G protein-coupled receptors mobilize intracellular calcium via the Gs-βγ-PLCβ module

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für meine Mama

Abstract

Accurate regulation of calcium is essential for many physiological processes. To fine-tune these processes, the cell can draw on a large portfolio of diverse mechanisms to govern calcium mobilization, in which GPCRs present an integral component. A key element of how the cell mobilizes calcium using GPCRs involves the large group of phospholipases Cβ. These enzymes are canonically activated by Gq-coupled receptors, but it has been found that Gi-coupled receptors can also activate PLCβ2 and PLCβ3 via their Gβγ subunit. Notably, this Gβγ-specific effect is fully dependent on active Gq. Upon activation, PLCβ enzymes hydrolyze the membrane component PIP_2 to DAG and IP_3. While DAG triggers various cellular effectors, IP_3 mediates a cytosolic increase in calcium via activation of endoplasmically located $InsP_3R$.

By studying the Gi-βγ-PLCβ-Ca^{2+} pathway and identifying active Gq as the driver of Gi-βγ-mediated Ca^{2+} release, we came up with a new hypothesis. Could Gq be the missing link that would allow even other members of the G protein family to release calcium via their Gβγ subunit? Therefore, we hypothesized that activation of Gq may permit Gs-βγ to activate PLCβ enzymes, resulting in the release of intracellularly stored calcium. To test this hypothesis, we used a variety of CRISPR/Cas9-edited cells, engineered molecules, and inhibitors alongside illuminating techniques such as fluorescence-based calcium measurement, HTRF-based cAMP accumulation, label-free whole-cell biosensing, real-time BRET and Nanobit measurements.

However, examination of Gq-dependent Gs-calcium revealed that Gs-GPCRs trigger more than one Gq-dependent calcium release pathway in non-excitable HEK293 cells. This exploration led us to discriminate two distinct pathways utilized by Gs-GPCRs: i) a cAMP-dependent and ii) a cAMP-independent, previously unrecognized pathway. Both pathways are tightly governed by active $G\alpha_q$, yet they diverge in terms of the underlying action. Because $G\alpha_s$ mainly orchestrates cAMP-mediated effects, we focused on the unknown cAMP-independent Gs-mediated calcium release and were able to identify Gs-βγ as the driving force. It appears that the Gs-βγ module activates Gβγ-sensitive PLCβ isoforms that mediate a calcium release from intracellular stores. Given the cell- and tissue-specific nature of calcium regulation, we extended our investigation to cells of greater physiological relevance. Thereby, we revealed a Gq-dependent Gs-mediated calcium response even in primary cell lines, including mouse brown adipose tissue and mouse embryonic fibroblasts, expanding the physiological significance of Gs-βγ-mediated calcium release. Therefore, our results illustrate the remarkable adaptability of GPCR calcium signaling and highlight its capacity to rapidly adapt to ever-changing conditions (externally & internally). The Gs-GPCR system appears to be a competent regulator of calcium dynamics, whether through $G\alpha_s$-mediated, cAMP-dependent mechanisms or through a Gs-βγ-mediated and cAMP-independent mechanism.

Zusammenfassung

Für viele physiologische Prozesse ist eine genaue Kontrolle des Kalziumhaushalts unerlässlich. Zur Feinabstimmung dieser Prozesse verfügt die Zelle über ein großes Portfolio verschiedenster Mechanismen. Eine wichtige Komponente sind die GPCRs, über die diese Kalziummobilisierung gesteuert werden kann. Dabei stellt die große Gruppe der Phospholipasen Cβ eines der Schlüsselelemente der GPCR vermittelten Kalziummobilisierung dar. Normalerweise werden diese Enzyme durch die Aktivierung von Gq-gekoppelten Rezeptoren aktiviert, aber es konnte gezeigt werden, dass auch Gi-gekoppelte Rezeptoren über ihre Gβγ-Untereinheit die PLCβ Isoformen 2 und 3 aktivieren können. Interessanterweise hängt dieser Gβγ-vermittelte Effekt vollständig von der Anwesenheit von aktivem Gq ab. Durch die Aktivierung der PLCβ Enzyme wird der Membranbestandteil PIP_2 zu DAG und IP_3 hydrolysiert. Während DAG verschiedene zelluläre Effektoren aktiviert, vermittelt IP_3 einen cytosolischen Kalziumanstieg, indem die auf dem endoplasmatischen Retikulum lokalisierten $InsP_3$-Rezeptoren aktiviert werden.

Durch die Untersuchung des Gi-βγ-PLCβ-Ca^{2+}-Wegs und die Identifizierung von aktivem Gq als Treiber der Gi-βγ-vermittelten Ca^{2+}-Freisetzung stellten wir eine neue Hypothese auf. Könnte Gq das fehlende Bindeglied sein, das es auch anderen Mitgliedern der G Protein Familie ermöglicht, Kalzium über ihre Gβγ-Untereinheit freizusetzen? Daher hypothetisierten wir, dass die Aktivierung von Gq es auch Gs-βγ ermöglichen könnte, PLCβ Enzyme zu aktivieren, was zur Freisetzung von intrazellulär gespeichertem Kalzium führt. Um diese Hypothese zu testen, haben wir eine Vielzahl an CRISPR/Cas9-editierten Zellen, modifizierten Molekülen und Inhibitoren zusammen mit aufschlussreichen Techniken wie fluoreszenzbasierter Kalziummessung, HTRF-basierter cAMP-Akkumulation, markierungsfreiem Ganzzell-Biosensing, Echtzeit-BRET und Nanobit-Messungen verwendet.

Bei der Untersuchung des Gq-abhängigen Gs-Kalziums stellten wir fest, dass Gs-GPCRs mehr als einen Gq-abhängigen Kalziumfreisetzungsweg in nicht erregbaren HEK293 Zellen auslösen. Wir identifizierten zwei unterschiedlicher Wege, die von Gs-GPCRs genutzt werden: i) ein cAMP-abhängiger und ii) ein cAMP-unabhängiger Weg. Beide Wege werden durch aktives $G\alpha_q$ gesteuert, unterscheiden sich jedoch in Bezug auf die zugrunde liegende Wirkung. Ausgehend davon, dass $G\alpha_s$ hauptsächlich cAMP-vermittelte Effekte orchestriert, haben wir uns auf die unbekannte cAMP-unabhängige Gs-vermittelte Kalziumfreisetzung konzentriert und konnten Gs-βγ als treibende Kraft identifizieren. Es scheint, dass das Gs-βγ-Modul die Gβγ-empfindlichen PLCβ Isoformen aktiviert und so eine Kalziumfreisetzung aus intrazellulären Speichern vermitteln kann. Angesichts der zell- und gewebespezifischen Natur der Kalziumregulation haben wir unsere Untersuchung auf Zellen von größerer physiologischer Relevanz ausgeweitet. Dabei konnten wir eine Gq-abhängige Gs-vermittelte

Kalziumreaktion sogar in primären Zelllinien, einschließlich des braunen Fettgewebes der Maus und embryonaler Fibroblasten der Maus, nachweisen, was die physiologische Bedeutung der Gs-βγ-vermittelten Kalziumfreisetzung erweitert. Unsere Ergebnisse veranschaulichen daher die bemerkenswerte Anpassungsfähigkeit der GPCR-Kalzium-Signalgebung und unterstreichen ihre Fähigkeit, sich schnell an die sich ständig ändernden Bedingungen (äußere wie innere) anzupassen. Das Gs-GPCR-System scheint ein kompetenter Regulator der Kalziumdynamik zu sein, sei es durch $G\alpha_s$-vermittelte, cAMP-abhängige Mechanismen oder durch einen Gs-βγ-vermittelten und cAMP-unabhängigen Mechanismus.

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Abbreviations

AC adenylyl cyclases

ATP adenosine-5´-triphosphate

AUC area under the curve

AVP arginine-vasopressin

BRET Bioluminescence resonance energy transfer

cAMP 3‘,5‘-cyclic adenosine monophosphate

CCh Carbachol

CICR calcium-induced calcium release

CRISPR Clustered Regularly Interspaced Short Palindromic Repeats

DAG diacylglycerol

DMEM Dulbecco's Modified Eagle's Medium

DMR Dynamic Mass Redistribution

EC_{50} concentration of half maximum effect

EPAC exchange protein activated by cAMP

ER endoplasmic reticulum

FR FR900359

FRET Förster resonance energy transfer

GAP GTPase-activating protein

GDI guanine nucleotide dissociation inhibitor

GDP Guanosindiphosphat

GEF guanosine triphosphate exchange factor

GPCR G protein-coupled receptor

GTP guanosine triphosphate

HBSS Hanks' Buffered Saline Solution

HEK human embryonic kidney

HTRF homogeneous time-resolved fluorescence

$InsP_3R$ inositol-1,4,5-trisphosphate receptor

IP_3 inositol-1,4,5-trisphosphate

Iso Isoproterenol

mBA murine brown adipocytes

MEF mouse embryonic fibroblasts

Nb17 nanobody 17

Nb5 nanobody 5

NCX Na^+/Ca^{2+} exchanger

NECA 5'-N-ethylcarboxamidoadenosine

PBS phosphate-buffered saline

PGD_2 prostaglandine G_2

PGE_1 prostaglandine E_1

PIP_2 phosphoinositol-4,5-diphosphate

PKA protein kinase A

PLC phospholipases

PLCβ phospholipase Cβ

PMCA plasma membrane Ca^{2+}-ATPase

PTX pertussis toxin

RFU relative fluorescence units

RTK receptor tyrosine kinase

RyR ryanodine receptor

SERCA sarco(endo)plasmic reticulum Ca^{2+}-ATPase

SR sarcoplasmic reticulum

TIRF Total internal reflection fluorescence

VGCCs voltage-gated calcium channels

β_1AR β1-adrenergic receptor

β_2AR β2-adrenergic receptor

Introduction

Calcium signaling

One of the key elements for a broad range of physiological processes in cellular life is the divalent cation calcium. Spatial and temporal changes in calcium gradients are essential triggers of cellular signal transduction. While the cytoplasmic calcium concentration during the resting state is around 100 nM, the extracellular calcium concentration is much higher (~2 mM). Furthermore, cells also contain calcium in internal stores in a high mM range (Figure 1). These include the endoplasmic/sarcoplasmic reticulum (ER, SR) as the most important calcium store in the cell and mitochondria (Michael J. Berridge et al., 2003; David E. Clapham, 2007). During various stimulations of the cell, such as membrane depolarization, stretch and extracellular agonists calcium can enter the cytoplasm from the extracellular space through plasma membrane channels (Michael J. Berridge et al., 2003). Additionally, the calcium stored in the ER/SR is released by the entering extracellular Ca^{2+} itself (calcium-induced calcium release), direct activation of calcium channels on the ER/SR or by the second messenger inositol-1,4,5-trisphosphate (IP_3), which binds to its receptors (inositol-1,4,5-trisphosphate receptor). The rising concentrations of intracellular calcium enable the cell to respond with acute and long-term cell activity, including muscle contraction, exocytosis, cell migration, gene transcription, and many more (David E. Clapham, 2007). Following a calcium response, cytoplasmic calcium is rapidly removed to the outside via a series of exchangers and pumps, such as the Na^+/Ca^{2+} exchanger (NCX) or the plasma membrane Ca^{2+}-ATPase (PMCA). In addition, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) or the mitochondrial uniporter continuously pumps calcium back into the intracellular stores (Michael J. Berridge et al., 2003; Strehler & Treiman, 2004). Withdrawal of Ca^{2+} from the intracellular space maintains the electrochemical gradient of the cell, recharging the internal stores and returning the cytoplasm to its resting state. Thus, calcium ions appear to regulate numerous cellular processes over a wide dynamic range, providing intricate control and coordination of various physiological responses.

Endoplasmic/sarcoplasmic calcium release

The main provision of calcium stored intracellularly is in the ER/SR, contributing significantly to calcium signaling. When a stimulus is applied to induce calcium mobilization, ion channels in the ER/SR membrane open. Subsequently, calcium rapidly flows into the cytosol, binding to its effectors, which mediate various Ca^{2+}-dependent processes (David E. Clapham, 2007). The two main calcium release channels on the ER/SR responsible for the calcium influx are the inositol-1,4,5-trisphosphate receptor ($InsP_3R$) and the ryanodine receptor (RyR) (Figure 1).

$InsP_3R$ are large proteins comprising tetramers of four closely related subunits. They are divided into three receptor subtypes, $InsP_3R1$, $InsP_3R2$ and $InsP_3R3$, with tissue-specific expression patterns, with at least one subtype expressed in each cell type (Santulli et al., 2017; C. W. Taylor & Tovey, 2010; S. J. Taylor et al., 1991). Furthermore, each receptor subtype has a different level of affinity for its endogenous ligand IP_3, with $InsP_3R2$ ranking the highest and $InsP_3R1$ ranking the lowest (Iwai et al., 2007). Processes involving membrane hydrolysis due to activation of phospholipases C, yield high levels of the endogenous ligand IP_3. Four IP_3 molecules are needed, each binding to one of the four subunits of $InsP_3R$, to activate the ion channel allowing calcium to cross the ER/SR membrane (Alzayady et al., 2016; Iwai et al., 2007). Not only does IP_3 lead to the opening of the $InsP_3R$, but it also sensitizes $InsP_3R$ to calcium. Thus, the effluxing calcium binds to the $InsP_3$ receptor and mediates a biphasic effect in which it initially promotes the efflux but then becomes inhibitory after high intracellular concentrations have been reached (Michael J. Berridge et al., 2003; David E. Clapham, 2007). These tissue-independent processes, called calcium-induced calcium release (CICR), already an established feature of RyR (Endo et al., 1970), allow an active $InsP_3R$ to propagate its activity to neighboring $InsP_3R$. The ability to control the spatial and temporal calcium release via $InsP_3R$ allows the cell to customize the physiological effect. While local Ca^{2+} clusters are thought to be responsible for fast actions (e.g. muscle contraction, neurotransmitter release) which reacts on pulses within the micro- to millisecond range, Ca^{2+} oscillations or waves propagating within the cell are attributed to be responsible for coordinating multicellular responses (e.g. gene transcription, cell migration and cell proliferation) (M. J. Berridge, 1993; Michael J. Berridge et al., 2003; Prole & Taylor, 2019).

The other type of intracellular Ca^{2+} release channels are RyRs. These channels show up to 40% similarity to $InsP_3R$, which is also reflected in their appearance, since RyR also form a massive tetrameric shape (Zalk et al., 2015). There are three different subtypes of RyRs, with RyR1 being the predominant form in skeletal muscle, RyR2 in cardiac muscle, and RyR3 in the brain (Santulli et al., 2017). Upon membrane depolarization of the transverse tubule (T-tubule), voltage-gated calcium channels (VGCCs) can either i) open, allowing extracellular Ca^{2+} to enter and activate RyRs (chemical coupling, cardiac muscle), or ii) directly activate RyRs on the SR (physical coupling, vertebrate skeletal muscle) (David E. Clapham, 2007; Di Biase & Franzini-Armstrong, 2005; Santulli et al., 2017). Both forms of RyR activation generate local Ca^{2+} sparks that, like $InsP_3Rs$, can propagate in waves throughout the cell (Michael J. Berridge et al., 2003; Guatimosim et al., 2002). RyR, together with $InsP_3R$, represent the major signaling pathway for intracellular calcium formation and regulation of a variety of Ca^{2+}-dependent processes.

Activation of one of the aforementioned calcium channels thus ensures constant activation of RYR or $InsP_3R$, which amplify the prevailing calcium signal. In this way, appropriate levels of

cytosolic calcium are achieved, both temporally and in concentration, to meet the requirements of a physiological response (David E. Clapham, 2007).

Abnormal changes in Ca^{2+} homeostasis and regulation, including $InsP_3R$ or RyR dysfunction, are implicated in a number of major diseases including hypertension, heart disease, diabetes, manic depression and Alzheimer's disease (Michael J. Berridge et al., 2003; Santulli et al., 2017). Therefore, special attention will be given to the further study of the intracellular pathways of calcium mobilization, including these channels, which are the most important players in this process.

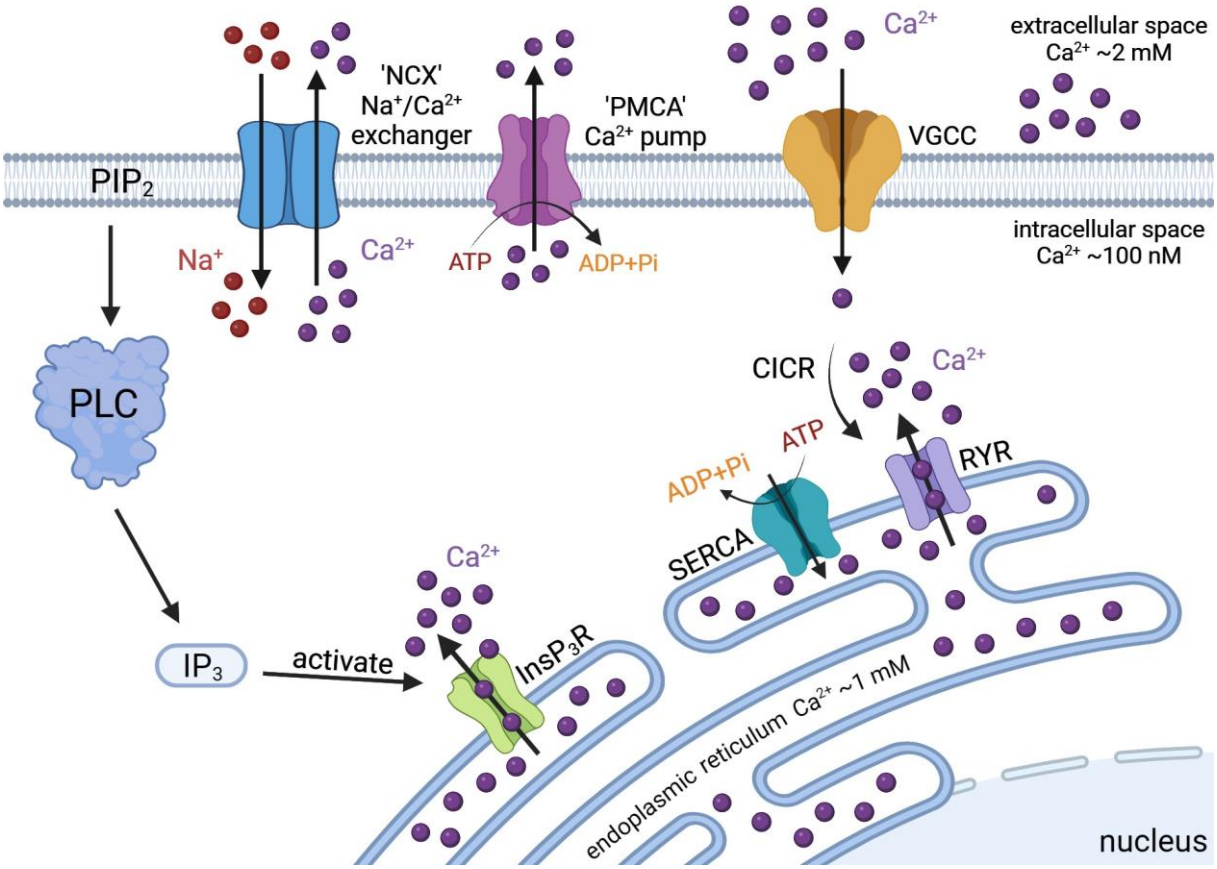

Figure 1. Intracellular mobilization and regulation of calcium. At the resting state, the low cytosolic calcium concentration is about 100 nM, while the extracellular calcium and the calcium stored in the ER/SR are in the mM range. Calcium pumps such as SERCA, PMCA and exchangers (NCX) maintain this low intracellular calcium level and create a constant gradient between the intracellular and extracellular domains. In order to maintain this gradient, the pumps require ATP, which they convert into ADP and Pi. To respond appropriately to physiological stimuli, the cell changes this gradient within micro- to milliseconds. Receptors such as the $InsP_3R$ and/or the RYR or membrane proteins such as VGCC are used for this purpose. $InsP_3$ receptors (green) are activated by their cognate ligand IP_3, which in turn is produced by large PIP_2-hydrolyzing enzymes known as phospholipases (PLC). In addition to $InsP_3Rs$, RYRs (light purple) are activated either by VGCCs due to i) CICR in cardiac muscle or ii) direct interaction with VGCCs in skeletal muscle (Michael J. Berridge et al., 2003; David E. Clapham, 2007; Di Biase & Franzini-Armstrong, 2005; Santulli et al., 2017).

Phospholipases C

Phospholipases are enzymes by which phospholipids are cleaved into fatty acids and other lipophilic substances. They are divided into four main groups: A_{1-2}, B, C, D.

The family of phospholipases C (PLC) is a central regulator of intracellular calcium signal transduction. As a phosphodiesterase, it hydrolyzes the membrane component phosphoinositol-4,5-diphosphate (PIP_2) into the PKA-activating messenger diacylglycerol (DAG) and the Ca^{2+}-mobilizing messenger IP_3 (Gresset et al., 2012; Kadamur & Ross, 2013). Mammalian cells express six families of PLCs that differ in their tissue distribution and cellular regulation. Strikingly, the enzyme-mediated reaction is the same for all PLCs. To ensure efficient Ca^{2+} signaling and respond appropriately to any demand from the body, cells express multiple PLCs that they use in combination (Figure 2). Regulation of PLC-δ enzymatic activity is largely controlled by the abundance of PIP_2 and intracellular Ca^{2+} concentrations. Thus, PLC-δ may further enhance Ca^{2+} signaling by responding to increased cytosolic Ca^{2+} levels induced by the action of other PLCs (Guo et al., 2010). The same behavior was observed for the closely related PLCη, which is mainly found in neuronal cells and is also activated by rising cytoplasmic Ca^{2+} (Kim et al., 2011; Popovics et al., 2011). Furthermore, a possible activation via Gβγ subunits seems to be considered (Zhou et al., 2008). Almost all growth factor receptors with its intrinsic tyrosin kinase (RTK) activity and some soluble RTKs lead to the phosphorylation/activation of PLCγ upon stimulation (Kamat & Carpenter, 1997). A special role in fertilization is assigned to PLCζ. This PLC isoform is expressed only in sperm and, after fusion with an egg, provides Ca^{2+} oscillations necessary for the fertilized egg to mature and grow (Saunders et al., 2002). Small amounts of Ca^{2+} appear to activate PLCζ, although some constitutive activity is also suggested (Gresset et al., 2012; Kadamur & Ross, 2013). While PLCε can be activated by all signaling pathways that activate Ras, Rap, and Rho, including activation of G protein-coupled receptors (GPCRs), PLCβ is primarily activated by GPCRs. Both PLC families have a particular impact on G protein-mediated cellular effects. Whereas the CDC25-homology domain of PLCε functions as a Ras- Guanosine triphosphate exchange factor (GEF), thus maintaining its own activity (feedforward loop) (Bunney et al., 2009; Kadamur & Ross, 2013; Alan V. Smrcka et al., 2012), PLCβ with its C-terminal coiled-coil domain, functions as a GTPase-activating G protein (GAP) that accelerates the hydrolysis of GTP-bound $G\alpha_q$, promoting its deactivation (Berstein et al., 1992; Ross, 2008).

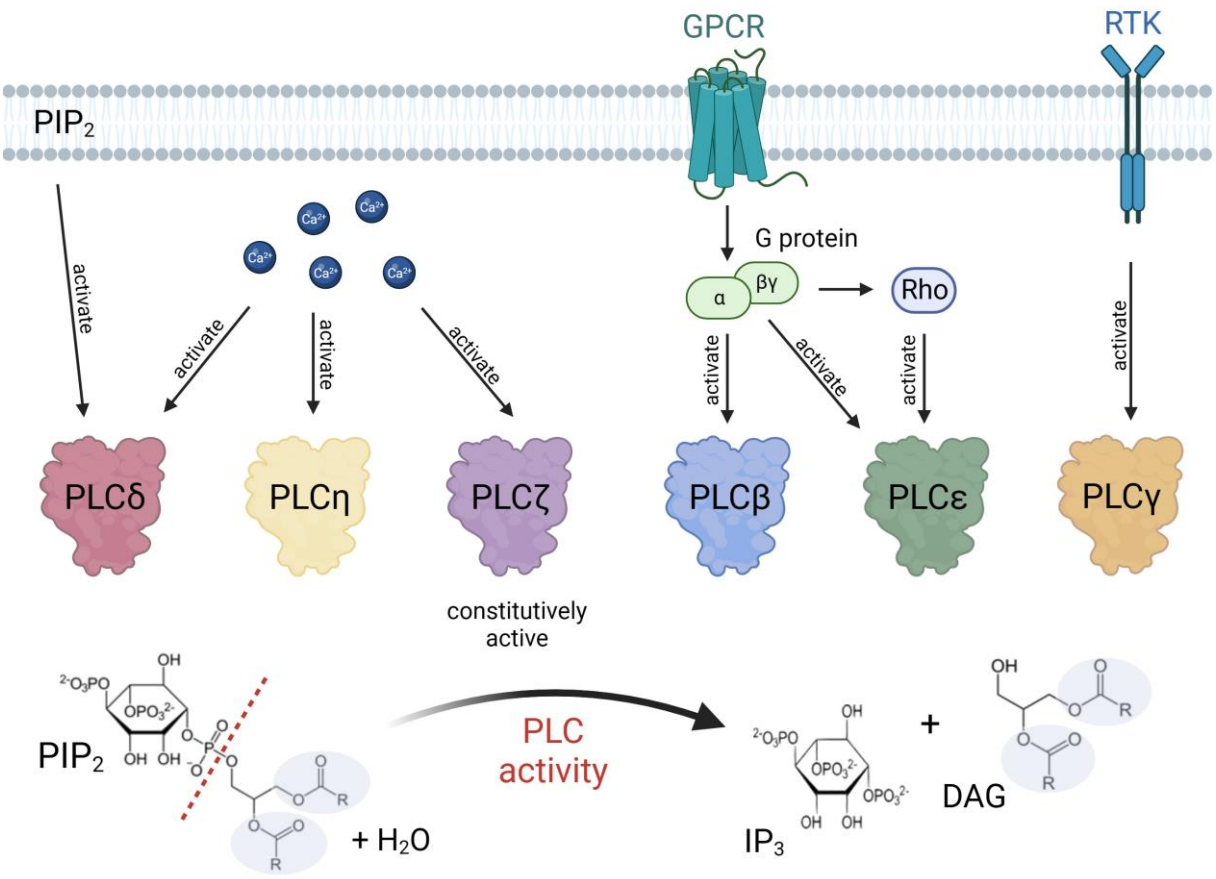

Figure 2. The phospholipase C family. Phospholipases C are a large family of membrane-cleaving enzymes divided into six isoforms (Kadamur & Ross, 2013). Despite structural differences within the regulatory domain, all phospholipase C isoforms share a similar active site that catalyzes the hydrolysis of PIP_2 to DAG and IP_3, while their activation is controlled by different cellular signals or stimuli (Gresset et al., 2012).

PLCβ structure and activation

The phospholipase C-beta (PLCβ) family plays a central role in G protein-mediated calcium release. Consisting of over 1000 amino acids (Kadamur & Ross, 2013), the PLCβ family is divided into four subtypes PLCβ 1-4 that share a highly conserved catalytic core structure. This core structure consists of a N terminal-pleckstrin homology (PH) domain, four tandem EF-hand repeats, a triosephosphate isomerase (TIM)-like barrel domain divided into X and Y halves containing the active site, and a C2 domain (Figure 3A, B) (Hicks et al., 2008; Lyon & Tesmer, 2013). Starting with the PH domain, which can bind activating and anchoring ligands (PIP_2, Rac-GTPase, Ca^{2+}, Gβγ), it is also predicted to contribute to membrane binding, since PH domains have high specificity and affinity for certain phospholipids (Kadamur & Ross, 2013; Lemmon, 2004). Four tandem EF-hand repeats, primarily used for calcium binding in other proteins (e.g., calmodulin), accelerate GTP hydrolysis by $G\alpha_q$ in PLCβ. This domain is followed by the TIM barrel, a catalytic domain, which in turn is divided into an X and a Y domain (Essen

et al., 1996). Both domains are connected by an acidic linker called the X-Y linker, which occludes the active site of the enzyme. The C-terminal extension of the C2 domain, which is responsible for Ca^{2+} and membrane surface binding, is divided into the proximal C-terminal domain, containing the primary $G\alpha_q$ binding site (Hα1-Hα2), and the distal C-terminal domain, which has a coiled-coil structure and serves as the primary membrane binding factor (Gresset et al., 2012; Kadamur & Ross, 2013; Lyon & Tesmer, 2013). To regulate its activity, PLCβ has two autoinhibitory domains that prevent membrane association and thus provide low basal activity. These include the X-Y linker within the TIM barrel and the Hα2' helix in the C-terminal extension (Hicks et al., 2008; Lyon et al., 2014; Lyon et al., 2011)

Activation of PLCβ involves the direct interactions with the G protein subunits $G\alpha_q$ and Gβγ. (Camps et al., 1992; Harden et al., 2011; A. V. Smrcka et al., 1991; A. V. Smrcka & Sternweis, 1993; Gary L. Waldo et al., 2010). Crystal structure analysis of GTP bound $G\alpha_q$ reveals its allosteric activation of PLCβ by binding the helix-turn-helix motif (Hα1-Hα2, HTH) of the proximal CTD. The binding displaces the Hα2' helix from the catalytic core (Lyon et al., 2011), leading to a re-localization of the PLCβ core structure towards the membrane (Gary L. Waldo et al., 2010). By the consequent approach of the core structure, the acidic stretch of the X-Y linker is displaced via electrostatic repulsion with the negatively charged membrane interface (Hicks et al., 2008; Lyon et al., 2014), ultimately exposing the active site of the enzyme and initiating substrate hydrolysis. Unlike $G\alpha_q$, which can activate any PLCβ family isoform, Gβγ selectively activates PLCβ2 and PLCβ3, and to some extent PLCβ1 (Fisher et al., 2020; A. V. Smrcka & Sternweis, 1993). Due to lacking crystal structure analysis of Gβγ in complex with PLCβ3, the binding mode of Gβγ is not entirely clear. However, Gβγ is thought to interact with regions of the N-terminal PH domain, the EF hands, or the Y domain as part of the TIM barrel. (Barr et al., 2000; Lyon & Tesmer, 2013; A. V. Smrcka, 2008).

PLCβ2 and PLCβ3 can be simultaneously activated by $G\alpha_q$ and Gβγ resulting in a synergistic activation of the enzymes (Philip et al., 2010; Rebres et al., 2011). On a physiological basis, simultaneous activation ensures the provision of high cellular calcium levels for adequate cellular function when needed (Abrams, 2005; Rebres et al., 2011; Roach et al., 2008; Shah et al., 1999). Interestingly, both $G\alpha_q$ and Gβγ are able to activate PLCβ, but there are significant differences in their potency (A. V. Smrcka & Sternweis, 1993). To mimic the $G\alpha_q$-PLCβ effect, approximately tenfold higher concentrations of Gβγ are required. The provision of the necessary Gβγ is mainly attributed to Gi-coupled receptors due to their high cellular expression (Kadamur & Ross, 2013). However, some studies have shown that simultaneous and/or sequential activation of a Gq- and a Gs-coupled receptor also results in increased Ca^{2+} levels (Short & Taylor, 2000; Tovey et al., 2008; Werry et al., 2002). Therefore, a possible contribution of Gs-βγ to the synergistic activation of PLCβ cannot be excluded. Consequently, not only

Gi-GPCRs but also other G protein families (Gs, Gq, G12/13) must be considered as Gβγ suppliers for PLCβ activation.

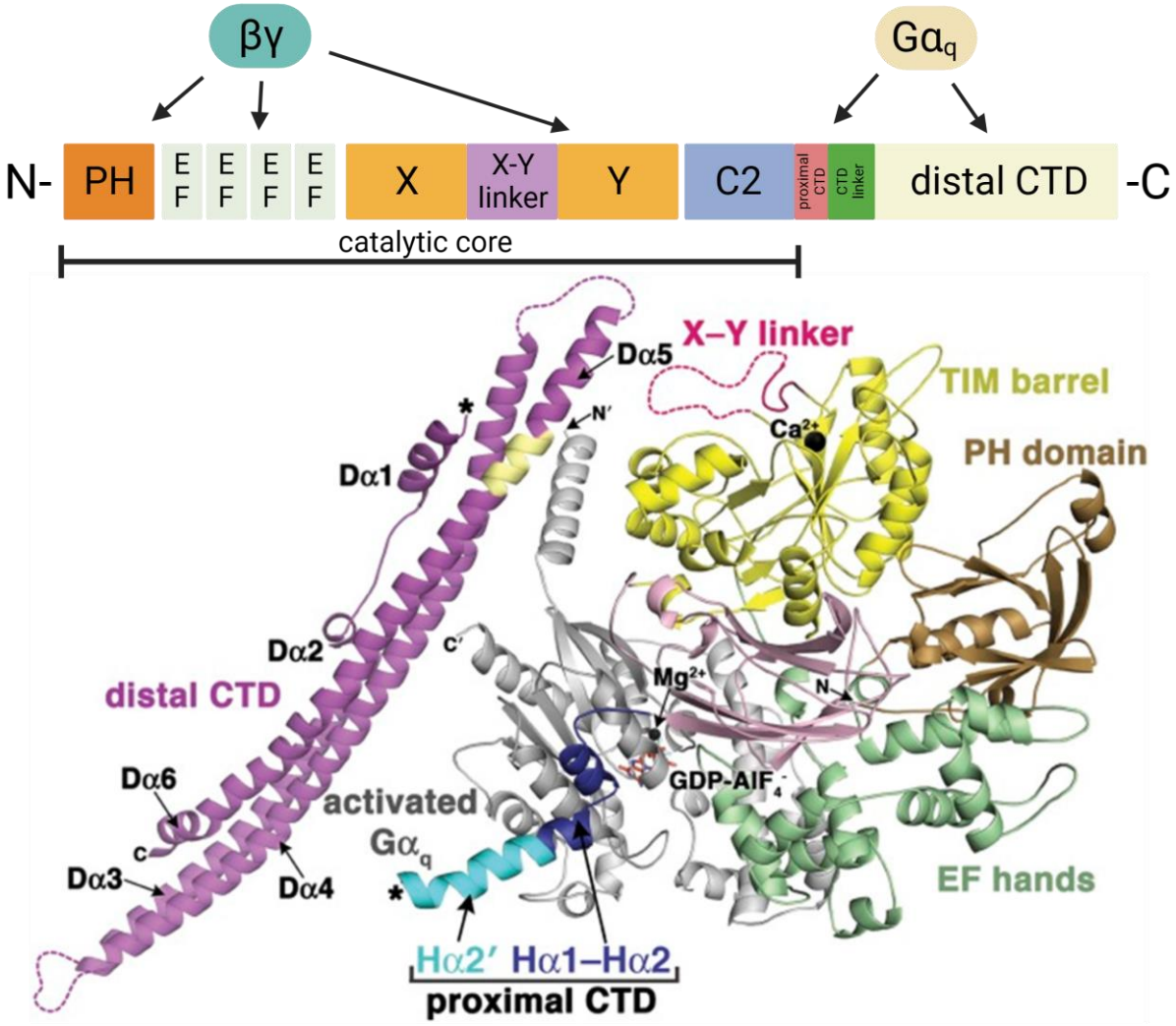

Figure 3. Structural insight of Phospholipase Cβ. Based on (Hicks et al., 2008; Lyon & Tesmer, 2013; Gary L. Waldo et al., 2010) (A) The structural domains of PLCβ from the N- (left) to the C-terminus (right). The domains interacting with Gβγ or $G\alpha_q$ are indicated by black arrows. (B) Complex of PLCβ3 with $G\alpha_q$ (Lyon et al., 2011). The active site of the enzyme is located within the TIM barrel structure (yellow). It is partially concealed by an X-Y linker region (pink) that exhibits disorder. $G\alpha_q$ interacts with the helix-turn-helix motif (Hα1-Hα2, HTH) and part of the distal C-terminal domain (CTD), highlighted in light yellow. To date, there is no X-ray structure of a PLCβ-Gβγ complex.

G protein-coupled receptors and G proteins

Every facet of cellular existence is influenced and altered by the outside world. In addition to the harmful influences from which the cell must be protected, many of these impacts are of vital importance to the cell. Regulatory access to the environment is provided by a lipid bilayer, the cell membrane, which protects the cell and allows communication through the proteins within it. One group of these proteins that run cellular function and translate extracellular stimuli into intracellular stimuli are GPCRs.

With well over 800 members encoded in the human genome, GPCRs are the largest family of membrane proteins divided into five main classes: i) the rhodopsin family (class A), ii) the secretin family (class B), iii) the glutamate and GABA family (class C), iv) the frizzled class, and v) the adhesion receptors. Although sub-divided, they share a common structure. Seven transmembrane (TM) α-helices with an extracellular N-terminus and an intracellular C-terminus connected by three loops on each side of the membrane (Hauser et al., 2017; Rosenbaum et al., 2009). A large variety of extracellular ligands such as photons, ions, small molecules, and peptides bind to the extracellular transmembrane region and activate the GPCR. Upon activation the GPCR undergoes a conformational change that results in a shift of the TM6 and TM5 regions and some microswitches within these transmembrane segments. The movement of TM6 and TM5 creates a cleft capable of binding a G protein that carries the extracellular signal to intracellular effectors and triggers a complex network of cellular responses (Katritch et al., 2013; Nygaard et al., 2009; Rosenbaum et al., 2009).

GPCRs are involved in a wide range of physiological and pathophysiological processes, including the neurologic, metabolic, pulmonary, and cardiovascular system. Additionally, cancer is also strongly affected by GPCRs, which in turn represent important targets for current and potential therapies. This explains why GPCRs play a vital role in research and are the subject of numerous clinical trials. Their relevance to these processes, but also their drugability through extracellular accessibility and abundance, has ensured that up to 30% of US Food and Drug Administration (FDA)-approved drugs directly target them (Hauser et al., 2017).

The signal transduction of GPCRs is canonically linked to the activation of guanine-nucleotide-binding proteins (G proteins) (Oldham & Hamm, 2008). G proteins are composed of three subunits, Gα, Gβ, and Gγ, which combine in various combinations to form a heterotrimer. The Gα subunit, on which the classification of G proteins is based, exists as a highly conserved protein fold consisting of a GTPase domain and a helical domain. These two domains form a nucleotide binding site capable of binding the guanine nucleotides GDP or GTP giving G proteins their name (Oldham und Hamm 2008; Syrovatkina et al. 2016). The Gβ and Gγ subunit form a functional dimer (Gβγ) within the heterotrimer due to nearly inseparable interactions between the Gβ and Gγ protein. Both, Gα and the Gβγ undergo post-translational

modifications that endow them with lipid moiety's serving as regulators of membrane localization and protein-protein interactions (Fogg et al., 2001; Oldham & Hamm, 2008). This structural division into a Gα and a Gβγ subunit also applies to G protein-mediated signaling, as both Gα and the Gβγ dimer function as intracellular mediators.

Depending on the guanine nucleotide, GDP or GTP, bound to the alpha subunit, two states of G proteins are described. In the resting GDP-bound state (inactive) Gα heterotrimerizes with Gβγ due to its high affinity (Figure 4A). Activation of a GPCR with subsequent change in Gα conformation dissociates GDP from its Gα-binding pocket (Figure 4B). Notably, the GPCR itself acts as a guanine-exchange factor that promotes GDP release. This paves the way for binding of equally high-affinity GTP to the empty Gα pocket, whose high cellular concentration exceeds that of GDP many times. Moreover, the binding of GTP to Gα (active) leads to a structural rearrangement of the heterotrimers, resulting in the loss of the Gβγ-binding surface and the uncoupling of the reversible bound Gβγ from Gα. Consequently, Gα (GTP) and Gβγ can bind and trigger downstream effectors, initiating the intracellular signaling cascade (Figure 4C) (Oldham & Hamm, 2008; Rosenbaum et al., 2009). Once the effectors are activated, the intrinsic GTPase activity of the Gα subunit hydrolyses GTP to GDP terminating the signal (Figure 4D). This deactivation process is accelerated by GTPase-activating proteins, which are often the Gα effector proteins. With the loss of GTP, Gα also loses its affinity for the effector and regains its affinity for Gβγ. Thus, the heterotrimer is once again ready to bind and transmit the extracellular signal upon activation of a GPCR (Syrovatkina et al., 2016). The rapid on/off rates of the GPCR-G protein complex are of paramount importance for cellular homeostasis, as the GPCR circuitry provides the cell with the ability to sense, process, and regulate environmental influences.

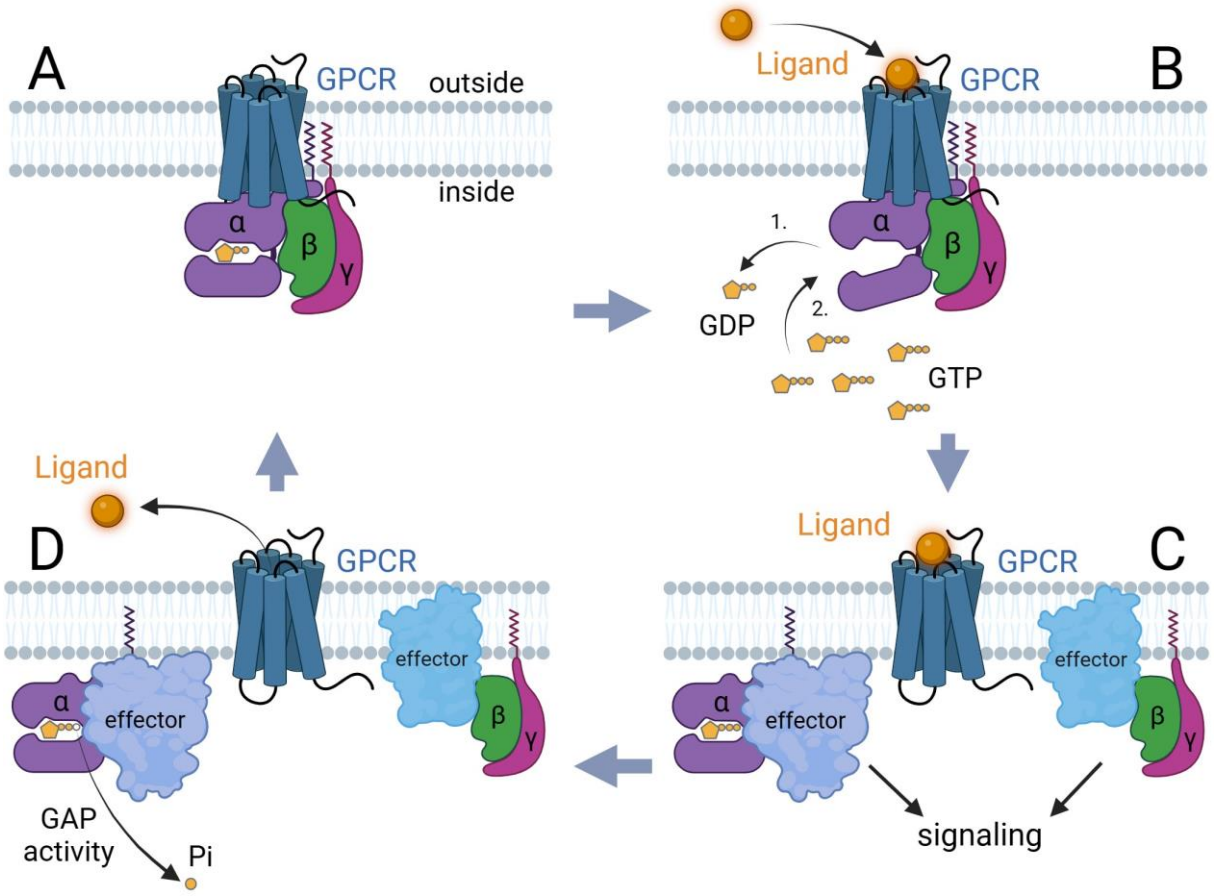

Figure 4. Activation of G proteins via G protein-coupled receptor. Based on (Oldham & Hamm, 2008; Rosenbaum et al., 2009; Syrovatkina et al., 2016). (A) In the inactive state, a heterotrimeric G protein consisting of the GDP-bound Gα and Gβγ subunits is bound to the cell membrane via prenylated residues of the Gα and Gγ subunits. (B) Activation of the GPCR leads to conformational changes in the Gα subunit. This results in the release of GDP, which is replaced by GTP, which is highly abundant in the cell. (C) The GTP-bound Gα dissociates from its Gβγ subunit, thus allowing both subunits to bind to their respective effectors. (D) The intrinsic GTPase activity of the Gα subunit, assisted by GTPase-activating proteins, hydrolyzes GTP to GDP and Pi. Upon hydrolysis of Pi from GTP, the GDP-bound Gα subunit regains its affinity for the Gβγ subunit, resulting in the reassembly of a heterotrimeric complex.

G protein signaling

The specificity of GPCR coupling and downstream target regulation is largely determined by the character of the Gα subunit (Figure 5). In the human genome, 16 genes encode 23 known Gα proteins (McCudden et al., 2005). There are four different families of Gα isoforms: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$, all of which trigger individual canonical cellular processes in their GTP-bound state (Oldham & Hamm, 2008). A major function of the $G\alpha_{q/11}$ family is the activation of PLCβ, thereby affecting intracellular calcium events (A. V. Smrcka et al., 1991; S.

J. Taylor et al., 1991; G. L. Waldo et al., 1991). In addition, $G\alpha_{q/11}$ proteins activate the small GTPase (small G protein) Rho (Williams et al., 2007), as does the $G\alpha_{12/13}$ family. However, the G12 and G13 signaling pathway is the major pathway for the activation of Rho (Hains et al., 2006; Seifert et al., 2004; Wing et al., 2001). By initiating the Rho signaling network Gq and G12/13 appear to regulate PLCε activity (Syrovatkina et al., 2016). The $G\alpha_{i/o}$ proteins are inhibitors of the membrane-bound adenylyl cyclases (ACs) that convert adenosine-5´-triphosphate (ATP) to the second messenger 3‘,5‘-cyclic adenosine monophosphate (cAMP), while $G\alpha_s$ proteins are the counterpart of Gi that stimulate the enzyme (Campbell & Smrcka, 2018). Next to the G protein alpha subunits, the Gβγ heterodimer is also a bona fide signal transducer that activates various effector targets such as K^+ inward channels (GIRK), PLCβ, ion channels, and many more (D. E. Clapham & Neer, 1993; A. V. Smrcka, 2008).

Gβγ signaling

Since Gα was initially shown to trigger the activation of intracellular effectors, it was rational to assume that the G alpha subunits were the primary transducers. For this reason, it has long been thought that Gβγ only acts as a guanine nucleotide dissociation inhibitor (GDI), inhibiting free Gα, thereby terminating Gα-mediated effects and facilitating the binding between the heterotrimer and the GPCR (D. E. Clapham & Neer, 1993). However, Gβγ subunits also appear to be signal transducers, either modulating, activating or inhibiting effectors (D. E. Clapham & Neer, 1993; McCudden et al., 2005; A. V. Smrcka, 2008).

In the human genome, 5 different Gβ and 12 Gγ subunits are expressed, resulting in 60 possible combinations, although not all combinations occur (Oldham & Hamm, 2008). There is little evidence that specific Gβγ combinations causes certain Gα coupling or effector activation. Nevertheless, some signaling pathways are activated by certain Gβγ pairs (Jones et al., 2004; Maier et al., 2000). Gβγ signaling in general is less potent than Gα effector activation. Approximately 10-fold higher concentrations of Gβγ (EC_{50} of 20-200 nM) are required to mimic the Gα effects (A. V. Smrcka & Sternweis, 1993). However, EC_{50} values of G proteins such as $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12/13}$ are at concentrations of 1-5 nM and in most cases are expressed at similarly low concentrations in cells, well below the required effector-activating Gβγ concentration. Consequently, only the most abundant Gi heterotrimers have the stoichiometric capacity to release enough Gβγ to activate the effectors (Kadamur & Ross, 2013). Furthermore, many physiological processes attributed to Gβγ are inhibited by PTX treatment (Pfaffinger et al., 1985; A. V. Smrcka, 2008), next to the findings, that Gi proteins do not bind their Gβγ subunit as strongly as other G proteins (“Clamshell” Model) (Chung & Wong, 2020). Thus, there is much to suggest that Gβγ derived exclusively from Gi heterotrimers are capable of signal transduction. These assumptions must take into account that cellular

processes are much more complicated and that the levels of G proteins vary in a tissue-specific and subcellular manner (A. V. Smrcka, 2008). Also, simultaneous activation of GPCRs coupled to different G proteins could additively provide stoichiometric Gβγ levels for effector activation, next to the pre-coupling of receptors, G proteins, and effectors which could also confer a Gβγ specificity (Riven et al., 2006). To date, the regulation of Gβγ signaling is still not fully understood and remains of great importance. Still, it seems increasingly clear that the signal transmission of Gβγ is in no way inferior to that of Gα proteins.

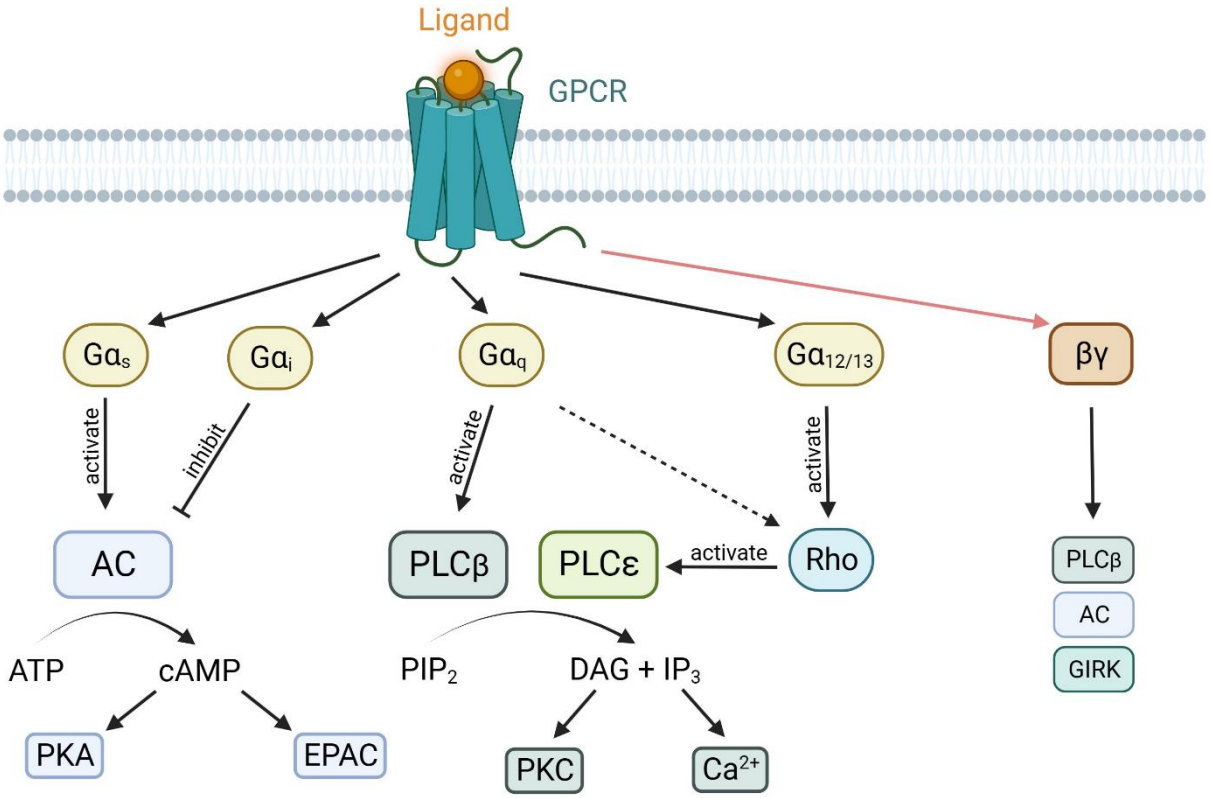

Figure 5. G protein signal transduction. Based on (McCudden et al., 2005; A. V. Smrcka, 2008). G proteins are classified into four families ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$) based on the structural and functional properties of their Gα subunit. The canonical signaling pathways of each G family are indicated by black arrows next to the effectors that are either activated or inhibited. Second messenger targets are also indicated. The dashed gray arrow represents a non-canonical pathway. In addition to the Gα families, the Gβγ subunit also functions as a bona fide signal transducer.

Gs-mediated calcium

Calcium signaling is mainly attributed to Gq-coupled proteins, as $G\alpha_q$ binds and activates PLCβ isoforms (A. V. Smrcka et al., 1991; S. J. Taylor et al., 1991; G. L. Waldo et al., 1991). In addition to $G\alpha_q$, Gβγ subunits can also trigger the same signaling cascade by activating PLCβ2 or PLCβ3, the isoforms most sensitive to Gβγ (Kadamur & Ross, 2013). This pathway is

thought to be more specific for Gi-derived Gβγ, thus linking activation of Gi-GPCRs to calcium mobilization (Kadamur & Ross, 2013; Pfeil et al., 2020; A. V. Smrcka, 2008). Moreover, also Gs-GPCRs are involved in intracellular calcium mobilization through various pathways. These include cAMP-dependent PKA phosphorylation of L-type calcium channels in cardiomyocytes (Kamp & Hell, 2000), cAMP-dependent sensitization of $InsP_3R$ channels (Konieczny et al., 2017), and cAMP-EPAC-dependent activation of PLCε (Schmidt et al., 2001). All these pathways are canonically triggered by initiating AC activity and a rise of second messenger cAMP. However, in several studies, Gs-GPCR calcium was detected only after pre- or costimulation of a Gq-GPCR and cAMP did not contribute significantly to this calcium (Short & Taylor, 2000; Tovey et al., 2003; Werry et al., 2002). Perfectly in line with this Gq requirement is the newly discovered Gi-βγ-calcium by Pfeil et al., 2020, raising the question of whether Gs-βγ also release calcium.

Goal of the study

Based on the discovery of a Gq-dependent Gi-βγ-PLCβ mediated calcium release, this study aims to investigate whether calcium events could be triggered by Gs-coupled receptors in a similar manner. In addition to $G\alpha_s$ increasing cytosolic calcium levels (Kamp & Hell, 2000; Konieczny et al., 2017; Schmidt et al., 2001), there is growing evidence that the Gβγ subunit derived from Gs also contributes to it (Short & Taylor, 2000; Tovey et al., 2003; Werry et al., 2002). The exact mechanism and factors responsible for the contribution of Gs-βγ to intracellular calcium events are not yet fully understood. This study aims to elucidate the underlying mechanism behind Gs-βγ-Ca^{2+} in order to gain new insights into the calcium signaling machinery. If we succeed, we will not only gain a deeper understanding how Gs-GPCRs impact cellular calcium events, but also support the role of Gβγ as a bona fide signal transducer that regulates calcium. This, in turn, will lay the foundation for new discoveries in a wide range of disease research, given the profound impact of calcium on various physiological activities.

Material

Table 1: Chemicals and Reagents

Name	Source	Ref. No.
5-HT	Sigma Aldrich	Cat# H9523-25mg
Isoproterenol (Iso)	Sigma Aldrich	Cat# I5627
A23187	Sigma Aldrich	Cat# C7522
ATP	Sigma Aldrich	Cat# A1852-1VL
Carbachol (CCh)	Sigma Aldrich	Cat# C4382-1g
Coelenterazine	Carbosynth Limited	Cat# EC14031
Coelenterazine h	NanoLight	Cat# 301-1
Forskolin (Fsk)	Bachem	Cat# TRC-F701800
FR900359	G. König lab	N/A
PKi 14-22	Sigma Aldrich	Cat# 476485
Isobutylmethylxanthine (IBMX)	Sigma Aldrich	Cat# I5879
AVP	Sigma Aldrich	Cat# 113-79-1
NECA	Sigma Aldrich	Cat# E2387
PGD_2	Biomol	Cat# Cay12010-1
PGE_1	Sigma Aldrich	Cat# 745-65-3
poly-D-lysine	Sigma Aldrich	Cat# P2636
poly-ethylene imine	Polysciences	Cat# 24313-2
ICI 118.551	Sigma Aldrich	Cat# I127
CGP 20712A	Sigma Aldrich	Cat# C231
PKI 14-22 amide	Sigma Aldrich	Cat# 476485
HJC0197	Biolog	Cat# C136
PTX	Thermo Fisher Scientific	Cat# PHZ1174
Gallein	Sigma Aldrich	Cat# 2103-64-2
thapsigargin	Sigma Aldrich	Cat# T9033
2-APB	Sigma Aldrich	Cat# 524-95-8

EGF	Sigma Aldrich	Cat# E9644
UTP	Sigma Aldrich	Cat# U4125
SNAP-Lumi4-Tb labeling reagent	Cisbio International	Cat# SSNPTBD
SNAP-Surface® Alexa Fluor® 647	New England Biolabs	Cat# S9136S

Table 2: Cell Culture Media

Name	Source	Ref. No.
DMEM-Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	Cat# 11965092
Hanks' Buffered Salt Solution (HBSS)	Thermo Fisher Scientific	Cat# 14175129

Table 3: Media Supplements

Name	Source	Ref. No.
Penicillin-Streptomycin- Solution	Thermo Fisher Scientific	Cat# 15140
Penicillin-Streptomycin-Amphotericin B Solution	Thermo Fisher Scientific	Cat# 15240
Fetal Bovine Serum (FBS)	Sigma Aldrich	Cat# -0804
4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES)	Carl Roth GmbH + Co. KG	Cat# HN77.4

Table 4: Antibodies

Name	Source	Ref. No.
mouse anti-PLCβ3	Santa Cruz Biotechnology	Cat# sc-133231; RRID: AB_2299534
rabbit anti-β-actin	BioLegend	Cat# 622102; RRID: AB_315946
goat anti-rabbit IgG Antibody HRP	antikoerper-online	Cat# ABIN102010; RRID: AB_10762386
goat anti-mouse IgG antibody HRP	Sigma Aldrich	Cat# A4416; RRID: AB_258167

Table 5: Plasmids

Name	Source	Ref. No.
pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1218
pCAGGS	Asuka Inoue Lab, Sendai	Plasmid #1440
M3-receptor-pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1205
V2-receptor-pcDNA3.1	Asuka Inoue Lab, Sendai	Plasmid #1518
LgBiT-PLCβ3-pCAGGS	Asuka Inoue Lab, Sendai	Plasmid #1452
SmBiT-Gγ2-pCAGGS	Asuka Inoue Lab, Sendai	Plasmid #1468
Gβ1-pCAGGS	Asuka Inoue Lab, Sendai	Plasmid #1455
$G\alpha_q$-pcDNA3.1	Asuka Inoue Lab, Sendai	Plasmid #1531
$G\alpha_s$-pcDNA3.1	Asuka Inoue Lab, Sendai	Plasmid #1530
PLCβ1-pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1515
PLCβ2-pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1124
PLCβ3-pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1354
PLCβ4-pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1516
PLCβ3^{F715A}-pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1533
PLCβ3$^{\Delta XY}$-pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1532
IP_3-sensor	Gulyás et al., 2015	Plasmid #1561
pGloSensor™-22F cAMP plasmid	Promega Corporation	Cat# V1501
masGRK3ct-pcDNA3.1	Nevin Lambert Lab, Georgia	Plasmid #1581
Nanobody5-pcDNA3.1	Jan Steyaert Lab, Brussels	Plasmid #1582
Nanobody17-pcDNA3.1	Jan Steyaert Lab, Brussels	Plasmid #1583
SNAP-ß2-AR pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1362
SNAP-ß1-AR pcDNA3.1	Evi Kostenis Lab, Bonn	Plasmid #1361

Table 6: Bacterial strains

Name	Source	Ref. No.
DH5α Competent Cells	Thermo Fisher Scientific	Cat# 18265-017
XL1-Blue Competent Cells	Stratagene	Cat# 200130

Table 7: Cell lines

Name	Source	Ref. No.
Human: native HEK	Asuka Inoue Lab, Sendai	N/A
Human: DP1 HEK	Evi Kostenis Lab, Bonn	N/A
Human: ΔGs HEK	Asuka Inoue Lab, Sendai	N/A
Human: ΔGq/11/12/13 HEK	Asuka Inoue Lab, Sendai	N/A
Human: Δ7 HEK	Asuka Inoue Lab, Sendai	N/A
Human: PLCβ1-4 mut. HEK	Asuka Inoue Lab, Sendai	N/A
Human: ΔAC3/6 HEK	Val J. Watts Lab, West Lafayette	N/A
Human: native HEK	Val J. Watts Lab, West Lafayette	N/A
Mouse: brown adipocytes	Alexander Pfeifer Lab, Bonn	N/A
Mouse: embryonic fibroblasts - Ttc21b	Dagmar Wachten Lab, Bonn	N/A

Table 8: Commercial Assay Kits

Name	Source	Ref. No.
ECL Prime Western blotting detection reagent	GE Healthcare	Cat# RPN2236
FLIPR® Calcium 5 Assay kit	Molecular Devices	Cat# R8186
HTRF-cAMP dynamic 2 kit	Cisbio International	Cat# 62AM4PEC
cAMP-Glo™ Assay	Promega Corporation	Cat# V1501
Pierce BCA Protein Assay	Thermo Fisher Scientific	Cat# 23225

Methods

Cell culture

Culture conditions

All cell lines were cultured in standard sterile cell culture flasks at 37°C in a 5% CO_2 humidified atmosphere. Depending on the growth rate, cells were passaged approximately 2 to 3 times per week. In this way, confluence was maintained below 80% and cells were replenished with media. Removal of the medium, washing of the cells several times with phosphate-buffered saline (PBS) (3-5 ml), and subsequent addition of the cell-detaching trypsin (1-2 ml) were performed under laminar airflow conditions. To terminate cell trypsinization, 10 mL of medium was added after complete cell detachment.

Cell culture medium

Different media mixtures were used to create optimal growth conditions depending on the cell line.

HEK parental, **HEK-DP1**, **CRISPR/Cas9-edited** cells lines**, murine brown preadipocytes** and **mouse embryonic fibroblasts** were kept in the following media mixture:

Constituent	Volume (ml)	Final concentration
Dulbecco's Modified Eagle Medium (DMEM)	500	
FBS	50	10%
Penicillin-Streptomycin mixture	5	100 U/ml Penicillin, 0.1 mg/ml Streptomycin

HEK-ΔAC3/6 cells were kept in the following media mixture:

Constituent	Volume (ml)	Final concentration
Dulbecco's Modified Eagle Medium (DMEM)	500	
FBS	50	10%
Penicillin-Streptomycin-Amphotericin B Solution	5	10,000 U/ml Penicillin, 10 mg/ml Streptomycin, 25µg/ml Amphotericin B

Transient transfection

Cells were transiently transfected with the cationic polymer polyethyleneimine (PEI, 1 mg/ml) 48 hours prior to measurement. PEI forms a positively charged complex with DNA, which binds to negatively charged surfaces and enters the cytoplasm by endocytosis. Two approaches were prepared for transfection: i) the DNA mixture containing 10 µg of the desired plasmid DNA in 300 µl of Opti-MEM and ii) the transfection reagent mixture containing 30 µl of PEI in 300 µl of Opti-MEM (DNA:PEI ratio 1:3). Both approaches were mixed and inverted. After 15 to 20 minutes of incubation, the approach was gently pipetted into a 10 cm dish containing 4 million cells in 9 ml HEK medium. If the number of cells for the experiments performed was different, the components were always adjusted to keep the ratio the same.

Isolation of primary cells

Murine brown preadipocytes (mBA):

Newborn pups of wild type mice were sacrificed and the interscapular BAT was harvested for the collection of mesenchymal stem cells. For the subsequent digestion, Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 123 mM Na^+, 5 mM K^+, 1.3 mM Ca^{2+}, 131 mM Cl^-, 5 mM glucose, 1.5% (w/v) bovine serum albumin (BSA), 100 mM Hepes, and 0.2% (w/v) collagenase type II (pH 7.4) was used for 30 minutes at 37°C. Digested tissue was filtered through 100 µm nylon mesh to remove cell debris and placed on ice for 30 min. This was followed by filtering through a 30 µm nylon sieve and centrifuging at 700 g for 10 minutes. The harvested preadipocytes were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin, streptomycin (100 µg/ml) (P/S), 4 nM insulin, 4 nM triiodothyronine, 10 mM Hepes, and sodium ascorbate (25 µg/ml). For immortalization, the cells were incubated at 37°C and 5% CO_2 at 60,000 cells per cm^2 in a 6 cm dish. Transduction was performed with lentivirus containing SV40 large T antigen. The cells were then cultured in DMEM supplemented with FBS, penicillin, and streptomycin, as previously described (Klepac et al., 2016).

Mouse embryonic fibroblasts (MEF):

For isolation of mouse embryonic fibroblasts, embryos are collected from a pregnant mouse. The embryos are washed with PBS and the head, viscera and extremities are removed, followed by a second washing step with PBS and cutting of the embryo into smaller pieces. The resulting tissue is incubated with 1 ml of trypsin-EDTA solution at 37 °C for 5 to 10 minutes until the tissue is completely dissociated. Stop trypsinization with the addition of 1-2 ml of DMEM supplemented with 10% FBS. To obtain a cell suspension, the cells are centrifuged at

1,500 rpm for 5 minutes. The cell pellet is then resuspended in DMEM/10% FBS and placed in a cell culture flask. Now, the cells are incubated at 37°C in 5% CO_2 with medium changes every 2 to 3 days (Xu, 2005).

Cell-based assays

Population-based Ca^{2+} mobilization:

For HEK parental, HEK-DP1, CRISPR/Cas9-edited and MEF cells, 60,000 cells were seeded in poly-D-lysine-coated (PDL) 96-well plates and cultured overnight. The next day, the medium was removed and 50 µl of calcium-5 dye (Molecular Devices, Sunnyvale, CA, USA) was added to each well. Prior to measurement, the wells filled with the dye were incubated at 37°C for 45 to 60 minutes. A total of 150 µl of Hanks' Buffered Saline Solution (HBSS) containing 20 mM Hepes was added to the wells (HBSS + Hepes) for the single addition Ca^{2+} assay and 100 µl for the double addition Ca^{2+} assay. Ca^{2+} mobilization was measured as the increase in fluorescence over time using the FlexStation 3 multimode benchtop instrument (Molecular Devices, Sunnyvale, CA, USA). An initial baseline reading was taken during the first 20 seconds (s) of the measurement. Then, either once at 20 s or twice at 20 s and 140 s, 50 µl of the compound was added.

For the mBA cell line, approximately 16,000 cells were seeded into PDL-coated 96-well plates. These plates were equipped with a ring of PBS in the outermost wells to protect the cells from large temperature fluctuations, so that only 60 wells per plate were filled with mBA. Cells were incubated at 37°C for 48 hours, and PTX was added 16 hours before measurement.

For experiments in the presence of PTX, a PTX concentration of 100 ng/ml was used with an incubation time of ~16 h. PTX was usually added overnight. FR-treated cells were exposed to a concentration of 1 µM FR for 1 hour (h). FR was either added directly to the wells and incubated for 1 h before adding the dye, or mixed directly with the dye and incubated for 45 min. All Ca^{2+} assays used 5 µM calcium ionophore A23187 as viability control. Fluorescence increase is expressed as $Ca^{2+}_{(i)}$ relative fluorescence units (RFU) over time.

cAMP accumulation:

The Cisbio HTRF kit (Cisbio Codolet, France) was used to measure cAMP according to the manufacturer's instructions. 5,000 HEK cells/well were stimulated with increasing concentrations of receptor agonists or Fsk for 30 minutes. After incubation, lysis buffer and homogeneous time-resolved fluorescence (HTRF) components were added to the wells and incubated for 1 hour at room temperature in the absence of light. HTRF values were recorded using a Mithras LB 940 multimode plate reader (Berthold Technologies, Bad Wildbad,

Germany). HTRF values were converted to cAMP concentrations in nM using the standard curve, which was first recorded using the cAMP standard solution provided by the manufacturer for each new kit.

cAMP-BRET:

BRET measurements were performed with the cAMP GloSensor™ (Promega Corporation, Wisconsin, USA) according to the manufacturer's instructions to determine real-time cAMP production by direct activation of a Gs-coupled receptor or adenylyl cyclase. 0.8 million HEK cells were transfected with 1.5 µg of pGloSensor™-22F cAMP plasmid in a 6 cm dish. 24 hours post transfection, the cells were harvested, washed with PBS, centrifuged, and resuspended with HBSS + HEPES. 50 µl of 50,000 cells/well were seeded into a flat-bottomed 96-well plate followed by the addition of 50 µl of GloSensor™ cAMP substrate (2%) in HBSS + HEPES. The cells were then incubated for 2 hours at room temperature. A PHERAstar microplate reader (BMG labtech, Ortenberg, Germany) was used to measure cAMP-BRET at an emission wavelength of 562 nm, after two additions of 50 µl compounds at 20 s and 120 s. To detect real-time cAMP formation, we measured triplicates for each compound with 3 s acquisition time per data point. The first 20 seconds were used as a baseline. The luminescence signals were normalized to the solvent primed iso addition.

IP_3-BRET:

To determine IP_3 generated by activation of Gs-coupled receptors, BRET measurements were performed using the IP_3-BRET sensor described in Gulyás et al., 2015. For this purpose, 1.2 million HEK cells were transfected into 6 cm dishes 30 h prior to the measurement. On the day of the assay, cells were trypsinized, washed twice with PBS, and centrifuged to obtain a cell pellet. The cell pellet was then resuspended with HBSS + HEPES to seed 80,000 cells in 80 µl in each well of a flat 96-well plate with a white bottom. Next, 10 µl of the BRET substrate coelenterazine h was added. To ensure a uniform distribution of the substrate with the cell suspension, the treated plate was gently tilted. A PHERAstar microplate reader (BMG labtech, Ortenberg, Germany) was used to measure BRET, with emission measured at 485 and 535 nm for 50 s before the addition of 10 µl agonist or buffer. For two-addition assays, a second addition was performed 120 s after the first, similar to the calcium assays. Data were buffer-corrected and quantified by determining the mean BRET decrease after compound addition, as previously described (Gulyás et al., 2015).

Dynamic Mass Redistribution (DMR):

Dynamic mass redistribution studies were performed using the Corning EPIC biosensor (Corning, NY, USA) based on the protocol published by Schröder et al., 2011. Briefly, a 384-well plate containing an optical biosensor was seeded with 18,000 HEK cells or

18,000 MEF cells per well and cultured overnight. On the day of measurement, the cells were washed with HBSS containing 0.4% dimethyl sulfoxide (DMSO) and 20 mM HEPES, followed by an equilibration step for 1 hour until the detected baseline was almost stable. A new measurement was then started and agonists were added after a baseline measurement of at least 5 minutes using the Cybi-SELMA semi-automated electronic pipetting system (Analytik Jena AG, Jena, Germany). After addition, measurements were recorded for a further 60 minutes at 37°C. Compound addition induced a change in cell shape (dynamic mass redistribution) measured as a pm shift in the reflected wavelength over time.

Nanobit:

Nanobit measurements were performed according to previously published protocols (Dixon et al., 2016; Shihoya et al., 2018), starting with cell preparation. HEK cells were seeded in a 6-well plate and transfected with all required constructs 24 hours before the experiment. On the day of the assay, all wells were removed from the medium and washed twice with 2 ml PBS before adding 2 ml PBS-EDTA solution (1:10) to detach the cells. The reaction was stopped by adding the same volume of HBSS supplemented with 5 mM HEPES and 0.01% BSA (2 ml). Cells were counted and the appropriate number was transferred to a 15 ml tube. The cell suspension was centrifuged and the cell pellet was resuspended in assay buffer. 80 µl of the cell suspension was added to a 96-well plate, followed by the addition of 20 µl assay buffer containing 50 µM of the substrate coelenterazine. After substrate addition, cells were incubated for 2 hours at room temperature. Using a SpectraMax L reader (Molecular Devices, Sunnyvale, CA, USA), background luminescence was measured for 5-10 cycles of 40 seconds, followed by manual addition of 20 µl ligand. After the addition of the ligand, the measurement was continued for a minimum of 15 cycles. Luminescence signals were normalized to baseline, which is the average of the first 5-10 cycles before ligand addition, and presented as the averaged fold-change values of 15 cycles after ligand addition.

Western Blot:

Lysates were obtained from the same transfected cell batch used for calcium measurement. On the day of the calcium measurement, 48 hours after the transfection, the cells were lysed and the amount of extracted protein was determined. Gradient gels were run and blotted to nitrocellulose membranes. Protein detection was performed as follows with β-actin as loading control: Nitrocellulose membranes were washed, incubated with RotiBlock for 1 hour, placed in a 50 ml falcon, and treated overnight with anti-β-actin antibody in RotiBLock (1:10,000) shaken at 4°C. The next day, the membranes were washed and the second anti-rabbit antibody diluted in RotiBlock (1:20,000) was applied to the membranes. After incubation for one hour, the β-actin band was detected with the ECL detection reagent. The membranes were then washed again and incubated with the RotiBlock for additional 60 minutes. To detect PLCβ

expression, the membranes were incubated with mouse anti-PLCβ3 monoclonal antibody in RotiBlock (1:500) overnight at 4°C on a shaker. The following day, all steps were repeated as described above for β-actin, including a secondary anti-mouse antibody and ECL detection reagent.

Internalization assay:

To verify the uniform expression of β_1AR and β_2AR, we treated the SNAP-tagged β_1AR and SNAP-tagged β_2AR transfected cells with the SNAP-Lumi4-Tb reagent, a Tb^{3+} complex that acts as an energy donor. On the basis of a previously published protocol (Levoye et al., 2015), we modified the DERET internalizing assay. 24 hours after transient transfection, cells were washed, trypsinized, and seeded at 60,000 cells/well in a PDL-coated 96-well plate. The medium was then removed and 50 µl/well of 100 nM SNAP-Lumi4-Tb reagent diluted in HBSS containing HEPES (20 mM) was added to the cells and incubated for 1 hour at 4°C. After one hour incubation, SNAP-Lumi4-Tb reagent was removed and 100 µl HBSS + HEPES was added to each well. Emission was measured at 620 nm using a PHERAstar microplate reader (BMG labtech, Ortenberg, Germany). Data are presented as relative fluorescence units for each transfected cell line.

Fluorescence Microscopy:

Cells transiently expressing SNAP-tagged β_1AR and SNAP-tagged β_2AR were seeded into 8-well PDL slides. After 24 hours, the medium (DMEM) was replaced with fresh medium containing 5 µM SNAP-Surface 649 (New England Biolabs, Ipswich, MA, USA) and incubated for 30 minutes at 37°C in 5% CO_2. The medium was then removed, the cells washed, and HBSS + HEPES was added. Fluorescence was detected at a wavelength of 676 nm using a Zeiss fluorescence microscope equipped with a xenon flash lamp, GFP or Cy5 filter cube, and Zeiss Plan Apo 63× (1.40 oil) objective.

In parallel with the Ca^{2+} assay, internalization and microscopy were performed on the same batch of cells to check for uniform expression of SNAP-tagged β_1AR and SNAP-tagged β_2AR.

Data evaluation:

All data were processed with Microsoft Excel and analysis was performed with GraphPad Prism 8 or 9. Representative curves are presented as mean + SEM, whereas quantified data are presented as mean ± SEM. Some of the kinetic data were baseline-corrected to a buffer control. This was done by subtracting the value of the buffer-stimulated curve from the corresponding ligand-stimulated curve for each measured time point. For quantification, baseline-corrected values were further processed as indicated in the plot Y-axis heading. Statistical analyses were performed using either two-way ANOVA with the Dunnett's correction method or a paired t-test. P values were determined and expressed as: ns = not significant,

*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. The graphical illustrations were created with Biorender.com.

Results

1. Do Gs-coupled receptors mobilize intracellular calcium?

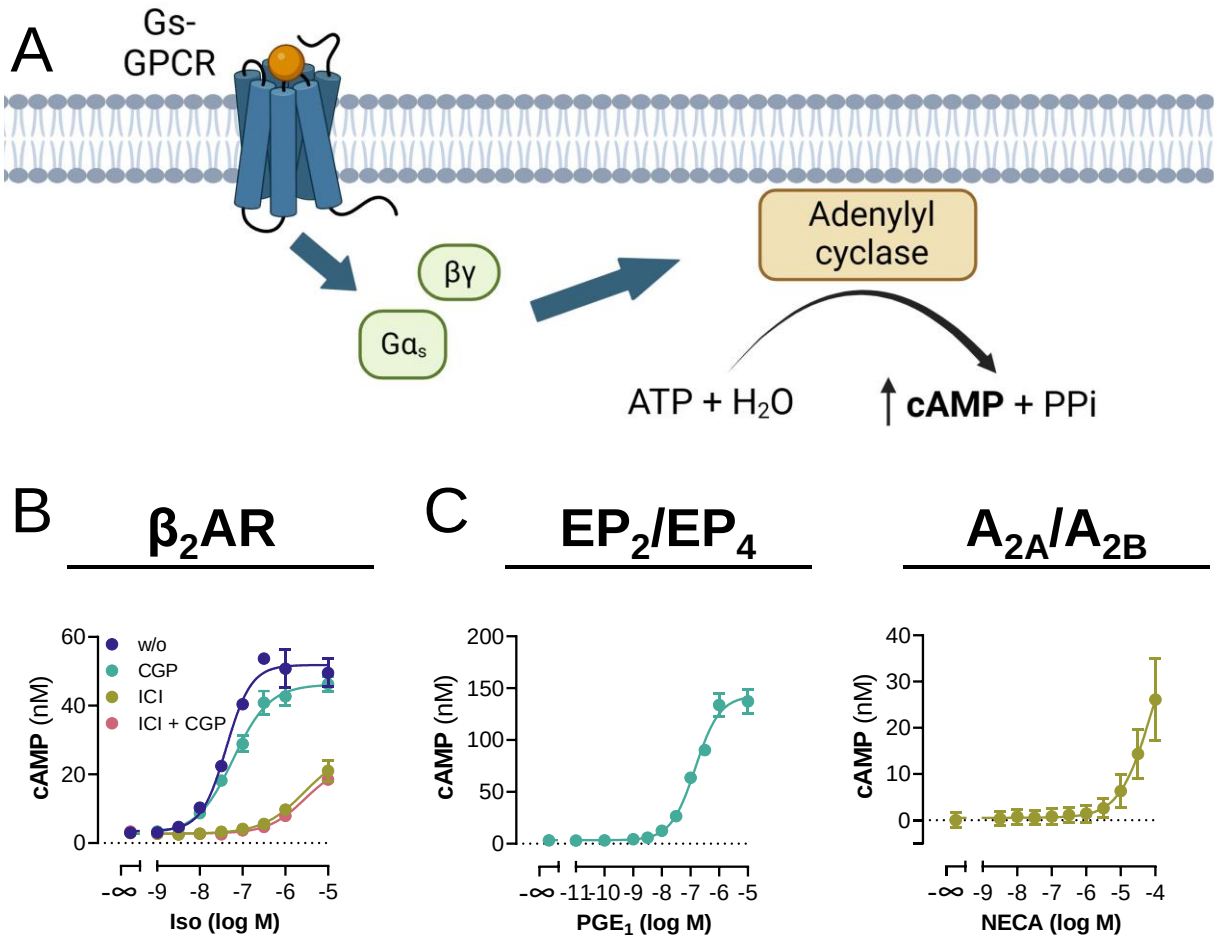

Figure 6. Gs-coupled β_2AR, EP_2/EP_4, and A_{2A}/A_{2B} receptors increase cAMP levels in HEK293 cells. (A) Upon stimulation with their cognate agonist, Gs-GPCRs activate adenylyl cyclases via their alpha subunit, resulting in an increase in cytosolic cAMP levels. (B) Stimulation of endogenous beta-adrenergic receptors with Isoproterenol (Iso) increased intracellular cAMP levels. Treatment of cells with the β_2AR-specific inhibitor ICI 118.551 (100 nM) blocked the Iso-mediated cAMP increase, whereas the β_1AR-specific inhibitor CGP 20712A (300 nM) did not significantly reduce the cAMP increase. (C) Another pair of Gs-coupled receptors, also endogenously expressed in HEK293 cells, were stimulated with their respective agonists, and responded with an increase in cytosolic cAMP levels. To exclude confounding effects of Gi on AC, all cells were pretreated with 100 ng/ml PTX. Data are presented as mean ± SEM of two independent performed experiments for β_2AR and A_{2A}/A_{2B} and as mean ± SEM of three independent performed experiments for the prostanoid EP_2/EP_4, all performed in triplicate.

Gs-coupled β_2AR, EP_2/EP_4, and A_{2A}/A_{2B} receptors increase cAMP levels in HEK293 cells

In order to investigate Gs-mediated calcium release in HEK293 cells, we first tested for endogenously expressed Gs-GPCRs with a classical Gs readout, the mobilization of intracellular cAMP (Figure 6A). Canonically, activation of Gs-GPCRs activates adenylyl cyclases. Due to the increased enzyme activity, the cytosolic level of the second messenger cAMP rises (Wettschureck & Offermanns, 2005). We took advantage of these cAMP-enhancing properties of various Gs-coupled receptors to infer functional expression in our HEK293 cell background. HEK293 cells are known to express several endogenous adrenergic receptors, including the β_2-adrenergic receptor (β_2AR), a well-known Gs-coupled receptor (Atwood et al., 2011; Rasmussen et al., 2011). Stimulation of the cells with Isoproterenol (Iso), a non-selective β-adrenergic agonist, resulted in an increase in cAMP levels (Figure 6B). To test whether β_2AR is the sole driver of Iso-induced cAMP, we treated the cells with either β_1-adrenergic receptor (β_1AR) selective antagonist CGP 20712 A (CGP) (Baker, 2005), β_2AR selective antagonist ICI 118.551 (ICI) (Baker, 2005; Skeberdis et al., 1997) or both inhibitors together. Iso-mediated cAMP production was almost insensitive to CGP treatment, whereas treatment of cells with ICI had an inhibitory effect on cAMP production and shifted the Iso-response curve to the right. The combination of the two inhibitors CGP and ICI did not further reduce cAMP levels, demonstrating that Iso mediates its effect in HEK293 cells exclusively through β_2AR and not β_1AR. Furthermore, the prostanoid EP_2/EP_4 and adenosine A_{2A}/A_{2B} receptors responded to the addition of the corresponding agonist by increasing cAMP levels (Figure 6C). Pretreatment with the Gi/o inhibitor pertussis toxin (PTX) was necessary to mask a possible effect of Gi-receptor subtypes on Gs-specific signaling. Taken together, all three receptors were found to mediate cAMP and were therefore suitable for further testing of our hypothesis of a Gs-βγ-mediated calcium release.

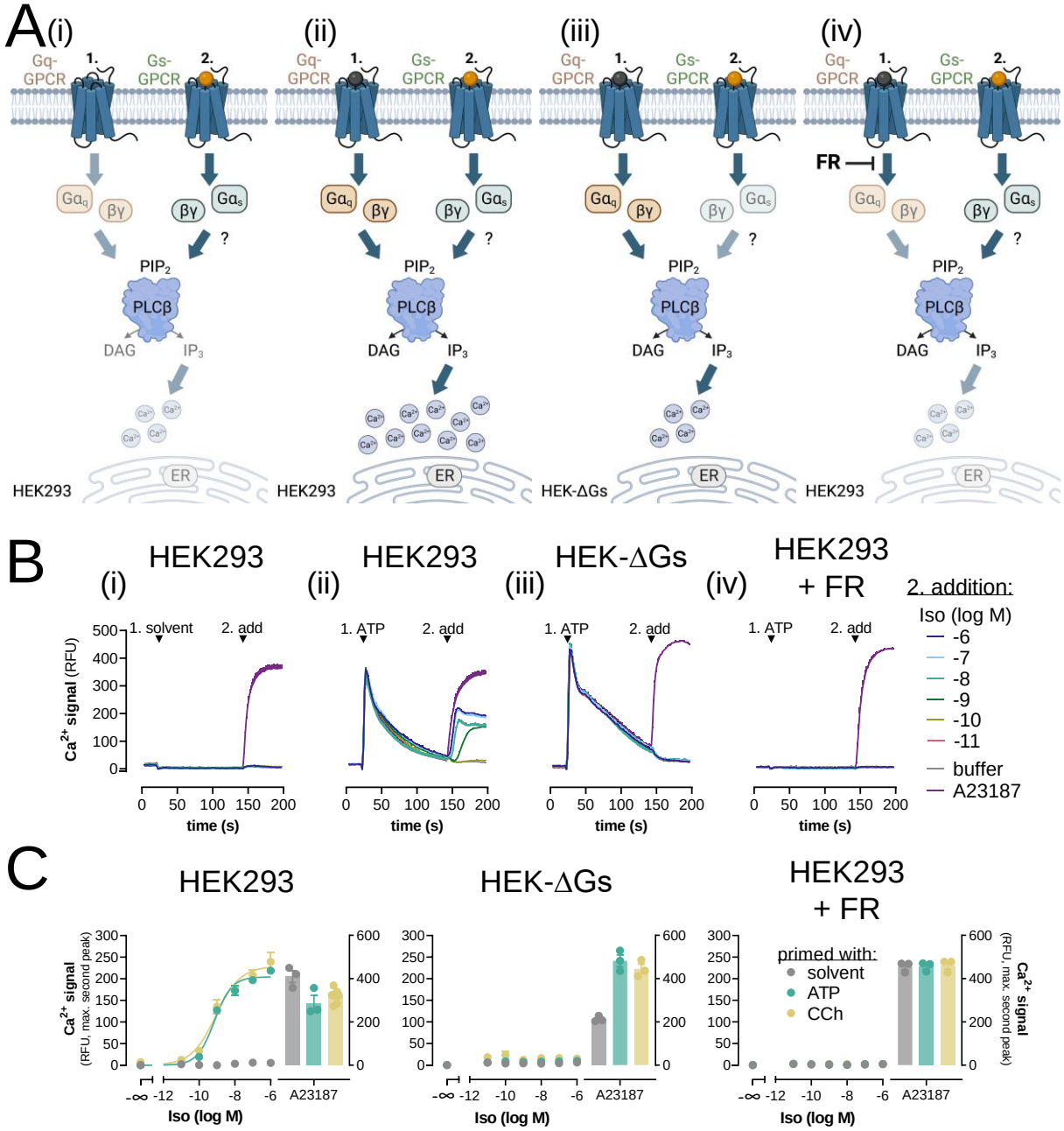

Figure 7. Gs-coupled β_2AR mediate calcium only after Gq activation. Calcium mobilization was recorded using a two consecutive addition protocol in either HEK293 or HEK-ΔGs cells. Prior to the Gs-stimulus Iso either solvent, Gq stimulus ATP (100 µM) or CCh (100 µM) was added to the cells in the presence or absence of 1 µM FR. ($A_{(i-iv)}$) Postulated mechanism related to the kinetics below. ($B_{(i-iv)}$) Representative calcium recordings shown as mean + SEM. Solvent-primed cells did not mobilize β_2AR-mediated calcium when activated with Iso ($B_{(i)}$), unless cells were primed with P2Y-receptor agonist ATP ($B_{(ii)}$). Changing the cellular background to HEK-ΔGs abolished the Iso-mediated calcium mobilization, while the Gq-mediated calcium peak remained ($B_{(iii)}$). All calcium transients in HEK293 cells were silenced by pretreatment with 1 µM of the Gq/11/14 inhibitor FR900359 ($B_{(iv)}$). (C) Concentration-response curves derived from the maximum calcium response of the second addition of Iso stimulating β_2AR for different cellular backgrounds, inhibitor treatment,

and different priming reagents. Averaged data are mean ± SEM of at least three biologically independent experiments, each performed in duplicate.

Gs-coupled β_2AR mediate calcium only after Gq activation

Active β_2AR signaling elicited concentration-dependent cAMP formation but did not produce detectable Ca^{2+} transients. These findings appear to contradict previous studies suggesting a pathway involving cAMP-PLCε-Ca^{2+} release (Schmidt et al., 2001) or the transactivation of nucleotide P2Y receptors as a downstream event of β_2AR in non-excitable cells (Stallaert et al., 2017). On the other hand, some groups reported the need for Gq priming, which allows Gs receptors to release Ca^{2+} (Ali & Bergson, 2003; Konieczny et al., 2017; Kurian et al., 2009; Short & Taylor, 2000; Tovey et al., 2003). Despite elevated cAMP levels we were unable to detect calcium for the tested β_2AR leading us to hypothesize that there must be additional mechanisms for Gs-Ca^{2+} (Figure 7B$_{(i)}$). Due to our findings in the previous Gi-project (Pfeil et al., 2020), Gi-coupled receptors elicited detectable calcium only when i) the receptor itself coupled Gq in addition to Gi proteins or when ii) a Gq receptor was stimulated prior to Gi receptor activation (heterologous Gq priming). The mere presence of Gq proteins was not sufficient to cause a calcium increase. We assumed that similar to Gi-mediated calcium, calcium mediated via Gs-GPCRs also requires activation of the Gq pathway. To test whether activation of the Gq pathway grants Gs-mediated calcium, we stimulated cells with ATP a Gq-stimuli to activate endogenously expressed Gq-coupled P2Y receptors prior to the addition of Iso. ATP priming triggered a robust first calcium peak followed by an Iso-mediated second calcium peak in a concentration-dependent manner (Figure 7B$_{(ii)}$). To determine the contribution of Gq and Gs, we used the Gq/11/14-specific inhibitor FR900359 (FR) and genetically modified HEK293 cells lacking all functional alleles of $G\alpha_s$ and $G\alpha_{olf}$ (hereafter HEK-ΔGs). In HEK-ΔGs cells, the Gq-mediated calcium spike prevailed, whereas Iso-triggered responses were undetectable, indicating Gs as the essential mediator of the second calcium recordings (Figure 7B$_{(iii)}$). Incubation of the cells with FR abrogated ATP-mediated Gq activation and consequently the subsequent Iso-mediated calcium (Figure **7**B$_{(iv)}$). Not only ATP priming, but also priming with Carbachol (CCh), an agonist of Gq-coupled muscarinic M3 receptors, allowed an Iso-mediated calcium increase (Figure 7C). In all experiments, FR treatment or $G\alpha_s$ depletion did not adversely affect cell viability, as the calcium ionophore A23187 caused an increase in Ca^{2+}, indicating an intact receptor-independent calcium response. This suggests that active Gq is required for Gs-calcium mobilization via the Gs-specific β_2AR receptor, regardless of which Gq-coupled receptor is activated.

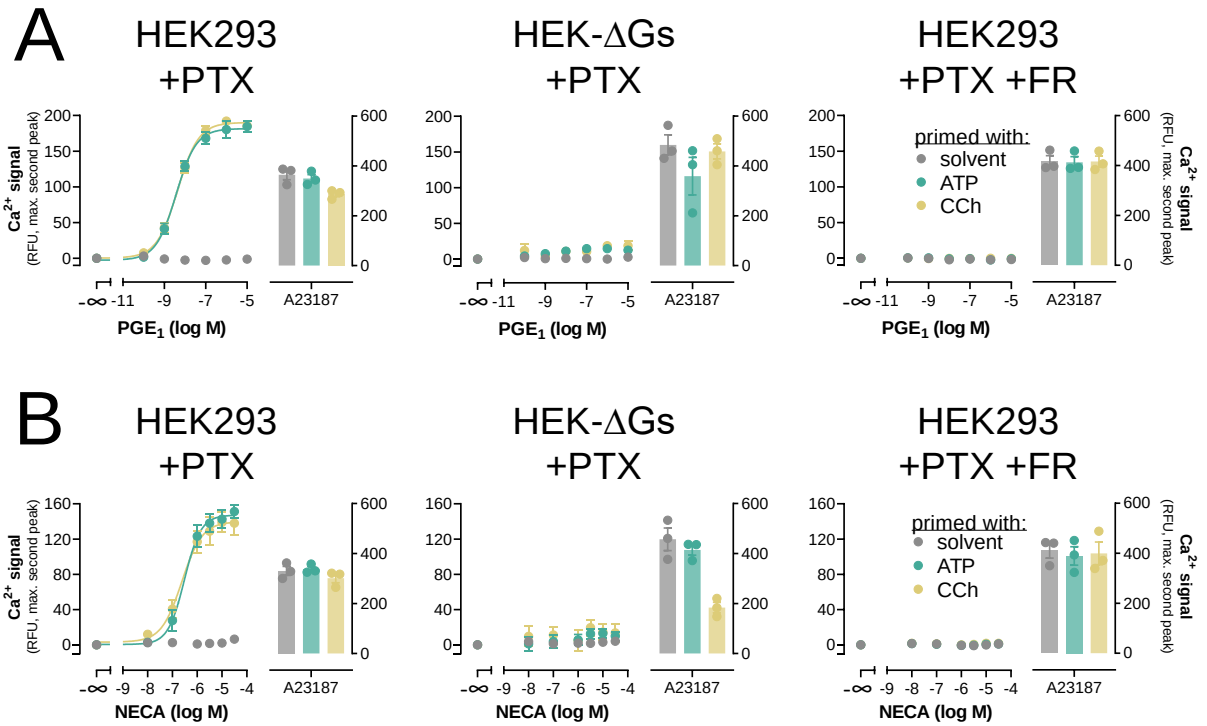

Figure 8. Gs-coupled receptors contribute to the increase in intracellular calcium levels. (A, B) Maximum calcium responses plotted as concentration-response curves of the addition of PGE_1 for stimulation of the prostanoids EP_2 and EP_4, NECA for stimulation of the A_{2A} and A_{2B} receptors, or the viability control ionophore A23187 (5 µM) as a bar graph after prior addition of solvent (no priming), ATP (100 µM), or CCh (100 µM). Cells were treated overnight (16 hours) with 100 ng/ml of the Gi inhibitor PTX to exclude the contribution of Gi-coupled prostanoid and adenosine receptor subtypes to Gs-Ca^{2+}. The averaged data represent the mean ± SEM of at least three biologically independent experiments, each performed in duplicate.

Gs-coupled receptors contribute to the increase in intracellular calcium levels

To understand whether Gs-mediated calcium release is unique to the β_2AR receptor or a general property of Gs-coupled GPCRs in the presence of active Gq, we examined two additional receptors: the prostanoid EP_2/EP_4 and adenosine A_{2A}/A_{2B} receptors. Before measuring calcium, all cells underwent PTX pretreatment to mask any Gi-mediated calcium elevation (Katada, 2012). As with β_2ARs, activation of these receptors by their respective agonists was unable to trigger Ca^{2+} release from intracellular stores unless cells were primed with a Gq stimulus. Notably, this Gs-mediated calcium elevation was again prevented by removal of Gs proteins or by inhibition of Gq (Figure 8A, B). In conclusion, the effect of Gq-primed Gs-calcium does not seem to be an exclusive feature of the β_2AR, since EP_2/EP_4 and A_{2A} and A_{2B} also rely on functional Gs and Gq proteins. In general, while Gs-GPCR

activation led to the formation of intracellular cAMP, additional Gq input was unavoidable for Gs-mediated calcium.

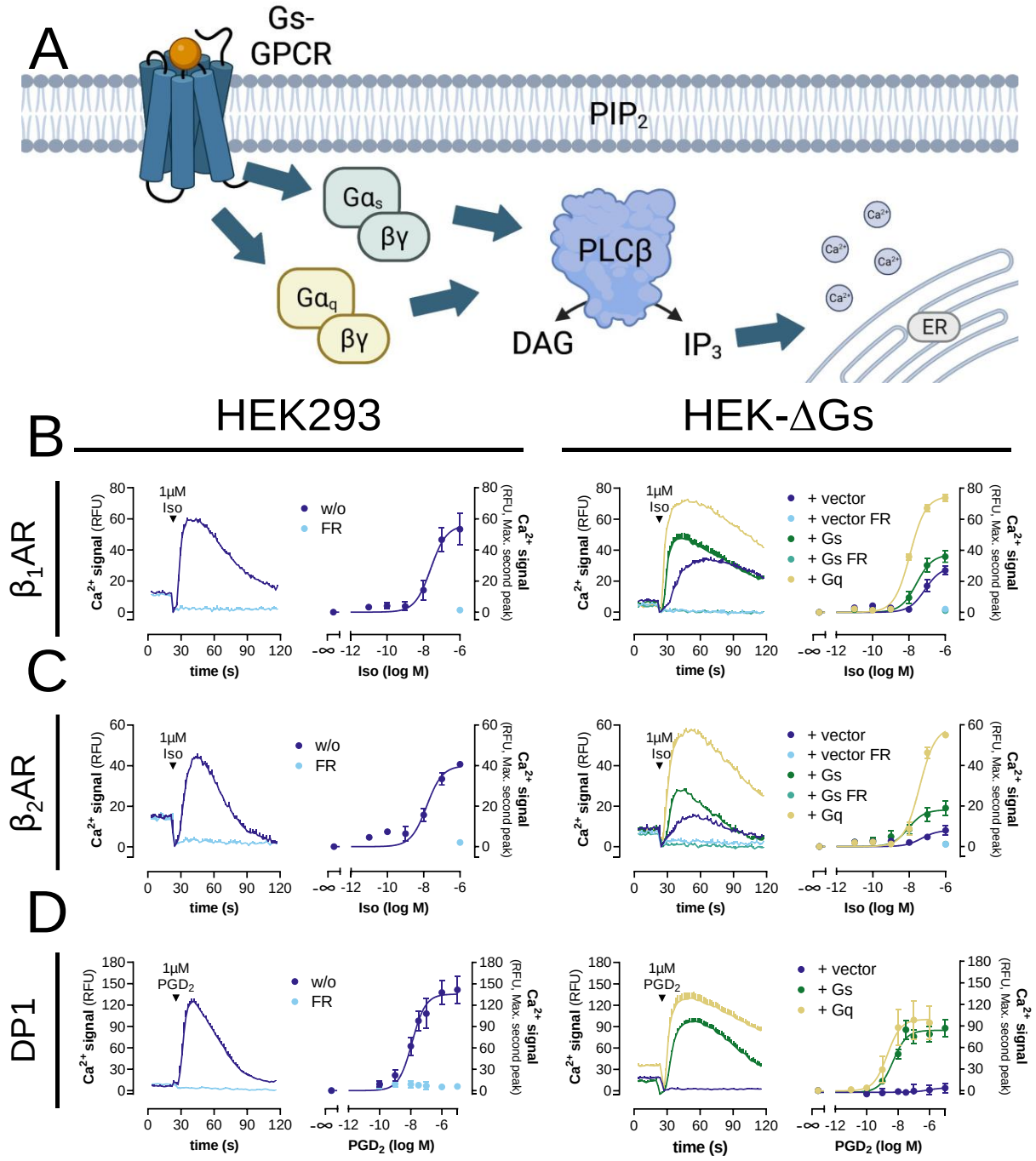

Figure 9. Gs-calcium is mobilized by Gs-GPCRs that bind to both Gq and Gs. (A) By altering the receptor amount or the signaling protein equipment Gs-coupled receptors engage secondary Gq coupling, which we investigated in HEK293 and HEK-ΔGs. (B-D) HEK293 and HEK-ΔGs were transiently transfected with the indicated receptors, while HEK-ΔGs were additionally transfected with either Gs, Gq, or vector. Shown are representative Ca^{2+} kinetics (left panel) and summary as maximal Ca^{2+} response (right panel) to compound addition in the presence or absence of 1 µM FR. Representative traces are mean + SEM, summary data are mean ± SEM of at least three biologically independent experiments, each performed in duplicate.

Gs-calcium is mobilized by Gs-GPCRs that bind to both Gq and Gs

Our results are consistent with several research articles describing the requirement of activated Gq for Gs-calcium (Buckley et al., 2001; Konieczny et al., 2017; Kurian et al., 2009; Leaver & Pappone, 2002; Tovey et al., 2010; Werry et al., 2002). Whether active Gq must be provided by another Gq-GPCR by heterologous Gq priming or whether Gs-GPCR can also provide their own Gq had to be investigated. First, we overexpressed SNAP-tagged β_1AR, SNAP-tagged β_2AR, and SS-flag-tagged DP1, which is known to increase calcium levels in recombinant and primary cells without prior Gq activation (Jandl et al., 2016), in our non-excitable HEK293 cell system. Stimulating the overexpressed βARs with Iso and the DP1R with its agonist PGD_2 promoted a concentration-dependent calcium mobilization even without previous activation of a Gq-GPCR (Figure 9C-D). Noteworthy, and in line with our previous results, this calcium signal remained fully sensitive to the Gq inhibitor FR. Since Gq priming was no longer necessary and FR treatment abolished Ca^{2+} signaling, we assumed that the overexpressed receptors were producing their own active Gq. To further investigate this assumption, we used HEK-ΔGs expressing the three different receptors and checked whether they can mediate calcium in the absence of $G\alpha_s$ proteins. Upon Iso stimulation β_1AR and β_2AR responded with calcium transients, which were enhanced by re-expression of Gs. In contrast, the DP1 receptor showed a detectable Ca^{2+} increase only after re-expression of the Gs subunit. And again, all responses remained fully FR-sensitive, suggesting that the observed calcium in HEK-ΔGs is dependent on activated Gq, even though prior Gq stimulation is not required. Overexpression of $G\alpha_q$ enhanced the residual Gs-independent calcium signal for βARs and restored the calcium response to DP1 in HEK-ΔGs cells, suggesting the capacity of each Gs-GPCR to couple to Gq if a high amount of Gq is available. It should be noted that the expression of β_1AR and β_2AR receptors was comparable between the different cell lines and co-transfection, as the luminescence measured after terbium labeling of SNAP-tagged β_1AR and SNAP-tagged β_2AR was comparable in all transfections (Figure 10A). A nearly uniform membrane distribution of the SNAP-tagged receptors was also confirmed by fluorescence microscopy images (Figure 10B). Taken together, these data indicate that Gs-GPCRs can either mobilize calcium facilitated by an external Gq input (heterologous priming) or activate Gq and Gs simultaneously, thereby providing their own Gq activation to facilitate Gs-mediated calcium mobilization. Therefore, we concluded that the requirement of prior Gq stimulus to generate Gs-calcium depends on the cellular background and on its receptor and signaling protein equipment. Importantly, a Gs-mediated calcium signal occurs exclusively in the presence of activated Gq pathway no matter if activated by the same receptor or by another Gq-GPCR.

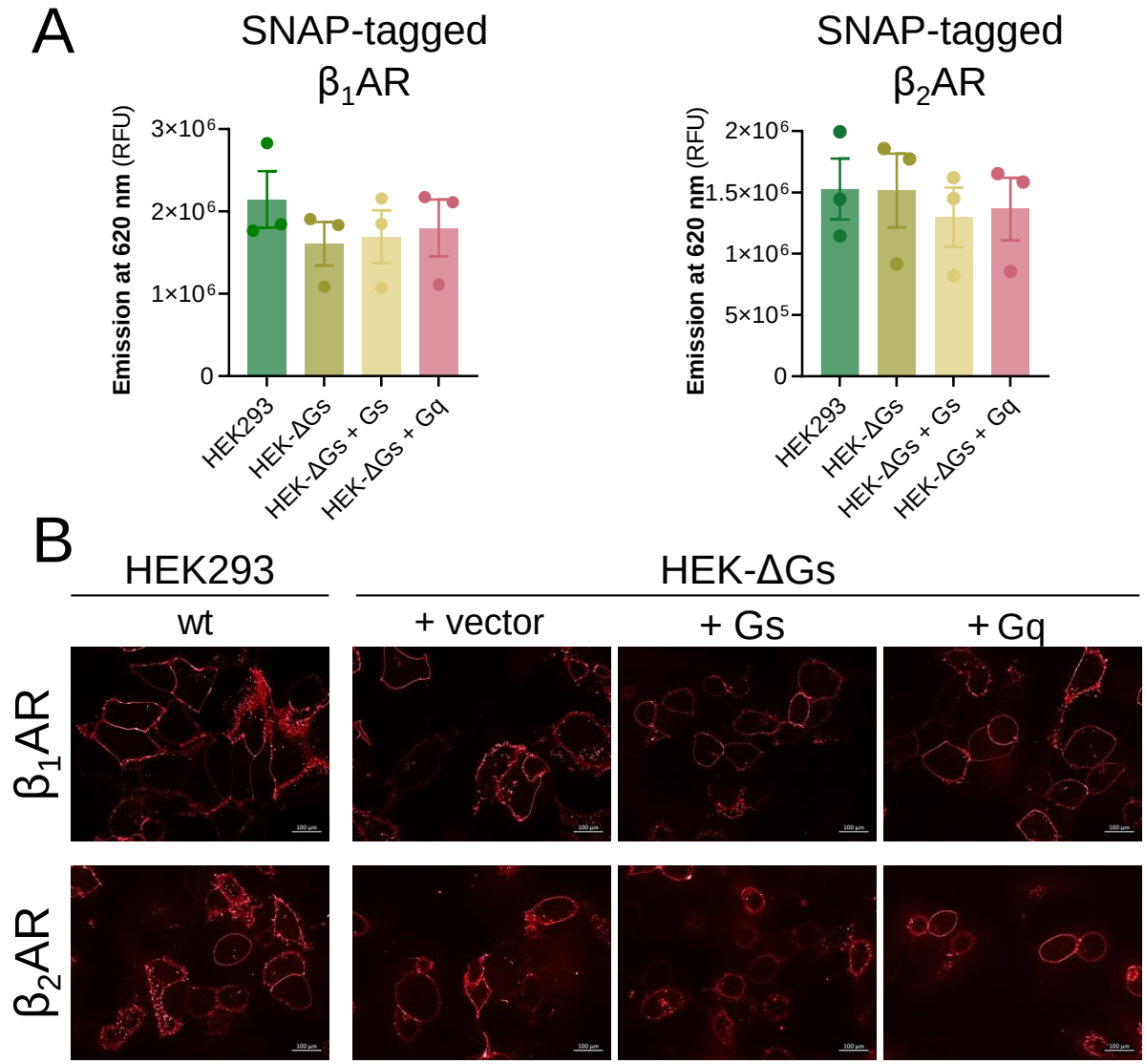

Figure 10. β_1AR and β_2AR are equally expressed in different cell lines and co-transfections. (A) Maximum fluorescence intensities of SNAP-tagged β_1AR and SNAP-tagged β_2AR after labeling with SNAP-Lumi4®-Tb, shown as mean + SEM of three independently performed experiments. (B) Structured fluorescence micrographs of cells transiently expressing SNAP-tagged β_1AR and SNAP-tagged β_2AR in: HEK293 wt (first column), vector-transfected HEK-ΔGs (second column), $G\alpha_s$-transfected HEK-ΔGs (third column), and $G\alpha_q$-transfected HEK-ΔGs (fourth column). Images were acquired using 63x magnification (Plan-Apochromat 63x/1.4 Oil M27). To improve visibility, the images were post-exposed with a common factor using Adobe Photoshop. The raw images were subjected to line scan analysis using the Zeiss-Zen image processing program. Images are representative of three independent biological replicates. The scale is 100 micrometers.

2. Does the Gq requirement for Gs-calcium of HEK293 cells extend to physiological systems?

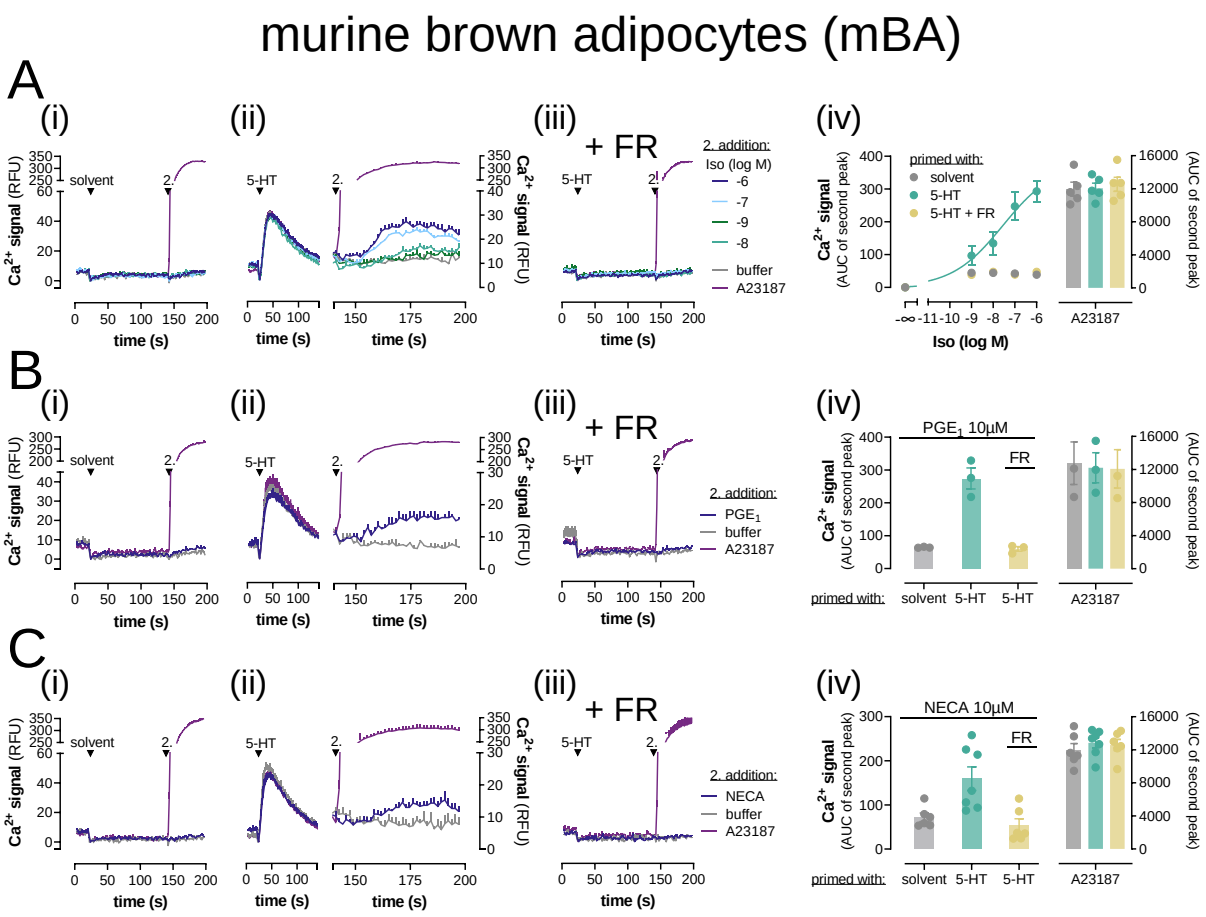

Figure 11. Gs-calcium demands Gq input in murine brown adipocytes (mBA). (A-C$_{(i-iii)}$) Representative calcium traces of primary brown murine adipocytes, pretreated with 100 ng/ml of the Gi inhibitor PTX. mBA were primed with either solvent (HBSS) or 10 µM 5-hydroxytryptamine (5-HT) followed by Gs-agonist Iso, PGE_1 (10 µM) and NECA (10 µM) in the presence or absence of 1 µM FR. (A$_{(iv)}$) Concentration-response relationships of Iso addition and bar graph of viability control A23187 (5 µM) plotted as area under the curve (AUC). Quantification of (B) and (C) shown as a bar graph (B$_{(iv)}$, C$_{(iv)}$) for the respective agonist as well as for the viability control A23187. Representative kinetics are mean + SEM, summarized data are mean ± SEM for the concentration-effect graph and mean ± SEM for the bar graphs of at least three biologically independent experiments, each performed in duplicate.

Gs-calcium demands Gq input in murine brown preadipocytes and mouse embryonic fibroblasts

So far, we have only studied the requirement of activated Gq for Gs-mediated calcium in HEK293 cells. HEK293 cells are a recombinant cell system that offers many advantages, as they are easy to transfect and culture, and can be used to generate knockout cell lines using CRISPR/Cas9. However, there are some concerns as to whether HEK293 cells are suitable to accurately represent physiological situations. For this reason, we checked on Gq-dependent Gs-calcium in a more physiological cell system. As a non-excitable primary cell system, we exploited mBA because they express all three types of βARs, the prostanoid EP_4 receptors, and both isoforms of the A_2R (Klepac et al., 2016). Consistent with our data collected in the HEK cell system, preadipocytes failed to release calcium upon agonist addition without prior Gq pathway activation (Figure 11A-$C_{(i)}$). Following the activation of the Gq-coupled 5-HT receptor, Iso, PGE_1, and NECA induced a Gs-mediated calcium peak (Figure 11 A-$C_{(ii)}$), that was abolished by FR introduction (Figure 11A-$C_{(iii)}$). Interference by Gi/o-GPCRs was excluded by pretreatment with PTX, which, like the Gq inhibitor FR, had no effect on cell viability as the control ionophore A23187 remained unaffected. Collectively, mBA require an activated Gq pathway for Gs-mediated calcium release.

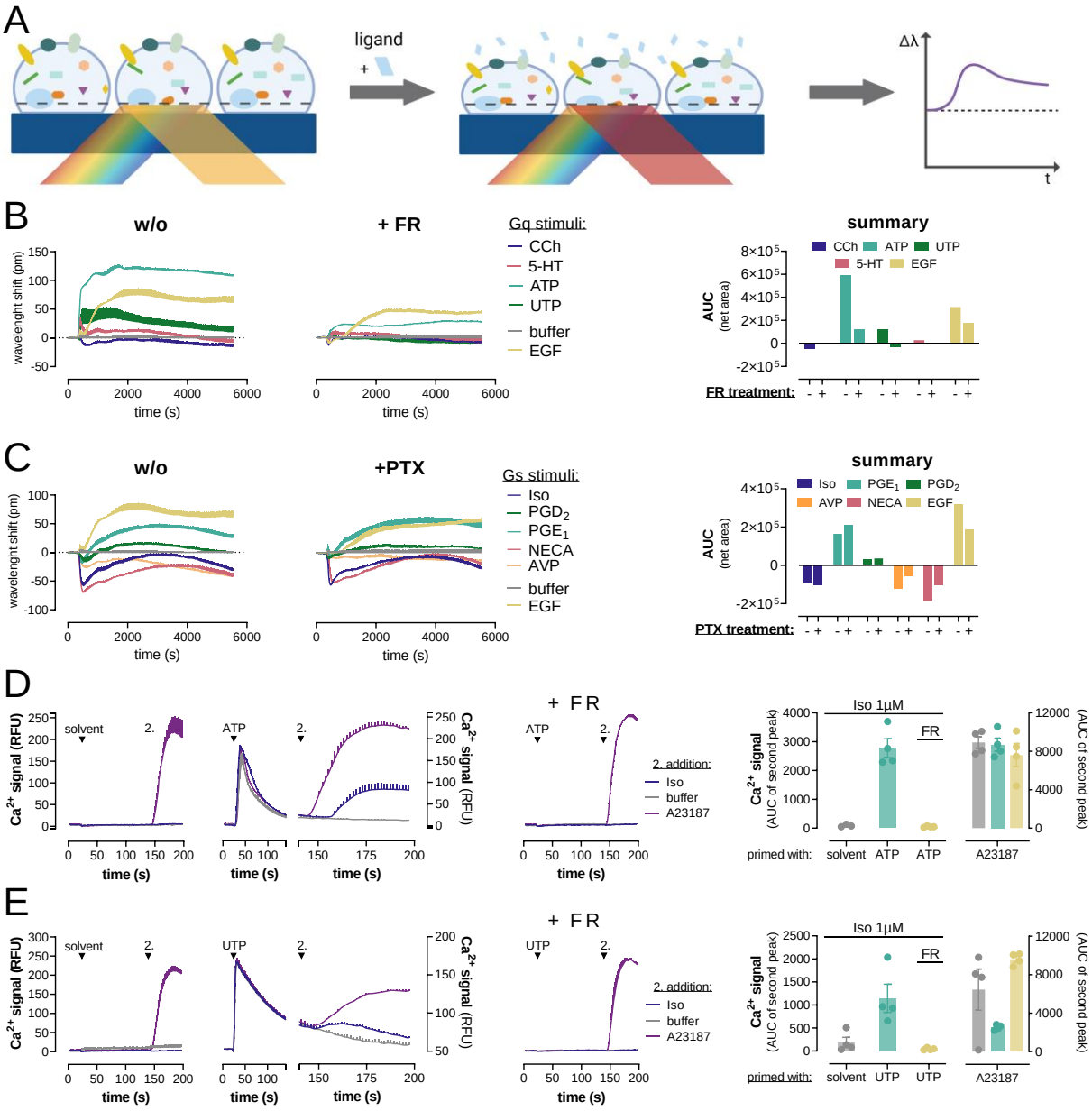

Figure 12. Mouse embryonic fibroblasts (MEF) generate Gq-primed Gs-calcium. (A) Activation of GPCRs trigger a signaling cascade that induces a change in cell shape. This change can be detected by an optical biosensor as a wavelength shift representing dynamic mass redistribution (DMR). Inhibitor effects such as FR or PTX on the DMR response confirms either contribution of Gq or Gi. Evidence for a possible Gs contribution was provided by the remaining PTX signals. (B, C) Representative DMR kinetics shown as mean + SEM and quantified data as the mean of a single performed DMR measurement to different (B) Gq stimuli (100 µM of CCh, ATP, UTP and 10 µM 5-HT) ± 1 µM FR or (C) Gs stimuli (1 µM Iso and 10 µM PGE_1, PGD_2, AVP, NECA) ± 100 ng/ml PTX, each performed in triplicates. (D, E) Representative calcium kinetics as mean + SEM in the presence or absence of 1 µM FR either primed with solvent, 10 µM ATP or 100 µM UTP followed by 1 µM of Iso. Right panels represent the quantification of four independent experiments as a bar graph ± SEM, each performed in duplicates.

Besides mBA, we studied MEF as another non-excitable primary cell system. Before we started with the calcium measurements, we performed dynamic mass redistribution to identify proper Gq- and Gs-GPCRs in these cells. DMR measurements provide a holistic, real-time overview of cellular cytoskeletal changes in response to ligand stimulation and thus helped us to find appropriate stimuli for our calcium assays (Figure 12A). All selected Gq stimuli provided cell deformation that was sensitive to FR treatment (Figure 12B). Since the largest cell shape change obtained was for ATP and its close relative UTP, we preferred both stimuli for further use. We also obtained shape changes for different Gs stimuli that were not significantly affected by PTX treatment (Figure 12C). The remaining PTX-insensitive signals indicate that the tested GPCRs signal via Gs. Based on these findings, we identified some Gq- and Gs-GPCRs for the upcoming calcium assay. MEF cells did not respond with a Gs-calcium response under solvent-primed conditions until the Gq-coupled P2Y receptor was activated by either 10 µM ATP or 100 µM UTP. As with recombinant HEK293 and mBA, both calcium spikes were abolished by FR treatment, indicating that MEFs did not respond until Gq activation. All endogenous cell systems were treated with PTX to exclude interfering effects of Gi/o-GPCRs on Gs signaling, because Gi/o-GPCRs also require Gq priming to mobilize calcium (Pfeil et al., 2020). Interestingly, the calcium spike triggered by A23187 was reduced when primed with UTP, whereas it was normally high when primed with ATP. It seems that UTP depletes the ER rapidly, leaving too little time for replenishment and thus for proper calcium release by the second addition of A23187 or Iso. This suggests that Iso and UTP use the same calcium stores and that the response to Iso may be reduced due to the lower amount of calcium in stock (Tovey et al., 2008). Taken together, with mBA and MEF, representing non-excitable primary cell lines, we revealed the Gq-dependent Gs-calcium in a more endogenous environment. Thus, it appears that Gq-dependent Gs-calcium is not exclusive to HEK293 cells and emerges as a potentially important mechanism by which Gq may act as an on-off switch that can regulate cytosolic calcium levels by requiring the involvement of Gs.

3. Which are the molecular players enabling a Gs-mediated calcium release?

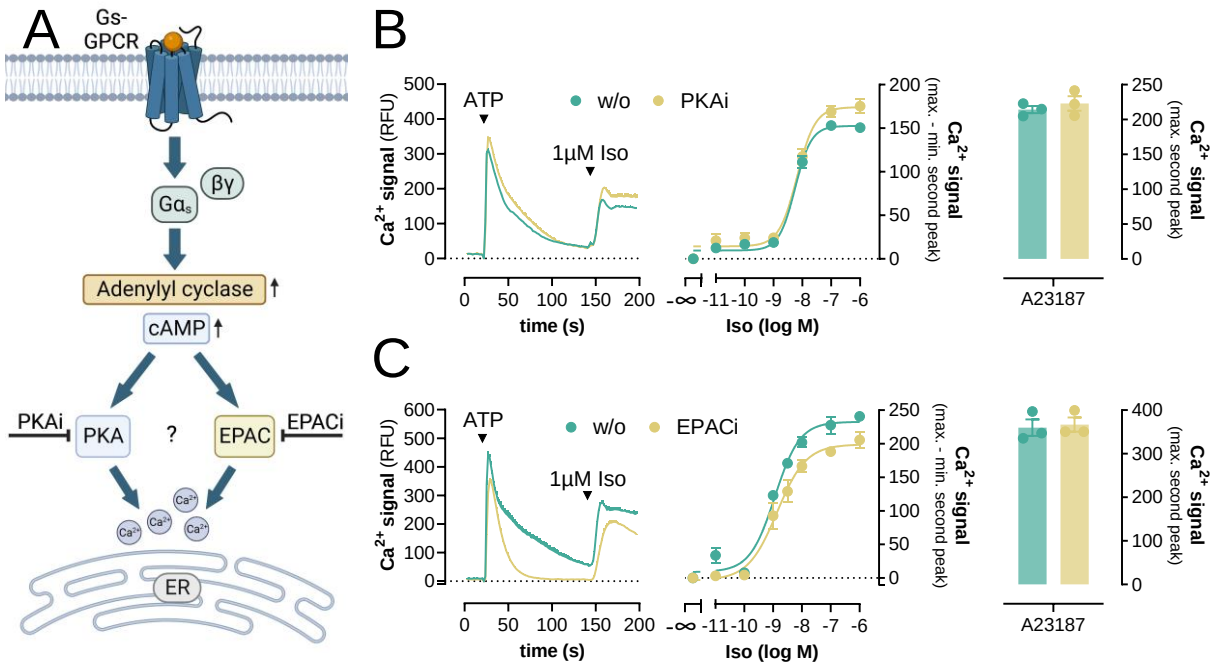

Figure 13. Neither PKA nor EPAC contribute to Gs-calcium. (A) To test the contribution of PKA or EPAC to Gs-calcium, we used specific inhibitors (PKAi; EPACi) in the calcium assay setup. (B, C) HEK293 cells were treated either with 10 µM of PKA inhibitor PKI 14-22 or 25 µM EPAC inhibitor HJC0197. Whereas cells treated with PKA respond with an Iso-mediated rise in calcium, the Iso response in EPAC treated cells was not profoundly inhibited. Viability control remains intact for both treatments. Representative traces are mean + SEM, quantified data are the mean ± SEM of three independent experiments each performed in duplicates.

Neither PKA nor EPAC contribute to Gs-calcium

Calcium is a regulator of a wide range of physiological functions and has different modes of its own mobilization. Therefore, an understanding of the molecular players that are involved in this process is enormously important. To achieve this goal, we focused on effector proteins known to induce calcium through activation of Gs. Activated Gs-GPCRs dissociate into their $G\alpha_s$ and Gβγ subunits, both of which are capable transducers that mediate their effects through direct protein-protein interaction. The extent to which either $G\alpha_s$ or Gβγ is involved in heterologous Gq-primed Gs-calcium release is still unclear and remains to be investigated. We took advantage of the canonical activation of ACs via stimulation of Gs-GPCRs (Figure 6), which is associated with a subsequent increase in intracellular second messenger cAMP. The secondary messenger cAMP in turn activates its two main effectors: protein kinase A (PKA)

and exchange protein activated by cAMP (EPAC) (Figure 13A). Both effectors are known to increase calcium levels by different mechanisms (Kamp & Hell, 2000; Schmidt et al., 2001; Supattapone et al., 1988; C. W. Taylor, 2017). Treatment of HEK293 cells with the PKA inhibitor PKI 14-22 resulted in a slight increase in the Iso-mediated calcium response (Figure 13B), consistent with a negative feedback regulation by the active form of the PKA enzyme (Yue et al., 1998). Inhibition of EPAC with HJC0197, a specific EPAC inhibitor, had no profound effect on the Iso-mediated calcium response, but significantly shortened the duration of the ATP-mediated calcium response (Figure 13C). The removal of intracellular calcium appears to be associated with reuptake into the ER or other organelles. As observed by other groups (Schmidt et al., 2001), we assume that EPAC affects the calcium dynamics of Gq-GPCRs. Despite the treatment with the inhibitors, the viability of the cells was not affected, since the calcium ionophore A23187 remained unchanged. In conclusion, we hypothesize that neither PKA nor EPAC contribute to our observed Gs-mediated calcium release, but that EPAC seems to be important for the calcium release mediated by Gq.

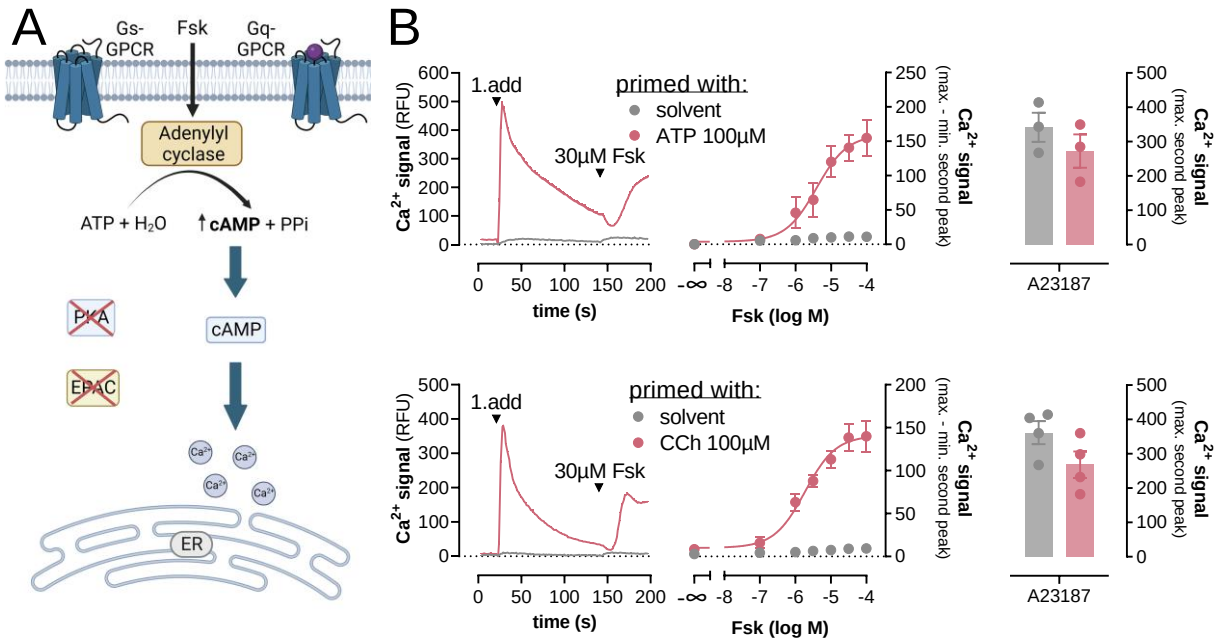

Figure 14. GPCR-independent cAMP elevation induces calcium only after Gq priming. (A) Forskolin (Fsk) increases cytosolic cAMP levels through receptor-independent activation of adenylyl cyclases. (B) Representative traces of HEK293 cells stimulated as indicated responded with a cAMP-dependent calcium increase. The viability control was intact and is shown as a bar graph for solvent priming (grey) and ATP/CCh priming (red). Representative data are mean + SEM whereas the quantification is shown as mean ± SEM of at least three independent experiments, each performed in duplicates.

GPCR-independent cAMP elevation induces calcium only after Gq priming

Next, we tested for direct cAMP-mediated effects using forskolin (Fsk) after establishing that the two major sensor effector proteins were not responsible for Gs-calcium (Figure 14A). Fsk is able to increase cAMP levels in a receptor-independent manner through the activation of ACs (Sutkowski et al., 1994). Contrary to our expectations, Fsk mimicked the Iso-mediated signaling when the Gq pathway was activated, regardless of the selected Gq-GPCR (Figure 14B). We concluded that Gs-mediated calcium must be directly triggered by cAMP (cAMP-dependent Ca^{2+}). An immediate effect of cAMP is the direct sensitization of $InsP_3R$ to elevate cytosolic calcium (C. W. Taylor, 2017). $InsP_3Rs$ play an important role in intracellular calcium mobilization by releasing calcium stored in the ER after binding their corresponding ligand IP_3 (C. W. Taylor & Tovey, 2010). Remarkably, signal junctions have been found that link $InsP_3R$ to AC type 6 and enable the provision of high local cAMP concentrations (Konieczny et al., 2017; C. W. Taylor, 2017). Using HEK293 cells lacking functional AC isoforms 3 and 6 (hereafter HEK-ΔAC3/6), we tested this correlation (Soto-Velasquez et al., 2018). Knockout of AC3/6 reduced the maximum intracellular amount of cAMP released after addition of Iso and Fsk (Figure 15B), providing a cell line ideally suited to study the effect of cAMP on calcium release. Strikingly, lower basal levels of cAMP affected Gq-GPCR signaling, as the first Gq-mediated calcium peak was enhanced upon activation. In contrast, the Iso calcium responded with a lower maximum amplitude and slower kinetics when the cellular background was changed from HEK293 to HEK-ΔAC3/6, whereas the response of Fsk was almost completely absent (Figure 15C). Because Iso elicited a receptor-dependent calcium response via activation of β_2AR, whereas Fsk increased calcium independently of receptor activation, we concluded that an additional, molecularly separable mechanism exists only for Iso. This additional mechanism required higher Iso concentrations (lower potency) compared to the cAMP-dependent Ca^{2+}. Of note, the lower levels of activated PKA as the amount of basal cAMP is reduced in HEK-ΔAC3/6 may account for the increase in Gq-mediated calcium (Yue et al., 1998). Overall, it appears that Iso may utilize two distinct pathways of calcium release, one that it shares with Fsk, a cAMP-dependent calcium release, and another not yet fully understood pathway that is cAMP-independent.

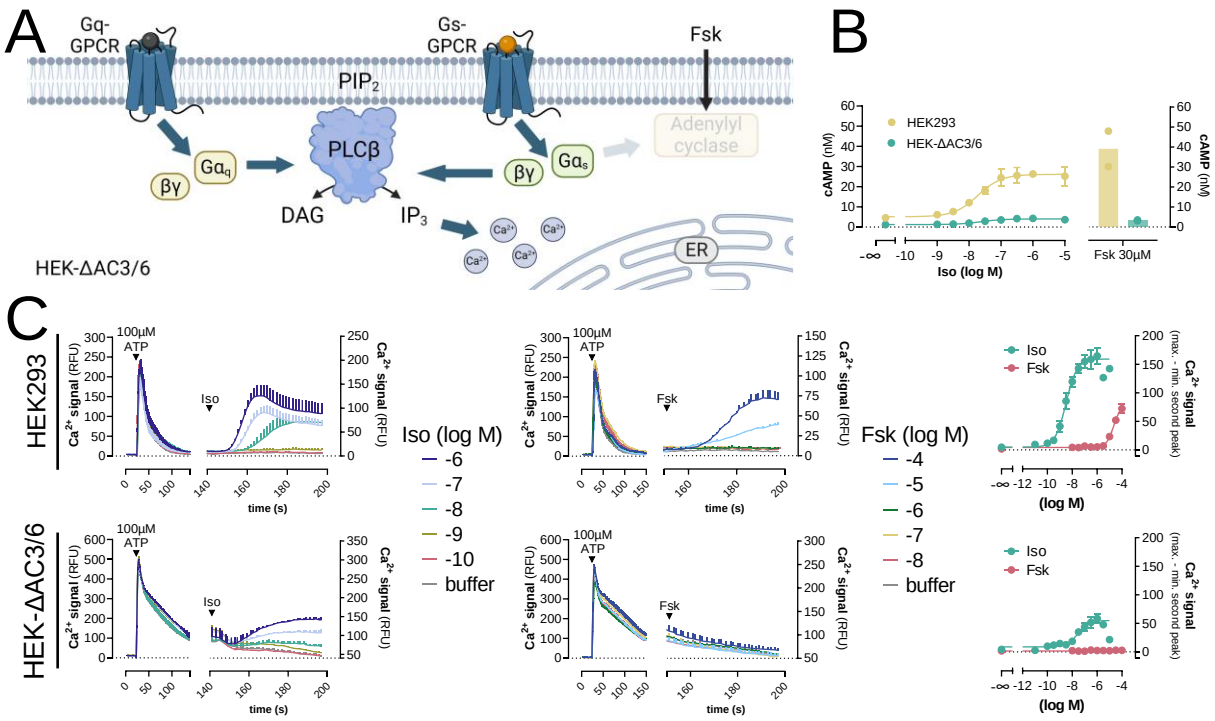

Figure 15. There are two different pathways that Gs-GPCRs use to release calcium. (A) HEK cells lacking functional AC3 and 6 isoforms (HEK-ΔAC3/6) were stimulated with Iso in addition to Fsk to determine the cAMP-mediated calcium component. (B) Mean ± SEM of two experiments confirming lower cAMP levels in HEK-ΔAC3/6 for Iso and Fsk. (C) Representative traces + SEM of HEK293 and HEK-ΔAC3/6 primed cells stimulated with Iso next to Fsk. Summarized data are the mean ± SEM of at least four experiments each performed in duplicates.

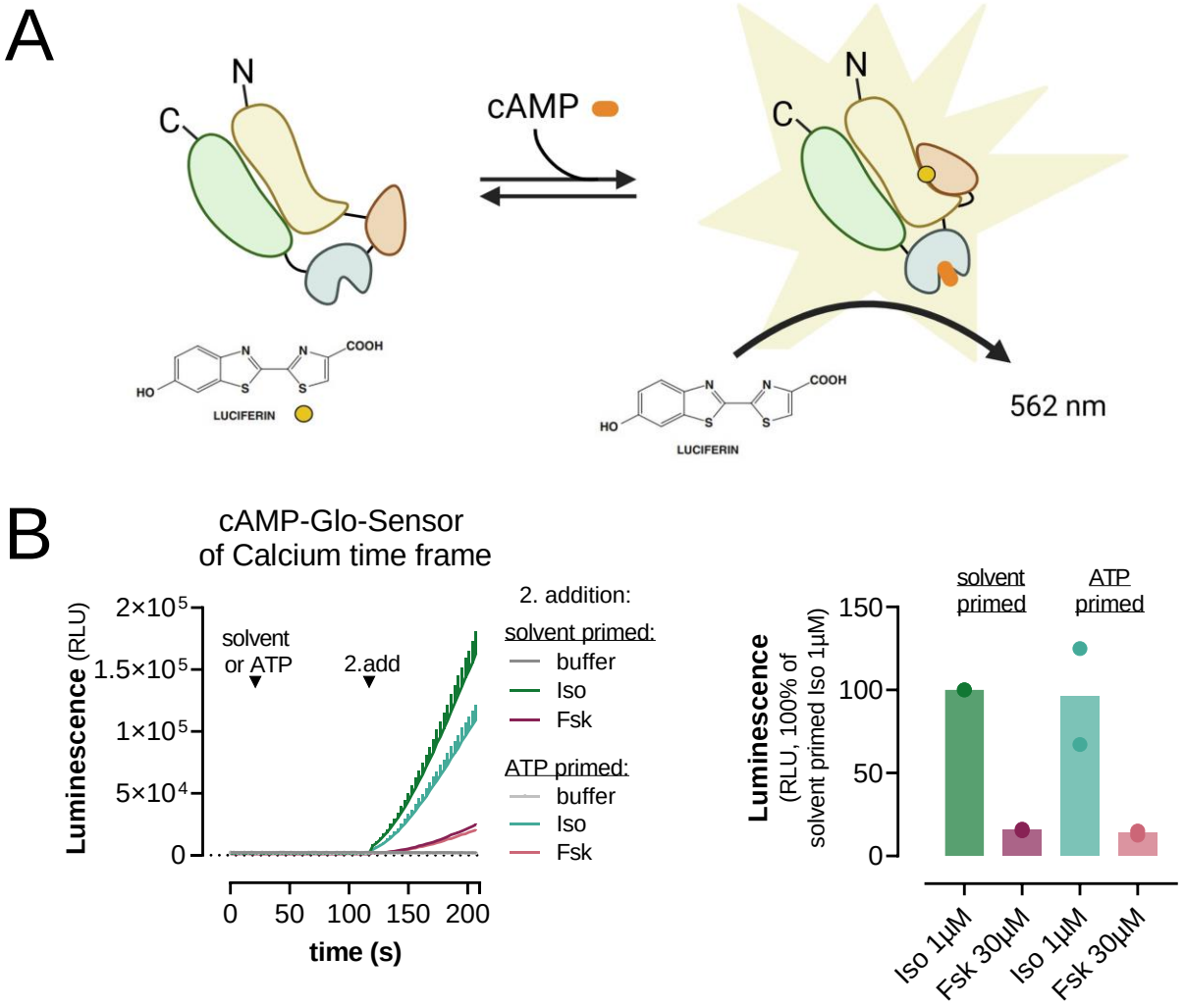

Figure 16. Fsk mimicked cAMP-dependent Gs-Ca^{2+} within the calcium detection window. (A) Conformational shift of the cAMP-BRET-based sensor and conversion of the substrate luciferin upon binding of cAMP based on Buccioni et al., 2011. (B) cAMP levels in HEK293 cells were monitored in real time using the BRET based cAMP sensor. Iso and Fsk increased cAMP independently of priming with either solvent or 100 µM ATP. Representative kinetics are shown as mean ± SEM, while quantified data are the mean of two independently performed cAMP measurements normalized to Iso 1 µM with solvent as primer.

Fsk mimicked cAMP-dependent Gs-Ca^{2+} within the calcium detection window

Since Fsk is a membrane-permeable compound that triggers direct AC activation, one must assume that it could activate AC much more slowly than Iso, which acts via rapid receptor activation. To ensure that Fsk increased the cAMP level within the Ca^{2+} detection window and thus the cAMP-dependent Ca^{2+} component of Iso could be mimicked by Fsk, we used a cAMP-Glo sensor (Figure 16A) (Buccioni et al., 2011). Both Iso and Fsk increased the cAMP level within the calcium detection window, independent of the activated Gq pathway,

suggesting that Fsk is a valid tool to mimic the cAMP-dependent calcium component by Iso (Figure 16B).

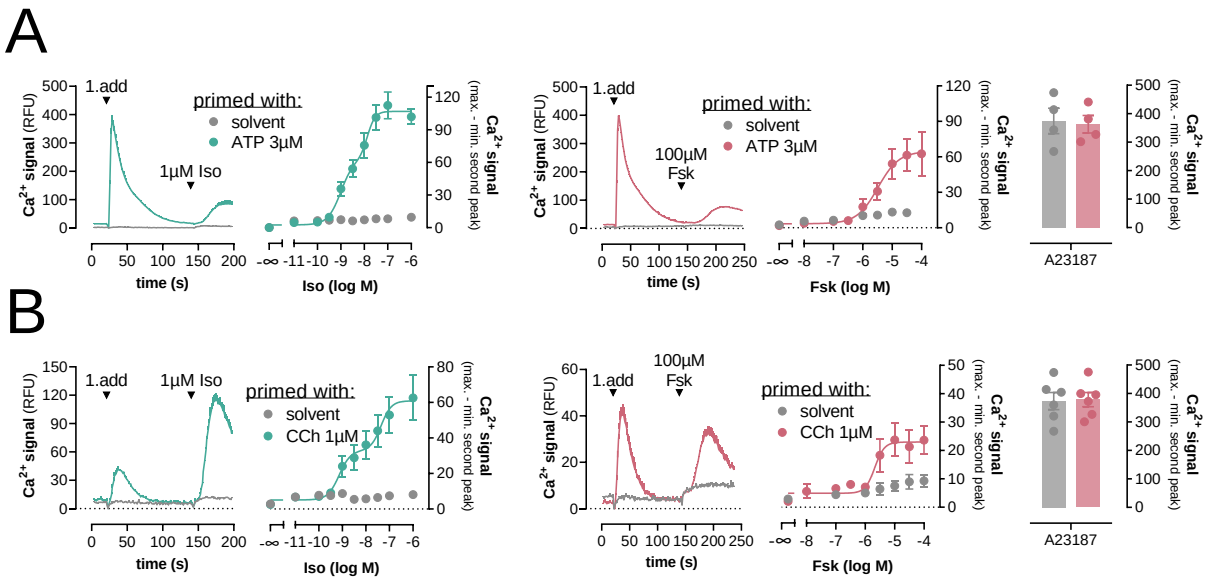

Figure 17. The amount of cAMP-dependent Ca^{2+} is reduced by lowering Gq input. (A, B) HEK293 cells were stimulated with 3 µM ATP or 1 µM CCh followed by adding either Iso or Fsk. Iso-addition showed a subtle biphasic tendency observed after pre-stimulation with 3 µM ATP and turned into a more pronounced biphasic curve after pre-stimulation with 1 µM CCh. Fsk remained monophasic regardless of the respective pre-stimulus. The reduction in Gq input did not affect the control stimulus A23187. Representative traces are the mean + SEM and quantified data are the mean ± SEM of at least 4 independent experiments, each performed in duplicate.

The amount of cAMP-dependent Ca^{2+} is reduced by lowering Gq input

The direct sensitization of $InsP_3R$ by cAMP is triggered by the amount of IP_3 produced (Tovey et al., 2008). Therefore, the Gs-cAMP-Ca^{2+} component should be reduced by lowering the Gq activity. Indeed, priming the cells with a low concentration of ATP (3 µM) or CCh (1 µM) reduced the Iso-mediated calcium response and revealed a biphasic curve, which was more prominent for CCh as compared to that of ATP (Figure 17A, B). The biphasic nature of calcium suggests that Gs proteins are capable of inducing calcium by two distinct molecular mechanisms. If both mechanisms are mediated by cAMP, Fsk should also induce a biphasic concentration response curve after decreasing the Gq stimuli. However, lowering the Gq pre-stimulus only reduced the maximum Fsk signal but did not cause the monophasic curve to become biphasic. Our experimental results suggest that Gs-Ca^{2+} consults a second calcium signaling pathway in addition to the well-known cAMP-dependent calcium signaling pathway, which is independent of cAMP.

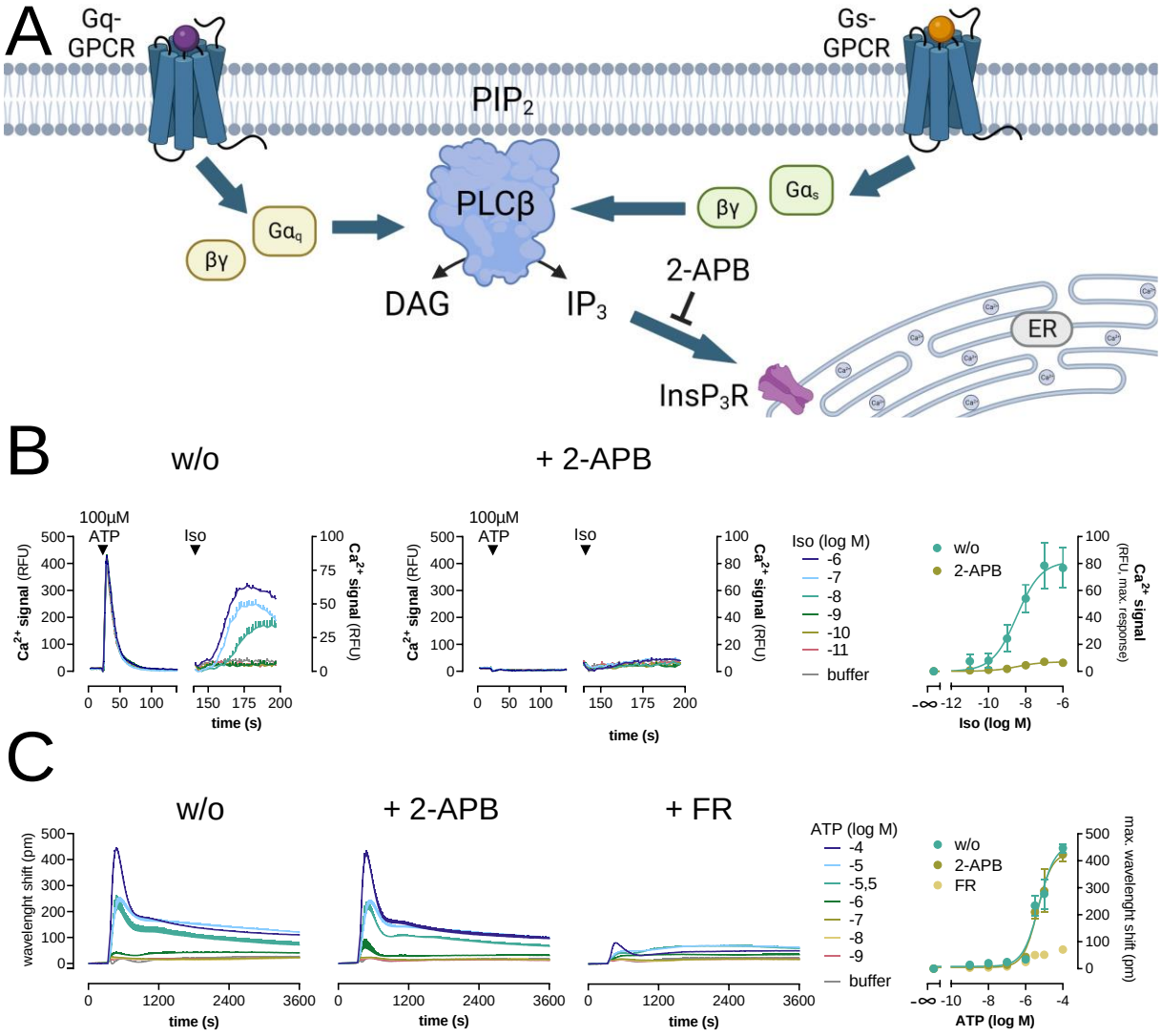

Figure 18. cAMP-independent Gs-calcium depends on $InsP_3R$-mediated calcium release. Calcium mobilization and DMR measurement in HEK293 cells under $InsP_3R$ inhibitor 2-APB treatment, to verify the involvement of $InsP_3R$. (A) 2-APB treatment prevents calcium release from the ER by blocking $InsP_3R$ to its agonist IP_3. (B) Representative Ca^{2+} traces and summary of averaged signals stimulated as indicated in the absence or presence of 50 µM $InsP_3R$ antagonist 2-APB. (C) Representative P2Y receptor-induced DMR traces in untreated (w/o), 50 µM 2-APB-treated, and 1 µM FR-treated cells, including the summary as maximum wavelength shift (pm). Representative traces of calcium and DMR assays are shown as mean + SEM, summaries are mean ± SEM for concentration-response curves of at least three independent biological experiments. Experiments were performed in duplicate for Ca^{2+} measurements and in triplicate for DMR.

cAMP-independent Gs-calcium depends on $InsP_3R$-mediated calcium release

Although there are obvious differences in the requirements for the input of Gq, the prior activation of Gq is essential for both the cAMP-dependent and the cAMP-independent calcium release pathways. As we have shown by reducing the Gq pre-stimulus, the amount of IP_3 produced is of particular importance for the cAMP-dependent sensitization of the $InsP_3R$ (Tovey et al., 2008) which we assume does not count for the cAMP-independent calcium. In this context, sole activation of a Gq-GPCR was sufficient and did not depend on the level of IP_3. 2-Aminoethoxydiphenylborate (2-APB) is a potent inhibitor of $InsP_3R$ and can block the binding of IP_3 to its receptor (Figure 18A). Pretreatment of HEK293 cells with 2-APB abolished Gs-Ca^{2+} after Gq priming, but also blocked total Gq-Ca^{2+} elicited by ATP, supporting the need of IP_3 for both calcium release pathways (Figure 18B). With the disappearance of Gq and Gs, it must be assumed that the Gs-calcium peak is exclusively dependent on the Gq-mediated peak and is therefore cAMP dependent. For this reason, we tested whether Gq activation still occurred in APB-treated cells despite a non-visible calcium response. We consulted a DMR analysis of APB-treated cells showing that even though ATP failed to increase calcium, it allowed the Gq pathway to be activated (Figure 18C). Almost all responses were quenched by the control stimulus FR, deployed to visualize Gq influence in this setup. Slight changes in cell shape under FR can be attributed to the Gi/o component. Taken together, we concluded that cAMP-independent calcium must also depend on a $InsP_3R$-mediated calcium release because the Gs-calcium transients were lost after APB treatment despite active Gq signaling. Of note, the Gq input required for cAMP-independent calcium is somehow different compared to the cAMP-dependent Gq input (Figure 17A, B) and requires active Gq rather than the formation of IP_3.

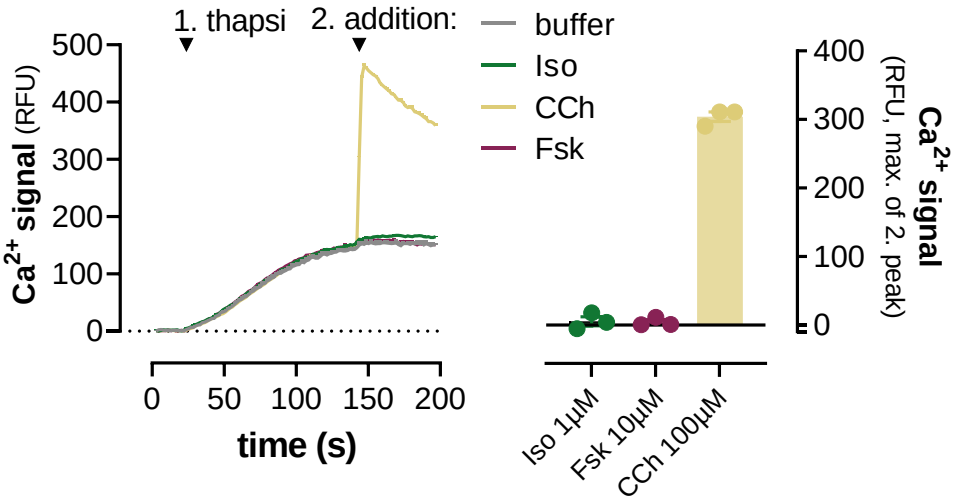

Figure 19. Elevation of intracellular calcium does not mobilize Gs-calcium. Increase of intracellular calcium independent of Gq-GPCR activation in HEK293 cells. (A) HEK293 cells were stimulated with 1 µM SERCA inhibitor thapsigargin, which replaced the Gq pre-stimulus, followed by a second addition of either 1 µM Iso, 30 µM Fsk, 100 µM CCh or buffer. The left panel shows a representative curve as mean + SEM, while the right panel shows the buffer-corrected summary of three independent experiments as the mean ± SEM.

Elevation of intracellular calcium does not mobilize Gs-calcium

Next, as APB did not allow Gs-calcium, the urgent question arises whether the increase in calcium was the key element in the activation of the Gs-mediated calcium. Calcium ions are known to regulate the activity of a large number of target proteins. One of these is the major Gs-effector protein adenylyl cyclase (Dessauer et al., 2017; Sadana & Dessauer, 2008), or the PLCβ isoform family, which contains calcium-binding domains, such as two EF hands or the catalytic center (Gresset et al. 2012; Kadamur und Ross 2013). Calcium mediated by prior activation of Gq can therefore cause a wide variety of different effects, including being the contributor for Gs-calcium. By inducing a Gq-GPCR independent calcium increase via the SERCA inhibitor thapsigargin, we tested, if high cytosolic calcium concentrations can trigger the Gs-mediated calcium release. SERCA is a calcium-ATPase located in the sarcoplasmic/endoplasmic membrane, shuttling cytosolic Ca^{2+} back to their storage site (Primeau et al., 2018). Instead of the usual addition of the Gq stimulus, the HEK293 cells were now treated with 1 µM of thapsigargin. When the inhibitor was added, the intracellular calcium concentration increased immediately. The subsequent addition of Iso and Fsk did not induce a detectable calcium response, whereas CCh caused an increase in cytosolic calcium (Figure 19). In conclusion, these data reemphasize the need for an active Gq as a simple

increase in intracellular calcium by SERCA inhibitor thapsigargin was not sufficient to promote Gs-mediated calcium.

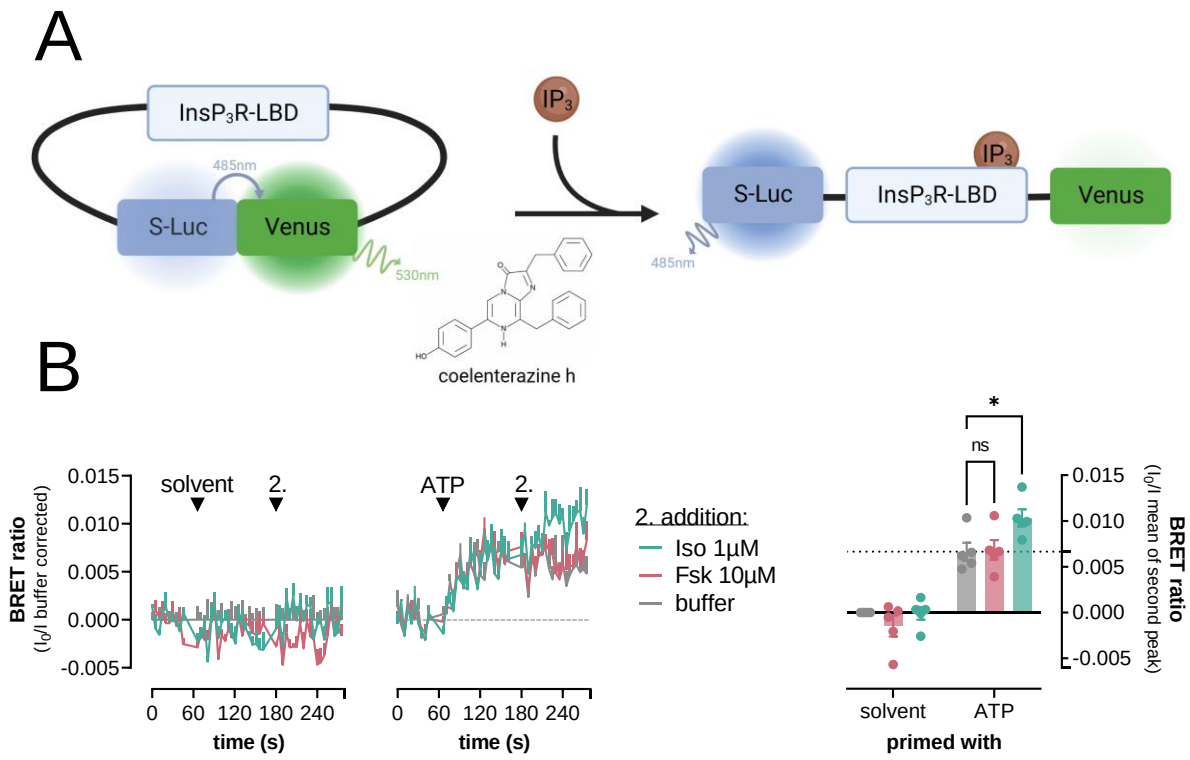

Figure 20. Gs-agonist Iso induces IP_3 formation after Gq priming. (A) Schematic drawing of the inositol-1,4,5-trisphosphate ($InsP_3$)-sensor. The BRET sensor contains a S-Luc at the N-terminus, the ligand-binding domain of the human type-I $InsP_3$ receptor and Venus at the C-terminus. Binding of IP_3 results in a decrease in the BRET signal as S-Luc and Venus separate. (B) Real-time IP_3 formation in HEK293 cells following the two-step addition protocol of the calcium assays. Solvent priming did not allow any formation of IP_3 whereas Gq priming with ATP enable an Iso-mediated increase. Representative traces of IP_3 are shown as the mean + SEM, the summary is depicted as a bar graph ± SEM of five independent biological experiments, performed in triplicates. Statistical significance was determined using a two-way ANOVA, with ns = not significant, *p<0.05.

Gs-agonist Iso induces IP_3 formation after Gq priming.

Since Gs-calcium only appears after activation of the $InsP_3R$, all evidence to date suggests that Gs-GPCRs must also be able to mobilize IP_3. In order to test this theory, we measured the intracellular formation of IP_3 in real-time using a BRET-based IP_3 sensor (Gulyás et al., 2015). The conformational drift of N-terminal S-Luc and C-terminal Venus upon binding of IP_3 to the ligand-binding domain of the human type-1 $InsP_3$-receptor allows the measurement of

cytosolic changes in IP_3 (Figure 20A). In agreement with our calcium data, Iso did not induce a detectable production of IP_3 in solvent primed cells. However, ATP pre-stimulation resulted in a rapid increase in IP_3 production after the addition of Iso, but did not result in any detectable changes in IP_3 production by Fsk (Figure 20B). Therefore, these data suggest that Gs-GPCRs can directly induce IP_3 formation after Gq activation and that Fsk and the associated change in AC activity or the accompanying change in cAMP levels do not induce IP_3 formation. Taken together, Gs-GPCRs control calcium after Gq pre-stimulation i) by a cAMP-dependent pathway, which could involve a cAMP sensitization of $InsP_3Rs$ (Konieczny et al., 2017) and ii) by a cAMP-independent IP_3 formation. Interestingly, the direct formation of IP_3 by Gs suggests that Gs-GPCRs, like Gq-GPCRs, contribute to membrane phospholipid hydrolysis. We hypothesize that phospholipase Cβ enzymes are activated not only by Gq-GPCRs but also by the family of Gs-coupled receptors.

4. Are phospholipases Cβ involved in calcium regulation of Gs?

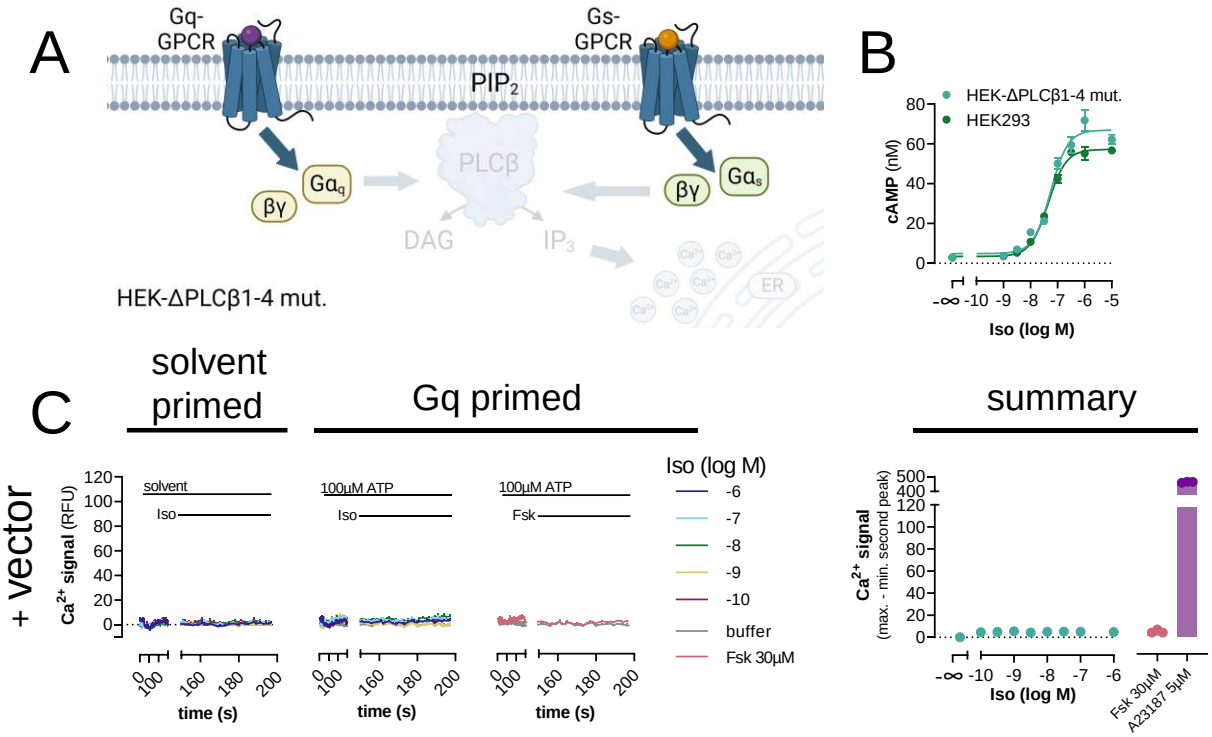

Figure 21. HEK-ΔPLCβ1-4 mut. cells do not mobilize calcium upon stimulation. (A) Deletion of the alleles encoding PLCβ isoforms in HEK293 cells resulted in the HEK-ΔPLCβ1-4 mut. cell line, which does not mobilize calcium. (B) Quantified cAMP data ± SEM of β_2AR activation in HEK293 or HEK-ΔPLCβ1-4 mut. cells. The results shown are from one experiment performed in triplicates. (C) Representative traces + SEM of vector transfected HEK-ΔPLCβ1-4 mut. cells stimulated as indicated are shown in the three left panels. The summary of three independent experiments ± SEM, each performed in duplicate is shown in the right panels. The rise in control ionophore indicates viable cells, although no calcium mobilization was observed upon Gs- or AC-agonist.

Gs activates phospholipases Cβ to mediate a calcium release

GPCR-induced IP_3 formation is attributed to increased activity of phospholipases Cβ, which hydrolyze PIP_2 to IP_3 and DAG. Canonically, these enzymes are activated by either Gq-coupled or Gi/o-coupled GPCRs. Interestingly, while Gq activates PLCβ via its alpha subunit, Gi-GPCRs require prior Gq alpha binding for activation by their Gβγ subunit (Gresset et al., 2012; Kadamur & Ross, 2013; Pfeil et al., 2020; A. V. Smrcka, 2008; A. V. Smrcka & Sternweis, 1993). It should be noted that prior Gq priming appears to be as imperative for Gi/o-mediated Ca^{2+} responses as for Gs-mediated responses. Based on our observations regarding Gs-GPCR mediated IP_3 generation, we were particularly intrigued by the possibility that PLCβ enzymes are involved in this process. This potential involvement could imply a novel role for PLCβ as an effector protein for Gs-GPCRs. To address this question, we used

HEK-ΔPLCβ1-4 mut. cells generated using CRISPR/Cas9 technology (Milligan & Inoue, 2018), targeting the alleles encoding the four isoforms of PLCβ (Figure 21A). While ATP stimulation or subsequent addition of Iso or Fsk did not mediate a detectable calcium response in HEK-ΔPLCβ1-4 mut. cells (Figure 21C), the Gs-β_2AR-mediated increase in cAMP was still intact (Figure 21B). This was also found for the receptor-independent calcium response elicited by the viability control A23187. Reintroduction of each individual PLCβ isoform (1-4) restored the ATP-mediated and the subsequent Iso and Fsk response, suggesting that the observed Gs-GPCR calcium is indeed PLCβ-mediated (Figure 22A-D). It was also striking that the Iso-mediated calcium signal followed a PLCβ isoform-specific pattern. Reintroduction of PLCβ isoforms 1 or 4 triggered a monophasic concentration-response curve to Iso with a high potency which could be mimicked in its maximal amplitude by a saturating concentration of Fsk (Figure 22A, B). In contrast, cells transfected with PLCβ2 or PLCβ3 and treated with Iso revealed a biphasic curve with two inflection points (Figure 22C, D). The EC_{50} values of the first inflection point, with the higher potency, is comparable to the inflection point of the concentration-response curve induced by Iso in cells transfected with PLCβ1 or β4, whereas the second inflection point appeared at higher Iso concentrations (lower potency Ca^{2+}). Strikingly, the maximal calcium response mediated by Fsk was similar to the first plateau of the biphasic Iso-curve. Our results provide compelling evidence that PLCβ1 and PLCβ4 exclusively trigger a single cAMP-driven Ca^{2+} release pathway, as Fsk mimics the Iso-mediated response. In contrast, PLCβ2 and β3 appear to use two distinct pathways for calcium release. One pathway involves an increase in intracellular cAMP levels, whereas the other, which has a lower potency, appears to involve an unidentified cAMP-independent mechanism. Therefore, PLCβ1 and β4 enzymes appear to differ from PLCβ2 and β3 in their mode of calcium delivery.

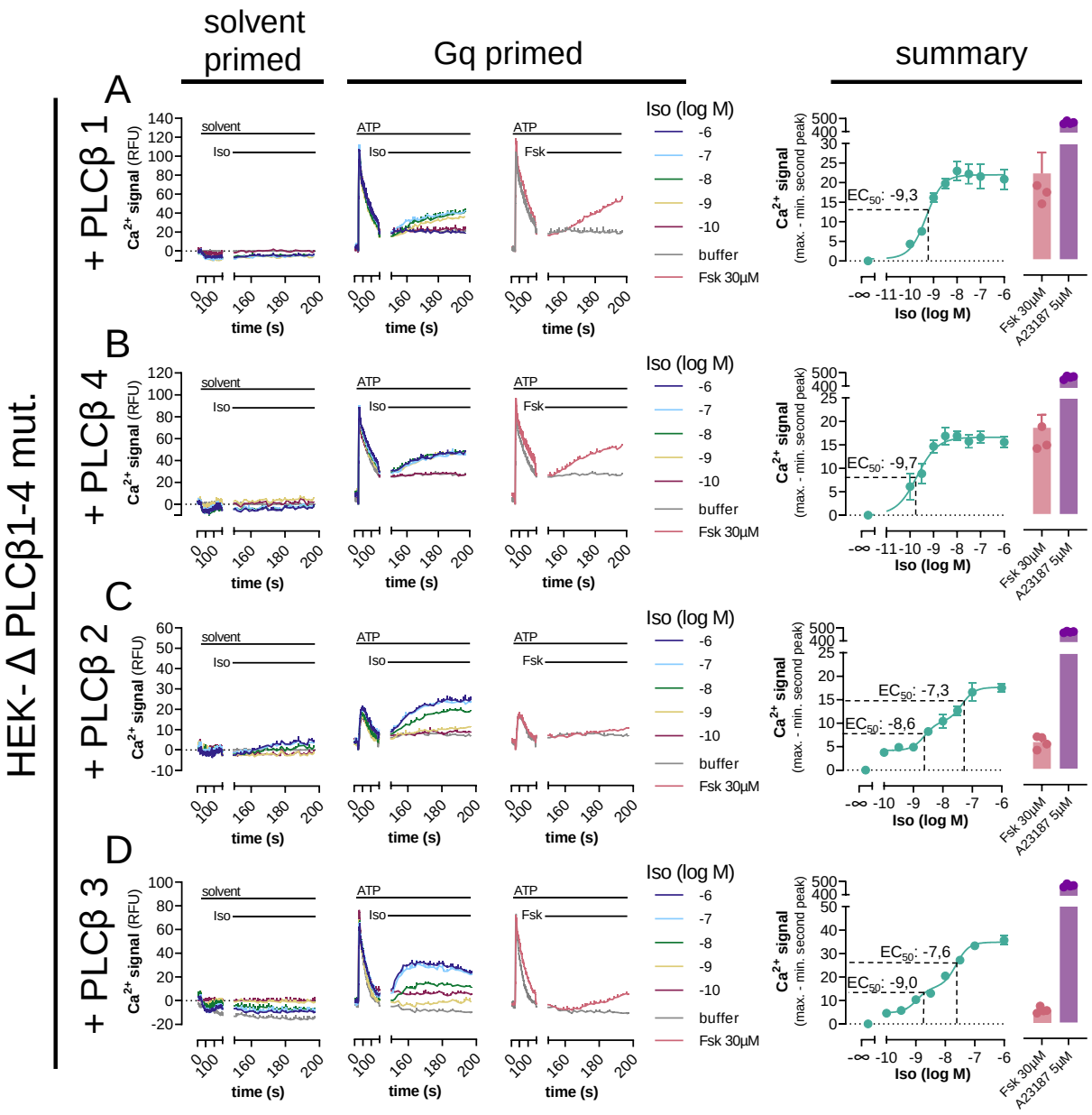

Figure 22. Gs activates phospholipases Cβ to mediate calcium release. (A-D) HEK-ΔPLCβ1-4 mut. cells transiently transfected with each of the individual PLCβ isoforms 1-4. Representative calcium kinetics + SEM (three left panels), summarized as mean ± SEM of at least three experiments (two right panels), of calcium mobilization in response to the β_2AR agonist Iso and the AC activator Fsk, when cells were primed with 100 µM ATP. The control ionophore A23187 induced calcium independent of priming (shown is the Gq-primed response). Reintroduction of PLCβ1 or β4 (A, B) shows a monophasic curve in response to Iso, whereas PLCβ2 and β3 (C, D) show a biphasic curve. The inflection points of the curves are marked with the corresponding EC_{50} values.

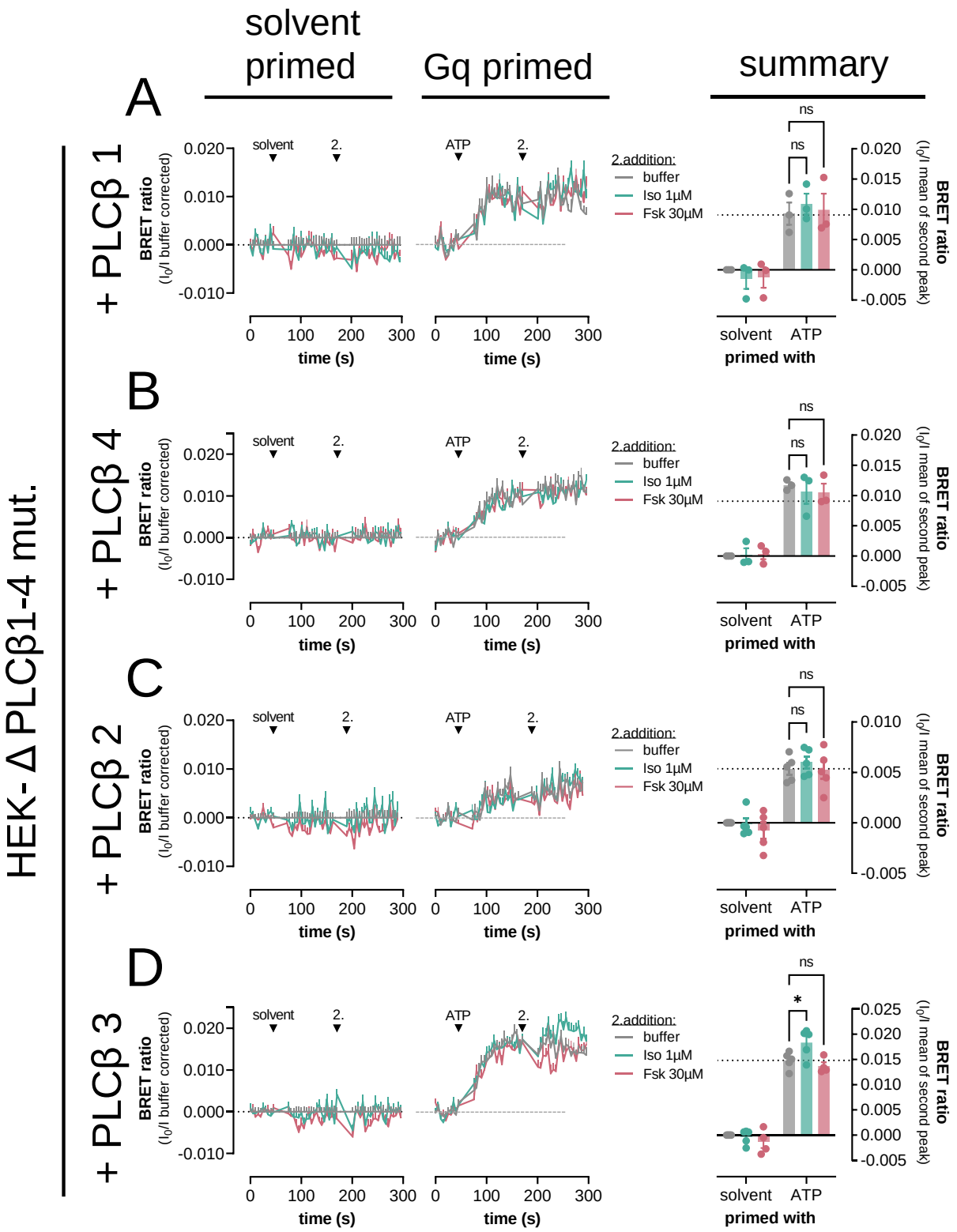

Figure 23. Gs-mediated IP_3 formation is PLCβ isoform specific. For real-time detection of IP_3 levels, HEK-ΔPLCβ1-4 mut. cells were transiently transfected with one of each of the four PLCβ isoforms and the IP_3-BRET sensor. Cells were primed with either solvent or 100 µM ATP followed by a second addition as indicated. (A-D) While Gs-agonist Iso significantly increased IP_3 levels for cells transfected with PLCβ3, no significant IP_3 increase was observed for cells transfected with one of the other PLCβ isoforms. (C) It should be noted that the IP_3 increase

mediated by PLCβ2 was not significant, but an increasing trend for Iso could be observed in the kinetics. Representative traces are the mean + SEM, while the left panel is the summary as mean ± SEM of at least three independent experiments. Statistical significance was determined using a two-way ANOVA, with ns = not significant, *p<0.05.

Gs-mediated IP_3 formation triggered by phospholipases Cβ3

Previous results in HEK-ΔPLCβ1-4 mut. cells showed individual differences in the provision of calcium between the four isoforms of PLCβ. While all the isoforms provide calcium in a cAMP-dependent manner, PLCβ isoforms 2 and 3 use an additional cAMP-independent calcium release pathway. Moreover, by knowing that Gs-GPCRs utilize IP_3 formation to release calcium via a cAMP-independent pathway (Figure 20B), we wanted to test whether PLCβ isoforms 2 and 3 are responsible for the formation of IP_3. To test our assumption, we expressed the BRET based IP_3 sensor next to each individual PLCβ isoform in the HEK-ΔPLCβ1-4 mut. cell line. Each individual PLCβ isoforms responded with an increase in the BRET ratio upon addition of the Gq agonist ATP (100 µM), indicating functional expression of the isoforms. Notably, the subsequent second addition of Iso elicited a significant increase in BRET response only for PLCβ3, while a rudimentary but non-significant upward trend was observed for PLCβ2 (Figure 23C, D). No production of IP_3 was detected when PLCβ isoform 1 and 4 were expressed (Figure 23A, B). Regardless of the PLCβ isoform chosen, the addition of Fsk did not result in an increase in the amount of IP_3. However, it is conceivable that the possible upward trend of Fsk towards the end of the measurement can be attributed to the AC-EPAC-PLCε axis (Schmidt et al., 2001) rather than a contribution of PLCβ. In conclusion, the Gs-coupled β_2AR causes an increase in IP_3 levels exclusively in cells re-expressing PLCβ3. Thus, Gs-GPCR mediated calcium release appears to be PLCβ isoform-dependent. While all isoforms facilitate the cAMP-dependent calcium pathway, PLCβ3 additionally consults an IP_3-dependent, cAMP-independent signaling pathway. We also suspected this additional pathway for PLCβ2 based on the biphasic calcium pattern but did not find it to be statistically significant in the BRET assays performed.

5. Can Gs-βγ bind to and activate the phospholipase Cβ3?

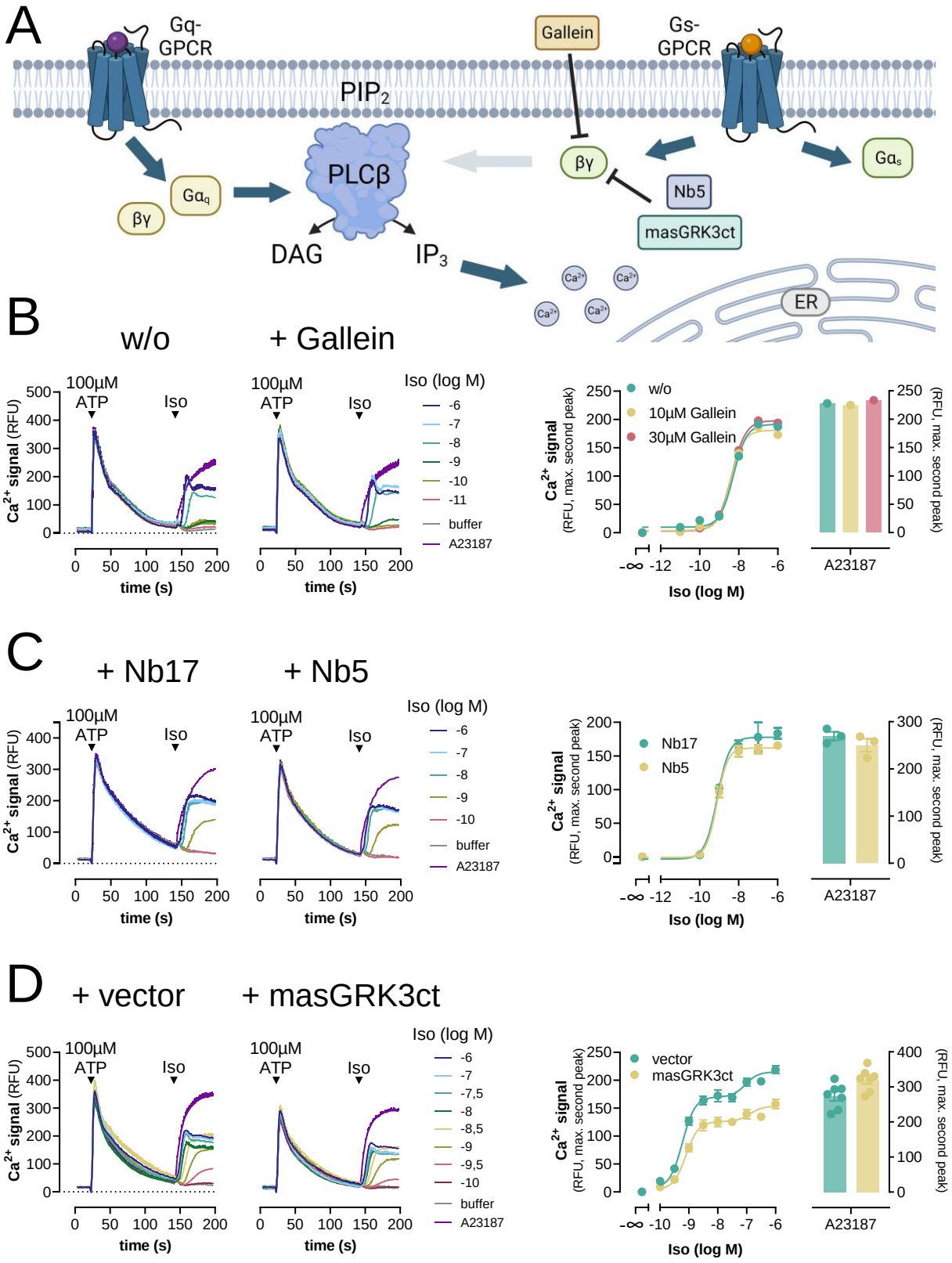

Figure 24. Neither Gallein, Nb5 nor masGRK3ct blocked Gs-βγ-mediated calcium. (A) Schematic representation of the different approaches to block Gβγ signaling. HEK293 cells

were either treated with (B) 10 µM or 30 µM of Gallein 45 minutes before the measurement or transfected with (C) control Nanobody 17 (Nb17) or Nanobody 5 (Nb5), respectively vector control or masGRK3ct. Neither Gallein nor Nb5 affected the Gs-Iso response that followed Gq priming. The reduced Gs-Iso response in the presence of masGRK3ct is attributed to the effect of masGRK3ct on the first Gq peak and not to an effect of the scavenger on the Iso-mediated calcium. Viability control A23187 was intact despite slight reduction of Nb5 transfected cells. Shown are the representative traces as mean + SEM on the left, followed by quantification as mean ± SEM on the right. Gallein kinetics shown are those treated with 10 µM. Except for Gallein-treated cells, all experiments were repeated independently at least three times in duplicates.

Gs-βγ subunits emerge as a novel activator of PLCβ3

Interestingly, our findings indicate that PLCβ isoform 3 and presumably PLCβ2, both of which can be naturally regulated by G protein-coupled βγ-subunits (Kadamur & Ross, 2013), facilitate a cAMP-dependent and cAMP-independent calcium release pathway, whereas PLCβ isoform 1 and 4 only trigger a cAMP-dependent mobilization of Gs-calcium. Due to high cellular abundance, it has been assumed that only Gi/o-GPCRs are able to signal through Gβγ (Chung & Wong, 2020; Kadamur & Ross, 2013; A. V. Smrcka, 2008). Strikingly, Gi-GPCRs were able to release Gβγ-calcium only after previous Gq activation, the same prerequisite that seems to be indispensable for Gs-calcium. Furthermore, as for Gi-βγ-Ca^{2+}, the Gβγ-sensitive isoform PLCβ3 is of particular importance for Gs-Ca^{2+}. However, while Gi mobilizes its calcium only through a cAMP-independent, Gβγ-dependent pathway (Pfeil et al., 2020) the activation of a Gs-GPCR mobilizes calcium in a cAMP-dependent and cAMP-independent manner. Based on these findings, we hypothesized that Gs-βγ induces cAMP-independent Ca^{2+} after Gq priming. To test this hypothesis, we took advantage of i) the small molecule inhibitor Gallein, ii) the single antigen fragment nanobody 5 (Nb5) and iii) the protein-based inhibitor masGRK3ct, which are all described to scavenge free Gβγ (Figure 24A) (Gulati et al., 2018; Hollins et al., 2009). HEK293 cells were either treated with Gallein or transfected with Nb5 or masGRK3ct to test whether the cAMP-independent part of Iso-mediated calcium is indeed Gβγ-mediated. After Gq priming the Gs-coupled β_2AR was stimulated by the addition of Iso. Neither Gallein, Nb5 nor masGRK3ct showed a significant effect on Iso-mediated calcium when primed with 100 µM of ATP compared to untreated/Nb17- or vector-transfected cells (Figure 24B-D). The modest reduction in Iso-response in cells transfected with masGRK3ct is likely caused by its effects on the initial Gq-mediated peak rather than by interception of Gs-βγ. This is reasonable because by stimulating purinergic receptors, free Gq-βγ can support Gq-alpha-mediated calcium release, providing a target for masGRK3ct. As a result, masGRK3ct is preoccupied with Gq-βγ, leaving less masGRK3ct available for scavenging free Gs-βγ. According to this hypothesis, reducing the first Gq peak can decrease free Gq-βγ as a target for masGRK3ct,

resulting in more free masGRK3ct to sequester Gs-βγ. In addition, low concentrations of the Gq agonist ATP or CCh (Figure 17) improved the visualization of low-potency, cAMP-independent Gs-calcium, while high-potency, cAMP-dependent Gs-calcium decreased. Thus, we modified the pre-stimulus from 100 µM ATP to a concentration of 1 µM CCh and reintroduced masGRK3ct (Figure 25A). Under the altered priming conditions, masGRK3ct considerably diminished the low-potency segment of the Iso-mediated biphasic concentration-response curve in comparison to the entire signal (decrease from 40% to 20% of total signal). Furthermore, in HEK-ΔPLCβ1-4 mut. cells re-expressing PLCβ3 in addition to masGRK3ct, thereby eliminating the need for Gq priming at low CCh as a biphasic curve emerged, the Iso-mediated low potency component of the signal was significantly attenuated (reduced from 50% to 10% of the total signal) (Figure 25B). Note that masGRK3ct also induces a slight reduction in the CCh-mediated peak, potentially linked to a decrease in the amount of IP_3 necessary for the cAMP-dependent Gs-calcium. Consequently, the cAMP-dependent Gs-Iso component may be slightly smaller. Gallein and Nb5 had no effect on the Gq-mediated first peak, indicating neither scavenger is suitable for detecting Gβγ effects in the chosen assay setup. However, it should be noted that under appropriate assay conditions, such as Gq priming at lower concentrations, Gallein or Nb5 may be suitable for detecting Gβγ effects on Iso-mediated calcium. In conclusion, masGRK3ct in combination with the right conditions proves to be an effective method for detecting Gβγ signaling. This approach allowed us to identify Gs-βγ as the driver of low-potency, cAMP-independent calcium. Furthermore, we propose the activation of PLCβ3 by Gs-βγ after prior activation of Gq as this isoform can be naturally regulated by Gβγ.

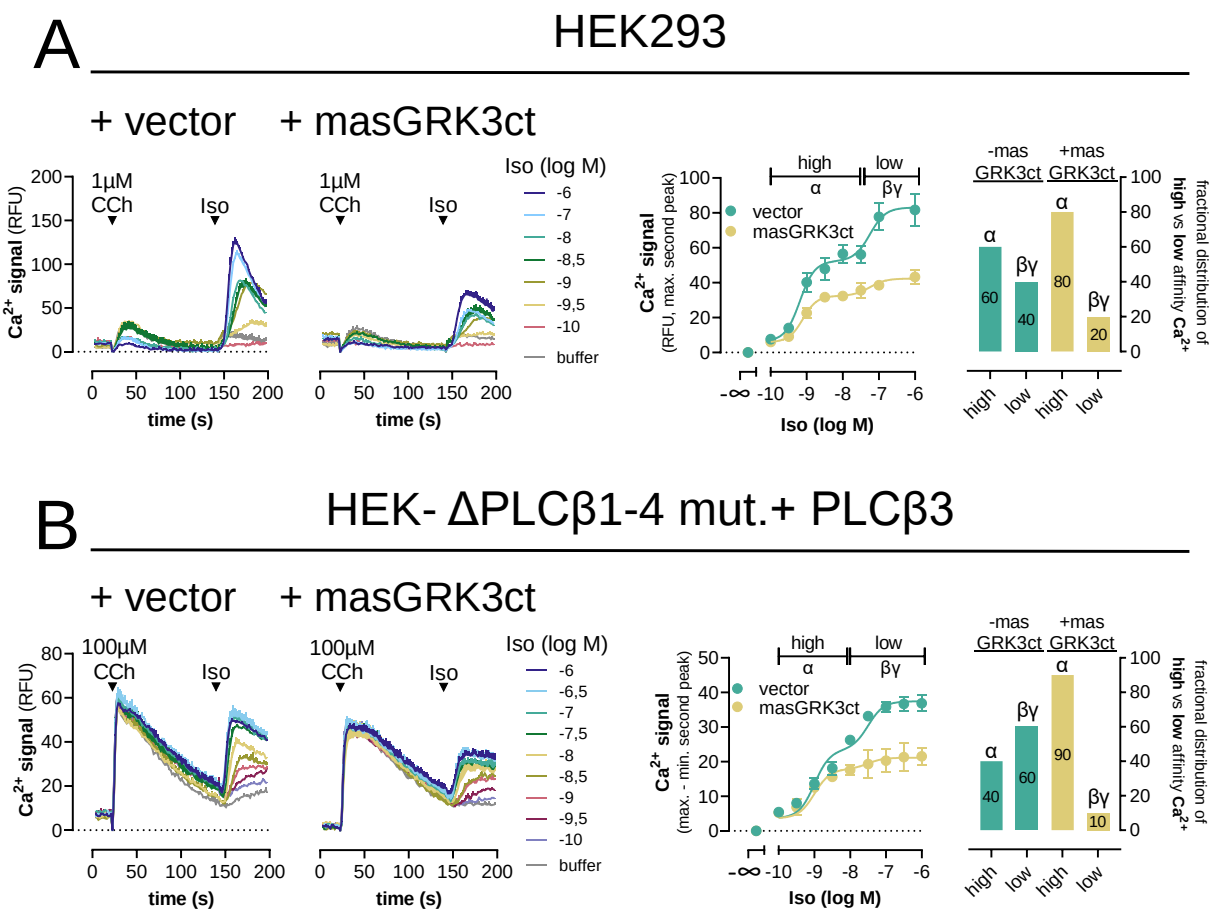

Figure 25. Gs-βγ subunits emerge as a novel activator of PLCβ3. Intercepting Gs-βγ-mediated calcium with the masGRK3ct. Transfection of either HEK293 cells (A) with vector or masGRK3ct or HEK-ΔPLCβ1-4 cells (B) transfected with PLCβ3 along with vector or masGRK3ct. While cells in A were exposed to a low concentration of CCh (1 µM), cells in B were exposed to a high concentration of CCh (100 µM). As usual, the addition of the Gs-agonist Iso followed the previous Gq activation. Regardless of the cellular context, masGRK3ct transfection abolished the cAMP-independent part of the biphasic curve, indicating the contribution of Gs-βγ. Fractional distribution in "high" potency Iso-calcium ($G\alpha_s$) and "low" potency Iso-calcium (Gs-βγ) was shifted by the scavenger towards the $G\alpha_s$-mediated part. Shown are the representative traces as mean + SEM on the left, followed by quantification as mean ± SEM on the right. The fractional distribution is determined by a nonlinear fit of the data points in the summary, resulting in no error bars. All experiments were repeated independently at least four times in duplicates.

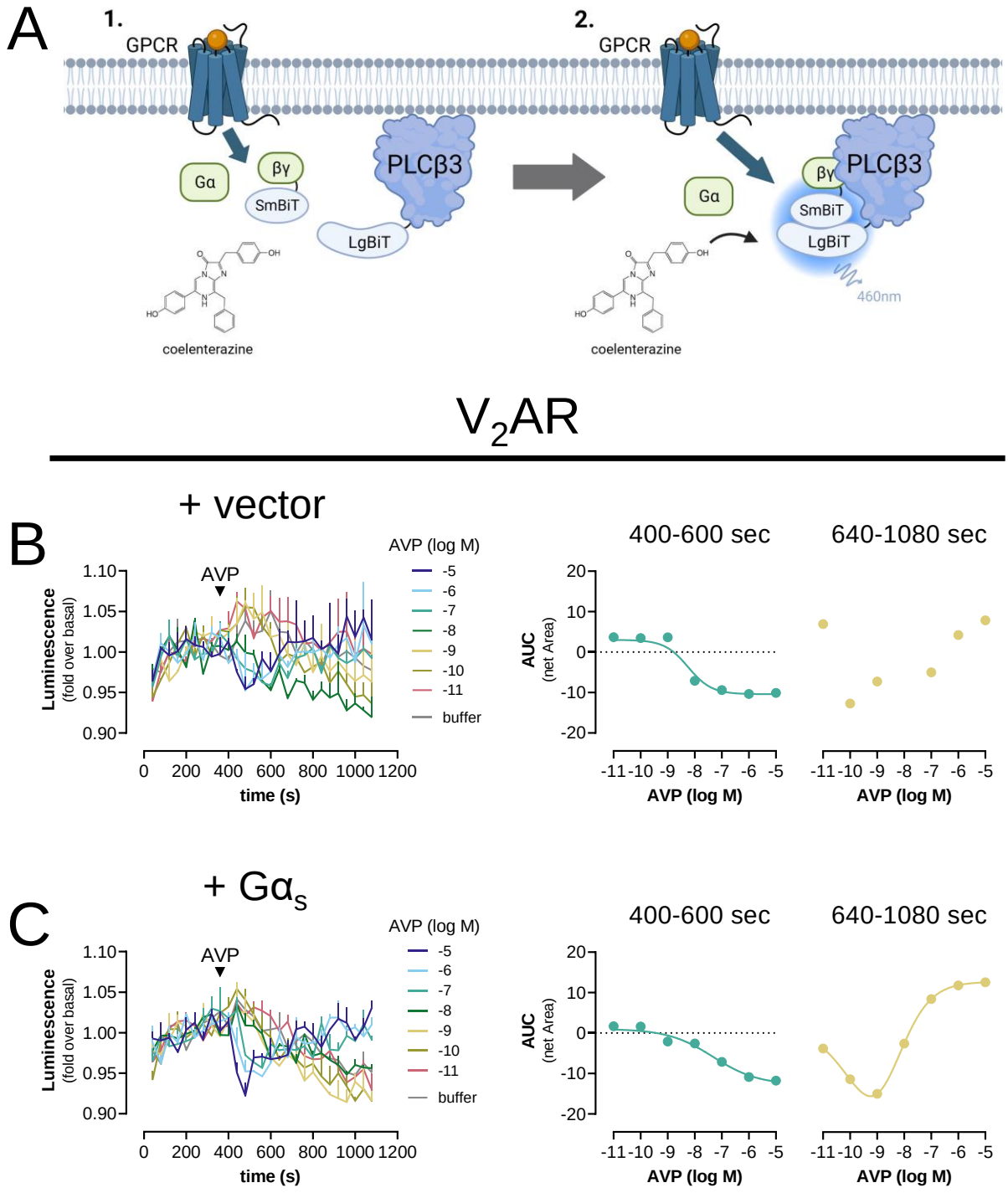

Figure 26. Dynamic interaction of Gs- and Gq-βγ with the PLCβ3 isoform. (A) By cleaving a nanoluciferase into a small fragment fused to Gγ2 (SmBiT-Gγ2) and the corresponding large fragment fused N-terminally to PLCβ3 (LgBiT-PLCβ3), we attempted to visualize V_2AR-mediated Gs-βγ-PLCβ3 binding. (B, C) Exemplary Nanobit traces as mean + SEM of HEK293 cells expressing either vector or $Gα_s$ along with SmBiT-Gγ2, LgBiT-PLCβ3, V_2AR and Gβ1 stimulated with V_2AR agonist AVP. Quantified data are averaged duplicates depicted in a downward trend section of 400-600 sec and an upward trend section of 640-1080 sec, suggesting Gs-βγ signaling in the early phase of V_2AR activation, followed by a shift to Gq-βγ signaling (data generated by S. Bravo, Master thesis 2021).

Nanobit complementation assay does not visualize proper Gs-βγ-PLCβ3 binding

All the data obtained so far indicate that Gs-βγ can activate PLCβ3 enzymes and there is also some evidence for PLCβ2 as well. For this kind of activation, a direct protein-protein interaction of the Gs-βγ subunit with its effector is required. To test whether Gs-βγ can bind to PLCβ, we took advantage of the so-called Nanobit complementation assay. The Nanobit assay indicates spatial proximity of two proteins by increasing luminescence (Figure 26A). For this purpose, a nanoluciferase (Nluc) is split into two parts: A small part bound to Gγ2 (SmBiT-Gγ2) and the corresponding large part bound to the N-terminus of PLCβ3 (LgBiT-PLCβ3). Besides SmBiT and LgBiT, HEK293 cells were also transfected with the V_2AR, Gβ1, $G\alpha_s$ or a control vector replacing $G\alpha_s$. The peculiarity of the selected V_2AR is its Gq and Gs coupling property, making it the appropriate positive control. At the beginning of the measurement the luminescence of vector expressing cells, representing endogenous $G\alpha_s$ levels, and $G\alpha_s$-transfected cells were quite similar. Stimulation of the V_2AR receptor with high concentrations of V2-agonist AVP (only for concentrations above 10 nM) produced an initial downtrend (Figure 26B, C), which conspicuously changed to an uptrend after 10 minutes (600 seconds) (Figure 26C). Low concentrations of AVP (below 10 nM) caused the luminescence to steadily decrease over time and failed to transition to an upward trend. We speculate that the separation of Gs-βγ from PLCβ3 – SmBiT and LgBiT are spatially separated – acts as the trigger for the downward trend. Additionally, this trend is triggered by even low concentrations of AVP. On the other side, high concentrations of AVP favor the coupling of low affinity Gq to V_2AR. This "secondary" GPCR signaling occurs exclusively after application of high concentrations of AVP and only after the Gs protein has detached from the receptor. Therefore, the subsequent increase in luminescence may be due to the binding of Gq-βγ to PLCβ3. Our results indicate that the V_2AR-mediated signal is partly due to Gs-βγ activity and partly due to Gq-βγ activity.

To verify this assumption, we transfected HEK293 with $G\alpha_q$ instead of $G\alpha_s$ and still observed a decrease for low concentrations of AVP whereas high concentrations did not induce any changes in luminescence (Figure 27A). However, all AVP-mediated signals changed to a downward pattern with FR pretreatment (Figure 27B). These findings support the conclusion that lower concentrations of AVP may only allow V_2AR to couple to Gs whereas high concentrations allow V_2AR to couple to both. Since the luminescence remained almost unchanged without FR treatment and clearly shows a decrease in the presence of FR, we assume that the emerging Gs-βγ-PLCβ3 separation and Gq-βγ-PLCβ3 binding masked each other (downward trend for Gs-βγ and upward trend for Gq-βγ). By changing the cellular background to PTX-treated HEK-Δ7 cells followed by re-transfecting Gs or Gq proteins, we hoped to specifically attribute V_2AR-mediated responses to Gq or Gs (Figure 27B). Utilization of HEK-Δ7 in the presence of PTX did not respond to the addition of AVP in a change in luminescence, establishing a cellular background without functional G proteins (Figure $27B_{(i)}$).

$G\alpha_q$-transfected cells responded with a concentration-dependent increase in luminescence to the addition of the agonist, with no evidence of a decreasing trend (Figure $27B_{(ii)}$). Conversely, a consistent downward trend was observed in cells transfected with $G\alpha_s$ and subjected to stimulation (Figure $27B_{(iii)}$). Thus, by switching to PTX-treated HEK-Δ7 cells, we were able to decipher cross-over effects and attribute specific changes in luminescence to the mediated G protein.

So far, we observed a Gs-βγ-PLCβ-Ca^{2+} release after Gq activation but were not able to visualize the binding between Gs-βγ to PLCβ3 with the Nanobit. We hypothesize that there is indeed a direct interaction between Gs-βγ and PLCβ3, but it is difficult to detect because either i) Gs-βγ simply reassociates too quickly with its Gs alpha subunit, or ii) it does not dissociate properly at all. Several studies have already shown poor heterotrimer dissociation due to the high affinity of Gs-βγ for its alpha subunit, making it difficult to observe Gs-derived βγ effects in cells (Chung & Wong, 2020; Digby et al., 2008; Masuho et al., 2021). Based on these results, the Nanobit assay does not appear to be suitable for the detection of Gs-βγ-PLCβ3 interaction, although we still hypothesize an interaction. However, we were able to detect the Gq-βγ-PLCβ3 interaction because Gq-βγ-PLCβ3 appears to interact for a period, long enough to form a functional luciferase.

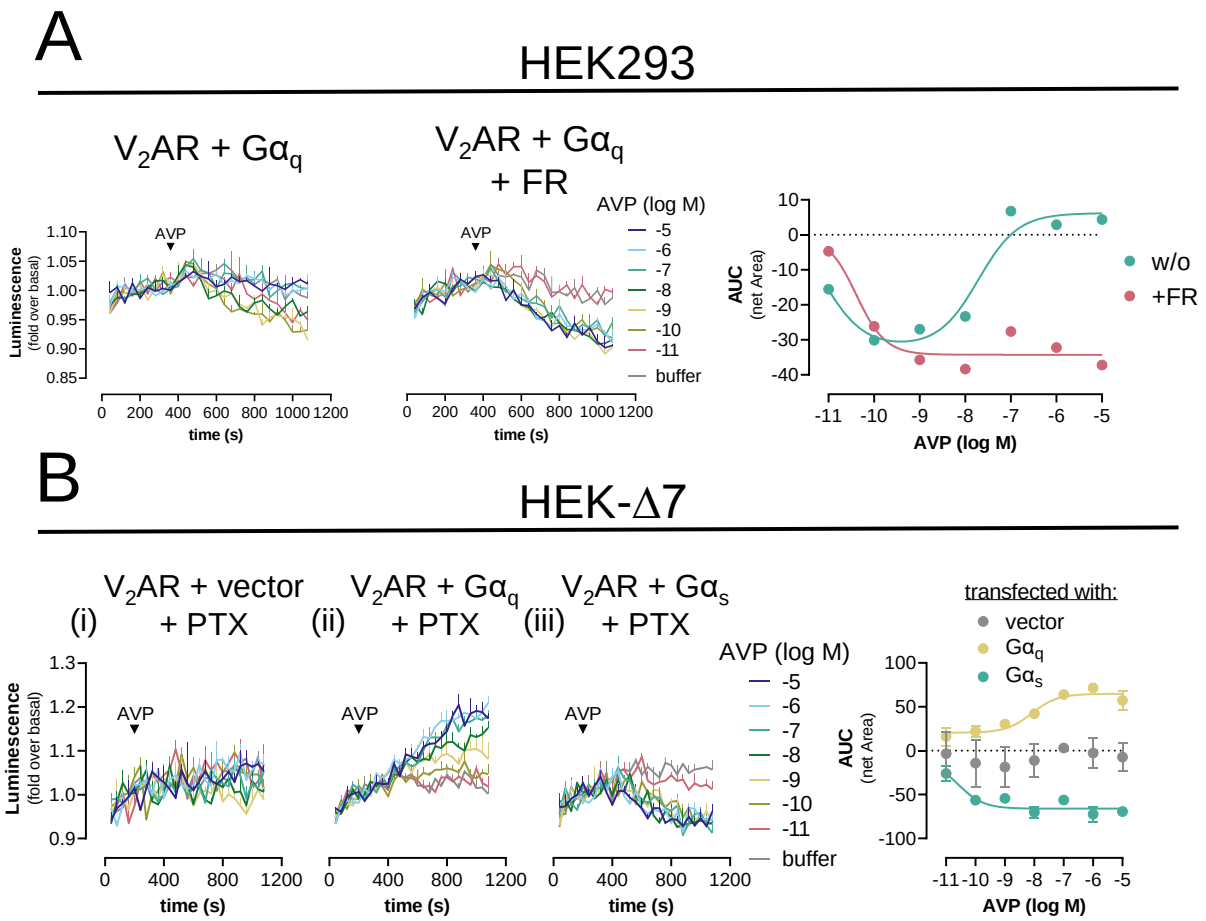

Figure 27. Separation and/or binding of Gβγ to PLCβ3 in a G alpha subunit dependent manner. (A) Representative kinetics + SEM and quantification as averaged duplicates of HEK293 cells transfected with V_2AR, $G\alpha_q$, SmBiT-Gγ2, LgBiT-PLCβ3 and Gβ1 in the absence or presence of 1 µM FR. While FR treated data represents Gs-βγ-PLCβ3 separation, omission of FR represents a mixed signal of Gs-βγ separation and Gq-βγ binding. ($B_{(i\text{-}iii)}$) HEK-Δ7 cells lacking all G alpha subunits except Gi/o were transfected with vector, $G\alpha_q$ or $G\alpha_s$ alongside V_2AR, SmBiT-Gγ2, LgBiT-PLCβ3 and Gβ1 and stimulated as indicated. To exclude the contribution of Gi/o, assays were performed in the presence of 0,1 ng/ml PTX. Expression of the G alpha subunits alone allows unambiguous attribution of specific G alpha-mediated βγ effects. Shown are exemplary Nanobit traces as mean + SEM and quantified as mean or mean ± SEM after averaging two duplicated measures (data generated by S. Bravo, Master thesis 2021).

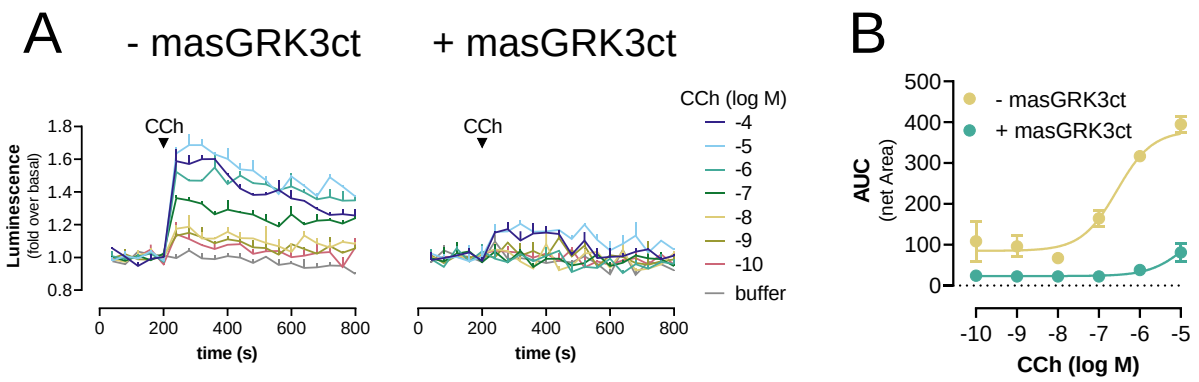

Figure 28. Gq-βγ-PLCβ3 interaction successfully inhibited by masGRK3ct. (A) Representative kinetics as mean + SEM recorded in HEK293 cells expressing M3R, $G\alpha_q$, SmBiT-Gγ2, LgBiT-PLCβ3 and Gβ1. Furthermore, either vector or Gβγ scavenger masGRK3ct was introduced into the cells. Introduction of masGRK3ct blocked Gq-βγ-PLCβ3 binding. (B) Summary of data shown in A as mean ± SEM of three independent experiments, each performed in duplicates (data generated by S. Bravo, Master thesis 2021).

Gq-βγ-PLCβ3 interaction successfully inhibited by masGRK3ct

It became clear that the Nanobit assay was ineffective in detecting the Gs-βγ-PLCβ3 interaction but effective in detecting the Gq-βγ-PLCβ3 interaction. Therefore, we attempted to abrogate Gq-βγ-PLCβ3 binding by applying the Gβγ scavenger masGRK3ct. Demonstration of the scavenging properties of masGRK3ct on the Gq-βγ-PLCβ3 interaction would help substantiate specific Gβγ inhibition effects on Gs-βγ in the calcium assay setup. Moreover, it was interesting to anticipate a possible Gq-βγ-PLCβ3-Ca^{2+} involvement alongside the conventional $G\alpha_q$-PLCβ3-Ca^{2+} paradigm based on the binding data, thus creating a broader acceptance of Gβγ-mediated Ca^{2+} release mechanisms. This time HEK293 cells were transfected with the M3 receptor next to $G\alpha_q$, SmBiT-Gγ2, LgBiT-PLCβ3 and Gβ1. Stimulation of M3 with different concentrations of its cognate agonist carbachol resulted in a concentration-dependent increase in luminescence, which almost completely disappeared when masGRK3ct was also expressed (Figure 28A, B). Thus, it appears that masGRK3ct is a perfect inhibitor of Gβγ-dependent signaling, because in the presence of masGRK3ct, Gq-βγ failed to interact with PLCβ3 and Iso-mediated calcium was strongly inhibited. Nevertheless, these results support the hypothesis of a Gq-derived βγ calcium, akin to what we postulate for Gs-βγ, because priming with a Gq stimuli should also enable Gq-βγ to activate PLCβ enzymes. The supporting nature of Gq-βγ-Ca^{2+} in addition to $G\alpha_q$-Ca^{2+} seems to be logical since calcium spikes and oscillations with their rapid in-/decrease are of particular importance for proper physiological functions. Overall, Gβγ subunits appear to support calcium mobilization in cells, at least for Gq- and Gi-coupled receptors, and further studies are needed to determine whether Gs-GPCRs also contribute.

6. Sole regulation of intracellular calcium levels by Gs-GPCRs

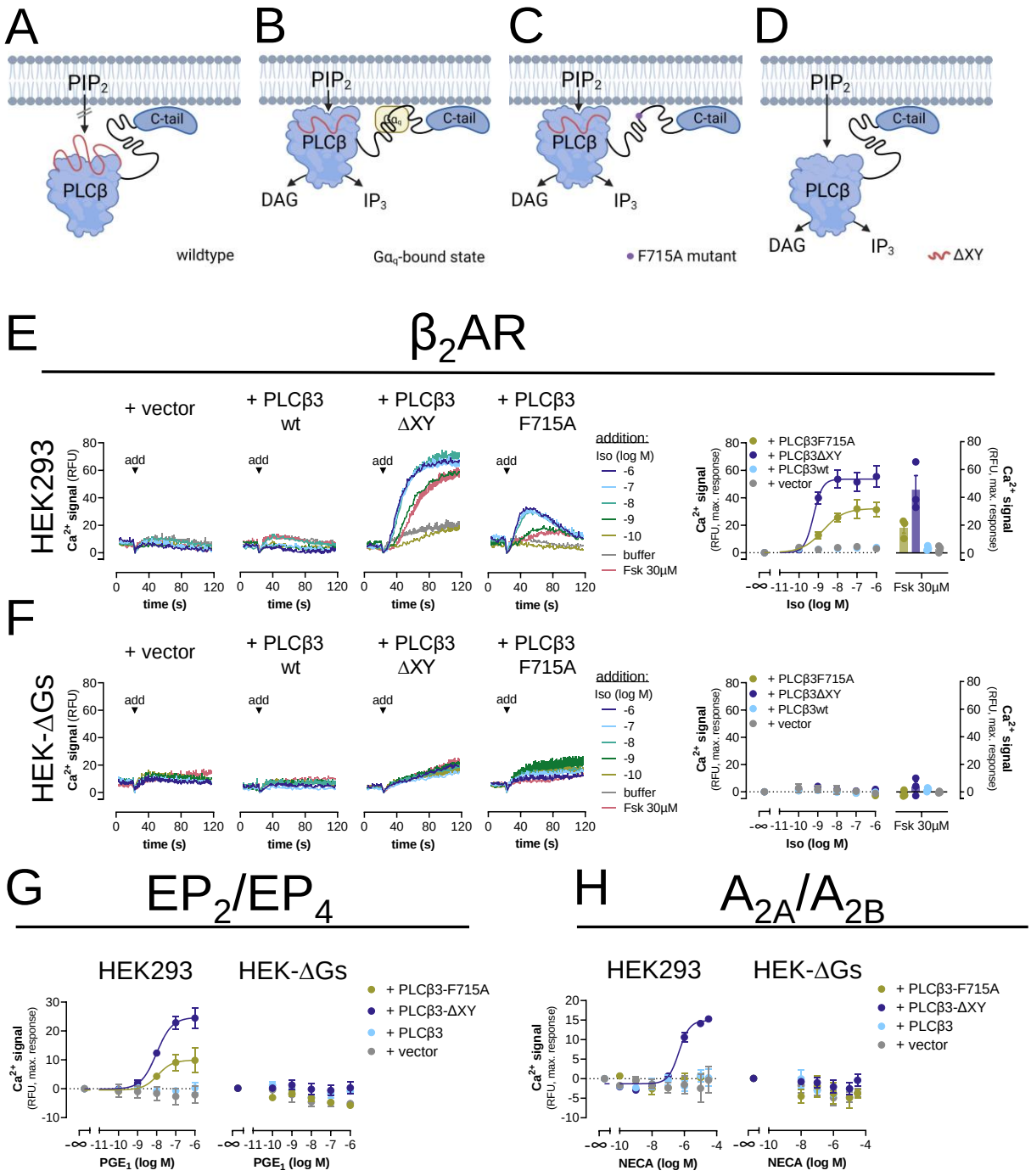

Figure 29. PLCβ3 constructs with impaired autoinhibition no longer require active Gq for Gs-βγ-mediated Ca^{2+}. Phospholipase Cβ3 wildtype (PLCβ3wt) contains an autoinhibitory domain called the XY linker (red), which closes the catalytic site and protects the phospholipid membrane from being cleaved by the enzyme. In addition, the HTH motif in the vicinity of the proximal C-terminal domain also contributes to the autoinhibition by blocking the access to the membrane. These two structural domains reduce the basal activity of PLCβ3 to almost no activity (Charpentier et al., 2014; Hicks et al., 2008; Lyon et al., 2014; Lyon et al., 2011). (B) Activation of the Gq-GPCR canonically leads to binding of $G\alpha_q$ (yellow) near the HTH motif.

The accompanying conformational change rearranges PLCβ3 at the membrane, leading to repulsion of the acidic region of the XY linker from the negatively charged phospholipids and opening of the catalytic side of the enzyme. (C) The PLCβ3F715A mutant contains a single point mutation in the HTH region (purple), making it more flexible and allowing the enzyme to approach the membrane with the XY linker repelled. (D) The PLCβ3ΔXY mutant does not contain a capped catalytic site due to the absence of the XY linker. Basal activity is more dominantly characterized by deleting the XY linker compared to the single-point mutated variant. (E-H) Either HEK293 or HEK-ΔGs cells were transfected with vector, PLCβ3wt, PLCβ3F715A or PLCβ3ΔXY variant and stimulated with different concentrations of Iso, PGE_1, NECA and a saturating concentration of Fsk. (E, F) Shown are the representative calcium traces as mean + SEM and quantification as mean ± SEM. (G, H) represents the quantification of PGE_1- and NECA-treated cells that were incubated with PTX to exclude any Gi/o contribution. All experiments were independently performed for three times and in duplicates.

PLCβ3 constructs with impaired autoinhibition no longer require active Gq for Gs-βγ-mediated Ca^{2+}

A Gs-GPCR mediated calcium increase can only be observed when Gq is preactivated. However, we hypothesize that even in the absence of Gq priming, the activation of Gs-GPCRs results in the binding of Gs-βγ to PLCβ2 and 3. The activation of PLCβ3 by Gs-βγ can only occur after binding of active $G\alpha_q$ to the helix-turn-helix (HTH) domain, resulting in relocating of the enzyme to the plasma membrane and concomitant removal of the XY linker, exposing the catalytic site. The conformational change is accompanied by an increase in PIP_2 hydrolysis which in turn is enhanced by Gβγ subunits. Disruption of the Gq-binding domain of the HTH region by single point mutation (PLCβ3F715A) or genetic deletion of the XY linker (PLCβ3ΔXY) also exposes the catalytic site of the enzyme without requiring active $G\alpha_q$ (Charpentier et al., 2014; Lyon et al., 2011). As a result of these functional changes, the constitutive activity of the PLCβ3 constructs increased (Hicks et al., 2008; Lyon et al., 2011). We postulate that expression of either PLCβ3F715A or PLCβ3ΔXY in HEK293 would allow Gs-βγ calcium without heterologous Gq priming. All cells were FR pretreated to block basal Gq and then stimulated with β_2AR (Figure 29E), EP_2/EP_4 (Figure 29G), and A_{2A}/A_{2B} (Figure 29H) cognate agonists. Despite comparable protein expression of PLCβ3wt, PLCβ3F715A and PLCβ3ΔXY (Figure 30A, B) only the autoinhibitory impaired constructs granted Gs-GPCR calcium without Gq priming in HEK293. Furthermore, Fsk-mediated a robust calcium signal in cells transfected with PLCβ3ΔXY but only a negligible signal in cells transfected with PLCβ3F715A. Differences in effect of Fsk could be due to strong constitutive activity of PLCβ3ΔXY and weak constitutive activity of PLCβ3F715A. Therefore, Fsk-induced calcium mobilization was stronger upon PLCβ3ΔXY expression compared to PLCβ3F715A expression, as the putative cAMP-$InsP_3R$ sensitization depends on the amount of IP_3 produced (Tovey et al., 2008). To verify the sole Gs contribution for the detected calcium by the mutants, we repeated the assays by using

HEK-ΔGs. While cell viability was not affected, the absence of Gs proteins abolished all calcium peaks for each transfectant (Figure 29F-H). It is noteworthy that the slight increase in fluorescence over time for both constructs in HEK-ΔGs could be attributed to their constitutive activity (Charpentier et al., 2014). In summary, the data from the PLCβ3 mutants once again support the Gs-βγ-PLCβ module as a driver of intracellular calcium.

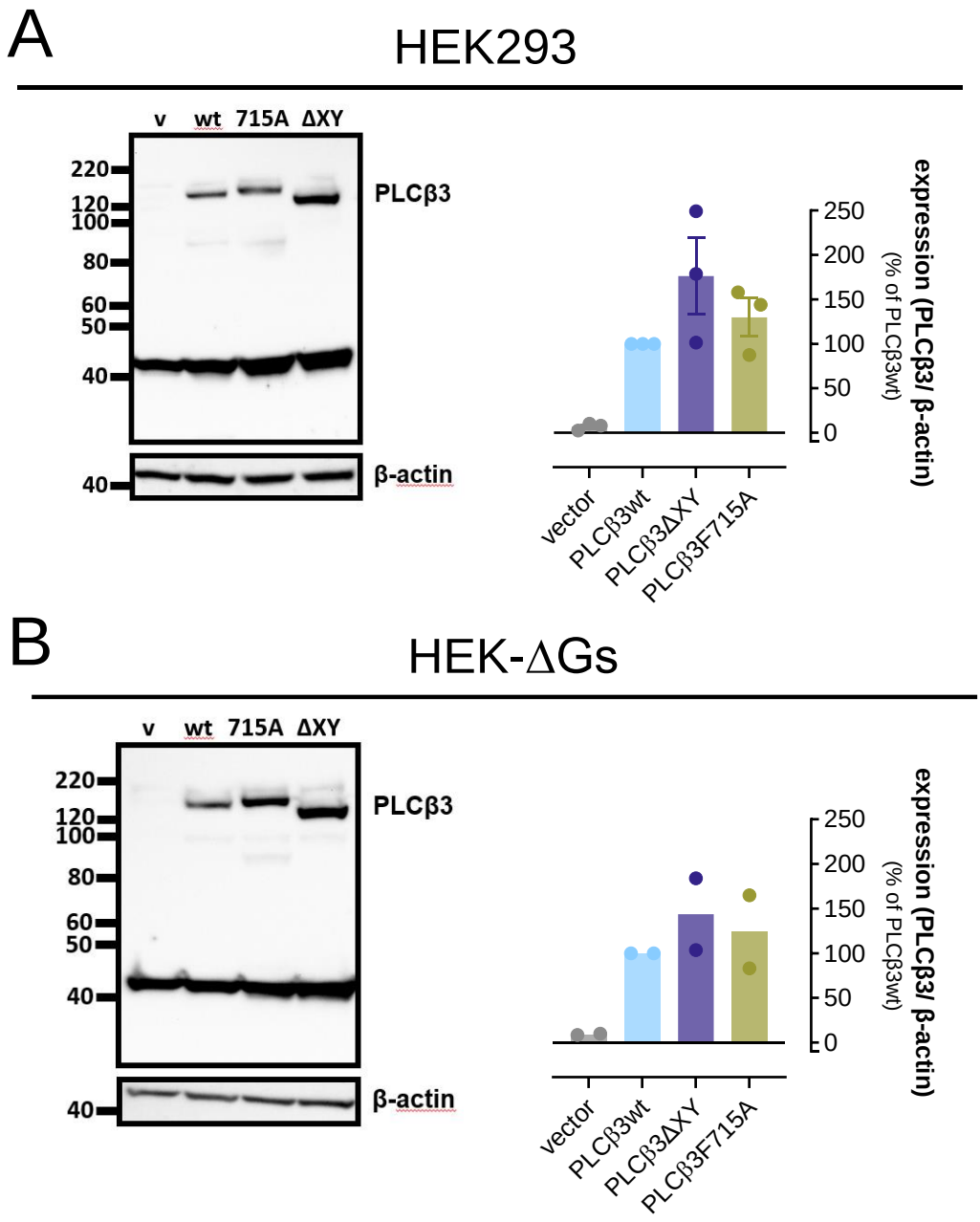

Figure 30. PLCβ3 mutants are expressed at similar levels in HEK293 and HEK-ΔGs. (A, B) Representative Western blot analysis showing comparable expression of PLCβ3wt, PLCβ3F715A and PLCβ3ΔXY in cell lysates obtained from calcium assays in (Figure 29E-H). β-Actin was used as a loading control. Molecular weight is given in kDa (kiloDalton). The quantification of the Western blots performed is shown in the right panels, with three replicates for the HEK293 cells and two replicates for the HEK-ΔGs cells. For HEK293 the mean is shown ± SEM, whereas HEK-ΔGs represent the mean.

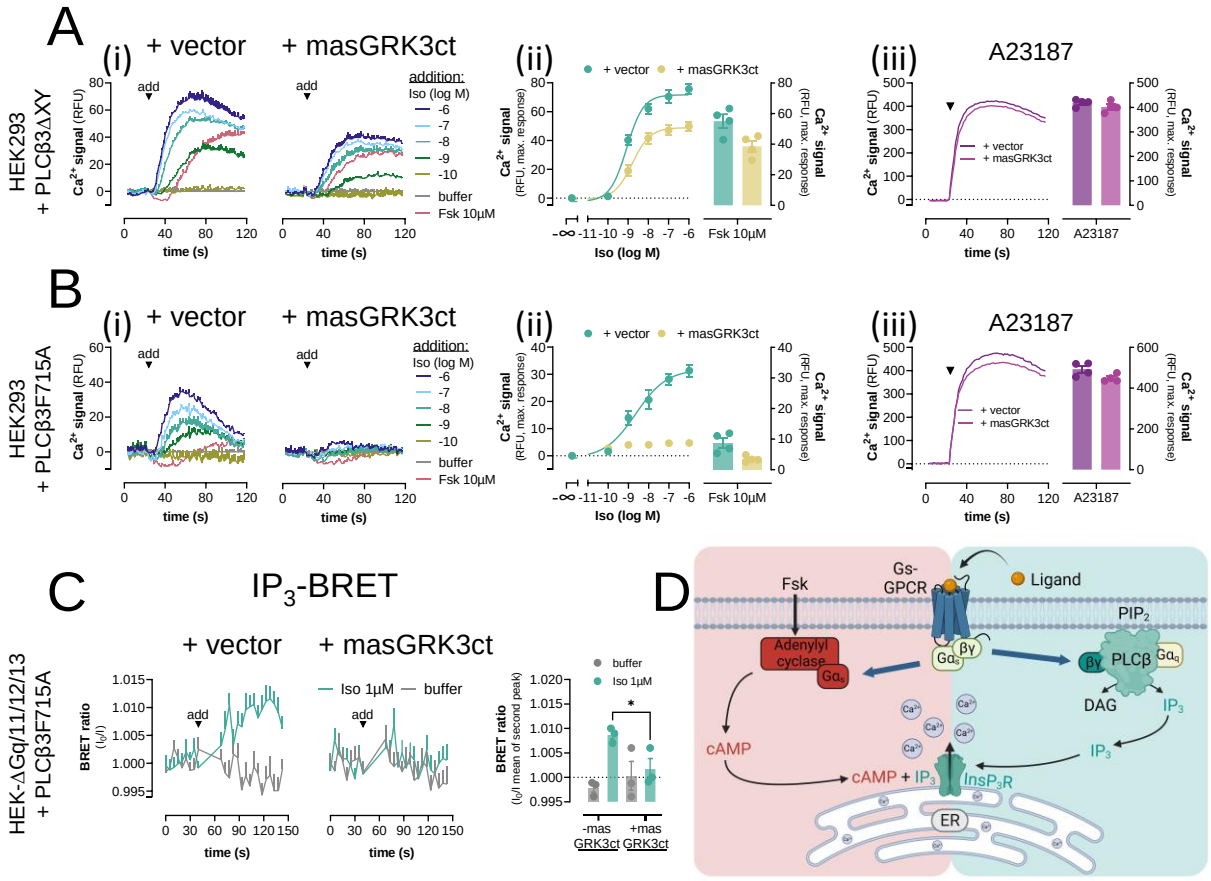

Figure 31. Gs-βγ activates PLCβ-mediated calcium. (A, B) HEK293 cells transfected with either PLCβ3ΔXY or PLCβF715A in the presence or absence of masGRK3ct. ($A_{(i)}$, $B_{(i)}$) Representative calcium kinetics as mean + SEM of cells stimulated with Iso or Fsk. ($A_{(ii)}$, $B_{(ii)}$) Quantification of the data shown in $A_{(i)}$ and $B_{(i)}$ as the mean ± SEM of four independent performed experiments. ($A_{(iii)}$, $B_{(iii)}$) Representative kinetics as mean + SEM and quantification as mean ± SEM of 5 µM calcium ionophore A23187 indicating intact cell viability. Cells in A, B were treated with 1 µM FR before the measurement to exclude contribution from Gq-Ca^{2+}. (C) Representative IP_3 real-time traces and the summary of averaged signals in HEK-ΔGq/11/12/13 cells, transfected with IP_3-BRET sensor, PLCβF715A and vector or masGRK3ct upon addition of Iso or buffer. MasGRK3ct abolished both IP_3 and calcium, confirming that the Gs-βγ-PLCβ3 calcium module contributes to the regulation of cytosolic calcium. Statistical significance was determined using a paired t-test, with *p< 0.05. (D) Gs-GPCR act on the cytosolic calcium level via its $Gα_s$ (red) and Gs-βγ subunit (green).

Gs-βγ activates PLCβ-mediated calcium

So far, we have shown that the calcium response of Gs is mediated by a cAMP-dependent part, which we hypothesize to be Gs-cAMP-$InsP_3R$ sensitization (Konieczny et al., 2017; C. W. Taylor, 2017), and a cAMP-independent part mediated via Gs-βγ-PLCβ activation. The detection of the unknown Gs-βγ-PLCβ-mediated calcium has been difficult to study due to the confounding effect of the cAMP-dependent Ca^{2+}. Our calcium readout has always shown two

intermixed signaling pathways, both of which are dependent on Gq activation. By transfecting masGRK3ct alongside PLCβ3ΔXY in HEK293, Iso-mediated calcium was reduced by approximately 50%. We attributed the remaining calcium response to the strong constitutive activity of PLCβ3ΔXY and the associated $InsP_3R$ sensitization. This assumption was supported by using Fsk to demonstrate the cAMP-dependent calcium portion (Figure $31A_{(i-ii)}$). The apparent similarity in shape and amplitude of the maximal Iso-concentrations to those of the supramaximal Fsk concentration indicates a complete trapping property for the Gs-βγ calcium by masGRK3ct. The lower constitutively active construct PLCβ3F715A mediated an almost undetectable increase in calcium upon Fsk addition, whereas Iso-mediated a concentration-dependent response that was completely abolished in the presence of masGRK3ct (Figure **31**$B_{(i-ii)}$). Notably, cell viability was not affected by the expression of PLCβ3 constructs and masGRK3ct (Figure 31$A_{(iii)}$, $B_{(iii)}$). All our results strongly suggest that activation of Gs-GPCRs after heterologous Gq priming triggers calcium release mediated partly by the Gs-βγ-PLCβ module. Because the measurement of calcium levels is not suitable for detecting Gs-βγ effects alone, we returned to the IP_3 real-time BRET assay. The IP_3 real-time BRET assay allowed us to study Gs-βγ effects in isolation, since intracellular increases in cAMP should not affect IP_3 generation by PLCβ. This time we used HEK-ΔGq/11/12/13 cells transfected with the IP_3-BRET sensor, PLCβF715A, vector or masGRK3ct. Upon Iso-stimulation, an increase in intracellular IP_3 was detected, whereas introduction of masGRK3ct abolished IP_3 formation (Figure 31C). These IP_3-BRET data confirmed Gs-βγ as a major driver of PLCβ-IP_3 formation, resulting in a cytosolic calcium increase due to activation of $InsP_3R$ channels on the ER. Thus, it appears that Gs-GPCRs influence cytosolic calcium levels not only via $G\alpha_s$, but also via the newly discovered Gs-βγ-PLCβ3-Ca^{2+} module (Figure 31D).

Discussion

The Gs-βγ-PLCβ calcium module

Calcium signaling pathways have become a prominent area of research due to their pervasive influence on nearly all facets of cellular life. Hence, cells possess a diverse array of calcium signaling machinery. One of these is the large family of G protein-coupled receptors, which cause calcium release by activating PLCβ enzymes via their $G\alpha_q$ or Gβγ moiety (Boyer et al., 1992; Camps et al., 1992; A. V. Smrcka et al., 1991; S. J. Taylor et al., 1991; G. L. Waldo et al., 1991). Although the activation of PLCβ by $G\alpha_q$ is well characterized and understood, there are still several unanswered questions regarding Gβγ-dependent activation. Among these questions, the origin of Gβγ is particularly intriguing and warrants further investigation. To date, it is believed that heterotrimeric Gi proteins are primarily responsible for Gβγ-mediated effects, including Gβγ-dependent PLCβ activation (Boyer et al., 1992; Kadamur & Ross, 2013; A. V. Smrcka, 2008). Our study has now revealed Gβγ derived from Gs-GPCR to activate PLCβ, and we have elucidated the mechanism underlying this activation. It appears that Gs-βγ-PLCβ-Ca^{2+} is only visible with prior or simultaneous activation of Gq proteins, as we have observed for Gi-βγ-PLCβ-Ca^{2+} (Pfeil et al., 2020). At the molecular level, Gβγ alone is not sufficient to overcome the strong autoinhibition of PLCβ enzymes. Overcoming autoinhibition can be achieved either by binding $G\alpha_q$ to PLCβ (canonical) or by mutating the autoinhibitory domains of the enzyme (Charpentier et al., 2014; Gresset et al., 2012; Lyon et al., 2014; Lyon & Tesmer, 2013). These findings are not restricted to recombinant HEK293 cell system as it also appears in a more physiological setting using mBA and MEF cells. Thus, our study sheds new light on the precise mechanism of Gs-βγ-mediated calcium release and reveals the intricate crosstalk interactions between Gs- and Gq-coupled receptors. This highlights the role of Gs-GPCRs as efficient modulators fine-tuning calcium homeostasis.

Gs-GPCRs calcium release

Gs-GPCRs are known to affect intracellular calcium levels through a variety of signaling pathways. cAMP, the main second messenger of Gs-GPCRs, is involved in almost all of them. This second messenger triggers the activation of its key effectors, PKA and EPAC, and subsequently initiates the associated signaling cascade, which has been the subject of numerous studies (Cheng et al., 2008; Pereira et al., 2007; Reiken et al., 2003). Our investigations have shown that the observed Gs-calcium response is not due to either of these two key effectors, as treatment with the respective inhibitors had barely any effect on the observed calcium levels. The inhibition of PKA even increased Ca^{2+} mobilization. This is consistent with some studies postulating an inhibitory effect of PKA on PLCβ (Yue et al., 1998).

It should be noted that Ca^{2+} regulation can show strong cell- and tissue-specific differences. In contrast to cardiomyocytes, non-excitable HEK293 cells used in our study are not equipped with L-type Ca^{2+} channels (Pérez-García et al., 1995; Zong et al., 1995), which can be phosphorylated by PKA, leading to the entry of extracellular Ca^{2+} into the cell (Kamp & Hell, 2000). In addition, an extracellular Ca^{2+} contribution has been excluded by us and others (Pfeil et al., 2020; Short & Taylor, 2000; Werry et al., 2002) as data obtained with the $InsP_3R$ inhibitor APB, heparin or xestospongin (Schaloske et al., 2000; Short & Taylor, 2000; Stallaert et al., 2017) and the extracellular Ca^{2+} chelator BAPTA (Tovey et al., 2008) suggested an intracellular Ca^{2+} origin. There are increasingly many approaches to explain calcium effects solely triggered by PTH activation (Short & Taylor, 2000; Tovey et al., 2003) by increased PKA activity leading to sensitization of $InsP_3Rs$ to IP_3 (Betzenhauser & Yule, 2010; Bird et al., 1993; J. I. E. Bruce et al., 2002; J. I. Bruce et al., 2003; Short & Taylor, 2000; Tovey et al., 2008). Despite these findings, we exclude a PKA-$InsP_3R$ sensitization as well as a general PKA involvement in the Gs-calcium observed by our group, as PKA inhibition enhanced the Ca^{2+} response. Rather, we explain Gs-GPCR mediated calcium peaks in the absence of heterologous Gq priming with promiscuous Gq coupling and activation (Rooney et al., 1991; Wenzel-Seifert & Seifert, 2000; Wu et al., 1995), as shown by our data overexpressing Gs-GPCRs (Figure 9). Although EPAC may increase intracellular calcium levels, we did not detect any effect on Gs-calcium. It is likely that the cAMP-mediated signaling cascade involving EPAC activation and the concomitant steps to induce a calcium release (Schmidt et al., 2001) need more time than the 200 seconds of our chosen calcium readout. In contrast to Schmidt et al., 2001, Tovey et al., 2008 excluded a contribution of EPAC, as direct activation did not lead to an observable calcium increase. In conclusion, we have excluded the influence of EPAC and PKA on our Gs-calcium.

Consistent with our findings on the requirement for active Gq, several studies report on a Gq-dependent Gs-calcium (Buckley et al., 2001; Konieczny et al., 2017; Kurian et al., 2009; Leaver & Pappone, 2002; Stallaert et al., 2017; Werry et al., 2002). Some of these reports even suggest a calcium signal that is mediated by a cAMP-dependent sensitization of $InsP_3R$ (Konieczny et al., 2017; Kurian et al., 2009; Tovey et al., 2010), which we also demonstrate using Fsk. According to previous studies (Konieczny et al., 2017; Tovey et al., 2010), the calcium pathway involving cAMP-dependent $InsP_3R$ sensitization does not directly induce IP_3 formation (Babich et al., 1991; Seuwen & Boddeke, 1995), which is consistent with our findings. This finding supports the notion that active Gq or constitutively active PLCβ isoforms are required to generate IP_3, which subsequently activates the cAMP-sensitized $InsP_3R$. In contrast to the belief that Gq-dependent Gs-GPCR calcium signal is exclusively mediated by cAMP, recent evidence suggests that multiple pathways are involved (Buckley et al., 2001; Stallaert et al., 2017). Our study shows that the activation of a Gs-GPCR induces the formation of IP_3 after prior activation of a Gq-coupled receptor. Notably, the introduction of a Gβγ

scavenger abolished this Gs-mediated IP_3 formation as well as a subsequent calcium release through the Gs-βγ-PLCβ module. Consistent with these studies, our results provide evidence that the Gs-cAMP-$InsP_3R$ sensitization calcium pathway and the recently discovered Gs-βγ-PLCβ calcium pathway are distinct but overlapping mechanisms involved in Gq-dependent Gs-GPCR calcium signaling.

Gβγ-dependent activation of PLCβ

The newly discovered Gs-βγ-PLCβ activation is contrary to conventional wisdom, which has long assumed that PLCβ is activated exclusively by Gi-GPCRs (Camps et al., 1992; Cowen et al., 1990; A. V. Smrcka & Sternweis, 1993; Sternweis & Smrcka, 1992). This assumption was based on the fact that Gi/o are the most abundant endogenous cellularly expressed G proteins, and thus the only G protein family member able to provide the high levels of Gβγ needed to activate the PLCβ enzymes (Boyer et al., 1992; Camps et al., 1992; Kadamur & Ross, 2013; A. V. Smrcka, 2008; A. V. Smrcka & Sternweis, 1993). However, transcriptomic analysis of HEK293 cells, which is our usual cell line, revealed comparable expression levels of Gi as Gs or Gq according to Atwood et al., 2011. Therefore, it is important to note that HEK293 cells may not be suitable to mimic physiological relevant processes. Despite the comparable expression in HEK293 cells, we also observed Gs-calcium in different cell lines, such as mBA and MEF cells representing a cell line with more physiological relevance. And indeed, it remains to be noted that without examining the expression levels, the attribution of the Gβγ mediator cannot be unambiguously established, as cell- and tissue-specific expression of the GPCR is relevant.

The lower affinity of activated GTP-$G\alpha_i$ for its Gβγ subunit, and thus the robust $G\alpha_i$-βγ dissociation compared to the other members of the G protein family, was another finding that led to the hypothesis that Gi-GPCRs are the only mediators of Gβγ (Chung & Wong, 2020; Masuho et al., 2021; Touhara & MacKinnon, 2018). Furthermore, several studies have shown that Gβγ-specific effects are effectively abolished by the Gi/o inhibitor PTX (Camps et al., 1992; Cowen et al., 1990; Moriarty et al., 1989; Okajima & Ui, 1984). Our data now reveal the Gq-specific binding of Gβ1γ2 to PLCβ3, but do not provide any evidence for the binding of Gs-Gβ1γ2 to PLCβ3. Based on these data, it must be assumed that other members of the G protein family next to Gi are also capable of activating PLCβ through its Gβγ subunit. The lack of detection of the Gs-βγ-PLCβ interaction calls into question the suitability of the Nanobit method to detect the protein-protein interaction by Gs, since Gq-βγ-PLCβ interaction was detectable, and our calcium data indicate the presence of a Gs-βγ-PLCβ-Ca^{2+} release. In general, because all signaling components are constantly overexpressed and thus do not represent an endogenous basis, a critical approach must be taken when interpreting Nanobit

data. It is also worth mentioning that other G proteins may bind different Gβγ subunits and thus activate different Gβγ effectors, as up to 60 Gβγ combinations are possible (A. V. Smrcka, 2008). Up to now, it is not known whether there are specific pairs of Gα-Gβγ combinations or whether each Gα family only assembles a heterotrimer with specific pairs of Gβγs (A. V. Smrcka, 2008).

Despite the assumption of an exclusive activation of PLCβ by Gi-βγ, a contribution of other G proteins could never be completely excluded (Jiménez et al., 1999; Werry et al., 2002; Zeng et al., 1996; Zhu & Birnbaumer, 1996). This leads to the question why the observed Gs-βγ-PLCβ calcium could not be identified until now. Since Gs-βγ-PLCβ-Ca^{2+} signaling requires active Gq (Gq priming), it was difficult to establish a direct link between activation of Gs-GPCRs and calcium release as no calcium was emitted in the absence of Gq. In addition, the lack of a Gs inhibitor made it difficult to attribute Gβγ-PLCβ-Ca^{2+}, or even calcium in general, to Gs. Gs-GPCRs can trigger calcium release through different pathways, leading to an overlap in calcium signaling (Kamp & Hell, 2000; Konieczny et al., 2017; Schmidt et al., 2001). Although Gs-βγ is known to bind and activate its effectors, observing Gs-derived Gβγ effects has proven challenging. It is generally accepted that Gβγ has a strong affinity for $G\alpha_s$ and therefore only stays in contact with its activated effector for a short time. Furthermore, a limited dissociation of the heterotrimer might also be reasonable (Digby et al., 2008; Leaney & Tinker, 2000; Masuho et al., 2021; Wellner-Kienitz et al., 2001). Considering these points, it seems almost obvious why the study of Gs-βγ-PLCβ calcium has been so difficult and suggests why Gi-βγ has long been considered the only Gβγ-PLCβ activator. With the availability of different cells lines i) HEK-ΔGs ii) HEK-ΔPLCβ1-4 mut. iii) HEK-ΔAcKO and the finding of the Gq requirement we could retrieve the Gs-βγ-PLCβ-Ca^{2+} module, despite the lacking Gs inhibitor. Our findings are consistent with recent studies suggesting crosstalk between Gq and Gs receptors, as well as a potential cAMP-independent Gs-calcium requiring Gq priming (Galaz-Montoya et al., 2017; Jiménez et al., 1999; Stallaert et al., 2017; Werry et al., 2002). Crosstalk interactions and underlying signaling mechanisms appear highly diverse, and precise mechanisms are difficult to define. We propose a crosstalk between Gq-GPCR and Gβγ subunits transcending their respective Gα-families, manifested by enhanced calcium mobilization, drawing from the similarity of Gi- next to the Gs-βγ-PLCβ pathway (Pfeil et al., 2020). Considering physiological aspects related to the complexity of spatiotemporal calcium regulation, crosstalk interactions between Gq-coupled GPCRs and other GPCRs such as Gi and Gs seem to be essential, as both are contradictory but together elicit a rapid response when calcium concentrations need to be upregulated (Gq activation).

Struggling to visualize Gs-βγ-PLCβ interaction

The interaction between Gi-βγ subunits and PLCβ3 has been well documented in studies (Camps et al., 1992; A. V. Smrcka & Sternweis, 1993; Sternweis & Smrcka, 1992), and our group (Pfeil et al., 2020) was able to demonstrate it using the Nanobit method. This interaction is a critical step in a Gi-βγ-mediated calcium release from the ER. Since both events Gi-Ca^{2+} and Gs-Ca^{2+} share its Gq-dependence, we hypothesized that this interaction is also relevant for Gs-βγ-mediated calcium.

However, despite our best efforts, we were unable to visualize the binding of Gs-βγ-PLCβ3 using the Nanobit method. Regardless of the cellular system used (HEK293, HEK-Δ7), the luminescence consistently decreased after stimulation of the Gs-GPCR in the presence of overexpressed $G\alpha_s$. This contrasts with our expected binding of Gs-βγ to PLCβ3 as observed for Gi-GPCRs. The decrease in luminescence over time indicates a progressive spatial separation of Gs-βγ and PLCβ3. We hypothesize that after a brief interaction of Gs-βγ with PLCβ3, Gs-βγ rapidly reassociates with GDP- and even GTP-bound $G\alpha_s$. This would hint towards a high affinity of Gs-βγ for its Gs alpha subunit, as reported in the literature (Galés et al., 2006). Yet, this phenomenon may not be the only explanation for our observation. An alternative possibility is the incomplete dissociation of the Gs-βγ subunit from $G\alpha_s$. This is supported by studies reporting that the classical dissociation model may not be applicable to all G proteins (Chung & Wong, 2020). Thus, the $G\alpha_s$-Gβγ complex may not bind to PLCβ3 due to spatial hindrance and inability of the Gβγ-PLCβ3 binding site. Of particular interest are recent findings by Inoue et al., 2019, who observed a $G\alpha_s$-βγ dissociation lasting at least 10 minutes, indicating $G\alpha_s$-Gβγ separation and thus contradicting this hypothesis. The strong affinity between Gβγ and $G\alpha_s$, along with the challenges associated with heterotrimer dissociation, may explain the difficulty in observing Gβγ-mediated effects derived from Gs, which has also been reported in the literature (Digby et al., 2008; Leaney & Tinker, 2000; Masuho et al., 2021; Wellner-Kienitz et al., 2001).

Despite the lack of a Gs-βγ-PLCβ3 interaction, we detected an interaction between Gq-βγ and PLCβ3 that was abrogated by introduction of masGRK3ct. This suggests the involvement of Gq-βγ-Ca^{2+} in Gq-mediated calcium release. Once more, it appears that while other G protein families such as Gi and Gq allow Gβγ-PLCβ3 Nanobit binding, the introduction of $G\alpha_s$ into our assay setup renders the Gβγ-PLCβ3 interaction undetectable. Taken together, we conclude that the Nanobit assay is not suitable to detect Gs-βγ-PLCβ3 interaction and we can only speculate about the reasons. Although we lack important evidence for our postulated Gs-βγ-PLCβ-Ca^{2+} module, we are confident that this module contributes to Gs-mediated calcium release. Just as we struggled to provide this significant evidence, this may be one of the reasons why Gs-βγ-mediated calcium release has long been disregarded or overlooked.

What can we learn from these results and what might be future approaches to the visualization of Gs-βγ interaction? i) Enzyme formation and substrate oxidation of the Nanobit technique outlast the Gs-βγ-PLCβ3 complex and therefore do not seem suitable for detecting it. Therefore, exploring alternative detection methods with faster onset kinetics, such as FRET or BRET, would be a logical approach for future visualization of this interaction. Nevertheless, it is worth noting that currently, no appropriate FRET or BRET sensors have been known to fully unravel this interaction. Overall, single-molecule imaging provides another way to study dynamic molecular interactions, in a more native environment. Real-time detection of GPCR di-/oligomerization (Calebiro et al., 2013) or GPCR-G protein interactions (Sungkaworn et al., 2017) was achieved using fluorophore-labeled proteins and TIRF microscopy, providing a potential method to detect Gβγ-PLCβ3 interaction. Of note, TIRF microscopy is limited to near-surface regions in a range ≤100 nm, since the evanescent electromagnetic field decays exponentially in its intensity to the distance of the surface and thus cannot excite fluorophores further away. Consequently, only interactions within this range will be detectable (Axelrod, 2003; Mattheyses et al., 2010). ii) Efforts to increase the lifetime of the Gs-βγ-PLCβ3 complex could be considered in addition to changing the detection method. One reason for the lack of detectability may be the high affinity of Gβγ for $G\alpha_s$, both in the GDP- and GTP-bound states (Digby et al., 2008). Hence, reducing the affinity of Gβγ to $G\alpha_s$ and prolonging the Gs-βγ-PLCβ3 interaction seems to be a viable approach to facilitate the detection of Gs-βγ-PLCβ3 complex. For this purpose, the use of macrocyclic peptides that antagonize the active or inactive state of $G\alpha_s$ (Dai et al., 2022) could be an elegant choice. It would be interesting to investigate whether inhibiting $G\alpha_s$ would allow even the Nanobit to detect Gs-βγ-PLCβ3 binding. Consequently, either by modifying the detection method or by manipulating the kinetics in reducing the affinity to $G\alpha_s$, it may be possible to make Gs-βγ-PLCβ3 detectable.

Gq affecting Gs- and Gi-GPCR outcome

Signal transduction is carefully regulated to provide appropriate and accurate responses to complex and constantly changing situations. The canonical signaling pathway of Gs-GPCRs involves the stimulation of the adenylyl cyclases via $G\alpha_s$, resulting in an increase in cellular cAMP levels. Conversely, Gi-GPCRs inhibit this process. Consequently, Gs and Gi exert opposing roles in modulating cellular outcomes. Therefore, the Gβγ signaling pathway that unites Gs and Gi in a common cellular outcome, the mobilization of calcium, must depend on an additional factor, the activation of Gq.

In physiological contexts, the necessary involvement of Gq can be achieved through a variety of different cellular mechanistic scenarios. As we have shown by overexpressing β_1AR, β_2AR

or DP1 (Figure 9) i) the upregulation of Gs-GPCRs can lead to coupling to Gq proteins thus facilitating Gs-βγ calcium mobilization. This phenomenon is not exclusively restricted to Gs-GPCRs as receptor overexpression can lead to promiscuous coupling (Dittman et al., 1994; Eason et al., 1992). ii) Viral infection or oncogenic mutations can lead to constitutive active Gq proteins enabling Gs-βγ calcium without Gq pre-stimulation (Bakker et al., 2004; van Raamsdonk et al., 2009; Vischer et al., 2006). (iii) Furthermore, hetero- or oligomerization of GPCRs can lead to intertwined signaling cascades that influence each other, such as the modulation of Gs-βγ calcium release (Werry et al., 2003). vi) An alternative scenario involves the localization of Gs- or Gi-GPCRs in close proximity to Gq-effector signaling nodes such as the Gq-PLCβ signaling complex (Harden et al., 2011). This compartmentalization, which is especially possible in developmental stages or diseases, could cause Gβγ derived from Gs/Gi to release calcium (Cordeaux & Hill, 2002).

It seems evident that Gq plays a pivotal role in enabling calcium release via Gβγ derived from both Gs- and Gi-GPCRs. This mechanism has significant physiological implications, as the cellular acquisition of Gs- and Gi-GPCRs and their subsequent participation in calcium release may facilitate a faster and enhanced functional response. The extensive crosstalk between Gq and Gs/Gi (Cordeaux & Hill, 2002) resulting in enhanced calcium signaling, is observed ubiquitously due to the widespread expression of GPCRs in various cell types. This interplay enhances the versatility of cellular functions, making them even more diverse.

GPCRs-mediated calcium in pathophysiology and disease

Dysregulation of GPCR-mediated calcium release contributes to many diseases such as chronic heart failure, neurodegeneration, respiratory diseases, and cancer (Campbell & Smrcka, 2018; Dhyani et al., 2020; Janssen et al., 2015; Koch et al., 1995; Pchitskaya et al., 2018). It is not surprising that disrupting the precise spatiotemporal regulation of calcium release can be detrimental. To develop pharmacological agents targeting GPCRs or their downstream signaling events to restore Ca^{2+} homeostasis, it is necessary to identify and understand the underlying pathways affecting Ca^{2+} dynamics. Besides the well-established $G\alpha_q$, $G\alpha_i$, and $G\alpha_s$ pathways of calcium release (Dhyani et al., 2020; Konieczny et al., 2017; Pfeil et al., 2020) our studies have expanded the spectrum of Gs-mediated calcium release with the discovery of the Gs-βγ-PLCβ-Ca^{2+} module. More specifically, this discovery reveals a hierarchical control of active Gq over Gs next to the Gi calcium release pathway (Pfeil et al., 2020) and reveals a novel signaling mechanism depending on Gβγ. Thus, our study provides a mechanistic understanding of how intracellular Ca^{2+} dynamics are regulated and simultaneously exposes $G\alpha_q$ and Gβγ subunits as therapeutic targets affecting Ca^{2+} dysregulation.

Based on the aforementioned findings, an interesting approach could be the treatment of airway epithelial inflammatory processes such as asthma or COPD (Adriaensen & Timmermans, 2004; Basoglu et al., 2005; Idzko et al., 2007; Mortaz et al., 2010). Inhibiting Gq while stimulating Gs in vivo may reduce the vasoconstrictive effect of Ca^{2+} while promoting Gs-cAMP-mediated dilation in airway epithelia. This could potentially result in a shift towards the non-calcium mediated effects of Gs-cAMP, thereby even enhancing the vasodilator responses. Consequently, improved airway function and symptom relief may be achievable with this approach but need to be established. This example, while purely theoretical, illustrates how our new understanding of Gs-βγ calcium facilitates new opportunities for targeted intervention in signaling cascades, offering potential advances in the treatment and prevention of associated diseases.

Environment-dependent biphasicity

We hypothesized that the total Gs-calcium consists of a cAMP-dependent and a cAMP-independent module, both fully sensitive to FR and thus dependent on Gq priming. However, these two modules differ in the mode of Gq action and so in the intensity of Gq input. While a high level of Gq input significantly enhances the cAMP-dependent response due to increased IP_3 production (Konieczny et al., 2017; Tovey et al., 2008) the Gs-βγ-PLCβ-Ca^{2+} module requires significantly less Gq input. This minimal Gq input is necessary only for binding $G\alpha_q$ to PLCβ to abolish autoinhibition, as shown for Gi-βγ-Ca^{2+} (Pfeil et al., 2020). Now, by reducing the intensity of Gq priming by lowering the agonist concentrations, we selectively reduced the cAMP-dependent component to the total Gs-calcium revealing a biphasic curve. Of note, altering the stimulated Gq receptor also directly affected the E_{max} and EC_{50} of the Gs-mediated response (Tovey et al., 2003; Werry et al., 2002). The appearance of the biphasic curve facilitated the introduction of the Gβγ scavenger masGRK3ct to highlight the Gs-βγ component. Nevertheless, the introduction not only reduced the Gs-mediated, but also the Gq-mediated calcium peak. Importantly, Nanobit data demonstrated successful abolition of Gq-βγ-PLCβ3 binding by masGRK3ct. Considering the putative Gq/Gs crosstalk, the question arises as to whether masGRK3ct effectively scavenges Gs-βγ or instead reduces Gq-βγ-mediated PLCβ activity, thus affecting Gs-calcium. This contrasts with the findings of Werry et al., 2002, who postulate that the mechanism of potentiation appears to be independent of the size of the initial Ca^{2+} spike. Besides, our data obtained with the IP_3 sensor indicate a Gs-βγ-mediated but not Fsk-mediated increase in IP_3 after Gq priming. Furthermore, introduction of the PLCβ3F715A mutant in the presence of masGRK3ct abolished Gs-βγ IP_3 formation, indicating a scavenger-specific effect on Gs-βγ calcium. Our data are consistent with findings by Abou-Samra et al., 1992 that Gs receptors can mediate IP_3 formation involving

PLCβ. In contrast to many groups that have not seen a Gs-mediated IP_3 formation (Babich et al., 1991; Seuwen & Boddeke, 1995), which we attribute to the lack of Gq priming conditions.

With the discovery of Gq as a common requirement for calcium signaling by $G\alpha_s$ and Gs-βγ, it became clear that the observed calcium response of Gs involved two overlapping signaling pathways. However, the mixed nature of these signals in the calcium display, combined with the fact that Gβγ effects are exclusively attributed to Gi-GPCRs, made it difficult to consider Gs-βγ as a potential calcium mediator. Nevertheless, a contribution of Gs-βγ to Gs-calcium either by direct IP_3 formation or by regulation of IP_3 sensitization has been considered by some groups (Tovey et al., 2003; Werry et al., 2002). Moreover, the deciphering process seemed difficult because $G\alpha_s$ and Gs-βγ both contribute to intracellular calcium and both rely on the same intracellular target channel $InsP_3R$, although they regulate it differently. The introduction of the Gβγ scavenger masGRK3ct for the selective separation of $G\alpha_s$ and Gs-βγ effects in combination with the detection of the Gs-βγ effect at the level of IP_3 provided valuable information on the possible involvement of Gs-βγ-PLCβ-Ca^{2+}. In conclusion, based on calcium effects always being attributed to cAMP as the major second messenger of $G\alpha_s$, in addition to Gβγ effects being attributed to Gi-GPCR, it was understandable that other groups attributed the calcium effects exclusively to $G\alpha_s$. Thus, with the identification of the missing key element Gq, we have contributed to the unification of recent studies that have assumed pure $G\alpha_s$-calcium effects or have speculated on a Gs-βγ contribution. Studies that have been contradictory in the past now seem to have much more in common than was originally thought.

Conclusion

In conclusion, our study expands our current knowledge about the cooperative role of GPCRs in the regulation of calcium mobilization. By unraveling the molecular mechanism underlying a Gq-dependent Gs-GPCR calcium, we deciphered the versatile role of Gs-GPCRs in calcium processes and highlighted Gq as a prerequisite for Gβγ-PLCβ activation. Despite the lack of Gs-βγ-PLCβ binding data and additional data in primary cells, which require further research, our findings help to harmonize the diverse and sometimes even conflicting studies on Gs-mediated calcium signaling. These new insights into Gs-calcium regulation enlarge our understanding of how intricate cellular crosstalk occurs and pave the way for new avenues of research into potential therapeutic targets associated with calcium dysregulation in a variety of diseases and conditions.

Summary

The interplay of a multitude of mechanisms in the cellular mobilization of the vital divalent ion calcium remains a fascinating mystery. Unraveling these highly dynamic and highly conserved mechanisms has occupied scientists for decades. This is primarily due to the essential regulatory functions of calcium in a variety of physiological processes. Without these finely tuned calcium spikes, oscillations, or waves, life as we know it would not be possible.

A known mechanism for calcium release is the stimulation of Gq- and Gi-coupled receptors, which triggers a cascade of events leading to the activation of PLCβ enzymes. While $G\alpha_q$ is capable of directly activating all isoforms of PLCβ, Gi-βγ selectively activates PLCβ2 and PLCβ3, and only when $G\alpha_q$ has been previously or simultaneously activated. The increased activity of PLCβ, which hydrolyzes the membrane component PIP_2, results in enhanced intracellular levels of IP_3 and DAG. Second messenger IP_3 in turn activates endoplasmically located $InsP_3$ receptors that function as calcium channels, leading to rising levels of cytosolic Ca^{2+}. While this signal transduction is mediated by the direct interaction of $G\alpha_q$ and Gi-βγ with PLCβ, the remaining G protein families: Gs and G12/13, are not thought to interact with PLCβ enzymes.

This study is based on the hypothesis of a Gs-βγ-mediated PLCβ activation resulting in an intracellular calcium mobilization. For a long time, Gs-GPCRs were thought to affect intracellular calcium levels only via stimulating AC with its second messenger cAMP. Over the years, evidence has accumulated indicating that Gs-GPCRs increase intracellular calcium levels also by crosstalking with Gq-GPCRs. This crosstalk has been deciphered as requiring the removal of PLCβ autoinhibition by $G\alpha_q$ for direct Gs-βγ-PLCβ activation, as observed for Gi-βγ-Ca^{2+}. Thus, this work makes an important contribution to the understanding of a Gs-mediated calcium regulation and demonstrates once again that the Gβγ subunits are bona fide signal transducers participating in calcium homeostasis.

To decipher our postulated Gs-βγ-PLCβ-Ca^{2+} pathway, we analyzed the putative signaling cascade step by step. For this purpose, various CRISPR/Cas9-edited cells, engineered molecules, and inhibitors either at the G protein or effector level were used in numerous assays. Our findings are that the molecular mechanism of action by which Gs-βγ can mediate calcium is dependent on active Gq, as is the case for Gi-βγ. It is not important whether the active Gq is provided via a pure Gq-coupled receptor or via promiscuous Gs receptor coupling, but rather its binding with concomitant release of the autoinhibited PLCβ enzyme. The domain occluding the catalytic site is called the XY linker and prevents binding of the enzyme to PIP_2 moieties in the membrane. Mutation or deletion of the autoinhibitory domains (PLCβ3ΔXY or PLCβ3F715A) renders Gq binding redundant and enables Gs-βγ calcium. While the

Gs-βγ-PLCβ3 interaction remains imperceptible, we propose that a protein interaction takes place, probably with a faster kinetic than that observed with Gi-βγ. However, this interaction does not eliminate autoinhibition and, therefore, does not result in an immediate increase in IP_3. This changes in the presence of active Gq or one of the mutants, as Gs-βγ now responds by synergistically increasing the calcium level. Despite the lack of data demonstrating Gs-βγ-PLCβ3 interaction, the biphasic calcium response curve, the partial inhibition of Gs-calcium by masGRK3ct, and the data generated with the IP_3 sensor suggest that Gs-βγ contributes to the overall Gs-calcium response. Finally, we demonstrate that Gs-GPCRs do not only mediate calcium via an indirect, cAMP-dependent pathway, as has long been suspected, but also directly and cAMP-independently via the Gs-βγ-PLCβ-Ca^{2+} module.

The present study separates Gs-mediated calcium release into a cAMP-independent and a cAMP-dependent component, thereby disentangling the various studies on Gs-mediated calcium release. This has long been overlooked as the two compartments are mixed at the calcium level, further complicated by differences in calcium regulation in different cell types (excitable and non-excitable) and tissues. These new findings bring Gs-GPCRs into focus as true calcium mediators, expand the portfolio of cellular calcium signaling cascades, and highlight the complexity of signaling in terms of GPCR crosstalk. Furthermore, we have successfully demonstrated our mechanism in primary cellular context, providing a fundamental basis for further in vivo studies. In addition to the importance of GPCRs for therapeutic applications, calcium plays a central role in numerous physiological and pathophysiological processes. Therefore, our study provides a small contribution to a deeper molecular understanding of the cellular processes involved, which is essential to identify potential avenues for developing new drug therapies.

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Publication

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