Location-Biased Signaling of Proton-Sensing Receptor GPR65 Within the Endocytic Pathway

by

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Dedication

This dissertation is dedicated to my loving parents:

David Morales Acevedo

and

Iris Loyda Rodríguez Román

who gave all they had to ensure I would have the opportunity of an education.

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Table of Contents

List of Figures

Abstract

G protein-coupled receptors (GPCRs) transduce distinct extracellular signals, including light, hormones, neurotransmitters, and ions, into diverse cellular signaling responses. These cellular responses underlie an array of physiological processes, ranging from the control of blood pressure, immune response, and neurological diseases to the progression of cancer. Considering the implications of GPCR signaling, understanding how GPCRs are regulated in human physiology and disease is very important.

The location of GPCRs within the cell is an increasingly recognized variable shaping signaling diversity at a cellular level. GPCR location at endosomes shapes downstream transcriptional responses. Endosomal signaling of prototypical GPCRs induces gene transcriptional responses with distinct cellular functions than surface receptor signaling.

The subcellular location of GPCR signaling presents an interesting context for proton-sensing GPCRs because they are more likely to be activated at acidic endosomal compartments than at the plasma membrane on the cell surface. In this dissertation, I have used the proton-sensing receptor GPR65 as a prototype to study how acidic environments at distinct cellular compartments, with an elevated proton concentration, change the signaling patterns of proton-sensing GPCRs. GPR65 is highly overexpressed

in many solid tumors and is emerging as an attractive target to treat cancer since its response to acidic environments is implicated in tumor signaling and immune function. Because GPR65 is a physiologically relevant but understudied receptor, GPR65 is an ideal candidate to study how receptor location and acidic environments shape the signaling of proton-sensing GPCRs.

I first investigated whether GPR65 follows the tight coupling of receptor signaling and trafficking and whether it can be selectively activated in endosomal compartments. Through confocal microscopy, receptor mutagenesis, and biochemical assays, I show that the trafficking of the prototypical proton-sensor GPR65 is fully uncoupled from signaling, unlike that of other known mammalian GPCRs. GPR65 internalizes and localizes to early and late endosomes, from where it can signal at steady state, irrespective of extracellular pH. Receptor mutants that were incapable of signaling trafficked normally, internalize, and localize to endosomal compartments. These findings show that GPR65 is constitutively active in endosomes and suggest a model where changes in extracellular pH reprogram the spatial pattern of receptor signaling and bias the location of signaling to the cell surface.

Next, I determined the effect of spatial organization on GPR65 signaling using a biosensor of the second messenger signaling molecule cAMP together with inhibitors of intracellular signaling proteins and effectors. I show that GPR65 increases intracellular second messenger cAMP presumably via two distinct signaling pathways which require soluble adenylyl cyclase activation. cAMP production by surface GPR65 requires EPAC and PLC, while cAMP generated by internalized receptors requires PLC activation dependent on the release of Gβγ subunits. These results suggest a model where

activation of GPR65 elicits diverse and distinct signaling pathways at different cellular locations.

This work adds to our understanding of how receptor location inside the cell is intricately linked to receptor signaling and lays the groundwork for one day targeting receptor location to influence cellular responses, with greater efficacy and fewer adverse effects for patients suffering from life-threatening diseases such as cancer. Defining factors that modulate the spatial organization of GPR65 signaling together with the identification of compounds with functional selectivity, may result in clinically valuable tools for diseases involving proton-sensing receptor GPR65.

Chapter 1 The Roles of Compartmentalized Proton-Sensing GPCR Signaling in Physiology and Disease

1.1 Abstract

G protein-coupled receptors (GPCRs) constitute eukaryotes' largest family of transmembrane signaling receptors. Canonical GPCR biology indicates receptor signaling is tightly coupled to trafficking. This idea states that receptors remain on the cell surface until they are activated, after which they are desensitized and internalized into endosomal compartments. An exciting and emerging idea in GPCR biology indicates that receptors can signal from intracellular compartments in addition to signals originating from the plasma membrane (PM). This emerging idea presents an interesting context for proton-sensing GPCRs because they are more likely to be activated in acidic intracellular compartments than at the cell surface. Like most GPCRs, proton-sensing GPCRs coupled to G proteins at the PM are internalized in the cell and trafficked through the endolysosomal system. Compartments within the endolysosomal system vary in pH and provide a novel acidic environment in which proton-sensing GPCRs may be uniquely active and signal. This review will discuss the biological roles of proton-sensing GPCRs at the cellular level. We will present evidence for compartmentalized GPCR signaling from intracellular membranes such as endosomes and highlight how receptor activation at endosomes may be exploited to develop new therapeutics with greater efficacy and less adverse effects for patients suffering from diseases associated with acidic environments.

1.2 Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane signaling receptors in eukaryotes that transduce various environmental inputs, including light, hormones, neurotransmitters, and ions, into intracellular signaling responses (Pierce et al., 2002). Following interaction with an environmental input, GPCRs transduce signals via distinct heterotrimeric G protein effectors, which modulate diverse downstream signaling pathways and physiological processes (Rosenbaum et al., 2009; Weis & Kobilka, 2018). In humans, these intracellular signaling responses underlie complex processes ranging from analgesia, mood, and reward to smooth muscle relaxation and bronchodilation (Gendron et al., 2016; Weinberg et al., 2019). Although GPCRs were once thought to only signal from the cell surface, these membrane proteins are now increasingly recognized as capable of signaling from internal cellular compartments (Lobingier & von Zastrow, 2019).

An exciting and emerging idea in GPCR biology indicates that signals originating from the plasma membrane (PM) have distinct profiles from those generated at internal compartments, demonstrating that signaling and trafficking are highly integrated events (Gorvin, 2018; Tsvetanova et al., 2015; Tsvetanova & von Zastrow, 2014). Once GPCRs initiate a signaling cascade at the PM, these membrane receptors are removed from the cell surface and trafficked through the endolysosomal system. Upon internalization and trafficking to endosomes, prototypical GPCRs such as the β_2-adrenergic receptor (B2AR) and the parathyroid hormone receptor (PTHR) can initiate a second wave of signaling from early endosomes (Bowman et al., 2016a; Ferrandon et al., 2009; Irannejad et al., 2013; Tsvetanova et al., 2015). B2AR and PTHR, which both couple to stimulatory Gαs

proteins, elevate the second messenger cAMP even after robust internalization of receptors. B2AR cAMP signaling also generates gene expression states that occur at endosomes that are distinct from the PM (Bowman et al., 2016a; Irannejad et al., 2013). This cAMP production mediated by receptors at the endosome persists even when the agonist is removed from the extracellular space and is disrupted by inhibition of endocytosis, indicating that endocytosis is required for endosomal cAMP production for these receptors (Ferrandon et al., 2009).

Signaling from endosomes is an emerging idea and especially relevant for a class of understudied receptors, the proton sensing GPCRs, which allow cells to sense and respond to elevated proton concentrations in acidic environments with a pH <7.0 (Ludwig et al., 2003). Like most GPCRs, proton-sensing GPCRs coupled to G proteins at the PM are internalized in the cell and trafficked through the endolysosomal system (Fukunaga et al., 2006). Compartments within the endolysosomal system vary in pH and provide a novel acidic environment in which proton-sensing GPCRs may be uniquely active and signal (Ko et al., 2020). In this review, we will examine the biological functions of the proton-sensing GPCRs and highlight how receptor activation at endosomes may be exploited to develop new therapies for diseases associated with acidic environments, like cancer and exacerbated inflammation.

1.3 Physiology of Proton-Sensing GPCRs

Proton-sensing GPCRs are membrane proteins that sense and respond to acidic environments with an elevated proton concentration (Ludwig et al., 2003). This family of receptors consists of three members: GPR4, GPR65 (TDAG8), and GPR68 (ORG1)

(Ludwig et al., 2003; Sisignano et al., 2021). Although these GPCRs remain categorized as orphan or “pharmacologically dark” receptors, the only known endogenous ligand is hydrogen ions or protons (X. P. Huang et al., 2015; Rowe et al., 2020; Sriram & Insel, 2018). Lipids, such as psychosine and other related glycosphingolipids have been reported as agonists (D. Im et al., 2001; D. S. Im, 2005; Tomura et al., 2005; J. Q. Wang et al., 2004), but these findings have been directly contested (Silva et al., 2022). Stimulation of proton sensing GPCRs occurs via protonation of histidine (His, H) residues (Ludwig et al., 2003). Other ionizable amino acids such as aspartic acid (Asp, D), glutamic acid (Glu, E), arginine (Arg, R), and lysine (Lys, K) can detect pH changes (Rowe et al., 2020). For instance, proton-sensor GPR65 detects protons via His 10, 14, and 243 (Ludwig et al., 2003). A recent study identified three other proton-sensing residues contributing to the activation of GPR65: D60, E142, and D286 (Rowe et al., 2020). These proton-sensing residues are responsible for agonist detection and signal transduction of extracellular pH changes to activate intracellular signaling pathways and generate distinct, context-dependent cellular responses, such as fibroblast proliferation in cancer and insulin secretion (Mogi et al., 2014; Wiley et al., 2019).

1.3.1 Biological Roles of GPR65

GPR65 senses and responds to acidic environments via histidine (H10, H14, and H243) and acidic triad residues (D60, E142, and D286) (Ludwig et al., 2003; Rowe et al., 2020). A phylogenetic analysis investigating the evolutionary origins of proton-sensing revealed that the pH sensing mechanism first emerged in GPR65, making GPR65 the oldest proton-sensor evolutionarily (Rowe et al., 2020). These residues on GPR65 detect

and transduce extracellular signals to increase cAMP production via G_s proteins and ras homolog-gene-family-member A (RhoA) activation through G_{13}, subsequently eliciting distinct, context-dependent cellular responses (**Figure 1.1**) (Justus et al., 2017; Ludwig et al., 2003; X. D. Ma et al., 2017; Ryder et al., 2012).

GPR65 is implicated in inflamed tissue and immune cell function. Acidic extracellular pH is a typical feature of inflamed tissue (Damaghi et al., 2013; Hanahan & Weinberg, 2011). This acidic environment is predominantly due to the increased metabolic demand from infiltrating immune cells. Immune cells like neutrophils increase oxygen consumption and glucose uptake for glycolysis and oxidative phosphorylation. As oxygen availability decreases, cells undergo anaerobic glycolysis, increasing lactic acid and acidifying the surrounding environment (Grinstein et al., 1991). Since GPR65 is highly expressed in immune cells, acidic pH stimulates GPR65 signaling in infiltrating immune cells (Okajima, 2013). Upon activation of GPR65 in mouse peritoneal macrophages, GPR65 inhibited pro-inflammatory cytokine secretion, specifically IL-6, and TNF-α, via stimulatory Gs protein activation and the protein kinase A (PKA) signaling pathway (Mogi et al., 2009; Okajima, 2013). In type II collagen-induced arthritis, GPR65 is a negative regulator of inflammation (Onozawa et al., 2011). This study reported increased arthritis in GPR65-null mice when compared to wild-type mice. Apart from anti-inflammatory roles, GPR65 in immune cells functions as a positive modulator in inflammation (Kottyan et al., 2009). Kottyan et al. (2009) reported that GPR65 increased the viability of eosinophils within an acidic environment by reducing apoptosis through cAMP pathway activation. This finding suggests GPR65 may increase asthmatic inflammation since eosinophils are vital players in asthmatic inflammation and allergic airway disease.

GPR65 senses acidic extracellular pH within the skeletal system. The skeletal system contributes to restoring normal physiological pH during metabolic acidosis in addition to respiratory CO_2 and renal acid excretion. One mechanism to restore pH levels is osteoblast inhibition by acidic extracellular pH, which reduces bone resorption of minerals and Ca^{2+} (Brandao-Burch et al., 2005; Krieger et al., 1992, 2004). On the other hand, osteoclasts activated by acidosis increase Ca^{2+} release in vitro, helping to buffer protons and restore physiological pH systemically (Bushinsky et al., 1985; Krieger et al., 1992). However, prolonged metabolic acidosis can reduce total volumetric bone density and, over time, can result in osteoporosis (Bushinsky et al., 1985). Proton sensor GPR65 has been reported to sense acidosis in bone cells. GPR65 is expressed in osteoclasts, and its activity can inhibit Ca^{2+} resorption (Hikiji et al., 2014). Decreased expression of GPR65 aggravates osteoclastic bone resorption in ovariectomized mice (Hikiji et al., 2014). In cultured osteoclast mice cells lacking GPR65, normal levels of inhibition of osteoclast formation in response to low pH were abolished (Hikiji et al., 2014). Together, these findings suggest that GPR65 activation and signaling may play a key role in osteoporosis and other bone density disorders.

Apart from involvement in the skeletal system, GPR65 activation displays opposing roles in tumor biology. The tumor microenvironment is highly acidic due to its altered metabolism, termed the "Warburg Effect" (Damaghi et al., 2013; Goldstein et al., 2010; Hanahan & Weinberg, 2011; Kato et al., 2013). This unique metabolic phenotype allows cancer cells to preferentially utilize glycolysis and produce vast quantities of lactic acid, which serves as the proton source for proton-sensitive proteins. Once lactic acid is dissociated into one lactate molecule and one proton, mono-carboxylate transporters and

proton transporters export lactate and protons into the extracellular space and surrounding cells. Proton-sensing receptors like GPR65 are activated by the acidic pH released into the tumor microenvironment and modulate tumor activities (Justus et al., 2013; Ludwig et al., 2003; Sisignano et al., 2021). GPR65 was reported to play a role in favor of cancer cell survival by transforming the mouse NMuMG mammary epithelial cell line and improving the survival of NCI-H460 human non-small cell lung cancer cells in an acidic microenvironment (Wun et al., 2004). On the other hand, GPR65 also has displayed tumor-suppressing properties. GPR65 suppresses c-Myc oncogene expression in human lymphoma cells and increases glucocorticoid-induced apoptosis in murine lymphoma cells (Z. Li et al., 2013b).

1.3.2 Biological Roles of GPR68

Initially identified in a human ovarian cancer cell line, GPR68 senses and responds to low pH through different G proteins, including G_i, G_q, $G_{12/13}$, and G_s proteins (Kotake et al., 2014; J. Li et al., 2013; Ludwig et al., 2003; Pera et al., 2018; Saxena et al., 2012). Its coupling preferences differ between cell types, tissues, and the pathophysiological states in which the receptor is studied. Low pH-mediated activation of GPR68 was reported to stimulate inositol trisphosphate (IP_3) production in CCL39 hamster fibroblast and HEK293 cells (Ludwig et al., 2003). This research group also found that GPR68-mediated production of IP_3 required histidine residues (H17, H20, H84, and H269) (Ludwig et al., 2003). A different group also reported GPR68 acid-sensing capabilities via the shared triad of buried acidic residues, specifically D67, E149, and D282 (Rowe et al., 2020). This pH-sensing mechanism of GPR68 is observed in various cells and tissues,

including peripheral sensory neurons in dorsal root ganglia (DRG), cardiomyocytes, endothelial cells, and osteoclasts, as well as diseases associated with acidic extracellular environments.

GPR68 has been identified as a candidate acid sensor within the cardiovascular system. This body system delivers oxygen and nutrients to tissues and removes metabolic waste. Without proper function, vascular occlusion can lead to tissue ischemia and acidification due to oxygen deprivation, anaerobic metabolism, and the inability to remove acid byproducts. Acidosis within the cardiac system disrupts the regulation of action potential duration in cardiac excitability, impairs sodium channels, and leads to life-threatening events (such as arrhythmia, myocardial infarction, and cardiac death) (Antzelevitch & Belardinelli, 2006; Ju et al., 1996; Yatani et al., 1988). GPR68 increases the heart's cardiomyogenic and pro-survival genes in the cardiovascular system while mediating gene expression in aortic smooth muscle cells (J. P. Liu et al., 2010; Russell et al., 2012; Tomura et al., 2005). In a rodent model of myocardial infarction, GPR68 expression levels were high in cardiomyocytes. These cells also formed a proton-sensing cellular zone surrounding the myocardial infarction. Russell et al. (2012) also identified GPR68-mediated activation by 3,5-disubstitued isoxazoles (Isx), which are cardiomyogenic small molecules targeting Notch-activated epicardium-derived cells. GPR68 activation by Isx increased the expression of cardiomyogenic and pro-survival genes. In human aortic smooth muscle cells, GPR68 was found to be the primary receptor responsible for extracellular acidic pH-induced production of inositol phosphate, PGI_2, and cAMP. GPR68 activation in aortic smooth muscle cells also induced COX-2 and

MAPK phosphatase-1 expression. While GPR68 regulates cardiac system function, it has also been reported to sense pH in the respiratory system.

GPR68 is implicated in respiratory disorders and airway contraction within the respiratory system. The respiratory system's primary function is to provide sufficient oxygen supply to tissues while removing carbon dioxide (CO_2). The delivery of oxygen to tissues occurs by hemoglobin binding oxygen, while the removal of CO_2 occurs through bicarbonate transport. This transport mechanism maintains acid-base homeostasis and serves as a buffer to prevent respiratory acidosis. Proton accumulation has been observed in several respiratory disorders, such as asthma. Asthma is a chronic inflammatory disease associated with bronchial hyper-responsiveness, airway inflammation, remodeling, and acidic features (Aoki et al., 2013). Aoki et al. (2013) demonstrated that GPR68 in dendritic cells is crucial for the onset of asthmatic inflammation. By knocking out GPR68, mice were resistant to asthma, inhibiting Th2 cytokine and immunoglobulin E production. In human airway smooth muscle cells, GPR68 activation induced the production of pro-inflammatory cytokine IL-6 and increased intracellular Ca^{2+} (Ichimonji et al., 2010; C. Liu et al., 2013; Matsuzaki et al., 2011; Saxena et al., 2012). Acidic extracellular pH of 6.3 induced the expression of CTGF, involved in the formation of extracellular matrix proteins and associated with airway remodeling, through the GPR68/Gq/11/IP_3/Ca^{2+} pathway. Further, GPR68 mediated airway smooth muscle cell contraction when exposed to a low pH extracellular environment.

Apart from implications in respiratory disorders and airway contraction, GPR68 plays a role in the skeletal system. Distinct bone cells express high levels of GPR68 and may regulate systemic pH by controlling the release of minerals from the bone. Bone cells

such as osteoblasts, osteoclasts, and chondrocytes highly express GPR68. GPR68 expression is also found during osteoclastogenesis and could be involved in osteoclast differentiation (Komarova et al., 2005). Low extracellular pH levels were found to increase the accumulation of the NFATc1 protein, which regulates osteoclastogenesis and osteoblastogenesis, through NF-kappa B ligand (RANKL) in the nuclei of rat and rabbit osteoclasts. This effect was thought to occur through prolonged Ca^{2+} release and activation of the calcineurin/NFAT pathway in response to GPR68 activation by low pH levels. In healthy human osteoblasts, low pH has been found to stimulate the expression of COX2 and PGE_2 through activation of GPR68, G_q coupling, PLC activation, and intracellular Ca^{2+} release (Tomura et al., 2008). In addition, neonatal calvarial osteoblasts endogenously expressing the receptor and Chinese hamster ovary (CHO) cells heterologously expressing GPR68 stimulate intracellular Ca^{2+} release after low pH exposure (Frick et al., 2009). In rat chondrocytes, low pH mediated GPR68 activation-induced apoptosis potentially reduces collagen production leading to detrimental effects in the spinal cord, specifically intervertebral disk degeneration (Yuan et al., 2014).

Apart from regulating the release of minerals from bone cells, GPR68 is implicated in tumor biology. GPR68 displays roles as a tumor suppressor. A recent study showed that GPR68 inhibits cancer metastasis, reduces cell proliferation, and inhibits cell migration. GPR68 overexpression in prostate cancer cells suppresses metastasis to other nearby body organs in mice (Sanderlin et al., 2015). Overexpression is also known to inhibit ovarian cancer cell proliferation and migration (Singh et al., 2007). On the other hand, GPR68 can also promote tumor development. GPR68 activation by low extracellular pH induces immunosuppression in mice and promotes cancer development

(Yan et al., 2014). Although GPR68 displays opposing roles in tumor biology, tumor-suppressing and tumor-promoting activities depend primarily on cell type and biological setting.

1.3.3 Biological Roles of GPR4

GPR4 senses and responds to protons through histidine residues, specifically H79, H165, and H269, and the shared triad of buried acidic residues (Ludwig et al., 2003; Rowe et al., 2020). These pH-sensing residues are protonated within a broad pH range of 5.6-7.6, and some are shared with proton-sensor GPR68. Each of these His residues are required for proton-dependent activation of GPR4 via Gs coupling leading to elevated cAMP levels in cells (Ludwig et al., 2003). Other reports identified different coupling preferences for GPR4. For example, proton-dependent activation of GPR4 requires G_s coupling, G_q, and $G_{12/13}$ (Krewson et al., 2020; Tobo et al., 2007); however, these coupling preferences vary per cell type and biological function.

Like GPR68 and GPR65, GPR4 displays several roles in the inflammatory response. GPR4 is highly expressed in endothelial cells and plays a role in the inflammatory loci in endothelial cells that line blood vessels. The inflammatory response by vascular endothelial cells facilitates the induction of inflammatory cytokines in the recruitment of white blood cells. Proton-dependent activation of GPR4 in human umbilical vein endothelial cells increases the expression of distinct pro-inflammatory genes such as chemokines, cytokines, PTGS2, NF-KB pathway genes, and adhesion molecules (Dong et al., 2013). These cells also increased GPR4-mediated white blood cells adhesion to endothelial cells via Gs-cAMP-Exchange protein activated by cAMP (Epac)

activation in response to acidic extracellular pH (A. Chen et al., 2011; Dong et al., 2013). Apart from exacerbating inflammation in vascular endothelial cells, GPR4 is a crucial regulator of blood vessel function.

GPR4 regulates blood vessel stability and integrity within the cardiovascular system and drives angiogenesis (Wyder et al., 2011; Yang et al., 2007). GPR4-Knockout (KO) neonatal mice displayed a higher perinatal mortality rate partially correlated with spontaneous hemorrhaging and respiratory distress. Pathology revealed that these mice exhibited disorganized and tortuous blood vessels. Another research group found that a different GPR4-KO mouse strain possessed fragmented and fragile blood vessels in tumor tissue, further supporting the role of GPR4 in blood vessel stability and integrity (Wyder et al., 2011; Yang et al., 2007). Acidic pH-mediated GPR4 activation is also implicated in driving angiogenesis or the formation of new blood vessels from pre-existing vessels. GPR4-KO mice reduced the formation of new blood vessels by decreasing the angiogenic response of the potent angiogenic factor VEGF (Vascular Endothelial Growth Factor). Although GPR4 is a positive regulator of angiogenesis, low pH-mediated GPR4 activation has biological implications in the renal system.

In the renal system, GPR4 has important implications for maintaining pH homeostasis. GPR4 is highly expressed throughout the kidney, specifically in the kidney cortex, kidney collecting ducts, and inner and outer medulla. Expression in these areas suggests that GPR4 activity could be necessary for renal acid excretion to buffer pH successfully. A recent study confirmed that renal acid excretion and the ability to respond to metabolic acidosis were reduced in GPR4-deficient mice (Codina et al., 2011; Sun et al., 2010). In addition, inner and outer medullary collecting duct cells elevated cAMP

levels in cells through GPR4-Gs activity after exposure to acidic extracellular pH. Finally, in renal epithelial cells, GPR4 overexpression increased the activity of PKA and subsequent protein expression of H^+-K^+-ATPase α-subunit (HKα2), a key regulator of pH in the stomach (Codina et al., 2011). GPR4 may be necessary for successful systemic pH buffering by controlling renal acid excretion.

Low pH-mediated activation of GPR4 is also implicated in insulin secretion and tissue sensitivity to insulin. GPR4-KO mice developed increased glucose tolerance by increasing their sensitivity to insulin (Giudici et al., 2013). GPR4 has also been implicated in modulating the expression of inflammatory molecules (Giudici et al., 2013). A balance between pro-inflammatory and anti-inflammatory molecules is crucial for maintaining insulin sensitivity. The absence of GPR4 has been shown to reduce the expression of several inflammatory molecules, such as IL-6, PPAR, TNF-, and TGF1B (Giudici et al., 2013). Apart from modulating insulin sensitivity, activation of GPR4 stimulates tumor-suppressing and promoting activities in distinct cell types and tissues.

GPR4 plays a role in cell migration, metastasis, and proliferation. Low pH-mediated activation of GPR4 leads to tumor-suppressing activities. In B16F10 melanoma cells, GPR4 overexpression (OE) and activation suppress tumor metastasis by obstructing migration and invasion of tumor cells (Castellone et al., 2011; Zhang et al., 2012). GPR4 OE also inhibits lung metastasis B16F10 melanoma cells in mice. On the other hand, GPR4 displays tumor-promoting activities. GPR4 malignantly transformed immortalized NIH3T3 fibroblasts (Wun et al., 2004). These data show that GPR4 functions as a tumor suppressor and promoter depending on the cellular context and biological systems expressed.

1.4 Proton-Sensing GPCR Activation in Acidic Intracellular Compartments

Extracellular acidic pH is a driver of pathological conditions and biological functions mediated by proton-sensing GPCR activation (Damaghi et al., 2013; Hanahan & Weinberg, 2011; Kato et al., 2013). However, the role of acidic intracellular pH in proton-sensing receptor activation, signal propagation, and physiology has only recently been investigated (Morales Rodríguez et al., 2023). This section will examine the early evidence for GPCR signaling from intracellular compartments and how this signaling differs from distinct cellular compartments. We will also suggest how internalized proton-sensing GPCR activation may be exploited to improve our understanding of receptor physiology and develop new therapies.

1.4.1 Evidence of well-characterized, prototypical GPCRs signaling from endosomes

Receptor activation and signaling from intracellular compartments is an emerging concept in the GPCR field. GPCR signaling from intracellular membranes can occur through G and non-G protein effectors. The first evidence of GPCR signaling from intracellular membranes suggested that this signaling is mediated via β-arrestins, which are non-G protein effectors. β-arrestins are a family of adaptor proteins that regulate the signaling and trafficking of various GPCRs (Luttrell & Lefkowitz, 2002). In addition to their roles in GPCR signal termination and receptor internalization from the plasma membrane, β-arrestins interact with GPCRs and scaffold kinase signaling pathways at the cell surface and internal compartments, specifically endosomes (Lohse et al., 1990; Lohse & Calebiro, 2013). G-protein effectors mediate GPCR signaling from intracellular compartments, and the signaling outcomes from the same G protein in different locations produce distinct

signaling responses. Early insight into endosomal G protein signaling stemmed from prolonged signaling responses even after the receptor was removed from the cell surface. For example, parathyroid hormone receptor (PTHR) activation elevates cellular cAMP via Gαs even after robust internalization of receptors from the plasma membrane (Ferrandon et al., 2009). Apart from prolonged signaling responses, GPCR internalization and endocytosis could contribute to distinct downstream signaling outcomes.

Endosomal G protein signaling might produce distinct signaling profiles for various GPCRs. For example, inhibition of β_2-adrenergic receptor (B2AR) endocytosis partially decreases second messenger cAMP production (Irannejad et al., 2013). Inhibiting B2AR endocytosis decreases cAMP production at later time points, specifically 5 minutes after agonist addition. This finding differs from the Gαs-coupled dopamine receptor D1 (DRD1), which measurably decreases cAMP after one- or two minutes following agonist treatment (Kotowski et al., 2011). The difference in dynamics between B2AR and DRD1 endosomal G protein signaling might be due to the time required for B2AR localization to specific endosomal domains.

1.4.2 GPCR signaling outcome differences between cellular compartments

Endosomal G protein signaling diverges from surface signaling at the molecular level. At the molecular level, PTHR and the vasopressin receptor 2 (V2R) display prolonged G protein signaling due to prolonged arrestin-Gβγ, GPCR-Gβγ, or GPCR-arrestin interactions on endosomes (Feinstein et al., 2011, 2013; Ferrandon et al., 2009; Wehbi et al., 2013). A single-particle electron microscopy structure of a chimeric GPCR bound to both arrestin-1 and a heterotrimeric G protein also supports the hypothesis that

a subset of GPCRs which strongly bind B-arrestins to sustain endosomal signaling could support multiple rounds of Gs protein activation (Thomsen et al., 2016; Wehbi et al., 2013).

Distinct cellular locations shape GPCR downstream transcriptional responses and may result from spatiotemporal regulation of GPCR-effector interactions and second messengers. For example, B2AR endosomal cAMP production is required and sufficient for the transcription of genes not upregulated by B2AR signaling from the cell surface (Bowman et al., 2016a; Tsvetanova & von Zastrow, 2014). Other GPCRs, such as the calcium-sensing receptor (CaSR) and the neurokinin 1 receptor (NK1R), require endosomal signaling to induce gene transcription from serum response elements (Gorvin, 2018; Jensen et al., 2017). Apart from endosomal signaling driving distinct gene transcriptional responses, endosomal GPCR signaling is linked to physiology.

GPCR signaling from endosomes can affect physiology and be modulated explicitly by new spatially-targeted pharmacology. For example, endosomal signaling of the luteinizing hormone receptor (LHR) has been linked to fertility (Lyga et al., 2016). Prolonged cAMP signaling and endocytosis of LHR are required for meiosis in the oocyte of ovarian follicles. NK1R and the calcitonin-like receptor (CLR) use spatially targeted ligands to inhibit endosomal signaling (Jensen et al., 2017; Yarwood et al., 2017). In animal models of inflammatory pain, these antagonists, conjugated to the lipid cholestanol, incorporated into the plasma membrane, are internalized and accumulate in endosomes to inhibit sustained signaling in spinal cord neurons and provide greater and longer lasting pain relief than traditional antagonists (Jensen et al., 2017; Yarwood et al., 2017). These endosomal-targeted ligands demonstrate how GPCR endosomal signaling

is linked to physiology and disease and highlight potential therapeutic approaches which exploit location bias in signaling.

1.4.3 Internalized proton-sensing receptors contribute to the cellular signaling response and present new treatment avenues for diseases

The array of responses mediated by proton-sensing GPCR activation is implicated in human physiology and disease (**Figure 1.1**) (Justus et al., 2013; Sanderlin et al., 2015; Sisignano et al., 2021). This array of responses, or what's known as the receptor's signaling profile, can be shaped by the location of the receptors within the cell (**Figure 1.2**). The subcellular location of proton-sensing GPCR activation and signaling has been recently identified as a contributor to the GPCR signaling profile (Morales Rodríguez et al., 2023).

Proton-sensing receptor GPR65 is active and able to signal from endosomes in addition to the PM (**Figure 1.2**) (Morales Rodríguez et al., 2023). We have previously reported that internalized GPR65 is required for a full signaling response. GPR65 is constitutively active in endosomes, but a change to acidic extracellular pH biases the localization pattern of signaling to the cell surface. Signaling for several GPCRs from endosomes is associated with gene transcription but the ways in which subcellular signaling outcomes alter downstream transcriptional responses and the signaling profile of clinically relevant GPCRs such as the proton-sensors is not fully understood. Defining how the receptor's signaling profile is altered by subcellular localization can improve our understanding of receptor physiology and will allow us to leverage receptor location to fine-tune the targeting of this family of GPCRs for better therapeutics.

1.4.3.1 Therapeutic avenues for internalized proton-sensing receptor signaling

Proton-sensing receptor variants have been linked to the onset of disease. A variant of proton-sensing receptor GPR65 has recently been associated with inflammatory bowel disease (IBD) susceptibility by gene mapping efforts (Lassen et al., 2016). The GPR65 I231L genetic variant reduces the activity and signaling of the receptor. The variant alters lysosomal pH and confers lysosomal dysfunction, increasing susceptibility to colitis and inflammatory bowel disease (IBD) risk. Another single nucleotide polymorphism in the GPR65 gene, associated with disease in atopic dermatitis patients, is rs8005161 (Xie et al., 2021). How these genetic variants affect receptor function from distinct cellular compartments such as endosomes or lysosomes is unknown. Examining the effect of these receptor variants on two key molecular aspects of receptor function, trafficking, and signaling, will provide a deeper understanding of the molecular consequences of genetic variations in the understudied family of proton-sensing GPCRs. This information will give insight into how these mutations affect proton-sensing receptor signaling and trafficking and how it differs from the canonical receptor.

1.4.3.2 Expanding therapeutic avenues to other proton-sensitive proteins

Parallels can be drawn between the previous findings and the therapeutic avenues to other proton-sensitive proteins. Increasing evidence supports the idea that GPCRs can signal from multiple cellular compartments (Crilly & Puthenveedu, 2021; Lobingier & von Zastrow, 2019). In addition to PM GPCR signaling, GPCRs can also signal from acidic intracellular compartments like endosomes. Signaling from these compartments is relevant to GPR4 and GPR68, as well as other proton-sensitive membrane proteins, such as GPR132, which displays weak proton-sensitivity, and other orphan GPCRs like GPR31

and GPR151, recently identified as proton-sensitive. These proton-sensitive proteins like many other prototypical GPCRs, contain ionizable residues that can be protonated when exposed to acidic environments (Rowe et al., 2020). Protonation due to acidic pH can result in a variety of physiological responses that can be modulated by subcellular localization. Subcellular localization biases and changes downstream transcriptional responses, either as a consequence of or in addition to differential interactions with G protein effectors (Crilly & Puthenveedu, 2021; Eichel & von Zastrow, 2018; Weinberg et al., 2019). Further studies should define how the receptor's signaling profile is altered by subcellular location. Characterization of the receptor's subcellular signaling patterns will improve our understanding of receptor physiology and therapeutic targeting of these proton-sensitive proteins.

Another avenue to exploit is genetic variations of proton-sensitive GPCRs. Gene mapping studies can help identify new disease-associated genes involving proton-sensitive proteins. Further studies on how these genes and genetic variations affect physiology in native systems and influence disease can guide the therapeutic targeting of proton-sensitive proteins.

1.4.4 Emerging areas of proton-sensing GPCR research

1.4.4.1 Modulating and targeting proton-sensing GPCRs

Proton-sensing GPCRs, including GPR65, GPR68, and GPR4, display a remarkable variety of biological functions and have been identified as potential drug targets for several pathophysiological states and diseases. However, there is a lack of compounds targeting proton-sensing receptors. Only a few compounds are available that

target the three proton sensors, either as allosteric modulators, agonists, or antagonists. Many of these pharmacological modulators lack selectivity and specificity. Specific examples on the types of pharmacological modulators available targeting each proton-sensing receptor are listed in these comprehensive reviews (Imenez Silva & Wagner, 2022; Silva et al., 2022). Identification and characterization of novel pharmacological tools are necessary to fully understand receptor physiology.

1.4.4.2 Unveiling novel Proton-Sensing GPCR Signaling Pathways

With some understanding of the roles in physiology and disease, proton-sensing GPCRs remain understudied when it comes to basic functional properties. Rowe et al. (2020) reported new G protein coupling preferences of proton-sensing receptors at the PM using a bioluminescence resonance energy transfer (BRET) mini-G protein assay. Like a few other prototypical GPCRs, proton-sensing GPCRs can exhibit a variety of G-protein coupling preferences (Hill & Baker, 2003; X. Ma et al., 2020; Wenzel-Seifert & Seifert, 2019). These coupling preferences can also exist at subcellular locations in cells and vary between cell type, tissue, and organ system (Hill & Baker, 2003; Xiao et al., 1999). Future studies should define how these coupling preferences differ upon subcellular localization and how these changes affect downstream signaling outcomes and generate distinct, context-dependent cellular responses. These new ideas can further illuminate the dark pharmacology of GPR4, GPR65, and GPR68 and significantly advance our understanding of receptor biology in health and disease.

1.5 Conclusion, remaining challenges, and future perspectives

Although significant advances have been made in understanding how compartmentalized GPCR signaling contributes to human physiology and disease, the precise biological roles of proton-sensing GPCR activation and signaling from intracellular compartments remain to be determined. Acidic intracellular compartments, such as endosomes, have been recognized as a critical signaling hub for cancer cells and other diseases associated with acidic microenvironments (Ko et al., 2020). More studies are needed to distinguish the links and underlying mechanisms between compartmentalized GPCR signaling, physiology, and disease. In addition to endosomes, GPCR signaling has also been reported on other acidic intracellular compartments, including the Golgi apparatus, secretory vesicles, and other internal membranes from the biosynthetic pathway (Crilly & Puthenveedu, 2021). Further studies on how other acidic intracellular compartments contribute to health and pathological conditions remain to be explored.

These new ideas indicate an exciting time for studying the cell biology of understudied GPCRs. For example, endosome signaling, specifically signaling from early endosomes, is an emerging idea. However, signal initiation from late endosomes and lysosomes, as well as other unexpected intracellular membranes, is a concept that has yet to be explored in GPCR biology. Further, selectively modulating signaling from specific compartments is an exciting and emerging prospect for developing therapeutics with greater efficacy and fewer adverse effects.

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1.7 Figures

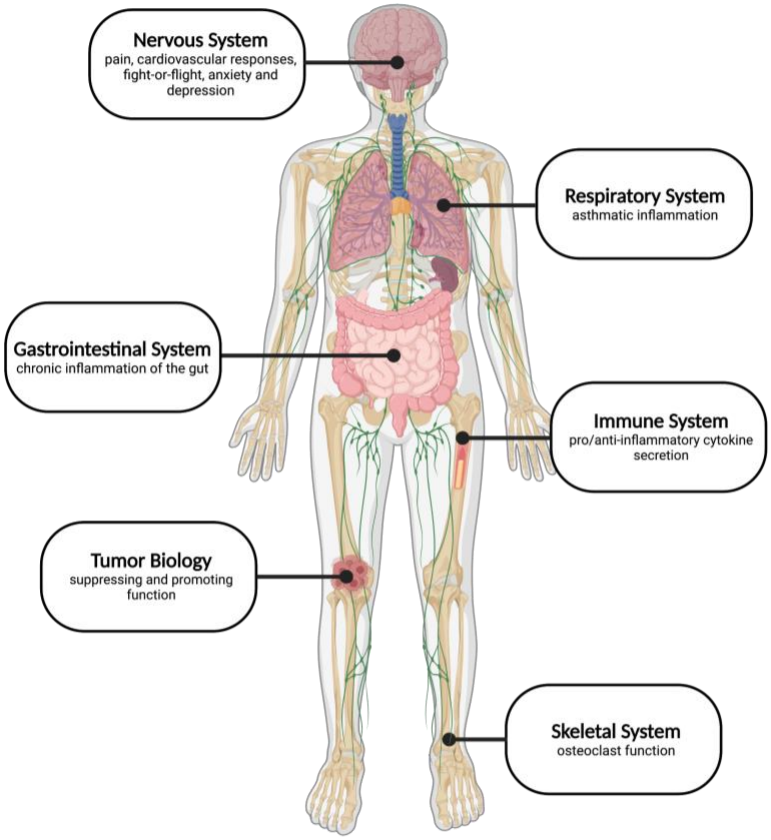

Figure 1.1 Physiology of proton-sensing receptor GPR65 function.

Schematic of distinct human body tissues presenting the main biological functions of proton-sensor GPR65. GPR65 function is implicated in the nervous, respiratory, gastrointestinal (GI), immune and skeletal systems. The role of GPR65 in tumor biology is complex. Both tumor-suppressing and tumor-promoting activities have been reported, which are primarily dependent on cell type and the surrounding environment.

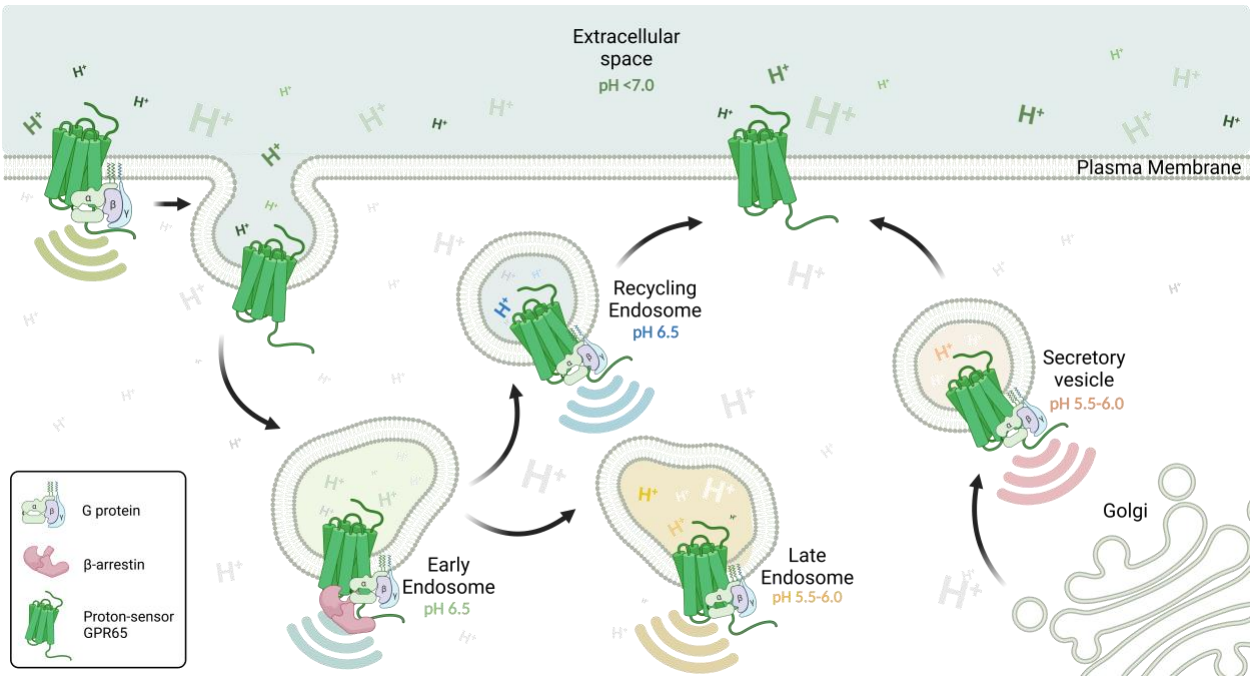

Figure 1.2 Potential intracellular signaling outcomes of proton-sensing GPCRs.

Proton-sensing GPCRs (including GPR65, GPR68, and GPR4) allow cells to sense and respond to acidic environments with a pH<7.0. Proton-sensing receptor GPR65 initiates a G_s-cAMP or $G_{12/13}$-RhoA signaling pathway at the PM. Once a GPCR signals at the PM on the cell surface, the receptor is removed from the cell surface and trafficked through the endocytic pathway. In addition to canonical PM signaling, the endocytic pathway varies in pH and provides a new environment in which proton-sensing receptors like GPR65 can be active and signal. GPR65 endocytosis can contribute to distinct downstream cAMP-dependent transcriptional control. Because GPR65 is a class A GPCR, many class A receptors recruit β-arrestins which interact with GPCRs and promote signaling pathways at the cell surface and endosomes. GPR65 could potentially initiate β-arrestin-mediated signaling at endosomes. Apart from the endocytic pathway, the biosynthetic pathway possesses adequate pH conditions to potentially activate proton-sensing receptors.

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Chapter 2 Location-Biased Activation of the Proton-Sensor GPR65 is Uncoupled from Receptor Trafficking

2.1 Abstract

The canonical view of G protein-coupled receptor (GPCR) function is that receptor trafficking is tightly coupled to signaling. GPCRs remain on the plasma membrane (PM) at the cell surface until they are activated, after which they are desensitized and internalized into endosomal compartments. This canonical view presents an interesting context for proton-sensing GPCRs because they are more likely to be activated in acidic endosomal compartments than at the PM. Here we show that the trafficking of the prototypical proton-sensor GPR65 is fully uncoupled from signaling, unlike that of other known mammalian GPCRs. GPR65 internalizes and localizes to early and late endosomes, from where they signal at steady state, irrespective of extracellular pH. Acidic extracellular environments stimulate receptor signaling at the PM in a dose-dependent manner, although endosomal GPR65 is still required for a full signaling response. Receptor mutants that were incapable of activating cAMP trafficked normally, internalize and localize to endosomal compartments. Our results show that GPR65 is constitutively active in endosomes, and suggest a model where changes in extracellular pH reprograms the spatial pattern of receptor signaling and biases the location of signaling to the cell surface.

2.2 Introduction

Activation and membrane trafficking are tightly coupled for all known members of the physiologically and clinically important G protein-coupled receptor (GPCR) family (Pierce et al., 2002; Sorkin & Von Zastrow, 2009; Sriram & Insel, 2018; Vilardaga et al., 2014). GPCRs activated at the plasma membrane (PM) on the cell surface are rapidly desensitized and internalized into endosomal compartments, from where they can either recycle back to the PM or be degraded in the lysosome. This sorting determines the further responsiveness of cells to ligands (Lobingier & von Zastrow, 2019; Weinberg & Puthenveedu, 2019a). Endosomes also serve as signaling stations for many GPCRs. The same signals originating from GPCRs endosomes can produce distinct downstream consequences from those originating in the plasma membrane (Bowman et al., 2016b; Irannejad et al., 2013; Sutkeviciute & Vilardaga, 2020; Tsvetanova et al., 2015; Vilardaga et al., 2014). These observations have led to an emerging model that the GPCR signaling is spatially encoded, where the integrated GPCR response is a balance of both surface and internal signals, determined by rates of trafficking of receptors to and from the PM.

Proton-sensing GPCRs present an interesting family of physiologically important GPCRs (Silva et al., 2022). The canonical view is that these receptors sense acidic extracellular environments by protonation and coordination of several amino acids, including extracellular histidines and buried amino acid triads containing aspartic acid and two glutamic acids (Ludwig et al., 2003; Rowe et al., 2020; Silva et al., 2022). There are 3 GPCRs - GPR4, GPR65, GPR68 - currently thought of as being the primary receptors that sense pH changes (Ludwig et al., 2003; Silva et al., 2022). Members of this family of GPCRs are highly overexpressed in many cancers (Insel et al., 2020). Acidic

environments are defining hallmarks of cancer and inflammation as well as many physiological processes (Erra Díaz et al., 2018; Kato et al., 2013; Ludwig et al., 2003; Okajima, 2013), and these GPCRs have generated interest as potential therapeutic targets.

The relationship between trafficking and signaling is especially interesting for proton-sensing GPCRs. Endocytosis of proton-sensing GPCRs transports them to endosomal compartments that are acidic and more likely to activate these receptors. Considering the intrinsic signaling potential of proton sensing GPCRs in intracellular compartments, whether these receptors follow the tight coupling of trafficking and signaling that have been described for most known GPCRs, or whether they can be selectively activated in endosomal compartments, are unanswered questions that are fundamental to understanding how cells respond to pH.

Here we addressed these questions using the proton-sensing receptor GPR65 as a prototype. We show that GPR65 internalizes from the PM irrespective of extracellular pH, and that receptor internalization is required for a full cellular signaling response. Together, our findings show that GPR65 dynamically traffics to and signals from multiple cellular compartments, and that, unlike for most known GPCRs, activation of GPR65 is uncoupled from receptor trafficking. Characterizing proton-sensing receptor trafficking and signaling will improve our understanding of how these receptors integrate responses from multiple locations in the cell, and how these responses influence physiology and disease states.

2.3 Results

2.3.1 GPR65 stimulates cAMP accumulation at neutral and acidic pH

We first determined the pH-dependent activation of GPR65, by measuring levels of the second messenger cAMP in HEK293 cells stably expressing GPR65. We used the cAMP biosensor GloSensor, which exhibits increased luminescence when bound to cAMP, to quantitatively measure cAMP levels (**Figure 2.1A**) (Fan et al., 2008; F. I. Wang et al., 2021). Upon exposure to proton concentrations from pH 6.0 to 8.0, GPR65-expressing cells displayed a dose-responsive elevation of cAMP levels (**Figure 2.1B and C**). This elevation in cAMP saturated at pH 6.4. Interestingly, cAMP levels were elevated at both acidic and neutral pH ranges. As negative controls, HEK293 cells not expressing GPR65 did not show a response (**Figure 2.1D and E**). Similarly, a GPR65 mutant where three key histidines -H10, H14 and H243 - were mutated, which has been shown to not increase cAMP levels, did not show a cAMP response (**Figure 2.1D and E**) (Ludwig et al., 2003; J. Q. Wang et al., 2004). This GPR65-mediated cAMP increase was similar to the prototypical Gs-coupled GPCR beta-2 adrenergic receptor (B2AR), which we used as a positive control for cAMP activation (**Figure 2.1D and E**). When cells expressing GPR65 were treated with high pH (i.e, a low concentration of protons), GloSensor luminescence rapidly decreased below that of vehicle media at pH 7.0 (**Figure 2.1B and C**, **Figure supplement 2.1B**). Both acute and chronic basic pH treatments decreased GPR65-mediated GloSensor luminescence (**Figure 1B**, **Figure supplement 1B**). Both the absolute cAMP response and the changes were reduced substantially, with levels staying close to baseline, in HEK293 cells not expressing GPR65 (**Figure supplement**

2.1A and B), indicating that the cAMP response we observed in GPR65-expressing cells were a result of GPR65 activation. In HEK293 cells, forskolin-induced cAMP responses did not change until pH 8.0, indicating that the dose-response observed in GPR65-expressing cells was not a direct effect of pH on the sensor (**Figure supplement 2.1C**). Together, these data demonstrate that GPR65 increases cAMP levels at both acidic and neutral pH.

2.3.2 GPR65 internalizes from the plasma membrane irrespective of extracellular pH

Because GPR65 exhibited increased cAMP levels at a physiologically relevant pH range, we asked whether GPR65 was constitutively internalizing from the PM, and how the internalization compared to the prototypical B2AR. To test this, HEK293 cells expressing epitope-tagged FLAG-GPR65 were immunolabeled live (**Figure 2.2A**) and imaged by confocal microscopy after 10 minutes of labeling at 37˚C. At neutral and basic pH, FLAG-GPR65 localizes to the PM and internal compartments (**Figure 2.2B and C**), suggesting that surface receptors are internalized rapidly at these pH levels. When exposed to acidic pH, FLAG-GPR65 localization did not change noticeably and is again observed at the PM and internal compartments (**Figure 2.2B and C**). Quantification of the number of internal receptor spots shows a similar degree of internalization of FLAG-GPR65 exposed to pH 6.4-8.0 (**Figure 2.2C**). This agonist-independent internalization pattern was distinct from the prototypical GPCR B2AR. At baseline, B2AR was localized primarily to the PM (**Figure 2.2B**). When exposed to a saturating concentration (10 μM) of the agonist isoproterenol, B2AR internalized and localized almost entirely to internal compartments (**Figure 2.2B and C**). The expression levels of receptors in all cell lines

were comparable, as measured by fluorescence levels (**Figure supplement 2.2A**), confirming that at similar expression levels, GPR65 and B2AR localized to different compartments at baseline. Additionally, GPR65 tagged with the SNAP-tag at the N-terminus, labeled with a membrane-impermeable SNAP dye, displayed a similar localization pattern as FLAG-tagged GPR65 (**Figure 2.2D and E**). Together, these data demonstrate that GPR65 constitutively internalizes from the PM and localizes to internal compartments irrespective of extracellular pH.

To directly test whether the intracellular localization represented GPR65 that was internalized from the PM, we inhibited endocytosis prior to labeling surface receptors and tested whether this inhibition reduced GPR65 intracellular localization. To inhibit endocytosis, we expressed a dominant-negative dynamin mutant (Dyn K44A), which inhibits dynamin-mediated endocytosis (Altschuler et al., 1998). FLAG-GPR65 and FLAG-B2AR stable cells expressing either Dyn K44A or wild type dynamin (WT Dyn) were labeled live using fluorescent anti-FLAG antibodies as above, and imaged using confocal microscopy. FLAG-GPR65 cells expressing Dyn K44A showed almost no intracellular puncta, while cells expressing WT Dyn showed multiple puncta similar to cells in Figure 2B and 2D (**Figure 2.2F**). Quantification of the ratio of intracellular fluorescence over total cell fluorescence over time revealed that the fraction of intracellular fluorescence was substantially lower in GPR65 cells expressing Dyn K44A than in cells expressing WT Dyn (**Figure 2.2G**). As controls, FLAG-B2AR cells expressing Dyn K44A, but not WT Dyn, showed a loss of intracellular fluorescence after isoproterenol (**Figure 2.2G**). Together, these data demonstrate GPR65 internalizes from the PM in a dynamin-dependent manner irrespective of pH, and localizes to intracellular endosomal structures.

We next asked whether GPR65 was localized to a biochemically specific endosomal compartment, and whether the localization pattern of GPR65 changed between neutral and acidic extracellular pH. We treated HEK293 cells stably expressing FLAG-GPR65 with pH 7.0 or pH 6.4 for 20 min, immunolabeled live, fixed, and stained cells with markers for distinct compartments along the endosomal pathway: APPL1 (very early endosomes), EEA1 (early endosomes), Rab11 (recycling endosomes), and Lamp1 (lysosomes) (**Figure 2.2H**). By using spot detection and colocalization analysis, we quantified the fraction of GPR65 puncta that colocalized with each endosomal marker. At pH 7.0, GPR65 localized to multiple compartments, including EEA1, APPL1, Rab11 and Lamp1 endosomes, and this localization pattern did not change after cells were exposed to pH 6.4 (**Figure 2.2I-K**). These results show that GPR65 is distributed across early and late endosomal compartments across neutral and acidic pH ranges, which is surprising considering that GPCR activation and trafficking are usually highly integrated (Bowman et al., 2016b; Stoeber et al., 2018; Thomsen et al., 2018; Vilardaga et al., 2014).

2.3.3 Proton-dependent activation of GPR65 is uncoupled from receptor trafficking

Because GPR65 was localized in internal compartments across basic and acidic pH ranges, we next asked whether internalization and endosomal localization of surface-labeled GPR65 required the receptor to be able to signal. To do this, we compared the localization of WT GPR65 to that of GPR65 mutants that were deficient in activating cAMP. In addition to the histidine mutant (H10F, H14F, and H243F) described in Figure 1, which did not stimulate cAMP, we generated an independent GPR65 mutant deficient in cAMP signaling by mutating an arginine 112 in the DRY Motif, a conserved stretch of

amino acids that governs GPCR activation and G protein coupling (Rovati et al., 2007). When this mutant (R112A) was transiently expressed in HEK293 cells expressing the GloSensor cAMP sensor, at neutral pH, the mutant showed low levels of luminescence, comparable to that of HEK293 cells not transfected with the receptor (**Figure 2.3A**). This low level was comparable to the GPR65 histidine mutant GPR65 described in Figure 1. In contrast, cells expressing WT GPR65 showed a significantly higher level of luminescence (**Figure 2.3A**). Exposure to pH 6.4 did not increase cAMP levels in cells expressing GPR65 R112A, or in cells not transfected with the receptor, in contrast to cells expressing WT GPR65 (**Figure 2.3B and C**), showing that the R112A mutant GPR65 was not capable of stimulating cAMP in response to pH (**Figure 2.3A-C**). Receptor expression were comparable across all cells analyzed, as measured by fluorescence levels (**Figure supplement 2B**), confirming that the differences in cAMP levels were due to intrinsic differences in the ability of expressed receptors to stimulate cAMP and not due to differences in expression levels. Strikingly, under the same conditions, FLAG-R112A GPR65 and FLAG-H10, 14, 243F GPR65 mutants were both localized to intracellular endosomal structures at steady state, identical to WT FLAG-GPR65, when visualized via confocal microscopy (**Figure 2.3D and E**). These results show that the ability of GPR65 to be activated was not required for receptor internalization, and that GPR65 trafficking and signaling were uncoupled.

2.3.4 Acidic endosomal environments activate endosomal GPR65 irrespective of extracellular pH

Localization of GPR65 to distinct intracellular compartments at steady-state raised the possibility that GPR65 was persistently active at acidic endosomes. To test this possibility, we first asked whether internalization of GPR65 was required for the full cAMP response. We pretreated cells with the endocytosis inhibitor Dyngo-4a (**Figure 2.4A**) for 15 min, and measured cAMP signaling via GloSensor luminescence after exposing cells to pH 6.4. Inhibition of GPR65 endocytosis reduced the total cAMP response when compared to vehicle-treated cells (**Figure 2.4B and D**). This reduction in cAMP response of Dyngo-4a treated GPR65-expressing cells was similar to iso-activated B2AR cells exposed to Dyngo-4a (**Figure 2.4B-D**), where endosomal B2AR was required for the full cAMP response as previously described (Bowman et al., 2016b; Tsvetanova & von Zastrow, 2014). As a control, Dyngo-4a pretreatment did not change forskolin-induced cAMP activation in HEK293 cells not expressing GPR65 (**Figure 2.4E**), indicating that Dyngo-4a had no direct effect on cAMP activation.

To test whether endosomal GPR65 was active and signaling even when surface receptors are inactive, we incubated cells for 2 hours with basic pH (pH 8.0), treated cells with increasing concentrations of Dyngo-4a (40, 80, and 100µM) for 15 min, and measured cAMP levels using GloSensor luminescence. At pH 8.0, GPR65-expressing cells showed basal cAMP levels that decreased as the concentration of Dyngo-4a increased, suggesting that endosomal GPR65 was active even in basic extracellular pH, when surface GPR65 was inactive (**Figure 2.4F**). Together, these data suggest that endosomal GPR65 contributes to the persistent cAMP response observed in GPR65-expressing cells.

We next asked whether GPR65 signaling from endosomes was due to activation by protons in acidic environments of endosomes, or whether it was due to ligand-independent constitutive activity. To distinguish these possibilities, we tested whether the acidic environment in endosomes was required for GPR65-mediated cAMP activation, by pretreating cells with the endosomal deacidifying agent chloroquine (CQ) and measuring cAMP at acidic and basic extracellular pH. CQ pretreatment for 30 min did not change cAMP levels when the extracellular pH was 6.4 (**Figure 2.4G**), consistent with surface receptors contributing to the majority of cAMP response in this situation. In contrast, CQ pretreatment for 30 min significantly reduced cAMP levels when the extracellular pH was 7.2 (**Figure 2.4H**), where surface receptors are inactive and endosomal receptors contribute to the majority of cAMP response.

2.3.5 Uncoupled trafficking and endosomal GPR65 signaling is conserved in physiologically relevant Jurkat T cells

We next asked whether the uncoupling of trafficking and signaling that we observed with expressed GPR65 in HEK293 cells was conserved in physiologically relevant cells. We focused on Jurkat T cells, an immortalized acute T cell leukemia line, which expressed endogenous GPR65, (Z. Li et al., 2013a), as a physiologically relevant model cell line. FLAG-GPR65 expressed in these cells was localized to intracellular compartments at pH 7.2 (**Figure 2.5A**). This internal localization of FLAG-GPR65 did not change noticeably upon exposing cells to pH 6.4 (**Figure 2.5A**). Quantification of the number of internal receptor spots in Jurkat cells shows a similar level of endosomal

localization of FLAG-GPR65 (**Figure 2.5B**) as was observed in HEK293 cells (**Figure 2B-C**).

To test whether the endosomal pool of GPR65 contributed to the cAMP response in Jurkat cells, we pretreated Jurkat cells expressing FLAG-GPR65 with the endocytosis inhibitor Dyngo-4a for 15 min, and measured GloSensor luminescence before and after shifting cells to pH 6.4. Inhibition of GPR65 endocytosis significantly reduced the total cAMP response in Jurkat cells expressing FLAG-GPR65 (**Figure 2.5C and E**). Exposing Jurkat cells expressing endogenous GPR65 to pH 6.4 showed a cAMP response with more rapid kinetics. Importantly, inhibiting GPR65 endocytosis significantly reduced this cAMP response (**Figure 2.5D and E**), suggesting that endogenous endosome-localized GPR65 contributes to the endogenous cAMP response in these cells.

2.4 Discussion

Here we show that GPR65 localizes to endosomal compartments and stimulates cAMP production from endosomes independently from extracellular pH changes. Surprisingly, GPR65 activity was not required for receptor endosomal localization. Our results show that endosomal GPR65 sets the basal cAMP tone, and with acidic activation of GPR65 at the plasma membrane further increasing cAMP levels (**Figure 2.5F**). These two sources of cAMP likely result in distinct pools of cAMP with distinct cellular functions.

Our results suggest that, contrary to known examples of mammalian GPCRs, trafficking of GPR65 to endosomal compartments is fully uncoupled from receptor activation at the plasma membrane. Prototypical class A GPCRs, like B2AR, are activated by agonist at the PM inducing a cascade of events that cause receptors to internalize and

localize to internal compartments. GPR65 localized in endosomes even at extracellular pH conditions that did not stimulate cAMP above baseline (**Figure 2.2B-E**), indicating that GPR65 internalization is ligand- and activation-independent. Ligand-independent internalization has been reported for the viral GPCR US28, which is constitutively active and is localized to internal compartments (Casarosa et al., 2001; Fraile-Ramos et al., 2001, 2002). Similarly, cannabinoid receptors or delta opioid receptors, show relatively high basal activity in the absence of added external ligands, and therefore show higher internalization in the absence of activating ligands. In these cases, receptor internalization is tightly coupled to its activation state. When these receptors are inactivated either by inverse agonists or by mutations, receptor endocytosis is substantially inhibited (Gendron et al., 2016; Leterrier et al., 2006). In contrast, mutations in the GPR65 DRY motif that completely block signaling (**Figure 2.3A-C**) have no effect on the endosomal distribution of GPR65 (**Figure 2.3 D and E**).

How GPR65 is constitutively internalized and distributed in endosomes in the absence of activation is still not known. The conventional view is that GPCRs are unable to interact with the clathrin endocytic machinery before being activated by ligands. Ligand-binding causes G protein activation, which initiates a cascade of events that activate GPCR kinase 2 (GRK2), which phosphorylates the GPCR, allowing receptors to recruit arrestins, which act as endocytic adapters that sequester receptors in endocytic domains (Kunselman et al., 2021). For activation-independent endocytosis, GPR65 could recruit a different member of the GPCR kinase family (Q. Chen & Tesmer, 2022) which does not need to be activated by a GPCR-G protein pathway. Alternatively, GPR65 could constitutively interact with arrestins or other endocytic adapters, via sequence motifs in

the cytoplasmic elements, that allows ligand-independent sequestration in endocytic domains and uncoupling of activation from endocytic trafficking.

This uncoupling of GPR65 activation and endosomal localization suggests that GPR65 signaling is regulated differently from other GPCRs. Activation of most GPCRs by ligand binding, typically at the PM, switches receptors from an “off” state to an “on” state. The initial G protein activation on the PM induced by ligand binding is rapidly desensitized by phosphorylation and arrestin binding. After internalization, a second phase of G protein-mediated signaling is initiated on endosomes. Importantly, G protein signaling from the PM and endosomes, even though they activate the same second messengers such as cAMP, activate separate sets of genes downstream of signals (Bowman et al., 2016b; Tsvetanova et al., 2015, 2021). For example, B2AR activates second-messenger cAMP from the cell surface and endosomes, but only endosomal cAMP induces the transcription of specific genes including phosphoenolpyruvate carboxykinase 1 (PCK1) and nuclear receptor subfamily 4 group A member 1(NR4A1) (Bowman et al., 2016b; Tsvetanova & von Zastrow, 2014). Gi-coupled receptors, such as opioid receptors, can also be in active conformations on endosomes after ligand-dependent activation at the PM (Stoeber et al., 2018). However, the consequence of endosomal cAMP are likely to be receptor specific, as inhibition of cAMP by opioid receptors from endosomes does not have an opposite effect on the same genes (Stoeber et al., 2018). The physiological outcome of activating a receptor, therefore, is an integrated response of these multiple phases of signaling from the surface and along the endocytic pathway, separated by time and space (Crilly & Puthenveedu, 2021; Irannejad et al., 2017). Importantly, for these known examples, because the ligand is extracellular,

mechanisms exist to transport the ligand to the endosomes, either by transporters that allow movement of small ligands such as catecholamines across the membranes, or by trafficking mechanisms that internalize larger ligands such as peptides (Irannejad et al., 2017; Stoeber et al., 2018). Unlike these, GPR65 activation in endosomes is independent of specific transport or trafficking mechanisms for secreted ligands, as the high proton concentrations are generated as part of normal endosomal acidification.

Whether GPR65 requires consistently high concentrations of protons in endosomes for signaling is an interesting question. This question is unclear even for canonical GPCRs such as opioid or adrenergic receptors, as there is no strong evidence that ligands internalize with receptors into endosomes. In our experiments, inhibition of endosomal acidification by CQ inhibited the basal cAMP signaling seen under conditions where surface GPR65 was inactive, and endosomal GPR65 was presumably the main source of cAMP (**Figure 2.4H**). Interestingly, at acidic extracellular pH, CQ had minimal inhibitory effect on cAMP signaling (**Figure 2.4G**). Although this experiment is confounded by observations that acidic environments can decrease the efficiency of CQ (Pellegrini et al., 2014), the data could reflect the fact that the magnitude of surface signaling is high enough to mask relative differences in endosomal signaling caused by neutralization, or that there is a pH-independent constitutive component to GPR65-mediated cAMP activation. The latter is consistent with the presence of residual GPR65 signaling in chloroquine-treated cells at high extracellular pH (**Figure 2.4H**). Overall, however, our results suggest that endosomal GPR65 activation is primarily determined by constitutive internalization of the receptor to late endosomal compartments where the acidic environment results in receptor activation and cAMP production (**Figure 2.5F**).

Based on this unique uncoupling of trafficking and signaling, we propose a model where GPR65 is active in endosomes at all times, and where acidic extracellular environments, rather than globally turning receptors on, instead switch or bias the intensity, timing, and location of signaling to the PM (**Figure 2.4**). At a physiological pH of 7.4 GPR65 is inactive at the plasma membrane (**Figure 2.1C**). The steep dose-response in the pH range of 7.2 to 6.8 allows cells to rapidly switch signaling to the plasma membrane, which could induce rapidly variable signaling outcomes that depend on the conformational biases induced by the membrane environment at the plasma membrane vs. endosomes (Wingler & Lefkowitz, 2020). Therefore, acidic environments such as those observed in solid tumors could convert “tonic” endosomal signaling by GPR65, which is physiologically beneficial for immune cells, where GPR65 is highly expressed, to “spikes” of surface cAMP signaling, as seen in Jurkat cells expressing endogenous GPR65 upon switching to acidic media (**Figure 2.5D**).

The model suggests interesting new aspects of how GPR65 activation regulates signaling in physiological systems such as in immune cells. A role for cAMP in modulating immune cells is well established, mainly downstream of adrenergic receptors expressed in both innate and adaptive immune cells (Guereschi et al., 2013; Padro C. J & Sanders V. M, 2014). Activation of cAMP can drive the secretion of selected cytokines and reduce inflammatory responses and infiltration by innate immune cells. However, cAMP also inhibits immune cell activation and proliferation, chemokine-dependent cell migration, and secretion of other cytokines and interferons (S. Huang et al., 1997; Padro C. J & Sanders V. M, 2014), which could collectively inhibit the effectiveness of immune cells in tumor clearance. Immune cells, as they infiltrate different environments are exposed to different

extracellular pH, like in the acidic environment in solid tumors. It is possible that the baseline level of tonic cAMP signaling, via constitutive GPR65 signaling from endosomes, is critical for maintaining normal function of immune cells, and that spikes of surface signaling via adrenergic agonists and GPR65 enable rapid and specific responses, depending on the precise cell type and immune environment.

Overall, our results reveal a new facet of GPR65 receptor signaling, and open an exciting area in understanding GPR65 and the family of proton-sensing receptors, which are understudied compared to most other families of GPCRs (Roth & Kroeze, 2015a). Whether GPR4 and GPR68 also follow a similar paradigm of uncoupled signaling and trafficking, and how this uncoupling is important for the function of these receptors in physiology, are important and exciting areas to pursue in the future.

2.5 Materials and Methods

Cell culture and transfection

Cell lines used were validated, and cells were purchased from ATCC. Cells in the lab were routinely tested for mycoplasma contamination, and only uncontaminated cells were used and maintained at 37°C with 5% CO_2. Stable clonal HEK293 cells expressing either GPR65, H10,14,243F GPR65 or B2AR N-terminally tagged with FLAG were cultured in DMEM high glucose (Cytiva, SH3024301) supplemented with 10% fetal bovine serum (FBS; Gibco, 26140079). Stable cell lines (GPR65, H10,14,243F and B2AR cells) expressing one of the constructs were generated using Geneticin (Gibco, #10131035) as selection reagent. All stable cell line plasmid transfections were conducted with Effectene (Qiagen, #301425) as per manufacturer's instructions. HEK293 cells were also transiently

transfected with GPR65, H10,14,243F or R112A GPR65 fused to Flag on its N-terminus using Effectene as per manufacturer's protocol. Jurkat cells, gifted by Dr. Adam Courtney and Yating (Christina) Zheng, were cultured in RPMI-1640 medium (Gibco, #A1049101) supplemented with 10% fetal bovine serum (FBS) and 2mM glutamine. Jurkat cells were transiently transfected at 90% confluency according to manufacturer's guidelines with TransIT-LT1 (Mirus, #MIR2300) with 1.5 ug of each DNA construct to be expressed.

DNA constructs

FLAG-GPR65 construct consists of an N-terminal signal sequence followed by a FLAG tag followed by the human GPR65 sequence in a pcDNA3.1 vector backbone. To create SNAP-GPR65, the receptor sequence was amplified from the FLAG-GPR65 construct by PCR with compatible cut sites (BamHI and XbaI) and ligated into a pcDNA3.1 vector containing an N-terminal sequence, followed by a SNAP tag. FLAG-H10,14,243F GPR65 and FLAG-R112A GPR65 DRY mutant were created with a full-length receptor sequence gene block from Integrated DNA Technologies (IDT) with restriction sites (AgeI and XbaI) compatible to FLAG-GPR65 vector backbone and ligated into a pcDNA3.1 vector containing an N-terminal sequence, followed by a FLAG tag. FLAG-B2AR construct was described previously (Bowman et al., 2016). WT Dyn and Dyn K44A were gifts from Adam Linstedt. pcDNA3.1 empty vector was a gift from Drs. Alan Smrcka and Hoa Phan.

Reagents

Leibovitz L15 imaging medium (Gibco, #21083-027) was used as the vehicle (pH 7.0) to deliver the desired pH since the buffered medium covers a wide pH range. The pH was adjusted by adding either 0.1 M HCl (Fisher Scientific, A144S-500) or 1 M NaOH (Fisher Scientific, #S318-500) and measured using pH test strips (Fisher Scientific, #13-640-502). Isoproterenol (Iso, #I5627) was purchased from Sigma Aldrich and used at 10μM from a 10 mM frozen stock. Dyngo-4a was purchased from ApexBio (#B5997), dissolved in DMSO (Fisher Scientific, #BP231-100) and used at the noted concentrations. Chloroquine was purchased from ApexBio (#B5997), dissolved in DMSO and used at 4μM. Mouse anti-FLAG M1 monoclonal antibody (Sigma Aldrich, #F3040) conjugated to Alexa 647 (Invitrogen, #A20173) and SNAP-surface dye 647 (NEB, #S9102S) were purchased from Sigma Aldrich, Invitrogen and New England BioLabs, respectively. anti-APPL1 (1:200; CST, D83H4, #3858S), anti-EEA1 (1:50; CST, C45B10, #3288), anti-Rab11 (1:50; CST, C45B10, #3288) or anti-LAMP1 (1:100; CST, D2D11 XP, #9091) rabbit monoclonal antibodies were purchased from Cell Signaling Technology (CST). Alexa 488 goat anti-rabbit secondary antibody (1:1000; #A11008) was bought from Invitrogen.

GloSensor cAMP assay

HEK293 cells (5-7 × 10^4 cells per well) were plated in a 96-well plate (Costar Corning, #3917) coated with poly-D-lysine (Sigma Aldrich, #P6407) to allow for adherence of cells. The following amounts of DNA were used per well: 60 ng of pGloSensor-22F cAMP plasmid (Promega, E2301), 100 ng of receptor or empty vector (control, pCDNA3.1$^+$). Reverse transfection was performed using Effectene. Twenty-four

hours after transfection, cells were washed once with Leibovitz L15 medium (Gibco, #21083-027), and 100 µl of 500µ g/mL D-luciferin (Goldbio, LUCK-1G) in Leibovitz's L-15 medium was added for 2 hours at room temperature. Luminescence was measured for 30-50 min using a Varioskan LUX multimode microplate reader. For basic pH experiments, cells were treated with 5 µM Forskolin (Sigma Aldrich, #F3917). For Jurkat cells, wells in a 96-well plate were coated with Collagen IV (Sigma-Aldrich, #C5533) to allow for adherence. The following amounts of DNA were used per well: 60 ng of pGloSensor-22F cAMP plasmid, 100 ng of receptor or empty vector (control, pCDNA3.1^{+}). Reverse transfection was performed using TransIT-LT1. Twenty-four hours after transfection, cells were washed once with Leibovitz L15 medium and 100 µl of 500ug/mL D-luciferin in Leibovitz's L-15 medium was added for 2 hours at room temperature. Luminescence was measured for 30-50 min using a Varioskan LUX multimode microplate reader. Raw luminescence values or the values corrected to baseline before acute changes in pH are noted as described.

Live cell imaging

All confocal live cell imaging was conducted using an Andor Dragonfly multimodal microscopy system (Andor). HEK293 cells were plated onto 25mm coverslips (Electron Microscopy Sciences, #50949050) coated with poly-D-lysine (Sigma Aldrich, #P6407) to allow for adherence. Two days later, cells were labeled with M1-647 antibody (1:1000) for 10 min and imaged in Leibovitz L15 imaging medium at 37°C in a CO_2-controlled imaging chamber, using a spinning disk confocal microscope (Andor, Belfast, UK) and a 60× objective. Confocal images were acquired using an iXon +897 electron-multiplying

charge-coupled device camera (Andor, Belfast, UK) and solid-state lasers of 488 nm or 647 nm.

Immunofluorescence of endosomal markers

HEK293 cells stably expressing FLAG-GPR65 were plated to poly-d-lysine (Sigma Aldrich) coated 12 mm glass coverslips (Fisher Scientific, #1254580P) and grown for 24-48 hr at 37 °C. Cells were labeled with M1 647 antibody (1:1000) for 10 min, exposed to either pH 7.0 or pH6.4 for 20min at 37°C, then fixed with 4% formaldehyde (FB002, Invitrogen) for 20 min at room temperature. Cells were rinsed with wash solution (PBS containing 1.25mM calcium chloride, 1.25mM magnesium chloride, with 5% FBS, 5% 1M glycine) twice and then blocked in PBS containing 1.25mM calcium chloride, 1.25mM magnesium chloride, with 5% FBS, 5% 1M glycine, and 0.75% Triton X-100. After, FLAG-GPR5 cells were incubated with either rabbit anti-APPL1 (1:200; CST, D83H4, #3858S), anti-EEA1 (1:50; CST, C45B10, #3288), anti-Rab11 (1:50; CST, C45B10, #3288) or anti-LAMP1 (1:100; CST, D2D11 XP, #9091) endosomal marker antibodies for 1hr. Cells were washed three times with PBS containing calcium and magnesium and then labeled with Alexa 488 goat anti-rabbit secondary antibody (1:1000; Invitrogen, #A11008) in a blocking buffer for 1 hr. Cells were washed three times for 5 min and coverslips were mounted onto glass slides (Fisher Scientific, #12550123) using Prolong Diamond Antifade Mountant (Invitrogen, #P36961). Confocal imaging of cells was performed using a spinning disk confocal microscope (Andor) and 100× objective. Representative images were taken across 10–20 fields per condition.

Image analysis and quantification

Stacks and time-lapse images were collected and analyzed with either FIJI or Imaris (Schindelin et al., 2012). We quantified intracellular receptors in two ways. We determined the total number of receptor spots in the cytoplasm of cells, using the Imaris software (Andor) spot's function. To determine the internal receptor fluorescence, we also analyzed images with FIJI and quantified receptor fluorescence in a region of interest corresponding to the cytosplasm of the cell as a fraction of total receptor fluorescence. For the endosomal colocalization analysis, we acquired the percent colocalization of receptor spots with the endosomal marker over the total number of receptor-positive endosomes via the Imaris software (Andor) spot's function and the MATLAB-based colocalize spots extension. To measure receptor expression across cells, we analyzed images with FIJI and quantified mean cell fluorescence in a region of interest outlining each cell. Statistical tests and graphs were generated using Prism 9 (GraphPad Software). Schematics were made using BioRender.com (Toronto).

2.6 Acknowledgments

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2.7 Figures

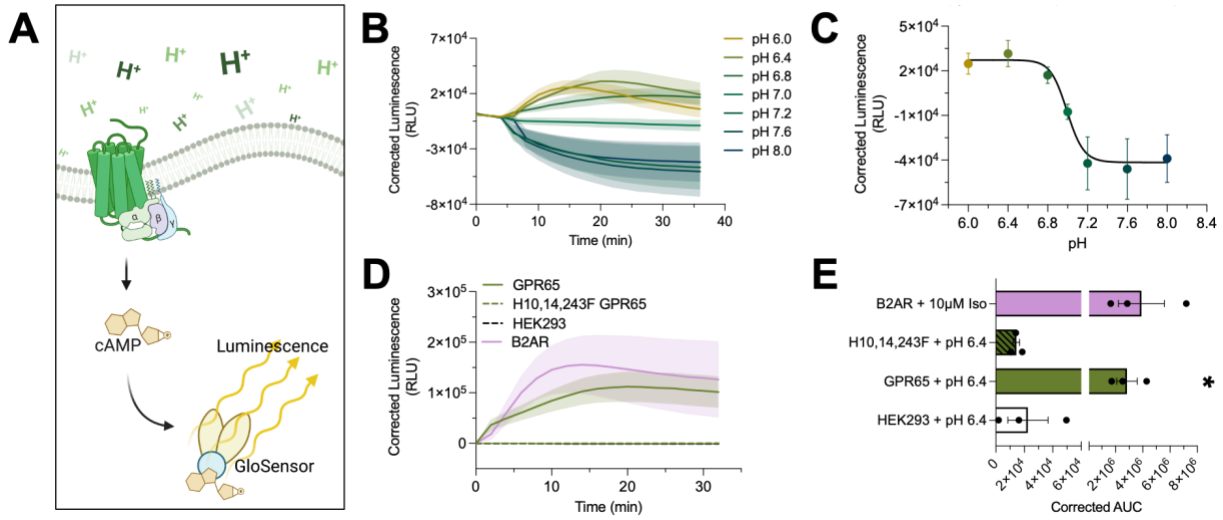

Figure 2.1 GPR65 increases cAMP levels at both neutral and acidic pH.

(**A**) Schematic diagram of the GPR65-GloSensor assay. Proton-dependent activation of GPR65 stimulates production of intracellular cAMP. cAMP binding to the GloSensor luciferase produces luminescence directly correlated to increased cAMP levels. (**B**) GloSensor luminescence over time in GPR65-expressing cells treated with pH 6.0, 6.4, 6.8, 7.0, 7.2, 7.6 or 8.0 (n=3 biological replicates). GPR65 displays elevated cAMP levels at neutral and acidic pH. (**C**) Proton-dependent changes in intracellular cAMP levels were measured by a concentration-response curve (pH 6.0-8.0) in HEK293 cells stably expressing FLAG-GPR65, and GloSensor (n=3 biological replicates, Non-linear regression fit [agonist] vs response-Variable slope). GPR65-mediated cAMP increase is saturable at pH 6.4. (**D**) GloSensor luminescence over time in HEK293 cells and HEK293 cells stably expressing FLAG-GPR65, FLAG-B2AR and FLAG-H10F, H14F, H243F-GPR65 mutant after addition of either pH 6.4 or Iso (n=3 biological replicates). Activation of GPR65 by pH 6.4 increases cAMP levels similar to positive control B2AR. (**E**) Bar graph of the area-under-curve (AUC) from figure 1D (n=3 biological replicates) (Ordinary one-way ANOVA, $p<0.05$). AUC of WT GPR65 is similar to positive control B2AR and significantly higher than negative controls.

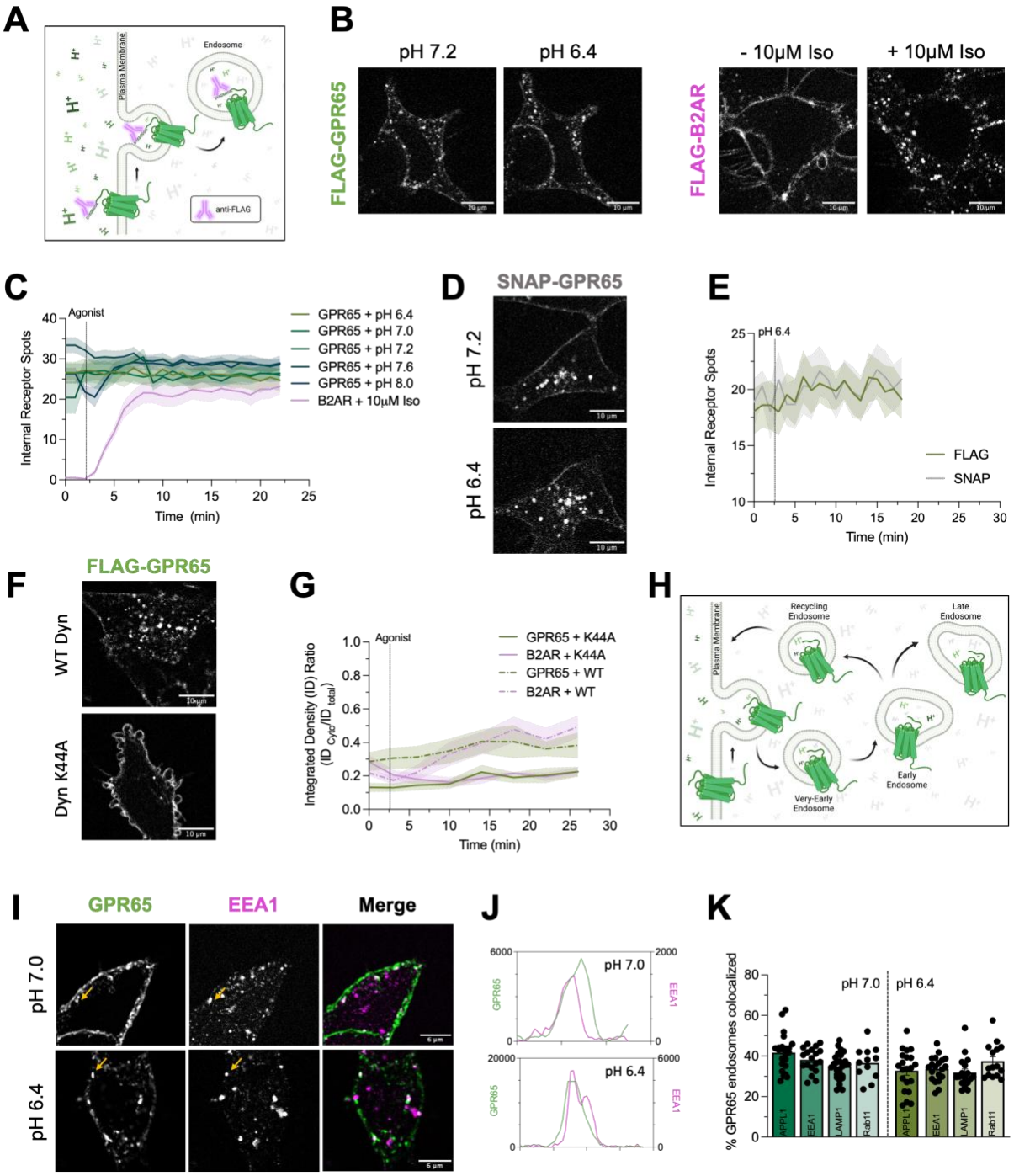

Figure 2.2 GPR65 internalization and endosomal localization is independent of extracellular pH.

(**A**) Schematic diagram of the FLAG internalization assay. A fluorescently tagged antibody will bind the FLAG tag (peptide sequence DYKDDDDK) at the N-terminus of the receptor. Immunolabeling live cells with the tagged antibody will allow for visualization of receptors that started at the PM when cells were labeled. (**B**) FLAG-tagged GPR65-expressing cells exposed to pH 7.2 (left) and pH 6.4 (right) were immunolabeled live and imaged by confocal microscopy. Localization of FLAG-GPR65 does not change noticeably. B2AR-expressing cells were immunolabeled live and imaged by confocal microscopy. After the addition of 10μM Iso, B2AR internalizes and localizes almost entirely to internal compartments. (**C**) Quantification of the

number of internal receptor spots over time (FLAG-GPR65, n=15-25 cells/condition; FLAG-B2AR, n=16 cells) (symbol indicates mean, shading is SEM). FLAG-GPR65 localization remains constant even after pH 6.4 addition. (**D**) SNAP-tagged GPR65-expressing cells exposed to pH 7.2 (top) and pH 6.4 (bottom) were immunolabeled live and imaged by confocal microscopy. Localization of SNAP-GPR65 is similar to FLAG-GPR65 (scale bars= 10 µm). (**E**) Quantification of the number of internal receptor spots over time. The FLAG-GPR65 internalization pattern is conserved in SNAP-GPR65 (SNAP-GPR65, n=15 cells; FLAG-GPR65, n=15 cells) (symbol indicates mean, shading is SEM). (**F**) FLAG-GPR65 cells expressing either dominant-negative Dyn K44A or WT Dyn were immunolabeled live and imaged by confocal microscopy. FLAG-GPR65 cells expressing Dyn K44A exhibit decreased endocytosis while cells expressing WT Dyn displayed increased internalization and endocytosis. **(G)** Quantification of the ratio of internal fluorescence over total cell fluorescence over time (WT Dyn, n=20 cells each; Dyn K44A, n=20 cells each) (symbol indicates mean, shading is SEM). FLAG-GPR65 cells expressing Dyn K44A exhibit a lower internal fluorescence while cells expressing WT Dyn display increased internal fluorescence. (**H**) Schematic of the endocytic pathway that allows for trafficking and transfer of cargoes between membrane-bound compartments. Once a membrane protein is internalized, the protein is transferred from very-early (APPL1+) endosomes to early (EEA1+) endosomes. From EEA1+ endosomes, the protein could either be inserted back into the PM via recycling endosomes (like Rab11+ endosomes) or it could be targeted for degradation via late endosomes (like LAMP1+ endosomes). (**I**) Representative confocal images of HEK293 cells expressing FLAG-GPR65 exposed to pH 7.0 or pH 6.4 for 20 min prior to fixing cells and staining for EEA1 with a 488 secondary antibody (scale bar= 6 µm). Yellow arrows denote GPR65 endosomes that colocalize with EEA1. (**J**) Fluorescence linear profile plots of GPR65 and EEA1, measured by lines drawn across regions of the cell with GPR65 endosomes after treatment with pH 7.0 or pH 6.4 for 20 min. Plots show EEA1 immunofluorescence increases along with GPR65 in both pH 7.0 and pH 6.4. (**K**) Quantitation of the percentage of GPR65 containing endosomes that colocalize with each of the endosomal markers. For this, FLAG-GPR65 cells treated with either pH 7.0 or pH 6.4 for 20 min were fixed and processed for immunofluorescence with the noted markers. GPR65 localizes in APPL1, EEA1 Rab11 and Lamp1 positive endosomes irrespective of pH (n = 21-23, 19-20, 13-16, and 17-28 fields for APPL1, EEA1, Rab11, and Lamp1, respectively).

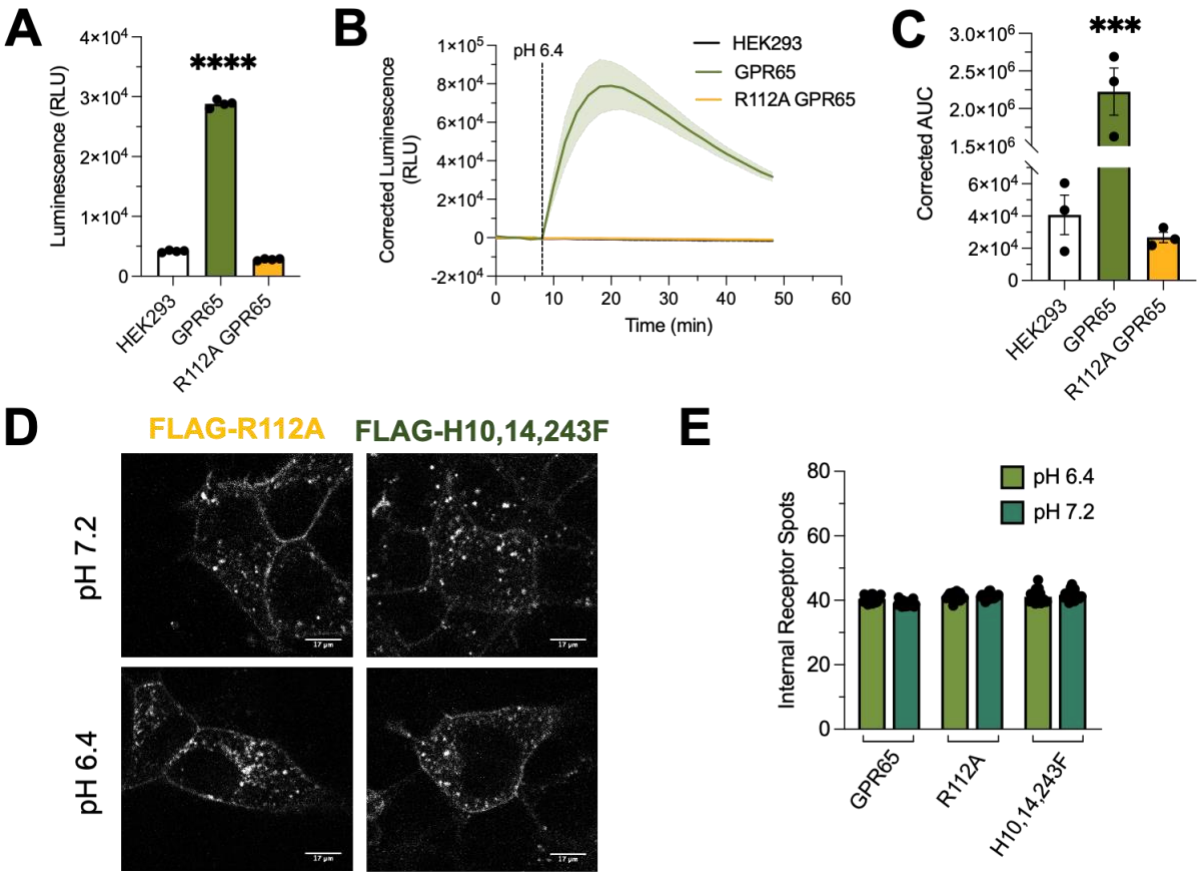

Figure 2.3 Activation of GPR65 is uncoupled from receptor trafficking.

(**A**) Luminescence at pH 7.0 of GPR65-expressing cells compared to R112A-GPR65 DRY Motif mutant and HEK293 cells (n=4 biological replicates) (Ordinary one-way ANOVA, $p<0.0001$). At neutral pH, WT GPR65 exhibits increased luminescence while R112A-GPR65 displays lower luminescence similar to HEK293. (**B**) Corrected luminescence trace of GPR65-expressing cells compared to R112A-GPR65 DRY Motif mutant and HEK293 cells exposed to pH 6.4 (n=3 biological replicates). After exposure to pH 6.4, WT GPR65 cells display increased luminescence while R112A-GPR65 DRY Motif mutant and HEK293 cells did not increase cAMP levels in response to acidic pH. (**C**) Bar graph of the area-under-curve (AUC) from figure 3B (n=3 biological replicates) (Ordinary one-way ANOVA, $p<0.05$). AUC of WT GPR65 is significantly higher than R112A-GPR65 DRY Motif mutant and the negative control, HEK293 not expressing GPR65. (**D**) FLAG-R112A and FLAG-H10,14,243F GPR65-expressing cells exposed to pH 7.2 (left) and pH 6.4 (right) were immunolabeled live and imaged by confocal microscopy. Localization of FLAG-R112A GPR65 and FLAG-H10, 14, 243F GPR65 do not change noticeably upon pH 6.4 addition. (**E**) Quantification of the number of internal receptor spots over time (FLAG-H10, 14, 243F GPR65, n=25-32 cells; FLAG-R112A GPR65, n=22-29 cells; GPR65, n=15-25 cells) (symbol indicates mean, error bars are SEM). FLAG-R112A and H10,14,243F GPR65 localization is similar to WT GPR65.

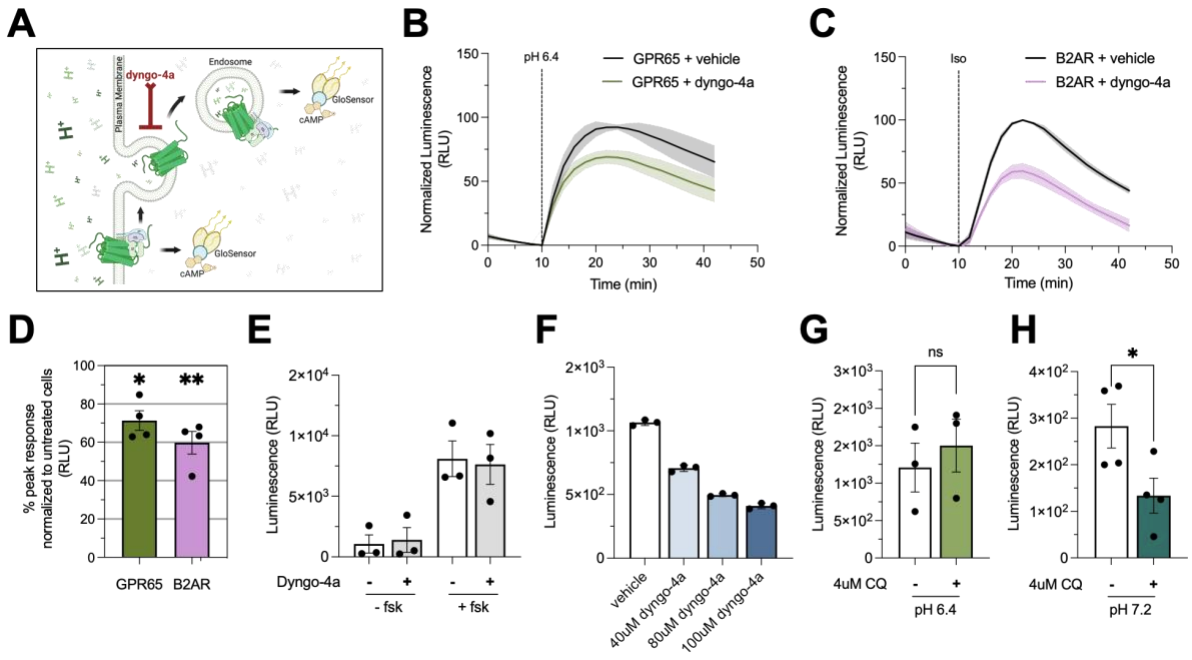

Figure 2.4 Internalized GPR65 contributes to cAMP response.

(**A**) Proton-dependent activation of GPR65 stimulates production of intracellular cAMP from multiple cellular compartments. Treatment of GPR65-expressing cells with endocytosis inhibitor Dyngo-4a will prevent internalization of the receptor leading to a decreased whole-cell cAMP output. (**B**) Corrected luminescence trace of GPR65-expressing cells pretreated with vehicle or Dyngo-4a for 15 min before addition of pH 6.4 (n=4 biological replicates). After exposure to pH 6.4, WT GPR65 cells treated with Dyngo-4a display lower luminescence and intracellular cAMP levels when compared to vehicle condition. (**C**) Corrected luminescence trace of B2AR-expressing cells pretreated with vehicle or Dyngo-4a for 15 min before addition of 10μM Iso (n=4 biological replicates). Positive control B2AR cells treated with Dyngo-4a display lower luminescence and intracellular cAMP levels when compared to vehicle condition. (**D**) Peak luminescence response of GPR65 and B2AR in cells pre-treated with Dyngo-4a, normalized to cells without Dyngo-4a (n=4 biological replicates) (Welch's t-test, $p<0.05$). WT GPR65 cells treated with Dyngo-4a display a significantly lower whole-cell cAMP output than vehicle. This decrease in GPR65 cAMP output is similar to B2AR. (**E**) Forskolin (Fsk)-stimulated cAMP accumulation in HEK293 pretreated with vehicle or Dyngo-4a (n=3 biological replicates), showing that Dyngo-4a does not directly affect cAMP accumulation. (**F**) Luminescence of GPR65 cells at pH 8.0 pretreated with vehicle or increasing concentrations of Dyngo-4a for 15 min, showing dose-dependent decrease in cAMP levels (n=3 biological replicates). (**G**) Peak cAMP luminescence response of GPR65-expressing HEK293 cells untreated or pretreated with chloroquine (CQ) for 30 minutes prior to exposure to pH 6.4, (n=3 biological replicates). CQ-treated GPR65-expressing cells display similar cAMP levels as untreated cells (Welch's t-test, $p>0.05$). (**H**) Peak luminescence response of GPR65-expressing HEK293 cells untreated or pretreated with chloroquine (CQ) for 30 minutes prior to exposure to pH 7.2 (n=4 biological replicates). GPR65 cells pre-treated with CQ display lower luminescence and intracellular cAMP levels when compared to untreated cells (Welch's t-test, $p<0.05$).

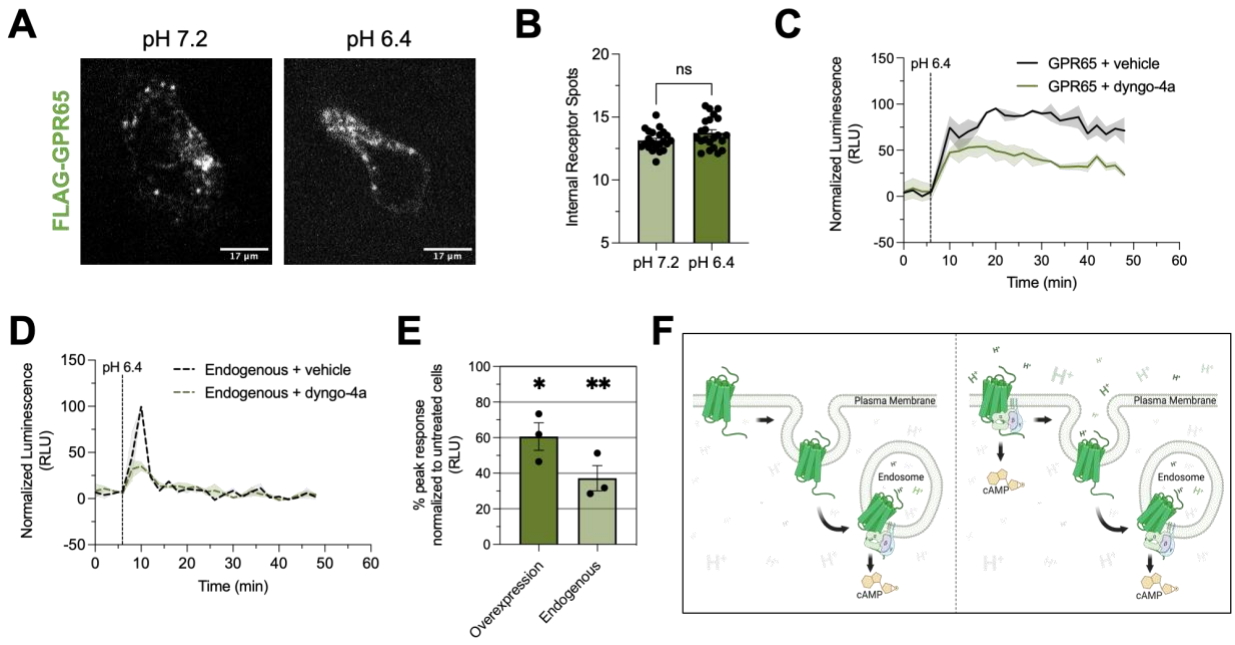

Figure 2.5 Internalized GPR65 contributes to cAMP signaling in Jurkat T cells.

(**A**) FLAG-tagged GPR65-expressing Jurkat cells exposed to pH 7.2 (left) and pH 6.4 (right) were immunolabeled live and imaged by confocal microscopy (Scale bar=17µm). Localization of FLAG-GPR65 does not change noticeably upon pH 6.4 addition. (**B**) Quantification of the number of internal FLAG-GPR65 spots at pH 7.2 and pH 6.4 in Jurkat cells, showing that the number of internal puncta do not differ between the two conditions (n=16) (Welch's t-test, $p>0.05$). (**C**) Corrected luminescence trace of FLAG-GPR65-expressing cells pretreated with vehicle or Dyngo-4a for 15 min before the addition of pH 6.4 (n=3 biological replicates). After exposure to pH 6.4, GPR65-expressing cells treated with Dyngo-4a display lower luminescence and intracellular cAMP levels when compared to vehicle conditions. (**D**) Corrected luminescence trace of plain Jurkat cells expressing endogenous GPR65 pretreated with vehicle or Dyngo-4a for 15 min before addition of pH 6.4 (n=3 biological replicates). After exposure to pH 6.4, plain Jurkat cells treated with Dyngo-4a display lower luminescence and intracellular cAMP levels when compared to vehicle conditions. (**E**) Peak luminescence response of FLAG-GPR65 and endogenous GPR65-expressing Jurkat cells with or without Dyngo-4a pretreatment (n=3 biological replicates) (Welch's t-test, $p<0.05$). GPR65-expressing cells treated with Dyngo-4a display a significantly lower whole-cell cAMP output than vehicle. (**F**) A model for location-switching of GPR65 signaling triggered by extracellular pH. WT GPR65 is constitutively (i.e. in a ligand- and activation-independent manner) internalized and localized to internal compartments even when the extracellular environment is at a basic pH. In the acidic environment of the endosome, endosomal GPR65 becomes activated and tonically increases cAMP levels. An acidic extracellular environment activates surface GPR65 increasing total cAMP levels, and switching the majority of cAMP production to the plasma membrane.

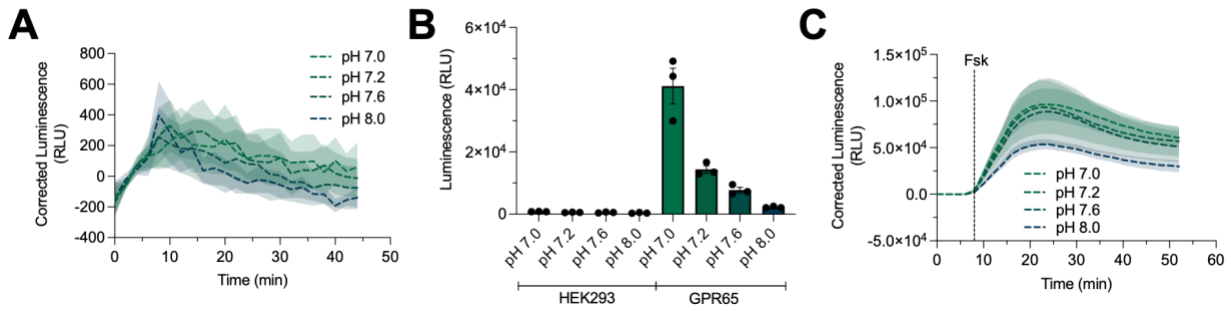

Figure supplement 2.1 Activation of GPR65 requires high proton concentrations.

(**A**) GloSensor luminescence trace in plain HEK293 cells exposed to a pH range from pH 7.0, to 8.0, after baseline readings (n=3 biological replicates). Exposure to a pH range from pH 7.0 to 8.0 over time does not significantly affect luminescence readings in HEK293 cells not transfected with GPR65 (untransfected). (**B**) GloSensor luminescence after two hours of neutral and basic pH (pH7.2, 7.6 and 8.0) pretreatment in HEK293 and GPR65-expressing HEK293 cells (n=6 biological replicates). Luminescence in GPR65-expressing cells depends on proton availability. (**C**) Forskolin (Fsk)-stimulated cAMP accumulation in untransfected HEK293 chronically treated with a pH range from 7.0 to 8.0 (n=3 biological replicates). Fsk-induced GloSensor luminescence in untransfected HEK293 does not change in the dose-responsive pH range up to 7.6, although a slight decrease is observed at pH 8.0.

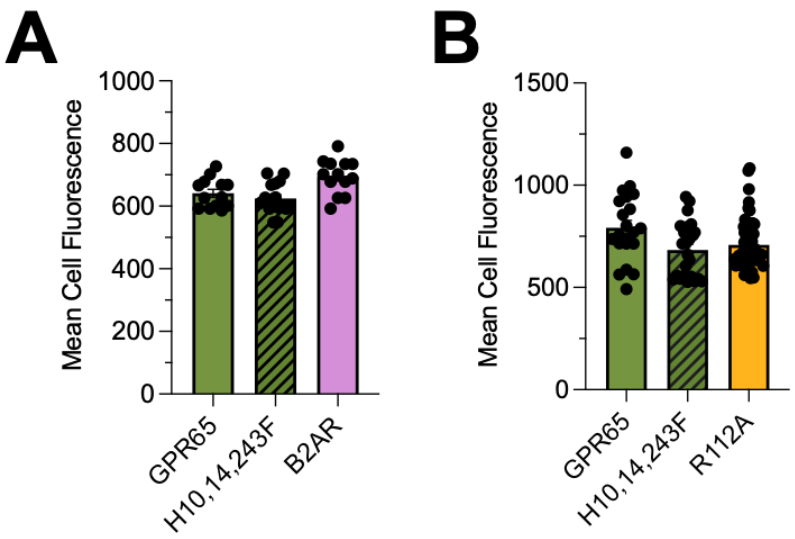

Figure supplement 2.2 Receptor expression is comparable across cells used for analysis.

(**A**) Mean cell fluorescence measurements in confocal images of HEK293 cells stably expressing either WT GPR65, H10,14,243F receptor mutant, or B2AR. (**B**) Mean cell fluorescence measurements in confocal images of HEK293 cells transiently expressing either WT GPR65, H10,14,243F receptor mutant, or R112A. The expression of receptors is comparable across the populations of cells that were compared.

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Chapter 3 GPR65 Activates Diverse and Distinct Signaling Pathways in Different Subcellular Locations

3.1 Abstract

GPR65 is a prototype for proton-sensing GPCRs, which are implicated in many physiological processes and are emerging as attractive drug targets to modulate the immune response and treat cancer. Despite their physiological importance, how acidic extracellular environments change the signaling patterns of proton-sensing GPCRs, and how this signaling differs in different subcellular locations are two understudied fundamental questions for this family of GPCRs. Here, we report that both surface and endosomal GPR65 stimulate cAMP production through a non-canonical soluble adenylyl cyclase (s-AC)-dependent mechanism. GPR65-mediated cAMP production requires distinct signaling effectors at different subcellular locations. cAMP generated by surface GPR65 requires Gs, EPAC, and PLC, while cAMP generated by internalized receptors requires PLC activation dependent on the release of Gβγ subunits. We propose a model where activation of GPR65 elicits diverse and distinct signaling pathways at different subcellular locations. This study will clarify how receptor location inside the cell is intricately linked to receptor signaling and lay the groundwork for one day targeting receptor location to influence cellular responses, with greater efficacy and fewer adverse effects for patients suffering from life-threatening diseases such as cancer.

3.2 Introduction

An exciting and emerging idea in G protein-coupled receptor (GPCR) biology indicates that signals originating from the plasma membrane (PM) have distinct profiles from those generated at intracellular membranes (Bowman et al., 2016; Irannejad et al., 2013; Lobingier & von Zastrow, 2019; Stoeber et al., 2018; Sutkeviciute & Vilardaga, 2020; Thomsen et al., 2018; Vilardaga et al., 2014; Weinberg & Puthenveedu, 2019). The intracellular location of GPCR signaling can shape the cellular response temporally by prolonging its overall duration (Lohse & Calebiro, 2013; Stoeber et al., 2018) and may shape the response spatially by moving the location of the intracellular second messenger relative to effectors.

Signaling from intracellular compartments, like endosomes, is especially relevant for proton-sensing GPCRs because these compartments are acidic environments in which proton-sensing GPCRs are uniquely active and signal (Hu et al., 2015). Like most GPCRs, proton-sensing GPCRs couple to G proteins at the PM, are internalized in the cell, and trafficked through the endolysosomal system (Lan et al., 2014; Tan et al., 2018). These membrane receptors have generated interest as potential therapeutic targets to treat cancer as they are highly overexpressed in many solid tumors and are thought to modify tumor signaling and the immune response (Imenez Silva & Wagner, 2022; Klatt et al., 2020; Silva et al., 2022; Sisignano et al., 2021; Wiley et al., 2019; Wun et al., 2004). Proton-sensing receptor location at subcellular compartments is a novel clinically valuable tool to influence cellular responses in tumor signaling and immune cell function (Crilly & Puthenveedu, 2021; Lobingier & von Zastrow, 2019; Morales Rodríguez et al., 2023; Weinberg et al., 2019).

Previously we have shown that prototypical proton-sensing receptor GPR65 is localized to endosomal compartments and stimulates cAMP production from endosomes independent of extracellular pH changes (Morales Rodríguez et al., 2023). Endosomal GPR65 sets the basal cAMP tone, and acidic activation of surface GPR65 further increases whole-cell cAMP levels. Considering GPR65 at endosomes is constitutively active irrespective of extracellular pH, how localization to distinct cellular compartments influences the GPR65 signaling profile is an unanswered question critical to understanding the physiological role of GPR65.

Here we use proton-sensing receptor GPR65 as a prototype to study how receptor location influences proton-sensing receptor signaling. We show that GPR65 increases intracellular second messenger cAMP presumably via two distinct signaling pathways which require soluble adenylyl cyclase (s-AC) activation. s-AC is an alternative source of cAMP mediated by activation of GPR65. We also show that cAMP production by surface GPR65 requires Gs, EPAC, and PLC, while cAMP generated by internalized receptors requires PLC activation dependent on the release of Gβγ subunits. Our results suggest a model where proton-dependent GPR65 activation elicits diverse and distinct signaling pathways at different cellular locations. Characterizing the GPR65 signaling profile at multiple cellular locations will improve our understanding of how proton-sensing receptors integrate cellular responses, and how these responses influence physiology and disease states.

3.3 Results

3.3.1 GPR65 is constitutively active and signals from endosomes.

To visualize GPR65 activation at endosomes, we pretreated HEK293 cells stably expressing GPR65 with basic pH (pH 7.2) and the endocytosis inhibitor Dyngo-4a, and measured cAMP signaling via GloSensor luminescence before and after pH 6.4 addition (**Figure 3.1A**). We used the cAMP biosensor GloSensor, which exhibits increased luminescence when bound to cAMP, to quantitatively measure cAMP levels (Fan et al., 2008; Wang et al., 2021). Upon exposure to acidic pH, inhibition of GPR65 endocytosis by Dyngo-4a reduced total cAMP luminescence when compared to vehicle-treated cells (**Figure 3.1B and D**). At basic extracellular pH, GPR65 displayed lower luminescence with increasing concentrations of endocytosis inhibitor, suggesting internalized GPR65 is the primary source of basal cAMP signaling (**Figure 3.1C**). Similarly, cells exposed to pH 6.4 and [Dyngo-4a] (40, 80, 100 μM) showed a dose-dependent inhibition of cAMP luminescence (**Figure 3.1D**). Because Dyngo-4a can be toxic to cells, we quantified cell viability after exposing cells in basic pH media to 100μM Dyngo-4a. Incubation of cells in basic pH media with Dyngo-4a suggests the observed findings are not a product of cell death but rather inhibition of endocytosis (**Figure supplement 3.1**). Together, these data indicate GPR65 is constitutively active at endosomes irrespective of extracellular pH and that internalized GPR65 is the primary source of basal cAMP signaling at basic extracellular pH.

We also asked whether engineered fluorescently tagged mini-G protein variants and nanobody biosensors report active GPR65 conformations at endosomes. mini-G protein variants are recruited to active receptors able to interact with G proteins, these variants mimic the interaction of the G protein with an active receptor (**Figure supplement 3.2A**) (Carpenter, 2018; Nehmea et al., 2017; Wan et al., 2018). To visualize

active GPR65 at endosomes, we first expressed mini-G_s proteins, in HEK293 FLAG-GPR65 stable cells, immunolabeled live, and imaged cells by confocal microscopy. mini-Gs variants are recruited to active receptors able to couple to stimulatory Gs proteins. mini-$G_{s/i}$ proteins, which are recruited to active receptors able to couple to inhibitory G proteins like Gi, were expressed in GPR65-expressing HEK293 stable cells as a negative control (Nehmea et al., 2017; Wan et al., 2018). Surprisingly, GPR65 at endosomes was able to recruit mini-$G_{s/i}$ protein variants and not mini-G_s (**Figure supplement 3.2B and C**). This result was unexpected given that a large body of GPR65 literature suggests the receptor increases cAMP levels via Gs.

To directly test whether GPR65 activates Gs proteins, we expressed nanobody Nb37-GFP, which selectively binds to active conformations of the Gs protein subunits (**Figure supplement 3.2D**) (Irannejad et al., 2013), in HEK293 FLAG-GPR65 stable cells, and imaged cells by confocal microscopy. Confocal images revealed GPR65 does not recruit Nb37-GFP, the biosensor for active Gs proteins **(Figure supplement 3.2E)**. Because mini-$G_{s/i}$ protein variants were recruited to active receptors, we thought GPR65 might be increasing intracellular second messenger cAMP through a Gs-independent mechanism. Gi-coupled receptors increase cAMP levels in neutrophils. Neutrophil polarization and migration require activation of pertussis-sensitive Gi-coupled receptors and release of Gβγ subunits followed by activation of adenylyl cyclase (AC) 9 to increase cAMP levels (**Figure supplement 3.2F**) (Liu et al., 2010, 2014; Mahadeo et al., 2007). To test whether the increase in cAMP levels required Gi coupling, we measured cAMP luminescence using the GloSensor and incubated cells overnight with the Gi protein inhibitor Pertussis toxin (PTX) **(Figure supplement 3.2F)**. GPR65-expressing cells

exposed to PTX, and low pH, displayed a higher increase in cAMP suggesting PTX-sensitive Gi proteins are not required for cAMP production **(Figure supplement 3.2G)**. As expected, fsk-induced GloSensor luminescence decreased in MOR-expressing cells, positive control for PTX-mediated Gi inhibition, following the addition of agonist DAMGO. Together, these data suggest GPR65 is actively signaling at endosomes but is unable to recruit biosensors for Gs protein subunit activity.

3.3.2 GPR65 signals through Gs and Gβγ protein subunits.

Because GPR65 was unable to recruit biosensors for Gs protein activity, we wanted to specifically test the requirement of Gs for cAMP signaling by GPR65. We expressed GPR65 in Gs-knock out (KO) HEK293T cells. Gs-KO eliminated the cAMP luminescence response upon exposure to acidic pH. Gs rescue in Gs-KO cells expressing GPR65 displayed an increase in cAMP luminescence similar to HEK293T cells expressing GPR65 and endogenous Gs (**Figure 3.2A and B**). This GPR65-mediated cAMP increase is similar to the prototypical Gs-coupled GPCR beta-2 adrenergic receptor (B2AR), which we used as a positive control for Gs activation (**Figure 3.2C and D**).

We next tested whether Gβγ was required for GPR65-meditated cAMP production to rule out the possibility of Gβγ subunits being involved. Gβγ subunits are major transducers of GPCR-dependent immune cell migration. Activation of Gi-coupled receptors and release of Gβγ subunits are required to initiate cAMP signaling through the direct activation of phosphatidylinositol 3-kinase γ (PI3Kγ) (Li et al., 2000; Stephens et al., 1994) and the subsequent generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which ultimately sets up a positive feedback loop involving guanine nucleotide

exchange factors that result in polarized accumulation of PIP3, actin polarization, and cell migration (Li et al., 2003; Surve et al., 2016; Yan et al., 2012). To test whether GPR65 requires Gβγ subunits to increase cAMP, we pretreated cells with the Gβγ inhibitor Gallein (Bonacci et al., 2006; Lehmann et al., 2008) for 30 minutes prior to measuring cAMP luminescence. Inhibition of Gβγ reduced cAMP response when compared to structurally similar, inactive analog, fluorescein-treated cells (**Figure 3.2E and F**). Together, these data demonstrate that GPR65 requires Gs and Gβγ protein subunits to increase cAMP.

3.3.3 Soluble adenylyl cyclase (s-AC) is involved in GPR65-mediated cAMP production.

The involvement of Gs and Gβγ subunits raised the possibility that GPR65 activates non-canonical signaling pathways to regulate cAMP. In the canonical cAMP pathway, activated GPCR couples to heterotrimeric Gs proteins to stimulate one or more isoforms of transmembrane-AC at the PM. The synthesized cAMP binds and activates a set of effectors, phosphorylates transcription factors, and initiates transcription of cAMP-specific genes. An additional non-canonical source of cAMP is soluble-AC (s-AC, AC10) which, unlike tm-AC which requires Gs, is activated by bicarbonate and Ca^{2+} (Pozdniakova & Ladilov, 2018). To test the possibility of a non-canonical s-AC-dependent cAMP pathway mediated by GPR65, we first measured cAMP signaling via GloSensor luminescence and exposed GPR65-expressing HEK293 cells to inhibitors of AC activity. We used 2', 5'-Dideoxyadenosine (dda), or SQ 22536 to inhibit transmembrane (tm)-AC, and KH7 or LRE1 to inhibit soluble (s)-AC (Caldieri & Sigismund, 2016; Ivonnet et al., 2015; Kriebel et al., 2018; Pizzoni et al., 2017). Exposure of GPR65-expressing cells to s-AC inhibitor KH7 following acidic pH results in a faster cAMP decay than vehicle-treated

cells, very different from our control B2AR, which requires tm-AC to increase cAMP levels in cells (**Figure 3.3A**). Additionally, we preincubated with AC inhibitors prior to measuring GloSensor luminescence in GPR65-expressing cells. The cAMP response of tm-AC inhibitor-treated GPR65-expressing cells was similar to vehicle-treated cells. GPR65 cells treated with s-AC inhibitors displayed a significant reduction in total cAMP response (**Figure 3.3B-E**). Together, these data demonstrate that s-AC but not tm-AC is involved in the increase in cAMP production after pH 6.4 addition.

3.3.4 Distinct signaling effectors -PLC and EPAC- are involved in GPR65 cAMP production.

Activation of EPAC (exchange protein activated by 3'-5'-cyclic adenosine monophosphate [cAMP]) and PLC (phospholipase C) enzymes promote signaling cascades that result in profound cellular changes. It is known that cAMP-binding to EPAC drives PLC activation to raise intracellular calcium ($[Ca^{2+}]i$) via Ca^{2+} store release (Ivonnet et al., 2015; Smrcka, 2015). An increase in $[Ca^{2+}]i$ leads to s-AC activation further increasing the prostaglandin E_2 receptor 4 (EP4) cAMP response (Ivonnet et al., 2015). Similarly, beta-adrenergic receptors (BARs) activation triggers an EPAC-PLC-dependent cAMP signaling pathway that targets presynaptic release machinery and regulates cardiac hypertrophy (Ferrero et al., 2013; Nash et al., 2019; Oestreich et al., 2009; Smrcka, 2015). To tease out the signaling pathways involved in GPR65-cAMP production (**Figure 3.4A**), we pretreated GPR65-expressing HEK293 cells with EPAC and PLC inhibitors prior to measuring cAMP luminescence. To test whether EPAC, a downstream effector of cAMP, increased cAMP levels in GPR65-expressing cells, we pretreated

GPR65-expressing HEK293 cells with selective EPAC inhibitor ESI-09 (5 μM) (Zhu et al., 2015) prior to measuring cAMP luminescence. Exposure of GPR65-expressing cells to ESI-09 and acidic pH reduced cAMP production when compared to vehicle-treated cells (**Figure 3.4B**). GPR65 cells treated with s-AC and EPAC inhibitors displayed a reduction in total cAMP response after pH 6.4 addition. The cAMP response of cells treated with tm-AC and EPAC inhibitors was similar to vehicle-treated cells. Exposure of GPR65-expressing cells to the PLC inhibitor U73122 (20μM) (Thompson et al., 1991) prior to acidic pH reduced cAMP production when compared to negative control U73343 (20μM), an analog of U73122 (**Figure 3.4C**). GPR65 cells treated with s-AC and PLC inhibitors displayed a reduction in total cAMP response after pH 6.4 addition. The cAMP response of cells treated with tm-AC and PLC inhibitors was similar to vehicle-treated cells. Together, these data demonstrate the involvement of EPAC and PLC effectors to increase cAMP production after pH 6.4 addition.

3.3.5 GPR65 activates distinct signaling pathways in different subcellular locations.

Because GPR65 is constitutively active and increases cAMP from endosomes, we wondered whether the cAMP signaling pathway differed upon subcellular location. To determine the subcellular cAMP signaling pathway differences, we pretreated GPR65-expressing HEK293 cells with Gβγ, AC, EPAC, or PLC inhibitors prior to measuring cAMP luminescence at basic extracellular pH. Basic extracellular pH will bias the location of cAMP signaling to endosomes and allow us to compare with acidic extracellular pH, which biases the location of cAMP signaling to the surface. At basic extracellular pH, exposure of GPR65-expressing cells to PLCi U73122 results in reduced GPR65-mediated cAMP

signaling (**Figure 3.5A**). Similarly, pretreatment with s-ACi KH7 decreased cAMP output at basic pH (**Figure 3.5A and B**). However, tm-AC inhibition at basic extracellular pH had a similar cAMP response as negative controls, vehicle-treated and analog-treated cells (**Figure 3.5A and B**). Exposure of GPR65-expressing cells to EPAC inhibitor ESI-09 did not reduce cAMP signaling at basic pH (**Figure 3.5B**). When exposed to acidic pH, cells treated with EPACi ESI-09 displayed a reduction in cAMP luminescence (**Figure 3.4B**). Gβγ inhibition by Gallein results in a significant reduction in cAMP luminescence (**Figure 3.5C**). Together, these data suggest a model where GPR65 activates diverse and distinct signaling pathways in different subcellular locations (**Figure 3.5D**). Internalized GPR65 signaling requires the release of Gβγ subunits to activate PLC and promote cAMP production via s-AC. Instead, surface GPR65 requires Gs, EPAC, and PLC activity to increase s-AC-dependent cAMP.

3.4 Discussion

Here we introduce s-AC as an alternative source of cAMP mediated by proton-dependent activation of GPR65 at the surface and endosomes. We also show that cAMP generated by surface GPR65 requires Gs, EPAC, and PLC, while cAMP generated by internalized receptors involves PLC activation dependent on the release of Gβγ subunits. Our results suggest a model where proton-dependent GPR65 activation elicits diverse and distinct signaling pathways at different subcellular locations. The functional effects of compartmentalized GPR65 signaling are not clear, but if it's like other mammalian GPCRs, endosome-generated cAMP can result in gene transcriptional responses with distinct cellular functions than surface-generated cAMP.

To our knowledge, we show for the first time the role of s-AC in proton-sensing receptor signaling. The role of s-AC in GPCR signaling is an emerging concept (Caldieri & Sigismund, 2016; Inda et al., 2016; Ivonnet et al., 2015; Pizzoni et al., 2017). s-AC is a unique AC due to its activation being dependent on bicarbonate and calcium (Han et al., 2005; Jaiswal & Conti, 2003). Unlike tm-ACs, s-ACs are cytosolic proteins with no membrane-spanning domains responsible for cAMP-mediated cellular functions (Pozdniakova & Ladilov, 2018; Schmid et al., 2014). The corticotropin-releasing hormone receptor 1 (CRHR1) engages s-AC activation, in addition to tm-AC activation. In hippocampal neuronal HT22 cells, CRHR1 activation elicits s-AC-dependent cAMP production predominantly from the endosomes (Inda et al., 2016). The CRHR1-mediated cAMP production at endosomes requires s-AC activation, and cAMP binding to EPAC leads to a sustained, late-phase ERK1/2 activation (Inda et al., 2016). These CRHR1 findings were the first to revise the canonical model in which GPCR-elicited cAMP production relies solely on tm-ACs. Like CRHR1, GPR65 cAMP production requires non-canonical ACs. However, this GPR65-cAMP increase occurs predominantly through s-AC and not tm-AC. Apart from CRHR1, other GPCRs such as prostaglandin receptors have been shown to require an EPAC-mediated s-AC-dependent cAMP amplification pathway (Ivonnet et al., 2015). In addition to activating a s-AC-dependent cAMP amplification pathway, the GPR65-mediated cAMP production pathway was also shown to differ upon subcellular location and involves Gs and non-Gs protein effectors.

cAMP production following GPR65 activation requires distinct downstream signaling effectors. GPR65-cAMP production relies on cAMP-binding to EPAC and PLC stimulation. Acidic extracellular pH activates surface GPR65 increasing cAMP levels via

Gs, EPAC, PLC, and s-AC activation. While endosome-generated cAMP requires Gβγ to stimulate PLC and increase cAMP through s-AC. Like GPR65, other mammalian Gs-coupled receptors require cAMP-binding to EPAC to stimulate PLC, increase $[Ca^{2+}]i$ release, and activate s-AC. Prostaglandin E_2 receptor 4 (EP4) activation has been shown to increase cAMP levels through the traditional Gs -tm-AC pathway, and while increasing $[Ca^{2+}]i$ release to promote s-AC activation (Ivonnet et al., 2015). Stimulation of the EP4 receptor was previously shown to activate EPAC and inhibition of EPAC supported a role for EPAC in increasing $[Ca^{2+}]i$ that results in stimulation of both s-AC and tm-AC. Another Gs-coupled receptor known to stimulate PLC, elicit $[Ca^{2+}]i$ release and s-AC-dependent cAMP production is the thyroid-stimulating hormone receptor (TSHR). TSHR increases cAMP levels at three different waves and subcellular locations (Pizzoni et al., 2017). Following TSHR surface-mediated cAMP production, cAMP generated in the endocytic compartment (second cAMP wave) triggers PLC-mediated Ca^{2+} release from the endoplasmic reticulum (ER) through the inositol triphosphate receptor (InsP3R). Unlike cytosolic cAMP generated by TSHR, Ca^{2+} can reach the nuclear compartment and rapidly activate local s-AC which leads to transcription of cAMP-dependent genes and thyroid cell proliferation (Pizzoni et al., 2017). Internalized B2AR is another GPCR whose subcellular location influences physiology. B2AR protects against hypertrophy through the inhibition of PLC epsilon signaling at the Golgi apparatus (Wei & Smrcka, 2023). Localization of B2AR at endosomes and activation of Gi, Gβγ subunit signaling at endosomes, and ERK activity are required to inhibit PLCe activity (Wei & Smrcka, 2023). The cardioprotective effect of B2AR inhibits the detrimental cardiac remodeling signaling of B1AR at the Golgi apparatus (Nash et al., 2019) resulting in decreased phosphorylation

and protection against cardiac hypertrophy. THSR and B2AR exemplify how GPCR cAMP signaling can differ upon subcellular location and require distinct downstream signaling effectors to generate distinct gene transcriptional responses and cellular functions.

It is known GPCR cAMP production does not solely rely upon Gs protein activation (Ivonnet et al., 2015; Liu et al., 2010, 2014; Subramanian et al., 2018). Prostaglandin E_2 receptor 1 (EP1), a Gq-coupled receptor, was shown to amplify the cAMP/PKA pathway by increasing $[Ca^{2+}]i$ resulting in s-AC and tm-AC activation (Ivonnet et al., 2015). PTX-sensitive Gi-coupled receptors increase cAMP levels in neutrophils. Increased cAMP levels in neutrophils occur mainly through the release of Gβγ followed by activation of tm-AC 9 (Liu et al., 2010, 2014; Subramanian et al., 2018). Gβγ plays a significant role in immune cell migration through the direct activation of PI3Kγ (Li et al., 2000; Stephens et al., 1994) and guanine nucleotide exchange factors (Li et al., 2003; Surve et al., 2016; Yan et al., 2012). This Gβγ-dependent activation of PI3Kγ and the subsequent generation of PIP3 set up a positive feedback loop that ultimately results in the polarized accumulation of PIP3, actin polarization, and formation of the leading edge of the cell (Li et al., 2003; Surve et al., 2016; Yan et al., 2012). In our study, the data suggest a model where GPR65 presents Gs coupling at the cell surface. How surface GPR65 increases cAMP at the PM following activation by acidic extracellular pH remains unclear. We speculate that tm-AC activation is required for initial cAMP production, but small amounts of tm-AC generated cAMP are enough to amplify the surface GPR65 signaling pathway. In addition, our data suggest GPR65 requires the release of Gβγ subunits to activate PLC and s-AC at endosomes. To confirm whether internalized GPR65 does not require Gs,

the rescue of Gs activity in Gs-KO cells and pretreatment of cells at basic pH can provide stronger evidence to support Gs mediating surface and not endosome-generated cAMP.

Our present findings reveal GPR65 increases intracellular second messenger cAMP presumably via two distinct signaling pathways involving soluble adenylyl cyclase (s-AC) activation. GPR65-mediated cAMP production from endosomes requires distinct signaling effectors than cAMP production at the PM but whether these two cAMP sources result in different physiological roles is not known. Future work needs to focus on defining factors that modulate the spatial organization of the GPR65-mediated cAMP response which could have significant physiological implications. Understanding the role of G protein– and endosome-dependent signaling, together with the identification of compounds with functional selectivity, may result in clinically valuable tools for diseases involving proton-sensing receptor GPR65.

3.5 Materials and Methods

DNA constructs

B2AR, MOR, and GPR65 N-terminally tagged with Flag were cloned as described previously (Morales Rodríguez et al., 2023; Soohoo & Puthenveedu, 2013). Nb37-GFP was a gift from Dr. Mark Von Zastrow. Venus mini$G_{s/i}$ and Venus-miniG_s were gifts from Dr. Greg Tall and Nevin Lambert. pcDNA3.1+ and Gs short chain were gifts from Dr. Alan Smrcka and Dr. Hoa Phan.

Cell culture, transfection, and reagents

HEK293 cells were purchased from ATCC and maintained at 37°C with 5% CO_2. Cells in the lab were routinely tested for mycoplasma contamination, and only uncontaminated cells were used. Stable clonal HEK293 cells expressing B2AR, MOR, or GPR65, N-terminally tagged with Flag were cultured in DMEM high glucose (Cytiva, SH3024301) supplemented with 10% fetal bovine serum (FBS; Gibco, 26140079). Stable cell lines expressing one of the constructs were generated as previously described (Morales Rodríguez et al., 2023; Soohoo & Puthenveedu, 2013). HEK293T and Gs-KO HEK293T (gifts from Dr. Alan Smrcka and Dr. Hoa Phan) were also transiently transfected with FLAG-B2AR and FLAG-GPR65 using Effectene as per manufacturer's protocol. Leibovitz L15 imaging medium (Gibco, #21083-027) was used as the vehicle (pH 7.0) to deliver the desired pH since the buffered medium covers a wide pH range. The pH was adjusted by adding either 0.1 M HCl (Fisher Scientific, A144S-500) or 1 M NaOH (Fisher Scientific, #S318-500) and measured using pH test strips (Fisher Scientific, #13-640-502). Isoproterenol (Iso, #I5627) and DAMGO (#E7384) were purchased from Sigma Aldrich and used at 10μM from a 10 mM frozen stock. Dyngo-4a was purchased from ApexBio (#B5997), dissolved in DMSO (Fisher Scientific, #BP231-100) and used at 40, 80 and 100 μM. For Gi recruitment experiments, cells were treated with 5 μM Forskolin (Sigma Aldrich, #F3917) and pertussis toxin (Sigma Aldrich, #P2930, PTX (100 ng/ml, overnight treatment). Mouse anti-FLAG M1 monoclonal antibodies (Sigma Aldrich, #F3040) conjugated to Alexa 647 (Invitrogen, #A20173) were purchased from Sigma Aldrich and Invitrogen, respectively. ESI-09 (#4773), Gallein (#3090), U73122 (#1268), U73343 (#4133), and Fluorescein sodium salt (#F6377), KH7 (#K3394), LRE1

(#SML1857), dda (#288104), and SQ 22536 (#568500) were purchased from Tocris Bioscience and Sigma Aldrich, respectively.

GloSensor cAMP assay

HEK923 cells (5-7 × 10^4 cells per well) were plated in a 96-well plate (Costar Corning, #3917) coated with poly-D-lysine (Sigma Aldrich, #P6407) to allow for adherence of cells. The following amounts of DNA were used per well: 40 or 20 ng of pGloSensor-22F cAMP plasmid (Promega, E2301), 40 or 50 ng for GPR65 or empty vector, and B2AR, or empty vector (control, pCDNA3.1$^+$) respectively. Reverse transfection was performed using Effectene. Twenty-four hours after transfection, cells were washed once with Leibovitz L15 medium (Gibco, #21083-027), and 100 µl of 500ug/mL D-luciferin (Goldbio, LUCK-1G) in Leibovitz's L-15 medium was added for 2 hours at room temperature. Luminescence was measured for 30-50 min using a Varioskan LUX multimode microplate reader. Raw luminescence values or the values corrected to baseline before acute changes in pH are noted as described.

Live cell imaging

All confocal live cell imaging was conducted using an Andor Dragonfly multimodal microscopy system (Andor). Cells were plated onto 25mm coverslips (Electron Microscopy Sciences, #50949050) coated with poly-D-lysine (Sigma Aldrich, #P6407) to allow for adherence. Two days after transfection, non-permeabilized cells were labeled with M1-647 antibody (1:1000) for 10 min to label surface receptors and imaged in Leibovitz L15 imaging medium with desired pH at 37°C in a CO-controlled imaging

chamber, using a spinning disk confocal microscope (Andor, Belfast, UK) and a 60× objective. Confocal images were acquired with a 20% laser power for a total of 22. minutes with an iXon +897 electron-multiplying charge-coupled device camera (Andor, Belfast, UK) and solid-state lasers of 488 nm or 647 nm.

Image quantification, statistics, and data analysis

Stacks and time-lapse images were collected as TIFF images and analyzed with FIJI and Imaris (Zürich). To determine recruitment of biosensors to active receptor, we acquired the percent colocalization of receptor spots with the biosensors over the total number of receptor-positive endosomes via using an ImageJ Macro: Object.picker (Weinberg, 2020; doi.10.5281/ zenodo.3811031). Statistical tests and graphs were generated using Prism 9 (GraphPad Software). Exact p values, statistical tests used, and sample sizes are provided in the figure legends. Schematics were made using BioRender.com (Toronto).

3.6 Acknowledgments

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3.7 Figures

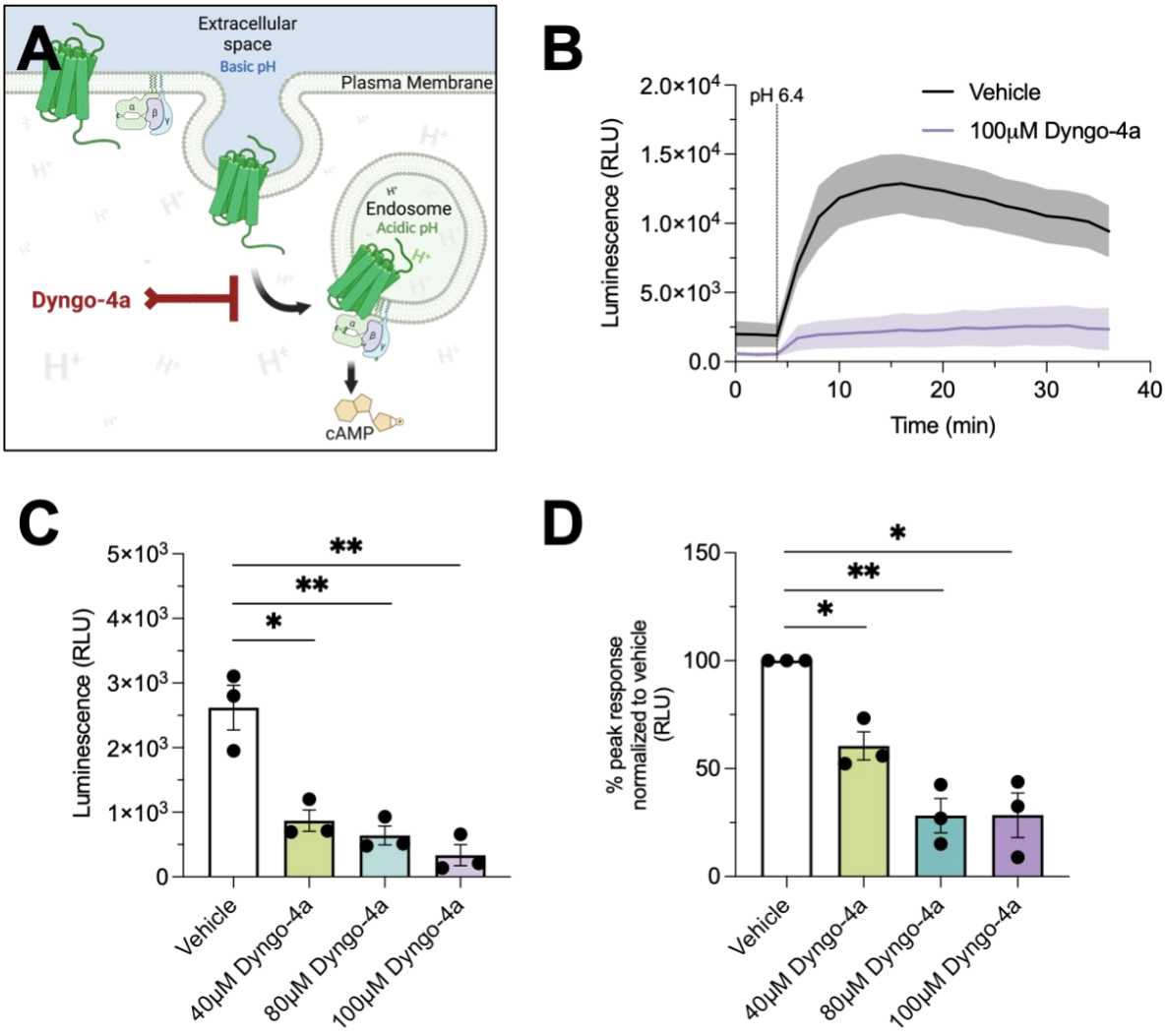

Figure 3.1 GPR65 is constitutively active and signals from endosomes.

(**A**) At basic extracellular pH, proton-dependent activation of endosomal GPR65 stimulates the production of intracellular cAMP. cAMP binding to the GloSensor luciferase produces luminescence directly correlated to increased cAMP levels. Treatment of GPR65-expressing cells with the endocytosis inhibitor Dyngo-4a will prevent internalization of the receptor leading to a decreased cAMP output. (**B**) Raw luminescence trace of HEK293 cells stably expressing GPR65 pretreated with vehicle or 100 μM Dyngo-4a for 30 min before the addition of pH 6.4 (n=3 biological replicates). Cells were incubated with basic extracellular pH for 2 hours prior to baseline readings. Before and after pH 6.4 addition, GPR65 cells treated with Dyngo-4a exhibited lower levels of cAMP luminescence than vehicle-treated cells at distinct concentrations. (**C**) Luminescence baseline readings at basic pH of GPR65-expressing cells with or without Dyngo-4a pretreatment (n=3 biological replicates) (Welch's t-test, $p<0.05$). At basic pH, GPR65-expressing cells pretreated with increasing amounts of Dyngo-4a exhibit decreased intracellular cAMP levels in a dose-dependent manner. (**D**) Peak luminescence response of GPR65-expressing cells pre-treated with Dyngo-4a, normalized to cells without Dyngo-4a (n=3 biological replicates) (Welch's t test, $p<0.05$). At pH 6.4, WT GPR65 cells treated with increasing amounts of Dyngo-4a display a significantly lower whole-cell cAMP output than vehicle in a dose-dependent manner.

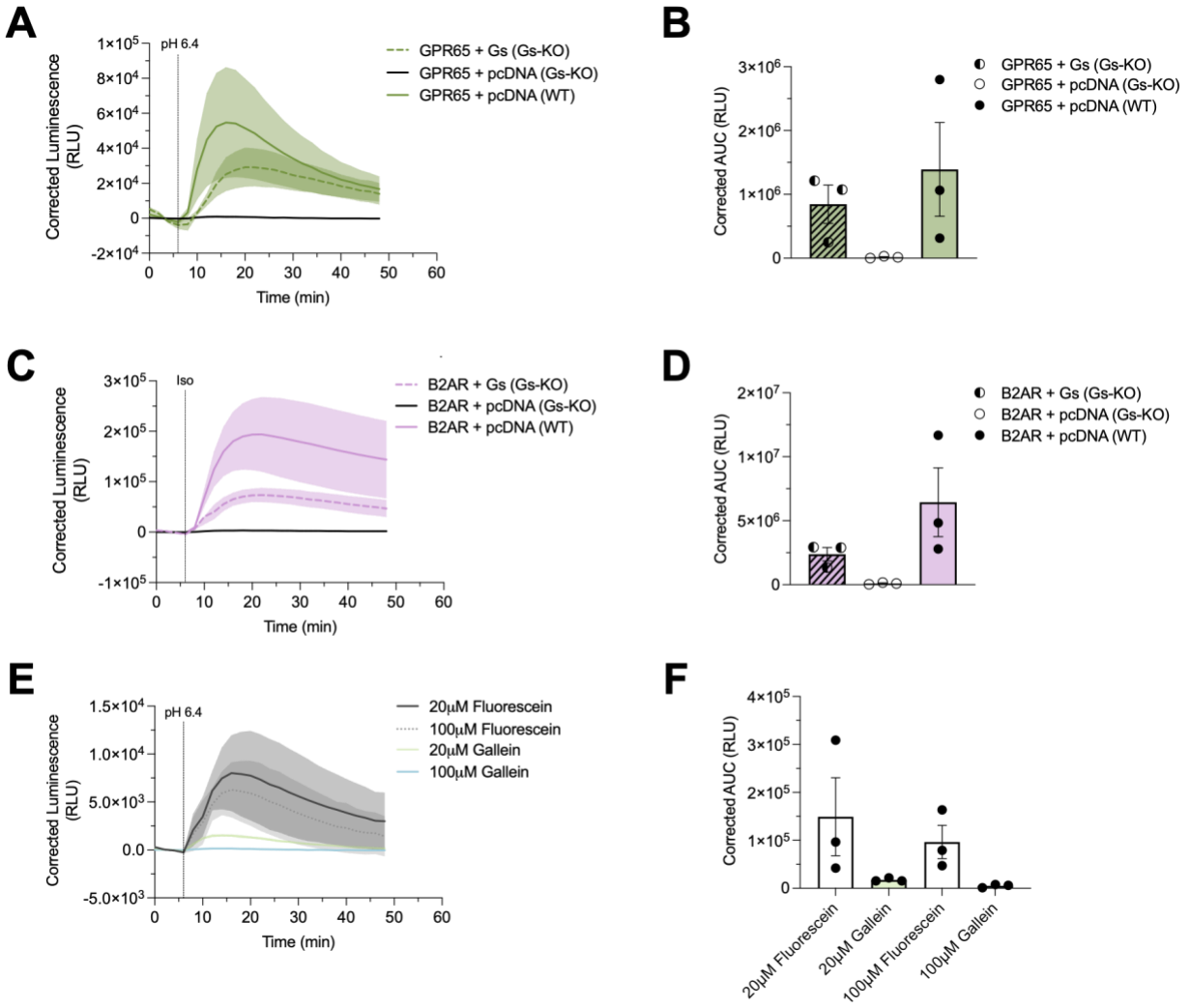

Figure 3.2 GPR65 signals through Gs and Gβγ protein subunits.

(**A**) Gs-knock out (KO) HEK293T and plain HEK293T cells expressing GPR65 and transfected with Gs or empty vector DNA (n=3 biological replicates). GPR65 cells expressing exogenous Gs rescued receptor activity and cAMP luminescence similar to HEK293T cells expressing endogenous levels of Gs protein. (**B**) Bar graph of the area-under-curve (AUC) from Figure 2A (n=3 biological replicates). The AUC of GPR65-Gs rescue is similar to HEK293T cells expressing endogenous Gs. (**C**) Gs-knock out (KO) HEK293T and plain HEK293T cells expressing positive control B2AR and transfected with Gs or empty vector DNA (n=3 biological replicates). B2AR cells expressing exogenous Gs rescued receptor activity and cAMP luminescence similar to HEK293T cells expressing endogenous levels of Gs protein. (**D**) Bar graph of the area-under-curve (AUC) from Figure 2C (n=3 biological replicates). The AUC of B2AR-Gs rescue is similar to HEK293T cells expressing endogenous Gs. (**E**) GloSensor luminescence over time in GPR65-expressing cells treated with increasing concentrations of Gallein (n=4 biological replicates). GPR65-expressing cells pretreated with increasing amounts of Gallein exhibit decreased intracellular cAMP levels in a dose-dependent manner. Fluorescein negative control at 20μM does not have an effect on cAMP. (**F**) AUC of HEK293 cells stably expressing GPR65 exposed to Gallein at distinct concentrations for 30 minutes prior to GloSensor readings (n=4 biological replicates). GPR65-mediated cAMP levels decrease in a Gallein-dependent manner.

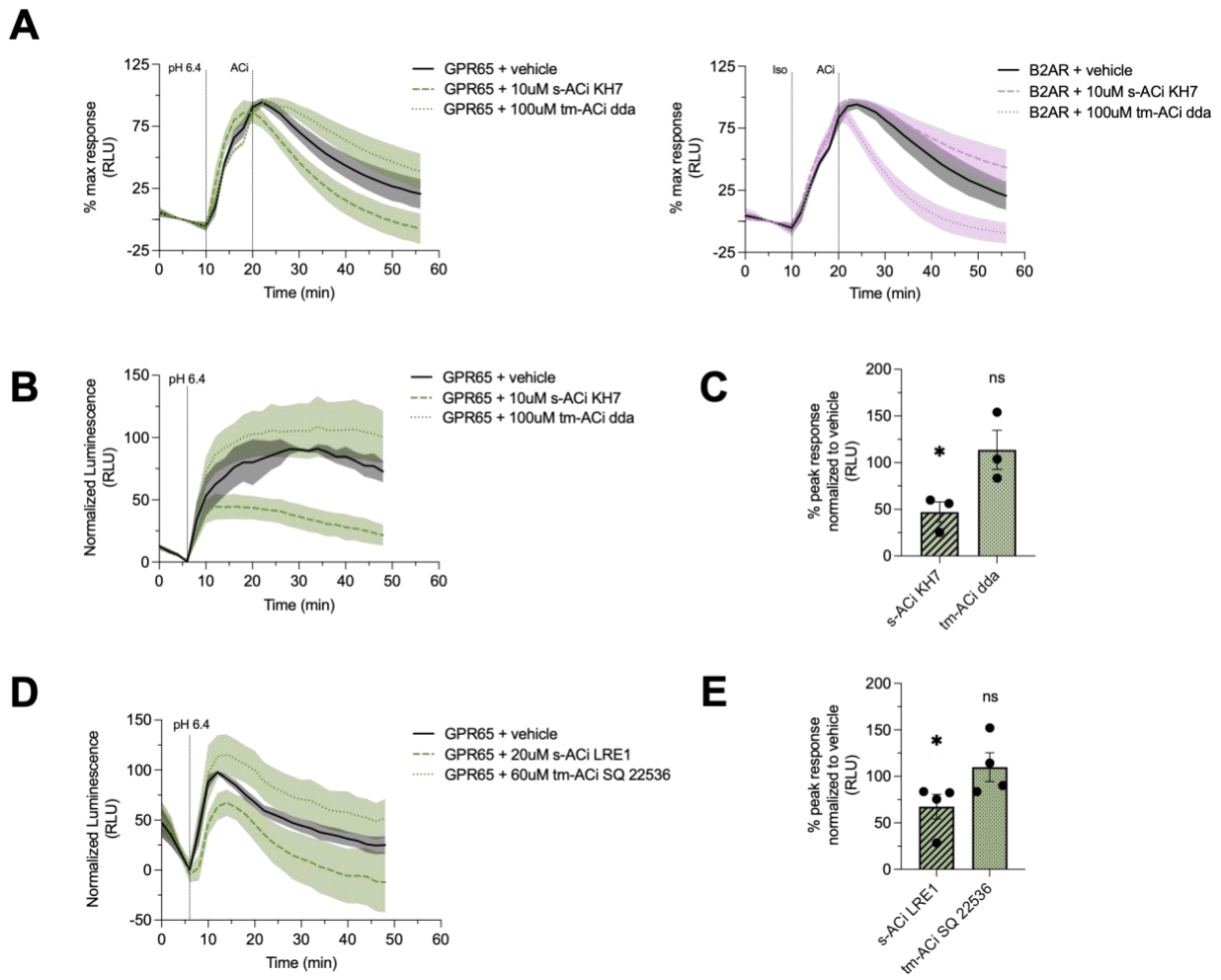

Figure 3.3 Soluble adenylyl cyclase (s-AC) is involved in GPR65-mediated cAMP production.

(**A**) Corrected luminescence trace of GPR65- and B2AR-expressing cells exposed to vehicle, KH7 or dda 10 minutes after acidic pH exposure (n=3 biological replicates). At pH 6.4, GPR65 treated with s-ACi KH7 displays a faster cAMP decay than vehicle, while tm-ACi positive control B2AR displays a faster decay in cAMP luminescence when exposed to dda. (**B**) Corrected luminescence trace of GPR65-expressing cells pretreated with vehicle or AC inhibitor (ACi) KH7 or dda for 15 min prior to baseline readings (n=3 biological replicates). After exposure to pH 6.4, WT GPR65 cells treated with s-ACi display lower luminescence and intracellular cAMP levels than vehicle-treated cells. (**C**) Peak luminescence response of GPR65 pre-treated with ACi, normalized to untreated cells (n=3 biological replicates) (Welch's t-test, $p<0.05$). WT GPR65 cells treated with s-ACi KH7 display a significantly lower whole-cell cAMP output than vehicle. (**D**) Corrected luminescence trace of cells pretreated with vehicle or AC inhibitor (ACi) LRE1 or SQ 22536 for 15 min (n=4 biological replicates). At pH 6.4, GPR65 treated with s-ACi displays lower luminescence and intracellular cAMP levels than vehicle. (**E**) Peak luminescence response of GPR65 pre-treated with LRE1 or SQ 22536, normalized to untreated cells (n=4 biological replicates) (Welch's t-test, $p<0.05$). WT GPR65 cells treated with s-ACi LRE1 display a significantly lower cAMP output than vehicle.

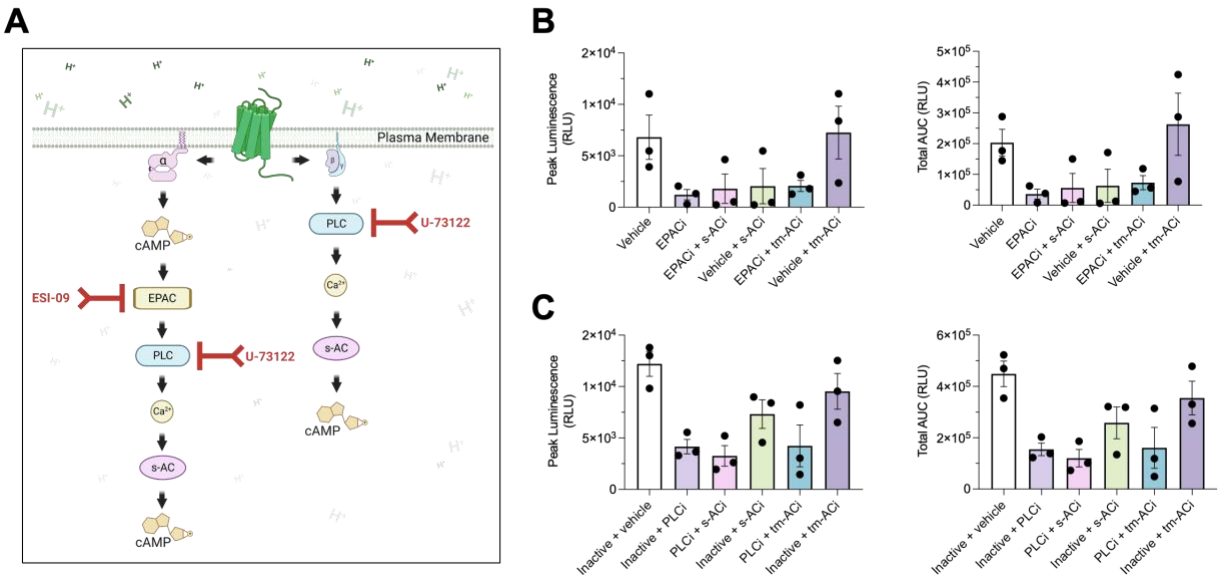

Figure 3.4 GPR65 activates s-AC via EPAC and PLC.

(**A**) Schematic of the hypothesized s-AC activation and cAMP production pathway. cAMP-binding to EPAC drives PLC activation to raise intracellular calcium ([Ca2+]i) via Ca2+ store release. An increase in [Ca2+]i leads to s-AC activation further increasing the cAMP response. (**B**) Peak luminescence and total AUC of GPR65 pre-treated with EPACi and ACi (n=3 biological replicates). At pH 6.4, WT GPR65 cells treated with EPACi ESI-09 display a lower whole-cell cAMP output than the negative control. (**C**) Peak luminescence and total AUC of GPR65 pre-treated with PLCi and ACi, (n=3 biological replicates). At pH 6.4, WT GPR65 cells treated with PLCi U73122 display a lower whole-cell cAMP output than analog-treated cells.

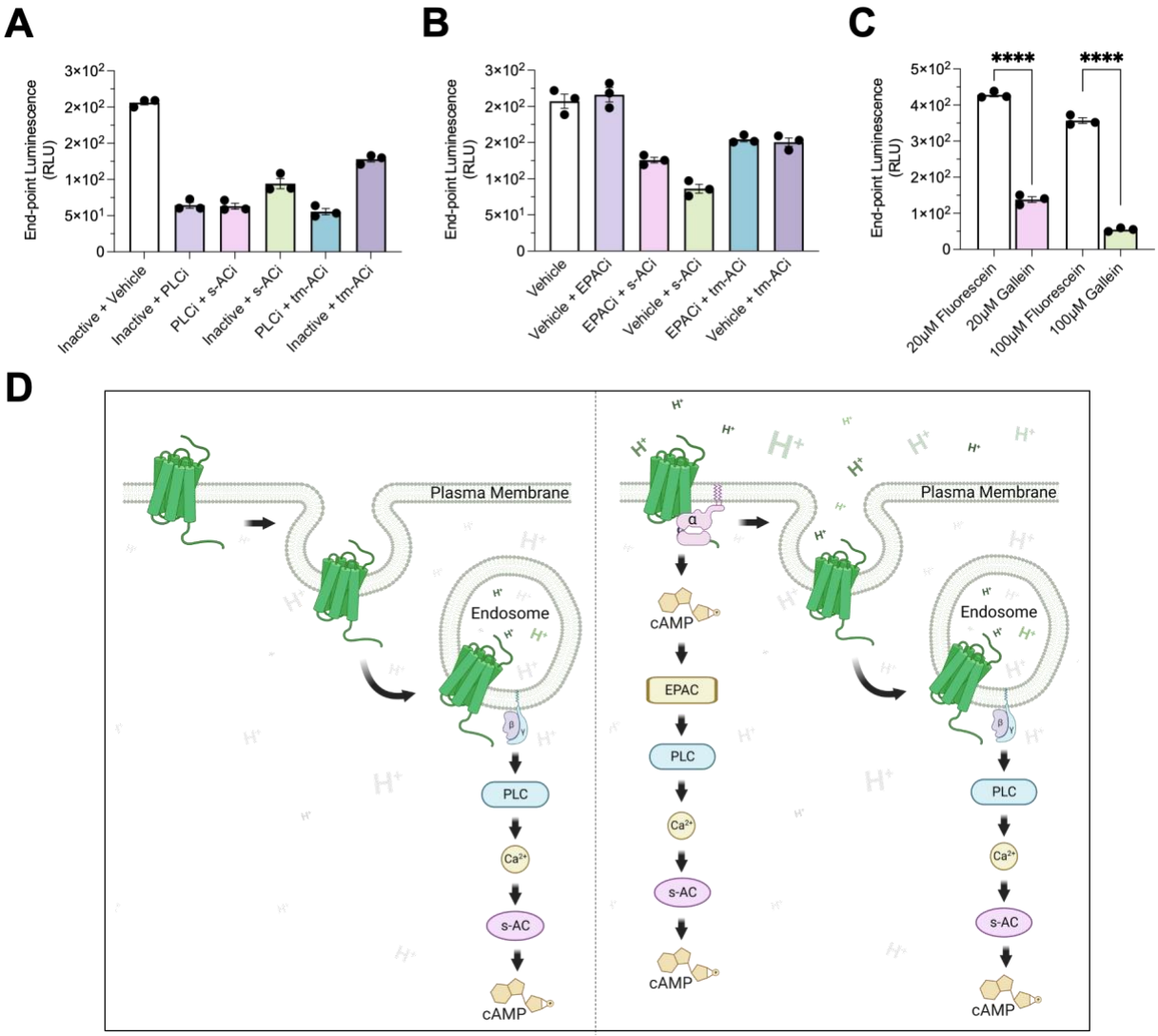

Figure 3.5 GPR65 activates s-AC via a Gs-EPAC-PLC-dependent pathway at acidic extracellular pH but not at basic extracellular pH.

(**A**) End-point luminescence of GPR65-expressing cells exposed pH 7.2 and pretreated with PLCi U73122 (20μM) for 20 minutes prior to GloSensor readings (n=3 biological replicates). After exposing cells to an extracellular pH of 7.2, GPR65-mediated cAMP levels decrease when pretreated with PLCi U71322 and s-ACi KH7. Although, a slight cAMP decrease is observed when GPR65 is exposed to pH 7.2 and tm-ACi dda. (**B**) End-point luminescence of GPR65-expressing cells exposed pH 7.2 and pretreated with EPACi ESI-09 (5μM) for 20 minutes prior to GloSensor readings (n=3 biological replicates). After exposing cells to an extracellular pH of 7.2, GPR65-mediated cAMP levels decrease when pretreated with s-ACi KH7, but not EPACi ESI-09. Similarly, a slight cAMP decrease is observed when GPR65 is exposed to pH 7.2 and tm-ACi dda. (**C**) End-point luminescence of GPR65-expressing cells exposed pH 7.2 and pretreated with Gβγ inhibitor Gallein (20μM) for 30 minutes prior to GloSensor readings (n=3 biological replicates). After exposing cells to an extracellular pH of 7.2, GPR65-mediated cAMP levels decrease when pretreated with Gβγ inhibitor Gallein and s-ACi KH7. (**D**) A model for location-switching of GPR65 signaling triggered by extracellular pH. Endosomal GPR65 is constitutively active and increases cAMP levels irrespective of extracellular pH. In the acidic environment of the endosome, endosomal GPR65 cAMP production requires Gβγ and PLC. Exposure to acidic extracellular pH activates surface GPR65 further increasing cAMP levels

via Gs, EPAC, PLC, and s-AC activation. An increase in $[Ca^{2+}]i$ can lead to s-AC activation and cAMP production irrespective of extracellular pH.

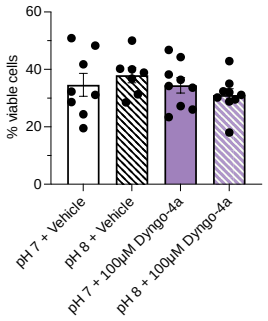

Figure supplement 3.1 Dyngo-4a pretreatment does not affect cell viability.

Quantitation of cell viability after exposing GPR65-expressing cells in basic pH media to 100µM Dyngo-4a for 15 minutes. Incubation of cells in basic pH media with 100µM Dyngo-4a suggests the observed findings are not a product of cell death but rather inhibition of endocytosis.

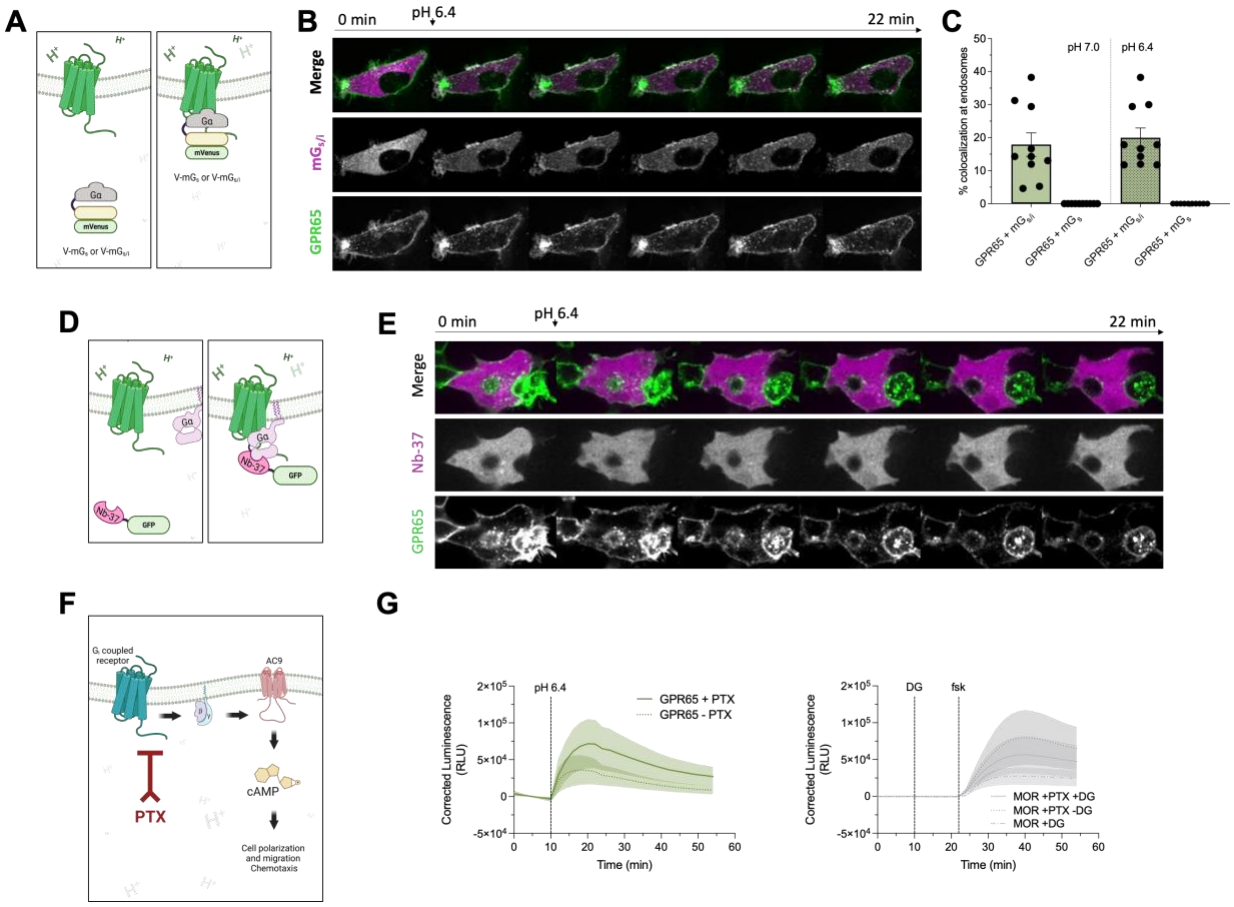

Figure supplement 3.2 Biosensors for GPCR activity reveal GPR65 is active at endosomes.

(**A**) Schematic diagram of mini-G protein variants which mimic the interaction of the G protein with an active receptor. (**B**) FLAG-tagged GPR65-expressing cells exposed to pH 7.0 prior to pH 6.4, immunolabeled live, and imaged by confocal microscopy. Active GPR65 at endosomes was able to recruit mini-$G_{s/i}$ protein variants irrespective of extracellular pH. (**C**) Quantitation of the percentage of GPR65-positive endosomes that colocalize with each of the mini-G protein variants at pH 7.0 and pH 6.4. GPR65 colocalizes with mini-$G_{s/i}$ at endosomes irrespective of extracellular pH (n=10 cells for mini-G_s and mini-$G_{s/i}$). (**D**) Schematic of nanobody Nb37-GFP, which selectively binds to active conformations of Gs protein subunits. (**E**) FLAG-GPR65 stable cells expressing Nb37-GFP were immunolabeled live and imaged by confocal microscopy. Confocal images revealed GPR65 does not recruit Nb37-GFP, the biosensor for active Gs proteins. (**F**) Schematic of Gi-coupled GPCR increasing cAMP levels via release of Gβγ subunits followed by activation of AC9. Inhibition of Gi proteins via PTX will test whether the increase in cAMP levels required Gi coupling. (**G**) GloSensor luminescence over time in HEK293 cells stably expressing FLAG-GPR65, and FLAG-MOR after the addition of either pH 6.4, DAMGO (DG), and forskolin (fsk) (n=3 biological replicates). GPR65-expressing cells exposed to PTX, and acidic pH, displayed a higher increase in cAMP suggesting PTX-sensitive Gi proteins are not required for cAMP production. Fsk-induced GloSensor luminescence in MOR-expressing HEK293 stable cells, positive control for Gi inhibition, displayed a decrease in cAMP luminescence following the addition of agonist DG.

3.8 References

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Chapter 4 Concluding Remarks and Future Directions

4.1 Significance Statement

Proton-sensing GPCRs are implicated in a variety of biological processes and disease states including ischemia, inflammation, and signaling in tumor microenvironments (Imenez Silva & Wagner, 2022; Insel et al., 2020; Sanderlin et al., 2015; Silva et al., 2022; Sisignano et al., 2021; Sriram & Insel, 2018; Wiley et al., 2019). The acidic environment generated by these processes stimulates GPCR signaling known to contribute to a vast array of context-dependent cellular responses. Despite these physiological insights, how different acidic environments in cells change the signaling patterns of proton-sensing GPCRs is still not known. Characterization of how subcellular localization influences proton-sensing receptor signaling, and function will expand our understanding of the role of subcellular signaling in physiology and disease.

This work contributes to the importance of understanding compartmentalized GPCR signaling and physiological implications of the proton-sensing receptor family, which have been less studied compared to other families of GPCRs. Using GPR65 as a prototype, I have made novel discoveries regarding how the trafficking and signaling of these clinically relevant receptors are regulated and how the uncoupling of these two aspects could play key roles in physiology and disease. The general principles by which GPR65 trafficking and signaling are regulated could be shared with other proton-sensing

receptors. GPR65 specifically is a promising potential target for the treatment of cancer (Justus et al., 2013). Therefore, these findings not only provide a new perspective on GPR65 function but will also aid in understanding the physiology and developing strategies to target this understudied family of clinically relevant membrane receptors in diseases.

4.2 Discussion and Future Directions

4.2.1 Proton-dependent activation of GPR65 is uncoupled from receptor trafficking

In chapter two, I examined how acidic environments change the signaling and trafficking patterns of proton-sensing GPCRs. I observed that GPR65 internalizes irrespective of extracellular pH. Acidic extracellular environments stimulate receptor signaling at the cell surface in a dose-dependent manner. However, GPR65 activity at the cell surface was not required for receptor endosomal localization. These findings show that GPR65 dynamically traffics to multiple cellular compartments and that, unlike most other mammalian GPCRs, activation of GPR65 is uncoupled from receptor trafficking. However, how these two aspects of receptor function are uncoupled for GPR65 and what physiological implications could be mediated by this uncoupling is still an open question.

The canonical view of GPCR function is that receptor trafficking is coupled to signaling (Sorkin & Von Zastrow, 2009). Following surface activation and signaling, some mammalian GPCRs, like the B2AR, are desensitized by proteins including the GPCR kinases (GRKs) and B-arrestin proteins, which respectively phosphorylate agonist-activated GPCRs and bind phosphorylated GPCRs to physically disrupt the receptor-G

protein complex (Bowman, Shanna L, 2017; Drake et al., 2006; Lobingier & von Zastrow, 2019). This process leads to receptor desensitization followed by the clathrin-mediated endocytosis and trafficking of GPCRs through the endolysosomal pathway. Within the endolysosomal pathway, GPCRs can initiate further signaling and later undergo dephosphorylation and recycling back to the cell surface or be sorted to lysosomes and degraded (Bowman et al., 2016a; Kunselman et al., 2021; Weinberg & Puthenveedu, 2019a). Trafficking of GPR65 to endosomal compartments from the surface is fully uncoupled from receptor activation at the plasma membrane (Morales Rodríguez et al., 2023). Because the trafficking of mammalian GPCRs is determined partly by the C-terminal tail, I speculate that the observed constitutive trafficking phenotype of GPR65 could be mediated by a trafficking motif.

GPCRs contain C-terminal sequences that can regulate the dynamics of clathrin-mediated endocytosis. Amino acid sequences such as the postsynaptic density 95/disc large/zonula occludins-1 (PDZ) domain (T. T. Cao et al., 1999) in the C-terminal tail of the receptor can change the rate and lifetime of receptor endocytosis. Receptor chimeras with an exchange in the C-terminal trafficking sequence can extend the rate of endocytosis (Weinberg & Puthenveedu, 2019a). The delta-opioid receptor (DOR) lasts ~40 seconds at the PM before undergoing dynamin-dependent scission. DOR with either the Beta-1 adrenergic receptor (B1AR) or B2AR C-terminal PDZ ligand last longer at the plasma membrane. PDZ ligands extend the receptors lifetime at the plasma membrane and delay dynamin recruitment, but this is not the only mechanism. The Mu-opioid receptor (MOR) promotes long lifetimes at the plasma membrane by delaying scission after dynamin recruitment (Soohoo & Puthenveedu, 2013). Interaction of kinases (e.g.,

Src and GSK3B kinases) with dynamin could also regulate GPCR clathrin-mediated endocytosis (H. Cao et al., 2010; Reis et al., 2015). Additionally, GPCR interactions with scaffolding proteins can regulate clathrin-mediated endocytosis. PDZ ligands of the serotonin 2A receptor (5HT2AR) and the corticotropin-releasing factor receptor 1 (CRFR1) bind the PDZ-containing protein synapse associated protein 97 (SAP97), and overexpression of SAP97 slowed the endocytic rate for both receptors (Dunn et al., 2013, 2014). GPCR PDZ ligand domains can also affect endosomal sorting preferences.

GPCRs contain trafficking motifs that determine endosomal sorting preferences (Bowman, Shanna L, 2017; Hanyaloglu & Zastrow, 2008; Kunselman et al., 2021; Marchese, Adriano; Paing, May; Temple, 2008; Romero et al., 2011). Endosomal sorting preferences displayed by GPCRs are determined in large part by specific amino acid sequences in the C-terminal tail of the receptor. For example, B2AR undergoes sequence-dependent recycling back to the cell surface and contains a recycling sequence on its C-terminus that interacts with (PDZ)-domain-containing proteins (T. T. Cao et al., 1999). Other GPCRs such as the related beta-1 adrenergic receptor (B1AR) and the kappa opioid receptor (KOR) contain similar sequences that conform to classical type I PDZ-ligand sequences (He et al., 2006; P. Huang et al., 2004; J. G. Li et al., 2002). For these receptors, these PDZ C-terminal sequences are required and sufficient for their recycling to the cell surface. Similarly, the mu-opioid receptor (MOR) recycles following agonist-induced endocytosis but requires a different C-terminal sequence to undergo sequence-dependent recycling. MOR contains a unique, seven amino-acid recycling sequence in its C-terminal tail, LENLEAE (Tanowitz & Von Zastrow, 2003; Weinberg et al., 2017). In contrast to adrenergic receptors, KOR, and MOR, the delta-opioid receptor

(DOR) does not recycle and is degraded in lysosomes following agonist-induced endocytosis (Tanowitz & Von Zastrow, 2003). Mutations of these c-term sequences and the development of GPCR chimeras reroute the endosomal sorting preferences displayed by these receptors and can serve as one approach to studying the mechanism behind the uncoupling of signaling and receptor trafficking. Sequence-dependent endosomal sorting mutations can shed light on the trafficking motifs and preferences of other less-studied GPCRs. Transplanting specific C-term sequences onto less well-known GPCRs has not only allowed the identification of endosomal sorting preferences and trafficking motifs but has also aided in the understanding of C-terminus residues enabling interaction with intracellular signaling effectors and transducers.

Apart from C-term motifs that regulate receptor trafficking, C-term sequences can also enable interactions with intracellular signaling effectors and adaptor proteins, such as GPCR Kinases (GRKs) and B-arrestins, which are proteins that regulate receptor signaling, amplify, and elicit cellular responses to extracellular signals (Bowman, Shanna L, 2017; Marchese, Adriano; Paing, May; Temple, 2008). GPCR chimeras and C-term mutations on phosphorylation sites can shed light on downstream regulators of receptor activation. Alanine substitutions of serine and threonine phosphorylation sites can help us determine the effect on GRKs and B-arrestin recruitment. Manipulating protein-protein interactions through pharmacological approaches (e.g. GRK inhibitors), and heterologous expression of GPCR effectors can help tease out whether the observed GPR65 constitutive trafficking is due to some constitutive level of phosphorylation or baseline interaction with arrestin. The interaction of GPR65 with arrestin and GRKs is an open

question that could be addressed by studying the mechanisms of receptor desensitization, trafficking, and signaling pathways mediated by acidic pH.

Visualization of B-arrestin recruitment to active GPR65 and receptor mutants can help determine potential interactors mediating the uncoupling of signaling and trafficking. I have performed preliminary experiments to visualize the recruitment of B-arrestin to active WT GPR65 at the PM using total internal fluorescence microscopy (TIRF-M). In GPR65-expressing HEK293 cells, B-arrestin sensor fluorescence at the PM did not change noticeably upon acidic pH exposure (**Figure 4.1**). The GPR65 arrestin sensor fluorescence at baseline is higher than the baseline fluorescence by prototypical GPCR B2AR. At baseline, B2AR does not display sensor fluorescence at the PM. When exposed to a saturating concentration (10 μM) of the agonist isoproterenol, sensor fluorescence at the PM rapidly and transiently increased (**Figure 4.1**). These preliminary findings suggest active GPR65 recruits B-arrestin but how WT GPR65 differs from receptor mutants, and what role B-arrestin plays in the uncoupling of receptor activation and trafficking is to be determined.

The mechanism underlying the uncoupling of GPR65 activation from receptor trafficking remains unclear. Future work would focus on studying potential proteins involved in receptor desensitization and developing receptor chimeras to identify trafficking motifs involved in mediating the unique phenotype of proton-sensor GPR65. In addition, identifying the role of uncoupling of signaling and trafficking in specific physiological scenarios is still an open question that could shed light on the development of novel therapies for diseases associated with acidic microenvironments.

4.2.2 Location-Biased Signaling of GPR65 Influences the Receptor's Net Signaling Response

Although extracellular acidic pH displays a cellular signaling response, endosomal GPR65 is required for, and further increases the cAMP signaling response. GPR65 localizes to distinct endosomal compartments and stimulates second-messenger cAMP production from endosomes independently from extracellular pH changes. These results show endosomal GPR65 sets basal cAMP levels, while extracellular acidic pH activation increases cAMP. These two sources of cAMP could result in distinct pools of cAMP with distinct cellular functions.

Endocytosis contributes to distinct cAMP signaling profiles for distinct GPCRs. For example, B2AR endocytosis is required for the full repertoire of downstream cAMP-dependent transcriptional control (Bowman et al., 2016a; Tsvetanova & von Zastrow, 2014). This subcellular cAMP response increased the expression of three endosomal-specific genes-PCK1, CGA and NR4A1. Similarly, GPCRs such as CaSR and NK1R also require endosomal signaling to induce gene transcription (Gorvin, 2018; Jensen et al., 2017). These reports make a strong case for the model where endosomal-generated cAMP is a specialized pool that has distinct downstream effects on gene activation.

Apart from location-biased downstream transcriptional outcomes, GPCR endosomal signaling is linked to physiology. For example, LHR endosomal signaling is linked to fertility (Lyga et al., 2016). Sustained cAMP signaling and endocytosis of LHR are required for meiosis in the oocyte of ovarian follicles. Similarly, endosomal signaling of the NK1R and CLR inhibit sustained signaling in spinal cord neurons and provide pain relief in animal models of inflammatory pain (Jensen et al., 2017; Yarwood et al., 2017).

This pain relief is greater and longer lasting than traditional receptor antagonists. GPCR signaling from endosomes is well documented across the field. However, the specific impact of GPCR endosomal signaling on physiological functions remains to be fully understood, especially for the understudied families of GPCRs such as the proton-sensing receptors.

The present findings reveal endosomal proton-sensing receptor GPR65 is required for the full cAMP signaling response but downstream signaling consequences for gene activation remain to be determined. GPR65 specifically is a promising potential target for the treatment of cancer (Justus et al., 2013; Sanderlin et al., 2015; Sisignano et al., 2021; Wun et al., 2004) although the impact of endosomal signaling on physiology is not yet known. From the literature, GPR65 expression and function are tightly coupled to c-myc oncogene downregulation (Z. Li et al., 2013b). Endosome-based downstream transcriptional measurements of known GPR65 gene and protein targets such as the c-myc oncogene can help us understand the implications of endosomal signaling in physiology. Similarly, analysis of the phosphoproteomic effects induced by endosome-based cAMP signaling (Tsvetanova et al., 2021) is another approach to show that endocytosis of GPR65 and other proton-sensors is required for not only the full transcriptional response but demonstrate that these GPCRs play a significant role in physiology as it was observed for B2AR. Future studies on the impact of endosome-based cAMP signaling in transducing the full repertoire of transcriptional responses upon receptor activation in native systems such as lymphomas and solid tumors will allow us to leverage receptor location to fine-tune the targeting of this family of GPCRs for better therapeutics.

4.2.3 Novel GPR65 signal transducers and effector proteins influence the GPR65 signaling profile from distinct cellular compartments.

In chapter three, I determined the effect of spatial organization on GPR65 signaling. I have shown that GPR65 in different cellular locations increases intracellular cAMP through different signal transducers and effector proteins. Specifically, GPR65 in both the PM and endosomes increases cAMP via the non-traditional s-AC, whereas EPAC function is specific to GPR65 signaling at the PM. These data reveal how the localization of GPR65 influences the signaling response at a cellular level. However, the mechanisms underlying localized GPR65 signaling specificity remain unclear.

Our data suggest surface GPR65 requires Gs, EPAC, PLC, and s-AC to increase cAMP. How surface GPR65 and Gs proteins increase cAMP to activate EPAC and promote s-AC activation is not clear. We speculate that tm-AC activation is required for initial cAMP production, but small amounts of tm-AC generated cAMP are enough to amplify the surface GPR65 signaling pathway.

At endosomes, GPR65 requires the release of Gβγ to activate PLC increasing cAMP through Ca^{2+}-dependent s-AC activity. Future studies should confirm whether internalized GPR65 does not require Gs. The rescue of Gs activity in Gs-KO cells and pretreatment of cells at basic pH can provide stronger evidence to support Gs mediating surface and not endosome-generated cAMP. Additionally, it is unclear if PLC activity mediated by GPR65 requires Ca^{2+} release from intracellular stores to activate s-AC. To confirm whether GPR65-cAMP activation of EPAC initiates a Ca^{2+} signal through PLC stimulation, direct measurement of calcium levels using biosensors (e.g., GCaMP or Fluo-

4) and exposing cells to basic pH before agonist exposure can help clarify the mechanism leading to endosomal cAMP production.

This work adds to our understanding of how receptor location inside the cell is intricately linked to receptor signaling and lays the groundwork for one day targeting receptor location to influence cellular responses. The present findings reveal that GPR65-mediated cAMP production from endosomes requires distinct signaling effectors than cAMP production at the PM but whether these two cAMP sources result in differences in gene expression with distinct physiological roles is not known. Future work should focus on defining factors that modulate the spatial organization of the GPR65-mediated cAMP response which could have significant physiological implications. Defining factors that modulate the spatial organization of GPR65 signaling together with the identification of compounds with functional selectivity, may result in clinically valuable tools for diseases involving proton-sensing receptor GPR65.

4.2.4 Exploring other avenues of GPR65 function

4.2.4.1 A GPR65 genetic variant is implicated in intestinal inflammation.

Proton-sensing receptors, like many other mammalian GPCRs, are susceptible to many genetic variations that dictate changes in physiology. The GPR65 I231L polymorphism displays decreased signaling, alters lysosomal pH, and increases colitis and inflammatory bowel disease (IBD) risk (Lassen et al., 2016). How these polymorphisms affect receptor function from distinct cellular compartments is unknown. Examining the effect of the GPR65 genetic variation on two key molecular aspects of receptor function, trafficking and signaling, will provide a deeper understanding of the

molecular consequences of genetic variations in the understudied family of proton-sensing GPCRs. Specifically, it will give insight into how the genetic variants of GRP65 signaling and trafficking differ from the canonical receptor.

4.3 Final Thoughts

Over the past decade, the characterization of proton-sensing receptor function has not been the focus of the GPCR field (Roth & Kroeze, 2015b). From the literature, we know proton-sensing GPCRs are implicated in a variety of biological functions but a clear explanation as to how these receptors function at a cellular level, how their signaling contributes to these responses, or even how their signaling is regulated is not clear. Development of small molecules -specifically agonists, antagonists, and inverse agonists- targeting GPR65 and other proton-sensors may help in characterizing proton-sensing receptor function. Similarly, the future directions highlighted in this dissertation are a step forward to understanding how these understudied GPCRs function. Characterizing GPR65 trafficking and activation will help us understand how proton-sensing receptors contribute to normal physiology and disease and provide new strategies to treat pathological conditions associated with acidic microenvironments.

4.4 Figures

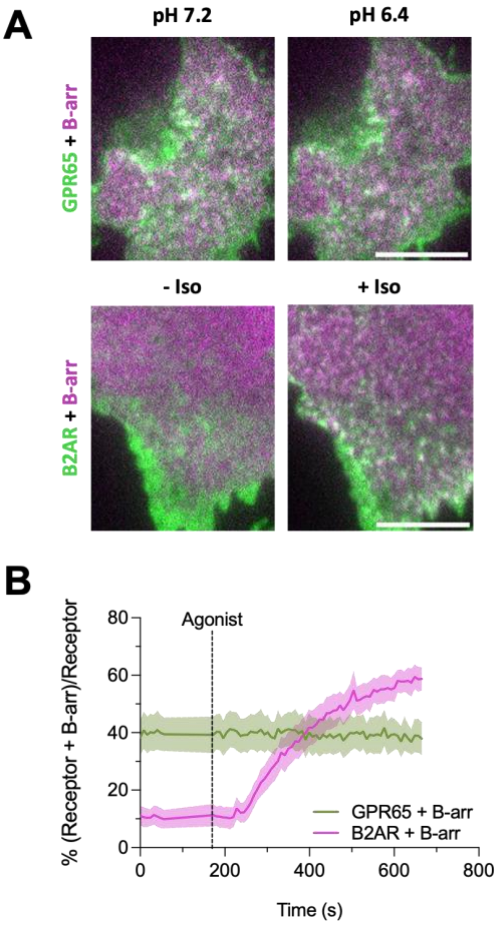

Figure 4.1 Active GPR65 recruits B-arrestin irrespective of extracellular pH.

(**A**) HEK293 cells expressing FLAG-GPR65 or FLAG-B2AR and imaged using TIRF-M to capture recruitment of B-arrestin (B-arr) to the PM after the addition of pH 6.4 and 10μM Iso, respectively (scale bar=17μm). B-arr sensor fluorescence to active GPR65 at the PM did not change noticeably upon acidic pH exposure. (**B**) Time course of the percentage of receptor spots that colocalize with the B-arrestin spots over total receptor spots. For this, FLAG-GPR65 cells preincubated with pH 7.2 prior to pH 6.4 exposure were labeled live and imaged using TIRF-M. GPR65 colocalizes with B-arrestin-containing spots irrespective of pH (n=8 fields for both GPR65 and B2AR).

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