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Succinate receptor GPR91, a Gα$_i$ coupled receptor that increases intracellular calcium concentrations through PLCβ

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ABSTRACT

Succinate has been reported as the endogenous ligand for GPR91. In this study, succinate was confirmed to activate GPR91 resulting in both 3′–5′-cyclic adenosine monophosphate (cAMP) inhibition and inositol phosphate formation in a pertussis toxin (PTX)-sensitive manner. GPR91 agonist-mediated effects detected using dynamic mass redistribution (DMR) were inhibited with PTX, edelfosine and U73122 demonstrating the importance of not only the Gα$_i$ pathway but also PLCβ. These results show that GPR91 when expressed in HEK293s cells couples exclusively through the Gα$_i$ pathway and acts through Gα$_i$ not only to inhibit cAMP production but also to increase intracellular Ca^{2+} in an inositol phosphate dependent mechanism via PLCβ activation.

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1. Introduction

G-protein-coupled receptors (GPCRs) constitute the largest family of cell-surface proteins and are activated by a diverse range of extracellular stimuli or ligands including amino acids, peptides, lipids, nucleotides and photons. As a result GPCRs play an essential role in regulating many physiological functions including neurotransmission, metabolism, secretion and cell growth. GPCRs are so named as stimulation by a ligand results in the receptor coupling to a trimeric G-protein which in turn dissociates into its subunits Gα and Gβγ that subsequently modulate downstream effectors. There are at least 18 different subtypes of Gα belonging to four subfamilies that in turn activate a small number of effector molecules [1]. Studies on the function of members of the Gα$_i$/Gα$_o$ family have often been performed using a toxin from Clostridium botulinum pertussis toxin (PTX) that inhibits activity of this family. Whilst many other G-protein-dependent signalling processes are resistant to PTX [2], some Gβγ downstream signalling events dependent on Gα$_i$ activation are inhibited by PTX. GPCR-dependent phospholipase C (PLC) activation is mediated by PTX-sensitive and -insensitive mechanisms [3,4]. The PTX-insensitive pathways are primarily mediated by Gα$_q$-dependent activation of PLCβ or pathways involving Rho and PLCε [4–6], whilst PTX-sensitive pathways are mediated by members of the Gα$_i$ family [7,8].

Succinate has been reported as an endogenous ligand for GPR91, a class A GPCR [9]. Succinate is a well-known intermediate in the tricarboxylic acid (Krebs) cycle, where it is formed from succinyl-CoA by succinyl-CoA synthetase and subsequently converted by succinate dehydrogenase to generate fumarate. Because the succinate dehydrogenase complex is part of the electron transport chain in the mitochondrial membrane, its activity indirectly depends on the availability of oxygen. As such, in situations when oxygen tension is low, succinate accumulates because of low activity of succinate dehydrogenase or other enzymes in the electron transport chain that affect its activity [10–12]. Transport of succinate to the extracellular compartment is likely to occur through porins and the succinate-fumarate/malate

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transporter [13]. The extracellular succinate then serve as an agonist for GPR91 [14,15]. GPR99 is the closest homologue, sharing 33% protein sequence identity with GPR91. GPR99 has also been "deorphanized" with another Krebs cycle intermediate, α-ketoglutarate which appear to be its ligand of choice [9]. GPR91 is expressed in a range of different tissues such as kidney, liver, adipose, spleen, heart, retina and intestine [9,16]. In the kidney, succinate-induced activation of GPR91 is reported to regulate the renin-angiotensin system and suggested to be important in the development of renovascular hypertension and diabetic nephropathy [9,17]. Succinate effect on GPR91 in neuronal retinal ganglion cells has been reported to have a pro-angiogenic effect involved in retinopathy of prematurity [18]. In a recombinant system expressing GPR91, succinate is claimed to activate both $G\alpha_i$- and $G\alpha_q$-mediated signalling pathways, inhibiting forskolin-stimulated 3′–5′-cyclic adenosine monophosphate (cAMP) production and stimulating Ca^{2+}-mobilization [9,19]. In contrast to this succinate is reported to induce inositol phosphate accumulation exclusively through the $G\alpha_i$ pathway in an erythroleukemic cell line TF-1 [20]. HEK293s cells are routinely used to recombinant express GPCRs and the resulting cell lines are often used to identify synthetic ligands, however this can be misguided if the signalling property of the system is altered. The purpose of the present study therefore was threefold: (1) to determine if GPR91 receptor activation in our recombinant HEK293s cell line couples through the $G\alpha_i$, $G\alpha_q$ or both pathways; (2) to examine the PTX sensitivity of GPR91-mediated cAMP and IP_3 formation; (3) to examine the mechanism of GPR91 agonist-mediated effects on IP_3 formation.

2. Materials and methods

2.1. Cloning of hGPR91, cell culture and membrane preparation

Full length DNA for human succinate receptor 1 (SUCNR1) (NM_033050) (GeneArt (Life Technologies)) was sub cloned into a pIRESneo3 vector (Clontech) and HEK293s cells (ATCC) were transfected using Lipofectamine™ 2000 (Life Technologies). A single clone was selected using fluorescence-activated cell sorting (FACS) sorting and a Ca^{2+}-mobilization assay. Cells were cultured at 37 °C, 5% CO_2 and 95% humidity in DMEM supplemented with 10% FBS and 800 µg/ml G418 (Invitrogen). Membranes were prepared using standard methods. Protein concentrations were determined with BCA protein assay kit (Pierce biotechnology).

2.2. Treatment with inhibitors

Cells were treated with 100 ng/ml PTX (Sigma) for 18–24 h before being used in cAMP, inositol-1-phosphate (IP1) or dynamic mass redistribution (DMR) assays, or for membrane preparation and guanosine 5′-[γ-^{35}S]-triphosphate (GTPγS) binding. Treatment with 10 µM edelfosine (Tocris) or 10 µM U73122 (Tocris) was done 1 h before DMR assays.

2.3. cAMP assay

Four thousand cells/well were incubated for 5 min with succinate in buffer (Hank's balanced salt solution (HBSS) (Invitrogen), 20 mM HEPES (pH 7.4) (Invitrogen), 0.1% bovine serum albumin (BSA) (Sigma)) in small-volume 384-well plates (Greiner). cAMP production was stimulated for 30 min with 10 µM forskolin (Sigma), 1 mM IBMX (Sigma) and 250 µM Alexa Fluor® 647-anti cAMP antibody (PerkinElmer, LANCE cAMP kit). The reaction was stopped with detection buffer containing 10 µM Biotin-cAMP and 15 µM

LANCE Eu-W8044 labelled streptavidin (PerkinElmer, LANCE cAMP kit). cAMP production was detected by homogeneous time resolved fluorescence (HTRF) (λ_{ex} = 340 nm, λ_{em} = 665 and 615 nm) using a Pherastar (BMG Labtech). All reactions were performed at room temperature.

2.4. IP_1 assay

IP_1 assays were conducted for 1 h at 37 °C with 25,000 cells/well (small-volume 384-well plates (Greiner)). IP_1 production was detected by HTRF as described above. All buffers and reagents according to manufacturers protocol (Cisbio).

2.5. $[^{35}S]$-GTPγS binding assays

Assays were performed at 30 °C for 45 min in 200 µl of 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 25 mM HEPES (pH 7.4), 0.05 µg/µl of membrane protein, 0.01% BSA, 25 µM guanosine 5′-diphosphate, 100 µM dithiothreitol and 0.53 nM $[^{35}S]$GTPγS (PerkinElmer). Non-specific binding was determined in the presence of 20 µM GTPγS. The reaction was terminated by addition of 50 mM Tris–HCl, 5 mM $MgCl_2$, 50 mM NaCl (pH 7.4) and filtration through 96-well GF/B filter plates (PerkinElmer) using a Biomek FX (Beckman Coulter). Filters were dried (30 min at 50 °C), scintillation liquid (PerkinElmer) was added and the bound radioactivity was determined using a scintillation counter (Wallac). Peptide blocking of $[^{35}S]$-GTPγS binding was performed as described previously [21]. All chemicals from Sigma unless stated otherwise.

2.6. Label-free assays – DMR

12,000 cells/well were cultured for 24 h in 384-well fibronectin-coated Epic biosensor plates (Corning). FBS was then removed to increase expression of receptors, and cells were cultured for an additional 24 h. Medium was exchanged for buffer (HBSS, 20 mM HEPES (pH 7.4) and 0.01% BSA) and cells were incubated for 1 h inside the Epic Biosensor (Corning) at 26 °C. A baseline was read for 5 min; succinate was added using a CyBi-Well vario (CyBio) and DMR was detected during 30 min. All buffer exchanges were done with an ELx (Bio-Tek).

2.7. Fluorescent imaging plate reader (FLIPR) Ca^{2+}-mobilization assays

10,000 cells/well were cultured in 384-well poly-D-Lysine coated plates (Greiner) for 24 h. Cells were washed with 1× HBSS and 20 mM HEPES (pH 7.4), loaded with Calcium 4 (Molecular Devices) and incubated for 1 h. The fluorescence ((λ_{ex} = 488 and λ_{em} = 540 nm) was detected in a FLIPR^{TETRA} (Molecular Devices) before and after addition of succinate. When studying succinate induced Ca^{2+}-mobilization in the absence and presence of 7 µM forskolin the assay was performed with and without extracellular Ca^{2+} by using HBSS without Ca^{2+}/Mg^{2+} (Invitrogen). For all other applications HBSS contained Ca^{2+}/Mg^{2+}.

2.8. Calculations and statistical analysis

Data were fitted with a four parameter logistic fit using the equation $y = A + ((B - A)/1 + ((C/x)^D)))$ where A is no activation, B is full activation, C is the EC_{50} and D is the Hill slope. All data were based on at least three independent experiments. Values shown are mean +/− S.E.M. Statistical significance of data for Ca^{2+}-mobilization and G-protein C-terminal peptide competition in GTPγS was determined with paired *t*-test analysis ($P < 0.05$).

3. Results

3.1. Succinate induced GPR91 signalling

To evaluate different downstream signalling events dependent on GPR91 we examined the activity of succinate at human GPR91 expressed in HEK293s cells, using a range of assay formats. Succinate inhibited cAMP production measured using HTRF with an EC_{50} of 50 ± 14 μM ($n = 3$) (Fig. 1). This inhibition was GPR91-dependent since succinate was not able to reduce an equivalent forskolin-mediated cAMP elevation in untransfected HEK293s cells (data not shown). Using an HTRF IPone assay as a surrogate for IP_3 production we found that formation of IP_1 was mediated by succinate with an EC_{50} of 421 ± 26 μM ($n = 3$) (Fig. 2). No formation of IP_1 could be detected in untransfected HEK293s cells with succinate (data not shown). Succinate increased Ca^{2+}-mobilization as detected using a FLIPR with an EC_{50} of 45 ± 6 μM ($n = 3$) (data not shown). Succinate did not cause an increase in Ca^{2+}-mobilization in untransfected HEK293s cells (data not shown).

3.2. Inhibition of $G\alpha_i$-protein coupling

To investigate the $G\alpha_i$-protein coupling we examined the effect of PTX on the ability of GPR91 to inhibit cAMP formation. Pre-treatment of hGPR91-HEK293s cells with PTX prevented succinate to mediate an inhibition of cAMP, indicating a role for $G\alpha_i$ (Fig. 1). We evaluated the effect of PTX on succinate-induced IP_1 formation to determine the relationship between $G\alpha_i$ and $G\alpha_q$ contribution to succinate-dependent Ca^{2+}-mobilization. Pre-treatment with PTX also completely abolished the ability of GPR91 to mediate a succinate-induced IP_1 production (Fig. 2) demonstrating that this downstream signal is unlikely to be $G\alpha_q$-dependent but instead mediated by a PTX-sensitive mechanism. HEK293s cells expressing the $G\alpha_i$ coupled receptor RXFP3 and the mainly $G\alpha_s$ coupled receptor RXFP1 were used as PTX-sensitive and -resistant controls, respectively (data not shown) and showed appropriate responses as reported previously [22,23].

The direct increase in G-protein GTP-binding by GPR91 was detected by [^{35}S]-GTPγS-binding. Succinate increased GTPγS-binding in membranes from hGPR91-HEK293s with an EC_{50} of 51 ± 6 μM ($n = 3$). The response was GPR91 specific since succinate did not mediate an increase in GTPγS-binding in membranes from untransfected HEK293s cells (data not shown). Membranes pre-
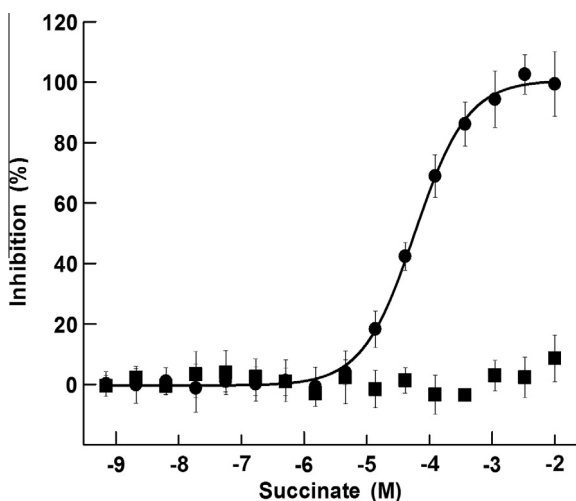

Fig. 2. Succinate stimulated IP_1 production in hGPR91-HEK293s cells untreated (●) and PTX-treated (■). Data is shown as % effect, defined as IP_1 production normalized to maximal effect of succinate.
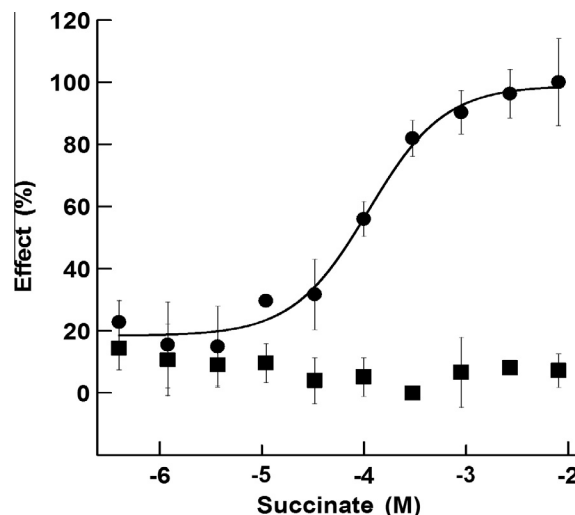
Fig. 3. Succinate mediated GTPγS-binding in membranes from untreated (●) and PTX-treated (■) hGPR91-HEK293s cells. Data is shown as % effect, defined as specific GTPγS-binding normalized to maximal effect of succinate.

pared from hGPR91-HEK293s cells pre-treated with PTX were not able to mediate a succinate-stimulated GTPγS-binding (Fig. 3). In addition we assessed the ability of G-protein C-terminal peptides to compete the G-protein interaction of GPR91 in our GTPγS assay. Whilst pre-treatment with a peptide equivalent to the last twelve amino acids of $G\alpha_{i1/2}$ reduced the GTPγS signal an equivalent peptide based on the C-terminus of $G\alpha_q$ did not (Fig. 4).

To further investigate other possible signalling pathways downstream of GPR91 we used a label-free technology (Corning Epic biosensor) to look at the total cellular response. Succinate stimulation of hGPR91-HEK293s cells showed a dose-dependent increase in DMR with an EC_{50} of 124 ± 7 μM ($n = 3$). Succinate produced no response in untransfected HEK293s cells (data not shown). Treating the hGPR91-HEK293s cells with PTX completely removed the ability for succinate to induce a DMR signal in these cells (Fig. 5) confirming that GPR91 signalling in HEK293s cells are solely $G\alpha_i$-dependent.

Fig. 1. Succinate inhibition of forskolin-stimulated cAMP production in untreated (●) and PTX-treated (■) hGPR91-HEK293s cells. Data is shown as % inhibition of forskolin-stimulated cAMP, normalized to maximal inhibition by succinate.
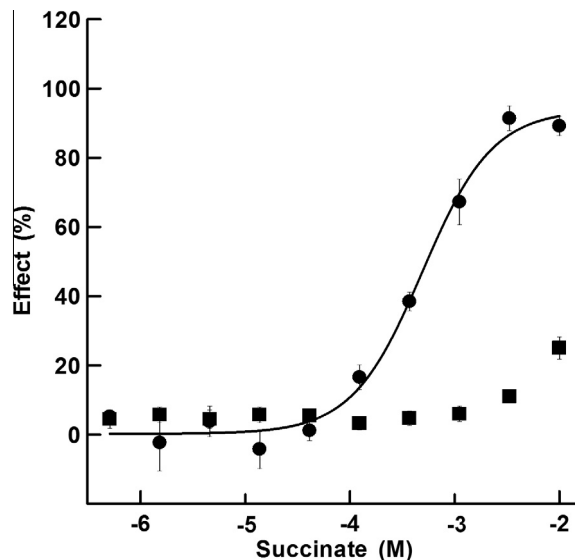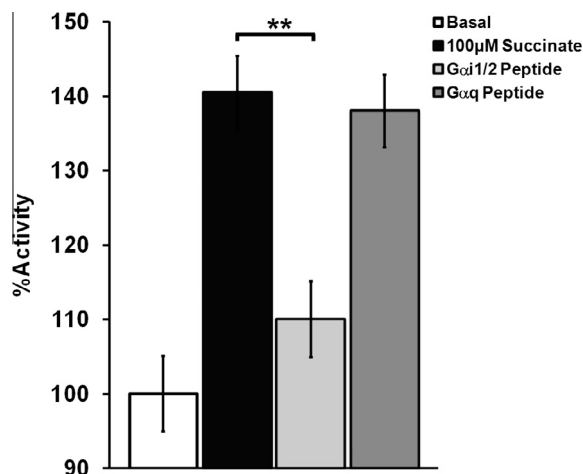
Fig. 4. Mapping G-protein coupling of GPR91. Basal and 100 μM succinate stimulated GTPγS-binding (% activity, mean ± S.E.M.) in hGPR91-HEK293s membranes in the absence and presence of peptides equivalent to the C-termini of $G\alpha_{i1/2}$ and $G\alpha_q$. Data were analysed using paired t-test (**$P < 0.05$; $n = 3$).

Fig. 6. PLCβ involvement in GPR91 signalling. Succinate mediated DMR response in untreated (●), edelfosine-treated (▲) and U73122-treated (■) hGPR91-HEK293s cells. The DMR response is normalized to maximal succinate response and shown as % effect.
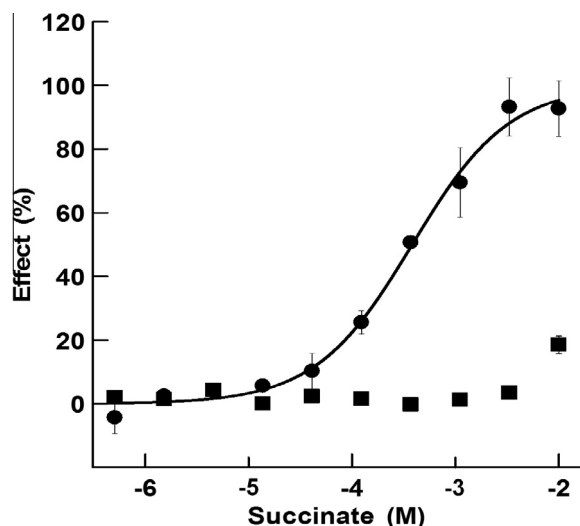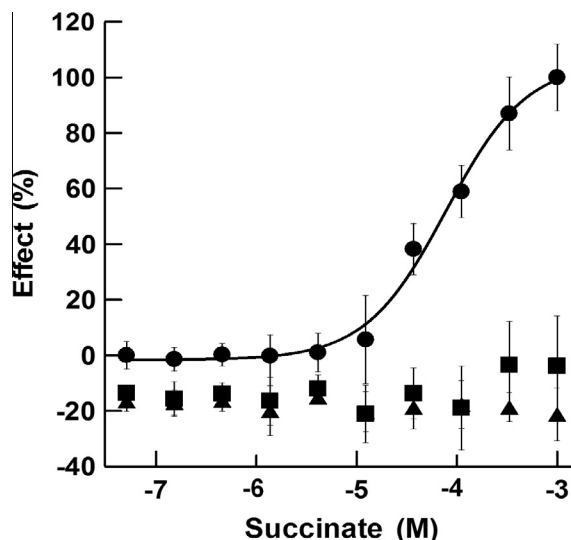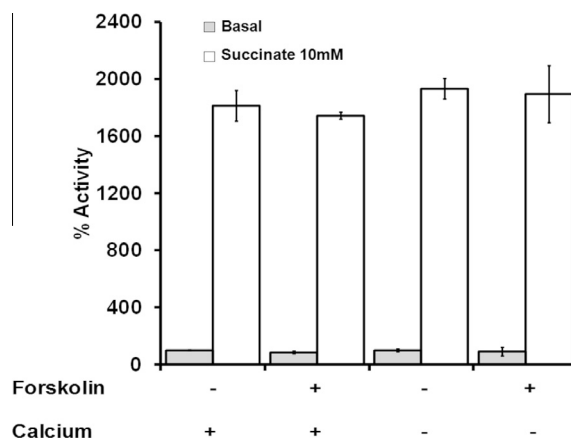

Fig. 5. Succinate induced DMR response in untreated (●) and PTX-treated (■) hGPR91-HEK293s cells. The DMR response is normalized to maximal succinate response and shown as % effect.

Fig. 7. Dependence of extra cellular Ca^{2+} and co-dependence of Ca^{2+} and cAMP on succinate mediated Ca^{2+}-mobilization in hGPR91-HEK293s. Ca^{2+} was included or excluded from the extra cellular buffer and 7 μM forskolin was included to stimulate cAMP. No significant ($P > 0.2$) influence of either forskolin stimulation or removal of extracellular Ca^{2+} was seen on succinate mediated GPR91 effect on Ca^{2+}-mobilization. Effect is presented as effect over basal (basal = 100%). Basal effect is shown in grey bars and succinate effect in blank bars.

3.3. Inhibition of PLCβ and dependence of extracellular Ca^{2+} and cAMP on Ca^{2+} release

To evaluate if PLCβ was involved in the Ca^{2+}-dependent processes we used edelfosine and U73122 as inhibitors of PLCβ and studied their effect on the total GPR91-mediated cell response in the DMR assay. Previous work in our lab has determined that the optimal inhibitory concentration of both edelfosine and U73122 is 10 μM, which in this study blocked the succinate-induced response completely, indicating that PLCβ is involved in GPR91 downstream signalling (Fig. 6).

We examined the dependence on extracellular Ca^{2+} for GPR91-mediated Ca^{2+} signalling by studying succinate-induced activation in hGPR91-HEK293s cells both in presence and absence of extracellular Ca^{2+} using FLIPR. The succinate induced Ca^{2+}-mobilization mediated by GPR91 was not affected by removal of extracellular Ca^{2+} demonstrating that GPR91 is mediating Ca^{2+}-mobilization from intracellular stores (Fig 7). To determine if the cAMP inhibition activity and Ca^{2+}-mobilization showed any co-dependence we used 7 μM of forskolin to initiate increased cAMP production.

However, we found that the succinate-dependent increase in Ca^{2+}-mobilization was unaltered (Fig. 7).

4. Discussion

It is a common physiological process that an extracellular stimulus can activate multiple signalling pathways through coupling to different G-proteins. This type of dual signalling may result from receptors interacting with multiple G-proteins, i.e., activation of the FFAR2 receptor leads to inhibition of adenylyl cyclase through $G\alpha_i$ and stimulation of PLC through $G\alpha_q$ [24]. However, this may not be true in all cases; multiple signalling events may occur via a single G-protein in response to stimulation by a given agonist. For example, some investigators have found that several structurally unrelated $G\alpha_s$ coupled receptors shared the ability to stimulate PLC in addition to stimulating adenylyl cyclase [25,26]. Previously it has been reported that GPR91 signals through two G-protein

pathways, $G\alpha_i$ and $G\alpha_q$. In this study we show that PTX completely abolished the succinate-induced cAMP inhibition and IP_1 production suggesting that GPR91 act exclusively through the $G\alpha_i$ pathway. This was also seen when a peptide equivalent to the C-terminus of $G\alpha_{i1/2}$ reduced the GTPγS signal whilst a peptide based on the C-terminus of $G\alpha_q$ did not. Whilst it is unlikely that $G\alpha_q$ would contribute to the measured GTPγS signal, the lack of effect of this peptide suggests that in this experimental system $G\alpha_q$ does not bind to GPR91. Furthermore, in a DMR assay that works independently of specific G-protein coupling and agonist trafficking, the succinate response at GPR91 expressed in HEK293s cells was entirely PTX-sensitive. These results demonstrate that GPR91 in our expression system acts through $G\alpha_i$ not only to inhibit cAMP production but also to induce Ca^{2+}-mobilization.

Receptor-induced activation of a G-protein is accomplished by the dissociation of its α subunit from the βγ dimer, both of which may in turn modulate target effectors. Evidence has been generated showing that the free βγ dimers play an important role in signal transduction processes [27]. Furthermore, it is widely accepted that Gβγ is the signal transducer for the PTX-sensitive PLC stimulation found for many $G\alpha_i$ coupled receptors [28] as well as being the mediator for stimulation of the extracellular-signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway [29]. The extensively characterized adrenergic α_{2A} receptor has been shown to give a Ca^{2+}-mobilization in a PTX-sensitive manner with involvement of both PLCβ and Gβγ subunits [30]. Activation of ERK1/2 and Akt mediated by the α_{2A} receptor is also suggested to proceed through the Gβγ subunits [31–33]. Purified PTX-sensitive $G\alpha_i$ family subunits are unable to reconstitute activation of PLC. On the other hand purified Gβγ subunits are able to activate PLCβ isoforms in vitro, albeit at relatively high concentrations compared to typical activation by $G\alpha_q$ subunits [28,34,35]. Many of the GPCR-dependent physiological processes inhibited by PTX are mediated by Gβγ rather than Gα [36–39]. Thus, most Gβγ dependent signalling appears to arise from $G\alpha_i$ proteins.

It is recognized in many cellular systems that the receptor/G-protein activation of PLC and IP_3 production is the transduction pathway regulating the release of Ca^{2+} from internal stores [40]. The amino steroid compound U73122 and edelfosine (ET-18-OCH_3) are reported to act as specific inhibitors of PLC and they have become important tools in establishing the link between PLC activation and cellular Ca^{2+}-mobilization [41,42]. We have shown that the succinate-induced GPR91 signalling detected by DMR is sensitive to both edelfosine and U73122, establishing that PLCβ is involved in GPR91 signalling. This is consistent with PLC activation being mediated through βγ subunits derived from PTX-sensitive $G\alpha_i$ proteins as opposed to classical $G\alpha_q$ stimulated Ca^{2+}-mobilization.

It has previous been shown that PLCδ activation by $G\alpha_{i/o}$-coupled receptor agonists are mediated by Ca^{2+} influx via store operated Ca^{2+} channels, by showing that agonist responses are not observed in the absence of extracellular Ca^{2+} [43]. The methacoline induced Ca^{2+}-mobilization by the $G\alpha_i$ coupled M2 receptor was on the other hand not reduced upon removal of extracellular Ca^{2+} [44]. To investigate if succinate-induced Ca^{2+}-mobilization for GPR91 was dependent on extracellular calcium we studied Ca^{2+}-mobilization upon succinate activation in the absence of extracellular Ca^{2+}. Activation of GPR91 was not impacted by removal of extracellular Ca^{2+} indicating that membrane Ca^{2+} channels are not involved in the signalling through Gβγ and PLCβ. This implies that GPR91 is mediating Ca^{2+}-mobilization from intracellular stores, this has also been seen for other $G\alpha_{i/o}$ receptors [30]. Our data suggests that there is no co-dependence on Ca^{2+}-mobilization and cAMP since stimulation of adenylate cyclase with forskolin did not alter the succinate-induced Ca^{2+}-mobilization.

In earlier studies recombinant GPR91 has been shown to activate both $G\alpha_i$ and $G\alpha_q$ pathways through which $G\alpha_i$ inhibits cAMP production and $G\alpha_q$ activates Ca^{2+}-mobilization [9,45]. These conflicting results may be due to the different cell systems or promiscuous G-protein coupling. However, results similar to ours have been reported in the erythroleukemic cell line TF-1 with endogenous expressed GPR91 [20]. Here the $G\alpha_i$ pathway was shown to mediate succinate-induced activation of ERK1/2 and inositol phosphate accumulation. Succinate has been shown to regulate lipolysis in white adipose tissue in a PTX-sensitive manner [16]. Succinate also induced platelet aggregation and inhibited cAMP production in platelets, but did not have any effect on Ca^{2+}-mobilization in this cell type [46]. The author's hypothesis for this is the lack of Gβγ−mediated activation of PLCβ in platelets. In primary hepatic stellate cells succinate did accelerate stellate cell activation but did not inhibit cAMP production or increased cytocolic Ca^{2+} [47]. There are evidence for differences in de-sensitization and internalization of GPR91 in different cell types. In HEK293s cells recombinant GPR91 was shown to internalize upon stimulation with succinate [9] whereas in polarized renal Madin-Darby Canine Kidney (MDCK) cells succinate did not induce internalization of recombinant GPR91 but instead a temporal desensitization of the receptor [45]. The GPR91 signalling seems to be highly specific depending on cell type and this may give some answer to the heterogeneity in signalling pathways that have been observed also in recombinant systems. This study demonstrates that GPR91 activates G_i and PLCβ signalling, which we believe is mediated by the βγ complex. It is clear that GPR91 can induce different signalling in different cell types not only by recruiting different Gα subunits but also through the βγ complex. This work clearly demonstrates the importance of investigating not only the second messengers (i.e. Ca^{2+} or inositol phosphate) but also the actual G-proteins involved in the signalling. A switch in G-protein coupling is not limited to engineered cells but can also occur with native receptor expression in host cells. Endogenous dopamine D1/D2 receptor responses couples to different G-proteins in different cell types further highlighting the importance to determine the G-protein coupling in each cell type [48]. In conclusion, when analyzing the signalling properties of agonists at GPR91 caution is required to ensure that the cellular system used has the expected signalling capability.

Acknowledgement

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