ROMANIAN ACADEMY
School of Advanced Studies of the Romanian Academy
Institute of Biochemistry

PhD THESIS SUMMARY

Modulators of GLP-1 secretion, a hormone involved in metabolic disease.
Signaling mechanism of GPR27, a novel adrenergic and atypical receptor

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Aim of the study

Unhealthy eating habits and a sedentary lifestyle characterize contemporary society. The devastating consequence is reflected in uncontrolled blood glucose levels, which can ultimately lead to weight gain, obesity, and diabetes mellitus (Calella et al., 2024, Hu, 2011). Blood glucose levels are regulated by the main glucoregulatory hormones: insulin, glucagon, and the incretin glucagon-like peptide-1 (GLP-1). Depending on the physiological context, insulin lowers blood glucose levels, while glucagon increases them. Meanwhile, the GLP-1 hormone has a dual action in combating hyperglycemia: insulinotropic and glucagonostatic (Aronoff et al., 2004). While the insulin receptor belongs to the receptor tiyrosine kinase class, glucagon and GLP-1 mediate their action through G protein-coupled receptors (GPCRs) (Habegger, 2022). Additionally, the activation of GPCRs can further enhance hormone secretion (Thor, 2022, Spreckley, 2015). These receptors are one of the largest families of membrane proteins, with seven transmembrane domains as their fundamental signature. They are ubiquitously expressed and respond to a wide range of extracellular signals, such as neurotransmitters and hormones (Lefkowitz, 2007). However, within this family, certain GPCRs have unidentified functions and ligands and are therefore classified as orphan receptors. Considering that over 34% of drugs approved by the FDA (U.S. Food and Drug Administration) or EMA (European Medicines Agency) target GPCRs, the discovery of therapeutic targets among orphan receptors is a priority for the pharmaceutical industry and academic research (Jobe and Vijayan, 2024).

Current antidiabetic therapy, which includes GLP-1 receptor agonists such as semaglutide (Ozempic), has proven effective in both glycemic control and reducing obesity risk (Lexchin and Mintzes, 2023). However, identifying natural secretagogues of the GLP-1 peptide and targeting orphan GPCR receptors expressed in pancreatic β-cells could lead to a more comprehensive approach in the treatment of type II diabetes. GLP-1 natural secretagogues offer the potential to stimulate the endogenous production of this hormone, facilitating physiological glycemic control and reducing dependence on exogenous medications (Bodnaruc et al., 2016). Additionally, orphan GPCRs, upon activation by specific ligands, could play a significant role in regulating metabolism and appetite, offering novel strategies for addressing metabolic dysfunctions (Shore and Reggio, 2015). SREB1 (GPR27) is an orphan receptor which belongs to the Superconserved Receptors Expressed in the Brain (SREBs) family (O'Dowd et al., 1998, Matsumoto et al., 2000). The GPR27 receptor is of particular interest because it is expressed in

numerous tissues (https://www.proteinatlas.org/ENSG00000170837-GPR27/tissue (Human Protein Atlas)) and although limited, existing studies suggest a potential role in metabolism, specifically in the regulation of glucose homeostasis (Ku et al., 2012, Chopra et al., 2020). Therefore, the study has been structured into two parts: Part I focuses on the **identification of new secretagogues of the GLP-1 hormone**, while Part II is dedicated to the **deorphanization and characterization of the GPR27 receptor, which has a potential role in insulin secretion**. Each part includes an INTRODUCTION chapter, which covers general aspects and theoretical relevance that support the experimental section; a MATERIALS AND METHODS chapter, which describes the techniques used; a RESULTS AND DISCUSSIONS chapter; a CONCLUSIONS AND PERSPECTIVES chapter, which summarizes the findings and discusses future research directions; and a FINAL CONCLUSIONS chapter, which integrates both research directions explored in the study.

The first part, titled "Modulators of GLP-1 secretion, a hormone involved in metabolic disease" involves the use of a bioluminescent method to identify potential modulators of GLP-1 hormone secretion and to characterize selected compounds. In this approach, a clone of the GLUTag cell line (GLUTag-NLuc), developed at the Institute of Biochemistry of the Romanian Academy was used. The first step focused on optimizing the luciferase activity assay, which reflects GLP-1 peptide secretion. The second step involved testing a library of 173 natural compounds using the GLUTag-NLuc cell clone. This functional analysis led to the identification of natural secretagogues, primarily from the flavonoid class. The third step aimed to determine the mechanism of action of the identified GLP-1 peptide secretagogues. Quercetin, a flavonoid, increased GLP-1 peptide secretion only in the presence of glucose. Additionally, quercetin reduced intracellular glucose transport and enhanced metabolism, as reflected by an increase in intracellular ATP levels. Thus, flavonoids – naturally found in various foods – could be incorporated into the daily diet, providing a potential preventive role against metabolic diseases (Anghel et al., 2022).

The second part, titled "Signaling mechanisms of the GPR27 receptor, a novel atypical adrenergic receptor," aimed to functionally characterize the orphan receptor GPR27 and its involvement in glucose-stimulated insulin secretion. Activity 1 focused on verifying the expression of the GPR27 receptor on the cell membrane surface. Activity 2 consisted of identifying GPR27 receptor ligands and the cellular signaling pathway involved. We tested

canonical intracellular signaling pathways, including the modulation of secondary messengers (cAMP and calcium) triggered by the activation of heterotrimeric G proteins. The hypothesis behind these experiments was based on the possible ligand-independent activation of the GPR27 receptor, a phenomenon known as constitutive activity. Indeed, a decrease in cAMP levels was observed, suggesting that GPR27 might be a constitutively active receptor. Using a set of specific inhibitors, we demonstrated that the observed cellular effects were not mediated by inhibitory Gi proteins, thereby refuting our initial hypothesis. Based on these results, we hypothesized that the C-terminal domain of the GPR27 receptor could play a key role in the observed functional characteristics. To investigate this, we generated several chimeric receptors by replacing the C-terminal domain of GPR27 with homologous domains from well-characterized receptors. Functional analysis of these chimeric receptors led to an unexpected discovery: the GPR27-β1AR chimera, in which the C-terminal domain of GPR27 was replaced with that of the β1-adrenergic receptor, behaved identically to the β1-adrenergic receptor – recognizing the same set of ligands and activating the stimulatory G protein (Gs). Through integrative analysis, we demonstrated that GPR27 functions as an adrenergic receptor. Moreover, GPR27 signaling in response to the adrenergic ligand isoproterenol exhibited a highly atypical mechanism, as it was independent of both G proteins and β-arrestin. Instead, GPR27 was found to be involved in transinhibition of a receptor tyrosine kinase, specifically the epidermal growth factor receptor (EGFR). Finally, in Activity 3, we demonstrated a potential role of the GPR27 receptor in modulating glucose-stimulated insulin secretion.

Part I

Modulators of GLP-1 secretion, a hormone involved in metabolic disease

1. INTRODUCTION

Intravenous administration of glucose results in lower insulin concentrations compared to oral administration. This phenomenon is attributed to the incretin effect – L cells in the intestine secrete GLP-1, while K cells release GIP. Incretins have an insulinotropic effect; however, GLP-1 also suppresses glucagon secretion (Kazafeos, 2011). By binding to its receptor GLP-1R, which is coupled with stimulatory G proteins (Gs), GLP-1 slows gastrointestinal motility, regulates appetite and satiety, and provides cardiovascular benefits (Krieger, 2020, Ferhatbegovic et al., 2023). In β-cells, it promotes insulin biosynthesis and secretion, enhances proliferation, reduces endoplasmic reticulum stress, and inhibits apoptosis (Koole, 2013).

The hormone GLP-1 is synthesized from preproglucagon. Enteroendocrine L cells are distributed throughout the entire small intestine (duodenum, jejunum, ileum) as well as the colon. The proximal L cells are responsible for the rapid phase of GLP-1 secretion, which is mediated by nutrients, while colonic L cells are stimulated by bile acids and bacterial metabolites (Kuhre et al., 2021). GLP-1 secretion in response to nutrients occurs via transporters or GPCR activation (Dolanc et al.). The regulation of GLP-1 secretion by glucose closely resembles insulin secretion from pancreatic β-cells (Figure 1), though notable differences exist, particularly between species. In the GLUTag cell line, a murine model derived from colonic tumor, GLP-1 exocytosis depends on glucose metabolism, involving K_{ATP} channel closure, as well as SGLT1, which co-transports two sodium ions along with one glucose molecule into the cell (Reimann and Gribble, 2002, Gribble et al., 2003). Similarly, GLP-1 secretion occurs in primary L cells isolated from mice (Reimann et al., 2008) as well as in the perfused small intestine of rats (Kuhre et al., 2015). In humans, SGLT1 plays a major role in glucose-stimulated GLP-1 secretion, involving membrane depolarization, which also engages voltage-dependent calcium and sodium channels (Sun et al., 2017).

Current antidiabetic therapy utilizes GLP-1-based medications, which fall into two categories: GLP-1 receptor agonists (incretinomimetics), such as exenatide, liraglutide, lixisenatide, taspoglutide, albiglutide, dulaglutide, and semaglutide, along with inhibitors of the enzyme that degrades GLP-1, namely DPP-4 inhibitors (gliptins), including sitagliptin, vildagliptin, saxagliptin, alogliptin, and linagliptin (Gilbert and Pratley, 2020). Both classes enhance insulin secretion leading to reduction of blood glucose only in hyperglycemic conditions (DeFronzo et al., 2014). Additionally, GLP-1R agonists provide beneficial effects on weight management and the cardiovascular system. In recent years, sales of Ozempic (Novo Nordisk, semaglutide) have surged due to its off-label use as a weight loss drug, demonstrating promising results (Han et al., 2023). Given the therapeutic benefits of GLP-1, the identification of new secretagogues remains a key research focus for developing more effective strategies in the treatment of diabetes and obesity.

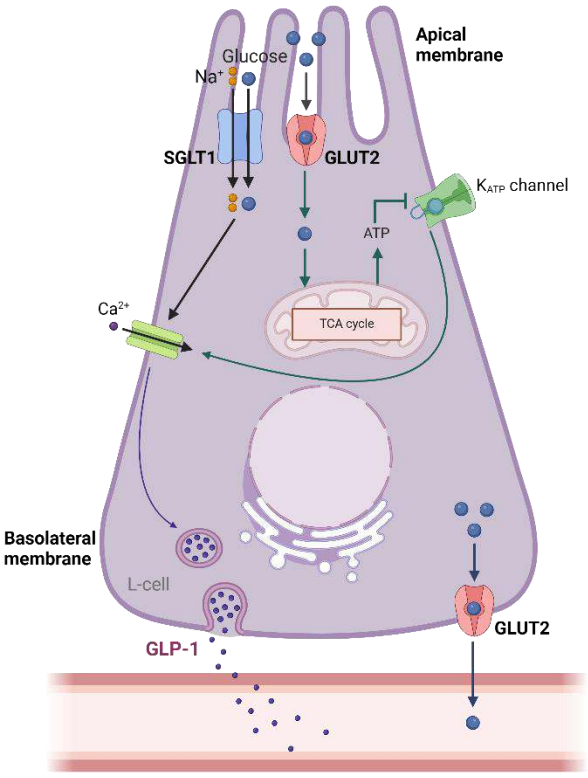

Figure 1. Glucose-stimulated GLP-1 secretion. Postprandially, glucose reaches the intestinal lumen, from where it can enter L-type enteroendocrine cells through two apically located transporters, GLUT2 and SGLT1. In humans, SGLT1 appears to play a more significant role (Sun et al., 2017). The glucose within the cell is metabolized, enhancing secretion, and in some cases (depending on the species), leading to the closure of K_{ATP} channels (Sun et al., 2017). Additionally, GLUT2 seems to be expressed at the basolateral membrane as well, where it is involved in glucose transport into circulation (Schmitt et al., 2017). Created in BioRender. Anghel, S. (2025) https://BioRender.com/p28a062.

2. MATERIALS AND METHODS

To identify GLP-1 secretagogues, we employed a reporter cell line (GLUTag-NLuc) developed at the Institute of Biochemistry of the Romanian Academy, where the hormone is co-

secreted with NanoLuc luciferase (Figure 2A). Thus, by measuring luminescence, the amount of hormone secreted is indirectly quantified. The luminescent secretion assay was validated by enzyme-linked immunosorbent assay (ELISA). Subsequently, the optimized method was applied to screen a library of natural compounds. Furthermore, the mechanism of action of the secretagogues was investigated using the established luciferase-based assay, as well as by measuring glucose uptake and ATP levels (Anghel et al., 2022).

3. RESULTS AND DISCUSSIONS

Initially, the luciferase-based GLP-1 secretion assay was optimized using known secretagogues under various experimental conditions (e.g., different cell numbers, different secretion media). Once the method was fully optimized, we tested a library of 173 natural compounds. Among the identified secretagogues, flavonoids were the most predominant, namely quercetin, luteolin, apigenin, baicalein, myricetin, chrysin, and fisetin (Figure 2B). Dietary flavonoids have been associated with a reduced risk of developing diabetes, as well as other benefits related to prediabetes or obesity, such as increased insulin sensitivity (Dinda et al., 2020, Martin and Ramos, 2021). Next, we investigated the cellular mechanism involved in GLP-1 secretion induced by flavonoids using the reporter cell line, focusing primarily on quercetin. In the presence of the L-type voltage-dependent calcium channel inhibitor, nifedipine, the stimulation of GLP-1 secretion by the compounds was abolished (Figure 3A). Furthermore, the increase in GLP-1 secretion by quercetin was observed only in the presence of glucose (Figure 3B). Glucose-mediated GLP-1 secretion involves two mechanisms: 1) glucose entry into cells via facilitated transport by GLUT, increasing intracellular metabolism and modifying the ATP/ADP balance, which allows the closure of K_{ATP} channels, or 2) glucose entry via the electrogenic transporter SGLT1; both events leading to a change in membrane potential, which opens L-type calcium channels (Sun et al., 2017, Gribble et al., 2003). Using specific glucose transporter inhibitors – phloridzin (SGLT1 inhibitor) and phloretin (GLUT inhibitor) – quercetin failed to stimulate GLP-1 secretion with the same efficacy (Figure 3C). Therefore, quercetin enhance glucose-induced GLP-1 secretion by involving glucose transporters, GLUT and SGLT1, and modulation of intracellular calcium via the opening of L-type voltage-dependent calcium channels (Anghel et al., 2022). The potentiation of glucose-induced GLP-1 secretion by quercetin could result from increased glucose uptake or an amplified glucose metabolism. Glucose uptake was assessed using a non-radioactive luminescence-based assay, revealing that

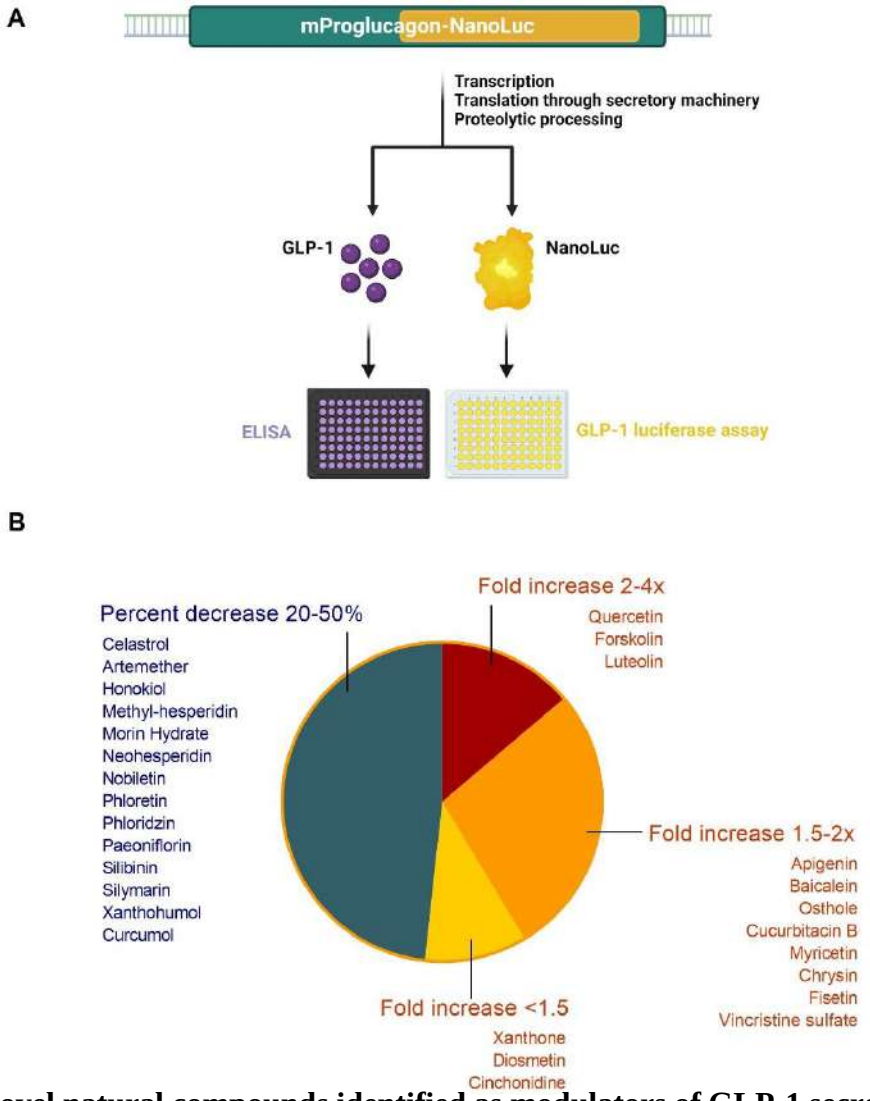

Figure 2. Novel natural compounds identified as modulators of GLP-1 secretion using the GLUTag-NLuc reporter cell line. A. Schematic representation of the GLP-1 luciferase-based secretion. The fusion protein proglucagon-NanoLuc undergoes proteolytic cleavage, generating GLP-1 and luciferase, which are subsequently secreted into the extracellular medium. The luciferase activity in the medium can be quantified using a plate reader, reflecting the amount of GLP-1 secreted. Created in BioRender. Anghel, S. (2025) https://BioRender.com/z42r753. **B.** Graphical representation of the identified compounds based on their effect on secretion levels compared to the control (DMSO). Adapted from (Anghel et al., 2022).

quercetin and luteolin (another identified flavonoid) reduced glucose entry into the cell (Figure 3D). However, in the presence of mitochondrial respiratory chain inhibitors – sodium azide (complex IV inhibitor) and oligomycin (ATP synthase inhibitor) – the effect of quercetin on

GLP-1 secretion was abolished (Figure 3E). Additionally, quercetin increased ATP levels, an effect that was blocked by the aforementioned inhibitors (Figure 3F). Therefore, quercetin stimulates GLP-1 secretion only in the presence of glucose by enhancing its metabolism, leading to increased ATP levels (Anghel et al., 2022).

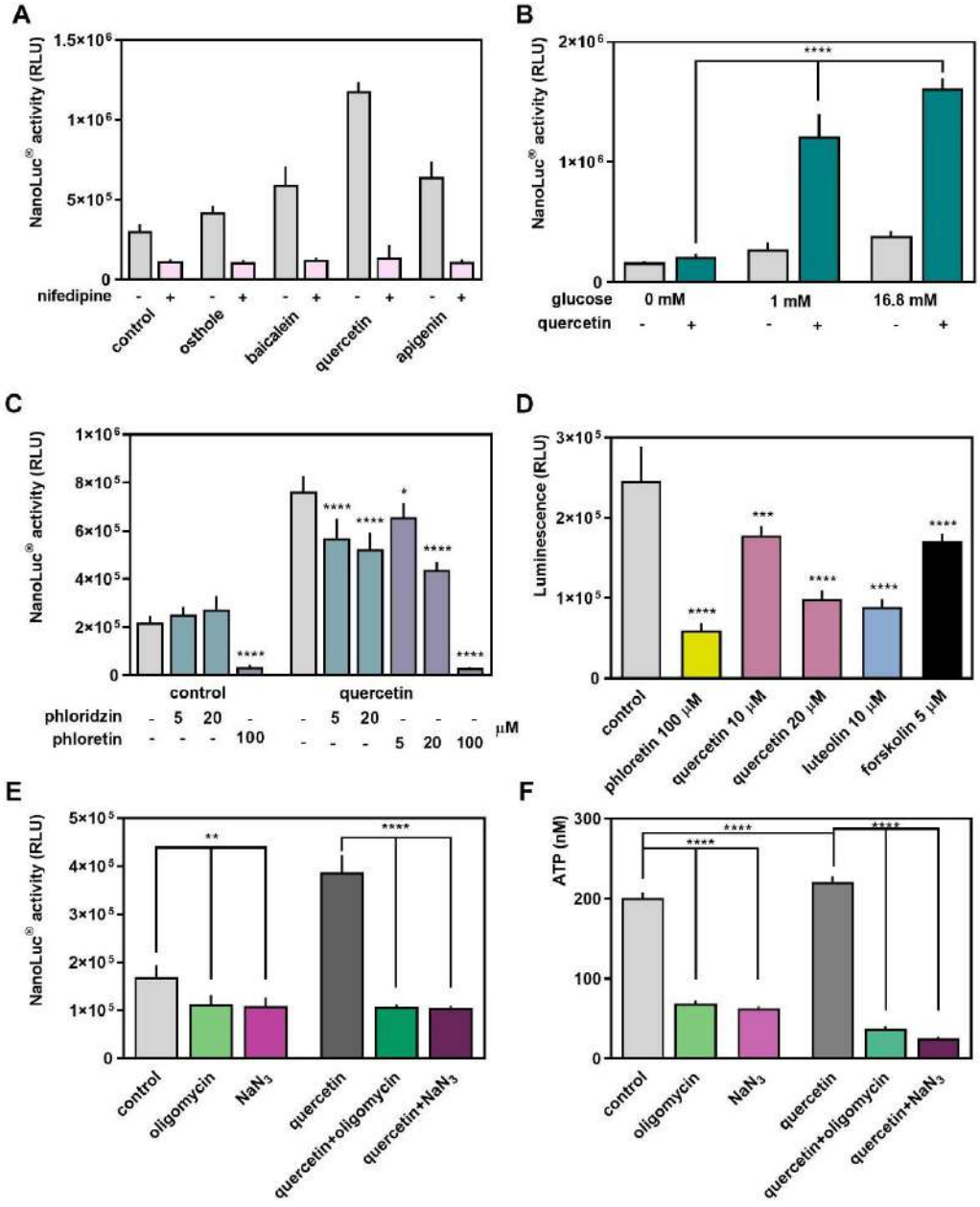

Figure 3. Quercetin modulates cellular glucose metabolism. A. Luciferase activity in response to compounds in the presence of nifedipine (5 μM). Data are presented as mean ± SD (n = 5-6). **B.** Glucose dependence of quercetin. Data are presented as mean ± SD (n = 6); ANOVA followed by Tukey's post-hoc test comparing quercetin in 0 glucose with quercetin at 1 mM and 16.8 mM glucose. **C.** Luciferase activity induced by quercetin in the presence of

glucose transport inhibitors, phloretin and phlorizin. Data are presented as mean ± SD (n = 5, except for control, 20 µM phlorizin, and quercetin at 20 or 100 µM phloretin, where n = 6); ANOVA followed by Tukey's post-hoc test comparing control or quercetin with/without inhibitor. **D.** Glucose uptake in GLUTag-NLuc cells under the influence of quercetin and luteolin. Phloretin (100 µM) and forskolin (5 µM) were used as positive controls. Data are presented as mean ± SD (n = 5); ANOVA followed by Tukey's post-hoc test comparing each compound with the control (DMSO). **E.** Luciferase activity measured in the presence of quercetin and mitochondrial respiratory chain inhibitors (sodium azide, 2 mM) or ATP synthase inhibitor (oligomycin, 2 µM). Data are presented as mean ± SD (n = 5); ANOVA followed by Tukey's post-hoc test comparing control with inhibitors and quercetin with quercetin plus inhibitors. **F.** ATP levels measured in 16.8 mM glucose in the presence of quercetin and mitochondrial respiratory chain inhibitors (sodium azide, 2 mM) or ATP synthase inhibitor (oligomycin, 2 µM). Data are presented as mean ± SD (n = 5); ANOVA followed by Tukey's post-hoc test comparing control with inhibitors and quercetin with quercetin plus inhibitors. Adapted from (Anghel et al., 2022). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Part II

Signaling mechanism of GPR27, a novel adrenergic and atypical receptor

1. INTRODUCTION

G protein-coupled receptors (GPCRs) constitute one of the largest and most essential families of cell membrane receptors, with ubiquitous expression and involvement in nearly all physiopathological processes (Lefkowitz, 2007). Their canonical activation involves interaction with heterotrimeric G proteins, composed of three subunits (α, β, γ), which exhibit intrinsic GTPase activity. Based on the effectors involved and the type of α subunit, G proteins are classified into Gαs, Gαi/o, Gαq, and Gα12/13 (Figure 4) (Milligan, 2006).

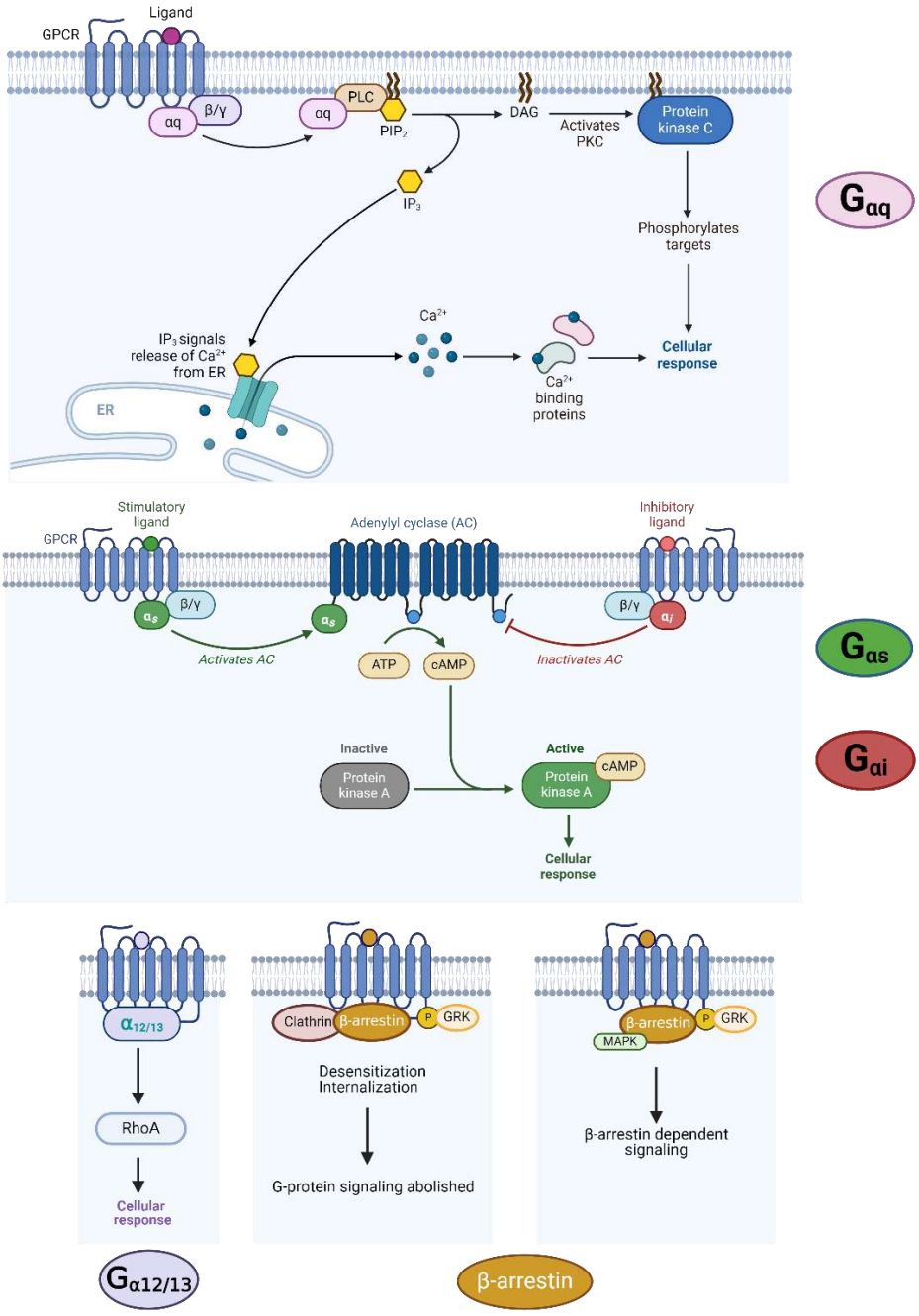

Figure 4. GPCR signaling pathways (Jean-Charles et al., 2017, Milligan, 2006). Created in BioRender. Anghel, S. (2025) https://BioRender.com/x53t279.

The human GPCR-ome consists of over 800 members (including sensory receptors), though some of them are classified as orphan receptors, as their endogenous ligand and function remain unknown (Tang et al., 2012). Given their ubiquitous expression and significance as therapeutic targets, orphan receptors represent an unexplored frontier in pharmacology that demands further investigation (Hauser et al., 2017).

GPR27 (SREB1) belongs to the SREB (Superconserved Receptors Expressed in the Brain) subfamily, alongside GPR85 (SREB2) and GPR173 (SREB3), which are classified as class A (Rhodopsin-like) receptors and are considered orphans. These three receptors share 52-63% sequence similarity and also exhibit approximately 25% identity with aminergic receptors (O'Dowd et al., 1998, Matsumoto et al., 2000). To emphasize the receptor's versatility and explore its potential functions and known pharmacology, a literature search was conducted across multiple databases (Web of Science, PubMed, and Scopus) using "GPR27" and "SREB1" as keywords. Only original research articles were selected for analysis (Table 1-1 from the full doctoral thesis). After removing duplicates and excluding articles that did not specifically address GPR27, 27 studies were analyzed. Among them, 3 focused on the conservation and evolution of GPR27 (Ribrioux et al., 2008, Breton et al., 2021, Breton et al., 2023), 8 investigated its pharmacology, including potential ligands and signaling pathways (Martin et al., 2015, Yanai et al., 2016, Hossain et al., 2016, Dupuis et al., 2017, Jin et al., 2018, Pillaiyar et al., 2021, Pillaiyar et al., 2023, Kaafarani et al., 2023), 5 explored its role in metabolism (insulin production, insulin sensitivity, lipid, and glucose homeostasis) (Ku et al., 2012, Chopra et al., 2020, Nath et al., 2020, Dolanc et al., 2022, Rutkove et al., 2023), 5 examined its involvement in cancer (Hallett et al., 2012, Lando et al., 2015, Wang et al., 2022, Pan and Gao, 2023, Cai et al., 2024), 3 reported the consequences of gene deletions in the GPR27 region (Petek et al., 2003, Pariani et al., 2009, Oh et al., 2023), and 3 provided evidence of its role in inflammatory states, including allergic reactions (Livingston et al., 2017, Sharma et al., 2023, Zhang et al., 2023). These findings indicate that GPR27 participates in a wide range of cellular processes, consistent with its widespread expression pattern. However, despite the identification of several potential ligands for GPR27 (Dupuis et al., 2017, Pillaiyar et al., 2021, Pillaiyar et al., 2023, Yanai et al., 2016), it is still considered an orphan receptor. However, it may recruit β-arrestin 2 through a ligand-dependent mechanism (Dupuis et al., 2017, Pillaiyar et al., 2021, Pillaiyar et al., 2023), but it can also couple Gq proteins (Ku et al., 2012) or Gi proteins (Martin et al., 2015), in a ligand independent manner. At the same time, a markedly inconsistent result is observed regarding its potential role in cancer, as its expression has been associated with both increased and decreased risk, as well as improved or reduced survival, depending on the cancer type. At the same time, data from The Cancer Genome Atlas Program (The Cancer Genome Atlas Program) (https://portal.gdc.cancer.gov/) indicate that GPR27 is more frequently

associated with loss-of-function rather than gain-of-function mutations in various cancers (The Cancer Genome Atlas Program). This suggests that GPR27 may be inactivated in cancer cells, possibly due to a potential protective role. Furthermore, its involvement in metabolism highlights the importance of its deorphanization and characterization, as it could serve as a pharmacological target promoting glucose homeostasis by modulating insulin secretion and/or action.

2. MATERIALS AND METHODS

The functional characterization of the GPR27 receptor was conducted through heterologous expression in the HEK293T cell line. Initially, its presence at the plasma membrane was confirmed using a whole-cell ELISA assay. Subsequently, reverse pharmacology approaches and the generation of chimeric constructs led to the identification of its ligand. By employing integrative methods, including assays detecting the activation of transcription factor response elements (Cheng et al., 2010), a panel of inhibitors, and a β-arrestin recruitment assay based on Firefly luciferase complementation (Dupuis et al., 2017), the signaling pathway of the GPR27 receptor was elucidated. The role of GPR27 in insulin secretion was evaluated by knocking down the receptor in the INS1E insulinoma cell line and measuring insulin secretion using an HTRF assay kit (Revvity).

3. RESULTS AND DISCUSSIONS

By using a whole-cell ELISA, the expression of GPR27 was determined and cofirmed its plasma membrane localization (Figure 5A). Using a cytosolic cAMP-sensitive probe (the Glosensor-22F system), we tested the potential constitutive activity of receptors belonging to the SREB family (Figure 5B). GPR27 and GPR173 reduce both basal and forskolin-stimulated cAMP levels, with GPR27 showing a stronger inhibition, suggesting coupling with Gi/o proteins. However, using specific inhibitors that ADP-ribosylate Gi/o family proteins and block GPCR-protein G interactions, namely pertussis toxin (PTX, Figure 5C) (Locht and Antoine, 1995) or OZITX (Figure 5D) (Keen et al., 2022), GPR27 inhibitory effect was not modified.

Next, we constructed chimeric receptors to characterize the orphan receptor GPR27: a) by restriction enzyme cloning, a truncated version of the GPR27 receptor was generated in which

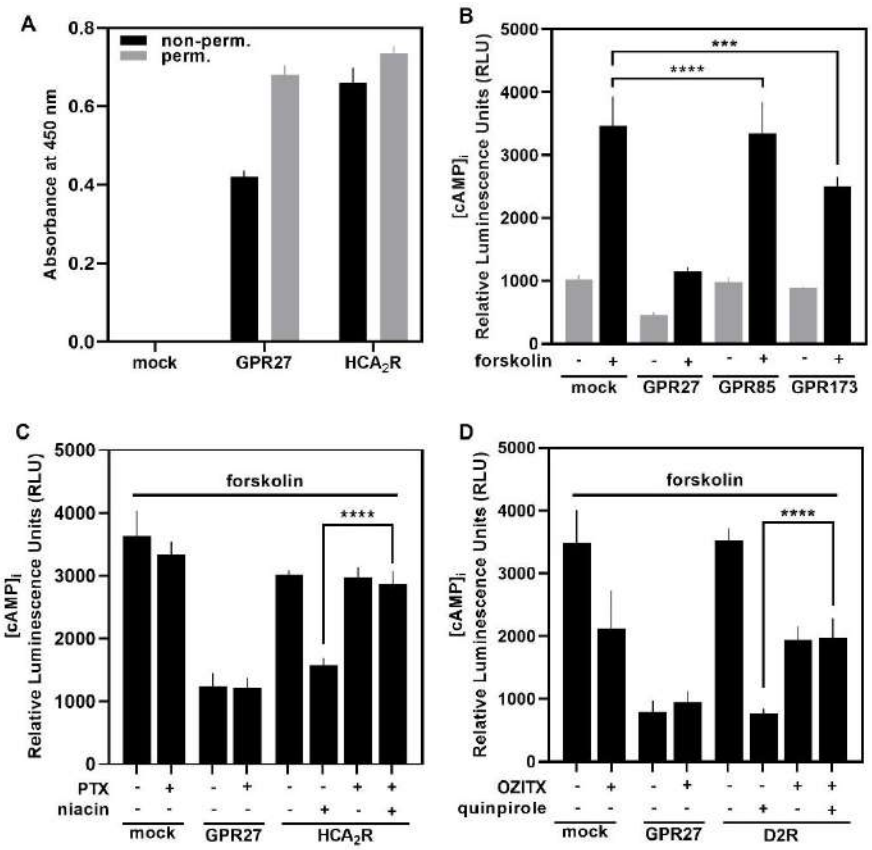

Figure 5. GPR27 decreases cAMP intracellular level without involving Gi/o proteins. A. Whole-cell ELISA in mock-transfected and GPR27- and HCA2R-expressing cells. **B.** The cAMP level determined by SREBs family in the absence/presence of foskolin (10 µM). **C., D.** cAMP levels measured in GPR27-expressing cells in the presence of PTX (Gi/o inhibitor, w/o w/o Gαz, 100 ng/mL) (**C**), respectiv OZITX (GαO, GαZ and Gαi inhibiting ToXin (Keen et al., 2022) (**D**). The HCA2R receptor (niacin receptor) was used as a positive control in the PTX experiment, while the D2 receptor was used in the OZITX experiment. Data are presented as mean ± SD (n = 4-6); ANOVA followed by Tukey's post-hoc test comparing the signal determined by each receptor with mock in the presence of forskolin (**A**) or the signal given by ligands minus/plus toxins (**C**, **D**). ***p<0.001, ****p<0.0001.

the C-terminal domain is absent (GPR27ΔCt), b) the C-terminal domain of the β1-adrenergic receptor (β1AR) was replaced with that of GPR27, resulting in the β1AR-GPR27 chimeric receptor, and c) several other receptor chimeras were synthesized replacing the C-terminal domain of the GPR27 receptor with the homologous domain of other known receptors, resulting in the following chimeric receptors: GPR27-β1AR, GPR27-α1AR, GPR27-β2AR, GPR27-M1,

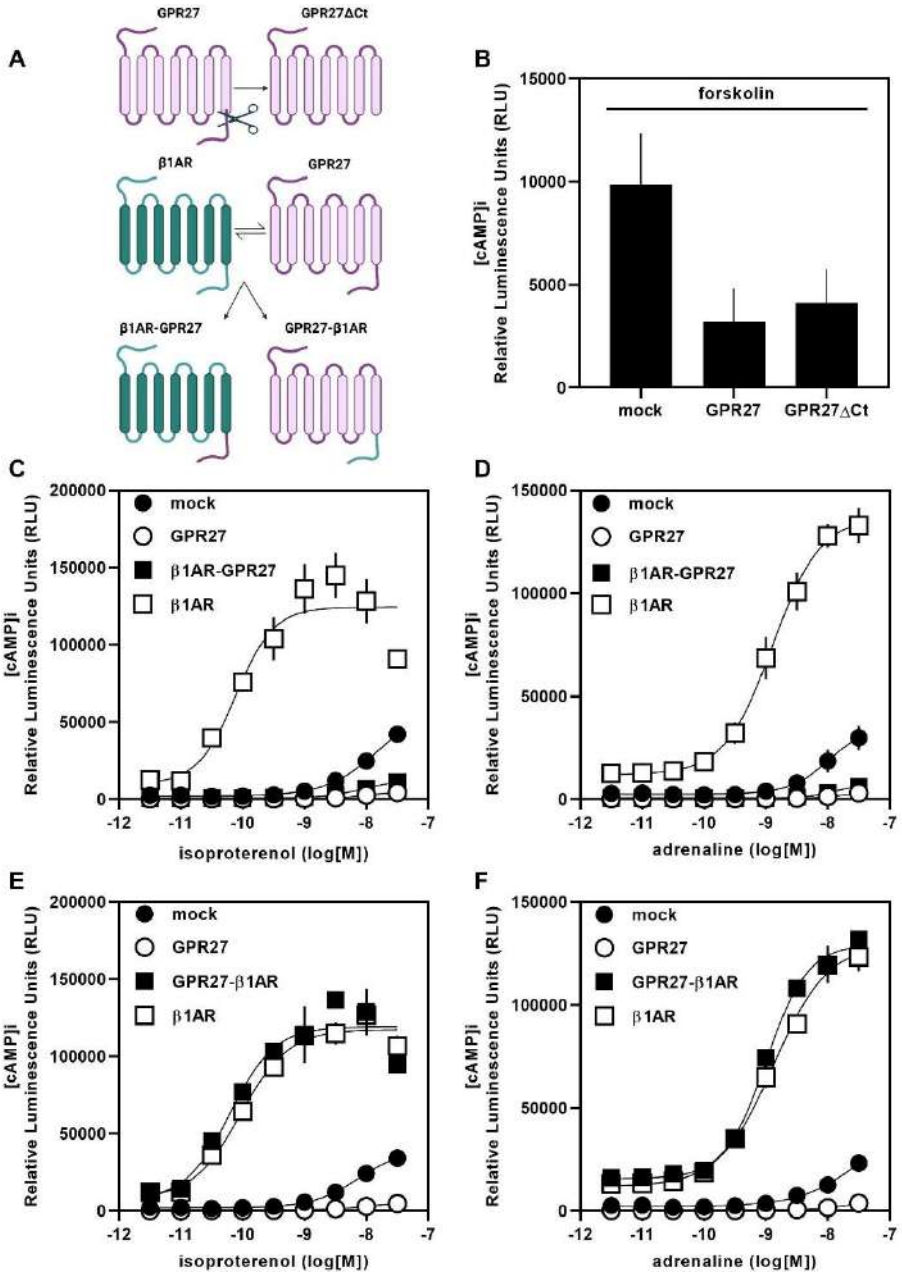

Figure 6. The chimeric GPR27-β1AR receptor responds to adrenergic ligands. **A.** Schematic representation of the truncated GPR27 receptor (GPR27ΔCt) and the chimeric receptors β1AR-GPR27 and GPR27-β1AR. Created in BioRender. Anghel, S. (2025) https://BioRender.com/a27p278. **B.** cAMP levels determined in mock, GPR27-expressing cells, and GPR27ΔCt-expressing cells. **C.**, **D.** cAMP levels induced by isoproterenol (**C**) and adrenaline (**D**) in cells expressing the β1AR-GPR27 chimera. **E.**, **F**. cAMP levels induced by isoproterenol (**E**) and adrenaline (**F**) in cells expressing the GPR27-β1AR chimera. Data are presented as mean ± SD (n = 3).

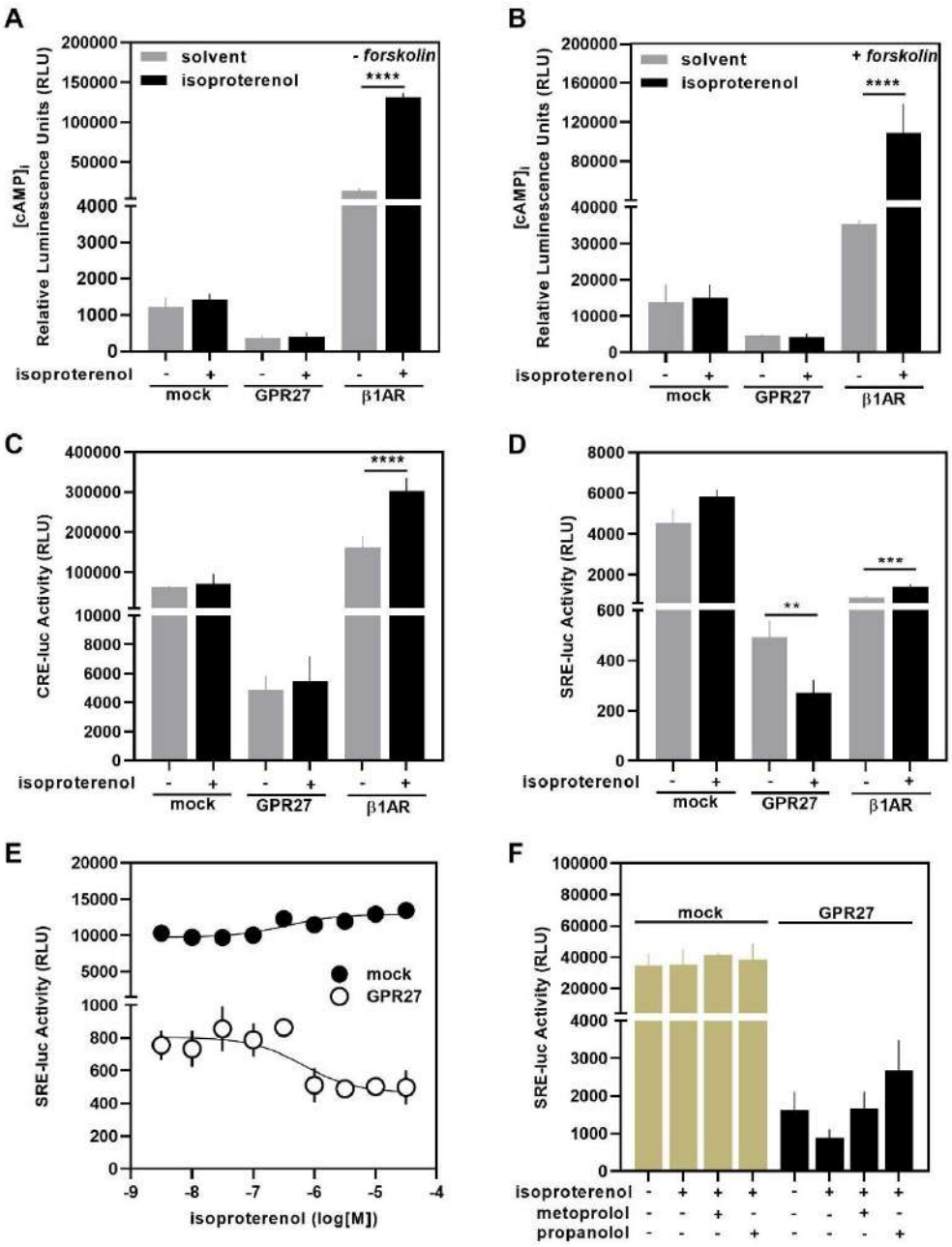

Figure 7. GPR27 receptor inhibits SRE activity in the presence of isoproterenol. A., **B.** cAMP levels induced by isoproterenol (1 nM) in the absence (**A**) or presence of forskolin (10 μM) (**B**) in cells transfected with an empty vector (mock), GPR27, or the β1-adrenergic receptor. **C.** CRE activity modulated by isoproterenol (1 nM) in cells expressing GPR27 or the β1AR receptor. **D.** SRE activity induced by isoproterenol (1 μM) in cells expressing GPR27 or the β1AR receptor**. E.** SRE activity determined by increasing concentrations of isoproterenol in mock-transfected and GPR27-expressing cells. **F.** SRE activity induced by isoproterenol (1 μM) and β-adrenergic antagonists (metoprolol, propranolol 10 μM) in GPR27-expressing cells.

Data are presented as mean ± SD (n = 4-6); Student's t-test compares the signal induced by each receptor in solvent versus in the presence of isoproterenol (**A**, **B**, **C**, **D**). **p<0.01, ***p<0.001, ****p<0.0001.

and GPR27-GPR75 (Figure 6A). GPR27 and the truncated version, GPR27ΔCt, induced similar cAMP levels (Figure 6B). The C-terminal domain of GPR27 within the β1AR-GPR27 chimera significantly reduces the cAMP signal in the presence of adrenergic ligands (isoproterenol and adrenaline), compared to the β1AR receptor, possibly due to the disruption of coupling with Gs proteins (Figure 6C, 6D). The GPR27-β1AR chimeric receptor was then used to test a library of FDA-approved compounds (TargetMol) consisting of 1403 substances, using the GloSensor-22F system (Table 3-1 full doctoral thesis). The screening revealed several findings: 1) similar to the β1-AR receptor, a constitutive activity was recorded, 2) numerous ligands were identified that increased the cAMP level, suggesting coupling with Gs proteins, 3) most of these ligands were aminergic, particularly adrenergic, 4) any activator of the GPR27-β1AR chimera was also an activator of the β1AR receptor, and 5) the concentration-response curves for isoproterenol (Figure 6E) and adrenaline (Figure 6F) exhibited similar patterns in cells expressing the β1AR receptor versus those expressing the GPR27-β1AR chimera. Thus, in terms of intracellular cAMP levels, the β1AR-GPR27 chimera behaves like GPR27, while the GPR27-β1AR chimera is like the β1-AR receptor. The other obtained chimeras did not elicit a response to isoproterenol.

GPR27 did not alter cAMP levels with isoproterenol (1 nM) in neither Gs mode (Figure 7A) or Gi mode (Figure 7B). Thus, only in the presence of the C-terminal domain of the β1-AR receptor, GPR27 can signal by recruiting Gs proteins, leading to the stimulation of cAMP formation (Figure 6E). To overcome the limitations imposed by measuring secondary messengers resulting from GPCR activation, we opted for a more integrative method of signaling pathway analysis, namely, the activation of response elements by transcription factors (Cheng et al., 2010). Isoproterenol activates cAMP response elements (CRE) only in cells expressing the β1AR receptor, not in GPR27-expressing cells (Figure 7C). In contrast, isoproterenol (1 μM) inhibits the activation of serum response elements via GPR27 (Figure 7D). Similarly, the inhibition of SRE activity induced by isoproterenol in GPR27-transfected cells is concentration-dependent (Figura 7E). Beta-adrenergic antagonists, metoprolol (β1-selective) and propranolol (non-selective), reverse the inhibition of SRE activity induced by isoproterenol through GPR27, providing further evidence that GPR27 is an adrenergic receptor (Figure 7F).

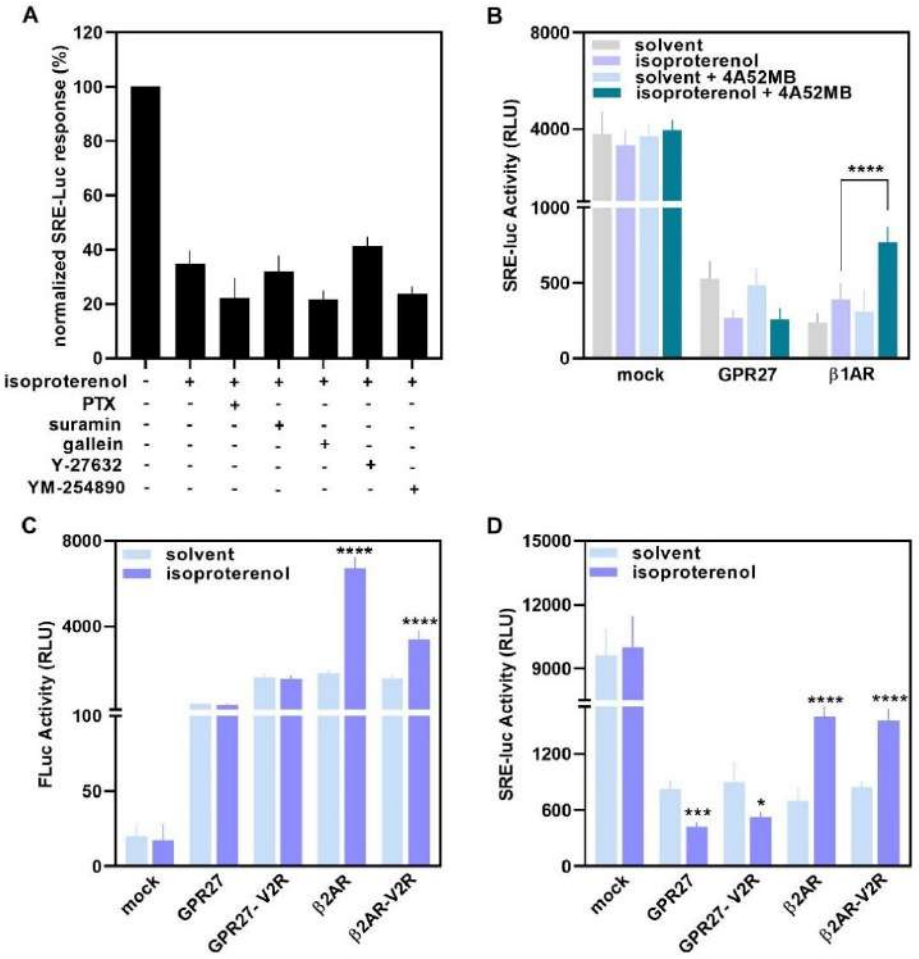

Figure 8. Inhibition of SRE activity by isoproterenol through the GPR27 receptor is independent of G proteins and β-arrestin. A. SRE activity induced by isoproterenol in the presence of G protein inhibitors, namely PTX (100 ng/mL), suramin (100 µM), gallein (100 µM), Y-27632 (10 µM), and YM-254890 (100 nM) in cells expressing GPR27. **B.** SRE activity induced by isoproterenol in the presence of the GRK inhibitor (4A52MB, 100 µM) in cells expressing GPR27 or β1AR receptor. **C.** Firefly luciferase activity in the complementary assay in the presence of isoproterenol on mock cells or cells expressing GPR27-FLuc, GPR27-V2R-FLuc, β2AR-FLuc, β2AR-V2R-FLuc, and FLuc-β-arrestin 2. **D.** SRE activity determined by isoproterenol in cells expressing the corresponding receptors from the firefly luciferase complementary assay in (**C**). Data are presented as mean ± SD (n = 4-6); ANOVA followed by Tukey post-hoc test comparing the signal determined by each receptor in the presence of the GRK inhibitor (4A52MB) minus/plus isoproterenol (**B**). Student's t-test compares the signal determined by each receptor in the absence of isoproterenol (solvent) with that induced by isoproterenol (**C**, **D**). *p<0.05, ***p<0.001, ****p<0.0001.

The classical signaling pathways of GPCRs involve both G protein-dependent and G protein-independent pathways governed by β-arrestin, although the contribution of G proteins in β-arrestin-dependent signaling is still a subject of debate (Grundmann et al., 2018). The inhibition of SRE activity induced by isoproterenol can be mediated by G proteins; their involvement was probed pharmacologically using specific inhibitors (Figure 8A). PTX (Gi/o inhibitor (Locht and Antoine, 1995)), suramin (blocks the receptor-G protein coupling (Chung and Kermode, 2005)), gallein (Gβγ inhibitor (Karuppagounder et al., 2018)), Y-27632 (ROCK inhibitor – G12/13 pathway) (Siehler, 2009)) și YM-254890 (Gq inhibitor (Nishimura et al., 2010)) failed to reversed isoproterenol-induced inhibition of SRE activity mediated by GPR27 (Figure 8A). The phosphorylation of the receptor by GRKs precedes the recruitment of β-arrestin (Jean-Charles et al., 2017). In the presence of a non-selective inhibitor for GRKs (Wang et al., 2016), the activation of SRE by isoproterenol in the case of β1AR is increased due to the blocking of receptor internalization, while the effect of GPR27 is being preserved (Figure 8B). The involvement of β-arrestin 2 was also investigated through a luciferase complementation assay (Dupuis et al., 2017). Additionally, two other chimeric receptors, GPR27-V2R and β2AR-V2R, were used (C-terminal domain replaced by that of vasopressin 2 (V2R) is rich in phosphorylation sites, thus favoring the recruitment of β-arrestin) (Dupuis et al., 2017). While β2AR and β2AR-V2R recruit β-arrestin 2 in the presence of isoproterenol, GPR27 and GPR27-V2R do not interact with it (Figure 8C). At the same time, the GPR27-V2R chimera behaves like the GPR27 receptor in terms of SRE activity (Figure 8D).

Another important signaling pathway of GPCRs is the interaction with receptor tyrosine kinases (RTKs). Specifically, the term transactivation is often found in the literature – the activation of GPCRs by a ligand leads to the activation of RTKs, a phenomenon that can also be reciprocal (RTKs can lead to GPCR activation) (Delcourt et al., 2007). The first receptor tyrosine kinase reported to be transactivated by a GPCR is the epidermal growth factor receptor (EGFR) (Daub et al., 1996) which promoves cell survival, proliferation and cell migration (Arteaga and Engelman, 2014). Isoproterenol (1 μM) inhibits the EGF-induced SRE activation in GPR27-expressing cells (Figure 9A). Moreover, the inhibition caused by isoproterenol (Figure 9B) or adrenaline (Figure 9C) is concentration-dependent. In the presence of erlotinib, an EGFR tyrosine kinase inhibitor (Anghel et al., 2024), the isoproterenol-induced SRE inhibition

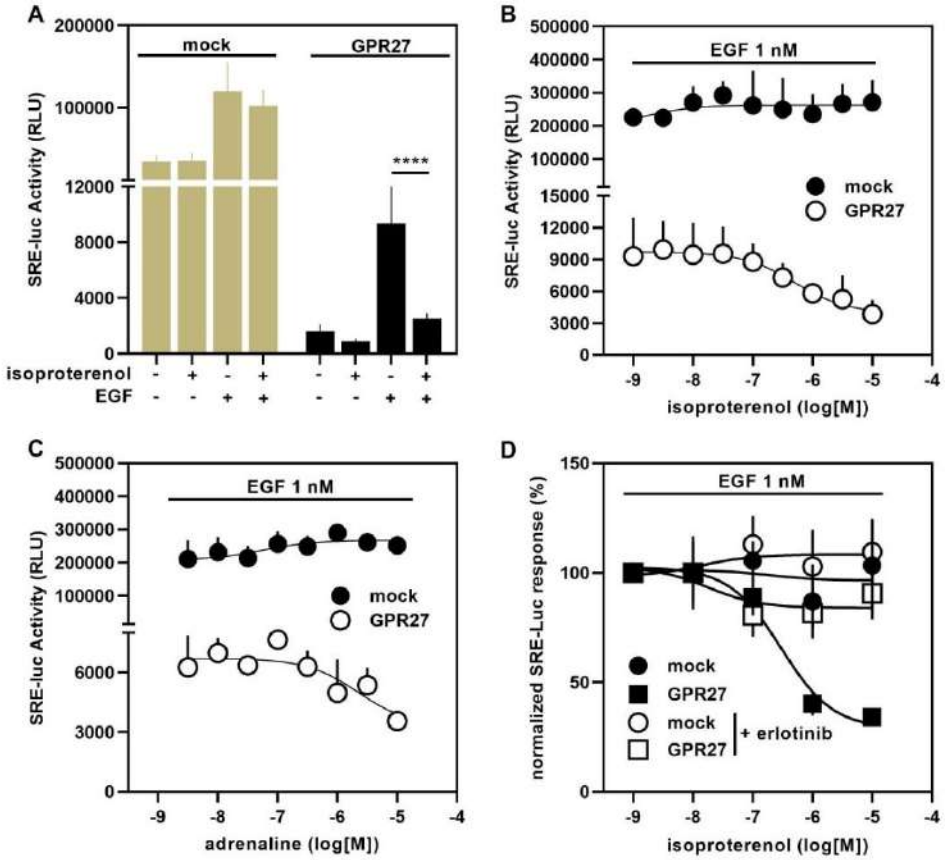

Figure 9. Isoproterenol and adrenaline inhibit epidermal growth factor (EGF)-induced SRE activity via GPR27. A. SRE activity induced by EGF (1 nM) and isoproterenol (1 μM) in cells transfected with an empty vector (mock) and cells expressing GPR27. **B., C.** Concentration-response curve of isoproterenol (B) or adrenaline (C) in the presence of 1 nM EGF in both cells transfected with an empty vector (mock) and those transfected with GPR27. **D.** SRE activity induced by EGF and increasing concentrations of isoproterenol in the absence or presence of erlotinib (10 μM) in mock cells and cells expressing GPR27. Data are presented as mean ± SD (n = 6); ANOVA followed by Tukey post-hoc test comparing the signal determined by the presence of EGF minus/plus isoproterenol (**A**). ****p<0.0001.

of SRE activity through GPR27 is abolished (Figure 9D). Therefore, it can be concluded that GPR27 transinhibits the EGFR receptor, a phenomenon rarely described in the literature as transinactivation (Duchene et al., 2002).

The chimeric receptors β1AR-GPR27 and GPR27-β1AR, as well as the truncated version of the GPR27 receptor (GPR27ΔCt) modulate SRE activity (Figure 10A) in accordance with their effects on cAMP levels (Figure 6B, C, and E). In the case of the β1AR receptor, the

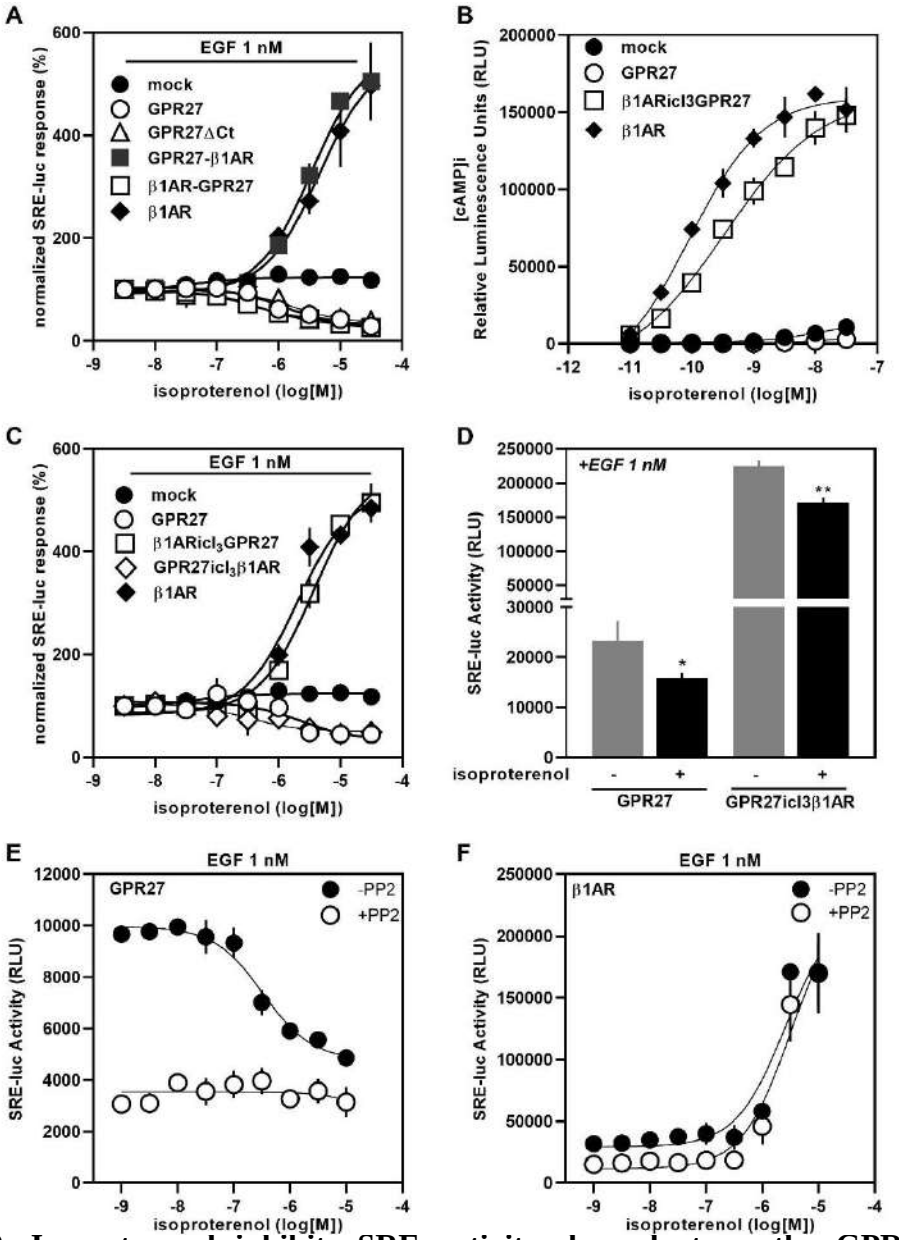

Figure 10. Isoproterenol inhibits SRE activity dependent on the GPR27 receptor, involving a kinase from the Src family. A. SRE activity induced by progressively increased concentrations of isoproterenol in the presence of EGF in cells expressing GPR27, the truncated version of the GPR27 receptor (GPR27ΔCt), β1AR receptor, and the chimeric receptors β1AR-GPR27 and GPR27-β1AR. **B.** cAMP levels generated by progressively increasing concentrations of isoproterenol in cells expressing GPR27, β1ARicl3GPR27, and β1AR. **C.** SRE activity induced by progressively increasing concentrations of isoproterenol in the presence of EGF, in cells expressing GPR27, the β1AR receptor, and the chimeric receptors β1ARicl3GPR27 and GPR27icl3β1AR. **D.** SRE activity induced by isoproterenol (1 μM) in

cells expressing GPR27 and the chimeric receptor GPR27icl3β1AR (raw data from **C**). **E., F.** SRE activity induced by progressively increasing concentrations of isoproterenol in the presence of PP2 (10 μM) and EGF (1 nM) in cells expressing GPR27 (**E**) and cells expressing the β1AR receptor (**F**). Data are expressed as mean ± SD (n = 3). Student's t-test compares the signal determined by each receptor minus/plus isoproterenol (**D**). *p<0.05, **p<0.01.

third intracellular loop (ICL3) has been shown to be essential for coupling the receptor with Gs proteins (Qiu et al., 2024). Thus, the ICL3 of the β1AR receptor was replaced with that of the GPR27 receptor, creating the chimeric receptor β1ARicl3GPR27. In the presence of increased concentrations of isoproterenol, the level of cAMP produced by the chimeric receptor β1ARicl3GPR27 is similar to that induced by the β1AR receptor, with only a slight rightward shift of the curve, indicating a minor reduction in functionality (Figure 10B). Therefore, it can be considered that the ICL3 region of GPR27 is compatible with that of the β1AR receptor. We generated the reciprocal chimeric receptor, GPR27icl3β1AR (GPR27 receptor with the ICL3 of the β1AR receptor). The SRE activity induced by this chimera is similar to that of the GPR27 receptor (Figure 10C). Furthermore, the similarity between the β1ARicl3GPR27 chimera and β1AR is once again noted. However, the level of SRE activity produced by the GPR27icl3β1AR chimera is 10 times higher than that induced by GPR27 (Figure 10D). Consequently, the ICL3 of β1AR causes a reversal of the inhibitory effect of GPR27.

The data presented so far reveal two important characteristics of GPR27 signaling: 1) it inhibits SRE activity in the presence of isoproterenol independent of G proteins or β-arrestin, and 2) it transinhibits EGFR. However, what is the underlying mechanism of this phenomenon? The inhibition of EGFR by GPR27 through isoproterenol may be explained by a mechanism of EGFR inactivation, specifically through dephosphorylation mediated by a tyrosine phosphatase (Ceresa and Vanlandingham, 2008) or by the inhibition of potential phosphorylation triggered by a kinase. Direct interaction between a GPCR and Src kinases, it occurs through the C-terminal domain or ICL3, provided that these regions contain proline clusters (Berndt and Liebscher, 2021). The ICL3 of GPR27 has proline clusters, which is why we chose to test an Src kinase inhibitor, PP2 (10 μM). The inhibition induced by isoproterenol, in the presence of EGF, dependent on GPR27 was abolished by PP2 (Figure 10E). Similarly, treatment with PP2 on cells expressing the β1 adrenergic receptor did not produce a significant effect (Figure 10F).

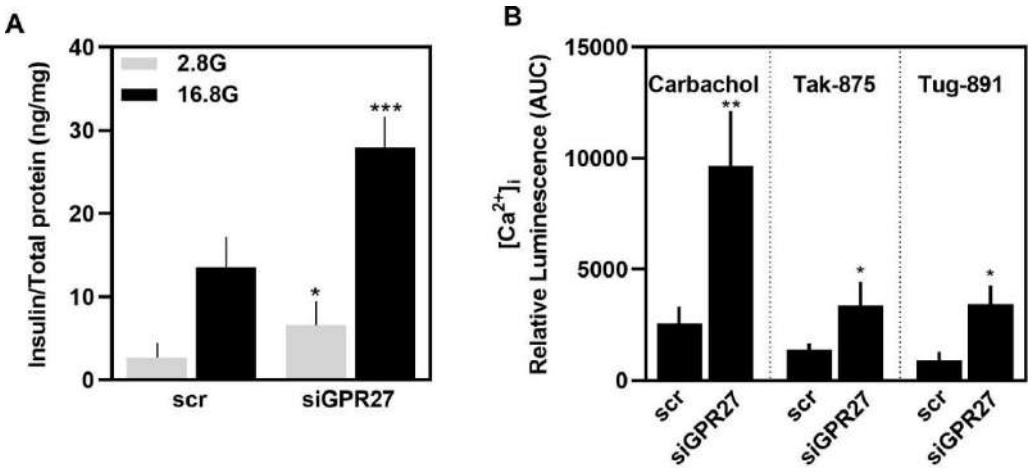

Figure 11. GPR27 knockdown in INS1E cells enhances glucose-stimulated insulin secretion. A. Glucose-stimulated insulin secretion under two conditions – 2.8G (2.8 mM glucose) and 16.8G (16.8 mM glucose) in cells transfected with scrambled RNA (scr) or with siRNA specific for the GPR27 receptor (siGPR27). **B.** Intracellular calcium levels determined by carbachol (muscarinic acetylcholine receptor ligand), Tak-875 (fatty acid receptor 1, FFAR1 ligand), and Tug-891 (fatty acid receptor 4, FFAR4 ligand) in cells transfected with scr or siGPR27. Data are presented as mean ± SD (n = 4-6). Student's t-test compares insulin secretion in cells transfected with scr versus those with siGPR27 at 2.8 mM glucose (2.8G) and 16.8 mM glucose (16.8G) (**A**); Student's t-test compares the signal determined by carbachol, Tak-875, or Tug-891 in cells transfected with scr versus those with siGPR27 (**B**). *p<0.05, **p<0.01, ***p<0.001.

Furthermore, the reduction of GPR27 receptor expression in INS1E cells led to an amplification of glucose-dependent insulin secretion (Figure 11A). Additionally, silencing the GPR27 receptor enhances the activity of receptors involved in glucose-stimulated insulin secretion. (Figure 11B). These data align with previously obtained results, further highlighting the inhibitory effect of GPR27.

Final Conclusions

The first part of this study highlights the **discovery of novel natural secretagogues of the intestinal hormone GLP-1**, based on the following observations:

- The luciferase-based GLP-1 secretion assay is fast and efficient.
- GLUTag-NLuc cells recapitulate the physiological secretion of GLP-1.

- The newly identified secretagogues cover a wide range of natural compounds, predominantly flavonoids. These are found in numerous fruits and vegetables (onion, tomato, apple, berries).
- Out of the library of 173 compounds, only one showed toxicity, namely dioscin (a saponin).
- The identified secretagogues function only in the presence of extracellular calcium (proven by calcium removal from the medium and by using the L-type calcium channel inhibitor, nifedipine).
- Quercetin, one of the most potent flavonoids identified, functions only in the presence of glucose, involving two glucose transporters, SGLT1 and GLUT1. Pharmacological blocking of these transporters resulted in a decrease in GLP-1 secretion induced by quercetin.
- Quercetin inhibits glucose absorption in cells, as demonstrated by a non-radioactive glucose uptake assay.
- Quercetin stimulates glucose-induced GLP-1 secretion by increasing ATP levels, suggesting an enhancement of glucose metabolism as the primary effect (Anghel et al., 2022).

The second part of this study demonstrates the **inclusion of the GPR27 receptor in the adrenergic receptor family** and illustrates one of the contemporary challenges of deorphanization – namely, **the unconventional, atypical signaling of certain GPCRs**. The key findings of this study are:

- Heterologous expression of the GPR27 receptor in the HEK293T cell line decreases cAMP levels in the absence of a ligand and independently of Gi protein activity.
- Chimeric receptors were constructed by replacing the C-terminal domain of GPR27 with the C-terminal domain of other known receptors to determine the importance of the C-terminal region in cellular signaling.
- cAMP and SRE assays demonstrated the similarity between GPR27 and the chimeric receptor β1AR-GPR27 (the β1-adrenergic receptor with the C-terminal domain of GPR27), both having an inhibitory effect. On the other hand, similarity was observed between β1AR and the chimeric receptor GPR27-β1AR (GPR27 with the C-terminal domain of β1AR), both showing a stimulatory effect in the presence of adrenergic ligands (adrenaline and isoproterenol).

- A library of 1,403 FDA-approved compounds was tested by determining cAMP levels in cells expressing the chimeric receptor GPR27-β1AR. The main identified ligands belong to the aminergic class, with adrenergic compounds standing out.
- The C-terminal domain of the β1-adrenergic receptor was identified as critical for the observed effect in the GPR27-β1AR chimeric receptor since chimeric GPR27 receptors containing the C-terminal domain of other receptors (GPR27-α1AR, GPR27-β1AR, GPR27-β2AR, GPR27-M1, GPR27-GPR75) do not respond to isoproterenol.
- The C-terminal domain of the β1-adrenergic receptor does not confer responsiveness to isoproterenol to other receptors, as demonstrated by the functional analysis of the chimeric receptors GPR75-β1AR and GPR173-β1AR, which had their C-terminal domain replaced with that of β1AR.
- The ICL3 region of the GPR27 receptor is compatible with the ICL3 region of the β1AR receptor, and the reverse is also true.
- Adrenergic ligands do not induce changes in secondary messengers such as cAMP and calcium, convincingly demonstrating that GPR27 does not function through G protein activation (Gi, Gs, Gq/11).
- Through an integrative functional analysis strategy based on measuring the activity of transcription factor response elements, it was successfully shown that GPR27 receptor stimulation induces a relatively new cellular effect, sparsely documented in the scientific literature, namely transinhibition of RTK function, such as EGFR.
- Reduced expression of the GPR27 receptor leads to increased glucose-stimulated insulin secretion.

GPR27 is an adrenergic receptor with atypical signaling, having the potential to open a new research field for unconventional GPCRs. Given the absolute novelty of this discovery and considering the complexity of the phenomenon, the in vivo analysis of the GPR27/adrenergic ligand pair will continue after the completion of the doctoral program.

List of published papers

1. **Anghel, S. A.,** Badea, R. A., Chiritoiu, G., Patriche, D. S., Alexandru, P. R., & Pena, F. (2022). Novel luciferase-based glucagon-like peptide 1 reporter assay reveals naturally occurring secretagogues. British journal of pharmacology, 179(19), 4738–4753. https://doi.org/10.1111/bph.15896, **IF = 9.47 , Q1, AIS = 1.62**

2. Trif, C., Banica, A. M., Manolache, A., **Anghel, S. A.,** Huţanu, D. E., Stratulat, T., Badea, R., Oprita, G., Selescu, T., Petrescu, S. M., Sisignano, M., Offermanns, S., Babes, A., & Tunaru, S. (2024). Inhibition of TRPM8 function by prostacyclin receptor agonists requires coupling to Gq/11 proteins. British journal of pharmacology, 181(9), 1438–1451. https://doi.org/10.1111/bph.16295, **IF = 6.8 , Q1, AIS = 1.682**

3. **Anghel, S. A.,** Dinu-Pirvu, C. E., Costache, M. A., Voiculescu, A. M., Ghica, M. V., Anuța, V., & Popa, L. (2024). Receptor Pharmacogenomics: Deciphering Genetic Influence on Drug Response. International journal of molecular sciences, 25(17), 9371. https://doi.org/10.3390/ijms25179371, **IF = 4.9, Q1, AIS = 1.053**

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