

Regulation of Ral GTPase Activity in Megakaryocyte by the
T2R4 Agonist, Quinine

By

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LIST OF ABBREVIATIONS

BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BCML	N α ,N α -bis(carboxymethyl)-L-lysine
Ca ²⁺	Calcium ion
CaM	Calmodulin
Ca ²⁺ /CaM	Calcium bound Calmodulin
cAMP	Cyclic Adenosine Monophosphate
DAG	Diacylglycerol
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
GAP	GTPase Activating Protein
GDP	Guanosine Diphosphate

GEF	Guanine nucleotide Exchange Factor
G _{gust}	Gustducin G-Protein
GPCR	G Protein-coupled receptor
G-Protein	Guanosine Nucleotide-binding Protein
GRK	G protein-coupled receptor kinases
GST	Glutathione-S-Transferase
GTP	Guanosine Triphosphate
IP ₃	Inositol trisphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Dalton
PBS	Phosphate-buffered saline
PLC	Phospholipase C
PMSF	Phenylmethane sulfonyl fluoride
PVDF	Polyvinylidene difluoride
RalBP1	Ral binding protein 1
RIP1	Ral interacting protein 1

RGS	Regulators of G-Protein Signaling
RLIP76	76 kDa Ral interacting protein
RRBD	Ral binding domain of RIPI
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
T2R	Bitter Taste Receptor/Taste Receptor Type 2
T2R4	Bitter Taste Receptor/Taste Receptor Type 2 Member 4
TAS2R	Bitter Taste Receptor Gene
TM	Transmembrane

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Abstract

Platelets are anucleated cells derived from megakaryocytes and play a crucial role in circulation including, blood clotting and repair during blood vessel injury. Quinine is one of the most bitter compound known and acts as an agonist for several bitter taste receptors (T2Rs) including, T2R4. When used as an antimalarial drug, quinine is known to cause thrombocytopenia. RalA is a small GTPase that has been shown to play a role in platelet function. RalA activity is regulated by calcium and the calcium binding protein, calmodulin. The objective of this study is to investigate if quinine regulates RalA activity in platelets independently and/or through T2R4.

The immortalized megakaryocyte cell line, CHRF-288-11, was utilized in these studies. Pull-down assays using Ral-binding domain of Ral-interacting protein 1 were employed to assess RalA activation. To investigate if CHRF cells express bitter taste receptor, T2R4, RT-PCR and western blot analysis was carried out.

Treatment of CHRF cells with quinine resulted in the activation of RalA. The results from PCR and western blot analysis demonstrated the presence of T2R4 in CHRF cells. It has been shown that calmodulin and calcium are required for RalA activation. First, we investigated if quinine altered the interaction between calmodulin and RalA. Incubation of CHRF cell lysate with quinine prior to incubation with CaM-Sepharose beads demonstrated that quinine did not affect the interaction between RalA and calmodulin. Next, we investigated if quinine mediated RalA activation is through a calcium dependent pathway. CHRF cells were treated with quinine and calcium chelator (BAPTA-AM) or calmodulin antagonist (W7). The results obtained demonstrated that quinine activated RalA independently of calcium. Treatment of CHRF cells with BCML,

a T2R4 antagonist, inhibited quinine mediated activation of Ra1A suggesting that quinine action is dependent on this bitter taste receptor. The information gained from these studies may lead to the development of an approach to control thrombocytopenia during therapeutic intervention where quinine is prescribed as the drug of choice.

Introduction

Overview

Cells respond to the external environment via cellular communication processes that regulate their activities and the magnitude of the response. How cells perceive and respond to their microenvironment is the basis of development, tissue repair, and immunity as well as normal tissue homeostasis (Vlahopoulos, Cen et al. 2015). Any defect in signaling interactions at the molecular level and its processing can have unhealthy consequences for the body. Diseases such as cancer, autoimmunity, and diabetes are the results of abnormal or failed cellular signal processing (Solinas, Vilcu et al. 2007; Wang, Grivennikov et al. 2013). Diseases can be treated effectively by understanding cell signaling pathways and their networks (Smith, Koobatian et al. 2015). Most of the extracellular signals are transmitted via receptors on the surface of cells. These receptors are protein molecules that recognize and respond to these external stimuli (Alberts, Bray et al. 2013).

Protein receptors can be classified depending on their cellular location into two predominant types: cell surface transmembrane receptors and intracellular receptors. Cell surface transmembrane receptors include ion channels, G protein-coupled receptors (GPCRs), and enzyme-linked hormone receptors (Hall 2015). Intracellular receptors are those found inside the cell, and include cytoplasmic receptors and nuclear receptors (Hall 2015). Each receptor is linked to specific cellular biochemical pathway. When a ligand binds to its corresponding receptor, it activates or inhibits the receptor

and affects the associated biochemical pathway (Hall 2015). G protein-coupled receptors (GPCRs) have seven-transmembrane domains and are activated by different external stimuli to cause varied intracellular responses (Trzaskowski, Latek et al. 2012). GPCRs are very important therapeutic and drug discovery targets (Wise, Gearing et al. 2002). Approximately 50% of prescription and medicinal drugs, including those with a bitter taste such as some antibiotics and quinine, target the GPCR family (Wise, Gearing et al. 2002; Davies, Secker et al. 2007; Jaggupilli, Singh et al. 2018). Quinine is a very intense bitter compound and acts as an agonist for several bitter taste receptors T2Rs, including T2R4 (Upadhyaya, Chakraborty et al. 2016). Quinine is known to inhibit platelet aggregation which causes severe thrombocytopenia (Aster, Curtis et al. 2009). Ral is a small GTPase that belongs to the Ras subfamily of low molecular mass GTP-binding proteins (Chardin and Tavitian 1989). Ral plays a central role in diverse biological processes through its interactions with specific effector proteins. Ral GTPase participate in the regulation of exocytosis and other related platelet activity (Shirakawa and Horiuchi 2015).

G protein-coupled receptors

G protein-coupled receptors (GPCRs) represent the largest and the most diverse family of membrane proteins and constitute ~3-4% of the human genome (Fredriksson, Lagerström et al. 2003). A wide variety of molecules can bind to GPCRs and act as ligands including odors, pheromones, ions, hormones, neurotransmitters, peptides and proteins (Milligan and Kostenis 2006). All GPCRs consist of seven transmembrane helices, three extracellular loops (EL1-3), three intracellular loops (IL1-3) as well as the

extracellular N-terminus and intracellular C-terminus. The transmembrane segments form seven alpha-helices in a flattened two-layer structure seen in all GPCRs (Milligan and Kostenis 2006). The predominant mechanism of signal transduction by a GPCR present on the cell surface is as follows: the binding of signal molecule (ligand) on the extracellular surface of a GPCR triggers movement of the transmembrane helices. These movements are then transmitted to the intracellular surface of the GPCR resulting in changes to a number of proteins that are attached, including the guanine nucleotide-binding G-proteins. These intracellular proteins activate or inhibit different effector proteins, causing second messenger responses and relaying the signal.

Guanine nucleotide-binding proteins (G-Proteins)

Guanine nucleotide-binding proteins (G-proteins) are a family of proteins located within the inner side of the plasma membrane, and some of these are activated by GPCRs. The G protein activates a cascade of further signaling events that finally result in a change in cell function (Pierce, Premont et al. 2002). G-proteins belong to the larger group of enzymes called, GTPases, and work as molecular switches. They cycle between the inactive guanosine diphosphate (GDP) and the active guanosine triphosphate (GTP) form. G-proteins belong to two classes; heterotrimeric G-proteins and monomeric small GTPases (small G-proteins).

Heterotrimeric G-proteins

Heterotrimeric G-proteins also called large G-proteins, consist of three subunits: alpha (α), beta (β) and gamma (γ) (Hurowitz, Melnyk et al. 2000). These are derived

from 35 genes with 16 encoding α -subunits, 5 β subunits and 14 γ subunits. The molecular weight of the α subunit ranges between 41-45 kDa while β and γ subunits are ~35 kDa and 8 –10 kDa respectively (Milligan and Kostenis 2006). The following is the generalized and simplified sequence of events in the GPCR-heterotrimeric G-protein signaling cascade. When GPCR is in the resting state, the α subunit is bound with GDP (inactive form) and is associated with the $\beta\gamma$ subunits. However, when extracellular signaling molecules or the first messengers bind to GPCRs, it leads to conformational change in G-protein. The α subunit releases its bound GDP and is replaced by GTP (active form) which leads it to dissociate into two components: α subunit and the $\beta\gamma$ complex. These subunits can activate distinct downstream effector proteins such as adenylyl cyclase, phospholipase C, inwardly rectifying K^+ channels, phospholipase $\beta 1$, $\beta 2$ and $\beta 3$, Src tyrosine kinase, Ca^{2+} channels and other ion channels (Milligan and Kostenis 2006). The effector in turn causes the production of second messengers such as, cyclic AMP, cyclic GMP, inositol trisphosphate, diacylglycerol, arachidonic acid, sodium, potassium and calcium. These second messengers ultimately lead to physiological responses (Ford, Skiba et al. 1998). The cycle is completed by the hydrolysis of alpha subunit-bound GTP to GDP, resulting in the re-association of the α and $\beta\gamma$ subunits and their binding to the GPCR, (Uings and Farrow 2001).

Monomeric G-proteins

Small G-proteins also called small GTPases (guanosine triphosphatases) are proteins with molecular weight of ~20-40 kDa and consist of a single polypeptide chain. These

small GTPases fall under the RAS superfamily, and comprise of more than 150 known members divided into subfamilies based on their structure, sequence and function (Wennerberg, Rossman et al. 2005). The main subfamilies are Ras, Rho, Ran, Rab and Arf GTPases (Table 1) (Goitre, Trapani et al. 2014). They act as molecular switches cycling between the inactive GDP bound form and the active GTP bound form to regulate many essential cellular processes such as: proliferation, cellular motion, cell division, apoptosis, exocytosis, and intracellular transport (Chardin 1988; Csépanyi-Kömi, Lévy et al. 2012). GDP/GTP cycling is controlled by two main classes of regulatory proteins; Guanine-nucleotide-exchange factors (GEFs) that promote formation of the active GTP-bound form (Schmidt and Hall, 2002), and GTPase-activating proteins (GAPs) which accelerate the intrinsic GTPase activity to promote conversion into the inactive GDP-bound form (Figure 1) (Bernards and Settleman, 2004). GTPases within a branch use shared and distinct GAPs and GEFs. GTPases in different branches exhibit structurally distinct but mechanistically similar GAPs and GEFs (Wennerberg, Rossman et al. 2005).

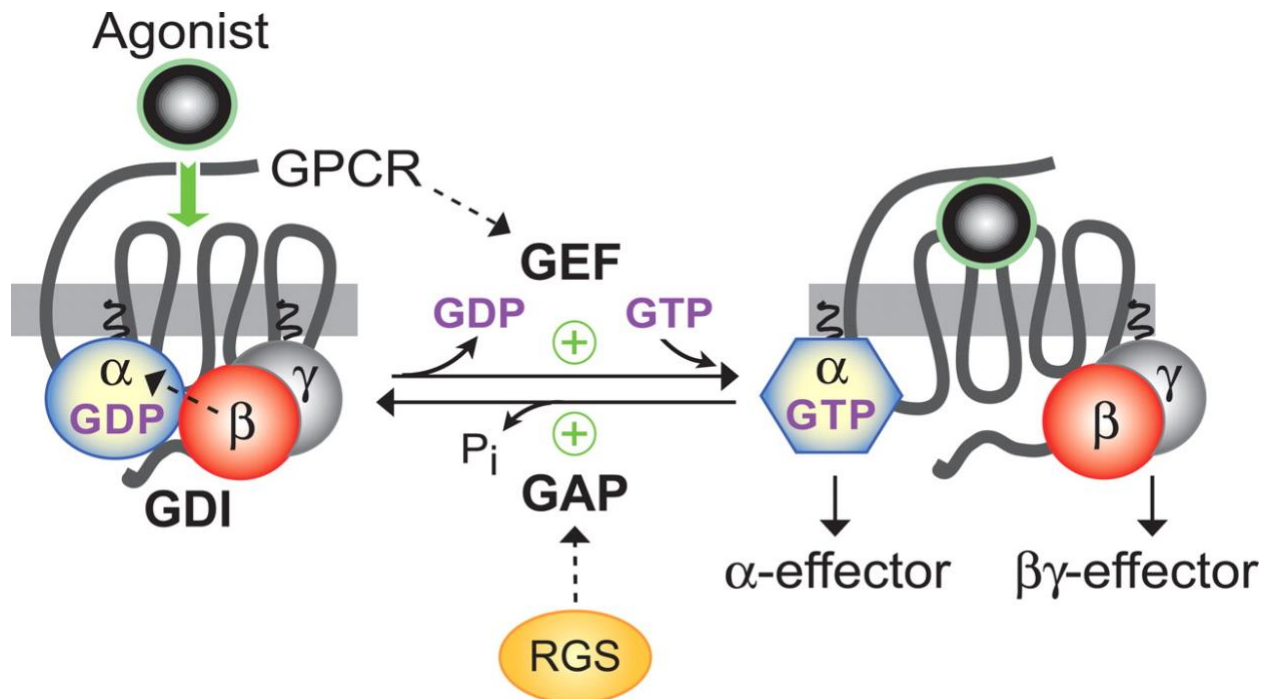


Figure 1. GTPase cycle of heterotrimeric G-proteins (Johnston and Siderovski 2007)

The Ras protein subfamily

The Ras sarcoma (Ras) oncoproteins or Ras p21 are the founding members of the Ras family and contain 36 members (Repasky, Chenette et al. 2004). Ras proteins act in response to diverse extracellular stimuli to regulate cytoplasmic signaling that control gene expression and regulation of cell proliferation, differentiation, and survival (Wennerberg, Rossman et al. 2005).

The Rho protein subfamily

Like Ras, Ras homologous (Rho) proteins have ~35% amino acid sequence homology with Ras p21. They also act as regulators of extracellular stimulus mediated signaling networks which regulate actin organization, cell cycle progression and gene expression (Etienne-Manneville and Hall 2002). Rho proteins have 20 members, and the most well

studied among all Rho members are RhoA, Rac1 and Cdc42 (Wennerberg, Rossman et al. 2005).

The Ran subfamily

The Ras-like nuclear (Ran) protein is the most abundant small GTPase in the cell, and it has a role in nucleocytoplasmic transport of both RNA and proteins (Weis 2003).

The Rab subfamily

The Ras-like proteins in brain (Rab), share ~30% amino acid sequence homology with Ras p21. Rab proteins are the largest branch of the Ras superfamily consisting of 61 members (Pereira-Leal, Hume et al. 2001). Rab GTPases are regulators of intracellular vesicular transport and the trafficking of proteins between different organelles of the endocytic and secretory pathways (Zerial and McBride 2001).

The Arf subfamily

Arf proteins called ADP-ribosylation factor (Arf) family proteins, are involved in regulation of vesicular transport (Memon 2004).

Monomeric G – Protein subfamily	Members
Ras	H-Ras, K-Ras, N-Ras, R-Ras, RalA, RalB, Rap1A, Rap1B, Rap2A, Rap2B, TC21, RERG, RRAD
Rho	RhoA, RhoB, RhoC, RhoD, RhoF, RhoG, RhoJ, RhoH, RhoU, Rac1, Rac1B, Rac2, Rac3, Cdc42, TC10, TCL, RhoBTB, RhoBTB ₂
Ran	Ran, TC4
Rab	Rab1A, Rab1B, Rab2, Rab3A, Rab3B, Rab3C, Rab3D, Rab4, Rab5, Rab6, Rab7, Rab8, Rab9, Rab10, Rab11, Rab13, Rab14, Rab25
Arf	ARF1, ARF2, ARF3, ARF4, ARF5, ARF6, ARLs, Trim26, ARL4D, ARFRP

Table 1. Classification of small G-Protein family

Ras p21 Protein Subfamily

Most protein members in this group are located near or attached to the plasma membrane with lengths of 183 to 340 amino acids. All members share significant amino acid identity with Ras p21 and are arranged into conserved branches such as; RAS oncoprotein (HRAS, KRAS, and NRAS), RRAS (Related to RAS), RAP (Ras-Proximal), RAL (RAS-Like), RIT (RAS-like Protein in All Tissues), ERAS (Embryonic Stem Cell–Expressed Ras), DIRAS (Distinct Subgroup of RAS) and ARHI, RASD (Ras Induced by Dexamethasone, NKIRAS (NFKB Inhibitor–interacting RAS-like, also called kB-Ras), REM (Rad and Gem–related, RERG (RAS-related and Estrogen-Regulated Growth inhibitor), and RHEB (Ras Homolog Enriched in Brain) (Colicelli 2004). Some of these

proteins are involved in control of mitogenesis, cytoskeleton reorganization and function as regulators of integrin-mediated cell adhesion and cell spreading (Colicelli 2004).

Ral GTPase

The focus of the studies in this thesis is the Ral (Ras-like) GTPase, a 27 kDa protein, which is involved in a wide spectrum of functions including: mitogenic responses, differentiation, protein trafficking, exocytosis, and cytoskeleton dynamics (Feig 2003). Ral GTPase is a member of Ras subfamily and shares 46%-51% amino acid identity with human Ras P21 proteins. Ral protein is widely expressed but is particularly abundant in brain (Olofsson, Chardin et al. 1988), testes (Jiang, Luo et al. 1995), and platelets (Bhullar, Chardin et al. 1990). It is located bound to the plasma membrane, and in endocytic and synaptic vesicles (Polakis, Weber et al. 1989), specifically in dense granules (Mark, Jilkina et al. 1996). It is involved in signal transduction pathways that regulate cell proliferation (Reuther and Der 2000), in Ras-induced oncogenic growth, and in induction of DNA synthesis (Miller 1997; Rodriguez-Viciano, Warne et al. 1997).

Ral isoforms

Ral has two isoforms: RalA and RalB. They share ~85% overall amino acid sequence homology with a 100% match in their effector-binding region. The difference between the two isoforms which lead to diverging subcellular localization and biological function lies in the amino acid variability in the C-terminal membrane targeting region (Kashatus 2013). The two Ral isoforms have crucial roles in oncogenic transformation of human cells, and in mediating both oncogenic proliferation and survival signals (Chien and

White 2003). Also they have a role in epithelial cell polarization (Orlando and Guo 2009). RalB specifically is required for survival of tumor cells, while RalA is required for anchorage-independent cell proliferation (Chien and White 2003).

The N-terminal 180 residues, includes G domain that is involved in GTP binding and hydrolysis. The strongest sequence identity is seen in the N-terminal 90 residues (98%) that include sequences involved in effector interaction. Residues 36–56 correspond to Ras and residues 25–45 are involved in effector interaction (Figure 2). The switch I (residues 41–51) and switch II (69–81) sequences are responsible for conformation change during the GDP-GTP cycle and one or both sequences are involved in binding to specific effectors (Mott and Owen 2010). The effector interaction sequences are conserved 100% in both RalA and RalB. But the greatest divergence (50%) is in the C-terminal membrane-targeting sequence. This membrane targeting sequence terminates with a CAAX tetrapeptide sequence that is a signal for post-translational modification by addition of a geranylgeranyl isoprenoid lipid to the cysteine residue. Point mutations in the effector interaction sequences (36–56) cause differential impairment in effector binding (Martin and Der 2012).

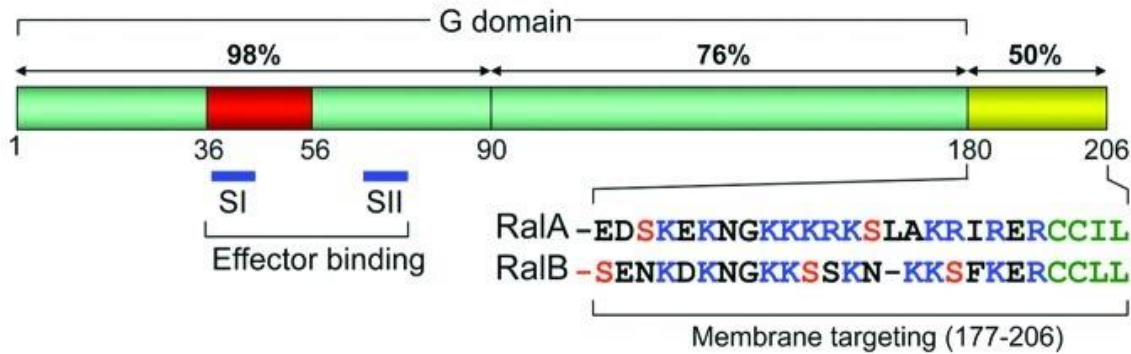


Figure 2. The characteristics of the two Ral isoforms (Martin and Der 2012)

Ral Effectors

Similar to other small GTPases, Ral proteins interact with several effector proteins to initiate downstream signal transduction when activated. To date, a large number of Ral effectors have been identified (Table 2). However, the most well-known and best characterized effectors are RIPI/RalBP1/RLIP76 and the Sec5 and Exo84 subunits of the octameric exocyst complex (He and Guo 2009). Some of the less characterized ones are phospholipase D1 (PLD1) (Kim, Lee et al. 1998; Luo, Liu et al. 1998) and zonula occludens 1-associated nucleic acid binding protein (ZONAB) (Frankel, Aronheim et al. 2005). Sec5 and Exo84 are known to engage in exocytosis interaction with Ral but the interaction occurs according to subcellular localization, with Sec5 at the plasma membrane and Exo84 at intracellular vesicles (Bodemmann, Orvedahl et al. 2011; Hazelett, Sheff et al. 2011).

It is well known that PLD1 catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. Agonists acting through GPCRs stimulate this hydrolysis. In HeLa cells, it has been shown that PLD1 is involved in cell cytokinesis through either

RalA or RalB (Cascone, Selimoglu et al. 2008). ZONAB is shown to be involved in gene transcription when activated by RalA in a cell density dependent manner in MDCK cells (Frankel, Aronheim et al. 2005). In addition, RalA has been shown to engage the effector filamin in actin cytoskeleton, actin crosslinking and lamellipodia formation (Gorlin, Yamin et al. 1990; Takafuta, Wu et al. 1998; Ohta, Suzuki et al. 1999). The Ral effector proteins; RIPI/RalBP1/RLIP76 have GAP region similar to RhoGAP domains, and have GAP activity upon activation by CDC42 and Rac. They are also named as Ral interacting protein 1 from mouse (RIPI), 76 kDa Ral interacting protein from human (RLIP76) and Ral binding protein-1 (RalBPI) from rat (Cantor, Urano et al. 1995; Jullien-Flores, Dorseuil et al. 1995; Park and Weinberg 1995).

RalBPI has a Ral binding domain in its C-terminal region and binds to the GTP-bound but not the GDP-bound form of Ral. In addition, Ral binding proteins participate in the cross-talk between Ras, Ral and Rho downstream cascades affecting activity of the Cdc42/Rac/Rho pathway in response to activation by RAS (Jullien-Flores, Dorseuil et al. 1995). RalBPI also regulates endocytosis of EGF and insulin receptors by recruiting RalGDS to the plasma membrane when Ras is stimulated (Nakashima, Morinaka et al. 1999). Although RalA and RalB can interact with the same set of effectors as described previously, the distinct biological functions of RalA and RalB are mediated by differences in subcellular localization, leading to their interaction with distinct subsets of effectors.

Ral Effector	Function
RalBP1 Sec5, Exo84 Filamin ZONAB Plc&1 RIP1	RhoGAP, Scaffold for other proteins Exocytosis dependent and independent Actin cytoskeleton, actin crosslinking, lamellipodia Gene transcription IP3 signaling Converting phosphatidylcholine to Phosphatidic acid

Table 2. Ral effector proteins

Ral A

Ral A is a 27 KDa small G-protein in humans and is encoded by the RalA gene on chromosome 7 (Rousseau-Merck, Bernheim et al. 1988). Like all other GTPases, RalA switches between the inactive GDP-bound and the active GTP-bound forms and regulates Ral-selective GEFs and GAPs.

RalA Activation in a Ras-independent manner

RalA may regulate both endocytosis and exocytosis at nerve terminals in a Ca^{2+}

dependent manner. *In vitro* studies have been shown that Ral is activated by the Ca^{++} /calmodulin (CaM) complex in response to elevated levels of Ca^{++} (Wang and Roufogalis 1999; Park 2001). Of relevance to exocytosis in platelets, Ral is activated by factors that stimulate the secretion system in platelets. For example, thromboxane and α -thrombin which are platelet agonists induce the fusion of dense granules with the plasma membrane and open the canalicular system (Morgenstern 1995). α -thrombin causes activation of Ral A via Ca^{++} signaling pathway (Wolthuis, Franke et al. 1998).

The activation of platelets by agonists requires activation of the heterotrimeric G protein, which activates PLC (Offermanns 2006). Then, PLC hydrolyzes phosphatidylinositol-4,5- biphosphate (PIP₂), releasing the second messenger's diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP₃). IP₃ in turn, stimulates the release of Ca^{++} from Endoplasmic Reticulum (ER), which may trigger the influx of Ca^{++} (Prenzel, Fischer et al. 2001).

Role of Calmodulin in Ras-independent pathway

CaM is a calcium binding protein, which acts as an intracellular calcium sensor and is involved in regulation of ion channels, the cell cycle, and cytoskeletal reorganization and development (Heiman, Atkinson et al. 1996). In GPCR signaling, CaM is involved in the intracellular signal transduction cascade as a second messenger to regulate cell proliferation (Klee and Vanaman 1982; Lu and Means 1993; Herget, Oehrlein et al. 1995), gene expression, protein translation and protein phosphorylation (Villalonga, Villalonga et al. 2006). RalA contains a putative C-terminal CaM-binding domain

(CaMBD) which includes basic/hydrophobic residues and that readily forms an amphiphilic alpha-helix (Wang, Roufogalis, 1999). RalA can be activated via Ca^{++} /CaM signalling pathways (Park 2001).

In vivo, RalA and RalB interact specifically and directly with CaM *in* eukaryotic systems (Clough, Sidhu et al. 2002). While *in vitro* it has been demonstrated that RalA was bound to CaM in a calcium dependent manner (Wang and Roufogalis 1999), in human platelets thrombin has been shown to induce the activation of both RalA and RalB in a CaM-dependent manner (Clough, Sidhu et al. 2002).

RalA activation in a Ras-dependent manner

The best known Ral signaling pathway that has been characterized is the Ras signaling pathway. Ral is activated by the epidermal growth factor receptor tyrosine kinase through the RalGEF (Repasky, Chenette et al. 2004). EGF binds to EGFR which activates Ras p21 and in turn binds to the Raf serine/threonine kinase in the plasma membrane. Subsequently this leads to phosphorylation events that promote full Raf kinase activation. This in turn leads the cell to enter mitosis resulting in cell division (Carpenter and Cohen 1979; Schlessinger 1986).

Other Ras family proteins, including Rap, R-Ras, Ral and Rheb proteins, also regulate signaling networks. Several Ras family proteins also appear to act as tumor suppressors, rather than as oncogenes (e.g. Rerg, Noey2 and D- Ras), in cancer development (Colicelli 2004).

EGF binding to the EGFR leads to activation of c-Src through the Ras/RalGEF/Ral pathway. C-Src in turn, leads to activation of the transcription factor STAT3 and the actin binding protein Cortactin-p (Goi, Shipitsin et al. 2000).

Role of RalA in platelet

Ras-like protein, RalA, has been shown to strongly inhibit GTP-dependent exocytosis (Wang, Li et al. 2004). However, the critical role for endogenous RalA in this process remains to be fully understood. An interesting, study suggested that CaM interacts with Ral GTPase and regulate its activity in platelets (Clough, Sidhu et al. 2002). As mentioned previously, it has been shown that both RalA and RalB become maximally activated after platelet stimulation by a number of platelet agonist (e. g. thrombin) (Wolthuis, Franke et al. 1998). Other studies demonstrated that quinine inhibited platelet aggregation induced by weak agonists (e. g. ADP and adrenaline) (Siess 1989).

Taste signal transduction cascade

Taste signaling predominantly originates in the oral cavity with the activation of taste receptors by the tastants (taste molecules or agonists). Humans can sense five basic types of tastes, which are salt, sour, sweet, bitter and umami. Ion channels are suggested to be involved in sensing the salt and sour tastes, whereas, sweet, bitter, and umami are sensed by GPCRs. There are 25 bitter taste receptors (T2Rs) in humans (Nelson, Hoon et al. 2001). A heterodimer of T1R1 and T1R3 detects sweet, and a heterodimer of T1R2 and T1R3 detects umami. The T1Rs belong to the Class C family of GPCRs, and T2Rs are suggested to be similar to Class A family of GPCRs. As is the

case for the global mechanism of GPCR activation, when a bitter agonist binds to extracellular surface of the T2R it causes conformational changes in the receptor, which in turn activates the heterotrimeric G-protein complex: α -gustducin and $\beta\gamma$ -subunits on the intracellular surface of the receptor. Then, $\beta\gamma$ -subunits activate the enzyme phospholipase C β 2 (PLC β 2) which hydrolyzes inositol phospholipid (PIP₂) resulting in the production of 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). Generation of IP₃ activates IP₃ receptors on the membrane of endoplasmic reticulum (ER), this process leads to opening of the calcium release channels and causes transient increase in intracellular calcium. This results in opening of the monovalent selective TRPM5 channels, leading to sodium influx, membrane depolarization and thus release of ATP as a neurotransmitter to activate the gustatory afferents (Finger, Danilova et al. 2005).

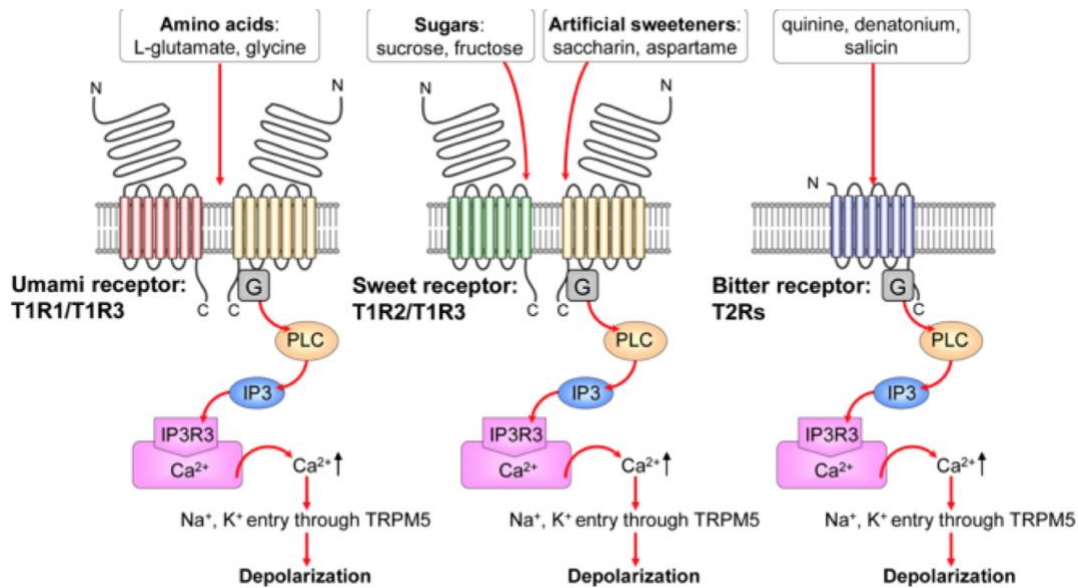


Figure 3. Umami, sweet and bitter tastes are mediated via G protein-coupled receptors (Kobayashi, Habara et al. 2010)

Bitter Taste Receptor (T2R4)

In humans, the 25 T2Rs have 25-90% amino acid identity (Chandrashekar, Mueller et al. 2000). This variability is the reason that each receptor can interact with chemically diverse ligands associated with bitter tastes. A single bitter compound can activate multiple T2Rs and each T2R can be activated by multiple bitter compounds (Meyerhof, Batram et al. 2010). T2Rs have a low affinity for their respective bitter ligands, with EC₅₀ values in the high micromolar to low millimolar range (Meyerhof, Batram et al. 2010). Thus, bitter compounds activate various T2Rs in different concentration ranges, differences usually being in the range of 10-100-fold. Among all known bitter compounds, quinine was shown to be one of the bitterest and is an agonist to at least

seven T2Rs including T2R4 (Prasad Pydi, Upadhyaya et al. 2012; Jaggupilli, Howard et al. 2016). Among the 25 T2Rs, T2R4 is expressed at significant levels in different oral and extra-oral tissues, and has pharmacologically well characterized ligands (agonists and antagonists). Previous structure-function studies on T2R4 extensively characterized quinine-T2R4 interactions (Prasad Pydi, Upadhyaya et al. 2012; Jaggupilli, Howard et al. 2016).

Cellosaurus CHRF 288-11 cell line

While there are a number of different models available to study platelet activation or function, each of them have their own advantages and drawbacks. For example, ethical concerns, the inability to culture in vitro and experimental costs make it a challenge to use platelets for studies. Immortalized cell lines offer an unparalleled advantage for pursuing feasibility and proof-of-principle studies. The CHRF 288-11 cell line is one such widely used cell line as a model for platelet studies. It is a human megakaryoblastic cell line established in vitro through the use of adherent stromal cells in long- term human bone marrow culture (Fugman, Witte et al. 1990). CHRF 288–11 cells are the late stage megakaryocytic cells that can mature into platelets (van der Vuurst, van Willigen et al. 1997). These cells contain markers and growth factors like platelets and synthesize most of the proteins found in platelets. Under the microscope, CHRF 288– 11 cells appear round approximately 15 to 20 microns in diameter with granular cytoplasm and oval nucleus having 2 -3 nucleoli. They have been used as a model for studying platelet signal transduction processes (Fugman, Witte et al. 1990).

Thrombocytopenia

Thrombocytopenia is a platelet disorder defined as “a platelet count of less than 150,000/mm³ or less than 150 ×10⁹ /L (Cherry-Bukowiec and Napolitano 2010). The normal range for platelet count in adult humans is 150 to 450 ×10⁹ /L. Thrombocytopenia may result from decreased production or increased destruction of platelets. A patient is at risk for spontaneous bleeding when the platelet count falls below 20,000 and may warrant platelet transfusion (Cherry-Bukowiec and Napolitano 2010). Thrombocytopenia is a significant clinical problem, which can develop as a side effects to drugs such as quinine (Aster, Curtis et al. 2009). Therefore, understanding the fundamental mechanism(s) by which drugs such as quinine induce thrombocytopenia is very much needed.

Hypothesis

Study rationale

Quinine is one of the bitterest compounds known, and acts as an agonist for several bitter taste receptors including, T2R4. Previous studies have shown that quinine inhibits platelet aggregation causing severe drug-induced thrombocytopenia. Ral plays a central role in diverse biological processes through its interactions with specific effector protein. Ral GTPase participates in the regulation of exocytosis and other related platelet activities. It has been demonstrated that both RalA and RalB become maximally activated after platelet stimulation with various agonists (e.g. thrombin). Previously studies from our lab have demonstrated the inhibitory effects of quinine predominantly acting through T2R4 on Rac1 function (Sidhu C et al., 2017). Ral protein is abundantly present in human platelets which are anucleated cells derived from megakaryocytes and play a crucial role in circulation including, blood clotting and repair during blood vessel injury. Previous Ral-mediated exocytosis is involved in many biological processes including platelet activation which lead to pathological condition such as thrombosis. However, the biological function of Ral protein and the signaling pathway in which Ral is involved are largely unknown. Further investigation will help to understand the role of Ral proteins in the presence of quinine in downstream signal transduction of T2R4 and its pathophysiological roles.

Hypothesis

Quinine regulates RalA activity in platelets independently and/or through T2R4.

Objectives

To test the above hypothesis, two objectives are proposed:

1) To determine the pathway for RalA activation mediated by quinine in CHRF cells

- a) To assess the effect of quinine on RalA activity.
- b) To evaluate the role of calcium in quinine mediated Ral A activation.
- c) To evaluate the role of CaM in quinine mediated Ral A activation.

2) To investigate the role of T2R4 in RalA signaling pathway in CHRF cells

- a) To confirm the expression of T2R4, in CHRF cells
- b) To investigate if quinine regulates RalA activity in platelets independently and/or through T2R4.

Materials and Methods

Materials

The Roswell Park Memorial Institute (RPMI) 1640 medium and DMEM-F12 were purchased from ThermoFisher Scientific (Ottawa, ON, Canada). Fetal bovine serum (FBS, Canadian Origin and cell culture tested), penicillin/streptomycin (100x) and trypsin-EDTA (0.5% or 10x) were purchased from Invitrogen (Oakville, ON, Canada). Quinine HCL, $\text{N}\alpha,\text{N}\alpha$ -Bis(carboxymethyl)-L-lysine (BCML), BAPTA-AM, thrombin, lysozyme and glutathione- agarose beads were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl (W7.HCl) was purchased from Calbiochem (La Jolla, CA, USA). Anti-Ral A monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY, USA). Horseradish peroxidase-conjugated secondary goat anti-mouse antibody was purchased from ThermoScientific. T2R4 Polyclonal antibody was obtained from Abcam (Cambridge, MA, USA). Prestained low range SDS PAGE molecular weight standards and horseradish peroxidase-conjugated secondary goat anti- rabbit antibody were purchased from Bio Rad Laboratories (Mississauga, ON, Canada). Polyvinylidene difluoride (PVDF) membrane and isopropylthio-B-D-galactopyranoside (IPTG) were purchased from Roche Diagnostics (Laval, QC, Canada). Enhanced Chemiluminescent (ECL) reagents and calmodulin-Sepharose 4B were purchased from Amersham Pharmacia Biotech (Montreal QC, Canada). PCR primers were purchased from Invitrogen (Burlington, ON, Canada). Restriction enzymes and 1Kb DNA ladder were from New England Biolabs. RNeasy mini kit was purchased from Qiagen. CHRF cells were a kind gift from Dr. Bing Xu (Brandeis University, MA, USA). Human embryonic kidney 293 cells containing the

SV40 T-antigen (HEK293T) were purchased from ATCC (Manassas, VA, USA). HEK293T cells stably expressing T2R4 and reported previously were used in this study (Pydi, Jaggupilli et al. 2015). GST-RRBD mutant bacterial expression plasmids were previously generated (Jilkina & Bhullar, 1996).

Methods

Buffers

NETT buffer

20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl and 1% Triton X-100.

NT buffer

20 mM Tris-HCL (pH 8.0), and 100 mM NaCl.

Phosphate buffer saline (PBS)

137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄.

Running buffer

25 mM Tris base, 190 mM glycine and 0.1% SDS.

Transfer buffer

48 mM Tris, 39 mM glycine and 20% methanol.

Ral lysis buffer

50 mM Tris (pH 7.5), 200 mM NaCl, 2.5 mM MgCl₂, 1% Norident, 10% Glycerol, protease inhibitor cocktail and 1 mM PMSF.

TBS-Tween 20

50 mM Tris (pH 7.5), 150 mM NaCl and 0.1% Tween 20

Blocking buffer

1X TBST with 5% nonfat dry milk.

Lysis buffer

200 mM HEPES (pH 7.4), 200 mM KCl, 1 mM MgCl₂, 0.55% triton X-100, protease inhibitor cocktail and 1 mM PMSF.

Solubilization buffer

50 mM Tris, 150 mM NaCl, 10% Glycerol (pH 7.6), protease inhibitor cocktail and 1% DM.

GST fusion protein expression in *E.coli* and purification using glutathione agarose beads

GST-RRBD were expressed in *Escherichia coli* AD202 cells by inoculating 50 µl of bacterial stock into 5 ml LB media supplemented with 50 µg/ml ampicillin and incubated for 16 - 18 hours at 37°C with shaking. 1 mM IPTG was subsequently added and

incubated for 2 hours at room temperature with shaking to stimulate the expression of the GST fusion protein. After 2 hours, the bacterial culture was centrifuged at 6,000 rpm for 20 minutes at 4°C. Then, the supernatant was discarded and the cell pellet was collected and re-suspended in NETT buffer. The bacterial cells were homogenized using a glass homogenizer, and 10 mg/ml lysozyme and 1 mM PMSF were added. The cells were lysed by sonication twice for 30 second each time. The lysate was centrifuged at 15,000 rpm for 30 minutes at 4°C. The supernatant containing the GST fusion protein was incubated with glutathione agarose beads prepared in 1:1 (v/v) ratio with NT buffer for 30 minutes at 4°C with constant rocking to isolate and purify the GST fusion recombinant protein. The beads were then washed 3 times with NETT buffer, followed by washing 2 times with NT buffer to remove unbound proteins. The final pellet was suspended in NT buffer in 1:1 (v/v) and stored at 4°C. Then 20 µl of the beads were used to check the purity of the final preparation using 12% Acrylamide SDS-PAGE followed by Coomassie staining.

Preparation of glutathione agarose beads

Glutathione agarose (GSH-agarose) beads (0.1 g) were suspended in 1.5 ml of water and allowed to settle overnight at 4°C. The excess water was removed by centrifugation and the beads were washed 3 times with NT buffer. Finally, 1.5 ml NT buffer was added to the beads and stored at 4°C until use.

Cell culture

CHRF 288-11 cells were cultured in 75 cm² flasks in RPMI 1640 medium supplemented with 10% FBS (heat inactivated at 55°C for 40 min) (v/v) and 1% Penicillin-Streptomycin

at 37°C and 5% CO₂. HEK293T cells stably expressing T2R4 were cultured in DMEM-F12 medium supplemented with 10% FBS (heat inactivated at 55°C for 40 min) (v/v) and 1% Penicillin-Streptomycin at 37°C and 5% CO₂ in 100 mm plates.

CHRF-288-11 cells treated with Thrombin, BCML, BAPTA-AM, W7 and/or Quinine

After CHRF-288-11 cells were cultured and reached a proper density, CHRF cells were seeded and serum starved in 6 well cell culture plates at a density of 1×10^6 one day before the experiment. The cells were washed with phosphate buffer saline (PBS) and treated with Quinine (1mM final concentration) for 15 mins, or Thrombin (0.2 U/ml final concentration) for 3 minutes, or BCML (60 nM) for 15 minutes or Quinine plus Thrombin or Quinine plus BCML at 37°C. When cells were treated with BAPTA-AM and W7, the cells were washed with PBS and then treated with BAPTA-AM (50 μ M final concentration) for 30 minutes, or W7 (150 μ M final concentration) for 10 minutes followed by quinine (1mM final concentration) or quinine plus W7 for 15 minutes, or quinine plus BAPTA-AM for 30 minutes at 37°C.

Pull-down of active Ral A using Ral-binding domain of Ral-interacting protein 1

After treatment with Thrombin, BCML, BAPTA-AM, W7 or/and quinine, cells were lysed in Ral lysis buffer containing protease inhibitor cocktail (consisting of 5 μ g/ μ l leupeptin, 5 μ g/ μ l aprotinin and 1 mM PMSF). The lysate was sonicated for 30 seconds at 4°C followed by centrifugation at 17,000 x g at 4°C for 10 minutes. Then the supernatant was collected and incubate with 50 μ l GST-RRBD beads for 2 hours at 4°C with

constant rocking. After incubation, the beads were washed 3 times with Ral lysis buffer. The final bead pellet was suspended in 25 µl Laemmli's sample buffer and heated at 100°C for 5 minutes. The proteins were separated on 12% Acrylamide SDS-PAGE and transferred to PVDF membrane overnight at 4°C. Next day, Western blotting was performed using mouse RalA monoclonal Antibody (1:1000) after incubating the membrane for 30 minutes at room temperature in blocking buffer. The membrane was washed three times with TBST buffer (15 minutes each) and incubated with RalA antibody. Following washing, the membrane was incubated with horseradish peroxidase-conjugated secondary goat anti-mouse antibody (1:5000 dilution). Finally, the membrane was washed three times with TBST buffer (15 minutes each) and chemiluminescence was used to visualize the antigen antibody complex. The bands were quantified using Fluor-Chem band-analysis program. Total RalA lysate after treatment was used to assess equal protein loading.

Calmodulin affinity binding assay

CHRF cells were washed with phosphate buffer saline and lysed in buffer consisting of (200 mM HEPES (pH 7.4), 200 mM KCl, 1 mM MgCl₂ 0.55% triton X-100 and containing protease inhibitor cocktail (5 µg/µl leupeptin, 5 µg/µl aprotinin and 1 mM PMSF). The lysate was sonicated for 30 seconds at 4°C followed by centrifugation at 17,000 x g at 4°C for 10 minutes. Treatment condition included dilution of the samples in; buffer alone, buffer plus quinine (1mM final concentration) for 15 minutes at 30°C. Then 50µl CaM- sepharose beads were added to the reaction and incubated for 2 hours at 4°C

with constant rocking. The beads were collected by centrifugation and washed. The proteins bound to CaM- sepharose beads were suspended in Laemmli's sample buffer and heated at 100°C for 5 minute, separated by 12% SDS-PAGE and transferred to PVDF membrane at 4°C (25 V overnight). Next day, Western blotting was performed. The membrane was blocked using blocking buffer for 30 minutes at room temperature. Then the membrane was incubated with mouse monoclonal Ral-A Antibody (1:1000) in TBST buffer containing 5% skim milk for 2 hours. After washing the membrane three times using TBST buffer (15 minutes each), the membrane was incubated with horseradish peroxidase-conjugated secondary goat anti-mouse antibody (1:5000) at room temperature for 1 hr. After washing in TBST buffer (3X, 15 min each), the antigen antibody complex was visualized using enhanced chemiluminescence. The bands were quantified using Fluor-Chem band-analysis program.

Western blot analysis for T2R4 in CHRF cells

CHRF-288-11 cells (transiently expressing T2R4) and HEK293T-T2R4 stable cell line were lysed in solubilization buffer. The lysate was sonicated for 30 seconds at 4°C followed by centrifugation at 17,000 x g at 4°C for 10 mins. The samples were aliquoted and stored in -80°C for future use. The samples suspended in Laemmli's sample buffer and heated at 100°C for 5 minutes. 12% SDS-PAGE was used to separate the eluted protein and transferred to nitrocellulose membrane at 4°C (100 Volts for 1 hour). Western blotting was performed using primary polyclonal T2R4 Antibody (1:1000) at 4°C, overnight with constant rocking using TBST buffer consisting of 1% skim milk. Next

day, washed the membrane three times using TBST buffer for 15 minutes each time followed by incubation with secondary antibody (1-5000) for 1 hour at Room Temperature. The membrane was washed three times with TBST for 15 minutes each time. Chemiluminescence based detection and Vilber Lourmat Fusion FX7 imager were used to visualize the bands.

RNA extraction and polymerase chain reaction (PCR)

Total RNA was isolated from CHRF cells using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNA concentration was determined by using a Nanodrop 2000 (Thermo Scientific, Canada) and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA. About 1 µg of purified RNA was reverse transcribed using SSIII RT (superscript III reverse transcriptase, Invitrogen), dNTPs, Oligo-dT primer and first strand buffer. The synthesized cDNA was used as template for amplification by PCR. PCR was performed in a total volume of 30 µl containing 1 µl reverse transcribed cDNA, 500 nM of each primer in the master mix. An initial denaturation step of 94°C for 10 min was followed by 52 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 20 seconds and finished with a final extension at 72°C for 5 minutes on a thermocycler (MJ mini cycler, Bio rad). The housekeeping gene GAPDH was used as a positive control in the PCR reactions. Negative RT reaction was used as an internal control and no reverse transcriptase added to the cDNA synthesis reaction. All PCR products were separated

on 2% agarose gel stained with ethidium bromide. Gel images were recorded and photographed under UV light.

Statistical Analysis

Fluor-Chem Band analysis program was used to quantify the bands. One-way ANOVA program of Graph pad Prism6 software was used for analysis and to determine statistical significance.

Results

Expression of GST-RRBD (RIP1 Ral binding domain)

The Ral-interacting protein 1 (RIP1) is a downstream effector for RalA and interacts specifically with Ral-GTP. Therefore, GST-tagged Ral-binding domain (GST-RRBD) of Ral-interacting protein 1 was expressed in *E. coli* AD202 cells. Glutathione agarose beads were added to the bacterial lysate to purify the fusion protein. The purity of the expressed fusion protein was checked by SDS-PAGE (Figure 4).

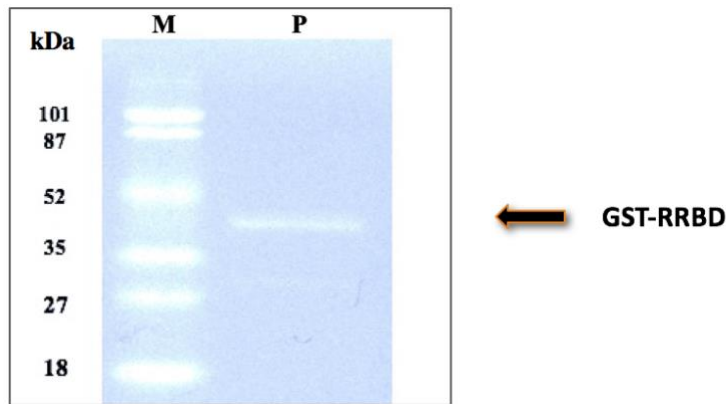


Figure 4. SDS-PAGE analysis of purified recombinant GST-RRBD fusion protein

The fusion construct was expressed in *E. coli* and glutathione agarose beads (as mentioned in materials and methods) were used to isolate and purify the fusion protein. Purity of the expressed protein was checked using 12% acrylamide SDS-PAGE followed by Coomassie blue staining (lane P). The expected size of the fusion protein is ~40 kDa and is indicated by an arrow in the above figure. The first lane (M) represent the protein markers in kDa.

Treatment of CHRF-288-11 cells with bitter agonist quinine and thrombin

To evaluate the effect of bitter agonist quinine on Ra1A activation, CHRF-288-11 were serum starved for 24 hours and then treated with quinine (1mM final concentration) for 15 mins followed by treatment with thrombin (0.2 U/ml) for 3 minutes. The concentration of 1 mM quinine was selected, as it is the EC₅₀ for T2R4. Pull-down assays using GST-RRBD were used to assess Ra1A activation. The results indicate that quinine causes a

significant increase in the activation of RalA when compared to control (Figure 5). Thrombin was used as a positive control because it is known to cause rapid activation of Ral A in platelets (Wolthuis, Franke et al. 1998).

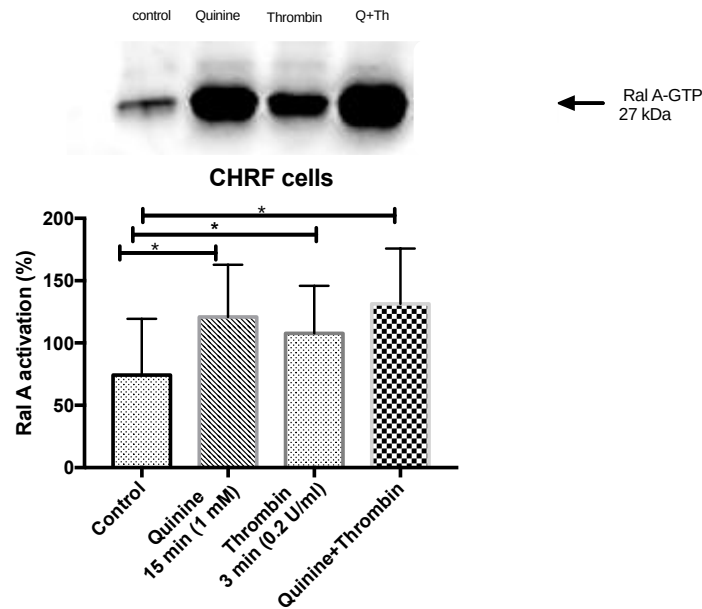


Figure 5. Quinine induced activation of Ral A in CHRF-288-11 cells

CHRF cells were cultured in RPMI media enriched with 10% FBS and 1% penicillin streptomycin in 5% CO₂ and 95% air at 37°C. When cells reached confluence, they were seeded at a density of 1×10^6 and serum starved in RPMI basal media for 24 hours. The cells were then washed with PBS and treated with quinine (1mM) for 15 min followed by treatment with thrombin (0.2 U/ml) for 3 minutes or quinine (Q) plus thrombin (Th). After treatment with quinine or thrombin, cells were lysed in Ral lysis buffer. The lysate was sonicated for 30 seconds at 4°C followed by centrifugation at $14,000 \times g$ at 4°C for 10 min. The supernatant was collected and incubated with 50 μ l GST-RRBD beads for 2 hr at 4°C with constant rocking. After incubation, the beads were washed 3 times with Ral buffer. The final bead pellet was suspended in 25 μ l Laemmli's sample buffer and heated at 100°C for 5 min. 12% SDS-PAGE was used to separate the eluted proteins and transferred to PVDF membrane overnight at 4°C. Next day, Western blotting was performed as described in the methods and using mouse RalA monoclonal antibody (1:1000 v/v). The bands were quantified using Fluor-Chem band-analysis program. Each result was normalized against the total RalA in the sample. The statistical significance of the data was analyzed using one-way ANOVA in

Graphpad Prism6 software, *p-value < 0.05. The experiment was repeated a minimum of three times, and a representative blot is shown

Effect on RalA activation upon treatment with bitter agonist quinine and calcium chelator, BAPTA-AM

To evaluate the role of calcium in quinine mediated Ral A activation, the following experiment was conducted. CHRF-288-11 cells were serum starved for 24 hrs and treated with BAPTA-AM (50 μ M final concentration) for 30 minutes followed by quinine (1mM) for 15 min. Pull-down assays using GST-RRBD were used to assess RalA activation. No significant change in Ral A activation was observed when the cells were treated with BAPTA-AM and BAPTA-AM plus quinine. This suggests quinine induced Ral A activation is independent of calcium (Figure 6).

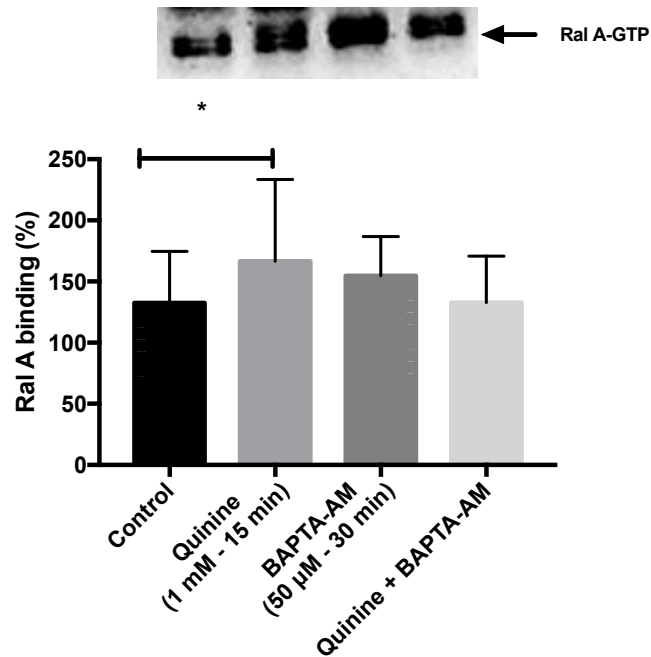


Figure 6. Effect of quinine and BAPTA-AM on Ral A activation in CHRF-288-11 cells

CHRF cells were cultured in RPMI media. The experiments were as described for Figure 5 except for treatment with BAPTA-AM (50 μ M final concentration) for 30 min followed by Quinine (1mM final concentration) for 15 min at 37°C. The bands were quantified using Fluor-Chem band-analysis program. The statistical significance of the data was done using one-way ANOVA in Graphpad Prism6 software, *p-value < 0.05. Each result was normalized against the total RalA in the sample. The experiment was repeated a minimum of three times and a representative blot is shown.

Treatment of CHRF-288-11 cells with bitter agonist quinine and CaM antagonist W7

To evaluate the role of CaM in quinine mediated Ral A activation, CHRF-288-11 cells were serum starved for 24 hours and treated with W7 (150 μ M) for 10 minutes followed

by quinine (1 mM) or quinine plus W7 for 15 minutes. Quinine induced Ral A activation was not reduced in the presence of the CaM inhibitor W7, which suggests quinine activates Ral A independently of CaM (Figure 7).

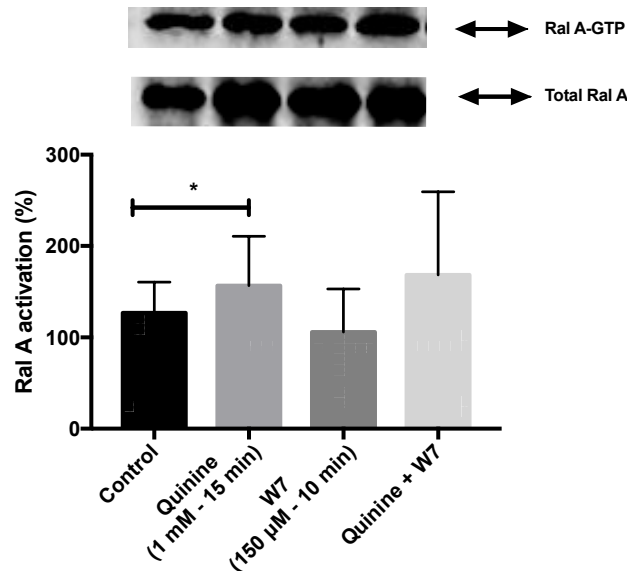


Figure 7. Effect of quinine and W7 on Ral A activation in CHRF-288-11 cells

CHRF cells were cultured in RPMI media. The experiments were as described for Figure 5 except for treatment with W7 (150 μ M) for 10 minutes followed by treatment with quinine (1mM) for 15 minutes and quinine plus W7. The bands after the Western blot were quantified using Fluor-Chem band-analysis program. The statistical significance of the data was done using one-way ANOVA of Graphpad Prism6 software, *p-value < 0.05. Each result was normalized against the total RalA in the sample. The experiment was repeated a minimum of three times, and a representative blot is shown.

Effect of quinine on interaction between RalA and Calmodulin

To examine if quinine influences Ral / CaM interaction, CHRF cells were lysed and then treated as follows: buffer alone, quinine (1 mM final concentration) with 50 μ l CaM-

Sepharose beads, and 50 μ l CaM-Sepharose beads alone (control) in buffer. The results suggest quinine causes Ral A does not affect Ral-CaM interaction (Figure 8).

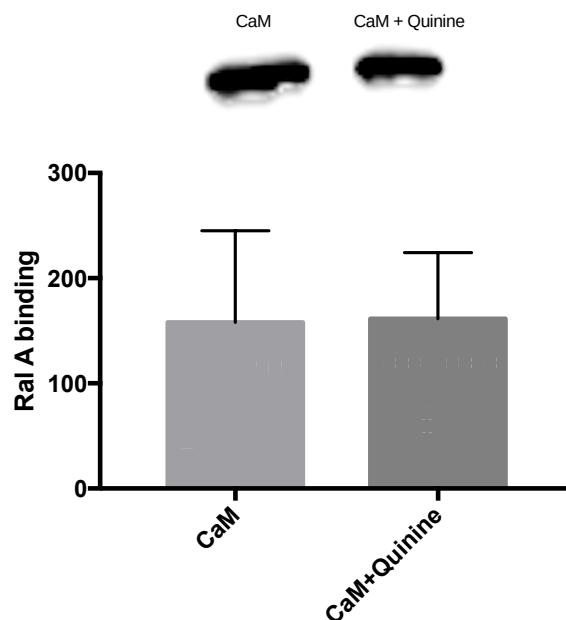


Figure 8. Ral A interaction in presence of quinine and CaM

CHRF cells were lysed in solubilization buffer. The lysate was sonicated for 30 sec at 4°C followed by centrifugation at 14,000 x g at 4°C for 10 min. Treatment conditions include the samples in buffer alone, quinine (1mM) in buffer for 15 min at 30°C. At the end of the incubation, 50 μ l CaM-Sepharose beads were added to both samples. The reactions were incubated for 2 hr at 4°C. The proteins bound to CaM-Sepharose beads were suspended in Laemmli's sample buffer and heated at 100°C for 5 min, separated by 12% SDS-PAGE and transferred to PVDF membrane at 4°C (25 V overnight). Next day, Western blotting was performed using mouse RalA monoclonal Antibody (1:1000). The bands were quantified using Fluor-Chem band-analysis program. Significance of the data was checked by t-test using Graphpad Prism6 software. The experiment was repeated a minimum of three times.

Treatment of CHRF-288-11 cells with bitter agonist quinine and T2R4 antagonist BCML

To determine whether quinine induced Ral A activation is through the bitter taste receptor T2R4, competition assays using T2R4 antagonist BCML were pursued. CHRF-

288-11 cells were serum starved for 24 hours, treated with T2R4 antagonist BCML (60 nM final concentration) for 15 minutes and with quinine (1 mM final concentration) for 15 minutes or quinine plus BCML. The results show that quinine causes a significant increase in the activation of RalA compared to control, while significant decrease in RalA activation was observed when cells were treated with quinine and BCML. This suggests quinine induced activation of RalA is through the bitter receptor, T2R4 (Figure 9).

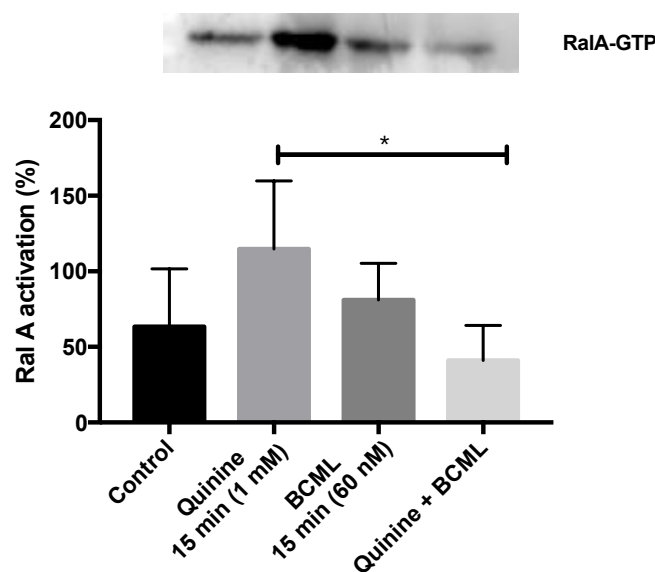


Figure 9. Biochemical characterization of T2R4 specific activation of Ral A in CHRF-288-11 cells

CHRF cells were cultured in RPMI media, and rest of the experiments were as mentioned earlier for Figure 5 except for treatment with quinine (1mM) for 15 min and BCML (60 nM) for 15 min or quinine plus BCML. Cells were lysed in Ral lysis buffer. The bands after the Western blot were quantified using Fluor-Chem band-analysis software. The statistical significance of the data was done using one-way ANOVA of Graphpad Prism6 software, *p-value < 0.05. The experiments were repeated a minimum of three times, and a representative blot is shown.

Characterization of endogenous T2R4 bitter receptor in CHRF-288-11 cells

To examine the expression of T2R4 in CHRF cells, reverse transcriptase (RT)-PCR and Western blot analyses were performed. To analyze the expression of TAS2R4 gene, RNA was isolated from CHRF cells and RT-PCR was performed (Figure 10). To characterize the T2R4 protein, western blot was performed using Anti-T2R4 antibody on CHRF cell lysates. As a positive control, lysates from HEK293T cells stably expressing T2R4 were used. Western analysis suggests expression of T2R4 in CHRF cells with molecular weight 34 kDa (Figure 11)

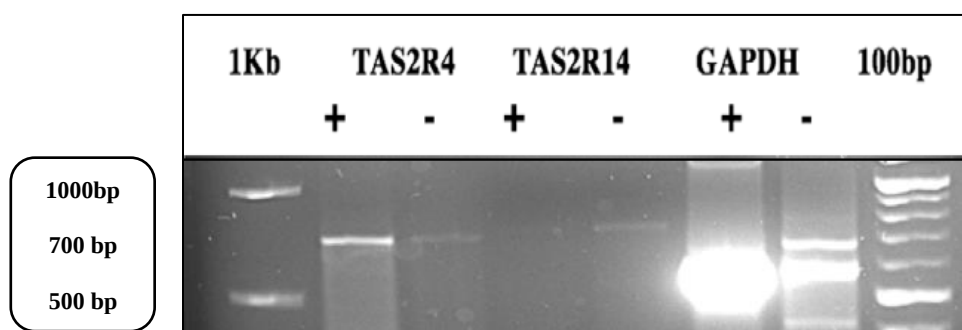


Figure 10. Reverse transcriptase (RT)-PCR analysis for the expression of bitter taste receptor gene TAS2R4.

Agarose gel electrophoresis (2%) analysis of the RT- PCR products showed that TAS2R4 was expressed in CHRF cells. Another bitter taste receptor gene TAS2R14 was also run as a control, no expression was observed for this gene. GAPDH was used as an internal control for the PCR reactions. + and – represent the addition and omittance of reverse transcriptase in the PCR reactions, respectively. Different DNA standards (ladders, 1 Kb and 100 bp) are run at the first and last lanes of the gels.

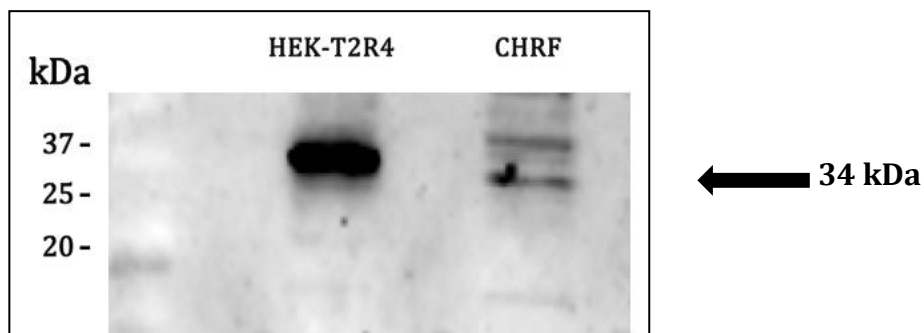


Figure 11. Western blot analysis of endogenous T2R4 in CHRF cells

Cell lysates of CHRF-288-11 cells and HEK239-T2R4 stable cell line were subjected to Western blot analysis as described in materials and methods, and using primary anti-T2R4 polyclonal Antibody (1:1000) at 4°C overnight with constant rocking. Next day, the blot was incubated with secondary antibody (1-5000) for 1h at RT. Chemiluminescence based detection using Vilber ourmat Fusion FX7 imager were used to visualize the bands. Molecular weight standards (left side) and T2R4 molecular weight (~34 kDa) are indicated in the figure above.

Discussion and conclusion

RalA is a 27 kDa GTP-binding protein that belongs to the Ras subfamily of Ras GTPase superfamily. As mentioned earlier, RalA like all GTPases acts as a molecular switch between the inactive GDP and active GTP forms (Park 2001). In platelets, Ral GTPases are found in dense granule, which are specialized secretory organelles and serve in regulating the release of storage contents from granules (Polakis, Weber et al. 1989; Mark, Jilkina et al. 1996). Thus, Ral GTPases have been suggested to play a crucial role in human platelet function (Bhullar and Seneviratne 1996; Frankel, Aronheim et al. 2005). The binding of growth factors, or agonists such as α -thrombin and platelet activating factor to receptors like receptor tyrosine kinase and GPCRs activate RalA in platelets (Wolthuis, Franke et al. 1998; Clough, Sidhu et al. 2002). Further, previously it has been demonstrated that RalA binds CaM, a calcium binding protein activated by the well-known second messenger, calcium. This binding of CaM to Ral has been shown to occur in both a calcium-dependent and a calcium-independent manner and is required for Ral activation (Clough, Sidhu et al. 2002). In addition to this pathway for RalA activation, Ras p21 can also participate in activation of RalA via epidermal growth factor which binds to EGFR leading to activation of Ras p21 and PLC pathways. Ras p21 upon activation recruits Ral-GEF which can lead to activation of Ral (Clough, Sidhu et al. 2002).

As mentioned earlier, all the bitter taste receptors including T2R4 belong to the GPCR family. However, recently, T2Rs have been shown to be expressed in several extra-oral tissues where they participate in mediating signal transduction other than the canonical bitter taste pathway (Gilca and Dragos 2017). Also, it has been demonstrated previously

that due to cell-permeant nature of quinine it can cause direct activation of G-protein (Naim, Seifert et al. 1994; Peri, Mamrud-Brains et al. 2000). T2R4 is activated by several bitter compounds (Meyerhof, Batram et al. 2010). The best pharmacologically characterized ligand for T2R4 is quinine (Prasad Pydi, Upadhyaya et al. 2012). Additionally, a recent study has discovered that BCML acts as an inverse agonist for T2R4 (Pydi, Sobotkiewicz et al. 2014). Previous studies have shown that the bitter agonist quinine inhibits platelet aggregation and it can also cause severe thrombocytopenia (Aster, Curtis et al. 2009). In the current study, we investigated if quinine and T2R4 play a role in regulating RalA activity in CHRF cells. We have discovered a novel effect of quinine on RalA function and this study is the first to show that quinine activates of RalA. The results also suggest that quinine activates RalA independently of calcium.

G-Protein/ T2R4 mediated RalA signaling

GPCRs function through both G-protein dependent and G-protein independent pathways (Wettschureck, Moers et al. 2005; Hoefen and Berk 2006). However, the G-protein dependent pathway for T2Rs is the only pathway that has been extensively studied so far.

The T2Rs are expressed at high levels in the circumvallate and fungiform taste buds and involved in taste perception in the gustatory system (Hevezi, Moyer et al. 2009).

It has been demonstrated previously that all bitter taste receptors including T2R4 signal predominantly through a calcium dependent pathway. The activation of T2R4 by a bitter ligand leads to release of calcium from ER via IP₃ secretion that leads to increase in intracellular calcium ions resulting in opening of transient receptor potential cation channel member 5 (TRPM). This leads to membrane depolarization which relays the taste signal to the brain (Finger, Danilova et al. 2005; Smrcka 2008). As stated above the bitter compound quinine is the best pharmacologically characterized ligand for T2R4 and is used clinically as an antimalarial drug. It has also been shown to inhibit platelet aggregation and cause severe thrombocytopenia (Aster, Curtis et al. 2009). However, we have shown for the first time using CHRF that quinine activates RalA through T2R4 independently of calcium. We confirmed these results by using quinine as an agonist and BCML as blocker for T2R4. In response to quinine stimulation, RalA underwent activation whereas BCML blocked quinine mediated action on T2R4 and inhibited RalA activation.

As discussed previously, it has been shown that CaM binds RalA in calcium-dependent and calcium-independent manner. Additionally, the binding of agonists such as α -thrombin and platelet activating factors to GPCRs activates RalA in platelets in a calcium dependent manner (Wolthuis, Franke et al. 1998; Wang and Roufogalis 1999; Park 2001; Clough, Sidhu et al. 2002). From our results, it has been established that using CaM affinity binding assay quinine does not inhibit Ral-CaM interaction. Western blot analysis demonstrated that the activation of RalA by quinine was not affected by the calmodulin-specific inhibitor, W7. This suggests that quinine activates RalA independently of CaM. Furthermore, we used BAPTA-AM, an intracellular calcium

chelator to demonstrate the calcium independency of the quinine mediated activation on RalA. Our data suggests an alternative pathway to the previous studies which demonstrated that the elevation of second messenger calcium is essential for activation of Ral when challenged with thrombin (Wolthuis, Franke et al. 1998; Clough, Sidhu et al. 2002). However, this support previous studies from our laboratory that have shown that quinine inhibits Rac1 activation through T2R4/G-protein and this is only partially calcium dependent (Sidhu, Jaggupilli et al. 2017). Further studies are required to elucidate the mechanism responsible for the quinine mediated activation of RalA (Figure 12).

In conclusion, this study is the first to show the effect of a bitter agonist acting through a T2R on RalA function. The treatment of CHRF cells with quinine causes a significant increase in the activation of Ral A, and the work in this thesis suggests that it is predominantly through T2R4. Results from PCR and western blot analysis demonstrated the presence of T2R4 in CHRF cells. Although it has been shown that calmodulin is required for RalA activation, my results suggest that quinine does not act through CaM. Thus, it can be concluded from this thesis work that quinine may have an effect on platelet function through activation of small G- protein, Ral. The information gained from these studies may lead to the development of an approach to control thrombocytopenia during therapeutic intervention where quinine is prescribed as the drug of choice.

Future directions

1. Investigate the role of DAG/PKC pathway in activation of RalA that is mediated by bitter taste receptor agonist quinine.

As mentioned earlier, there are two known pathways that play a role in downstream signal transduction of bitter taste receptors: PLC-IP₃ and the DAG pathways. But less is known about DAG which has been shown to play a role in signal transduction of Ral. Thus, further experiments are required to determine the function of DAG/PKC pathway in possible activation of RalA.

2. Investigate the role of Ras-dependent pathway in activation of RalA that is mediated by bitter taste receptor agonist quinine.

As stated above, RalA can be activated through Ras-dependent and Ras-independent pathway (calcium dependent). In this study, we have investigated only the calcium dependent pathway. Further experiments are required to determine the Ras/Ral-GEF pathway in activation of RalA in response to quinine (Figure 12).

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