Quinine Regulates Rac1 GTPase Activity through the Bitter Taste Receptor T2R4 and G-Protein

by

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ABSTRACT

Rac1 is a member of the Rho family of low molecular mass GTP binding proteins (GTPases). It regulates the dynamics of actin cytoskeleton by causing membrane ruffling, chemotaxis, and lamellipodia formation. As is the case with other GTPases, Rac1 cycles between the active GTP-bound form and the inactive GDP-bound form. T2R4 is a bitter taste receptor that belongs to the GPCR (G-protein coupled receptor) family of proteins. In addition to mediating bitter taste sensations from the tongue, T2R4s have been recently found in tissues other than the oral cavity e.g. nasal epithelium, airways, brain, gastrointestinal tract and male reproductive system suggesting a much broader physiological function for these receptors. Quinine, an antimalarial drug is one of the most bitter tasting compounds known. Quinine is a known agonist for T2R4s whereas BCML (Nα,Nα-Bis(carboxymethyl)-L-lysine) acts as an inverse agonist.

Since Rac1 is activated via various receptors like tyrosine kinase (RTKs), integrins and GPCRs; we have investigated the potential role of T2R4 in regulating Rac1 activity. In this study, HEK293T cells stably expressing T2R4/$G\alpha_{16/44}$, T2R4 or $G\alpha_{16/44}$ were transiently transfected with HA-Rac1 followed by treatment with quinine or quinine plus BCML for 15 min. After incubation, active Rac1 was pulled-down from cell lysate using GST-PAK1 and anti-HA monoclonal antibody was used in Western blots to quantify amount of active Rac1. The results demonstrated that quinine treatment resulted in significant ($p<0.001$) reduction in the amount of active Rac1 whereas in the presence of BCML, quinine failed to cause any significant change in the amount of active Rac1 when compared to untreated cells. There was

no change in the level of active Rac1 when HEK293T cells stably expressing T2R4 were treated with quinine.

This study is the first to show an inhibitory downstream action of a T2R4 agonist on Rac1 function. Further investigation will help in better understanding the downstream signal transduction network of T2R4 and its extra-oral physiological roles.

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I would like to dedicate this thesis to my family and friends like family.

TABLE OF CONTENTS

Page No.

LIST OF TABLES

Page No.

LIST OF FIGURES

Page No.

LIST OF ABBREVIATIONS

APC	Allophycocyanin
BAPTA-AM	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)
BCML	Nα,Nα-bis(carboxymethyl)-L-lysine
BitterDB	Bitter ligand Database
Ca^{++}	Calcium ion
CAAX	C-cysteine, A-aliphatic amine, X- any amino acid
CaM	Calmodulin
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
CRIB	Cdc42/Rac1 Interactive Binding Motif
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EL	Extra-cellular Loop

ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
Gα16/44	Gα16/44 gustducin chimera
GABA	γ-Aminobutyric acid
GAP	GTPase Activating Protein
GDI	Guanine-nucleotide Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
G_{gust}	Gustducin G-Protein
GIRK	G-protein regulated inward K^+ channel
GIT	Gastro-intestinal Tract
GLP-1	Glucagon Like Peptide-1
GPCR	G Protein-coupled receptor
GPCRdb	G Protein-coupled receptor database
G-Protein	Guanosine Nucleotide-binding Protein
GRK	G protein-coupled receptor kinases
GST	Glutathione-S-Transferase
GTP	Guanosine Triphosphate

HA-Tag	Human influenza hemagglutinin Tag
HEK 293T	Human Embryonic Kidney Cells T-antigen
IC50	Half Maximal Inhibitory Concentration
IL	Intra-cellular Loop
IP_3	Inositol trisphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K^+	Potassium ion
kDa	Kilo Dalton
PAK	p-21 Activating Kinase
pCMV	Cytomegalovirus Plasmid
PDE	Phosodiesterase
PKA	Protein Kinase A
PLC	Phospholipase C
PMSF	Phenylmethane sulfonyl fluoride
PVDF	Polyvinylidene difluoride
RGS	Regulators of G-Protein Signaling
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase

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

1. INTRODUCTION

1.1 Overview

Signal transduction is the fundamental mechanism through which the cell interacts with the external environment. A number of complex processes and pathways are involved in relaying the outside information to the inside of the cell. Receptors constitute one of the major routes in cell signaling and are classified broadly into internal receptors and cell-surface receptors. In humans the cell surface receptors are further subdivided into G-protein coupled receptors, ion-channel linked receptors and enzyme linked receptors (Boundless 2016). A receptor is a protein that is embedded in the cell membrane and communicates with both external and internal environments through intrinsic and extrinsic domains i.e. a transmembrane receptor. These receptors can be activated by a wide variety of stimuli usually called the first messengers or ligands which can be electrical, physical, light or chemical molecules (Hendrickson 2005). Once activated, these receptors undergo conformational change leading to activation of a signaling cascade such as production of second messengers and subsequent induction of physiological response due to changes in cellular function as a result of stimulation. In humans and several other animal species, taste perception constitutes one of the most vital mechanism of signal transduction because the ability to differentiate between various compounds in the food based on taste serves as a protective system against ingestion of harmful or poisonous substances. The sensation of taste is perceived when a compound binds to the protein present in the taste receptor cells (TRCs) on the taste buds in the oral cavity. The information is then relayed to the brain where the taste sensation is recognised into bitter, sweet, salty, umami or sour.

1.2 G Protein-Coupled Receptors

The G protein-coupled receptors or GPCRs are the most abundant of the five classes of cell surface receptors. As the name suggests, these receptors function by coupling to the heterotrimeric G-protein for signal transmission. GPCRs are a target for approximately 50% of the drugs on market (Davies, Secker et al. 2007). Rhodopsin and β-adrenergic receptors were the first to be sequenced and studied for the seven transmembrane structures (Hendrickson 2005). A wide range of receptors fall in the category of GPCRs including taste, olfactory, chemokine and various hormonal receptors (such as angiotensin, epinephrine, serotonin and gonadotropin-releasing hormone) (Uings and Farrow 2000). The GPCRdb classifies human GPCRs into 7 different classes: Class A (Rhodopsin), Class B1 (Secretin), Class B2 (Adhesion), Class C (Glutamate), Class F (Frizzled), Class T (Taste 2) and Class O (other) GPCRs (Isberg, Mordalski et al. 2016).

1.3 Bitter taste receptors (T2Rs)

Of the five taste sensations, bitter (Adler, Hoon et al. 2000), umami and sweet tastes are mediated via GPCRs (Gilbertson, Damak et al. 2000; Sainz, Korley et al. 2001; Andres-Barquin and Conte 2004). Whereas the salty (Heck, Mierson et al. 1984) and sour (Kinnamon, Dionne et al. 1988; Ugawa, Mianami et al. 1998; Andres-Barquin and Conte 2004) tastes are believed to be transmitted through cation channels. In humans the T2Rs (Taste Receptor Type 2) are encoded by intron less genes known as TAS2R (Adler, Hoon et al. 2000). Chromosomes 5, 7 and 15 have been shown to contain the TAS2R genes (Conte, Ebeling et al. 2002). Although there are 25 bitter taste receptors in humans, also knowns as

T2Rs, they are capable of identifying multiple bitter compounds and the bitter compounds are also able to bind to multiple T2Rs. (Meyerhof, Batram et al. 2010). Similar to GPCRs the structure of T2Rs consists of seven hydrophobic transmembrane α-helices with extracellular N-terminal domain, intracellular C-terminal domain, three extracellular loops (ECL1-3) and three intracellular loops (ICL-1-3) (Hendrickson 2005) (**Figure 1**).

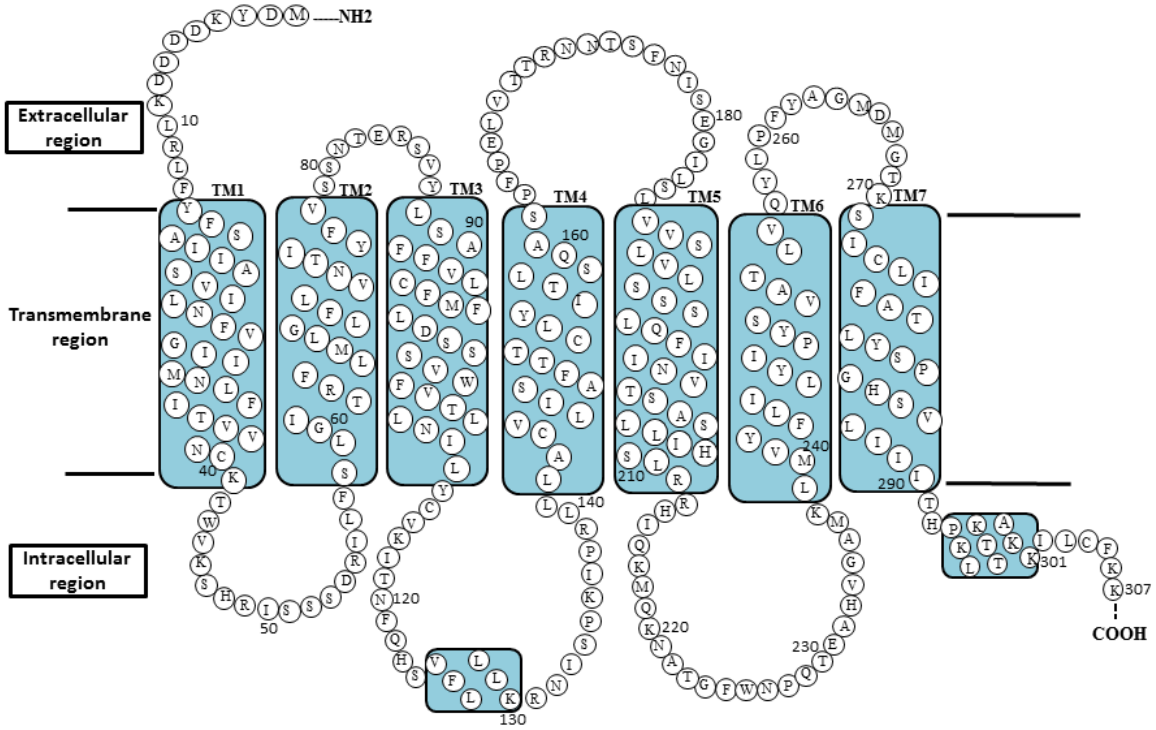


Figure: 1 Two-dimensional schematic representation of amino acid sequence of T2R4: T2R4 contains 307 amino acids with extracellular N-terminus, intracellular C-terminus and seven transmembrane loops (TM1-7). (Personal communication with Dr. Chelikani)

1.3.1 Extra-oral expression of T2Rs

Apart from bitter taste perception in the oral cavity these receptors have been recently shown to be expressed in various extra-oral tissues (**Figure 2**) (Shaik 2016; Avau and Depoortere 2016). Several regions of the rat brain have been shown to contain neurons that express functional bitter taste receptors (Singh, Vrontakis et al. 2011; Dehkordi, Rose et al. 2012; Voigt, Bojahr et al. 2015). However, recently increased expression of T2Rs in cerebral cortex has been implicated in Parkinson's disease and human neuroblastoma (Garcia-Esparcia, Schluter et al. 2013; Ansoleaga, Garcia-Esparcia et al. 2015). Expression of T2Rs has also been demonstrated in the gastrointestinal tract (GIT) of humans (Widmayer, Kuper et al. 2012; Janssen, Laermans et al. 2011; Kaji, Karaki et al. 2009). T2Rs have been implicated in the regulation of appetite as well as in maintaining homeostasis in the intestine by modulating insulin and glucose levels via GLP-1 hormone (Dotson, Zhang et al. 2008; Avau and Depoortere 2016). Also, recently, T2Rs have been implicated in breast cancer, where they are shown to have differential expression among different breast cancer cell lines in comparison to normal mammary tissue (Singh, Chakraborty et al. 2014). Nasal epithelium, upper and lower respiratory tract express T2Rs which mediate the movement of cilia and clearing of the airways (Finger, Bottger et al. 2003; Shah, Ben-Shahar et al. 2009). Additionally, T2Rs bind to bitter ligand and cause dilation of the airway smooth muscles (Deshpande, Wang et al. 2010). Also, it has been shown that the bitter compound, dextromethorphan causes vasoconstriction of the pulmonary circuit whereas it causes relaxation of the airways; thus highlighting the differential regulatory function of T2Rs (Upadhyaya, Singh et al. 2014). Expression of T2Rs has been demonstrated in the male reproductive system and has a role during spermatogenesis (Li 2013; Xu, Cao et al. 2013).

The vast distribution of T2Rs opens up new avenues for research and additional beneficial therapeutic targets (Avau and Depoortere 2016).

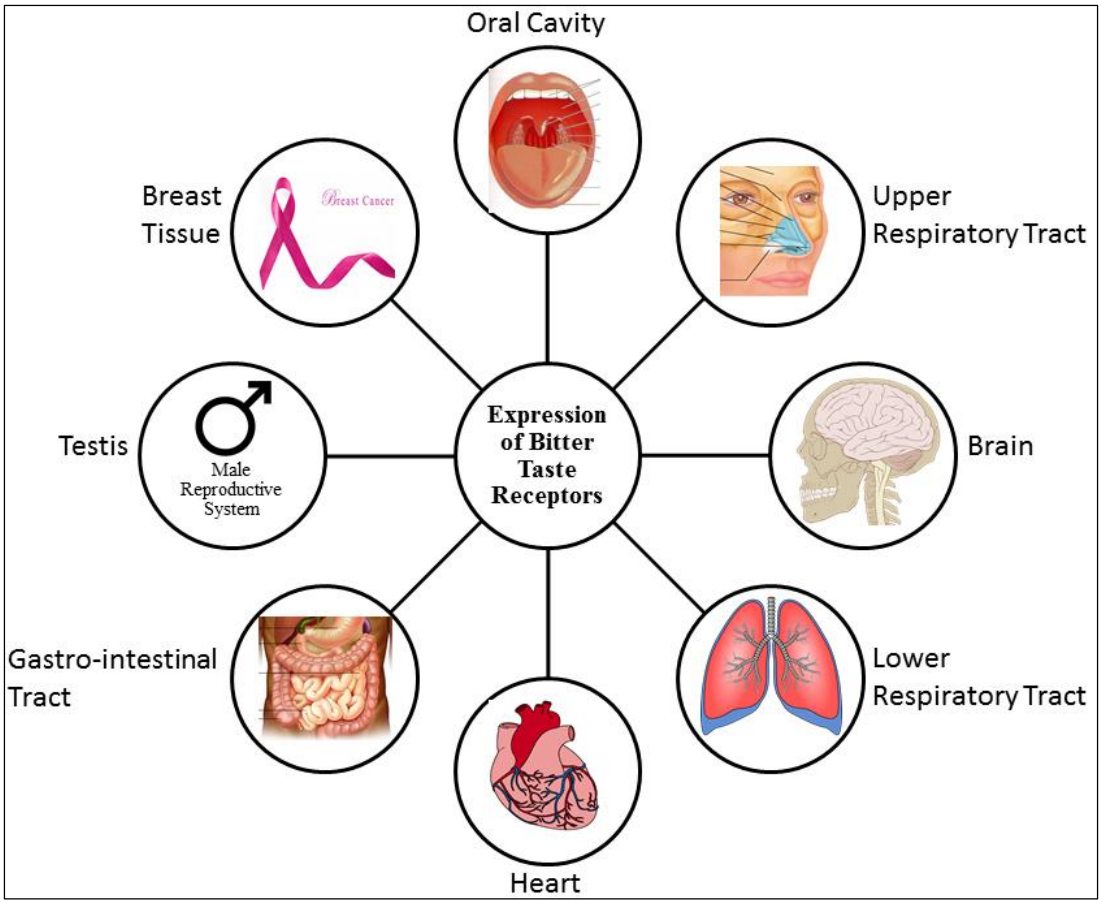


Figure 2: Schematic representation of distribution of T2Rs in various regions (oral and extra-oral) of human body.

1.3.2 Bitter Ligands for T2Rs

T2Rs are activated by over 700 structurally diverse bitter ligands and they have been classified into synthetic or natural compounds (Meyerhof, Batram et al. 2010; Jaggupilli,

Howard et al. 2016). The list of these compounds can be found in the bitter ligand database called BitterDB (Wiener, Shudler et al. 2012). Structurally the bitter compounds include a wide range of molecules such as amines, amides, terpenoids, steroids, fatty acids, esters and glycosides to name a few (Meyerhof, Batram et al. 2010; Jaggupilli, Howard et al. 2016). Among these a well-known and one of the bitterest compounds, quinine, is a natural alkaloid that has two major fused-ring systems consisting of the aromatic quinolone, and the bicyclic quinuclidine (**Figure 3**). In addition to its bitter taste and anti-malarial properties it also acts as an anti-pyretic, anti-inflammatory and analgesic drug (Meyerhof, Batram et al. 2010; Pydi, Sobotkiewicz et al. 2014).

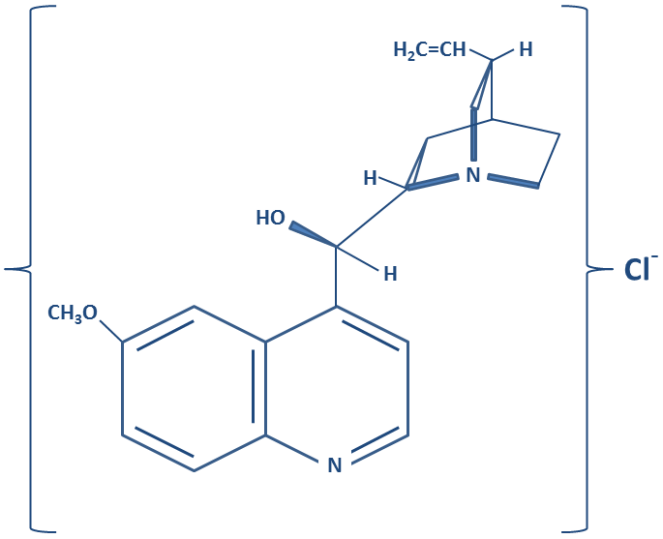
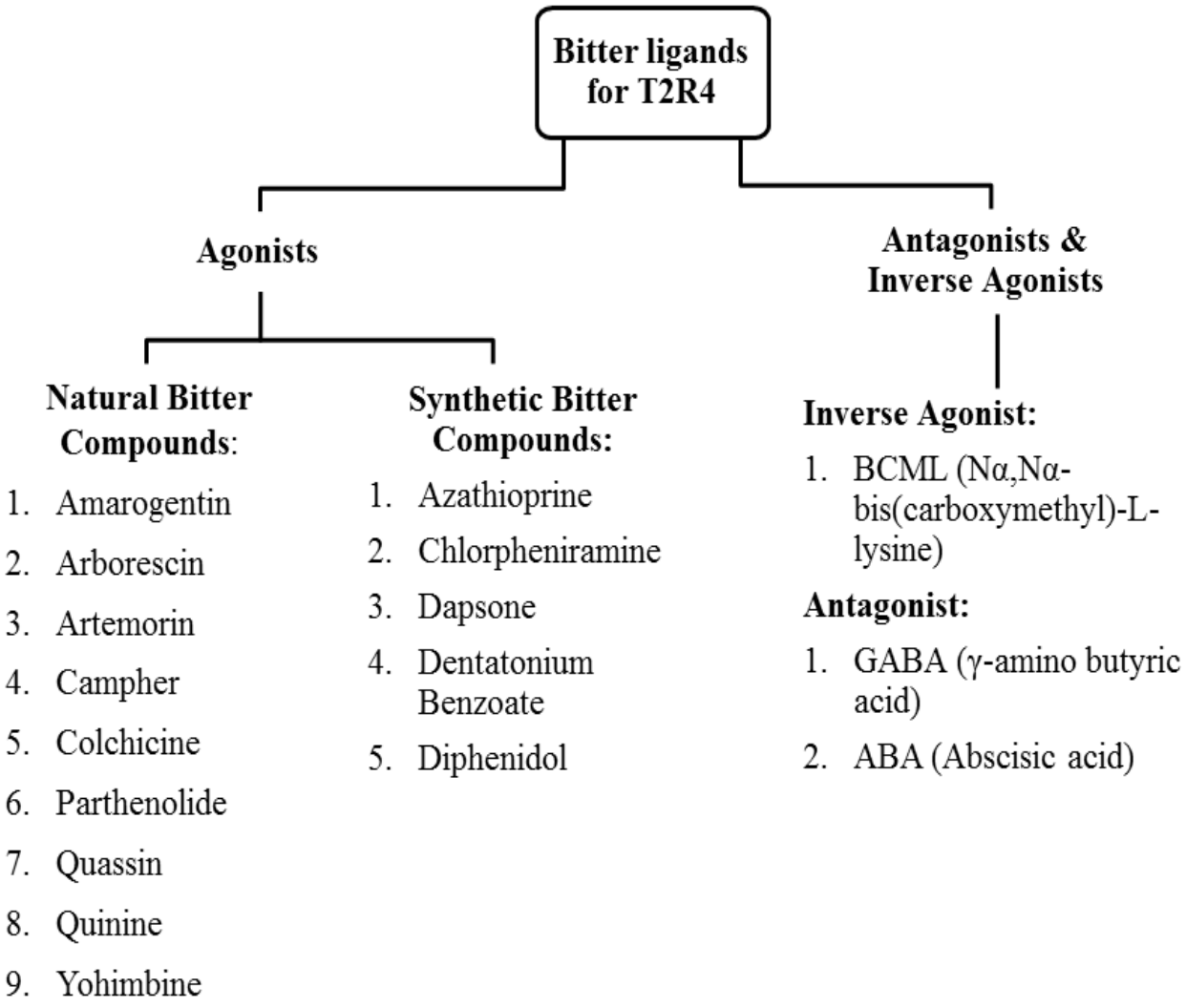


Figure 3: Structure of Quinine Hydrochloride (Empirical formula:$C_{20}H_{24}N_2O_2$.HCl)

Quinine was suggested to activate nine T2Rs (Meyerhof, Batram et al. 2010), however its efficacy (EC_{50} of quinine = 1mM) was determined only for T2R4. In our study, T2R4 was

targeted because it was the only T2R that had been well-characterized with an inverse agonist, Nα,Nα-bis(carboxymethyl)-L-lysine (BCML) with an IC_{50} of 59 ± 18 nM (Pydi, Sobotkiewicz et al. 2014) (**Table 1**).

Table 1: Tabular representation of agoinsts, antagonists and inverse agonist for the receptor T2R4



1.4 G-Proteins

The G-proteins are also called guanine nucleotide proteins or GTP-binding proteins because these proteins have the ability to bind GDP or GTP. The G-proteins participate in signal transduction by converting from the inactive GDP-bound form to the active GTP-bound form (McCudden, Hains et al. 2005). G-proteins can be classified into 'large' or heterotrimeric G-proteins and the 'small' or monomeric G-proteins (Konstantinopoulos, Karamouzis et al. 2007).

1.4.1 Heterotrimeric G-Proteins

α, β and γ subunits constitute the heterotrimeric G-proteins which upon activation dissociates into Gα and βγ complexes. The α subunit is the largest of the three with a molecular weight ranging from 41-45 kDa whereas β and γ have molecular weights of 35kDa and 8-10kDa respectively (Milligan and Kostenis 2006). There are 23 known Gα proteins in humans which are encoded by 16 genes. Broadly, G-proteins are divided into 5 families and they are named after the Gα subunit **(Table 2)**. The large numbers of combinations of βγ dimers are encoded by five Gβ and twelve Gγ genes in the human genome **(Table 2)** (McCudden, Hains et al. 2005). Our study explores the gustducin G-protein (G_{gust}) which is involved in the bitter taste perception. It has been shown that mRNA of α-gustducin is expressed in the taste buds and knockout studies in mice have been shown to cause a 70% decrease in bitter taste perception (Mclaughlin, Mckinnon et al. 1992; Wong, Gannon et al. 1996). Although, G-gustducin shares 80% sequence homology with G-

transducin, the proteins are very different functionally. Intracellularly, α-gustducin has been found to co-localize with β_3 γ_{13} complexes (Yan, Sunavala et al. 2001; Margolskee 2002).

Table 2: Types of heterotrimeric G-proteins and α,β,γ subunits

G-protein Family	α subunit
Gs(stimulatory)	α_s , α_{olf}
Gi (inhibitory)	$\alpha_{i(1-3)}$, $\alpha_{o(1-2)}$, α_t(transducin), α_g(gustducin), α_z
Gq	α_q, α_{11}, α_{14}, α_{15}, α_{16}
$G_{12/13}$	α_{12}, α_{13}

βγ Complex	βγ Subunits
$G_{\beta\gamma}$	β (1-5), γ (1-12)

The heterotrimeric G-protein complex is bound to the intracellular part of the GPCR. However, upon ligand binding the GPCR is activated and the conformational changes in the receptor trigger the activation of the G proteins (Oldham and Hamm 2007). This further leads to dissociation of G protein into active GTP bound α subunit and βγ complex. This results in transduction of upstream signal to the downstream effector proteins (Oldham and Hamm 2007; Oldham and Hamm 2008). The reformation of heterotrimeric complex occurs due to the intrinsic GTPase activity of α subunit and through the regulators of G-protein signaling (RGS) proteins (De Vries, Zheng et al. 2000).

Activated G protein leads to production of a range of second messengers including IP_3 (inositol 1,4,5-triphosphate), cyclic AMP (cAMP) and DAG (diacylglycerol) which cause further initiation of intracellular signaling pathways (Smrcka 2008). The G proteins co-ordinate the production of these second messengers through the activation of its effector proteins such as phospholipase C, adenylyl cyclase, Ca^{++} channels and G-protein regulated inward K^+ (GIRK) channels (McCudden, Hains et al. 2005).

1.4.2 Monomeric Small G-Proteins

Small GTPases are similar to the alpha subunit of heterotrimeric G-proteins (Yang 2002) with molecular weight ranging from 20-40 kDa (Takai, Sasaki et al. 2001; Csepanyi-Komi, Levay et al. 2012). Eukaryotes have more than 150 known small G-proteins also known as the Ras superfamily (Wennerberg, Rossman et al. 2005). Ras superfamily is further classified into five subfamilies based on their structure, function and sequence homology: Ras, Rho, Ran, Rab and Sar1/Arf (Goitre, Trapani et al. 2014) (Paduch, Jelen et al. 2001) (**Table 3**). Breifly, description of each subfamily is described hereafter:

i. The *Ras subfamily* derives its name from the founding member of the family, Ras p21. It was discovered as a retroviral oncogene from **Ra**t **s**arcoma and it encodes a 21 kDa protein called Ras p21 (Cox and Der 2010). Members of Ras family regulate gene expression leading to cell growth, differentiation and survival (Corbett and Alber 2001; Wennerberg, Rossman et al. 2005; Cox and Der 2010).

ii. *Rho subfamily* gene was discovered upon screening a cDNA library of abdominal ganglia of Aplysia (Madaule and Axel 1985). It was named Rho since it shared 35% amino acid

homology with H-Ras of the **Ra**s **ho**mology (Etienne-Manneville and Hall 2002). Rho subfamily participates in cytoskeletal dynamics such as cell movement and adhesion (Corbett and Alber 2001). In this study, we focus on the Rac1 member of the Rho subfamily.

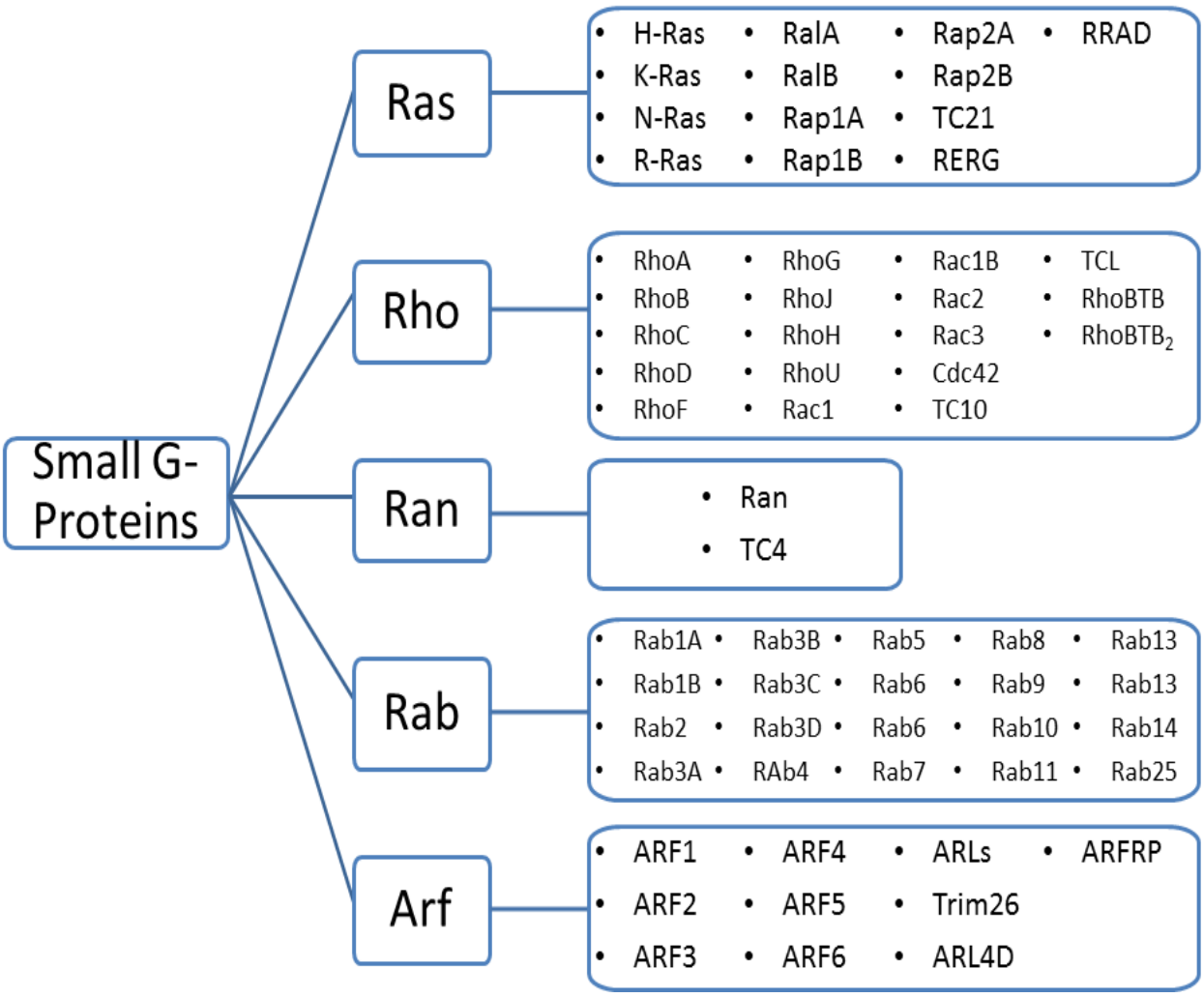
iii. *Ran subfamily* is known as **Ra**s related **n**uclear protein and the corresponding gene was first reported in cDNA library of human teratocarcinoma (Drivas, Shih et al. 1990; Cox and Der 2010). This subfamily plays a role in nuclear transport of both RNA and proteins (Weis 2003).

iv. *Rab subfamily* shares 30% amino acid homology with Ras hence it is called **Ra**s-like protein in **b**rain and is the largest subfamily comprising over 60 proteins (Pereira-Leal and Seabra 2001). It was discovered in cDNA library of Rat brain by using the conserved amino acids of Ras p21 as a probe (Touchot, Chardin et al. 1987). They are known to regulate intracellular vesicle trafficking and protein trafficking within organelles (Wennerberg, Rossman et al. 2005).

v. *Arf subfamily* is called the **A**DP **r**ibosylation **f**actor and they were isolated from the rat liver membrane by protein purification (Kahn and Gilman 1984). They regulate vesicle trafficking and actin remodelling (Memon 2004).

Similar to heterotrimeric G-proteins, small G-proteins act as a molecular switch cycling between the active (GTP-bound) and inactive (GDP-bound) forms (**Figure 4**). However, the GTP-bound form leads to signal transduction by interacting with specific effectors. This activity of the small G-proteins is regulated by two main classes of proteins known as GEFs (guanine nucleotide exchange factors) and GAPs (GTPase activating proteins).

Table 3: Types of small/monomeric G-proteins



1.5 Rho GTPase subfamily

Molecular weight of Rho GTPases is ~21 kDa and they are further divided into 8 subfamilies containing 20 members: Rho, Rac, Cdc42, RhoBTB, Rnd, Rho D/F, Rho U/V and Rho H (Vega and Ridley 2008; Tang, Olufemi et al. 2008). However, Rac, Cdc42 and Rho are the most extensively studied as well as highly conserved members of the family (Vega and Ridley 2008). There are 3 types of Rho GTPases: RhoA, RhoB and RhoC and they affect stress fibre and focal adhesion formation (Burridge and Wennerberg 2004). Cdc42 participates in the formation of filopodia (Phillips, Calero et al. 2008). Rac subfamily contains the following members: Rac1, Rac2, Rac3 and RhoG and plays an important role in lamellipodia formation, chemotaxis and cell polarity (Nobes and Hall 1999).

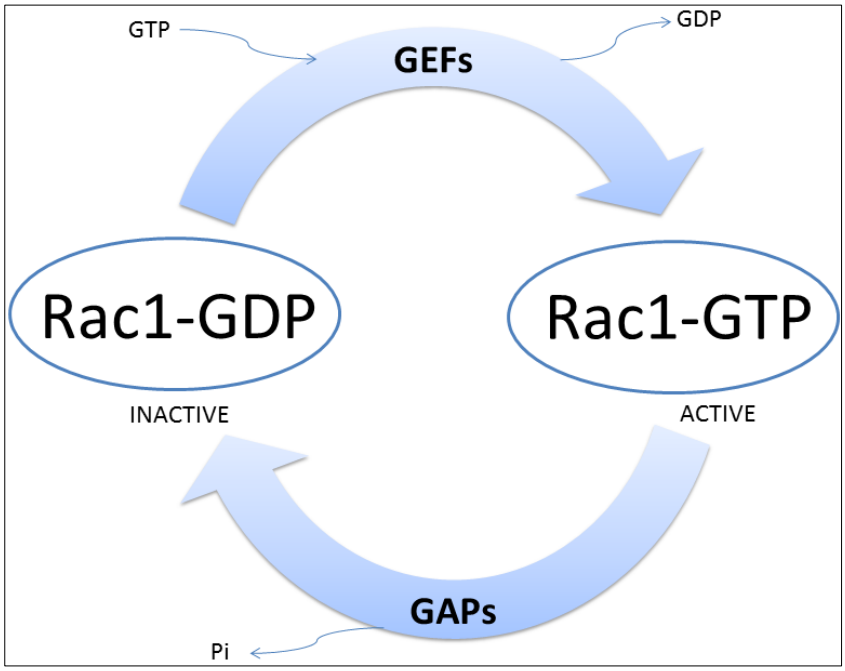


Figure 4: GTPase cycle of Rac1 small G-protein. GEFs and GAPs catalyse the cycling of Rac1 between active (GTP-bound) and inactive (GDP-bound) forms.

1.6 Rac1 GTPases

Rac1 (Ras-related C3 botulinum toxin substrate 1) is a ~21 kDa protein that belongs to the family of small G-protein called Rho GTPases. It has been shown to play an important role in modulating actin cytoskeletal dynamics like membrane ruffling, chemotaxis and lamellipodia formation (Hall 1998; Ridley 2006). Other functions of Rac1 include regulation of cell cycle and gene expression (Burridge and Wennerberg 2004; Jaffe and Hall 2005).

1.6.1 Regulators of Rac1- GEFs and GAPs

Rac1 cycles between the active GTP bound form and inactive GDP bound form similar to the other GTPases (Marei and Malliri 2016) (**Figure 4**). This process is controlled by two types of regulatory molecules: GEFs (Guanine Nucleotide Exchange Factors) which cause the exchange of GDP to GTP and GAPs (GTPase Activating Proteins) which augment the slow intrinsic GTPase activity of the protein (Marei and Malliri 2016; Rossman, Der et al. 2005). Third type of regulators called the GDIs (Guanine-nucleotide Dissociation Inhibitors) are also known to play a role in inactivating GTPases by inhibiting the GDP dissociation (Bishop and Hall 2000). The GEFs are classified into two: Dbl family containing over 70 members and DOCK family containing 11 members (Lin and Zheng 2015). Dbl and DOCK families are structurally and functionally different because Dbl has DH/PH (Dbl homology/pleckstrin homology) domain whereas DOCK includes the DOCK180 homology domains, DHR1 & 2 (Heasman and Ridley 2008). The well-known effectors for Rac1 are: VAV1, VAV2, DBL and Tiam1 (Bid, Roberts et al. 2013). On the other hand there are over 70 GAPs in eukaryotes which contain a 150 amino acid conserved RhoGAP catalytic

domain (Lin and Zheng 2015). The most common negative regulators of Rac1 include RhoGAP1, RacGAP1, ABR and BCR (Bid, Roberts et al. 2013). The third type of regulators, the GDIs, contain two domains: N-terminal domain that prevents nucleotide exchange and hydrolysis as it confines the GTPases and the C-terminal domain which binds to the GTPases as it contains the geranyl-geranyl binding pockets (Bid, Roberts et al. 2013). There are three known GDIs: Rho GDI1, Rho GDI2 and Rho GDI3 with 1 being ubiquitously expressed, 2 in B & T lymphocytes and 3 in brian, kidneys, lungs and pancreas (Dovas and Couchman 2005).

1.6.2 Rac1 Effector Proteins

Once activated Rac1 interacts with its downstream effectors to initiate signal transduction. The majority of the effectors of Rac1 contains a CRIB (Cdc42/Rac1 interactive binding motif) domain which binds to the active form of Rac1 (Hakoshima, Shimizu et al. 2003). Structurally, the CRIB domain binds to the switch I and II regions of the Rac1 protein (Bustelo, Sauzeau et al. 2007) and the well-known effectors of Rac1 are listed in **Table 4**.

Table 4: Summary of Rac1 effector proteins

Effectors of Rac1	Type of Protein
p70 S6 Kinase	Ser/Thr kinase
Mlk 2, 3	Ser/Thr kinase
MEKK 1,4	Ser/Thr kinase
PAK1,2,3	Ser/Thr kinase
PI3K	Lipid kinase
PI-4-P5K	Lipid kinase
DAG kinase	Lipid kinase
PLD	Lipase
PLC-β2	Lipase
WAVE	scaffold
POSH	scaffold
POR-1	scaffold
p140Sra-1	scaffold
p67phox	scaffold
IQGAP1,2	scaffold

From the above list, the most well studied effector is the p-21 activating kinases (PAK), which participates in membrane ruffling and cytoskeletal reorganization. It is a serine/threonine kinase comprising of six members which are broadly subdivided into two groups based on their homology: Group I (1-3) and Group II (4-6) (Lin and Zheng 2015). Among these, the Group I PAKs are more widely studied than the other. They are found as homodimers in the inactive form and upon activation they dissociate (Lin and Zheng 2015). Rac1-GTP causes activation of the group I PAKs, for example, upon activation PAK 1 causes phosphorylation of MLC (myosin light chain), LIMK (**L**in11, **I**sl-1 & **M**ec-3

Kinases), dynein light chain 1 and filamin A which in turn leads to actin stabilization (Bokoch 2003). Also, during migration, Rac1-induced actin reorganization at the cell's leading edge requires PAK1/LIMK pathway (Yang, Higuchi et al. 1998). In this study, we have used GST-PAK1 fusion protein to pull out-active Rac1 from the total lysate.

1.6.3 Structure of Rac1

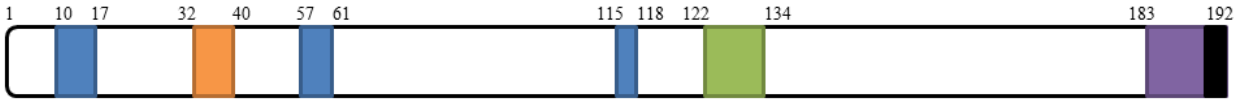


Figure 5: Linear schematic diagram representing the functional domains of Rac1: GTP binding domain; Effector domain; Rho insert domain; Membrane localization domain; CAAX motif.

Similar to the other Rho GTPases, Rac1 also contains the G-domain comprising of six-stranded β-sheets enclosed by α-helices (Hakoshima, Shimizu et al. 2003). There are four functional domains in Rac1: GTP binding domain (three in number), effector domain, Rho insert domain and the membrane localization domain (Naji, Pacholsky et al. 2011; Hakoshima, Shimizu et al. 2003) (**Figure 5**). The Rho insert is unique to only Rho GTPases (Johnson 1999). The Rho GTPases have switch I and switch II which correspond to the Ras switch I (32-38) and II (59-97) respectively (Hakoshima, Shimizu et al. 2003). However, the switch I being elastic in nature exposes the effector binding domain which allows the effector proteins to recognize the active form of Rac1 (Bishop and Hall 2000; Johnson 1999). Residues 32-40 comprise the effector domain or switch I of Rac1 (**Figure 5 & 6**).

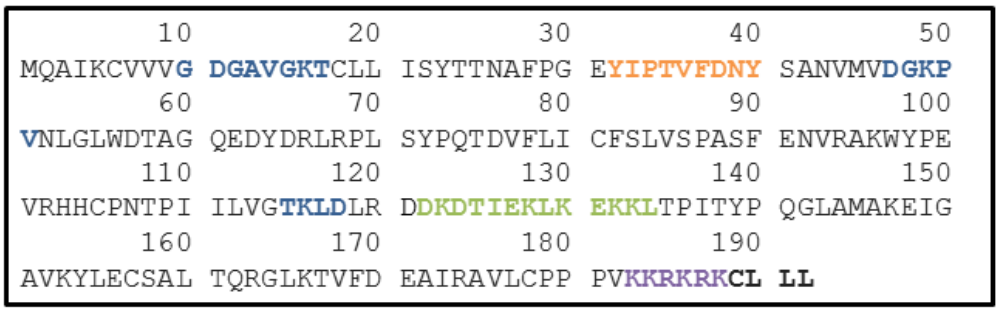


Figure 6: Peptide sequence of Rac1 with various domains highlighted.
Blue- GTP binding domain; Orange- Effector domain; Green- Rho insert domain; Purple: membrane localization domain; Black (bold)- CAAX motif.

1.6.4 Localization of Rac1

Rac1 protein, in addition to GTP/GDP exchange can also dock to the membranes in order to perform their functions. This is achieved by post-translational modifications of their CAAX (C-cysteine, A-aliphatic amine and X- any amino acid) motif which is present at the C-terminal and helps in localization of the protein to the membranes (Roberts, Mitin et al. 2008; Johnson 1999). Firstly, the geranyl-geranyl group is added to the cysteine residue by GGTase enzyme followed by its translocation to the endoplasmic reticulum (ER). Next in the ER the protease Rce1 cleaves the AAX motif by proteolysis leading to exposure of α-carboxyl group; which undergoes further methyl-esterification by a carboxy methyltransferase (Bustelo, Sauzeau et al. 2007). Finally, the weak polybasic region just upstream of the CAAX motif mediates the localization of Rac1 to the membranes of large

pinocytic vesicles and lipid rafts in addition to the plasma membrane (Prieto-Sanchez and Bustelo 2003; Jou, Leung et al. 2000) .

1.6.5 Role of Rac1 in Cancer

As mentioned earlier, Rac1 modulates actin cytoskeletal dynamics such as lamellipodia formation, chemotaxis and membrane ruffling. Due to these processes mediated by Rac1, it has been associated with mechanisms implicated in malignant transformations that include tumorigenesis, angiogenesis, invasion and metastasis in various cancer cells (Bid, Roberts et al. 2013). Recently, it has been shown that mutations leading to overexpression and over activation of Rac1 correlates well with characteristics of malignancy and aggressive growth patterns of tumors or cancer cells (Rathinam, Berrier et al. 2011). Rac1 has been implicated in several cancer cells such as breast cancer, lung carcinoma, squamous cell carcinomas of head & neck and melanoma. Overexpressed Rac1 is found in breast cancer cells and is localized to the plasma membrane as a result of possible deregulation of transcription (Schnelzer, Prechtel et al. 2000). Somatic mutation of low frequency (<0.5%) in Rac1 have been shown to be present in several tumor/cancer cells such as melanoma (Lin and Zheng 2015). In 5-6% of melanomas P29S mutation of Rac1 may be responsible for resistance to Raf inhibitors (Watson, Li et al. 2014). Rac1 signaling pathways have been shown to be required for leptin-induced invasion in colon adenocarcinomas (Attoub, Noe et al. 2000). Also, Rac1 is over expressed in lung adenocarcinomas associated with epithelial mesenchymal transitions which are induced by matrix metalloproteinase in lung cells (Stallings-Mann, Waldmann et al. 2012).

1.6.6 Mechanism of Activation of Rac1

It has been shown that Rac1 is activated by a wide variety of receptors including Receptor Tyrosine Kinase (RTKs), integrins and GPCRs as well as by stress (Wertheimer, Gutierrez-Uzquiza et al. 2012). Further, it lead to the release of a well-known intracellular second messenger Ca^{++}. Intracellularly, the concentration of the ubiquitous second messenger, Ca^{++} is only around 100 nM in the resting state. In order to maintain such low concentration of Ca^{++} it is pumped out of the cell actively into the ER, extracellular space and mitochondria (Clapham 2007; Demaurex and Nunes 2016). However, upon activation of the cell, the concentration of cytoplasmic Ca^{++} may increase to about 500 to 1000 nM. Phospholipase C pathway (PLC) is the most common pathway involved in release of Ca^{++} from the intracellular stores (Clapham 2007) (**Figure 7**). Calcium then relays the signal through various Ca^{++} binding proteins such as, Calmodulin (CaM) (Shen, Valencia et al. 2005) which has been shown to interact with Rac1 leading to its activation (Elsaraj and Bhullar 2008; Xu and Bhullar 2011). Calmodulin is ~ 17kDa protein which is known to act as a calcium sensor and it participates in calcium dependent signal transduction pathways (Kumar, Chichili et al. 2013). Calmodulin causes phosphorylation of Rac1 GEF, Tiam1 leading to Rac1 activation (Fleming, Elliott et al. 1999). Also, Protein Kinase C (PKC) has been shown to participate in activation of Rac1 (Price, Langeslag et al. 2003). Once activated, Rac1 regulates both cytoskeletal and non-cytoskeletal cellular processes through various downstream effectors. Rac1 activates WAVE which further regulates actin cytoskeleton leading to membrane ruffling and cell migration through the Arp 2/3 protein (Wertheimer, Gutierrez-Uzquiza et al. 2012). Rac1 has also been shown to activate the

JAK/STAT pathway which is involved in cell cycle regulation including proliferation, transcription and apoptosis (Wertheimer, Gutierrez-Uzquiza et al. 2012).

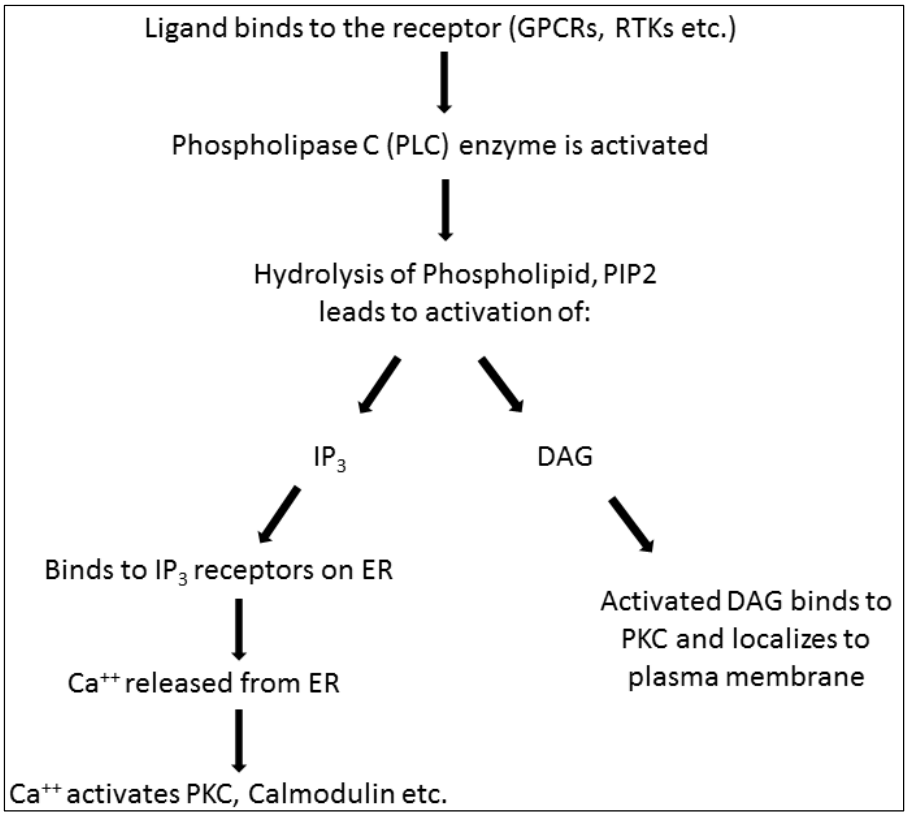


Figure 7: PLC Pathway Activation leading to Intracellular Calcium Release

2. HYPOTHESIS

2.1 Study rationale

Extra-oral expression of T2Rs has led to the exploration of their role as potential therapeutic targets in treatment of various diseases. Moreover, activation of T2Rs has been shown to cause the release of intracellular calcium and hence the activation of various downstream calcium dependent signaling pathways. Also, calcium has been shown to play a vital role in Rac1 activation mediated through receptors such as GPCRs and RTKs. However, no information exists if the bitter taste receptors, T2Rs which are well-known GPCRs play any role in regulating Rac1 activation. In this study T2R4 has been used to explore the role of bitter taste receptors in Rac1 activity.

2.2 Hypothesis

T2R4 plays a role in regulating Rac1 activity.

3. OBJECTIVES

1. To demonstrate the regulatory effects of T2R4 on Rac1 activity as a result of quinine treatment:

    i) To assess the effect of quinine on Rac1 activity.

    ii) To confirm the role of T2R4 in Rac1 signaling pathway.

    iii) To determine the role of Gα16/44 in mediating Rac1 signaling.

2. To explore the role of calcium in Rac1 function:

    i) To examine the effects of quinine on Rac1 activity in the absence of calcium by using intra-cellular calcium chelator, BAPTA-AM.

4. MATERIALS AND METHODS

4.1 MATERIALS

Quinine HCL (Cat# Q1125), BCML (Cat# 14580), BAPTA-AM (Cat# A1076), glutathione-agarose beads (Cat# G4510), EGTA (Cat# E8154-10G) and DMSO (Cat# D8418) were purchased from Sigma. Anti-HA monoclonal antibody (Cat# sc-7392) was purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated secondary goat-anti mouse antibody (Cat# 31430) was purchased from Thermo Scientific. ECL prime western blotting detection reagent (Cat# RPN2232), Amersham Hybond P 0.45 μm PVDF transfer membrane (Cat# 10600023) and Hyperfilm (Cat# RPN3114K) were purchased from GE Healthcare. 30% Bis-acrylamide (Cat# 161-0156) and counting slides-dual chamber for cell counter (Cat# 145-0011) were from BioRad Laboratories. FBS (Cat# 16140071), trypsin-EDTA (Cat# 25300-062), penicillin-streptomycin (Cat# 15140), Opti-MEM (Cat# 11058021) and lipofecatmine 3000 (Cat# 3000015) were purchased from Invitrogen. Dulbecco's Modified Eagle Medium nutrient mixture F-12 (Ham) (DMEM/F-12) (Cat# 12400-024) was obtained from Life Technologies. Miniprep kit (Cat# 27104) and Maxiprep kit (Cat# 12163) were purchased from Qiagen. Tris-base (Cat# BP152-5), glycine (Cat# BP-381-5), SDS (Cat# BP166-500), sodium bicarbonate ($NaHCO_3$) (Cat# S233-500), LB Broth (Cat# BP1426-500) and methanol (Cat# A454-4) were from Fisher BioReagents. GST-PAK1 bacterial plasmid and pCMV-HA vector were a kind gift from Dr. M Hosini (National Center of Neurology and Psychiatry, Tokyo, Japan) and Dr. X-J. Yao (University of Manitoba, Canada) respectively. The G protein, Gα16/44, harboring pcDN3.1 was a gift from Dr. T. Ueda (Nagoya City University, Nagoya, Japan).

4.2 METHODS

4.2.1 Isolation of GST Fusion Protein

DH5-α *Escherichia coli* cells were used for expressing the recombinant fusion protein GST-PAK1 induced by 1 mM IPTG as described previously (Clough, Sidhu et al. 2002; Elsaraj and Bhullar 2008; Xu and Bhullar 2011). Briefly, 200-400 ml bacterial culture transfected with GST-PAK1 plasmid was grown overnight at 37°C followed by addition of 1 mM IPTG for 2 h at room temperature to allow expression of the fusion protein. The cells were centrifuged at 6,000 rpm for 20 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in NETT buffer (20 mM Tris-HCL, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 % Triton X-100) followed by glass homogenisation. The homogenised cells were collected in a 50 ml tube and 1 mM PMSF and lysozyme (10 mg/ml) mix was added. The cells were further lysed by ultrasonic cell disruption twice for 30 sec each. The lysate was centrifuged at 15,000 rpm for 30 min to remove cell debris. 20% (final concentration) glycerol was added to the supernatant and stored at -80°C in aliquots for future use. Glutathione agarose beads prepared in 1:1 ratio of NT buffer (20 mM Tris-HCL, pH 8.0 and 100 mM NaCl) was used to purify the GST-PAK1 fusion protein by incubating at 4°C for 30 min. Next, the beads were collected by centrifugation at 14,000 x g for 1 min and washed thrice with NETT buffer and twice with NT buffer. The final pellet was suspended in NT buffer (1:1) and stored at 4°C. SDS-PAGE was used to determine the purity of the fusion protein.

4.2.2 Preparation and Purification of HA-Rac1 Plasmid

HA-Rac1 plasmid was prepared by Dr. Bing Xu (Xu et al. 2012). Briefly, GST-Rac1 (full length) was used as a template to generate the plasmid. PCR was performed as described previously (Xu et al. 2012). The protocol followed for PCR is: initial denaturation was done for 2 min at 95°C, and then 18 cycles of denaturation were performed for 15 sec at 95°C. Followed by, 30 sec annealing at 55°C and 5 min elongation at 68°C. Sequencing was done for validation (Robarts Research Center, London, Canada). ECoRI and BamHI restriction sites were used to subclone Rac1 into pCMV-HA vector. DH5α *E. coli* competent cells were used to transform the ligated plasmid. Transformed cells were incubated overnight at 37°C on ampicillin (100 µg/ml) containing LB agar plates. Further, the HA-Rac1 plasmid was purified. The bacterial culture containing the plasmid was grown overnight at 37°C and the cells were collected by centrifugation at 6000 rpm at 4°C for 20 min. The Qiagen protocol for maxiprep or miniprep kits was followed to purify the plasmid DNA and the purity was checked using Nanodrop with 260/280 ratio between 1.8-1.9.

4.2.3 HEK 293T cells as Model System

HEK (Human Embryonic Kidney) 293 cells were developed in Alex Van der Eb's lab in Netherlands in 1973 from a healthy fetus legally aborted under Dutch law (Graham, Smiley et al. 1977). They are called HEK 293 because these clones were produced as a result of experiment number 293. Since then these cells have been used in cell biology research and HEK 293 cells are easy to culture and transfect. In this study an adherent variant of these

cells called HEK 293T cells has been used. These cells have SV-40 large T-antigen which promotes high expression of the desired gene (DuBridge, Tang et al. 1987).

4.2.3a Heterologous System for T2Rs

Endogenously, T2Rs are expressed in low levels in the cells, so in order to perform structural-functional studies T2Rs need to be over-expressed. Various methods have been developed to over-express GPCRs in heterologous systems that include codon optimization of the respective genes (Tate and Grisshammer 1996; Ames, Nuthulaganti et al. 2004 and Chakraborty, Xu et al. 2015). In this study we used, HEK 293T stable cells produced by transfecting codon-optimized T2R4 or by co-transfection of T2R4 and Gα16/44 chimera, developed by Dr. Chelikani's lab.

4.2.3b Stable Cell Generation

HEK 293T cells stably expressing T2R4 and T2R4/Gα16/44 chimera were generated by Dr. Chelikani's lab following the method described previously (Chakraborty, Xu et al. 2015). Briefly, HEK 293T cells were co-transfected with T2R4 in pcDNA4/HisMaxB/Zeo and Gα16/44 chimera in pcDNA3.1/Hygro vectors (each 3 μg/well) in a 6 well plate. After 24 hours, the cells were plated in growth medium at different dilutions (1:100, 1:200 and 1:500). After 48 hours, the growth medium was replaced with selection medium containing zeocin and hygromycin at 300 μg/mL. Selection medium was changed every 2-3 days until clear isolated clones were observed. The isolated colonies were selected as individual clones and characterized for the high expression of extracellular T2R4 and intracellular Gα16/44 using flow cytometry analysis. The cells were stained with APC conjugated anti-FLAG

antibody at 1:300 dilutions for $1x10^5$ cells. The best clone was further characterized for its functionality in response to quinine using calcium mobilization assay as described previously (Pydi, Jaggupilli et al. 2015).

4.2.4 Glutathione Agarose Beads Preparation

1.5 ml of water was added to 0.1 g of glutathione agarose beads and left overnight at 4°C for beads to swell. The following day beads were centrifuged and the water was discarded. Then, the beads were washed in NT buffer three times and final bead pellet was re-suspended in 1.5 ml of NT buffer and stored at 4°C for future use.

4.2.5 Cell Culture

HEK 293T cells stably expressing T2R4, T2R4-Gα16/44 or Gα16/44 were cultured in 100 mm plates in DMEM enriched with 1.5 g/L $NaHCO_3$, 10 % FBS (v/v) and 100 U penicillin-streptomycin in 5 % CO_2 and 95 % air (v/v) at 37°C.

4.2.6 Transient Transfection of Cells

The cells were transfected using the Lipofectamine 3000 transfection reagent protocol by Invitrogen. In brief, the HEK 293T cells stably expressing T2R4-Gα16/44, T2R4 or Gα16/44 were seeded in 6 well plates at $1x10^6$ cells/well 24 h prior to transfection in order to obtain 70-80 % confluent cells. Next day, the cells were transfected with HA-Rac1 plasmid (3 µg/ml) using Lipofectamine 3000 (2 µl/µg DNA). 6-7 h after transfection, media

containing lipofectamin and plasmid mix was removed and the cells were replenished with fresh DMEM media (supplemented with 1.5 g/L $NaHCO_3$, 10 % FBS and 100 U penicillin-streptomycin).

4.2.7 Treatment of Cells with BAPTA-AM, Quinine and BCML

24 h post-transfection the cells were treated with BAPTA-AM (50 µM final concentration, dissolved in DMSO) for 30 min followed by quinine (1 mM final concentration, dissolved in double distilled water), BCML (60 nM final concentration, dissolved in double distilled water) or quinine plus BCML for 15 min.

4.2.8 GST-PAK1 Pull-down of Active Rac1 from Cell Lysate

Following treatment with quinine or BCML, cells were lysed in Rac1 lysis buffer (50 mM Tris-HCl, pH 7.4, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 2.5 mM EGTA, and a protease inhibitor cocktail). The lysate was sonicated for 30 sec at 4°C followed by centrifugation at 14,000 × *g* at 4°C for 10 min. After centrifugation, the supernatant was collected and incubated with 50 µl GST-PAK1 beads for 2 h at 4°C with constant rocking. After incubation, the beads were washed four times with Rac1 washing buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1 % Triton X-100, 5 mM EGTA, and a protease inhibitor cocktail). The final bead pellet was suspended in 25 µl of Laemmli's sample buffer and heated at 100°C for 5 min. 12 % SDS-PAGE was used to separate the eluted protein and transferred to PVDF membrane by overnight electrophoresis at 4°C. Following day, Western blotting was performed using mouse anti-

HA monoclonal antibody (1:1000). Total Rac1 in the starting sample after various treatments were also determined by Western blotting.

4.2.9 Statistical Analysis

The bands were quantified using Fluor-Chem Band-analysis program. The output of each sample was normalized using the respective total Rac1 in various samples. Statistical analysis of the data was done using one-way ANOVA program of graph pad prism6 software. $P < 0.05$ was considered significantly different.

5. RESULTS

5.1 Expression of GST-Pak1 (Pak binding domain)

One of the downstream effector proteins of activated Rac1 is called the p21 activated kinase 1 (PAK1). The GTPase binding domain of PAK1 was expressed in the form of GST tagged fusion protein in DH5α competent *E.coli.* Further, glutathione agarose beads were used to purify the fusion protein and its purity was assessed prior to experiment. The molecular weight of the fusion protein is ~34 kDa and can be seen in the figure below (**Figure 8**).

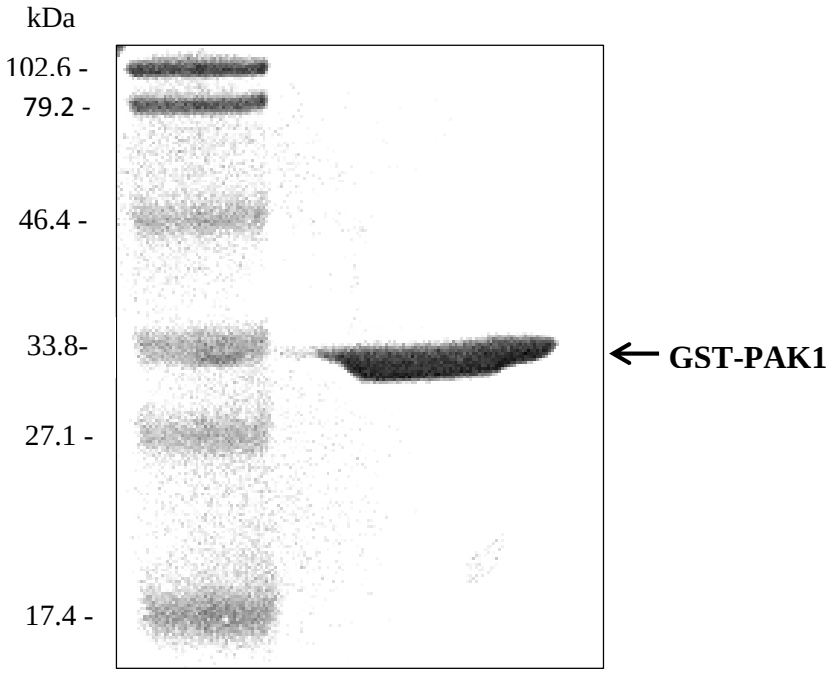


Figure 8: Purification of recombinant GST-PAK1 fusion protein: GST-PAK1 was purified from *E.coli* using glutathione agarose beads after stimulation with IPTG for 2h at room temperature. Purified protein was analysed on 12% SDS PAGE followed by Coommasie blue staining.

5.2 Effect of quinine, BAPTA-AM or BCML on Rac1 activity in HEK 293T stable cells over expressing T2R4/G$\alpha_{16/44}$ and transiently transfected with HA-Rac1

To evaluate the effect of quinine on Rac1, HEK 293T stable cells over expressing T2R4/G$\alpha_{16/44}$ were treated with 1 mM quinine for 15 min. Active Rac1 was pulled-down using GST-PAK1. The results indicated that quinine causes a significant decrease in the activation of Rac1 when compared to control (**Figure 9, Page No. 33**).

In the above experiment, the cells were also treated with BAPTA-AM (50 μM) for 30 min prior to quinine treatment to investigate the role of calcium in quinine mediated Rac1 inactivation. The results show a decrease in Rac1 activation in BAPTA-AM and BAPTA-AM plus quinine treated cells in comparison to control, but the decrease was not significant when compared to quinine treated cells. Thus, indicating a partial role of calcium in quinine mediated inactivation of Rac1.

Similarly, the cells were also treated with BCML (60 nM), which is an inverse agonist for T2R4 and competes with quinine for binding to the receptor. It can be seen from the results **Figure 9, Page No. 33**) that BCML blocks quinine mediated inactivation of Rac1 and suggesting a role of T2R4 in this pathway.

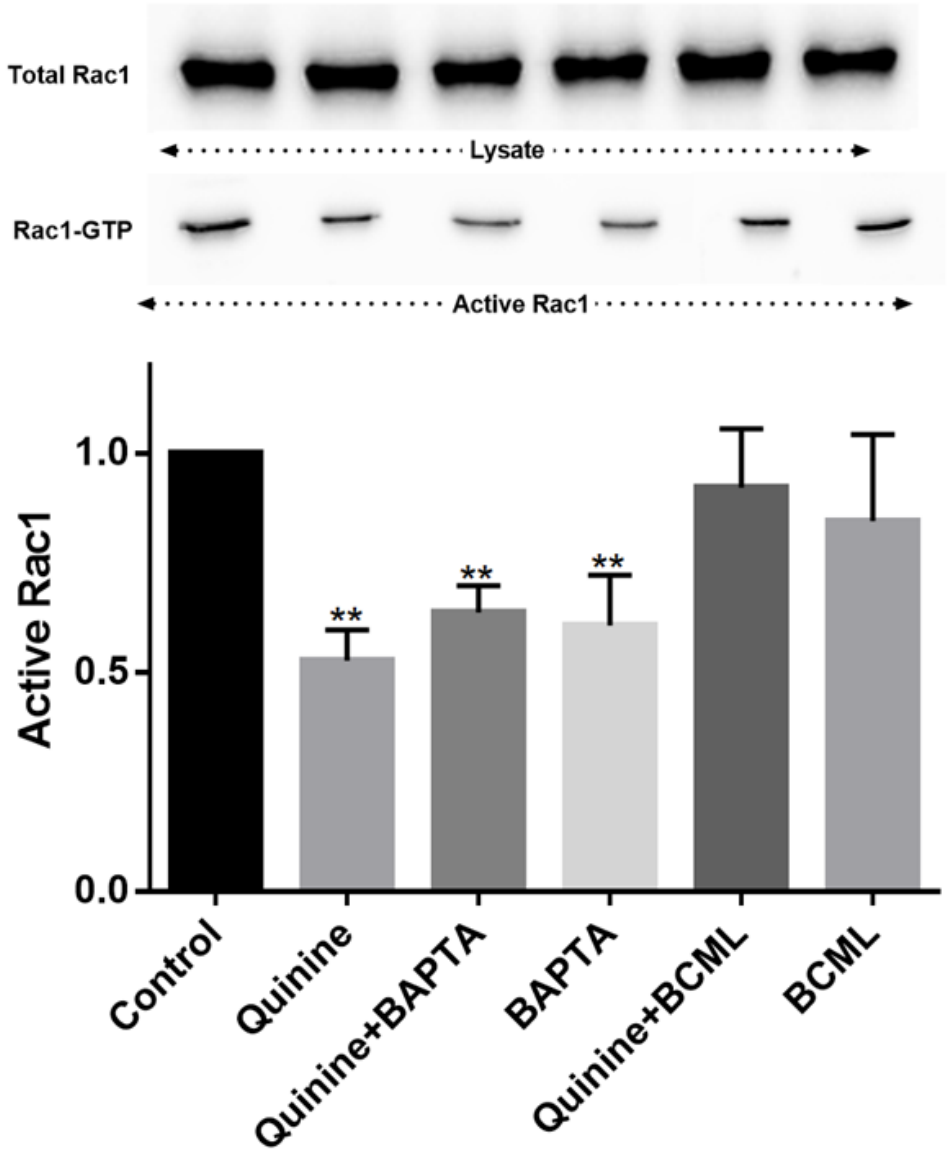


Figure 9: Quinine causes inactivation of Rac1 in cells overexpressing T2R4 and Gα16/44. HEK293T cells stably expressing T2R4/G$\alpha_{16/44}$ were grown in DMEM/F12 plus 10% FBS and 100 U penicillin/streptomycin solution at 37°C in 5% CO_2 and 95% air (v/v). Cells were transiently transfected with HA-Rac1 and treated with BAPTA-AM (50 μM) for 30 min followed by treatment with quinine (1 mM) or quinine plus BCML (60 nM) for 15 min. Cells were lysed in Rac1 lysis buffer and active form of the GTPase (HA-Rac1) was pulled out using GST-PAK1 as described in Materials and Methods section. The final bead pellet was suspended in 25 μl of Laemmli's sample buffer and heated at 100°C for 5 min. Western blotting was performed using mouse anti-HA antibody. Quantification was carried out using Fluor-Chem program and values with *$p<0.05$, **$p<0.01$, were considered significantly different. The results were normalized against the basal levels of Rac1 in various samples. The experiments were repeated a minimum of 3 times. A representative autoradiograph is shown.

5.3 Effect of quinine or BCML on HEK 293T control cells transiently transfected with HA-Rac1

Quinine is known to be a cell permeant compound (Peri, Mamrud-Brains et al. 2000). Hence, to rule-out any direct effects of quinine on Rac1, HEK 293T control cells were treated with 1 mM quinine for 15 min or BCML (60 nM) or BCML (60 nM) plus quinine (1 mM). The results (**Figure 10, Page No. 35**) showed no significant effect on Rac1 activity in response to quinine or quinine plus BCML when compared to control or BCML treated cells.

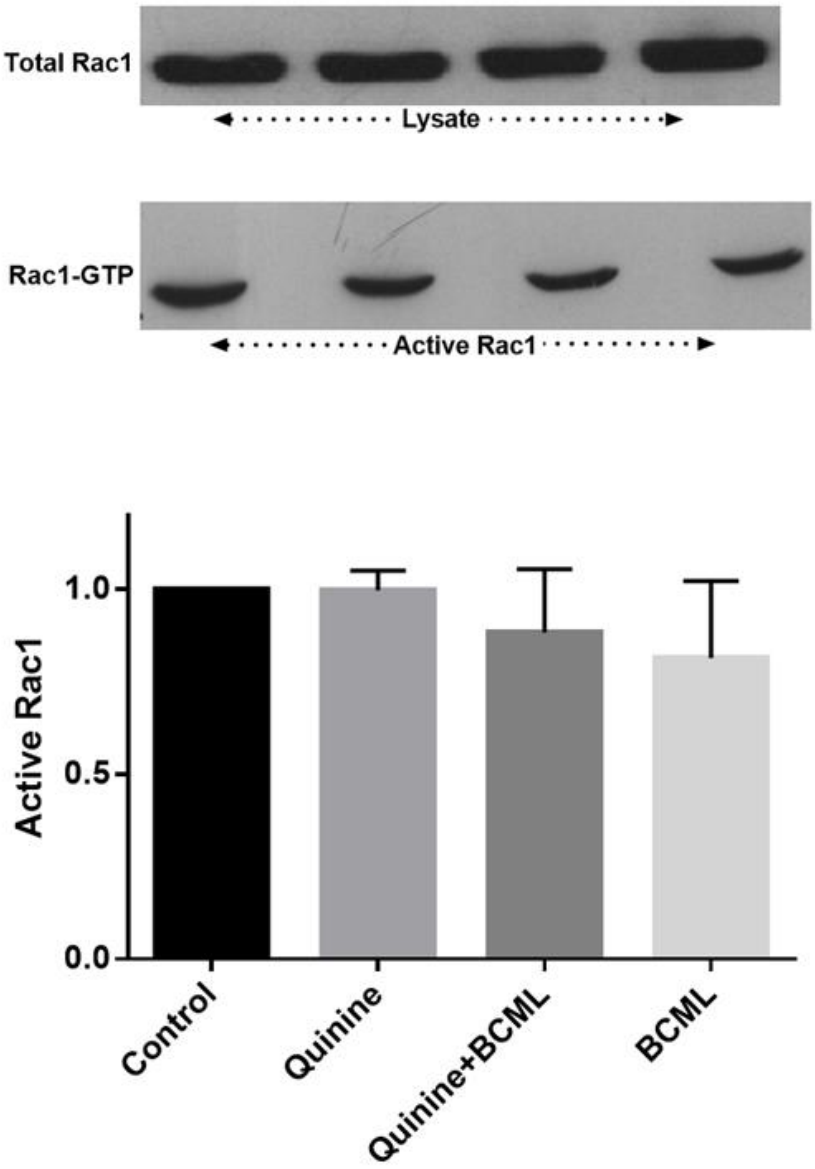


Figure 10: Effect of Quinine on Rac1 activation in control cells. HEK293T cells were grown in DMEM/F12 plus 10% FBS and 100 U penicillin/streptomycin solution at 37°C in 5% CO_2, 95% air (v/v) and transiently transfected with HA-Rac1. Cells were treated with or without quinine (1 mM) or quinine plus BCML (60 nM) or BCML (60 nM) for 15 min. Cells were lysed in Rac1 lysis buffer and active form of HA-Rac1 was pulled out using GST-PAK1 as described in Materials and Methods section. The final bead pellet was suspended in 25 µl of Laemmli's sample buffer and heated at 100°C for 5 min. Western blotting was performed using mouse anti-HA antibody. Quantification was carried out using Fluor-Chem program and values with *$p<0.05$, **$p<0.01$, were considered significantly different. The results were normalized against the basal levels of overexpressed Rac1 in various samples. The experiments were repeated a minimum of 3 times. A representative autoradiograph is shown.

5.4 Effect of quinine on HEK 293T stable cells over expressing T2R4 transiently transfected with HA-Rac1

It has been shown that GPCRs signal transduction occurs not only through G-protein dependent pathway (Wettschureck and Offermanns 2005) but also through G-protein independent pathways such as, β-arrestins, GRKs (GPCR-kinases) and Srcs (non-receptor tyrosine kinases) (Hoefen and Berk 2006). However in the case of T2Rs, only G-protein dependent pathway has been shown to be involved so far (Margolskee 2002; Chandrashekar, Mueller et al. 2000; Mclaughlin, Mckinnon et al. 1992). To confirm the involvement of G-protein, HEK 293T cells stably expressing only T2R4 were treated with 1 mM quinine for 15 min. The results (**Figure 11, Page No. 37**) showed no significant effect on activity of Rac1.

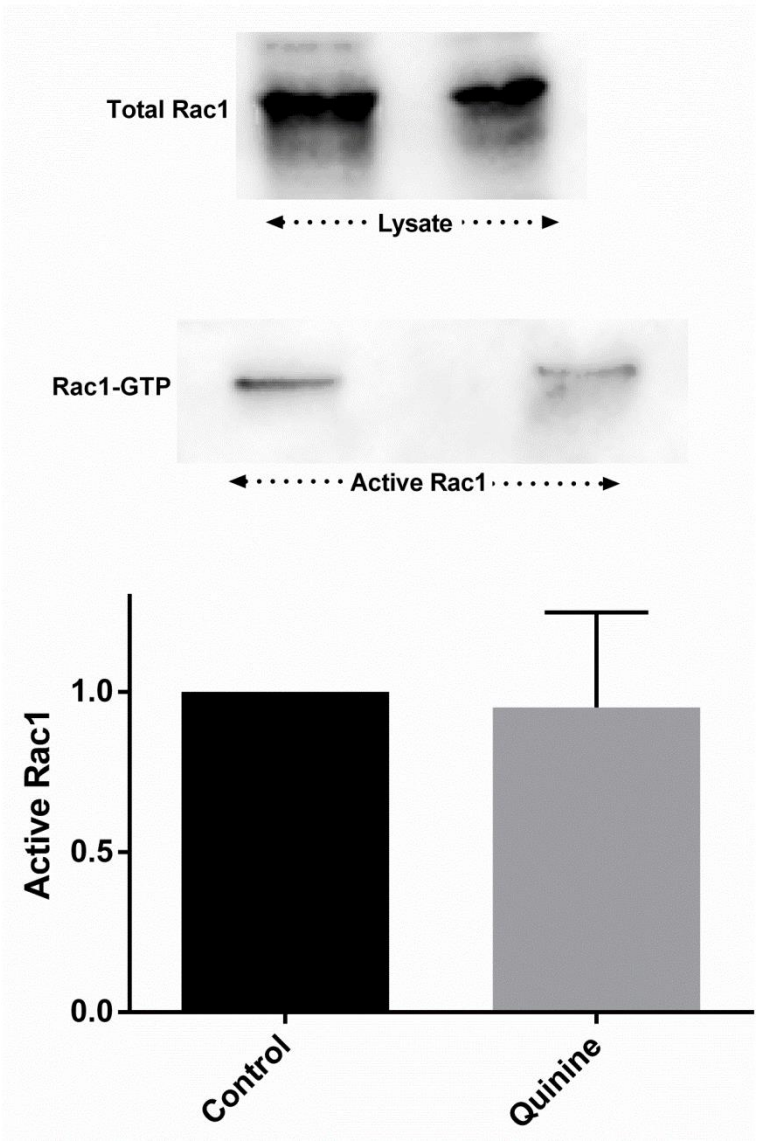


Figure 11: Effect of Quinine on Rac1 activation in cells overexpressing T2R4. HEK293T cells stably expressing T2R4 were grown in DMEM/F12 plus 10% FBS and 100 U penicillin/streptomycin solution at 37°C in 5% CO_2, 95% air (v/v) and transiently transfected with HA-Rac1. Cells were treated with or without quinine (1 mM) for 15 min followed by lysis in Rac1 lysis buffer and active form of HA-Rac1 was pulled out using GST-PAK1 as described in Materials and Methods section. The final bead pellet was suspended in 25 µl of Laemmli's sample buffer and heated at 100°C for 5 min. Western blotting was performed using mouse anti-HA antibody. Quantification was carried out using Fluor-Chem program and values with *$p<0.05$, **$p<0.01$, were considered significantly different. The results were normalized against the basal levels of overexpressed Rac1 in various samples. The experiments were repeated a minimum of 3 times. A representative autoradiograph is shown.

5.5 Effect of quinine, BAPTA-AM, or BCML on HEK293T stable cells over expressing $G\alpha_{16/44}$ transiently transfected with HA-Rac1

Quinine has been shown to activate the heterotrimeric G-protein directly (Peri, Mamrud-Brains et al. 2000; Naim, Seifert et al. 1994) independent of the receptor. So HEK293T cells stably expressing $G\alpha_{16/44}$ were treated with 1mM quinine for 15 min to investigate its effect on activation of Rac1. Interestingly, the results (**Figure 12, Page No. 39**) showed a significant decrease in activation of Rac1 when compared to control. This suggested a direct interaction between quinine and the G-protein.

Also, cells were treated with BAPTA-AM (50μM) and BCML (60nM). The results (**Figure 12, Page No. 39**) indicated a decrease in activation of Rac1 in BAPTA-AM plus quinine treated cells when compared to control. Also, BCML was unable to block the quinine from causing inactivation of Rac1 which further verified that quinine can potentially act directly on G-protein.

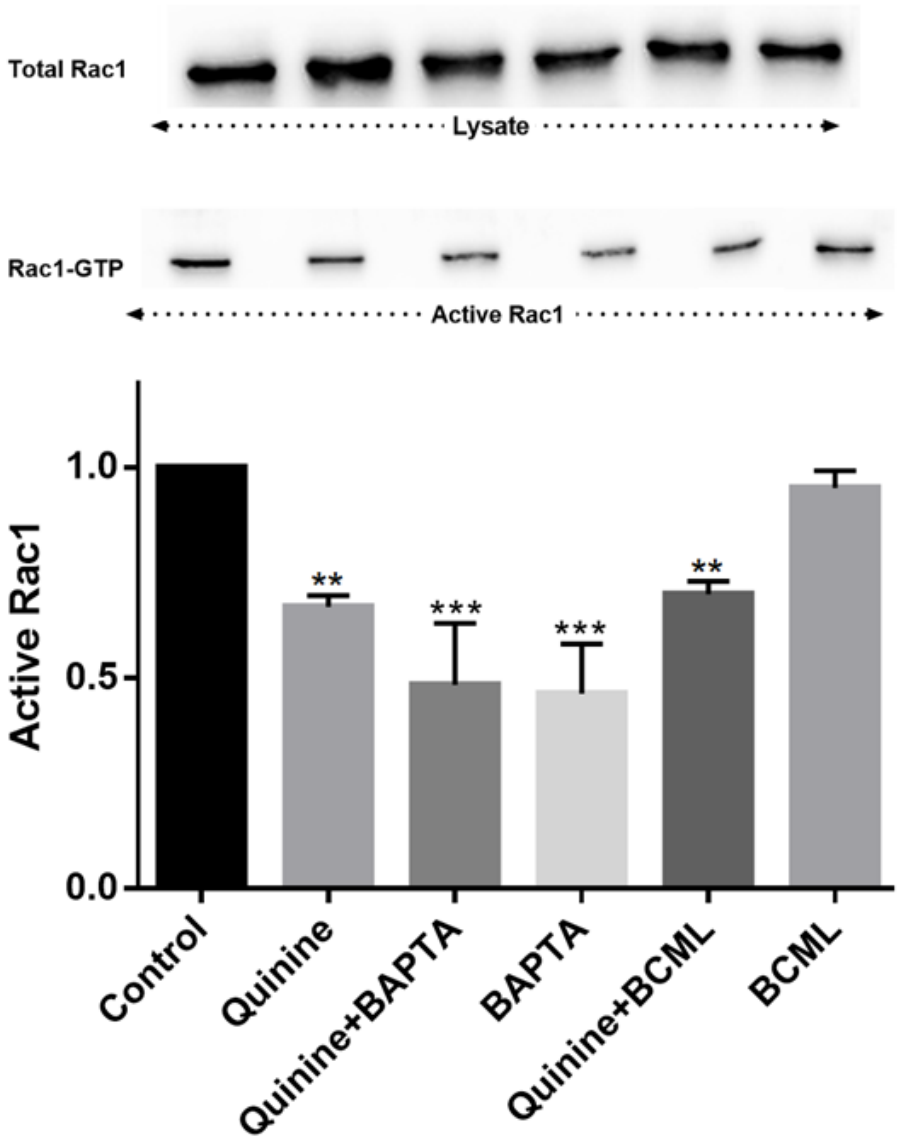


Figure 12: Effect of Quinine on Rac1 activation in cells overexpressing Gα16/44. HEK293T cells stably expressing $G\alpha_{16/44}$ were grown in DMEM/F12 plus 10% FBS and 100 U penicillin/streptomycin solution at 37°C in 5% CO_2, 95% air (v/v) and transiently transfected with HA-Rac1. Cells were treated with BAPTA-AM (50 μM) for 30 min followed by treatment with or without quinine (1 mM) or quinine plus BCML (60 nM) for 15 min. Cells were lysed in Rac1 lysis buffer and active form of HA-Rac1 was pulled out using GST-PAK1 as described in Materials and Methods section. The final bead pellet was suspended in 25 μl of Laemmli's sample buffer and heated at 100°C for 5 min. Western blotting was performed using mouse anti-HA antibody. Quantification was carried out using Fluor-Chem program and values with *$p<0.05$, **$p<0.01$, ***$p<0.001$ were considered significantly different. The results were normalized against the basal levels of overexpressed Rac1 in various samples. The experiments were repeated a minimum of 3 times. A representative autoradiograph is shown.

6. DISCUSSION

Rac1 is a small GTPase that acts as a molecular switch cycling between the active GTP bound form and inactive GDP bound form. As mentioned earlier, Rac1 participates in mediating actin cytoskeleton, axonal growth, cell to cell adhesion, mesenchymal like migration and cell polarity (Bid, Roberts et al. 2013). Additionally, Rac1 also regulates gene expression, cell cycle and transcriptional dynamics (Bustelo, Sauzeau et al. 2007). Stimulation of receptors like GPCRs, receptor tyrosine kinase, integrins and factors such as stress results in activation of Rac1 that is partially mediated via Calmodulin (Xu and Bhullar 2011). Further, Calmodulin (CaM) which is a highly conserved and ubiquitously expressed calcium binding protein is activated by the well-known second messenger, calcium (Yamniuk and Vogel 2004). Once activated Rac1 mediates the downstream signaling through its effector proteins. Amongst all the effectors (**Table 4**), Rac1 most commonly causes activation of p-21 activating kinases (PAKs) with PAK1 being the primary effector. This leads to activation of LIM kinases which further results in growth of actin filaments via cofilin inactivation (Ridley 2006). Also, Rac1 participates in actin polymerisation by dissociating WAVE1 resulting in activation of APR2/3 (Eden, Rohatgi et al. 2002). Additionally, Rac1 plays a role in mediating processes such as proliferation by interacting with Jak/STAT pathway of the MAP kinase system, ROS (Reactive Oxygen Species) production and cell killing via NADPH oxidase and response to inflammation through the regulation of NF-κB (Bid, Roberts et al. 2013; Bishop and Hall 2000). The activity of Rac1 is regulated by GEFs and GAPs. GEFs catalyse the formation of Rac1-GTP (active form)

whereas GAPs catalyse the reformation of Rac1-GDP (inactive form) (Bos, Rehmann et al. 2007).

T2R4 is a bitter taste receptor (T2Rs) which belongs to the GPCR family of membrane receptors. Similar to GPCRs, T2R4 is a seven-transmembrane protein and functions in association with the heterotrimeric G-proteins upon activation (Uings and Farrow 2000; Hendrickson 2005). Furthermore, activation of G-proteins leads to release of intracellular calcium from the endoplasmic reticulum via PLC and IP_3 and results in activation of downstream signal transduction pathways (Smrcka 2008). It participates in mediating bitter taste perception upon bitter ligand binding. 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 (Avau and Depoortere 2016). It has been shown thus far that T2R4 receptor is activated by a number of bitter compounds: five synthetic bitter compounds and nine natural bitter compounds (Meyerhof, Batram et al. 2010) (**Table 1**). Also, recently antagonist and inverse agonist for the T2R4s have been discovered (Pydi, Jaggupilli et al. 2015; Pydi, Sobotkiewicz et al. 2014) (**Table 1**). Among the listed ligands for T2R4, so far the efficacy (EC_{50}) has been determined only for the agonist, quinine (Pydi, Sobotkiewicz et al. 2014). Additionally, T2R4 is the only receptor among the nine T2Rs activated by quinine, which has been characterized with antagonist and inverse agonist (Pydi, Sobotkiewicz et al. 2014; Pydi, Jaggupilli et al. 2015). BCML has been shown to have a very low IC_{50} value i.e. 59 ± 18 nM when compared to the other blocker such as GABA with an IC_{50} value of 3.2 µM ± 0.3 µM (Pydi, Sobotkiewicz et al. 2014). In the current study we have investigated if quinine and T2R4 play a role in Rac1 activity and used BCML as a blocker.

We have discovered a novel effect of quinine on Rac1 function. This study is the first to show an inhibitory downstream action of a T2R4 agonist on activation of Rac1. Also, partial role of calcium in mediating the pathway has been shown by using intracellular calcium chelator, BAPTA-AM. T2R4 and heterotrimeric G-protein were also shown to play a pivotal role in the pathway leading to inactivation of Rac1 by quinine. BCML which is a recently discovered inverse agonist for T2R4 was used to show the function of the receptor in the pathway.

6.1 T2R4 Mediated Rac1 Signaling

Quinine is an amphiphilic substance i.e. it is both water and fat soluble which makes it a cell-permeant compound (Peri, Mamrud-Brains et al. 2000). However, the results of our study show that activation of T2R4 by quinine is required to cause inactivation of Rac1. This is in line with previous data which shows that quinine is a bitter agonist of T2R4 (Meyerhof, Batram et al. 2010). Thus, bitter taste receptor T2R4 is important in mediating the decrease in activity of Rac1 in response to quinine stimulation. Furthermore, it can be observed from our results that BCML successfully blocks quinine's action on T2R4 and prevents inactivation of Rac1. It has been described previously that BCML is a potent inverse agonist of T2R4 which competes with quinine for same binding site of the receptor (Pydi, Sobotkiewicz et al. 2014). Quinine's inability to cause any significant change in the activation of Rac1 in the absence of T2R4 further emphasises the importance of the receptor in quinine mediated signal transduction which results in inhibition of Rac1 activation.

6.2 G-Protein mediated Rac1 Signaling

Gustducin is a heterotrimeric G-protein that is involved in taste perception in the gustatory system. It comprises of α-gustducin and β3 and γ13 subunits (Wong, Gannon et al. 1996). Association between bitter taste perception and Gα-gustducin (taste specific G-protein) was demonstrated prior to the discovery of bitter taste receptors (Wong, Gannon et al. 1996). Moreover, it was also shown that there was 70% reduction of bitter taste perception in mice deficient in α-gustducin protein (Wong, Gannon et al. 1996). In addition to Gα-gustducin, the second messengers and other elements in the downstream signaling pathways of T2Rs were already known before they were discovered as bitter taste receptors (Chandrashekar, Mueller et al. 2000). Moreover, in general GPCRs function through both G-protein dependent and G-protein independent pathways including GRKs, β-arrestins and Srcs (Wettschureck and Offermanns 2005; Hoefen and Berk 2006). However, as stated above involvement of only G-protein dependent pathway for T2Rs has been shown so far and our results also support the same. It has been indicated by our data that Gα16/44 plays a significantly important role in causing a decrease in Rac1 activity in response to quinine because in the absence of heterotrimeric G-protein, Rac1 inactivation is abrogated. Also, it has been demonstrated previously that due to cell-permeant nature of quinine it can cause direct activation of G-protein (Peri, Mamrud-Brains et al. 2000; Naim, Seifert et al. 1994). Similar results were obtained in our study where quinine caused inactivation of Rac1 in the presence of only Gα16/44. In contrast to that, in the absence of Gα16/44 quinine failed to cause any change in Rac1 activation. Thus, this supported the previous studies showing the critical function of Gα16/44 in T2R mediated signaling pathway. Another interesting finding was that BCML was unable to block quinine in the absence of receptor T2R4 which

highlighted the specificity of BCML for the receptor. Additionally, it could be inferred from the data that effects of quinine on Rac1 were slightly higher in cells expressing both T2R4 and Gα16/44 when compared to only Gα16/44 expressing cells. It can be implied from the current results that T2R4 does play a role in quinine mediated inactivation of Rac1, although Gα16/44 seems to play a more critical role.

6.3 Role of Calcium in quinine mediated Rac1 inactivation

Calcium is the most well-known and extensively studied intracellular second messenger (Berridge, Lipp et al. 2000). The calcium ion concentration gradient between extracellular and intracellular regions is very high which means that cells when stimulated have a rapid influx of calcium ions into the cells (Berridge, Lipp et al. 2000; Yamniuk and Vogel 2004). In the gustatory system, this increase in intracellular calcium ions results in opening of transient receptor potential cation channel member 5 (TRPM) leading to membrane depolarization which relays the taste signal to the brain (Hofmann, Chubanov et al. 2003; Finger, Danilova et al. 2005). Similarly in the non-gustatory system such as muscles, the rise in intracellular calcium is important for causing muscle contraction (Clapham 2007). Also, it has been shown previously that Rac1 plays a role in Rac1 activation via the calcium binding protein, Calmodulin (Elsaraj and Bhullar 2008; Xu et al. 2012). In this study we used BAPTA-AM, an intracellular calcium chelator to demonstrate the calcium dependency of the pathway. In T2R4 and Gα16/44 expressing cells it could be seen that in the absence of calcium there was only a slight increase in Rac1 activation thus suggesting the participation of calcium dependent pathway in controlling the Rac1 activity. Also, we observed that in only Gα16/44 expressing cells, the chelation of calcium by BAPTA-AM was unable to

prevent the inactivation of Rac1 by quinine. This demonstrates that T2R4 play a role in activating the calcium dependent pathway. However, it could be inferred from the results that the effects of quinine were cumulative with the effects of BAPTA-AM. This highlighted the significance of T2R4 in mediating the partial calcium dependent pathway. The slight variation in Rac1 inactivation by quinine among cells expressing T2R4 plus Gα16/44 and only Gα16/44 could be due the activation of different pathway by quinine which requires T2R4 for mediating the calcium dependent pathway. However, direct activation of Gα16/44 by quinine does not activate this pathway. Further study is required to delineate the potential route of Rac1 inactivation by quinine.

From the current study it can be seen that T2R4 follows a heterotrimeric G-protein dependent pathway to cause inactivation of Rac1. Further, Gα16/44 plays the pivotal role in the signal transduction pathway and it is partially dependent on calcium. This suggests the activation of another potential second messenger that has inhibitory effects on Rac1. Studies on bitter taste signaling pathway have shown that there are two opposing pathways that are activated simultaneously upon binding of bitter ligand to a T2R. Upon activation T2Rs lead to dissociation of heterotrimeric G-protein (gustducin) into Gα subunit and Gβγ subunits (Margolskee 2002). The gustducin βγ units cause the generation of IP_3 and DAG which further leads to release of intracellular calcium from the endoplasmic reticulum (Yan, Sunavala et al. 2001; Margolskee 2002). However, the Gα gustducin subunit is believed to cause activation of a phosphodiesterase (PDE) including PDE1A and this causes a decrease in cyclic nucleotides such as cAMP and cGMP (Andres-Barquin and Conte 2004; Yan, Sunavala et al. 2001; Kolesnikov and Margolskee 1995). This function of Gα gustducin is in contrast to the Gα subunits of other G-proteins where the stimulation of Gα subunit leads to

activation of cyclic nucleotides and inhibition of the phosphodiesterase (Li, Liu et al. 2016; Kobayashi, Tsubosaka et al. 2013). This could possibly explain the inhibitory effect of quinine on Rac1 mediated via T2R4 and Gα16/44 as reported in our study. Supplementary to the above findings, stimulation of cAMP further causes activation of protein kinase A (PKA) which in turn leads to induction of GEFs for Rac1 such as Tiam1 and VAV2, hence causing activation of Rac1 (Schlegel and Waschke 2014). Henceforth, it can be concluded from the literature and our data that Gα16/44 can be causing the inactivation of Rac1 by supressing the cAMP/PKA/Tiam1 pathway. Further investigation in this direction is needed to better understand the mechanism underlying the quinine mediated inhibition of Rac1 activation.

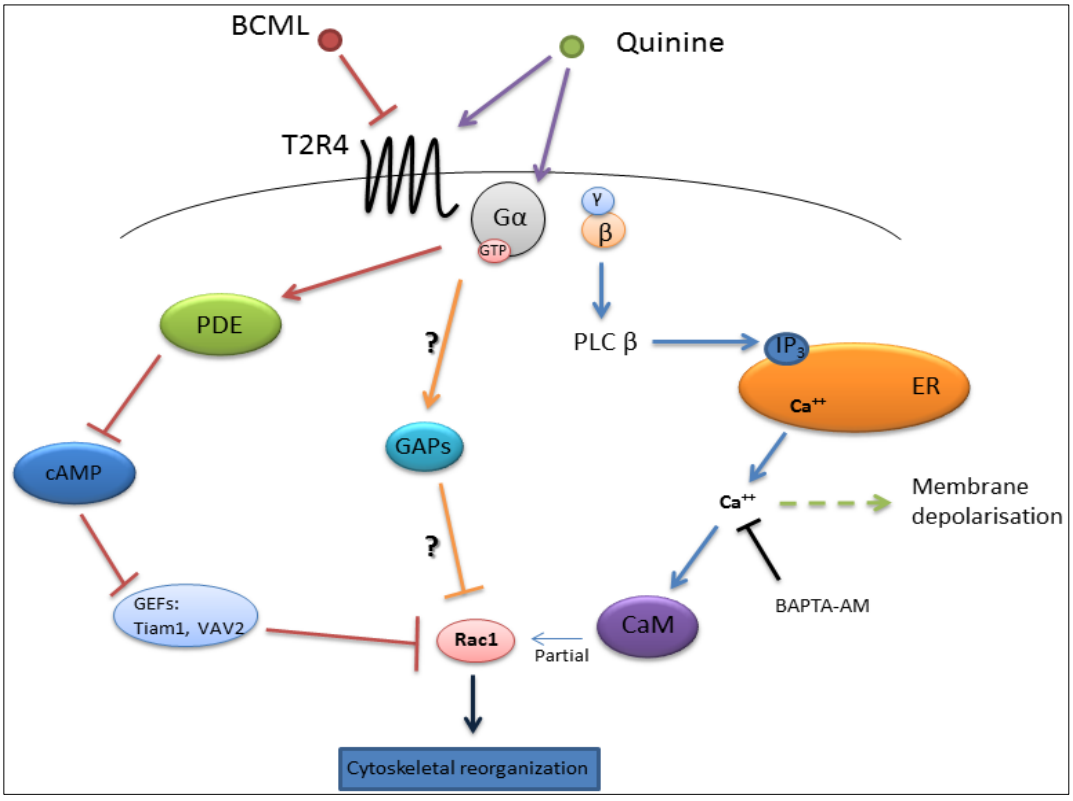


Figure 13: Proposed model for Rac1 inactivation mediated by quinine via T2R4/G-Protein pathway.

7. CONCLUSION

It can be inferred from the above that quinine plays a role in inhibiting activation of Rac1 that is mediated through T2R4 and $G\alpha_{16/44}$ and is partially calcium dependent. It can also be concluded from the findings that T2R4 plays an important role in activation of calcium pathway but is not necessarily required in downstream signaling of quinine mediated inactivation of Rac1. However, it is apparent from the data that G-protein is pivotal in causing inactivation of Rac1 by quinine since in the absence of G-protein quinine becomes ineffective. Though BCML is able to successfully block inhibitory action of quinine on Rac1 activation, it is unable to do so in the absence of T2R4. Hence, it can be proposed that combined activation of T2R4 and G-protein have a cumulative effect on quinine's action on Rac1 activity. Rac1 has been shown to be activated by calcium directly or through calcium binding proteins and T2Rs activation also involves an increase in calcium ions intracellularly. The results of our study also indicate that the effects are partially dependent on calcium however quinine may be causing the activation of a different pathway in addition to the usual calcium dependent pathway. These additional possibilities can be triggering a negative feedback mechanism or activation of GAP proteins (**Figure 13**) which in turn cause the downregulation of Rac1. This is a novel study which demonstrates the inhibitory effects of an agonist of T2R4, thus indicating functional selectivity of quinine. Since T2R4s have been found to participate in signal transduction in extra-oral tissues, the decreased activity of Rac1 in response to quinine can have various clinical implications especially in cancers associated with Rac1 mutations. Further study is required to delineate the possible pathway that is functional in leading to Rac1 inactivation.

8. FUTURE DIRECTIONS

8.1 Determine the effects of other known bitter agonists of T2R4

This study was done using only one bitter ligand, quinine. Further investigation can be done for the remaining agonists of T2R4, for example diphenidol to examine if similar effects are produced. Quinine is an amphiphilic compound, which makes it promiscuous in nature and it is known to cause direct activation of G-protein. Hence, it poses a hindrance in studying the role of the receptor.

8.2 Investigate the role of cAMP/GEF pathway in inactivation of Rac1

As mentioned earlier, there are two known second messengers that play a role in downstream signal transduction of bitter taste receptors: calcium and cAMP. In this study only the role of calcium has been examined and the results indicate that Rac1 activity is partially regulated by the calcium dependent pathway. Thus, further experiments are required to determine the function of cAMP/PKA/Tiam1/VAV2 pathway in inactivation of Rac1.

8.3 Evaluate the effects of quinine on Rac1 in a time-course manner

In this study cells were treated with quinine for only 15 minutes. However, recent literature demonstrates that bitter taste receptors unlike other receptors are not internalized, instead more receptors appear on the membrane with prolonged exposure to the ligand (Upadhyaya, Chakraborty et al. 2016). Thus, time course treatment could be done to evaluate the effects on Rac1 activity.

8.4 Determine the expression of T2R4 in an endogenous system and confirm if quinine causes effects in the endogenous system similar to the heterologous system

This research was done using heterologous system whereby, T2R4 and Gα protein was overexpressed in HEK 293T cells. However, to further validate the findings of this study, similar experiments could be performed in an endogenous system. It is already established that human platelets have an abundant source of Rac1 (2-3 μM) and GPCRs are also known to activate Rac1 (Azim, Barkalow et al. 2000; Schmidt, Scudder et al. 2003). Additionally, T2Rs have been found to be expressed in the extra-oral tissues including the vasculature. From this information it can be deduced that platelets or their precursors, CHRF-2811 cells could be used as a potential endogenous system to study the effects of quinine and/or bitter agonists on Rac1 function.

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