathrin, the major structural component of the clathrin-based endocytic machinery (Figure 1.1.5) [226,227]. Phosphorylated and β-arrestin bound receptors are then internalized within clathrin-coated vesicles. The budding of clathrin-coated vesicles from the plasma membrane is regulated by dynamin GTPases, which are also activated by β-arrestin *via* the action of c-Src. In the GTP-bound form, dynamin is thought to form a collar at the neck of the clathrin-coated pit and to catalyze the fission of the vesicles [228]. Expression of a dominant-negative β–arrestin 1 mutant, who binds with high affinity to clathrin but is significantly impaired in its ability to interact with phosphorylated GPCRs, significantly suppresses internalization of M_1, M_3 and M_4 mAChRs in HEK293 cells [75,229]. Moreover, expression of another dominant-negative β-arrestin, β-arrestin S412D, which binds to phosphorylated GPCRs with similar affinity, but not to clathrin, inhibits M_1 mAChR in HEK293 cells [58]. These results are consistent with observations that internalized M_1 receptors colocalize with β-arrestin and clathrin in HEK293 and RBL-2H3 cells [230,231]. These studies concluded that in HEK293 cells, M_1, M_3 and M_4 mAChRs are internalized in a β-arrestin dependent manner *via* clathrin-coated vesicles. On the other hand, M_2 mAChRs have been shown to internalize in a β-arrestin and clathrin-independent manner [230,232]. It has also been shown that a peptide sequence derived from the third cytoplasmic loop of the M_2 mAChR that contains the GRK2 phosphorylation sites and a putative β-arrestin binding site, does not bind β-

arrestin, whereas a peptide sequence derived from the third cytoplasmic loop of the M_3 mAChR is able to bind to β-arrestin [233]. These observations indicate that other cytosolic proteins can associate with phosphorylated M_2 mAChRs to mediate clathrin-independent internalization.

It is important to note that receptor desensitization does not necessarily require receptor phosphorylation. The activation of α_{1B}-adrenergic receptors in SH-SY5Y human neuroblastoma cells reduces M_3 receptor-$G\alpha_{q/11}$ protein coupling without an increase in M_3 receptor phosphorylation [234]. Direct activation of M_3 receptors, in contrast, was found to induce M_3 receptor phosphorylation and desensitization. It has been postulated that this heterologous desensitization of mAChRs is caused by sequestration of $G\alpha_{q/11}$ following activation of the more abundant α_{1B}-adrenergic receptor. In opposite manner, receptor phosphorylation may not be associated with receptor desensitization. CK1α binds to the Loop3I of the human M_3 receptor in a Gβγ subunit-independent manner (putative CK1α binding site is contained in the amino acid sequence His374-Val391) and robustly phosphorylates serine and threonine residues in the Loop3I of agonist-stimulated human M_3 receptors [219,235]. These putative CK1α phosphorylation sites are downstream from the putative Gβγ binding site and GRK2 phosphorylation sites [236]. In accordance with this, CK1α-mediated phosphorylation is not accompanied by significantly reduced M_3 receptor coupling to PLC or Ca^{2+}-ions increases in CHO cells. Very interestingly, CK1α-mediated phosphorylation of the M_3 receptor contributes to the mechanism of M_3 receptor-mediated activation of the mitogen-activated protein kinase pathway ERK1/2 [237].

In many cells, exposure to muscarinic agonists induces rapid desensitization and internalization of the cell surface mAChRs that is followed by a loss of total number of cellular receptors in the following hours. This process, also termed down-regulation, can only be overcome by de novo receptor synthesis. This process requires an intact cytoskeleton, as demonstrated by the inhibitory effect of colchicines, on agonist-induced mAChR down-regulation [238]. Until now, only a few domains in the M_1–M_3 receptors have been implicated in regulating mAChR down-regulation. First, deletion of 123 of the 156 amino acids in the Loop3I of the mouse M_1 receptor (i.e., residues 221–343) decreases the magnitude of (but does not abolish) agonist-induced receptor down-regulation by ~55% in Y1 adrenal carcinoma cells [239,240]. Second, four mutations in the M_1 receptor

specifically impair M_1 receptor down-regulation in CHO cells [241]. These mutations are located either in the second intracellular loop (i.e., Val127Ala) or in the N- and C-terminal regions of the Loop3I (i.e., Ile211Ala, Glu360Ala, and Lys362Ala). Deletion of a large part of Loop3I of the human M_2 receptor (residues 234–381) significantly (but not completely) inhibits M_2 receptor down-regulation in CHO cells [242]. In addition to residues in the third cytoplasmic loop, the lone tyrosine residue in the C-terminus of the porcine M_2 receptor (Tyr459) has been identified as another important amino acid for M_2 receptor down-regulation in JEG-3 cells [243]. Down-regulation of human M_3 receptors in CHO cells requires three threonine residues (Thr550, Thr553, and Thr554) in the C-terminus of the receptor protein. Interestingly, the down-regulation-defective phenotype of all mAChR mutants in the above studies is not correlated with reduced signalling to PLC or adenylyl cyclase. Thus, "classical" signalling of the M_1–M_3 receptors to $G\alpha_{q/11}$ and $G\alpha_i$ proteins does not seem to be required for down-regulation of these mAChR subtypes [244].

SPECIFIC LIGANDS AND THERAPEUTIC DRUG TARGETING

To date, there have been many therapeutic drugs targeting muscarinic acetylcholine receptors [reviewed in [245,246], see Figure 1.2.2]. Among them, the oldest and the most common are atropine and scopolamine. Atropine, which is a non-specific antagonist for muscarinic acetylcholine receptor, is an alkaloid extracted from deadly nightshade (*Atropa belladonna*). It is a secondary metabolite of these plants and a drug with a wide variety of effects. Preparations of belladonna were known to the ancient Hindus and have been used by physicians for many centuries. During the time of the Roman Empire and in the Middle Ages, the deadly nightshade shrub was frequently used to produce obscure and often prolonged poisoning. This prompted Linnaeus to name the shrub *Atropa belladonna*, after Atropos, the oldest of the three Fates, who cuts the thread of life. The name belladonna derives from the alleged use of this preparation by Italian women to dilate their pupils. Today, belladonna is still used to dilate pupils (mydriasis) before eye examinations. The mechanism of this action involves blocking the contraction of the pupillary sphincter muscle. Atropine is often used as a premedication for anesthesia because it reduces bronchial and salivary

secretion (which are also directed by the parasympathetic system). Injections of atropine are used in the treatment of bradycardia and pulseless electrical activity (PEA) in cardiac arrest in order to block the vagus nerve termini and to accerelate the beating of the heart. Atropine is also used in the treatment of poisoning by organophosphate insecticides and nerve gases (chemical weapons) [reviewed in [247]]. Scopolamine (l-hyoscine) is found chiefly in *Hyoscyamus niger* (henbane). In India, the root and leaves of the jimson weed plant were burned and the smoke inhaled to treat asthma. British colonists observed this ritual and introduced the belladonna alkaloids into western medicine in the early 1800s.

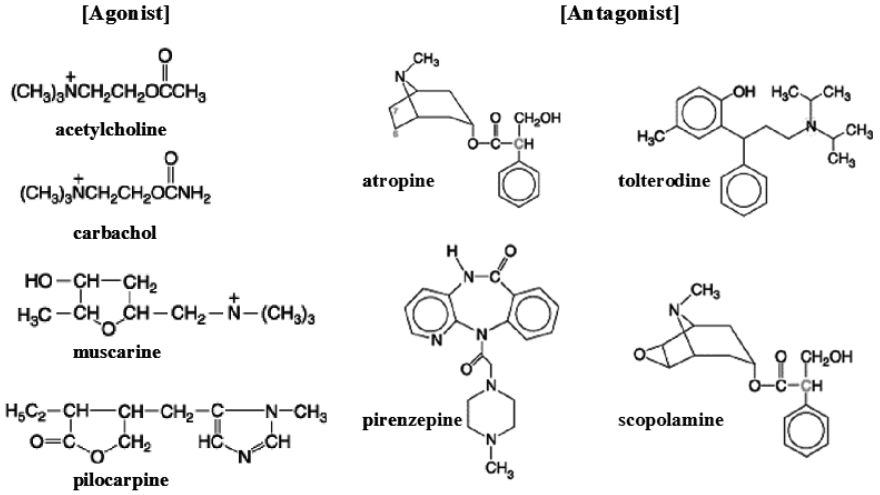

Figure 1.2.2: *Chemical structure of several muscarinic receptor ligands.*

Another kind of muscaric antagonist, butylscopolamine, is a commonly used anti-muscarinic drug for treating cramps in gastrointestinal, urinary, uterine and biliary ducts. It exhibits antiemetic and antispasmodic effects by preventing the hypercontractions in visceral smooth muscles. Pirenzepine is an M_1/ M_3-selective anti-muscarinic agent that inhibits gastric acid secretion at relatively lower doses than are required to affect gastrointestinal motility, salivery secretion, central nervous system, cardiovascular, ocular and urinary functions [248].

Pilocarpine is the chief alkaloid obtained from the leaflets of South American shrubs of the genus *Pilocarpus*. Although it was long known by the natives that chewing of the leaves of *Pilocarpus* plants caused salivation, the Brazilian physician Coutinhou apparently performed the first experiments in 1874. The

alkaloid was isolated in 1875, and shortly thereafter Weber described the actions of pilocarpine on the pupil and on the sweat and salivary glands. The muscarinic agonist pilocarpine has been used for treatment of chronic open-angle glaucoma and acute angleclosure glaucoma for more than 100 years. Intraocular pressure is determined by the balance between production of aqueous humor production by the ciliary body cells and drainage at the Canal of Schlemm. Muscarinic receptor activation leads to both an increase in drainage and a decrease in humor production [249]. The former mechanism involves dilation of the smooth muscle in the Canal of Schlemm, whereas the latter effect results from the muscarinic activation-induced production of nitric oxide and subsequent inhibition of Na+/K+-ATPase [250].

The poisonous effects of certain species of mushrooms have been known since ancient times, but it was not until Schmiedeberg isolated the alkaloid muscarine from Amanita muscaria in 1869 that its properties could be systematically investigated [251]. Arecoline is the chief alkaloid of areca or betel nuts, the seeds of Areca catechu. The red-staining betel nut is consumed as a euphoretic by the natives of the Indian subcontinent and East Indies in a masticatory mixture known as betel and composed of the nut, shell lime, and leaves of Piper betle, a climbing species of pepper [252].

The accumulated knowledge of the muscarinic receptor subtypes and their specific roles has begun to stimulate the development of novel, specific and therapeutically useful muscarinic receptor ligands.

Cevimeline, originally described in the neuroscience literature as AF102B, is an M_1/ M_3R-selective agonist that is clinically used for treating the dry mouth and eyes in Sjögren's syndrome [253]. Sjögren's syndrome is a systemic autoimmune disorder with relatively high incidence (0.5-1% in adult women) that is characterized by the infiltration of lymphocytes and destruction of grandular tissues. The major complaint of the patients suffering from Sjögren's syndrome is dry mouth/eyes, due to the decrease in saliva and eyewater. The M_3R is present and stimulates glandular secretion in both salivary gland and lacrimal glands, and therefore cevimeline is therapeutically beneficial for alleviating the symptoms in Sjögren's syndrome [254]. Recently, anti-muscarinic agents have been emerging as novel therapeutic drugs for disorders of smooth muscle hyperactivity, including overactive bladder (incontinence) and chronic obstructive pulmonary diseases [255].

For the treatment for overactive bladder, several compounds (oxybutynin [256], tolterodine [257], solifenacin and darifenacin [258]) have been used clinically. These drugs are muscarinic receptor antagonists that are relatively specific for the M_2 and M_3 receptors, and inhibit bladder responsiveness without any side effects. The antagonist tiotropium has been suggested as a firstline approach for the treatment of chronic obstructive pulmonary disorders, such as emphysema pulmonum [259], because its effect is superior to the conventional therapeutics using bronchodilators, such as theophilline [260].

PHYSIOLOGICAL ROLES AND FUNCTION OF EACH RECEPTOR SUBTYPE

The precise physiological roles of the individual mAChR subtypes have not previously been fully identified because of the nonselective effect of the ligands and the redundant expression of multiple receptor subtypes in many tissue and cell types [183]. In order to circumvent these limitations, the gene targeting techniques have been exploited to generate mutant mice that are deficient in one of the five mAChR subtypes [261]. M_1 – M_5 receptor knockout mice are viable, fertile and essentially healthy (Table 1.2.2).

M_1 Muscarinic Receptor

The M_1 receptor gene was the first gene encoding for a muscarinic receptor that was ablated in mice [262]. M_1 receptors are predominantly expressed in the forebrain, including the cerebral cortex, hippocampus and corpus striatum, where this sub-type contributes by 50-60% to the total of the muscarinic receptors. Consistent with this observation, some behavioral deficits and altered responses to pharmacological agents have been detected in M_1R knockout mice [262-264]. Systemic administration of pilocarpine, a muscarinic receptor agonist, causes epileptic seizures in wild-type, but not in M_1R knockout mice, suggesting that M_1R might contribute to the pathogenesis of some kinds of epileptic seizures [262]. Behavioral studies revealed that M_1R-deficient mice exhibited a remarkable increase in locomotor activity [263], which was probably caused by an increase in dopamine release in the striatum [264]. These findings suggest that pharmacological blockade of the M_1 receptor in the central nervous system might be therapeutically effective for Parkinson's disease by increasing dopamine release

from the striatum. In contrast to the behavioural disorder, there were little obvious cognitive deficits (in learning and memory) in M_1R-knockout mice, although long-term potentiation (LTP) in response to burst stimulation in the hippocampus was slightly reduced [265].

Table 1.2.2: *Phenotype of Muscarinic Acetylcholine Receptor-Deficient Mice.Adapted from* [266]

Rptor subtypes	Phenotype in central nervous system	Phenotype in periphery
M_1R	Absence of pilocarpine-induced seizure activity Increase in locomotors activity Lack of mAChR-mediated inhibition of M current in sympathetic ganglion neurons LTP in hippocampus is slightly reduced	No obvious deficits are detected
M_2R	Absence of oxotremorine-mediated tremor and akinesia Reduction of mAChR-mediated analgesia	Slight reduction in carbachol-mediated contraction of smooth muscle Lack of carbachol-mediated bradycardia
M_3R	No obvious deficits are detected	~25% reduction in body weight Significant reduction (~40-95%) in carbachol mediated contraction of smooth muscle Enlarged pupils Slight reduction in pilocarpine induced salivary secretion
M_4R	Increase in basal locomotors activity	Reduced auto-inhibition of ACh-release in heart atria and urinary bladder
M_5R	Reduction of oxotremorine mediated dopamine release in the striatum	Reduction in ACh-induced dilation of cerebral blood vessels

The "M current" is a constitutively active, voltagedependent potassium channel current that can be inhibited by mAChR agonists [199,267]. It was identified in sympathetic ganglion cells and in hippocampal pyramidal neurons. Electrophysiological analysis has demonstrated that inhibition of the M current by carbachol (CCh) in sympathetic ganglion neurons was completely abolished in M_1R knockout mice [262]. However, no significant change was observed in inhibition of the M current in hippocampal CA1 pyramidal neurons in the same mice [268].

M_2 Muscarinic Receptor

Previous studies have suggested that the administration of centrally active muscarinic agonists such as scopolamine exhibits remarkable analgesic effects [269]. Muscarinic agonist-induced analgesia can be as effective as morphine-induced analgesia in animal models, and muscarinic analgesia is of considerable interest because it leads to less addiction and tolerance [269,270]. In M_2R-deficient mice, the oxotremorine (muscarinic receptor agonist)-mediated antinociceptive response was greatly attenuated in both tail-flick and hot plate tests [271], suggesting that the M_2R plays a crucial role in muscarinic agonist-induced analgesia. On the other hand, electrophysiological examination demonstrated that the muscarinic receptor-dependent inhibition of the neuronal (Nand P/Q- type) calcium channel was abolished in sympathetic ganglion neurons from M_2R-deficient mice [272]. The muscarinic agonist, carbachol (CCh), induced bradycardia in spontaneously beating sinoatrial node cells and atria; an effect that was completely abolished in M_2R deficient mice [271,273], indicating that the M_2R is responsible for cholinergic deceleration of the beating heart. In M_2R knockout mice, the carbachol-mediated contraction of a smooth muscle preparation (from stomach, urinary bladder and trachea) was slightly attenuated [273].

M_3 Muscarinic Receptor

M_3 muscarinic receptors are broadly expressed in the brain, although the expression level is not high when compared to that of M_1 and M_2 receptors. In the periphery, M_3 receptors are predominantly expressed in smooth muscle and glandular tissues [274]. No obvious behavioural or cognitive defects were observed in M_3R-deficient mice [275]. Interestingly, M_3R-deficient mice exhibited a remarkable (~25%) reduction in body weight. The body weight loss could be reversed when the mice were offered a wet paste diet in place of a standard dry

diet [276], suggesting that the body weight loss may partially due to reduced salivary flow during the intake of food. In fact, in M_3R-deficient mice, the pilocarpine-induced salivary secretion was decreased [276]. In M_3R-knockout mice, a significant reduction (~40-95%) in carbachol-induced contraction of smooth muscle (urinary bladder, ileum, stomach fundus, trachea and gallbladder) was detected, and especially in urinary bladder, carbacholinduced contraction of smooth muscle was almost absent in M_3R-knockout mice, but present in M_2R-knockout mice, suggesting the crucial role of the M_3 receptor for inducing contraction in this tissue [276,277]. Mydriasis was observed due to the lack of parasympathetic control of the papillary sphincter muscle [276].

M_4 Muscarinic Receptor

The Muscarinic M_4 receptor is known to be abundantly expressed in the striatum [274]. Consistent with this fact, locomotor activity was significantly increased in M_4R-deficient mice [278]. This may also be caused by an increase in dopamine-release from the striatum, as administration of a dopamine D1 receptor agonist enhanced the locomotor activity [278,279].

M_5 Muscarinic Receptor

The M_5 receptor was the last muscarinic acetylcholine receptor cloned. Localization studies have revealed that the M_5R is abundantly expressed in dopamine-containing neurons of the substantia nigra par compacta, an area of the midbrain providing dopaminergic innervation to the striatum [274].

Concordantly, oxotremorine-mediated dopamine release in the striatum was markedly decreased in M_5R-deficient mice [280]. More intriguingly, in M_5R-deficient mice, acetylcholineinduced dilation of cerebral arteries and arterioles was greatly attenuated [280], suggesting that the M_5 receptor might be a suitable target for the treatment of cerebrovascular ischemia.

1.3 SCOPES AND AIM OF THIS THESIS

Muscarinic receptors comprise a large family of G protein-coupled receptors (GPCRs) involved in regulating the activity of many important functions of the central and peripheral nervous system. In order to relay such diversity of physiological outcomes, muscarinic receptors not only interact with heterotrimeric G proteins to activate or inhibit classical downstream effector molecules, but also with accessory molecules termed GPCR interacting proteins (GIPs). Such proteins are diverse in nature, involving scaffold molecules, ion channels, enzymatic activities as well as other GPCRs, thus leading to receptor homo- or heterodimerization and multiprotein complex formation (also called signalsome or receptosome). By means of these novel interactions, muscarinic receptors can acquire unappreciated abilities for ligand recognition, signalling and regulation. However, the dynamics of such a complex are not well understood. Many questions remain unsolved. For instance: Is the signalsome stabilized by agonist binding to the receptor? Or is the agonist the responsible for the initiation of the signal transduction complex *de novo?* Can signalsome organization be influencing the duration or magnitude of the signal? Which of the various identified protein interactors share a mutually exclusive site? And what are their roles?

The overall aim of this thesis was to gain insight into molecular aspects of the protein-protein interaction of the muscarinic receptor family. Specifically, the present work is focused on the recognition of novel interacting proteins of the M_3 subtype. The questions outlined above are addressed throughout the next chapters, and some of them are answered or partially answered.

The widely recognized role of dimerization for many cell surface proteins contrasts with the classic models proposed for GPCRs, which were generally believed to function as monomers and to signal through downstream G-proteins in a 1:1 stoichiometric ratio. Thus, the firsts aim for this thesis has therefore been:

1. To characterize the molecular interaction between M_3 and M_5 muscarinic receptor subtypes and to get insight into the physiological role of muscarinic receptor heterodimerization.

Topology of GPCRs allows several potential regions for protein-protein interaction (Extracellular (N-terminal) and intracellular loops (3Iloop and C-terminal tail)). A large number of interactions between aminergic receptors and their intracellular faces have been described in addition to those classically involved in signal transduction.

Therefore, the second aim of this thesis has been:

2. To identify interacting proteins of M_3 and M_5 muscarinic receptor subtypes within the large 3Iloop using Tandem Affinity Purification methods and mass spectrometry analysis.

3. To delineate the molecular interaction and functional roles of the C-terminal tail and its function as an antiapoptotic peptide, as well as determined their therapeutic uses and pharmacological implications.

Accumulating evidence indicates that signalling efficiency/specificity for mAChRs is determined in part by accessory proteins that physically interact with key domain/motif or are found in the microenvironment of the receptor. The extent of these GPCR-protein interactions varies, ranging from transitory interactions (i.e. signalling purposes) to more stable interactions. Therefore, GPCR-protein assemblies might be considered as dynamic complexes that contribute to the intricate process of downstream signalling and their regulation.

4. To characterize subtype-specific receptor signalling, both in the mechanisms involved in receptor regulation, as well as in the unveiling of novel receptor-interacting proteins mediated by the conserved motif (NPxxY (5,6) F) in the M_3 muscarinic receptor subtype.

CHAPTER 2

Oligomerization of muscarinic receptor family

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2.1 Homo-and heterodimerization of muscarinic M_3-M_5 receptors in non-neuronal cholinergic system.

2.1 Homo-and heterodimerization of M_3-M_5 muscarinic receptors in non-neuronal cholinergic system

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ABSTRACT

Although previous pharmacological, biochemical and biophysical data supports the notion that muscarinic receptors form homo- and heterodimers; and non-neuronal cholinergic system in lymphocytes, in both T- and B-cells, is characterized by highly expression of M_3 and M_5 muscarinic receptor subtypes; direct and functional interactions between M_3 and M_5 receptors and their underlying mechanism(s) have not been well explored today. By means of coimmunoprecipitation and bioluminescence resonance energy transfer, we demonstrated that M_3 and M_5 muscarinic receptors could form constitutive homo- and heterodimers in the cell surface of cotransfected cells. We find that co-expression of both receptors leads to a significant increase in the level of BRET signals with similar affinity values when compared to M_3 homodimerization, but with relatively higher affinity values with respect to M_5 homodimerization. Oligomerization statuses, which are not seen to be modified upon agonist stimulation, are, however, affected by the expression of minigenes derived from the third intracellular loop of each subtype. Signal transduction analysis in lymphocytes T-cells revealed that a proper receptor heteromer is required for mitogen-activated protein kinase phosphorylation and the positive modulation of cell growth. This suggests that the oligomeric association of muscarinic receptors provides a molecular mechanism for the increased diversity of cholinergic signalling in lymphocytes cells, and can be consider during therapeutic treatment of the immune system.

INTRODUCTION

Over the last decade, G protein-coupled receptors (GPCRs) have been shown to have a constitutive association with each other or with members of a related family to form homo- or hetero-oligomers [281,282]. GPCR oligomerization leads to alterations in receptor function, and has been implicated in many aspects of receptor pharmacology, such as maturation, ligand binding, activation, signaling, desensitization, endocytosis, and trafficking [78,105,106].

Recently, several studies showed that muscarinic acetylcholine receptors (mAChRs), like other GPCRs, may be arranged in oligomeric complexes [168,283,284]. Complexes binding curves of muscarinic receptors expressed in rabbit heart, rat brain stem, and computer simulations of agonist binding properties at M_2 muscarinic receptors expressed in cultured cells were consistent with the notion of binding sites located on dimeric receptor molecules [285,286]. Likewise, studies using purified muscarinic receptors suggest that the complex binding properties of muscarinic agonists are due to multiple states of affinity of the receptor proteins themselves, and are not caused by the association of receptors with different classes of G proteins [287]. Maggio et al., using chimeric α2-adrenergic/M_3 muscarinic receptors, not only provided additional pharmacological evidence that mAChRs can form dimmers, but also demonstrated that intermolecular interactions between two inactive receptors could result in a dimeric complex with the restoration of receptor function properties [288,289]. Site-directed mutagenesis and coimmunoprecipitation of solubilized epitope-tagged M_3 mAChR has also been employed to demonstrate the existence of mAChRs oligomers in a direct fashion [290]. This biochemical approach revealed that the M_3 mAChR is capable of forming disulfide-linked as well as noncovalent dimers/multimers [291]. More recently, using bioluminescence resonance energy transfer (BRET), the occurrence of constitutive homo- and hetero-oligomers among M_1, M_2, and M_3 mAChRs has been demonstrated in living cells [163].

Previous studies have provided evidence of the existence of a non-neuronal cholinergic system [292]. RT-PCR and pharmacology analysis have revealed that lymphocytes express most of the cholinergic components which expressed in

neurons, and constitute an independent cholinergic system that can be regulated in an autocrine fashion [293]. Stimulation of T and B cells with muscarinic receptor agonists elicits intracellular calcium signalling, up-regulation of c-fos expression, and increased in nitric oxide synthesis and cytokine production [294-296]. Furthermore, wide arrays of combination of mAChR subtypes are expressed among lymphocytes cells. Immunohistochemical, western-blot and RT-PCR studies show that the M_3, M_4 and M_5 subtypes are the most highly expressed and are responsible for immune cell function [297,298]. It has also been revealed that different muscarinic agonists affect immune functions in different ways. For instance, oxotremorine treatment enhances and arecoline suppresses IL-2 production and cell proliferation [299,300]. Another study showed that pilocarpine did not inhibit cytokine production, but decreased the number of interleukin receptor bearing cells [301]. M_5 KO mice suggest that the muscarinic receptor is involving in the modulation of antibody classes switching from IgM to IgG1 [296]. Also, in vivo experiments have shown that direct interaction between T-lynphocites and target cells (B-lynphocytes or antigen presenting cells) leads to up-regulation of the gene expression of M_5 receptor subtypes [302]. Thus, M_3 and M_5 muscarinic receptors are expressed on the same lynphocytes elements, displaying a complex signalling interaction. To date, however, no studies have examined the molecular mechanism underlying the interactions between these two receptors and their functional role in signal transudation modulation in lymphocytes cells.

In this study, we examined the M_3 muscarinic receptor as a potential partner for association with the M_5 receptor subtype, as well as each receptor subtype's ability to associate with the other in order to form homodimers. Using MAPK phosphorylation and cytokine production assays we determined the functional implications of heterodimer formation, as well as demonstrated that the activation of signalling cascade requires co-activation of both receptors.

RESULTS

Functional characterization of the tagged receptors

The affinities of the GFP^2- or Rluc-tagged receptors for the receptor-specific ligands were indistinguishable from those of the non-fused receptors, as shown in Table 2.1.1. The expression levels of the various receptors ranged between 400-

600 fmol/mg. And although co-expression of these fused or non-fused receptors showed no significant changes in their ligand binding affinities, the expression level of M_3-GFP^2 decreased a little by co-expression with M_3-Rluc.

Coupling of the different muscarinic constructs to the G_q/PLC pathway was assessed as their ability to mediate carbachol stimulated IP accumulation. The IP accumulation mediated by the differently tagged M_3 or M_5 was similar overall to that of the wild type receptor.

Table 2.1.1: *Ligand binding properties of M_3 and M_5 mAChR constructs. Radioligand binding studies of transfected HEK-293 cells with RLuc-tagged, GFP^2-tagged, or nontagged (WT) mAChR were carried out as described under "Experimental Procedures." Curves were fitted by nonlinear regression analysis by assuming a single binding site, and K_D values were determined by using GraphPad Prism software. Results represent means ± S.E. (n = 4).*

Receptor	**Antagonist binding [^{3}H]-NMS**	
	B_{max} (fmol/mg)	K_D (pM)
3 x HA-M_3	529.1 ± 42.5	63.2 ± 20.8
3 x HA-M_3-Rluc	483.0 ± 39.3	58.3 ± 19.2
3 x HA-M_3-GFP^2	513.1 ± 41.6	60.6 ± 21.4
3 x HA-M_5	520.2 ± 27.8	86.2 ± 16.5
3 x HA-M_5-Rluc	541.5 ± 32.7	92.1 ± 15.3
3 x HA-M_5-GFP^2	557.8 ± 37.5	89.9 ± 22.7

M_3/M_5 colocalization in the cell surface of Molt-3 cells

A number of behavioral studies in lymphocytes cells have previously demonstrated functional interactions between muscarinic receptors; the mechanism(s) underlying these are not clearly understood. One possible mechanism could be direct interactions between receptor subtypes.

Using immunocytochemistry and a co-immunoprecipitation approach we confirmed the preferential colocalization of the M_3 and M_5 muscarinic receptor in both co-transfected HEK-293 cells and endogenously expressing Molt-3 cells (Figure 2.1.1).

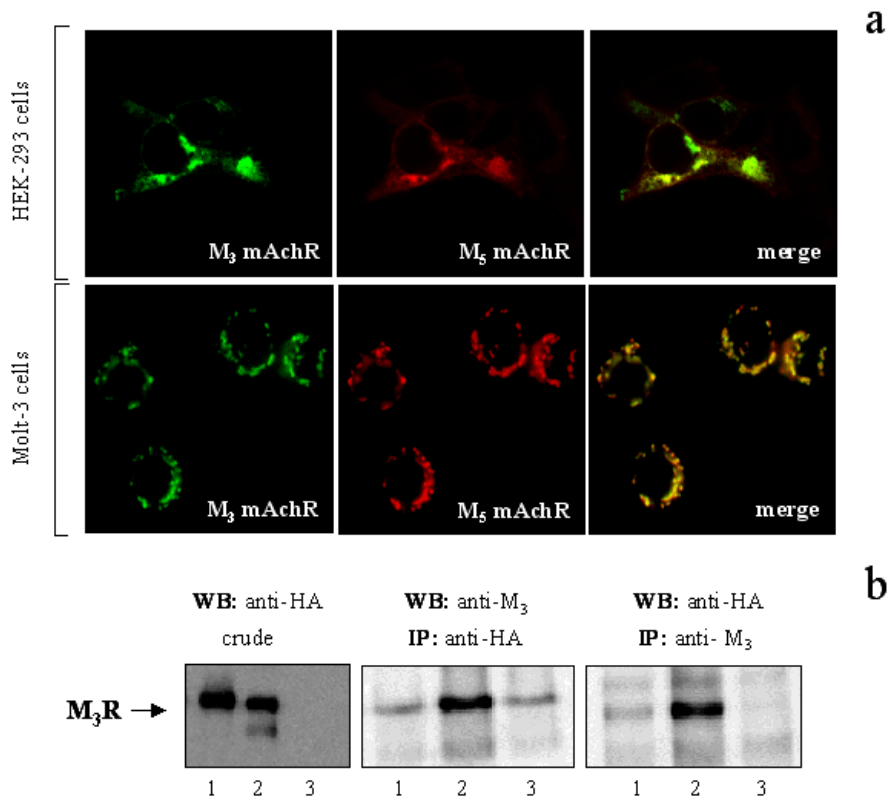

Figure 2.1.1 ***a)*** *Co-immunoprecipitation and colocalization of M_3 and M_5 muscarinic receptors. Non-permebilized Molt-3 cells endogenously expressing M_3 and M_5 receptor subtypes and HEK cells transfected with M_3, M_5, or both receptors simultaneously (coexpression) were immunostained with rabbit anti-M_3 monoclonal antibody and rabbit anti-M_5 monoclonal antibody. The bound primary antibodies were detected using either Alexa Fluor 488-conjugated goat anti-mouse IgG antibody or Texas Red-conjugated goat anti-rabbit. Cells were analyzed by double immunofluorescence with confocal microscopy. Superimposition of images reveals M_3/M_5 cell surface colocalization in yellow (merge).* ***b)*** *Coimmunoprecipitation of M_3 and M_5 receptors. HEK-293 cells transiently expressing HA-M_5 alone (lane 1), HA-M_5 plus M_3 (lane 2), or M_3 (lane 3) were washed, solubilized, and processed for immunoprecipitation using anti-HA monoclonal antibody (2 µg/ml; IP: HA) or anti-M_3 monoclonal antibody (2 µg/ml; IP: M_3). Solubilized membranes (Crude) and immunoprecipitates (IP) were analyzed by SDS-PAGE and immunoblotted using rabbit anti-HA polyclonal antibody (1:2000) or rabbit anti-M_3 polyclonal antibody (1:1000) and HRP-conjugated goat anti-rabbit IgG as a secondary antibody. These blots are representative of four different experiments with similar qualitative results. IB, Immunoblot.*

Quantification of images of specific immunostaining of M_3 and M_5 muscarinic receptor in Molt-3 cells (n = 4) showed there to be a 70 ± 5% (in means ± SEM)

of Molt-3 cytoplasm membrane cell and internal compartments that are immunoreactive for both M_3 and M_5, and whereas 14 ± 3% are endowed with M_3 but not with M_5, only 3 ± 1% are endowed with M_5 but not with M_3 (Figure 2.1.1, top). The number of cytoplasm membrane cells that do not express any of the receptors is < 15%. Therefore, distribution of both receptor subtypes in co-transfected HEK-293 cells was localized in the vicinity of the plasma membrane and internal compartments (Figure 2.1.1, bottom). These merged images revealed that a striking overlap in the distribution of both receptors likely happens in Molt-3 cells.

Immunoprecipitation reactions were carried out on HEK 293 cells transiently expressing 3x HA-tagged M_5 receptors, M_3 wild type receptors, or both, using anti-HA antibody resin. After separation of the proteins by SDS-PAGE, Western immunoblotting was performed using a rabbit anti-HA antibody (Sigma-Aldrich). As shown in Figure 2.1.1, an antibody against 3x HA-M_5 was able to immunoprecipitate M_3 receptor from solubilized cells and vice verse. This band did not appear in immunoprecipitates from cells only transfected with the cDNA for either 3x HA-M_5 or M_3 (Fig. 2.1.1).

Homo-oligomerization of M_3 and M_5 muscarinic receptors

In HEK-293 cells we examined the possibility of direct receptor-receptor interaction by constructing quantitative BRET saturation curves in cells co-transfected with a constant amount of receptor-Rluc construct and increasing concentrations of the receptor-GFP^2 plasmids.

Although from curves generated by fluorescence- and luminescence- directed measurements provide the theoretical behaviour sufficient to predict receptor oligomerization complexes, they do not provide enough information about the binding parameters required for proper quantitative analysis of receptor-receptor interactions. Because of this, we decided to do BRET analysis in a quantitative fashion. To complete this analysis, we conducted saturation experiments in which the amount of each receptor effectively expressed in transfected cells was monitored for each individual experiment by correlating both total luminescence and total fluorescence in the number of [^{3}H]-NMS-binding sites in permeabilized cells. Total luminescence and total fluorescence emitted by the Rluc and GFP^2 fusion proteins were measured following the addition of the Rluc substrate

coelenterazine H and direct excitation of the GFP^2 at 400 nm, respectively. Correlation obtained between the numbers of total binding sites and either the luminescence or fluorescence emitted by each of the receptor fusion molecules was linear, and the slopes were similar for the two receptors considered (Appendix 2). The linear regression equations derived from "Appendix 2" were thus used to transform the luminescence and fluorescence value in the receptor number. BRET signals were plotted as a function of the ratio between the receptor-GFP^2/receptor-Rluc numbers.

As shown in Figure 2.1.2 top, significant quantitative BRET signals were observed for the M_3/M_3 and M_5/M_5 pairs, confirming previous findings that M_3 receptor subtypes can form homodimers [163,303]. Albeit to a higher extent, in respect to M_5 receptor subtype homodimers, co-expression of the two subtypes also led to a sizable BRET signal (Figure 2.1.2 bottom). In all cases, BRET increased as a hyperbolic function of the concentration of the GFP^2 fusion construct increased (assessed by the fluorescence emitted upon direct excitation at 400 nm), reaching an asymptote at the highest concentrations used. Co-expression of M_3-Rluc with soluble GFP^2 led to marginal signals that increased linearly with an increasing amount of GFP^2 added.

When comparing the BRET saturation curves obtained for the M_3 homo- and heterodimers (Table 2.1.2), similar BRET50 values were obtained, indicating that the receptors had similar relative affinities with one another. However, BRET50 of M_5 heterodimers showed little high affinity with respect to M_5 homodimers. This has important implications, because it suggests that, under basal conditions, M_3 homo- and heterodimers have a similar probability of forming when the two receptors are heterologously expressed. Instead, it is very probably that M_5 receptor subtypes might form heterodimers when are co-expressed with M_3 receptor subtypes. Previous reports highlight that heterotrimeric formation between homologous receptors is highly probable [108].

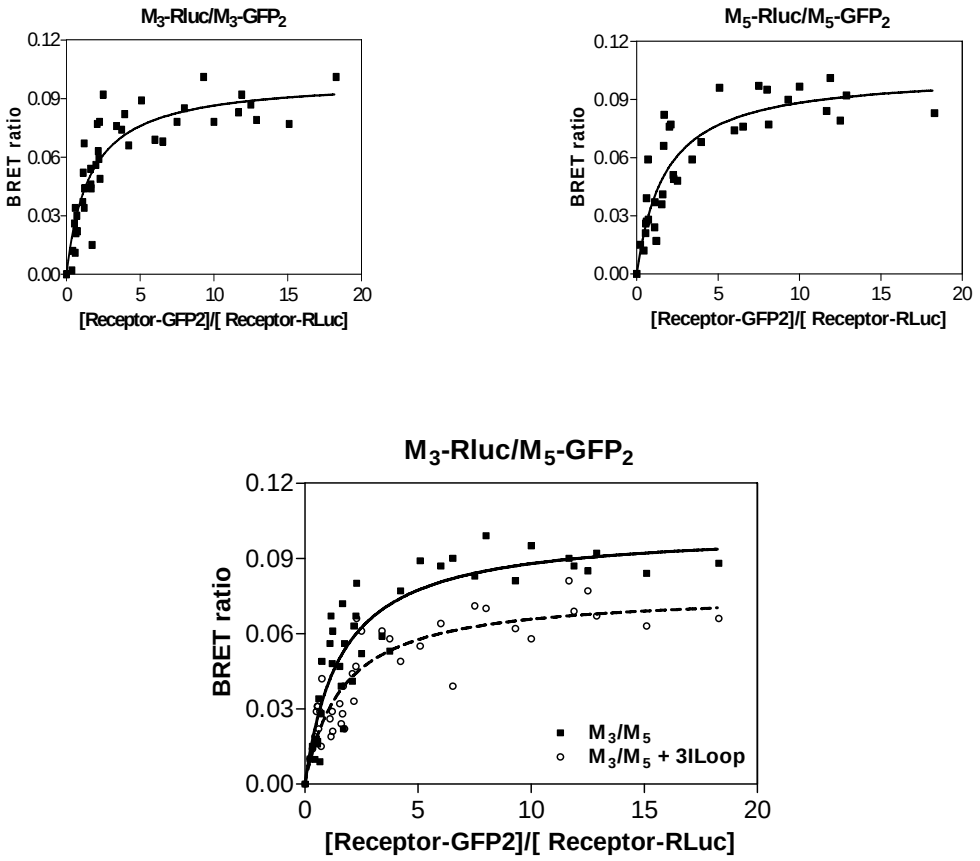

Figure 2.1.2 *Quantitative analysis of M_3 and M_5 homo- and hetero-dimerization. BRET donor saturation curves of M_3 and M_5 mAChR homo (top, middle)- and hetero (bottom)-dimers were performed by transfecting HEK 293 cells with a constant DNA concentration of acceptor receptor-Rluc and increasing concentrations of donor receptor-GFP^2 constructs. BRET, total fluorescence, and total luminescence values were determined as described under "Experimental Procedures." Fluorescence and luminescence values were transformed into receptor numbers using slope values from standard titration curves shown in supplemental material. Heterodimerization analysis was also performed in cells co-expressing both receptor subtypes in addition to the 3-5μg of minigene construction that codifies for a peptide of the third intracellular loop of the M_5 muscarinic receptor. BRET values were plotted as a function of the (receptor-GFP^2)/(receptor-RLuc) ratio. The curves represent 9 saturation curves that were fitted using a non-linear regression equation assuming a single binding site.*

In addition, the BRETmax value for each donor-acceptor pair was found to be significantly similar. This could indicate that energy transfer between RLuc and GFP^2 within each donor-acceptor pair were similarly efficient.

Table 2.1.2 *Parameters from BRET saturation curves.*

Pair Transfected	BRET max	BRET 50
M_3-Rluc/M_3-GFP^2	0.10 ± 0.05	1.59 ± 0.29
M_5-Rluc/M_5-GFP^2	0.10 ± 0.01	1.76 ± 0.32
M_3-Rluc/M_5-GFP^2	0.10 ± 0.01	1.58 ± 0.27
M_3-Rluc/M_5-GFP^2 + 3ILoop	0.07 ± 0.01	1.78 ± 0.33

The $BRET_{max}$ is the maximal BRET ratio obtained for a given pair. The $BRET_{50}$ represents the acceptor/donor ratio required to reach half-maximal BRET values. Results are the mean ± S.E. of 9 experiments performed in duplicate.

Several interesting findings regarding the effect of ligand binding on the formation of homo- or hetero-oligomers have been reported [116,133,304]. Eventually, changes in the BRET signal reflect either variations in dimer formation or simply conformational changes within pre-existing dimmers. In contrast, other studies have failed to show BRET changes upon agonist treatment [107,123,153,162]. In our experiments, stimulation with the agonist carbachol (10 μM) did not promote any consistent change in the BRET saturation curves, indicating that the dimers form constitutively and that receptor activation does not affect their oligomerization state (Supplementary materials).

Structural determinants potentially involved in hetero-oligomerization

So far, the mechanism by which muscarinic receptors form oligomers has been poorly elucidated at a molecular level. Several reports have suggested transmembrane domain swap, disulfide cross linking, or dimerization motifs within the transmembrane helices and third intracellular loop [305]. Among the structural determinants of the muscarinic receptor potentially involved in receptor oligomerization, we investigated the role of its large third intracellular loop in M_3 and M_5 hetero-dimerization.

To investigate whether the third intracellular loop played a role in receptor oligomerization, we co-expressed a constant among M_3-Rluc cDNA and a variable among M_5-GFP^2 with or without a minigene construct encoding for a peptide of the third intracellular loop. Previous reports have described that the use of peptides from regions implicated in receptor dimerization might induce reduction in BRET levels as result of either a dissociation of the protomers leading to monomerization or a conformational change within the dimer that increases the distance or negatively affects the dipole orientations of the energy donor and acceptor without disrupting the

dimmer [306]. In an effort to distinguish between these two possibilities, BRET titration experiments were carried out to determine the relative affinity between each of the protomers, as described previously [120]. Indeed, if the peptide promotes a dissociation of the protomers, one would predict that third intracellular loop should decrease the apparent affinity of the receptors for one another. As shown in Figure 2.1.2 bottom, the expression of the peptide of the third intracellular loop of the M_5 receptor led to a reduction in the BRETmax and a little, but significant, reduction in value of BRET50 (see Table 2.1.2). These results strongly indicate that the third intracellular loop of the M_5 muscarinic receptor (and by analogy, the third intracellular loop of M_3) acts by affecting the conformational organization of the dimmer, and also by promoting a little dissociation into monomers of the hetero-complex.

M_3/M_5 heteromeric complexes; receptor signal and T-cell immune response

Given that both M_3 and M_5 mAChR are coexpressed in many lymphocytes cells and that they were also found to form heterodimers (Fig. 2.1.2), we asked whether M_3 and M_5 heterodimerization could modify carbachol enhanced IL-2 production, which is the first in a series of lymphocytotrophic hormones and has a pivotal role in the generation and regulation of immune response. Previous reports described that muscarinic agonist oxotremorine M (Oxo-M) enhanced phytohemagglutinin (PHA)-induced IL-2 production in T-cells. PHA-induced IL-2 production was enhanced when cell were pretreated > 1hr with Oxo-M, and a 24 hr Oxo-M pre-treatment doubled PHA-induced cytokine production. As shown in Figure 2.1.2 bottom, transfection of cells with a peptide of the third intracellular loop resulted in a low affinity of M_3 and M_5 receptors to form a heteromer. Thus, we treated Molt-3 cells endogenously expressing M_3 and M_5 mAChR with 10-µM carbachol for 24 hr in the presence or absence of the minigene (5-µg cDNA) and determined the extent of cytokine production by ELISA (IL-2). Incubation of Molt-3 cell with PHA (10 -µg/ml) and 10-µM of CCh for 24 hr enhanced IL-2 production by 74 %. However, Molt-3 cell transfected with minigene construction only showed and agonist-enhancement of 57% (1.3–fold in less, ANOVA, $P<0.01$) (figure 2.1.3).

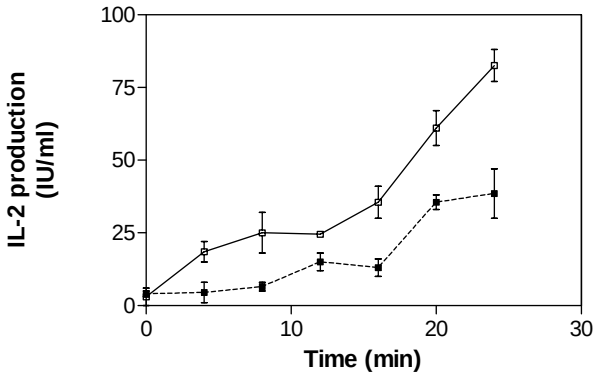

Figure 2.1.3 *Effect of heterodimerization disruption in carbachol enhanced PHA- IL-2 production. Molt-3 cells were transfected with vehicle (■) or minigene construction (□), preincubated with 10-μM CCh for 0-24 hr, following 24 hr of 10 -μg/ml PHA treatment; subsequently, the amount of IL-2 was measured. Values are expressed as IU (1IU/ml= 0.2 ng/ml). Data are means ± S.D. from a representative experiment preformed in triplicate.*

Next, we examined if the modulation of the receptor-enhanced cytokine production seen in Molt-3 cells endogenous expressing M_3/M_5 receptors can be observed using another GPCR signaling assay. For this, we monitored the extent of phosphorylation of extracellular signal-regulated kinases (ERK1/2) in response to carbachol in the absence or presence of the minigene construct. We find that treatment with carbachol (1-10 μM) leads to a significant increase in the extent of phosphorylation of ERK1/2 (Figure 2.1.4). However, carbachol stimulation of Molt-3 cells transfected with minigene (3-5 μg of cDNA) leads to a small but significant decrease in the extent of ERK1/2 phosphorylation (Figure 2.1.4). As a control, we examined the effect of the minigene construct in HEK-293 cells expressing M_3 homodimers on ERK1/2 phosphorylation. We find that expression of the minigene construct does not affect agonist-increase in ERK1/2 phosphorylation as in vehicle cells (pcDNA3.1). Results appear to be associated with an increased lower affinity of receptor heterodimerization with a consequent decrease in receptor signal. It has shown that mAChR subtypes exhibit different sensitivities to agonist-induced ERK½ phosphorylation. More exactly, in COS cells it has been demonstrated that the paired activation of the two components of the M_3 muscarinic receptor dimmer is required for induction of MAPK activation.

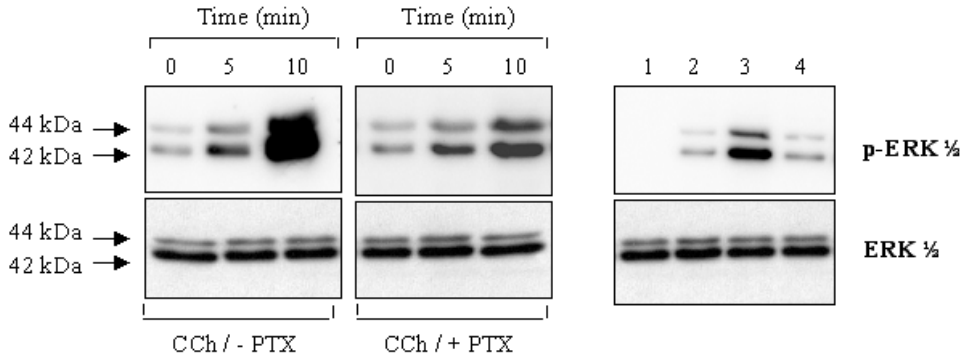

Figure 2.1.4 *Activation of ERK½ in Molt-3 cells endogenously expressing M_3 and M_5 muscarinic receptors. Cells were treated with agonist for 5 min at 37°C and then lysed directly into SDS-PAGE loading buffer. After Western immunoblot, phospho- ERK½ immunoreactivity was determined by densitometry. (A and B) Time-dependent course of ERK ½ activation on carbachol-stimulated Molt-3 cells and after overnight pretreatment with 100-ng/ml pertussis toxin. Cells were incubated with 1 mM of CCh for the indicated time points. (C) Activation of ERK½ in Molt-3 cells after 5 min of agonist stimulation in absence (lane 3) or presence (lane 4) of the minigene construct (5μg). Molt-3 cells non-stimulated with carbachol (Lane 1) and carbachol-stimulated in presence of MAPK specific inhibitor PD-98059 (lane 2) were performed as an internal control. Equal loading was confirmed with antibodies against total ERK ½. Means ±S.E.M is shown; n= 4. *: Significantly different when compared to Molt-3 minigene transfected cell (ANOVA: $p<0.05$).*

DISCUSSION

Muscarinic receptor families are capable of forming homo- and hetero-oligomers, which has been demonstrated by a wide variety of approaches, including pharmacological analysis of ligand-receptor interactions, coimmunoprecipitation, or, more recentl,y resonance energy transfer approaches (fluorescence resonance energy transfer and BRET) [163,307,308]. However, the existence of homo- and hetero-oligomers constituted by M_5 muscarinic receptor subtypes has not been reported yet, despite the large amount of experimental evidence that supports the active role of this receptor subtype in the non-neuronal cholinergic system.

In the present study we provide for a first time biochemical, biophysical, and functional evidence of the existence of receptor-receptor interaction between M_3 and M_5 muscarinic receptors and with each other. Our results from quantitative BRET saturation analysis show that M_3 and M_5 can form homoligomers in intact HEK 293 cells. Analysis of BRET parameters (BRET50 and BRETmax) supported the existence of constitutive M_3 and M_5 mAChR homodimers in live cells (Table 2.2). Results in agreement with previous pharmacological and BRET signal demonstrated M_1, M_2 and M_3 muscarinic receptor homodimerization [163], although BRET50 values

for M_5 muscarinic receptors were significantly higher than those determine for M_3 homodimers. This indicated that the likelihood of homodimer formation of the M_3 subtype appears to be slightly but consistently higher than with M_5 receptors subtypes (Table 2.1.2). Therefore, our data suggests that both mAChR subtypes in a given tissue results in a combination of homodimers, whose proportions depend on both the relative affinity between interacting species and their expression levels, and a minor complement of monomers.

Following the quantitative BRET saturation curve approach, we also demonstrated that co-expression of M_3 and M_5 muscarinic receptor subtypes can form heterodimers in HEK-293 living cells. Our results from BRET saturation curves clearly indicate that M_3 and M_5 receptors can form heterodimers. These results are also confirmed by co-immunoprecipitation experiments. BRET50 values from M_3/M_5 heterodimerization did not differ from values found for M_3 mAChR homodimerization curves. However, they were significantly lower than those determined for M_5 mAChR homodimers (Table 2.1.2). These results support the idea that the M_5 muscarinic receptor subtype exists as a combination of a high affinity homo- and hetero-dimers receptor population, but with a high affinity to form heterodimers when it is co-expressed with M_3 subtypes.

Although previous reports have demonstrated that GPCR agonist binding induces or stabilized receptor oligomerizatio, we cannot observe any change in the BRET parameters when cells were incubated in the presence of a carbachol agonist. It seems that for the muscarinic receptor family, agonist binding does not promote any conformational changes that disestablish the receptor dimerization state. By using SDS-PAGE and Western blot analysis of membrane extracts from COS-7 cells expressing a mutant M_3 mAChR, Zeng and Wess showed that agonist-induced receptor stimulation had no significant effect on M_3 mAChR dimerization [291]. In addition, carbachol treatment of HEK 293 cells expressing homologous or heterologous combinations of M_1, M_2, and M_3 mAChR fusion proteins revealed no significant changes in the BRET signal, as compared with control untreated co-transfected cells [163].

In an attempt to better understand the structural determinants involved in M_3/M_5 heterodimerization, we investigated the effects of the third intracellular loop on structural dimer organization. Previous studies using peptide strategies have identified structural determinants that can affect both GPCR dimers and function. For instance, a peptide derived from the TM6 domain of the β_2-AR was found not only to decrease the amount of receptor dimers detected in co-immunoprecipitation studies, but also to

inhibit receptor-stimulated AC activity, leading the authors to conclude that dimerization may be important in receptor function [309]. Likewise, Banères *et al.* found that a peptide mimicking the TM6 domain inhibited the dimerization of the BLT 1 receptor and affected the ability of the receptor to interact with the G protein [310]. In addition, Granier et al. demonstrated that a peptide mimicking the V2 receptor third intracellular loop inhibits the receptor function through a modification of its dimeric structural organization and direct action on G proteins [306]. In our studies, cells co-expressing M_3-Rluc and M_5-GFP^2 in the presence or absence of minigene construction encoding for a peptide for the third intracellular loop of the M_5 receptor subtype revealed a low but significant difference in BRET50. Therefore a significant inhibition in the $BRET_{max}$ signal was observed. These results strongly suggest that the minigene construct would modify the distance and/or orientation between M_3-Rluc and M_5-GFP^2 engaged in dimer formation affecting the oligomerization state itself. It also supports the notion that the peptide changes the conformation of a pre-existing dimer with promoting disassembly of protomers.

However it is important to notice that this effect was not higher (increased in 1,1 fold respect to BRET50 of cell without minigene expression). Probably indicating that in muscarinic receptor heterodimerization, specifically for M_3 and M_5, other structural components can actively participate in protomer organization and assembly. Based on mutagenesis and cross-linking experiments, helix VI for β-AR [309], helix IV for dopamine D_2 [137], or helices I and II for Ste2 pheromone receptor oligomers [311] have been proposed to provide inter-monomeric contacts. In the prototypical GPCR, rhodopsin, intradimeric contacts involving helices II, IV and V, as well as the cytoplasmic third intracellular loop are too involved in the formation of dimer rows [312]. Whereas the C terminus is implicated in the $GABA_B$-R1/$GABA_B$-R2 receptor oligomerization [313], the glycophorin motif seems to be involved in the formation of β_2-AR homo-oligomers [309], and the role of the N terminus int the oligomerization of the bradykinin and calcium receptors was reported [314,315]. Also, in the muscarinic receptor family, specifically the M_3 subtype, the functional role of the third intracellular loop in receptor dimerization has been demonstrated [316]. Altogether, these results support the notion that GPCR oligomerization involves the participation of different structural determinants whose role might differ among receptors.

The documented coexpression of M_3 and M_5 receptors in many acetylcholine-sensitive tissues like striatal dopamine neurons [317], the gastrointestinal tract [318] and T and B lymphocytes cells [297], suggests that heterodimerization could indeed occur in native mammalian tissues, assuming simultaneous expression of both receptors in the

same cells. As shown for the M_5 receptor, some receptors may form homodimers but preferentially engage with heterodimers. This may also be the case for the 1D-adrenergic receptor (1D-AR) [113], reflecting that a large spectrum of affinities is likely to exist for the formation of different GPCR heterodimers.

The determination of functional consequences of muscarinic receptor heterodimerization is difficult to achieve using classic radioligand competition binding assays and a specific pharmacological profile, particularly for heterodimers composed of M_3-M_5 subtypes that display similar affinities for the same radioligand. Understanding, in part, the structural determinants of M_3-M_5 heterodimers allows us to study the role of heterodimerization in signal transduction and cytokine production in lymphocytes T-cells by the use of a minigene construct which promotes a disestablishing effect in M_3/M_5 heterodimerization.

When Molt-3 cells endogenously expressing M_3 and M_5 receptor subtypes were transfected with the minigene construct, agonist stimulation led to a low level in ERK phosphorylation. Similar extensions were observed in agonist enhanced PHA-induced-IL-2 production. Theses results revealed that proper receptor heteromer is required for proper acetylcholine receptor response and function.

Our previous results demonstrated that the heterogeneous population of both M_3 and M_5 mAChR is composed of homodimers together with a significant fraction of heterodimers, whose proportion depends on both receptor subtypes and the relative affinity values for both homodimers and the M_3/M_5 heterodimer. Such a heterogeneous population of receptors oligomerization will provide a molecular mechanism for an increased diversity of cholinergic signalling in lymphocytes cells, a fact which might be considered during therapeutic treatment of the immune system.

In summary, we investigated M_3 and M_5 muscarinic receptor homo- and hetero-dimerization using BRET technology. The relative propensity for muscarinic receptor homo- and heterodimer formation was determined in quantitative BRET donor saturation assay and showed that M_3/M_5 heterodimers are formed at low expression levels and at a probability higher than the corresponding homodimers. We also determined the role of the third intracellular loop as a structural determinant in M_3/M_5 heterodimerization. In addition, our data suggest that agonist-induced mAChR signals in T lymphocyte cells are enhanced as a result of M_3/M_5 heterodimerization.

MATERIALS AND METHODS

Materials. [^{3}H]-Quinuclidinyl benzilate ([^{3}H]-QNB, 42 Ci/mmol) and N-[^{3}H]-methylscopolamine ([^{3}H]-NMS, 81 Ci/mmol) were purchased from Amersham Biosciences. Dulbecco's modified Eagle's medium, penicillin/streptomycin, and fetal bovine serum were purchased from Invitrogen. Restriction enzymes were from New England Biolabs (Beverly, MA), and coelenterazine h was obtained from Promega (Madison, WI). The anti-HA polyclonal antibody (HA.11) was purchased from Covance (Berkeley, CA). Carbamylcholine chloride (carbachol), atropine sulfate and all other reagents were purchased from Sigma.

cDNA Construction. The constructs presented herein were made using standard molecular biology techniques employing PCR and fragment replacement strategies. The cDNAs of the human M_3 muscarinic receptor (kindly provided by T. Bonner, NIH, USA) and human M_5 muscarinic receptors (provided by D. Bello, ETH, Switzerland) were subcloned into the mammalian expression vector pcDNA-3.1-3xHA (gift from P. Calvo, SFU, CA) giving the 3xHA-M_3-pcDNA and 3xHA-M_5-pcDNA vectors. Basically, 1.9 and 1.7-kb fragments encoding the human M_3, M_5 receptors were respectively amplified from their cDNAs using sense and antisense primers harboring unique EcoRV and XbaI sites, then subcloned into the mammalian pcDNA3.1-3xHA vector using EcoRV/XbaI.

The cDNAs fragment encoding a fragment of the third intracellular loop of M_3 and M_5 muscarinic receptor (123 and 100 amino acid residues respectively) were subcloned into the InterPlay® mammalian TAP system, pNTAP-B vector (Stratagene, USA) resulting in the pTAP-3IL-frag-M_3 and pTAP-3IL-frag-M_5 vectors. 3ILoop was amplified from the 3xHA-M_3-pcDNA vectors using the Expand High Fidelity PCR System (Roche, USA) and spliced into BamHI/EcoRI sites of the pNTAP-B vector.

M_3-Rluc and M_5-Rluc were constructed by ligating the Renilla luciferase (RLuc) to the C-terminal tail of each receptor subtype. Human M_3 and M_5 mAChRs coding sequences without their stop codons were amplified from 3xHA-M_3-pcDNA and 3xHA-M_5-pcDNA vectors using sense and antisense primers harboring unique XhoI and EcoRI sites. The fragments were then subcloned in-frame into humanized pRluc-N2 (h) vectors (Packard Bioscience). In a similar manner, M_3-GFP^2 and M_5-GFP^2 were constructed using sense and antisense primers harbouring unique EcorRI and BamHI sites and subcloned into the pGFP^2-N1 vector (Packard Bioscience). The reading frame and PCR integrity of all cloned constructs were confirmed by DNA sequencing.

Cell Culture and Transfection. HEK-293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100-µg/ml streptomycin, 2 mM L-glutamine (all from Invitrogen) streptomycin at 37°C in a humidified 5% CO2 incubator. For transfections, 2×10^6 cells were seeded into 100-mm dishes. About 24 h later, cells were transfected with 4 µg of plasmid DNA/dish by using the LipofectAMINETM Plus kit (Life Technologies, Inc.) according to the manufacturer's instructions. For BRET assays, HEK-293 cells were transfected using PolyEthylenImine reagent (PEI, Sigma-Aldrich) as described previously [319].

Molt-3 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM GlutaMAX-1, 1 mM sodium pyruvate, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in a humidified 10% CO2 environment. Molt-3 cells were transiently transfected using DEAE-dextran as described previously [320].

Membrane Preparation and Radioligand Binding Assay. About 48 h after transfection, HEK-293 cells were washed twice with cold PBS, harvested and homogenized in binding buffer (25 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM EDTA), using a Polytron tissue homogeniser. Cell membranes were collected by centrifugation at 20, 000g for 15 min and homogenized as above. After centrifugation at 40, 000g for 20 min at 4ºC, the final pellet was resuspended in binding buffer, and membranes were either used immediately or frozen in liquid nitrogen and stored at -80 °C until required. Protein concentration was determined by using the Bradford protein assay kit (Bio-Rad, USA). In order to determine the affinity of NMS for each sample, membranes were incubated with different concentrations of [3H]-NMS (ranging from 12.5 pM to 1.5 nM) in 5 mM PBS at 25°C for 60 min. The incubations were stopped by filtration through Whatman GF/B filters and washed extensively with ice-cold 40 mM PBS prior to scintillation counting. Non-specific binding was determined in the presence of 10μM atropine.

Co-immunoprecipitation and western blot. Immunoprecipitation and western blot experiments were carried out as described previously (canals M., Burgueño j. 2004). Protein immunodetection on membranes was assessed using anti-M_3 (1: 1000), anti-M_5 (1: 1000) and anti-HA (1: 2000) as primary antibodies; and then horseradish-peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:60 000) as secondary antibody.

Immunocytochemistry Staining. For immunocytochemistry, Molt-3 cells (endogenously expressed M_3 and M_5 receptor subtypes) or transiently co-transfected HEK-293 cells were grown on poly-D-lysine-coated glass coverslips and fixed with 4% formaldehyde solution for 20 min followed two washes with PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. Then, after permeabilization with buffer A containing 0.2% Triton X-100 for 5 min, cells were treated with PBS containing 1% bovine serum albumin (buffer B). After 1 h at room temperature, cells were labelled with the indicated primary antibody for 1 h, extensively washed, and stained with the indicated fluorescently labelled secondary antibody. Samples were rinsed and visualized on a Leica SP1/MP confocal microscope (HEK-293 cells) or Leica DMRB fluorescence microscope (Molt-3). The primary antibodies used were as follows: rabbit anti-HA monoclonal antibody (2 μg/ml; Sigma), mouse anti-M_3 polyclonal antibody and rabbit anti-M_5 polyclonal antibody (1:500, Santa Cruz Biotechnology). The secondary antibodies used were as follows: Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200; Invitrogen, Eugene, OR), Texas Red-conjugated goat anti-rabbit IgG (1:1000; Invitrogen).

BRET assays. Forty-eight hours after transfection HEK-293 cells with a constant (2 μg) or increasing amounts of cDNA of Receptor-Rluc (M_3 or M_5) and Receptor-GFP^2 (M_3 or M_5)

respectively were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspension (20 µg of protein) was distributed in duplicated into 96-well microplates (either clear-bottomed or white opaque Isoplates) for fluorescence and luminescence determinations). The total fluorescence of cell suspensions was quantified and then divided by the background (mock-transfected cells) in a Fluostar Optima Fluorimeter (BMG, Labtechnologies, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter of 400 nm and an emission filter of 510 nm. And *Renilla luciferase*, total luminescence, was determined on samples incubated 10 min with 5-µM coelenterazine H (Molecular Probes, Invitrogene, USA) using a Mithras LB 940 (Berthold Technologies, Germany) as described bellow. The background values for total luminescence were negligible, and they were subtracted from sample values.

For BRET measurement, 20 µg of cell suspensions were distributed in duplicate in 96-well white opaque microplates (Corning, NY) and incubated for 10 min at 25°C in the absence or presence of carbachol. DeepBlueC substrate (Molecular Probes, OR) was added at a final concentration of 5 µM, and readings were performed immediately after, using a Mithras LB 940 (Berthold Technologies, Germany) that allows the sequential integration of the signals detected with two filter settings [485 nm (440-500 nm) and 530 nm (510-590 nm)]. The BRET ratio is defined as [(GFP^2 emission at 500–530)/(Rluc emission 440–500)] – cf., where cf. corresponds to (emission at 510–590)/(emission at 440–500) for the Receptor-*R*luc construct expressed alone in the same experiment.

Luminescence and fluorescence levels of several receptor-RLuc and receptor-YFP (or receptor-GFP) fusion proteins have been found to be linearly correlated with receptor numbers (McVey et al., 2001; Ayoub et al., 2002; Mercier et al., 2002; Jockers R, 2004). Because this correlation is an intrinsic characteristic of each fusion protein, correlation curves have to be established for each construct. HEK 293 cells were transfected with increasing cDNA concentrations of the Receptor-Rluc or GFP^2 fusion protein constructs. Maximal luminescence and fluorescence was determined as described above and correlated with relative receptor number determined in the same cells as described under "Radioligand Binding Experiments". Luminescence and fluorescence were both plotted against binding sites, and linear regression curves were generated. The standard curves generated for each single experiment were used to transform fluorescence and luminescence values into femtomoles of receptor. Thus, the fluorescence/luminescence ratios were transformed into (receptor-YFP)/(receptor-RLuc) ratios, which allowed us to determine accurate BRETmax and BRET50 values (Mercier JF. 2002). To control the number of cells and also to express receptor numbers in fmol/mg of total cell protein, protein concentration was determined using a Bradford assay kit (Bio-Rad).

ERK phosphorylation. Transfected Molt-3 cells endogenously expressing the M_3 or M_5 receptor subtype with or without the 3ILoop-minigene were grown to 80% confluence and rendered quiescent by serum starvation overnight prior to ERK phosphorylation; an

additional 2h incubation in fresh serum-free medium was performed to minimize basal activity. Cells were subsequently stimulated by addition of medium with or without the muscarinic agonist carbachol. Rapid rinsing with ice-cold PBS finished stimulation, and cell lysis was performed during 10 min by the addition of 500 µl ice-cold lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1% TritonX-100, protease and phosphatase inhibitor cocktail). The cellular debris was removed by centrifugation at 13, 000g for 5 min at 4°C, and the total protein content was measured using BCA Protein Assay Reagent (Pierce, USA). Aliquots corresponding to 5 µg of protein were mixed with sodium dodecyl sulfate (SDS) loading buffer, applied to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot. Extracellular signal regulated kinase ½ (ERK ½) activation was assayed by incubating PVDF blots with a mouse anti-phospho-ERK ½ antibody (Sigma-Aldrich, Germany). Control blots were also run in parallel, and probed with rabbit anti-ERK ½ antibody (Sigma-Aldrich, Germany) that recognized both unphosphorylated and phosphorylated forms. The immunoreactive bands were visualized using horseradish peroxidase-linked secondary anti-mouse and anti-rabbit antibodies (Dako, USA) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) and then measured by quantitative densitometry.

IL-2 production. The amount of IL-2 produced by Molt-3 cells (transfected with the 3ILoop-minigene or mock pcDNA3.1) was determined using and ELISA kit for human IL-2 (DuPont-New England Nuclear). In brief, cell-free culture supernatants were obtained by centrifugation at 500-x g for 5 min. Samples were assayed fallowing manufacture's instruction and analysed on an automated ELISA plate reader. Data is expressed as IU (1 IU/ml = 0.2 ng/ml).

Data analysis. All binding data were analysed using the commercial program GraphPad PRISM 4.0 (GraphPad Software, USA). BRET saturation curves were analyzed using Prism 4 software. Isotherms were fitted using a nonlinear regression equation assuming a single binding site, which provided BRETmax and BRET50 values. The correlation between fluorescence or luminescence and receptor density was analyzed by a linear regression curve fitting with the same software. For statistical evaluation of the biochemical data, unless otherwise specified, one-way analysis of variance (ANOVA) was used. Group differences after significant ANOVAs were measured by post hoc Bonferroni´s Multiple Comparison test.

CHAPTER 3

The third intracellular loop as a key structural determinant in protein-protein interaction

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3.1 Impaired multiprotein complex formation and signal transduction of the M_3 muscarinic receptor by its soluble third intracellular loop.

3.2 Systematic identification of muscarinic receptor family interacting proteins by mass spectrometry.

3.1 Impaired multiprotein complex formation and signal transduction of the M_3 muscarinic receptor by its soluble third intracellular loop

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ABSTRACT

Several motifs located in the third intracellular loop of the M_3 muscarinic receptor are critical for G protein activation and scaffold protein interaction. To date, however, the dynamic feature of multiprotein complex formation is not fully understood. We have constructed a minigene encoding the third intracellular loop of the M_3 muscarinic receptor in order to explore the feasibility of peptides from the intracellular regions of G protein-coupled receptors to act as inhibitors of muscarinic multiprotein complex formation and signalling. We find that this construct, when co-expressed with the M_3 receptor, shows the capacity to act as a competitive antagonist of the receptor for G protein and receptor-scaffold/accessory protein interactions. Transient transfection of human embryonic kidney-293 cells with DNA encoding the human M_3 and M_5 receptor subtypes results in a carbachol-dependent increase of inositol phosphate. Co-transfection of the third cytoplasmic loop minigene dramatically reduces inositol phosphate levels and blocks agonist-induced activation of $G_{q/11}$ protein for M_3 and M_5 muscarinic receptors. The effect of the minigene expression on the regulation of the mitogen-activated protein kinases pathway is suggested by to the lack of ERK½ protein activation. Furthermore, minigene expression led to reduced AKT activation and deficient NF-κB translocation to the nucleus. These data, together with the results of co-immunoprecipitation of different scaffold and kinase proteins, provide experimental evidence for the role for the third cytoplasmic loop

of the human M_3 muscarinic receptor in multiprotein complex formation and G-protein activation.

INTRODUCTION

Muscarinic acetylcholine receptors (mAChRs) comprise a class I subfamily of heptahelical, transmembrane G-protein coupled receptors (GPCRs) and are represented by five distinct subtypes, denoted as M_1, M_2, M_3, M_4 and M_5 [183,266]. The functional response of mAChRs is through heterotrimeric guanine nucleotide-binding proteins (G proteins), involving primarily the third intracellular loop (3ILoop). Muscarinic M_1, M_3 and M_5 receptors preferentially couple to $G_{q/11}$ thereby activating phospholipase C-β, and inducing a subsequent increase in intracellular calcium concentration [321]. In contrast, M_2 and M_4 couple primarily to G-proteins of the $G_{i/o}$ classes, typically leading to adenylate cyclase inhibition and the activation of inward-rectifier potassium conductance [202,203]. Among different other possible responses, in a suitable cellular context, all mAChR subtypes can regulate a wide network of signalling intermediates, including small GTPase Rho, phospholipase D, phosphoinositide-3 kinase, non-receptor kinases and mitogen-activated protein kinases [322-325].

Evidence has been mounting in the past years to indicate that signalling efficiency/specificity for mAChRs is determined in part by accessory proteins that physically interact or are found in the microenvironment of the receptor [326]. A number of proteins have been shown to interact with mAChRs, including other GPCRs, kinases, and scaffold proteins such as β-arrestin [64,327,328]. These proteins together with the classical core signalling entities (receptor, G protein and effectors) contribute to the formation of a signalsome complex at the cytoplasmatic face of the receptor [64]. Understanding the nature and dynamic features of such a complex, which have not been unambiguously established, may be a key step in designing novel strategies for the development of next generation of drugs. The sequence similarities of the different subtypes, at the ligand binding sites, is the main hurdle for designing and identifying proper subtype-selective ligands [329,330]. However, important differences in size and homology of the intracellular loops across different muscarinic receptors, derived from sequence alignment, can be responsible for the specificities of each subtype.

Previous studies on muscarinic receptor signalling mechanisms have demonstrated that the cytoplasmic domain of these receptors, particularly the second and third intracellular loops, is critical in mediating signal transduction by G proteins [331]. Indeed, previous observations using receptor-derived peptides from specific regions of the M_1 and M_2 receptors have shown that the C-terminal tail of the 3ILoop is critical for functional receptor-G-protein interaction [332]. These observations have confirmed that there are different structural determinants for receptor-G-protein coupling and G-protein activation. More recently, specific motifs in the 3ILoop of M_1 and M_3 receptor have been shown to bind some accessory proteins with high affinity (calmodulin, oncogenic SET, and small GTPase Rho) [64,333-335].

These observations point to a specific role of 3ILoop in receptor-G protein coupling, signal transduction and multiprotein complex formation. Thus, we hypothesized that the soluble expressed 3ILoop could act as an analogue of the receptor 3ILoop, and as a scaffold protein as well, competing with the receptor for its interacting proteins, and therefore inhibiting the specific G-protein-mediated downstream effects.

To verify this hypothesis, we developed a minigene construct capable of expressing the human M_3 muscarinic receptor 3Iloop (3ILoop-minigene), and measured its ability to inhibit downstream signalling of receptors that couple to $G_{q/11}$ proteins. Our results suggest that the presence of the 3ILoop-minigene construct not only prevents G-protein coupling to M_3 receptors but also impairs the functional coupling of other GPCRs that selectively interact with the same G protein population. We also find that the construct can inhibit the M_3 muscarinic signal transduction pathway and its functionality, probably by recruiting interacting proteins as confirmed by co-immunoprecipitation experiments.

These results highlight the functional relevance of the interplay among GPCRs and selective G-protein pools, a key process for cell regulation. Moreover, muscarinic receptor-derived peptides could be used as selective inhibitors for the protein-protein interaction in which these receptors are involved. This approach could contribute to the development of novel pharmacological strategies aimed at suppressing non-desired GPCR hyperactivity (or altered physiological function) in some pathological conditions.

RESULTS

Design, construction and expression of 3ILoop-minigene.

The 3ILoop of the muscarinic receptor family is one of the largest in the GPCR superfamily (156-239 amino acids) and shares a low degree of sequence homology between mAChRs subtypes, with the exception of amino acids located at the C and N flanking regions of the loop (Figure 3.1.1 A). The 3ILoop has been shown to be critical for G protein coupling and activation and is one of the main target domains for interacting proteins [64]. These physical interactions with a wide range of proteins often lead to the formation of a multiprotein complex that is able to elicit a signal cascade, bypassing the need to couple with the heterotrimeric G-proteins for signalling [79].

To determine whether the expression of the 3ILoop could selectively antagonize G protein signalling and work as an analogue protein, competing by the same pool of scaffold and interacting proteins, we generated a minigene vector that encodes a set of peptides (Calmodulin Binding Peptide/Streptavidin Binding Peptide/3ILoop) as described under Materials and Methods (Figure 3.1.1 B).

HEK-293 cells were transiently co-transfected with the minigene and the M_3 or M_5 muscarinic receptor. Total RNA was isolated 48 hours post-transfection and analyzed by RT-PCR, using a set of primers that spanned the vector (SBP) and inserted sequence (3ILoop), to confirm the transcription of the 3ILoop-minigene. Our results confirmed the presence of a single 541-base pair band corresponding to the RT-PCR product of the minigene in the transfected cells (Figure 3.1.1C).

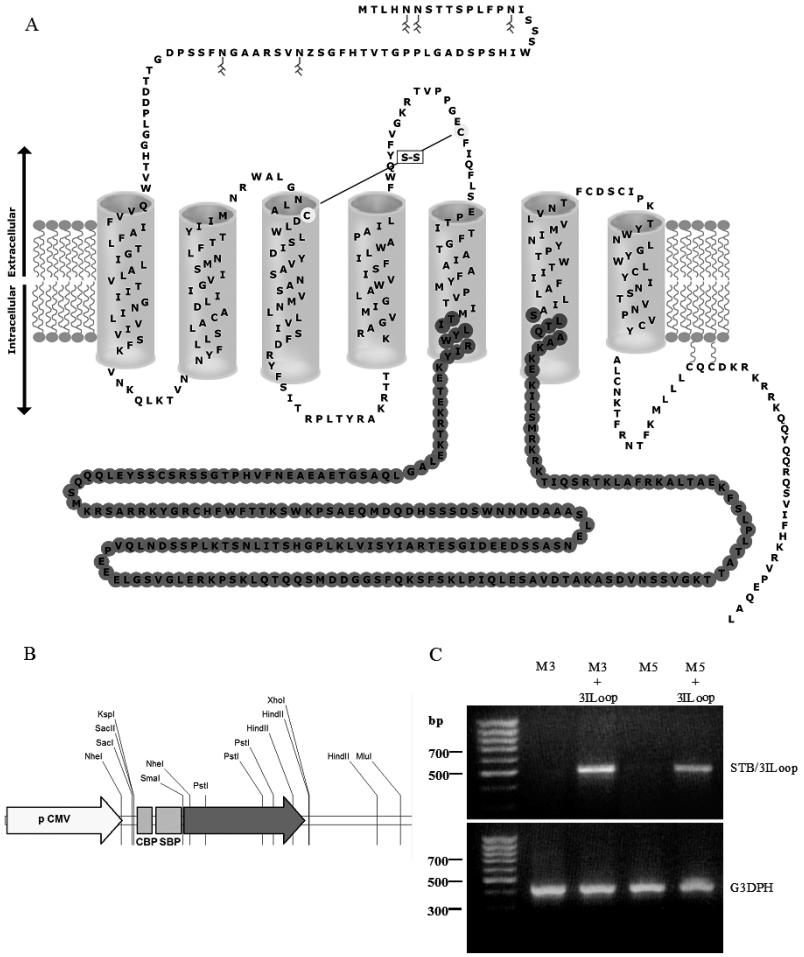

Figure 3.1.1 *Topological model of the M_3 human muscarinic receptor and the minigene construction. (A) A two-dimensional topology of the M_3 human muscarinic receptor sequence is represented (extracellular space at the top and the intracellular space at the bottom). Filled circles represent the amino acid sequence of peptide 3ILoop employed to construct the minigene. (B) Design of the 3ILoop-minigene vector (884 bp) is also depicted: CBP, Calmodulin Binding Peptide; SBP, Streptavidin Binding Peptide; 3ILoop (630 bp), the Third Intracellular Loop of the M_3 human muscarinic receptor. (C) HEK 293 cells were transiently co-transfected with M_3 or M_5 receptor subtypes and 3ILoop-minigene vector. To confirm the transiently transfection of minigene in HEK 293 cells, total RNA was isolated and subjected to RT-PCR. The PCR analysis was achieved using primers specific for a CBP + 3ILoop fragment. Separation of the PCR products on 1.5% agarose gels indicates the presence of the 3ILoop-minigene RNA by a single 541-bp band. Lane 1 is a 1-kilobase pair DNA ladder; lane 2 is a PCR product from cells transfected with pcDNA-3xHA-M_3; lane 3 correspond to cells co-transfected with pcDNA-3xHA-M_3 and 3ILoop-minigene; lane 4 correspond to cells transfected with pcDNA-3xHA-M_5; and lane 5, to cells co-transfected with pcDNA-3xHA-M_5 and 3ILoop-M_3 minigene. G3DPH gene was used as housekeeping control.*

We also attempted to demonstrate expression of the minigene construct by Western blotting, but due to the poor quality of available antibodies against the third intracellular determinants of the M_3 subtype, we were not able to detect expression of the minigene protein product (data not shown).

3ILoop-minigene effect on receptor binding and expression levels.

The K_d values for [^{3}H]-NMS binding to membranes obtained from cells expressing the M_3 receptor in the presence and in the absence of the minigene were not significantly different (Table 3.1.1).

Table 3.1.1 *Ligand binding and functional properties of M_3/M_5 muscarinic receptor expressed alone or together with the 3Iloop-minigene.*

Receptor	**Biding studies**		**PI assays**
	[^{3}H]-NMS K_D (pM)	B_{max} (fmol/mg)	Folds increased respect basal level
M_3-WT	85.3 ± 17.7	1250 ± 106	7.1 ± 2.2
M_3 + 3ILoop	86.8 ± 27.9	1219 ± 66	3.93 ± 2.3
M_5-WT	164.9 ± 41.4	934 ± 46	6.1 ± 1.1
M_5 + 3ILoop	163.7 ± 55.03	908 ± 32	4.6 ± 2.5

Affinity constants (K_D) for radioligand [^{3}H]-NMS was determined in saturation binding assays. B_{max} values indicate maximum number of binding sites/mg of membrane protein. Data is presented as means ± SEM for two to five (binding studies) or three to six (PI assays) independent experiments, each performed in duplicate s.

Parallel experiments were performed with a HEK-293 cell line expressing M_5 muscarinic receptor to determine whether the 3ILoop-minigene has the same behaviour when expressed in cells with another $G_{q/11}$-coupled receptor. The M_5 subtype showed similar specific [^{3}H]-NMS binding either in the presence or in the absence of the 3ILoop (Table 3.1.1). These results indicate that no major changes or modulation of the conformational state of the orthosteric binding site of the receptors are detected as a result of the presence of the minigene, and are in agreement with those obtained for M_3-expressing cells.

3ILoop-minigene inhibits agonist-mediated stimulation [^{35}S]-GTPγS binding. The 3ILoop is known to be critical for G-protein coupling and activation, and the expression of the minigene seems to compete for the same pool of G proteins or target the receptor-G protein interface. Therefore, a reasonable step forward was to determine whether the presence of the minigene, co-transfected in M_3 or M_5 transfected cells, had any effect on G-protein activation. To this aim, agonist-induced stimulation of [^{35}S]-GTPγS binding to membranes from M_3 or M_5-HEK-293 cells after transfection with the minigene or vector in the presence of 1 μM GDP was carried out. Cells co-transfected with M_3 or M_5 receptor subtype with the empty vector revealed a 10-fold increase of CCh-stimulated [^{35}S]-GTPγS binding for both receptors, but with a lower potency (pEC-50_{M3}= 4.84 ± 0.04 and pEC-50_{M5}= 5.77 ± 0.05) in the case of the M_5 receptor (Figure 3.1.2).

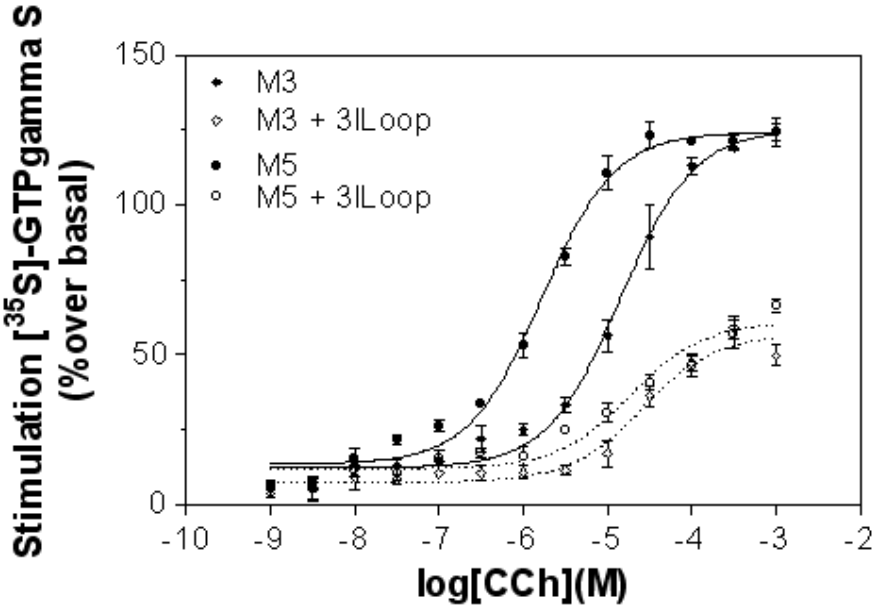

Figure 3.1.2 *3ILoop-minigene effect on agonist-stimulated [^{35}S]-GTPγS binding to membranes from cells expressing the M_3 or M_5 receptor subtypes. Bindings of [^{35}S]-GTPγS binding (0.3 nM) to membranes prepared from transient expressing M_3-HEK-293 or M_5-HEK-293 cells were measured after incubation at different concentrations of carbachol for 1h at 30°C in the presence (●) or absence (■) of 3ILoop-minigene (5μg). Values are expressed as percentage (%) of specific binding to the sample in the absence of ligand (control). Data are means ± S.D. from a representative experiment preformed in triplicate.*

The co-expression of the minigene with the M_3 subtype resulted in a decrease of 45% in agonist-dependent stimulation of [^{35}S]-GTPγS binding, and a relative efficacy decay of 2.2 fold with regard to the M_3 control vector (*Emax* % over basal of 56.9 ± 1.9 and 125.3 ± 2.2, respectively). Similar results were obtained in cells expressing the M_5 subtype. The presence of the minigene reduced carbachol-mediated stimulation of [^{35}S]-GTPγS binding in about 50% (Figure 3.1.2) ($Emax_{M5+3ILoop}$= 60.82 ± 2.4, and $Emax_{M5}$=124.4 ± 2.0). This suggests that the 3ILoop-minigene inhibits the agonist-mediated stimulation of [^{35}S]-GTPγS binding to $G_{q/11}$ proteins mediated by M_3 receptor or M_3-closely related GPCR (for instance the M_5 subtype).

Effect of 3ILoop-minigene Expression on PLC Activation.

We also analyzed agonist-stimulated phosphatidylinositol (1,4,5) trisphosphate (Ins-(1,4,5)-P3) formation in HEK-293 cells co-expressing each receptor subtypes with the minigene or the empty vector in order to assess whether the presence of the minigene could compete and recruit G protein-mediated activation of receptors that interact with the same population of G proteins. First, we characterized the time- and concentration dependence of carbachol-stimulated [^{3}H]-myo-InsPs accumulation in the transfected M_3 and M_5-HEK-293 cells (data not shown). [^{3}H]-myo-InsPs accumulation reached a peak between 5-10 min after agonist exposure. Complete desensitization occurred within 30 min of high dose stimulation. Non-appreciable carbachol-stimulated [^{3}H]-myo-InsPs accumulation was detected in non-transfected cells.

Figure 3.1.3 shows the concentration-response curves for carbachol-dependent stimulation of [^{3}H]-myo-InsPs in M_3 and M_5-HEK-293 cells co-expressing the minigene or the empty vector. Carbachol produced a seven fold stimulation of inositol phosphate over basal, suggesting that the presence of endogenous G proteins in HEK293 cells can effectively activate PLC. In contrast, expression of the minigene vector abolished M_3-agonist [^{3}H]-myo-InsPs accumulation essentially at all agonist concentrations, with a reduction in relative efficacy in approximately 50%. A similar change was observed in cells expressing the M_5 receptor, in which Ins- (1,4,5) P3 release upon carbachol stimulation produced an increased of 6 folds in [^{3}H]-myo-InsPs accumulation in contrast with the

reduction observed in relative efficacy values (46%) when co-expressed the minigene vector.

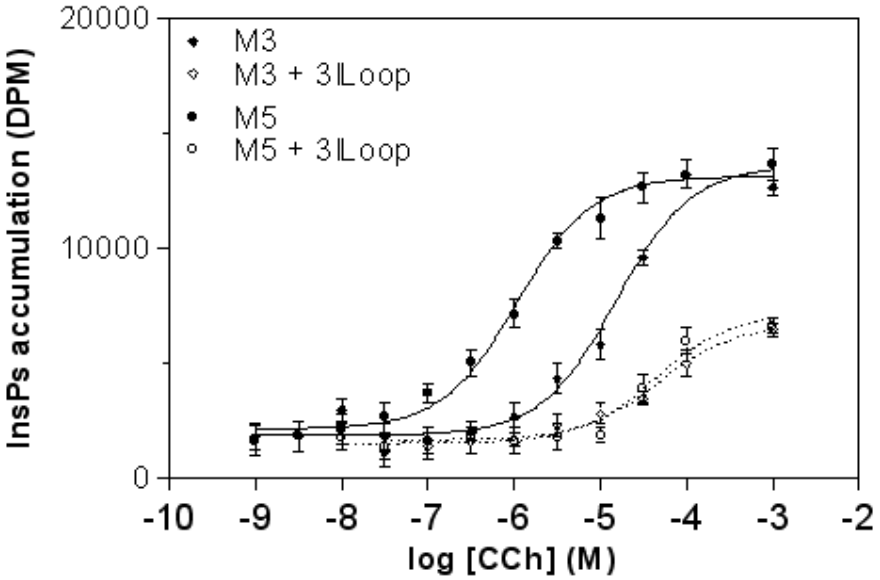

Figure 3.1.3 *Effect of 3ILoop-minigene vector and muscarinic receptor subtypes co-expression on PLC activity in CCh-stimulated HEK-293 cells. Cotransfected cells with M_3 or M_5 receptor subtypes and 3ILoop-minigene (●, ○) or empty vector (■, □) were stimulated with CCh as indicated, for 1 hr at 37°C in presence of LiCl as described in Materials and Methods. Data are presented as increase of InsPs (DPM) above basal levels in the presence of carbachol. Basal levels observed with the various receptors were not significantly different. Data represent the average ± S.E.M. values of triplicate determinations of three independent experiments.*

In addition to the change observed for both subtypes, in the maximum PLC response between minigene and control transfection we also observed consistent and statistically significant increases in the carbachol concentration needed for 50% stimulation of Ins- (1,4,5) P3 release for both receptors when co-transfected with the minigene (pEC_{50} in presence of 3ILoop-minigene construct referred to the control, decreased in 1.1 ± 0.6 fold for M_3 receptor and 1.4 ± 0.5 fold for M_5 receptor).

In order to ensure that EC_{50} and *Emax* changes observed were not due to alterations in the receptor density, we plotted the negative logarithm of EC_{50} *vs.* receptor density for experiments with co-expression of the minigene. This plot

shows that receptor expression was rather variable but that the EC_{50} shifts were independent of receptor density (data not shown).

Effect of 3ILoop-minigene expression on ERK ½ activation.

Previous experimental evidence supports the idea that at least two distinct mechanisms are involved in the activation of ERK ½ pathway by muscarinic receptors [219,235]. The first mechanism is PKC-dependent and is essential for the activation of ERK ½, whereas the second mechanism would be dependent on the phosphorylation state of the receptor. This second mechanism operates along with PKC-dependent activation and could be associated with the activity of a series of kinases (CK1α and GRKs) that recognize a specific domain in the third intracellular loop (His374-Val391 for M_3 subtype) [235]. As mentioned above, the expression and binding capacity of M_3 and M_5 receptor subtypes were unaffected by the presence of the minigene vector, whereas coupling to $G_{q/11}$ proteins was altered, and as a consequence carbachol failed to stimulate phosphatidylinositol production. We evaluated whether expression of the minigene could further alter ERK ½ signal transduction after agonist stimulation, and if this vector could be used as a potential inhibitor of this type of GPCR signalling.

First, a time course for ERK ½ activation was performed (Figure 3.1.4 A). Stimulation of both M_3 receptors co-transfected with the empty vector or with the minigene, by 20-μM carbachol, caused maximal activation of ERK ½ at 5 min. However, figure 3.1.4 B shows that ERK ½ activation by carbachol in cells co-transfected with minigene was significantly lower than ERK ½ activation in cells transfected with the empty vector (4–fold in less, $P<0.01$).

Figure 3.1.4 C shows the effects of minigene co-transfection on MAPK activity in cells expressing either M_3 or M_5 receptor subtypes. A significant decrease in ERK ½ phosphorylation was observed in both cases when compared to activation in the presence of the minigene vector. Preincubation with the MEK specific inhibitor PD-98059 inhibited carbachol-induced ERK ½ phosphorylation to the same extent as that obtained in cells co-transfected with the minigene construct. This confirmed that the 3ILoop-minigene vector acts as an efficient inhibitory protein of the MAPK signalling pathway after agonist stimulation.

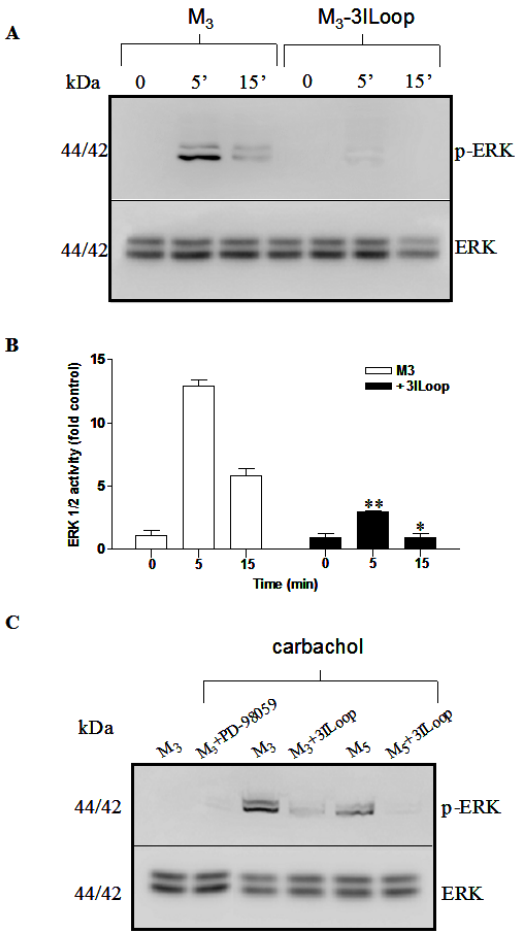

Figure 3.1.4 *Expression of the 3ILoop-minigenes alters agonist-mediated ERK ½ phosphorylation upon activation of M_3 and M_5 receptor subtypes. (A) Time-dependent course of ERK ½ activation on carbachol-stimulated M_3- HEK-293 cells transfected or not with 3ILoop-minigene. Cells were incubated with 1 mM of CCh for the indicated time points. (B) Densitometry analysis of time course of ERK ½ activation following carbachol exposition is shown. Phosphorylation was quantified by scanning densitometry using the NIH Image program and normalized against total ERK ½ protein. Means ±S.E.M is shown; n= 6. *: Significantly different compared to M_3-15min (ANOVA: $p<0.05$); **: Significantly different compared to M_3-5min (ANOVA: $p<0.01$). (C) Western blot analysis for phospho-ERK ½ (p-ERK) following incubation with 1 mM of carbachol for 5 min. in HEK-293 cells transiently expressing M_3 or M_5, co-transfected with the 3ILoop-minigene (5µg) (lane 4 and 6 respectively) or empty vector (lane 3 and 5). M_3-HEK-293 cells non stimulated with carbachol (Lane 1) and carbachol-stimulated M_3-HEK-293 in presence of MAPK specific inhibitor PD-98059 (lane 2) were performed as internal control. Equal loading was confirmed with antibodies against total ERK ½.*

Taking into account this latter result, where a critical decrease in ERK ½ phosphorylation in cells expressing the minigene construct was observed, we decided to analyse whether scaffold/associate proteins or protein kinases were involved in the observed behaviour. As suggested above, at least one of the two mechanisms described for MAPK activation by muscarinic receptor family would

involve scaffold proteins, like β-arrestin and kinases (GRK-2, GRK-3 and CK1-α) in some form of direct complex with the receptor.

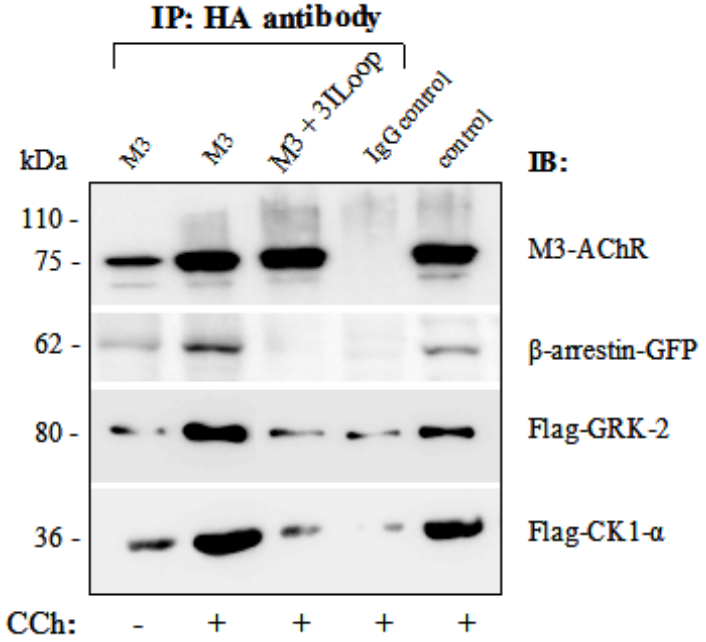

Figure 3.1.5 *Effect of 3Iloop-minigene in receptor association with different protein kinas and scaffold protein by co-immunoprecipitation experiments. HEK-293 cells co-transfected with M_3 receptor, in the presence or absence of the 3Iloop minigene, plus each of the following proteins (β-arrestin-GFP, Flag-GRK-2 or Flag-CK1-α) were stimulated with carbachol (0.5 mM, 5min) prior to solubilization. Extracts were immunoprecipitated with anti-HA antibody (or non-immune IgG control) before SDS-PAGE and Western blotting. At the top sections, the immunoprecipitation was probed with an antibody against the M_3 muscarinic receptor (anti-M_3 SCB). The middle sections show specific co-immunoprecipitation with β-arrestin-GFP (anti-GFP antibody, SCB). A low level of specific pulldown of β-arrestin-GFP can be seen when the cells co-expressed with the 3Iloop minigene construct. Similar effects were observed in the bottom sections when immunoprecipitations were probed with anti-FLAG antibody for both kinases (GRK-2 and CK1-α) under the same conditions. The left panel (control) represents the input levels of immunoreactive M_3 receptor, β-arrestin-GFP, Flag-GRK-2 or Flag-CK1-α in original extracts as a positive control of each antibody quality. The amount of interacting protein co-immunoprecipitated was normalized for the amount of 3xHA-M_3 receptor immunoprecipitated. The blots are representative of at least three separate experiments.*

We carried out co-immunoprecipitation experiments to ascertain if the direct complex formation had been altered or blocked by the presence of the cytoplasmatic third intracellular loop. Figure 3.1.5 shows co-immunoprecipitation

data from HEK-293 cells co-transfected with HA-M_3 receptor in the presence or absence of the 3ILoop-M_3 minigene, plus each of the following vectors (β-arrestin-GFP, Flag-GRK-2 or Flag-CK1-α).

Input levels of each protein and the efficiency of M_3 receptor immunoprecipitation were monitored to ensure a balance between samples (data not shown). In addition, we also analysed the input level of immunoreactive M_3 receptor, β-arrestin-GFP, Flag-GRK-2 or Flag-CK1-α in original extracts as a positive control of each antibody quality (Figure 3.1.5 right lane). In all cases, low levels of immunoreactivity were associated with the receptor in basal conditions. However, preincubation of the cells with carbachol caused increased association for each protein, as monitored by densitometry of the immunoblots (11 folds respect control, means ± S.E., n=2). Exposure of the cell to the 3ILoop-M_3 minigene, and to agonist incubation as well, caused a decrease in the level of immunoreactivity for each protein, similar to the immunoreactivity observed when the receptor is kept in basal conditions. These low levels of co-immunoprecipitation probably resulted from the competition of the 3ILoop-minigene with the same pool of proteins interacting with the receptor, and from their capability to block their association. Co-immunoprecipitation results would also account for the low signal levels obtained in ERK ½ phosphorylation assays in the presence of the 3Iloop.

Inhibition of AKT activation is mediated by the expression of minigene construct.

Carbachol binding to the $G_{q/11}$ muscarinic receptors stimulated AKT promoting cell growth and survival in many cell types [336]. Therefore, the effect of minigene expression on carbachol–induced AKT-activation was determined.

The activation of M_3 or M_5-expressing cells by carbachol incubation reaches a maximum at 10 min after stimulation (Figure 3.1.6). No activation occurred in cells preincubated with the PI3K antagonist LY294002 as show in Figure 3.1.6. Western blot analysis revealed that, as previously observed in ERK ½ signalling, co-transfection of the M_3 or M_5-HEK-293 expressing cells with the minigene vector abolished the phosphorylation of AKT after agonist stimulation (Figure 3.1.6).

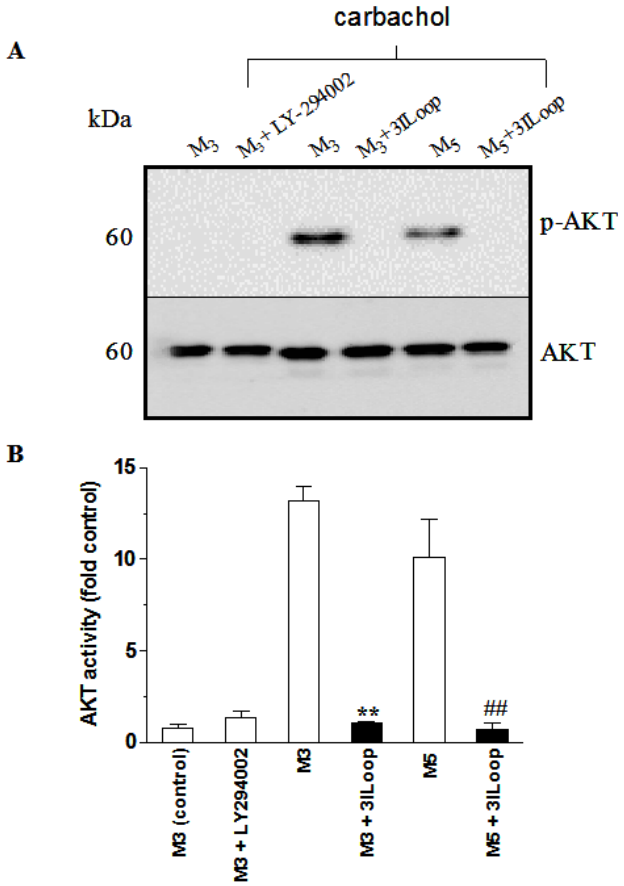

Figure 3.1.6 *3ILoop effect on AKT phosphorylation mediated upon activation of M_3 and M_5 receptor subtypes. (A) HEK-293 cells co-transfected with each receptor subtype and the 3ILoop-minigene (lane 4 and 6 respectively) or empty vector (lane 3 and 5) were exposed to 1 mM of carbachol for 10 min after 48 h of transfection. Cell lysates were resolved in SDS-PAGE (12%) and Western blots performed with an antibody against phosphorylated AKT. (p-AKT). M_3-HEK-293 cells non stimulated (Lane 1) and carbachol-stimulated M_3-HEK-293 in the presence of a selective PI3K inhibitor, LY294002 (lane 2) were performed as internal controls. Equal loading was confirmed with rabbit anti-AKT antibody. (B) The extent of AKT phosphorylation was quantified by scanning densitometry. Means ±S.E.M is shown; n= 4. **: Significantly different compared to M_3 (ANOVA: $p<0.01$). ##: Significantly different compared to M_5 (ANOVA: $p<0.01$).*

DISCUSSION

The precise structural determinants important for mediating intermolecular interactions in the acetylcholine muscarinic receptor family are being recognized

and found to be prominent in signalling cascades [170,216,337]. Sequence analysis of the third intracellular loop of mAChRs showed that these loops are some of the largest in the GPCR superfamily (156-239 amino acids) and contain no homology between the mAChRs subtypes except for ~20 amino acids at the N and C terminal regions (Appendix 3). Furthermore, the 3ILoop is recognized not only as one of the main target domains for interaction with G proteins but also as a binding site for direct interaction of accessory proteins that regulate alternative signalling pathways [229,338,339].

As part of an effort to define the potential role of the third intracellular loop in protein-protein interactions and multiprotein complex organization, we report here the co-expression of a minigene construction that encoded the 3ILoop of the M_3 human muscarinic receptor along with the intact receptor.

The rationale of this approach was to explore the ability of this structural determinant to interfere with G protein interaction or to compete for other interacting proteins that participated in a putative multiprotein complex formation, and to analyze its capacity to compete for functionally relevant processes. Our efforts were specifically focused on the third intracellular loop of the M_3 muscarinic receptor subtype, because this region was reported to be involved in direct G protein binding and activation, and the putative site for interaction of a group of scaffold or accessory proteins (arrestin binding, calmodulin and small G proteins interactions) [169,340,341]. The antecedent for our experimental design can be found in previous studies of cellular expression of fragments, or in vitro G protein activation assays, which reported the ability of the intracellular loops --or peptides derived from these loops-- to interact with the same molecular partners as the intact receptor [342-344]. Experimental evidence was available showing that these co-expression experiments could act mimicking the intact receptor, or coupling with and activating the relevant G protein[345,346]. In the current series of experiments, the third intracellular loop domain of the M_3 receptor was shown to have a recognizable impact on the function of the intact M_3 receptor subtype. In our experiments, proximal events occurring at the level of the plasma membrane were dramatically affected as shown by the impairment of receptor-accessory protein co-immunoprecipitation in cells co-expressing the minigene construct. The co-expression of the minigene construct along with the wild-type receptor exhibited decreased G protein activation, phosphatidylinositol production, and subsequent

signalling, but normal ligand binding and receptor membrane expression (Figure 3.1.7). Therefore, our results indicate that the co-expression of the 3ILoop of the M_3 receptor with the M_5 subtype produce similar effects, as with previous studies in which the expression of the third intracellular loop of the μ-opioid receptor altered the functionality not only when co-expressed with the intact μ -opioid receptor but also when co-expressed with other classes of GPCR [347].

While we only focused on the study of the effect of the third intracellular loop, we cannot exclude the possible involvement of the first and second cytoplasmic loops or the C-terminal tail. Thus, although the 3ILoop is the largest intracellular loop, and is usually proposed to be one of the main sites for intracellular interaction, this does not obviously exclude specific domains in other loops from playing a role in protein-protein interactions or from participating in a multiprotein complex formation or function. In fact, the C-terminal tail is recognised to be an important structural determinant with anti-apoptotic properties within the muscarinic receptor family [348]. On the other side, the second intracellular loop, together with the DRY motif, has also been described to bind the G protein [349].

In our experimental design, we chose to express the intracellular domain with two-epitope tags (CBP-SBP: TAP-system) at the N-terminal domain in order to purify the recombinant receptor and for subsequent study by mass spectrometry in future experiments to determine the nature of the interacting proteins. The possibility of conformational and accessibility changes in potentially critical regions adjacent to these tags was not likely due to their small size (less than 15 aa). However, in order to ensure that the appropriate construct was correctly incorporated into the cell, we used a RT-PCR strategy that involved primers spanning vector and insert sequences that would have been absent in the intact receptor construct. Unfortunately, we were unable to measure the protein expression of the minigene construct by Western blot due to the lack of an antibody that specifically recognized this sequence.

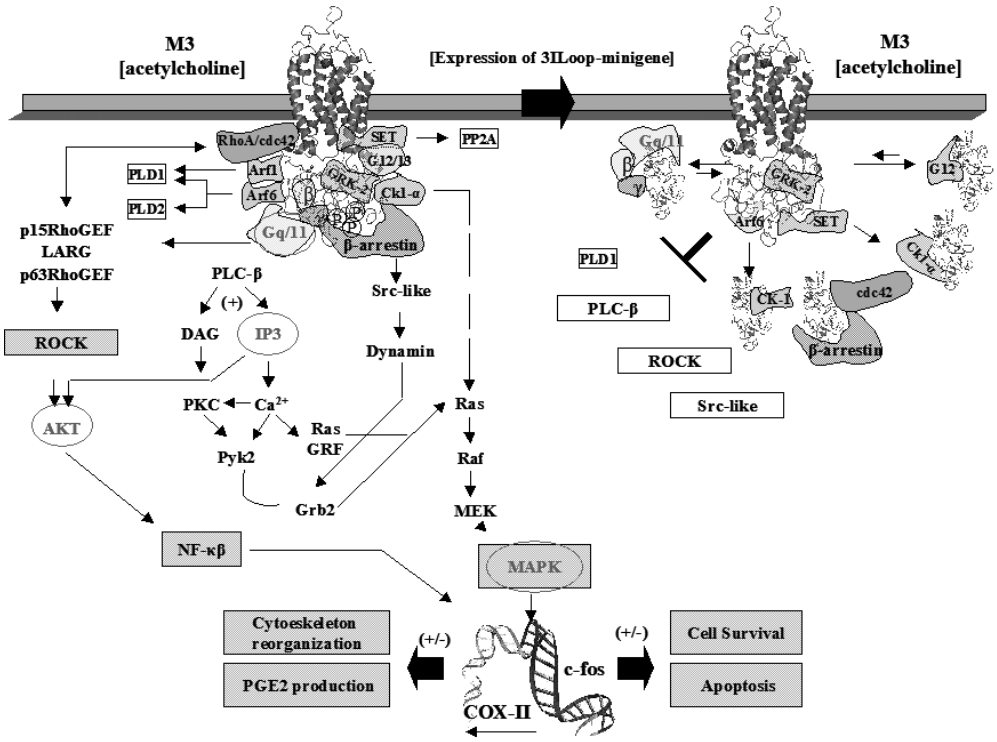

Figure 3.1.7. *3ILoop-minigene expression could interfere with the recruitment of specific M_3/M_5-interacting proteins to the plasma membrane. After receptor activation by neurotransmitter (ACh), or pharmacological agonists (carbachol), a large number of proteins participate in a receptor-cantered scaffolding complex (β-arrestin, kinases, small G protein and others). (A) These multiprotein complexes, specifically for each receptor, can be formed by the stable or transiently bound with the third intracellular loop and/or the carboxyl terminus of the receptor. (B) Expression of the 3ILoop-minigene probably works as inhibitor protein, competing for the proper formation of mutiprotein complexes with M_3 receptor at membrane level, and resulting in down-activation of the signal transduction after agonist stimulation.*

We found that our minigene-system was able to block receptor-$G_{q/11}$ protein coupling as much as 45 % with regards to control, as demonstrated by the low efficacy in agonist-induced stimulation of [^{35}S]-GTPγS binding. These observations are consistent with the finding of other groups using a similar experimental approach with other GPCRs, such as δ-opioid, μ-opioid and α_2-adrenergic receptors [347]. Upon co-expression of the minigene with the M_5 subtype, we also observed low affinity levels with agonist-stimulated [^{35}S]-GTPγS, and a reduction in efficacy. These experiments suggest that the minigene interferes with the specific $G_{q/11}$ protein pool that couples with M_3 as well as with

M_5 subtypes, probably competing with the same pool based on the loop specificity. Supporting this observation, we proceeded to show alterations in the second messenger's levels following expression of the minigene in intact cells. Agonist-mediated activation of Ins (1,4,5) P3 accumulation was blocked in the presence of the minigene in cells expressing either the M_3 or M_5 subtypes. Nevertheless, in the case of the M_5 subtype, the inhibitory effect of the 3ILoop was less pronounced. We also observed a reduction, not only in the level of maximal accumulation of phosphatidylinositol, but also in the potency of carbachol concentration-response curves, indicative of a reversible and competitive process. Our results are consistent with those previously reported for the angiotensin receptor, where the presence of the second intracellular loop of the angiotensin AT1a receptor resulted in a change in the angiotensin II concentration needed for 50% stimulation for Ins (1,4,5) P3 release [346].

These observations can be also interpreted as reflecting the specificity in the inhibitory effect of the minigene for receptor coupling to a certain G protein pool, or alternatively as a result of cross-reactivity between intracellular loops of the M_3 or M_5 receptors at the G protein level. Probably, the low sequence homology within these receptors cannot account for the significant effect detected for the minigene impairment of the receptor-G protein coupling process, suggesting that G-protein interaction sites are similar in both receptors [350]. This idea is in agreement with the observed blockage of Ins (1,4,5) P_3 production and G protein activation, which did not decrease more than 50% with regards to the wild type, considering that multiple distinct structural domains could be involved in this interaction, as observed for dopamine and M_1 muscarinic receptors [351]. An alternative explanation is that the relevant domain that interacts with the G protein is exposed in the natural receptor, and that the modified minigene does not represent or contain this structure, and therefore does not effectively compete for the binding with its homologous partner.

Several lines of evidence show that muscarinic receptors can activate the MAPK pathway in a variety of ways, depending on or independent of the classical G-protein activation pathway [235]. One of these mechanisms is PKC-dependent and is essential in ERK activation; it can involve βγ-subunits and $G_{\alpha q/11}$-subunits. Other mechanisms depend more on the phosphorylation state, and are mediated by a group of different kinases, like GRK2/3 or CK1α, recruiting β-arrestin scaffold

protein or other adaptor proteins respectively. Our experiments showed that cells co-expressing the minigene construct completely failed to phosphorylate ERK ½ upon receptor activation, suggesting that PKC-dependent mechanisms, as well as other mechanisms, which depend upon a group of kinases and accessory proteins, have been blocked.

In addition, using immunoprecipitations, we analyzed whether or not the intact receptor, once activated, loses its ability to recruit and unite CK1α or β-arrestin in the presence of the minigene in order to further analyze if the dramatic blockade of ERK ½ phosphorylation is or is not the result of the inhibitory effects of the minigene construct at the level of the G protein. Our experiments showed a loss of binding proteins CK1α and β-arrestin, with regard to the wild type receptor. Taking this observation into consideration, one plausible scenario is that the minigene construct not only disrupts the G subunit association, as confirmed by the different experimental approaches undertaken, but can also act as an inhibitory or competitor subunit that potentially disrupts the mutiprotein complex formation, thus resulting in a dramatically reduced ERK ½ phosphorylation upon receptor activation.

The third intracellular loop is 256 residues long, containing multiple motifs of basic and acid residues and some currently recognized functional sequence motifs. This primary sequence pattern is not conserved throughout the G protein-coupled receptor superfamily and not even within the class A rhodopsin-like receptor subfamily that represents the closest structurally-related class of GPCR. It is possible that this will ultimately constitute a conformational structural motif that will be more broadly representative and determine part of the specificity for each receptor in these families. However, no structural data is currently available to confirm or refute this hypothesis.

Interacting proteins for such motifs appearing in modular form can be identified by affinity purification approaches, such as yeast two-hybrid screening and immunoprecipitation. Our current work, in which the minigene construct representing the third intracellular loop of the M_3 muscarinic receptor specifically influences the intracellular signalling of the intact receptor, probably blocking a putative multiprotein complex formation, supports the notion that important motifs could be present in this region. The fact that this loop is significantly long and that it is a recognized site for post-translational modifications should make it

an ideal tool for exploring potential molecular partners that might mediate the observed effect under different cellular conditions. The use of a TAP-system strategy that complements the co-expression experiments of the construct with the intact receptor under different physiological condition, together with immunoaffinity purification followed by mass spectrometry analysis, is believed to be a productive approach to the investigation of protein-protein interactions, not only for muscarinic receptors but also for other types of GPCR. As we learn more about the molecular details of multiprotein complex formation events, it should help unravel the patterns of the interacting proteins important in the control and definition of the receptor's physiological behavior in time and space under each cellular condition.

MATERIALS AND METHODS

Materials. [^{35}S]-GTPγS (1,202 Ci/mmol), [^{3}H]-myo-inositol (3,0 Ci/ml) and [^{3}H]-NMS (77 Ci/mmol) were from Amersham Biosciences (Germany). FuGene® transfection reagent was purchased from Roche (USA). All receptor ligands, protease and phosphatase inhibitor cocktails, and all other reagents were purchased from Sigma-Aldrich (Germany). The pcDNA3-Flag-GRK2 encoding the bovine G receptor kinase-2 was kindly provided by Y. Chen (FUMC, Shanghai, China) and the pcDNA3-Flag-CK1-α encoding the human casein kinase 1-α was provided by M. Bini (Palermo University, Italy).

DNA Constructs. The cDNAs of the human M_3 muscarinic receptor (kindly provided by T. Bonner, NIH, USA) and human M_5 muscarinic receptors (provided by D. Bello, ETH, Switzerland) were subcloned into the mammalian expression vector pcDNA-3.1-3xHA (gift from P. Calvo, SFU, CA) giving the 3xHA-M_3-pcDNA and 3xHA-M_5-pcDNA vectors. Basically, 1.9 and 1.7-kb fragments encoding the human M_3, M_5 receptors were respectively amplified from their cDNAs using the Expand High Fidelity PCR System (Roche, USA) with primer pairs (forward: 5'- ACC GAT ATC TTG CAC AAT AAC AGT ACA AC-3'; reverse: 5'-CCC TCT AGA CTA CAA GGC CTG CTC GGG TGC-3' and forward: 5'- ACC GAT ATT CAA GCT TGG TAC CAC CAT G-3'; reverse: 5'-CCC TCT AGC CCG GGT AGC TTG CTG TTC CCC-3'). Each fragment was purified by agarose gel electrophoresis (Bio-Rad, USA) and then subcloned into the mammalian pcDNA3.1-3xHA vector using EcoRV/XbaI.

3ILoop-minigene construct. The cDNAs fragment encoding the entire third intracellular loop of M_3 muscarinic receptor (315 amino acid residues) were subcloned

into the InterPlay® mammalian TAP system, pNTAP-B vector (Stratagene, USA), resulting in the pTAP-3ILoop-M_3 vector. The 3ILoop was amplified from the 3xHA-M_3-pcDNA vectors using the Expand High Fidelity PCR System (Roche, USA) with primer pairs (forward: 5'- GCG GAT CCA CTG GAG GAT CTA TAA GG-3'; reverse: 5'- GCG AAT TCC TCC TTG ACC AGG GAC ATC C -3') and spliced into BamHI/EcoRI sites of the pNTAP-B vector. The reading frame and PCR integrity of all cloned constructs were confirmed by DNA sequencing.

Cell Culture and Transfection. HEK-293 cells were grown to 70% confluence in Dulbecco's modified Eagle's medium (DEMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen, USA), 2 mM L-glutamine, 100-IU/ml penicillin and 100-µg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Cells were co-transfected with pTAP-3ILoop-minigene and the corresponding human muscarinic plasmid, by means of the calcium phosphate precipitation method or using FuGene® transfection reagent (Roche, USA). Equivalent amounts of empty vector pcDNA-3.1 were added, in all co-transfection experiments, to normalize the total amount of cDNA. Cells were harvested 48 h after transfection and centrifuged at 30, 000g for 30 min to obtain two separated fractions (membranes and supernatant). These fractions were frozen as aliquots in 5 mM phosphate buffer saline (PBS), pH 7.4, and stored at -80°C until use.

3ILoop-minigene transcript analysis. HEK-293 cells were washed with PBS 48h post-transfection, and total RNA was purified using Quick Prep™ total RNA extraction kit (Amersham Bioscience, Germany) and subject to RT-PCR (Access RT-PCR system; Promega, USA). Primers were selected according to Gene Bank database resource (forward: 5'- GCG GAT CCA CTG GAG GAT CTA TAA GG-3'; reverse: 5'- GCG AAT TCG ACC AGG GAC ATC C-3) in order to amplify a segment of 541 bp. A RT-PCR negative control was performed loading DEPC water instead of cDNAs and a positive control was performed using G3DPH primers.

Membrane Preparation and Radioligand Binding Assay. About 48 h after transfection, HEK-293 cells were washed twice with cold PBS, harvested and homogenized in a binding buffer (25 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA), using a Polytron tissue homogeniser. Cell membranes were collected by centrifugation at 20, 000g for 15 min and homogenized as above. After centrifugation at 40, 000g for 20 min at 4ºC, the final pellet was resuspended in binding buffer, and membranes were either used immediately or frozen in liquid nitrogen and stored at -80 °C until required. Protein concentration was determined by using the Bradford protein assay kit (Bio-Rad, USA). In order to determine the affinity of NMS for each sample, membranes were incubated with different concentrations of [^{3}H]-NMS (ranging from 12.5 pM to 1.5 nM) in 5 mM PBS at 25°C for 60 min. The incubations were stopped by filtration through

Whatman GF/B filters and washed extensively with ice-cold 40 mM PBS prior to scintillation counting. Non-specific binding was determined in the presence of 10μM atropine.

Inositol Phosphate Determination. Transfected HEK-293 cells were labelled for 18–24 h with [^{3}H]-myo-inositol (Amersham Biosciences, Germnay) at 3,0 Ci/ml in DMEM (high glucose, w/o inositol (Invitrogen, USA). After labelling, cells were washed and pre-incubated for 5 min in PBS at 37 °C, and subsequently incubated in FCS free medium with different concentrations of carbachol -or without carbachol- in the presence of 10 mM LiCl for 5 min. Reactions were terminated by perchloric acid addition. Inositol phosphates were extracted and separated on Dowex AG1-X8 columns (Bio-Rad, USA). Total labelled inositol phosphates were then counted by liquid scintillation.

[^{35}S]-GTPγS binding assay. HEK-293 cell membranes were diluted in an ice-cold buffer containing 10 mM HEPES and 0.1 mM EDTA, 5Mm deoxycholate (pH 7.4) as previously described, pelleted and resuspended in a binding buffer consisting of 10 mM HEPES, 10 mM $MgCl_2$, and 100 mM NaCl (pH 7.4) at a final protein concentration of 125 μg/ml. Incubations were conducted in a final assay volume of 1 ml (125μg total protein) for 1 h at 30°C in the presence of 1 μM GDP and 0.3 nM [^{35}S]-GTPγS (1,202 Ci/mmol) and the appropriate ligand concentration (carbachol from 1 nM to 1 mM). The reaction was stopped by addition of 5 ml of ice-cold buffer containing HEPES/NaOH (10 mM) (pH 7.4) and $MgCl_2$ (1 mM), immediately followed by rapid filtration through glass fibre filters (Whatman GF/C) presoaked in the same buffer. The filters were washed twice with 5 ml of buffer and the radioactivity was measured by scintillation counting. Nonspecific binding was determined in the presence of 10 μM GTPγS. Assays were performed in triplicate.

MAPK and AKT assay. Co-transfected HEK-293 cells expressing M_3 or M_5 receptor subtypes with or without the 3ILoop-minigene were grown to 80% confluence and rendered quiescent by serum starvation overnight prior to MAPK or AKT phosphorylation assay; an additional 2h incubation in fresh serum-free medium was performed to minimize basal activity. Cells were subsequently stimulated by the addition of a medium with or without the muscarinic agonist carbachol. Rapid rinsing with ice-cold PBS completed the stimulation, and cell lysis was performed during 10 min by the addition of 500 μl ice-cold lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1% TritonX-100, protease and phosphatase inhibitor cocktail). The cellular debris was removed by centrifugation at 13, 000g for 5 min at 4°C, and the total protein content was measured using BCA Protein Assay Reagent (Pierce, USA). Aliquots corresponding to 5 μg of protein were mixed with sodium dodecyl sulfate (SDS) loading buffer, applied to

12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot. Extracellular signal regulated kinase ½ (ERK ½) and protein kinase B (AKT) activation were assayed by incubating PVDF blots with a mouse anti-phospho-ERK ½ antibody (Sigma-Aldrich, Germany) and phospho-AKT antibody (New England Biolabs, UK) respectively. Control blots were also run in parallel, and probed with rabbit anti-ERK ½ antibody (Sigma-Aldrich, Germany) and total-AKT antibody that recognized both unphosphorylated and phosphorylated forms. The immunoreactive bands were visualized using horseradish peroxidase-linked secondary anti-mouse and anti-rabbit antibodies (Dako, USA) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA).

Immunoprecipitation. In order to immunoprecipitate the wild-type M_3 muscarinic receptor with its associated proteins, plasmids encoding the HA-M_3 receptor and the β-arrestin-GFP, Flag-GRK-2 or Flag-CK1-α expressing vectors were transiently co-transfected into HEK-293 cells. 48 h later, cells were serum-deprived for 4 h., then exposed to carbachol (20 μM) -or no drug- for 10 min and washed once with PBS before being solubilized in immunoprecipitation buffer (PBS, pH 7.5, 1% CHAPS, 0.75% sodium deoxycholate, protease and phosphatase inhibitor cocktail). Carbachol was re-added where appropriate. Extracts were centrifuged at 15, 000g for 15 min at 4°C to remove particulate material and precleared with Protein G/A Sepharose (Santa Cruz Biotechnology, USA). After centrifugation, the supernatant was transferred to tubes containing either rabbit monoclonal anti-HA (Santa Cruz Biotechnology, USA) or nonimmune mouse IgG (Sigma-Aldrich, Germany) with the required amount of Protein G/A Sepharose suspension, before rolling at 4°C overnight. Beads were collected by centrifugation and washed twice with immunoprecipitation buffer before 35 μl of 2x Laemmli buffer (2% SDS, 5% mercaptoethanol, 20 Mm Tris, pH 7.4) was added per ml of original supernatant. After SDS-PAGE and electroblotting, Western blot was carried out on the sample and the original supernatants to detect immunoprecipitated proteins and monitor input levels. The primary antibodies used were an anti-Flag M_5 mouse monoclonal antibody (Sigma-Aldrich, Germany) and a rabbit polyclonal anti-GFP (gift from Y. Mesa; UB, Spain), followed by preabsorbed secondary antibodies to horseradish peroxidase (Santa Cruz Biotechnology, USA). Bands were visualized by ECL (Kodak, USA) and then measured by quantitative densitometry.

Data analysis. All binding data were analysed using the commercial program GraphPad PRISM 4.0 (GraphPad Software, USA). In the [^{35}S]-GTPγS binding assay, basal binding was defined as [^{35}S]-GTPγS binding in the absence of agonist. For each agonist concentration, the percentage of binding over basal was calculated to determine the agonist-stimulated [^{35}S]-GTPγS binding. Data were fit to a sigmoidal dose-response

curve. For statistical evaluation of the biochemical data, unless otherwise specified, one-way analysis of variance (ANOVA) was used. Group differences after significant ANOVAs were measured by post hoc Bonferroni´s Multiple Comparison test.

3.2 Systematic identification of muscarinic receptor family interacting proteins by mass spectrometry

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ABSTRACT

G protein-coupled receptors (GPCRs) constitute one of the most important families of membrane receptors through which cells respond to extracellular stimuli; they likely function in the context of a signal transduction complex. The identification and analysis of their components provides insight into a better understanding of these receptors' function and regulation. We used tandem-affinity purification (TAP) and mass spectrometry as a systematic approach to characterizing multiprotein complexes in the muscarinic receptor family as a test case. To overcome the limitations of detergent solubilization, we developed a strategy in which receptors are co-expressed with a cytoplasmic minigene construct, encoding the third intracellular loop and the C-terminal tail tagged to the TAP-cassette of each receptor subtype. Numerous protein complexes were identified, including many new interactions in various signalling pathways and apoptotic responses; some of which were common to both receptors, while others were specific for each subtype. Systematic identification data set together with interactions reported in the literature revealed to us a high degree of connectivity, allowing for the first time an outline of the muscarinic interactome as a network of protein complexes at a level of organization beyond binary interactions. This comprehensive map of networks contains fundamental biological information and offers context for a more reasoned and informed approach to drug discovery and muscarinic receptor subtype specificities.

INTRODUCTION

GPCRs constitute the largest family of membrane receptors through which cells communicate with each other and respond to a wide variety of extracellular stimuli [352]. Their medical importance is reflected in their being targeted by about half of the drugs prescribed for human diseases, making them extremely valuable targets for the pharmaceutical industry [353]. In humans, this family of receptors has been proposed to contain approximately 1000 members that control physiological processes, such as neurotransmission, cellular metabolism, secretion, cell differentiation, and growth [1].

The prototypic topology of GPCRs consists of seven transmembrane domains linked by three extracellular and three intracellular loops [354]. Amino acids within the extracellular and/or hydrophobic 7TM core of the receptor are involved in ligand binding, allowing the initiation of conformational change within the receptor upon agonist stimulation. These conformational changes are then propagated to the intracellular domains of the receptor [355]. This results in G protein binding and activation, as well as initiation of intracellular events by multiprotein complex reorganization[356].

As GPCR-researchers enter the so-called "Post-Genomic" era, they have begun to embrace the exciting opportunity of investigating protein-protein interaction and multiprotein complex reorganization in high-throughput experiments [357,358]. However, our knowledge regarding the identity of the interacting proteins of specific complexes is limited, and is based on selected biochemical approaches and genetic analyses. The only comprehensive protein-interaction studies on GPCRs are based on ex vivo and in vitro systems, such as two-hybrid systems and proteomics analysis of GPCR-associated protein complexes using receptor-specific antibodies [359-361]. They are limited by the availability of adequate tools for each GPCR, and need to be integrated with more physiological approaches, which have been fundamental for the biological understanding of their function.

Despite this limitation, several interacting proteins of GPCRs have been recently identified [362-364]. Most of these proteins interact with the C-terminal domain via their PDZ, Src homology 2 (SH2) and SH3, pleckstrin homology, or Ena/VASP homology (EVH) containing domains [359]. And in other receptors is the third

intracellular loop, which is related to and contains sites that may be implicated in protein interactions [365].

Recently, a tandem affinity purification (TAP) method has been successfully used for high throughput identification of soluble proteins engaged in interacting complexes in bacterial, yeast and mammalian cells [366-368]. This strategy allows for fast purification with a high yield of protein complexes under native conditions and overcomes the aforementioned limitations [369]. In essence, this method is based upon the two-step affinity chromatography purification of complexes formed in intact cells using a TAP-cassette tagged to the protein concerned [370]. The protein of interest can be expressed in mammalian cells where subcellular localization and post-translational modifications are conserved. In addition, the recruitment of protein complexes may be induced by treatment of the cells with variety pharmacological compounds or under different physiological conditions [371,372].

As part of a broader effort to define protein complexes within GPCRs, and taking into consideration previous experimental evidence showing that co-expression of intracellular loops of GPCR -or peptides derived from these loops- could mimic the intact receptor, coupling with and activating the relevant G protein or interacting with the same molecular partners [347,373-375]. We modified a TAP method suitable for the purification of associated proteins of the muscarinic receptor family, chosen as a model. Essentially, the idea was to co-express a cytoplasmic minigene construct (TAP cassette tagged to the 3Iloop and C-terminal tail of each receptor subtype) along with the intact receptors in human SK-N-MC neuroblastome cells, and, after large-scale tandem affinity purification of the minigene, identify the interacting protein by LC-MS/MS (An approach that we termed Intracellular Loop Tandem Affinity Purification (Iloop-TAP)).

Using this approach, which provides a global characterization of the interacting protein with the intracellular domains of membrane-bound receptors, we have performed a comprehensive analysis of the muscarinic receptor interactome as a network of protein complexes at an organizational level, a model system relevant to other GPCRs.

RESULTS

ILoop Tandem affinity Tag for Purification of Muscarinic receptor Interacting proteins

The ILoop-TAP strategy for purifying and identifying the muscarinic receptor associated proteins is illustrated in Figure 3.2.1. We used as TAP tag support the modified version TAP system commercialised by Stratagene, composed of a Calmodulin binding peptide sequence followed a Streptavidin binding peptide. This TAP system combined two high-affinity purification steps as the basic concept. This is similar to strategies developed previously, but differs from these strategies in the lack of a mild elution using a site-specific protease, although protein complex purification with high efficiency and specificity is guaranteed. To use a tandem affinity purification that is easy compatible with receptor membrane proteins, we chose to construct the minigene tagged to the TAP tag instead of the WT receptor (pTAP-mini-$M_{1/5}$ vectors). For each receptor subtype, only the large third intracellular loop and the C-terminal tail were tagged to the TAP cassette. As described previously, this resulted in a vector-containing minigene proteins that are soluble expressed and keep their capacity to bind interacting proteins by mimicking the endo-facial side of the receptor.

Each pTAP-mini-$M_{1/5}$ vectors were co-expressed along with each receptor subtypes in human SK-N-MC neuroblastome cells following TAP purification procedures. Amicon concentrated elutes proteins were separated by one-dimensional gel electrophoresis, and proteins were detected either by Coomassie Blue ($1x10^9$ cell) or by silver staining ($1x\ 10^7$ cells). Whereas only a few bands were visible in non-transfected cells, several specific protein bands were reproducibly present in each specific-subtype expressing cells. Individual bands were excised and digested with trypsin, and the resulting peptides were analysed by nano-LC-MS/MS and identified by database search algorithms (Mascot software in Swiss-Prot databases).

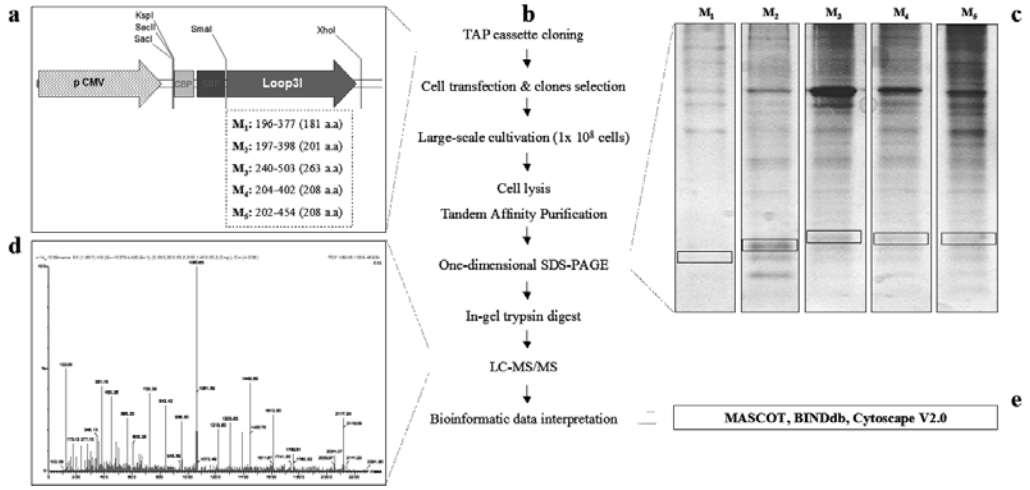

Figure 3.2.1 *The ILoop-TAP strategy for identification of muscarinic receptor family interacting proteins.* ***a****, Schematic representation of minigene construction. Stratagene TAP cassette is inserted at the N-terminal of a given 3ILoop and C-terminal tail.* ***b****, Overview of complexes purification and sample processing.* ***c*** *and* ***d****, examples of TAP complexes purified from different receptor subtypes on denaturing protein gels and stained with Coomassie and fragment spectra of selected peptide ion.* ***e****, Bioinformatic tools used for protein analysis. The MS/MS data was analysed and matched to all human protein sequences in the Swiss-Prot database using the MASCOT search engine following formatting for import into BIND, the Biomolecular Interaction Network Database. The arbitrary molecular interaction network file obtained was then visualized as a connectivity organic distribution network using Cytoscape software.*

To evaluate the purification efficiency of the receptor complexes, we monitored each purification step by immunoblotting. As shown in Figure 3.2.2, TAP-tagged minigene-M_5 was bound efficiently on calmodulin resin because there was no visible amount left in the flow-through after the binding. Proteins eluted from the calmodulin resin were subsequently incubated with immobilized Streptavidin resin for the second purification step. And the TAP-tagged minigene M_5-containing complexes were efficiently captured on Streptavidin resin as shown in Figure 3.2.2, *lane 5* (flow-through after Streptavidin binding), demonstrating that the TAP-tagged minigene-M_5 can be expressed and purified efficiently with the condition established in our experiments. Similar results were observed from cells expressing other receptor subtypes.

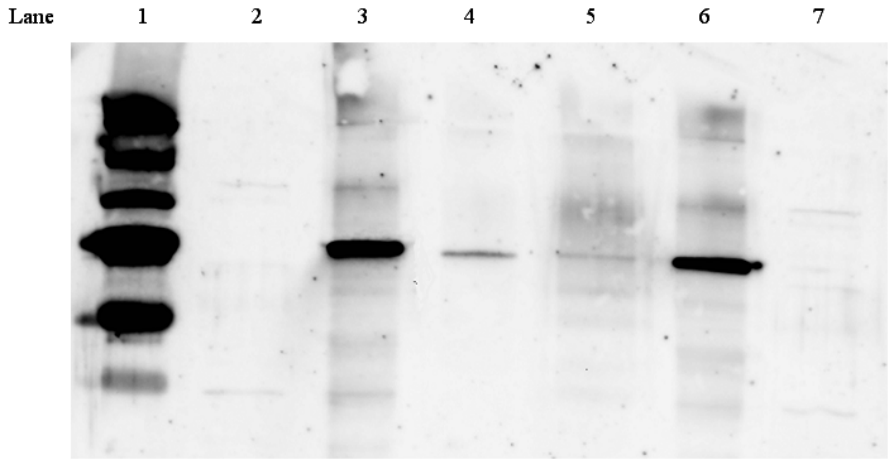

Figure 3.2.2. *Small-scale ILoop-TAP-M_5 purification. The M_5 muscarinic receptor complexes were efficiently isolated using two-step affinity purification as shown by immunoblotting using anti-CBP antibody. Lane 1, Santa Cruz Biotechnology molecular weight;* lane 2*, without;* lane 3*, cell lysate;* lane 4*, unbound fraction after incubation with streptavidin resin;* lane 5*, wash with streptavidin binding buffer;* lane 6 *elution from streptavidin resin;* lane 7*, unbound fraction after binding to calmodulin resin. The samples were separated by 15% SDS-PAGE, and the minigene was detected by western blot using the anti-CBP antibody.*

Categorization of detected proteins

To detect proteins that specifically interact with the intracellular loops of each receptor subtype, we performed differential analyses of gel protein patterns obtained with each specific (TAP tagged-minigene) and mock (TAP alone) baits. A comparison of protein patterns in the gels obtained indicated a selective recruitment of different proteins. Also present were several abundantly mitochondrial and ribosomal origin proteins unambiguously identified by MS/Ms and present in all lanes (control, M_1 to M_5), which we classified as non-specific or contaminant proteins (see Appendix 3.1). We also decided to pragmatically exclude from our list purified heat-shock proteins, as these proteins appeared with higher frequencies in our analysis. However, it's important to take care when it comes time to interpret this data, as the active role of heat-shock proteins in the function of some GPCRs has been reported [376-378]

We generated 252 samples for mass spectrometry and subsequently identified 142 unique proteins. Ontology classification of identified peptides revelled proteins of various subcellular compartments and functions, most of them known to have an important role in GPCR signalling (Figure 3.2.3 a, b). The majority of the

peptides identified were from proteins known be localized to the plasma membrane or cytosol, followed by protein localized in the Reticulum and Golgis apparatus. In addition, proteins identified from mass spectrometric determination yielded 62% signalling proteins in agreement with classical function of GPCR as transducers.

Figure 3.3.3 c, showing an average of 50% of proteins identified as $G_{q/11}$ and 30% of those identified as $G_{i/0}$ muscarinic receptor associated proteins. Identification of specific associated proteins for each receptor subtype from 7 independent purifications resulted in mayor specificities for M_4 and M_3 subtypes, followed by M_2, M_5 and M_1 (Figure 3.2.3 d).

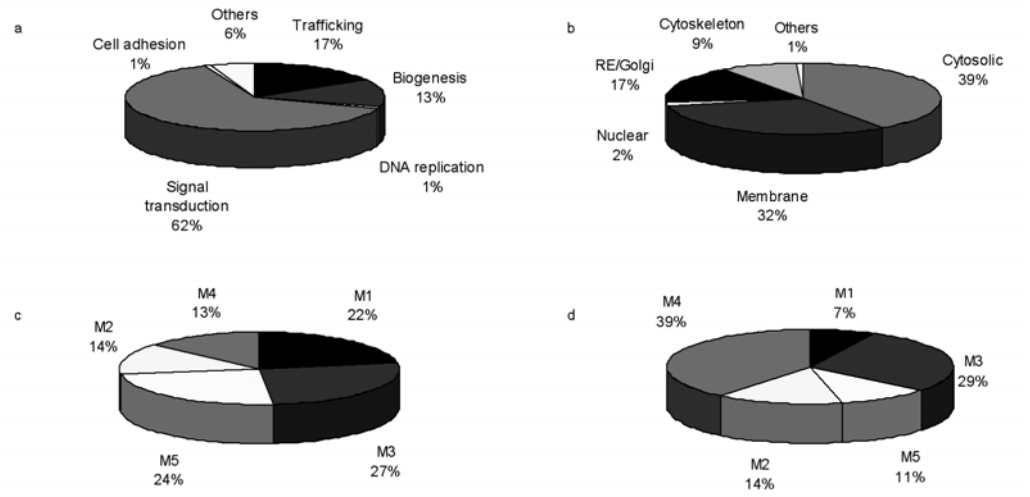

Figure 3.2.3. *Statistic and distribution of protein identified. Numbers outside pie charts represent the percentages of total proteins.* **a**, *classification of proteins according to function;* **b**, *subcellular localization;* **c**, *number of proteins per receptor subtypes;* **d**, *number of specific proteins per receptor subtypes.*

Sensitivity, specificity and reliability of ILoop-TAP approach

Of the 142 purified associated proteins, approximately 80% presented as specific interacting proteins, showing that the ILoop-TAP method is very efficient for the large-scale retrieval and identification of receptor interacting proteins. Although it is difficult to estimate the percentage of interaction proteins, which are covered by our data set, a rough estimation, can be made based in the observation that many identified proteins (at least 42 %) are likely to interact with other GPCRs and

have been associated with their function (crude estimate). We also observed that the method failed to detect some known interactions (RGS, GRK). Proteins, which likely participate in transient interaction, low stoichiometric complexes, and/or interactions that occur only in specific physiological states that were either not present or under-represented in our experimental conditions probably cannot be identified using this method.

There are several possible reasons why the remaining 20% of identified proteins are non-specific proteins. Particular proteins like ribosome's protein are ubiquitously expressed in the cytosol, and can persistently interfere during purification. In other cases, the 12K (relative molecular mass Mr 12,000) TAP tag probably binds with some degree of affinity to some proteins. Quality and reproducibility of the results obtained for purifications were assessed by systematic comparison of our data to the literature (see below) and the purification of each receptor complex at least four times.

Dynamic of protein complexes

It is becoming increasingly clear that GPCRs exist as a large complex of proteins involving dimers or oligomers of receptor and associated proteins that, depending in their variable composition, determine their function. The ILoop-TAP strategy revealed a dynamic composition of muscarinic receptor subtype interactome. The dynamics of complex composition are well illustrated by the cellular signalling complexes formed around each subtype (see Appendix 3.2). For instance, TAP-mini-$M_{3/5}$ purification resulted in the identification of the known G-protein-coupled receptor (GPCR)-kinase interacting proteins 1 (GIT1), and of the three small GTPase, RhoA, Rac1 and Cdc42. GIT1 are ubiquitous multidomain proteins involved in diverse cellular processes through interactions with proteins including ARF, Rac1 and Cdc42, GTPases, p21- activated kinase (PAK), PAK-interacting exchange factor (PIX), the kinase MEK1, phospholipase Cγ (PLCγ) and paxillin [379]. The TAP-mini-$M_{1/3/5}$-purified complexes were found to additionally contain the multidomain paxillin protein, known to participate in the organization of actin cytoskeleton and cell motility [380], revealing enough plasticity among the different complexes and a cascade of recruitments and association, which take the receptor as a reference point.

A particular complex is not necessarily of invariable composition nor are all its building blocks uniquely associated with that specific complex. Figure 3.2.4 shows small-scale TAP-mini-M_1 complex purification before and upon agonist stimulation. Comparing identified proteins in both cases revealed the presence of distinct bands corresponding to different associated proteins. Using the Iloop-TAP approach, we determined that some associated proteins (likely core components) could be identified and validated independently of cellular state. Whereas more dynamic, perhaps regulatory components, may be present differentially depending cellular pharmacological treatment or physiological conditions.

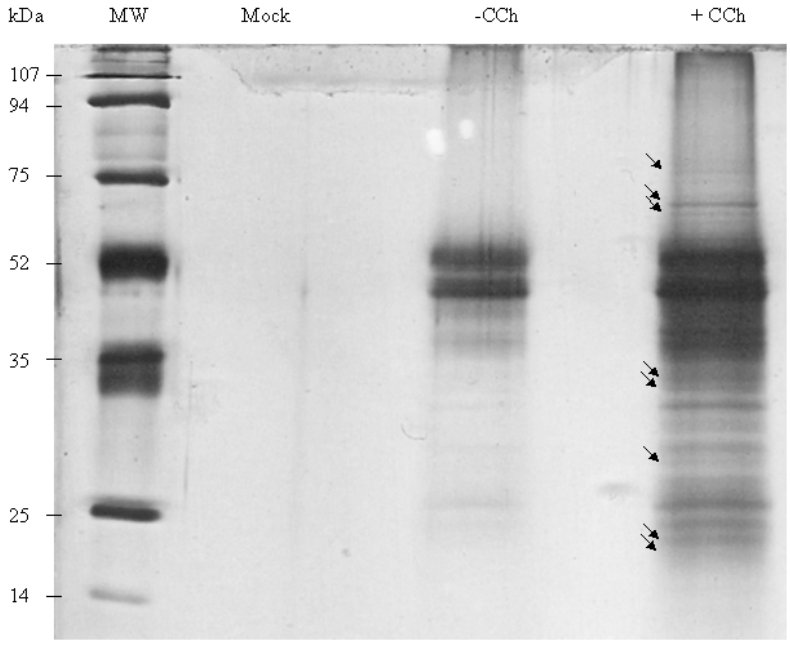

Figure 3.2.4. *Purification of TAP-mini-M_1 associated complexes. Approximately 1x 10 ^{7} SK-N-MC-TAP-mini-M_1 cells (with or without carbachol 10μM treatment) and SK-N-MC-TAP cells (Mock) were submitted to the ILoop-TAP strategy protocol. Elutes were separated by 15% SDS-PAGE gel and proteins were detected by silver staining.*

Examples of proteins that have been identified as associated proteins present before and upon agonist stimulation have been α-synuclein, serine/threonine-protein phosphatase 2A (PP2A) and, surprisingly, the Regulatory G protein Signalling 2 (RGS2). RGS proteins have previously been reported to interact with GPCRs and their heterotrimeric G-proteins. They were selectively recruited upon agonist stimulation of the plasma membrane by G proteins and

correspondingly by receptors that activate those G proteins. However, based on our results, it seems that M_1 muscarinic receptors keep their association with specific RGS2 proteins independently of receptor activation. We also identified a wide number of proteins that were selectively recruited only upon agonist stimulation (see Appendix 3.2), thus indicating that signalling efficiency/specificity for mAChRs is determined in part by dynamics change in multiprotein complexes reorganization.

Network map of Muscarinic receptor Interactome

After assigning individual proteins to each receptor subtype complex, we investigated relationships between complexes to understand the integration and coordination of the muscarinic receptor process. We represented these relationships by linking complexes that share components (Figure 3.2.5). By plotting all the data of set interactions described here, together with interactions detected by other biochemical methods reported in the literature, we obtained a comprehensive view of the network of complexes.

Connections in this network not only reflect the physical interactions of complexes, but may also represent common regulation, localization, turnover or architecture. All data sets were imported into BIND database, a database built around an ASN.1 specification standard that stores all relevant information about the interacting partners, including experimental evidence for the interaction, subcellular localization, biochemical function, associated cellular processes and links to the primary literature. Using Cytoscape software and the export file containing a arbitrary molecular interaction network obtained from the BIND database, we constructed and visualized a single large network of muscarinic receptor interactome.

By analysing this large network containing 400 interactions among 100 proteins, as well as several smaller networks, we could detect some interesting features. First, receptors of similar functions tend to cluster together within the network, suggesting that sharing of components reflects functional relationships. Second, similar behaviours can be observed with assessor proteins that share a common subcellular localization. And third, the network of interaction reveals sets of interactions that link cellular process and cross-connections reflecting the central role of some proteins in regulating other cellular processes.

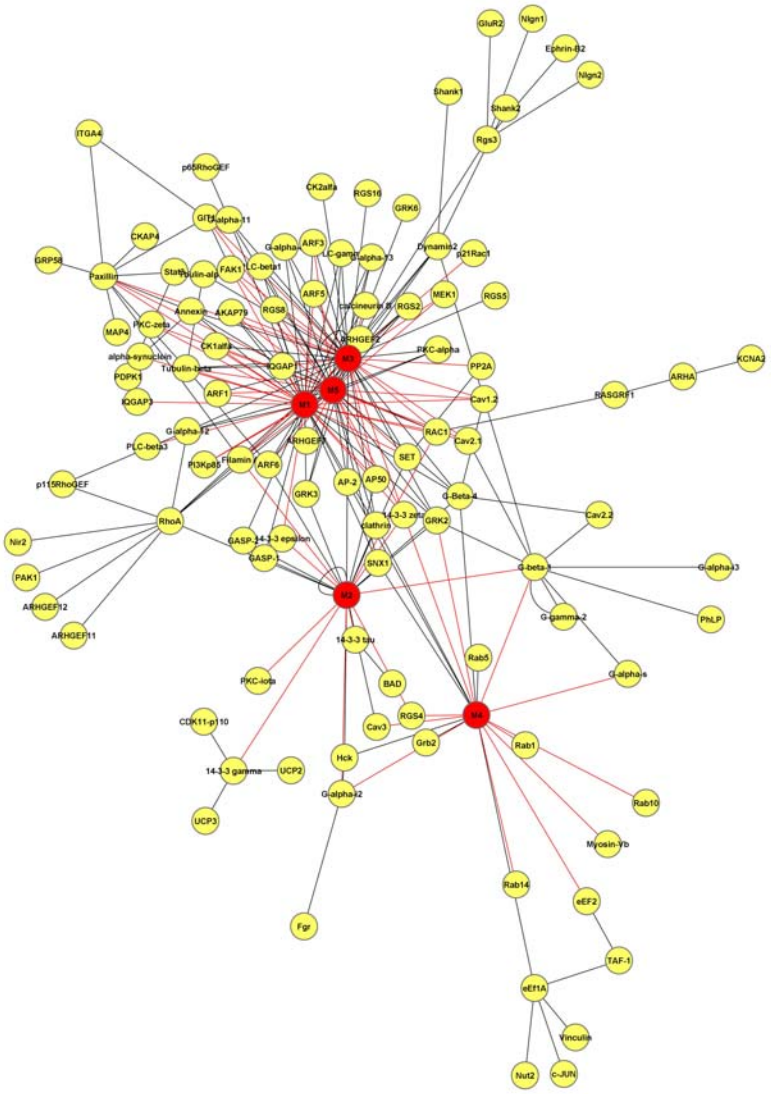

Figure 3.2.5. *The muscarinic receptor family interactome. Graphs were generated automatically by organic algorithm using Cytoscape software. Line thickness represents the number of experiments describing a given interaction and illustrates the connection between proteins. Lines are color-coded according to the experiments: ILoop-TAP results (red), reported in the literature (black). Muscarinic receptors subtypes nodes are highlighted in red. Each protein is labeled using BIND annotation.*

Validation of selected identified proteins

Functional protein–protein interactions were assessed by co-immunoprecipitation experiments. In spite of the fact that a comprehensive, step-by-step verification of all proteins in our data set will be undertaken using complementary methods, we selected and analysed here only several uncharacterised muscarinic-interacting proteins for their ability to form a complex with muscarinic receptors.

GIT-1 and paxillin were selected because of their capacity to interact with each other and are involved in diverse cellular processes. While AKAP-150 has been described as a scaffold molecule of the prototypical β2-adrenergic receptor, orchestrating the interactions of various protein kinases (including tyrosine kinases), protein phosphatases (e.g. calcineurin) and cytoskeletal elements enabled multivalent signalling.

Lysates of SK-N-MC cells exogenously expressing the 3xHA-$M_{2/3/5}$ muscarinic receptor subtype were subjected to immunoprecipitation with anti-HA beads and analysed by Western blotting with the anti-GIT-1, anti-paxillin, anti-FAK antibodies. GIT-1 and paxillin did not coimmunoprecipitate with M_2 muscarinic receptor subtypes in correspondence with results obtained by ILoop-TAP approaches (Fig. 3.2.6). However, bands corresponding to each interacting protein were observed in cells expressing M_3 and M_5 subtypes. Detection of the Focal Adhesion kinase (FAK) protein in immunoprecipitated $G_{q/11}$ receptor subtypes is in agreement with previous reports, which described paxillin and GIT protein as multiplatform components that can actively recruit tyrosin kinases such as FAK protein (data not shown). This demonstrates dynamic complex formation around muscarinic receptors by the cascade recruitment of theses scaffold proteins.

A similar set of experiments was performed using immunoprecipitation and Western blot analysis with the anti-AKAP antibody. Like GIT-1 and paxillin, AKAP copurified with $G_{q/11}$-coupled receptor subtypes. However, it did not coimmunoprecipitate with the M_2 muscarinic receptor subtype, a $G_{i/0}$-coupled receptor (Figure 3.2.6). This confirms a specific interaction also reported by TAP purification.

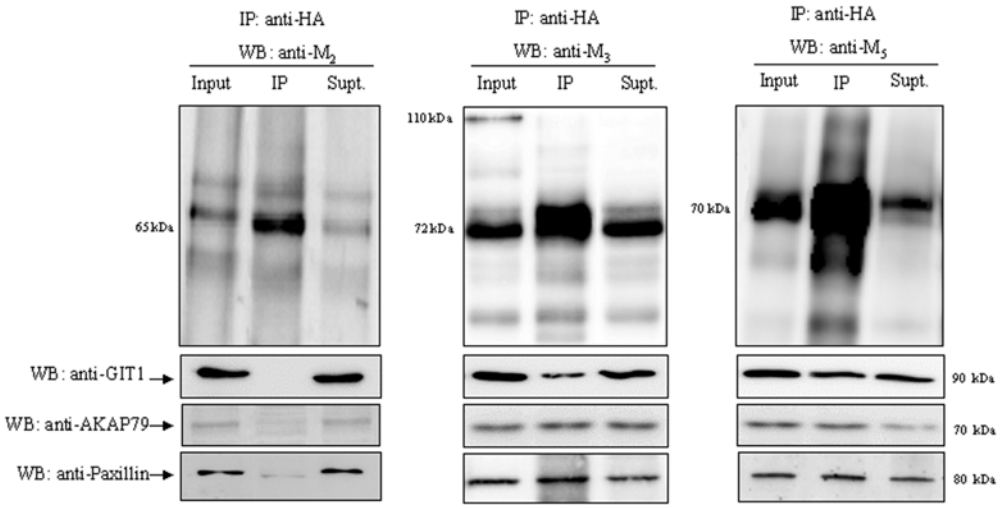

Figure 3.2.6. *Interaction of the intact muscarinic receptor subtypes with endogenous selected interacting proteins (GIT-1, paxillin, AKAP79). SK-N-MC cells transiently transfected with pcDNA3-3xHA-M_2, or pcDNA3-3xHA-M_3, or pcDNA3-3xHA-M_5 were lysed in Nonidet-p40 buffer, and the receptor was immunoprecipitated as described under "Material and Methods". The membrane transfer was first probed with an anti-GIT1, anti-Paxillin or anti-AKAP79 antibody followed by striping and reprobing with anti-HA monoclonal antibody to confirm effective receptor immunoprecipitation. The input and supernatant (Supt.) lanes represent ¼ of the lysate volume used for each immunoprecipitation (IP). The data are representative of two to three independent experiments.*

Previous experiments have demonstrated that muscarinic acetylcholine receptor-stimulation suppress M- type potassium channel current, inhibition which is mediated by the multivalent AKAP-150 protein. This protein is particularly interesting because, in addition to its role in the inhibition of current channels, it also associates with protein kinases, phosphatases and cytoskeleton elements enabling multivalent signalling.

DISCUSSION

To assign cellular functions and identify new interacting proteins of GPCRs, and to understand the context in which receptor-protein interactions operate, several large-scale approaches have been undertaken. These include co-immunoprecipitation experiments, yeast two-hybrid screens [381], resonance energy transfer studies [382], protein ship and computational *in silico* methods [383].

The ILoop-TAP proteomic approach presented here may well contribute to the largest analysis of protein-protein interaction in GPCRs. Maximizing sensitivity and reproducibility over other methods to detect receptor binding partners and using muscarinic receptor family as a model system, we identified 148 different interacting proteins, of which 57 had been detected previously as muscarinic receptor-binding partners.

The GO classification of the proteins identified by Iloop-TAP strategy revealed some interesting avenues of further consideration: first, the presence of heterotrimeric G proteins in all receptor subtype-associated protein purifications. Consistent with the known coupling of muscarinic receptors to G proteins, we identified four Gα-protein for $M_1/M_3/M_5$ receptor subtypes (Gα-q, Gα-11, Gα-12 and Gα-13) and three Gα-i isoforms (Gα-i1, Gα-i2 and Gα-i3) for M_2/M_4 receptor subtypes, as well as two different Gβ isoforms (Gβ1 and Gβ4) and three Gγ isoforms (Gγ2, Gγ7 and Gγ11). This demonstrates that our approach can clearly be used as a method for large-scale protein-protein interaction detection, not only for the muscarinic receptor family, but also for other GPCRs. Second, we identified several cytoskeletal proteins, including those associated with GPCRs' reorganization and regulation (α- and β-tubulin, filamin A, annexin A2, dynamin 2, clathrin, IQ-GAP1/3 and paxillin), some of them existing in a common complex with specific receptor subtypes. This suggests that muscarinic receptors selectively bind to different structural components in the cell and may have a role in receptor internalisation and the regulation of cell spreading, migration, and the attachment at sites of focal adhesion [384]. In support of this notion are recent reports pointing toward different mechanisms of internalisation using M_2 and M_4 receptors depending of the cellular type [385,386], as well as the active roles of tubulin and microtubule reorganization in PLD activation mediated by $G_{q/11}$ muscarinic receptor subtypes [387]. More interestingly, using drugs that stabilized or destabilized microtubule organization, it was found that the activation of muscarinic leads to modulation of ion currents in cardiac myocyte cells [388], functions mediated by the direct interaction of the receptor and the G protein with the microtubule cytoskeleton. The third and last consideration is the identification of several muscarinic receptor subtypes-specific signalling proteins such as ADP-ribosylation factors, elongation factor 1-A (eEF-1A), oncogenic SET protein,

Rac1, and different isoforms of phospholipase C and the protein kinase C (β-1, β-3, γ and α, δ, τ respectively). Interestingly, the small GTPases Rac1 has been shown to function downstream of M_1 and M_3 receptors. In the context of Rac 1 activity but not RhoA activity, M_3 muscarinic receptor-mediated activation of PLC and PKC triggers cell death [389]. The ADP-ribosylation factors are members of the Arf arm of the Ras superfamily of guanosine triphosphate (GTP)-binding proteins. Physiologically, Arfs regulate membrane traffic and the actin cytoskeleton. However, Arf function likely involves many additional biochemical activities. Arf activates phospholipase D and phosphatidylinositol 4-phosphate 5-kinase with the consequent production of PA and PIP2, respectively [390]. It has been shown that vasopressin V2 receptor and M_2 muscarinic receptor processing and trafficking are under ARF-6 control [391]. Also, Arf 1 and 6 mediate PLD1/2 activation by M_3 muscarinic receptor [334]. eEF-1A and other elongation factors have been reported to modulate M_4 muscarinic receptor subtype function by direct interaction with the receptor [392,393]. Several serine/threonine phosphatases participate in the dephosphorylation of activated GPCRs. Phosphatases of the PP2A and PP2B subfamilies have been reported to target GPCRs. M_1 receptor subtypes stimulate the formation of a multiprotein complex centred on TRPC6 channels where PP2B plays an active role in the disassociation of the muscarinic receptor from the complex [394].

The most important outcome of our study is the possibility to study and identify specific interacting proteins under native conditions. Although we used only one set of experimental parameters here for the evaluation of complex composition, we will, in the future, systematically modify experimental parameters (both pharmacological and physiological conditions) to evaluate the impact of a changing environment on complex variability. These studies should help to elucidate the dynamics of complex assembly and disassembly for each receptor subtype. Moreover, it may be a starting point in deciphering receptor specifies and a molecular context for the choice and evaluation of drug targets.

Another important outcome of our experiments is that, comparing the experimental results in the literature with our data-set, we were able to construct a muscarinic receptor family network, which allowed us to group interacting proteins into cluster complexes. The network that resulted is a functional description of the muscarinic receptor interactome at a higher level of

organization and revealed some interesting information. Although comparison of our data set with literature is straightforward, it is important to keep in mind that our IloopTAP approach yields complexes composition data that, in any case, produces binary interactions, just as the results from two hybrid methods. This supports the view that receptor complex formation is more than the sum of binary interactions.

However, binary analysis methods are of exceptional value for the detection of pairwise and transient associations of GPCRs. The success of our approach in the characterization of receptor complexes relies on the conditions used for the assembly and retrieval of the complexes. These include localization and post-translational modifications in a manner that closely approximates normal physiology. Therefore, because the ILoopTAP method does not provide information on the orientations of complex components, complex characterization by two-hybrid analysis, or resonance energy transfer methods are ideally complementary.

In summary, our ILoopTAP protocol allowed a relatively fast large-scale purification of GPCR-associated proteins under native conditions by mass spectrometry with low number of non-specific proteins. Specific interacting proteins from each receptor subtype were identified systematically, representing a major methodological advance in the identification of GPCR-interacting protein complexes. In addition, collected data allowed for the first time the construction of a muscarinic receptor network (interactome), reflecting receptor complex properties and tendencies. Through the 'interactome' concept, we are able to propose signal roles for interacting partners that had not been previous described. This methodology biochemical and theoretical approach can be used systematically in the future for the understanding and analysis of other classes of receptor and subfamilies of GPCR.

MATERIALS AND METHODS

Materials. [[^{3}H]-NMS (77 Ci/mmol) were from Amersham Biosciences (Germany). The FuGene® transfection reagent was purchased from Roche (USA). The cDNAs of the human M_1 and M_4 muscarinic receptor were kindly provided by F. Ciruela (UB, Spain), human M_3 muscarinic receptors was provided by T. Bonner (NIH, USA), and human M_2

and M_5 muscarinic receptors was provided by D. Bello (ETH, Switzerland). The InterPlay® mammalian TAP system, pNTAP-B vector was purchased from Stratagene (USA). Mouse anti-paxillin monoclonal antibodies were purchased from Calbiochem. Rabbit anti-GIT1 monoclonal antibodies were purchased from Transduction Laboratories. Mouse anti-AKPA79 monoclonal antibodies and rabbit anti-HA monoclonal antibodies were purchase from Santa Cruz Biotechnology. Goat anti-mouse and anti-rabbit secondary antibodies were obtained from DAKO (Glostrup, Denmark). All receptor ligands, protease and phosphatase inhibitor cocktails, and all other reagents were obtained from Sigma-Aldrich (Germany).

DNA constructs. The cDNAs encoding the entire 3ILoop and the C-terminal tail of the each human muscarinic receptor subtypes (M_1-M_5) were subcloned into the InterPlay® mammalian TAP system, pNTAP-B vector (Stratagene, USA) resulting in the pTAP-mini-$M_{1/5}$ vectors. Basically, the fragments encoding the 3Iloop and C-terminal tail of human M_3 to M_5 receptors were amplified from their cDNAs using the Expand High Fidelity PCR System (Roche, USA) with their respective primer pairs (see supplementary information Table 1) and spliced into BamHI/EcoRI sites of the pNTAP-B vector after agarose gel electrophoresis (Bio-Rad, USA) fragment purification. The reading frame and PCR integrity of all cloned constructs were confirmed by DNA sequencing.

Cell Culture and Transfection. SK-N-MC neuroblastome cells were grown to 70% confluence in Dulbecco's modified Eagle's medium (DEMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen, USA), 2 mM L-glutamine, 100-IU/ml penicillin and 100-µg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Cells were co-transfected with pTAP-mini-$M_{1/5}$ and the corresponding human muscarinic plasmid, by means of the calcium phosphate precipitation method or using FuGene® transfection reagent (Roche, USA). Equivalent amounts of empty vector pcDNA-3.1 were added, in all co-transfection experiments, to normalize the total amount of cDNA. Cells were harvested 48 h after transfection and collected by centrifuge at 5 000g for 5 min. This fraction was frozen and stored at -80°C until needed.

Purifying the protein complexes. All purification steps were conducted at 4 °C in the presence of a protease and phosphatase inhibitor mixture (Roche Applied Science) and using the Interplay ™ TAP Purification kit following the instructions of the manufactures (Stratagene, USA). Briefly, for large-scale experiments, carbachol treated and untreated $1x10^9$ SK-N-MC cells were resuspended in lysis buffer and subjected to successive rounds of freeze-thawing (dry ice/ -80°C/cold water). The supernatant was recovered after centrifugation at 16,000 x g for 10 min and incubated under rotation for 4 h with washed Streptavidin resin (50 µl of resin by 1ml of supernatant). The resin was colleted by centrifugation at 1,500 x g for 5 min and washed three times with 1 ml of Streptavidin

binding buffer (SBB), resuspended into 100 µl of Streptavidin elution buffer (SEB), and incubated for 30 min to elute the protein complexes. The supernatant was collected and mixed with 2µl of Streptavidin supernatant supplement and 400 µl of calmodulin binding buffer (CBB) per 100 µl of supernatant. It was then incubated for 2 h under rotation with 25 µl of calmodulin resin per 500 µl of the eluted following centrifugation and washed five times using 1 ml CBB. Retained proteins from this second purification step were eluted after 30 min of incubation with a 100-µl calmodulin elution buffer (CEB). Elutes from three successive purifications were pooled together and concentrated with an Amicon Ultra-4 10 000 MWCO centrifugal filter device (Millipore). A sample buffer was added to the concentrated sample proteins, which were then separated on a 10% SDSPAGE gel and visualized with a colloidal Coomassie staining kit (Invitrogen).

Mass Spectrometry and Protein Identification. Coomassie Blue-stained bands were excised and subjected to in-gel trypsinization as previously described [17]. Digestions were carried out overnight at 37 C with sequencing grade trypsin (Promega, USA). Resulting peptides were extracted under basic and acidic conditions and subjected to LC-MS/MS analysis. Nanoflow LC was performed using an Ultimate HPLC system (LC Packings, San Francisco, USA) at a flow rate of 200 nL/min over Zorbax SB-C18 reverse phase resin (Agilent, Wilmington, USA) packed into 75-µm inner diameter PicoFrit columns (New Objective, Woburn, USA) using a 0–50% linear acid gradient of solvent B in 50 min (solvent A was 0.2% formic acid in 5% acetonitrile, and solvent B was 0.2% formic acid in 90% acetonitrile). The LC effluent was electrosprayed into the sampling orifice of a QSTAR quadrupole-TOF mass spectrometer (ABI/MDS Sciex, Concord, ON, Canada), which operated to collect the MS/MS spectra in a data-dependent manner. The MS/MS data was analysed and matched to all human protein sequences in the Swiss-Prot database using the MASCOTsearch engine (Matrix Sciences, London, UK) [18]. Searches were performed without constraining protein molecular weight or isoelectric point. Cysteine carbamidomethylation, methionine oxidation and one missed trypsin cleavage were set as variable modifications. Identification was considered positive if the protein was identified with at least one peptide with an ion score greater than the Mascot significance threshold of 36 ($p < 0.05$). For the protein with a score close to the threshold value, the identification was confirmed by manual interpretation of corresponding MS/MS data.

As a consequence of both isolation methods used to recover protein complexes from concentrated extracts and the sensitive mass spectrometry used to identify proteins in each bands, we detected non-specific contaminants in each purifications. These recurrent background species were filtered from the dataset, taking into consideration two criteria: structural components of the ribosome and mitochondrion, which were detected in many

preparations (see Supplementary Table), and all proteins that detectably bound to TAP-system alone.

Network Management. To gain a more comprehensive view of muscarinic receptor interactome, interaction data from TAP experiments was combined with interaction detected by other biochemical methods and reported in the scientific literature. All data was searched using PreBIND (http://bioinfo.mshri.on.ca/prebind/), a support vector machine and natural language-processing-based algorithm that can identify publications that describe protein-protein interactions. Interactions found in this way were manually verified by reading the original abstract. The resulting data sets containing non-redundant interactions, combined with our experimental results, were formatted for import into BIND, the Biomolecular Interaction Network Database. BIND is built around an ASN.1 specification standard that stores all relevant information about the interacting partners, including experimental evidence for the interaction, subcellular localization, biochemical function, associated cellular processes and links to the primary literature. BIND is an open source public database implemented by the Blueprint consortium and is freely available at http://www.bind.ca and can export an arbitrary molecular interaction network as a Cytoscape network file. Visualization and connectivity distribution of the muscarinic receptor interactome network was calculated using the Cytoscape software by partitioning the network by node degree (protein).

Validation of the Selected Specific Interacting Proteins. Several newly identified putative interacting proteins were validated by co-immunoprecipitation experiments. Plasmids encoding the HA-M_2, HA-M_3 or HA-M_5 receptors were transiently transfected into SK-N-MC cells. 48 h later, cells were serum-deprived for 4 h. They were then exposed to carbachol (20 μM) for 10 min and washed once with PBS before being solubilized in immunoprecipitation buffer (PBS, pH 7.5, Nonidet P-40, DTT 2mM, phosphatase inhibitors (NaF 50 mM, Na_3VO_4 0,1mM, $Na_4P_2O_7$ 10 mM, EDTA 5 mM, EGTA 5 mM), and protease inhibitor complete (Roche Diagnostics). Extracts were centrifuged at 15, 000g for 15 min at 4°C to remove particulate material and precleared with Protein G/A Sepharose (Santa Cruz Biotechnology, USA). After centrifugation, the supernatant was transferred to tubes containing either rabbit monoclonal anti-HA (Santa Cruz Biotechnology, USA) or nonimmune mouse IgG (Sigma-Aldrich, Germany) with the required amount of Protein G/A Sepharose suspension, before rolling at 4°C overnight. Beads were collected by centrifugation and washed twice with immunoprecipitation buffer before 35 μl of 2x Laemmli buffer (2% SDS, 5% mercaptoethanol, 20 Mm Tris, pH 7.4) was added per ml of original supernatant. After SDS-PAGE and electroblotting, Western blot was carried out on the sample and the original supernatants to detect immunoprecipitated proteins and monitor input levels. The

primary antibodies used were an anti-GIT1 rabbit monoclonal antibody, a mouse anti-paxillin monoclonal antibody and a mouse anti-AKAP monoclonal antibody, followed by preabsorbed secondary goat anti-rabbit or anti-mouse antibodies to horseradish peroxidase (DAKO, USA). Bands were visualized by ECL (Kodak, USA) and then measured by quantitative densitometry.

Data Annotation. Proteins in our dataset were annotated using terms from the Gene Ontology (GO) project (http://www.geneontology.org). A subset of terms from the Biological Process and Cellular Component GO ontologies were selected to form a generalized categorization of muscarinic receptor interactome cellular localizations and biological processes. Some related GO terms were collapsed into a single category. For example, "endoplasmic reticulum" and "Golgi apparatus" were combined to form the "endoplasmic reticulum/Golgi" category.

CHAPTER 4

The four intracellular loop and the C-terminal tail

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4.1 The Inhibitor Effect of the C-terminal Tail of Muscarinic Receptor on Rhodopsin Mutants R135L and L46R in SHSY5Y Human Neuroblastome Cell Line

4.1 The Inhibitor Effect of the C-terminal Tail of Muscarinic Receptor on Rhodopsin Mutants R135L and L46R in SHSY5Y Human Neuroblastome Cell Line

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ABSTRACT

Rhodopsin mutations are the most common cause for autonomic dominant retinitis pigmentosa. A common mechanism of retina degeneration is the activation of apoptotic events. Some important biochemical events in light-induced photoreceptor apoptosis have been identified, but the therapeutic utility of blocking cell death remains unclear. However, it is now clear that G-protein-coupled receptors can regulate programmed cell death through a variety of mechanisms that are dependent on receptor subtype. It has been demonstrated that $G_{q/11}$-coupled subtypes of the muscarinic receptor family are able to protect against apoptotic cell death. In particular, the polybasic region conserved within the C-terminal tail appears to be the structural determinant of coupling to the anti-apoptotic pathway. In order to investigate whether the anti-apoptotic properties of this epitope can be used as a blocking system to inhibit apoptotic effects promoted by rhodopsin mutants, we engineered a group of quimeric receptors where the C-terminal tail of the human M_3 muscarinic receptor was inserted in the C-terminal tail of two rhodopsin mutants (R135L and L46R), previously reported for patients with severe retinitis pigmentosa. We provide evidence that quimeric construction protects cells from H_2O_2-induced apoptosis. Promoting the early expression of Bcl-2 decreased Bax expression and reduced caspase-3 activation. Correspondingly, cytochemical evaluation revealed an absence of cytochrome c into cytoplasm and an increased of NF-κβ translocation to the nucleus. These effects were blocked only by HA-1077 Rho kinase inhibitor, indicating that the insertion of the anti-apoptotic epitope of the muscarinic receptor remains functional, reducing many apoptotic markers, and can be used as a therapeutic method for late-stage inhibition of apoptosis.

INTRODUCTION

Retinal degenerations, including retinitis pigmentosa (RP) are a leading cause of human blindness [395]. In these diseases, initially functional photoreceptor cells of the eye are

irreplaceably lost over time [396]. There are two main mechanisms by which cells can die: necrosis, which is a lytic response to overwhelming stress, and apoptosis [397]. Apoptosis is non-lytic, morphologically distinct, and its core elements are apparently conserved in all cells. Previous results indicate that cell death of RP is apoptotic and about 30% of all autosomal dominant RP is caused by mutations in the photoreceptor protein rhodopsin [398]. This suggests that the activation of cell death by such mutations occurs by the common mechanism of apoptosis.

Also, there is a growing amount of evidence that activation of some G-protein-coupled receptors can provide protection from a wide variety of potentially lethal toxic insults [399-402]. Prominent among the GPCRs that protect cells from apoptotic stimuli are the Gq/11 subtypes of the muscarinic receptor family [403,404]. For example, activation of endogenous muscarinic M_3 receptors protected human neuroblastoma SH-SY5Y cells and primary neuronal cultures from apoptosis caused by three common insults: DNA damage, oxidative stress, and impaired mitochondrial function [405]. In fact, the anti-apoptotic effect of muscarinic receptors in neuronal models has led to the suggestion that this process may play a role in neuronal development, and lesions in cholinergic innervation observed in neurodegenerative diseases such as Alzheimer's may result in the loss of the survival stimulus that contributes to neuronal cell death [348]. Side-directed mutagenesis revealed that a polybasic region in the C-terminal tail of the M_3-muscarinic receptor, which is conserved amongst the members of the Gq/11-coupled muscarinic receptor subtypes, is responsible for and involved in coupling the receptors to the anti-apoptotic pathway [406].

Muscarinic and rhodopsin receptors belong to the class A subfamily of the large family of G-protein coupled receptors [407]. They share a common structure and signal transduction mechanisms in spite of the differences that exists between them [408]. This discovery raises the possibility that transmission of the ligand-binding signal to the cytpoplasmic surface and cytoplasmic loops is similarly conserved. Therefore, a quimeric construction where the C-terminal region of the rhodopsin mutants were replaced by the anti-apoptotic C-terminal region of the M_3 muscarinic receptor might keep its functional properties, and provide protection to the cell. Engineering the quimeric receptor of two rhodopsin mutants (R135L and L46R), previously reported for patients with severe retinitis pigmentosa [409,410], we set out our hypothesis and showed evidence of protection against apoptosis and common activation mechanisms among GPCRs.

RESULTS

Expression in Vitro of quimeric construction in the presence of 11-cis-Retinal

Construction and analysis of chimeric receptor, consisting of the replacement of part of the C-terminal region of the rhodopsin with the anti-apoptotic C-terminal region of M_3 muscarinic receptor, was carried out to determine if the functional domain of the muscarinic receptor might keep its functional properties, and provide protection to the cell in an opsin receptor background. Engineered quimeric receptors of two rhodopsin mutants (R135L and L46R), previously reported for patients with severe retinitis pigmentosa [410], and wild type were used (Figure 4.1.1)

Expression of quimeric constructions in human neuroblastome cells SH-SY5Y was carried out in the presence of 11-cis retinal or 9-cis retinal. Upon reconstitution with 11-cis retinal, all chimeras showed chromophore formation with absorption maxima at close to 500 nm. In addition, immunocytochemistry of all chimeras expressed in the presence of 11-cis or all-trans retinals were studied. While the chimeric-WT and chimeric-L46R receptors exhibited proper plasma membrane localization in presence of 11-cis retinal, the R135L-quimeric displayed a pattern consistent with endoplasmic reticulum accumulation in presence of 11-cis-retinal (Figure 4.1.1B). Under identical culture conditions and in the presence of all-trans-retinal or without any retinal, little if any chimera receptors could be detected.

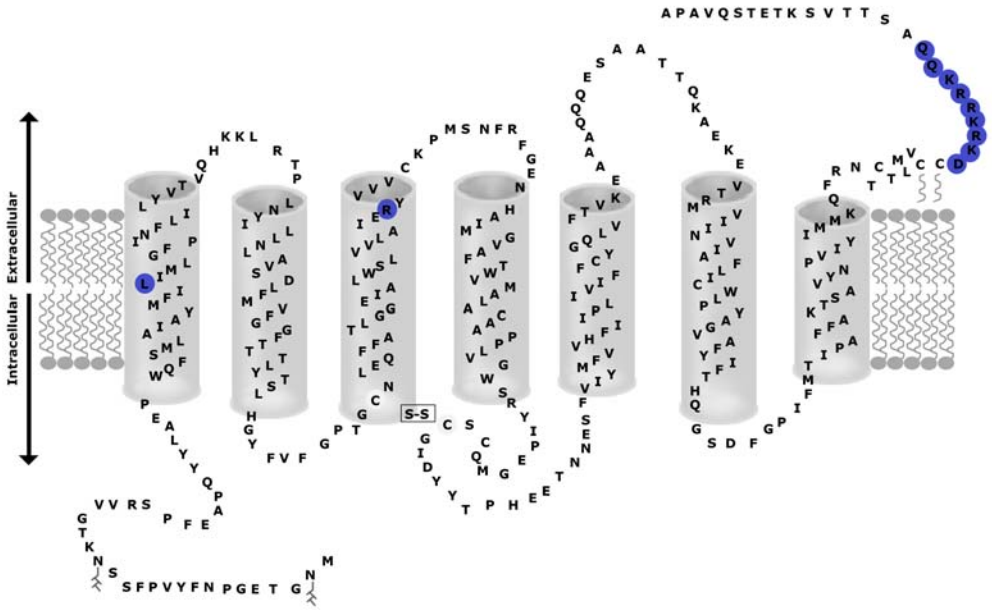

Figure 4.1.1 *Secondary structure model of bovine rhodopsin. In blue (cluster), C-terminal region replaced in this study; (alone point), RP mutants studied (R135L and L46R).*

Replacement of the C-terminal tail of rhodopsin by the anti-apoptotic C-terminal region of M_3 muscarinic receptor promotes cell survival

To determine whether the replacement of the C-terminal tail of rhodopsin by the homologue and polybasic region of the C-terminal tail of human M_3 muscarinic receptor promotes cells survival, SH-SY5Y culture cells expressing chimeras were incubated with hydrogen peroxide to induce cell death. 48h after transfection, SH-SY5Y cells expressing chimeras and maintained in a medium containing 11-cis retinal were photo-bleached in the presence of H_2O_2 at a concentration of 100 μM for 18 h. Then the protective effects of each construction were determined using MTT assay. The viability of cells expressing WT (Rho-WT) and growing in a medium containing 100 μM H_2O_2 and 11-cis retinal was approximately 55% of that for cells expressing rhodopsin chimeric (Rho-M_3), indicating that replacement of the C-terminal region did in fact protect again peroxide-induced cell death. Similar effects were also observed with respect to chimeras of severe retinitis pigmentose mutants (L46R-M_3 and R135L-M_3) when compared to cells expressing receptors mutants alone (L46R and R135L) (Figure 4.1.2).

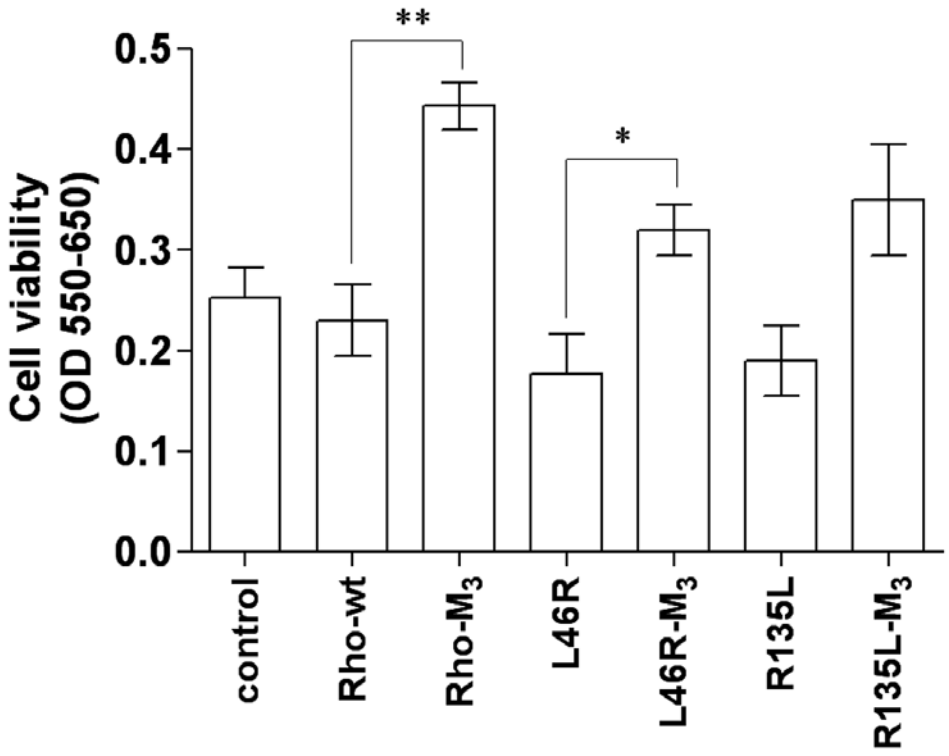

Figure 4.1.2 *Replacement of the C-terminal tail of rhodopsin by the anti-apoptotic C-terminal region of M_3 muscarinic receptor promotes cell survival. Cells expressing each quimeric receptor were grown in the absence of or presence of hydrogen peroxide (100 μM) for 18 h. MTT assays were carried out to determine cell viability. Means ± S.E.M is shown; n=4. **: Significantly different compared to Rho-wt (ANOVA: $p<0.01$). *: Significantly different compared to L46R (ANOVA: $p<0.05$)*

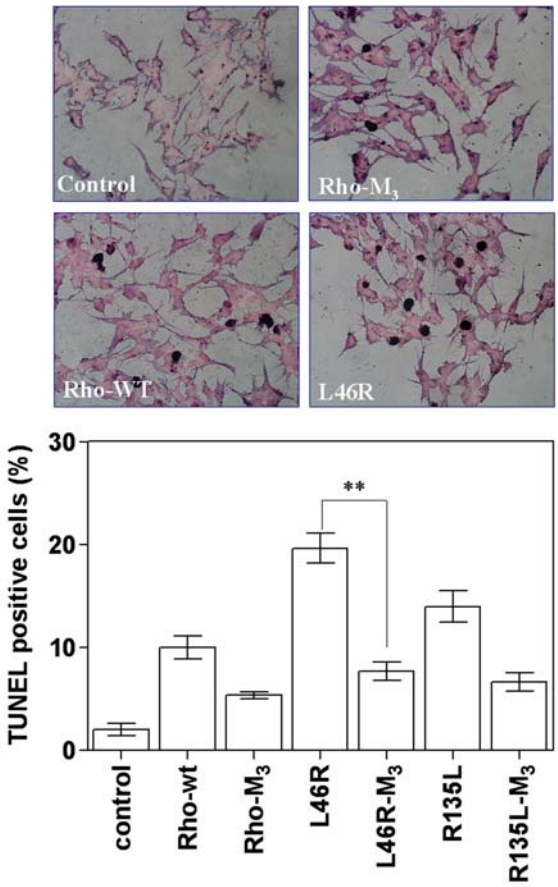

Figure 4.1.3 *In vitro expression of the opsin quimeric constructions reversed apoptotic effects induced by peroxide. Transfected cells were grown in DEMEM in the presence of 11-cis-retinal and peroxide for 18 h. TUNEL assays were carried out to determine DNA fragmentation. TUNEL-positive cells were very darkly stained with a condensed nucleus as visualized by light microscopy. Means ± S.E.M is shown; n=7. **: Significantly different compared to L46R (ANOVA: p<0.01).*

Since the MTT assay measures cell viability and accounts for changes in cell survival and proliferation, TUNEL staining was used to assess the number of cells dying by apoptosis. About 10-22 % of cells expressing rhodopsin receptor and mutants alone respectively were TUNEL-positive when grown in the presence of peroxide, whereas cell-expressing chimeras significantly reduced the number of apoptotic cells to only 5 to 8%, a value similar to that which is obtained for cells growing in presence of carbachol (10 μM).

Chimera's expression protects against H2O2-induced cell death

Incubation of cells with peroxides triggers the activation of caspase-3, a potent effector of apoptosis, by proteolytic cleavage of the 32 kDa pro-caspase into two active fragments. Subsequently, activated caspase-3 cleaves susceptible substrates, such as PARP, an enzyme involved in DNA repair [411].

The effects of H2O2-induced caspase-3 activation were reflected in measurements of the proteolysis of PARP, which is mediated by caspase-3 and results in the production of a stable 85-kDa product cleaved from the 116-kDa intact PARP. Treatment with 100

μM H2O2 caused a concentration-dependent increase in PARP proteolysis (Fig. 2B) and in caspase-3 activity in all receptor-transfected cells (data not shown). However, cells expressing chimera receptors showed a great reduction in both PARP and caspase-3 proteolysis, induced by 100 μM H2O2 in the presence of 11-cis retinal (Figure 4.1.3 right panel). Therefore, time-course analysis of caspase-3 activity revealed a significant reduction in cells expressing the chimeric Rho-M_3 receptor compared with the WT (Figure 4.1.3 left panel). Time-course caspase-3 activity also showed that for both receptors, WT and Rho-M_3 chimeric, caspase-3 activity increased from 2 h to 6 h and reached a plateau after 6h. We also evaluated the significance of the protective effect of chimeras by compared it with that of a general caspase inhibitor, BAF. These experiments demonstrated that the substantial protection provided by chimeras against 100 μM H2O2-induced PARP proteolysis was similar to that afforded by 1μM BAF caspase inhibitor (data not shown).

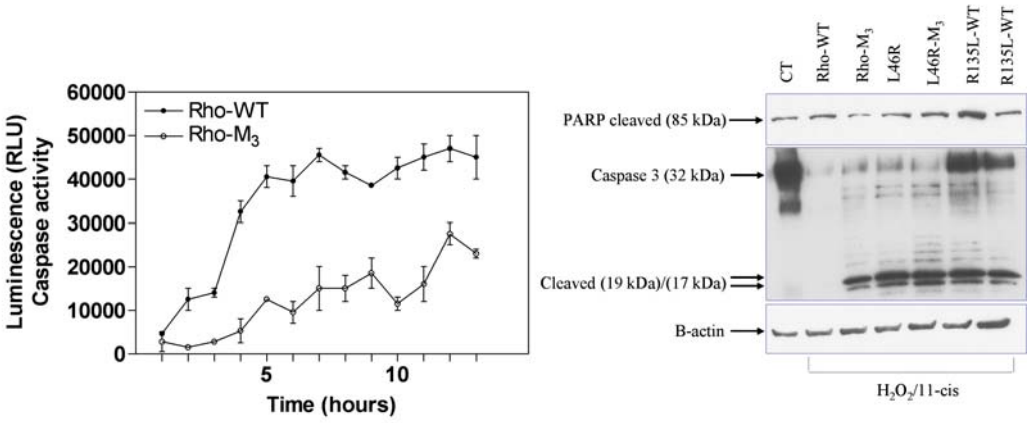

Figure 4.1.4 *Inhibition of peroxide-induced apoptosis by cells expressing quimeric constructions.* ***Left****, hydrolysis of caspase-3 selective fluorogenic substrate Ac-DEVD-MCA in peroxide-treated SH-SY5Y cells was monitored over time. Cells expressing both WT rhodopsin or the quimeric construction were treated with 100 μM peroxide for up to 15 h. Whole cell lysate sampled directly from the treatment wells was incubated with 40 mM Ac-DEVD-MCA in 250 μl final volume. Data are presented as mean ± SEM of three independent experiments, each performed in duplicate.* ***Right****, Western blot analysis of caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage. Procaspase 3 processing was verified after 48h treatment with peroxide in the presence of the 17 kDa active subunit on the blot. Detection of 85 kDa PARP fragment confirmed caspase 3 activation.*

Furthermore, we assessed whether chimera constructions up-regulate anti-apoptotic Bcl-2 and Bcl-xL proteins. The members of the Bcl-2 protein family are widely thought to represent a central point of regulation of caspase-mediated apoptosis [412]. The balance of pro- and anti-apoptotic members of the Bcl-2 family, particularly in relation to their mitochondrial membrane localization, determines whether a cell enters apoptosis. In the present study, we show that levels of the anti-apoptotic protein, Bcl-2, fall dramatically following H_2O_2 treatment. The fall in Bcl-2 levels correlates closely with an increase in caspase activity. Similar results were found in cells expressing receptors and mutants.

However, in cells expressing chimeras, significant prevention of the reduction of Bcl-2 expression levels was observed (Figure 1.4.4). Interestingly, levels of Bcl-2 were not significantly affected by R135L-M_3 chimeric construction.

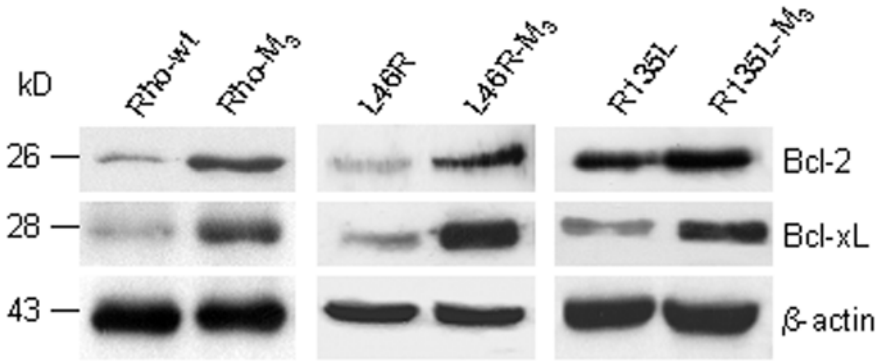

Figure 4.1.5 *Peroxide induces an increase in the expression of Bcl-xL and Bcl-2 in SH-SY-5Y cells expressing the quimeric constructions. This shows the protective effect of the M_3 muscarinic polybasic sequence in the RP rhodopsin mutants background.*

Chimera construction prevents cyto c release during apoptosis

Previous studies have shown that translocation of cyto c into the cytosol occurs in response to multiple apoptotic stimuli and plays an important role in inducing apoptosis [413,414]. Release of cyto c from mitochondria is a potential signaling component for regulating apoptosis in many cell types, including neuroblastome cells induced into apoptosis by oxidative stress. Therefore we wished to examine next the release of cyto c in cytosolic and mitochondrial cellular compartments in cells expressing receptor mutants and chimeras following exposure to peroxide. Cyto c was undetectable in the cytosol in immunofluorescence images from untreated cells. In contrast, cytosols from peroxide-treated cells contained increased cyto c levels starting at 2 h, as detected by immunofluorescence. Cyto c levels increased moderately at 4 and 8 h, and more significantly at 16 h after peroxide treatment (data not shown). Examination of cyto c release from mitochondria compartment in cells expressing the WT rhodopsin receptor revealed only a slight reduction in cyto c levels up to 8 h following H2O2 treatment. In contrast, there was a dramatic increase of cyto c levels in the cytosol at 16 and 24 h, indicating a depletion of cyto c from mitochondria. This effect was prevented in cells expressing the chimeric Rho-M_3 receptor, where a 35% reduction in Cyto c release was observed at 16h of H2O2 incubation.

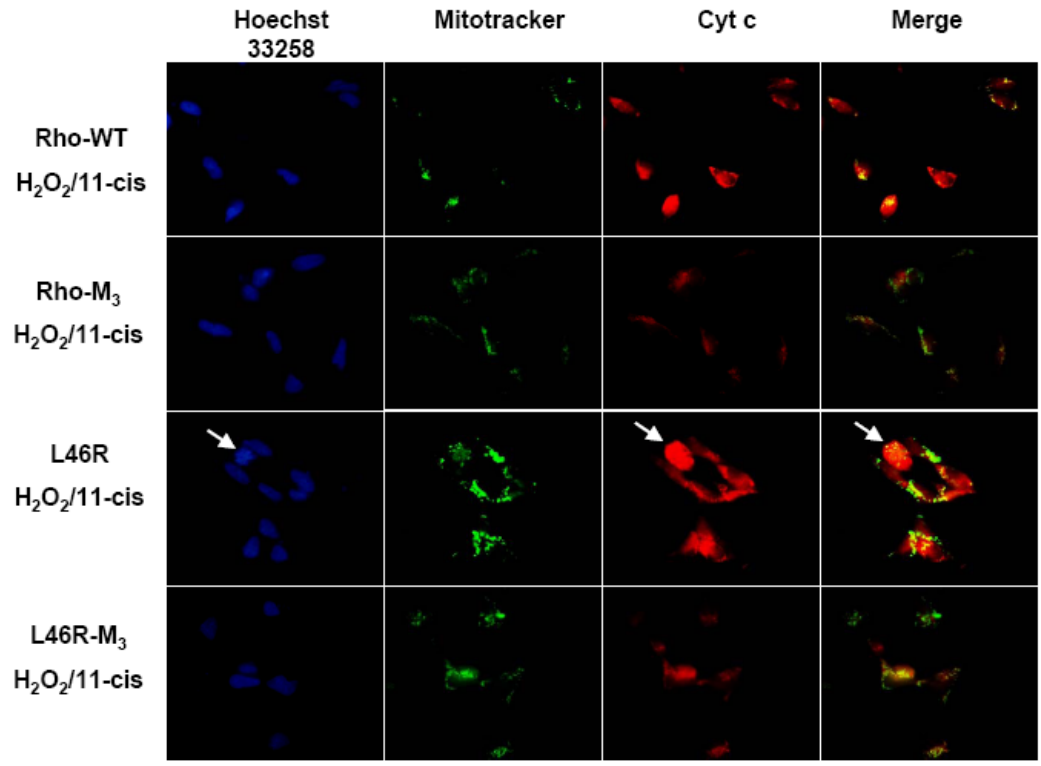

Figure 4.1.6 *Cytocrome c undergoes subcellular redistribution in peroxide-treated cells. Cells expressing each quimeric and WT construction were incubated in the presence of 100 μM H_2O_2 for 15 h. Cells were analyzed by immunocytochemistry using an anti-cytochrome c-specific antibody and an Alexa-488-coupled secondary antibody. MitoTracker staining was performed before fixation. Loss of or diffuse cytochrome c staining in apoptotic cells is indicative of cytochrome c release.*

This result was not limited to the Rho-M_3 chimeric, since the L46R-M_3 chimeric receptor also is characterized by a reduction in cytosolic levels of cyto c release from mitochondria (Figure 1.4.5). Interestingly, cells expressing the L46R mutant showed a sustained and early release of cyto c following H2O2 when compared to rhodopsin WT receptor expression.

MATERIALS AND METHODS

Material. U72122, coenzyme C3 and BFA were from Calbiochem (Nottingham, U.K.).). All tissue culture reagents were purchased from Gibco BRL (Glasgow, U.K.). Carbachol was purchased from Sigma (Poole, U.K.). Anti-Bcl-2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cloning of chimeric rhodopsin-M_3-C terminal tail. Constructions of chimeric receptors and receptor mutants (L46R and R135L, kindly provided by E. Ramon.) were carried out based on the nucleotide sequence of the synthetic opsin and human M_3 muscarinic receptor genes. Basically, using a QuikChange site-directed mutagenesis kit and routine PCR methods, regions between the helix-8 and the C-terminal tail of the opsin gene were replaced by a peptide corresponding to a polybasic region of the C-terminal tail of human M_3 muscarinic receptor.

The sense primers used were: 5-GCGGAAAAGGATGTCACTAGTCAAGGAGAAGAAAGCGGCCC-3; 5-GCGTACCAAAGAGCTAGCTGGCCTGCAAGCC-3. The reading frame, PCR integrity of the cloned construct and the sequences of the quimeric, were confirmed by DNA sequencing.

Cell Culture, transfection and treatment. SH-SY5Y human neuroblastome cells were grown on 12-well plates to confluences (~2 x 106 cells per well) at 37 °C, 5% CO2 in a humidified atmosphere with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. To discover whether or not quimeric constructions protect cells from apoptosis, we studied each construct expressed in vitro in the presence of 11-cis retinal with or without 100 µM H2O2. Plasmid cDNAs were transfected in human neuroblastome cells (SH-SY-5Y) by using Fugene transfection reagent (Roche). 11-cis-Retinal was delivered into the cells as a carrier prepared according to Jones et al. (14), except that phosphatidylcholine was used at 5 mg/ml. All experiments involving retinals were conducted under dim red light or in the dark. 11-cis-retinal was added to cells at 125 µl per 150-mm dish at 3 h after transfection and a second aliquot was added 12 h later. A second control received liposome only. At the beginning of the experiment, cultures were washed three times with serum-free DMEM. Apoptosis was then induced by the addition of H2O2 at a concentration of 100 µM, for periods of 6–24 h (usually 16 h). To avoid artefacts in time course experiments, all conditions were washed with serum-free DMEM at time 0 and were treated (100 µM H2O2) at the appropriate time so that all time points were collected simultaneously.

Visualization of apoptotic nuclei (TUNEL labelling). Transfected SH-SY5Y cells, growing in 24-well tissue culture plates with poly-D-lysine-coated glass coverslips, treated as described above, were washed with PBS and then fixed with 4% paraformaldehyde for 20 min at room temperature. Fragmented DNA (high molecular weight or internucleosomal) was detected by incorporating fluorescein-12-dUTP at 3´-OH ends using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, as described [Cui, QL., Almazan G. 2005]. Anti-fluorescein antibody Fab fragments conjugated with horseradish peroxidase detected incorporated dUTP. Stained cells were visualized by light microscopy. Labelled nuclei and the total number of cells were counted in at least five different fields.

MTT assay of cell viability. The number of living cells in 96-well plates (2□104 cells per well) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was dissolved in PBS at a concentration of 5 mg/ml. From this stock solution, 10 µl per 100 µl of medium was added to each well, and the plates were incubated at 37°C for 3 h. Acid-isopropanol (100 µl of 0.04 M HCl in isopropanol) was then added to the well and mixed in. After 15 min at room temperature, the plates were read on a Bio-Rad micro-ELISA spectrophotometer at a test wavelength of 550 nm and a reference wavelength of 650 nm. SH-SY5Y cell growth in DEMEN alone (defined as 100%) were used to normalize absolute MTT values.

Assay for caspase-3/7 activity. Caspase-3/7 activity was measured with the Apo-ONE homogeneous assay kit using a synthetic fluorometric substrate for caspase, Z-DEVD-Rhodamine 110. After treatment with H2O2 for the time indicated, 100 l of homogeneous

caspase-3/7 reagent (buffer+substrate) was added to each well of the SH-SY5Y cells in 96-well plates. The 96-well plate was incubated at room temperature and assayed on a Cytofluor II multiwell fluorescence spectrometer (excitation 485 nm, emission 520 nm).

SDS-PAGE and Western blot. Transfected and treated cells were harvested, washed twice with phosphate-buffered saline, and lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 5 µg/ml pepstatin, 1 nM okadaic acid, and 0.5% Nonidet P-40). The lysates were sonicated for 10 s on ice and centrifuged at 16,000 x g for 15 min, and supernatants were collected. Protein concentrations were determined using the Bradford method or the bicinchoninic (BCA) method (Pierce). Proteins were resolved in SDS-polyacrylamide gels (12%), transferred to nitrocellulose membranes, and incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The primary antibodies used were: poly (ADP-ribose) polymerase (PARP), cytochrome c (BD Pharmingen), bcl-2, Bax, tubulin (Upstate Biotechnology, Lake Placid, NY), cytochrome oxidase (Molecular Probes, Eugene, OR). Proteins were visualized by ECL® (enhanced chemiluminescence; Amersham Biosciences) and were quantified by densitometry.

Immunofluorescence analyses. Cells were grown on Vitrogen-coated coverslips and cultured in the presence of 11-cis-, or 9-cis-retinal. 24 h after transfection, they were washed with PBS, fixed in ice-cold methanol for 5 min, and stained with anti-rhodopsin antibodies rho-1D4 (C-terminal specific), followed by Cy3-conjugated goat anti-mouse IgG.

Cyto c subcellular localization: cells were grown on Vitrogen-coated coverslips, washed with PBS, fixed with 2% paraformaldehyde, incubated with anti-cyto c mAb, followed by FITC-conjugated secondary mAb. MitoTracker Red (100 nM) was used for mitochondrial localization, and Hoechst 33258 (10 µg/ml) for nuclear staining in confocal and conventional microscopy, respectively. The immunostained slides were examined using a Nikon FXA epifluorescence microscope or a Leica TCS-NT confocal laser-scanning microscope with a 63´objective lens (Leica Lasertechnik GmbH, Heidelberg, Germany). For comparison of triple-stained patterns the three images were superimposed. MitoTracker Red and Hoechst 33258 were from Molecular Probes.

Statistical analysis. Statistical significance was determined by one-way ANOVA and, if significant, group means were compared by post hoc analysis using Tukey multiple comparison of means test. Values shown are the mean s.d. of three experiments (three to four cultures per experiment).

CHAPTER 5

Roles of Receptor-interacting proteins in subtype-specific receptor signaling

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5.1 NPxxY (5,6) F motif as a key step in conformational requirements for G proteins activation and multiprotein complex reorganization of the human M_3 muscarinic receptor.

5.1 NPxxY (5,6) F motif as a key step in conformational requirements for G proteins activation and multiprotein complex reorganization of the human M_3 muscarinic receptor

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ABSTRACT

Activation of the M_3 Muscarinic receptor by agonist binding is followed by conformational changes and recruitment of accessory and scaffold proteins, that together with the classical core signalling entities contribute to the formation of a signalsome complex. The dynamics of such a complex is not well understood but a conserved NPxxY (5,6) F motif located in the transmembrane VII of the receptor was found to be critical in G protein activation. We have generated receptor mutants (N540A, N540D and F551A) to determine the effect of the mutation on binding, G protein coupling, signalling and multiprotein complex reorganization after the expression of the mutated receptor in COS-7 cells. The mutated receptor displayed similar expression levels and Ligand binding properties compared with the wild type, except receptor mutant N540A, which showed a high reduction in level expression and slightly reduced antagonist binding sites. In addition, the N540A receptor mutant showed a dramatic reduction in G protein activation and completely failed to elicit PLD activation and PI response to carbachol stimulation. However, the N540D mutant activates G proteins and stimulates PLC and PLD, but with different time course and desensitization compared with the WT. Co-immunoprecipitation experiments allowed us to show that each mutant retains a different capacity to form stable complexes with different populations of G proteins and accessory proteins (ARF1, ARF6, RhoA). Different levels of ARF, RhoA and G proteins were observed in the immunoprecipitation complexes for N540D mutant. Indicating the role of the NPxxY (5,6) F motif as a structural determinant during conformational and selectively activation of G proteins and multiprotein complex reorganization.

INTRODUCTION

The M_3 muscarinic acetylcholine receptor (M_3) is a member of a family of muscarinic receptors that subserve diverse roles both in central and peripheral nervous system [415]. It is a class A, rhodopsin-like, G protein-coupled receptor (GPCR) that signals primarily through the heterotrimeric G protein $G_{q/11,}$ activating phospholipase Cβ, with a corresponding increasing in intracellular calcium [266]. M_3 receptors also regulate a

network of signalling intermediates, including PKC, small GTPase Rho, phospholipase D, phosphoinositide-3 kinase, non-receptor kinases and mitogen-activated protein kinases [416].

Intracellular loop interactions and conserved residues have been described as critical in maintaining normal receptor conformation, G protein coupling, signalling and regulation. Chimeric construction and site directed mutagenesis indicated that both the M_3 second intracellular loop and the C-terminal portion of the third intracellular loop are important for coupling to the $G_{q/11}$ protein [417]. In addition, activation is mediated by a conformational rearrangement that includes the TM7 and probably, as described for rhodopsin, movement in the C terminal tail (Helix-8) [215].

A sequence alignment of GPCRs of the rhodopsin family reveals that the majority of GPCRs, as well as the M_3 receptor, contain a conserved NPxxY (5,6) F motif at the end of transmembrane VII and the beginning of Helix-8 [338]. Mutagenesis studies, rhodopsin crystal structure and molecular modelling have shown that mutation of residues in the motif of different GPCRs is implicated in a network of interactions that modulate receptor activity, expression and signalling [418-422]. A theoretical model based on a computational modelling study has been proposed, which explains that the special structural property of the conserved N/DP motif differs from a regular Pro-kink, of ideal α-helix, allowing for a major flexibility in the TM7 [423]. The flexibility introduced by the NPxxY (5,6) F motif may play a role in GPCR activation by functioning as a sensitive conformational switch and can determine the selectivity and magnitude of signalling, regulation and scaffolds protein interaction with the receptor.

The role of the NPxxY (5,6) F motif in the muscarinic receptor family has been associated more directly with translocation of both native ARF1 and transfected ARF1-HA protein to the plasma membrane following activation of M_3 receptor [424,425]. Interaction between the M_3 muscarinic receptor and small-G proteins, ARF and RhoA, has been described as critical during the activation of the PLD and ROCK pathway [426-428].

Based on the precept that NPxxY (5,6) F motif is involved in the activation and potentially in the signalsome complex formation of GPCRs in general and the M_3 muscarinic receptor in particular, we generated a series of mutants. In essence, the Asn located in the conserved NPxxY motif was replaced by an Asp (N540D) and Ala (N540A), and Phe located at the putative helix-8, by Ala ((F551A). Mutation of Asn to Ala switches the M_3 receptor to a high affinity inactive conformation for the transmission of a signal to different effectors. However, the mutated receptor retains a capacity to form stable complexes with $G\alpha_q$ subunits in response to agonists, as

demonstrated by coimmunoprecipitation. By contrast, the N540D mutant results in slightly higher levels of ERK phosphorylation than those displayed by the WT and similar levels of PLC and PLD activity. In addition, this mutant showed different time course and desensitisation with respect to the wild type. Co-immunoprecipitation and activation assays for some scaffold proteins that can potentially bind to the muscarinic receptor were also analysed. These experiments allowed us to observe that each mutant retains a different capacity to form stable complexes with different populations of G proteins and accessory proteins (ARF1, ARF6 and RhoA) and the bases of different desensitisations can be found here.

RESULTS

To explore the role of the NPxxY (5,6) F motif in multiprotein complexes formation and assembly by human M_3 muscarinic receptors upon agonist stimulation, a group of single mutations were generated. Exactly, Asp (N540D) and Ala (N540A) replaced Asn located in the conserved NPxxY motif, and Ala (F551A) replaced Phe located at the putative helix-8. The antagonist and agonist binding properties, phosphoinositide hydrolysis stimulation, G protein activation, small G protein RhoA activation and co-immunoprecipitation of the receptor and mutants with some scaffold protein were studies after transient expression in COS-7 cells.

N540D and F551A mutants are expressed on the cell surface and binding ligand with affinities similar to the wild type receptor.

As mentioned above, to determine if the conserved motif NPxxY (5,6) F plays a role in the rearrangement, assembly and multiprotein complex formation of the M_3 receptor, we have mutated the ASN 540 position to Ala and Asp, and also the Phe 551 position to Ala (Figure 5.1.1).

All mutants and the WT receptor were transiently expressed in COS-7 cells and assayed for their ability to bind antagonist [^{3}H]-NMS, and the agonist, carbachol. The results of radioligand binding studies are summarized in Table 5.1.1. Saturation binding experiments showed that all mutant receptors were expressed at densities comparable with the WT, with Bmax values ranging from 890 to 924 fmol/mg of protein; with the exception of N540A mutant that showed a four-fold reduction in level expression. In addition, the various mutant receptors displayed antagonist-binding affinities that did not vary more from the values determined for the WT. However, N540A bound antagonists with lower affinities than the WT receptor. Agonist-binding parameters showed for all mutants non-significantly lower agonist binding affinities than the WT, and an important increase in agonist affinity with respect to the WT for the N540A mutant. In all cases, agonist-binding studies were carried out in the absence of GTP and

GTP analogues, since previous studies had shown that such agents did not affect the agonist binding properties of the M_3 muscarinic receptor when transiently expressed in COS-7 cells [429].

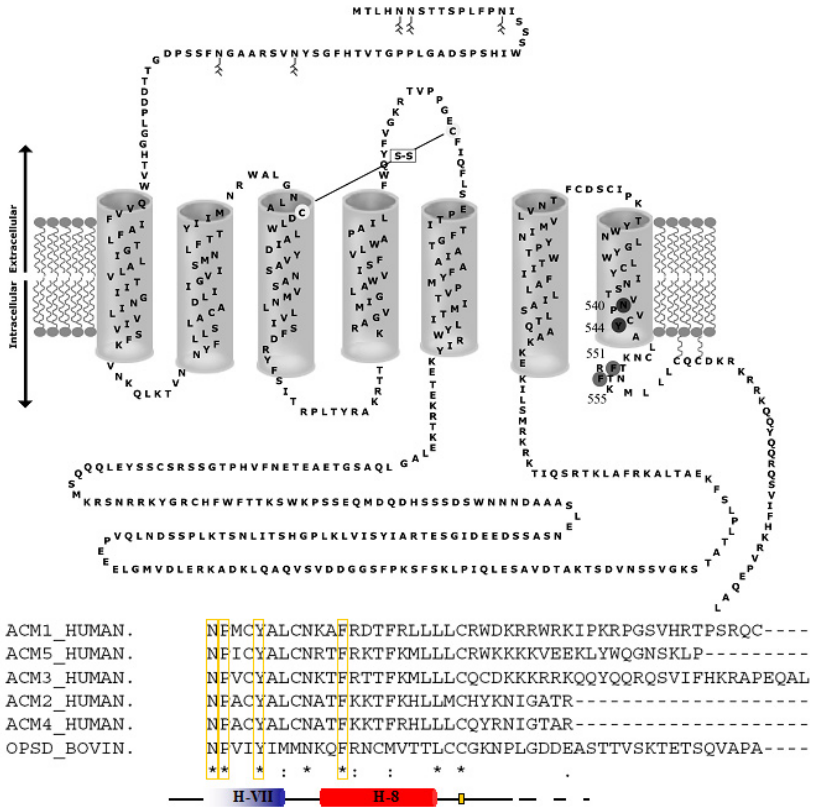

Figure 5.1.1 *Location of the NPxxY (5,6) F motif in the M_3 model. A two-dimensional model, with the extracellular domain on top and the intracellular domain at the bottom, of the human M_3 sequence is depicted. Mutated residues are highlighted. Mutations were generated using QuikChange site directed mutagenesis kit from Stratagene. A schematic representation of the multiple alignments of the muscarinic receptor family and rhodopsin is represented. Residues upstream and downstream of the conserved Pro at the end of TM7 (P7.50) are shown in yellow. The region conserved among the family is depicted with an asterisk and the putative H-8 is showed in red.*

Immunoprecipitation of the WT and mutant receptors was performed using an antibody directed against the hemagglutinin epitope (HA) fused to the amino terminus of each one and transiently expressed in COS-7 cells. Epitope-tagged WT and mutant receptors expressed in COS-7 cells displayed similar agonist affinities and maximal-binding capacities compared with untagged WT and mutated receptors. In addition, to confirm that the transfection and expression efficiencies of each tagged receptor were similar, we performed western blot of COS-7 cells membranes expressing HA-tagged WT and mutated receptors with anti-HA antibodies. The human M_3 muscarinic receptor was expected to migrate at a mass of 67-75 kDa based on their primary sequence and on previous reports (Figure 5.1. 6) [430].

Table 5.1.1 *Ligand binding and functional properties of M_3 muscarinic receptor mutants expressed in COS-7 cells.*

Receptor	Binding studies			PI assays	
	[3H]-NMS		Carbachol	Carbachol	Maximum increase in IP levels (WT taken as 100%)
	K_D (pM)	B_{max} (fmol/mg)	K_{app} (μM)	EC_{50} (μM)	
M_3-WT	90 ± 17	924 ± 37	8.4 ± 2.3	3.4 ± 0.5	100
N540D	87 ± 15	910 ± 33	12.2 ± 2.8	3.6 ± 0.2	91 ± 4
N540A	105 ± 69	226 ± 33	1.1 ± 0.8	-	-
F551A	86 ± 14	890 ± 30	9.2 ± 2.1	5.8 ± 0.2	52 ± 3

Affinity constants (Kd)for the radioligand [3H]NMS were determined in saturation binding assays. Inhibition constants (Ki) for carbachol agonist were obtained in competition binding experiments as described in Materials and methods (nH= Hill coefficients). Bmax values indicated a maximum number of binding sites/mg of membrane protein. Data are presented as means ± SEM of three to four independent experiments, each performed in duplicate. Agonist binding parameters were obtained in competition binding assays as described in M&M using [3H]NMS (200pM) as a radioligand. Kapp values were calculated from IC50 values using Cheng and Prussof ecuation and can be consider an approximation of Ki values. In all cases, Hill coefficients (nH) was significantly smaller than 1 ($P< 0.05$). EC50 values for carbachol-induced IP1 accumulation were determined graphically from plos of log (carbachol) versus percentage responses. Data are presented as means ± SEM of three to four independent experiments, each performed in duplicate.

The N540A mutant is unable to activate PLC, PLD and MAPK pathway.

We then investigated the effects of each mutation on receptor function in COS-7 cells by quantifying the activation of three of the main signalling pathways activated by M_3 muscarinic receptor [416].

PLC activation mediated by M_3 muscarinic receptor has been described previously and it is the main pathway activated by M_3 receptor upon agonist stimulation [184,334]. To assess the ability of the wild type and mutant receptors to activate the PLC-signalling cascade, we measured the accumulation of total inositol phosphate (IP). Non-appreciable carbachol-stimulated [^{3}H]-myo-InsPs accumulation was detected in un-transfected cells. Concentration-response curves for the WT and mutant receptor are represented in Figure 5.1.2 and Table 5.1.1. A small change in IP accumulation was measured between the WT and the N540D mutant receptor, even at highly agonist concentration (10^{-3} M CCh). However, under similar experimental conditions, the F551A mutant receptor showed a two-fold reduction with respect to the WT.

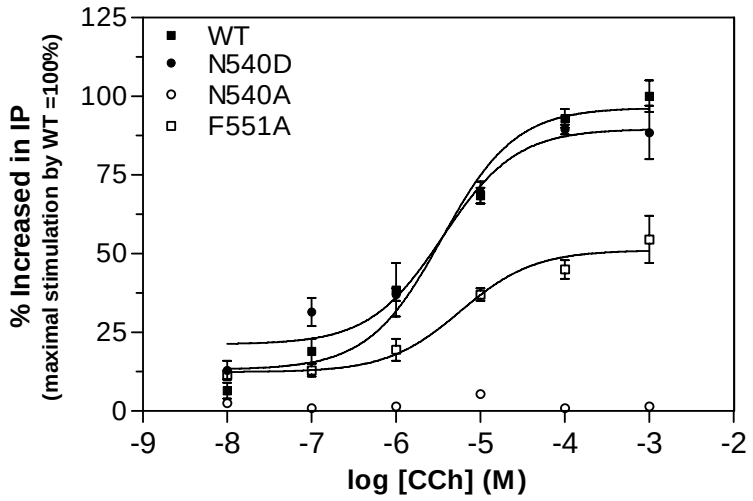

Figure 5.1.2 *Stimulation of PI hydrolysis mediated by wild-type (WT) and mutants expressed in COS-7 cells.* **.** *Effect of 3ILoop-minigene vector and muscarinic receptor subtypes co-expression on PLC activity in CCh-stimulated HEK-293 cells. Cotransfected cells with M_3 or M_5 receptor subtypes and 3ILoop-minigene (●, ○) or empty vector (■, □) were stimulated with CCh as indicated, for 1 hr at 37°C in presence of LiCl as described in Materials and Methods. Data are presented as an increase of InsPs (DPM) above basal levels in the presence of carbachol. Basal levels observed with the various receptors were not significantly different. Data represents the average ± S.E.M. values of triplicate determinations of three independent experiments.*

In addition, agonist-mediated [^{3}H]-myo-InsPs accumulation was abolished in cells expressing the N540A mutant essentially at all agonist concentrations. Time-course of carbachol-stimulated [^{3}H]-myo-InsPs accumulation in WT and mutant transfected cells reached a peak between 20 to 60 minutes after agonist exposure, and fifteen percent of receptors desensitised 2 hours after agonist stimulation (2 min, 1mM CCh).

Although the best-established signalling pathway from the M_3 receptor is the pertussis toxin-insensitive activation of PLC via the heterotrimeric $G_{q/11}$ protein, PLD is also strongly activated via both the $G_{q/11}$ and $G_{12/13}$ protein [431,432]. We then investigated the ability of the WT receptor and mutants to activate PLD activity, measured as a formation of [^{3}H]-phosphatidylbutanol ([^{3}H]-Ptdbut).

As illustrated in Figure 5.1.3, carbachol stimulated [^{3}H]-Ptdbut formation in the WT with an efficacy of six-fold over basal and a maximal potency of 1.43 ± 0.18 nM. The N540D and F551A mutants activated PLD in a similar way with respect to the WT as shown in PLC activation, but with lower potency. In addition, no detectable [^{3}H]-Ptdbut production was measured with the N540A mutant, even when exposed to 10^{-4} M CCh.

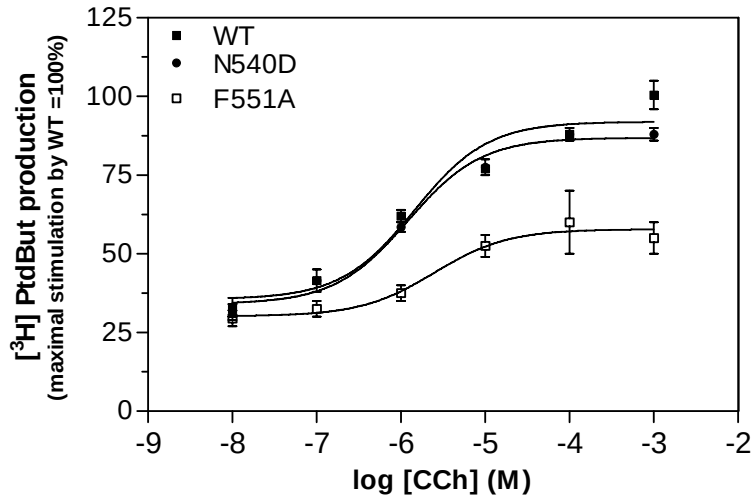

Figure 5.1.3 *Stimulation of Pbutnol mediated by wild-type (WT) and mutants expressed in COS-7 cells.*

To assure that the EC_{50} and E max change observed were not due to alterations in the receptor density, we plotted the negative logarithm of EC_{50} *vs.* receptor density for each mutant and WT in both experiments. This plot shows that receptor expression was rather variable but that the EC_{50} shifts were independent of receptor density.

Finally we decided to test the MAPK pathway that can be activated by M_3 receptors in a protein kinase C-dependent and –independent fashion [235,303].

Since not many studies have been performed on COS-7 cells, we first assessed whether the WT receptor expressed in COS-7 cells was able to activate MAPK in response to carbachol. A maximal increase of 20 folds in ERK½ phosphorylation over basal was reached when WT-transfected cells were incubated with 10 μM CCh. In addition, a time course study of ERK½ phosphorylation in WT-COS-7 cells using 10 μM of CCh resulted in a maximal activation at 5 min (data not shown).

Figure 5.1.4 shows effects of carbachol-stimulated MAPK activation in cells expressing WT and mutant receptors. A significant decrease (up to approximately 85%) in ERK½ phosphorylation was observed in the case of the N540A mutant with a less extended decrease for F551 mutant when compared to the activation mediated by the WT. Also, a non-significant statistic increase for N540D mutant was observed.

Agonist-stimulated N540A mutants are coupled to $G_{q/11}$ proteins without activating them.

Previous studies on HEK-293 cells expressing M_3 muscarinic receptors showed that a G protein of the $G_{q/11}$ class mediated carbachol-stimulated PLC activation and PLD activation occurred via a G protein of the $G_{12/13}$ class [433]. Co-expression of both, $G\alpha_q$ or $G\alpha_{12}$ subunits with the WT receptor and N540D/F551A mutants increased [^{3}H]-myo-

InsPs and [^{3}H]-Ptdbut productions in a concentration dependent manner that reached a maximum when cells were transfected with 5 µg of Gα DNA plasmids. In similar conditions, co-expression of both Gα subunits with the N540A mutant did not induce any change, in agreement with results described above where this mutant did not stimulate PLC and PLD activities. Also, co-immunoprecipitation experiments with COS-7 cells co-expressing the 3x HA-WT or 3x HA-mutants and Gα subunits were performed. A non-basal association between $G\alpha_{12}$ subunits was observed in carbachol-stimulated N540A-COS-7 cells, correlating with PLD activation results. In contrast, as shown in Fig.5.1.6, *upper panel*, when cells were stimulated with 10 µM CCh and subjected to immunoprecipitation with anti-HA antibodies, $G\alpha_q$ subunits co-immunoprecipitated with both the 3x HA-M_3-WT and 3x HA-M_3-mutants as a band that migrated at 42 kDa. The effect of agonists on $G\alpha_q$-3x HA-M_3 and $G\alpha_q$-3x HA-N540A complex formation was 4.3 ± 1.1 and 4.7 ± 1.8 fold of the basal level respectively (mean ± S.E. of five experiments).

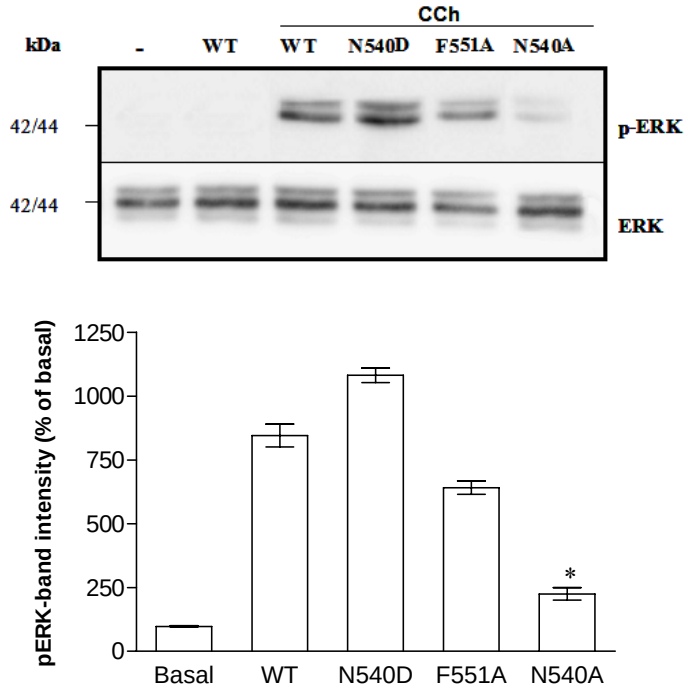

Figure 5.1.4 *Agonist stimulated MAPK activation in COS-7 cells expressing WT and mutants. Confluent cultures were exposed to serum-free medium 12 h before the addition of carbachol (1mM) for 5 min. Cells were washed extensively after agonist treatment and prepared for MAPK extraction. Extracts corresponding to 1x10^5 cells was run on 12.5% SDS-PAGE gel. The proteins were transferred onto nitrocellulose, and MAPKs p44 and p42 were detected using a mouse monoclonal antibody against human p42/44 MAPK phosphorylation on Thr-202 and Tyr-204, or a rabbit polyclonal antibody against total human p42/44 MAPK. The proteins were detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence. The intensity of the bands was quantified using Imagen Quand Program. Bars represent the means ±S.E.M. of the phospho-ERK (p-ERK) immunopositive band intensity expressed as the percentage change from the basal (serum-free medium-*

*treated) level calculated from four separate experiments. *P≤ 0.05 compared to basal conditions in WT-COS-7.*

Moreover, as the N540A does not display increased basal level IP accumulation and the last results suggest that the mutated receptor forms stable complexes with Gqα subunits upon agonist stimulation, we precede to monitored basal guanine nucleotide exchange, using [^{35}S] GTPγS assay. In non-stimulated cells, [^{35}S] GTPγS assay gave almost identical values for basal binding in both WT receptors and mutants. However, 1 μM of carbachol induced an increase in [^{35}S] GTPγS binding (43% over basal) for high and low WT receptors and N540A/F551A mutant expression in COS-7 cells. No change was observed when cell expressing N540A mutant (data not shown).

A different time-course and desensitization patron characterizes agonist-stimulated mutant N540D.

M_3 muscarinic receptors activated both PLC and PLD upon agonist stimulation using different molecular mechanisms and time kinetics [434]. While PLD stimulation showed a rapid and long lasting desensitization, agonist stimulation of PLC is not desensitized following short-term treatment. 2 min of pre-exposure to agonists led to an about 2-fold increase in receptor-mediated PI formation, supersensitivity that disappeared 2 h after carbachol removal.

We then decided to analyze whether or not each mutant (N540A and F551A) could maintain its capacity to stimulate both phospholipase, as well as maintain the same patron of desensitization and time-course upon agonist incubation.

The time course of PLD and PLC activation by carbachol in transfected COS-7 cells is shown in Figure 5.1.5. There was rapid desensitization of the PLD, but not the PLC response, over the times examined. The time-course of carbachol-stimulated [^{3}H]-myo-InsPs accumulation in the transfected WT and mutants-COS-7 cells reached a peak between 20-60 min after agonist exposure and fifteen percent of receptors desensitised 2h after agonist stimulation (2 min, 1mM of carbachol). In contrast, maximal levels of labeled [^{3}H] Ptdbut were already obtained after 2 min of incubation with carbachol (1mM), followed by a stable plateau up to at least 30 min of incubation for the WT receptor. An identical time-course of [^{3}H]-Ptdbut formation, although at a lower level, was obtained in F551A COS-7 cells. Interestingly, COS-7 cells expressing N540D mutant showed a time course patron more similar to the patron showed by WT and mutants in agonist-stimulated PLC activation. We also observed that the N540D mutant was not desensitized following short-term agonist treatment in the same manner as the WT. On the contrary, 2 min of preexposure to carbachol led to about 2-folds increase in receptor-mediate [^{3}H]-Ptdbut formation 20-30 min after exposition. However, after that

time, this supersensitivity disappeared with a long lasting desensitization similar to the WT.

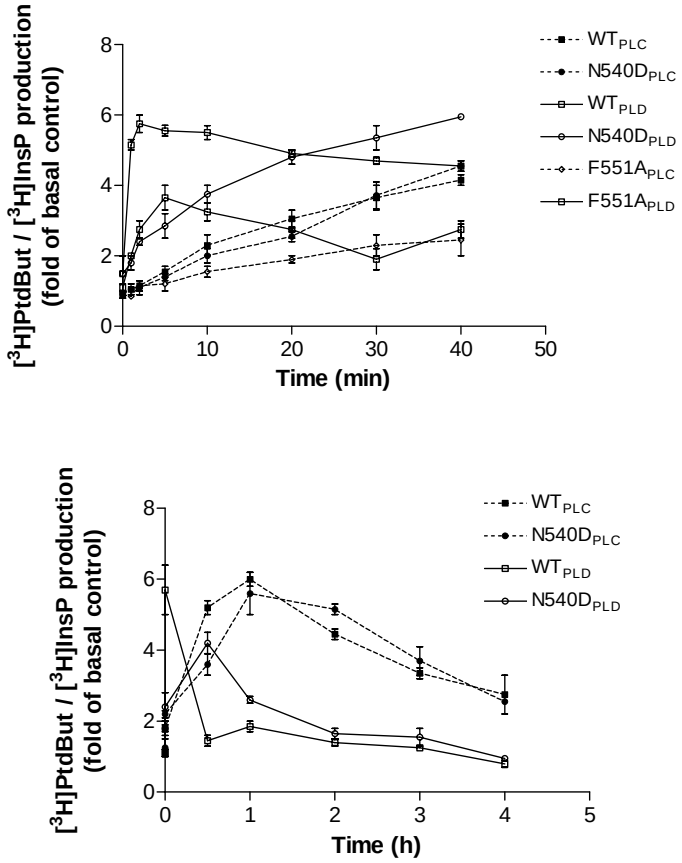

Figure 5.1.5 *PLD and PLC responses of wild type, N540D mutant receptors expressed in COS-7 cells. Upper panel, show the time course of [^{3}H]-myo-inositol phosphate ([^{3}H]-myo-InsPs) and [^{3}H]-phosphatidylbutanol ([^{3}H]-Ptdbut) production evoked by 1 mM carbachol. Values are folds of basal control (means ± s.e.m; n= 4-6). Lower panel, desensitisation of muscarinic-stimulated PLD and PLC. COS-7 cells expressing wild type and N540D mutant receptors prelabeled with [^{3}H]-myo-InsPs and [^{3}H]-Ptdbut were pretreated for 2 min with 1mM carbachol. After wash-out of carbachol, the carbachol (1mM) induced formation of [^{3}H]-myo-InsPs and [^{3}H]-Ptdbut was measured at the indicated periods of time for 10 min. Basal accumulation of [^{3}H]-Ptdbut was unchanged. Basal accumulation of [^{3}H]-myo-InsPs, on the other hand, was different depending on whether carbachol-pretreated cells or untreated were utilized. Data are from one experiment (means ± s.e.m, triplicate culture dishes). Similar results were obtained in three separate experiments.*

Different interacting proteins are associated with agonist-stimulated mutant receptors.

Taking into consideration the last result, where a WT receptor and N540D mutant showed different desensitization and time-course in PLD activation, as well as, in

various cell types ARF and Rho proteins were specifically implicated in M_3 receptor-mediated PLD activation; we decided to analyze weather G proteins ($G_{q/11}$ and $G_{12/13)}$ or scaffold/associate proteins were affected and might be responsible for the variability of phospholipase regulation.

First, by western blot analysis we demonstrated that in WT cells, both subtypes of G protein ($G\alpha_q$ and $G\alpha_{12}$) remain associated with the M_3 muscarinic receptor. Our studies using an antibody specific for $G\alpha_{q/11}/G\alpha_{12/13}$ (Santa Cruz Biotech) did not detect endogenous Gα in the COS-7 cells consistent with previous report (Ref). Both mutant receptors, N540D and F551A were found to be associated with Gαq and Gα12 to the same extent as WT. In contrast, as mentioned above, the coupling of N540D mutant receptor with Gα12 was severely abrogated (Figure 5.1.6).

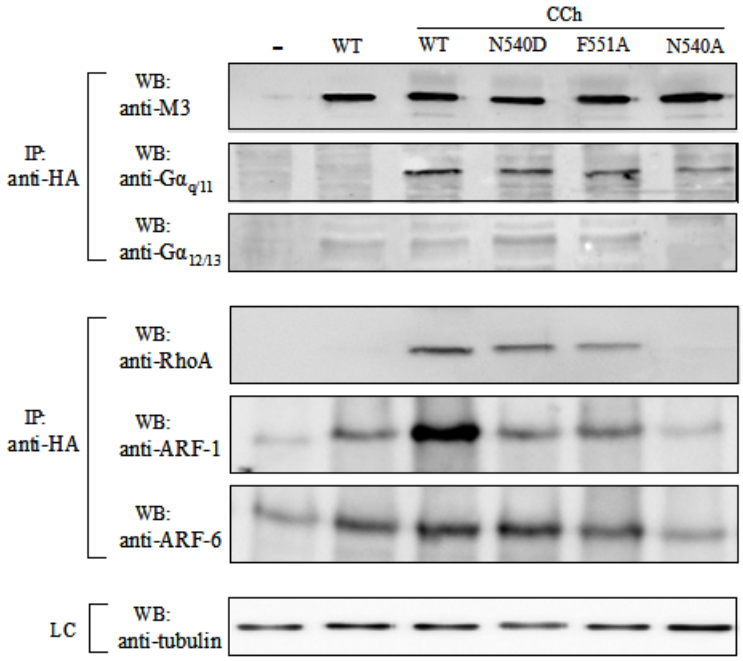

Figure 5.1.6. *Differential coupling of WT and mutants to $G_{q/11}$, $G_{12/13}$, RhoA and ARF1/ARF-6 proteins. Receptors present in crude membrane preparations of non-stimulated and stimulated cells expressing WT or mutants were solubilized with 4 mM CHAPS and immunoprecipitated with anti-HA. The immunoprecipitates were subjected to SDS-PAGE and Western blotting using anti-M_3, anti-$G_{q/11}$, anti-$G_{12/13}$, anti-RhoA and anti-ARF 1/6 antibodies. The experiment shown was repeated twice with identical results.*

Also, Figure 5.1.6 shows that a solubilized M_3 receptor can be immunoprecipitated using an anti-HA antibody, which causes co-precipitation of immunoreactive ARF and Rho A after priming with carbachol. Co-immunoprecipitation of WT-RhoA complex required pre-exposure to agonists. Little co-immunoprecipitation was observed when using control immunoreagents (non-immune IgG) or in the presence of excess peptide antigen. However, ARF1 and ARF6 proteins seem to be specifically associated with the WT receptor without activation. In addition, densitometry analysis indicated that after carbachol priming, the WT-ARF1 complex co-immunoprecipitated with increase of on

average 4.21 ± 1.36-fold (n=4) over WT under basal conditions. The corresponding value for WT-ARF6 was lower (1.03 ± 0.57-fold (n=6) increase over Wt under basal conditions) but only the increased ARF1 was statistically significant ($p<0.05$, n=4). We observed that the carbachol induced on ARF1 coimmunoprecipitation with the WT receptor was reduced to basal levels in COS-7 cells expressing N540D mutant. As before, the concentration of ARF1-myc present in the lysates was very similar between the different conditions and the number of receptors immunoprecipitated was also even (Figure 5.1.6. top). However, upon agonist stimulation ARF6 coimmunoprecipitation levels are closing similar for both the WT and N540D mutant. Observations using immunoprecipitation of the WT and mutants receptor indicate that ARF1, and to a lesser extent ARF6, form a stable complex with the WT receptor that can be increased in the presence of an agonist, correlating with the functional studies on PLD activation. But, these levels can be varied when receptors is mutated in key domain or position, as shown in results for the N540D mutant, where the associated of the ARF1 isoform is, in part, abrogated after agonist priming.

We then asked whether the apparent change in affinity for ARF isoforms and small G proteins caused by the N540D mutation affects the signalling function of the WT and N540D mutant receptors. To examine interacting protein-independent and interacting protein-dependent receptor activation we measured agonist-induced activation of PLC and PLD in COS-7 cells transfected with either WT or N540D receptor under different inhibitory conditions.

The activation of PLD by WT and N540D mutant in COS-7 cells was differentially inhibited by brefeldin A (BFA), an inhibitor of guanine-nucleotide exchange in ARF, and C3 exoenzyme, an inhibitor of small G protein RhoA. Activation of PLD by WT receptors in these cells (as for WT receptors expressed in HEK 293 and endogenous expressed in 1321N1 human astrocytoma cells) was sensitive to BFA (100 μM) whereas activation of PLD by the N540D mutant was BFA-resistant (Figure 5.1.7 upper panel). The WT receptor but not the N540D mutant responses were attenuated by a C3 exoenzyme inhibitor (Figure 5.1.7 upper panel). In previous work, it has been showed that changing the sequence of the carboxyl-terminal tail domain (CT) of the 5-HT2A receptor in a GST-fusion protein construct from N^{376}PxxY to D^{376}PxxY reduced the affinity of the construct for ARF1 isoform [422,435]. PLC activation by the WT receptor was inhibited by U73122 (IC_{50} of 11± 1μM) but not the PLD activation, whereas with either PLC or PLD, N540D mutant response was sensitive to U73122 in concentrations of up to 20 μM (Figure 5.1.7 upper and lower panel).

We decided to also test the effect of inhibition of the βγ subunit in both WT and N540D mutants. Co-transfection of the WT or N540D mutant with pREP4-GRK2-Ct plasmid (plasmid containing an inhibitory peptide of βγ subunit) resulted in a decrease in PLD

activation for the WT receptor and no significant statistical change in N540D mutants. Neither change has been observed for both WT and mutant in PLC activation.

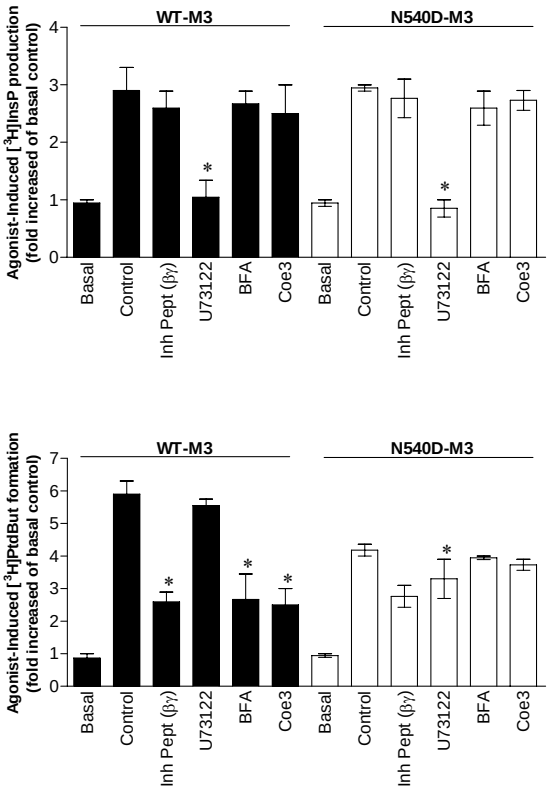

Figure 5.1.7 *Properties of agonist-evoked PLD and PLC responses in COS-7 cells expressing wild type and N540D mutant receptors. Upper panel and lower panel 10 mM carbachol-induced PLC and PLD signalling responses of wild type and N540D mutant receptors respectively. Comparison of their susceptibility to inhibition by co-transfection of pREP4-GRK2-Ct plasmid (plasmid containing an inhibitory peptide of βγ subunit), U73122 inhibitor (15 μM), BFA inhibitor (100 μM), C3 exoenzyme (4,8 μg/ml: Coe3). In control and inbibitor-treated cells, an amount of empty vector equivalent to that used for pREP4-GRK2-Ct was contransfected with the receptor constructs. Values are means ± s.e.m, n=5, *p<0.05.*

With the discrepancy between the variable increased in ARF1/ARF6 isoform binding to agonist stimulated WT receptors and mutants, our results suggest that there exists a correlation between M_3 multiprotein complex formation and PLD/PLC extension of activation and regulation. These results concur with the idea that agonists produce a conformation change in the receptor during activation that can selectively induces translocation of ARF1/RhoA/ βγ subunit to the membrane, promoting their involvement in some form of complex with the muscarinic receptor, and may ultimately enhance

receptor mediated activation of PLC/PLD, and determine their time-course and desensitization.

DISCUSSION

A sequence alignment of GPCRs of the rhodopsin family reveals that the majority of GPCRs contain an NPxxY (x) $_{5,6}$F sequence [408]. Mutation in this domain affects ligand affinity, receptor expression, G protein coupling, and receptor association with interacting proteins such as small G proteins ARF and Rho [418,422,436-439]. The function of this motif during multiprotein complex organization by the M_3 muscarinic receptor is not well understood. Therefore, we set out to elucidate the role of this region by using site direct mutagenesis in combination with coimmunoprecipitation experiments and signal transduction assays. This study demonstrates for the first time that change at the level of residue N540D/F551A in M_3 muscarinic receptors contributes to heterotrimeric G protein ($G_{q/11}$ and $G_{12/13}$) regulation but not G protein binding itself. Residue N540D is also implicated in the receptor desensitization process and also in PLC/PLD signal transduction efficacy. Furthermore, given the variety of affects observe in these studies, as well as previously results which demonstrated that change from NPxxY (x) $_{5,6}$F to DPxxY (x) $_{5,6}$F in some GPCRs produced selective interaction with small G proteins [422], we can conclude that this region, perhaps in cooperation with other intracellular loops, controlled interacting protein association/selectivity and determined in part, the mechanism of activation of PLD.

Our results support and expand upon previous studies of GPCRs that these residues, because they are located deep inside in transmembrane helix-VII and seem to allow a local flexibility in the conformational rearrangement, are not directly involved in ligand-binding sites but are key positions in hydrogen bound formation and stability upon agonist stimulation [440,441]. Agonists could activate the N540D and F551A mutant receptor with a non-lower agonist binding affinity, as the antagonist-binding affinities did not vary more from the values determined for the WT. However, compared with the WT, N540A mutation resulted in a significant reduction in receptor level expression, bound antagonists with lower affinities, and with an important increased in agonist affinity. An explanation would be that the drastic change of Asn by Ala completely abrogates the formation of hydrogen bound than can keep receptor under stable conformation before and after activation. Ala replacement in position N7.49 in rhodopsin M_1 muscarinic receptor and the TRFR receptor did not facilitate conformational organization of the active state showing variable change in agonist/antagonist affinity with respect to Wt ().

With the N540A/D and F551A mutations, we demonstrated a selective regulation of downstream signal transduction by a different conformational arrangement of M_3

receptor. The WT and N540D mutant resulted in a small change in IP accumulation even at highly agonist concentration. However, the F551A mutant receptor showed a two-fold reduction with respect to WT and the N540A mutants completely lost their capacity to activate the PLC signal. Also, carbachol stimulated [^{3}H] Ptdbut formation resulted in a six-fold increase for the WT receptor and N540D mutant, with lower activation for the WT receptor and N540D mutant, lower activation for the F551A mutant, and no detectable [^{3}H] Ptdbut production for the N540A mutant, but with a lower potency in all cases. We also corroborated that theses results were observed at the level of the MAPK PLC/PLD pathway and the changes of Asn by Asp keep a relatively stable conformation that does not substantially perturb the conformation change produced during receptor activation upon agonist stimulation.

Schmidt and Jakobs showed that activation of M_3 muscarinic receptor in HEK cells leads to stimulation of both phospholipase (PLC/PLD), but with distinct efficacies and with very distinct durations of each response, but this study did not identify the essential transducing component responsible for the variable desensitisation [442]. Mitchell and Johnson demonstrated in COS-7 cells that the M_3 muscarinic receptor substantially utilized an ADP-ribosylation factor (ARF)-dependent route for PLD activation [443]. The M_3 receptor displayed a major ARF1-dependent route for PLD1 activation, whereas the ARF-6 dependent pathway activates both PLD1 and PLD2 [444]. However, these studies did not show in detail if the selective coupling by different ARF factors is responsible for desensitization.

Comparing the time course of PLD and PLC activation of the WT and N549D/F551A mutant, we demonstrated the active role of the NPxxY (x) $_{5,6}$F motif during the desensitization process of the receptor, and more specifically the importance of residue position 7.49. We observed, as previously reported, that the time-course of carbachol-stimulated [^{3}H]-myo-InsPs accumulation in the transfected WT-COS-7 cells reached a peak between 20-60 min after agonist exposure and fifteen percent of receptors desensitized 2 h after agonist stimulation (2 min, 1 mM of carbachol). In contrast, maximal levels of [^{3}H] Ptdbut were already obtained after 2 min of incubation with carbachol (1mM), followed by a stable plateau of at least 30 min of incubation for the WT receptor. Although at a lower level, the F551A mutant showed similar time-course and desensitization, and the N540D mutant did not desensitize following short-term agonist treatment in the same manner as the WT for PLD response. On the contrary, 2 min of pre-exposure to carbachol led to about a two-fold increase in receptor-mediate [^{3}H] Ptdbut formation 20-30 min after exposition.

Our co-immunoprecipitation studies demonstrated that although WT and mutants are associated to a same extent with the two subtypes of g protein ($G\alpha_q$ and $G\alpha_{12}$) and RhoA protein, different levels of association were observed for ARF1/6 factors. We observed, using co-immunoprecipitation experiments, that WT and mutants can bind ARF1, and

to a lesser extent ARF6, forming a stable complex which is increased upon agonist priming. But these levels can be varied when the receptor is mutated in a key domain or position, as shown in the result for the N540D mutant, where the association of the ARF1 isoform does not increase to a similar extent as the WT after agonist incubation. Previous experiments reported that N376 to D mutation in the conserved NPxxY (x) $_{5,6}$F motif of the 5-HT_{2A} receptor alters the binding preference of GST-fusion protein constructs of the Ct domain from ARF1 to an alternative isoform of ARF6 [422,445]. They also reported that the presence of an N or D residue within the conserved motif cannot be the sole determinant of ARF1 or ARF6 selectivity, since some NPxxY (x) $_{5,6}$F containing receptors such as the M_3 receptor utilized both isoforms. We are in agreement, in part, with previous results; because we find that despite that the M_3 receptor can bind both isoforms (ARF1/6), with a change in the N7.49 position they lost their capacity to increase the binding level of ARF1 isoform upon agonist stimulation. In the case of M_3 muscarinic receptor, we confirm that the presence of the DPxxY (x) $_{5,6}$F motif does not promote any coupling with ARF6, as happens for other receptors, but determines the magnitude of the ARF1 coupling upon agonist activation. The activation of PLD/PLC by the WT and N540D mutant was differentially inhibited by brefeldin A (BFA), an inhibitor of guanine-nucleotide exchange on ARF1, and C3 exoenzyme, an inhibitor of small G protein Rho. WT receptor PLD response was sensitive to BFA (IC_{50}=100 μM), C3 exoenzyme inhibitor (IC_{50}=100 mM), and no changes were observed when the PLC specific inhibitor U73122 up to 20 μM of concentration was used. However, the N540D mutant showed a PLD response sensitive to the PLC specific inhibitor U73122 (IC_{50}=100 μM), but with resistance to BFA (IC_{50}=100 μM), and to the C3 exoenzyme inhibitor. In addition, for both the WT and N540DD mutant receptor, the U73122 inhibitor reduced PLC activity. All inhibitors' sensitivity was seen at low and high agonist concentrations, indicating that coupling to this pathway was not restricted to a particular level of agonist occupancy.

The long lasting desensitization of the M_3 receptor-stimulated PLD observed in our experiments and described in previous reports [446] suggests that either an inhibitory factor is formed upon receptor activation or that the activated receptor induced the loss or permanent inactivation of an essential coupling component. Since ARF1 and PLD1 are not normally localized to a large extent at the plasma membrane, either the receptor or theses mediators may need to undergo translocation to enable WT receptor-induced PLD activation. Our findings showed that ARF1 and PLD1 were clearly and selectively increased in plasma membranes following agonist stimulation (data not shown). Also Mitchell et al., using a dominant negative mutant dynamin 1, inhibited endoncytosis of agonist-occupied M_3 receptor without any inhibitory effect on PLD response [447]. These results seem to be inconsistent with the idea of endoncytic mechanism.

More recently, Kumar-Saini et al. demonstrated that the spatiotemporal response of G protein subunits in living cells to receptor activation showed 6 of the 12 members of the G protein γ subunit family translocating specifically from the plasma membrane to the endomembrane [448]. The γ subunit translocated βγ complexes, where the α subunit retained on the PM. Chisiari and Gautam, using fluorescent recovery after photobleaching, also demonstrated the rapid shuttle between plasma membrane and intracellular membranes ($t_{1/2}$<1min) [449]. Azpiazu and Gautam also reported that G protein activation by M_3 muscarinic receptor caused a rapid reversible translocation of the G protein γ11 subunit from the plasma membrane to the Golgi complex [450]. The rate of γ11 subunit translocation is sensitive to the rate of activation of the G protein α subunit. In addition, in rhodopsin the coupling of the γ subunit in the junction between the end of the TM-VII and the C terminal tail was demonstrated. We tested the effect of inhibition of the βγ complex and its translocation on PLD response. We observed that for the WT receptor inhibition of both, the βγ complex on their translocation significantly affected [^{3}H] Ptdbut production and ARF1 co-immunoprecipitation. However, the N540D mutant showed a resistant-sensitivity to βγ complex inhibition.

The data presented here indicates that stimulation of PLD/PLC in COS-7 cells by transfected M_3 muscarinic receptors are affected by change in the NPxxY (x) $_{5,6}$F motif. These changes can cause different degrees of structural flexibility, resulting in the regulation of the interacting proteins, such as ARF factors, and the selective regulation of desensitization mechanisms. Mutation in the N 4.79 position resulted in a very rapid change (less than 2min) in PLD response upon agonist-stimulation. This phenomenon may be the result of the different recruitments of ARF1/ARF6 factors, and the resistant-sensitive βγ complex inhibition and translocation (Figure 5.1.8.). This demonstrates for the first time the active role of the NPxxY (x) $_{5,6}$F motif in the selective process of multiprotein complex formation and reorganization by human M_3 muscarinic receptors, and their role during G protein regulation and desensitization.

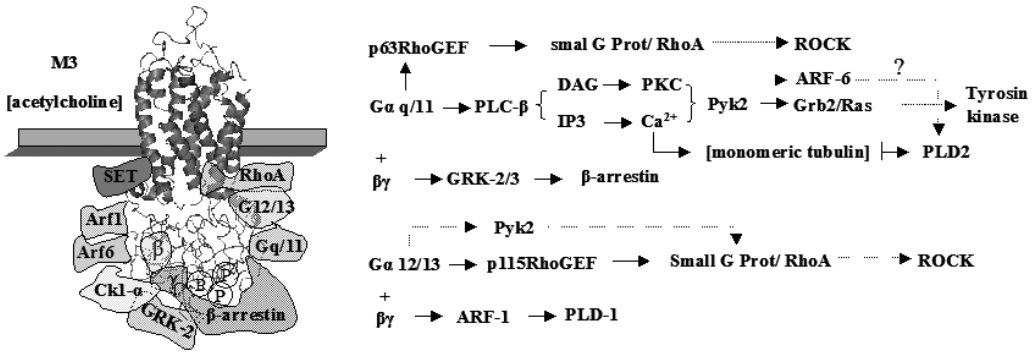

Figure 5.1.8 *Recruitment of specific proteins to the plasma membrane by M_3. After receptor activation by neurotransmitter acetylcholine (ACh), or pharmacological agonists, many proteins may participate in a larger receptor-centered scaffolding complex (β-arrestin, kinases, small G-proteins and others).*

Flexibility introduced by the NPxxY motif, upon activation, may play a role in GPCR activation by functioning as a sensitive conformational switch, and can determine the selectivity and magnitude of signalling, regulation and scaffold-protein binding to the receptor.

MATERIALS AND METHODS

Material. [^{35}S]-GTPγS (1.202 Ci/mmole), [^{3}H]-myo-inositol (3.0 Ci/ml), and [^{3}H]-NMS (77 Ci/mmole), were from Amersham Biosciences (Germany). FuGene tranfection reagent was purchased from Roche (USA). All receptor ligand, protease and phosphatase inhibitor cocktails, and all other reagents were purchased from Sigma-Aldrich.

Numbering of Residues: Residues were numbered according to their position in the human M_3 receptor sequence. We also indexed residues relative to the most conserved residue in the TM, which is according to a consensus-numbering scheme described in detail previously (Ballesteros)

DNA Constructs: The cDNAs of the human M_3 muscarinic receptor (kindly provided by T. Bonner, NIH, USA) were subcloned into the mammalian expression vector pcDNA-3.1-3xHA (gift from P. Calvo, SFU, CA) giving the 3xHA-M_3-pcDNA vectors. Basically, a 1.9-kb fragment encoding the human M_3 receptor was amplified from their cDNA using primer pair Fm3EcoRV, 5_-ACCGATATCTTGCACAATAACAGTACAACCTCGCC-_3 AND Rm3XbaI, 5_-CCCTCTAGA-CTACAAGGCCTGCTCGGGTGC-3_, utilizing the Expand High Fidelity PCR system (Roche Applied Science, USA) according to the manufacturer' instructions. The 1.9kb fragment was purified by agarose gel electrophoresis (Bio-Rad, USA) and the subcloned into the mammalian pcDNA-3.1-3xHA vector using EcoRV/XbaI. All single point mutations were made in the NPxxY (x) $_{5,6}$F motif located in the TM7 and at the beginning of the putative H-8. Exchanging the endogenous residues to Alanine or aspartic in the 3xHA-M_3.-cDNA sequences with the QuikChange site directed mutagenesis kit from Stratagene generated mutants. The sense primers used were: N540A, 5_-CTGTTT CTG CTG GGC CCC CGC CTT TG_-3; N540D, 5_-ACC TCT CTG GCT GTC CTA CTC ACC_-3; F551A, 5_- CTG TTT CTG CGC GGCCCC CTT CTT_-3. The reading frame, PCR integrity of the cloned construct and the sequences of the mutants were confirmed by DNA sequencing.

Cell Culture and Transfection. HEK 293 cells were grown to 70 % confluence in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf bovine serum (Invitrogen, USA), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. Cells were transfected with xHA-M_3.-cDNA, by means of the calcium phosphate precipitation method or using FuGene tranfection reagent (Roche, USA). Cells were harvested 48 h after transfection and centrifuged at 3000xg for 30 min to obtain two separate fractions (membrane and supernatant). These fractions were frozen as aliquots in 5mM phosphate buffer, pH7.4, and stored at -80^{o}C until required.

Membrane Preparation and Radioligand Binding Assays. About 48 h after transfection, HEK 293 cells were washed twice with of cold phosphate buffer saline (PBS), harvested and homogenized in binding buffer (25mM HEPES, pH 7.4, 5 $Mgcl_2$, 1mM EDTA) using Polytron tissue homogenizer. Cells membranes were colleted by centrifugation at 20000xg for 15 min

and homogenized as above. After centrifugation at 40000xg for 20 min at 4°C, the final pellet was resuspended in binding buffer, and membranes were either used immediately or frozen in liquid nitrogen and stored at -80 °C until required. Protein concentration was determined by using the Bradford protein assay kit (Bio-Rad, USA). In order to determine the affinity of NMS for each sample, membranes were incubated with different concentrations of [^{3}H]-NMS (ranging from 12.5 pM to 3.2 nM) in 5 mM phosphate buffer at 25 °C for 60 min. The incubations were stopped by filtration through Whatman GF/B filters and washed extensively with ice-cold 40 mM PBS prior to scintillation counting. Non-specific binding was determined in the presence of 10 μM atropine. Agonist binding potencies were determined in competition with [^{3}H]-NMS. Membranes were incubated with 0.1 nm [^{3}H]-NMS and concentration from 10pM to 10 mM of carbachol in 5 mM phosphate buffer at 25 °C for 3h. The assays were terminated by filtration and counting, as described above.

[^{35}S]-GTPγS binding assay. HEK-293 cell membranes were diluted in an ice-cold buffer containing 10 mM HEPES and 0.1 mM EDTA, 5mM deoxycholate (pH 7.4) as previously described, pelleted and resuspended in a binding buffer consisting of 10 mM HEPES, 10 mM $MgCl_2$, and 100 mM NaCl (pH 7.4) at a final protein concentration of 125 μg/ml. Incubations were conducted in a final assay volume of 1 ml (125μg total protein) for 1 h at 30°C in the presence of 1 μM GDP and 0.3 nM [^{35}S]-GTPγS (1,202 Ci/mmol) and the appropriate ligand concentration (carbachol from 1 nM to 1 mM). The reaction was stopped by addition of 5 ml of ice-cold buffer containing HEPES/NaOH (10 mM) (pH 7.4) and $MgCl_2$ (1 mM), immediately followed by rapid filtration through glass fibre filters (Whatman GF/C) presoaked in the same buffer. The filters were washed twice with 5 ml of buffer and the radioactivity was measured by scintillation counting. Nonspecific binding was determined in the presence of 10 μM GTPγS. Assays were performed in triplicate.

PLD and PLC assay activities. PLD and PLC activities were monitored as production of [^{3}H] phosphatidylbutanol and [^{3}H] inositol phosphate respectively, as described previously (): in brief, tranfected COS-7 cells in 12-well plates were labelled by incubation for 18-24 h with [^{3}H] palmitate (2μCi/well) and myo-[^{3}H] inositol (3μCi/ml) in serum-free medium. Agonist responses (100 μM carbachol) were measured usually over 30-40 min in the presence of 10 mM liCl before cells were lysed in ice-cold 10mM formic acid and [^{3}H] inositol phosphate was separated on Dowex AG1-X8 ion exchange columns (Bio-Rad, USA). For [^{3}H] phosphatidylbutanol ([^{3}H] Ptdbut) production, agonist response (100 μM carbachol) was measured usually over 30-40 min in the presence of 30 mMbutan-1-ol. Assays were terminated, phospholip was extracted into chloroform/methanol, and [^{3}H] Ptdbut was separated on Whatman LK5D thin layer chromatography plates (Whatman, UK). For desensitization experiments, in both assays, prelabelled cells were treated with 2 min of agonist in HBSS and then washed twice with HBS. Then, agonist was added in the conditions described above for the indicated periods of time. Inhibitory agents and the butan-1-ol were added 30 min prior to and immediately before agonist, respectively. For assays in acute permeabilized cells, prelabelled COS-7 cells were primed with agonist (100 μM carbachol), 10 min at 37 oC or control before intracellular buffer was added (2mM MgATP, 10 μM NAD, 8 μM digitonin, and 30 mM butan-1-ol) as well as the test agents (BFA, C3 exoenzyme, GPTγS, $GPPCH_2P$ or F^-(NaF in the

presence of 30 μM $BeCl_2$)). All data were colleted from scintillation counted and are expressed as means ± S.E from between 4 and 6 separate determinations.

MAPK assay. Co-transfected HEK-293 cells expressing M_3 receptor subtypes were grown to 80% confluence and rendered quiescent by serum starvation overnight prior to MAPK phosphorylation assay; an additional 2h incubation in fresh serum-free medium was performed to minimize basal activity. Cells were subsequently stimulated by the addition of a medium with or without the muscarinic agonist carbachol. Rapid rinsing with ice-cold PBS completed the stimulation, and cell lysis was performed during 10 min by the addition of 500 μl ice-cold lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1% TritonX-100, protease and phosphatase inhibitor cocktail). The cellular debris was removed by centrifugation at 13, 000g for 5 min at 4°C, and the total protein content was measured using BCA Protein Assay Reagent (Pierce, USA). Aliquots corresponding to 5 μg of protein were mixed with sodium dodecyl sulfate (SDS) loading buffer, applied to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot. Extracellular signal regulated kinase ½ (ERK ½) and protein kinase B (AKT) activation were assayed by incubating PVDF blots with a mouse anti-phospho-ERK ½ antibody (Sigma-Aldrich, Germany) and phospho-AKT antibody (New England Biolabs, UK) respectively. Control blots were also run in parallel and probed with rabbit anti-ERK ½ antibody (Sigma-Aldrich, Germany) and total-AKT antibody that recognized both unphosphorylated and phosphorylated forms. The immunoreactive bands were visualized using horseradish peroxidase-linked secondary anti-mouse and anti-rabbit antibodies (Dako, USA) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA).

Immunoprecipitation. In order to immunoprecipitate the wild-type M_3 muscarinic receptor with its associated proteins, plasmids encoding the HA-M_3 receptor and the Gαq, Gα12, Gβ, Gγ, β-arrestin-GFP, Flag-ARF-1 or Flag-ARF-6 expressing vectors were transiently co-transfected into HEK-293 cells. 48 h later, cells were serum-deprived for 4 h., then exposed to carbachol (20 μM) -or no drug- for 10 min and washed once with PBS before being solubilized in immunoprecipitation buffer (PBS, pH 7.5, 1% CHAPS, 0.75% sodium deoxycholate, protease and phosphatase inhibitor cocktail). Carbachol was re-added where appropriate. Extracts were centrifuged at 15, 000g for 15 min at 4°C to remove particulate material and precleared with Protein G/A Sepharose (Santa Cruz Biotechnology, USA). After centrifugation, the supernatant was transferred to tubes containing either rabbit monoclonal anti-HA (Santa Cruz Biotechnology, USA) or nonimmune mouse IgG (Sigma-Aldrich, Germany) with the required amount of Protein G/A Sepharose suspension, before rolling at 4°C overnight. Beads were collected by centrifugation and washed twice with immunoprecipitation buffer before 35 μl of 2x Laemmli buffer (2% SDS, 5% mercaptoethanol, 20 Mm Tris, pH 7.4) was added per ml of original supernatant. After SDS-PAGE and electroblotting, Western blot was carried out on the sample and the original supernatants to detect immunoprecipitated proteins and monitor input levels. The primary antibodies used were an anti-Flag M_5 mouse monoclonal antibody (Sigma-Aldrich, Germany) and a rabbit polyclonal anti-GFP (gift from Y. Mesa; UB, Spain), followed by preabsorbed secondary antibodies to horseradish peroxidase (Santa Cruz Biotechnology, USA). Bands were visualized by ECL (Kodak, USA) and then measured by quantitative densitometry.

Data analysis. All binding data were analysed using the commercial program GraphPad PRISM 4.0 (GraphPad Software, USA). In the [^{35}S]-GTPγS binding assay, basal binding was defined as [^{35}S]-GTPγS binding in the absence of agonist. For each agonist concentration, the percentage of binding over basal was calculated to determine the agonist-stimulated [^{35}S]-GTPγS binding. Data were fit to a sigmoidal dose-response curve. For statistical evaluation of the biochemical data, unless otherwise specified, one-way analysis of variance (ANOVA) was used. Group differences after significant ANOVAs were measured by post hoc Bonferroni´s Multiple Comparison testing.

CHAPTER 6

Molecular aspect of protein-protein interaction

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6.1 Muscarinic acetylcholine receptors interacting proteins

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ABSTRACT

Muscarinic acetylcholine receptors (mAChRs) comprise a large family of G protein-coupled receptors (GPCR) involved in regulating the activity of many important functions of the central and peripheral nervous system. In order to relay such a diversity of physiological outcomes, mAChRs not only interact with heterotrimeric G proteins to activate or inhibit classical downstream effectors, but also with a number of accessory proteins that physically interact with their intracellular domains. Such proteins are diverse in nature, involving scaffold molecules, ion channels, and enzymatic activities, as well as other GPCRs, thus leading to receptor homo- or heterodimerization and playing a critical role in their distribution in specific areas of the cells, their trafficking, and the fine modulation of their signal transduction properties. Extensive characterization of proteins interacting with some mAChRs by means of proteomic approaches using pull-down show that receptor-interacting proteins and the function of their association with muscarinic receptors differs from one receptor subtype to another. The specificity of receptor association with such proteins may be largely determined by the occurrence of protein-interacting specific motifs, posttranslational modifications and the differential tissue distribution of the GPCR-interacting proteins. Such receptor interactions can explain key physiological differences among muscarinic subtypes as well as puzzled functional antagonisms and synergisms characteristic of certain muscarinic receptors.

INTRODUCTION

Muscarinic acetylcholine receptors (mAChRs) have been shown to mediate various functions in the central and peripheral nervous systems (CNS, PNS) [451]. These include modulation of exocrine glandular secretion, vasodilatation and smooth muscle contraction [452], cell proliferation or survival [453], neural development and synaptic plasticity [454]. Muscarinic receptors are activated by both endogenously produced acetylcholine and exogenously administered muscarinic compounds. Pharmacological, anatomical and molecular studies have demonstrated the existence of five muscarinic receptor subtypes, denoted as muscarinic M_1, M_2, M_3, M_4 and M_5, which belong to class I family of heptahelical, transmembrane G-protein coupled receptors (GPCRs) [183].

Each receptor subtypes are characterized by a distinct selectivity for heterotrimeric G protein coupling. Thus, M_1, M_3 and M_5 are coupled to $G_{q/11}$ proteins and stimulate phospholipase C activity, resulting in the generation of the second messengers inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG), the mobilization of intracellular Ca2+ and the activation of protein kinase C (PKC) [184]. On the other hand, M_2 and M_4 are coupled to $G_{i/0}$ proteins, which results in the inhibition of adenilate cyclase, as well as prolonging potassium channel, non-selective cation channel, and transient receptor potential channel opening [455,456]. By means of this differential set of G protein partners, muscarinic receptors can initiate distinct signalling pathways within a same cell in order to trigger diverse, even opposed, functional outcomes in response to the same stimuli. It has been proven as well that mAChRs regulate a baste network of signalling intermediates, including small GTPase Rho, phospholipase D, phosphoinositide-3 kinase, non-receptor kinases and mitogen-activated protein kinases [322,457,458].

Although the first proteins found to have functional interactions with muscarinic receptors were, of course, G proteins, an increasing amount of evidence in the field suggests that this simplistic model defined as "one receptor -one G protein -one effector no longer exists. A great number of proteins have been identified as interacting with mAChRs, including GPCRs, kinases, and scaffolding proteins such as arrestin [459]. Determining in part the signalling efficiency/specificity for mAChRs [64]. Thus, receptors are now considered as complex signalling units, or signalosomes, that dynamically couple to multiple G proteins or other molecular entities or scaffold proteins in a temporally and spatially regulated manner, and even can form homodimers or heterodimers with distinct GPCRs or other non-GPCR membrane receptors, resulting in pharmacologically and functionally distinct receptor populations [79].

In this review, effort will be made to discuss novel muscarinic receptor interacting partners that link the receptors to alternative signalling pathways beyond G proteins. We will put emphasis on explaining how muscarinic receptors regulate signal transduction pathways mediated by these proteins, including receptor dimerization. Such knowledge will allow us to address some fundamental questions concerning the importance of molecular mechanisms hidden behind the pharmacology properties for each receptor subtype. It may also uncover factors that modulate synaptic transmission and provide insights into a variety of physiologic and pathophysiologic processes.

Physiological roles of muscarinic receptors

Uncovering the physiological roles of the distinct muscarinic subtypes has been possible thanks to the gradual development of more selective receptor agonists and blockers, and with the detailed phenotypic characterization of mice knockout [460]. Different experimental approaches, including immunohistochemical and mRNA hybridization studies, show that mAChRs are present in virtually all organs, tissues, or cell types

(Table 6.1.1) [461-463]. Muscarinic acetylcholine receptors are expressed pre- and postsynaptically and have discrete distribution throughout the central and peripheral nervous systems [464].

Peripheral mAChRs mediate the classical muscarinic actions of acetylcholine on organs or tissues that are innervated by parasympathetic nerves. Muscarinic receptors are found in visceral smooth muscle, in cardiac muscle, in secretory glands, and in the endothelial cells of the vasculature [280,465]. Except for endothelial cells, each of these sites receives cholinergic innervation. The most common responses mediated by these peripheral mAChRs include reduction of heart rate, stimulation of exocrine glandular secretion, vasodilatation and smooth muscle contraction. Central mAChRs are involved in regulating an extraordinarily large number of cognitive, behavioural, sensory, motor, and autonomic functions [460].

Table 6.1. 1. *Signal transduction and specificities of muscarinic receptor subtypes in neural and non-neuronal system.*

Subtype	G proteins	Organ and Tissue	Functional Rol (es) or Response	Refs.
M_1	$G\alpha_{q/11}$	Autonomic ganglion including myenteric plexus, cerebral cortex, canine saphenous vein, Lymphocyte and keratinocyte	Depolarization, contraction, increased gastric acid secretion, IL-2 production, proliferation	460,466-470
M_2	$G\alpha_{i/0}$	Heart, atrium, ileum smooth muscle, sinu-atrial node	Reduce Ach release, contractile force and rate	471-476
M_3	$G\alpha_{q/11}$	Urinary bladder, iris circular muscle, blood vessels-endothelium, Smooth muscle and salivary glands, pancreatic β cells, Lymphocyte and keratinocyte, colon and thyroid cancer cells	Contraction, vasodilatation via release of NO, inhibition of apoptosis, inhibition of cell migration, proliferation	477-480
M_4	$G\alpha_{i/0}$	Corpus striatum, Uterus (guinea pig), lung, muscle (rabbit), keratinocyte	Contraction, NO-dependent relaxation, cell migration	460,469
M_5	$G\alpha_{q/11}$	Substantia nigra pars compacta, ventral tegmental area, brain microvasculature	Growth and proliferation	481,482

There is also evidence that mAChRs are expressed in several non-innervated tissues [483]. They are expressed in primary and metastasis tumour cells which act as the inductors of transformation, growth and proliferation [484,485]. This has been described in vascular

endothelium, keratinocytes, lymphocytes and placenta [486-488], whereas acetylcholine acts, via mAChRs activation, in an autocrine fashion [489].

Structural basis of interactions

A critical question in GPCRs signalling is what determines the specificity of signal transduction processes. One of the most important steps in answering this question is the analysis of control signal specificity by a multiprotein complex formation, in contrast with the classical model, one receptor -one G protein -one effector [490]. Extended observations related to GPCR signalling mechanisms have demonstrated that the cytoplasmic face of these receptors and, particularly, the third intracellular loop and the C-terminal tail, are critical in mediating the signal transfer to G proteins and play specific roles in mediating protein-protein interactions [491].

Sequence analysis of the cytoplasmic hydrophilic domains of the five muscarinic receptor subtypes reveals high sequence similarity in the case of the first and second intracellular loops, which comprise about 90% of amino acid identity. Nevertheless, fewer similarities are observed when analysing the third intercellular loop, which has a sequence similarity near 50% and a high degree of variability in length or size, depending on the receptor subtype. A deeper analysis of the potential positive and negative charge distribution showed a great number of cluster formations at the level of the third intracellular loop and the C terminal tail (Figure 6.1.1)

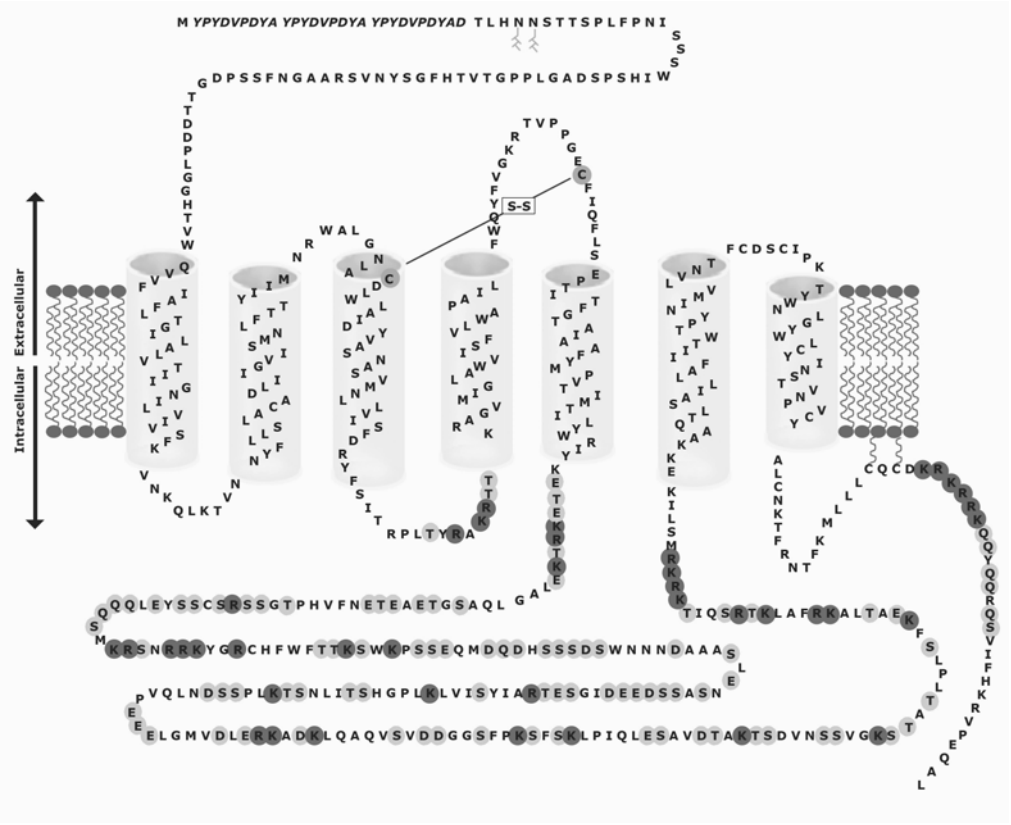

Figure 6.1.1 *Representation of ionisable amino acid on the cytoplasmic face of the M_3 muscarinic receptor 2D-topology model (basic (dark circles) and acidic (light circles) residues).*

Site-directed mutagenesis studies showed that the N-terminal portion of the third intracellular loop of muscarinic and other G protein-coupled receptors represents one of the most fundamental structural determinants for direct G protein interaction [349,492]. Insertion mutagenesis studies with the rat M_3 muscarinic receptor suggested that this region forms an amphiphilic alpha helix and that the hydrophobic side of this helix represents an important G protein recognition surface. Further analysis of this receptor segment showed that Tyr254 located at the N-terminus of the 3ILoop and specific residues located in the second intracellular loop of the M_3 muscarinic receptor play a key role in muscarinic receptor-induced Gq activation [493].

Other experimental strategies involving the coexpression of hybrid M_2/M_3 muscarinic receptors with hybrid G alpha-subunits correlated with the proposed model [338]. Using this approach, it was demonstrated that the C-terminus of G protein alpha i/o-subunits is recognized by a short sequence element in the M_2 muscarinic receptor ("VTIL") which is located at the junction between the sixth transmembrane domain (TM VI) and the 3ILoop. This interaction is a critical part of determining coupling selectivity and triggering G protein activation.

On the other hand, expression of a peptide encompassing the entire 3ILoop of muscarinic receptors abrogated the interaction of receptors with G proteins and disrupted signalling of other GPCRs that couple to the same G protein population [494]. This suggests that the 3ILoop of muscarinic receptors can be considered an inhibitor that targets and interacts directly with Gq/11 proteins. In addition, this domain also serves for the direct binding of Gβγ complexes, calmodulin and RGS protein [495,496].

The C-terminal domain of GPCRs which has been termed “the magic tail” is recognized as one of the most important domains for the regulation of GPCR function [497]. This region is also implicated in post-translation modifications and is crucial for receptor phosphorylation, desensitization and trafficking in some GPCRs [498]. The sequence, the length, and the different recognition motifs involved in protein-protein interactions are specific to each GPCR C-terminus. Despite the number of protein interactions associated with the C terminal tail of many GPCRs (as in the case of muscarinic receptors), as so far as we know only a few groups of putative interactions have been reported [64]. The predicted C-terminal tails of the muscarinic receptors is a highly conserved domain both in sequence similarity and length. This domain contains a conserved region following the seventh transmembrane domain composed of a cluster of basic amino acids that confer anti-apoptotic properties to the receptor, but molecular details remain known [348].

Figure 6.1.1 shows examples of sequence motifs associated with protein interaction in the muscarinic receptor family. The ability of mAChRs to interact with different proteins probably begins at the sequence motif level of the cytoplasmic face, where a great number of charged amino acid clusters have been observed and a quantity of post-

transductional modification takes place. As mentioned above, special interest is given to the 3Iloop, which represents a docking site for the protein-protein interaction of many receptor subtypes.

Transmembrane muscarinic receptor interacting proteins

Homo and Heterodimerization

It is well established that a variety of cell surface receptors interact with each other to form dimers, and that this is essential for their activation [499]. Although the existence of muscarinic receptor dimers was predicted from early pharmacological and biochemical analysis, solid evidence supporting dimerization has come within the past few years using different experimental approaches such as co-immunoprecipitation of distinctly tagged versions of the receptor, light resonance energy transfer techniques FRET and BRET (fluorescence and bioluminescence resonance energy transfer, respectively), as well as analysis of cooperative ligand binding [500,501]. Pioneering studies by Wess and colleagues have demonstrated that in a heterologous expression system of COS-7 cells, M_3 receptor are able to form functional homodimers [502,503]. Purification and co-immunoprecipitation experiments in Sf9 cells also showed oligomers formation by muscarinic M_2 acetylcholine receptors [504]. In addition, as different subtypes of muscarinic receptors are coexpressed in the same cell type in several tissues, for example, M_1 and M_2 receptors in neurons and M_2 and M_3 receptors in smooth muscle cells, it has been proposed that mAChR subtypes may heterodimerize [505]. Maggio and Wess showed that M_3 dimerization/oligomerization is receptor subtype specific and occurs in both transfected cells and native tissues [506]. Using bioluminescence resonance energy transfer, it has been demonstrated that M_1, M_2, and M_3 mAChR can form constitutive homo- and heterodimers in living HEK 293 cells. And that the cell receptor population for theses single subtypes is predominantly composed of high affinity homodimers [507] (Figure 6.1.2). In the same report, saturation curve analysis of cells expressing two receptor subtypes demonstrates the existence of high affinity M_1/M_2, M_2/M_3, and M_1/M_3 mAChR heterodimers, although the relative affinity values were slightly lower than those for mAChR homodimers.

A relatively controversial issue concerning GPCR oligomerization is the effect of ligand binding on the formation of oligomers. Although for many GPCRs, the role of agonists in dimerization for the muscarinic receptor M_3 has been described, the activation of thse receptors by the agonist produces no change or effect on the dimerization process. [508] Similar behaviour has been described for the M_2 receptor subtype, where dimerization/oligomerization of the M_2 receptor is independent of the presence of the agonist carbachol or the inverse antagonist atropine [509]. In addition, FRET and BRET experiments showed that short-term agonist treatment did not modify the oligomeric status of homo- and heterodimers for M_1/M_2 and M_3 subtypes. Nevertheless, when

expressed in JEG-3 cells, the M_2 receptor exhibits much higher susceptibility than the M_3 receptor to agonist-induced down-regulation. Coexpression of M_3 mAChR with increasing amounts of the M_2 subtype in JEG-3 cells resulted in an increased agonist-induced down-regulation of M_3, suggesting a novel role of heterodimerization in the mechanism of mAChR long-term regulation [510]. Whereas in some cases agonist induced increases were observed in the level of dimers, in others no change at the level of receptor dimers has been observed.

At present, it has been firmly established that there are some classes of GPCRs where oligomerization already occurs [511]. From the evidence gathered so far, it appears that class A hetero-oligomers are formed before or during translocation form endoplasmic reticulum/Golgi to the plasme membrane [512]. However, measuring BRET or FRET signals in cell fractions collected from sucrose gradient, used to separate cell surface proteins from intracellular material, revealed that homo-oligomers could already be detected in light weight fractions for the β_2-AR [513], α_{1A}-AR [514] as well as hetero-oligomers between D_1R and D_2R [515]. With respect to the muscarinic receptor family, there have to this day been no reports analyzing the oligomerization of these receptors from a biogenesis perspective.

In principle, dimerization/oligomerization of mAChRs can result from either noncovalent (ionic or hydrophobic) or covalent association of the receptor protomers. In the M_3 receptor, a cysteine pair (Cys140 and Cys220) is thought to be critically involved in the formation of covalently linked M_3 receptor [516]. These two cysteine residues are highly conserved within GPCRs of the rhodopsin family, and are able to form an intramolecular disulphide bond, thus covalently linking the first and second extracellular loops. But they may also participate in the formation of intermolecular disulfide bonds. Apart from covalent interactions mediated by cysteine residues, it has been demonstrated that the 3ILoop actively participates in the heterodimerization of M_2 and M_3 muscarinic receptors. Deleting 196 amino acids from the 3ILoop of short chimeric alpha2 adrenergic/M_3 muscarinic receptors resulted in the loss of intermolecular interaction compared with receptors that kept the entire loop [517] (Figure 6.1.2).

At the moment, the functional implications and biological significance of (homo and hetero) dimerization of mAChRs is still poorly understood. A large body of evidence has progressively substantiated the role of receptor homodimerization and heterodimerization in different functional aspects of GPCRs [281]. Dimerization can be required for receptor maturation and correct transport from the endoplasmic reticulum to the plasma membrane. Receptor dimerization can also influence ligand recognition by each of the constituent protomers [518]. Thus, ligand binding to one protomers might increase or decrease binding affinity to the other protomers [519]. As ligand-binding sites for muscarinic receptors are buried in a hydrophobic pocket formed by transmembrane

domains, it is possible that interactions between protomerss through such domains might rearrange ligand-binding sites (domain swapping) and modify ligand selectivity and affinity [520].

Ionic channels interaction

Interaction amongst members of divergent families of receptors presents an additional layer of complexity when considering the mechanisms of receptor cross talk in cells. Recent reports provide compelling biochemical and functional information about direct interaction between GPCRs and receptor channels [521,522]. However, modulation of ion channels by mAChR agonists appears increasingly complex. It was observed that K(+) channels may be activated by M_2 and M_4 receptor subtypes after agonist stimulation, although in the rat superior cervical ganglion, topographical constraint effects appear to be limited the effect to the M_2 mAChR [523].

G-protein-gated inwardly rectifying K^+ channels (GIRK1) were first identified in atrial myocytes where they are activated by acetylcholine at muscarinic M_2 receptors [524]. It was subsequently shown that this activation was membrane-delimited, mimicked by non-hydrolysable GTP analogues, and involved a direct interaction with the Gβγ dimmer, not the Gα subunit [525,526]. Using the techniques two-hybrid and pull-down assay, it was demonstrated that Gβγ interacts directly with the protein GIRK1, an interaction which takes place at the C Terminal. [527] In parallel, it has been observed that Regulators of G protein Signalling proteins (RGSs) accelerate the activation and deactivation kinetics of GIRK channels [528]. Co-expression of RGS4 accelerated all the PTX-insensitive $G\alpha_{i/0}$-coupled GIRK currents to a similar extent. In contrast to RGS4, RGS7 selectively accelerated $G\alpha_0$-coupled GIRK currents. Co-expression of Gbetagamma, in addition to enhancing the kinetic effects of RGS7, caused a significant reduction in steady-state GIRK currents indicating RGS7-Gbetagamma complexes disrupt $G\alpha_0$ coupling. Altogether these results provide further evidence for a GPCR-Galphabetagamma-GIRK signalling complex that is revealed by the modulatory effects of RGS proteins on GIRK channel gating. These functional experiments demonstrate that the formation of this signalling complex is markedly dependent on the concentration and composition of G protein-RGS complexes.

Other example of complex regulation between muscarinic receptors and receptor channels is the activation of endogenous transient receptor potential-canonical subtype 6 channels (TRPC6) in neuronal PC12D cells [529] by the M_1 muscarinic subtype. Activation of TRPC6 channels correlates with the formation of a multiprotein complex containing M_1 mAChRs, TRPC6 channels, and protein kinase C (PKC). Formation of the M_1 mAChR-TRPC6-PKC complex is transient, with the highest levels reached approximately 2 min after stimulation of M_1 mAChRs PKC in the complex phosphorylates TRPC6 on a conserved serine residue in the carboxyl-terminal domain (S768 in the TRPC6A isoform and S714 in the TRPC6B isoform). The immunophilin

FKBP12, the phosphatase calcineurin, and Ca2+-binding protein calmodulin are also recruited to the M_1 mAChR-TRPC6-PKC complex following activation of M_1 mAChRs and remain stably associated with the TRPC6 channels after M_1 mAChRs and PKC have disassociated. The activated TRPC6 channels form the center of a dynamic multiprotein complex that includes PKC and calcineurin, which respectively phosphorylate and dephosphorylate the channels. Phosphorylation of the TRPC6 channels by PKC is required for the binding of FKBP12, which in turn is required for the binding of calcineurin and calmodulin. Subsequent dephosphorylation of the channels by calcineurin is required for the disassociation of M_1 mAChRs [530,531].

But in all of these examples, many of the details related to the pathway, second messengers, or interacting protein remain unclear. In none of these cases has a direct interaction between divergent receptors been observed; only a functional modulation mediated by second messengers or interacting proteins, as is the case with betagamma dimmer and the RGS protein[532,533]. As far as we know, only activation by the muscarinic M_3 receptor of the Ca2+ release channels, present in the entoplasmic reticulum membrane, suggests that there is an underlying a mechanism in which receptor-channel coupling is independent of intracellular messengers [534].

Scaffolding and soluble interacting proteins with intracellular loops

3ILoop: the predominant target of muscarinic receptor interacting proteins

Nowadays, it is well established that the third intracellular loop of muscarinic receptors is not only one of the main target domains for interaction with G proteins, but also a binding site for direct interaction with different proteins. The physical interaction of the third intracellular loop with a large number of proteins, in some cases, forms a multiprotein complex bypassing the need to couple with the heterotrimeric G-proteins for signalling. One of theses cases is the activation of small G proteins by a large number of the GPCR vie Gα subunit activation or by direct interaction with the receptor [535,536].

The muscarinic-mediated regulation of small G-proteins has been viewed typically as a downstream consequence of heterotrimeric G-protein activation, particularly $G_{\alpha q}$, $G_{\alpha 11}$ or $G_{\alpha 12}$ activation, as ocurrs in many other GPCR [537]. $G_{12/13}$-mediated RhoA activation involves direct interactions with RhoGEF proteins (RhoGEF, LARG and p115-RhoGEF) [538-540]. In a similar manner, $G\alpha_{q/11}$-coupled receptors can couple with activation of Rho, via direct association of $G_{q/11}$ with Rho GEF [541,542], albeit with less efficacy when compared with $G\alpha_{12}/G\alpha_{13}$ [543]. However, there are evidences to show direct activation of small G-proteins by mAChRs. One of the examples, is the activation of the enzyme phospholipase D (PLD) by the small G-proteins Arf and RhoA via M_2 and M_3 muscarinic receptor [322,544,545]. Both Arf and RhoA can be coimmunoprecipitated with the 3Iloop of muscarinic acetylcholine M_3 receptor. Dominant negative constructs

of ARF1/6 and PLD1/2 proved that the characteristic BFA-sensitive PLD activation shown by the M_3 receptor appears to involve ARF1-mediated activation of PLD1, where additional ARF6-mediated component may involve PLD1 or PLD2 [546,547]. Mutation in the conserved NPXXY amino acid sequence in their transmembrane VII domain prevent the association of these receptors with ArF or Rho and also signalling to PLD [548]. All the experimental results lead to the conclusion of a complex formation, stabilized by exposition of a docking site for the Arf and Rho protein between the carboxyl terminal of the 3ILoop and the NPXXY conserved motif localized in the transmembrane domain VII.

However, the muscarinic receptor coupled to Gq can activate RhoA, it is generally proposed that Gi coupled receptors activate Rac1, but not RhoA, a process involving Gbetagamma-dimers and phosphatidylinositol 3-kinase (PI3K) [549]. It has been proposed that this selectivity can be redirected by the Regulatory G-protein signalling 3 (RGS3L), which can function as a molecular switch, changing Gi-coupled receptor via Gbetagamma-dimers and PI3K from Rac1 to RhoA activation [550].

In addition, the binding of the SET protein to M_2 and M_3 mAChRs is another case that illustrates that muscarinic receptor-mediated signalling is triggered by the 3ILoop and does not always depend on G-protein coupling. The SET protein, also called TAF-1 for template activating factor I, was first described as part of the SET-CAN fusion gene in patients with acute undifferentiated leukemia [551]. The cellular role of SET is not clearly defined, but many functions have been described for this protein. It is involved in the control of gene transcription as a component of the INHAT complex (inhibitor of histone acetyltransferase), and is also considered an inhibitor of PP2A, which is involved in cell cycle progression and GPCR traffiking and regulation [552,553]. Co-immunoprecipitation experiments and mass spectrometry demotrated that SET interacts directly with the 3Iloops of M_2 and M_3 muscarinic receptor, and that for the latter, the interaction involves the last 17 amino acids of the 3Iloop (I474-Q490) [554] (Figure 6.1.3). Using siRNA knockdown, Violaine et al. demonstrated that SET acts to provide a brake on M_3 muscarinic signalling. It has been proposed that SET interaction could impede G-protein coupling to the receptor. The last 17 amino acids found to interact with SET in the C-terminus of the third intracelullar loop are also involved in G-protein coupling and the activation of various GPCR [555,556]. A second possibility is that SET, by virtue of its ability to inhibit PP2A, may increase the basal level of receptor phosphorylation and can regulate the magnitude or duration of agonist-induced receptor phosphrylation. Theses changes in the phosphorylation status may play an important role in the maximal response observed following receptor activation, as well as in receptor trafficking [557,558]. The isolation of SET as a receptor's binding partner, as well as the functional characterization of this interaction, represents a totally unappreciated mechanism for regulation of muscarinic receptor signalling and it capacities.

Likewise, using the 3ILoop as bait, Bernstein et al. examined whether GPCRs selectively recruit RGS proteins to modulate linked G protein signalling, and demonstrated a direct interaction between RGS2 and the 3Iloop of the M_1 muscarinic receptor [559]. Based on the fact that closely related RGS16 does not bind to the M_1 receptor, they postulated that GRS2-M_1 interacts in a selective way. Probably, RGS2 strongly inhibits M_1 muscarinic-mediated phosphoinositide hydrolysis in the cell membrane by acting as an effector's antagonist. A model has been postulated in which the 3Iloop of mAChRs, and the larger GPCR family, selectively recruit specific RGS protein(s) via their N-termininal to regulate the linked G protein, a critical step in the activation/ desensitization mechanism [560].

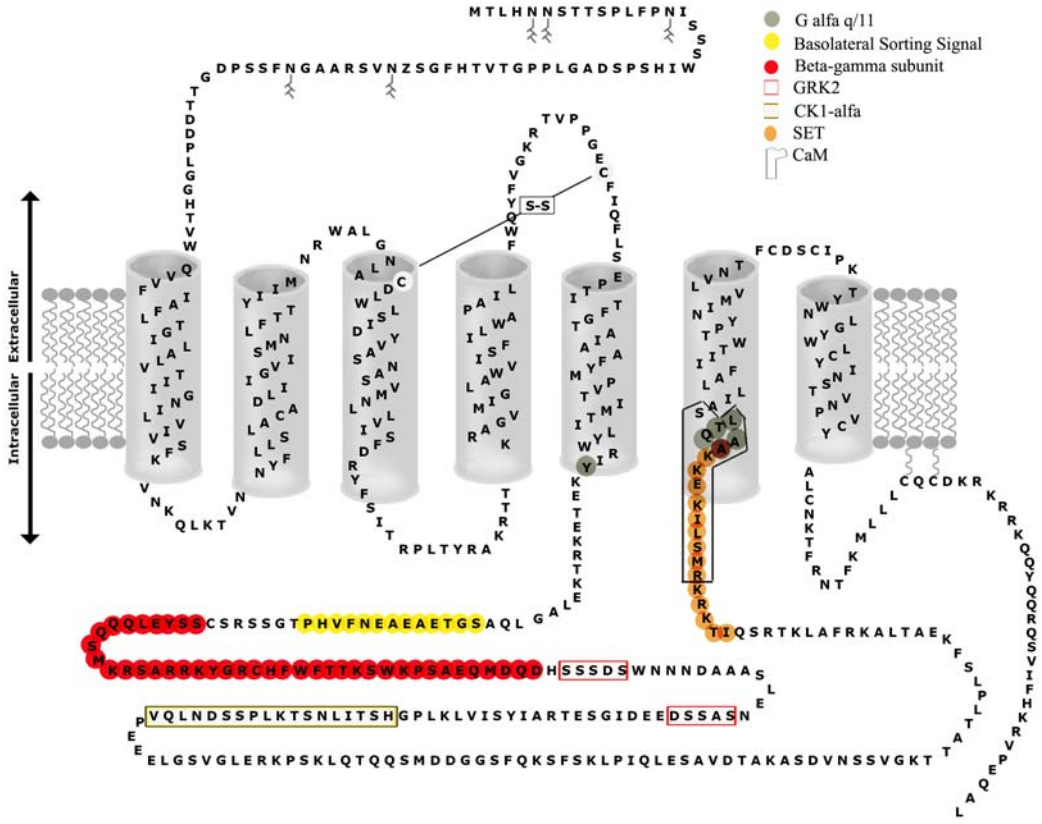

Figure 6.1.3. *Molecular dissection of sequence motifs those are associated with protein interaction in M_3 muscarinic receptor. A two-dimensional topology, with the extracellular space at the top and the intracellular space at the bottom, of the M_3 human muscarinic receptor sequence are represented. The residues highlighted and described in the legend are sites of recognition by protein interacting with the intracellular face of the receptor. Note that in the case of the N-termine of the 3Iloop coincides more than one recognition site by protein interacting. This particularity can be the response to many question related with the desensitization and regulation process of muscarinic receptor activation and signal transduction mechanism. Also, the consensus motif that can be phosphorylated are specifically and different for each protein kinase.*

McClatchy et al found a novel interaction between the M_4 mAChR and elongation factor 1A2 (eEF1A2) both *in vitro* and *in vivo* [561]. eEF1A is a GTP-binding protein that

is essential in protein synthesis mediating the binding of the aminoacyl-tRNA to the acceptor site of the ribosome [562,563]. It is also only expressed in skeletal muscle, heart muscle, and brain in adult mammals [564,565]. The M_4, via the 3Iloop, can modulate translation process as well as protein synthesis through its interaction with eEF1A2.
In the last years it has been demonstrated that CaM can interact with a motif located in the C-termini juxtamembrane regions of the M_1 and M_3 muscarinic receptor 3ILoop[566] (Figure 6.1.3). Binding of CaM to muscarinic receptors was found to be antagonistic with PKC-mediated receptor phosphorylation, suggesting a role in receptor desensitization [229]. This interaction may also be critical for muscarinic receptor-mediated activation of the ERK signalling pathway, which is dependent on Ca2+/CaM and involves agonist-induced receptor internalization and arrestins [567]. In this regard, a recent study has demonstrated a direct interaction of all arrestin subtypes with CaM bound to Ca^{2+} [568]. The CaM binding site on arrestins overlaps with the binding site for GPCRs, indicating that receptors and CaM should compete for interacting with arrestins. This also suggests that arrestin-CaM interaction may regulate the availability of both proteins for interacting with receptor partners. The specific implication of this interaction on elucidates receptor-mediated induction of ERK1, 2 signalling pathway and internalization remains to be determined.

The C terminal tail

Although there is increasing evidence that the "magic tail" of GPCRs constitutes the main anchoring domain for soluble interacting proteins (more than 50 GIPs interacting with GPCR C-termini have been identified [569]), in the case of muscarinic receptor family only the third intracellular loop has been reported as critical domain capable of binding adaptor and scaffold proteins. Nevertheless, a few examples have been reported with a functional role in receptor regulation and signalling[570].
Many proteins proposed to be involved in post-endocytotic sorting of receptors were probed for interactions with the C terminal tail of muscarinic receptors using GST pull-down assays [571], which routinely have been used to confirm protein interactions identified by co-immunoprecipitation and yeast two-hybrid screening. Interaction with *N*-ethylmaleimide-sensitive factor (NSF) was identified in all members of the muscarinic receptor family, which has been suggested to be responsible for the recycling of the β_2-adrenergic receptor [572]. In contrast, sorting nexin 1 (SNX1), which was originally demonstrated to be required for the lysosomal sorting of the epidermal growth factor receptor, was only suggested to be involved in the lysosomal sorting of M_1, M_4 and M_5 subtypes [573]. Another protein called G protein-coupled receptor-associated sorting protein (GASP) was recently suggested to be involved in the preferential lysosomal sorting of the δ-opioid receptor and confirmed to bind to all subtypes of muscarinic receptors [574,575].

Despite the small role of the C terminal tail of muscarinic receptor in the recruitment and anchoring of adaptor and scaffolds protein, it is well established that the C-terminal tail of the M_3 muscarinic receptor is an essential structural element for signalling to the anti-apoptotic pathway [348]. Experimental evidence has shown that the removal of the distal portion of the C-terminal tail results in a receptor that is coupled normally to the $G_{q/11}$/phospholipase C pathway and the mitogen-activated protein kinase pathway, but is unable to couple to the anti-apoptotic pathway [576]. Furthermore, it has been demonstrated that a poly-basic region conserved within the C-terminal tail of the $G_{q/11}$-coupled muscarinic receptor subtypes (CDKRKRRKQ) appears to be the structural determinant of coupling to the anti-apoptotic pathway [348]. Today it is still unclear what protein or proteins are implicated in the connection of the muscarinic receptor with the anti-apoptotic pathway, and which anti-apoptotic signalling is implicated.

Muscarinic receptor interactome

Until a few years ago it was thought that the interactions between GPCRs, G-proteins and their effectors could describe the whole system. However, we now know that it is much more complex, with many other proteins binding to GPCRs and influencing their activity [577]. Indeed, several reports have shown that a particular complex is not necessarily of invariable composition, nor are all its building blocks uniquely associated with that specific complex. One complex may be the result not only of physical interaction between the receptor and the partners' protein, but also of the participation of many non-"direct" associations resulting in the formation of a network that interconnects the receptor with a number of other pathways, determining receptor specificities [578]. This is a phenomenon that has received several names: interactome, signalsomes or receptosome; but essentially describes the complexity of receptor association, signalling and their regulation throughout the formation of multiprotein complexes [578,579].

A well-documented example of networks and their receptor-specificities is the description of the internalization process of muscarinic receptors upon agonist stimlation. For mAChRs and most other GPCRs, agonist-induced phosphorylation of the receptor causes receptor internalization [580]. Alanine substitution mutagenesis of the putative phosphorylation sites in the third cytoplasmic loops of the human M_1, M_2 and M_3 mAChR robustly decreases receptor internalization [581,582]. In addition, three threonine residues in the C-terminus of the human M_3 mAChR (T550, T553, and T554) have been identified as being important in the internalization and desensitization of the receptor in HEK293 cells [583]. The role of the various GRKs in mAChR internalization is critically dependent on mAChR subtype, as well as on the cell type involved. For instance, expression of GRK2 K220W mutant in COS-7 cells increase M_1 mAChR internalization, whereas M_3 mAChR internalization in the same cell type is unaffected

[584]. While expression of GRK2 K220W reduces M_2 mAChR internalization in COS-7 cells [585], it does not change M_2 mAChR internalization in BHK-21 and CHO cells [586,587].

GRK mediated arrestin binding also initiates receptor internalization/sequestration, which occurs via the association of the receptor-arrestin complex with components of clathrin-coated pits [588-590]. Various groups have confirmed the role of β-arrestins and clathrin-coated vesicles in mAChR internalization. Expression of a dominant-negative β-arrestin-1 mutant, which binds with high affinity to clathrin, but is significantly impaired in its ability to interact with phosphorylated GPCRs, significantly suppresses internalization of M_1, M_3, and M_4 mAChRs in HEK293 cells [591,592]. In similar way, expression of another dominant-negative β-arrestin mutant, β-arrestin S412D, which binds to phosphorylated GPCRs with unchanged affinity, but not to clathrin, inhibits M_1 mAChR in HEK293 cells [593]. These results are consistent with the observations that expression of a dominant-negative clathrin mutant strongly inhibits M_1, M_3, and M_4 mAChR internalization [594]. Interestingly, in HEK293 cells, M_2 mAChRs internalize in a β-arrestin- and clathrin-independent manner [595,596]. These experiments suggest that phosphorylated M_2 mAChRs do not readily interact with β-arrestin in HEK293 cells. Indeed, Wu et al. proved that a peptide sequence derived from the 3Iloop of the M_2 mAChR, which contains the GRK2 phosphorylation sites and a putative β-arrestin binding site, does not bind β-arrestins derived from a brain cytosolic fraction. In contrast, a peptide sequence from the thirdintracellular loop of the M_3 mAChR is capable to do so [597]. These observations increase the possibility that other cytosolic adaptor proteins associate with phosphorylated M_2 mAChRs to mediate non-clathrin-mediated internalization of the receptor.

The ability of β-arrestins to promote GPCR internalization does not arise solely as a consequence of their ability to bind clathrin. The binding of arrestins to clathrin is, per se, not capable of triggering internalization. Endocytosis and trafficking are enhanced by the fact that arrestin recruits phosphoinositides, adapter molecule AP-2, another endocytic protein, and intracellular trafficking proteins such as the *N*-ethylmaleimide-sensitive factor (NSF), the ADP-ribosylation factor ARF6, and its exchange factor ARNO [598]. ARF6 regulates vesicle budding by recruiting vesicle-coat proteins including COP1 coatomers. It is possible that mAChR internalization requires the interaction of the receptors with other proteins as well. Wu et al. have shown that the Gβγ subunit is able to bind to a peptide (C289-D329) corresponding to the 3Iloop of the M_3 mAChR. Although M_3 mAChR mutants lacking the Gβγ binding site exhibit unchanged ligand binding properties and functional responsiveness, they are unable to promote receptor internalization [599]. Downstream of the Gβγ binding motif are the putative GRK2 phosphorylation sites (332SSS334 and 349SASS352). It has been proposed that the

marked reduction in internalization of the Gβγ subunit binding-defective M_3 mAChR mutants may be due to impaired phosphorylation of the receptor by GRK2 [600].

The network of proteins that interact with GPCRs may be particularly important for signalling, trafficking and targeting these receptors to particular cellular compartments. Different types of connections to different types of receptors allow both positive and negative cross talk, and this fine-tuning allows the cell to respond differently to ligand binding. The fact that we have so far been unable to model this complexity in assays may explain why we are still unable to reliably design GPCR-targeted drugs that will be free of side effects.

Compartimentalization of muscarinic receptors. The cell context

Much has been written concerning the influence of receptors on cell function. Discussed far less often is the reverse relationship, the influence of the cellular context on receptor function and regulation. Spatial organization of muscarinic receptor complexes into specific membrane domains has been shown to influence or determine signal transdcution [601]. The cell background imposes phenotypic selectivity, by concentrating components of the cellular signalling cascade within microdomains and, in specific cases, determining the spatial and temporal-relationship of interacting proteins with the resulting change in receptor pharmacology properties [602].

The localization of GPCRs and their downstream signalling partners to specific membrane lipid compartments (so-called "lipid rafts" and in particular caveolae) perhaps provides a more widely applicable paradigm for the regulation of receptor signalling [603]. An interesting property of caveolae is that they selectively accumulate a variety of signal components, including GPCRs, G-protein and second messenger-regulated kinases, prompting the proposal that they might regulate signal transduction [604]. In cardiac myocytes, as well as specialized conduction and pacemaker cell, the M_2 muscarinic receptor has been demonstrated to translocate out and into caveolae upon agonist stimulation, resulting in a clear demonstration that multiprotein complex composition and regulation may be tightly linked to their localization in the lipid microdomain [605,606]. Also, in airway smooth muscle cells, caveolae facilitated the muscarinic receptor–medited intracellular Ca^{2+} mobilization and cell contraction [607]. And the loss of caveolin-1 has been associated with disruption of M_3 muscarinic receptor activity in bladder [608]. It has been recently demonstrated that caveolin 2 has an active role in the regulation of M_1 muscarinic receptor endocytosis and trafficking in MDCK epiteail cells. Association of the M1 muscarinic receptor with caveolin 2 inhibits receptor endocytosis through the clathrin-medited pathway or retains the receptor in an intracellular compartment [609]. This intracellular association and the attenuation of receptor trafficking is rescued by the co-expression of caveolin 1.

One of the most striking examples of the effect of cellular embiroment on muscarinic receptosome is observed in striatal neurons. Intraneuronal trafficking of M_4 muscarinic receptors has been observed in vivo-- that is, under regulation of the cholinergic environment [610]. M_4 receptor subcellular compartimentalization and function will be dependant on the phenotype of the cholinoceptive neuron and on its neurochemical environment. On the other hand, acute and chronic acetylcholinesterase inhibition regulates in vivo the localization and quantity of M_2 and M_4 receptor subtypes at the cell surface and in the cytoplasme [611].

The bad face of muscarinic receptor interactions

We are now discussing the active role of the muscarinic receptor interacting protein in receptor regulation and function. We discussed above the importance of assesory protein in receptor specificities and signal efficiency/efficacy. And we determined that improved molecular understanding of the mechanism underlying receptor-protein interaction is likely to provide us with previously unrealized opportunities to achieve greater muscarinic pharmacology specificity and drug development. But receptor interacting protein doesn't always show us the best face of the physiological behaviour of the cells. Evidence accumulated over the last decade gives adequate proof of the existence of circulating antibodies in different autoimmune diseases, which binds to muscarinic receptors, converting the cells in pathologically active cells.

One of these examples is Chagas' disease, caused by a parasite, Trypanosoma cruzi, which is widely distributed in Latin America [612]. Dysautonomias (that is, derangements of sympathetic and parasympathetic nervous system functions), are seen fairly often during the chronic course of Chagas' disease. Many infected patients develop, in the course of the disease, neurogenic cardiomyopathy or digestive damage [613]. Existence of circulating antibodies that bind to beta-adrenergic and M_2 muscarinic receptors has been reported as a basis for Chagas' disease [614]. The anti-M_2 receptor antibodies recognize an epitome on the second extracellular loop. The neurotransmitter receptor-autoantibody interaction triggers in the cells intracellular signal transductions that alter the physiological behavior of the target organs, leading to tissue damage. Moreover, the deposit of autoantibodies on the myocardial receptors, behaving like an agonist, could induce desensitization and/or down regulation of the receptors. This in turn can lead to a progressive blockade of them with sympathetic and parasympathetic denervation [615].

Sjögren syndrome (SS), the second most common autoimmune rheumatic disease, refers to keratoconjunctivitis sicca and xerostomia resulting from immune lymphocytes that infiltrate the lacrimal and salivary glands [616]. However, differential diagnosis remains confusing due to the high prevalence of vague symptoms of dryness, fatigue, and myalgias in the general population. Previous studies have demonstrated that antibodies against muscarinic receptors from exocrine glands correlate with Sjögren

syndrome in the majority of patients [617]. Experimental evidence has demonstrated that serum IgG antibodies-stimulating M_3, M_4, and M_1 cholinoreceptors exerted an increase in COX-2 mRNA without affecting COX-1 mRNA expression and increased PGE (2) production [618]. This results in the chronic inflammation and cognitive dysfunction characteristic of SS patients [619]

Isolated congenital heart block may be associated with autoimmune disorders such as Sjögren Syndrome and systemic lupus erythematosus [620]. It has been demonstrated that the circulation of autoantibodies against neonatal heart M_1 muscarinic acetylcholine receptors in the sera of children correlated with congenital heart block [621]. Autoantibodies were able to react with the second extracellular loop of the human M_1 receptor and decreased contractility, activated nitric oxide synthase activity, and increased production of cyclic GMP [622].

CHAPTER 7

Discussion and conclusions

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G-protein-coupled receptors (GPCRs) form the largest superfamily of proteins in the human body. About 1% of the human genome encodes for over 1000 GPCRs [1]. They are located at the cell surface and are expressed ubiquitously. GPCRs regulate diverse intracellular responses and are therefore involved in all kinds of diseases, making them interesting therapeutic targets [2]. Acetylcholine, an endogenous amine, acts on a subfamily of these GPCRs, the muscarinic receptors, causing physiological effects such as increase in basal locomotors activity and gastric acid release [266].

Muscarinic acetylcholine receptors (mAChRs) have been shown to mediate various functions in the central and peripheral nervous systems (CNS, PNS) [623]. These include modulation of exocrine glandular secretion, vasodilatation and smooth muscle contraction [624], cell proliferation or survival [625], neural development and synaptic plasticity [626]. Muscarinic receptors are activated by both endogenously produced acetylcholine and exogenously administered muscarinic compounds. Pharmacological, anatomical and molecular studies have demonstrated the existence of five muscarinic receptor subtypes, denoted as muscarinic M_1, M_2, M_3, M_4 and M_5, which belong to class I family of heptahelical, transmembrane G-protein coupled receptors (GPCRs) [183].

Each receptor subtypes are characterized by a distinct selectivity for heterotrimeric G protein coupling. Thus, M_1, M_3 and M_5 are coupled to $G_{q/11}$ proteins and stimulate phospholipase C activity, resulting in the generation of the second messengers inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG), the mobilization of intracellular Ca2+ and the activation of protein kinase C (PKC) [184]. On the other hand, M_2 and M_4 are coupled to $G_{i/0}$ proteins, which results in the inhibition of adenilate cyclase, as well as prolonging potassium channel, non-selective cation channel, and transient receptor potential channel opening [627,628]. By means of this differential set of G protein partners, muscarinic receptors can initiate distinct signalling pathways within a same cell in order to trigger diverse, even opposed, functional outcomes in response to the same stimuli. It has been proven as well that mAChRs regulate a baste network of signalling intermediates, including small GTPase Rho, phospholipase D, phosphoinositide-3 kinase, non-receptor kinases and mitogen-activated protein kinases [322,629,630].

Although the first proteins found to have functional interactions with muscarinic receptors were, of course, G proteins, an increasing amount of evidence in the field suggests that this simplistic model defined as "one receptor -one G protein - one effector no longer exists. A great number of proteins have been identified as interacting with mAChRs, including GPCRs, kinases, and scaffolding proteins such as arrestin [631]. Determining in part the signalling efficiency/specificity for mAChRs [64]. Thus, receptors are now considered as complex signalling units, or signalosomes, that dynamically couple to multiple G proteins or other molecular entities or scaffold proteins in a temporally and spatially regulated manner, and even can form homodimers or heterodimers with distinct GPCRs or other non-GPCR membrane receptors, resulting in pharmacologically and functionally distinct receptor populations [79].

In this work, it is discuss novel muscarinic receptor interacting partners that link the receptors to alternative signalling pathways beyond G proteins. Emphases on explaining how muscarinic receptors regulate signal transduction pathways mediated by these proteins, including receptor dimerization have been putting out. This allows us to address some fundamental questions concerning the importance of molecular mechanisms hidden behind the pharmacology properties for each receptor subtype.

Homo- and heterodimerization of M_3 and M_5 muscarinic receptor subtypes

It is well established that a variety of cell surface receptors interact with each other to form dimers, and that this is essential for their activation [499]. Although the existence of muscarinic receptor dimers was predicted from early pharmacological and biochemical analysis, solid evidence supporting dimerization has come within the past few years using different experimental approaches such as co-immunoprecipitation of distinctly tagged versions of the receptor, light resonance energy transfer techniques FRET and BRET (fluorescence and bioluminescence resonance energy transfer, respectively), as well as analysis of cooperative ligand binding [632,633]. Pioneering studies by Wess and colleagues have demonstrated that in a heterologous expression system of COS-7 cells, M_3 receptor are able to form functional homodimers [634,635]. Purification and co-immunoprecipitation experiments in Sf9 cells also showed oligomers formation by muscarinic M_2 acetylcholine receptors [636]. In addition, as

different subtypes of muscarinic receptors are coexpressed in the same cell type in several tissues, for example, M_1 and M_2 receptors in neurons and M_2 and M_3 receptors in smooth muscle cells, it has been proposed that mAChR subtypes may heterodimerize [637]. Maggio and Wess showed that M_3 dimerization/oligomerization is receptor subtype specific and occurs in both transfected cells and native tissues [638]. Using bioluminescence resonance energy transfer, it has been demonstrated that M_1, M_2, and M_3 mAChR can form constitutive homo- and heterodimers in living HEK 293 cells. And that the cell receptor population for theses single subtypes is predominantly composed of high affinity homodimers [639] (Figure 6.1.2). In the same report, saturation curve analysis of cells expressing two receptor subtypes demonstrates the existence of high affinity M_1/M_2, M_2/M_3, and M_1/M_3 mAChR heterodimers, although the relative affinity values were slightly lower than those for mAChR homodimers. However, the existence of homo- and hetero-oligomers constituted by M_5 muscarinic receptor subtypes has not been reported yet, despite the large amount of experimental evidence that supports the active role of this receptor subtype in the non-neuronal cholinergic system.

In **chapter 2**, we provide for a first time biochemical, biophysical, and functional evidence of the existence of receptor-receptor interaction between M_3 and M_5 muscarinic receptors and with each other. Our results from quantitative BRET saturation analysis show that M_3 and M_5 can form homoligomers in intact HEK 293 cells. Analysis of BRET parameters (BRET50 and BRETmax) supported the existence of constitutive M_3 and M_5 mAChR homodimers in live cells (Table 2.2). Results in agreement with previous pharmacological and BRET signal demonstrated M_1, M_2 and M_3 muscarinic receptor homodimerization [163], although BRET50 values for M_5 muscarinic receptors were significantly higher than those determine for M_3 homodimers. This indicated that the likelihood of homodimer formation of the M_3 subtype appears to be slightly but consistently higher than with M_5 receptors subtypes. Therefore, our data suggests that both mAChR subtypes in a given tissue results in a combination of homodimers, whose proportions depend on both the relative affinity between interacting species and their expression levels, and a minor complement of monomers.

Following the quantitative BRET saturation curve approach, we also demonstrated that co-expression of M_3 and M_5 muscarinic receptor subtypes can

form heterodimers in HEK-293 living cells. Our results from BRET saturation curves clearly indicate that M_3 and M_5 receptors can form heterodimers. These results are also confirmed by co-immunoprecipitation experiments. BRET50 values from M_3/M_5 heterodimerization did not differ from values found for M_3 mAChR homodimerization curves. However, they were significantly lower than those determined for M_5 mAChR homodimers. These results support the idea that thr M_5 muscarinic receptor subtype exists as a combination of a high affinity homo- and hetero-dimers receptor population, but with a high affinity to form heterodimers when it is co-expressed with M_3 subtypes.

Although previous reports have demonstrated that GPCR agonist binding induces or stabilized receptor oligomerizatio, we cannot observe any change in the BRET parameters when cells were incubated in the presence of a carbachol agonist. It seems that for the muscarinic receptor family, agonist binding does not promote any conformational changes that disestablish the receptor dimerization state. By using SDS-PAGE and Western blot analysis of membrane extracts from COS-7 cells expressing a mutant M_3 mAChR, Zeng and Wess showed that agonist-induced receptor stimulation had no significant effect on M_3 mAChR dimerization [291]. In addition, carbachol treatment of HEK 293 cells expressing homologous or heterologous combinations of M_1, M_2, and M_3 mAChR fusion proteins revealed no significant changes in the BRET signal, as compared with control untreated co-transfected cells [163].

In an attempt to better understand the structural determinants involved in M_3/M_5 heterodimerization, we investigated the effects of the third intracellular loop on structural dimer organization. Previous studies using peptide strategies have identified structural determinants that can affect both GPCR dimers and function. For instance, a peptide derived from the TM6 domain of the β_2-AR was found not only to decrease the amount of receptor dimers detected in co-immunoprecipitation studies, but also to inhibit receptor-stimulated AC activity, leading the authors to conclude that dimerization may be important in receptor function [309]. Likewise, Banères *et al.* found that a peptide mimicking the TM6 domain inhibited the dimerization of the BLT 1 receptor and affected the ability of the receptor to interact with the G protein [310]. In addition, Granier et al. demonstrated that a peptide mimicking the V2 receptor third intracellular loop inhibits the receptor function through a modification of its dimeric structural

organization and direct action on G proteins [306]. In our studies, cells co-expressing M_3-Rluc and M_5-GFP^2 in the presence or absence of minigene construction encoding for a peptide for the third intracellular loop of the M_5 receptor subtype revealed a low but significant difference in BRET50. Therefore a significant inhibition in the $BRET_{max}$ signal was observed. These results strongly suggest that the minigene construct would modify the distance and/or orientation between M_3-Rluc and M_5-GFP^2 engaged in dimer formation affecting the oligomerization state itself. It also supports the notion that the peptide changes the conformation of a pre-existing dimer with promoting disassembly of protomers.

However it is important to notice that this effect was not higher (increased in 1,1 fold respect to BRET50 of cell without minigene expression). Probably indicating that in muscarinic receptor heterodimerization, specifically for M_3 and M_5, other structural components can actively participate in protomer organization and assembly. Based on mutagenesis and cross-linking experiments, helix VI for β-AR [309], helix IV for dopamine D_2 [137], or helices I and II for Ste2 pheromone receptor oligomers [311] have been proposed to provide inter-monomeric contacts. In the prototypical GPCR, rhodopsin, intradimeric contacts involving helices II, IV and V, as well as the cytoplasmic third intracellular loop are too involved in the formation of dimer rows [312]. Whereas the C terminus is implicated in the $GABA_B$-R1/$GABA_B$-R2 receptor oligomerization [313], the glycophorin motif seems to be involved in the formation of β_2-AR homo-oligomers [309], and the role of the N terminus int the oligomerization of the bradykinin and calcium receptors was reported [314,315]. Also, in the muscarinic receptor family, specifically the M_3 subtype, the functional role of the third intracellular loop in receptor dimerization has been demonstrated [640]. Altogether, these results support the notion that GPCR oligomerization involves the participation of different structural determinants whose role might differ among receptors.

The documented coexpression of M_3 and M_5 receptors in many acetylcholine-sensitive tissues like striatal dopamine neurons [317], the gastrointestinal tract [318] and T and B lymphocytes cells [297], suggests that heterodimerization could indeed occur in native mammalian tissues, assuming simultaneous expression of both receptors in the same cells. As shown for the M_5 receptor, some receptors may form homodimers but preferentially engage with heterodimers. This may also be the case for the 1D-adrenergic receptor (1D-AR) [113], reflecting that a large

spectrum of affinities is likely to exist for the formation of different GPCR heterodimers.

The determination of functional consequences of muscarinic receptor heterodimerization is difficult to achieve using classic radioligand competition binding assays and a specific pharmacological profile, particularly for heterodimers composed of M_3-M_5 subtypes that display similar affinities for the same radioligand. Understanding, in part, the structural determinants of M_3-M_5 heterodimers allows us to study the role of heterodimerization in signal transduction and cytokine production in lymphocytes T-cells by the use of a minigene construct which promotes a disestablishing effect in M_3/M_5 heterodimerization.

When Molt-3 cells endogenously expressing M_3 and M_5 receptor subtypes were transfected with the minigene construct, agonist stimulation led to a low level in ERK phosphorylation. Similar extensions were observed in agonist enhanced PHA-induced-IL-2 production. Theses results revealed that proper receptor heteromer is required for proper acetylcholine receptor response and function.

Our previous results demonstrated that the heterogeneous population of both M_3 and M_5 mAChR is composed of homodimers together with a significant fraction of heterodimers, whose proportion depends on both receptor subtypes and the relative affinity values for both homodimers and the M_3/M_5 heterodimer. Such a heterogeneous population of receptors oligomerization will provide a molecular mechanism for an increased diversity of cholinergic signalling in lymphocytes cells, a fact which might be considered during therapeutic treatment of the immune system.

The third intracellular loop as a key structural determinant in receptor-protein interactions

The third intracellular loop of muscarinic receptors is not only one of the main target domains for interaction with G proteins, but also a binding site for direct interaction with different proteins. Indeed, previous observations using receptor-derived peptides from specific regions of the M_1 and M_2 receptors have shown that the C-terminal tail of the 3ILoop is critical for functional receptor-G-protein interaction [641]. These observations have confirmed that there are different structural determinants for receptor-G-protein coupling and G-protein activation. More recently, specific motifs in the 3ILoop of M_1 and M_3 receptor have been

shown to bind some accessory proteins with high affinity (calmodulin, oncogenic SET, and small GTPase Rho) [64,334,642,643].

These observations point to a specific role of 3ILoop in receptor-G protein coupling, signal transduction and multiprotein complex formation. In **chapter 3,** using a minigene construct capable of expressing the human M_3 muscarinic receptor 3Iloop (TAP-3ILoop-minigene), it was demonstrated that the soluble expressed 3ILoop could act as an analogue of the receptor 3ILoop, and as a scaffold protein as well, competing with the receptor for its interacting proteins, and therefore inhibiting the specific G-protein-mediated downstream effects.

The rationale of our approach was to explore the ability of this structural determinant to interfere with G protein interaction or to compete for other interacting proteins that participated in a putative multiprotein complex formation, and to analyze its capacity to compete for functionally relevant processes. Our efforts were specifically focused on the third intracellular loop of the M_3 muscarinic receptor subtype, because this region was reported to be involved in direct G protein binding and activation, and the putative site for interaction of a group of scaffold or accessory proteins (arrestin binding, calmodulin and small G proteins interactions) [169,340,644]. The antecedent for our experimental design can be found in previous studies of cellular expression of fragments, or in vitro G protein activation assays, which reported the ability of the intracellular loops --or peptides derived from these loops-- to interact with the same molecular partners as the intact receptor [342,344,645]. Experimental evidence was available showing that these co-expression experiments could act mimicking the intact receptor, or coupling with and activating the relevant G protein[346,646]. In the current series of experiments, the third intracellular loop domain of the M_3 receptor was shown to have a recognizable impact on the function of the intact M_3 receptor subtype. In our experiments, proximal events occurring at the level of the plasma membrane were dramatically affected as shown by the impairment of receptor-accessory protein co-immunoprecipitation in cells co-expressing the minigene construct. The co-expression of the minigene construct along with the wild-type receptor exhibited decreased G protein activation, phosphatidylinositol production, and subsequent signalling, but normal ligand binding and receptor membrane expression (Figure 3.1.7). Therefore, our results indicate that the co-expression of the 3ILoop of the M_3 receptor with the M_5 subtype produce similar effects, as

with previous studies in which the expression of the third intracellular loop of the μ-opioid receptor altered the functionality not only when co-expressed with the intact μ -opioid receptor but also when co-expressed with other classes of GPCR [347].

The third intracellular loop is 256 residues long, containing multiple motifs of basic and acid residues and some currently recognized functional sequence motifs. This primary sequence pattern is not conserved throughout the G protein-coupled receptor superfamily and not even within the class A rhodopsin-like receptor subfamily that represents the closest structurally-related class of GPCR. It is possible that this will ultimately constitute a conformational structural motif that will be more broadly representative and determine part of the specificity for each receptor in these families. However, no structural data is currently available to confirm or refute this hypothesis.

Interacting proteins for such motifs appearing in modular form can be identified by affinity purification approaches, such as yeast two-hybrid screening and immunoprecipitation. Our current work, in which the minigene construct representing the third intracellular loop of the M_3 muscarinic receptor specifically influences the intracellular signalling of the intact receptor, probably blocking a putative multiprotein complex formation, supports the notion that important motifs could be present in this region. The fact that this loop is significantly long and that it is a recognized site for post-translational modifications should make it an ideal tool for exploring potential molecular partners that might mediate the observed effect under different cellular conditions.

The use of this TAP-system strategy that complements the co-expression experiments of the construct with the intact receptor under different physiological condition, together with immunoaffinity purification followed by mass spectrometry analysis, allow us to systematically identified new interacting protein of muscarinic receptor family (**chapter 3.2**).

The ILoop-TAP proteomic approach presented in this work contributes to the largest analysis of protein-protein interaction in muscarinic receptor family. Maximizing sensitivity and reproducibility over other methods to detect receptor binding partners, we identified 148 different interacting proteins, of which 57 had been detected previously as muscarinic receptor-binding partners.

The GO classification of the proteins identified by Iloop-TAP strategy revealed some interesting avenues of further consideration: first, the presence of heterotrimeric G proteins in all receptor subtype-associated protein purifications. Consistent with the known coupling of muscarinic receptors to G proteins, we identified four Gα-protein for $M_1/M_3/M_5$ receptor subtypes (Gα-q, Gα-11, Gα-12 and Gα-13) and three Gα-i isoforms (Gα-i1, Gα-i2 and Gα-i3) for M_2/M_4 receptor subtypes, as well as two different Gβ isoforms (Gβ1 and Gβ4) and three Gγ isoforms (Gγ2, Gγ7 and Gγ11). This demonstrates that our approach can clearly be used as a method for large-scale protein-protein interaction detection, not only for the muscarinic receptor family, but also for other GPCRs. Second, we identified several cytoskeletal proteins, including those associated with GPCRs' reorganization and regulation (α- and β-tubulin, filamin A, annexin A2, dynamin 2, clathrin, IQ-GAP1/3 and paxillin), some of them existing in a common complex with specific receptor subtypes. This suggests that muscarinic receptors selectively bind to different structural components in the cell and may have a role in receptor internalisation and the regulation of cell spreading, migration, and the attachment at sites of focal adhesion [384]. In support of this notion are recent reports pointing toward different mechanisms of internalisation using M_2 and M_4 receptors depending of the cellular type [385,386], as well as the active roles of tubulin and microtubule reorganization in PLD activation mediated by $G_{q/11}$ muscarinic receptor subtypes [387]. More interestingly, using drugs that stabilized or destabilized microtubule organization, it was found that the activation of muscarinic leads to modulation of ion currents in cardiac myocyte cells [388], functions mediated by the direct interaction of the receptor and the G protein with the microtubule cytoskeleton. The third and last consideration is the identification of several muscarinic receptor subtypes-specific signalling proteins such as ADP-ribosylation factors, elongation factor 1-A (eEF-1A), oncogenic SET protein, Rac1, and different isoforms of phospholipase C and the protein kinase C (β-1, β-3, γ and α, δ, τ respectively). Interestingly, the small GTPases Rac1 has been shown to function downstream of M_1 and M_3 receptors. In the context of Rac 1 activity but not RhoA activity, M_3 muscarinic receptor-mediated activation of PLC and PKC triggers cell death [389]. The ADP-ribosylation factors are members of the Arf arm of the Ras superfamily of guanosine triphosphate (GTP)-binding proteins. Physiologically, Arfs regulate membrane traffic and the actin

cytoskeleton. However, Arf function likely involves many additional biochemical activities. Arf activates phospholipase D and phosphatidylinositol 4-phosphate 5-kinase with the consequent production of PA and PIP2, respectively [390]. It has been shown that vasopressin V2 receptor and M_2 muscarinic receptor processing and trafficking are under ARF-6 control [391]. Also, Arf 1 and 6 mediate PLD1/2 activation by M_3 muscarinic receptor [334]. eEF-1A and other elongation factors have been reported to modulate M_4 muscarinic receptor subtype function by direct interaction with the receptor [392,647]. Several serine/threonine phosphatases participate in the dephosphorylation of activated GPCRs. Phosphatases of the PP2A and PP2B subfamilies have been reported to target GPCRs. M_1 receptor subtypes stimulate the formation of a multiprotein complex centred on TRPC6 channels where PP2B plays an active role in the disassociation of the muscarinic receptor from the complex [394].

The most important outcome of our study is the possibility to study and identify specific interacting proteins under native conditions. Although we used only one set of experimental parameters here for the evaluation of complex composition, we will, in the future, systematically modify experimental parameters (both pharmacological and physiological conditions) to evaluate the impact of a changing environment on complex variability. These studies should help to elucidate the dynamics of complex assembly and disassembly for each receptor subtype. Moreover, it may be a starting point in deciphering receptor specifies and a molecular context for the choice and evaluation of drug targets.

Another important outcome of our experiments is that, comparing the experimental results in the literature with our data-set, we were able to construct a muscarinic receptor family network, which allowed us to group interacting proteins into cluster complexes. The network that resulted is a functional description of the muscarinic receptor interactome at a higher level of organization and revealed some interesting information. Although comparison of our data set with literature is straightforward, it is important to keep in mind that our IloopTAP approach yields complexes composition data that, in any case, produces binary interactions, just as the results from two hybrid methods. This supports the view that receptor complex formation is more than the sum of binary interactions.

However, binary analysis methods are of exceptional value for the detection of pairwise and transient associations of GPCRs. The success of our approach in the characterization of receptor complexes relies on the conditions used for the assembly and retrieval of the complexes. These include localization and post-translational modifications in a manner that closely approximates normal physiology. Therefore, because the ILoopTAP method does not provide information on the orientations of complex components, complex characterization by two-hybrid analysis, or resonance energy transfer methods are ideally complementary.

The C terminal tail

Although there is increasing evidence that the "magic tail" of GPCRs constitutes the main anchoring domain for soluble interacting proteins (more than 50 GIPs interacting with GPCR C-termini have been identified [648]), in the case of muscarinic receptor family only the third intracellular loop has been reported as critical domain capable of binding adaptor and scaffold proteins. Nevertheless, a few examples have been reported with a functional role in receptor regulation and signalling[570].

Despite the small role of the C terminal tail of muscarinic receptor in the recruitment and anchoring of adaptor and scaffolds protein, it is well established that the C-terminal tail of the M_3-muscarinic receptor is an essential structural element for signalling to the anti-apoptotic pathway [348]. Experimental evidence has shown that the removal of the distal portion of the C-terminal tail results in a receptor that is coupled normally to the $G_{q/11}$/phospholipase C pathway and the mitogen-activated protein kinase pathway, but is unable to couple to the anti-apoptotic pathway [649]. Furthermore, it has been demonstrated that a poly-basic region conserved within the C-terminal tail of the $G_{q/11}$-coupled muscarinic receptor subtypes (CDKRKRRKQ) appears to be the structural determinant of coupling to the anti-apoptotic pathway [348]. Today it is still unclear what protein or proteins are implicated in the connection of the muscarinic receptor with the anti-apoptotic pathway, and which anti-apoptotic signalling is implicated. However, **in chapter 4**, we demonstrated by using quimeric receptors where the C-terminal tail of the human M_3 muscarinic receptor was inserted in the C-terminal tail of two rhodopsin mutants (R135L and L46R), previously reported for patients with severe retinitis pigmentosa, that the anti-apoptotic properties of this epitope can

be used as a blocking system to inhibit apoptotic effects promoted by rhodopsin mutants. We provide evidence that quimeric construction protects cells from H_2O_2-induced apoptosis. Promoting the early expression of Bcl-2 decreased Bax expression and reduced caspase-3 activation. Correspondingly, cytochemical evaluation revealed an absence of cytochrome c into cytoplasm and an increased of NF-κβ translocation to the nucleus. These effects were blocked only by HA-1077 Rho kinase inhibitor, indicating that the insertion of the anti-apoptotic epitope of the muscarinic receptor remains functional, reducing many apoptotic markers, and can be used as a therapeutic method for late-stage inhibition of apoptosis.

Roles of Receptor-interacting proteins in subtype-specific receptor signalling

A sequence alignment of GPCRs of the rhodopsin family reveals that the majority of GPCRs contain an NPxxY (x) $_{5,6}$F sequence [408]. Mutation in this domain affects ligand affinity, receptor expression, G protein coupling, and receptor association with interacting proteins such as small G proteins ARF and Rho [418,422,650-653]. Activation of the M_3 Muscarinic receptor by agonist binding is followed by conformational changes and recruitment of accessory and scaffold proteins, that together with the classical core signalling entities contribute to the formation of a signalsome complex. The dynamics of such a complex is not well understood but a conserved NPXXY (5,6) F motif located in the transmembrane VII of the receptor was found to be critical in G protein activation. In **chapter 5**, we set out to elucidate the role of this region by using site direct mutagenesis in combination with coimmunoprecipitation experiments and signal transduction assays. We have generated receptor mutants (N540A, N540D and F551A) to determine the effect of the mutation on binding, G protein coupling, signalling and multiprotein complex reorganization after the expression of the mutated receptor in COS-7 cells. The mutated receptor displayed similar expression levels and ligand binding properties compared with the wild type, except receptor mutant N540A, which showed a high reduction in level expression and slightly reduced antagonist binding sites. In addition, the N540A receptor mutant showed a dramatic reduction in G protein activation and completely failed to elicit PLD activation and PI response to carbachol stimulation. However, the N540D mutant activates G proteins and stimulates PLC and PLD, but with different time course and desensitization compared with the WT. Co-immunoprecipitation experiments

allowed us to show that each mutant retains a different capacity to form stable complexes with different populations of G proteins and accessory proteins (ARF1, ARF6, RhoA). Different levels of ARF, RhoA and G proteins were observed in the immunoprecipitation complexes for N540D mutant. Indicating the role of the NPXXY (5,6) F motif as a structural determinant during conformational and selectively activation of G proteins and multiprotein complex reorganization.

With the N540A/D and F551A mutations, we demonstrated a selective regulation of downstream signal transduction by a different conformational arrangement of M_3 receptor. The WT and N540D mutant resulted in a small change in IP accumulation even at highly agonist concentration. However, the F551A mutant receptor showed a two-fold reduction with respect to WT and the N540A mutants completely lost their capacity to activate the PLC signal. Also, carbachol stimulated [^{3}H] Ptdbut formation resulted in a six-fold increase for the WT receptor and N540D mutant, with lower activation for the WT receptor and N540D mutant, lower activation for the F551A mutant, and no detectable [^{3}H] Ptdbut production for the N540A mutant, but with a lower potency in all cases. We also corroborated that theses results were observed at the level of the MAPK PLC/PLD pathway and the changes of Asn by Asp keep a relatively stable conformation that does not substantially perturb the conformation change produced during receptor activation upon agonist stimulation.

Schmidt and Jakobs showed that activation of M_3 muscarinic receptor in HEK cells leads to stimulation of both phospholipase (PLC/PLD), but with distinct efficacies and with very distinct durations of each response, but this study did not identify the essential transducing component responsible for the variable desensitisation [654]. Mitchell and Johnson demonstrated in COS-7 cells that the M_3 muscarinic receptor substantially utilized an ADP-ribosylation factor (ARF)-dependent route for PLD activation [655]. The M_3 receptor displayed a major ARF1-dependent route for PLD1 activation, whereas the ARF-6 dependent pathway activates both PLD1 and PLD2 [656]. However, these studies did not show in detail if the selective coupling by different ARF factors is responsible for desensitization.

We observed, as previously reported, that the time-course of carbachol-stimulated [^{3}H]-myo-InsPs accumulation in the transfected WT-COS-7 cells reached a peak

between 20-60 min after agonist exposure and fifteen percent of receptors desensitized 2 h after agonist stimulation (2 min, 1 mM of carbachol). In contrast, maximal levels of [^{3}H] Ptdbut were already obtained after 2 min of incubation with carbachol (1mM), followed by a stable plateau of at least 30 min of incubation for the WT receptor. Although at a lower level, the F551A mutant showed similar time-course and desensitization, and the N540D mutant did not desensitize following short-term agonist treatment in the same manner as the WT for PLD response. On the contrary, 2 min of pre-exposure to carbachol led to about a two-fold increase in receptor-mediate [^{3}H] Ptdbut formation 20-30 min after exposition.

Stimulation of PLD/PLC in COS-7 cells by transfected M_3 muscarinic receptors are affected by change in the NPxxY (x) $_{5,6}$F motif. These changes can cause different degrees of structural flexibility, resulting in the regulation of the interacting proteins, such as ARF factors, and the selective regulation of desensitization mechanisms. Mutation in the N 4.79 position resulted in a very rapid change (less than 2min) in PLD response upon agonist-stimulation. This phenomenon may be the result of the different recruitments of ARF1/ARF6 factors, and the resistant-sensitive βγ complex inhibition and translocation (as demonstrated by co-immunoprecipitation experiments). This demonstrates for the first time the active role of the NPxxY (x) $_{5,6}$F motif in the selective process of multiprotein complex formation and reorganization by human M_3 muscarinic receptors, and their role during G protein regulation and desensitization.

Concluding remarks

Until a few years ago it was thought that the interactions between muscarinic receptor, G-proteins and their effectors could describe the whole system. However, we now know that it is much more complex, with many other proteins binding to muscarinic receptors and influencing their activity. Indeed, in these five years of work, we demonstrated by different approach (from resonance energy transfer to tandem affinity purification and mass spectrometry) the active role of interacting protein in muscarinic receptor regulation and signalling. We shown that a particular complex is not necessarily of invariable composition, nor are all its building blocks uniquely associated with that specific complex. One

complex may be the result not only of physical interaction between the receptor and the partners' protein, but also of the participation of many non-"direct" associations resulting in the formation of a network that interconnects the receptor with a number of other pathways, determining receptor specificities.

1. We demonstrated that M_5 muscarinic receptor subtype form homodimers. These homodimers are the functional units at the cell surface. In addition to homodimerization, M_5 receptor subtype is able to form heterodimers with M_3 receptor subtype. M_3/M_5 heterodimers have been detected in living cells and agonist activation of the two receptors does not seem to modify the degree of heterodimerization. BRET analysis showed that the third intracellular loop is an important domain in receptor interaction and heterodimers formation.
2. In T-cell M_3/M_5 heteromeric receptor complexes seem to play an active role in modulation of regulation of immune response
3. Using a novel TAP-3ILoop approach it was systematically identified interacting protein of each muscarinic receptor subtype.
4. Comparative analysis of experimental results together with scientific literature, allowed the representation of muscarinic receptor network interactome.
5. Quimeric construction where the anti-apoptotic C-terminal region of the M_3 muscarinic receptor was used keep its functional properties, and provide protection to the cell. Engineering the quimeric receptor of two rhodopsin mutants (R135L and L46R), previously reported for patients with severe retinitis pigmentosa [410,657], it was demonstrated the evidence of protection against apoptosis and common activation mechanisms among GPCRs.
6. NPXXY (5,6) F motif is involved in the activation and potentially in the signalsome complex formation of the M_3 muscarinic receptor subtype. The flexibility introduced by the NPXXY (5,6) F motif play a role in muscarinic receptor activation by functioning as a sensitive conformational switch and also determine the selectivity and magnitude of signalling, regulation and scaffolds protein interaction.

Much progress has been made in the understanding of the molecular pharmacology and function of muscarinic receptor family, but still many

questions remain unanswered, while new finding, often add more questions. Therefore, the muscarinic receptor family deserves to receive the same (if not more) attention and continuous research efforts it has received so far, for many years to go.

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APPENDIX

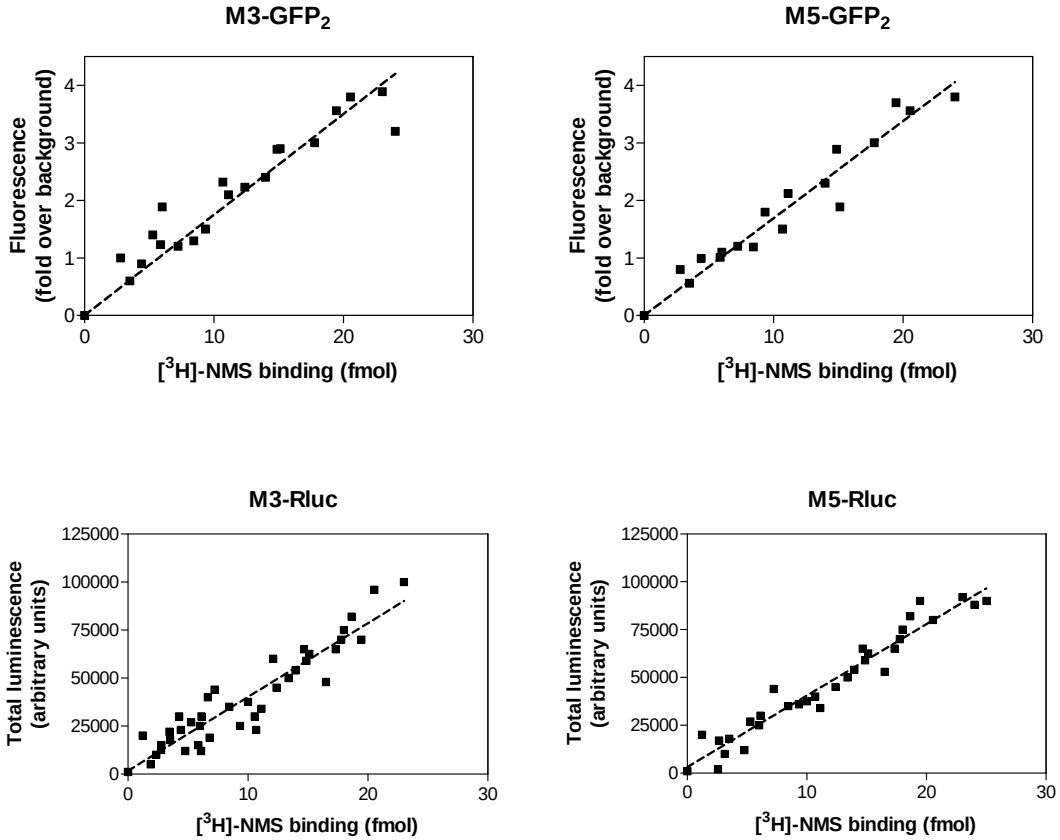
Appendix 1:

Most conserved residues located in rhodopsin family (A). Residues number is based on the sequence of bovine rhodopsin.

TM Domain	Residue type	Residue number
H-I	N	55
H-II	D	83
H-III	R	135
H-IV	W	161
H-V	P	215
H-VI	P	267
H-VII	P	303
Note also the disulfide bridge between C110 and C187		

Appendix 2:

Titration of Donor and Acceptor Fusion Proteins—Expression levels of mAChR-RLuc and mAChR-GFP constructs were monitored by measuring luminescence and fluorescence, respectively, as described in MATERIALS AND METHODS. This procedure is based on the observation that luminescence and fluorescence levels of several receptor-RLuc and receptor-GFP (or receptor-YFP) fusion proteins have been found to be linearly correlated with receptor numbers. Because this correlation is an intrinsic property of each fusion protein, we tested whether our mAChR fusion proteins followed a linear correlation pattern. Thus, we expressed each mAChR-RLuc or mAChR-GFP at different levels in HEK 293 cells and assessed the relationship between luciferase activity or fluorescence, respectively, and the amount of mAChR-binding sites in the same cells. Luminescence and fluorescence (arbitrary units) were plotted against total binding sites, and linear regression curves were obtained. These standard curves generated for each single experiment were used to transform fluorescence and luminescence values into femtomoles of receptor. Thus, the fluorescence/luminescence ratios were transformed into (receptor-GFP)/(receptor-RLuc) ratios, which allowed us to determine accurate BRETmax and BRET50 values.



Appendix 3.1 and 3.2:

APPENDIX

Appendix 1:

Most conserved residues located in rhodopsin family (A). Residues number is based on the sequence of bovine rhodopsin.

TM Domain	Residue type	Residue number
H-I	N	55
H-II	D	83
H-III	R	135
H-IV	W	161
H-V	P	215
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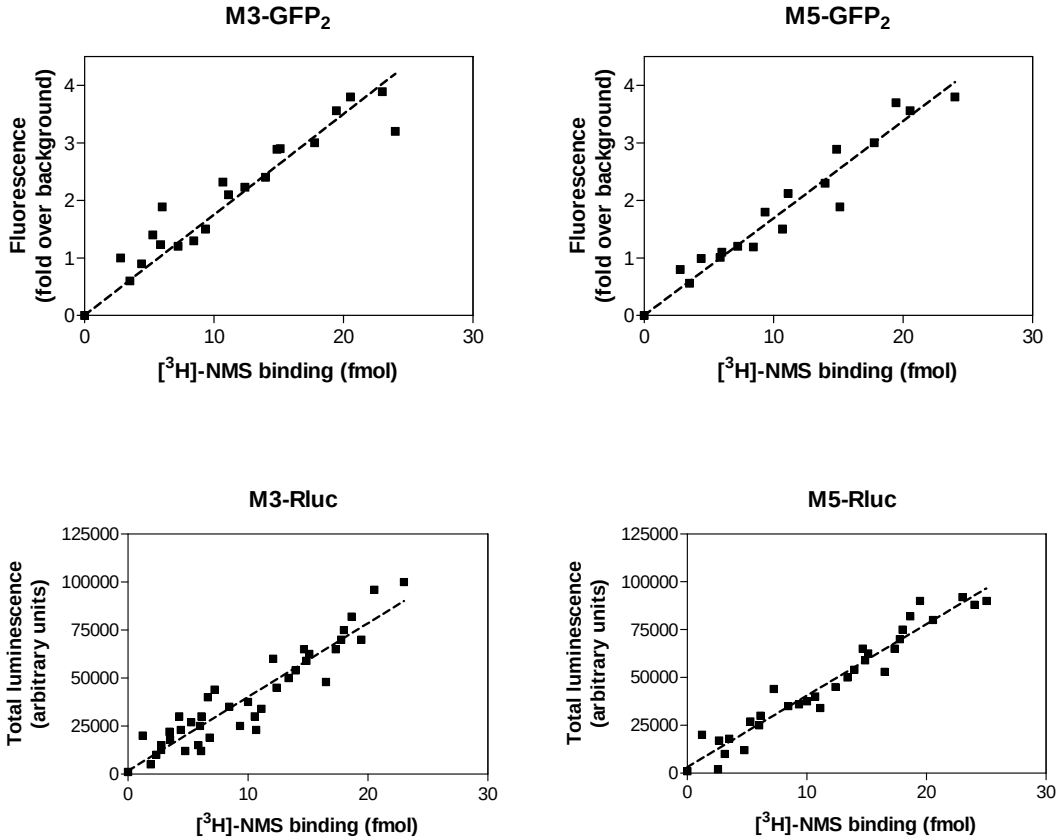

Appendix 3.1 and 3.2:

Acc # IPI	Acc # (hyperlink: Swissprot/NCBI)	Protein name (yellow: Background contaminant, red: Known receptor interaction partner)	Lenght (aa)	Mr (Da)	Function/Classification
IPI00014978	Q15172	alpha isoform of regulatory subunit A, protein phosphatase 2	486	56194	Kinase/phosphatase
IPI00215914	P84077	ADP-ribosylation factor 1	181	20697	Signal Transduction/Trafficking
IPI00256684	O95782	Adaptor protein complex AP-2	977	107555	Trafficking
IPI00219518	P40616	ADP-ribosylation factor-like protein 1	181	20418	Signal Transduction/Trafficking
IPI00215917	P61204	ADP-ribosylation factor 3	181	20601	Signal Transduction/Trafficking
IPI00215919	P84085	ADP-ribosylation factor 5	180	20530	Signal Transduction/Trafficking
IPI00215920	P62330	ADP-ribosylation factor 6	175	20082	Cell Motility/Trafficking
IPI00307794	P24588	AKAP 79	227	47072	Signal Transduction
IPI00455315	P07355	Annexin A2	339	38604	Signal Transduction
IPI00027464	P63098	Calcineurin subunit B isoform 1	170	19300	Kinase/phosphatase
IPI00334271	Q13554	Calcium/calmodulin-dependent protein kinase type II beta chain	664	72727	Signal Transduction
IPI00334344	Q13555	Calcium/calmodulin-dependent protein kinase type II gamma chain	558	60629	Signal Transduction
IPI00020984	P27824	Calnexin precursor	592	67568	Protein Secretion
IPI00022256	Q96CW1	Clathrin coat assembly protein AP50	345	49655	Trafficking
IPI00024067	Q00610	Clathrin heavy chain	1675	191615	Trafficking
IPI00183400	P48729	Casein kinase I isoform alpha	337	38915	Kinase/phosphatase
IPI00011102	P48730	Casein kinase I isoform delta	415	47330	Kinase/phosphatase
IPI00744507	P68400	Casein kinase II subunit alpha	391	45144	Kinase/phosphatase
IPI00010865	P67870	Casein kinase II subunit beta	215	24942	Kinase/phosphatase
IPI00009236	Q03135	Caveolin-1	178	20472	Signal Transduction/Biogenesis
IPI00019870	P51636	Caveolin-2	162	18291	Signal Transduction
IPI00012011	P23528	Cofilin-1	166	18502	Signal Transduction
IPI00033022	P50570	Dynamin 2	870	98064	Trafficking
IPI00396485	P68104	Elongation factor 1-alpha 1	462	50141	Translation
IPI00000875	P26641	Elongation factor 1-gamma	437	50119	Translation
IPI00024911	P30040	Endoplasmic reticulum protein ERp29 precursor	261	28993	Transport
IPI00333541	P21333	filamin A, alpha	2647	280739	Signal Transduction
IPI00216217	Q05397	Focal adhesion kinase 1	1052	119233	Kinase/phosphatase
IPI00641153	Q92896	Golgi apparatus protein 1 precursor	1179	134593	Other
IPI00217906	P04899	Guanine nucleotide-binding protein G(i), alpha-2 subunit	355	40451	Binding Protein
IPI00384861	Q9Y2X7	ARF GTPase-activating protein GIT1	761	84341	Signal Transduction
IPI00003362	P11021	78 kDa glucose-regulated protein precursor	654	72333	Binding Protein
IPI00328744	Q03113	Guanine nucleotide-binding protein alpha-12 subunit	381	44279	Signal Transduction
IPI00290928	Q14344	Guanine nucleotide-binding protein G alpha13	377	44050	Cell Motility/Signal Transduction
IPI00848226	P63244	Guanine nucleotide-binding protein beta subunit-like protein 12.3	317	35077	Signal Transduction

IPI00514055	P63092	Guanine nucleotide-binding protein G(s), alpha subunit	394	45665	Signal Transduction
IPI00026268	P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	340	37377	Signal Transduction
IPI00012451	Q9HAV0	Guanine nucleotide-binding protein beta subunit 4	340	37567	Signal Transduction
IPI00220578	P08754	Guanine nucleotide-binding protein G(k), alpha subunit (G(i)apha-3)	354	40532	Signal Transduction
IPI00337415	P63096	Guanine nucleotide-binding protein G(i), alpha-1 subunit	354	40391	Signal Transduction
IPI00305551	P29992	Guanine nucleotide-binding protein G(11), alpha subunit	359	42123	Signal Transduction
IPI00288947	P50148	Guanine nucleotide-binding protein G(q), alpha subunit	353	41467	Signal Transduction
IPI00012497	P25098	G-protein coupled receptor kinase 2	689	79574	Kinasa/phosphatase Signal transducti
IPI00019926	P35626	G-protein-coupled receptor kinase 3	688	79710	Kinase/phosphatase Signal transducti
IPI00017330	P43250	G-protein-coupled receptor kinase 6	576	65991	Kinase/phosphatase
IPI00328905	A2RRC9	IQ motif containing GTPase activating protein 3	1631	184519	Signal Transduction
IPI00029446	O95248	Isoform 1 of SET-binding factor 1	1867	208315,00	Kinase/phosphatase
IPI00003479	P28482	Mitogen-activated protein kinase 1	360	41390	Signal Transduction
IPI00479962	Q9ULV0	Myosin-Vb	1849	213756,00	
IPI00220030	P49023	Paxillin	591	64533	Cell Adhesion
IPI00216920	B1AK73	Phospholipase C beta 1	232	26197	Signal Transduction
IPI00010400	A5PKZ6	Phospholipase C beta 3	1234	138799	Signal Transduction
IPI00383849	A2A284	Phospholipase C gamma	1291	148660	Signal Transduction
IPI00290738	Q13492	Phosphatidylinositol-binding clathrin assembly protein	652	70755	Trafficking
IPI00644989	Q15084	Protein -disulfide isomerase A6		48091	Biogenesis
	P30101	Protein disulfide-isomerase A3	505	56782	Signal transduction
IPI00299571	Q15084	Protein disulfide-isomerase A6 precursor-Isoform 2	440	48121	Protein Folding
IPI00021448	P27986	PI3K p85 alpha	724	83598	Signal Transduction
IPI00011736	O00459	PI3k p85 beta	728	81624	Signal Transduction
IPI00016639	P41743	Protein kinase C iota	587	67258	Signal Transduction
IPI00013749	Q05513	Protein kinase C zeta	592	67660	Kinase/phosphatase/Signal Transducti
IPI000887297	B0LPH5	Protein kinase C alfa	152	17441	Signal Transduction
IPI00056334	Q969G5	Protein kinase C, delta binding protein	261	27642	Other
IPI00220642	P61981	14-3-3 protein gamma	247	28303,00	Signal Transduction
IPI00000816	P62258	14-3-3 protein epsilon	255	29174	Signal Transduction
IPI00018146	P27348	14-3-3 protein tau	245	27764,00	Transcription
IPI00021263	P63104	14-3-3 protein zeta/delta	245	27745	Signal Transduction
IPI00010271	P63000	Rac-1 (Ras-related C3 botulinum toxin substrate 1)	192	21450	Signal Transduction
IPI00009342	P46940	Ras GTPase-activating-like protein IQGAP1	1657	189252	Signal Transduction
IPI00647786	Q92888	p115 Rho guanine nucleotide exchange factor (GEF) (Rho guanine nucleotide exc	912	102435	Signal Transduction
IPI00412782	Q92974	Rho/rac guanine nucleotide exchange factor (GEF)	893	101174	Signal Transduction
IPI00022542	Q13464	Rho-associated protein kinase 1	1354	158175	Kinase/phosphatase/Biogenesis
IPI00013177	P41220	RGS2	211	24382	Signal Transduction
IPI00028112	P49798	RGS4	205	23256	Signal Transduction
IPI00183077	O15539	RGS5	181	20946	Signal Transduction

IPI00855716	P57771	RGS8	180	20917	Signal Transduction
IPI00289948	Q5VYN9	RGS16	202	22749	Signal Transduction
IPI00010270	P15153	p21-Rac2 (Ras-related C3 botulinum toxin substrate 2	192	21429	Signal Transduction
IPI00010271	P63000	p21-Rac1(Ras-related C3 botulinum toxin substrate 1)	192	21450	Signal Transduction
IPI00449906	Q14155	Rho guanine nucleotide exchange factor 7	803	92012	Signal Transduction
IPI00332511	P63151	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform	447	51692	Kinase/phosphatase
IPI00008380	P67775	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	309	35594	Kinase/phosphatase
IPI00429689	P62714	Serine/threonine protein phosphatase 2A, catalytic subunit, beta isoform	309	35575	Kinase/phosphatase
IPI00072377	Q01105	SET translocation (myeloid leukemia-associated)	290	33489	DNA replication/Transport
IPI00006408	Q9Y314	Similar to eNOS interacting protein	301	33172	Signal Transduction
	P61106	Ras-related protein Rab-14	215	24110/23897	Trafficking/Signal transduction
	P20339	Small GTP-binding protein rab5	215	23659	Trafficking
IPI00220527	Q13596	Sorting nexin 1	522	59070	Trafficking
IPI00289876	O15400	Syntaxin 7	261	29816	Trafficking
IPI00478231	P61586	Transforming protein RhoA	193	21768	Signal Transduction
IPI00007750	P68366	Tubulin alpha-1 chain	448	49924	Cell motility
IPI00011654	P07437	Tubulin beta chain	444	49671	Other
IPI00031370	Q9BVA1	Tubulin beta-2B chain	445	49953	Other
IPI00013683	Q13509	Tubulin beta-3 chain	450	50433	Other
IPI00290566	P17987	T-complex protein 1, alpha subunit	556	60344	Protein Folding
IPI00297779	P78371	T-complex protein 1, beta subunit	535	57488	Protein Folding
IPI00302927	P50991	T-complex protein 1, delta subunit	539	57924	Protein Folding
IPI00027626	P40227	T-complex protein 1 subunit zeta	531	58024	Protein Folding
IPI00015228	Q99418	Cytohesin-2	400	46546	Biogenesis
IPI00021257	P53365	ADP-ribosylation factor-interacting protein 2	341	37856	Biogenesis
IPI00166301	Q8N264	Rho GTPase-activating protein 24	748	84258	Trafficking
IPI00784156	P63010	Adapter-related protein complex 2 beta-1 subunit	937	104553	Trafficking
IPI00003865	P11142	Heat shock cognate 71 kDa protein		71082	Chaperone
IPI00329351	P10809	60 kDa heat shock protein, mitochondrial precursor		61346	Chaperone
IPI00018206	P00505	Aspartate aminotransferase, mitochondrial precursor		47844	
IPI00291006	P40926	Malate dehydrogenase, mitochondrial precursor		35965	
IPI00303476	P06576	ATP synthase beta chain, mitochondrial precursor		56525	
IPI00000874	Q06830	Peroxiredoxin 1		22324	
IPI00005132	Q9NVN8	Hypothetical protein FLJ10613		66216	Unknown function
IPI00005160	O15143	ARP2/3 complex 41 kDa subunit		41722	
IPI00019912	P51659	Peroxisomal multifunctional enzyme type 2		80092	
IPI00019962	Q96EY4	Hypothetical protein FLJ11184		24018	Function unknown
IPI00021439	P02570	Actin, cytoplasmic 1		42052	

IPI00024145	P45880	Voltage-dependent anion-selective channel protein 2		38639	
IPI00106491	Q9BPV9	Hypothetical protein		27657	Function unknown
IPI00216308	P21796	voltage-dependent anion channel 1		30868	
IPI00217966	P00338	lactate dehydrogenase A		36950	
IPI00218849	Q9H7B2	similar to hypothetical protein FLJ21087		38775	Function unknown
IPI00219018	P04406	Glyceraldehyde-3-phosphate dehydrogenase		36201	
IPI00290982	Q14204	Dynein heavy chain, cytosolic		534768	
IPI00294398	Q16836	Short chain 3-hydroxyacyl-CoA dehydrogenase		34313	
IPI00219150	P39026	ribosomal protein L11		20468	Ribosomal proteins
IPI00219178	P26373	ribosomal protein L13		24304	Ribosomal proteins
IPI00221088	P46781	ribosomal protein S9		22635	Ribosomal proteins
IPI00002821	P50914	60S ribosomal protein L14		23258	Ribosomal proteins
IPI00294181	P53025	60S ribosomal protein L10a		24856	Ribosomal proteins
IPI00031691	P32969	60S ribosomal protein L9		21964	Ribosomal proteins
IPI00008433	P46782	40S ribosomal protein S5		23033	Ribosomal proteins
IPI00025091	P04643	40S ribosomal protein S11		18590	Ribosomal proteins
IPI00027270	Q02877	60S ribosomal protein L26		17248	Ribosomal proteins
IPI00012772	P25120	60S ribosomal protein L8		28104	Ribosomal proteins

Abbreviations

AC	Adenylyl cyclase
ATP	Adenosine 5’-triphosphate
BRET	Bioluminescence resonance energy transfer
CaMK	Ca2+/calmodulin dependent protein kinase
cAMP	Adenosine 3’,5’-cyclicmonophosphate
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
DMSO	Dimethylsulfoxide
EDTA	Ethylendiaminetetraacetic acid
ER	Endoplasmatic reticulum
ERK-1/2	Extracellular regulated kinase-1/2
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GRK	G-protein coupled receptor kinase
GST	Glutathione-S-transferase
GTP	Guanosine 5’-triphosphate
MAPK	Mitogen-associated protein kinase
mRNA	Messenger ribonucleic acid
PD98059	2-(2-Amino-3-methoxyphenyl)-4-H-1-benzopyran-4-one
PKC	Protein kinase C
PLC	Phospholipase C
PVDF	Polyvinylidene difluoride
Rluc	Renilla luciferase
RT-PCR	Retro-transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrilamide gel electrophoresis
YFP	Yellow fluorescent protein

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Currículo vitae

Dasiel Oscar Borroto Escuela was born November 14 1979 in Yaguajay (Cuba). He attended the " Bachillerato" at the Instituto Preuniversitario Vocacional de Ciencias Exactas in Sancti Spiritus. After his graduation he studied Biochemistry at the Faculty of Biology, University of Havana in 1998. During the final stage of his study he performed two year in Accelerated Bioinformatic Program within the Center for Genetic Engenierring and Biotechnology , Physical-Chemistry Division under supervision of Dr. Rolando Pajon Feyt. He performed an additional six month internship in the Center for Molecular Immunology, led by Dr. Eduardo Suárez.

After graduation in 2003, he immediately continues his study with his PhD. Project in the lab of Dr. Pere Garriga (, Industrial and Molecular Biotechnology Group (GBMI) AND Center for Molecular Biotechnology (CBIM), Barcelona, Spain). The performed work, described with this thesis, focused on the study of muscarinic receptor family, with emphasis on their structural-functional relationship and protein-protein interactions of the receptors. Within the PhD. Project he collaborated with Dr. Francisco Ciruela, Nueromolecular Group, University of Barcelona. He also conducted nine-month internship at the Biomedical Proteomics Research Group (BPRG), Department of Structural Biology and Bioinformatics, University Medical Centre (CMU), University of Geneva, Switzerland under the supervision of Dr Jean-Charles Sanchez.

Since June 2007, he attended the finalization of his thesis and hopes to continue to work in Dr. Francisco Ciruela Group at University of Barcelona as a postdoctoral researcher.

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- **Borroto-Escuela DO**, Couté J, Perez Alea M, Ciruela F, Sánchez JC, Garriga P. Impaired multiprotein complex formation and signal transduction of the M3 muscarinic receptor by it soluble third intracellular loop. [Manuscript in preparation].
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