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A Perspective On A Promising Role For Caffeine In Modulating The Calcium-Sensing Receptor In Pancreatic Beta-Cells.

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Abstract:
The extracellular calcium-sensing receptor (CaSR) is a G-protein-coupled receptor (GPCR) that plays a crucial role in the parathyroid glands and kidneys in maintaining calcium (Ca^{2+}) homeostasis. The extracellular CaSR is present in various cell types not implicated in controlling plasma Ca^{2+} levels. There is accumulating evidence that CaSR plays a significant role in regulating the function of β-cells in the pancreas. Divalent cations and insulin are released together during exocytosis, and their concentration in the restricted intercellular compartments of the pancreatic islet increases to activate CaSR in neighboring cells. Subsequently, the activated CaSR stimulates insulin vesicle release in the adjacent cells, and by repeating these steps, the signal is amplified in the whole islet. Recent work developed a photoaffinity-tagged glutathione derivative (DAZ-G) as a chemical tool. The study demonstrated that DAZ-G can identify and analyze CaSR ligands. The use of DAZ-G identified caffeine as a positive allosteric modulator of the CaSR, suggesting that the physiological effects of caffeine are partly attributed to its stimulation of the CaSR. Future work may utilize caffeine to activate the CaSR in pancreatic β-cells to improve their secretory function.

Keywords: *Calcium-sensing receptor, Caffeine, photoaffinity-tagged glutathione, pancreatic β-cells*

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I. The Calcium-Sensing Receptor (CaSR) And Calcium Homeostasis:

The CaSR belongs to a class C G protein-coupled receptors (GPCRs). CaSR comprises a homodimer that represents its extracellular domain (ECD). The ECD is comprised of the "Venus flytrap (VFT)" domain, which consists of two lobes (LB1 and LB2), as well as the cysteine-rich domain (CRD). VFT and CRD are linked to the seven transmembrane domains (7TMD) and the C-terminal tail (region). CaSR is highly expressed in the parathyroid glands and kidneys, which play a key role in maintaining calcium (Ca^{2+}) homeostasis [1].

CaSR was initially cloned in cells that secrete parathyroid hormone (PTH) from cows [2]. Once activated, the receptor reduces PTH secretion, leading to decreased renal reabsorption of Ca^{2+}, lowered intestinal Ca^{2+} absorption, reduced Ca^{2+} resorption from the bone, and diminished synthesis of 1, 25-dihydroxyvitamin D_3 (the active form of vitamin D) by the kidney. In addition, the activated CaSR promotes the release of the Ca^{2+}-lowering hormone "calcitonin" from C-cells in the thyroid [3]. The vital role of the CaSR in Ca^{2+} homeostasis was confirmed by studies on the functional mutations in the CaSR gene that lead to the loss or gain of functions, resulting in hypercalcemic and hypocalcemic disorders, respectively [4].

CaSR expression in different body tissues:

The expression of CaSR is not restricted to the parathyroid glands. It is also expressed in a wide variety of body tissues (elegantly reviewed in Squires et al., 2014) [5]. The expression of extracellular CaSR was detected for the first time on rodent and human pancreatic β-cells by the work of Rasschaert and Malaisse, 1999, and Squires et al., 2000, and Bruce et al., 1999, who evidenced the role of CaSR in monitoring Ca^{2+} in pancreatic juice and concluded that CaSR prevents calcium accumulation which may lead to the formation of calcium carbonate stones [6, 7, 8]. In gastrin-secreting cells, CaSR is involved in sensing the alterations in dietary Ca^{2+} [9].

The role of CaSR in pancreatic β-cells

A logical illustration of the CaSR's role comes from its ability to sense local alterations in Ca^{2+} levels and transmit this response to the adjacent cells in a paracrine fashion. This hypothesis was later elegantly confirmed by work in a kidney epithelium model where Ca^{2+} release from fibroblasts activated the CaSR on co-cultured HEK293 cells. Subsequently, HEK293 cells responded independently to the alterations in extracellular Ca^{2+} originating from adjacent fibroblasts [10]. Squires et al., 2014, suggested that glucose-evoked insulin secretion in one cell is linked to the simultaneous release of divalent cations (e.g., Ca^{2+} and Mg^{2+}). The Ca^{2+} in the intercellular space activates the CaSR of adjacent cells. Through this process, the response is amplified across the whole islet, which ultimately enhances β-cell-to-β-cell communication and improves the secretory function of the islet (**Figure 1**).

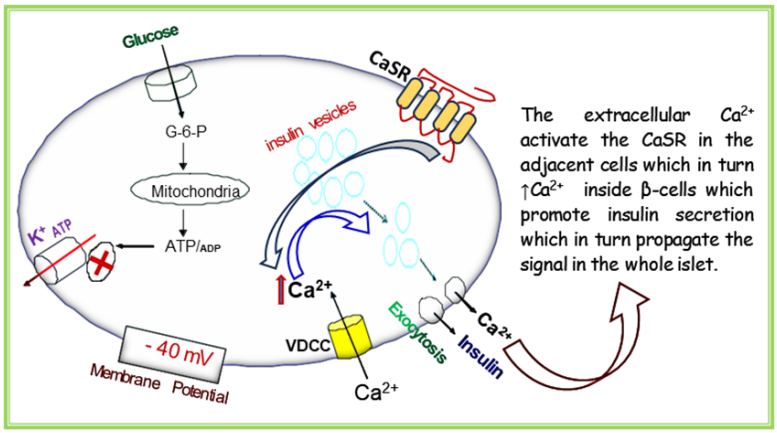

Figure 1: Schematic presentation shows glucose-evoked insulin secretion and the paracrine nature of the CaSR activation in a pancreatic β-cell. Ca^{2+} and insulin-containing vesicles (blue circles) in cells are secreted. The extracellular Ca^{2+}, in turn, activates the CaSR, which increases the intracellular Ca^{2+}, leading to enhanced insulin secretion. In the occurrence of these steps, the signal is amplified to include the whole islet. K^{+}_{ATP}: ATP-sensitive K^{+} channels. VDCC: voltage-dependent Ca^{2+} channel. G-6-P: Glucose-6-Phosphate. Glut-2: Glucose transporter.

Previous work demonstrated that an allosteric activation of CaSR using calcimimetic in a clonal mouse insulin-secreting cell line (MIN6) significantly enhanced E-cadherin expression. The CaSR-mediated increase in cadherin-mediated cell-to-cell contact has been beautifully confirmed using atomic force microscopy. The technique measured the magnitude of the intercellular adhesion forces provided by E-cadherin. Moreover, calcimimetic-mediated allosteric activation of the CaSR increased the expression of voltage-dependent calcium channels (VDCC) responsible for enhancing insulin secretion via increasing intracellular Ca^{2+} [11].

Moreover, the CaSR is implicated in regulating cellular proliferation as demonstrated by many studies involving various cell types. In fibroblasts, [12] found improvements in cell turnover triggered by low $[Ca^{2+}]_e$ (0.5 mM) that were considerably boosted by R568 (1 μM) activation of CaSR. The CaSR-driven proliferation occurs by stimulating the p42/44 MAPK pathway. This mechanism was demonstrated by the inhibition of MAPK using MEK inhibitor that caused a substantial reduction in cell growth. Chattopadhyay et al., 1999, also showed that CaSR stimulated increases in the turnover of proliferating astrocytoma cells. This increase in proliferation is caused by activating the nonselective-cation channels [13]. Moreover, using rodent osteoblastic cells, Yamaguchi et al., (2000) reported that the mechanism of CaSR-mediated increase in proliferation was believed to be caused by the p42/44 and p38 MAPK cascades [14].

The research studies on the proliferation of primary β-cells and islets faced a technical challenge owing to the low mitotic index of these cells. However, researchers used transformed cell lines, especially MIN6 cells, to overcome this issue. MIN6 cells are a cell line of choice for measuring cellular turnover because the turnover of MIN6 cells can be regulated by external stimuli [15, 16]. In MIN6 cells, the CaSR activation stimulated improvements in proliferation even in normal levels of extracellular Ca^{2+}, $[Ca^{2+}]_e$. Furthermore, increases in $[Ca^{2+}]_e$ can increase β-cell proliferation, and the mechanism involved stimulation of p38 and p42/44 MAP kinase pathways [17, 18]. Interestingly, the research work of Hills, Younis, and their colleagues in 2012 revealed that CaSR promotes cell-to-cell adhesion via increased expression of E-cadherin, positively impacting MEK-driven turnover of pancreatic β-cells.

Allosteric modulators of CaSR

For the CaSR to perform multiple physiological functions even in situations with reduced $[Ca^{2+}]_e$ concentrations, many intracellular allosteric regulators bind to multiple distinct sites on the receptor. Structural investigations demonstrated the presence of four Ca^{2+} binding sites located within ECD of the receptor. Geng et

al., 2016, and Huang et al., 2009, found that the binding of Ca^{2+} to these sites demonstrates cooperativity, meaning that the binding of Ca^{2+} to one site stimulates the binding of Ca^{2+} to the other sites. Similarly, Geng et al., (2016) explained how other divalent cations, such as Mg^{2+}, can bind to the ECD of CaSR [19].

The extracellular CaSR also binds to positive allosteric modulators (PAMs) including L-amino acids, especially aromatic amino acids, such as phenylalanine (Phe) and tryptophan (Trp). The amino acid binding site (ABS) is found in the cleft between the two lobes LB1 and LB2 of ECD. Large-size PAMs, such as gamma-glutamate-containing peptides, and both forms of glutathione (reduced "GSH" and oxidized "GSSG") can also bind to the ABS [20, 21].

The CaSR also reacts to other naturally occurring positively charged ligands that allosterically bind to the receptor. These compounds include polyamines like spermine, spermidine, and histamine. Polyamines can bind and activate the CaSR even when extracellular Ca^{2+} is depleted. On the other hand, CaSR's activity can be inhibited by various factors including anions such as phosphate and sulfate. Elevated plasma phosphate levels are observed in chronic kidney disease [22].

Clinical applications of allosteric modulation of the CaSR

The CaSR plays a vital role in the negative modulation of PTH secretion. Therefore, PAMs of CaSR are used to treat hyperparathyroidism. Hyperparathyroidism is commonly caused by parathyroid adenoma or carcinoma, resulting in primary hyperparathyroidism (PHPT). Chronic kidney disease could also be a secondary cause of PHPT, where clearing phosphate out of the body is defective, and activation of 1,25-dihydroxyvitamin (D_3) in the kidneys is reduced, leading to diminished Ca^{2+} levels. These changes subsequently elevate the PTH production and release, ultimately causing parathyroid hyperplasia. Two FDA-approved drugs (Cinacalcet and Etelcalcetide) work by activating the CaSR, which in turn reduces the PTH to treat individuals who have secondary hyperparathyroidism [23, 24].

It is worth mentioning that the CaSR signaling is associated with NLRP3-mediated inflammatory rheumatoid arthritis and autoinflammatory disease [25, 26]. Furthermore, the activation of CaSR leads to constriction of the lung airways, remodeling of the pulmonary system, and fibrosis, all of which are connected to the development of asthma. As a result, negative allosteric modulators (NAM) of CaSR, such as NPS2143, are seen as a promising treatment for airway hyperresponsiveness and allergic asthma [7]. The stimulation of the CaSR is also linked to the progression of prostate and breast cancers to the stage of metastasis [10].

Polyamine derivatives such as spermine, spermidine, and histamine, known to induce inflammation and asthma in the lung airway, act as CaSR agonists without specific binding sites known [23, 28]. As mentioned earlier, Cinacalcet is the first CaSR PAM approved by the FDA to treat hyperparathyroidism. It binds to the pocket in the 7TMD, referred to as the cinacalcet-binding site (CBS) [29]. Etelcalcetide is another positive allosteric modulator of the CaSR that is approved by the FDA. It binds between the two LB2 lobes of the VFT, creating the etelcalcetide binding site (EBS). Etelcalcetide binds through a disulfide bond with C482 and forms salt bridges at EBS [30, 31]. Additionally, the CaSR can detect anions such as phosphates and chlorides with a phosphate ion binding at ECD [32]. Therefore, identifying CaSR ligands and their modes of CaSR interactions is important to understand and control CaSR-mediated physiology and pathology.

The possible effect of caffeine on CaSR regulation

The detection and verification of CaSR ligands are primarily accomplished through CaSR's downstream functional measurements (i.e., Ca^{2+} flux) and radioactive ligand binding studies [33], which have the potential to impede their utility. Matarage Don et al., (2024) developed a photoaffinity-tagged glutathione derivative ("DAZ-G" or photoaffinity diazirine and glutathione) as a chemical tool that enables the analysis of CaSR ligands. Their work demonstrated that DAZ-G binds to CaSR allosterically mimicking the binding of other PAMs such as gamma-glutamyl tripeptides [34]. Subsequently, it efficiently increases intracellular Ca^{2+} $[Ca^{2+}]_i$ with an EC_{50} of 115 nM. Furthermore, this study showed that DAZ-G can identify and analyze other CaSR ligands and their binding sites.

DAZ-G was utilized to identify new ligands for the CaSR. Matarage Don and other researchers demonstrated that caffeine is a strong PAM for CaSR. These findings revealed that the CaSR is a new target for caffeine which is commonly consumed as a main component of coffee. This discovery suggests that caffeine's powerful effects could be partially mediated through its activation of CaSR. Additionally, the enormous physiological roles of CaSR in our bodies may be regulated by regular consumption of caffeine in the form of coffee or other drinks.

A new chemical probe for CaSR using a photoaffinity tag

To investigate how certain substances bind to CaSR, Matarage Don et al. (2024) created a modified version of a CaSR ligand, GSH, containing a photoaffinity tag, diazirine. Previous research has shown that

many di- or tripeptides containing gamma-glutamate maintain their binding specificity to the ABS in CaSR. Matarage Don and his colleagues have designed a GSH-derived probe called DAZ-G.

Previous research has shown that many di- or tripeptides containing gamma-glutamate maintain their binding specificity to the amino acid binding sites in CaSR. The binding and activation of CaSR by gamma-glutamyl tripeptides such as GSH and GSSG have been proven in recent literature through radio-labeled ligand binding assay, Ca^{2+} flux assay, and molecular docking studies. Matarage Don and her colleagues have designed a GSH-derived photoaffinity probe called DAZ-G to investigate the CaSR-ligand interactions **Figure 2**.

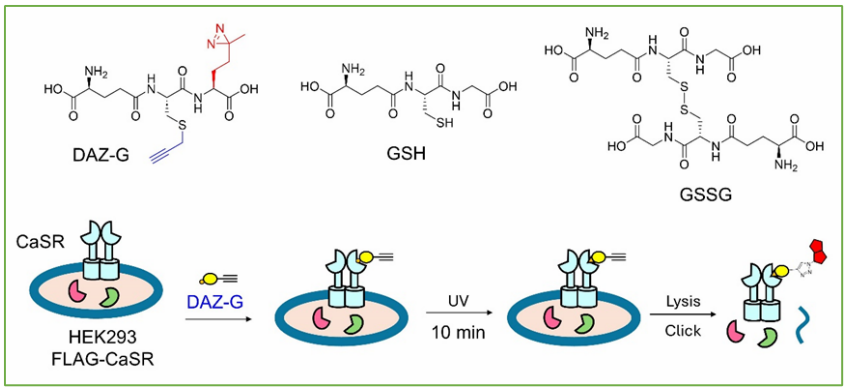

Figure 2. The photoaffinity-tagged glutathione-derived ligand. This ligand is designed to bind with CaSR in the Human Embryonic Kidney 293 (HEK293) cell line. The upper part of the diagram shows the structures of DAZ-G, reduced glutathione (GSH), and oxidized glutathione (GSSG). In the lower diagram, Matarage Don et al. employed an approach to observe the interaction between DAZ-G and CaSR: DAZ-G was exposed to cells containing FLAG-CaSR. Subsequently, the cells underwent UV irradiation, leading to the covalent binding of DAZ-G to CaSR. Following the click reaction of lysates with biotin-azide, The biotinylated CaSR was examined using Western blot analysis [34].

The binding of the DAZ-G to the CaSR was enhanced by elevating the concentration of DAZ-G (concentration-dependent). Furthermore, the work is accomplished by investigating the binding of DAZ-G to the receptor by adding competitors (reduced and oxidized glutathione). The results showed that both forms of glutathione compete with DAZ-G conjugation to the CaSR, suggesting that DAZ-G was conjugated to the receptor at the same ABS site in CaSR where glutathione binds as demonstrated by Matarage Don et al., 2024.

The new CaSR probe, DAZ-G, induces the release of intracellular Ca^{2+} $[Ca^{2+}]_i$

The CaSR is GPCR, it connects to $G_{i/o}$ and $G_{q/11}$ proteins. These proteins decrease the cAMP and activate phospholipase C (PLC). The stimulation of PLC cleaves phosphatidylinositol biphosphate (PIP2) into 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), both acting as second messengers. These messengers cause the release of intracellular Ca^{2+} $[Ca^{2+}]_i$ from the endoplasmic reticulum (ER) stores and activates protein kinase C (PKC), which in turn stimulates the opening of the membrane calcium channels [35].

In a previous study in the School of Life Sciences at Warwick University in UK, Hills, Younis, and their colleagues (2012) demonstrated that an increase in β-cell to β-cell interaction is accompanied by enhanced L-type voltage-dependent calcium channel (VDCC) expression. This results in amplified changes in $[Ca^{2+}]_i$ when stimulated by secretagogues, which was measured using Fura-2/AM as a probe. The elevated $[Ca^{2+}]_i$ via VDCC may activate the CaSR on adjacent cells, thereby aiding in the propagation and amplification of the signal across the intact islet [11]. This synchronization is crucial for ensuring appropriate insulin secretion. Similarly, the conjugation of DAZ-G to the ABS sites on the CaSR caused a significant (5.5 times) and rapid (in 28−90 seconds) release of $[Ca^{2+}]_i$ in HEK 293 cells. Matarage Don et al., (2024) showed that the increase in $[Ca^{2+}]_i$ levels was demonstrated by a calcium flux test utilizing a fluorogenic Ca^{2+} probe "Cal-520".

The use of DAZ-G identified caffeine as a PAM of the CaSR.

DAZ-G was utilized to identify new ligands for the CaSR. Matarage Don and other researchers demonstrated that caffeine is a strong PAM for CaSR. These findings revealed that the CaSR is a new target for caffeine that is commonly consumed as a main component of coffee. This discovery suggests that caffeine's physiological effects could be partially mediated through its activation of CaSR. Additionally, the multifaceted physiological roles of CaSR in our bodies may be regulated by regular consumption of caffeine in the form of coffee or other drinks.

A novel role of caffeine as a CaSR modulator

Caffeine is a white crystalline purine compound bitter-tasted and semi-alkaline, and its chemical name that indicates its composition is trimethylxanthine, **Figure 3** shows the chemical composition of caffeine and an image of caffeine powder, which is classified as a highly toxic substance. Caffeine metabolism actions are elegantly reviewed in "The Coffee World, 2024" by Mustafa YG Younis [36].

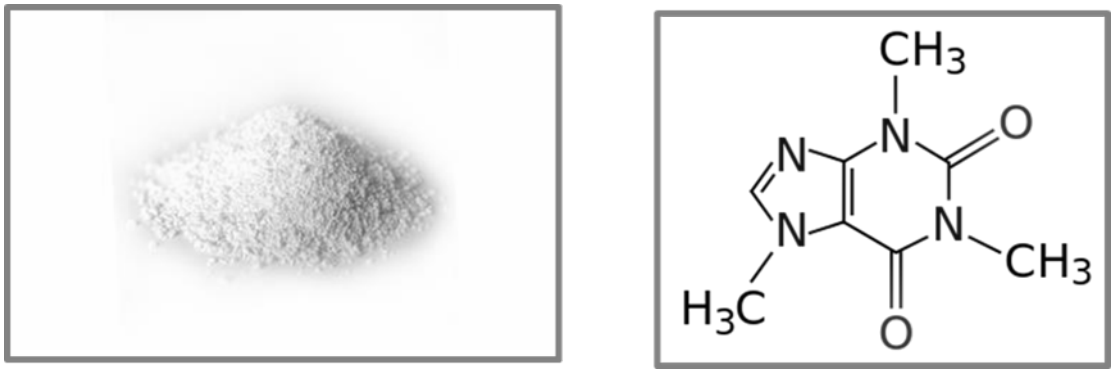

Figure 3: Diagram showing the chemical composition of caffeine (right) and a picture of white caffeine powder (left) "highly toxic substance" (Modified from http://ar.unp-solution.com).

Caffeine is well known for its influences such as sleeplessness, excitability, and palpitations. Other effects include dehydration and increased urination. These actions are suggested to be caused by blocking the adenosine receptors A1 and A2a. These receptors are involved in the secretion of several hormones such as norepinephrine, dopamine, serotonin, and acetylcholine [37]. Caffeine also increases lipolysis by inhibiting the cyclic nucleotide phosphodiesterase which transforms cAMP into AMP. This causes an increase in the levels of cAMP that subsequently activates the hormone-sensitive lipase and stimulates lipolysis [38].

The significant discovery of caffeine as a strong positive allosteric modulator for CaSR [34] may be utilized in future research work related to finding a cure for diabetes by using caffeine to activate the CaSR in pancreatic β-cells to improve the secretory capacity of these cells. A previous research study conducted by Lu and colleagues 2013 indicated that caffeine does not elevate $[Ca^{2+}]_i$ in the calcium microfluorimetry assay. However, the study used Fura-2 for the experiment. It has been previously reported that caffeine can interfere with Fura-2 when sensing $[Ca^{2+}]_i$, suggesting that the previous report may have produced a false negative result [39, 40, 41]. On the contrary, recent data collected from the DAZ-G competition tests and the Ca^{2+} microfluorimetric assay using a new Ca^{2+} ligand (Cal-520), revealed that caffeine stimulates the CaSR and increases the concentration of $[Ca^{2+}]_i$. Therefore, these findings agreed with an early report that hypothesized that caffeine suppresses PTH release in the human parathyroid gland through CaSR [34, 38].

Caffeine has been found to activate adenosine receptors at concentrations (10−50 μM) achievable through regular consumption of coffee and other caffeinated drinks. Additionally, the work by Matarage Don and colleagues demonstrated that caffeine is effective at activating the CaSR, suggesting that drinking coffee-containing caffeine can trigger CaSR at multiple tissue sites in the human body. However, more research is needed to fully cover caffeine's effectiveness in activating the CaSR.

II. Conclusion

All these findings suggested that caffeine could modulate various physiological functions by activating the CaSR. These effects resulting from the CaSR induction include Ca2+ regulation, bone development, osteoporosis, and pancreatic β-cell functions. Based on the research findings discussed, we suggest a new research proposal to investigate how caffeine could activate the CaSR in pancreatic β-cells. The activated CaSR leads to enhancing cell-to-cell contact and communication via increasing the [Ca2+]i flux that, in turn, enhances insulin granules secretion which is accompanied by an increase in Ca2+ ions in the intercellular spaces that consequently re-activate CaSR in neighboring cells that ultimately orchestrate the signal to activate and improve the whole islet. Other experimental work may include, calcium microfluorimetry to access the magnitude of [Ca2+]i release following CaSR stimulation by caffeine, studying the changes in insulin secretion, the effects of caffeine stimulation of the CaSR on the degree of cell-to-cell adhesion using Atomic force microscopy-single-cell force spectroscopy (AFM-SCFS) and finally studying the possible impact of this activation on β-cell proliferation within the pancreatic islet. All the suggested work could illustrate and shed light on the mechanism underlying the health benefits of coffee drinking that may be related to its caffeine content.

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