Functional expression of $P2Y_2$ receptor and trafficking of the receptor

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Dedicated:

To my Parents

For all the encouragement, love and support

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1 INTRODUCTION

Cells need to communicate with their environment, i.e. to receive signals from the surrounding environment and to respond to those signals appropriately as well as to send signals over shorter or longer distances. This intercellular communication will then determine whether a cell will proliferate, differentiate, or die, and whether it will adhere or migrate. Virtually all aspects of its behavior depend on the cells ability to accurately interpret signals. These extracellular stimuli are transmitted via their cell-surface receptors and ion channels. Cell surface receptors transduce a signal from a transmitter outside the cell across the plasma membrane to the appropriate targets within the cell. One large class of surface receptors are generally integral membrane proteins (or protein complexes) with transmitter binding sites located outside the cell, α-helical regions crossing the cell membrane and a cytoplasmic domain that generates a signal inside the cell upon transmitter binding (e.g. due to conformational change, dimerization). There are several major families of such receptors in the superfamily termed G protein-coupled receptors (GPCRs). Nucleotide activated purinergic receptors (P2Y receptors) belong to the classical family 1 of GPCRs (Fig. 1.1), which undergo internalization and desensitization after activation by their agonists. According to the classification made by Bockaert and Pin (1999), GPCRs can be subdivided into 6 families (family 1-5, and cAMP receptor family) based on their respective ligands and binding sites. Once GPCRs are activated by their agonists, these receptors associate with and promote activation of heterotrimeric G proteins bound to the inside of the cell membrane. G proteins play important roles in determining the specificity and temporal characteristics of the cellular responses. The G proteins consist of three subunits, α, β, and γ. The α subunit binds GDP or GTP**.** The agonists activating family 1 GPCRs include peptide and non-peptide neurotransmitters, hormones, growth factors, odorant molecules and light. The interaction of the specific ligand with its GPCR – via activation of heterotrimeric guanine nucleotide binding proteins - initiates a signaling cascade leading to e.g. enhanced gene expression through activation of the mitogen-activated protein kinase (MAP kinase; MAPK) pathway, and thus regulate cell proliferation, differentiation, and motility. Abnormal signaling or prolonged activation of G-protein signaling pathways impairs normal functioning of various cells and tissues of our body.

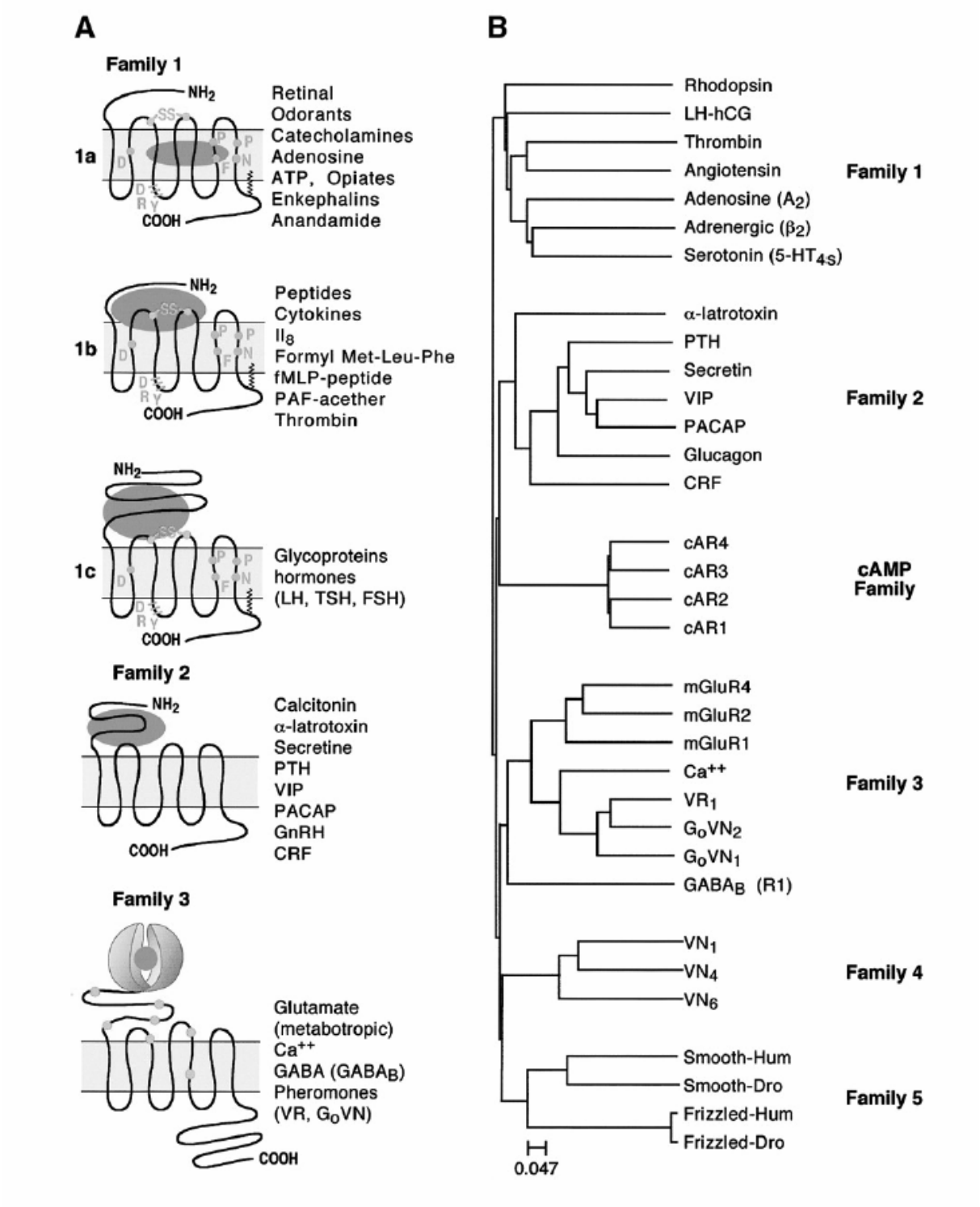


Figure 1.1. Classification and diversity of GPCRs (Bockaert and Pin, 1999). (**A**) Three main families (1, 2 and 3) can be easily recognized when comparing their amino-acid sequences. Receptors from different families share no sequence similarity, suggesting that GPCRs are a remarkable example of molecular convergence. Family 1 contains most GPCRs including receptors for odorants. Group 1a contains GPCRs activated by small ligands and includes the rhodopsin and β-adrenergic receptors. The binding site is localized within the seven TMs. Group 1b contains receptors for peptides whose binding site includes the N-terminal, the extracellular loops and the superior

parts of TMs. Group 1c contains GPCRs for glycoprotein hormones. They are characterized by a large extracellular domain and a binding site which is mostly extracellular but at least with contact with extracellular loops e1 and e3. Family 2 GPCRs have a similar morphology to group 1c GPCRs, but they do not share any sequence homology. Their ligands include high molecular weight hormones such as glucagon, secretin (Krasnoperov et al., 1997; Davletov et al., 1998). Family 3 contains mGluRs and the Ca^{2+} sensing receptors (Pin and Bockaert, 1995). However, GABA-B receptors (Kaupmann et al., 1997) and a group of putative pheromone receptors coupled to the G protein Go (termed VRs and Go-VN) became new members of this family (reviewed in Bargmann et al., 1997). (**B**) Family 4 comprises pheromone receptors (VNs) associated with Gi (Dulac and Axel, 1995). Family 5 includes the `frizzled' and the `smoothened' (Smo) receptors involved in embryonic development and in particular in cell polarity and segmentation. Finally, the cAMP receptors (cAR) have only been found in *D.discoïdeum* but its possible expression in vertebrate has not yet been reported.

1.1 Purinergic receptors

Purinergic receptors were first defined in 1976 and are now distinguished into two types of purinoceptors, named P1 and P2 for adenosine and ATP/ADP, respectively (Burnstock, 1978). All four subtypes of P1 receptors, namely A1, A2A, A2B and A3, are typical G protein-coupled (metabotropic) receptors and specific agonists and antagonists are available for each subtype (see Ralevic & Burnstock, 1998). The P2 receptors, activated by extracellular nucleotides, are currently composed of the ionotropic P2X family and the metabotropic P2Y receptor family. The P2Y recceptor consists of 8 members which all have been cloned and functionally characterized (Abbracchio et al, 2003). with eight members The P2X receptors with seven subtypes including the $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$ have been identified as ligand-gated ion channels (Boeynaems et. al., 2005). The P2X receptors are involved in a wide variety of functions, like neurotransmission, vascular relaxation or constriction.

1.1.1 The family of P2Y receptors

The metabotropic P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) are characterized by seven transmembrane spanning regions with a high level of sequence homology between some regions. The structural diversity of the intracellular loops and C-terminus among P2Y subtypes influences the degree of coupling with Gq/11, Gs and Gi proteins (Fig. 1.2). Each P2Y receptor binds to a single heterotrimeric G protein (typically Gq/11), although $P2Y_{11}$ can couple to both Gq/11 and Gs, whereas $P2Y_{12}$ couples to G_i. P2Y

receptors may form homo- and heteromultimeric assemblies under some conditions, and many cells express multiple P2Y receptor subtypes. P2Y receptors can be activated principally either by nucleotide diphosphates ($P2Y_{1,6,12,13}$) or mainly by nucleotide triphosphates ($P2Y_{2,4}$). Some P2Y receptors are activated by both purine and pyrimidine nucleotides ($P2Y_{2,4,6}$) with the human $P2Y_4$ and $P2Y_6$ receptors are preferentially activated by the uracil-nucleotides UTP or UDP. Other P2Y receptors are activated by purine nucleotides alone ($P2Y_{1,11,12,13}$). The $P2Y_2$ receptor exhibits equal internal calcium increase in response to ATP or UTP (Abbracchio et al., 2003). Nucleotide activation of recombinant P2Y receptors either activates phospholipase C and release of intracellular calcium or affects adenylyl cyclase and thus alters cAMP levels. The recently discovered $P2Y_{14}$ receptors does not show any preferential activation by purine or pyrimidine nucleotides, but is instead activated by UDP-glucose (Chambers et al., 2000; Freeman et al., 2001).

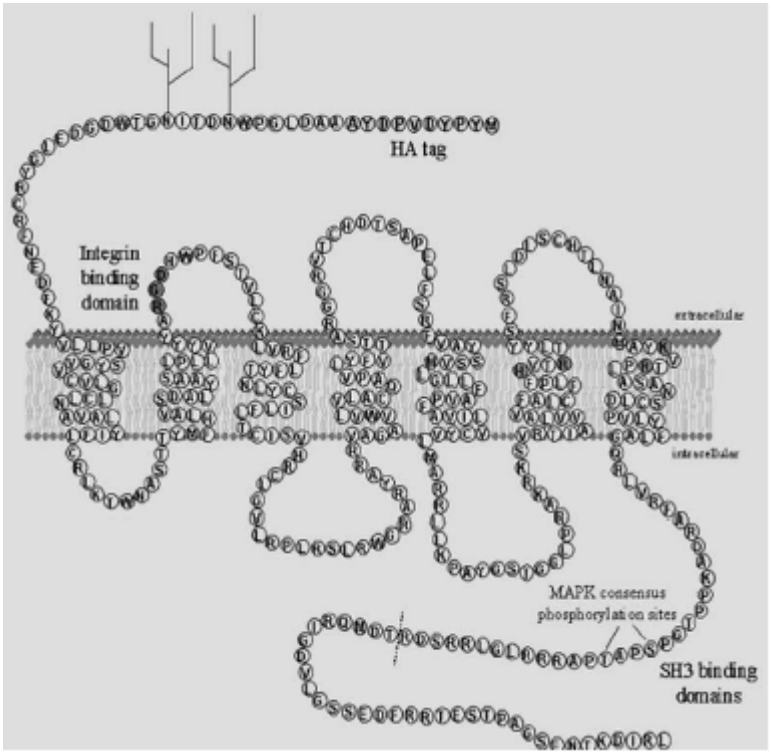


Figure 1.2: Two-dimensional structure of the human $P2Y_2$receptor (Weisman et. al., 2005) Highlighted features include the consensus RGD integrin-binding domain, positively-charged amino acid residues involved in agonist binding , two consensus PXXP SH3-binding domains , and an incorporated hemagglutinin (HA) tag used for immunofluorescence and immunoprecipitation of various $P2Y_2$ receptor constructs. The dotted line indicates the location of a truncation site for creation of a sequestration-resistant $P2Y_2$ receptor.

Site-directed mutagenesis of P2Y receptors, which has been carried out to probe for regions of agonist–receptor interactions, has suggested that four amino acid residues of the transmembrane (TM) regions TM6 and TM7 might be important for agonist potency and specificity (Barnard, E.A. and Simon, J. (2001), Erb et. al., 1995 and Jiang, et.al., 1997). All cloned P2Y receptors share the TM6 H-X-X-R/K motif, which has been proposed to be crucial for agonist activity (Fig. 1.3). In $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors, a Y-Q/K-X-X-R motif in TM7 has also been proposed to participate in ligand binding. In $P2Y_{12}$ and $P2Y_{13}$ receptors and in the UDP-glucose receptor ($P2Y_{14}$ receptor subtype), this motif is substituted with K-E-X-X-L.

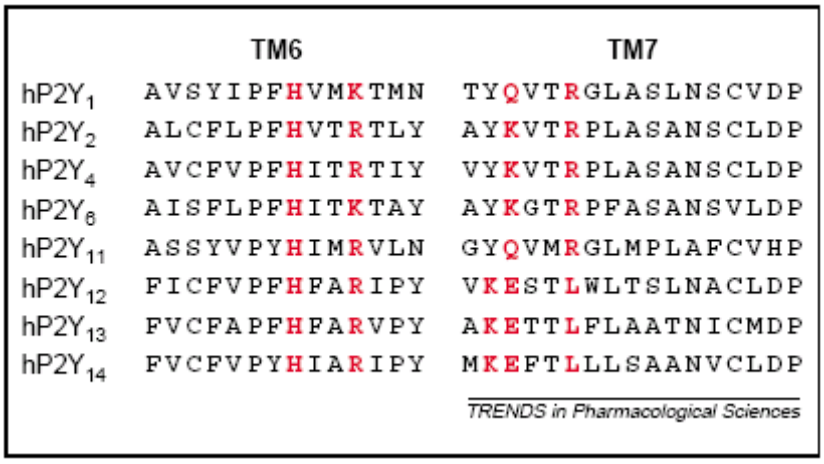


Fig. 1.3 Alignment of putative nucleotide binding motifs in human P2Y receptors. Binding motifs in transmembrane domain 6 (TM6) and TM7 of human (h) P2Y receptors are shown. Crucial amino acids for nucleotide binding are highlighted in red. Sequences were aligned using CLUSTALX. (From: Abbracchio et al., 2003)

1.1.2 Expression and function of the $P2Y_2$ receptor

The $P2Y_2$ receptor is expressed in a wide variety of cells and tissues, including airway epithelial cells, fibroblasts, thymocytes, vascular smooth muscle cells, leucocytes and cardiomyocytes. In cells derived from the peripheral nervous system and the CNS, $P2Y_2$ receptors have been identified in immortalized astrocytes, NG108-15 neuroblastoma glioma hybrid cells, Schwann cells, dorsal horn and cortical astrocytes, astrocytoma cells, rat cortical neurons, and oligodendrocytes (Berti-Mattera et. al., 1999, Gendron et. al., 2003, Ho et. al., 1995, Kirischuk et. al., 1995 and Weisman et. al., 1999). In situ hybridization and reverse transcriptase–polymerase chain reaction (RT-PCR) revealed that $P2Y_2$ receptor messenger RNA (mRNA) was expressed at relatively low levels in normal rodent (i.e., rat, mouse, and gerbil) brain slices, but was most

abundant in the hippocampus and cerebellum. In the hippocampus, $P2Y_2$ mRNA was highly expressed in the dentate gyrus and also detected in rat primary astrocytes, primary neurons, and microglial cells (Gendron et. al., 2003).

The $P2Y_2$ receptor subtype is distinguished in its ability to be upregulated under conditions of stress or injury in activated thymocytes, salivary gland epithelial cells, and models of vascular tissue injury (Koshiba et. al., 1997, Seye et. al., 2002 and Turner et. al., 1997). For example, $P2Y_2$ receptor expression is dramatically increased in smooth muscle cells under conditions, which lead to intimal thickening which are correlated with the increased expression of osteopontin and the proliferation of smooth muscle cells (Seye et. al., 2002). Furthermore, $P2Y_2$ receptor expression is upregulated by agents that mediate inflammation, including cytokines, interleukin (IL)-1β, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α (Hou et. al., 1999 and Hou et. al., 2000). Consistent with a role for $P2Y_2$ receptors in the proliferative phenotype, $P2Y_2$ mRNA expression has been found to be downregulated during cell differentiation (Martin et. al., 1997). The $P2Y_2$ receptor agonist UTP stimulates expression of mRNA of the cytokine transforming growth factor-β (TGF-β) in astrocytes (Gendron et. al., 2003). In urn, TGF-β regulates cell proliferation and differentiation that is dependent on activation of p38 and c-Jun NH_2-terminal kinase (JNK) in cultured astrocytes (Gendron et. al., 2003).

$P2Y_2$ receptors mediate a wide variety of cellular responses of the extracellular nucleotides ATP and UTP. The physiological and/or pathological actions activated include such different cellular responses like pro-inflammatory responses, airway epithelial mucociliary clearance, regulation of cell proliferation or cell motility, and expression of cell adhesion molecules and cytokines (Lethem et. al., 1993, Miyagi et. al., 1996, Seye et. al., 2002, Muscella et. al., 2003 and Seye et. al., 2003). In several cell types, upregulation of $P2Y_2$ receptors is associated with nucleotide-induced stimulation of PKC, cyclo-oxygenase, and MAPKs (Turner et. al., 1997, Turner et. al., 1998, Marrelli, et. al., 1999 and Kannan et. al., 2003). In primary murine astrocytes, $P2Y_2$ receptors mediate the activation of calcium-dependent and–independent PKCs and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) to regulate the activity of cytosolic phospholipase A2 ($cPLA_2$) (Xu et. al., 2002 and Gendron et. al., 2003), which hydrolyzes cell membrane phospholipids to produce arachidonic acid (AA) (Dennis, 1994), a precursor of pro-inflammatory chemokines, such as eicosanoids,

prostaglandins, and leukotrienes (Balsinde et. al., 2002). Prostaglandin E2 (PGE_2) is a mediator of inflammation in the neuroendocrine and immune systems (Kopp et. al., 2001, Vancheri et. al., 2004) and a potent vasodilator that acts with other chemokines to increase microvascular permeability in the peripheral nervous system (Karaki and Kuwahara, 2004, Ferreira et. al., 1997). Prostaglandins, including PGE_2, have been shown to promote inflammatory responses associated with a number of diseases (Kopp 2001 and Vancheri et. al., 2004). The ability of ATP and UTP to enhance cytokine-induced inflammation strongly suggests that $P2Y_2$ receptor modulate the actions of cytokines in neurodegenerative diseases. Furthermore, P2Y receptors mediated release of AA and activation of type 2 cyclooxygenase (COX-2) play a role in inflammation and reactive astrogliosis in neurodegenerative diseases (Brambilla et. al., 1999 and Brambilla and Burnstock 2000).

The $P2Y_2$ receptor does not only act as a single GPCR but has also been found to be functionally coupled with other P2 receptors. Together with the $P2Y_1$ receptor the $P2Y_2$ receptor activation caused arachidonic acid release from cultured astrocytes (Chen et. al., 1998) and also mediates neuroinflammatory responses in astrocytes and microglial cells together with coactivated $P2X_7$ nucleotide receptors (Gendron et. al., 2003).

1.1.3 Functional coupling of the $P2Y_2$ receptor to other receptors

The $P2Y_2$ receptor has been demonstrated to transactivate the epidermal growth factor receptor (EGFR) by interactions with its proline-rich consensus Src-homology-3 (SH3)-binding domain, PXXP (P is proline and X is any amino acid) (Liu et. al., 2004). Immunofluorescence experiments showed that the SH3 binding domains in the $P2Y_2$ receptor facilitated its colocalization with the EGFR in response to $P2Y_2$ activation by nucleotides. The proline-rich peptide sequences have been shown to play important roles in protein–protein interactions that occur in signal transduction pathways. For example, the SH3 domain in the β3 adrenergic receptor interacts directly with Src to activate ERK1/2 (Cao et. al., 2000). PXXP motifs in the β1 adrenergic and dopamine D4 receptor interact with endophilins, SH3 domain-containing proteins, to mediate receptor internalization and receptor coupling to G-proteins (Green and Liggett 1994, Tang et. al., 1999), adenylyl cyclase, and MAP kinase (Oldenhof et. al., 1998). Activation of GPCRs often causes the concomitant activation of growth factor receptors, although the mechanism controlling this transactivation is unclear (Luttrell et.

al., 1997, Andreev et. al., 2001). Previous studies have indicated that Src and Pyk2 are involved in the signaling pathway for growth factor receptor (GFR) transactivation by some GPCRs, including the $P2Y_2$ receptor and protease activated receptor-1 (PAR1 receptor) (Soltoff 1998 and Wang et.al., 2002), although researchers are divided in their opinion about the role of this pathway in the activation of downstream mitogenic signaling by GPCRs. For example, studies with EGFR/Src/Pyk2 dominant negative mutants or an EGFR kinase inhibitor demonstrated the importance of EGFR/Src/Pyk2 in $P2Y_2$–mediated MAPK activation in rat-1 fibroblasts and PC12 cells (Soltoff et. al, 1998). In contrast, studies with embryonic fibroblasts derived from Src–/–, Pyk2–/–, or Src–/–Pyk2–/– mice suggested that Src/Pyk2 are essential for GPCR-mediated transactivation of the EGFR but are dispensable for GPCR-mediated MAPK activation (Andreev et. al., 2001). Two PXXP motifs were identified in the intracellular carboxy-terminal tail of the human $P2Y_2$ receptor that mediate GFR transactivation induced by ATP or UTP (Liu et. al., 2004). Although activation of the $P2Y_2$ receptor mediates ERK1/2 phosphorylation, studies indicate that deletion of the SH3-binding motifs of the $P2Y_2$ receptor did not suppress ERK1/2 activation (Liu et. al., 2004), most likely because of the ability of the $P2Y_2$ receptor to also activate Src and ERK1/2 via $P2Y_2$ receptor-mediated transactivation of the integrin signaling pathway.

Another binding motif contained in the $P2Y_2$ receptor couples it to the thrombospondin receptor (CD47). The $P2Y_2$ receptor contains the consensus integrin binding motif, Arg-Gly-Asp (RGD) in its first extracellular loop (as shown in Fig.1.4) (Erb et. al., 2001). The RGD motif functionally couples the $P2Y_2$ receptor to $\alpha_v\beta_3/\beta_5$ integrins and it has been demonstrated that the $P2Y_2$ receptor colocalized with $\alpha_v\beta_3/\beta_5$ integrins when the recombinant $P2Y_2$ receptors was expressed in human 1321N1 astrocytoma cells. These cells are devoid of endogenous G protein-coupled P2Y receptors. In addition, as revealed with a mutant $P2Y_2$ receptor in which the RGD motif was replaced with Arg-Gly-Glu (RGE), the RGD motif does not only provide tight coupling to the integrins, but is also necessary to maintain the $P2Y_2$ receptor in a high-affinity ligand binding state, as the. EC_{50} for nucleotide-induced calcium mobilization was approximately 1000-fold greater for the RGE mutant (Erb et. al., 2001). Furthermore, the RGE-mutant $P2Y_2$ receptor is not any longer sensitive to pertussis toxin-mediated uncoupling of the receptor from Go-protein, suggesting that association with $\alpha_v\beta_3/\beta_5$ enabled access to the Go protein-coupled, integrin-associated thrombospondin receptor (CD47) and its signaling pathway.

The $\alpha_v\beta_3/\beta_5$ integrins are widely expressed in cells of the cardiovascular system and play critical roles in angiogenesis and inflammatory responses, including cell proliferation, migration, adhesion, and infiltration (Zhang et. al., 2002; Hutchings et. al., 2003, Li et. al., 2003 and Pidgeon et. al., 2003). Activation of $P2Y_2$ receptors by UTP and ATP induces proliferation and/or migration of human epidermal keratinocytes, lung epithelial tumor cells, and smooth muscle cells (Wilden et. al.,1998, Schäfer et. al., 2002 and Greig et. al.,2003).

The different pathways found to be activated by the $P2Y_2$ receptor are summarized in the schematic outline (Fig. 1.4).

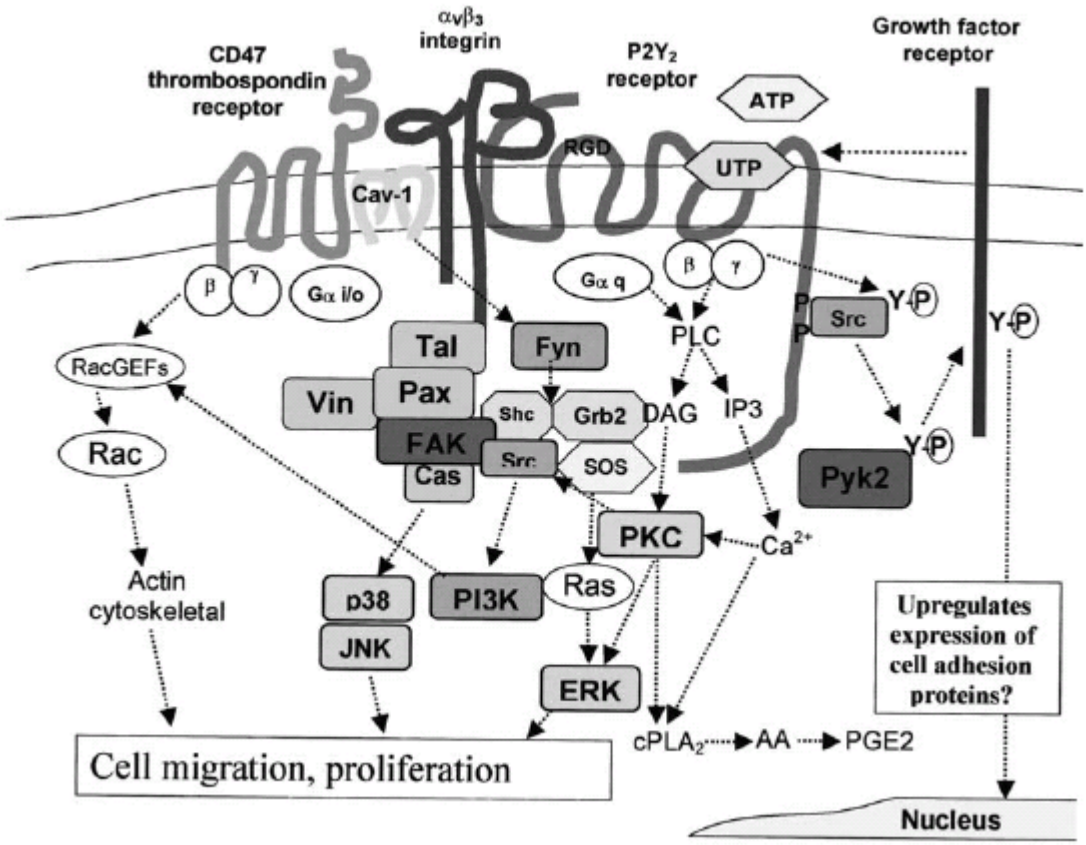


Figure 1.4 Schematic outline of the signaling pathways mediated by the activated $P2Y_2$ receptor. The activation of the $P2Y_2$ receptor by ATP or UTP activates a number of pathways and also leads to transactivation of the growth factor receptor signaling pathways. (From: Weisman et al., 2005)

1.1.4 Regulation of P2Y receptor-mediated signal transduction

The mechanism of internalization and the intracellular trafficking of P2Y receptors has not been studied in detail. Internalization pathways have been reported for the β_2-adrenergic receptor, including ligand-stimulated endocytosis (Claing et. al.,

2002) and downregulation that is independent of endocytosis (Jockers et. al., 1999) Receptor endocytosis is a complex process that involves the recruitment of a number of proteins to the plasma membrane, such as arrestins, clathrin and AP2 (Claing et. al., 2002), and translocation to the early endosomal compartment and lysosomes (McArdle et. al., 2002). This process is also known to be regulated by the cholesterol content and fluidity of the plasma membrane (Kwik et. al., 2003). Extraction of cholesterol from the plasma membrane with methyl-β-cyclodextrin (MβCD) has been reported to inhibit clathrin-mediated receptor endocytosis (Rodal et. al., 1999). Besides clathrin-mediated receptor endocytosis it can occur through caveolin-mediated pathways (McArdle et. al., 2002). Receptor endocytosis from the plasma membrane and intracellular trafficking require massive rearrangement of the cytoskeleton mediated by cytoskeleton-associated proteins (Qualmann et. al., 2000). Agonist stimulation of P2Y receptors has been reported to result in the reorganization of the actin cytoskeleton in myocytes (Sauzeau et. al., 2000). However, a role for the actin cytoskeleton in P2Y receptor endocytosis was not clearly established. The targeting of the mannose-6-phosphate receptor from early endosomes to other compartments, such as late endosomes, lysosomes, or recycling endosomes is directed via the multivesicular body (Miwako et. al., 2001). An interesting regulatory step that could play a role in directing receptors from endosomes to lysosomes is the proteasome complex. Ubiquitin and the proteasomal pathway have been implicated in lysosomal targeting and degradation of the growth hormone receptor (Kerkhof et. al., 2001).

The role of kinases has been postulated in the desensitization and resensitization of GPCR. In case of GPCRs the rate of internalization and reappearance is controlled by a number of factors, which include the association of the receptor with arrestin and the phosphorylation state of the receptor. It is know that in HEK 293 cells that express either the beta-2 adrnergic receptors or the vasopresin 2 receptor the rate of endocytosis of the receptor is different because of the differential association of the receptors which arrestin that cause the difference in the rate of trafficking though both of the receptors are phopshorylated before endocytosis (Oakley t. al., 1999). However it s know that for the delta opiod receptor there is a both a phosphorylation and phosphorylation-independent internalization of the receptor that takes place. The phosphorylation of the receptor in this case is mediated via the GPCR coupled kinases (GRK) (Zhang et. al, 2005). Stimulation of the $P2Y_2$ receptor results in the rise of intracellular calcium and the production of DAG. These can in turn activate kinases like Ca^{2+}/calmodulin-

dependent protein kinase II (CamK-II) or protein kinase C (PKC) or Phosphatidylinositol 3-kinase (PI3K). In case of $P2Y_2$ there are amino acid residues in the third intracellular loop and the C-terminal that are potential sited for phosphorylation by CaMK-II and PKCs (Garrad et. al., 1998). The family of CaMK-II consists of 4 different genes namely (α, β, γ, and δ) and there are to date 38 different isoforms of the CaMK-II (Tombes et. al., 2003). The CaMK-II is almost expressed in all tissue and plays a role in number of process like modulating the function of beta adrenergic receptor response (Zheng et. al., 2004) and also modulating the function of the SERCA pumps in the endoplasmic reticulum (Frank et. al., 2003). However the role of CaMK-II in modulating the trafficking of GPCRs and in endocytotis is yet to be clearly elucidated. CaMK-II is known to play a role in desensitization and endocytosis of the $P2Y_1$ receptor (Tulapurkar et. al., 2006) and also for other GPCRs (Kawakami et. al., 2003). The role of CaMK-II and PKC in modulating the desensitization and internalization of metabotropic glutamate receptor-1a is well known too (Mundell et. al., 2004). Protein kinase C is an important family of kinases that has been widely investigated for its role in modulating different cellular processes. The PKC family consists of a total of 19 members that have been subdivided into families depending on their requirement for Ca^{2+}, phosphatidylserine and diacylglycerol (DAG) for activation. The classical or conventional PKCs (α, βI, βII, γ) require all of the three mentioned factors for their activity, novel PKCs (μ, δ, ε, η,θ) are Ca2+ independent but are regulated by DAG and phosphotidylserine and the atypical (λ, ζ, τ) are onl dependent on phosphotidylserine for their activity.The role of these kinases in the endocytosis of the GPCRs is know (Namkung et. al., 2004), but the precise role of the different families is not been clearly studied. Prolonged stimulation of P2Y receptors results in agonist-induced receptor desensitization, but not known is whether this is the direct result of receptor internalization, or if it is due to changes in interactions with intracellular signaling modulators. Site-directed mutations of probable phosphorylation sites of the murine $P2Y_2$ receptor revealed different structural determinants for receptor desensitization and sequestration (Garrad et. al., 1998).

In addition these kinases that are activated upon activation of GPCRs PI3K kinase too is know to be activated or mediate the fine tuning of the response of GPCRs. In case of beta –2 adrenergic receptor it is know that the activated PI3K is responsible for the compartmentalization of the stimulus and modulating the protein kinase-A response in cardiac myocytes (Jo et. al., 2002). In addition to the role of clathrin in

associating with membrane lipids and the possible role of PI3K has been also postulated (Beck and Keen 1991). PI3K has been also implicated to work in close association with the actin cytoskeleton (Brachmann et. al., 2005) and to modulate endocytosis and transport of vesicles (Merrifield, 2004). In case of the $P2Y_2$ receptor it is known that in thyroid cells PI3K is now to modulate the ERK1/2 activation (Elia et. al., 2005).

In addition to the actin cytoskeleton the PI3K kinase is known to interacts with the Rho family of GTPases (Di Marzio, et. al., 2005) The Rho family of GTPases belong to a larger family that consist of Ras, Rho/Rac/cdc42,Rab, Sar1/Arf and Ran (Bhattacharya et. al., 2005). Rho proteins are known to be involved in modulating the trafficking of GPCRs (Sah et. al., 2000). In case of the $P2Y_2$ receptors it is now that it does interact with Rho kinases in vascular myocytes and modulate the actin cytoskeleton (Sauzeau et. al., 2000). However the direct role of Rho kinases in modulating the trafficking of the $P2Y_2$ receptor has not been demonstrated. In addition to the whole orchestra of the kinases and cytoskeletal proteins that are necessary for the endocytosis of the receptor the lipid content of the plasma membrane does play a role in endocytosis of the receptor (Rodal et. al., 1999).

The phospholipase are known to also modulate the lipd content of the membrane. Dynamin a large GTPase whose role in GPCR endocytosis is well established (Oakley et. al., 1999). In addition to dynamin, phospholipases have also been postulated to play a role in facilitating endocytosis (Shen et. al., 2001). The phopspholipases D (PLD) family includes two members namely PLD_1 and PLD_2. PLD1 is mainly present at the golgi membranes and the PLD_2 is mainly localized at the plasma membrane. Both the isoforms are palmitoylated and phosphorylated on Ser/Thr residues under basal conditions that help them anchor them to the respective membranes (Exton 2002).Thus it is reasonable to imagine that the PLD2 is the main isoform that is involved in the modulation of endocytosis of GPCRs. The activity of PLDs is modulated by the level of intracellular calcium and by PKCs (Siddiqi et. al., 2000). It has also been recently shown that PLD_2 plays a role in the de and resensitization of the mu opiod receptor (Koch et. al., 2004). However the role of the PLD_2 in modulating the endocytosis of the $P2Y_2$ receptor has not been well studied.

MAPK are known to be activated on stimulation of GPCRs (Crespo et. al., 1994) and growth factor receptor. The MAPK family consists of a series of kinases that are sequentially phosphorylated and the last kinase in the series is MAP kinase also known as ERK1/2. In case of the $P2Y_2$ receptor too it is know that stimulation of the receptor

induces phosphorylation of MAPK in different cell types and leads to proliferation (Hou et. al., 1999, Miyagi et.al., 1996, Wilden et. al., 1998). However the effect of MAPK on modulating receptor endocytosis and reappearance of the receptor have not been directly visualized and studied in detail.

Dephosphorylation of the endocytosed receptor is one of the steps that form a part of the complex process of recycling the receptor back to the plasma membrane. The process of recycling the receptor back to the plasma membrane restores the cells ability to respond to surface agonists again. Dephosporylation of the receptor is mainly mediated via protein phosphatases. Protein phosphatases are divided into serine/threonine-specific and tyrosine-specific enzymes, depending on the specificity of the residues that they dephosphorylated. They are further subdivided into type-1 (PP1) and type-2 (PP2) depending on their specificity for the b and a subunit of phosphorylase kinase respectively. The family of PP2 is divided into three subtypes, depending on ionic requirements: PP2A (not dependent on metal ions for activity); PP2B (calcineurin, Ca^{2+}-calmodulin-dependent) and PP2C (Mg^{2+} or Mn^{2+} dependent) (Fernandez et. al., 2002). The role of PP2A has mainly been studied in the modulation of cellular proliferation and thus as a potential taget for anti-cancer therapy, however its role in vesicle trafficking (Guatimosim et. al., 2002) and modulation of receptor endocytosis (Runnwgar et. al., 1997) has not been extensively studied.

In addition to the nucleotide triphosphates another class of molecules namely dinucleotide polyphosphates have been also postulated to function via the P2Y receptors. Dinucleoside polyphosphates have been discovered in the late 60's as probable signaling molecules (Zamecnik and Stephenson, 1969), but it was not till 1976 that their putative physiological role in modulating the proliferation of mammalian cells was demonstrated (Rapaport and Zamecnik, 1976). The most ubiquitous of the dinucleotide polyphosphates and the most studied are the diadenosine polyphosphates. The naturally occurring diadenosine polyphosphates are $Ap_{2\text{-}7}A$. These diadenosine polyphosphates exhibit a large number of physiological effects ranging like modulation of vascular tone(Schlüter et. al.,1998),function of K_{ATP} channels (Jovanovic et. al., 1996), glycealdehyde -3-phosphate dehydrogenase activity (Filonenko et. al., 1989), release of neurotransmitters like acetylcholine(McLennan et. al., 2000) and intracellular pH (Schulte et. al., 1999). Diadenosine polyphosphates have been also known to modulate the proliferation of different cells types like renal mesangial (Schulze-Lohoff, 1995). In spite of the large physiological functions that have been attributed to the

diadenosine polyphosphates it is till now always debatable if these substances act via their own class of receptors (Pintor et. al., 2000) or do they signal via the P2Y receptors (Laubinger et. al., 2003).

However, little is known about the pathways involved in $P2Y_2$ receptor trafficking. In addition to this there is also little evidence to the role of the diadenosine polyphosphates in modulating the trafficking of the $P2Y_2$ receptor and their role in modulating proliferation via the $P2Y_2$ receptor. Thus, we decided to investigate the trafficking of the $P2Y_2$ receptor and its interaction with intracellular compartments and the cytoskeleton to elucidate the subcellular localization of the $P2Y_2$ receptor upon agonist stimulation.

1.2. Aims of this study:

The cell system that we used for this study is Human embryonic kidney cells (HEK 293). These cells were chosen as an expression system for the $P2Y_2$ receptor as these cells do endogeneously express the P2Y receptors. This system thus provides a good model fro studying the trafficking of the receptor as the entire machinery for receptor trafficking is present in the cell.

The main aims of this study are:

1) Functional expression of the $P2Y_2$ receptor tagged to either GFP or the myc-His tag.
2) To investigate the factors that modulate the trafficking of the receptor and possible proteins that might be involved in the trafficking of the receptor.
3) To determine the role of different kinases in modulating the trafficking of the receptor.
4) To investigate if the diadenosine polyphosphates activate the $P2Y_2$ receptor and modulate its traficking.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Lab instruments and materials

Applied Biosystems divisions: (Foster city, CA, USA)	ABI PRISM^{TM} 310 Genetic analyzer
Biometra : (Göttingen, Germany)	T3 Thermocycler
Bio-Rad :	Electrophoresis power supply
	Gel electrophoresis system
	Semi-dry transfer cell
Carl Zeiss : (Jena, Germany)	LSM510meta Confocal laser scanning microscope
	Axiovert 135 fluorescence microscope
Eagle Eye : (Stratagene, Heidelberg, Germany)	Still video system
Eppendorf :	Thermomixer comfort
Heraeus :	Biofuge A, 13 R, 3.2 RS (centrifuge)
	LaminAir® (clean bench)
	Cell culture incubator
Molecular devices :	Microplate reader
Pharmacia Biotech :	UV/visible spectrophotometer
Tecan : (Berlin, Germany)	Genios plus (microplate reader)
TILL Photonics : (Munich, Germany)	Ca^{2+} imaging system

2.1.2 Chemicals

Alexis : Pertussis toxin (PTX)
(San diego, CA, USA)

Amersham Pharmacia Biotech : ECL plus (western blotting detection system)

Applied Biosystems : DNA sequencing kit
(Warrington, UK)

BD Transduction: Anti Clathrin heavy chain antibody, Anti Early endosome antigen (EEA-1), Anti lysosome associated marker protein-1 (LAMP-1)

Calbiochem : FTI-277, KN62, G 418, GÖ6976, GF 109203X, GÖ6983, ML-9, MG-132, U73122, Y-27632, Wortmannin,

Carl Zeiss: Immersol^{TM} 518N (Immersion oil for microscopy)
(Jena, Germany)

Clontech: pEGFPN2 (enhanced green fluorescencent protein expression vector)

Gibco/BRL DNA purification kit

Invitrogen: pcDNA 3.1 A(+) myc-His, anti myc antibody
(Groningen, Netherland)

Molecular Probes: Cholera toxin subunit B coupled to Alexa Fluor 555, Fura-2/AM, goat anti mouse antibody coupled to Alexa fluor 555, Lyso tracker Red and Phallodin coupled to Alexa Fluor 546

Quiagen DNA Mini/Midi preperation kit

Roche Molecular Biochemicals : Cell Proliferation ELISA BrdU (cheminuminiscence) kit, DOTAP
(Mannheim, Germany)

Sigma-Aldrich: ATP, chlorpromazine, cytochalasin-D (Cyto-D), filipin-III, UTP
(Deisenhofen, Germany)

2.1.3 Buffer and Solvent

All buffers were prepared with distilled water.

2.1.3.1 Cell culture medium and solvents

DMEM :	3.7 g/L $NaHCO_3$, 4.5 g/L D-glucose, 1.028 g/L N-Acetyl L-alanyl-L-glutamine
HAM'S F-12 :	1.17 g/L $NaHCO_3$; 1.802 g/L D-glucose, 0.146 g/L L-glutamine

2.1.3.2 Buffer

1x HBSS :	145 mM NaCl, 5.4 mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 20 mM HEPES, pH 7.4 adjusted with 1.67 M Tris (hydroxymethyl)-aminomethane
1x Ca^{2+} free HBSS:	120 mM NaCl, 5 mM KCl, 1mM $MgCl_2$, 25 mM glucose, 2mM Na_2HPO_4, 20 mM HEPES, pH 7.4
1x PBS:	137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4

2.2 Methods

2.2.1 Cell Culture

The HEK 293 cells were grown in medium consisting of DMEM/Ham's-F12 (1:1), supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin (referred to in subsequent text as culture medium) in a 5% CO_2/95% air, humidified atmosphere at 37°C (Vöhringer, et.al.,2000).

2.2.2 Subcloning of the $P2Y_2$ receptor DNA

The $rP2Y_2$ receptor cDNA was kindly provided by Dr. Rice (Rice et. al., 1995) The DNA that we received was subcloned from pBK-CMV-P2Y2 to pcDNA3.1A(+) Myc-His (Invitrogen) and pEGFPN2 (Clontech). The original vector was digested with EcoRI and XhoI or EcoRI and BamH-I to obtain DNA fragment encoding for the $P2Y_2$ receptor. The fragments that were obtained were ligated into the linearized Myc-His and EGFP vectors respectively. The ligated product was purified using the DNA purification kit from Gibco/BRL. The purified DNA was checked for the presence of the inset by digestion of the ligated DNA using EcoR-I and BamH-I. The successfully ligated DNA constructs were transformed into E.coli using the potassium chloride method. The E.coli were plated and allowed to grow for 24 hours at 37^0C on LB-agar plates containing 100 mg/ml ampicilin, as a selection marker. The positive clones were

picked , and cultivated in 5 ml of LB medium containing ampicilin for 24 hours. DNA was prepared form the above cultures using the DNA-Mini-prep kit (Qiagen). The DNA obtained from the mini prep was digest with EcoR-I and BamH-I to recheck for the presence of the inset of the DNA encoding $P2Y_2$ receptor with the appropriate tag. The DNA from the positive clones was sequenced using the DNA sequencing kit from ABI PRISM., using the following set of primers:

$P2Y_2$ forward-1: GGT GGC GTT GCC TTA GAT AC, $P2Y_2$ reverse-1: CCC CCT CCC AGG TGC CAT TG, $P2Y_2$ forward-2: CGC AGT ACA AGG GCT GGG AG, $P2Y_2$ reverse-2: CCT GCA TCA GCG TGC ACC GG and $P2Y_2$ forward-3 : CGC TTT GCC CGA GAT GCC AAG CC.

The clones that expressed the complete $P2Y_2$ receptor with the appropriate tag without any point mutations and frame shift, were retransformed in E.coli using the potassium chloride method, and the DNA was isolated using the DNA Midi-Prep kit (Qiagen).

2.2.3 Transfection and selection of cells

The $rP2Y_2$ receptor DNA was subcloned into pcDNA 3.1A(+) myc-his (MH) (Invitrogen) and pEGFPN2 (Clontech). Transfection of HEK 293 cells was carried out as described previously (Vöhringer, et.al.,2000) Briefly, HEK 293 wild-type cells were transfected with 5 µg/ml DNA in serum-free medium using DOTAP for 8 h. After this time, the medium was replaced with complete medium and the cells were selected with 1 mg/ml G418. Transfected cells were grown in medium consisting of DMEM/Ham's-F12 (1:1), supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin in a 5% CO_2/95% air, humidified atmosphere at 37°C.

2.2.4 Cytosolic Ca^{2+} measurements

The cells were plated on PLL-coated plates and single cell measurements were made after 3 days, when the cells were 40-60% confluent. The changes in free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured by preincubation of the cells with 2 µM Fura-2AM for 30 min in NaHBS (HEPES buffered saline solution: 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose and 20 mM HEPES/Tris pH 7.4). Then cells were stimulated under continuous superfusion of pre-warmed NaHBS at 37°C with different concentrations of UTP or other agonists at 37°C. Fluorescence intensity was recorded alternatively at 340 nm and 380 nm excitation and 520 nm emission. Changes were monitored in single cells bathed in a perfusion chamber which was placed on the microscope stage (Zeiss) of a fluorescence imaging

system from TILL Photonics with a 40X oil immersion objective and a flow rate of 1 ml/min (Vöhringer, et.al., 2000).

2.2.5 Live cell imaging

The cells were plated at a density of 5×10^5 to 1×10^6 cells/dish on poly-L-lysine (0.01%)-pre-coated coverslips (Ø =30 mm). The cells were stimulated with 100 μM UTP in medium in a 5% CO_2/95% air, humidified atmosphere at 37°C. Lysosomes were labeled by preincubating the cells for 30 min with 100 nM LysoTracker Red (Molecular Probes) prior to agonist stimulation. The cells were pre-incubated for 30 min with the appropriate concentration of the inhibitor, to investigate the role of specific proteins in the modulating the trafficking of the receptor.

2.2.6 Agonist-induced internalization

The cells were plated at a density of 5×10^5 to 1×10^6 cells/dish (Ø = 50 mm) on poly-L-lysine (0.01%)-pre-coated coverslips (Ø =22 mm). The cells were stimulated with 100 μM ATP or UTP in medium at 37°C. Labeling of other subcellular compartment markers such as LAMP-1 (lysosomes), and EEA-1 (early endosomes) was performed in fixed cells by immunofluorescence, as described below.

2.2.7 Cell staining for immunofluorescence

Immunohistochemistry was performed as suggested by the manufacturer (Pharmingen Heidelberg, Germany). Briefly, cells grown on coverslips were fixed using methanol:acetone (1:1) for 10 min at -20^0C after agonist stimulation. The cells were then placed in blocking buffer (1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h. Cells were then incubated with primary antibody (e.g., anti-clathrin heavy chain, anti-EEA-1, anti-LAMP-1, or anti-myc antibody) in blocking buffer for 1 h at room temperature, washed three times in PBS and incubated with Alexa Fluor 568 secondary antibody in blocking buffer for 1 h at room temperature. The cells were then washed three times in PBS and the coverslip was inverted onto a slide covered with mounting medium. For F-actin staining, the cells were stimulated with agonist, fixed as above, and incubated for 10 min with phalloidin-Alexa Fluor 546. The cells were then washed three times with PBS and the coverslip was placed onto a slide and covered with mounting medium.

2.2.8 Confocal imaging

Images were taken on a Zeiss inverted LSM 510 laser scanning confocal microscope equipped with a Plan-Apochromat 63X objective. The GFP was excited using a 488 nm argon/krypton laser, and the emitted fluorescence was detected with a

505-530 band pass filter. LysoTracker Red and Alexa Fluor 555/568 were detected using a 543 nm helium/neon laser for excitation and a 560 nm long pass filter. In live-cell imaging, the cells were incubated on stage in a chamber of 5% CO_2 at 37°C in complete culture medium. Images were processed with Zeiss confocal microscopy software, release 3.2. The amount of receptor that was endocytosed by stimulation of the cells with agonist was quantified by measuring the change in fluorescence intensity at the plasma membrane and in the cytoplasm. A three channel (green, red and transmission) simultaneous measurement was performed at each time point for the figures indicated, which also included a Z-scan. Green fluorescence detected the $P2Y_2$ receptor coupled to GFP and the red fluorescence detected the lysosomes in the cytoplasm. The Z-scan was used to detect the upper plasma membrane via an overly between the transmission picture and the GFP channel. The exact position of the lower plasma membrane can be also determined, but only changes in the upper plasma membrane were considered. The position of the ROIs for the plasma membrane was adjusted for every time point with reference to the transmission picture to encompass the plasma membrane and not the cytoplasm. To confirm that the ROI on the plasma membrane did not encompass minimal or any of the cytoplasmic regions a colocalization analysis was done between the green and the red channel (Weisshart et. al., 2004). The calculations took into consideration the relative intensities of the pixels in the respective ROI and the value obtained was the mean of the fluorescence intensities of the pixels in the ROIs.

2.2.9 Cell Proliferation ELISA by 5-bromo-2'-deoxyuridine (BrdU) incorporation

This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. After its incorporation into DNA, BrdU is detected by an immunoassay method using a kit from Roche.

HEK 293 cells stably expressing the $rP2Y_2$-GFP receptor or untransfected HEK 293 cells were plated at a density of 5 X 10^3 cells per well in 96-well plates. The cells were stimulated 24 h after plating them, with the respective test substances in an appropriate 96-well plate at 37°C for one hour. In case of pre-incubation with inhibitors, the incubators were added one hour before stimulation with the appropriate test substance. Subsequently, BrdU is added to the cells and the cells are reincubated (usually 15 h). During this labeling period, the pyrimidine analogue BrdU is incorporated in place of thymidine into the DNA of proliferating cells. After removing

the culture medium, the plate was dried at 60^0C for 1.5 hours and then the cells are fixed and the DNA is denatured in one step by adding FixDenat (the denaturation of the DNA is necessary to improve the accessibility of the incorporated BrdU for detection by the antibody). The anti-BrdU-peroxidase antibody binds to the BrdU incorporated in newly synthesized, cellular DNA. The immune complexes are detected by the subsequent substrate reaction.

2.2.10 Data analysis

Unless stated otherwise, results are presented as mean ± S.E.M., and statistical analysis was achieved by Student's unpaired t test using SigmaPlot (Jandel Scientific, Erkrath, Germany).

3. RESULTS:

3.1 Functional expression of GFP- and MH-tagged $P2Y_2$ receptors in HEK-293 cells

The $P2Y_2$-GFP receptor was directly visualized in live cells using a confocal laser scanning microscope (Fig. 3.1A). The receptor was localized mainly at the plasma membrane of the cells and not concentrated in any other cellular compartment. We also expressed the $P2Y_2$ receptor with a myc-his (MH) tag which was detected by immunostaining of fixed cells with anti-myc antibody as primary antibody and Alexa Fluor 488-conjugated secondary antibody. The myc-his tagged $P2Y_2$ receptor was similarly detected at the plasma membrane of the cells (Fig. 3.1B). Thus, incorporation of either the GFP or MH tag at the C-terminus of the $P2Y_2$ receptor did not prevent the targeting of the receptor to the plasma membrane.

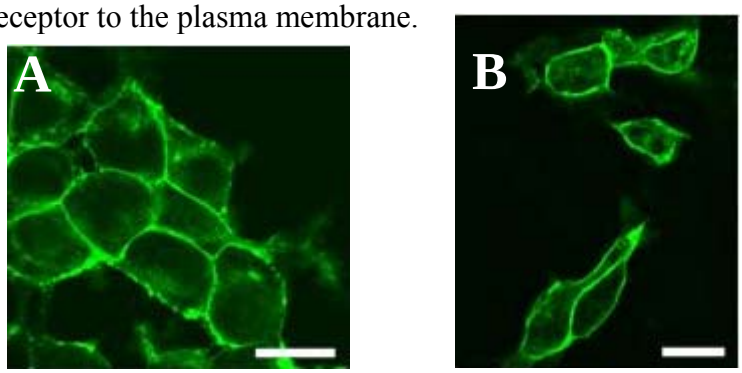
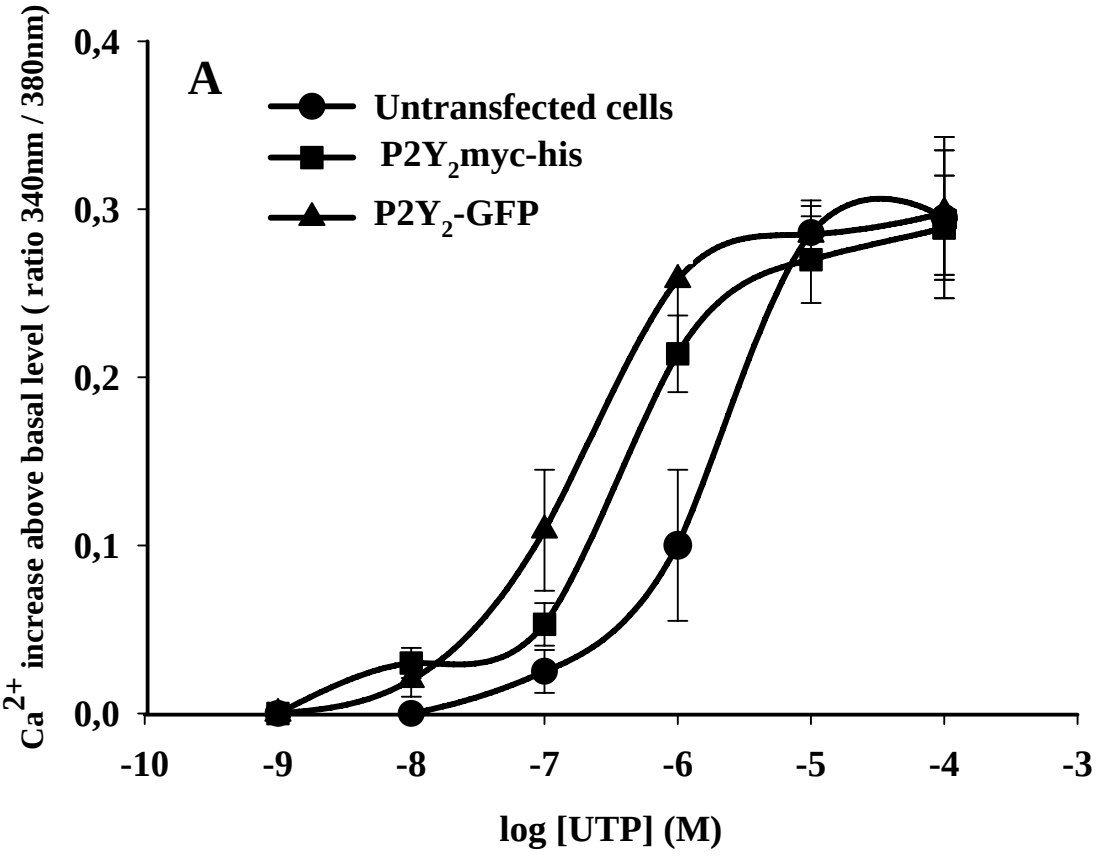


Figure 3.1: Stable expression of $rP2Y_2$ receptor in HEK-293 cells.
(A) HEK 293 cells were transfected with the DNA $rP2Y_2$GFP by the lipofection method. The expression of the receptor was visualized using the CLSM. The stably expressed receptor is found to be exclusively localized on the plasma membrane. This localization is similar to the endogenously expressed $P2Y_2$ receptor. (B) HEK 293 cells transfected with $rP2Y_2$MH. The expression pattern of the receptor was conformed in stably transfected cells by immunohistochemistry using anti-myc and Alexa Fluor 488. Scale bar 20 μm.

Functional coupling of the transfected $P2Y_2$-GFP receptor was determined by measuring the changes in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$)in fura-2 loaded cells upon stimulation with $P2Y_2$ receptor agonists. Transfected and untransfected cells were challenged with the nucleotide agonists UTP or ATP, at different concentrations ranging from 10^{-9} M to 10^{-4} M. The cells were visualized at 340 nm to monitor changes in $[Ca^{2+}]_i$ by fura-2 fluorescence and at 460 nm to detect the $P2Y_2$-GFP receptor. The simultaneous visualization of fura-2 and GFP fluorescence allowed the selection of double-positive, transfected cells with high receptor expression level for measurement of the Ca^{2+} response. In untransfected cells the calcium response

to nucleotide is due to stimulation of endogeneous $P2Y_2$ and $P2Y_1$ receptors, since mRNAs for these receptors were previously detected in HEK-293 cells by reverse transcriptase-polymerase chain reaction (RT-PCR) (Schäfer et. al.,2003; Vöhringer et. al., 2000). In untransfected cells, the agonist-induced calcium response was submaximal at agonist concentrations below 10^{-5} M, and at 10^{-7} M there was only a negligible response. The EC_{50} value for either UTP or ATP in untransfected cells was 1.8 µM. In $P2Y_2$-GFP receptor transfected cells, the sensitivity of the cells to the agonist was increased, as observed from the leftward shift of the curves in comparison to untransfected cells. The EC_{50} values for nucleotides in cells expressing the $rP2Y_2$-GFP receptor or the $rP2Y_2$-MH receptor, respectively, were 200 nM and 350 nM for UTP, and 200 nM and 150 nM for ATP (Fig 3.2, A and B). These results confirm that the tagged receptors were functional when expressed in HEK-293 cells.



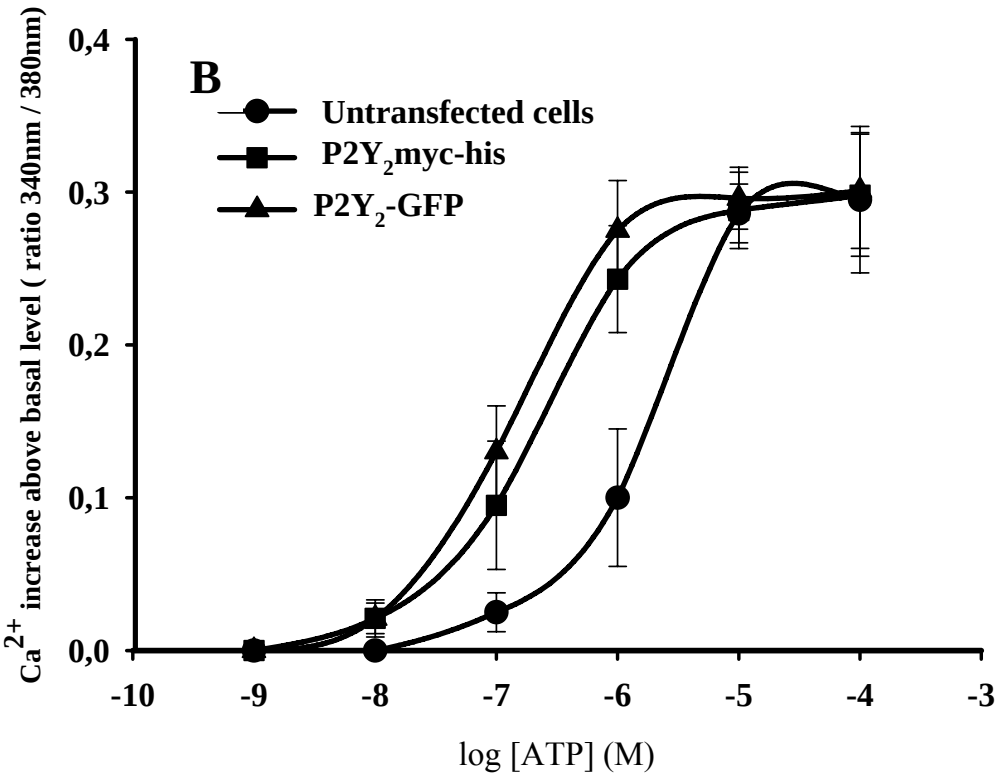
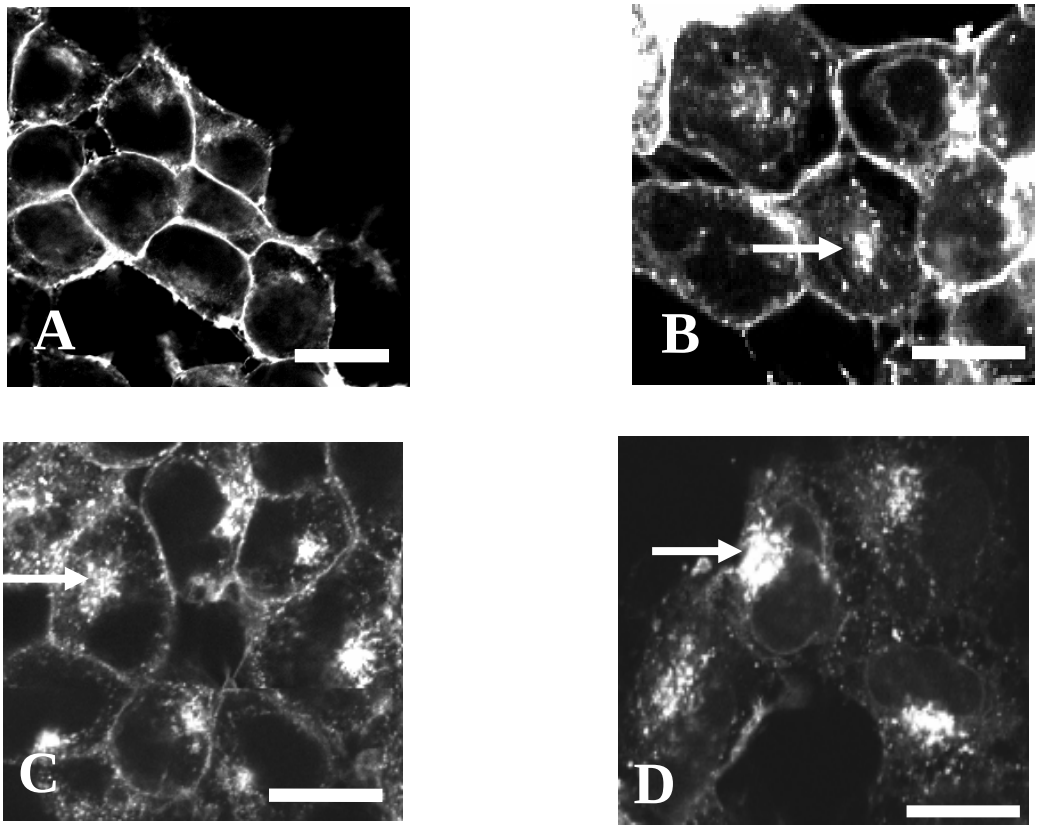


Figure 3.2 : Functional expression of the transfected rP2Y$_2$ receptor in HEK 293 cells.
The stably transfected HEK 293 cells expressing either rP2Y$_2$-GFP (triangles) or rP2Y$_2$myc-his (squares) were grown in serum-containing medium. For measurement the cells were preloaded with fura-2A and then perfused with NaHBSS containing Ca^{2+} and stimulated for 1 min with different concentrations of UTP (A) or ATP (B) The untransfected cells (circle) were stimulated with the same concentrations of agonists as control. The data are from single cell Ca^{2+} recordings and represent the mean ± S.E. for at least 50 cells for each value.

3.2 Effect of agonist concentration on receptor localization

The rP2Y$_2$-GFP receptor expressed in HEK-293 cells was stimulated with UTP, an agonist of the P2Y$_2$, but not the P2Y$_1$ receptor. In unstimulated cells, the receptor was localized mainly to the plasma membrane (Fig. 3.1A). Stimulation of rP2Y$_2$-GFP receptor transfected cells with 1-100 μM UTP resulted in a concentration-dependent increase in the extent of receptor internalization. This was observed as a loss of receptor-associated fluorescence from the plasma membrane and the simultaneous accumulation of fluorescence in intracellular organelles (Fig. 3.3 B-D). The increase in the amount of internalized receptor is indicated by bold arrows (Fig. 3.3 B-D). The loss of fluorescence from the plasma membrane was determined by quantitating the

fluorescence intensity in user-defined regions of interest (ROI) that encompass either the plasma membrane or cytoplasmic regions. As shown in Fig. 3.3E, at 100 μM UTP there was almost no receptor left at the plasma membrane but it appeared entirely in the cytoplasmic compartment. Qualitatively similar results were obtained for internalization of the rP2Y_2-MH receptor (data not shown), indicating that the presence of either a GFP or myc-his tag did not affect receptor internalization. In our further studies of receptor endocytosis we used the agonist UTP at 100 μM, because this concentration induced complete receptor endocytosis and the amount of receptor present at the plasma membrane and in the cytoplasm could be clearly distinguished.



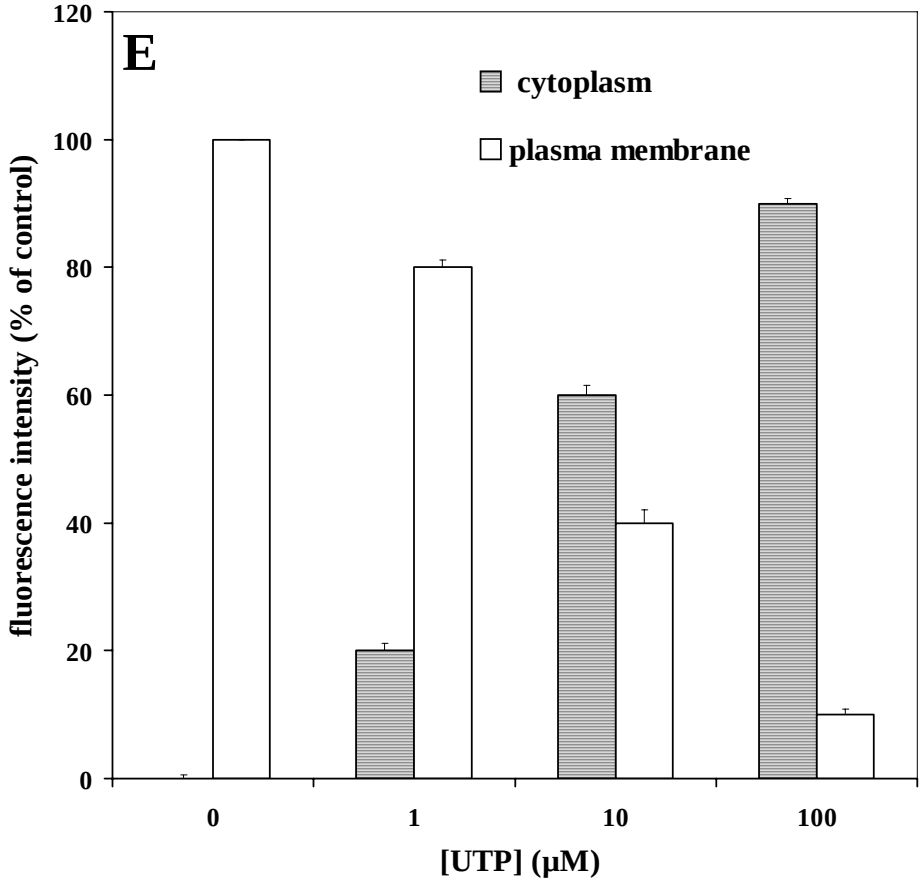


Figure 3.3: Stable expression of the rP2Y$_2$ receptor in HEK-293 cells and agonist concentration-dependent internalization of the rP2Y$_2$-GFP receptor.

Stable expression of the rP2Y$_2$ receptor in HEK-293 cells and agonist concentration-dependent internalization of the rP2Y$_2$-GFP receptor. (*A–D*) HEK-293 cells were stably transfected with cDNA encoding the rP2Y$_2$-GFP gene, as described in Materials and methods. Expression and localization of the receptor were visualized in live cells using confocal laser scanning microscopy (CLSM). The rP2Y$_2$-GFP receptor was visualized in stably transfected HEK-293 cells that were unstimulated (*A*, control cells), or stimulated for 30 min at 37°C with 1 μM (*B*), 10 μM (*C*) or 100 μM UTP (*D*). The increase in the amount of internalized receptor is indicated by bold arrows. Scale bar, 20 µm. (*E*) The fluorescence intensity at the plasma membrane and in the cytoplasm was calculated by selecting the appropriate region of interest, using Zeiss software. The distributions of cellular fluorescence intensity between the cytoplasm (hatched bars) and the plasma membrane (open bars) were determined in unstimulated and stimulated cells. The data are normalized to initial fluorescence intensity at the plasma membrane (100%). The data presented are the means ± SD from at least three independent experiments with 20 cells each.

3.3 Compartmentalization of the receptor after stimulation with agonist

The agonist-promoted loss of surface immunoreactivity in cells expressing the P2Y$_2$ receptor has been investigated previously (Sromek et. al.,1998), but the time

dependent compartmentalization of the $P2Y_2$ receptor and the mechanism responsible for internalisation have not yet been investigated. HEK-293 cells stably expressing $rP2Y_2$-GFP receptors were stimulated with 100 μM UTP for 30 or 60 min. In these experiments, after stimulation, the cells were fixed and incubated with antibodies raised against either the early endosome antigen–1 (EEA-1) (Mills et. al., 1998) or the lysosomal associated membrane protein-1 (LAMP-1). After 30 min with UTP, the internalized receptors (green) colocalized with early endosomes (red) (colocalization shown by yellow, and indicated by arrows in Fig. 3.4, A3, but not with lysosomes (data not shown). After 60 min with UTP, $P2Y_2$-GFP receptors colocalized with lysosomes (red), as indicated by the yellow signal and indicated by arrows in Fig. 3.4, A6, but were no longer detected in early endosomes (data not shown).

Lysosomal localization of $P2Y_2$-GFP receptors after stimulation with UTP for 60 min was seen in fixed cells (Fig. 3.4, A6). A similar colocalization was obtained when the cells were cells pre-loaded with Lyso Tracker were exposed to 100 μM UTP for 60 min (see large yellow structure in Fig. 3.9B indicated with bold arrow). This experiment thus confirmed the above immunohistochemistry results.

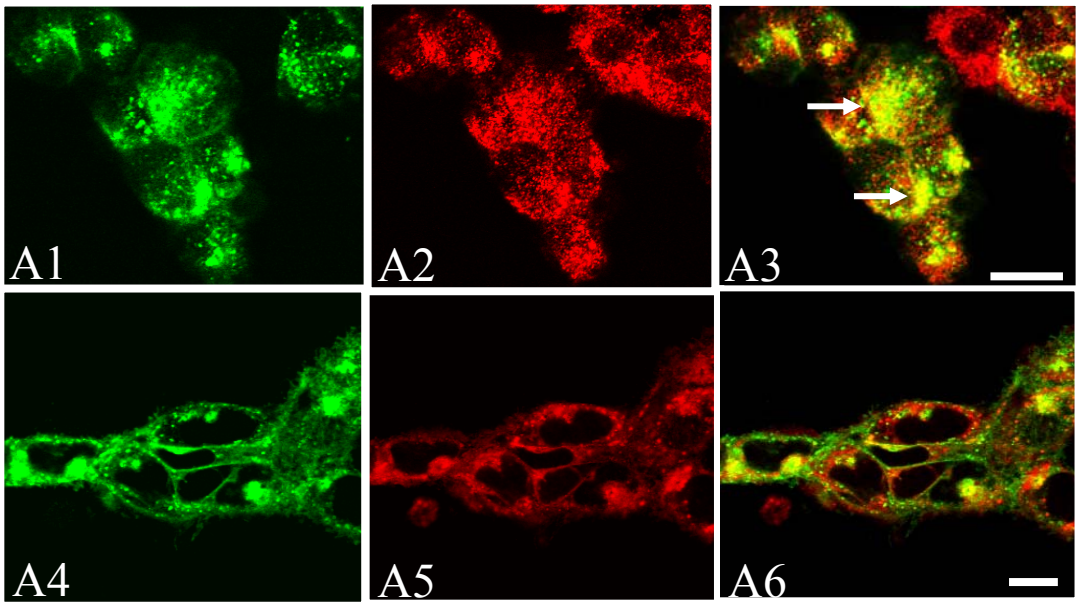


Figure 3.4 (A): Compartmentalization of the receptor after stimulation with agonist.
Colocalization of the $rP2Y_2$-GFP receptor with early endosomes and lysosomes after UTP stimulation. HEK-293 cells stably expressing the $rP2Y_2$-GFP receptor were stimulated with 100 μM UTP at 37^0C in culture medium for 30 min **(A1)** or 60 min (A4). Colocalization of the GFP-tagged receptor (green) with early endosomes or lysosomes was determined in fixed cells with mouse anti-EEA-1 antibody (A2; red) or anti-LAMP-1 antibody (A5, red), respectively, visualized with goat anti-mouse Alexa

Fluor 568 as secondary antibody. Colocalization is indicated in yellow (A3, A6), marked with arrows. Scale bar shown for each figure is 20 µm. Results shown are representative of at least 3 individual experiments.

Farnesylation of endosomal proteins is important in trafficking of the receptors from early endosomes and deciding the trafficking of the receptor. So we investigated if this factor affects the trafficking of the $P2Y_2$ receptor. Cells were preincubated for 30 min with 100 nM LysoTracker red to label lysosomes and 10 µM farnesyltransferase inhibitor FTI-277. This inhibitor blocks farnesylation of endosomal proteins and prevents trafficking of internalized membrane proteins between endosomes and lysosomes (Doisneau-Sixou et.al.,2003). Preincubation of the cells with FTI-277 did not affect the localization of the $rP2Y_2$-GFP receptor (Fig. 3.5, A1) and did not prevent the UTP-induced endocytosis of the receptor (Fig. 3.5, A2). However, FTI-277 prevented the colocalization of internalized receptors with lysosomes stained with LysoTracker red, even after 60 min of UTP stimulation (Fig. 3.5, A3), a time normally sufficient to target the $rP2Y_2$-GFP receptor to lysosomes (Fig. 3.4, A6).

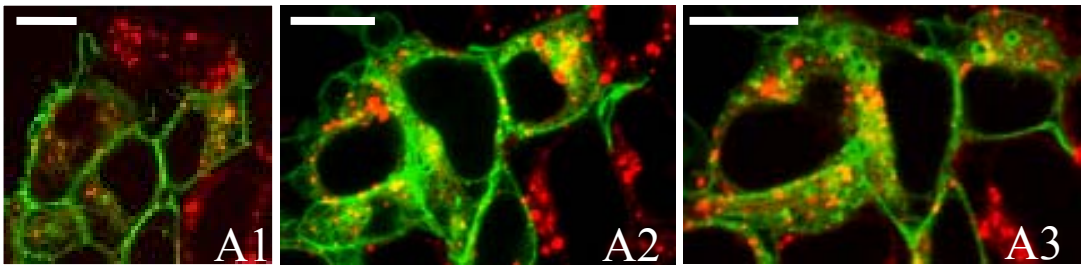


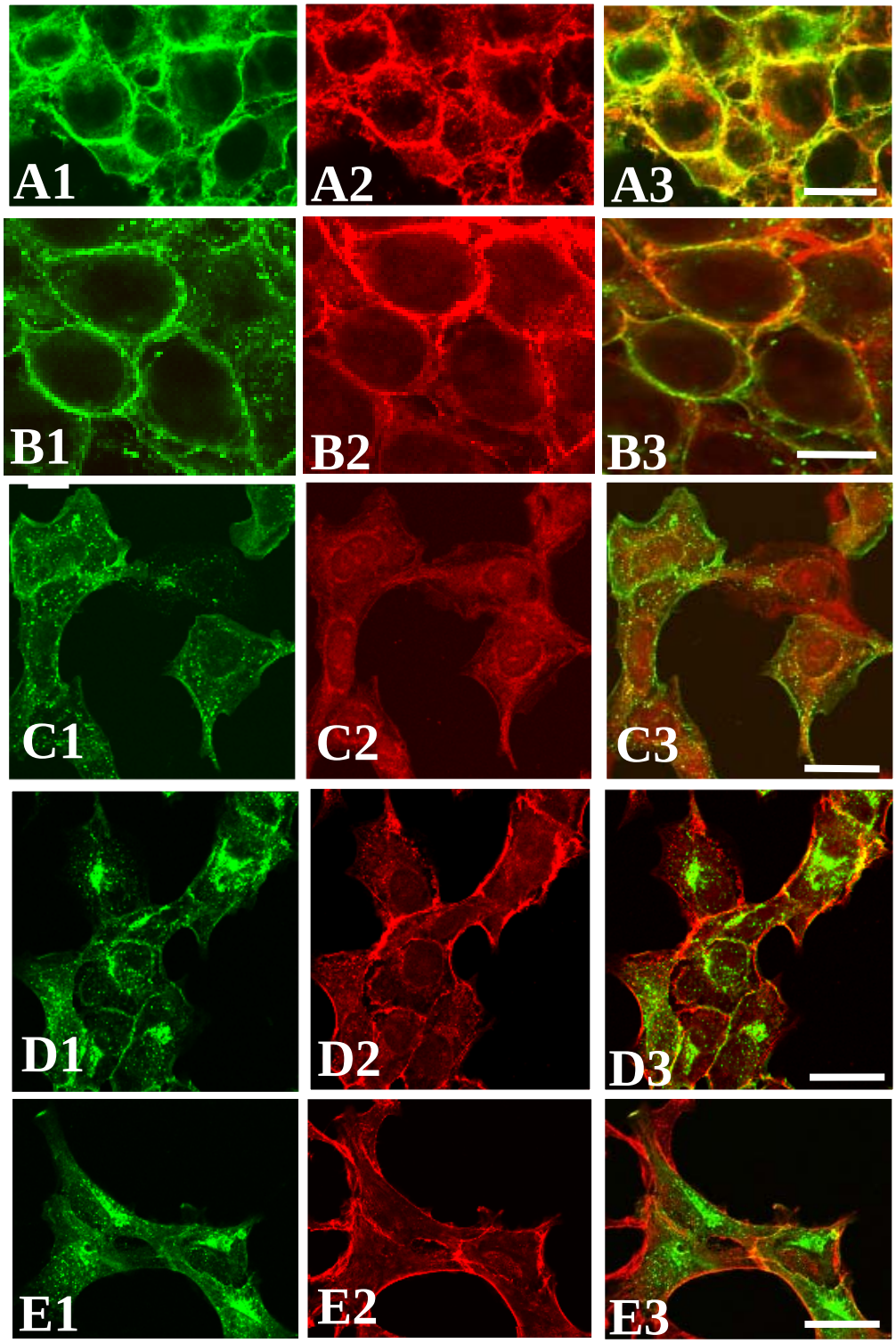
Figure 3.5: Effect of the farnesyltransferase inhibitor FTI-277 on trafficking of the $rP2Y_2$-GFP receptor.(also in Tulapurkar et. al., 2005).
Effect of the farnesyltransferase inhibitor FTI-277 on trafficking of the $rP2Y_2$-GFP receptor by live cell imaging. HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated with 100 nM LysoTracker (red fluorescence) and 10 µM FTI-277 for 30 min at 37°C. Cells were unstimulated (A1), or stimulated with 100 µM UTP for 30 min (A2) or 60 min (A3) and receptors and lysosomes were visualized. FTI-277 prevents colocalization of the the internalized receptor with lysosomes. The results shown are representative of 3 different experiments. Scale bar of 20 µm.

3.4 UTP induced actin cytoskeletal rearrangements

Rearrangements of the cytoskeleton play an important role in cellular processes such as endocytosis and exocytosis (Qualmann et. al., 2000). Stimulation of HEK-293 cells expressing the $rP2Y_2$-GFP receptor with 100 µM UTP caused massive reorganization of the actin cytoskeleton counterstained in fixed cells with Alexa Fluor 546-labeled phalloidin (red). In unstimulated cells the $rP2Y_2$-GFP receptor and actin were colocalized to the plasma membrane with few actin fibers in the cytoplasm (Fig.

3.6, A2) indicating that the cells were not under stress (Giancotti, et.al., 1999). For the first 5 min of UTP stimulation, the rP2Y$_2$-GFP receptor remained localized to the plasma membrane, whereas actin was redistributed to the cytoplasm (Fig. 3.6 B 1,2). After 10 min of UTP stimulation, initial receptor internalization was observed and actin was diffusely distributed in the cytoplasm (Fig. 3.6, C1,2). In case of Fig. 3.6 B3, it is seen that there is no colocalization of actin with the receptor in comparison to 3.6 A3, and a diffuse staining of actin is seen. This demonstrates that actin rearrangement preceded endocytosis of the receptor. After 20 min of UTP stimulation, only 20% of the receptors remained at the plasma membrane, as compared to unstimulated cells, whereas actin had redistributed to the plasma membrane (Fig. 3.6, D1,2). The rP2Y$_2$-GFP receptor was completely internalized at 30 min (data not shown), and 60 min after UTP stimulation, whereas actin reappeared concentrated at the plasma membrane (Fig. 3.6, E1,2).

Figure 3.6: Actin cytoskeleton rearrangement in UTP-stimulated HEK-293 cells expressing the rP2Y$_2$-GFP receptor. HEK-293 cells expressing the rP2Y$_2$-GFP receptor were incubated in culture medium at 37°C in the absence (*A*) or presence (*B–E*) of 100 μM UTP for 5 (*B*) 10 (*C*), 20 (*D*) or 60 (*E*) min. The cells were then fixed and counterstained with actin-binding phalloidin coupled to Alexa Fluor 546 (as described in Materials and methods) and actin (red) and rP2Y$_2$-GFP receptor (green) were visualized. In *A* to *E*, the respective picture with number 1 represents green fluorescence of P2Y$_2$-GFP receptor, the picture with number 2 represents red fluorescence of actin staining, and the picture with number 3 gives the overlay. Colocalization of actin and rP2Y$_2$-GFP receptor is indicated in yellow. Scale bar, 20 μm. Results shown are representative of at least three individual experiments.



To confirm the role of actin cytoskeleton in receptor endocytosis, HEK-293 cells expressing the rP2Y$_2$-GFP receptor were pretreated with an actin-disrupting agent, cytochalasin-D (Sergeeva M., et.al., 2000), prior to UTP stimulation. Fig. 3.7 indicates that a 30 min treatment with UTP was insufficient to induce receptor internalization in

cytochalasin-D-treated cells (Fig. 3.7, A2), whereas the receptor was completely internalized by a 20 min treatment with UTP in the absence of cytochalasin-D, as seen above in Fig. 3.6 D1. As compared to unstimulated cells (Fig. 3.6 A1), a 30 min stimulation with UTP, induced a punctate receptor distribution in the membrane (Fig. 3.7, A2). In cytochalasin-D-treated cells stimulated with UTP for 60 min, the receptor was internalized in small vesicles (Fig. 3.7, A3), in contrast to the large receptor aggregates internalized in cells treated with UTP in the absence of cytochalasin-D (Fig. 3.1D).

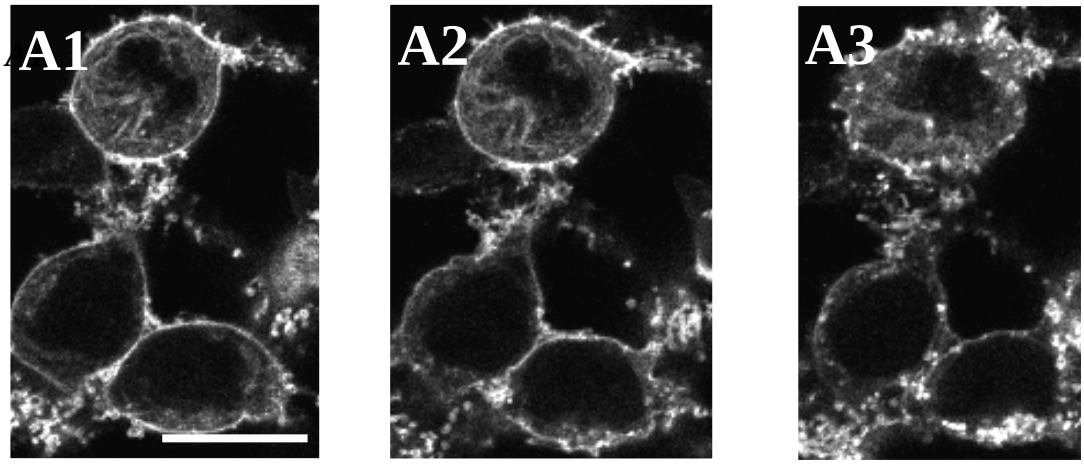


Figure 3.7: (A) Effect of actin disrupting agent cytochalasin-D, on endocytosis of the rP2Y$_2$-GFP receptor.
(*A*) HEK-293 cells stably expressing the rP2Y$_2$-GFP receptor were preincubated with 100 nM cyto-D at 37 °C for 30 min and live-cell confocal imaging was performed. Images were collected from cells before stimulation (*A1*), and stimulated with 100 µM UTP for 30 min (*A2*) or 60 min (*A3*). Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

We next examined the effect of the myosin light chain kinase inhibitor, ML-9 (Kawamura M., et. al., 2003), on P2Y$_2$ receptor internalization. Myosin is a small ATPase whose activity is important for the normal reorganization of the actin cytoskeleton. Myosin light chain kinase modulates the function of myosin by phosphorylation of specific residues. Thus, we decided to investigate the role of a subtle modulatory protein on the trafficking of the P2Y$_2$ receptor. HEK-293 cells expressing the rP2Y$_2$-GFP receptor were preincubated with 100 µM ML-9 and 100 nM LysoTracker Red for 30 min and then stimulated with 100 µM UTP. In comparison to the membrane localization of rP2Y$_2$-GFP receptors in ML-9-treated controls (Fig. 3.8, A1), a 30 min UTP stimulation in the presence of ML-9 induced receptor localization (green) in small vesicular clusters below the plasma membrane (marked by arrows in Fig. 3.8, A2), which is different from the effect of cytochalasin-D in UTP-treated cells

(Fig. 3.7, A2). For three-dimensional analysis, YZ- and XZ-images were constructed from XY-plane images, which were taken from the bottom to the top of each cell. The presence of the vesicles, as indicated by arrows in Fig. 3.8 A2, just below the plasma membrane is visible in these X-Z and the Y-Z projections. After a 60-min UTP stimulation in the presence of ML9, receptor endocytosis had occurred although there was little localization of receptor in lyososmes (red) in Fig. 3.8, A3, in contrast to results in the absence of ML-9 (cf. data for fixed cells shown in Fig. 3.4, A6). Taken together, these findings indicate that the actin cytoskeleton and cytoskeletal-associated proteins (e.g., myosin) mediate the endocytosis of the rP2Y_2-GFP receptor.

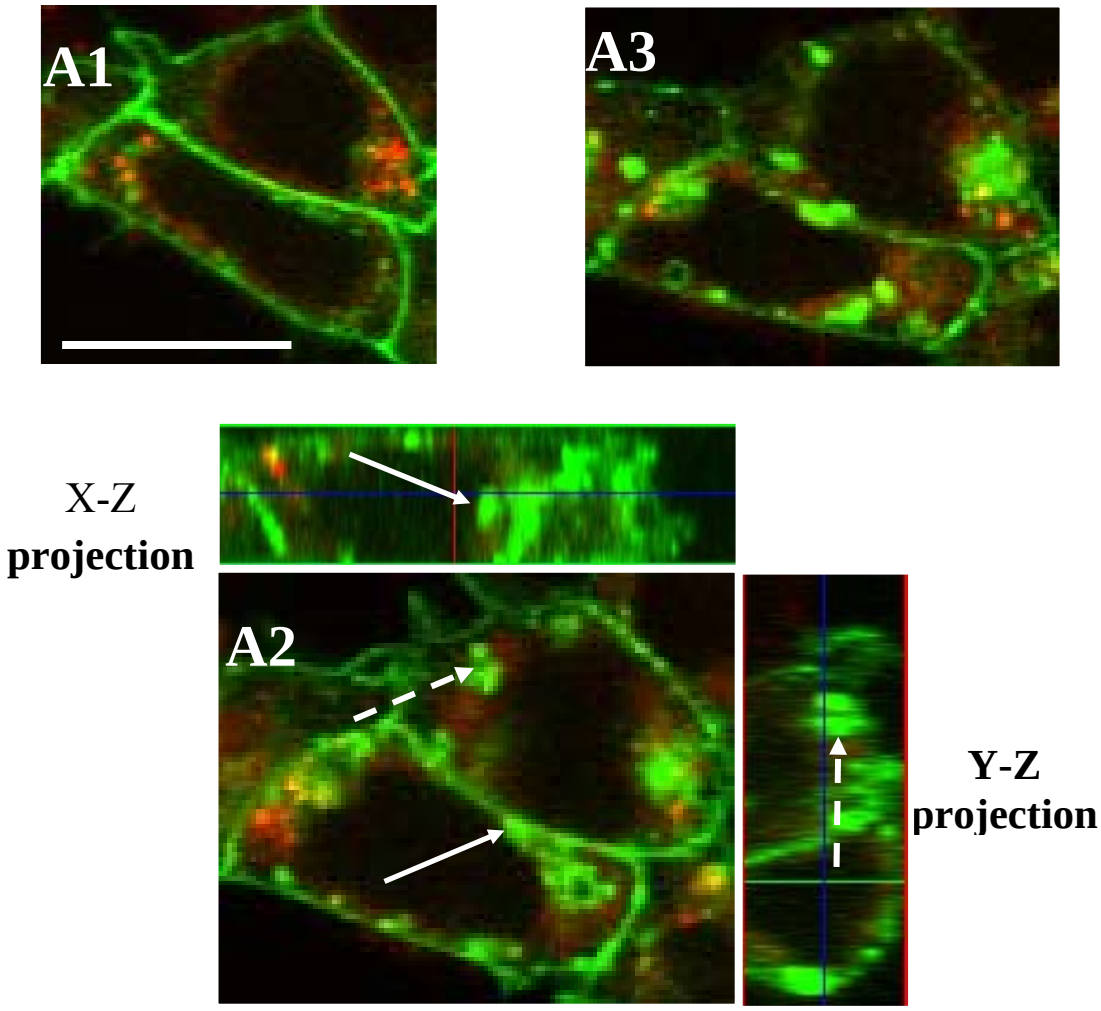


Figure 3.8: Role of myosin light chain kinase, in endocytosis of the rP2Y_2-GFP receptor. HEK-293 cells expressing the rP2Y_2-GFP receptor were preincubated with 100 µM ML-9 and 100 nM LysoTracker Red for 30 min at 37 °C and then live-cell imaging was performed. Images were collected from cells before stimulation (A1), and stimulated with 100 µM UTP for 30 min (A2) or 60 min (A3). In ML-9-treated cells, the receptor was clustered upon UTP treatment in small vesicles below the plasma membrane (arrows in A2). This is clearly visible in the X-Z and Y-Z projections. There was no colocalization of the endocytosed receptor with lysosomes (as seen in A3). Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

3.5 Inhibition of receptor internalization

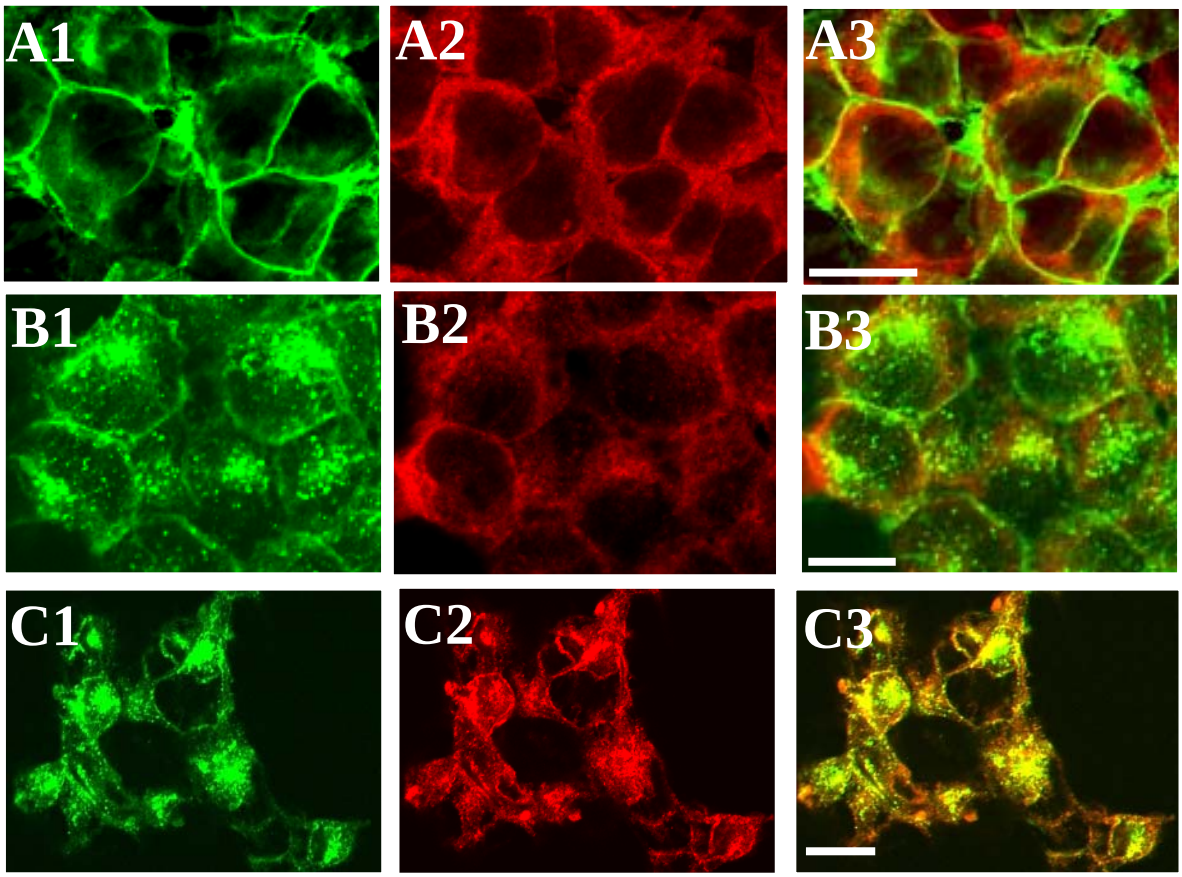
UTP-mediated rP2Y_2-GFP receptor internalization in HEK-293 cells was completely inhibited at 4°C (data not shown). In addition, there was very little receptor internalization at 14°C or 28°C (data not shown), indicating that P2Y_2-GFP receptor endocytosis is temperature-dependent. The temperature effect indicates that there is no constitutive endocytosis of the receptor.

P2Y_2 receptor endocytosis was also sensitive to the density of the medium, since addition of 0.45 M sucrose prevented UTP-induced receptor internalization (data not shown), and after a 30 min UTP stimulation most of the receptor remained localized to the plasma membrane in small punctate structures (data not shown). The inhibition of endocytosis of the receptor by 0.45 M sucrose suggests the role of clathrin-dependent mechanism (McArdle et. al., 2002).

3.6 Colocalization of the P2Y_2 receptor and clathrin

Unstimulated cells displayed a diffuse distribution of clathrin (red) in the cytoplasm, and the receptor (green) was localized to the plasma membrane (Fig. 3.9, A1,2). After a 10 min UTP stimulation, colocalization of the internalized receptor and clathrin was first detected (Fig. 3.9, B3). After a 20 min UTP stimulation, most of the receptor signal colocalized with clathrin (Fig. 3.9, C3). Interestingly, rP2Y_2-GFP receptor and clathrin colocalization was no longer detectable after 30 min UTP stimulation (data not shown). Thus, these results strongly suggest that UTP-dependent endocytosis of the P2Y_2-GFP receptor occurs via clathrin-coated pits, prior to receptor transport to early-endosomes or lysosomes depending on the duration of agonist exposure.

Figure 3.9: UTP induces colocalization of the rP2Y_2-GFP receptor with clathrin. HEK-293 cells expressing the rP2Y_2-GFP receptor were incubated in the absence (A) or presence (B, C) of 100 μM UTP at 37 °C for 10 min (B), or 20 min (C). The cells were fixed, immunolabeled with mouse monoclonal anti-clathrin antibody and with goat anti-mouse Alexa Fluor 568 as secondary antibody, and images were collected. The receptor is visualized by green fluorescence, the clathrin by red fluorescence. The pictures numbered 1, 2 and 3 in A to C show P2Y_2-GFP receptor in 1, clathrin staining in 2, and the overlay in 3. Colocalization is indicated by yellow. Scale bar, 20 μm. Results shown are representative of at least three individual experiments.



To confirm the involvement of a clathrin-mediated pathway in agonist-induced $P2Y_2$-GFP receptor endocytosis, cells were preincubated with 100 µM chlorpromazine, an inhibitor of clathrin-mediated endocytosis (Petris et. al., 2003), and then stimulated with 100 µM UTP for 30 min. Chlorpromazine by itself did not affect cell shape or $P2Y_2$-GFP receptor distribution in the absence of UTP (Fig. 3.10, A1), but it prevented UTP-induced receptor internalization (Fig. 3.10, A2). When cells were preincubated with 100 µM chlorpromazine for 30 min and 5 nM cholera toxin subunit-B coupled to Alexa Fluor 555, a marker for the caveolin-rich lipid rafts in the plasma membrane (Orlandi et. al., 1998), the $rP2Y_2$-GFP receptor colocalized with cholera toxin subunit B in unstimulated cells (Fig. 3.10, A3). After a 30 min stimulation with 100 µM UTP, neither receptor endocytosis nor redistribution of cholera toxin was detected (Fig. 3.10, C3), indicating that inhibition of clathrin-mediated endocytosis by chlorpromazine does not lead to $P2Y_2$-GFP receptor internalization by the caveolin-mediated pathway. Cholera toxin internalization is known to occur by a caveolin-mediated pathway.

However, cholera toxin was not internalized upon activation of the $P2Y_2$-GFP receptor in HEK-293 cells. Therefore, $P2Y_2$ receptors apparently do not couple to this pathway.

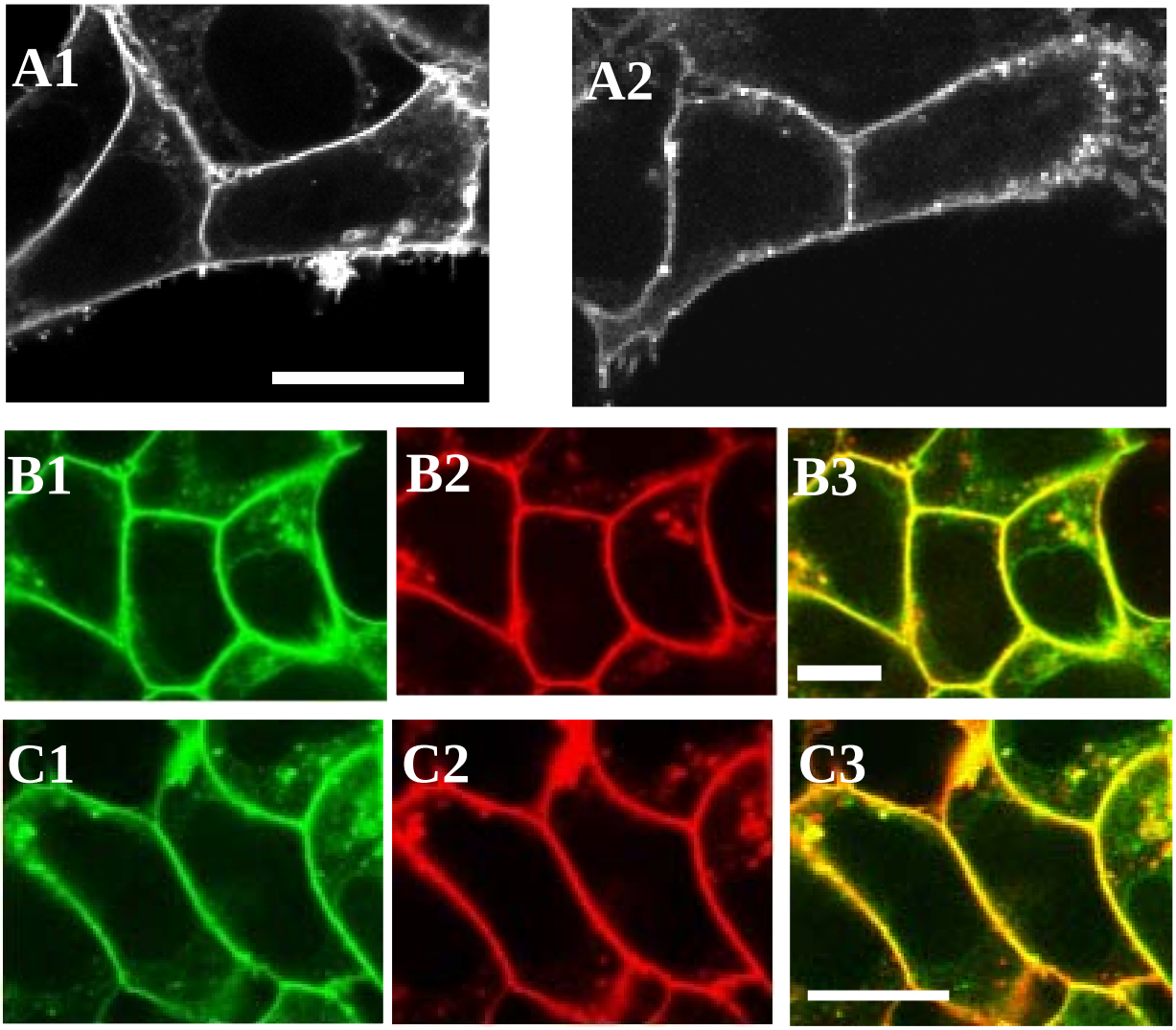


Figure 3.10: Effect of chlorpromazine and cholera toxin on internalization of the rP2Y$_2$-GFP receptor.
HEK-293 cells expressing the rP2Y_2-GFP receptor were preincubated with 100 µM chlorpromazine alone (A1) or together with 5 nM cholera toxin subunit B coupled to Alexa Fluor 555 (B) for 30 min at 37 °C. The cells were then incubated for 30 min at 37°C in the presence of 100 µM UTP (A2 and C), and images were collected. The numbering of pictures in B and C depicts, $P2Y_2$-GFP receptor (green fluorescence) in 1, cholera toxin subunit B staining (red fluorescence) in 2, and the overlay in 3. Cells that are shown in B and C are the same set of cells. Those depicted in (B) are before and (C) after incubation with UTP. Scale bar is 20 µm. Results shown are representative of at least three individual experiments.

The cholesterol content of the plasma membrane is important for receptor endocytosis. Methyl-beta-cyclodextrin (MβCD) is an agent, known to extract cholesterol from the plasma membrane (Rodal et. al., 1999), thereby inhibiting

invagination of clathrin-coated pits (Rodal et. al., 1999). When HEK-293 cells expressing the rP2Y_2-GFP receptor were pretreated with 5 mM MβCD to deplete plasma membrane cholesterol, the rP2Y_2-GFP receptor did not internalize either in unstimulated cells (Fig. 3.11, A1) or in cells stimulated with 100 μM UTP for 30 min (Fig. 3.11, A2). After a 60 min UTP stimulation in the presence of MβCD (Fig. 3.11, A3), however, internalized receptors were observed close to the plasma membrane (bold arrow) and in some cells there was a punctate appearance (broken arrow) of receptor-containing vesicles, indicating that depletion of plasma membrane cholesterol significantly altered the rate and extent of P2Y_2-GFP receptor endocytosis. The endocytosed receptor exhibits a punctate distribution indicating that the receptor is not targated to lysososmes. This punctate distribution of the receptor points to the fact that cholesterol depletion does affect the targeting of the receptor.

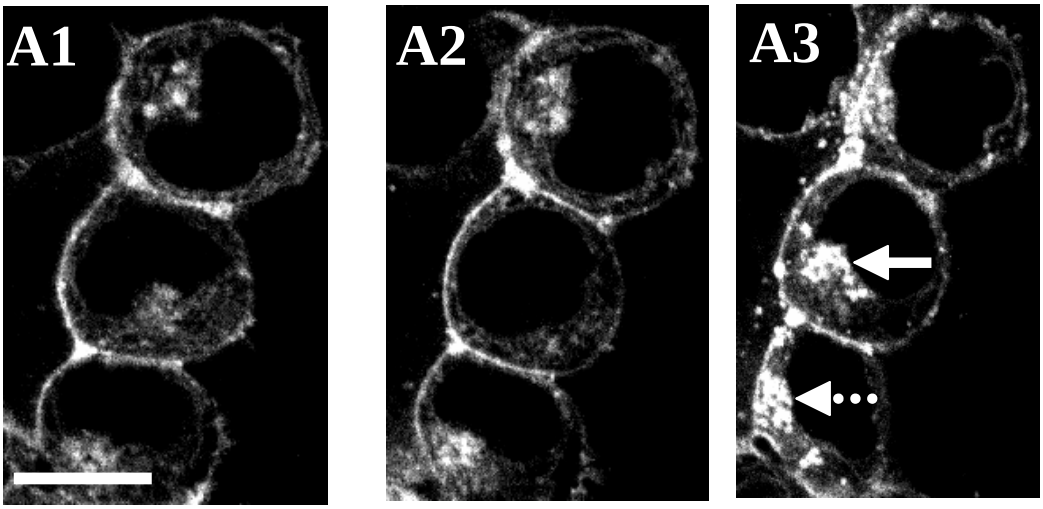


Figure 3.11: Effect of the cholesterol-extracting agent methyl-beta-cyclodextrin (MβCD), on rP2Y_2-GFP receptor endocytosis. HEK-293 cells expressing the rP2Y_2-GFP receptor were preincubated with 5 mM MβCD for 30 min at 37 °C (A1), then incubated in the presence of 100 μM UTP for 30 min (A2) and 60 min (A3), and images were collected. In MβCD-treated cells, receptor endocytosis is inhibited for the first 30 min (as seen in A2) and at the end of 60 min there is a punctate distribution of the endocytosed receptor (arrows in A3). Scale bar is 20 μm. Results shown are representative of at least three individual experiments.

To further confirm that endocytosis of the P2Y_2-GFP receptor was independent of caveolin, cells were preincubated with 10 μM filipin III, an inhibitor of caveolin-mediated pathways (Orlandi et. al., 1998), prior to stimulation with 100 μM UTP. Filipin III did not affect the shape nor induce receptor internalization in unstimulated

cells (Fig.3.12, A1), but receptors were completely internalized after a 30 min (Fig. 3.12, A2) or 60 min UTP stimulation (Fig. 3.12, A3), consistent with the conclusion that internalization of the $P2Y_2$-GFP receptor is mediated by a clathrin-dependent pathway.

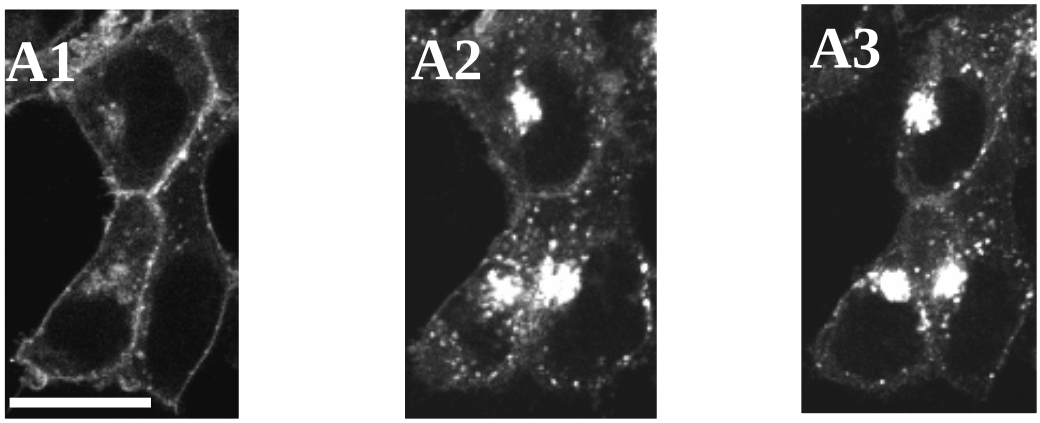


Figure 3.12: Effect of filipin-III, inhibitor of caveolin mediated endocytosis on $rP2Y_2$-GFP receptor endocytosis. HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated with 10 μM filipin III for 30 min at 37 °C (A1), then incubated in the presence of 100 μM UTP for 30 min (A2) and 60 min (A3), and images were collected. Filipin III is an inhibitor of caveolin- mediated endocytosis. It has no effect on the endocytosis of the receptor, as complete endocytosis of the receptor is observed after 30 min in filipin III-treated cells. Scale bar is 20 μm. Results shown are representative of at least three individual experiments.

3.7 Reappearance of the $P2Y_2$ receptor after receptor internalization

HEK-293 cells expressing the $rP2Y_2$–GFP receptor were preincubated for 30 min with 100 nM LysoTracker (Fig. 3.13A) and incubated for 60 min in the presence of 100 μM UTP to induce receptor internalization (Fig. 3.13B). It is observed that the internalized receptor is colocalized with lysosomes (Fig. 3.13B bold arrows). Then, the cells were washed and allowed to recover in agonist-free medium for 4 h 30 min prior to cell imaging, whereupon receptors remained internalized as aggregates outside of lysosomes (Fig. 3.13C, broken arrows).

Trafficking of receptors from endosomes to lysosomes is known to occur via an intermediate multivesicular body complex (Miwako, et.al., 2001). To investigate the proteasome-dependent trafficking of $rP2Y_2$-GFP receptors from endosomes to lysosomes, we preincubated the cells with 10 μM MG-132, a proteasome inhibitor (Haglund et. al., 2003). Pretreatment of the cells with MG-132 did not affect cell morphology or receptor distribution in unstimulated cells (Fig. 3.13D), whereas in cells

stimulated with 100 µM UTP for 60 min, there was complete endocytosis of the receptor in large aggregates outside of lysosomes (Fig. 3.13E, bold arrows). This is different from that seen in the absence of the inhibitor, where the endocytosed receptor is colocalized with lysososems (Fig.3.13B bold arrows). After a 4 h 30 min recovery period, $P2Y_2$-GFP receptors had reappeared at the plasma membrane (Fig. 3.13F, broken arrows). The pattern observed in Fig. 3.13F is different from that exhibited in 3.13C, as there are no vesicular structures that are present in the cytoplasm, indicating that MG-132 prevented trafficking of the endocytosed receptors to lysosomes and promoted receptor distribution in cytoplasmic vesicles that facilitated recycling of internalized receptors back to the plasma membrane. Thus, targeting of the $P2Y_2$-GFP receptor to lysosomes probably involves a proteasome-dependent pathway.

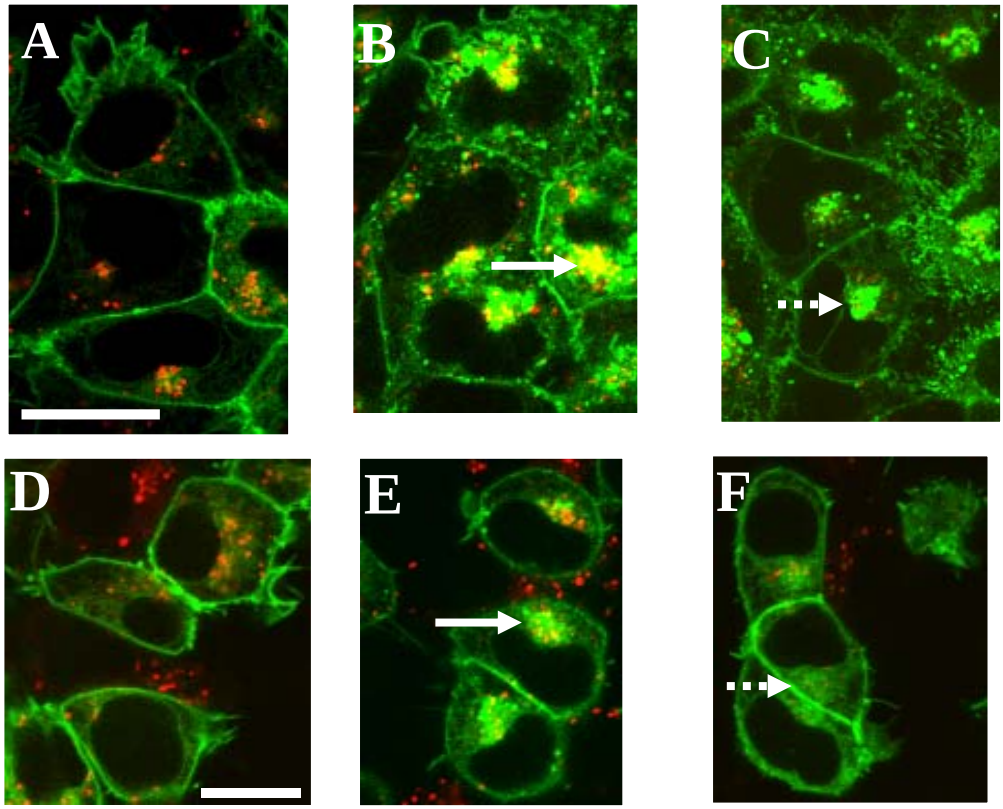


Figure 3.13: Recyling of the internalized $rP2Y_2$-GFP receptor. HEK-293 cells expressing the $rP2Y_2$-GFP receptor (green) were preincubated with 100 nM LysoTracker (red) for 30 min at 37 °C (A), then cells were incubated for 60 min in the presence (B) of 100 µM UTP, and images were collected. UTP-stimulated cells also were washed three times and allowed to recover for 4 h 30 min in agonist-free medium, and images were collected (C). Other cells were pretreated with 100 nM LysoTracker and 10 µM MG-132 (D), then incubated in the presence of 100 µM UTP for 60 min at 37 °C (E), allowed to recover for 4 h 30 min in the continued presence of MG-132 (F), and images were collected. MG-132 is a proteasome inhibitor. In cells treated with MG-132 there is an inhibition of the targeting of the endocytosed receptor to lysosomes (as seen in E, indicated by bold arrow), different from B with colocalization of endocytosed receptor with lysosomes (bold arrow). A complete reappearance of the receptor back to

the plasma membrane (as seen in F, broken arrow) was observed in MG-132-treated cells, whereas in untreated cells there are aggregates of endocytosed receptor (*C*, broken arrow). Scale bar, 20 μm. Results shown are representative of at least three individual experiments.

3.8 Role of kinases and phosphotases in modulating the translocation of the rP2Y$_2$-GFP receptor

Kinases play an important role in modulating the signaling mediated via GPCR's. In case of the P2Y receptors the role of kinases in modulating the translocation of the receptor is not well studied. In the P2Y$_2$ receptor it is know that there are a number of possible amino acid residues that could be phosphorylated by calcium/ calmodulin dependent kinase II (CaMK II) and protenin kinase –C (PKC) (Garrad et. al., 1998). The role of these kinases was investigated only on their ability to modulate the initial Ca^{2+} response (Garrad et. al., 1998). However the role of these kinases in modulating the translocation of the receptor has not been studied. Therefore we investigated the effect of different kinases in modulating the receptor translocation.

3.8.1 Role of Calcium / Calmodulin dependent protein kinase II (CamK II)

HEK-293 cells stably expressing the rP2Y$_2$-GFP receptor were pre-incubated for 30 min with 10 μM of KN-62, a specific inhibitor of CaMK-II (Davies et. al, 2001). The cells were stimulted with 100 μM UTP. 30 min of preincubation of the cells with 10 μM KN-62 did not alter the distribution of the receptor or the morphology of the cell (Fig. 3.14A). After 30 min of stimulation with 100 μM UTP, no endocytosis of the receptor was observed (3.14B). After 60 min of stimulation with UTP in presence of KN-62, endocytosis of the receptor could be visualized (Fig. 3.14C). The endocytosis of the receptor that visualized was not to the same extent as observed in untreated cells. The cells were washed free of the agonist and were further visualized for 90 min in the continued presence of 10 μM KN-62. It was seen that the receptor partially reappears to the plasma membrane (Fig 3.14D). The effect of 10 μM KN-62 on modulating the initial Ca^{2+} response was investigated and we observed that pretreated cells exhibited a Ca^{2+} response that was 50 % of that obtained in untreated cells, when they were challenged with 100 μM UTP. Thus we can conclude that KN-62 modulates the initial

Ca^{2+} response and also does play an important role in modulating the trafficking of the receptor.

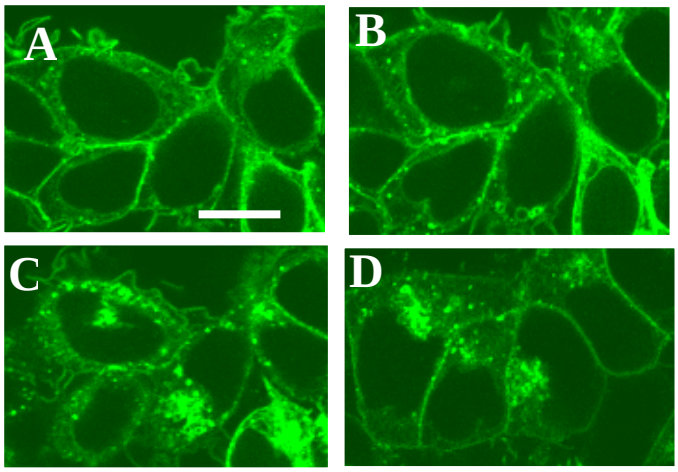


Figure 3.14: Role of CaMK II in modulating the trafficking of $P2Y_2$-GFP receptor. HEK-293 cells expressing the $P2Y_2$-GFP receptor were pre-treated for 30 min with 10μM KN-62 (A) and then stimulated for 30 (B) and 60 min (C) with 100 μM UTP. The cells were then washed free of agonist and visualized for further 90 min (D). Pre-incubation of the cells with KN-62 delays endocytosis of the receptor. Results shown are representative of at least three individual experiments. Scale bar 20 μm.

3.8.2 Effect of protein phosphatases on modulating the receptor translocation

De-phosphorylation of receptors too in know to play an important role in the recycling the GPCR's (Oakley et. al., 1999). However, in case of the $P2Y_2$ receptors this effect has till now not been investigated. We pre-incubated the cells for 30 min with 5 nM of okadaic acid, a specific inhibitor of protein phosphatase 2A (PP2A) at this concentration (Schonthal 1998). The effect of the inhibitor on the Ca^{2+} signaling in the cells was investigated. The pre-incubation resulted in a reduction of the initial Ca^{2+} response which was 49% compared to that obtained with untreated cells. At the level of endocytosis, pre-incubation of the cells with okadaic acid did not influence the shape of the cells, the distribution of the receptor (Fig 3.15A) and the endocytotic kinetics of the receptor. A complete endocytosis of the receptor was observed after 30 min of stimulation of the cells with 100 μM UTP (Fig 3.15B). The agonist was withdrawn and the cells were allowed to recover, in the presence of the inhibitor. After 90 min of removal of the agonist it was observed that there was a reduced reappearance of the

receptor on the plasma membrane (Fig 3.15C), in comparison to cells that were not exposed to the inhibitor. After 120 min of withdrawal of the agonist it was observed that there was only a partial recovery of the receptor. These results indicate that the dephosphorylation of the receptor is important for the trafficking of the receptor back to the plasma membrane.

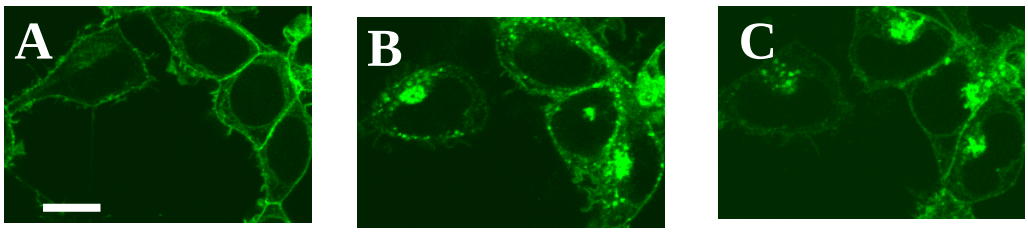


Figure 3.15: Role of Protein phosphatase 2A in modulating the trafficking of $P2Y_2$-GFP receptor. HEK 293 cells expressing the $P2Y_2$-GFP receptor were pre-treated for 30 min with 5nM okadaic acid, a specific inhibitor of protein phosphatase 2A (A) and then stimulated for 30 min(B) with 100 µM UTP. The cells were then washed free of agonist and visualized for further 60 min (C). Pre-incubation of the cells with okadaic acid does not affect the endocytosis of the receptor, but delays the reappearance of the receptor. Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

3.8.3 Role of Protein Kinase C (PKC) in trafficking of the receptor

Protein kinsae-C comprise of a family of kinases that play an important role in modulating activity of different protiens like Na+ K+ ATPase (Dada et. al., 2003), actin cytoskeleton (Larsson 2006) and GPCR trafficking (Namkung and Sibley 2004) The PKCs can be divided into three categories, namely Ca^{2+} dependent, Ca^{2+} independent and atypical. We investigated the role of these kinases in controlling the kinetics of endocytosis of the $P2Y_2$ receptor and its localization, using the commercially available subtype specific inhibitors for PKCs.

3.8.3.1 Effect of Inhibition of Ca^{2+} dependent PKCs

This subclass comprises of PKC alpha and beta. The cells were pre-treated for 1 hour with 1 µM GO 6976. After 60 min of pre-incubation with inhibitor, 100 nM of lysotracker red was added to the culture medium (Fig. 3.16A). The Ca^{2+} response observed to 100 µM UTP from pre-treated cells was 26% of that obtained from untreated cells. The cells were stimulated with 100 µM UTP in continual presence of the inhibitor. It is observed that in the first 30 min there is an inhibition of endocytosis of the receptor (Fig. 3.16B). Endocytosis of the receptor was observed at the end of 60 min, but there was no colocalization of the endocytosed receptor with lysosmes (Fig 3.16C) (bold arrows). When the cells were allowed to recover for 90 min in the absence of agonist, but in the continued presence of inhibitor it was observed that there is a

partial reappearance of the receptor on the plasma membrane (Fig. 3.16D). (bold arrows). In case of untreated cells a partial reappearance of the receptor (cf Fig. 3.13C) was observed after 4. hours 30 min of withdrawal of agonist. Thus it indicates that the Ca^{2+} dependent PKCs modulate both the endocytosis and reappearance of the $P2Y_2$ receptor.

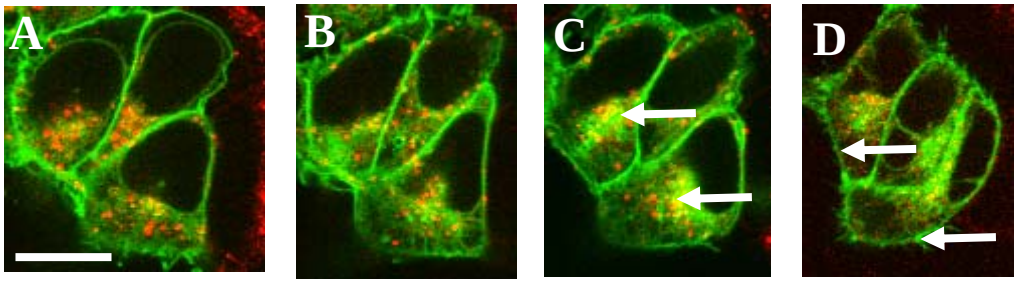


Figure 3.16: Role of Ca^{2+} dependent protein kinase C (PKCs) in modulating the trafficking of $P2Y_2$-GFP receptor.
HEK-293 cells expressing the $rP2Y_2$-GFP receptor (green) were preincubated with 1 µM GO 6976, a specific inhibitor of Ca^{2+} dependent protein kinases for and 100 nM LysoTracker (red) at 37 °C (*A*). Then cells were incubated for 30 min (B) and 60 min (C) in the presence of 100 µM UTP, and images were collected. UTP-stimulated cells also were washed three times and allowed to recover for 90 min in agonist-free medium, and images were collected (D). Pre-treatment of the cells with GO 6976 delays endocytosis of the receptor and prevents colocalization of the endocytosed receptor with lysosomes. It hastens up the reappearance of the receptor back to the plasma membrane. Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

3.8.3.2 Effect of a broad spectrum inhibitor of PKCs

The inhibitor GF 109203X is a broad spectrum inhibitor of the members of the PKC family mainly consisting of both the Ca^{2+}dependent and independent PKCs. At the concentration of 1 µM in inhibits PKCα,βI,βII,γ,δ,ε. The cells were pre-incubated for 1 h with 1 µM of the inhibitor (Fig.3.17 A) and for 30 min with 100 nM lysotracker red. and stimulated with 100 µM of UTP for 60 min. The initial Ca^{2+} response obtained from these cells was 51% of that obtained from untreated cells. A slightly delayed endocytosis of the receptor is observed (Fig. 3.17 B), as 40 min is required for complete endocytosis of the receptor in comparison to 30 min that is observable in the absence of the inhibitor (Fig. 3D). This indicates that a broad spectrum inhibitor does not drastically affect the endocytosis kinetics of the $P2Y_2$ receptor. After 60 min of stimulation with UTP there is a colocalization of the receptor with lysosomes is observed (Fig. 3.17C) (bold arrows). This is the second difference that is observed

between the trafficking of the $P2Y_2$ receptor when the cells were pre-treated with the Ca^{2+} specific inhibitor of PKCs and the broad-spectrum inhibitor of PKCs.

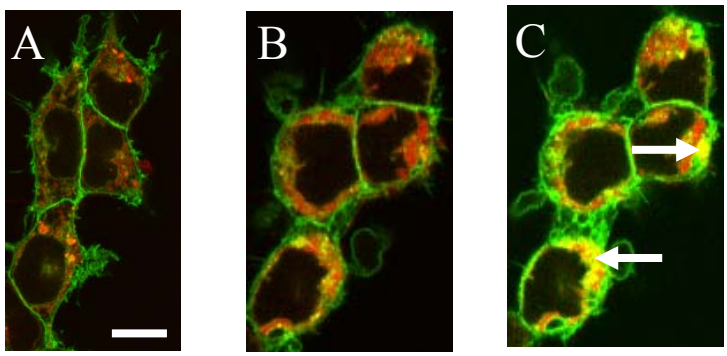


Figure 3.17: Role of protein kinase C (PKCs) in modulating the trafficking of $P2Y_2$-GFP receptor. HEK-293 cells expressing the $rP2Y_2$-GFP receptor (green) were preincubated with 1 µM GF 109203 X, a broad spectrum inhibitor of protein kinases for 60 min and 100 nM LysoTracker (red) 37 °C (A). Then cells were incubated for 60 min in the presence (C) of 100 µM UTP, and images were collected. Pre-treatment of the cells with GF109203 X resulted in delayed endocytosis of the receptor (B) 40 min is required for the complete endocytosis of the receptor in comparision to 30 min in untreated cells .It does not prevent colocalization of the endocytosed receptor with lysosomes. Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

3.8.3.3Effect of inhibition of atypical PKCs

This family of PKCs includes PKC ζand δ. At 1µM GO 6983 specifically inhibits only the atypical PKCs (Javed et. al., 2004). The protocol for the internalization experiments was identical to that used for the other inhibitors of the PKC family. In case of experiments with GO6983 the META channel and the technique of online emission fingerprinting was used for detection of the emitted fluorescence as the inhibitor is excitable at 543 nm, and emits at 620nm. The technique of online finger printing employs the principle of linear unmixing. In this method the emission spectrum of each of the putative fluorophore is initially recorded in exactly the same experimental configuration for which the technique would be applied. During the course of the experiment the pre-recorded spectra are used to calculate the exact percentual contribution of each of the flurophore in a give pixel (Dickinson et. al., 2001). Pre-incubation of the cells with the inhibitor did not affect the shape of the cells or the loading with lysotracker Red (Fig. 3.18A). However pre-treatment of the cells with

inhibitor did decrease the initial Ca^{2+} response by 47 % in comparison to that exhibited by untreated cells. Normal endocytosis of the receptor was observed after 30 min of stimulation with 100µM UTP (Fig.3.18B). It was interesting to observe that after 40 min of treatment with UTP a colocalization of the endocytosed receptor was observed with lysososmes (Fig.3.18C) (bold arrows). This is different from that observed in case of untreated cells, where colocalization was observed after 60 min of stimulation with UTP (cf. Fig. 3.13B). This indicates that the inhibiton of the atypical PKCs accelerates the translocation of the endocytosed receptor to lysososmes. When the cells were pre-incubated with 5 µM GO 6983, a colocalization between the endocytosed receptor and the lysosomes is observed 30 min after stimulation of the cells with 100 µM UTP. When the cells were allowed to recover for 90 min in the presence of 1 µM GO 6983 a weak reappearance of the receptor on the plasma membrane is observed and most of the endocytosed receptor is redistributed in the cytoplasm is large aggregates (Fig 3.18D) (bold arrows). There is no localization of the receptor with lysosomes. This is similar to the results obtained when normal endocytosis of the receptor is observed. This could lead us to speculate that the endocytosis of the receptor is modified, but the reappearance of the receptor is not drastically affected.

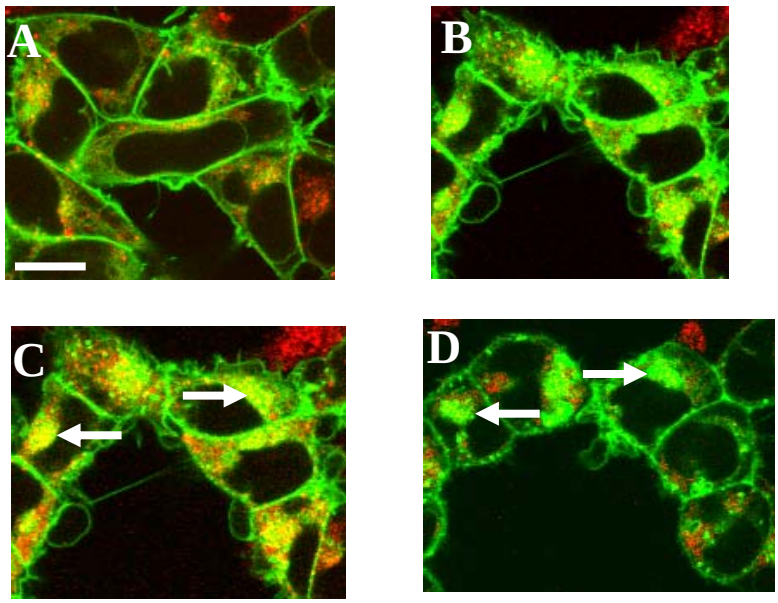


Figure 3.18: Role of atypical PKCs in modulating the trafficking of the P2Y$_2$-GFP receptor. HEK-293 cells expressing the rP2Y$_2$-GFP receptor (green) were preincubated with 1 µM GO 6983, a specific inhibitor of atypical protein kinases for 60 min and 100 nM LysoTracker (red) for 30 min and at 37 °C (*A*). Then cells were incubated for 30 min (B) and 40 min in the presence (C) of 100 µM UTP, and images were collected. UTP-stimulated cells also were washed three times and allowed to recover for 90 min in agonist-free medium, and images were collected (D). Pre-treatment of the cells with GO 6983 does not affect the rate of endocytosis of the receptor but hastens the

colocalization of the endocytosed receptor with lysosomes. There is a weak reappearance of the receptor back to the plasma membrane. Scale bar, 20 μm. Results shown are representative of at least three individual experiments.

3.8.4 Role of phosphoinositol-3-kinase (PI3K) in $P2Y_2$ receptor trafficking

The cells were simultaneously preincubated with 100 nM lysotracker Red and wortmannin, to label lysosmes and a specific inhibitor of PI3K, respectively (Fig. 3.19A). The initial Ca^{2+} signal that was observed on challenging the pre-treated cells with 100 μM UTP was 31% of that observed in untreated cells. After 30 min of stimulation with 100 μM UTP it is observed that there is complete endocytosis of the receptor (Fig. 3.19B), but there no colocalization of the endocytosed receptor with lysosomes. This is similar to that is observed in case of untreated cells. After further 30 min of stimulation it is seen that there is a aggregation of the receptor in form of large vesicles, but it is not colocalized with the lysosomes (Fig. 3.19C). This indicated that the pre-incubation of the cells with wortmannin did not affect the temporal kinetics of endocytosis of the receptor, but its targeting to lysosomes. Though a colocalization of the receptor with the lysosomes seems apparent from the figure (Fig. 3.19B), it is clear from the X-Z and Y-Z projections that the lysosomes and the endocytosed receptor vesicles are present in different cellular levels. After 360 min of removal of agonist, it is observed that the receptor did not reappear on the plasma membrane and is still present in large aggregates in the cytosol and it is not localized with the lysosomes (Fig. 3.19D). This is an additional difference that is observed to untreated cells, where a partial reappearance of the receptor is observed on the plasma membrane (ref. Fig. 3.13C).

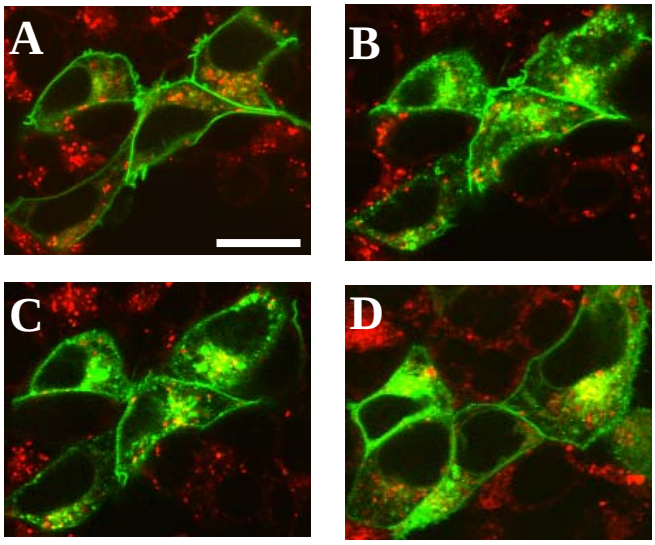


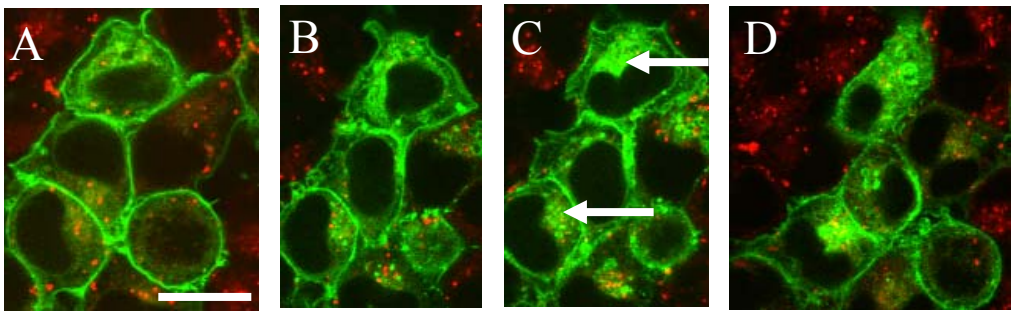
Figure 3.19: Role of PI3 kinase in modulating the trafficking of the $P2Y_2$ receptor. HEK-293 cells expressing the $rP2Y_2$-GFP receptor (green) were preincubated with 100 nM wortmannin, a specific inhibitor of PI3 kinases for 60 min and 100 nM LysoTracker (red) for 30 min at 37 °C (*A*). Then cells were incubated for 30 min (B) and 60 min in the presence (C) of 100 μM UTP, and images were collected. UTP-stimulated cells also were washed three times and allowed to recover for 360 min in agonist-free medium, and images were collected (D). Pre-treatment of the cells with wortmannin does not affect the rate of endocytosis of the receptor but prevents the colocalization of the endocytosed receptor with lysosomes. There is a weak reappearance of the receptor back to the plasma membrane. Scale bar, 20 μm. Results shown are representative of at least three individual experiments.

3.8.5 Modulation of $P2Y_2$ receptor endocytosis by Rho-A kinase

Rho kinase has been know to be involved in modulating the activity of GPCRs as the kinases in this family are actively involved in the remodeling of the actin cytoskeleton, which is one of the factors involved in endocytosis of the GPCRs (Mueller and Strange, 2004). We pre-incubated the cells with 10 μM of Y-27632, a specific inhibitor of RhoA kinase (Smith et. al., 2001). The pre-incubation of the cells with the inhibitor did not affect the loading of the lysotracker red and the localization of the receptor (Fig. 3.20A). After 30 min of stimulation of the cells with 100 μM UTP there was a minimal endocytosis of the receptor (Fig. 3.20B). This is similar to that which is observed in case of cells pre-incubated with 100 nM Cytochalasin-D (ref-Fig.3.6.A2), an inhibitor of actin cytoskeleton. However, preincubation of the cells with the inhibitor reduce the initial Ca^{2+} response exhibited by the cells to 100 μM UTP by 14% compared to untreated cells. This indicates that though the pre-treatment of the cells with Y-27632 affected the endocytotic kinetics of the receptor but not significantly the initial calcium response. After further continual stimulation with 100 μM UTP, endocytosis of the receptor was observed, but there was no colocalization of the endocytosed receptor with lysosomes (bold arrows) (Fig. 3.20C). The endocytosed receptor did not form the large aggregate that is observed in untreated cells. After withdrawal of the agonist it is observed that there is a partial reappearance of the receptor on the plasma membrane (Fig. 3.20D). This indicates that the inhibition of the RhoA kinase plays an important role in the trafficking of the receptor. This corroborates our data that is obtained with cytochalasin-D and ML-9, that in addition to actin, the supporting machinery that is involved in aiding the remodelling of the actin cytoskeleton has to be intact to promote normal trafficking of the receptor.

Figure 3.20: Role of RhoA kinase in modulating the trafficking of the $rP2Y_2$-GFP receptor. HEK-293 cells expressing the $rP2Y_2$-GFP receptor (green) were preincubated with 10 μM Y-27632, a specific inhibitor of RhoA kinases for 60 min and 100 nM

LysoTracker (red) for 30 min and at 37 °C (*A*). Then cells were incubated for 30 min (B) and 60 min (C) in the presence of 100 µM UTP, and images were collected. UTP-stimulated cells were then washed three times and allowed to recover for 90 min in agonist-free medium, and images were collected (D). Pre-treatment of the cells with Y-27632 caused a minimal internalization of the receptor after 30 min of stimulation and prevented co-localization of the internalize receptor with lysosomes at the end of 60 min. There is a partial reappearance of the receptor back to the plasma membrane. Scale bar, 20 µm. Results shown are representative of at least three individual experiments.



3.8.6 Role of Mitogen Activated Protein Kinase (MAPK) in modulating endocytosis of the $P2Y_2$ receptor

MAP kinases (MAPK) have been shown to be activated on stimualtion of a number of GPCRs (Schmidt et. al.,2000; Wang et. al, 2002), but their role in modulating the endocytosis of the P2Y receptor endocytois has not been well studied. The MAPK cascade comprises a number of kinases which are phosphorylated in a sequential manner finally resulting in the phosphorylation of MAPK. The activated MAPK can then result in stimulation of proliferation (Wang et. al., 2003). We wanted to investigate the role of the activated MAPK in modulating the trafficking of the $P2Y_2$ receptor. To study this, we inhibited the activation of the MAPK by inhibiting the activity of MAPK kinase (MEK) with 10 µM U0126. After 30 min of pre-incubation (Fig. 3.21A), the cells were treated for 60 min with 100 µM UTP. It is seen that after 30 min of stimulation, internalization of the receptor is observed (Fig. 3.21B), but the internalized receptor is present in small vesicles (indicated by arrows) distributed in the cytoplasm. This is different from the pattern that is observed in untreated cells (ref. Fig. 2A1). After further 30 min of stimulation an aggregation of the endocytosed receptor is observed (Fig. 3.21C). This indicates a similar pattern of aggregation of the receptor as in untreated cells. After 90 min of withdrawal of agonist there is a partial reappearance of the receptor on the plasma membrane (Fig. 3.21D). Thus we can infer that although the initial endocytosic pattern of the receptor was different from that in case of

untreated cells, the reappearance pattern of the receptor seems to be similar to that observed in untreated cells.

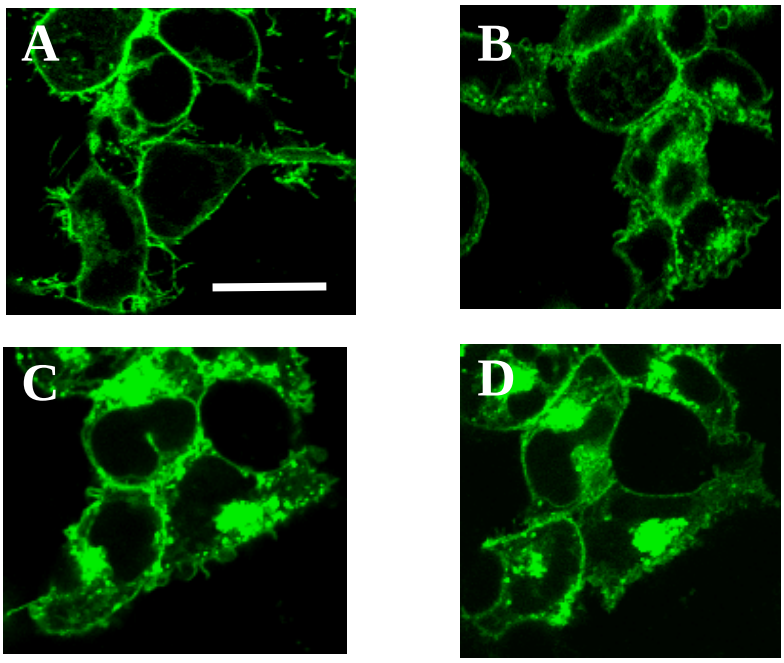


Figure 3.21: Role of MEK in modulation of $P2Y_2$ receptor trafficking. HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated with 10 μM U-0126, a specific inhibitor of MAPK kinases for 60 min and at 37 °C (*A*). Then cells were incubated for 30 min (B) and 60 min (C) in the presence of 100 μM UTP, and images were collected. UTP-stimulated cells also were washed three times and allowed to recover for 90 min in agonist-free medium, and images were collected (D). Pre-treatment of the cells with U 0126 does not affect the rate of endocytosis of the receptor. There is a weak reappearance of the receptor back to the plasma membrane. Scale bar, 20 μm. Results shown are representative of at least three individual experiments.

3.9 Effect of Phospholipase D2 (PLD_2) on endocytosis of the $P2Y_2$ receptor

Phospholipases have been implicated in modulation of the endocytosis of receptors (Du et. al., 2004; Shen et. al., 2001). To determine the role ofPLD_2 in endocytosis of the $P2Y_2$ receptor the cells were treated either with 1-butanol or 2-butanol. 1-butanol is used to check if PLD_2 has a role in a given process because of the virtue of the transphosphatidylation reaction, whereby the enzyme preferentially utilizes a primary alcohol over water to generate a phosphatidylalcohol instead of phosphatidic acid. Thus, the presence of a primary alcohol inhibits the production of phosphatidic acid by PLD. The reaction is highly specific for primary alcohols and secondary

alcohols are not utilized by PLD. Thus 2-butanol is used as a negative control (Morris et. al., 1997). The cells were pre-incubated with 0.75% 1-butanol (Fig 3.22A) for 30 min and then stimulated with 100 µM UTP. It is observed that for the first 30 min there is no endocytosis of the receptor (Fig. 3.22B). After 60 min of stimulation there is only a partial endocytosis of the receptor observed (Fig 3.22C). This indicates that inhibition of PLD_2 inhibits the endocytosis of the $P2Y_2$ receptor. After 30 min of withdrawal of the agonist, in the continual presence of the inhibitor, endocytosis of the receptor was observed (Fig 3.22D). The percentage of endocytosis further increased 60 min after withdrawal of the agonist (Fig 3.22E).This means that inhibition of the PLD_2 pathway does delay the endocytosis of the receptor but still the kinases could be still active the trafficking pattern still seems similar to that seem in the case of protein kinase-C mediated.

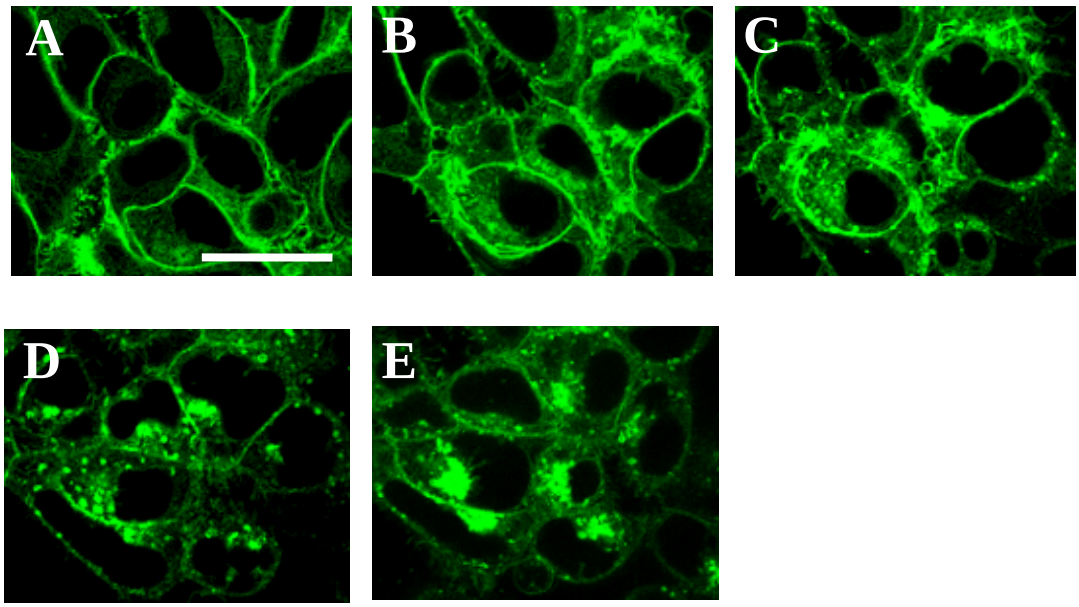


Figure 3.22: Role of PLD_2 in modulation of $P2Y_2$ receptor trafficking. HEK-293 cells expressing the $rP2Y_2$-GFP receptor were preincubated with 0.75% v/v 1-butanol, a specific inhibitor of PLD_2 for 60 min and at 37 °C (A). Then cells were incubated for 30 min (B) and 60 min (C) in the presence of 100 µM UTP, and images were collected. UTP-stimulated cells were washed three times and allowed to recover for 30 min (D) and 90 min (E) in agonist-free medium, and images were collected. Pre-treatment of the cells with 1-butanol delays the rate of endocytosis of the receptor. There is a weak reappearance of the receptor back to the plasma membrane after withdrawal of agonist. Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

To confirm the role of PLD_2 in modulating the translocation of the $P2Y_2$ receptor, the cells were preincubated for 30 min with 0.75% v/v 2-butanol (Fig. 3.23 A). 2-butanol is an inactive analogue of 1-butnol, as it does not inhibit the function of PLD_2.

It was seen on stimulation of the 2-butanol pre-treated cells with 100 µM UTP, complete endocytosis of the receptor is observed after 30 min (Fig.3.23B) and a translocation of the endocytosed receptor to the lysosomes (Fig 3.23C) (bold arrows) is seen after 60 min of stimulation with UTP. The endocytotic kinetic is similar to that exhibited by untreated cells. Thus we can infer that PLD_2 is involved in modulating the trafficking of the $P2Y_2$ receptor.

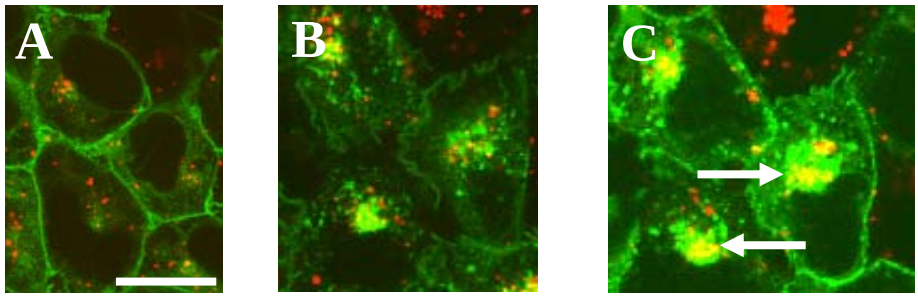


Figure 3.23: Effect of 2-butanol in modulating the endocytosis of the $P2Y_2$ receptor. HEK-293 cells expressing the r$P2Y_2$-GFP receptor (green) were preincubated with 0.75% (v/v) 2-butanol for 60 min and 100 nM LysoTracker (red) for 30 min at 37°C (*A*). Then cells were incubated for 30 min (B) and 60 min (C) in the presence of 100 µM UTP, and images were collected. Pre-treatment of the cells with 2-butanol does not affect the rate of endocytosis of the receptor and the colocalization of the endocytosed receptor with lysosomes. This indicates that 2-butanol does not affect the endocytosis of the receptor. Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

3.10 Effect of ATP on UTP on differential trafficking of the $P2Y_2$-GFP receptor

The cells were stimulated with 100 µM ATP or UTP for 30 min or 60 min. The cells were then washed and allowed to recover for 60 min. It is observed that there is a difference observed in the trafficking of the receptor depending on the duration of stimulation with the different agonists. In case of a 30-min stimulation with either 100 µM ATP (Fig. 3.24B) or UTP (Fig. 3.24C), a complete endocytosis of the receptor was observed. After the withdrawal of the agonist and after an hour's recovery it is observed that in both cases there is complete reappearance of the receptor on the plasma membrane (Fig. 3.24D and E). This indicates that when cells were stimulated for 30 min with 100 µM ATP or UTP, there was no observable difference in the pattern of endocytosis or reappearance of the receptor, corroborating the fact that ATP and UTP

function as equipotent agonists at the $P2Y_2$ receptor. So we stimulated the cells for 60 min with 100 µM of either ATP (Fig. 3.24F) or UTP (Fig. 3.24G) and allowed the cells to recover for 1 h. An interesting difference is observed in the trafficking of the receptor, although both agonists are equipotent on the $P2Y_2$ receptor. A 60 min stimulation with 100 µM UTP and ATP resulted in a complete endocytosis of the receptor. In case of ATP-stimulated cells there is a significant reappearance of the receptor on the plasma membrane after 1 h of withdrawal of the agonist, (Fig. 3.24H) whereas in UTP-treated cells there is a minimal reappearance of the receptor on the plasma membrane (Fig. 3.24I). This indicates that the stimulation of the cells with 100 µM UTP or ATP for 60 min leads to a different trafficking of the endocytosed receptor, which then leads to the different degrees of reappearance of the receptor back to the plasma membrane.

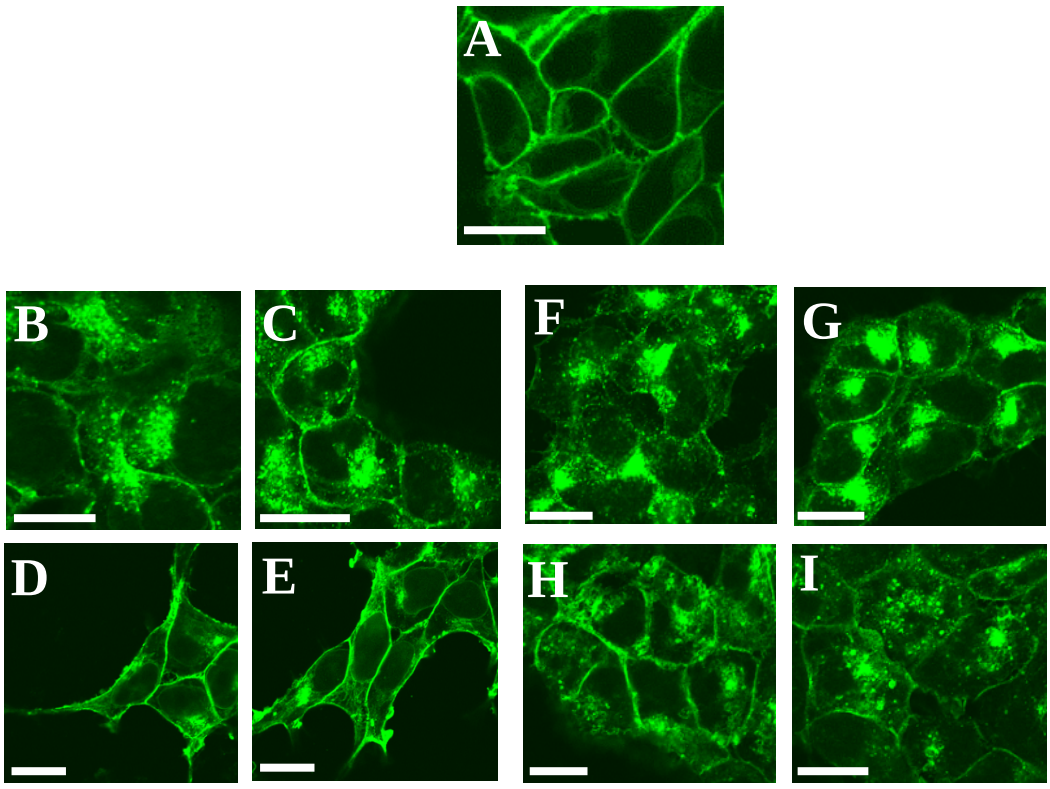


Figure 3.24: Differential trafficking of the $P2Y_2$-GFP receptor on stimulation with ATP and UTP. HEK-293 cells expressing the $rP2Y_2$-GFP receptor (A) were stimulated with 100 µM ATP (B) or UTP (C) for 30 or 60 min, respectively at 37 °C. Then the cells were allowed to recover for 60 min and then fixed (as described in Materials and Methods). In case of unstimulated cells (A) the receptor is localized on the plasma membrane and on stimulation for 30 min with 100 µM ATP (B) or UTP (C) a complete endocytosis of it is observed. A complete reappearance of the receptor at the plasma membrane is observed after 60 min of withdrawal of ATP (D) and UTP (E). Similarly 60 min stimulation of the cells with 100 µM ATP (F) or UTP (G) results in complete endocytosis of the receptor. A 1 h. withdrawal of the agonist results in significant

reappearance of the receptor in case of ATP (H) and not UTP (I). Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

This is an interesting difference that we observed in comparison to the $P2Y_1$ receptor. When HEK 293 cells stably expressing the $P2Y_1$-GFP receptor (Fig. 3.25A) were subjected to the above protocol, we observed that either in case of a 30 min (Fig. 3.25) or of 60 min (Fig. 3.25C) stimulation of the cells with 100 µM ATP the $P2Y_1$ receptor did completely recycle back to the plasma membrane one hour after withdrawal of the agonist (Fig. 3.25D and E). This indicates that in the same cell, stimulation of the cell with ATP results in different trafficking pathway of two types of receptors.

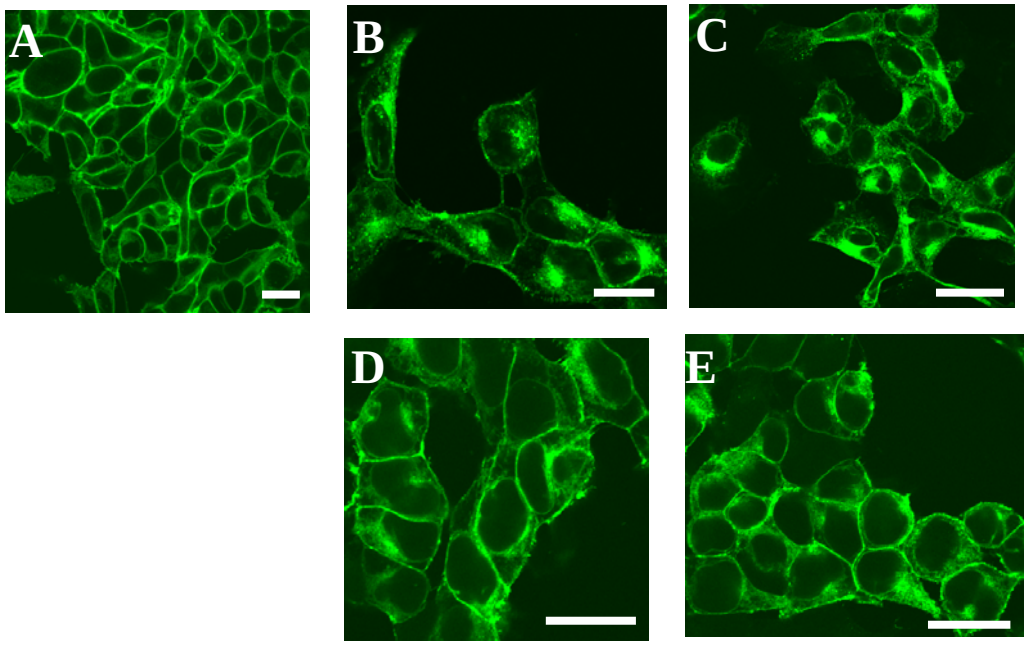


Figure 3.25: Trafficking of the $P2Y_1$-GFP receptor upon stimulation with ATP. HEK-293 cells expressing the $rP2Y_1$-GFP (A) receptor were stimulated with 100 µM ATP for 30 min (B) or 60 min (C) at 37 °C. Then the cells were allowed to recover for 60 min and then fixed (as described in Material and Methods). In case of unstimulated cells (A) the receptor is localized on the plasma membrane and stimulation with 100 µM ATP for 30 min (B) or 60 min (C) results in a complete endocytosis of the receptor. Irrespective of the initial duration of stimulation, a complete reappearance of the receptor at the plasma membrane is observed after 60 min of withdrawal of ATP (D and E). This indicates that the receptor reappeared on the plasma membrane after either 30 min (D) or 60 min of stimulation (E). Scale bar, 20 µm. Results shown are representative of at least three individual experiments.

3.11. Interaction of Diadenosine polyphosphates with $P2Y_2$ receptor

Diadenosine polyphosphates are an important class of molecules that play an important role physiological role both intracellularly and extracellularly in modulating cellular function. The current hypothesis is that the diadenosine polyphosphates exert their physiological actions via the P2 receptors or via a separate class of GPCRs. The theory for the presence of a separate class of receptors that are activated by the diadenosine polyphosphates is further supported by the fact that a residual cellular response to nucleotides is observed in cells that were pre-treated with P2 receptor antagonist. So we decided to investigate the role of diadenosine polyphosphates in signaling via the $P2Y_2$ receptor using HEK 293 cells stably transfected with the $P2Y_2$-GFP receptor. We investigated the role of these substances in their ability to stimulate an increase in intracellular calcium and possibly induce endocytosis of the receptor. We also investigated their role in modulating the targeting of the endocytosed receptor and receptor mediated cell proliferation as parameters to study the role of modulation of $P2Y_2$ receptor function by diadenosine polyphosphates.

3.11.1 Effect of diadenosine polyphosphates in stimulating a rise in $[Ca^{2+}]_i$

Changes in intracellular calcium were measured using the protocol that was used for confirming the functional expression of the $P2Y_2$-GFP receptor. The role of different diadenosine polyphosphates, namely $Ap_{2,3,4,5}A$, were investigated in their ability to influence the intracellular calcium levels.

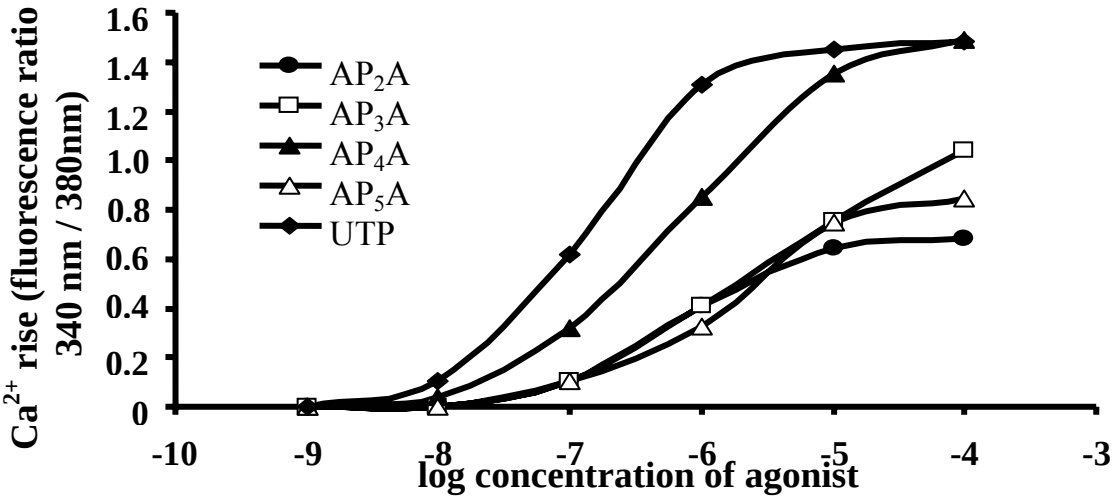


Figure 3.26: Concentration–response curves for Ca^{2+} rise induced by diadenosine polyphosphates (Ap_nAs) in HEK 293 cells expressing the $P2Y_2$-GFP receptor.
HEK 293 cells stably expressing the $rP2Y_2$-GFP receptor were preloaded with Fura-2/AM and then superfused with NaHBSS containing Ca^{2+} and stimulated for 1 min with the different concentrations of agonists. The concentration-dependent rise in Ca^{2+} is measured as the ratio between the fluorescence obtained at 340 nm and 380 nm upon stimulation with different concentrations of Ap_2A (closed circles), Ap_3A (open squares), Ap_4A (closed triangles), Ap_5A (open triangles) and UTP (closed diamonds). The data are from Ca^{2+} recordings in single cells and represent the mean ± s.d. from at least 50 cells for each value.

The dose response curve for the Ca^{2+} responses that was obtained on stimulating the HEK 293 cells stably expressing the $P2Y_2$-GFP receptor with different concentrations of diadenosine polyphosphates is shown in Fig. 3.26. We can observe from the dose response curve that the diadenosine polyphosphates exhibit different potencies in elevating the $[Ca^{2+}]_i$. The order of potencies that we observe from the dose response curves is UTP >Ap_4A>Ap_3A≥Ap_5A>Ap_2A. This indicates that in the family of diadenosine polyphosphates Ap_4A is the most potent in activating the $P2Y_2$ receptor. Since we observed this graded potency among the diadenosines in elevating the intracellular calcium we decided to investigate their ability to induce endocytosis of the $P2Y_2$ receptor and to compare the trafficking of endocytosed receptor after stimulating the cells with UTP.

3.11.2 Endocytosis and trafficking of the $P2Y_2$ receptor by diadenosine polyphosphates

The endocytosis experiments were carried on cells, that were maintained in complete growth medium at 37^0C in a humidified atmosphere of 5% CO_2. The cells were stimulated with 100 µM diadenosine polyphosphates for 30 min and the endocytosis of the receptor was observed. We quantified the percentage of endocytosis of the receptor. We observed that after 30 min of exposure to 100 µM diadenosine polyphosphates endocytosis of the receptor is observed. There was a minimal endocytosis of the receptor in case of cells stimulated with 100 µM Ap_2A for 30min (Fig.3.27 A). In case of cells exposed to 100 µM $Ap_{3,4,5}A$ endocytosis of the receptor was observed to different extents (Fig. 3.27 B,C and D).

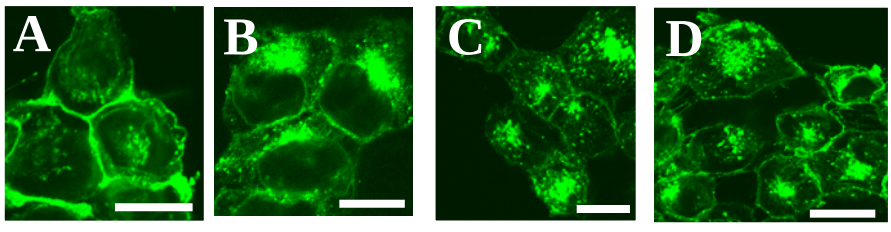


Figure 3.27: Visualization of $P2Y_2$-GFP receptor endocytosis induced by diadenosine polyphosphates. HEK 293 cells stably expressing $P2Y_2$-GFP receptor were stimulated with 100 μM of agonist and were visualized on stage for 30 min at 37^0C (A–D). The localization of the receptor was observed after 30 min of stimulation of HEK 293 cells expressing $P2Y_2$-GFP receptor that were stimulated with 100 μM of (A) Ap_2A, (B) Ap_3A, (C) Ap_4A and (D) Ap_5A.

We quantified the percentage of endocytosed receptor (Fig. 3.28) by measuring the loss of fluorescence intensity on the plasma membrane. The values of endocytosis, that were obtained were compared to the percentage of endocytosis of the receptor induced by 100 μM UTP. The most selective and potent agonist of the $P2Y_2$ receptor was UTP and among the diadenosine polyphosphastes the order of potency was Ap_4A > Ap_5A>Ap_3A>>Ap_2A. It is observed that the order of potency exhibited by the diadenosine polyphosphates is different for the order of potency exhibited in terms of the Ca^{2+} response. The difference in potency that is send between Ap5A and Ap3A at the level of endocytosis is not statistically significant and so it ca be considered that the diadenosie polyphosphates pretty much exhibit a similar order of potency both at the level of Ca^{2+} signaling and promoting endocytosis of the receptor.

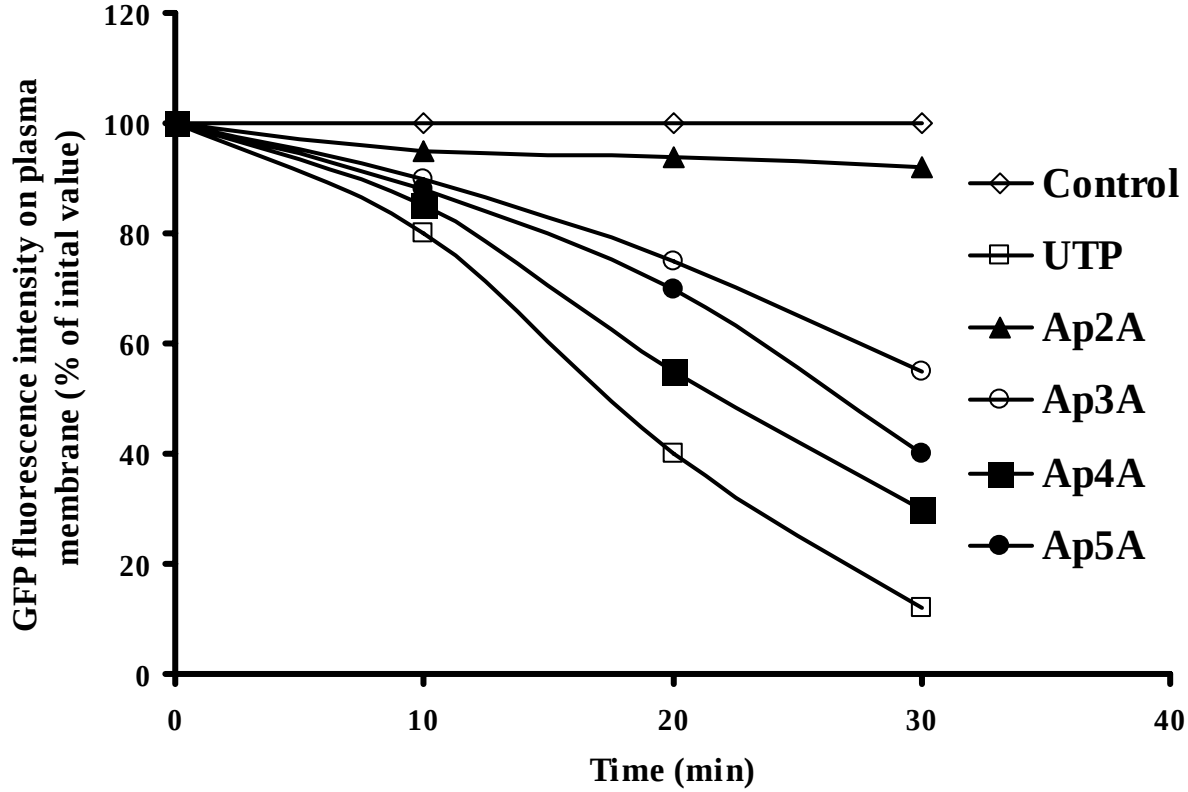


Figure 3.28: Quantification of fluorescence intensity in HEK 293 cells stably expressing $rP2Y_2$-GFP receptor. HEK 293 cells stably expressing $P2Y_2$-GFP receptor were stimulated with agonist for 30 min at 37^0C. The translocation of the receptor was observed using CLSM, and the change in the fluorescence quantitated in user-defined

regions of interest (ROI) that encompass the cytoplasmic regions of the same cell, using Zeiss software. The increase of fluorescence intensities in the cytosol were used as a measure of the endocytosis of the $P2Y_2$-GFP receptor. The changes in the fluorescence intensity after stimulation of the cells with 100 µM of UTP (open squares), Ap_2A (closed triangles), Ap_3A (open circles), Ap_4A (closed squares) and Ap_5A (closed circles). The data represent the mean ± s.d. from at least 30 cells.

We further investigated if the endocytosed receptor would co-localize with the lysosomes at the end of 60 min. as observed in the case of cells that were treated with 100 µM UTP. Cells pre-loaded with 100 nM lysotracker (Fig. 3.29A) were stimulated with 100 µM of the different diadenosine nucleotides and observed under conditions as described above. It was observed that in case of Ap_4A, 60 min after stimulation there was complete endocytosis of the receptor (Fig.3.29B) but after 80 min of continual presence of agonist the endocytosed receptor was colocalized with lysosomes (Fig. 3.29C) (bold arrows).

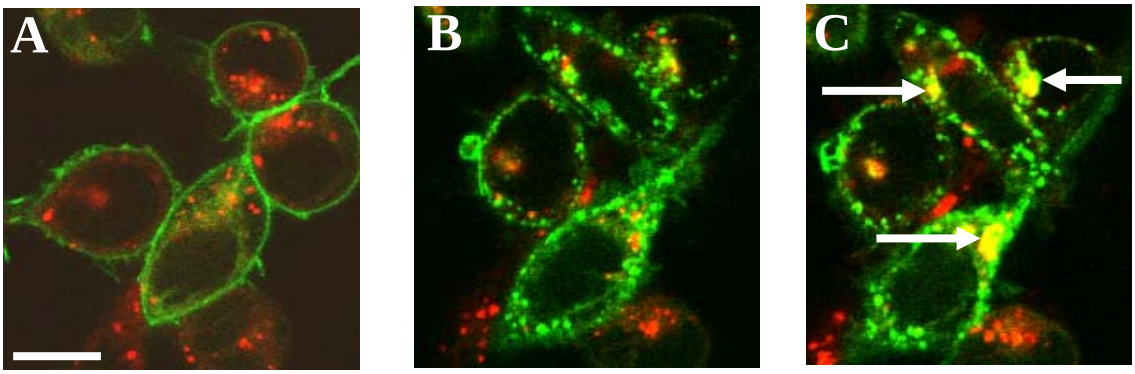


Figure 3.29: Modulation of endocytosis of $P2Y_2$-GFP receptor by diadenosine tetraphosphate. HEK-293 cells expressing the $rP2Y_2$-GFP receptor (green) were preincubated with 100 nM LysoTracker (red) for 30 min at 37 °C (*A*), then cells were incubated with 100 µM Ap_4A, and images were collected. It is observed after 60 min of stimulation (B) complete endocytosis of the receptor was observed but no colocalization of the endocytosed receptor with lysosomes. Further presence of Ap_4A resulted in colocalization of the endocytosed receptor with lysosomes (bold arrows) after 80 min (C).

The data regarding the duration required for the endocytosed receptor to be transported to the lysosomes on stimulation with the other diadenosine polyphosphates is indicated in Table 3.1.

Table 3.1: Localization of the endocytosed $P2Y_2$-GFP receptor on stimulation with 100 μM of diadenosine polyphosphate. HEK 293 stably expressing the $P2Y_2$-GFP receptor were preincubated with lysotracker and stimulated with 100 μM of diadenosine polyphosphates until a localization of the endocytosed receptor was observed with the lysosomes. The data indicated here are from a minimum of 4 experiments.

S.No	100 μM agonist	Time for colocalization of endocytosed $P2Y_2$ receptor with lysosomes
1	UTP	60 min
2	Ap_3A	120 min
3	Ap_4A	80 min
4	Ap_5A	100 min

Thus from the above data we can infer that though the diadaenosine polyphosphates do induce endocytosis of the $P2Y_2$ receptor, they stimulate different pathways that differently traffic the receptor in comparison to UTP. This could be one of the mechanisms in addition to the different Ca^{2+} response that is observed on exposing the cells to the diadenosine polyphosphates. This could serve as a cue to the cell to respond specifically in a modified manner to diadenosine polyphosphates in comparison to nucleotide triphosphates.

3.12 Role of nucleotides and diadenosine polyphosphates in modulation of proliferation of HEK 293 cells

HEK 293 cells stably expressing the $rP2Y_2$-GFP receptor were stimulated with different concentrations of UTP, ATP and Ap_4A for 16 h. The effect of the agonist on modulation of proliferation was monitored by detecting the changes in the amount of BrdU, that was incorporated into the cells. BrdU incorporation in the cells was detected using the ELISA kit from Roche (as described in Material and Methods). It is observed that in the case of stimulation with different concentrations of UTP, ATP and Ap_4A, there is a dose dependent increase in the BrdU incorporation (Fig. 3.30). ATP and UTP

are equipotent in terms of stimulation of proliferation in the stably transfected HEK 293 cells

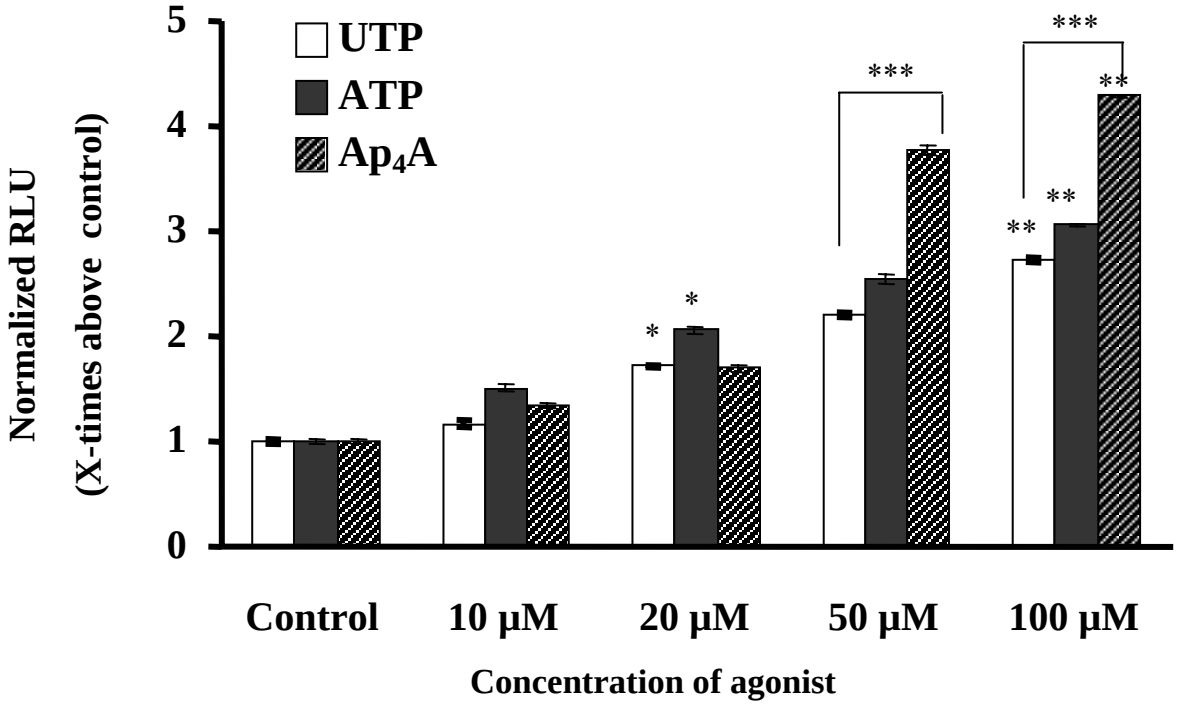


Figure 3.30: Modulation of proliferation of HEK 293 cells stably expressing rP2Y$_2$-GFP receptor with different agonist. 10–100 µM ATP or UTP or Ap$_4$A was added to proliferating HEK 293 cells, and bromodeoxyuridine (BrdU) incorporation was measured after 16 h. Values are means ± SD of 4–6 experiments run in triplicate. The statistical significance levels between the stimulated and control cells are * P <0.5, ** P < 0.001 and between UTP and Ap$_4$A is *** P <0.005

A 4-fold increase in the BrdU incorporation was observed in the Ap$_4$A stimulated HEK 293 cells stably expressing the rP2Y$_2$-GFP receptor. The effect of other diadenosine polyphosphates in modulating proliferation on these cells was also investigated using the BrdU incorporation method and the order of potency that we obtained was Ap$_4$A>Ap$_5$A≥Ap$_3$A>>Ap$_2$A. It is known from literature that diadaenosine polyphosphates do undergo hydrolysis to produce ATP or ADP (Vollmayer et. al.,2003) Thus to confirm that the increase in the BrdU was primarily due to activation of the cells by diadenosine polyphosphates or due to subsequent hydrolysis to ATP or ADP we preincubated the cells for 1 hr with 100 µM α,β-MeADP, a potent inhibitor of

ectonucleotidases before stimulation of the cells with diadenosine polyphosphates in the continued presence of the inhibitor. The results are summarized in Table 3.2.

S.No.	Agonist	Stimulation of proliferation (X-times above control) (mean ±S.D.)
1	100 μM α,β-Me-ADP	1.19 ± 0.014
2	100 μM Ap_3A	4.03 ± 0.032
3	100 μM α,β-Me-ADP + 100 μM Ap_3A	3.88 ± 0.046
4	100 μM Ap4A	4.82 ± 0.051
5	100 μM α,β-Me-ADP + 100 μM Ap_4A	4.54 ± 0.023
6	100 μM Ap_5A	3.17 ± 0.041
7	100 μM α,β-Me-ADP + 100 μM Ap_5A	4.64 ± 0.064

Table 3.2: Effect of diadenosine polyphosphates on BrdU incorporation in the presence of α,β-Me-ADP. HEK 293 cells stably expressing the $P2Y_2$-GFP receptor were pre-incubated for 1 h with 100 μM of α,β-Me-ADP and then stimulated with 100 μM of different diadenosine polyphosphates, and the bromodeoxyuridine (BrdU) incorporation was measured after 16 h. Values are mean ± SD n= 4–6 experiments run in triplicate.

Thus we observe that even in the presence of α,β Me-ADP the diadenosine polyphosphates did result in a higher BrdU incorporation than that observed when the cells were either stimulated with ATP or UTP. This indicates that the diadenosine polyphosphates do stimulate proliferation in HEK 293 cells via a signalling pathway that is different from that adopted by ATP or UTP. Thus taking into account the above sets of parameters namely modulation of intracellular Ca^{2+} levels, promoting endocytosis of the $P2Y_2$ receptor, differential trafficking of the endocytosed receptor and finally their effect on modulating proliferation in HEK 293 celss we can infer that the diadenosine polyphosphates do signal to a large extent via the purinergic receptors. The signalling that is exhibited by them is however different form the classical agonist of the $P2Y_2$ receptor namely UTP and ATP.

4. DISCUSSION:

P2Y receptors constitute an important family of metabotropic receptors through which extracellular nucleotides exert their effects. The role of extracellular nucleotides as signaling molecules was realized in 1929, from the work of Drury and Szent-Györgyi who published the first report of an extracellular action of adenine compounds. It was, however, not until the early 1970s that their role as extracellular messengers was demonstrated (Burnstock et. al., 1972). Finally, in 1993 the first P2Y receptors were cloned (Lustig et. al., 1993; Webb et. al., 1993). Currently the P2Y receptor family consists of 8 functionally characterized receptor subtypes namely $P2Y_{1,2,4,6,11,12,13,14}$ (Abbracchio et al., 2003) that are expressed in humans. The $P2Y_2$ receptor is a unique member of this receptor family as it responds equipotently to both UTP and ATP (Abbracchio et al., 2003). The $P2Y_2$ receptor has been the protein of therapeutic interest in this family as it has been implicated to play a role in modulation of transport of Cl^- ions and thus thought to be a potential target in treatment of cystic fibrosis. The modified agonists of the $P2Y_2$ receptor diquafosol (diuridine tetraphosphate) and denufosol (uracil-cytosine dinucleotide) are currently undergoing clinical trials for is use in treating dry eye disease (Brunschweiger & Müller, 2006). Thus, it is important to understand the mechanisms that regulate the trafficking of the $P2Y_2$ receptor. It is also crucial for understanding the proteins that modulate the trafficking of this receptor and the possible downstream proteins that are activated during the trafficking of the receptor.

4.1 Functional Expression of the tagged $rP2Y_2$ receptor in HEK 293 cells

In the HEK 293 cell system we were able to demonstrate a functional integration and correct localization of the $rP2Y_2$ receptor tagged with either a GFP or a myc-his tag at the C-terminus (as seen for example in Fig. 3.1). This can be concluded from the following experimental results. Since the expression pattern of the tagged $rP2Y_2$ was similar to that of the endogenously expressed $hP2Y_2$ receptor, we verified the functional expression of the receptor by measuring the calcium response obtained in the stably expressing cell on treating them with different concentrations of either ATP or UTP. We observed that there was an increase in sensitivity of the transfected cells to both ATP and UTP. The transfected cells exhibited an equipotent Ca^{2+} response to both ATP

and UTP independent of the tag attached to the $P2Y_2$ receptor (as demonstrated in Fig. 3.2A and B). This confirmed that there is functional expression of the $P2Y_2$ receptor in the HEK 293 cells. We used the HEK 293 cells as an heterologous expression system as it has been demonstrated that the HEK 293 cells do endogenously express the $P2Y_2$ receptor (Schäfer et. al., 2002). The presence of the normal signaling machinery that is required for the $P2Y_2$ receptor in HEK 293 cells due to the endogenous expression of the $hP2Y_2$ receptor make HEK 293 cells an ideal heterologous system for studying the trafficking of the $P2Y_2$ receptor. Thus, from the above two results we can infer that we have functional expression of the $rP2Y_2$ with either the GFP tag or the myc-his tag.

We had used this system successfully to study the internalization of the $rP2Y_2$-GFP receptor by novel adenosine 5'-O-(1-boranotriphosphate) derivatives (Tulapurkar et al., 2004), where we found that the rank order of potency of these substances in eliciting an increase in $[Ca^{2+}]_i$ was also apparent for the endocytosis induced by these substances. We therefore proceeded to study the trafficking of the $rP2Y_2$ receptor in this functional system. Stimulation of the cells expressing $rP2Y_2$-GFP receptor with 100 μM UTP, which is an agonist only for the $P2Y_2$ receptor induced internalization of the receptor (as seen in Fig. 3.3D). A similar internalization of the receptor was also observed in case of the myc-his tagged receptor. This indicates that the presence of either of the tags did not affect the endocytotic kinetics of the receptor. Thus, in all following studies we performed endocytosis experiments with the GFP tagged $P2Y_2$ receptor so as to aid in live cell visualization and observe the translocation kinetics of the receptor in the given cell population.

4.2 Endocytosis pathways of $P2Y_2$ receptor and involved proteins

One of the molecular players in receptor endocytosis is actin, which is part of the cytoskeleton that undergoes a massive reorganization to enhance endocytosis (Lamaze et. al., 1997). The role of the actin cytoskeleton and clathrin in modulating the endocytosis of GPCRs and specifically P2Y receptors has been described (Ferguson, 2001, Baurand et. al., 2005; Tulapurkar et. al, 2005). Clathrin-mediated endocytosis of many GPCRs is coupled to β-arrestin and GRKs (Reiter & Lefkowitz, 2006). We did not see actin stress fibers in unstimulated HEK-293 cells expressing the $rP2Y_2$-GFP receptor (Fig 3.6A2) , which is consistent with a normal distribution of the actin cytoskeleton in unstimulated HEK-293 cells (Giancotti and Ruoslahti 1999). In these

cells we studied rP2Y_2 receptor internalization. Our data indicate that in unstimulated HEK-293 cells, actin and the P2Y_2-GFP receptor are colocalized in the plasma membrane, most likely in a complex involving extracellular matrix binding proteins and integrins. Colocalization of actin and the P2Y_2-GFP receptor decreased after stimulation of cells with UTP, indicating that actin depolymerization precedes the endocytosis of the receptor and facilitates its endocytosis (Fig 3.6B3). Changes in the distribution of the actin cytoskeleton are necessary for endocytosis, since endocytotic vesicles have been shown to have an actin tail that helps in their propulsion (Qualmann et. al., 2000). In rP2Y_2-GFP receptor-expressing cells, there was complete reappearance of actin at the plasma membrane after 60 min of UTP stimulation (Fig 3.6E3). These observations strongly suggest that actin plays a role in the internalization of the P2Y_2 receptor. To confirm the role of actin in P2Y2-GFP receptor endocytosis, we demonstrated the inhibitory effects of Cyto-D, an actin-disrupting agent (Sergeeva et. al., 2000), and ML-9, a myosin light chain kinase inhibitor (Kawamura et. al., 2003). In astrocyte cultures, incubation of the cells with 1 μM Cyto-D for 3 h also decreased ATP-induced Ca^{2+} mobilization (Sergeeva et. al., 2000). Preincubation of the rP2Y_2-GFP receptor-transfected HEK-293 cells with a low inhibitor concentration (100 nM) for 30 min did not affect cell morphology. A clear inhibition of receptor endocytosis was observed 30 min after stimulation of the cells with UTP. In the presence of Cyto-D, the activated receptors were not internalized, but exhibited a vesicular appearance in the plasma membrane, probably due to the absence of nucleotide-induced actin depolymerization (Fig. 3.7A2). However, after 60 min of UTP stimulation there was internalization of the P2Y_2-GFP receptor in small vesicles (Fig. 3.7A3) despite of the presence of Cyto-D, suggesting that there are proteins other than actin which play a role in P2Y_2 receptor endocytosis.

Actin remodeling is rapid and is involved in processes like cell shape change, cell movement and transport of vesicles in the cytoplasm. Myosin, an enzyme that hydrolyzes ATP, aids in actin remodeling. Myosin light-chain kinase (MLCK) phosphorylates myosin and regulates its activities. ML-9 inhibited the internalization of the P2Y_2-GFP receptor after 30 min of UTP stimulation (Fig.3.8A2), indicating that MLCK plays an important role in remodeling the cytoskeleton. On further stimulation of the cells for 60 min, an inhibition in targeting of the endocytosed receptor to the lysososmes was also observed (Fig. 3.8A3). These facts indicate that a normal remodeling of the cytoskeleton plays an important role in the formation of the

endocytotic complex at the plasma membrane which further influences the targeting of the endocytosed receptor.

Endocytosis of receptors can take place via different pathways. The two most commonly known routes of endocytosis are via clathrin or caveolin (Sadir et. al., 2001). The clathrin dependent pathway involves the recruitment of arrestin and adapter protein-2 to form an internalization vesicle. In our study, $P2Y_2$ receptor internalization was inhibited in the presence of 0.45 M sucrose, indicating that internalization of the receptor occurs via the clathrin-mediated pathway that is sensitive to the density of the medium (McArdle et. al., 2002). Furthermore, $P2Y_2$-GFP receptor endocytosis in HEK-293 cells was prevented by chlorpromazine, an inhibitor of the clathrin-dependent pathway (Fig 3.10A2) (Petris et. al, 2003). This confirms the role for clathrin in the receptor internalization. Another possible pathway for endocytosis in HEK-293 cells is caveolin mediated. Cholera toxin subunit B is known to be endocytosed exclusively by the caveolin- mediated pathway (Orlandi and Fishman 1998). Stimulation of $P2Y_2$-GFP receptor-expressing cells with 100 μM UTP in the presence of cholera toxin subunit B and chlorpromazine revealed an absence of internalization of both the $P2Y_2$-GFP receptor and cholera toxin, suggesting that the caveolin mediated pathway cannot be activated by $P2Y_2$ receptors in HEK-293 cells (Fig 3.10C3). The lack of a contribution of the caveolin pathway was confirmed by the lack of effectiveness of filipin III, a known inhibitor of the caveolin-dependent process (Fig 3.12A3) (Orlandi and Fishman 1998). Thus, endocytosis of the $P2Y_2$ receptor clearly proceeds via a clathrin-mediated pathway. The role of plasma membrane lipids in the endocytosis of the $P2Y_2$ receptor was also investigated. These lipids are normally associated with the caveolin-mediated endocytosis pathway (Rodal et.al., 1999). However, pretreatment of the cells with MβCD resulted in delayed endocytosis of the receptor and a punctate distribution of the endocytosed receptor (Fig 3.11A3). These facts indicate that although the endocytosis of the receptor proceeds via the clathrin-mediated pathway, the plasma membrane cholesterol content does have an influence on this pathway and that there could be lipids involved in the clathrin-mediated pathway. The content of plasma membrane cholesterol does have an influence on actin reorganization in the cell, indicating that treatment of the cells with MβCD not only affects the cholesterol content but also alters the reorganization of actin cytoskeleton. This effect is revealed by the punctate

distribution of the endocytosed receptor, a pattern which resembles the distribution of the receptor observed after treatment of the cells with Cyto-D.

The role of the actin cytoskeleton and clathrin in modulating the endocytosis of GPCRs and specifically P2Y receptors has been described (Fergusson, 2001, Baurand et. al., 2005; Tulapurkar et. al, 2005). Clathrin-mediated endocytosis of many GPCRs is coupled to β-arrestin and GRKs (Reiter & Lefkowitz, 2006). Before endocytosis begins the invaginated membrane has to be pinched off from the plasma membrane. This process is mainly mediated by dynamin (Ferguson , 2001). In addition to dynamin, PLD has been postulated to play a role in modulating endocytosis of GPCRs (Shen et al., 2001). Especially PLD_2 has been found to regulate the endocytosis of GPCRs like the μ-opioid receptor (Koch et. al., 2004) and the transferrin receptor (Padrón et al., 2006). Moreover, PLD_2 has been found to play a role not only in the agonist-dependent, but also in the agonist-independent endocytosis of the μ-opioid receptor (Koch et al., 2006). Subsequent stimulation with 100 μM UTP for 30 min prevented the internalisation of the receptor (Fig. 3.22B). The different trafficking of the $P2Y_2$ receptor in cells where the PLD was inhibited indicates that PLD has a prominent in the early steps of internalisation and for further correct processing of the endocytosed receptor. However, future investigations have to explore the mechanism and the subtype of PLD involved. The possibility that beta arrestin might not be involved in the endocytosis of the P2Y receptors in HEK 293 cells has been postulated (Mundell and Benovic , 2000). Studies on platelets by Mundell (Mundell et al., 2006) revealed that the $P2Y_1$ receptor endocytosis is not dependent on beta–arrestin, whereas $P2Y_{12}$ endocytosis is. Our own studies on HEK293 cells stably expressing the $P2Y_2$-GFP receptor did also show no dependence of $P2Y_2$ endocytosis on beta-arrestin (data not shown). These observations indicate that, although internalization of the $P2Y_1$ and $P2Y_2$ receptor is mediated by clathrin, there seems to be a different or additional mechanism, which might not be linked to beta-arrestin, as has been found for most other GPCRs endocytosed by clathrin (Reiter & Lefkowitz, 2006).

4.3 Trafficking and compartmentalization of the $rP2Y_2$ receptor

The trafficking of the $P2Y_2$ receptor to different intracellular compartments determines the signals that are transmitted and the subsequent response of the cell to external stimuli. After chronic stimulation with agonist, the time required for receptor

resensitization is greater than after an acute stimulus. This effect might be due to differential targeting of the receptor. We observed that after 30 min of UTP stimulation there was targeting of the internalized $P2Y_2$-GFP receptor to early endosomes (Fig. 3.4A3) and upon prolonged stimulation there was colocalization of the receptor with lysosomes (Fig. 3.4A6). Thus, different receptor trafficking pathways are activated, depending on the duration of stimulation with agonist. The localization of the $P2Y_2$ receptor in early endosomes after a 30-min UTP stimulation indicated the possibility that the receptor could be recycled back to the plasma membrane. In contrast, stimulation of the cells for 60 min with the same concentration of UTP resulted in receptors targeting to lysosomes, where they would be degraded, requiring new receptor synthesis for resensitization. These observations could explain the differences in time necessary for resensitization of the cells and reappearance of the receptor at the plasma membrane after different lengths of receptor stimulation. The slower resensitization of the receptors stimulated for 60 min could also be attributable to the time required for transport of presynthesized receptors from the Golgi to the plasma membrane. Importantly, these variations in resensitization rate with duration of agonist stimulation are not due to the modification of $P2Y_2$-GFP receptors, since a similar dependency for receptor resensitization has been observed for endogenously expressed $P2Y_2$ receptors in human U937 cells (Santiago-Perez et. al., 2001).

Receptor endocytosis is an important process by which extracellular signals regulate cellular functions. Regulation by receptor endocytosis has been observed for the epidermal growth factor receptor that signals from endosomes to promote cell survival (Wang et. al., 2002). Trafficking of receptors from endosomes to lysosomes requires the presence of associated proteins, like Rabs, whereas the farnesylation of RhoB is necessary for targeting to early endosomes (Prendergast 2001). Incubation of $P2Y_2$-GFP receptor-expressing cells with the farnesyltransferase inhibitor FTI-277 did not affect UTP-induced internalization of the receptor, but due to the absence of farnesylation of the early endosomal coat proteins, the endocytosed receptor was not further trafficked to lysosomes (Fig.3.5A3). Thus, the absence of the appropriate vesicle coat proteins led to mistrafficking of the $P2Y_2$-GFP receptor. FTI-277 can affect the reorganization of actin via the inactivation of Ras, thus affecting the initial step of endocytosis, namely the reorganization of the cellular cytoskeleton (Lesh et. al., 2001). Trafficking of receptors from the early endosomes to other compartments, such as late endosomes, lysosomes, or recycling endosomes, is directed via the multivesicular body

(Miwako et. al., 2001; Sorkin and Von Zastrow, 2002). The proteasome-mediated degradation of GPCRs is one of the methods for termination of receptor signaling (Kerkhof and Strous 2001). For the first time, we give here indication for a proteasomal dependent degradation pathway for $P2Y_2$ receptor trafficking, as indicated by the blockage of receptor targeting to lysosomes by the proteasomal inhibitor MG-132 (Fig. 3.13E). The complete pathway that we have investigate is summarized below in Figure 4.1.

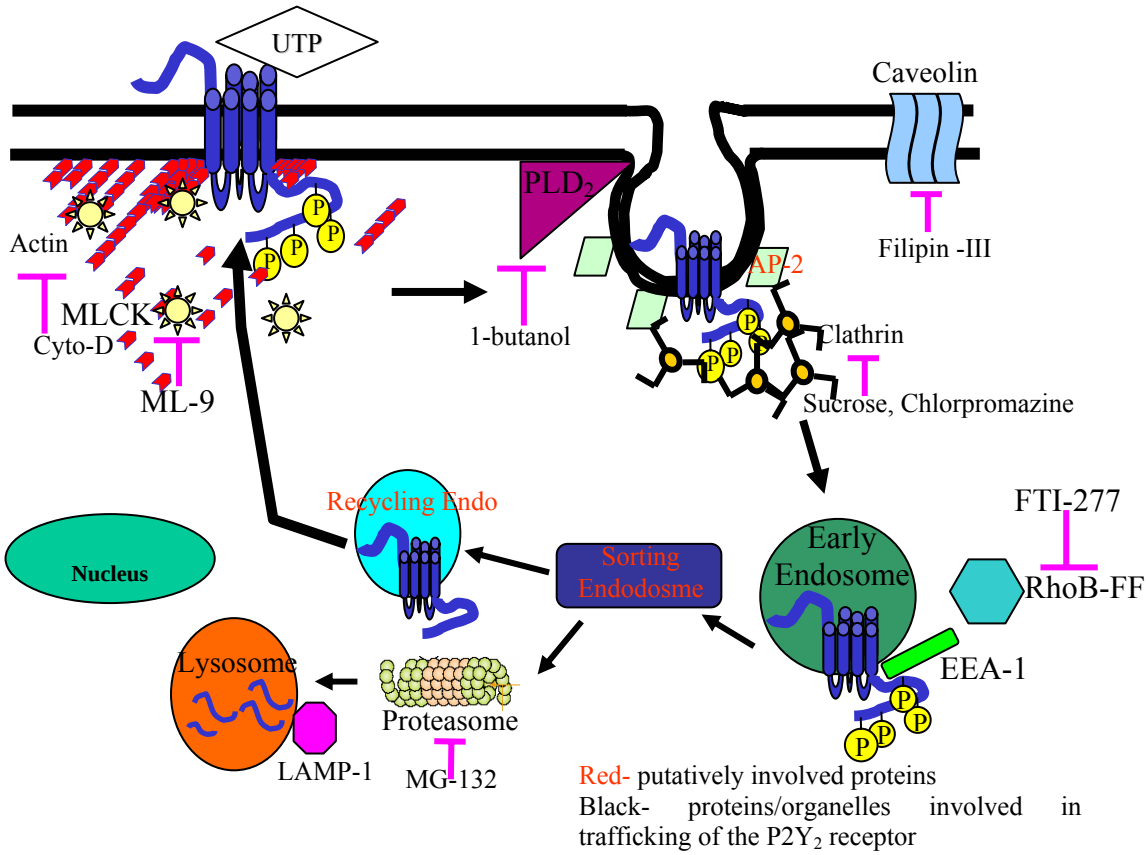


Figure 4.1: Endocytotic pathway for the $P2Y_2$ receptor. The rat $P2Y_2$ receptor tagged with GFP was stably expressed in HEK 293 cells. Activation of the $P2Y_2$ receptor with UTP results in the remodeling of the actin cytoskeleton followed by endocytosis of the receptor via clathrin coated pits. The endocytosed receptor is trafficked from the early endosomes to the lysosomes via a proteasome dependent pathway.An inhibition of this pathway results in recycling of the receptor back to the plasma membrane.

4.4 Regulation of $P2Y_2$ receptor internalization and trafficking by protein kinases and phosphatase PP2A

Our pharmacological investigation of the contribution of the protein kinases PKC, CaMKII, RhoA, PI3K, and MEK revealed that these kinases very likely regulate

the endocytosis of the $P2Y_2$ receptor at different levels of endocytosis or resensitization. Phosphorylation of the $P2Y_2$ receptor by CaMKII seems to be necessary for the correct processing of the receptor as pre-incubation of the cells with KN-62, results in a initial inhibiton of the endocytosis of the receptor. (Fig. 3.14B).This is similar to the observation obtained for the $P2Y_1$ receptor (Tulapurkar et. al., 2006). Prolonged stimulation of the cells results in the internalization of the receptor and probable localization to lysosomes. Localization of the endocytosed $P2Y_2$ receptor to lysosomes as well as the trafficking back to the plasma membrane is controlled by the Ca^{2+}-dependent PKCs isoforms α- and βI,βII. Interestingly, incubation of the cells with the broad spectrum PKC inhibitor GF 109203X, which inhibits PKCα,βI,βII,γ,δ and ε, does not interfere with the localization of the endocytosed receptor to lysosomes, but leads to a small delay in endocytosis. This obvious discrepancy in the effect compared to GO 6976 (Fig.3.16C) can be explained by the fact, that GF 109203X is a weaker inhibitor of the Ca^{2+}-dependent PKCs than GO6976 (Bartlett et al., 2005; Davis et al., 2000), which can also be seen in its lower inhibition of intracellular Ca^{2+} increase. The slight delay of the endocytosis by GF 109203X suggests that Ca^{2+}- independent PKCs may regulate the correct endocytosis of the receptor (Fig. 3.17C). In contrast to the Ca^{2+}-dependent or -independent PKCs, the atypical PKCs ζ and δ seem to be involved in controlling the trafficking of the receptor to the lysosomes (Fig.3.18C) as pre-treatment of the cells with GO6983 hastens the localization of the endocytosed receptor with the lysosomes. Thus, the different PKCs seem to control distinct steps of endocytosis and reappearance of the $P2Y_2$ receptor.

The $P2Y_2$ receptor contains amino acid residues for phosphorylation by MAPK and PKCs similar to other GPCRs. It has been demonstrated that the phosphorylation of the receptor by PKC plays an important role in the desensitization of the receptor (Garrad et. al;. 1998, Mundell et al., 2006). However, the role of receptor phosphorylation by MAPK and its effect on endocytosis has not been investigated. Especially most recent publications suggested a possible role of endocytosis in the proliferation signaling (Gobeil et al., 2006). We therefore investigated the role of MAPK in modulating the endocytosis of the $P2Y_2$ receptor by pre-incubation of the cells with 10 μM of U0126, a MEK inhibitor (Davies et. al., 2000). We observed that the pre-incubation of the cells with the MEK inhibitor did not affect the localization of the receptor. The different endocytosis pattern of the $P2Y_2$ receptor in pre-treated cells in the initial trafficking indicates an involvement of the phosphorylation by MAPKs in

the early phase of endocytosis (Fig.3.21B), but probably not the final destination (Fig. 3.21 C) and the recycling of the receptor. This is evident from the aggregates of the receptor (Fig. 3.21D) that are present after 90 min after the withdrawal of the agonist. This pattern of reappearance is similar to that seen in case of untreated cells (data not shown). Thus, we can infer that stimulation of the $P2Y_2$ receptor with UTP results in the activation of MAPK (Gao et. al., 1999, Elia et. al., 2005), which is responsible for further receptor signaling, but also for the proper internalization of the receptor. This is an interesting mechanism by which the signaling cascade initiated by the stimulated receptor, in addition to regulating other signaling pathways, regulates its own trafficking. This mechanism reveals a degree of fine tuning that is present at the level of cellular responses, as it is know that receptors can signal even after being endocytosed from the endosomes (Wang et. al., 2004).

In addition to the kinases that are capable of directly phosphorylating the $P2Y_2$ receptor, kinases that are involved in remodeling of the cytoskeleton have also been postulated to modulate the trafficking of GPCRs (Mueller and Strange, 2004).We observed that the kinetics of internalization of the receptor (Fig. 3.20B) exhibited a pattern that is similar to that exhibited by cytochalasin-D treated cells. The absence of co-localization of the endocytosed receptor with lysosomes (Fig.3.20C) is similar to that observed in the ML-9 treated cells. These facts indicate that the kinases that modulate rearrangement of the too play an important role in the proper endocytosis of the receptor and in its trafficking.

PI3K is a kinsae that has been implicated in modulating the endocytosis of GPCRs (Jo et. al., 2002) and also in the transport of vesicles (Merrifield, 2004). In case of the wortmannin pre-incubated cells the kinetics of endocytosis of the receptor were not affected but the endocytosed receptor was not trafficked to the lysosomes (Fig.3.19C) which then results in a prolonged delay in partial reappearance of the receptor. This is similar to the results obtained by Naga Prasad et. al., 2001 where they were able to show the involvement of the PI3K in the trafficking and sequestration of the β adrenergic receptor. Thus we can infer that the PI3K might modulate a variety of factors from its association with clathrin (Beck and Keen 1991) to vesicle transport which then finally influence the trafficking of the $P2Y_2$ receptor.

In contrast to the kinases, which control different steps in the endocytosis of the $P2Y_2$ receptor as well as the trafficking back to the plasma membrane, the phosphatase PP2A is only involved in the regulation of the reappearance of the $P2Y_2$ receptor at the

plasma membrane (Fig. 3.15C). An involvement of PP2A in the regulation of recycling of GPCRs has been described for the β-adrenergic receptor (Pippig et al., 1995) the parathyroid hormone receptor (Chauvin et al., 2002) and the $P2Y_1$ receptor too (Tulapurkar et. al., 2006).

The correct targeting of the $P2Y_2$ receptor to lysosomes as well as its recycling back to the plasma membrane is very likely regulated through the activity of PI3K. However, whether PI3K directly phosphorylates the $P2Y_2$ receptor and/or proteins involved in targeting to lysosomes or involved in the recycling of the receptor by Golgi vesicles is not known. This is also true for the action of CaMKII, PP2A and the PKCs. Furthermore, activation of RhoA kinase followed by phosphorylation of the actin cytoskeleton is necessary for the correct induction of $P2Y_2$ receptor endocytosis as well as activation of the MAPK pathway to guaranty the correct processing of the endocytosed vesicles. The role of the different kinases and protein phosphatase 2A is summarized in Figure 4.2.

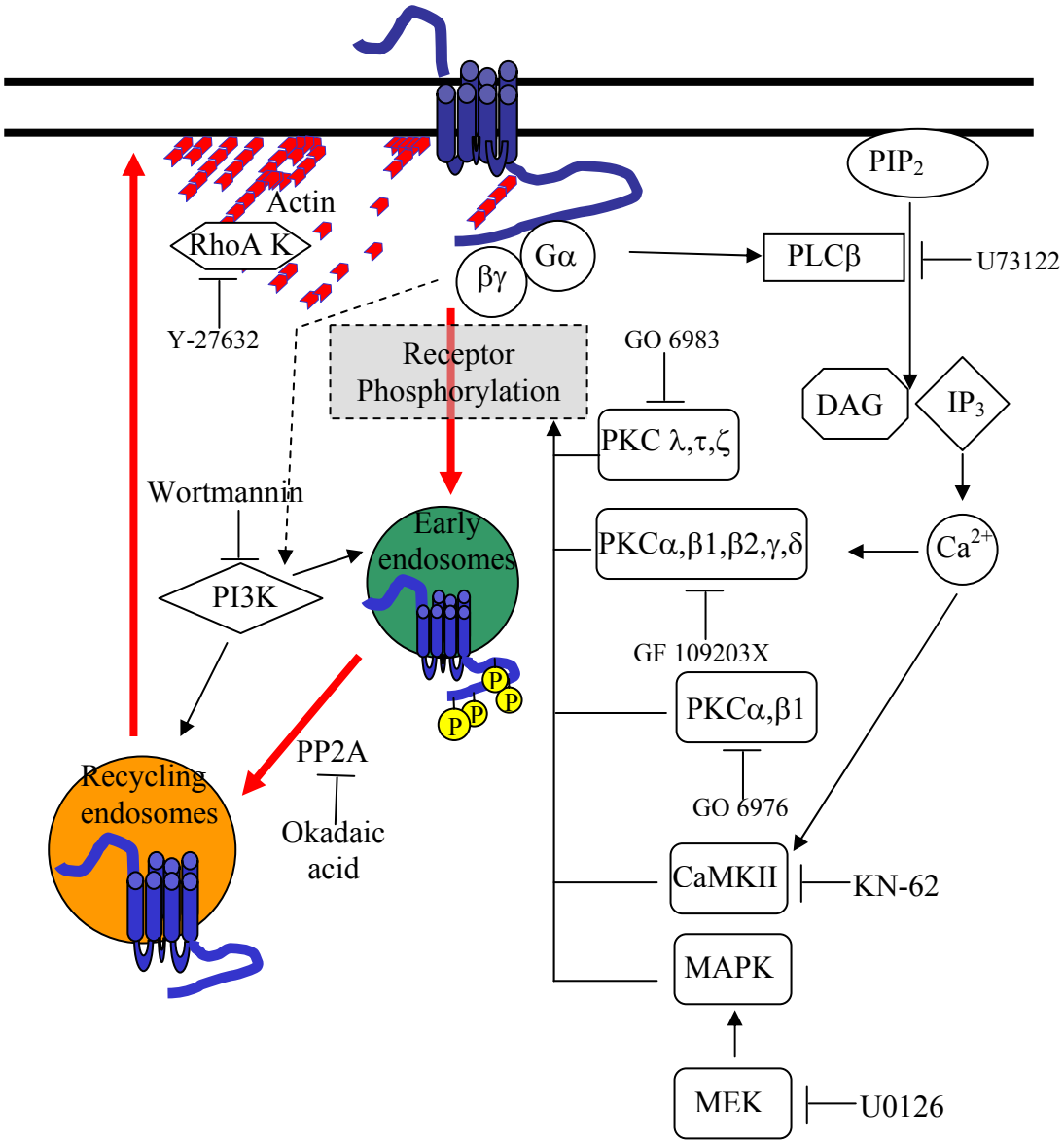


Figure 4.2: Role of protein kinases and protein phosphatase 2A in trafficking of the P2Y2 receptor. HEK 293 cells stably expressing the $P2Y_2$-GFP receptor were preincubated with inhibitors for different kinases and then stimulated with 100 μM UTP for 60 min. The cells were washed thrice with medium to remove agonist and further trafficking of the receptor was observed in the continual presence of the inhibitor

4.5. Different trafficking of $P2Y_2$ receptor

4.5.1 Trafficking by ATP and UTP

Prolonged stimulation of cells with agonist can lead to agonist-induced desensitization of the receptor and termination of downstream signals. Endocytosis is one of the pathways by which desensitization of a receptor can occur. Receptor internalization is a dynamic process that involves the modification and translocation of a number of proteins to the plasma membrane (Claing et. al., 2002). The mechanism of $P2Y_2$ receptor internalization, the trafficking of the receptor, and its compartmentalization after endocytosis have been investigated here.

We found a strong correlation between the concentration of the agonist and endocytosis of the $rP2Y_2$-GFP receptor (as seen in Fig. 3.3 E). Like UTP, stimulation of the cells with ATP exhibited a similar pattern of endocytosis of the $rP2Y_2$-GFP receptor. This indicates that both UTP and ATP are equipotent at the calcium level and do exhibit similar degrees of receptor endocytosis at a given concentration (Fig. 3.24 B and C). The time course of internalization of the human $P2Y_2$ receptor (Sromek and Harden, 1998) is similar to what we observe here with the $rP2Y_2$ receptor. Although both ATP and UTP induce complete internalization of the $P2Y_2$ receptor we observed a difference in the trafficking of the receptor when the cells were stimulated for longer time periods (60 min). Stimulation of cells for 30 or 60 min with 100 μM of ATP or UTP resulted in complete endocytosis of the receptor indicating trafficking of the receptor by the same endocytotic pathway. This is in agreement with our data where we could show that after 30 min of stimulation with 100 μM UTP there is a trafficking of the receptor to the early endosomes and that there was no colocalization of the receptor with lysosomes. However, in case of ATP-stimulated cells there seems to be a different pathway that is involved in the reappearance of the receptor. When the cells were allowed to recover for 60 min in the absence of agonist we observed a complete reappearance of the receptor in case of ATP-stimulated cells (Fig 3.24H) whereas only a partial reappearance in case of UTP-stimulated cells could be seen (Fig 3.24I). Here, most of the receptor remained inside the cell in vesicles, very likely lysosomes. This had been found in the case of UTP-mediated endocytosis of the receptor, where 60 min of stimulation with UTP leads to trafficking of the receptor via the proteasomal pathway to the lysosomes (Fig. 3.13B). In contrast, in ATP-stimulated cells, most of the receptors were recycled back to the plasma membrane. This is an interesting observation, as this seems to be one of the mechanisms by which the cells differentiate

in their physiological response between the stimulation of the receptor by ATP or UTP. This is one of the first differences that we were able to show between ATP and UTP, which were supposed to be equipotent agonists at the $P2Y_2$ receptor.

4.5.2. Trafficking by diadenosie polyphosphates

Diadenosine polyphosphates are the most ubiquitous members of the dinucleoside polyphosphate family. These are naturally occurring compounds that modulate a large number of physiological responses (Kisselev et. al., 1998). Till date it is not clear if there are specific receptors for the diadenosine polyphosphates (Pintor et. al., 2000) or act via the P2Y receptors (Laubinger et. al., 2003) we decided to investigate the effect of these substances in modulating the endocytosis of the $P2Y_2$ receptor. The different diadenosine polyphosphates did induce an increase in the intracellular calcium in the HEK 293 cells stably expressing the $rP2Y_2$-GFP receptor to different degrees and the order of potency being ATP=UTP ≥ Ap_4A >Ap_3A≥ Ap_5A >Ap_2A (Fig. 3.26). this was the first indication that the diadenosine polyphosphates signal via the P2Y2 receptor and exhibit different potencies at the same receptor. When the cells were stimulated for 30 min with 100μM of the different diadenosine polyphosphates the difference in potencies of these substances on stimulating the P2Y2 receptor became further clear. We observed that Ap_2A did not induce and endocytosis of the receptor (Fig. 3.27A), where Ap_4A and Ap_5A did induce a s significant amount of endocytosis of the receptor (Fig. 3.27 C and D).These results indicated that though all of the Ap_nAs were capable of causing an elevation of the intracellular calcium at 100 μM , they are not able to promote endocytosis of the $P2Y_2$ receptor. We further investigated the time required for the endocytosed receptor to be trafficked to the lysosomes on stimulation of the cells with the diadenosine polyphosphates. It was interesting to observe that though Ap_3A, Ap_4A and Ap_5A did induce complete endocytosis of the $P2Y_2$ receptor, the endocytosed receptor required different duration of time to co-localize with the lysosomes (Table 3.1). The order of potency in inducing endocytosis of the receptor and duration required to trafficked to the endocytosed receptor to the lysosome are similar to the potency exhibited by the Ap_nAs at the level of calcium signalling. Thus from the above results we can infer that the difference in the degree of endocytosis of the receptor and the duration required for the endocytosed receptor to be trafficked to the lysosomes could be specific signals used by the cell to distinguish

between the different ApnAs and between diadenosine polyphosphates and nucleotide triphosphates.

4.6 $P2Y_2$ receptor and proliferation

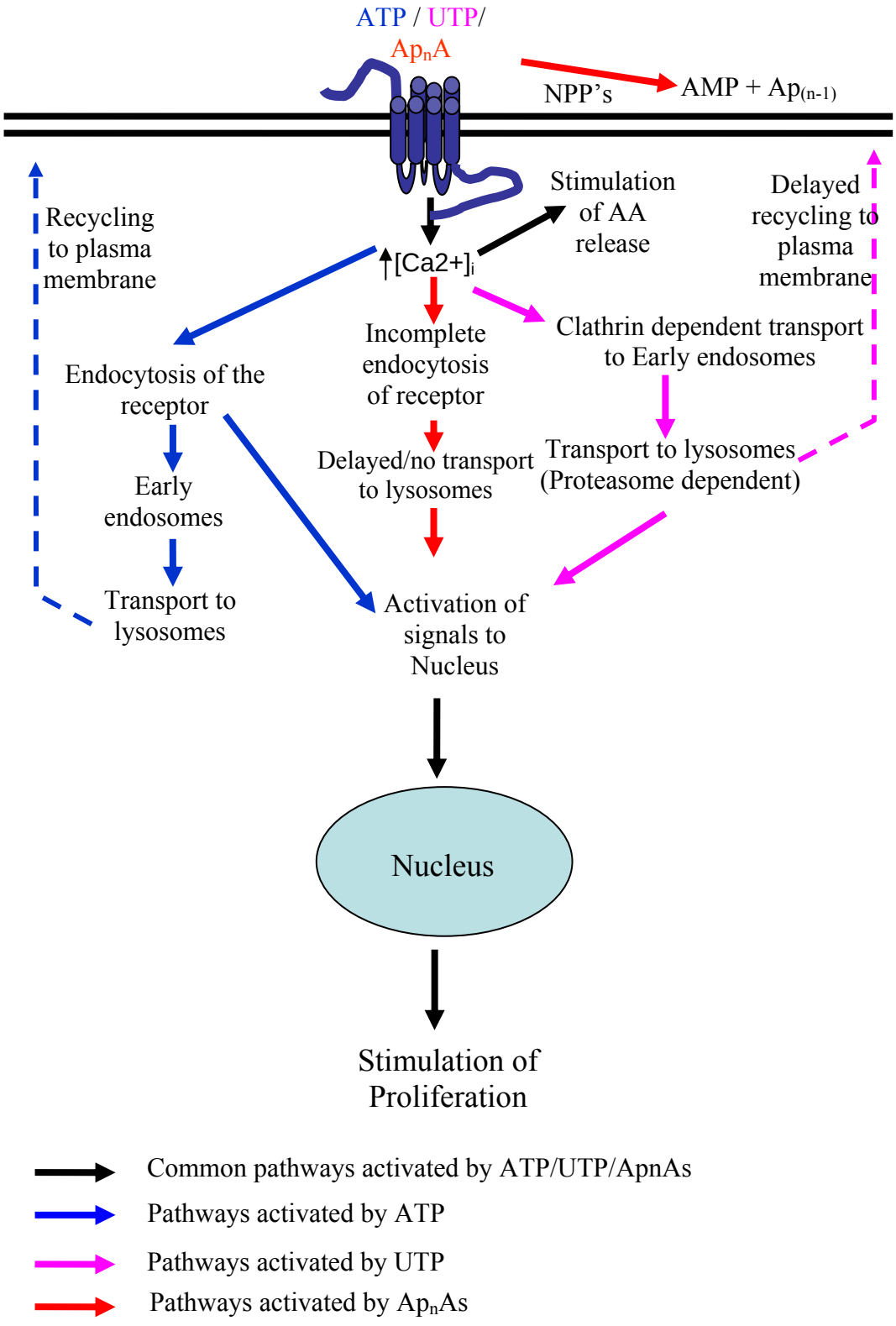
The $P2Y_2$ receptor is involved in modulation of proliferation, like the other P2Y receptors, namely $P2Y_1$, $P2Y_{12}$ (Czajkowski et. al., 2004) and $P2Y_6$ (Korcok et. al., 2005). The role of P2Y receptors modulating proliferation has been studied in detail. Activation of $P2Y_2$ receptors causes proliferation and/or migration of human epidermal keratinocytes, lung epithelial tumor cells, glioma cells, and smooth muscle cells (Wilden et al., 1998; Tu et al., 2000; Seye et al., 2002; Schäfer et al., 2003, Greig et al., 2003). Cell proliferation in response to $P2Y_2$ receptor activation is associated with the activation of PI3-K, ERK1/2 and the expression of the early response protein c-fos (Muscella et al., 2003, Neary et al., 2003), PKC (Muscella, et. al., 2004), CDKs (Lee and Han 2006) and NF-κB (Korcok et. al., 2005, Schäfer et al., 2006) which are regulated from the signals emanating from the stimulation of the receptor. In case of the $P2Y_2$ receptor, it has been shown to modulate the cell cycle (Miyagi et. al., 1996; Schäfer et al., 2006; 2003). The subtypes of P2 receptors involved in the anticancer activity of adenine nucleotides are largely unresolved, but $P2Y_1$ and $P2Y_2$ receptors are considered as targets for tumor cell antiproliferation. Consistent with a role for $P2Y_2$ receptors in cell proliferation, $P2Y_2$ receptor mRNA expression is down-regulated during cell differentiation (Martin et al., 1997). The $P2Y_2$ receptor is up-regulated in thymocytes as an immediate early gene response (Koshiba et al., 1997) and in injured and stressed tissues (Turner et al., 1997; Seye et al., 2002). Thus, it is very important to evaluate whether ATP and UTP induced activation of the $P2Y_2$ receptor leads to similar or different degrees of proliferation. In terms of endocytotic kinetics both 100 µM ATP or UTP resulted in complete internalization of the receptor. We therefore expected an equal degree of proliferation induced upon stimulation of the cells with either ATP or UTP. However, even in the presence of the $P2Y_1$ receptor antagonist MRS2179, a small, but significant difference in the proliferation potency of ATP as compared to UTP was observed, already starting at concentrations of 20 µM (data not shown). These data are in complete correlation with our previous data (Schafer et. al., 2003), where in the A549 cells we observed this difference too. In case of A549 cells this difference between ATP and UTP in modulating the proliferation is markedly observed as the A549 cells lack the $P2Y_1$ receptor.

When the cells were stimulated with diadenosine polyphosphates it was observed that the dadenosine polyphosphates stimulated proliferation to a larger extent that that induced by either ATP or UTP (Table 3.2). The reason for this increased level of proliferation could be either due to the activation of different signal transduction pathways or due to hydrolysis products of the diadenosine polyphopsphates which could act as an additional trigger for proliferation and thus result is increased proliferation that that obtained with ATP/UTP. We observed that even a pre-incubated of the cells with α,β-Me-ADP, an inhibitor of the extracellular ectonucleotidases (Vollmayer et. al.,2003), before stimulation with diadenosine polyphosphates did not significantly alter the proliferative response (Table 3.2). Thus form the above results we can infer that the diadenosine polyphosphates stimulate a different pathway or and additional pathway that is not activated by either ATP or UTP that results in the significantly increased proliferation induced by these substances.

Thus far, the strength of a signal elicited by an agonist could be correlated by its ability to induce a stronger degree of internalization. However, in the case of the $P2Y_2$ receptor, there is complete endocytosis of the receptor induced by both ATP and UTP and therefore, some thus far unknown signaling protein seems to be differently activated. Based on these results that one may speculate that the strength of the endocytosis is correlated with the strength of the physiological reposne of the cell which in this case is proliferation. One possible candidate could be ERK1/2, as it has been shown to be activated four-fold higher upon activation of the CCR7 chemokine receptor by CCL19 than by CCL21 (Kohout et al., 2004).

Whether ATP or UTP induce different internalization mechanisms of the $P2Y_2$ receptor, as has been found for the CC chemokine receptor (Kohout et al., 2004) and for the μ-opioid receptor (Johnson et al., 2006), is not known. The latter result is especially important, as it has been found that the μ-opioid agonist DAMGO ([D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin) induces arrestin-2 translocation to the plasma membrane, whereas morphin does not. This difference of the two agonists is consistent with their ability to induce internalization of the μ-opioid receptor (Johnson et al., 2006). Moreover, β-arrestins scaffold members of the MAPK signaling cascade (Reiter & Lefkowitz, 2006). Thus, the role of β-arrestins in the internalization and the modulation of proliferative signals of the $P2Y_2$ receptor needs further in-depth examination. A comparison of the different endocytotic/trafficking and signaling to the nucleus by ATP/UTP/Ap_nA are summarized in Fig. 4.3.

Figure 4.3: Activation of $P2Y_2$ receptor endocytosis/trafficking and signalling to the nucleus by ATP / UTP / Ap_nAs via the $P2Y_2$ receptor.



4.7 Conclusion

Taken together, our results support a model for endocytosis of the $P2Y_2$ receptor that involves active participation of the cytoskeleton via a clathrin-dependent pathway, and trafficking of the receptor from endosomes to lysosomes that most likely involves receptor ubiquitination. We also found evidence that in addition to the role of cytoskeleton and clathrin in the modulation of endocytosis of the $P2Y_2$ receptor, PLD regulates the endocytosis of the $P2Y_2$ receptor. Inhibition of PLD delays the endocytosis of the receptor, whereas the inhibition of the MAPK modulated the distribution of the internalized receptor. In addition to this we were also able to add a new aspect to the activation of MAPK by the $P2Y_2$ receptor. MAPKs do not only play an important role in modulating the proliferative activity by the $P2Y_2$ receptor (Shen et.al., 2004, Muscella et. al., 2003) but also affect the trafficking of the receptor. From the receptor trafficking and proliferation experiments presented here, we could demonstrate for the first time that at the $P2Y_2$ receptor ATP and UTP are equipotent agonists at the $P2Y_2$ receptor only at the level of calcium response whereas in the further downstream events, like trafficking of the receptor and modulation of proliferation by the $P2Y_2$ receptor they exhibit different regulatory mechanisms indicating that they stimulate different pathways in trafficking of the internalized receptor. In addition to the nucleotides ATP and UTP diadenosine polyphosphates also modulate the trafficking of the $P2Y_2$ receptor. The clear association of defined molecular steps in $P2Y_2$ receptor trafficking found here will enable us to design strategies to allow long-term stimulation and signaling of this receptor subtype. Thus, the $P2Y_2$ receptor is an intriguing new target for anti-inflammatory, immunomodulatory and antiproliferative therapies in neurodegenerative diseases, cancer and atherosclerosis (Abbracchio et al. 2006; Lazarowski et. al., 1997; Luttikhuizen et al., 2004) for which an understanding of the consequences of continuous stimulation of this nucleotide receptor is important.

5. ZUSAMMENFASSUNG

Extrazelluläre Nukleotide üben eine große Anzahl von physiologischen Effekten durch die Aktivierung von P2Y Rezeptoren aus. Die Aktivität der metabotropen P2Y Rezeptoren, die zu der Familie der G-Protein-gekoppelten Rezeptoren gehören, wird durch Endozytose reguliert. Von den acht Subtypen der P2Y Rezeptoren ($P2Y_{1,2,4,6,11,12,12,14}$) ist der $P2Y_2$ Rezeptor einzigartig, da er equipotent auf Adenin- und Uridin-Nukleotide anspricht. Zusätzlich zu Purin- und Pyrimidin-Nukleosidtriphosphaten können P2Y Rezeptoren auch durch Diadenosinpolyphosphate aktiviert werden.

Da die Proteine, die den intrazellulären Transport des endozytierten $P2Y_2$ Rezeptors regulieren, nicht bekannt sind, haben wir den $P2Y_2$ Rezeptor der Ratte mit grün-fluoreszierendem Protein (GFP) markiert und funktional in HEK-293 Zellen exprimiert, um die Translokation des Rezeptors in lebenden Zellen mittels konfokaler Mikroskopie zu beobachten. Die funktionelle Expression des Rezeptors wurde durch Messung des Anstiegs der intrazellulären Ca^{2+} Konzentration ($[Ca^{2+}]$) nach Applikation ansteigender Konzentrationen von ATP oder UTP bestätigt. Die Stimulation mit den Agonisten bewirkte eine zeitabhängige Translokation des $P2Y_2$ Rezeptors von der Plasmamembran in das Cytoplasma. Das Ausmaß der Endozytose des Rezeptors war von der Konzentration des Agonisten abhängig. Nach der Stimulation der Zellen mit UTP wurde zuerst eine Kolokalisation des endozytierten Rezeptors mit frühen Endosomen und dann mit Lysosomen beobachtet. Die Inhibierung der Rezeptor-Endozytose durch hoch-viskoses Medium oder Chlorpromazin in Gegenwart von UTP weist auf die Internalisierung des Rezeptors durch den Clathrin-vermittelten Signalweg hin. Die Nichtbeteiligung des Caveolin-vermittelten Signalweges wurde bestätigt, da Filipin-III nicht die Kinetik der Endozytose des $P2Y_2$-GFP Rezeptors beeinflußte. Die Steuerung des Rezeptors von den Endosomen zu den Lysosomen scheint den Proteasom-Signalweg einzuschließen, da die Inhibierung des Proteasomen-Komplexes das Recycling des Rezeptors zur Plasmamembran erhöhte. Ein interessanter Befund der Studie ist die Tatsache, dass ATP und UTP unterschiedliche Transport-Signalwege für den endozytierten Rezeptor auslösen, obwohl sie eine identische Potenz hinsichtlich der Ca^{2+}-Freisetzung besitzen. Dies ist aus den unterschiedlichen Kinetiken des Wiedererscheinens des endozytierten $P2Y_2$-GFP Rezeptors an der Zelloberfläche ersichtlich, je nach Stimulation durch ATP bzw. UTP. Am intrazellulären Transport des endozytierten $P2Y_2$ Rezeptors sind die Ca^{2+}-Calmodulin abhängige Proteinkinase II

(CaMKII), PP2A, Proteinkinase C, RhoA-Kinase, PI3-Kinase und Phospholipase D_2 (PLD_2) beteiligt. Dies konnte mittels Vorbehandlung von $P2Y_2$-GFP Rezeptor-exprimierenden Zellen mit spezifischen Inhibitoren dieser Proteine nachgewiesen werden, da diese den Zeitverlauf sowohl von der Endozytose als auch des Wiedererscheinens des Rezeptors an der Zelloberfläche veränderten. Die Diadenosinpolyphosphate haben unterschiedliche Potenz in der Erhöhung von $[Ca^{2+}]_i$, der Induzierung der Endozytose des $P2Y_2$ Rezeptors, seines intrazellulären Transportes und der $P2Y_2$ Rezeptor-vermittelten Proliferation. In der Reihenfolge $Ap_4A > Ap_3A \geq Ap_5A >> Ap_2A$ nahm die Wirksamkeit am $P2Y_2$ Rezeptor für die angeführten Prozesse ab.

Zusammenfassend kann gesagt werden, dass in dieser Studie zum ersten Mal gezeigt wurde, dass (i) eine starke Korrelation zwischen der Konzentration des Agonisten und der dadurch bedingten Endozytose des $P2Y_2$ Rezeptors besteht, (ii) ATP und UTP einen unterschiedlichen intrazellulären Transport des endozytierten Rezeptors auslösen, (iii) der Proteasom-Signalweg sehr wahrscheinlich an dem intrazellulären Transport des $P2Y_2$ Rezeptors beteiligt ist, (iv) dieser intrazelluläre Transport durch eine Reihe von Proteinkinasen und zytosolischen Proteinen kontrolliert wird, die dadurch letztendlich die weiteren Reaktionen der Zelle bestimmen und (v) Diadenosinpolyphosphate den intrazellulären Transport des $P2Y_2$ Rezeptors und auch die durch den Rezeptor stimulierte Proliferation modulieren.

Abstract

Extracellular nucleotides exert a large number of physiological effects through activation of P2Y receptors. The activity of the metabotropic P2Y receptors, which belong to the family of G protein-coupled receptors (GPCRs), is regulated by endocytosis. Out of the eight $P2Y_{(1,2,4,6,11,12,13,14),}$ subtypes that have been characterized, the $P2Y_2$ is unique in that it responds equipotently to adenine and uridine triphosphates in terms of Ca^{2+}-release. In addition to purine or pyrimidine nucleoside triphosphates, P2Y receptors can also be activated by diadenosine polyphosphates.

As the proteins which regulate trafficking of the endocytosed $P2Y_2$ receptor are not known, we investigated this by functional expression of the rat $P2Y_2$ receptor, tagged with green fluorescent protein (GFP) in HEK-293 cells and visualised receptor translocation in live cells by confocal microscopy. Agonist stimulation revealed a time- and concentration-dependent translocation of the receptor from the plasma membrane to the cytoplasm. Colocalization of the endocytosed $P2Y_2$ receptor with clathrin, early endosomes and lysosomes in the stated sequence upon stimulation of the cells with UTP reveals internalization through the clathrin-, but not the caveolin-mediated pathway: The proteasome pathway very likely is involved in targeting of the receptor from endosomes to lysosomes, because proteasomal inhibition increased receptor recycling back to the plasma membrane. An interesting finding of the study was that ATP and UTP initiated different trafficking pathways for the endocytosed receptor were equipotent at the $P2Y_2$ receptor in terms of the Ca^{2+} response. Pharmacological investigation of the trafficking revealed that CaMKII, PP2A, PKCs, RhoA kinase, PI3K and PLD_2 are involved in modulating the trafficking of the endocytosed $P2Y_2$ receptor. Different potencies in elevating $[Ca^{2+}]_i$, inducing endocytosis or modulating trafficking of the $P2Y_2$ receptor and proliferation with a potency order of $Ap_4A > Ap_3A \geq Ap_5A >> Ap_2A$ could be demonstrated.

In conclusion through this study we were able to demonstrate for the first time that (i) there is a strong correlation between the concentration of the agonist and endocytosis of the $P2Y_2$-GFP receptor; (ii) ATP and UTP induce different trafficking of the endocytosed receptor; (iii) the proteasomal pathway is involved in trafficking of the $P2Y_2$ receptor ; (iv) trafficking of the $P2Y_2$ receptor is controlled by a orchestra of kinases and cytosolic proteins which finally regulate the fate of the cell and (v) diadenosine polyphosphates also modulate the trafficking of the $P2Y_2$ receptor and also $P2Y_2$ receptor modulated proliferation.

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7 LIST OF ABBREVEATIONS

AC	Adenylyl cyclase
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
Ap_nA	Diadenosine polyphosphate
Ap_2A	Diadenosine diphosphate
Ap_3A	Diadenosine triphosphate
Ap_4A	Diadenosine tetraphosphate
Ap_5A	Diadenosine pentphosphate
BSA	Bovine serum albumin
BrdU	5-Bromo-2'-deoxyuridine
$[Ca2+]_i$	Intracellular calcium concentration
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosinemonophosphate
CNS	Central nerve system
CTxB	cholera toxin subunit B conjugated to Alexa Fluor 555
Cyto-D	Cytochalasin-D
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate
EDTA	Ethylene diamaine tetra acetic acid
EEA-1	Early endosome antigen-1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinase
FCS	Fetal calf serum
FTI-277	Farnesyl transferase inhibitor
Fura-2/AM	Fura-2/Acetoxymethyl Ester
GF-109203X	Bisindolylmaleimide
G418	Geneticin sulfate
GFP	Green fluorescent protein

GRK	GPCR coupled kinase
HEK-293	Human embryonic kidney cells
LAMP-1	Lysosome-associated marker protein-1
MAPK	Mitogen-activated protein kinase
MβCD	Methyl-beta-cyclodextrin
MEK	Mitogen-activated protein kinase kinase
MG-132	Inhibitor of Proteasomal complex
ML-9	Inhibitor of myosin light chain kinase
MLCK	Myosin light chain kinase
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PI 3-Kinase	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD_2	Phospholipase D2
PLL	Poly-L-lysine
Pyk2	proline-rich tyrosine kinase 2
Raf	A mitogen-activated protein kinase kinase kinase
Ras	A small GTPase; one of the oncogenes family
Rho A	A small GTPase
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
Src	A nonreceptor tyrosine kinase
UDP	Uridine diphosphate
UTP	Uridine triphosphate

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List of publications and conference abstracts:

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1. **Tulapurkar ME**, Laubinger W, Nahum V, Fischer B, Reiser G. Subtype specific internalization of P2Y1 and P2Y2 receptors induced by novel adenosine 5'-O-(1-boranotriphosphate) derivatives. Br J Pharmacol. 2004; 142(5):869-78.
2. **Tulapurkar ME**, Schafer R, Hanck T, Flores RV, Weisman GA, Gonzalez FA, Reiser G. Endocytosis mechanism of $P2Y_2$ nucleotide receptor tagged with green fluorescent protein: clathrin and actin cytoskeleton-dependence. Cell Mol Life Sci. 2005; 62(12):1388-99.
3. **Tulapurkar, M.E.**; Zündorf, G. and Reiser, G. Internalisation and desensitization of a green fluorescence protein-tagged $P2Y_1$ nucleotide receptor are differently controlled by inhibition of calmodulin-dependent protein kinase II. J Neurochem. 2006; 96(3):624-3 Nahum V,
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7. **Tulapurkar ME**, Schafer R and Reiser G. Differential role of $P2Y_2$ receptor agonists in modulating receptor translocation and role of PLD_2, MAP kinase in modulating receptor trafficking (submitted)
8. **Tulapurkar ME**, Schafer R, Hartig, R and Reiser G. Role of kinases in modulating $P2Y_2$ receptor trafficking and $P2Y_2$ receptor mediated proliferation (in preparation)

9. **Tulapurkar ME,** Laubinger W, Schafer R and Reiser G. Role of diadenosine polyphosphates in modulation of $P2Y_1$ and $P2Y_2$ receptor signaling. (in preparation)

Contributions to conferences:

1 **M. E. Tulapurkar**, G. Zuendorf, T. Hanck, G. Reiser. Visualization of agonist induced $P2Y_{1,2}$ receptor endocytosis: possible role of different signaling pathways.: presented at Fall meeting of GBM (Gesellschaft für Biochemie und Molecularbiologie) Bochum Germany, September 9-12, 2001.

2 **Tulapurkar M.E**., Zündorf G., Nahum V., Fischer B., Reiser G. Novel Adenosine 5'-O-(1-boranotriphosphate) derivatives induced subtype specific internalization of P2Y receptor. German Neuorscience Society, Göttingen, 2003

3 **Tulapurkar M.E.**, G. Zündorf, T. Hanck, G. Reiser Agonist-specific translocation and recycling of nucleotide-activated $P2Y_2$ receptor German Neuorscience Society, Göttingen 2003

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5 **Tulapurkar M.E**, G. Zündorf, T. Hanck, G. Reiser. Endocytosis of $P2Y_2$ receptor. Signaling Processes and Structures in Nervous System in Health and Disease Study Group of (Gesellschaft für Biochemie und Molecularbiologie) Dresden, Germany, 2003.

6 **Mohan Tulapurkar**, Theo Hanck, Denise Ecke and Georg Reiser. Sequestration and recycling pathway of $P2Y_2$ nucleotide receptor: Clathrin and actin cytoskeleton-dependent receptor endocytosis, determined by visualization of green fluorescent tagged receptor. Purines 2004.

7 **Tulapurkar, M.E**. and Reiser, G. Regulation of $P2Y_2$ receptor endocytosis by protein kinases and phosphatases. GBM (Gesellschaft für Biochemie und Molecularbiologie) Annual Meeting of the Study Group Neurochemistry Leipzeig 2004. The abstract was published in special issue of International Journal of Developmental Neuroscience (2004), 22(7), 571-599

8 **Tulapurkar, M.E**. and Reiser, G. Dynamics of P2Y nucleotide receptors: sensitivity and translocation with long-term stimulation 20th Biennial Meeting

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RESUME

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