PI3Kγ as a nodal point in μ-opioid receptor signaling

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von
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Dedicated to my father Late Mr. Rajamouli Madishetti

&

mother Mrs. Vijayalaxmi Madishetti

Contents

1. Introduction

1.1 Phosphoinositide 3-kinases (PI3Ks)

Phosphoinositide 3-kinases (PI3Ks) are a group of enzymes that catalyse the transfer of γ phosphate group of ATP to the D3-position of inositol ring of phospotidylinositols (PtdIns), PtdIns-4-phosphate (PtdIns4P) and PtdIns-4,5-biphosphate (PtdIns(4,5)P_2). PI3Ks can be activated by stimulation of cell surface receptors. The D-3 phosphorylated lipids produced by activated PI3Ks bind to PH domain containing signaling mediators that coordinate various signaling events regulating cellular processes like gene expression, protein synthesis, cell growth, metabolism, cytoskeletal rearrangements and vesicle trafficking. The termination of PI3K signaling involves degradation of phosphorylated lipids by lipid phosphatases. The Src-homology (SH2)-domain containing inositol 5-phosphatase type 2 (SHIP2) or inositol polyphosphate-5-phosphatase E (IPP5E) dephopshorylate PtdIns(3,4,5)P_3 at 5'-OH position of the inositol ring converting it to PtdIns(3,4)P_2. Another lipid phosphatase, such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN) dephosphorylates the PtdIns(3,4,5)P_3 at 3-OH position to produce PtdIns(4,5)P_2 (Cantley, 2002; Hawkins et al., 2006; Vanhaesebroeck et al., 2010).

Eight different PI3K catalytic subunits have been identified so far in mammals. On the basis of structural and functional homology, PI3Ks have been classified into three major classes, class I, II and III (Hawkins et al., 2006).

1.2 Class I PI3Ks

Class I PI3Ks are heterodimeric proteins that contain a catalytic subunit of 110 kDa and an associated adapter/regulatory subunits. Four types of closely related catalytic subunits and two distinct families of regulatory subunits were reported so far. Based on the type of regulatory subunits they bind to class I PI3Ks are subdivided into class IA and class IB. The class IA consists of p110α, p10β and p110δ catalytic subunits that bind to p50-55/p85 type of regulatory subunits to form tight dimers. Class IB consists of a dimer of catalytic subunit p110γ and regulatory subunit either of p101 or p84 (also

known as $p87^{PIKAP}$). Basically they phosphorylate PtdIns(4,5)P_2 to produce PtdIns(3,4,5)P_3 in vivo (Hawkins et al., 2006).

1.2.1 Class IA PI3Ks

Class IA PI3Ks consists of 3 catalytic isoforms – p110α, p110β and p110δ cloned by different groups (Hiles et al., 1992; Hu et al., 1993; Vanhaesebroeck et al., 1997). Human cells contain three genes *PI3KCA, PI3KCB, PI3KCD* that encode the catalytic subunits of class IA PI3K enzymes termed PI3Kα, PI3Kβ and PI3Kδ, respectively. The isoforms p110α and p110β are expressed ubiquitously whereas p110δ is expressed predominantly in leukocytes. They bind to regulatory subunit of p50-85 kDa, which has multiple isoforms-p85α, p85β, p55α, p50β and p55γ (Vanhaesebroeck et al., 2010).

1.2.1.1 Structure

The p110 subunits of class IA PI3Ks have five domains: an N-terminal domain called p-85 binding domain, also called ABD (adapter binding domain) that binds to regulatory subunits of p85 family members, a RAS-binding domain (RBD), a C2 domain that has been proposed to bind to cellular membranes, a helical domain of unknown function, and a kinase catalytic domain. The structure of the catalytic subunits of class I PI3Ks are presented in Fig.1.1 (Vanhaesebroeck et al., 2010).

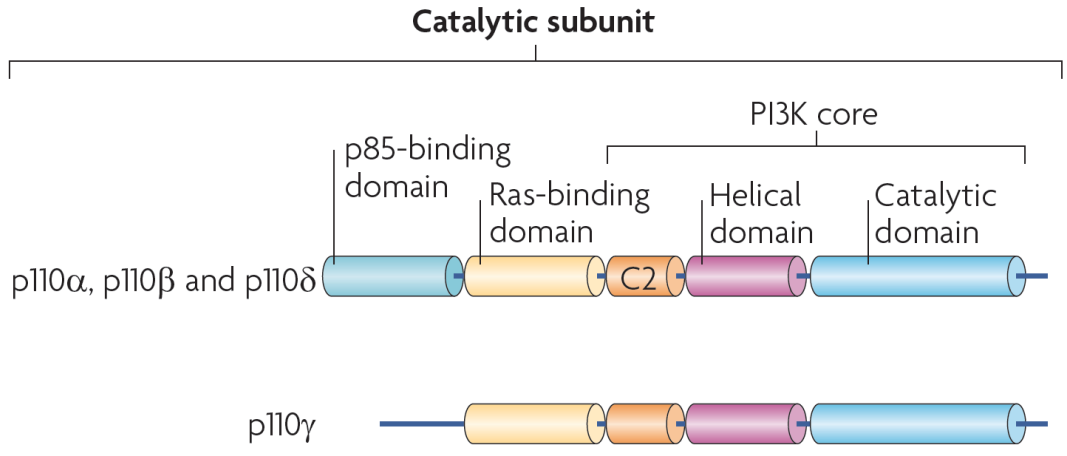

Fig. 1.1: Domain structure of the catalytic subunits of class I PI3Ks (Vanhaesebroeck et al., 2010).

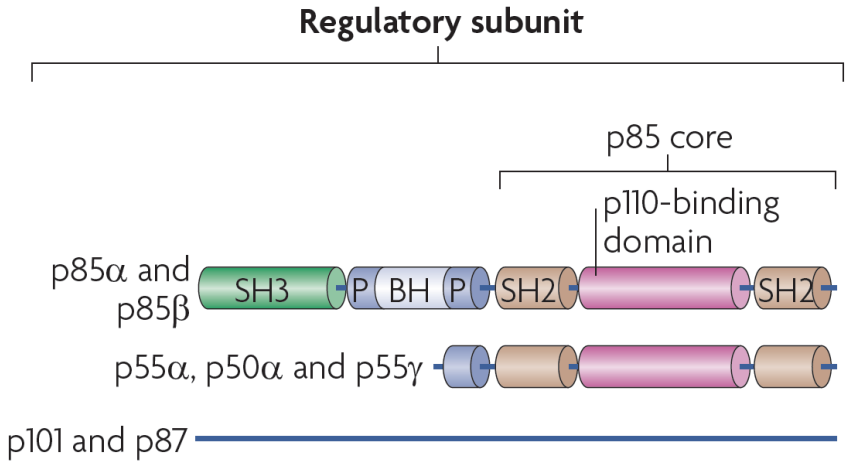

Fig. 1.2: Domain structure of the regulatory subunit of the class I PI3Ks (Vanhaesebroeck et al., 2010) p87 also called as p84 regulatory subunit was found to have two molecular weights (Suire et al., 2005; Voigt et al., 2006).

1.2.1.2 Regulatory subunits

The regulatory subunits of class IA PI3Ks are collectively referred to as p85 and are encoded by three genes in humans called as *PI3KR1*, *PI3KR2* and *PI3KR3*. *PI3KR1* encodes p85α, p55α and p50α, while *PI3KR*2 encodes only p85β and *PI3KR3* encodes only p55γ. The regulatory subunit p85 also contains five domains: an N-terminal SH3 domain, proline–rich region and BCR homology (BH) (breakpoint cluster region homology) domain, and two SH2 domains (the more N-terminal nSH2 and the C-terminal cSH2) separated by an iSH2 domain or p110-binding domain, which is 10 nm – long rigid coil (Wu et al., 2007) which is responsible for binding to the catalytic subunit at ABD site. In total, mammals have 5 distinct p85 isoforms potentially giving rise to fifteen distinct p85-p110 combinations. The amino acid sequences of p110β and p110δ are 55 % identical but they are only 40 % identical to p110α, which is more frequently found to be mutated in many cancers. The interactions of ABD and kinase domain of p110α are distinct from that of p110β or p110δ and mutations in these regions are more frequent (Amzel et al., 2008; Vanhaesebroeck et al., 2010).

Class IA PI3Ks are activated by receptor tyrosine kinases. Regulatory subunits translocate the catalytic p110 subunits to the plasma membrane where their substrate PtdIns(4,5)P_2 is located. SH2 domains of the p85 regulatory subunits direct the PI3K

complexes to phosphorylated Tyr-X-X-Met motifs (where X is an amino acid) on protein tyrosine kinases or their substrates (Wymann et al., 2003). Interaction between p85 and p110 catalytic subunit regulate their enzymatic activity (Huang et al., 2007; Miled et al., 2007). Lipid kinase activity of these enzymes catalyze the phosphorylation of the PtdIns(4,5)P_2 to produce PtdIns(3,4,5)P_3 which then bind to PH domain containing effector proteins including Ser/Thr protein kinases (AKT), Tyr protein kinase (BTK), adapter proteins like GAB2 and regulators of small GTPases like GAPs and GEFs, which mediate large range of cellular processes such as proliferation, survival, cytoskelatal remodeling and membrane trafficking. Five distinct p85 isoforms were recognized. They can bind non-specifically to any of the catalytic subunits, in vitro although their binding preference to distinct p110 catalytic units in vivo needs further understanding (Vanhaesebroeck et al., 2010). Genetic inactivation of p85 regulatory subunits has differential roles. Genetic deletion of p85α, by deletion of exon 1 of *PI3KRI*, resulted in attenuation of PI3K function downstream of B cell receptor (Fruman et al., 1999; Suzuki et al., 1999). Loss of P85α or p85β resulted in increased insulin signaling (Ueki et al., 2002).

1.2.2 Class IB PI3K-PI3Kγ

PI3Kγ was cloned in our laboratory (Stoyanov et al., 1995). It is encoded by *PIK3CG* (Hirsch et al., 2000a). It consists of a dimer of p110γ catalytic subunit and p101 (Stephens et al., 1997) or p84/87 (Suire et al., 2005; Voigt et al., 2006) regulatory subunit. Expression of P110γ has been thought to be limited to leukocytes (Hirsch et al., 2000b) but recent studies reported its expression not only in white blood cells but also in mastocytes (Bohnacker et al., 2009), endothelial cells (Puri et al., 2005), airway smooth muscle cells (Jiang et al., 2010), heart (Patrucco et al., 2004), brain (Narita et al., 2004) and neuronal cells (Cunha et al., 2010; König et al., 2010).

1.2.2.1 Structure

The crystal structure of PI3Kγ revealed that it contains defined protein modules starting from N terminal, consists of RAS binding domain, lipid binding C2 domain, helical

(PI3K homology) domain and the ATP binding kinase domain (Pacold et al., 2000; Walker et al., 2000) (Fig. 1.1 & 1.3).

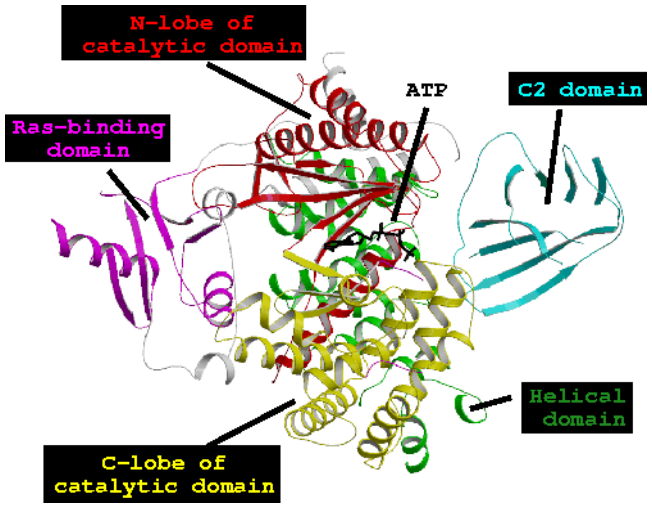

Fig. 1.3 The ATP binding site of PI3Kγ enzyme. The structure of the PI3Kγ enzyme catalytic subunit consists of a helical domain with ATP binding site, C2 catalytic domain, Ras-binding domain, N and C terminal catalytic domain (Walker et al., 2000).

1.2.2.2 Regulatory subunits

The p110γ catalytic subunit binds to the adapter/regulatory subunits p101 or its homolog p84 or $p87^{PIKAP}$ which have distinct sequences (Fig. 1.2). *PI3KR5* encodes p101 subunit whereas *PI3KR6* encodes p84/87 subunits. The nomenclature p84 or p87 was because the protein was found to have different molecular weights. The regulatory subunits bind p110γ upon stimulation of Gi subtype of G-protein coupled receptors (GPCRs) to form two types of complexes, namely, p110γ:p84 complex and p110γ:p101 complex. The non-redundant functions regulatory subunits were reported recently. Upon adenosine receptor stimulation binding of p84 to p110γ is required to produce PtdIns(3,4,5)P_3, phosphorylation of AKT, migration of cells and synergistic adenosine enforced degranulation of mast cells. In addition, PtdIns(3,4,5)P_3 pools produced by p110:p101 complex were rapidly endocytosed to motile microtubule-associated vesicles distinct from the PtdIns(3,4,5)P_3 pools produced by p84:p110γ complex. Moreover, p84:p110γ signaling was essential for full scale degranulation of mast cells as the

reconstitution of PI3Kγ null cells with p110γ and p101 was unable to restore this function (Bohnacker et al., 2009).

PI3Kγ is activated by Gi subtype of GPCRs. Its interaction with Gβγ subunits of heterotrimeric G-proteins result in activation of MAPK upon stimulation of muscarinic-m2 receptors was identified in our laboratory(Lopez-Ilasaca et al., 1997). Further, the direct interaction between Gβγ subunits and N, C terminal sites of p110γ was reported (Leopoldt et al., 1998). Later on, the dual kinase activities of p110γ were deduced, indicating that the protein kinase activity was responsible for the activation of MAPK whereas lipid kinase activity produces PtdIns(3,4,5)P_3 subsequently activating protein kinase B (AKT). In their experiments, Bondeva et al., showed that replacing the catalytic core of PI3Kγ with that of class II PI3Ks or calss III PI3Ks or FRAP (a member of the target of rapamycin family without assigned lipid kinase activity) affected the lipid kinase activity as measured by their ability to phosphorylate different phosphatidylinositol substrates. Moreover, the ability of these constructs to phosphorylate the MAPK was not affected suggesting the protein kinase activity was unaltered. Further, they reported that the PI3Kγ dependent phosphorylation of MAPK was not affected by mutations in RAS binding site of PI3Kγ (Bondeva et al., 1998).

1.3 Structural similarities of p110α and p110γ

The Ras binding domain (RBD) of free structure of PI3Kγ has a mobile loop. Residues of 255-267 within RBD of p110γ constitute this mobile loop i.e. which are not ordered and binding of Ras results in proper ordering of this mobile loop (Pacold et al., 2000). In case of p110α this loop is ordered and is in a distinct conformation from that of p110γ-RAS structure. Part of this mobile loop in p110α is locked in the ATP binding site of the kinase domain of a neighboring molecule in the p110α niSH2 crystal. Pacold et al., based on the comparison of structures of free p110γ and p110γ-RAS complex, suggested that in a Ras-p110γ complex the C2 domain and kinase domain were spread apart due to an allosteric effect. The binding of p85 to p110α has a similar effect. The C2 domain of p110α interacts with the iSH2 domain of p85α. One of the residues responsible for such interactions is Asn 345 of C2 domain of p110α is situated on a loop called CBR1. There is no sequence similarity between CBR1 regions of p110γ and

p110α. The CBR1 region of p110γ is incompatible to binding with p85 because it would clash with iSH2 domain. This explains why p110γ binding required another regulatory subunit p101. Mutations in the helical domain of p110α most commonly seen are at the residues E542K, E545K. E545 is conserved in all class IA PI3Ks whereas it is replaced by Ala in p110γ. The kinase domains of p110α and p110γ represent the most conserved residues in the two proteins. Helix αK12 in p110α and helix kα11 in p110γ, constitute one of the most divergent features of the two structures. The His 1047 Arg in helix αK12 in p110α undergoes oncogenic mutations. This residue might form a hydrogen bond with Leu956. The corresponding residues in p110γ are Arg 1076 and Leu987 indicating that this region is conserved in both the structures but they did not interact with each other as in the case of p110α, which means no oncogenic mutations were identified in p110γ. The residues that line the ATP binding pockets of p110α and p110γ are highly conserved and have similar three-dimensional structures. The binding of wortmannin forms covalent bond with Lys833 and makes hydrogen bonds with Asp964, Ile963, Val882 and Ser806 in p110γ. All the five residues are present in p110α but the binding of wortmannin to p110α is different. Moreover, the loop between residues 771-799 in p110α adopts a different conformation than the corresponding loop in p110γ (residues 803-811) (Pacold et al., 2000; Amzel et al., 2008).

1.4 G-protein coupled receptors

G-protein coupled receptors (GPCRs) represent the largest family of cell surface molecules involved in signal transmission, accounting for > 2 % of the total genes encoded by the human genome. All GPCRs are essentially composed of seven-transmembrane α-helices that intertwine in and out of the membrane. Upon stimulation by agonists, heterotrimeric intracellular sites containing α, β and γ subunits will be exposed and dissociation of GDP bound to Gα subunit and its replacement by GTP takes place. This leads to the dissociation of Gα and Gβγ subunits representing a functional dimer. Both Gα and Gβγ subunit complexes then stimulate several downstream effectors independently (Fig. 1.4) (Dorsam and Gutkind, 2007). GPCRs receive external signals from hormones, neurotrnasmitters, chemokines, paracrine and autocrine factors and relay them inside the cells via G-proteins initiating several

interconnecting signal transduction pathways and effecting many cellular processes like transcription, motility, contractility, secretion, cell proliferation, growth and differentiation (Neves et al., 2002).

The α-subunits of G-proteins are divided into four subfamilies: Gαs, Gαi Gα12, and Gαq. A single GPCR can bind several Gα proteins. Each G-protein activates several downstream effectors. Typically, Gαs stimulates adenylyl cyclase and increases intracellular cAMP levels, whereas Gαi inhibits adenylyl cyclase and decreases intracellular cAMP levels. Members of Gαq family bind to and activate phospholipase C which cleaves PtdIns(4,5)P_2 into diacylglycerol (DAG) and inositol triphosphate (IP3) (Fig. 1.4). In addition, β and γ subunits were more diverse, there are 5 β subunits (highly similar sequence), 12-14 γ subunits (more diverse). Gβγ subunits function as a dimer to activate many signaling molecules including phospholipases, ion channels and lipid kinases (Dorsam and Gutkind, 2007), of particular importance to mention, is its binding to p110γ and subsequent activation of MAPK (Lopez-Ilasaca et al., 1997).

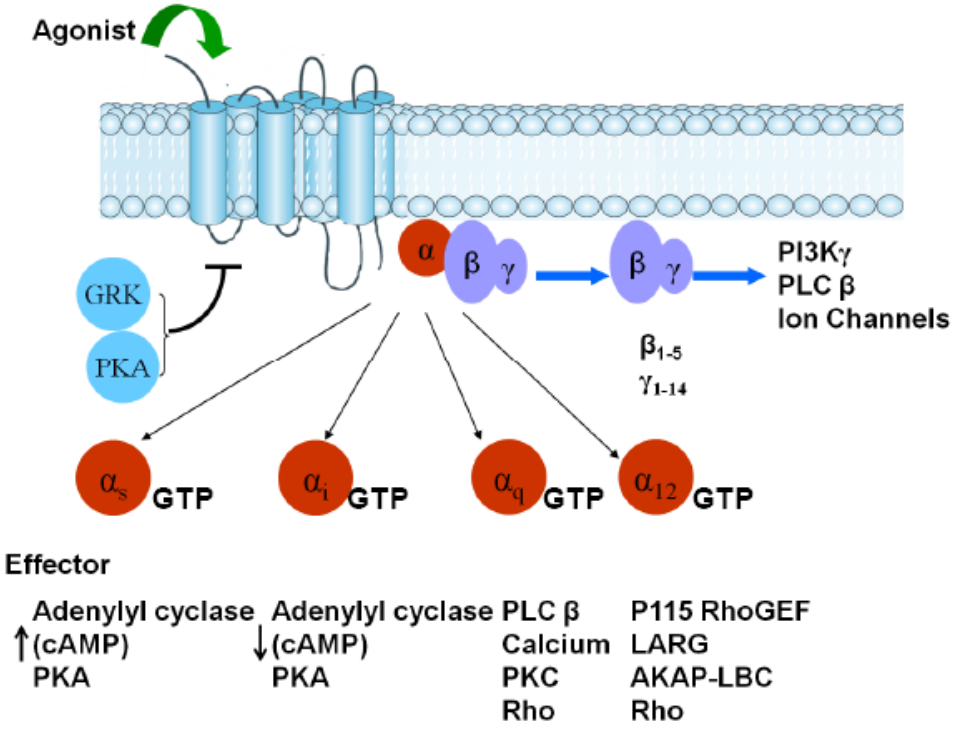

Fig. 1.4: Signaling via G-protein coupled receptors. Upon agonist stimulation GTP attaches to the Gα-subunit which then dissociates itself from Gβγ subunits. Gα and Gβγ are functional dimers that can signal independently. Four types of Gα-subunits mediate different signaling pathways. GRK, PKA phosphporylate the C-terminus of GPCR leading to uncoupling of GPCR from G-protein and termination of GPCR signaling (Modified from (Dorsam and Gutkind, 2007)).

GPCRs, in general, can couple to more than one type of G-protein. This could happen following phosphorylation of the receptor or chronic stimulation with the agonist. β_2-adrenergic receptors (β_2-AR) classically reported to be coupled to Gs type of proteins. Agonist stimulation of β_2-AR leads to increased adenylyl cyclase activation, rising intracellular cAMP levels and associated PKA levels. Daaka et al., reported the activation of MAPK following isoproterenol stimulation of HEK293 cells expressing β_2-AR. Interestingly, H-89 which inhibits PKA could inhibit the isoproterenol induced activation of MAPK. The authors indicated that PKA can phosphorylate the β_2-AR which then switches its coupling to Gi-protein resulting in non-conventional, pertussis toxin (PTX) sensitive inhibitory signal propagation via non-receptor tyrosine kinase C-Src and the G-protein RAS dependent activation of MAPK (Daaka et al., 1997).

Zamah et al., generated two types of mutant β_2-AR with mutations at consensus PKA binding site one containing alanine (Ala) substituted for serine (Ser) and another one containing Ser substituted with aspartate (Asp). They reported that mutant β_2-AR with Ala substituted for Ser at consensus PKA sites was able to activate adenylyl cyclase but not ERK where as the mutant β_2-AR with Ser substituted with Asp was able to activate adenylyl cyclase and ERK also. In the latter case, the activation of adenylyl cyclase was via a PTX insensitive pathway whereas activation of ERK was abolished by PTX suggesting that this mutant receptor was able to couple to Gi proteins. Further investigations carried out in completely reconstituted system of wild type or PKA site mutant β_2-AR, heterotrimeric Gs and Gi protein, indicated that β_2-AR with the PKA site mutants show reduced phosphorylation by PKA. Moreover, PKA mediated phosphorylation of purified wild type β_2-AR, in vitro, leads to reduced binding to Gs protein and increased binding to Gi protein upon stimulation with isoproterenol as measured by the G-protein activation by its ability to bind [^{35}S] GTPγS (Zamah et al., 2002).

1.4.1 μ-Opioid Receptor

μOR is a GPCR that signal by catalyzing the ligand-dependent nucleotide exchange on Gi and Go proteins. They act by inhibiting adenylyl cyclase, inhibiting N-type calcium channels and activating G protein-gated inwardly rectifying potassium (GIRK) – type

potassium channels (Whistler et al., 1999). Different endogenous peptides act as agonists at μOR. They included β-endorphine which is a non-selective agonist whereas endomorphin1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin2 (Tyr-Pro-Phe-Phe-NH_2) are highly selective for μOR (Zadina et al., 1997). External agonists of μOR consists of a class of analgesics called opioid analgesics acts predominantly on μOR (along with other opioid receptors) to exert their main physiological actions. Morphine, an opioid alkaloid, is one of the drugs used to treat moderate to severe pain acts as an agonist at μOR. Etonitazine, etorphine, methadone are some of the other opioid analgesics with relatively different levels of capabilities to produce analgesia, tolerance development and addiction/physical dependence (Martini and Whistler, 2007). Inhibition of adenylyl cyclase mediated by morphine or DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol, a synthetic peptide, μ-opioid receptor specific agonist) does not require full receptor occupancy (Chavkin and Goldstein, 1984; Costa et al., 1992). However, opioid mediated activation of inwardly rectifying potassium channel currents requires higher agonist occupancy at the receptor (Kovoor et al., 1998).

μOR is a GPCR that is classically known to bind to Gi proteins causing inhibition of adenylyl cyclase upon acute stimulation with agonists. However, chronic agonist stimulation of μOR causes super activation of adenylyl cyclase leading to abnormally higher cAMP levels. This phenomenon serves as a hallmark of addiction/withdrawal syndrome following chronic morphine/opiate treatment (Avidor-Reiss et al., 1996). The mechanistic basis of such initial inhibition followed by stimulation might involve signal switching from inhibitory type of G-proteins to stimulatory type of G-proteins. Several attempts to understand the molecular mechanisms involved in μOR signal switch were reported. Zhang et al., reported that association of Src kinase and μOR within the lipid raft domains and subsequent phosphorylation of μOR at Tyr336 by Src kinase leads to switching of its coupling to a stimulatory Gi2α subunit (Zhang et al., 2009). Chakrabarti et al., reported the association of μOR with Gsα protein. In their studies they showed that in Chinese hamster ovary (CHO) cells stably expressing μOR, the immunoprecipitates obtained with anti-Gsα antibodiy contain μOR coimmunoprecipitated with Gsα protein. The authors also reported that acute and chronic morphine treatment increased the magnitude of μOR coimmunoprecipitated with Gsα (Chakrabarti et al., 2005). Rozenfeld et al., reported that heterodimerization of

μOR with δOR leads to the switch in signaling with respect to ERK1/2 activation. In their studies with CHO cells expressing either μOR alone or μOR and δOR together, they demonstrated that stimulation with DAMGO resulted in differential patterns of phosphorylation of ERK1/2. CHO cells expressing μOR alone showed a peak phosphorylation of ERK at 5 min whereas the CHO cells expressing both μOR and δOR together, at high heterodimeriaztion states, showed a first peak at 3 - 5 min and a second peak at 15 min. SKNSH cells expressing heterodimers of μOR and δOR endogenously also showed similar behavior. Moreover, the second peak could be blocked by knockdown of β-arrestin2 or treating the SKNSH cells with a combination of μOR agonist and δOR antagonist suggesting that the effect was indeed due to heterodimerization of μOR and δOR. The authors concluded that heterodimerization of μOR and δOR leads to a constitutive recruitment of β-arrestin2 to the receptor complex regulating the ERK1/2 activation and subsequent signaling (Rozenfeld and Devi, 2007).

1.4.3 Desensitization and tolerance development

Desensitization is a process wherein sustained stimulation of the GPCR leads to diminished receptor responsiveness over time such that the receptor no longer responds to the agonist stimulation. Desensitization of GPCRs begins within seconds of agonist stimulation and involves phosphorylation of the receptor. Second messenger-regulated protein kinases like PKA/PKC phosphorylates the receptor at serine/threonine residues within cytoplasmic loops and C-terminal domains. This phosphorylation leads to abrogation of further GPCR-G-protein coupling. Phosphorylation of a receptor other than that stimulated could also happen, as the receptor phosphorylation is independent of agonist occupancy at the receptor, leading to heterologous desensitization. In this way receptors for different ligands can be affected simultaneously. Homologous desensitization is a two step process involving phosphorylation of the receptor by GRKs (GPCR kinases) and subsequent binding of β-arrestins leading to uncoupling of receptor–G-protein complex. Agonist occupancy is a prerequisite for homologous desensitization and endocytosis of the receptor-β-arrestin complex might occur in many cell types (Smith and Luttrell, 2006).

Antinonciceptive tolerance development following repeated administration of morphine is a peculiar problem that limits the clinical use of such an excellent pain relieving drug. Tolerance development to morphine induced analgesia is attributed mainly to cellular and molecular adaptations occurring following stimulation of μOR, because morphine predominantly acts on this receptor to elicit its analgesic activities. μOR knockout mice did not show morphine induced antinonciception, respiratory depression inhibition of gastrointestinal transit and addiction (Matthes et al., 1996; Sora et al., 1997; Bohn et al., 2003).

Molecular mechanisms involved in development of tolerance are quite complex and involves multiple signaling mediators and pathways. In a model of thermal antinonciception Bohn et al., showed that β-arrestin-2 knockout mice showed enhanced and prolonged antinonciception compared to wild type mice following short term and long–term treatment with morphine (Bohn et al., 1999; Bohn et al., 2000).

Internalization of μOR following acute agonist stimulation leading to removal of μOR from the cell surface (plasma membrane) and desensitization (uncoupling of receptor from G-protein) following chronic agonist stimulation might lead to tolerance development (Hsu and Wong, 2000). At signal transduction level, the mechanisms that contribute to the development of tolerance reported include phospolipase C mediated activation of NMDA (N-methyl-D-aspartate) receptor and nitric oxide synthase (NOS) leading to antiopioid effects (Hsu and Wong, 2000). Further, involvement of PKC in desensitization of μOR also was reported (Bailey et al., 2009).

μOR presents a peculiar phenomenon that it exhibits different grades of internalization and desensitization following stimulation with different agonists. For example Schulz et al., showed that in μOR expressing HEK293 cells, DAMGO induces rapid and robust phosphorylation and internalization of μOR whereas morphine induces negligible levels of phosphorylation and internalization. Moreover, the desensitization of μOR caused by chronic treatment with DAMGO is reversible whereas that produced by morphine is irreversible in this cell culture system (Schulz et al., 2004).

Whistler et al., reported that in μOR expressing HEK293 cells, the rank order of selected agonists for inducing endocytosis was DAMGO = methadone >> morphine which means DAMGO and methadone causes similar levels of endocytosis of μOR

whereas morphine causes negligible endocytosis. They also reported that other potent agonists like etorphine caused rapid endocytosis whereas mixed agonist/antagonist buprenorphine caused negligible receptor endocytosis. Further, GIRK–type potassium channels were over expressed in the HEK293 cells over expressing μOR and the ability of various agonists to activate these channel currents was tested to establish the rank order of the agonists wherein DAMGO and morphine produces similar levels of receptor-mediated activation of GIRK which is significantly greater than the GIRK activation produced by methadone (i.e. DAMGO ≥ Morphine >> methadone). The **r**elative **a**ctivity (as measured by the normalized current) **v**ersus **e**ndocytosis (RAVE) caused by various agonists were compared to reveal that when DAMGO has a RAVE value of 1, morphine has 3.8 (Whistler et al., 1999) which served as a guide to understand the ability of various agonists to cause tolerance development. Koch et al., demonstrated that the potency of a μ-opioid receptor agonist to induce receptor endocytosis is negatively correlated with its ability to cause development of tolerance in a cellular model (Koch et al., 2005). In line with this, Grecksch et al., demonstrated that in a rat model, buprenorphine and morphine which are known to cause very less or negligible internalization produced higher tolerance than etonitazine which is known to cause high internalization of μOR. They measured the antinonciceptive effects of these drugs at equieffective doses in a hot plate method and electric tail root stimulation method of nonciceptive stimulus (Grecksch et al., 2006).

The phenomenon, wherein, different agonists acting on same receptor activates different downstream signaling pathways and different cellular responses is termed as functional selectivity or biased agonism or collateral efficacy. One of the explanations for such behavior could be that the physical interaction between the receptor and agonist will have conformational constraints leading to preference for a signaling pathway over other pathways (Kenakin, 2007, 2009).

1.5 Functions of PI3Kγ

PI3Kγ is classically known to be activated by Gi protein coupled GPCRs. Hence, cellular functions of PI3Kγ mostly involved stimulation of various GPCRs in respective cells. Generation of PI3Kγ knockout mice largely enabled the studies to reveal its

function in vivo. Mice lacking PI3Kγ grow normally and they are fertile but when their immune system is stressed show clear-cut abnormalities (Hirsch et al., 2000b).

1.5.1 Immune system related functions

PI3Kγ is predominantly expressed in hematopoietic cells i.e. white blood cells – neutrophils, macorphages, T-cells and B- cells. N-formyl-Met-Leu-Phe (*f*MLP), a bacterial peptide, stimulates biphasic production of ROS (reactive oxygen species) in tumor necrosis factor-α (TNF-α)–primed human neutrophils. The initial phase of this biphasic response that occurs within 10 sec of exposure of *f*MLP is dependent on PI3Kγ as measured by the production of PtdIns(3,4,5)P_3 (Condliffe et al., 2005). This initial phase of response is important for the second burst of ROS production that is largely governed by p110δ (Condliffe et al., 2005). Neutrophils and macrophages derived from PI3Kγ null mice exhibits severely impaired chemotaxis in response to various GPCR-binding chemo-attractants like chemokines, *f*MLP, complement fragment C5a, in vitro. In vivo, in a mouse model of peritonitis, induced by injection of Gram-positive or Gram-negative bacteria, reduced accumulation of macrophages and reduced clearance of bacteria from peritoneal cavity observed in PI3Kγ null mice compared to wild type mice (Hirsch et al., 2000b). In a model of lung sepsis, broncho-alveolar leukocyte (neutrophils) recruitment required PI3Kγ (Yum et al., 2001) establishing the role of this class IB isoform in innate immunity and inflammation. Catalytic activity of p110γ is required for the neutorphil chemotaxis as the catalytically inactive PI3Kγ show chemotactic defect which is similar to that observed in the absence of PI3Kγ. The lipid kinase activity defines the direction of cell migration thus implying that PI3Kγ is required for directional sensing and function as a compass for inflammatory cells (Rickert et al., 2000).

GPCR mediated signals that cause apoptosis and differentiation of T cells in CD4+/CD8+ helper or effector cells are dependent on PI3Kγ (Sasaki et al., 2000; Rodríguez-Borlado et al., 2003). Thymus development is severely reduced in mice lacking PI3Kγ although another PI3K isoform, PI3Kδ found to govern the B cell maturation and function (Clayton et al., 2002) suggesting the role of PI3Kγ in adaptive immunity. Mastocytes are the first line of defense against invading pathogens releasing

many proinflammatory and vasoactive agents like histamine. Abnormal activity of mast cells was found in cases of allergy and asthma. Mast cell activation, triggered by antigen induced cross-linking of IgE immunoglobulin with FcεRI receptor triggers degranulation of mast cells, the initial phase of this event is controlled by PI3Kδ, whereas full degranulation of the mast cells required PI3Kγ (Sasaki et al., 2000; Laffargue et al., 2002; Ali et al., 2004; Hirsch et al., 2006). Essentially, PI3Kγ and δ plays a non-redundant, sequential role in mast cell activation.

1.5.2 Vascular functions

Expression of PI3Kγ in endothelial cells was reported earlier (Puri et al., 2005). Spingosine-1-phosphate (S1P), participates in endothelial cell migration and angiogenic differentiation by acting on Gi coupled receptor S1P1 and Gi/Gq/G12 coupled S1P3 receptors. Heller et al., reported that PI3Kγ and PI3Kβ isoforms are involved in this signaling. The S1P induced phosphorylation of AKT was mediated predominantly by PI3Kβ isoform whereas the endothelial migration mediated via activation of Rac1 which in turn dependent on both the isofroms PI3Kβ and PI3Kγ (Heller et al., 2008).

Vecchione et al., reported the expression of p110γ in mouse aortic smooth muscle cells. Angiotensin II (AngII) is a peptide that acts as an endogenous agonist of GPCRs named AT1A in smooth muscle cells causing vasoconstriction. PI3Kγ is required for the AngII mediated activation of Rac and the subsequent triggering of ROS production in vascular smooth muscle cells. In addition, AngII–evoked Ca^{2+} flux is impaired in vessels from PI3Kγ knockout mouse. PI3Kγ mediated phosphorylation of AKT in these cells governs the L-type Ca^{2+} channel-mediated extracellular Ca^{2+} entry. Taken together these two signaling events protect PI3Kγ knockout mouse from AngII induced hypertension (Vecchione et al., 2005). In rat portal veins, PI3Kγ can be activated by β1γ3 dimer of AT1A receptor upon stimulation with Ang II (Viard et al., 1999) as evidenced by blockade of production of PtdIns(3,4,5)P_3 and calcium influx by antibodies inhibiting PI3Kγ (Quignard et al., 2001).

PI3Kγ is expressed in airway smooth muscle cells of mouse trachea and lung. Acetylcholine (ACh) stimulated contraction of cultured precision–cut lung slices was mediated by PI3Kγ as evidenced by its inhibition by PI3Kγ specific inhibitor.

Moreover, Jiang et al., also showed that other PI3K isoforms do not inhibit ACh induced contraction of smooth muscle cells of lungs. At the cellular level, pharmacological inhibition of PI3Kγ inhibited the ACh induced Ca^{2+} oscillations in individual airway smooth muscle cells from mouse lung slices that are essential for sustained contraction (Jiang et al., 2010).

Platelet aggregation might be dependent on PI3Kγ, at least partially. Mouse platelets lacking PI3Kγ exhibit only partial aggregation defect. Generally, platelets contain Gαi as well as Gαq type of GPCRs. These receptors can be stimulated by agonists ranging from thrombin to thromboxane and ADP. PtdIns(3,4,5)P_3 production stimulated by P2Y12 (purinergic P2Y, G-protein coupled, 12) receptors which are coupled to Gi-protein was PI3Kγ dependent whereas PtdIns(3,4,5)P_3 production by P2Y1 receptors that are coupled to Gq-protein was not dependent on PI3Kγ. ADP stimulation of PI3Kγ null platelets causes weak but significant reduction of fibrinogen binding along with weak aggregation defect and thromboembolism which might have been mediated by Rap1B, a small GTPase. In addition, p110γ knockout platelets show increased cAMP levels (Hirsch et al., 2001; Hirsch et al., 2006).

Phenotypic differences between PI3Kγ knockout and PI3Kγ kinase dead versions were reported in case of endothelial progenitor cells (EPCs). PI3Kγ kinase dead mice develop vascular collaterals whereas PI3Kγ knockout mice present impairment in post–ishemic hind limb angiogenisis indicating a kinase-independent function of PI3Kγ (Madeddu et al., 2008).

1.5.3 Cardiac Functions

In cardiomyocytes, the platelet activating factor (PAF) induced negative inotropism is dependent on PI3Kγ. Binding of PAF to Gi-coupled GPCR in these cells induces activation of PI3Kγ followed by PtdIns(3,4,5)P_3 production and activation of AKT resulting in subsequent stimulation of NO synthesis (Alloatti et al., 2003) and negative inotropism. Both lipid kinase and protein kinase activities of p110γ play pivotal roles in β-adrenergic receptor endocytosis. PIK domain of P110γ interacts with β-ARK1 (β-adrenergic receptor kinase-1) and gets targeted to agonist occupied receptor triggering

localized production of PtdIns(3,4,5)P_3 for the organization of clathrin-coated pits driving receptor internalization (Naga Prasad et al., 2005).

Differences in phenotypes of PI3Kγ knockout and PI3Kγ kinase dead versions revealed scaffolding functions of p110γ. Transverse aortic constriction leads to cardiac pressure overload, subsequent cardiac remodeling and heart failure. In response to transverse aortic constriction, PI3Kγ kinase dead mice developed similar adaptive responses as of control mice whereas PI3Kγ knockout mice developed rapid, fatal cardiac myopathy. Cardiac contractility is affected by cAMP concentrations and PDE3B controls the local concentrations of cAMP by accelerating its degradation. p110γ binds to PDE3B and increases its phosphodiesterase activity leading to increased hydrolysis of cAMP, negatively regulating the cardiac contractility (Patrucco et al., 2004). In addition, cardiac myocytes from PI3Kγ knockout mice show increased cAMP levels whereas cardiac myocytes from PI3Kγ kinase dead mice and control mice show similar cAMP levels. IBMX treatment of cardiac myocytes from PI3Kγ kinase dead mice and control mice show increased cAMP levels that are comparable to the cAMP levels in cardiac myocytes from PI3Kγ knockout mice. Further, Patrucco et al., showed that the phosphodiesterase activity of PDE3B in immunoprecipitates from cardiac extracts of PI3Kγ knockout mice was reduced compared to that of PI3Kγ kinase dead or PI3Kγ wild type cardiac extracts. Taken together, they indicated that PI3Kγ interacts with PDE3B and regulates intracellular cAMP levels through kinase-independent functions (Patrucco et al., 2004). Subsequently, Perino et al., showed that p110γ forms a macro molecular complex tethering together p84/87-PKA-PDE3B. This association leads to phosphorylation of p110γ and PDE3B by PKA giving rise to increased phosphodiesterase activity of PDE3B and reduced lipid kinase activity of p110γ. This further demonstrates the scaffold functions of PI3Kγ in cardiomyocytes connecting cAMP-AKT pathways (Perino et al., 2011).

Cardiomyocytes lacking PI3Kγ show higher basal cAMP levels and higher basal intracellular Ca^{2+} densities. Forskolin induced activation of adenylyl cyclase is unaltered in wild type and PI3Kγ knockout cardiomyocytes suggesting that PI3Kγ does not affect adenylyl cyclase directly. Specific inhibition of PDE3B by milrinone or general inhibition of phosphodiesterases by IBMX increases the L-type calcium currents in wild type myocytes compared to PI3Kγ knockout myocytes, in basal

conditions. Moreover, lack of p110γ increases the sensitivities of myocytes to β-adrenergic receptor stimulation. Isoproterenol treatment of adult mouse ventricular myocytes increases the L-type calcium currents significantly higher in wild type cells compared to PI3Kγ knockout cells. This increase is further augmented by milrinone treatment only in wild type cells but not in PI3Kγ knockout cells. Taken together, these data suggests that phosphodiesterase activity of PDE3B is regulated by PI3Kγ in basal conditions as well as after β-adrenergic receptor stimulated conditions (Marcantoni et al., 2006).

1.5.4 Neuronal functions: Involvement of PI3Kγ in μOR mediated signal transduction

PI3Ks play pivotal role in various neuronal functions affecting neuronal plasticity, long-term potentiation (LTP), learning and memory (Opazo et al., 2003; Horwood et al., 2006; Ran et al., 2009). PI3K isoforms show different expression patterns in various parts of the brain suggesting involvement of specific isoforms in specialized neural functions, although experimental evidence is largely required to confirm this speculation. Towards this end, recent reports suggested involvement of PI3Kγ in NMDAR (N-methyl-D-glutamate aspartate receptor) dependent long-term depression (LTD) and behavioral flexibility. In their studies Kim et al., showed the differential expression of PI3K isoforms. PI3Kγ was detected in crude synaptosomal membranes and synaptosomal fractions whereas P110α was detected in crude cytosolic S2 fraction. In line with their differential expression pattern PI3Kγ selective inhibitor inhibited homosynaptic NMDAR LTD whereas PI3Kα selective inhibitor did not. Thus they reported that PI3Kγ was specifically involved in induction phase of NMDAR LTD as evidenced by its selective impairment in PI3Kγ knockout mice. At signal transduction level it was shown that NMDA induced p38 MAPK activation was impaired in PI3Kγ knockout mice compared to wild type mice. In hippocampal slices, NMDA stimulation of NMDA receptor leads to Gβγ independent, PI3Kγ dependent activation of p38 MAPK and it was reported as the possible mechanism of involvement of PI3Kγ in impairment of homosynaptic NMDA receptor LTD (Kim et al., 2011).

Expression of PI3Kγ in mouse olfactory epithelium was reported by Brunert et al., recently. They showed that PtdIns(3,4,5)P_3 production was abolished in olfactory receptor neurons (ORNs) from PI3Kγ knockout mice compared to ORNs from wild type mice, in response to odorant Henkel 100 stimulation. This suggests the involvement of PI3Kγ dependent signaling in olfactory neurons (Brunert et al., 2010).

Narita et al, reported the molecular evidence of activation of PI3Kγ by stimulation of μOR. In their experiments they showed the expression of PI3Kγ in mouse periaquiductal gray (PAG) at plasma membrane and cytoplasm. In addition, they showed the co-localization of PI3Kγ with μOR at the plasma membrane (Narita et al., 2004). They also reported that single subcutaneous injection of morphine could result in two fold increase in the immunoreactivity of PI3Kγ at the plasma membrane in mouse PAG. Moreover, the higher immunoreactivity of phosphorylated PLCγ1 (phospholipase Cγ1) was noticed and interestingly, this was co-localized with PI3Kγ immune reactivity at the plasma membrane. Since, PLCγ1 is known to contain PH domain and an effector protein of PtdIns(3,4,5)P_3 (Falasca et al., 1998), it might suggest possible involvement of PI3Kγ- PtdIns(3,4,5)P_3-PLCγ in morphine induced antinonciceptive effects. In in vivo experiments they found that intracerebroventricular injection of wortmannin or LY294002 in mice inhibited the morphine evoked antinonciceptive effects as evidenced by rightward shift of dose-response curves in a warm plate test (Narita et al., 2004).

Opioid analgesics are excellent pain relieving agents that acts predominantly via μOR which is a GPCR coupled to Gαi/o type of G-protein. Morphine and DAMGO, acting on μOR, can inhibit the Ca^{2+} currents through voltage-gated Ca^{2+} channels (VGCC). This requires direct interactions of Gβγ with Ca^{2+} channels. Long-term administration of DAMGO or morphine can cause desensitization of μOR resulting in abolition of inhibition of Ca^{2+} currents through VGCC in mouse DRG neurons. Chronic DAMGO induced desensitization of μOR was enhanced in PTEN knockout mice DRGs which result in constitutive activation of AKT. Moreover, pharmacological inhibition of PI3K and MAPK also attenuated the chronic DAMGO induced desensitization of μOR in mouse DRGs as measured by Ca^{2+} flux through VGCC (Tan et al., 2003). In line with this, long-term treatment (6 h bath application of DAMGO) produced desensitization of μOR in wild type DRG neurons but not in DRGs from PI3Kγ knockout mice as measured by the Ca^{2+} currents via VGCC (König et al., 2010). Similar behavior was

observed with DRGs from PI3Kγ kinase dead mice. In addition, reconstitution of PI3Kγ knockout DRGs with PI3Kγ lead to restoration of desensitization of μOR as evidenced by the response to acute administration of DAMGO, following long–term treatment with DAMGO to induce desensitization, which was comparable to wild type response. This suggested the requirement of enzymatic function of PI3Kγ for the long–term treatment induced desensitization of μOR. Further, pharmacological inhibition of PKC by bis-indolyl-maleinimide also abolished the DAMGO induced desensitization in wild type DRG neurons. Supporting the above data, administration of repeated escalating doses of morphine over a period of 4 days induced less tolerance development in PI3Kγ knockout mice compared to wild type mice in a tail-flick test (König et al., 2010). Since PKC is classically reported to be involved in desensitization of μOR (Kelly et al., 2008) and PKCα was shown to interact with PK3Kγ (Lehmann et al., 2009) it might be possible that μOR desensitization could be mediated by interactions of PI3Kγ and PKC.

Morphine causes increase in whole cell potassium currents in cultured mouse DRG sensory neurons. This hyperpolarizes the nonciceptive neurons and thus morphine attenuates PGE2 induced depolarization of neurons resulting in antinoncieptive effects. Morphine also stimulates activation of AKT, eNOS (nitric oxide synthase) and subsequently nitric oxide production leading to peripheral analgesia in a mouse model of PGE2 (prostaglandin E2) induced hypernonciception (Cunha et al., 2010).

The mechanism of peripheral analgesia induced by morphine involves on one hand activation of AKT leading to subsequent stimulation of NO synthesis (Cunha et al., 2010) on the other hand depends on cAMP mediated of inhibition of TRPV1 (transient receptor potential vanilloid receptor-1) currents (Endres-Becker et al., 2007). Thus two pathways lead to comparable physiological function.

In general, μOR is a GPCR that classically couples to Gi proteins exhibits signal switch and variable desensitization patterns leading to tolerance development. The molecular mechanisms of these processes are still under investigations by many investigators. PI3Kγ is proved to be involved in GPCR mediated signaling. Increasing evidence is accumulating in the direction of involvement of PI3Kγ in μOR signaling pathways that leads to peripheral analgesia. This motivated us to investigate the role of PI3Kγ in signal transduction mediated by μOR upon stimulation with morphine and forms the basis for our current investigations.

1.6 Aim of the current work

Previous reports indicates that PI3Kγ has kinase-independent activities in heart that exert a negative feed-back control on intracellular cAMP levels affecting myocardial contractility (Patrucco et al., 2004). In cardiomyocytes, the multiprotein complex of p84/87-p110γ-PKA-PDE3B results in phosphorylation of p110γ and PDE3B by PKA affecting the enzymatic functions of p110γ and PDE3B. The lipid kinase activity of p110γ was inhibited and phosphodiesterase activity of PDE3B was stimulated by this complex formation where PI3Kγ functions as an anchoring protein (Perino et al., 2011).

König et al., reported that PI3Kγ knockout mice showed reduced tolerance development in response to repeated morphine administration compared to wild type mice. Further, chronic DAMGO induced desensitization of μOR is mediated by PI3Kγ through its kinase dependent functions (König et al., 2010).

In the light of the discovery of kinase–independent functions of PI3Kγ in case of cardiomyocytes, we wanted to explore the kinase–independent functions of PI3Kγ in neuronal cells following stimulation of μOR with morphine. Hence, in the current work, we explored i) the crosstalk between cAMP – AKT pathways and ii) functioning of PI3Kγ as a nodal point connecting the two pathways following stimulation of μOR with morphine on a functional scale.

In order to study this, we used a model system of SK-N-LO cells (cells with normal levels of PI3Kγ and cells with down-regulated levels of PI3Kγ) expressing μOR and dorsal root ganglion neuronal cells from wild type and PI3Kγ knockout mice. We investigated the effects of altered PKA levels brought about by the activation of adenylyl cvclase and direct inhibition of PDEs or PKA, on morphine induced activation of protein kinase B (AKT) in presence and absence of PI3Kγ.

2. Materials and Methods

2.1 Materials

2.1.1 Plasmids

- pcDNA3.1-HA-mOPRM1 wt encoding mouse μOR plasmid (Kindly provided by Prof. Schultz Institute of Pharmacology and Toxicology, University Hospital, Jena, Germany).
- pLKO.1-puro derivative plasmids carrying shRNA target sequences (as shown in Table 2.1)

Table. 2.1 shRNA plasmid sequences

Plasmid (code)	shRNA sequence	Clone ID (Sigma Aldrich)
PI3Kγ (γ1)	CCGGGCAACCTTTGTTCTTGGAATACTCGAGTATTCCAAGAACAAAGGTTGCTTTTTG	NM_002649.2-3122s1c1
PI3Kγ (γ3)	CCGGGCCCTATCAAATGAAACAATTCTCGAGAATTGTTTCATTTGATAGGGCTTTTTG	NM_002649.2-2774s1c1
PI3Kγ (γ5)	CCGGCCTGTGGAAGAAGATTGCCAACTCGAGTTGGCAATCTTCTTCCACAGGTTTTTG	NM_002649.2-940s1c1
--------- (control)	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT	Lentiviral non targeting control vector (SHP002)

- Lentiviral packaging plasmids MDL, RSV and VSV were kindly provided by C. Stocking, HPI, Hamburg from their laboratory collection.

2.1.2 Cell lines

Cell line	Type	Source
SK-N-LO	Human neuroblastoma cell line, adherent	Childern's University Hospital, Tübingen, Germany (Volker et al., 2010)
HEK293T	Human embryonic kidney cell line, packaging cells, adherent	C. Stocking, HPI Hamburg

2.1.3 Antibodies

- PI3Kγ monoclonal antibody, directed against whole protein PI3Kγ (# 641) (Produced in the Institute of Molecular Cell Biology, CMB, FSU Jena)
- μOR monoclonal antibody (Lupp et al., 2011) (Kindly provided by Prof. Schultz Institute of Pharmacology and Toxicology, University Hospital, Jena, Germany)
- HA (tag) monoclonal antibody (New England biolabs GmbH, Frankfurt, Germany cat. no. 2367)
- Phospho AKT serine 473 antibody (Cell Signaling Technology, Frankfurt, cat. no. 9271S)
- Phosphorylated AKT threonine 308 antibody (Cell Signaling Technology, Frankfurt, cat. no. 9275S)
- Pan AKT antibody (Cell Signaling Technology, Frankfurt, cat. no. 9272S)
- Phospho ERK1/2 monoclonal antibody (Cell Signaling Technology, Frankfurt, cat. no. 9106S)
- Pan ERK1/2 monoclonal antibody (Cell Signaling Technology, Frankfurt, cat. no. 4695S)
- β-actin monoclonal antibody (Sigma, cat. no. A5441)
- Vinculin monoclonal antibody (Biozol, Eching, cat. no. BZL-03106)

- Peroxidase labeled affinity purified antibody to rabbit IgG (H+L) produced in goat (Kirkegaard & Perry laboratories, Weden, cat. no. 074-1506)
- Peroxidase labeled affinity purified antibody to mouse IgG (H+L) human serum adsorbed produced in goat (Kirkegaard & Perry laboratories, Weden, cat. no. 074-1806)
- AlexaFluor®488 – Immunofluorecesence secondary antibody (Invitrogen Life technologies)

Usage of antibodies for immunoblotting:

Antibody	**SK-N-LO cells**	**DRGs**
PI3Kγ	1:100	-
μOR	1:200	1:20 (Immunocytochemistry)
P-AKT serine 473	1:1000	-
P-AKT threonine 308	1:1000	1:500 (Zhuang et al., 2004)
AKT	1:1000	1:500 (Zhuang et al., 2004)
P-ERK1/2	1:1000	1:1000
ERK1/2	1:1000	1:1000
β-actin	1:5000	-
Vinculin	1:5000	-
Anti-mouse secondary	1:10,000	1:10,000
Anti-rabbit secondary	1:5000	1:5000
AlexaFluor®488 secondary	-	1:1000

2.1.4 Drugs and inhibitors

Table. 2.2 Drugs and inhibitors used.

Drugs/Inhibitors	Target	Source
Morphine	μOR agonist	Sigma
Naloxone	Specific μOR antagonist	Sigma
Pertussis toxin (PTX)	Gi – protein coupled GPCR inactivator	Enzo Life Sciences
Wortmannin	Pan PI3K inhibitor	Sigma
AS605240	PI3Kγ inhibitor	Axxora GmbH
A66 S isomer	PI3Kα inhibitor	Symansis
TGX221	PI3Kβ inhibitor	Baker Heart Institute
IC87114	PI3Kδ inhibitor	Baker Heart Institute
Forskolin	Adenylyl cyclase activator	Enzo Life Sciences,
IBMX	Non-selective Phosphodiesterase inhibitor	Sigma
H-89	PKA inhibitor	Enzo Life Sciences,

2.1.5 Chemicals

Substance	Source
HEPES	Roth
EGTA	AppliChem
β-Glycerophosphate	SERVA

$MgCl_2.6H_2O$	Roth
Microcystin	Axxora
Pepstatin	AppliChem
Leupeptin	AppliChem
Pefa bloc – AEBSF-HCl	AppliChem
Sodium orthovanadate	Sigma –Aldrich
PMSF	SERVA
NP40	Calbiochem
Sodium Azide	Roth
Bovine serum albumin – BSA	PAA
Non-fat dried Milk powder	AppliChem
β-Mercaptoethanol	Roth
Polyacrylamide – RotiPhorese® Gel 30 (37.5:1)	Roth
Tris	AppliChem
Glycine	Roth
Sodium dodecyl sulphate – SDS	AppliChem
Ammonium persulphate – APS	SERVA
TEMED	Promega
PVDF membrane	Immobilon®
Methanol	Roth
Tween-20	SERVA
Triton X-100	Roth
ECL solution	Perkin Elmer
Sodium Chloride	Roth
Lectin beads	Sigma

Bromophenolblue	Bio-Rad
Ampicillin	Roth

2.1.6 Cell culture media and additives

Substance	Source
• Fetal Calf Serum (FCS)	Gibco
• L-Glutamine	PAA
• Penicillin/Streptomycin	PAA
• Collagenase: type 2	PAA
• 1x Trypsin/EDTA	PAA
• Sigmacote	Sigma
• Poly-L-Lysine – PLL	Sigma
• Dimethylsulfoxide (DMSO) HUBRI MAX®	Sigma Aldrich
• IMDM	PAA
• HAM'S F12	PAA
• G418 – (G418 sulphate)	PAA
• Puromycin – (Puromycin dihydrochloride)	Sigma
• Normal goat serum	DAKO Diagnostika

For shRNA experiments

- Dulbecco modified Eagle medium (DMEM/F12) (GIBCO-Life Technologies, Germany)
- Defined fetal calf serum (Sigma-Aldrich, cat. no. F-7524)
- 0.2 µm low-protein binding syringe filter holders (Sartorius, Germany, cat.no. 16534 K)
- PEI, Polyethylenimine (Sigma-Aldrich, cat. no. 40872-7)
- Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, Sigma-Aldrich, cat. no. AL-118, cat. no. 10,768-9)

- EndoFree Plasmid Maxi Kit (Qiagen, Weden, Germany, cat. no. 12362) for plasmid isolation

2.1.7 General solutions and other materials

Lysis buffer	20 mM	HEPES buffer pH 7.5
	10 mM	EGTA
	40 mM	β-glycerophosphate
	1 %	NP-40
	2.5 mM	$MgCl_2$

Adjust the pH to 7.5

Added protease and phosphatase inhibitors, just before use (final concentration),

100 µg/ml	AEBSF
1 mM	PMSF
1 mM	Vanadate
1 µg/ml	Pepstatin A
1 µg/ml	Leupeptin
3.4 nM	Microcystin
1 mM	β-glycerophosphate

LB Medium	10 g	Bacto – tryptone
	5 g	Bacto-yeast extract
	10 g	NaCl

Milli Q Water to 1 Liter, autoclave to sterilize

10x TBS	0.05 M	Tris
	0.5 M	NaCl

TBS-Tween (TBS-T)	1x	TBS
	0.01 %	Tween 20

Separation gel buffer	2 M	Tris	pH 8.8
Stacking gel buffer	0.5 M	Tris	pH 6.8
20 % APS	20 % Ammonium per sulphate in Milli Q water		
10 x PAGE buffer	250 mM	Tris	
	2 M	Glycine	
	35 mM	SDS	
	pH is about 8.7 do not adjust		
Blocking solution	2.5 % Bovine serum albumin (BSA) in 1x TBS-T		
5 % Milk solution	5 % Non-fat dried milk powder in 1x TBS-T		
Transfer buffer	48 mM	Tris	
	39 mM	Glycine	
	0.037 %	SDS	
	20 %	methanol	
	pH is between 9-10 do not adjust the pH		
Stripping solution	100 mM	β- Mercaptoethanol	
	2 %	SDS	
	62.5 mM	Tris pH 6.7	
5x Laemmli buffer	Glycerin	4 ml	
	20 % SDS	3 ml	
	β-mercaptoethanol	3 ml	
	1 % Bromophenolblue	200 µl	
	0.5 M Tris	2 ml	
	Aliquots stored at -20 ^{o}C		

RIPA buffer-(Radio immuno precipitation assay buffer)

150 mM	NaCl
50 mM	Tris HCl pH7.4
5 mM	EDTA
1%	Nonidet P-40
0.5 %	Na-deoxycholate
0.1 %	SDS

Mix NaCl, EDTA and Tris, then adjust pH to 7.4 then add other ingredients

Zamboni Fixing solution

350 ml	Saturated picric acid
80 g	Paraformaldehyde

in 500ml bottle warm up to 60°C

add drop wise 2,5 % NaOH until PFA is dissolved totally

filter in 2 liter-bottle and fill up with PB-buffer up to 2 liter

PB-buffer Zamboni

200 mM	NaH_2PO_4	133 ml
200 mM	Na_2HPO_4	567 ml

Fill up to 1 liter with Milli Q H_2O

0,1 M PB-buffer for immunocytochemistry:

0,5 M	Na_2HPO_4	154.8 ml
1 M	NaH_2PO_4	22.6 ml

fill up with Milli Q H_2O to 1 liter,

Add 1 ml Triton-X 100 for immunocytochemistry

TE (10:1)Buffer

10 mM	Tris-HCl, pH 7.6,
1 mM	EDTA

5x PBS

500 mM	NaCl
100 mM	Na_2HPO_4
400 mM	NaH_2PO_4

Dilute to 1x with Milli Q Milli Q H_2O and adjust pH to 7.4

- **Poly-L-Lysine** (PLL) **–** Working solution 50 µg/ml in Milli Q water
- **Polyethylenimine (PEI)** – stock solution 10 µg/µl in H_2O
- **G418** – 100 mg/ml stock solution in 100 mM HEPES buffer pH 7.5 – filter sterilized using 0.22 µ filter
- **Puromycin** – 2 mg/ml stock solution in 100 mM HEPES buffer pH 7.5 – filter sterilized using 0.22 µ filter
- **Coating of Pasteur pipette:** Pipette 4-5 times up and down with the solution Sigma cote® and flame Pasteur pipette. Then sterilize them.
- Luminescent Image Analyzer-Image Reader (FUJI Film LAS 4000)
- Tissue culture flasks (Nunc, Germany)
- 6-well tissue culture plates (Nunc, Germany, cat. no. 140675)
- 12-well tissue culture plates (Nunc, Germany, cat. no. 150628)
- Whatmann filter Paper (Macherey-Nagel, Germany)
- Bio-Rad power supply (model no. 1000/500)
- Semi-dry transfer cell (Trans-Blot® SD)
- Laser scanning microscope (Zeiss LSM 510 Meta)

2.1.8 Animals

DRGs were collected from adult wild type and PI3K$\gamma^{-/-}$mice (C57/B6J). Animals were housed four to six in a cage and maintained in a 12 h day/light cycle in a temperature controlled environment with unrestricted access to food and water.12-14 week old mice were used for experiments. The wild-type and PI3K$\gamma^{-/-}$mice were derived by 10 generations of successive backcrosses of heterozygous male knockout mice from chimeric C57BL6/129Sv PI3K$\gamma^{-/-}$mice (Hirsch et al., 2000) with C57BL/6 females (Jackson Laboratories, USA).

2.2 Methods

2.2.1 m-μOR plasmid amplification

Medium scale plasmid preparation was performed using Qiagen Midi Prep Plasmid Kit, according to the manufacturer's instructions. *E. coli* TO1 pcDNA3.1-HA-mOPRM1 wt was grown in 100 ml LB medium overnight. The bacterial cells were harvested by centrifugation at 6000 x g in a Sorvall centrifuge for 15 min at 4 ^{o}C. The bacterial pellet was resuspended in 10 ml of resuspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mg/ml RNase A). 10 ml of lysis buffer (200 mM NaOH, 1 % SDS) was added, mixed gently and incubated at room temperature for 5 min. 10 ml of chilled neutralization buffer (3.0 M potassium acetate, pH 5.5) was added, mixed immediately, but gently and incubated on ice for 15-20 min. Samples were centrifuged once again. Supernatant was applied to a Qiagen-tip previously equilibrated with 10 ml of equilibration buffer (750 mM NaCl, 50 mM MOPS, pH 7.0, 1% ethanol) to bind plasmid DNA to the column and washed with another 10 ml to remove the carbohydrates of the bacterial strains. The DNA was eluted in 15 ml of elution buffer (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15 % ethanol) precipitated with 0.7 volumes of room temperature isporopanol and immediately centrifuged at 15000 x g for 30 min at 4 ^{o}C and the supernatant was removed carefully. The DNA was washed with 5 ml of 70 % ethanol. Air-dried for 5 min and redissolved in a 30 ml of TE Buffer. The DNA concentration was determined spectrophotometrically. Quiagen purified DNA was used for transfection of the cells.

2.2.2 Cell culture conditions

- General media for SK-N-LO cells: 1:1 mixture of IMDM : HAM'S F12 supplemented with 10 % FCS.
- Media for m-μOR SK-N-LO cells: 1:1 mixture of IMDM : HAM'S F12 supplemented with 10 % FCS and 1 mg/ml G418.
- Media for shRNA treated m-μOR SK-N-LO cells: 1:1 mixture of IMDM : HAM'S F12 supplemented with 10 % FCS and 1 mg/ml G418 and 1 μg/ml puromycin.

Cultivation of cells: SK-N-LO (and all the other cells generated) cells were grown in T-75 flasks with a filter containing screw capped lid, in a humidified atmosphere containing 5% CO_2 at 37 ^{o}C. The cells were maintained by regular splitting avoiding excessive densities. Handling of cells was done in sterile laminar air flow unit avoiding contaminations.

Trypsinization: Media was aspirated from the flask. The cells were washed once with 1x PBS. 1 ml trypsin was added to the cells in T-75 flask, incubated for 2 – 5 min, gently tapped to detach the cells from the flask surface and new medium was added. Generally, 1:5 or 1:8 split was done twice a week.

Storage of cells: The cells were grown to 80 % confluence in T-175 flasks. Following trypsinization, the cells were added with FCS containing 10 % DMSO and dispensed in storage vials in aliquots of 1 ml each containing around 1-2 X 10^6 cells. The vials were stored at -150 ^{o}C.

Thawing of the cells: A freeze stock of cells was warmed to 37 ^{o}C for 3 – 5 min, transferred to an Eppendorf's tube centrifuged at 1200 rpm for 5 min. The DMSO containing medium was removed and the cells were resuspended in appropriate growth media and transferred to T-75 tissue culture flask.

2.2.3 Transfection of SK-N-LO cells

SK-N-LO (Human neuroblastoma cell line) cells were grown in a 1:1 mixture of IMDM and HAM'S F12 media supplemented with 10 % FCS. The cells were split and 200,000-300,000 cells/well were seeded into a 6-well plate. Next day, sub confluent cells were transfected with 2 – 3 µg mouse µOR plasmid wild type following a PEI transfection protocol.

Mix of DNA was prepared in an Eppendorf tube (final volume 1 ml)

Tube 1: DNA (2-3 µg) + 500 µl of 1:1 mixture of IMDM:HAM'S F12 media without serum

Tube 2: PEI (2.5 µg/1 µg DNA) + 500 µl of 1:1 mixture of IMDM:HAM'S F12 media without serum

Tube 2 contents were added to Tube 1 content, mixed gently and kept at room temperature for 30 min.

Cells were washed twice with warm 1:1 mixture of IMDM:HAM'S F12 media without serum. DNA – PEI mixture was carefully added to the cells. The plate was incubated in a CO_2 incubator at 37 ^oC for 6 h. The PEI containing media was replaced with 1:1 mixture of IMDM:HAM'S F12 media containing 10 % FCS. Two days after the transfection SK-N-LO cells were selected with 1 mg/ml G418 for 2 weeks. Surviving cells were further propagated in tissue culture flasks and analyzed for expression of PI3Kγ and μOR.

2.2.4 Expression analysis of PI3Kγ

The cells (400,000-600,000) were lysed in 200 μl of ice cold lysis buffer containing protease and phosphatase inhibitors. The cells were incubated on ice for 10 min, then the samples were scraped and collected into Eppendorf's tubes and centrifuged at 14,000 rpm for 20 min at 4 ^oC. 160 μl of the cell lysate supernatants were added with 40 μl of 5x SDS-sample buffer. The samples were heated at 95 ^oC for 5 min. The samples were resolved by 10 % SDS-PAGE.

2.2.5 Expression analysis of μOR

800 μl of RIPA buffer with necessary protease and phosphotase inhibitors was added to the cells (400,000-600,000) in 6 well plates and incubated on ice for 10 min. The samples were scraped and collected into Eppendorf's tubes and centrifuged for 30 min at 14,000 rpm, at 4 ^oC. These cell lysate supernatants were transferred to new tubes containing 40 μl of lectin beads. The tubes were kept on rotation for 2 h at 4 ^oC. The tubes with cell lysate and beads were centrifuged for 5-9 sec and the supernatant was transferred to another tube and used for loading control analysis. The beads were washed with ice cold RIPA buffer three times. The remaining liquid was aspirated with insulin syringe. Subsequently, 60 μl of 2x SDS-sample buffer was added to the beads and heated at 60 ^oC for 20 min with constant mixing. Thus prepared samples were

resolved by 7.5 % SDS-PAGE. Additionally, 160 µl of above supernatant was added to 40 µl of 5x SDS sample buffer and the sample was cooked for 5 min at 95 ^{o}C, which was used for loading controls.

2.2.6 Stimulation experiments using SK-N-LO cells

SK-N-LO cells stably transfected with mouse µ-opioid receptor, further referred to as m-µOR SK-N-LO cells, were seeded in 6-well plates. After 24 h the medium was replaced with serum free media (containing 1:1 mixture of IMDM:HAM'S F12 + 1 mg/ml G418) and incubated for 12 h before the experiment (starvation period). In a typical experiment, serum starved cells were preincubated with selected inhibitors or vehicle in serum free media (containing 1:1 mixture of IMDM:HAM'S F12) for indicated time before stimulation with morphine. Incubation time for pertussis toxin was for 12 h (began with starvation period). Immediately following stimulation, the cells were lysed in 200 µl of ice cold lysis buffer containing protease and phosphatase inhibitors. The samples were processed for Western blot analysis as described under expression analysis of PI3Kγ. The proteins were resolved by 10 % SD-PAGE and immunoblotted with appropriate antibodies.

2.2.7 shRNA mediated down regulation of PI3Kγ in m-µOR SK-N-LO cells

Knockdown of PI3Kγ in m-µOR SK-N-LO cells was accomplished by shRNA method. The following steps were involved in this process.

- Production of lentiviral particles

- Transduction of m-µOR SK-N-LO cells

Production of lentiviral particles

- 7-8 x10^5 HEK293T cells per dish were seeded into seven 10 cm dishes. The cells were distributed evenly in the dish.

- The cells in 10 cm dishes were incubated at 37 °C overnight. Next day transfected with packaging plasmids.

Procedure of transfection as follows

- DMEM/F12 medium without FCS was prewarmed to room temperature. 10 µg lentiviral expression plasmid (pLKO.1-derivative plasmids encoding the shRNA genes) and the ready prepared lentiviral packaging mix were diluted in DMEM/F12 as mentioned below. In parallel pLKO.1 derivative plasmid encoding a nontargeting shRNA prepared, as described below.

  1) 3.5 ml of DMEM-F-12 media without FCS + 17.5 µl of PEI (10 µg/ml solution) (2.5 µg/ µg of DNA)

  2) 3.5 ml of DMEM-F-12 media without FCS + pMDL + pVSV + pRSV (packaging mix)

Plasmid	**amount (µg)/10 cm dish**
pMDL	10
pVSV	2
pRSV	5

- 500 µl of packaging mixture prepared above was dispensed into seven Eppendorf's tubes labeled as γ1, γ3, γ5, control & green (GFP encoding reference plasmid).
- Lentiviral expression plasmids were added to the 7 tubes, as mentioned below

Plasmid ID	**code**	**amount(µg)/ dish**
NM_002649.2-3122s1c1	γ1	2
NM_002649.2-2774s1c1	γ3	2
NM_002649.2-940s1c1	γ5	2
Lentiviral nontargeting control	Control	2
GFP encoding reference plasmid	Green	2

- 500 µl PEI solution prepared above was added to each tube containing lentiviral expression plasmid.
- The contents of the tube were mixed thoroughly immediately after addition and vortexed. Then incubated for 30 min at 37 ^{o}C.
- The FCS-containing media in HEK293T cells seeded dishes was replaced with DMEM/F12 media without FCS. DNA-PEI solution was added drop-wise to the cells, carefully spreading the solution over the entire surface of the well (i.e. each Eppendorf's tube was added to each plate).
- The dishes were incubated for 6 h in an incubator at 37 ^{o}C with 5 % CO_2.
- The PEI-containing media was replaced with DMEM/F12 media containing 10 % FCS and the cells were incubated.
- The supernatant containing viral particles on the cells was collected at 24, 48 and 72 h, filtered through 0.2 µ filter and stored at 4 ^{o}C. After removing the media from the cells, carefully prewarmed fresh FCS-containing DMEM/F12 medium was added. Care was taken to prevent the detachment of the cells from the surface while handling.
- The cumulative supernatant was centrifuged in 30-40 kDa filter containing tubes at 3000 g for 15 min and the supernatant was collected (500-1000 µl) into separate labeled Eppendorf's tubes. Samples were aliquoted and stored at -80 ^{o}C.

Transduction of m-µOR SK-N-LO cells and selection of stable clones

- 40,000-50,000 m-µOR SK-N-LO cells/well were seeded into a 12 well plate with growth media containing 1:1 mixture of IMDM:HAM'S F12 supplemented with 10 % serum and 1 mg/ml G418.
- The cells in each well were added with polybrene (final concentration 8 µg/ml). The plate was gently swirled to homogenize the medium while preventing the detachment of cells.
- 50 µl of lentiviral particles were added to the wells. The medium was gently distributed in the well.
- Subsequently, the plates were centrifuged at 500 x *g* for 60 minutes and further incubated at 37 °C in a humidified incubator in an atmosphere of 5 % CO_2.

- 6 to 8 hours post-infection polybrene containing medium was replaced with fresh growth medium. In order to raise the efficiency of transduction a second and a third infection round was carried out by adding new virus particles to the cells and spinning the plates again. Subsequently, the cells were incubated at 37 °C in a humidified incubator in an atmosphere of 5 % CO_2.
- After 24 to 48 hours the cell growth medium was replaced with the medium containing 1 µg/ml puromycin for selection. To monitor the efficiency of selection, one well was left uninfected and selected with the same amount of puromycin.
- Selection against puromycin was maintained 10 to 14 days (i.e. stopped when no viable cells were visible in the uninfected control wells).
- Cells were subsequently characterized for expression of PI3Kγ using SDS-PAGE and subsequent immunoblotting.

2.2.8 Preparation and culture of dorsal roort ganglia (DRGs)

- **DRG medium**: DMEM F12- Dulbecco's modified Eagle Medium (Gibco) with 1 mM Glutamine supplemented with 10 % FCS (heat inactivated) and 1 % Penicillin/Streptomycin
- Mouse was anesthetized and killed by overdose of anaethesia. Spinal cord was dissected out, cleared off extra tissues and kept in ice cold DMEM/F12 medium. The spinal cord was cut into two halves along the longitudinal axis. The Dura, a quite solid and clear membrane lying between spinal cord and DRGs was removed carefully. DRGs were pulled out by holding the two long horns (neruites) protruding outside gently and kept in a small culture dish with DMEM/F12 medium. DRGs from all spinal levels were collected. The long horns and additional films of the DRG were cut by holding it with tweezers and smoothly cutting around like peeling. Subsequently, DRGs were transferred into new small culture dish with 2 ml of DMEM/F12 medium.
- Collagenase (final concentration - 0.4 U/ml) was added to DRG containing culture dish and incubated at 37 ^{o}C for 45 min. After incubation, DRGs were

transferred to a 15 ml falcon tube with 2-3 ml of DMEM/F12 medium using a fire polished coated Pasture-pipette (coated with Sigmacote®).

- After the DRGs were settled down at the bottom, the medium was aspirated out with Pasture-pipette. 1 ml trypsin was added to the tube with DRGs and incubated at 37 ^{o}C for 10 min. Following incubation, the DRGs were allowed to settle down. Subsequently, trypsin was aspirated out with Pasteur-pipette.
- The DRGs were washed once with 2-3 ml of DMEM/F12 medium by aspirating the medium after the DRGs were settled down.
- 2 ml of DMEM/F12 medium was added to the tube. The DRGs were dissociated by beating the tube 10 times. The cells were allowed to settle down and the supernatant was collected into another falcon tube with Pasteur-pipette.
- Addition of 2 ml of DMEM/F12 medium and the beating process was repeated (4 – 5 times) until no visible flocks appear in the supernatant and each time the supernatant was collected into another tube using Pasteur-pipette.
- The collected supernatant was pipetted up and down with Pasteur-pipette for 10 – 20 times carefully avoiding the air bubbles. The tube was centrifuged at room temperature for 7 min at 1300 rpm in swinging bucket rotor (Heraus Megafuge 1.0).
- The supernatant was aspirated and 4 ml of DMEM/F12 medium supplemented with 10 % FCS and 1x Penicillin/Streptomycin was added to DRGs. The cells were resuspended by aspirating them using Pasteur-pipette.
- The cells were centrifuged again at room temperature for 7 min at 1300 rpm. The supernatant was aspirated and added with sufficient new medium containing 10 % FCS and 1x Pencillin/Streptomycin, to seed into either 12- well plates (20,000-30,000 cells/ well) for western blotting or into glass bottomed dishes coated with poly L-lysine (PLL) for microscopy (around 15,000-20,000 cells/dish).

2.2.10 Immunocytochemistry

The cells were washed with ice cold PBS. Added with 400 µl of ice cold Zamboni fixing solution and kept at room temperature for 30- 40 min. Zamboni fixing solution

was removed and the cells were washed with PB-buffer until the yellow color disappears (to wash off Zamboni fixing solution completely). The cells were permeabilized by incubating them with 1 ml of PB-buffer containing 0.1 % Triton X-100 and 3 % normal goat serum for 2 h at room temparature. Then the primary antibody (1: 20) in PB-buffer containing 0.1 % Triton X-100 and 3 % normal goat serum was added to the cells and incubated overnight at 4 ^{o}C. The cells were washed with PB-buffer for 4 times. Secondary antibody (Alexa 488-1:1000) in PB-buffer containing 0.1 % Triton X-100 and 3 % normal goat serum was added to the cells. The dishes were kept in a dark room for 2 h at room temperature with gentle shaking. The cells were washed with PB-buffer for 4 times. The cells were visualized using laser scanning microscope (Zeiss Meta 510 Microscope) at 488 nm excitation wavelength with 40x optical zoom.

2.2.11 Stimulation of DRGs

Immediately following the collection, the DRGs were seeded in a 12 - well plate with DMEM/F12 media containing 10 % FCS. After 5-6 h of incubation at 37 ^{o}C in a humidified incubator in an atmosphere of 5 % CO_2, the media was replaced with DMEM/F12 media without serum (starvation media). Following 10-12 h starvation period the cells were used for experiment. In a typical experiment, starved cells were preincubated with selected inhibitors or vehicle in serum free media for indicated time before stimulation with morphine. Immediately following stimulation, the cells were lysed in 100 µl of ice cold lysis buffer containing protease and phosphatase inhibitors. Incubated on ice for 10 min, then the cells were scraped and collected into Eppendorf's tubes. The cell lysates were centrifuged at 14,000 rpm for 20 min at 4 ^{o}C. 90 µl of the cell lysate supernatants were added with 23 µl of 5x SDS-sample buffer. The samples were heated at 95 ^{o}C for 5 min. The samples were resolved by 10 % SDS-PAGE.

2.2.12 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The prepared protein samples were resolved by SDS-PAGE. 10 % or 7.5 % polyacrylamide gels were prepared as mentioned below (Table. 2.3). Stacking gel was prepared on the separation gel. The protein samples were loaded in the slots made in collection gel.

Table 2.3 Gel composition

	10 % Separation gel	**7.5 % Separation gel**	**4 % Stacking gel**
Water	4.8 ml	8 ml	3.1 ml
30 % Polyacrylamide	3.33 ml	5.3 ml	0.66 ml
Separation gel buffer	1.9 ml	4 ml	--
Collection gel buffer	--	--	1.25 ml
20 % SDS	50 µl	80 µl	25 µl
20 % APS	40 µl	40 µl	30 µl
TEMED	4 µl	20 µl	2 µl

The prestained protein marker was used to assess the molecular weight of the proteins. Proteins were resolved by applying a constant voltage of 110 V for 80-90 min using 1x PAGE buffer.

2.2.12 Immunoblotting

The proteins were transferred on to PVDF transfer membranes using a semi-dry transfer blotting system. Briefly, a sandwich was prepared by placing the gel and membrane between transfer buffer soaked wet Whatmann filter papers (3x) each at the top and

bottom, and applied an electric field to allow the transfer of proteins on to the membrane under constant current 1 mA/ cm^2 for 2 h. Subsequently, the membranes were briefly kept in 1x TBS-T solution and then kept in blocking solution composed of 2.5 % bovine serum albumin (BSA) in 1x TBS-T for 2 h. The blocking solution was exchanged with respective primary antibody containing solution (2.5 % BSA in 1x TBS-T) and incubated overnight at 4 ^{o}C. Then the membranes were washed three times with 1x TBS-T and incubated for 1 h at room temperature with respective HRP-conjugated secondary antibody in 5 % non-fat dried milk powder solution. Then the membranes were washed three times with 1x TBS-T. The blots were visualized in ECL solution for 1 min and images were captured on Luminescent Image Analyzer (LAS-4000, FUJI FILM) for 1-10 min. Subsequently, the membranes were stripped using strip buffer at 56 ^{o}C for 30 min and blocked with 2.5 % BSA in 1 % TBS-T solution before probing with suitable primary antibodies and secondary antibodies for loading controls as mentioned above (reprobed).

2.2.12 Statistical Analysis

All data are given as mean ± SD of three to five individual experiments. To determine statistical significance of the described results, analysis of variance with Fischer's LSD was performed. A p value of < 0.05 was considered as statistically significant.

3. Results

3.1 Activation of AKT upon stimulation of μOR by morphine in SK-N-LO cells

3.1.1 Expression of μOR in SK-N-LO cells

We intended to study the involvement of PI3Kγ in μOR signaling. To facilitate such studies, we here describe the generation of a suitable cell culture model. SK-N-LO cells express PI3Kγ endogenously. Since the endogenous expression of μOR was undetectable, at least at protein level (Fig. 3.1A), plasmid pcDNA3.1 encoding HA-tagged mouse μOR was transfected into SK-N-LO cells. 48 h after transfection the cells were selected using G418 for 2 weeks and propagated further. The expression of μOR in SK-N-LO cells was confirmed immunologically (Fig. 3.1). Human μOR expressing SK-N-LO cells served as positive control. In parallel, the expression of PI3Kγ was demonstrated in Fig. 3.2. This cell line with endogenous PI3Kγ and stably expressing μOR will be further referred to as m-μOR SK-N-LO.

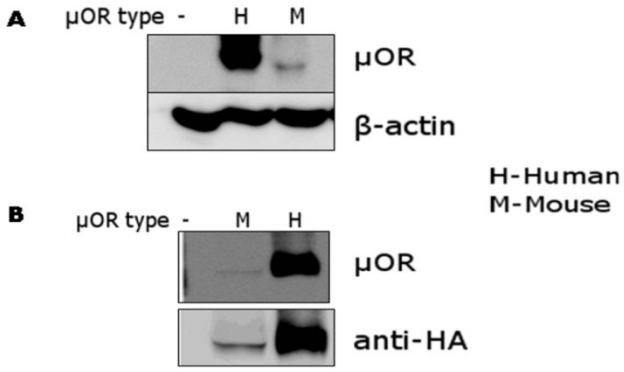

Fig. 3.1 Expression of μOR in SK-N-LO cells. Plasmid pcDNA3.1 encoding HA-tagged mouse μOR was transfected into SK-N-LO cells. Following selection, the cells were lsyed in RIPA buffer and glycosylated proteins were enriched using wheat germ lectin agarose beads. Proteins were eluted from the beads using SDS-sample buffer for 20 min at 60 ^{o}C. Supernatants from pull down samples were probed for β-actin. The samples were separated on a 7.5 % SDS-PAGE and immunoblotted, **A)** with specific antibodies recognizing μOR and β-actin, **B)** with specific antibody recognizing μOR and after stripping redeveloped with specific antibody recognizing anti-HA. Human μOR expressing SK-N-LO cells served as positive control.

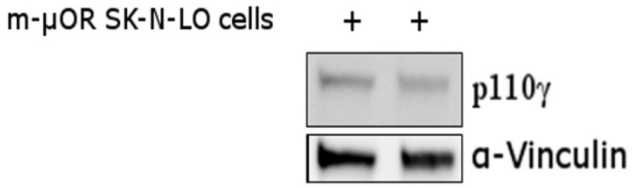

Fig. 3.2 Expression of PI3Kγ in m-μOR SK-N-LO cells. Whole cell lysates of m-μOR SK-N-LO cells were lysed. The cell extracts were separated on a 10 % SDS-PAGE and immunoblotted with specific antibody recognizing N-terminus of catalytic subunit of PI3Kγ named p110γ. The blot was stripped and redeveloped with specific antibody recognizing α-vinculin.

3.1.2 Morphine activates AKT and ERK1/2 in m-μOR SK-N-LO cells

To characterize the μOR mediated signaling activity, m-μOR SK-N-LO cells were stimulated with 10 μM morphine for indicated time periods (2.5 to 30 min) as shown in Fig. 3.3. After cell lysis, cell extracts were resolved by SDS-PAGE and immunoblotted. Treatment of cells with morphine evoked a transient activation protein kinase B (AKT). Maximum phosphorylation of AKT was observed at 2.5 min followed by a decline to reach control levels by 30 min. Phosphorylation of AKT at threonine 308 and serine 473 sites were observed. In addition, morphine stimulation resulted in a strong activation of ERK1/2. The phosphorylation of ERK1/2 also followed similar time-dependent kinetics as of AKT in these cells. Hence, 2.5 min stimulation was considered for evaluation of the effect of different stimuli on morphine induced activation of AKT and ERK1/2 in these cells. Wherever higher phosphorylation of ERK1/2 was observed recognition of ERK1/2 by pan ERK1/2 antibody was impaired. So β-actin was probed for as an additional control.

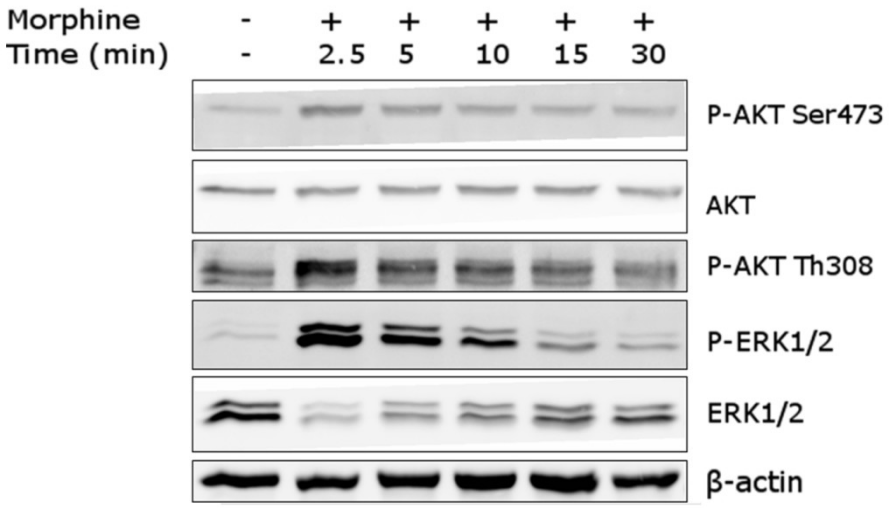

Fig. 3.3 Morphine activates AKT and ERK1/2 in m-μOR SK-N-LO cells. m-μOR SK-N-LO cells were treated with 10 μM morphine for indicated time periods. Subsequently, whole cell lysates were separated on 10 % SDS-PAGE and immunoblotted with specific antibodies recognizing phosphorylated AKT serine 473 and phosphorylated ERK1/2. The blots were stripped and redeveloped with specific antibodies recognizing phosphorylated AKT threonine 308 and total ERK1/2. Then the bolts were stripped again and redeveloped with specific antibodies recognizing total AKT and β-actin.

3.1.3 Involvement of μOR and PI3Ks in morphine induced AKT and ERK1/2 activation

We asked questions i) whether the morphine induced activation of AKT and ERK1/2 is mediated via μOR? Does it involve PI3Ks? In order to address these questions, m-μOR SK-N-LO cells were preincubated overnight with 100 ng/ml pertussis toxin (PTX), a Gi subtype of GPCR inhibitor, before being stimulated with 10 μM morphine for 2.5 min. Pretreatment with PTX blocked the morphine stimulated phosphorylation of AKT at sites threonine 308 and serine 473. In addition, inhibition of the morphine induced phosphorylation of ERK1/2 was also observed with this pretreatment (Fig. 3.4).

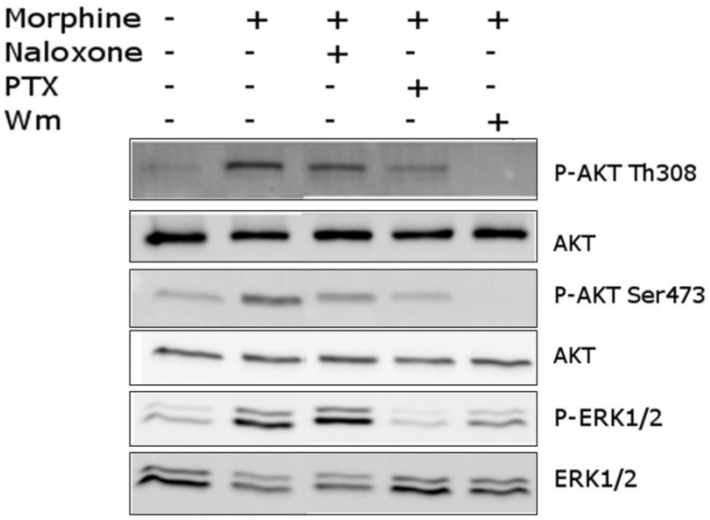

Fig. 3.4 Involvement of μOR and PI3Ks in morphine induced AKT activation. m-μOR SK-N-LO cells were preincubated with 10 μM naloxone (30 min) or 100 ng/ml pertussis toxin (overnight) or 100 nM wortmannin (Wm) (30 min) before being stimulated with 10 μM morphine for 2.5 min. Cells were lysed, whole cell extracts were resolved by 10 % SDS-PAGE and immunoblotted with specific antibodies recognizing phosphorylated AKT threonine 308, phosphorylated AKT serine 473, phosphorylated ERK1/2. Subsequently, the membranes were stripped and reprobed with specific antibodies recognizing total AKT and total ERK1/2.

Similarly, preincubation of m-μOR SK-N-LO cells for 30 min with 10 μM naloxone, a specific μOR antagonist, inhibited the morphine induced phosphorylation of AKT at both sites and ERK1/2. In addition, m-μOR SK-N-LO cells were pretreated with 100 nM wortmannin, a PI3K inhibitor, for 30 min before being stimulated with 10 μM morphine for 2.5 min. Pretreatment with wortmannin resulted in inhibition of phosphorylation of AKT at threonine 308 and serine 473 sites (Fig. 3.4). Morphine induced phosphorylation of ERK1/2 was also abolished by pretreatment with wortmannin. Taken together, these data indicate that morphine induced activation of AKT and ERK1/2 involved stimulation of Gi subtype of GPCRs, that the receptor was μOR and a pathway dependent on activation of PI3Ks.

3.2 Identification of PI3K isoforms involved in the activation of AKT by morphine

3.2.1 Pharmacological approach

In order to study PI3K selectivity for morphine induced activation of AKT, we here investigated the possible role of PI3Kγ in morphine induced activation of AKT via μOR. In a typical experiment, m-μOR SK-N-LO cells were preincubated with 100 nM AS605240, a specific PI3Kγ inhibitor, for 15 and 30 min prior to stimulation with 10 μM morphine for 2.5 min. After the cell lysis, the extracts were resolved by SDS-PAGE and immunoblotted. Pretreatment with AS605240 – either for 15 or 30 min – resulted in nearly complete inhibition of phosphorylation of AKT. The phosphorylation at threonine 308 and serine 473 sites was inhibited (Fig. 3.5). At 100 nM concentration AS605240 selectively inhibited PI3Kγ. Hence, this observation confirms the involvement of PI3Kγ in activation of AKT by stimulation of μOR with morphine. Nevertheless, other PI3K isoforms could be involved in this signaling process.

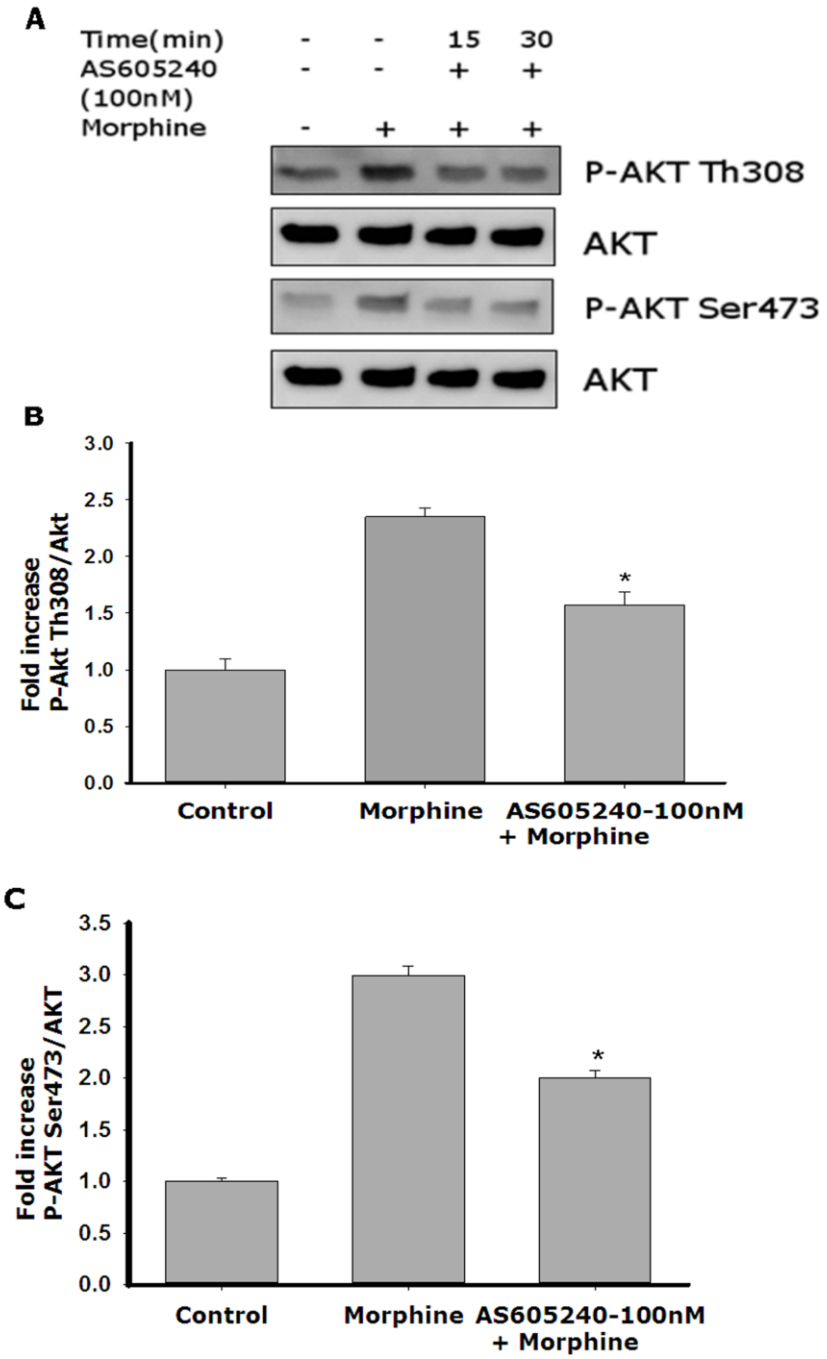

Fig. 3.5 Involvement of PI3Kγ in morphine induced activation of AKT. A. m-μOR SK-N-LO cells were preincubated with AS605240 for 15 or 30 min. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min. After cell lysis, cell extracts were subjected to 10 % SDS-PAGE and immunoblotting with specific antibodies as described in Fig: 3.4. **B, C.** Densitometric analysis of the Fig. A and similar experiments showing the ratio of P-AKT threonine 308/total AKT. (B) or P-AKT serine 473/total AKT (C). The data represent mean ± SD, n=3. The responses of 30 min preincubation with AS605240 were used for quantification. * $P<0.05$ compared to morphine treatment, n=3.

Class IA PI3K members, namely – p110α, β and δ isoforms, attached to p85 regulatory subunits, were conventionally reported to be associated with receptor tyrosine kinases and proved to have major role in insulin, growth hormone mediated signaling implying on cell growth, adhesion, survival and proliferation (Vanhaesebroeck et al., 2010). We investigated the possibility of their involvement in morphine induced activation of AKT by means of pharmacological inhibition using isoform specific inhibitors. In this study we used the following inhibitors.

Table. 3.1 PI3K Isoform specific inhibitors (Hawkins et al., 2006; Jamieson et al., 2011)

Compound	**Isoform selectivity**	**IC_{50}**	**Concentrations used**
A 66 S form	P110α	30 nM	30 nM
TGX221	P110β	20 nM	20 nM
IC87114	P110δ	200 nM	200 nM
AS605240	P110γ	100 nM	1, 10 μM
Wortmannin	PI3Ks	3 – 14 nM	100 nM

In a typical experiment, the cells were preincubated with isoform selective inhibitor for 30 min as shown in Table. 3.1 and then stimulated with 10 μM morphine for 2.5 min. After the cell lysis, the extracts were resolved by SDS-PAGE and immunoblotted. The activation of AKT was unaffected by pretreatment with either A66 or IC87114, as evidenced by the unaltered phosphorylation of AKT at threonine 308 and serine 473 sites in response to morphine stimulation (Fig. 3.6). In contrast, pretreatment with the selective PI3Kγ inhibitor AS605240 inhibited the morphine induced

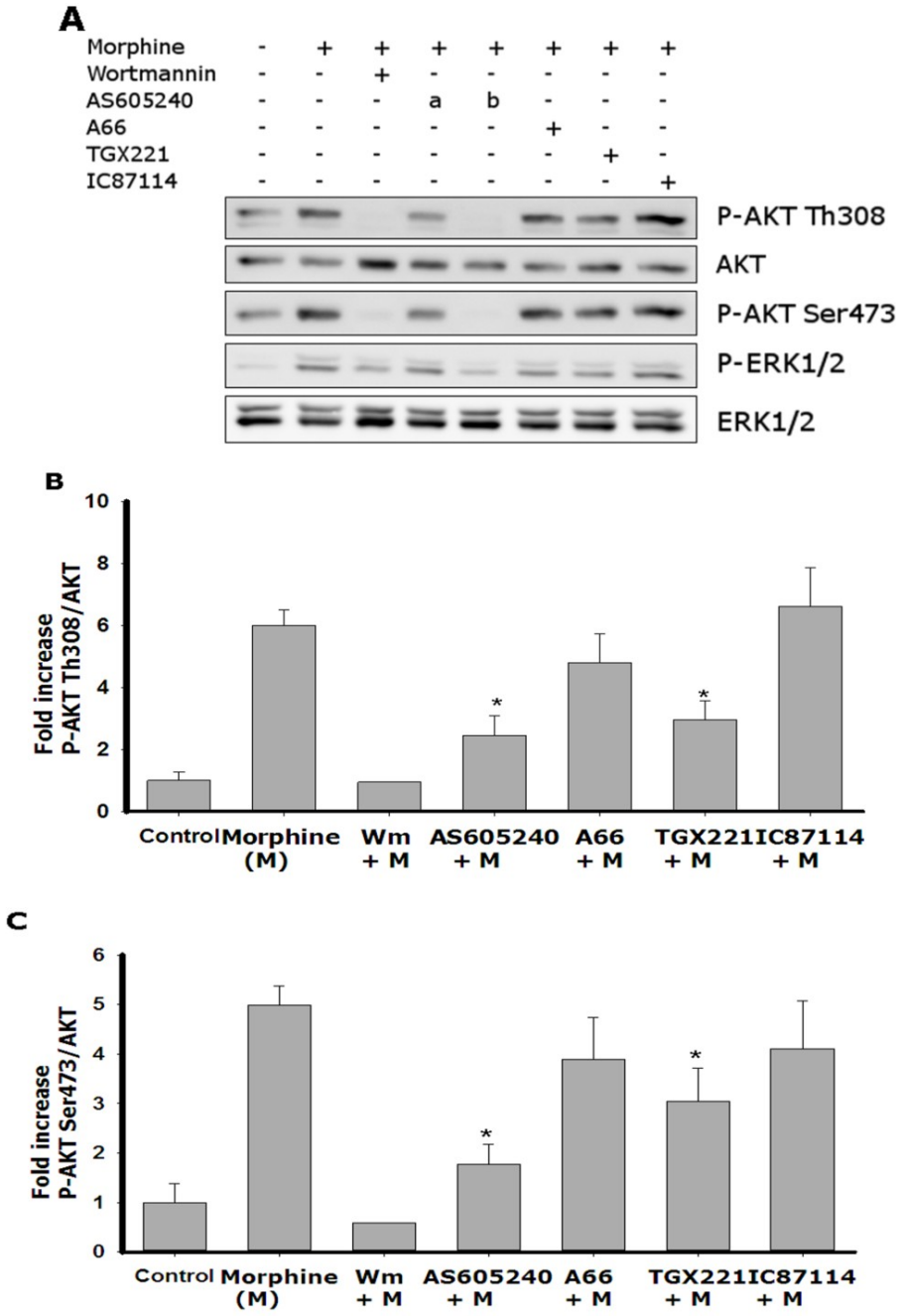

Fig. 3.6 Involvement of PI3Kβ in morphine induced activation of AKT. A. m-μOR SK-N-LO cells were pretreated with one of the PI3K isoform specific or non-specific inhibitors followed by stimulation with 10 μM morphine for 2.5 min. 1 μM and 10 μM concentrations of AS605240 represent a & b, respectively. Cell lysis, cell extract preparation, Western blot analysis and probing with indicated specific antibodies were done as described in Fig. 3.4. **B**, **C**. Densitometric analysis of Fig. A and similar experiments showing the ratio of phosphorylated AKT threonine 308/total AKT (B) or phosphorylated AKT serine 473/total AKT (C). The data represent mean ± SD, n=3. Data were compared to cells treated with 1 μM AS605240 pretreatment.*P<0.05 compared to morphine treatment.

activation of AKT in a dose-dependent manner as evidenced by the inhibition of phosphorylation at both the phosphorylation sites. In addition, pretreatment with TGX221 – selective PI3Kβ inhibitor – also inhibited the morphine induced phosphorylation of AKT. Nevertheless, these data demonstrated that the major contributor to morphine induced AKT activation was PI3Kγ. Abrogation of the activity of other PI3K isoforms – p110α and δ did not show any effect up on morphine induced phosphorylation of AKT. However, TGX221 might have an additional role in morphine induced activation of AKT. The phosphorylation of ERK1/2 was affected in a similar pattern as of phosphorylation of AKT. The higher concentration of AS605240 used was to compensate for the loss of effectiveness following repeated freeze-thaw cycles of the same aliquot of the stock solution.

3.2.2 Genetic approach

3.2.2.1 Knockdown of PI3Kγ in m-μOR SK-N-LO cells by shRNA

To investigate the requirement of PI3Kγ in morphine induced activation of AKT, we used RNAi approach to suppress the expression of PI3Kγ in m-μOR SK-N-LO cells. m-μOR SK-N-LO cells were transduced with lentiviral particles containing either non-targeting control shRNA or targeting PI3Kγ plasmids encoding different shRNA genes (γ1, γ3 and γ5). Following 48 h of transduction, the cells were selected with puromycin and expanded further. The cells were lysed, whole cell lysates were subjected to SDS-PAGE and immunoblotted to analyze the expression of PI3Kγ. Simultaneously, separate set of cells were processed to monitor the presence of μOR following viral transduction. As shown in Fig. 3.7A the cells expanded from γ3 and γ5 clones showed most effective suppression of PI3Kγ compared to control shRNA expressing cells. Cells derived from γ5 clone showed nearly 70 % suppression of PI3Kγ. These cells were used for further studies (Fig. 3.7B). Further, the presence of μOR was confirmed in the cells (non-targeting control shRNA treated cells) following viral transduction (Fig: 3.7C). The cell line treated with non-targeting control shRNA (without knock down PI3Kγ) will be further referred to as Ctrl-m-μOR SK-N-LO cells. The cell line with knockdown of PI3Kγ will be further referred to as γ5-m-μOR SK-N-LO cells. The analyses were carried out with cell pools propagated for short periods to avoid clonal drifts.

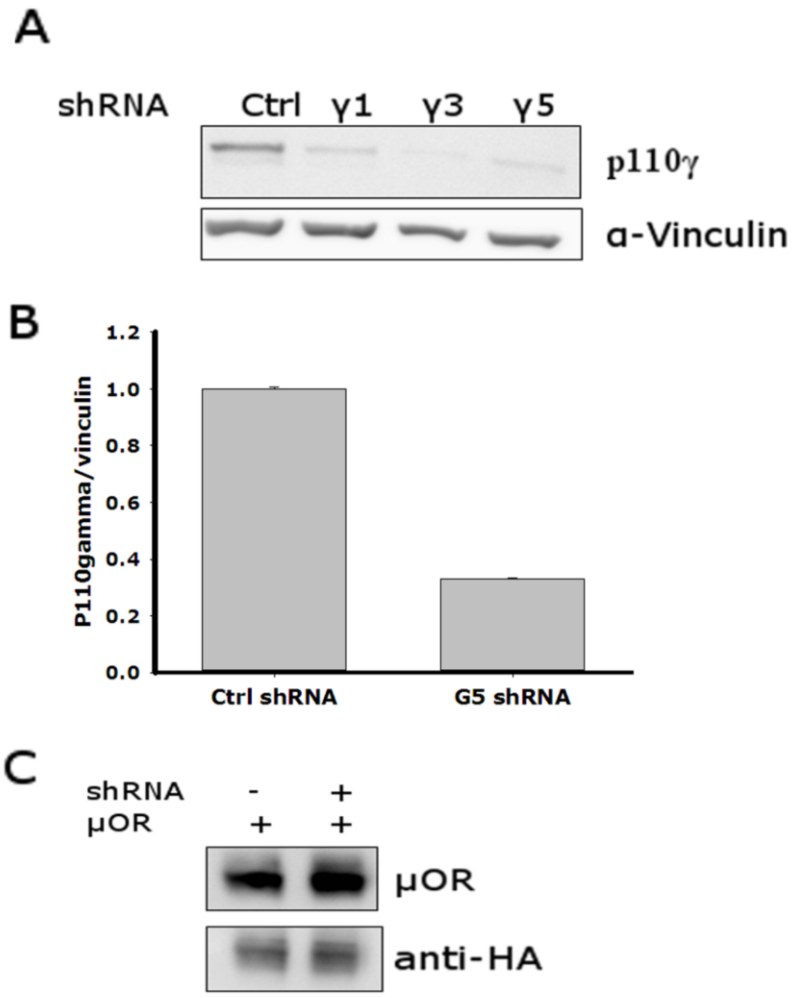

Fig. 3.7 Knockdown of PI3Kγ in m-μOR SK-N-LO cells by shRNA. A. Whole cell lysates of m-μOR SK-N-LO cells treated with non-targeting control shRNA or one of the PI3Kγ specific shRNA were subjected to 10 % SDS-PGAE and immunobolted with specific antibodies for PI3Kγ and vinculin. **B**. Densitometric analysis showing the ratio of p110 gamma (γ) /vinculin following treatment of m-μOR SK-N-LO cells with non-targeting control shRNA or γ5 (G5) PI3Kγ specific shRNA. Data represent mean ± SD, n=4. **C**. m-μOR SK-N-LO cells treated with non-targeting control shRNA or naive cells were lysed in RIPA buffer and glycosylated proteins were enriched using wheat germ lectin agarose beads. Proteins were eluted from the beads using SDS-sample buffer for 20 min at 60 ^{o}C, subjected to SDS-PGAE and immunoblotted with indicated specific antibodies as described Fig. 3.1B.

3.2.2.2 Effect of viral transduction on signaling activity m-μOR SK-N-LO cells

At first, we tested whether the cells behave the same after undergoing viral transduction and selection process. To test this, we compared the responses of the Ctrl-m-μOR SK-N-LO cells with that of m-μOR SK-N-LO cells to various stimuli. Expression of PI3Kγ in Ctrl-m-μOR SK-N-LO cells was comparable to that of m-μOR SK-N-LO cells (Fig. 3.8). In both types of cells, morphine stimulation evoked activation of AKT and ERK1/2 as evidenced by the phosphorylation of AKT and ERK1/2 which was completely blocked by pretreatment (overnight) of cells with PTX (100 ng/ml). This suggests that the basic responses of the cells to morphine stimuli and the pathway of the activation of AKT and ERK1/2 were similar in shRNA treated as well as untreated cells.

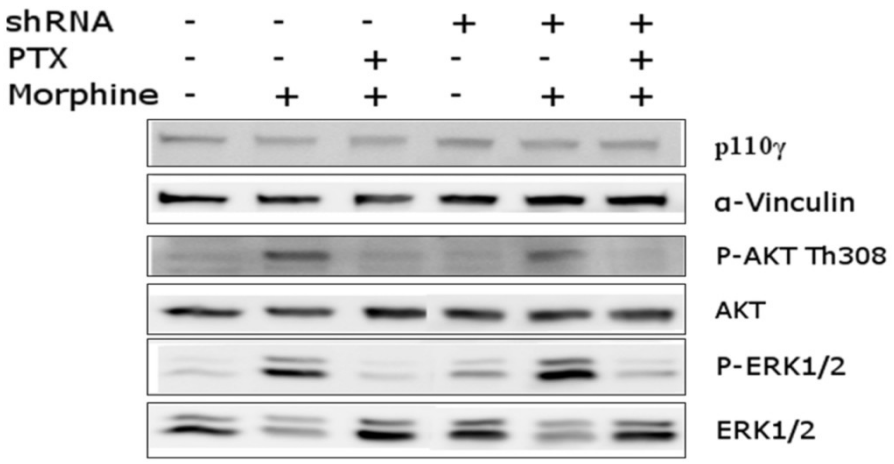

Fig. 3.8 Effect of viral transduction on signaling activity m-μOR SK-N-LO cells. Ctrl-m-μOR SK-N-LO and m-μOR SK-N-LO cells were preincubated either with vehicle or 100 ng/ml PTX (overnight). Then the cells were stimulated with 10 μM morphine for 2.5 min. After cell lysis, cell extracts were subjected to 10 % SDS-PAGE and immunoboltted with specific antibodies recognizing p110γ, phosphorylated AKT threonine 308 and phosphorylated ERK1/2. Then the membranes were stripped and redeveloped with specific antibodies recognizing vinculin, total AKT and total ERK1/2.

3.2.2.3 Requirement of PI3Kγ for morphine induced activation of AKT and ERK1/2

To test the requirement of PI3Kγ for morphine induced activation of AKT we stimulated the Ctrl-m-μOR SK-N-LO cells and γ5-m-μOR SK-N-LO cells with 10 μM morphine for 2.5 min. As expected, morphine stimulation elicited activation of AKT in Ctrl-m-μOR SK-N-LO cells but not in γ5-m-μOR SK-N-LO cells. The phosphorylation of AKT at threonine 308 and serine 473 sites was significantly reduced in γ5-m-μOR SK-N-LO cells compared to Ctrl-m-μOR SK-N-LO cells (Fig. 3.9). This was in line with our earlier observation using pharmacological inhibitors. Moreover, morphine induced phosphorylation of ERK1/2 was abolished in γ5-m-μOR SK-N-LO cells in contrast to the Ctrl-m-μOR SK-N-LO cells. These data demonstrate that PI3Kγ was indeed required for the activation of AKT and ERK1/2 upon stimulation of μOR with morphine. The residual activation seen in case of γ5-m-μOR SK-N-LO cells could be due to the residual PI3Kγ as the maximum suppression observed was nearly 70 %.

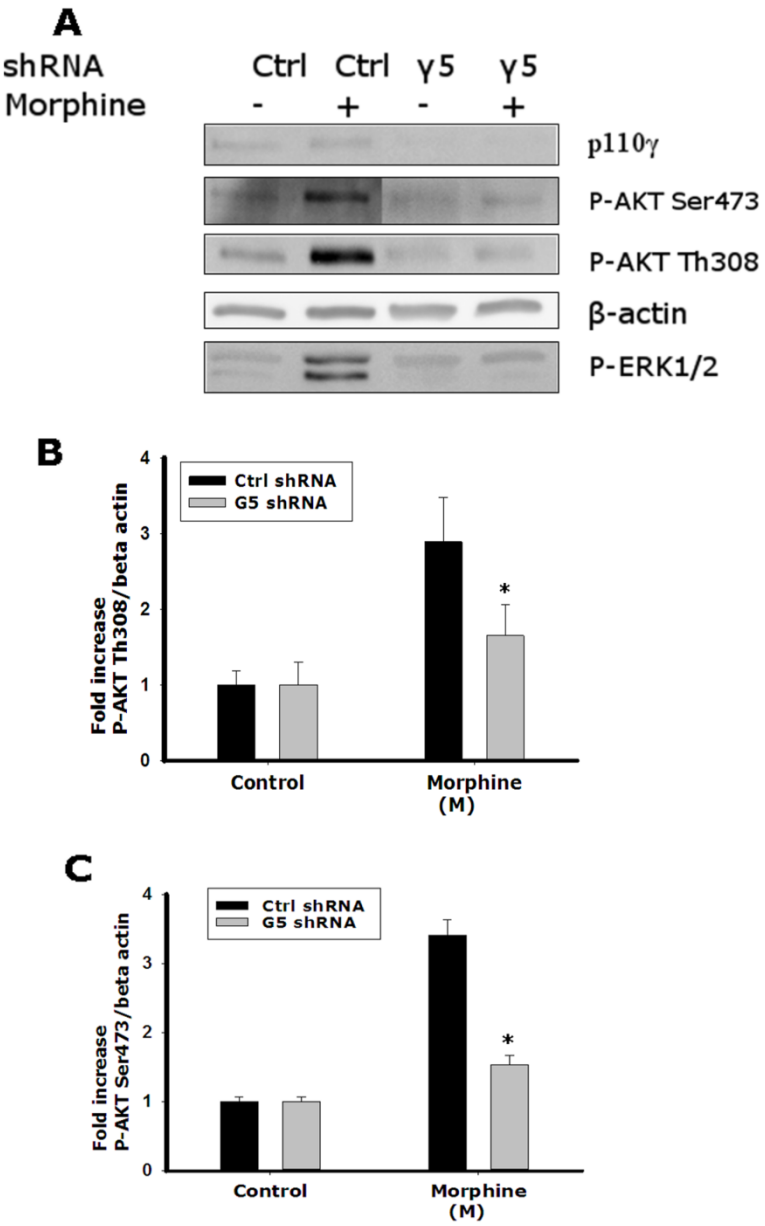

Fig. 3.9 Requirement of PI3Kγ for morphine induced activation of AKT. A. Ctrl-m-μOR SK-N-LO cells (Ctrl shRNA) and γ5-m-μOR SK-N-LO cells (G5 shRNA) were stimulated with 10 μM morphine for 2.5 min. After cell lysis, cell extracts were resolved by 10 % SDS-PAGE and immunoblotted with specific antibodies recognizing p110γ, phosphorylated AKT threonine 308 and phosphorylated ERK1/2. Then the membranes were stripped and redeveloped with specific antibodies recognizing phosphorylated AKT serine 473 and β-actin. **B**. Densitometric analysis of Fig: A and similar experiments showing the ratio of phosphorylated AKT threonine 308/total AKT. The data represent mean ± SD, n=5. *P<0.05 compared to morphine treatment of Ctrl-m-μOR SK-N-LO cells. **C.** Densitometric analysis of Fig: A and similar experiments showing the ratio of phosphorylated AKT serine 473/total AKT. The data represent mean ± SD, n=3. *P<0.05 compared to morphine treatment of Ctrl-m-μOR SK-N-LO cells.

3.3 Crosstalk between cAMP-AKT pathways: Functional interaction of PI3Kγ-PKA-PDE upon agonist stimulation of μOR

PI3Kγ has protein kinase and lipid kinase activities which result in activation of ERK1/2 and AKT, respectively (Bondeva et al., 1998). Kinase-independent activities of PI3Kγ regulate intracellular cAMP levels in cardiomyocytes (Patrucco et al., 2004) by stimulatory interaction with PKA and PDE3B. In cardiomyocytes, the multiprotein complex of p110γ-PKA-PDE3B enhances the phosphodiesterase activity of PDE3B, leading to increased breakdown of cAMP, and inhibits lipid kinase activity of p110γ, leading to reduced production of PtdIns(3,4,5)P_3 (Perino et al., 2011). These observations indicated a crosstalk between cAMP-AKT pathways in case of cardiomyocytes.

In our earlier experiments, we observed that PI3Kγ was involved in morphine induced activation of AKT. In line with the observations in caridomyocytes, we asked whether or not a crosstalk between cAMP and AKT pathways exists in neuronal cells? If so does it requires PI3Kγ?

In order to address this question we used two model systems.

1) SK-N-LO cell culture system: At first we explored the existence of a crosstalk between cAMP-AKT pathways in m-μOR SK-N-LO cells. Subsequently, we investigated the requirement of PI3Kγ using the cell lines Ctrl-m-μOR SK-N-LO (with PI3Kγ) and γ5-m-μOR SK-N-LO (with down regulated PI3Kγ) generated as described in section 3.2.2.1, and
2) Primary neuronal cell culture system: We used dorsal root ganglion cells (DRGs) from wild type and PI3K$\gamma^{-/-}$ mice for this purpose. Primary neuronal cells from mouse DRGs express μOR and PI3Kγ endogenously. Availability of knockout mice makes it an ideal model system to investigate the effects of presence and absence of PI3Kγ in μOR mediated signal transduction, although it poses considerable methodological challenge.

3.3.1 Investigations in SK-N-LO cell culture system

3.3.1.1 Effect of forskolin on the activation of AKT by morphine

Here, we investigated the effect of elevated intracellular cAMP levels on activation of AKT by morphine. In order to do this, m-μOR SK-N-LO cells were preincubated with 10 μM forskolin for 15, 30 and 60 min prior to stimulation with 10 μM morphine for 2.5 min. Forskolin activates adenylyl cyclase which in turn elevates the intracellular cAMP levels (Yu and Sadée, 1988) giving rise to increased levels of PKA. Forskolin pretreatment resulted in inhibition of morphine induced activation of AKT. The phosphorylation of AKT at both threonine 308 and serine 473 sites was inhibited at all time points tested (Fig. 3.10). Forskolin treatment for 2.5 min, *per se*, did not affect AKT phosphorylation. Furthermore, pretreatment with forskolin did not affect the phosphorylation of ERK1/2 induced by morphine in the same cells, at all the time points tested.

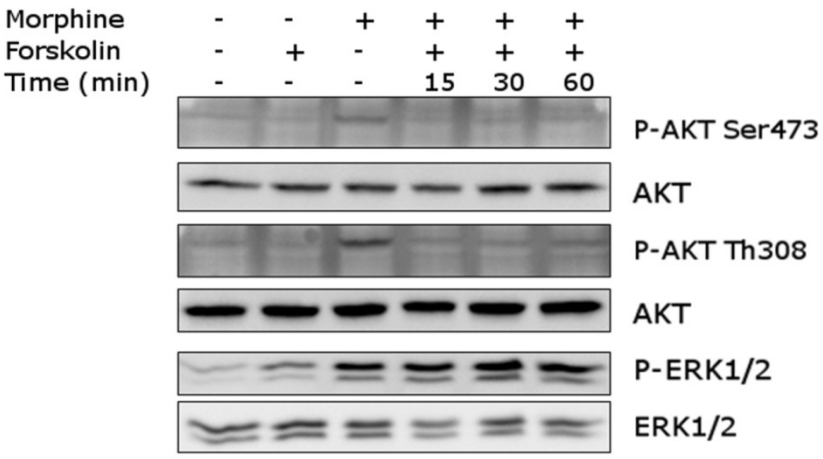

Fig. 3.10 Forskolin inhibits the morphine induced activation of AKT. m-μOR SK-N-LO cells were preincubated with 10 μM forskolin for 15 or 30 or 60 min as indicated. Subsequently, the cells were stimulated with 10 μM morphine or 10 μM forskolin for 2.5 min as indicated. After cell lysis, cell extracts were resolved by 10 % SDS-PAGE and immunoblotted as described in Fig: 3.4.

3.3.1.2 Effect of IBMX on the activation of AKT by morphine

Further we asked a question, whether elevation of intracellular cAMP resulting from inhibition of its breakdown by phosphodiesterases (PDEs) would have similar effect on morphine induced AKT phosphorylation? To test the effect of inhibition of PDEs on morphine induced activation of AKT, m-μOR SK-N-LO cells were pretreated with 500 μM IBMX for 15, 30 and 60 min prior to stimulation with 10 μM morphine for 2.5 min. IBMX is a non-selective phosphodiesterase inhibitor which enhances the intracellular cAMP levels by inhibiting its hydrolysis by PDEs (Weston et al., 1997). Pretreatment with IBMX inhibited the morphine induced phosphorylation of AKT at the phosphorylation sites, threonine 308 and serine 473. This effect was observed with a preincubation time of 15 min and persisted until 60 min (Fig. 3.11). IBMX treatment for 2.5 min, *per se*, did not have any effect on AKT phosphorylation. The phosphorylation of ERK1/2 was unaffected similar to the case of forskolin pretreatment.

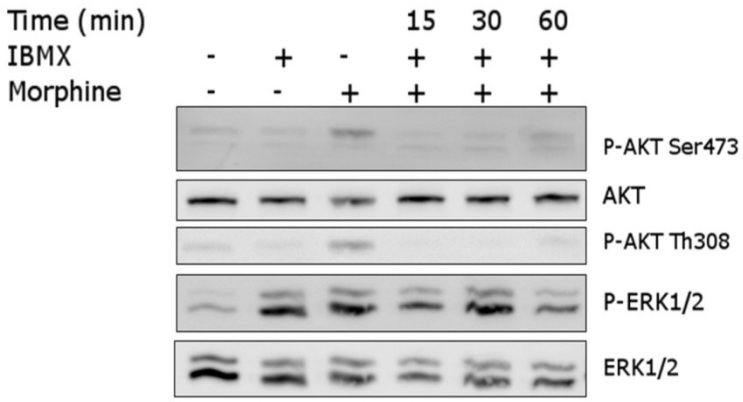

Fig. 3.11 IBMX inhibits morphine induced activation of AKT. m-μOR SK-N-LO cells were preincubated with 500 μM IBMX for 15 or 30 or 60 min as indicated. Subsequently, the cells were stimulated with 10 μM morphine or 500 μM IBMX for 2.5 min as indicated. After cell lysis cell extracts were resolved by 10 % SDS-PAGE and immunoblotted as described in Fig: 3.4.

In total, these data indicate that enhanced intracellular cAMP levels inhibited the activation of AKT upon stimulation of μOR with morphine and demonstrated the existence of crosstalk between cAMP and PI3K/AKT pathway in neuronal cells as exemplified for SK-N-LO cells.

3.3.1.3 Effect of H-89 on morphine induced activation of AKT

PKA is one of the effector proteins through which cAMP elicits its functions (Netherton et al., 2007). We hypothesized that the effects of elevated intracellular cAMP on morphine induced activation of AKT might have been mediated by PKA. In order to investigate this, m-μOR SK-N-LO cells were preincubated with 10 μM H-89, PKA inhibitor (Reuveni et al., 2002), for 15 and 30 min, prior to stimulation with 10 μM morphine for 2.5 min. The pretreatment of cells with H-89 enhanced morphine induced activation of AKT. Interestingly, the inhibition of PKA enhanced the phosphorylation of AKT at threonine 308 site but not at serine 473 site, at all the time points tested (Fig. 3.12). However, pretreatment of cells with H-89 did not alter the phosphorylation of ERK1/2 at all the time points tested. These data demonstrated that elevated cAMP levels indeed act via PKA to affect morphine induced activation of AKT.

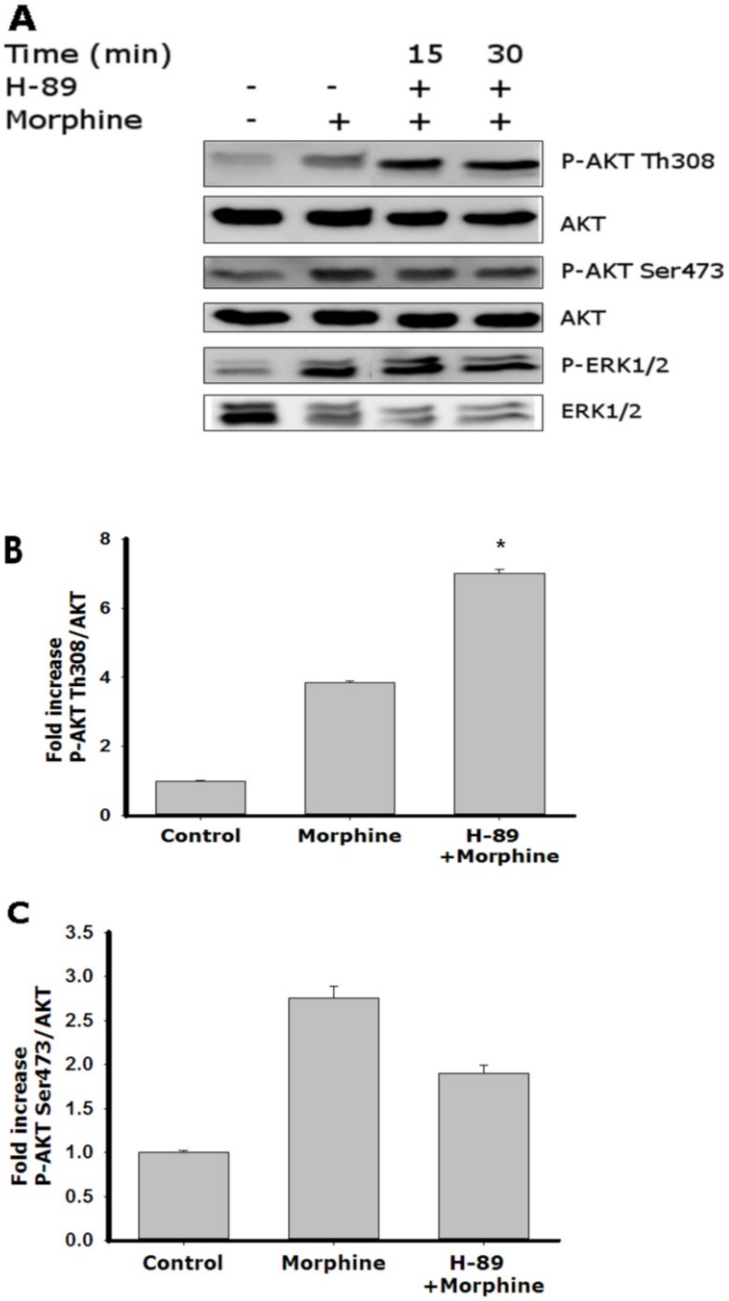

Fig. 3.12 H-89 enhances the morphine induced activation of AKT. A. m-μOR SK-N-LO cells were preincubated with 10 μM H-89 for 15 or 30 min as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min as indicated. After cell lysis, cell extracts were resolved by 10 % SDS-PAGE and immunoblotted as described in Fig: 3.4. **B, C**. Densitometric analysis of Fig. A and similar experiments showing the ratio of phosphorylated AKT threonine 308/total AKT (B) or phosphorylated AKT serine 473/total AKT (C). The data represent mean ± SD, n=3. *P<0.05 compared to the sample with morphine treatment. The responses of 30 min pretreatment with H-89 were used for quantification.

3.3.1.4 Effect of viral transduction on signaling activity in m-μOR SK-N-LO cells

Because we found that there is a crosstalk occurring between cAMP and AKT pathways in m-μOR SK-N-LO cells we further investigated the involvement of PI3Kγ in this crosstalk. At first, we explored whether the behavior of the cells to various stimuli following viral transduction is same or not? To test this, we compared the responses of m-μOR SK-N-LO cells to that of Ctrl-m-μOR SK-N-LO cells following stimulation with various agents. When stimulated with 10 μM morphine for 2.5 min, m-μOR SK-N-LO cells as well as Ctrl-m-μOR SK-N-LO cells, showed similar activation of AKT as evidenced by phosphorylation of AKT at threonine 308 (Fig. 3.13). Pretreatment with 10 μM forskolin for 30 min abolished the morphine induced phosphorylation of AKT at threonine 308 in m-μOR SK-N-LO cells as well as in Ctrl-m-μOR SK-N-LO cells, in a similar manner. Furthermore, pretreatment with 10 μM H-89 for 30 min, enhanced morphine induced activation of AKT, in both cell types, as evidenced by enhanced phosphorylation of AKT at threonine 308 site. These data suggested that the viral transduction did not alter the responses of the m-μOR SK-N-LO cells to various stimuli.

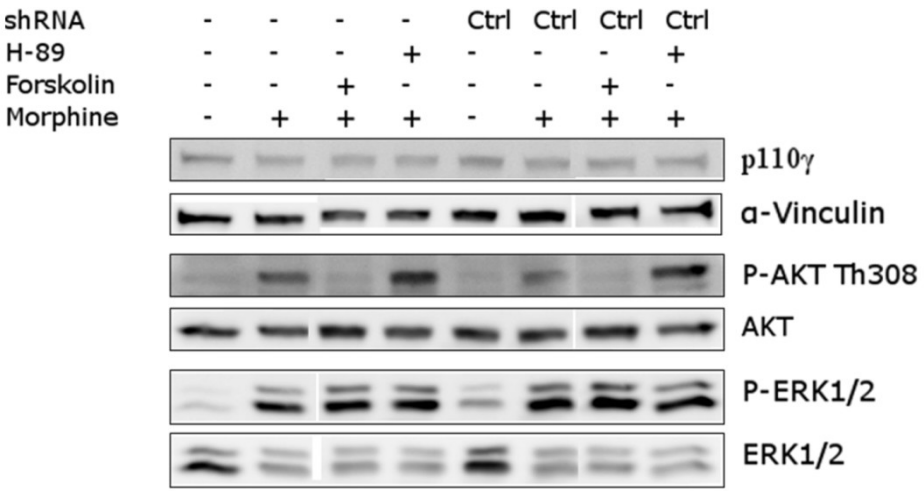

Fig. 3.13 Effect of viral transduction on signaling activity in m-μOR SK-N-LO cells. Ctrl-m-μOR SK-N-LO cells and m-μOR SK-N-LO were (cells expressing non-targeting control shRNA or naive cells) were preincubated with 10 μM forskolin (30 min) or 10 μM H-89 (30 min) as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min as indicated. After cell lysis, the lysates were subjected to SDS-PAGE and immunoblotted with specific antibodies as described in Fig. 3.9.

3.3.1.5 Role of PI3Kγ on the influence of cAMP/PKA signaling on morphine induced activation of AKT

In order to investigate the involvement of PI3Kγ in the influence of altered cAMP/PKA levels on AKT activation, Ctrl-m-μOR SK-N-LO cells and γ5-m-μOR SK-N-LO (depleted for PI3Kγ as shown above) cells were stimulated with 10 μM morphine for 2.5 min. Morphine stimulation induced activation of AKT and ERK1/2 in Ctrl-m-μOR SK-N-LO cells but not in γ5-m-μOR SK-N-LO cells suggesting the requirement of PI3Kγ for morphine induced activation of AKT.

We investigated the role of PI3Kγ in the effect of IBMX on activation of AKT by morphine. In order to do this, Ctrl-m-μOR SK-N-LO cells and γ5-m-μOR SK-N-LO cells were preincubated with 500 μM IBMX before being stimulated with 10 μM morphine for 2.5 min. The morphine induced phosphorylation of AKT at threonine 308 was significantly impaired in γ5-m-μOR SK-N-LO cells than that observed in Ctrl-m-μOR SK-N-LO cells (Fig. 3.14). Similar to the data obtained from m-μOR SK-N-LO cells, pretreatment of cells with IBMX resulted in inhibition of AKT in Ctrl-m-μOR SK-N-LO cells and this effect was diminished in γ5-m-μOR SK-N-LO cells.

Further, pretreatment with 10 μM forskolin for 30 min, abolished the AKT activation by morphine in Ctrl-m-μOR SK-N-LO cells which was absent in case of γ5-m-μOR SK-N-LO cells as there was no activation seen, in first case. Inhibition of PKA, by pretreatment with 10 μM H-89 for 30 min, enhanced the morphine induced phosphorylation of AKT at threonine 308 site in Ctrl-m-μOR SK-N-LO cells which was significantly higher than that observed in γ5-m-μOR SK-N-LO cells (Fig. 3.15). The phosphorylation at serine 473 site was not enhanced by H-89 pretreatment in response to morphine stimulation in Ctrl-m-μOR SK-N-LO cells. This was in line with the earlier observations with m-μOR SK-N-LO cells. These data indicate the requirement of PI3Kγ for the crosstalk between cAMP and AKT pathways involving PKA as a cAMP effector protein in SK-N-LO cells. The residual activity observed in case of H-89 pretreatment of γ5-m-μOR SK-N-LO cells might be a result of residual PI3Kγ as the maximum suppression achieved was ~70 % in these cells compared to Ctrl-m-μOR SK-N-LO cells. Moreover, morphine induced phosphorylation of ERK1/2 was unaffected by pretreatment with either forskolin or H-89 in Ctrl-m-μOR SK-N-LO cells whereas γ5-

m-μOR SK-N-LO cells did not show phosphorylation of ERK1/2 in response to morphine stimulation.

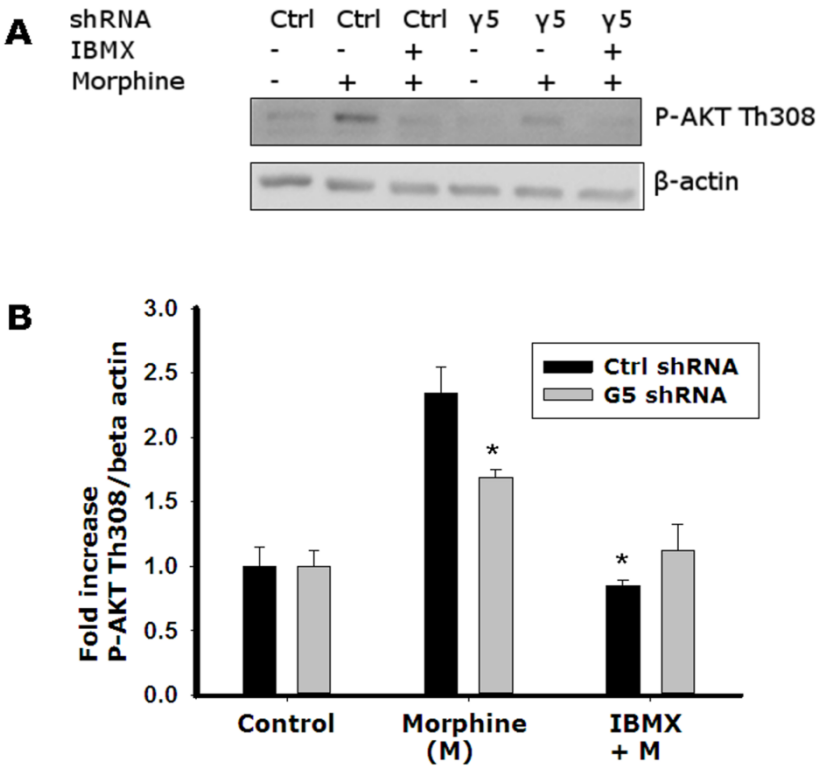

Fig. 3.14: Effect of PI3Kγ on influence of IBMX on morphine induced activation of AKT. **A.** Ctrl-m-μOR SK-N-LO cells (Ctrl shRNA) and γ5-m-μOR SK-N-LO cells (G5 shRNA) were preincubated with 500 μM IBMX for 30 min as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min as indicated. After cell lysis, cell extracts were resolved by 10 % SDS-PAGE and immunoblotted with specific antibodies as described in Fig. 3. 9. **B.** Densitometric analysis of Fig. A and similar experiments showing the ratio of phosphorylated AKT threonine 308/total AKT. The data represent mean ± SD, n=3. *P<0.05 compared to morphine treatment of Ctrl-m-μOR SK-N-LO cells.

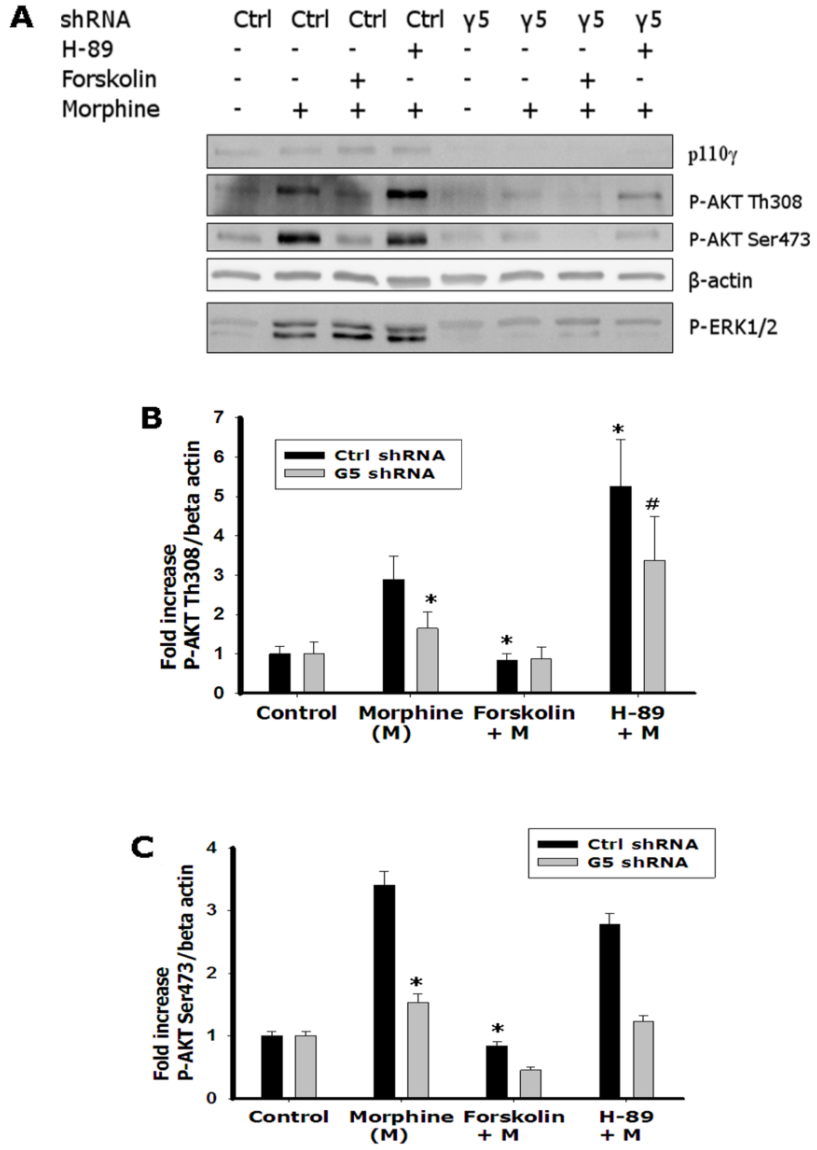

Fig. 3.15 Effect of PI3Kγ on the influence of cAMP/PKA signaling on morphine induced activation of AKT. A Ctrl-m-μOR SK-N-LO cells (Ctrl shRNA-in the legend) and γ5-m-μOR SK-N-LO cells (G5 shRNA-in the legend) were preincubated with 10 μM forskolin or 10 μM H-89 for 30 min as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min as indicated. After cell lysis, cell extracts were resolved by 10 % SDS-PAGE and immunoblotted with specific antibodies as described in Fig. 3.9. **B.** Densitometric analysis of Fig. A and similar experiments showing the ratio of phosphorylated AKT threonine 308/total AKT. The data represent mean ± SD, n=5. *P<0.05 compared to morphine treatment of Ctrl-m-μOR SK-N-LO cells. #P<0.05 compared to treatment of Ctrl-m-μOR SK-N-LO cells treated with H-89 and morphine. **C.** Densitometric analysis of Fig. A and similar experiments showing the ratio of phosphorylated AKT serine 473/total AKT. The data represent mean ± SD, n=3. *P<0.05 compared to morphine treatment of Ctrl-m-μOR SK-N-LO cells.

Elevation of intracellular cAMP levels by forskolin or IBMX inhibited the morphine induced activation of AKT in a PI3Kγ dependent way. In addition, enhancement in morphine induced activation of AKT by inhibition of PKA required PI3Kγ. Taken together, these data demonstrate the central role of PI3Kγ in the crosstalk between cAMP and AKT pathways. Further, the data indicate the functional interaction among PI3Kγ, PKA and PDE regulating the lipid kinase activity of p110γ in SK-N-LO cells upon stimulation of μOR with morphine.

3.3.2 Investigations in primary neuronal cell system

3.3.2.1 Expression of μOR and PI3Kγ in mouse DRGs

We conducted our investigations on the role of PI3Kγ in μOR mediated signaling and its possible involvement in orchestrating the crosstalk between cAMP-AKT pathways in primary cell culture of mouse DRG sensory neurons. At first we show that DRG neurons express μOR and PI3Kγ endogenously. Here, we show the expression of μOR in cultured DRG neurons and the expression of PI3Kγ in paraformaldehyde-fixed paraffin embedded sections of DRG neurons (Fig. 3.16).

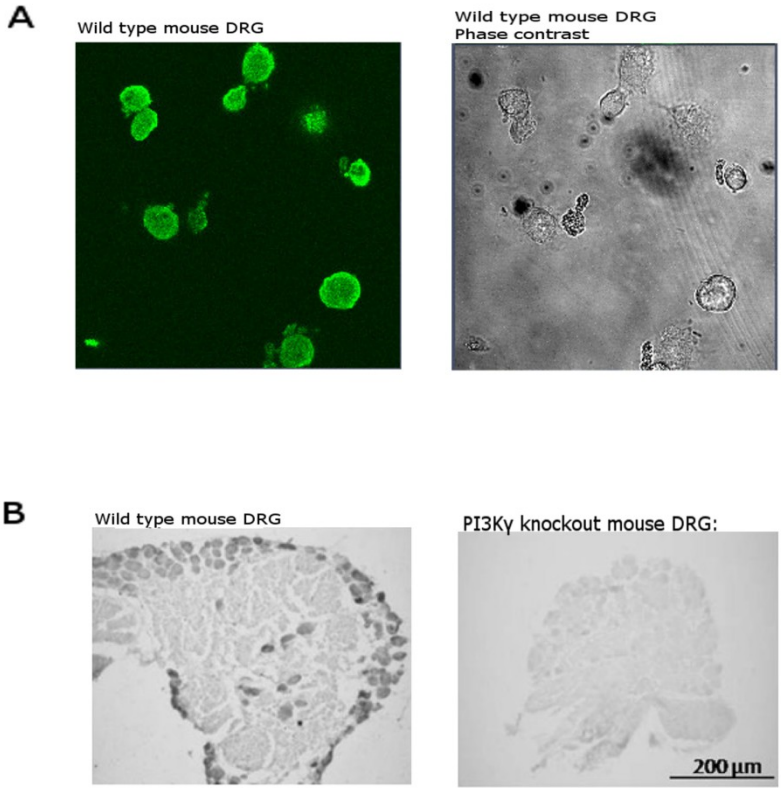

Fig. 3.16 Expression of µOR and PI3Kγ in mouse DRG. A. Confocal laser immunoflouroscence microscopy of cultured mouse DRG neurons examining the immunoreactivity for flourecently labeled anti-MOR monoclonal antibody. **B**. Immunohistochemical stainings were performed on sections from paraformaldehyde-fixed and paraffin embedded DRG from wild type and PI3Kγ knockout mice with anti-PI3Kγ specific antibody, mouse IgG2a isotype control (courtesy: Dr. C. König). DRGs from wild type mouse showing immunoreactivity for PI3Kγ in morphological small diameter neurons compared to IgG2a control staining in PI3Kγ knockout mouse DRGs.

3.3.2.2 Requirement of PI3Kγ for the effects of cAMP/PKA on morphine induced activation of AKT

To elucidate the role of PI3Kγ in signaling activity of µOR, DRG neuronal cells from wild type and PI3Kγ knockout mice were stimulated with 10 µM morphine for 2.5 min. After cell lysis, cell extracts were subjected to SDS-PAGE and immunoblotted. The treatment with morphine evoked an activation of AKT as evidenced by phosphorylation at threonine 308 site in wild type cells but not in knockout cells (Fig. 3.17). Morphine stimulation also elicited a slight activation of ERK1/2 in wild type cells, although not appreciably, as evidenced by phosphorylation of ERK1/2. In case of knockout DRG

neuronal cells, induction of phosphorylation of ERK1/2 was not observed in response to morphine

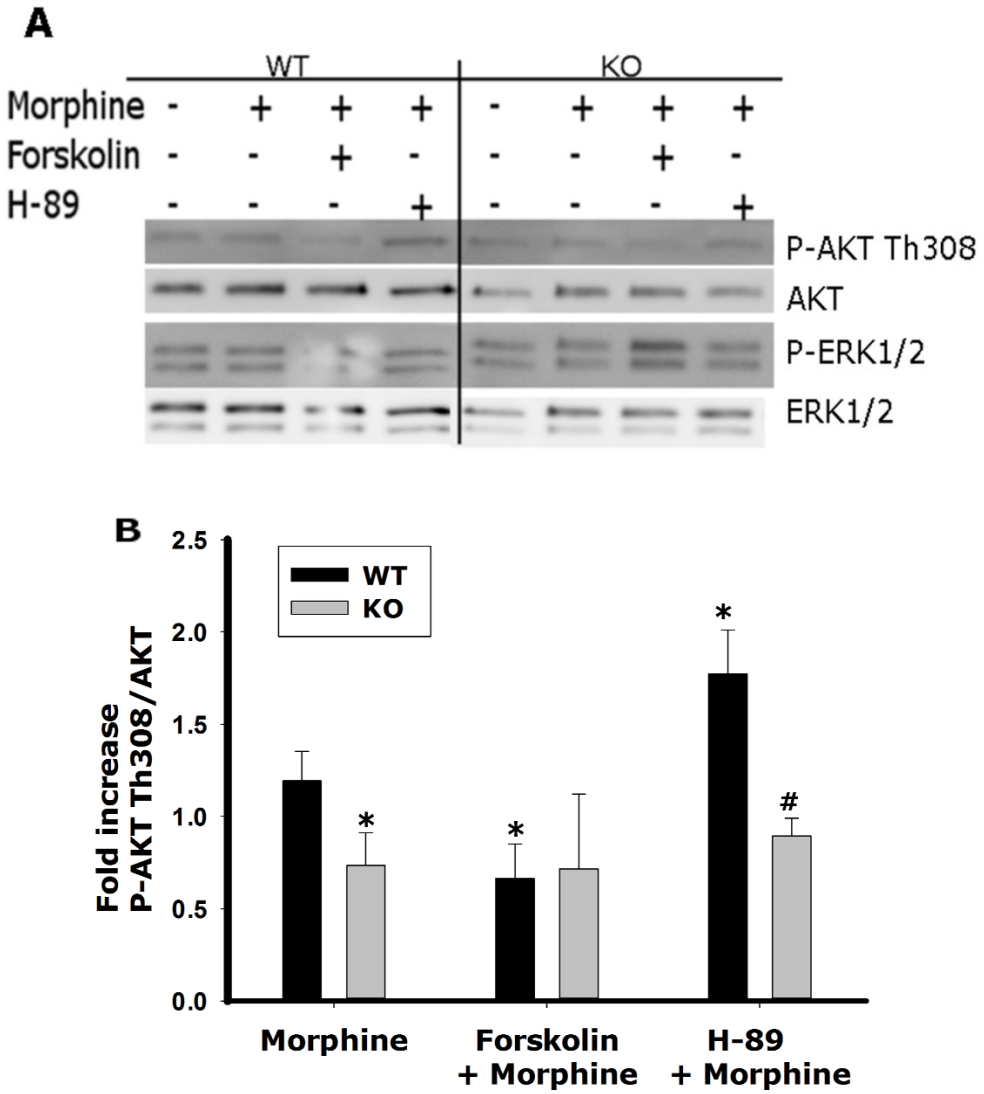

Fig. 3.17 Requirement of PI3Kγ for the influence of cAMP/PKA on activation of AKT upon stimulation of μOR with morphine. A. DRG neuronal cells from wild type and PI3Kγ knockout mice were preincubated (30 min) with either 10 μM forskolin or 10 μM H-89 as indicated. Subsequently, the cells were stimulated with 10 μM morphine for 2.5 min. After cell lysis, cell extracts were subjected to 10 % SDS-PAGE and immunoblotted with specific antibodies recognizing phosphorylated AKT threonine 308 and phosphorylated ERK1/2. Then the membranes were stripped and redeveloped with specific antibodies recognizing total AKT and total ERK1/2. **B.** Densitometric analysis of the Fig. A and similar experiments, showing the ratio of P-AKT threonine 308/total AKT. The data represents mean ± SD, n=3. *p <0.05 compared to morphine treatment of wild type DRG cells. # p<0.05 compared to H-89 treatment of wild type DRG cells.

stimulation. Taken together these data indicate that morphine induced activation of AKT in DRG neuronal cells was mediated via PI3Kγ.

Further, we investigated the functional interactions of PI3Kγ and PKA. i.e. the influence of PI3Kγ on the effects of cAMP on PI3K/AKT pathway in cultured DRG neuronal cells. In order to do this, DRG neuronal cells from wild type and knockout mice were preincubated with 10 μM forskolin for 30 min prior to stimulation with 10 μM morphine for 2.5 min. Pretreatment with forskolin inhibited the morphine induced activation of AKT in wild type cells as evidenced by inhibition of phosphorylation of AKT at threonine 308 (Fig. 3.17). In case of knockout cells, activation of AKT by morphine and inhibition by forskolin pretreatment was not observed. However, phosphorylation of ERK1/2 was unaffected by pretreatment with forskolin in case of the wild type cells but knockout cells showed a higher phosphorylation of ERK1/2 compared to morphine treatment.

cAMP elicits its cellular functions by tethering or binding to effector proteins like PKA or EPAC (Netherton et al., 2007). We hypothesized, that the effect of cAMP on AKT might have been mediated by PKA. In order to investigate this, DRG neuronal cells from wild type and knockout mice were preincubated with 10 μM H-89 (PKA inhibitor) for 30 min before stimulation with 10 μM morphine for 2.5 min. Interestingly, pretreatment with H-89 resulted in enhanced morphine induced activation of AKT in wild type cells compared to knockout cells, as evidenced by the enhanced morphine induced phosphorylation of AKT at threonine 308 site (Fig. 3.17). These data indicated that the effect of cAMP on AKT was mediated by PKA and PI3Kγ was essential for enhanced activation of AKT. However, the induction of phosphorylation of ERK1/2 was unaffected by H-89 pretreatment in the wild type cells as well as knockout cells.

Taken together, these data suggest that activation of adenylyl cyclase by forskolin, inhibition of PKA by H-89 altered lipid kinase activity of p110γ as measured by the phosphorylation of AKT at threonine 308 site that was activated upon stimulation of μOR by morphine.

4. Discussion

4.1 Generation of model systems

In order to facilitate elaborative studies to explore the mechanism of μOR signaling and the role of PI3Kγ in signaling pathways we needed a suitable and convenient model system. Thus SK-N-LO cells that express PI3Kγ endogenously were stably transfected with a plasmid encoding mouse μOR which will be referred to as m-μOR SK-N-LO. The stable production of μOR and PI3Kγ was confirmed in this cell line, subsequently. To study the function of PI3Kγ, RNAi mediated suppression of PI3Kγ was carried out. This was accomplished by transduction of m-μOR SK-N-LO cells with lentiviral particles containing non-targeting control shRNA and targeting PI3Kγ to generate two more cell lines, namely, Ctrl- m-μOR SK-N-LO and γ5 m-μOR SK-N-LO, with and without PI3Kγ, respectively. The synthesis of μOR and PI3Kγ was confirmed subsequently. In addition, we tested whether the viral transduction has any effect on the signaling activity of these cells. In our observations we found comparable levels of expression of μOR and PI3Kγ in m-μOR SK-N-LO cells and Ctrl m-μOR SK-N-LO cells i.e. before and following viral transduction. Moreover, the signaling activity initiated by these cells to various stimuli remained unchanged. Thus, these cell lines were a suitable tool to study the role of PI3Kγ in μOR mediated signal transduction.

Expression of PI3Kγ in murine bone marrow derived neutrophils, macrophages was reported earlier (Hirsch et al., 2000b). Presence of PI3Kγ in cardiomyocytes (Patrucco et al., 2004) airway smooth muscle cells (Jiang et al., 2010) and mast cells (Bohnacker et al., 2009) was reported subsequently. Expression of PI3Kγ in mouse brain tissue (mRNA and protein) was reported recently (Kim et al., 2011). Here, we show the expression of PI3Kγ in DRG neurons from wild type mice but not PI3Kγ$^{-/-}$ knockout mice. In the mean time, Cunha et al., reported the expression of PI3Kγ in wild type mouse and rat DRG neurons but not in PI3Kγ$^{-/-}$ mice DRG neurons (Cunha et al., 2010).

Xu, et al., reported the endogenous expression of μOR in cultured DRG neurons of rats (Xu et al., 2003). In addition, Becker et al., reported the co-expression of TRPV1 (transient receptor potential vanilloid receptor-1) and μOR in cultured rat DRG neurons along with the induction of expression of μOR in DRG neurons of rats with CFA induced inflammation (Endres-Becker et al., 2007). In line with the literature data we

here show the expression of μOR in cultured mouse DRG neurons. Thus, we confirmed the endogenous expression of μOR and PI3Kγ in mouse DRG neurons.

4.2 Activation of AKT by morphine

AKT also known as PKB (protein kinase B) is a 57 kDa serine /threonine protein kinase that is activated upon by various cell surface receptor stimuli. Various agonists, like chemokines and growth hormones etc., stimulate GPCRs to activate AKT which then propagates the signals inside the cells. Thus, AKT serves as a signaling mediator for many cellular processes like cell survival, adhesion, migration and proliferation, apoptosis and pain relief. AKT contains PH domain which binds to PtdIns(3,4,5)P_3/ PtdIns(4,5)P_2 produced upon activation of PI3Ks and thus serves as a marker for PI3Ks activation by cell surface receptors (Vanhaesebroeck and Alessi, 2000). Bondeva et al., reported the activation of AKT by PI3Kγ elucidating the lipid kinase activity of p110γ differentiating itself from the protein kinase activity (Bondeva et al., 1998). Generally, activation of AKT is monitored by phosphorylation of AKT at threonine 308 residue located in the activation loop and serine 473 residue located in the C-terminal hydrophobic motif. Phosphorylation of AKT at threonine 308 site occurs via phosphoinositide–dependent kinase-1(PDK1) which is also a PH domain containing protein kinase that binds to PtdIns(3,4,5)P_3. But PDK1 dependent phosphorylation of AKT is strictly dependent on prior binding of PtdIns(3,4,5)P_3 to AKT at PH domain because only then it relives the PH domain mediated inhibition to make the threonine 308 in the activation loop available for phosphorylation (McManus et al., 2004; Hawkins et al., 2006). Moreover, downstream protein kinase activity of AKT is best represented by the phosphorylation at threonine 308 site rather than at serine 473 site: Phosphorylation of downstream substrates of AKT kinase PRAS40, TSC2 which are important regulatory components of mTOR complex and TBC1D4 which is a Rab GTPase activating protein with a role in regulation of glucose metabolism, are best correlated with phosphorylation of AKT at threonine 308 than at serine 473 in samples of early-stage non-small cell lung carcinoma (NSCLC) (Vincent et al., 2011). Further, a better correlation was established between phosphorylation of AKT at threonine 308 site (but not serine 473 site) and poor prognosis in NSCLC and acute myeloid leukemia (Tsurutani et al., 2007; Gallay et al., 2009). Hence, in our studies, we monitored

phosphorylation of AKT at threonine 308 site preferentially over phosphorylation at serine 473 site.

Morphine which is an agonist at μOR stimulates survival signals by activation of AKT, p70S6k and 4E-BP1 in Chinese Hamster ovary cells expressing μOR where the authors reported the phosphorylation of AKT at serine 473 site (Polakiewicz et al., 1998). In agreement with these data, in our studies we found that stimulation of m-μOR SK-N-LO cells with morphine resulted in activation of AKT as monitored by phosphorylation of AKT at serine 473 site. In addition, we observed the phosphorylation of AKT at threonine 308 site in response to morphine stimulation in these cells. Phosphorylation at both sites followed similar kinetics, peaking at 2.5 min with decline to reach control levels by 30 min. Polcakwiz et al., reported that the activation of AKT by morphine occurs via a pathway sensitive to Gi subtype of GPCR inactivator, pertussis toxin (PTX), μOR specific antagonist, naloxone and pan PI3Ks inhibitor, wortmannin (Wm) in Chinese Hamster ovary cells expressing μOR (Polakiewicz et al., 1998). Similar to these data preincubation of m-μOR SK-N-LO cells with PTX, naloxone and Wm inhibited the morphine induced activation of AKT as evidenced by inhibition of phosphorylation of AKT at threonine 308 and serine 473 sites. This indicated that morphine induced activation of AKT in m-μOR SK-N-LO cells occurred via μOR which is a Gi type of GPCR and was mediated by the activation of PI3K.

A similar report about the involvement of PI3Kγ in Gi subtype of GPCR signaling was from our laboratory proving the activation of MAPK by stimulation of m2 receptors over expressed in COS-7 cells with carbachol (Lopez-Ilasaca et al., 1997). There are reports about the involvement of class IA PI3K isoform, PI3Kβ, in GPCR mediated activation of AKT. Cardiomyocytes from PI3Kβ knockout mice show inhibition of AKT compared to their wild type counterparts upon stimulation of β-adrenergic receptor with isoproterenol (Perino et al., 2011). In macrophages, PI3Kβ along with PI3Kγ contributed to the activation of AKT in response to complement fragment 5a (C5a) stimulation as evidenced by blockade of phosphorylation of AKT by preincubation of cells with isoform specific small molecule inhibitors (Guillermet-Guibert et al., 2008). In agreement with these literature data, our data indicated that PI3Kγ and PI3Kβ are the PI3K isoforms involved in mediation of morphine induced activation of AKT in m-μOR SK-N-LO cells. Pharmacological inhibition of PI3Kγ and β by preincubation of m-μOR SK-N-LO cells with 100 nM AS605240 and TGX 221,

selective inhibitors of PI3Kγ and β, respectively, inhibited the morphine induced phosphorylation of AKT at threonine 308 and serine 473 sites. Pharmacological inhibition of PI3Kα and δ by pretreatment with A66 and IC87114 - PI3Kα and δ selective inhibitors, respectively, did not interfere with morphine induced activation of AKT in m-μOR SK-N-LO cells. This is consistent with the literature reports indicating their involvement in receptor tyrosine kinase mediated signaling rather than GPCR mediated signaling (Vanhaesebroeck et al., 2010).

Morphine induced activation of AKT as evidenced by the phosphorylation of AKT at threonine 308 site and its inhibition by PTX pretreatment was observed in Ctrl-m-μOR SK-N-LO cells expressing non-targeting shRNA control, similar to the case of m-μOR SK-N-LO cells suggesting the unaltered signaling activity of the cells following viral transduction. In addition, γ5-m-μOR SK-N-LO PI3Kγ knock down cells showed significantly reduced activation of AKT compared to Ctrl-m-μOR SK-N-LO cells in response to morphine stimulation. Phosphorylation of AKT at threonine 308 and serine 473 sites was significantly inhibited by depletion of PI3Kγ suggesting the requirement of PI3Kγ for morphine induced activation of AKT.

In similar lines, we observed the phosphorylation of AKT at threonine 308 site following stimulation of wild type cultured DRG neurons with morphine which was not observed in PI3Kγ$^{-/-}$ DRGs in our studies, suggesting the requirement of PI3Kγ for morphine induced activation of AKT. Cunha et al., showed stimulation of cultured DRG neurons with morphine induced activation of AKT as shown by phosphorylation at serine 473 site which was mediated by μOR. This activation of AKT was not observed in DRG neurons that lack PI3Kγ (Cunha et al., 2010).

4.3 Activation of ERK1/2 by morphine

Earlier reports suggested the activation of MAPK by stimulation of μOR over expressed in Chinese hamster ovary cells. Activation peaked at 4 to 8 min and maintained till 1 h (Li and Chang, 1996). In line with this, Rozenfeld et al., reported the activation of ERK1/2 upon stimulation of Chinese hamster ovary cells expressing μOR with DAMGO (Rozenfeld and Devi, 2007). In agreement with these data, we found that morphine stimulation induced a strong phosphorylation of ERK1/2 in m-μOR SK-N-LO

cells but differing from the reported observations followed a time-course with strong and maximum activation at 2.5 min with a decline to reach control levels by 30 min. The time-course of morphine induced phosphorylation of ERK1/2 followed similar kinetics to that of activation of AKT in m-μOR SK-N-LO cells. Further, morphine induced phosphorylation of ERK1/2 in m-μOR SK-N-LO cells was inhibited by PTX, naloxone and Wm pretreatment suggesting that it was mediated by Gi subtype of GPCR. These data indicated that the μOR mediated activation of ERK1/2 by morphine was dependent on activation of PI3K which was in agreement with the observation from earlier reports.

Involvement of PI3Kγ in GPCR mediated activation of ERK1/2 representing the protein kinase activity of p110γ was reported earlier from our laboratory (Lopez-Ilasaca et al., 1997). Similar to this data, morphine induced ERK1/2 activation was mediated by PI3Kγ in m-μOR SK-N-LO cells. Pharmacological inhibition of PI3Kγ, by AS605240 inhibited the phosphorylation of ERK1/2 in a dose-dependent manner. Pharmacological inhibition of PI3Kα and δ did not interfere with the phosphorylation of ERK1/2. Pharmacological inhibition of PI3Kβ by preincubation of m-μOR SK-N-LO cells with TGX221 inhibited the phosphorylation of ERK1/2 suggesting an additional role for this class IA PI3K isoform in μOR signaling as in the case of activation of AKT. This was in agreement with recent reports indicating a role for PI3Kβ in GPCR mediated signaling as in the case of AKT activation.

Morphine induced activation of ERK1/2 as evidenced by the phosphorylation of ERK1/2 and its inhibition by PTX pretreatment was observed in Ctrl-m-μOR SK-N-LO cells expressing non-targeting shRNA control, similar to the case of m-μOR SK-N-LO cells suggesting the unaltered signaling activity of the cells following viral transduction. In addition, γ5-m-μOR SK-N-LO PI3Kγ down regulated cells showed almost complete inhibition of activation of ERK1/2 compared to Ctrl-m-μOR SK-N-LO cells in response to morphine stimulation, suggesting the requirement of PI3Kγ for morphine induced activation of ERK1/2.

However, we did not observe appreciable levels of activation of ERK1/2 upon morphine stimulation of wild type DRG neurons compared to PI3Kγ$^{-/-}$ DRGs. Moreover, forskolin and H-89 pretreatment did not alter this effect in wild type DRGs.

4.4 Central role of PI3Kγ in crosstalk between cAMP-AKT pathways

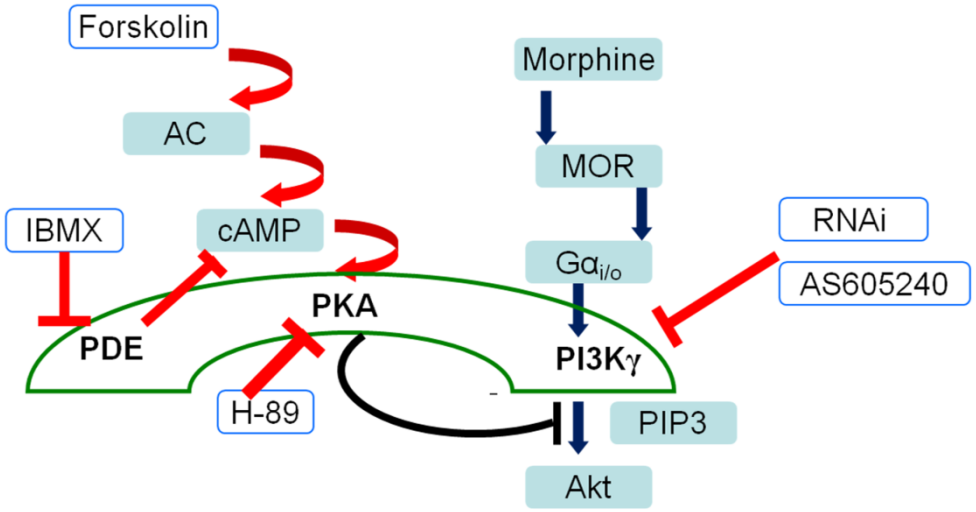

Fig. 4.1 Schematic representation of elucidation of the role of PI3Kγ in crosstalk between cAMP and AKT pathways. Role of PI3Kγ in morphine induced activation of AKT was investigated by pharmacological inhibition and genetic knockdown of PI3Kγ. Forskolin, IBMX elevates cAMP levels which in turn affected morphine induced activation of AKT. H-89 inhibits PKA which subsequently affected morphine induced activation of AKT, illustrating the crosstalk between cAMP-AKT pathways. Effects of forskolin, IBMX and H-89 on morphine induced activation of AKT in presence and absence of PI3Kγ were investigated to elucidate the role of PI3Kγ in the crosstalk between cAMP/PKA – PI3K/AKT pathways.

Because we observed that PI3Kγ mediates morphine induced activation of AKT we further explored the crosstalk between cAMP-AKT pathways and the role PI3Kγ plays in this crosstalk. In our cell culture model, we altered the intracellular cAMP levels either by activation of adenylyl cyclase or by inhibition of phosphodiesterases that catalyze the hydrolysis of cAMP and monitored the effects on morphine induced activation of AKT. Preincubation of m-μOR SK-N-LO cells with either forskolin, indirect activator of cAMP or IBMX, a non specific phosphodiesterase (PDEs) inhibitor, inhibited the morphine induced activation of AKT as evidenced by the inhibition of phosphorylation of AKT at threonine 308 and serine 473 sites. In similar lines, pretreatment of m-μOR SK-N-LO cells with H-89, PKA inhibitor, enhanced

morphine induced activation of AKT. Interestingly, the phosphorylation at threonine 308 site but not at the serine 473 site was enhanced significantly by the inhibition of PKA in these cells. This novel finding demonstrated that the H-89 effect was mediated indeed via PI3Ks. This observation was supported by earlier reported observations where staurosporine, a potent PDK1 inhibitor, attenuated the activation of AKT in cultured HEK293 cells stimulated by insulin which is manifested as inhibition of phosphorylation at threonine 308 site with no effect on phosphorylation of serine 473 site, which is a mTORC2 effector protein in many cell types (Hill et al., 2001; Hresko and Mueckler, 2005). Moreover, preincubation of m-μOR SK-N-LO cells with either forskolin, IBMX or H-89 did not alter morphine induced phosphorylation of ERK1/2 suggesting that the protein kinase activity of p110γ was not affected. Taken together, these data indicated a crosstalk between cAMP-AKT pathways arising out of functional interaction among PI3Kγ-PKA-PDE resulting in alteration of lipid kinase activity of p110γ in neuronal cells as exemplified by SK-N-LO cells. Interestingly, only the lipid kinase activity of p110γ was affected by these interactions but not the protein kinase activity as measured by the phosphorylation of AKT at threonine 308. However, elevation of cAMP by forskolin or IBMX resulted in inhibition of phosphorylation of AKT at both the phosphorylation sites but inhibition of PKA resulted in enhancement of phosphorylation of threonine 308 site only. The reason for such difference needs further investigations.

In similar lines, pretreatment of Ctrl-m-μOR SK-N-LO cells expressing non-targeting shRNA control with forskolin or IBMX inhibited the morphine induced phosphorylation of AKT at threonine 308 and serine 473 sites similar to m-μOR SK-N-LO cells. But as the morphine induced activation of AKT was not observed in case of γ5-m-μOR SK-N-LO PI3Kγ depleted cells further inhibition by forskolin could not be observed. However, the effect of IBMX pretreatment on morphine induced AKT activation was diminished in case of γ5-m-μOR SK-N-LO cells compared to Ctrl-m-μOR SK-N-LO cells. Furthermore, H-89 pretreatment of Ctrl-m-μOR SK-N-LO cells enhanced morphine induced phosphorylation of AKT at threonine 308 site but not at serine 473 site similar to the case of m-μOR SK-N-LO cells whereas γ5-m-μOR SK-N-LO cells showed significantly reduced enhancement of morphine induced phosphorylation of AKT. Taken together, these data indicates the functional interaction

between PI3Kγ, PKA and PDE in neuronal cells as exemplified by SK-N-LO cells and the requirement of PI3Kγ for the crosstalk between cAMP/PKA-PI3K/AKT pathways.

In our observations, elevation of intracellular cAMP levels by forskolin pretreatment resulted in inhibition of morphine induced phosphorylation of AKT in cultured DRG neurons. In addition, inhibition of PKA by H-89 pretreatment enhanced the morphine induced activation of AKT in cultured DRG neurons. These data suggest a crosstalk between cAMP/PKA and PI3K/AKT pathways. Furthermore, altered morphine induced phosphorylation of AKT by forskolin and H-89 was observed in case of DRG neurons from wild type mice but not PI3Kγ$^{-/-}$ mice. This novel observation indicated the requirement of PI3Kγ for PKA mediated effects on AKT resulting in crosstalk between cAMP and AKT pathways. Moreover, the altered PKA levels did not affect the phosphorylation of ERK1/2.

The direct interaction of PI3Kγ with PKCα regulating the *f*MLP induced production of reactive oxygen species in neutrophils was reported from our laboratory earlier (Lehmann et al., 2009). Perino et al., reported the functional and physical interaction between PI3Kγ and PKA earlier. In cardiomyocytes PKA binds to a site on N-terminal region of p110γ orchestrating the multiprotein complex containing regulatory subunits p84/87-p110γ-PKA-PDE3B. The association of PKA with p110γ leads to phosphorylation of p110γ and PDE3B by PKA. Further, the phosphodiesterase activity of PDE3B will be enhanced resulting in breakdown of cAMP negatively affecting myocardial contractility. Binding of regulatory subunits to the p110γ following PKA binding is essential for this stimulation of PDE activity of PDE3B (Perino et al., 2011). In cardiomyocytes lacking PI3Kγ higher cAMP levels were observed which was due to defective PDE3B functioning. Consequently, PI3Kγ$^{-/-}$ mice developed rapid and fatal cardiomyopathy in response to cardiac pressure overload induced by transverse aortic constriction (TAC). Interestingly, PI3Kγ$^{KD/KD}$ mice, unlike PI3Kγ$^{-/-}$ mice, showed similar adaptive responses to that of control mice indicating that PI3Kγ has kinase-indepenednt functions (Patrucco et al., 2004).

In addition, the lipid kinase activity of PI3Kγ will be affected negatively by PKA mediated phosphorylation of p110γ. In case of cardiomyocytes, agonist stimulation of β-adrenergic receptor, which is a Gs coupled GPCR, increases the intracellular cAMP levels giving rise to elevated PKA levels which then phosphorylate p110γ inhibiting its

lipid kinase activity. Under such circumstances, pretreatment with H-89 restored the lipid kinase activity of p110γ following isoproterenol treatment as evidenced by the increased PtdIns(3, 4, 5)P_3 production (Perino et al., 2011). However, it was not shown whether this was reflected down-stream of PtdIns(3, 4, 5)P_3 i.e. at the level of phosphorylation of AKT which is a bona-fide effector protein containing PH domain that is activated upon by PtdIns(3, 4, 5)P_3 and a marker for PI3Kγ lipid kinase activity downstream of PtdIns(3, 4, 5)P_3 (Bondeva et al., 1998).

In our experiments, absence of PKA mediated inhibition, brought about by pretreatment with H-89, might have resulted in enhanced lipid kinase activity of p110γ causing enhanced morphine induced phosphorylation of AKT at threonine 308 in case of SK-N-LO cells and DRG neurons with PI3Kγ, but not in those with down regulated PI3Kγ or without PI3Kγ. This might have downstream functional consequences.

Capsaicin induces activation of AKT and ERK1/2 in a PI3K dependent way in cultured rat DRG neurons. In a model of rat hind paw inflammation, intradermal injection of capsaicin produced heat hyperalgesia which was inhibited by pretreatment with wortmannin (PI3K inhibitor) or PD98059 or U0126 (MEK/ERK inhibitors). However, the early induction of hyperalgesia was inhibited only by inhibition of PI3K but not by the inhibition of MEK suggesting that the early induction of hyperalgesia is independent of ERK activation (Zhuang et al., 2004). Morphine induces peripheral analgesia in a PGE2 induced inflammatory pain model by activating AKT in a PI3Kγ dependent way which then activates nNOS (neuronal nitric oxide synthase) and NO (nitric oxide) production in DRG neurons resulting in increased K_{ATP} currents hyperpolarizing the nonciceptive neurons (Cunha et al., 2010). Morphine inhibits TRPV1 currents induced by capsaicin by acting on μOR and in a cAMP/PKA dependent pathway. In vivo manifestation of this was inhibition of capsaicin induced inflammatory pain (Endres-Becker et al., 2007). Taken together, these data illustrate that morphine induced peripheral analgesia involves the two signaling pathways cAMP pathway as well as AKT pathway. Our observations showed the effects of cAMP on AKT upon stimulation of μOR with morphine in SK-N-LO cells and cultured mouse DRG neurons. Hence, our observation that H-89 pretreatment enhanced morphine induced phopshorylation of AKT i.e. at a level down-stream of PtdIns(3, 4, 5)P_3 might indicate that this interaction of PI3Kγ-PKA would have functional implications downstream of AKT leading to peripheral analgesia induced by morphine.

In summary, our observations from SK-N-LO cell system and complementary data from primary neuronal cell system suggest that PI3Kγ mediates morphine induced activation of AKT and acts as a nodal point organizing a crosstalk between cAMP and AKT pathways in these model systems. This is in line with recent investigations in cardiomyocytes (Patrucco et al., 2004; Perino et al., 2011). Taken together, this is represented as a model in Fig. 4.2, wherein PI3Kγ functions as a nodal point in the signal transduction by μOR upon agonist stimulation.

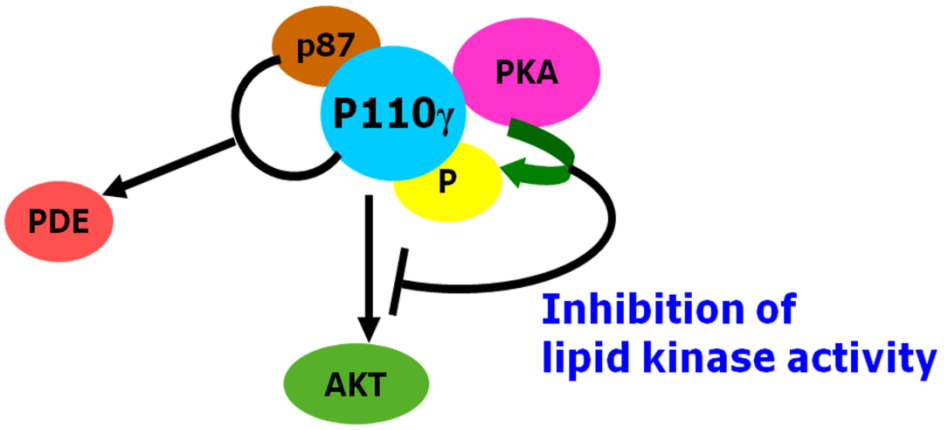

Fig. 4.2 Proposed model of interaction of p84/87, p110γ, PKA and PDE

5. Zusammenfassung

Phosphoinositide-3-kinase (PI3Ks) sind Enzyme, welche die Phosphorylierung der 3'-Position von Inositolen katalysieren. Durch diese Aktivität sind sie an der Regulation von vielen zellulären Prozessen wie Proliferation, Überleben und Migration beteiligt. Während die Isoformen PI3Kα, β und PI3Kδ in der Signaltransduktion von Rezeptor-Tyrosin-Kinasen beteiligt sind, regulieren die Isoformen PI3Kβ and PI3Kγ Signalwege, die über G-Protein-gekoppelte–Rezeptoren (GPCR) vermittelt werden.

Das Rezeptorprotein μOR ist ein GPCR, welches sich mit G_i-Proteinen assoziiert. Morphin ist eine opioide analgetische Droge, die als Agonist von μOR zur Schmerzlinderung eingesetzt wird. Durch permanente Behandlung dieses Agonisten kommt es zur Desensibilisierung des μOR und zu zellulären Anpassungen, welche die Wirkung dieses Agonisten einschränken. Die molekularen Mechanismen, die zu dieser Desensibilisierung führen sind noch nicht aufgeklärt.

Um die Funktion von PI3Kγ bei der μOR-vermittelten Signaltransduktion weitergehend beschreiben zu können, wurden geeignete Modell-Zellsysteme generiert. Die humane Neuroblastom-Zelllinie SK-N-LO, die endogen PI3Kγ exprimiert, wurde mit einem μOR-kodierenden Plasmid transfiziert und stabil μOR-produzierende m-μOR-SK-N-LO-Zellen propagiert. Durch stabile lentivirale Transduktion von shRNA wurde in diesen Zellen die Expression von PI3Kγ stabil unterdrückt. Die generierte Zelllinie γ5-m-μOR-SK-N-LO zeigt ein um 70% vermindertes Niveau von PI3Kγ im Vergleich zu der mit einer Kontroll-shRNA transduzierten Zelllinie Ctrl-m-μOR-SK-N-LO.

Die Stimulation von m-μOR-SK-N-LO mit Morphin resultierte in einer Aktivierung der Signalproteine AKT und ERK1/2. Das Maximum der Stimulation wurde 2,5 min nach Morphin-Zugabe erreicht. Die Aktivierung von AKT wurde durch die Phosphorylierung der Aminosäure-Reste $Serin_{473}$ und $Threonin_{308}$ demonstriert. Die Behandlung der Zellen mit dem Gi-Aktivator Pertussis Toxin, dem spezifischen m-μOR-Agonisten Naloxon bzw. dem pan-spezifischen PI3K-Inhibitor Wortmannin hemmte die Morphin-induzierte Aktivierung von AKT. Daraus lässt sich schlussfolgern, dass PI3K-Proteine bei der Aktivierung von μOR beteiligt sind.

Die pharmakologische Hemmung von PI3Kβ oder PI3Kγ blockierte die Morphin-induzierte Aktivierung von AKT in m-μOR-SK-N-LO-Zellen. Demgegenüber hatte in

diesen Zellen die pharmakologische Hemmung von PI3Kα oder PI3Kδ keinen Einfluss auf die Morphin-vermittelte Aktivierung von AKT. Die PI3Kγ-Knock down Zelllinie γ5-m-μOR-SK-N-LO zeigte eine signifikant reduzierte Phosphorylierung von AKT, woraus sich schlussfolgern lässt, dass PI3Kγ für die Morphin-vermittelte Aktivierung von AKT benötigt wird.

Forskolin und IBMX sind Agenzien, welche das intrazelluläre Niveau von cAMP erhöhen. Die Vorinkubation von m-μOR-SK-N-LO-Zellen mit diesen Substanzen inhibierte die Morphin-vermittelte Aktivierung von AKT. Die Hemmung der Proteinkinase A (PKA) mit H-89 erhöhte die Morphin-induzierte $Threonin_{308}$-Phosphorylierung von AKT. Diese Ergebnisse demonstrieren den cross talk der cAMP- und μOR-vermittelten Signaltransduktionswege. Während die Vorbehandlung von Ctrl-m-μOR-SK-N-LO-Zellen zu einer signifikanten Erhöhung der Morphin-vermittelten AKT-Aktivierung führte, war diese Stimulierung in PI3Kγ-depletierten γ5-m-μOR-SK-N-LO-Zellen drastisch eingeschränkt. Daraus lässt sich schlussfolgern, dass PI3Kγ am cross talk von cAMP und AKT-vermittelten Signalwegen beteiligt ist.

Durch die Phosphorylierung von AKT am Threonin-Rest 308 wurde in dieser Arbeit erstmalig gezeigt, dass Morphin AKT aktiviert. Diese Signalaktivität wurde als Marker eingesetzt, um den cross talk zwischen cAMP- und AKT-vermittelten Signalwegen weitergehend zu untersuchen. Die Erhöhung der AKT-Stimulierung durch die Hemmung von PKA über H-89 und Abhängigkeit dieser Stimulierung durch PI3Kγ liefern neue Einblick in die Regulation dieser Signalprozesse. Da AKT über PI3Kγ aktiviert wird, lässt sich spekulieren, dass die Hemmung von PKA in einer Erhöhung der spezifischen Lipid-Kinase-Aktivität resultiert.

Weitergehend wurde der Zusammenhang zwischen cAMP- und AKT-vermittelten Signalwegen in primären neuronalen Zellen untersucht, welche von PI3Kγ-Wildtyp und PI3Kγ-Knock out Mäusen isoliert wurden. Die Morphin-induzierte Aktivierung von AKT wurde in kultivierten DRG (dorsal root ganglia)-Wildtyp-Neuronen nachgewiesen. In DRG-Zellen von PI3Kγ-Knock out-Mäusen fand diese Aktivierung nicht statt. Außerdem führte die Vorbehandlung von DRG in Wildtyp-Zellen zu einer Inhibierung der Morphin-induzierten AKT-Aktivierung, die nicht in DRG-Knock out-Zellen beobachtet werden konnte. Die Hemmung von PKA durch H-89 resultierte in einer Stimulierung der Morphin-induzierten Aktivierung von AKT in Wildtyp-DRG, jedoch nicht in PI3Kγ-Knock out-DRG.

6. Summary

Phosphoinositide-3-kinases (PI3Ks) are enzymes that catalyze the phosphorylation of 3 position of the inositol ring of the phosphoinositol lipids. By this way they are implicated in many cellular processes like cell cycle progression, cell growth, survival and migration. Whereas PI3Kα, β and PI3Kδ isoforms were involved in receptor tyrosine kinase signaling, PI3Kβ and PI3Kγ are the PI3K isoforms involved in GPCR mediated signaling.

μOR is a Gi coupled GPCR. Morphine, an opioid analgesic drug, acts as an agonist at μOR to exert pain relief in moderate to severe pain conditions. Loss of analgesic effectiveness of morphine following repeated use is mainly attributed to the desensitization of the μOR and cellular adaptations that takes place following stimulation of μOR. The molecular mechanisms associated with desensitization of μOR need further understanding.

In order to investigate the role of PI3Kγ in μOR mediated signaling we generated suitable model cell systems. At first, SK-N-LO cells endogenously expressing PI3Kγ were transfected with a plasmid encoding μOR to generate a cell line stably expressing μOR (m-μOR SK-N-LO cells). Then, PI3Kγ gene was stably down regulated in m-μOR SK-N-LO cells using shRNA approach to generate γ5-m-μOR SK-N-LO cells with down-regulated PI3Kγ (~ 70 % suppression was observed) and Ctrl-m-μOR SK-N-LO cells expressing normal levels of PI3Kγ (shRNA control).

Stimulation of m-μOR SK-N-LO cells with morphine resulted in activation of AKT and ERK1/2. Maximum activation was observed at 2.5 min post stimulation. Activation of AKT was evidenced by phosphorylation of AKT at serine 473 and threonine 308. Pertussis toxin (Gi inactivator), naloxone (specific μOR antagonist) and wortmannin (pan PI3Ks inhibitor) pretreatment inhibited the morphine induced activation of AKT and ERK1/2 in m-μOR SK-N-LO cells, suggesting the involvement of Gi-coupled GPCRs, in particular μOR and a pathway dependent on activation of PI3Ks.

Pharmacological inhibition of PI3Kβ or PI3Kγ isoform impaired the morphine induced activation of AKT in m-μOR SK-N-LO cells. However, pharmacological inhibition of PI3Kα or PI3Kδ isoform did not interfere with morphine induced activation of AKT in m-μOR SK-N-LO cells. Moreover, in response to morphine, γ5-m-μOR SK-N-LO

PI3Kγ down-regulated cells showed significantly reduced phosphorylation of AKT at serine 473 and threonine 308 sites indicating the requirement of PI3Kγ for the morphine induced activation of AKT.

Preincubation of m-μOR SK-N-LO cells with intracellular cAMP elevating agents, forskolin or IBMX, inhibited the activation of AKT in response to morphine. In addition, inhibition of PKA by H-89 enhanced the morphine induced phosphorylation of AKT at threonine 308 site. Together, these data indicated a crosstalk between cAMP-AKT pathways in m-μOR SK-N-LO cells. Further, H-89 pretreatment resulted in significantly reduced enhancement of morphine induced AKT activation in γ5-m-μOR SK-N-LO down-regulated cells compared to Ctrl-m-μOR SK-N-LO cells indicating the requirement of PI3Kγ for the crosstalk between cAMP-AKT pathways.

Here we showed for the first time, morphine induced activation of AKT as measured by the phosphorylation of AKT at threonine 308 site. Taking this as a marker, the crosstalk between cAMP-AKT pathways was explored. The novel observations included the enhancement of morphine induced phosphorylation of AKT at threonine 308 site following inhibition of PKA by H-89 and its dependence on PI3Kγ. Because the activation of AKT might be considered as a marker for the lipid kinase activity of PI3Kγ, we could propose that the inhibition of PKA might have enhanced the lipid kinase activity of PI3Kγ, as measured by the phosphorylation of AKT at threonine 308 site.

Finally, the crosstalk between cAMP-AKT pathways has been explored in primary neuronal cells from wild type and PI3Kγ knockout mice. Morphine induced activation of AKT was observed in cultured DRG neurons of wild type but not PI3Kγ knockout mice as measured by the phosphorylation of AKT at threonine 308 site. Moreover, forskolin pretreatment of DRG neurons of wild type mice resulted in inhibition of morphine induced AKT activation which was not observed in DRG neurons from PI3Kγ knockout mice. Inhibition of PKA by H-89 resulted in enhanced morphine induced activation of AKT in wild type DRG neurons but not in PI3Kγ knockout DRG neurons.

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8. Appendix

Symbols

^{o}C	degree Celicus
g	Gramm
h	Hour
kDa	kilo Dalton
min	Minute
rpm	Rounds per minute

Abbreviations

µOR	Mu-opioid receptor
4E-BP	Eucaryotic translation initiation factor 4E binding protein 1
ABD	Adapter binding domain
ADP	Adenosine diphosphate
AKT (PKB)	Protein Kinase B
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
ERK	Extracellular signal-regulated Kinase
*f*MLP	Formyl-Methyl-Leucyl-Phenylalanin
GAP	GTPase activating proteins
GDP	Guanosine diphosphate
GEF	Guanine exchange factors
GFP	Green flourescent protein
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GSK3β	Glycogen synthase kinase-3β

GTP	Guanosine triphosphate
IBMX	3-Isobutyl-1-methyxanthine
LB	Luria Broth
MAPK	Mitogen-activated protein kinase
mRNA	messenger RNA
mTORC	mammalian target of rapamycin complex
NMDA	N-methyl-D-glutamate aspartate
NO	Nitric oxide
p70S6K	p70S6K kinase
PDE3B	Phosphodiesaterase 3B
PDK1	Phosphoinositide dependent kinase1
PH	Pleckstrin homology
PI3K	Phosphoinositide 3- kinase
PKA	Protein kinase A
PKC	Protein kinase C
PtdIns	Phosphoinositide
PtdIns(3)P	Phosphoinositide (3) Phosphate
PtdIns(3,4,5)P_3	Phosphoinositide (3,4,5) triphosphate
PtdIns(4,5)P_2	Phosphoinositide (4,5) diphosphate
PTEN	phosphatase and tensin homolog deleted on chromosome 10
RBD	RAS-binding domain
RNAi	RNA interference
ROS	Reactive oxygen species
SH2	Src homology 2
SH3	Src homology 3
SHIP	Src-homology (SH2)-domain containing inositol 5-phosphatase
shRNA	small hairpin RNA
TRPV1	Transient receptor potential vanilloid receptor-1
VGCC	Voltage-gated Ca^{2+} channels

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Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angeferitigt habe.

Jena, den 31 Mai 2012 Sreedhar Madishetti

Declaration of honor

I hereby declare that I am familiar with the course of examination for doctoral candidates of Faculty of Biology and Pharmacy of Friedrich-Schiller-Universität Jena.

I have composed and written the dissertation by myself and I have mentioned all the sources of information.

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I have not submitted this work or substantially similar work to any post secondary school.

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