Thromboxan A_2 Receptor (TPRα): A Potential Human Drug Target

Biochemistry
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The human thromboxane A_2 receptor (TPRα), a member of the superfamily of G protein-coupled receptors, is involved in platelet activation and vasoconstriction in response to binding of thromboxane A_2 (TXA_2). However, over-activation of TPRα can lead to the development of thrombotic cardiovascular events such as heart attacks and strokes. Currently the prevention of secondary cardiovascular events is carried out by the administration of aspirin. The TPRα is an important target for drug development due to its role in platelet aggregation and vasoconstriction. This review will focus the potential of TPRα as a human drug target.

Introduction

Thromboxane A_2 is a prostanoid that activates vasoconstriction and platelet aggregation through interaction with the thromboxane A_2 receptor (TPRα). The official name of this receptor is the thromboxane A_2 receptor (Magrane M. and the UniProt consortium 2011), but it is also known as the thromboxane/prostaglandin H_2 (PGH_2) receptor or the prostanoid TP receptor (Coleman *et al*. 1994, Ting *et al*. 2012). For the purpose of this review, thromboxane A_2 and the thromboxane A_2 receptor will be referred to as TXA_2 and TPRα, respectively.

TPRα is a G-protein coupled receptor (GPCR) expressed in human blood platelets and plays a vital role in platelet activation through coupling to the G-protein G_q (among others) (Ting *et al*. 2012). Although it binds both TXA_2 and PGH_2, the ligand with greater affinity for this receptor is TXA_2 (Devillier & Bessard 1997).

Activation of TPRα by TXA_2 triggers cellular signalling responses which result in platelet adhesion, vesicle trafficking, and changes in the cytoskeleton (Ting *et al*. 2012), all essential for haemostasis. However, if TPRα is over-activated serious thrombotic cardiovascular diseases may develop such as acute myocardial infarction, hypertension and unstable angina (Halushka & Halushka 2002).

Aspirin is currently used to inhibit platelet TXA_2 synthesis as a means of treating a variety of thrombotic cardiovascular diseases, such as acute myocardial infarction, unstable angina and secondary prevention of myocardial infarction (Morinelli & Halushka 1991, Mousa 1999). Aspirin inhibits cyclooxygenases (COXs), key enzymes involved in the biosynthesis of TXA_2 (Fig. 1) (Mousa 1999).

However, as mentioned, TXA_2 is not the only endogenous ligand for TPRα. Therefore aspirin inhibits just one of the many pathways that can result in platelet aggregation (Mousa 1999). Aspirin, despite its success in the treatment of secondary acute myocardial infarction, has efficacy issues. There is a proportion of the population who may experience "aspirin resistance", with explanations for this resistance still inconclusive (Halushka & Halushka 2002, Gasparyan *et al*. 2008). Furthermore, low doses of aspirin carry a risk of increased upper gastrointestinal side effects, such as bleeding and ulcers, which could become life-threatening (Cryer & Feldman 1999). Despite Bousser *et al.* (2011, p. 2013) concluding that aspirin is still "the gold standard anti-platelet drug for secondary stroke prevention in view of its efficacy, tolerance, and cost", it is responsible for a high rate of people being hospitalised due to a bad reaction to the drug (Pirmohamed *et al*. 2004).

Thus, the development of a new anti-platelet drug is necessary for the treatment of numerous diseases where platelet activity is promoted. The three-dimensional structure of TPRα at atomic resolution would aid the design of effective anti-platelet agents by providing a molecular insight into how the receptor functions.

This review aims analyse what is known about TPRα in current literature and highlight some considerations when targeting TPRα.

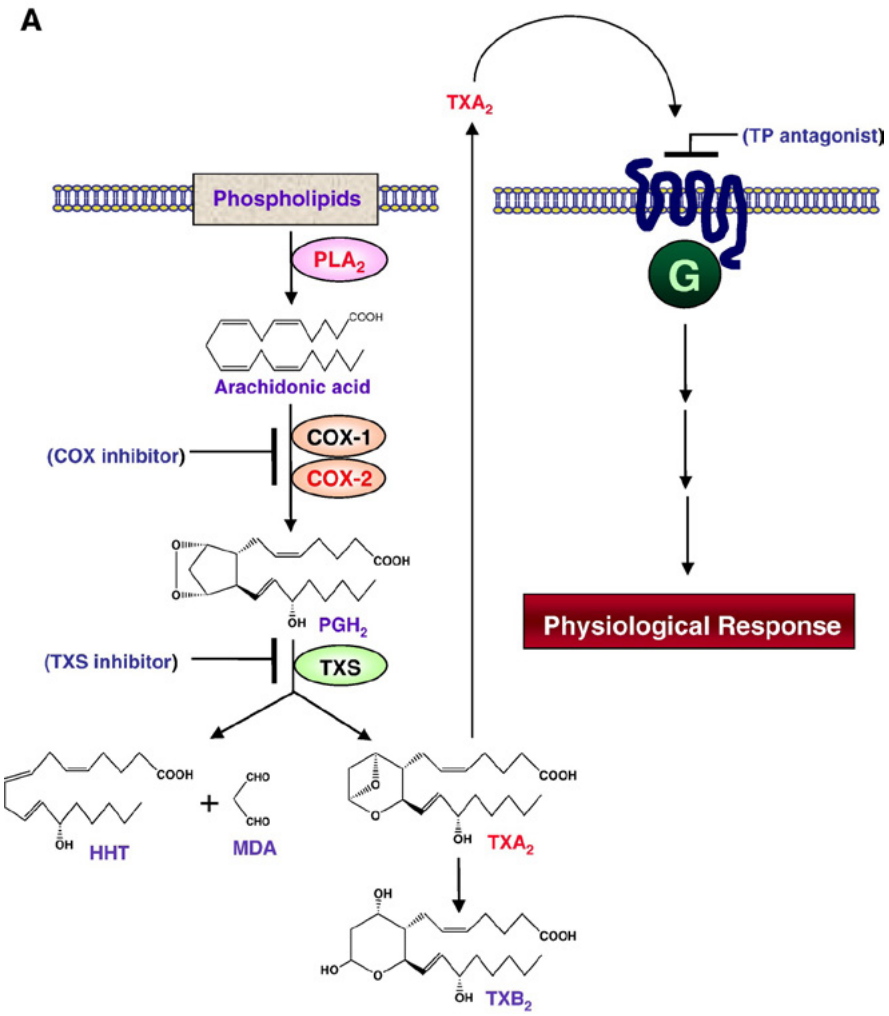

Figure 1: The human TXA_2 biosynthetic pathway. *The chronological steps of thromboxane A_2 (TXA_2) synthesis and the stages where drugs act, affecting TXA_2 function, are shown. TXA_2 is synthesised from arachidonic acid (AA) which is released from the membrane by phospholipase A_2 (PLA_2). Cyclooxygenases (COX-1 and COX-2) convert AA into important biological mediators known as prostanoids, the first being prostaglandin H_2 (PGH_2), a precursor of thromboxane A_2 (TXA_2). PGH_2 is converted to TXA_2, 12-L-hydrooxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde (MDA) by thromboxane synthase (TXS). Thromboxane B_2 (TXB_2) is a product of TXA_2 hydrolysis that is biologically inactive and removed from the body. (Nakahata 2008)*

1. Functions of Thromboxane A_2 and Thromboxane A_2 Receptor

1.1. Biosynthesis of TXA_2

TXA_2 is an unstable product of the cycloxygenase (COX) mediated metabolism of arachidonic acid (AA), catalysed by phospholipase A_2 (PLA_2). COXs synthesise the endoperoxide prostaglandin, prostaglandin H_2 (PGH_2), from AA which is in turn converted to TXA_2 by thromboxane synthase (TXS). TXA_2 has a short half-life of about 30 seconds (Fig. 1) before being converted into the inactive TXB_2 (Hamberg *et al*. 1975). Thus, TXA_2 acts on neighbouring cells in close proximity to the cell where it is produced in an autocrine or paracrine manner (Nakahata 2008).

1.2. TXA_2 and the TPRα Signalling Pathway

Binding of a TXA_2 to TPRα elicits several molecular responses which, overall, result in the elevation of intracellular Ca^{2+} levels which is the key to platelet activation (Fig. 2) (Brass *et al*. 1997).

Several G-protein isotypes are stimulated by TPRα upon ligand binding. In 2000, Perry V. Halushka outlined that there are at least nine G proteins which couple to TPRα; $G\alpha_q$, $G\alpha_{i2}$, $G\alpha_s$, $G\alpha_{11}$, $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{15}$, $G\alpha_{16}$ and G_h. The G_q isotype is of interest as TPRα-mediated platelet aggregation primarily associated with G_q (Offermanns *et al*. 1994). The G_q family activates Phospholipase C beta (PLC-β) which cleavesthe phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to form second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Ting *et al*. 2012). IP_3 releases intracellular calcium stores and DAG activates protein kinase C (Fig. 3) (Berridge & Irvine 1984, Kikkawa *et al*. 1986). Overall, these responses lead to platelet activation (Huang *et al*. 2004).

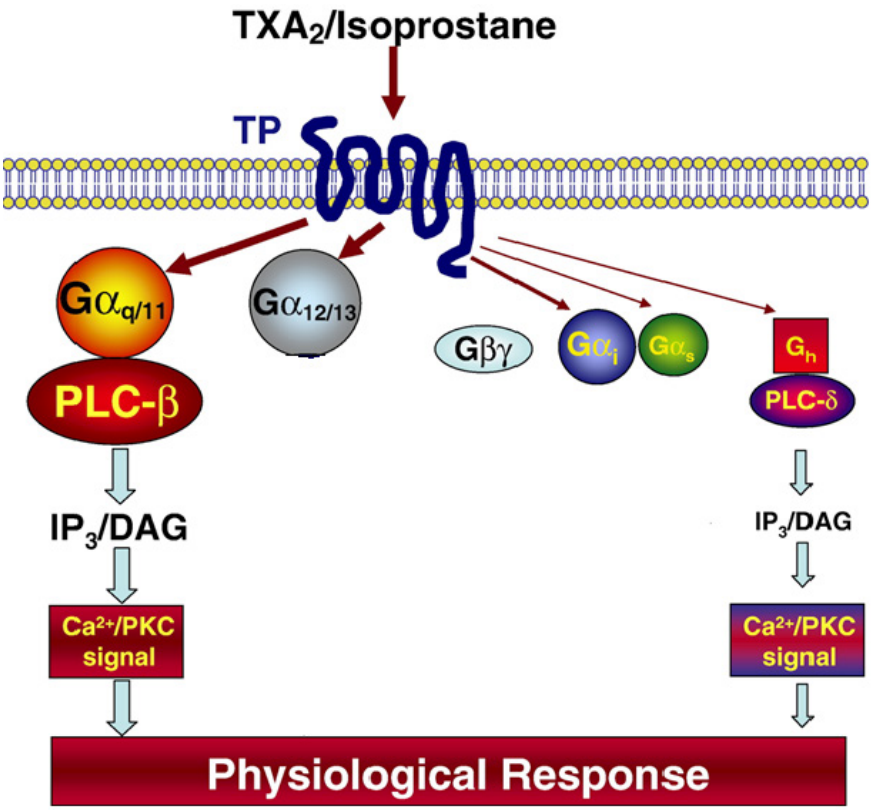

Figure 2: G protein coupling of thromboxane A_2 receptor and signal transduction *When ligand bound, TPRα can couple to several different G proteins. When TPRα is bound to $G_{\alpha/11/h,}$ phospholipase C-β (PLC-β) is activated, which converts phosphatidylinositol 4,5-bisphosphate ($PIP_{2)}$ into 2nd messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases intracellular calcium stores and DAG activates protein kinase C (Berridge & Irvine 1984, Kikkawa et al. 1986). (adapted from Nakahata 2008).*

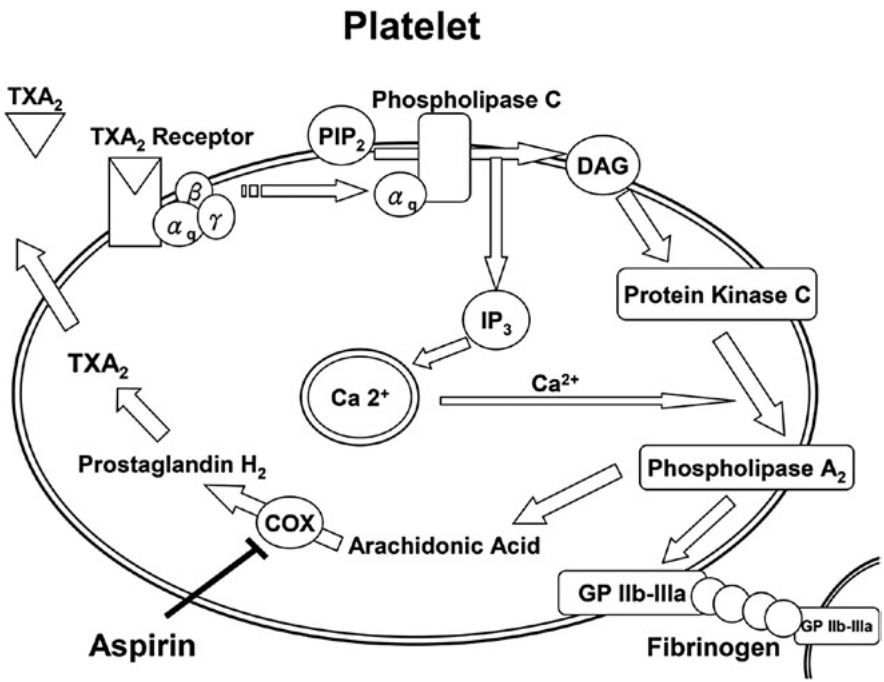

Figure 3: TXA_2 and TPRα intracellular signalling. *Gq protein activates phospholipase C, which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 elevates the cytosolic free Ca^{2+} concentration by inducing vesicular release of Ca^{2+} into cytosol. DAG activates protein kinase C and activates phospholipase A_2 stimulation of phospholipase A_2 is associated with the activation of glycoprotein IIb/IIIa complex, leading fibrinogen cross-linkage by binding to glycoprotein IIb/IIIa receptors present on each platelet (Jeng et al. 1998). Activation of PLA_2 also amplifies the signal transduction message by the generation of more TXA_2 from arachidonic acid by cyclooxygenase (COX). Aspirin inhibits COXs, down-regulating TXA_2 and thus platelet aggregations and clot formation. (Shiau et al. 2012)*

1.3. Physiological/Pathophysiological role of TXA_2 and TXA_2 Receptors in cardiovascular diseases

Platelet activation is a significant physiological response when TXA_2 binds TPRα. Platelet activation leads to alterations in platelet shape, aggregation and secretion. This in turn stimulates thrombus formation (Ally & Horrobin 1980, Dorn & DeJesus 1991, Spurney *et al*. 1991, Katugampola & Davenport 2001). TXA_2-induced platelet aggregation and vasoconstriction can lead to acute myocardial

infarction (Martin 1984, Hiraku *et al*. 1986, Dorn *et al*. 1990, Katugampola & Davenport 2001). These disease states are currently treated by TXA_2 synthesis inhibitors but not TPRα antagonists. TPRα antagonists could in theory be an effective means of treatment, but to date development of this class of drugs has been unsuccessful. TXA_2 is a ligand well-known to exert its actions through TPRα, but it is not the only endogenous ligand to do so. There is current evidence that suggests isoprostane 8-iso-prostaglandin $F_2\alpha$ (8-iso $PGF_2\alpha$) is another TPRα stimulant (Montuschi *et al*. 2004). Isoprostanes are peroxidative products of AAs, and are more stable when compared to TXA_2 (Montuschi *et al*. 2004). Therefore, TXA_2 may not by the primary ligand responsible for the cardiovascular diseases associated with TPRα-mediated signalling.

2. Physiological/Pathophysiological Effects of Targeting TPRα-Mediated Signalling

2.1. Marketed Drugs which Target TPRα-mediated Signalling

An effective antagonist targeting TPRα-mediated signalling has not been synthesised to date due, in part, to the fact not enough is known about what happens structurally when TPRα becomes activated. If the crystal structure was determined then it is possible that an effective drug could be synthesised based on the structure of the receptor. As the antagonist would be specific for the TPRα, it should not, in theory, have any serious side effects. To further qualify it as effective and successful in clinical trials, the proposed TPRα drug should be suitable for oral or intravenous administration, like that of aspirin. Also, the length of time of which the drug acts in the body should be adequate to therapeutically treat the disease state (Mousa 1999) i.e. it must be stable and not breakdown into a potentially dangerous by-product *in-vivo*.

The class of drugs which target TXA_2 production itself have been very successful to date (aspirin and dazoxiban). Aspirin and non-steroidal anti-inflammatory drugs (NSAIDS) are cycloxygenase inhibitors which inhibit TXA_2 at the early stages of biosynthesis.

These inhibitors stop TXA_2 production by blocking arachidonic acid conversion to PGH_2 (Fig. 1). There are three cycloxygenase (COX) isoenzymes identified; COX-1, COX-2 and COX-3. Platelets express COX-1 which can be inhibited non-selectively (i.e. by COX inhibitors which target both COX-1 and COX-2). Aspirin inhibits COX-1 by irreversibly acetylating a serine residue in the catalytic channel (Roth *et al*. 1977). At higher concentrations, COX-2 may also be inhibited by aspirin (Cipollone *et al*. 1997). This action leads to long-term down-regulation of TXA_2 synthesis in platelets as they don't possess a nucleus and cannot make their own COX-1.

Low dose aspirin remains the best standard of secondary prevention after myocardial infarction or stroke and more than 80% of such patients take aspirin regularly (Campbell *et al*. 2007). Despite the success of this class of drugs, none of the current anti-platelet agents available to the public meet all of the requirements outlined, as their lack of selectivity result in undesirable side-effects (Mousa 1999).

A negative side-effect of the non-selectivity of aspirin is that it may also inhibit PGI_2 synthesis, along with several other prostaglandins (Fig. 4) (Coccheri 2010). PGI_2 is a lipid essential for the maintenance of the endothelium and its inhibition can lead to deleterious side-effects such as disruption of the gastrointestinal lining. The ability of aspirin to inhibit PGI_2 production is believed to be relevant to explaining why some people are sensitive to aspirin while others are resistant (Pusch *et al*. 2008, Coccheri 2010). It is for these reasons that research efforts have shifted to focus on other targets in the signalling pathway.

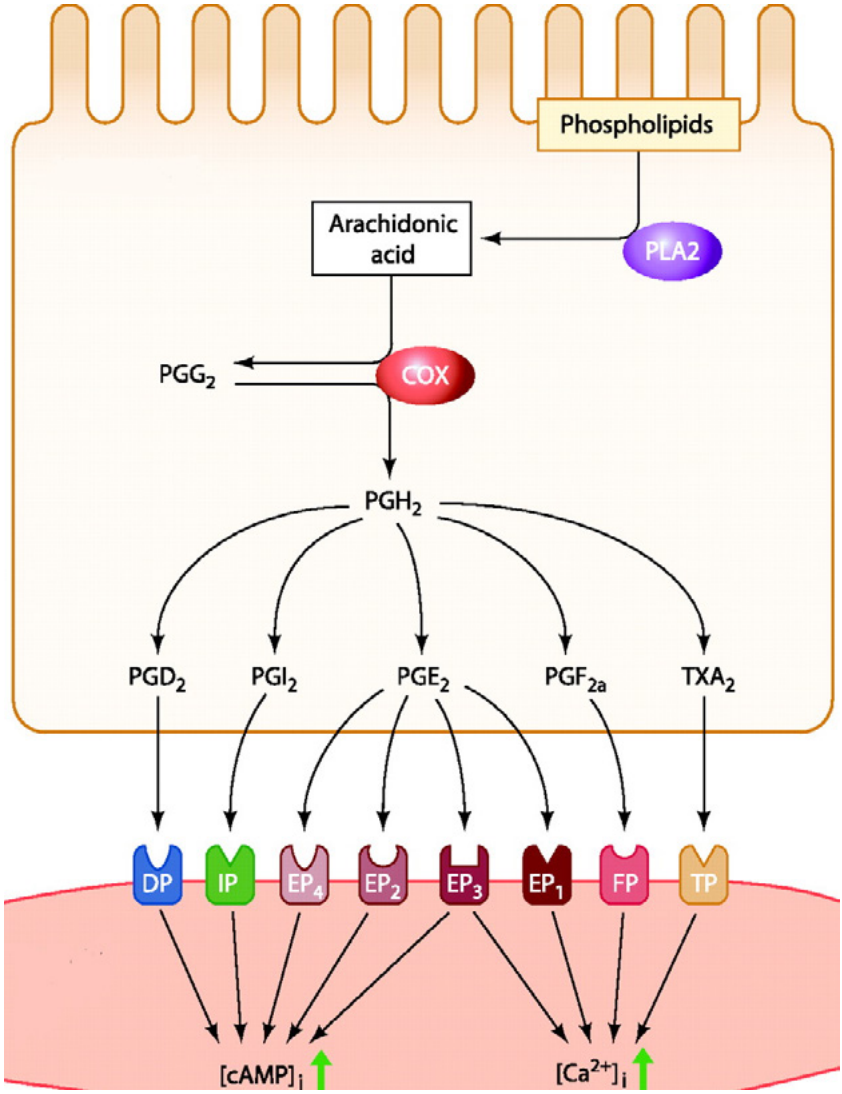

Figure 4: The cyclooxygenase (COX) and prostanoid biosynthetic and signalling pathways. *Arachidonic acid (AA) is released by phospholipase A_2 (PLA_2) and is acted on by COX enzymes and synthase enzymes (prostaglandin synthases and thromboxane synthase). These enzymes synthesise their respective prostaglandins and they move out of the cell through prostaglandin transporter (PGT). When aspirin blocks COX-1, it invariably inhibits the all of these signalling cascades. (adapted from Ruan et al. 2011)*

2.2. Thromboxane Synthase (TXS) Inhibitors

Thromboxane synthase (TXS) inhibitors were developed to block conversion of PGH_2 to TXA_2. These drugs proved successful as they reduced TXA_2 synthesis in platelets and improved TXA_2-related pathophysiological conditions (Dogne *et al*. 2005). Many drugs of this class were designed but unfortunately failed at clinical trials due to the accumulation of PGH_2, which increases platelet activity by acting as an agonist for TPRα (Ting *et al*. 2012). If a drug was produced which targeted the downstream receptor as opposed to the TXA_2 synthesis itself, it would reduce the effects of TXA_2 but PGI_2 production would be unaffected.

The added advantage of direct antagonism of TPRα over TXA_2 synthesis inhibition is that such drugs would block other ligands which have agonistic effects on the receptor, such as endoperoxides and isoprostanes (Gresele *et al*. 1987, Fiddler & Lumley 1990). There is evidence to show that TPRα mediates signalling through multiple effectors, including phospholipase C (PLC), some small guanosine triphosphate hydrolases (GTPases) and adenylyl cyclase (AC) (Ting *et al*. 2012). Furthermore, TPRα can recognise endogenous ligands other than eicosanoid thromboxane A_2 (TXA_2), such as the endoperoxide PGG_2 isoprostanes (Khasawneh *et al*. 2008). It will be important to considering the implications of the downstream pathways of these ligands when antagonising/blocking receptor.

Unfortunately, little progress has been made with successfully targeting these receptors through development of drugs due to problems with efficacy and toxicity. Most antagonists synthesised have not passed phase I/II clinical trials (Ting *et al*. 2012). In 2011, Bousser *et al.* studied terutroban. They interpreted no safety or efficacy advantages with terutroban versus aspirin. However, this study is inconclusive as it was prematurely stopped by the Data Monitoring Committee as it didn't meet the predefined criteria for non-inferiority (Bousser *et al*. 2011). Non-inferiority trials are intended to show that the effect of a new treatment is not worse than that of an active control, aspirin in this case, by more than a specified margin (Snapinn 2000). Therefore, research in developing drugs to target TPRα would benefit from the high resolution structure

determination.

3. Structural Features of TPRα

3.1. G-Protein Coupled Receptors

TPRα is one of five prostanoid receptor classifications recognised (Coleman *et al*. 1994). TPRα is a member of a large superfamily of transmembrane proteins, GPCRs, that are implicit in various cellular signals (Moreira 2014).

The GPCR superfamily is comprised of over 800 members, making it one of the largest protein families in the human genome. Not surprisingly it is associated with numerous human diseases and 30% of marketed drugs target GPCRs (Fredriksson *et al*. 2003, Hopkins & Groom 2002). Phylogenetic analysis has identified five main families that classify human GPCRs; rhodopsin-like (672 members), secretin (15 members), glutamate (22 members), adhesion (33 members) and frizzled-taste 2 (36 members) (Moreira 2014). Of these, TPRα belongs to the rhodopsin-like, also known as sub-family A.

The topology of sub-family A GPCRs comprises seven transmembrane α helices (TM1-7) in addition to an extracellular N-terminus and an intracellular C-terminus (Moreira 2014). These helices are connected by three extracellular (ECL) and three intracellular (ICL) loops.

3.2. Coupling of TPRα to G proteins

GRCR signalling involves the coupling of the activated receptor to a heterotrimeric GTP-binding protein (G-protein) (Jastrzebska *et al*. 2010). In the case of TPRα, binding occurs to the G-protein G_q among others. TPRα can only be associated with one G-protein at a time and receptor recycling regulates the specificity of the G-protein (Ting *et al*. 2012). The G_q protein is divided into two functional units, the guanine nucleotide-binding G_α subunit and the $G_{\beta\gamma}$ dimer (Fig. 5). When activated, the functional units dissociate and these in turn regulate a large number of downstream effects through association with enzymes and effector proteins (Hamm 1998). As

these receptors are so influential with regards to signal transduction in the cell in which they are expressed, it is important to structurally characterise them. However, only recently was the first GPCR/G-protein complex structure characterised (Rasmussen *et al*. 2011). This was a significant breakthrough as this was the first high-resolution view of transmembrane signalling by a GPCR in an activated state (R*) and in complex with its endogenous G-protein. The active state of a GPCR can be defined as that conformation that couples to and stabilises a nucleotide-free G protein (Rasmussen *et al*. 2011). Having a picture of the transmembrane protein in an active conformation provides crucial information of how ligand binding affects structure and thus enables design of better agonists/antagonists of the target protein.

Therefore, it would be beneficial to solve the structure not only of TPRα, but also that of TPRα-G_q complex. If the TPRα-Gq complex structure was determined then one would have a picture of the TPRα in R* state and it might be possible to study how TPRα responds to ligand binding and how it this changes the conformation of the receptor by comparison of the co-complex structure to that of the receptor on its own.

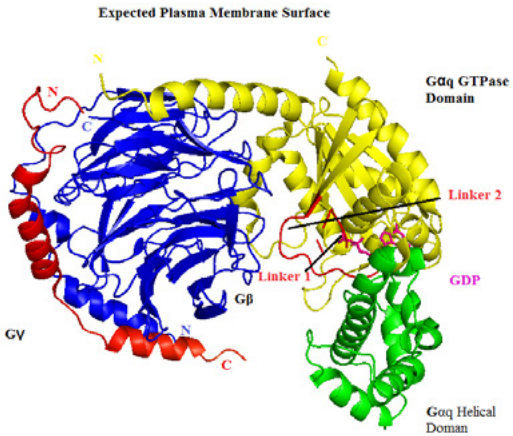

Figure 5: Structural representation of Heterotrimeric Gq-protein (PDB ID code 3AH8). *Generated using Pymol. G$\alpha_{i/q}\beta\gamma$ heterotrimer orientated with respect to the plasma membrane. G$\alpha_{i/q}$ consists of the GTPase and the helical domains connected by two linker*

regions, Linker 1 and Switch I (Linker 2) (Nishimura et al. 2010).

Conclusions

TPRα is not a "potential" drug target but it is in fact already a current human drug target for treatment of multiple thrombotic disorders. There has been a great deal of interest in regulating this receptor's activity as an alternative to circumvent the side-effects observed with aspirin (Halushka & Halushka 2002). Aspirin's side-effects are observed due to its lack of selectivity as it antagonizes both COX-1 and -2. Aspirin inhibits PGI_2 production, which is essential for maintenance of the endothelium which explains why patients who regularly take aspirin develop disruption of the gastrointestinal lining e.g. stomach ulcers. The next target in the pathway is thromboxane synthase (TXS), but inhibitors of this enzyme have been unsuccessful in clinical trials due to accumulation of PGH_2 which increases platelet activity (Ting *et al*. 2012). In theory, direct inhibition of downstream receptor itself, TPR**α,** presents many advantages as a drug target over the blocking of its upstream regulators. The advantage of an antagonist specific for TPRα is that all ligands which up-regulate the receptor would be blocked, not just TXA_2. Furthermore, PGI_2 production would be unaffected, thus limiting the possibility of side-affects, and PGH_2 accumulation would not be an issue. Unfortunately, the vast majority of compounds targeting TPRα failed to pass clinical trials or toxicity screenings as mentioned previously (Ting *et al*. 2012). The determination of the high resolution structure of TPRα may be a deciding factor to its success as a drug target as it will aid in rational, structure based drug design. While none of the antagonists developed for targeting TPRα to date have made it into clinical use, it may still prove a lucrative investment both intellectually and financially.

Acknowledgements

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