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1 **Insights into calcium-sensing receptor trafficking and biased signalling by studies of calcium**

2 **homeostasis**

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23 **ABSTRACT**

24 The calcium-sensing receptor (CaSR) is a class C G-protein coupled receptor (GPCR) that detects

25 extracellular calcium concentrations, and modulates parathyroid hormone secretion and urinary

26 calcium excretion to maintain calcium homeostasis. The CaSR utilises multiple heterotrimeric G-

27 proteins to mediate signalling effects including: activation of intracellular calcium release; mitogen-

28 activated protein kinase (MAPK) pathways; membrane ruffling; and inhibition of cAMP production.

29 By studying germline mutations in the CaSR and proteins within its signalling pathway that cause

30 hyper- and hypocalcaemic disorders, novel mechanisms governing GPCR signalling and trafficking

31 have been elucidated. This review focusses on two recently described pathways that provide novel

32 insights into CaSR signalling and trafficking mechanisms. The first, identified by studying a CaSR

33 gain-of-function mutation that causes autosomal dominant hypocalcaemia (ADH), demonstrated a

34 structural motif located between the third transmembrane domain and the second extracellular loop of

35 the CaSR that mediates biased signalling by activating a novel β-arrestin-mediated G-protein-

36 independent pathway. The second, in which the mechanism by which adaptor protein-2 σ-subunit

37 (AP2σ) mutations cause familial hypocalciuric hypercalcaemia (FHH) was investigated, demonstrated

38 that AP2σ mutations impair CaSR internalisation and reduce multiple CaSR-mediated signalling

39 pathways. Furthermore, these studies showed that the CaSR can signal from the cell surface using

40 multiple G-protein pathways, whilst sustained signalling is mediated only by the $G_{q/11}$ pathway. Thus,

41 studies of FHH and ADH associated mutations have revealed novel steps by which CaSR mediates

42 signalling and compartmental bias, and these pathways could provide new targets for therapies for

43 patients with calcaemic disorders.

44 **1   Introduction to the CaSR**

45   Extracellular calcium (Ca^{2+}_e) is required for diverse biological functions ranging from blood

46   coagulation, mineralisation of bone matrix, muscle contraction, and hormone secretion (Brown 1991).

47   Thus, calcium concentrations within the blood are tightly regulated. The parathyroid gland plays an

48   essential role in calcium homeostasis by detecting Ca^{2+}_e in the blood, and in response to

49   hypocalcaemia, secretes the parathyroid hormone (PTH) to normalize serum calcium concentrations.

50   PTH achieves this by: enhancing bone resorption; activating calcium reabsorption at the kidneys; and

51   stimulating the synthesis of 1,25-dihydroxyvitamin D_3, which mobilises intestinal calcium absorption

52   (Fig. 1A) (Riccardi and Brown 2010). The net effect of these three pathways is to increase Ca^{2+}_e,

53   which provides feedback inhibition to the parathyroid gland, to suppress PTH secretion (Conigrave

54   and Ward 2013). The parathyroid is able to detect Ca^{2+}_e concentrations in the blood using the cell-

55   surface expressed calcium-sensing receptor (CaSR), a class C G-protein coupled receptor (GPCR), for

56   which Ca^{2+} is the major ligand (Conigrave and Ward 2013; Riccardi and Brown 2010).

57   The CaSR is a 1078 amino acid protein that exists at cell surfaces as a disulphide-linked homodimer

58   (Ward, et al. 1998), although it is capable of forming heterodimers (Chang, et al. 2007; Gama, et al.

59   2001). The CaSR has a large extracellular domain, which was recently crystallized by two

60   independent labs, and consists of a bilobed venus fly-trap ligand binding domain (VFTD), and a

61   cysteine-rich domain (CRD) (Geng, et al. 2016; Zhang, et al. 2016) (Fig. 1B). Ca^{2+}_e binds between the

62   two lobes (lobe 1 and lobe 2) of the VFTD, which initiates a conformational change, facilitating lobe

63   2-lobe 2 dimerisation and allowing the cysteine-rich domains to interact (Geng et al. 2016; Zhang et

64   al. 2016). These conformational changes are predicted to re-orientate the seven transmembrane (TM)

65   domain (Geng et al. 2016), and consequently activate the associated G-proteins and initiate signal

66   transduction (Standfuss, et al. 2011). The CaSR has been reported to couple to multiple G-protein

67   subtypes, but predominantly signals by: the $G_{i/o}$ pathway, to suppress cAMP and activate mitogen-

68   activated protein kinase (MAPK) cascades (Kifor, et al. 2001; Thomsen, et al. 2012); the $G_{q/11}$-

69   phospholipase C (PLC)-mediated pathway, to generate inositol 1,4,5-trisphosphate (IP_3) and

70   diacylglycerol, that activate intracellular calcium (Ca^{2+}_i) mobilisations (Hofer and Brown 2003) and

3

71   MAPK pathways, respectively. The CaSR can also couple to a G-protein-independent mechanism

72   involving β-arrestin proteins to also activate MAPK signalling (Thomsen et al. 2012) (Fig. 2).

73

74   **2   Human disorders of the CaSR provide insights into receptor signalling mechanisms**

75   The importance of the CaSR in the regulation of Ca^{2+}_e is highlighted in patients with germline

76   mutations of the receptor that have disorders of calcium homeostasis. Heterozygous loss-of-function

77   mutations in the CaSR lead to the autosomal dominant condition familial hypocalciuric

78   hypercalcaemia (FHH) (Pollak, et al. 1993), which is characterised by lifelong mild-to-moderate

79   hypercalcaemia, normal or mildly raised serum PTH levels and low urinary calcium excretion (Firek,

80   et al. 1991; Marx 2015). FHH is considered to be a benign disorder as most patients are

81   asymptomatic, but it can be associated with chondrocalcinosis and pancreatitis in some cases (Hannan

82   and Thakker 2013; Pearce, et al. 1996a; Volpe, et al. 2009). Homozygous and compound

83   heterozygous loss-of-function mutations of the CaSR are the common cause of neonatal severe

84   hyperparathyroidism (NSHPT), which is characterised by marked elevations in serum calcium and

85   PTH, failure to thrive and hyperparathyroid bone disease (Chattopadhyay and Brown 2006; Hannan

86   and Thakker 2013; Pollak et al. 1993). On occasion, heterozygous CaSR mutations may lead to an

87   NSHPT-like phenotype, and this is largely due to the dominant-negative nature of the mutant receptor

88   on the wild-type CaSR in these cases (Obermannova, et al. 2009; Pearce, et al. 1995).

89   Gain-of-function mutations of the CaSR cause autosomal dominant hypocalcaemia (ADH)

90   characterised by mild-to-moderate hypocalcaemia and inappropriately low or normal PTH

91   concentrations (Hannan and Thakker 2013; Pearce, et al. 1996b). Up to 50% of patients present with

92   hypocalcaemic symptoms of paraesthesia, carpopedal spasms, seizures, and ectopic calcification of

93   the kidneys and basal ganglia (Hannan and Thakker 2013; Pearce et al. 1996b). Some patients with

94   gain-of-function mutations in CaSR may have Bartter syndrome type 5, which is characterised by

95   renal salt wasting, hypokalaemia, hyperreninaemia and hyperaldosteronaemia (Vargas-Poussou, et al.

96   2002; Watanabe, et al. 2002).

4

97 **2.1 Biased signalling of the CaSR**

98 Functional studies in HEK293 cells have demonstrated that disease-causing mutations may influence

99 CaSR signalling responses in a biased manner (Leach, et al. 2012). Despite the capability of the CaSR

100 to signal via multiple signalling pathways, *in vitro* studies in HEK293 cells have shown that it

101 preferentially couples to the Ca^{2+}_i signalling pathway (Leach et al. 2012). In contrast, disease-causing

102 CaSR mutants have been shown to switch this preferential signalling, with some FHH1-causing

103 mutations signalling equally via the Ca^{2+}_i and MAPK pathways, or predominantly via MAPK

104 pathways; while many ADH1-associated mutants couple more strongly to Ca^{2+}_i (Leach et al. 2012).

105 Furthermore, studies of positive and negative allosteric CaSR modulating compounds, revealed they

106 too can mediate a biased signalling response, with both classes of drugs influencing Ca^{2+}_i to a greater

107 extent than ERK1/2 phosphorylation (Leach, et al. 2013). Thus, these findings established that

108 agonist-induced CaSR signalling may occur in a biased manner, although the GPCR structural motifs

109 mediating ligand-dependent bias were not described.

110

111 **2.2 Novel insights into mechanisms by which CaSR can mediate signalling bias**

112 Recently, a novel ADH1-associated CaSR mutation, Arg680Gly, has provided some insights into the

113 molecular mechanisms mediating signalling bias (Gorvin, et al. 2018a). *In vitro* analyses showed that

114 Ca^{2+}_i signalling in cells expressing the Arg680Gly mutation was not different to that observed in wild-

115 type cells, in contrast to previously reported ADH1-associated CaSR mutants (Gorvin et al. 2018a).

116 However, the Arg680Gly mutation did enhance MAPK signalling (Gorvin et al. 2018a). Furthermore,

117 this type of signalling bias, in which an ADH1 mutation enhances MAPK signalling, but does not

118 affect Ca^{2+}_i has not previously been described and provided an opportunity to explore the role of the

119 Arg680 residue in CaSR structure-function. As MAPK acts as a convergence pathway for multiple

120 CaSR signalling pathways (Fig. 2) each was investigated using a single reporter assay measuring

121 luciferase upstream of a serum-response element (SRE), which acts as a measure for MAPK-mediated

122 transcription (Gorvin, et al. 2017b). By applying compounds that specifically block either the $G_{q/11}$

5

123   ((YM-245890 and UBO-QIC), the $G_{i/o}$ (pertussis toxin) or the β-arrestin (β-arrestin-1 and β-arrestin-2

124   targeting siRNA) pathways, it was shown that the Arg680Gly mutation enhanced MAPK signalling

125   by a β-arrestin1/2-mediated pathway (Gorvin et al. 2018a; Schrage, et al. 2015; Takasaki, et al. 2004).

126

127   The structural location of the mutant residue within the CaSR transmembrane domain (TMD) region

128   provides some insights into the likely mechanism mediating this bias. Homology modelling of the

129   CaSR TMD, based upon the structure of the closely related human metabotropic glutamate receptor 1

130   (mGluR1) (Gorvin et al. 2018a; Hu, et al. 2005), predicts that the Arg680 residue lies at the

131   extracellular side of TM3, and that the residue forms salt bridge connections with the side chain of

132   adjacent residues in extracellular loop 2 (ECL2) (Glu767) or TM7 (Glu837) (Fig. 3). Such

133   connections between residues within TMDs or with ECLs of GPCRs are known to be important in

134   receptor activity. For example, TM3 and TM6 of the β2AR forms ionic interactions which lock the

135   receptor in an inactive state, and conformational changes within these helices governs G-protein

136   coupling at the cytoplasmic face of the receptor (Ballesteros, et al. 2001; Rasmussen, et al. 2007).

137   Furthermore, disruption of a salt bridge in β2-AR allows lateral displacement of TM3 away from

138   TM4 and TM5, facilitating β-arrestin binding (Shukla, et al. 2014), and it was therefore hypothesised

139   that the CaSR Arg680Gly mutation may break a salt-bridge between TM3 and ECL2 or TM7

140   allowing β-arrestin to bind more readily at the CaSR cytoplasmic face (Gorvin et al. 2018a). The

141   importance of the Arg680, Glu767 and Glu837 residues in the CaSR has previously been recognised.

142   Mutation of Glu767 and Glu837 has been shown to increase signaling by the CaSR (Hu, et al. 2006;

143   Hu et al. 2005; Hu, et al. 2002; Uckun-Kitapci, et al. 2005), and previous structural homology models

144   that investigated the binding of allosteric modulator drugs with the CaSR demonstrated that the three

145   residues form critical contacts for drug binding (Miedlich, et al. 2004; Petrel, et al. 2004), indicating

146   their importance in CaSR structure-function. To investigate the hypothesis that a salt-bridge is

147   disrupted by mutation of Arg680, a series of mutations were engineered at the Arg680, Glu767 and

148   Glu837 residues. First, Glu767 and Glu837 were mutated to Arg residues, to introduce unfavourable

149   electrostatic interactions. Under these conditions, the Glu767Arg engineered mutant was shown to

6

150   enhance MAPK activity in a similar way to the Arg680Gly ADH1 mutant, a response that was also β-

151   arrestin sensitive (Gorvin et al. 2018a). In contrast, responses in cells expressing the CaSR Glu837Arg

152   engineered mutant were similar to those in cells expressing WT CaSR (Gorvin et al. 2018a). To

153   confirm that a salt-bridge is required between residues 680 and 767 in the CaSR, a double mutant was

154   generated by mutating the Glu767Arg mutant receptor with an additional missense mutation of

155   Arg680 to Glu680. This should allow the salt-bridge between the two residues to reform. Indeed, in

156   this double mutant (Glu680-Arg767) CaSR, MAPK signalling was restored to levels similar to those

157   seen in wild-type cells (Gorvin et al. 2018a).

158

159   Thus, these studies have revealed some of the important structural motifs of CaSR that mediate

160   signalling bias, and discovery of this novel β-arrestin-specific pathway may help facilitate the

161   development of targeted therapeutics for CaSR. Furthermore, it demonstrates the importance of

162   investigating multiple signalling outputs downstream of CaSR, to ensure that potentially disease-

163   causing mutations are not classified as benign polymorphisms (Gorvin et al. 2018a).

164

165   **3   Insights from human genetic mutations in components of the CaSR signalling pathway**

166   Both FHH and ADH are genetically heterozygous conditions with mutations in the CaSR accounting

167   for approximately 65% and 70% of cases, respectively (Hannan, et al. 2012; Nesbit, et al. 2013a).

168   Two further genetically distinct forms of FHH, and one further distinct form of ADH, have been

169   described (Mannstadt, et al. 2013; Nesbit et al. 2013a; Nesbit, et al. 2013b). Heterozygous loss- and

170   gain-of-function germline mutations in the α-subunit of the G-protein 11 (Gα_{11}), a component of the

171   CaSR signalling pathway, give rise to FHH type-2 (FHH2) and ADH type-2 (ADH2), respectively

172   (Mannstadt et al. 2013; Nesbit et al. 2013a). Only four FHH2 and six ADH2 mutations have been

173   described to date, and therefore these mutations account for only a small number of cases of these

174   disorders (Gorvin, et al. 2016; Gorvin, et al. 2017a; Li, et al. 2014; Mannstadt et al. 2013; Nesbit et al.

175   2013a; Piret, et al. 2016). FHH type-3 (FHH3) is due to mutations in the sigma subunit of the adaptor

7

176   protein-2 (AP2σ), which plays a fundamental role in clathrin-mediated endocytosis of transmembrane

177   proteins, such as GPCRs (Nesbit et al. 2013b). The AP2σ protein is ubiquitously expressed and

178   clathrin-mediated endocytosis is a critical cellular process; however, FHH is a largely benign

179   condition, and the phenotypes observed in FHH3 patients are largely CaSR-specific. Thus, by

180   studying the AP2σ mutations identified in FHH3, which have been described in a single residue

181   (Hannan, et al. 2015; Nesbit et al. 2013b), novel insights into the trafficking and signalling

182   mechanisms of CaSR have been elucidated, and indicate an important interplay between these two

183   processes (Gorvin, et al. 2018b).

184

185   3.1   Trafficking of the CaSR

186   The cell surface expression of the CaSR is important for detecting extracellular ligand and signalling

187   by the receptor, and therefore the plasma membrane expression of CaSR is carefully regulated. This

188   involves multiple pathways including: receptor synthesis and secretion, trafficking to the plasma

189   membrane, and removal of the CaSR from the cell surface by endocytosis.

190

191   3.1.1   Regulation of cell surface expression by functional desensitisation

192   In contrast to most GPCRs, which undergo agonist-dependent desensitisation by phosphorylation

193   and/or by β-arrestin proteins, functional desensitisation appears to have only a minimal impact on

194   CaSR expression, and there are inconsistencies between findings in these studies (Bouschet, et al.

195   2005; Lorenz, et al. 2007; Pi, et al. 2005; Thomsen et al. 2012). Initial studies of the CaSR showed

196   that several proteins desensitise the receptor. These include: PKC, which phosphorylates the receptor

197   following activation of signalling pathways and recruits β-arrestin; G-protein regulatory kinase (GRK)

198   2, which binds to $G\alpha_q$ and inhibits its signalling; and, GRK4 which phosphorylates CaSR to facilitate

199   desensitisation (Lorenz et al. 2007; Pi et al. 2005). Furthermore, GRKs and β-arrestin have

200   independent functions as combined treatment of cells with these proteins enhances desensitisation

201   (Lorenz et al. 2007). The traditional view of GPCR desensitisation and internalisation was one in

202   which the receiver is phosphorylated and β-arrestin recruited, followed by internalisation of the

203   receptor by clathrin-mediated endocytosis, which is facilitated by interactions between β-arrestin and

204   AP2 (Shukla, et al. 2011). While studies of CaSR showed that β-arrestin can be recruited, this did not

205   enhance receptor internalisation, which only occurred when cells were treated with high

206   concentrations of Ca^{2+}_e (10mM) (Lorenz et al. 2007). β-arrestin is also now recognised to have

207   another function, as a scaffold protein that facilitates signalling either at the plasma membrane, or for

208   some GPCRs, at the endosome (Shukla et al. 2014). This function of β-arrestin has been recognised

209   for CaSR in some studies, providing seemingly contradictory information to that in studies of

210   functional desensitisation. Thus, treatment of cells with dominant-negative forms of β-arrestin1 or β-

211   arrestin2, or with siRNA targeting β-arrestin1 or β-arrestin2, reduces the pERK and membrane

212   ruffling signals downstream of CaSR (Bouschet et al. 2005; Gorvin et al. 2018b; Thomsen et al.

213   2012). Further studies are required to determine whether the discrepancies within these data sets are

214   due to experimental differences, differences in cell type, or if both desensitisation and enhanced

215   signalling occur downstream of CaSR, but at different spatial or temporal points.

216

217   **3.1.2 Regulation of cell surface expression by the secretory pathway and agonist-driven**

218   **insertional signalling (ADIS)**

219   The cell surface expression of CaSR, and mechanisms by which the receptor is synthesised and

220   exported to the plasma membrane, are generally well understood. The CaSR is synthesised at the ER

221   where it forms homodimers (Pidasheva, et al. 2006) and undergoes quality control and immature

222   glycosylation steps prior to progression to the Golgi (Fan, et al. 1997). At the Golgi, the CaSR

223   undergoes further maturation. This forward trafficking through the secretory pathway involves a

224   number of regulatory proteins that have been well described in previous reviews (Breitwieser 2013;

225   Huang, et al. 2011). The core-glycosylated CaSR is then retained within pre-plasma membrane

226   compartments (Fan et al. 1997). Therefore CaSR is retained intracellularly within two large

227   intracellular reserves: one at the ER and one at pre-plasma membrane compartments (Breitwieser

228   2012).

9

229

230   The large pool of fully mature CaSR plays a unique role in regulation of Ca^{2+}_e. Unlike many GPCRs,

231   the CaSR is chronically exposed to its ligand at baseline concentrations, and thus the receptor

232   undergoes very little functional desensitisation (Gama and Breitwieser 1998). Furthermore, CaSR is

233   able to elicit signalling responses for as long as elevated Ca^{2+}_e is available (Grant, et al. 2011; Lorenz

234   et al. 2007; Pi et al. 2005). Experimental studies have sought to explain this apparent paradox and

235   using a combination of total internal reflection fluorescence microscopy (TIRFm) and a construct that

236   allows the simultaneous measurement of CaSR insertion within plasma membranes and endocytosis

237   (known as BSEP-CaSR), a new model for CaSR cell surface expression has emerged (Fig. 4). This

238   model proposes that in the basal state, CaSR is only weakly expressed at the plasma membrane, but

239   on exposure to increases in Ca^{2+}_e there is an increase in anterograde trafficking through the secretory

240   pathway (Grant et al. 2011) (Fig. 4). This increase in the secretory pathway involves both mature

241   CaSR located in pre-plasma membrane regions, and newly synthesised CaSR from the ER

242   (Breitwieser 2013; Grant et al. 2011), and at present the mechanisms that trigger the ADIS events are

243   not fully elucidated. However, studies in which ADIS and CaSR signalling were measured

244   simultaneously revealed that elevated Ca^{2+}_i and the $G\alpha_{q/11}$ pathway is likely involved (Gorvin et al.

245   2018b; Grant, et al. 2012). Furthermore, the 14-3-3 proteins, which bind CaSR at an Arg-rich site

246   within the C-terminus, limits the release of CaSR from the endoplasmic reticulum, and therefore

247   regulates CaSR cell surface expression by reducing the ADIS-mobilisable pool of the receptor (Grant,

248   et al. 2015). The presence of the ADIS system and constitutive endocytosis may also explain some of

249   the inconsistencies between studies of CaSR trafficking, as endocytosis of the receptor is balanced by

250   the continual insertion of new CaSR from the secretory pathway (Breitwieser 2013). Those studies in

251   which a tagged construct and cell surface labelling (e.g. FLAG) was used in isolation, without also

252   measuring insertion of new CaSR within the plasma membrane, may not reflect the physiological

253   state. Similarly, measurement of total cell surface expression of receptor cannot be used as a surrogate

254   for determining the rate of internalisation.

255

10

256 These studies have demonstrated that trafficking of the CaSR plays a critical role in receptor

257 signalling and calcium homeostasis. More recent studies, building upon these initial findings, have

258 identified further insights into CaSR cell surface expression and endocytosis, and recognised further

259 connections between the trafficking and signalling of this receptor.

260

261 **3.1.3   Regulation of cell surface expression by endocytosis**

262 The CaSR has been described to have two types of internalisation: a constitutive pathway (Grant et al.

263 2011), and an agonist-driven pathway (Gorvin et al. 2018b; Lorenz et al. 2007). The CaSR was shown

264 to internalise using clathrin-mediated endocytosis in early studies (Holstein, et al. 2004). In addition,

265 CaSR has been described to associate with several proteins that facilitate clustering at the plasma

266 membrane and therefore increase the efficiency of internalisation, or act as scaffolds to enable

267 signalling to occur. One such protein is caveolin-1, with which CaSR has been shown to

268 coimmunoprecipitate (Kifor, et al. 1998). CaSR has been described to be enriched in caveolae

269 structures in parathyroid chief cells and osteosarcoma cells (Jung, et al. 2005; Kifor et al. 1998; Sun

270 and Murphy 2010). CaSR-mediated signalling is impaired in cells treated with caveolin-1 targeting

271 siRNA, and it is likely that this signalling function is facilitated by caveolae acting as signalling hubs

272 allowing the CaSR, G-proteins and PKC to cluster together (Jung et al. 2005; Kifor et al. 1998; Sun

273 and Murphy 2010). However, it is currently unknown whether caveolin facilitates CaSR clustering

274 within caveolae structures to enhance signalling, or whether the signalling itself drives this clustering

275 to encourage endocytosis (Breitwieser 2013). Filamin is an actin binding cytoskeleton protein that is

276 important for protein scaffolding (Hjalm, et al. 2001) and may also facilitate clustering that aids in

277 receptor endocytosis. Filamin binds to the CaSR C-terminus and increases total cellular content of

278 CaSR by preventing its proteosomal degradation (Hjalm et al. 2001), with some studies showing

279 CaSR expression is reduced in cells treated with siRNA targeted against filamin. However, other

280 reports show no such changes in CaSR expression, and this likely requires further investigation

281 (Huang, et al. 2006; Mingione, et al. 2017). The net result of Filamin A binding to CaSR is increased

282 MAPK signalling by the receptor (Hjalm et al. 2001; Pi, et al. 2002).

11

283

3.1.4   Effect of AP2σ mutations on CaSR signalling and trafficking

285   Mutations in AP2σ have been demonstrated to cause FHH3, and are associated with impaired CaSR-

286   mediated Ca^{2+}_i signalling (Nesbit et al. 2013b). The AP2 complex is a ubiquitously-expressed

287   heterotetrameric protein which plays a fundamental role in the clathrin-mediated endocytosis of

288   transmembrane proteins, such as GPCRs. The two larger subunits, α and β, have appendages that bind

289   to the clathrin coat proteins, plasma membrane phospholipids, and endocytic accessory proteins (e.g.

290   β-arrestin); while the two smaller subunits, μ and σ, bind to endocytic motifs of cargo proteins

291   (Collins, et al. 2002; Jackson, et al. 2010; Kelly, et al. 2008; Kirchhausen, et al. 2014) (Fig. 4). The

292   AP2μ subunit recognises tyrosine-based motifs and the AP2σ subunit recognises dileucine-based

293   motifs (Haucke and De Camilli 1999; Kelly et al. 2008) (Fig. 4). FHH3-associated mutations in the

294   AP2σ protein have been reported to affect the Arg15 residue, and structural modelling studies using a

295   published structure of the AP2 complex, have shown these missense mutations (to Cys15, His15 and

296   Leu15) likely disrupt interactions with a putative dileucine motif in the CaSR C-terminus (Nesbit et

297   al. 2013b), and thus affect CaSR endocytosis.

298

299   Initially this hypothesis was tested using an ELISA assay of total CaSR at cell surfaces in HEK293

300   cells stably overexpressing CaSR (HEK-CaSR), and transiently transfected with AP2σ-mutant

301   proteins. This showed that AP2σ-mutant expressing cells had increased CaSR cell surface expression

302   when compared to AP2σ-WT cells following stimulation with 5mM Ca^{2+}_e (Nesbit et al. 2013b). Thus,

303   it was concluded that CaSR endocytosis is impaired in these cells resulting in increased total CaSR

304   cell surface expression (Nesbit et al. 2013b). More detailed studies characterising the ADIS and

305   endocytosis components of CaSR regulation in AP2σ mutant cells using TIRFm and the BSEP-CaSR

306   construct, showed both ADIS and CaSR endocytosis were impaired resulting in the net effect of an

307   increased total CaSR cell surface expression in cells expressing AP2σ mutant protein compared to

308   WT cells (Gorvin et al. 2018b). Additional TIRFm studies focussing on CaSR and clathrin

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309   demonstrated that CaSR and clathrin colocalise at plasma membranes, and that the duration of this

310   colocalisation is prolonged in AP2σ-mutant expressing cells (Gorvin et al. 2018b). Furthermore, the

311   vesicles containing both CaSR and clathrin in AP2σ-mutant cells, when compared to WT cells, were

312   less motile, which is an indication that the vesicles are less likely to result in viable endocytic events

313   (Gorvin et al. 2018b; Rappoport and Simon 2003). Thus, cells expressing AP2σ-mutant proteins have

314   delayed recruitment of clathrin, and colocalisation with CaSR is prolonged, resulting in impaired

315   CaSR endocytosis (Gorvin et al. 2018b).

316

317   The effect of the FHH3-associated AP2σ-mutant proteins on multiple CaSR-mediated signalling

318   pathways has been characterised in a number of cellular assays in HEK293 cells stable overexpressing

319   AP2σ WT and mutant proteins and lymphoblastoid cell-lines derived from blood samples from FHH3

320   patients with the AP2σ-Cys15 mutation (Gorvin et al. 2018b; Nesbit et al. 2013b). These studies

321   confirmed that AP2σ mutations of the Arg15 residue impair Ca^{2+}_i mobilisation, phosphorylated

322   ERK1/2 (pERK1/2) MAPK signalling, membrane ruffling and suppression of cAMP, and that all of

323   these pathways occur downstream of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ (Gorvin et al. 2018b; Nesbit et al. 2013b).

324   Therefore, these studies have shown that AP2σ mutations reduce endocytosis resulting in increased

325   CaSR cell surface expression, but paradoxically decrease CaSR-mediated signalling (Gorvin et al.

326   2018b). To explain this paradox, we hypothesised that CaSR may be able to continue signalling from

327   within the cell (i.e. sustained signalling) (Gorvin et al. 2018b). Such sustained signalling has been

328   previously reported for some class A (e.g. β2-adrenergic receptor (β2AR), dopamine receptor D1

329   (DRD1), thyroid-stimulating hormone receptor (TSHR), vasopressin receptor 2 (V2R) and luteinizing

330   hormone receptor (LHR)), and class B (e.g. parathyroid hormone 1 receptor (PTH1R)) GPCRs

331   (Calebiro, et al. 2009; Feinstein, et al. 2013; Ferrandon, et al. 2009; Irannejad, et al. 2013; Jean-

332   Alphonse, et al. 2014; Kotowski, et al. 2011). Thus, in cells with the AP2σ mutation, in which there is

333   impaired endocytosis, the availability of internalised receptors from which sustained signals could

334   emanate would be reduced and thus the net effect would be impaired overall CaSR-mediated

335   signalling. To test this hypothesis, a combination of imaging and biochemical analyses, along with

13

chemical inhibitors were used in HEK-CaSR, HEK-AP2σ and CRISPR-Cas generated β-arrestin

knockout cells to assess CaSR-mediated MAPK signalling (Gorvin et al. 2018b). To assess sustained

signalling two primary assays were used: 1) assessment of pERK1/2 over 60 minutes following

treatment of cells with a 5 min pulse of 5mM Ca^{2+}_e; and 2) analysis of SRE luciferase reporter

responses over 12 hours, following a 5 min pulse of 5mM Ca^{2+}_e. Using these methods, MAPK

sustained signals were demonstrated in HEK-CaSR cells, and evidence for an internal, likely

endosomal, source was shown in three ways (Gorvin et al. 2018b) (Fig. 5). First, addition of Dyngo, a

chemical inhibitor of dynamin, which is required for vesicle scission during clathrin-mediated

endocytosis, abolishes sustained signals, whilst rapid plasma membrane mediated signals remain

intact. Loss of this sustained response in Dyngo-treated cells was not due to increased apoptosis or

decreased proliferation, and was unaffected by inhibition of CaSR protein synthesis as the sustained

rise in pERK1/2 was not blocked by tunicamycin, an inhibitor of glycosylation of newly synthesised

CaSR. Second, sustained signals were reduced in cells expressing a dominant-negative Rab5 protein,

which delays maturation of early endosomes and therefore slows clathrin-mediated endocytosis.

Third, reduction or loss of these sustained signals was observed in cells expressing AP2σ mutant

proteins (Fig. 5).

The G-proteins involved in these sustained signals were also explored as MAPK signalling is a

convergence pathway for multiple CaSR-mediated signalling pathways (Fig. 2). The ability of the

$G\alpha_{q/11}$ and $G\alpha_{i/o}$ pathways to activate CaSR sustained signals was investigated using G-protein

specific inhibitors (UBO-QIC and PTx) and the SRE luciferase reporter assay, which showed that

inhibition of both $G\alpha_{q/11}$ and $G\alpha_{i/o}$ impaired the early, plasma membrane mediated CaSR signal, while

only $G\alpha_{q/11}$ was important for the later sustained signal. Furthermore, confocal microscopy confirmed

that $G\alpha_q$ and phosphatidylinositol 4,5-bisphosphate (PIP_2), the lipid hydrolysed by PLC, colocalise

with CaSR at plasma membranes and a subpopulation of endosomes (Gorvin et al. 2018b). In

addition, inhibitors of the PLC-DAG-IP_3 pathway (U73122, GF-109203X and 2-

aminoethoxydiphenyl borate (2-APB), which inhibit PLC, PKC and the IP_3-receptor, respectively)

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363 were shown to reduce sustained pERK1/2 signals, indicating that $G\alpha_{q/11}$ and its signalling pathway are

364 important for CaSR-mediated signalling (Gorvin et al. 2018b). Finally, the effects of the β-arrestin

365 scaffold proteins, which are important for the sustained endosomal signalling of some GPCRs such as

366 V2R and PTH1R (Feinstein et al. 2013; Wehbi, et al. 2013), were assessed in HEK-CaSR cells with

367 deletion of the β-arrestin1 and β-arrestin2 genes. In these cells both pERK and SRE reporter responses

368 were unaffected by deletion of the β-arrestin proteins (Gorvin et al. 2018b). Thus, the CaSR mediates

369 some MAPK signals from endosomes using $G\alpha_q$ and PLC, but does not require β-arrestin for this

370 pathway.

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372 Conclusions and important lessons for the future (or unanswered questions)

373 Since the cloning of the CaSR gene 25 years ago (Brown, et al. 1993), many insights have been

374 gained into the role of the CaSR in calcium homeostasis, and by studying patients with mutations

375 within this gene we have learnt much about CaSR signalling and trafficking mechanisms. The

376 discovery that CaSR can signal from within the cell, most likely from an endosomal source, and that

377 disruption of a specific structural motif can mediate G-protein independent signalling bias opens up

378 many new avenues of investigation. How do mutations in the CaSR affect these endosomal pathways?

379 Can drugs that target the CaSR affect these pathways? How are trafficking and signalling of the CaSR

380 so intimately linked? Future studies investigating these questions will undoubtedly reveal further

381 complexities into the regulation of CaSR, and could provide mechanisms relevant to other GPCRs.

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1   **Figures**

2   **Figure 1**   **Regulation of extracellular calcium at the parathyroid glands**

3   (**A**) Schematic illustrating how the parathyroid glands respond to changes in extracellular calcium

4   concentrations $[Ca^{2+}_e]$ in the blood. In the presence of low $[Ca^{2+}_e]$ in the blood, the parathyroid glands

5   secrete parathyroid hormone (PTH). PTH binds to PTH receptors at the kidney, resulting in calcium

6   reabsorption, and activation of 1,25-dihydroxyvitamin D_3; which in turn acts upon bone to mediate

7   calcium release, and the intestine to activate calcium resorption. PTH also acts directly on bone to

8   activate calcium release. The tissue-specific effects on calcium are shown in the hatched box with an

9   arrow to indicate that these are increased by low $[Ca^{2+}]$ in the blood. (**B**) Schematic of the

10   homodimeric calcium-sensing receptor (CaSR) showing the two protomers (CaSR1 and CaSR2)

11   within the plasma membrane (PM). The calcium-sensing receptor is expressed highly at the

12   parathyroid gland and its major function is to detect $[Ca^{2+}_e]$ and regulate PTH secretion accordingly.

13   The CaSR has a large extracellular domain (ECD) comprising the venus flytrap domain (VFTD) that

14   contains two lobes (lobe 1 and lobe 2), and a cysteine-rich domain (CRD). The CRD connects the

15   ECD to the 7 transmembrane domains (TMD), and the CaSR has a long cytoplasmic intracellular

16   domain (ICD). Ionised calcium binds between the two lobes of the VFTD. In the presence of high

17   calcium the CaSR is activated, leading to changes in its signal transduction and reduced PTH

18   secretion.

19   **Figure 2**   **Major signalling pathways of the calcium-sensing receptor (CaSR)**

20   Schematic diagram of the calcium-bound homodimeric CaSR and its major signalling pathways. The

21   CaSR is expressed at the plasma membrane (PM) where its function is to detect $[Ca^{2+}_e]$ in the blood.

22   The CaSR activates two major signalling pathways: $G_{q/11}$ and $G_{i/o}$. Activation of the $G_{q/11}$ pathway

23   leads to stimulation of its major effector protein phospholipase C (PLC), which hydrolyses

24   phosphatidylinositol 4,5-bisphosphate (PIP_2) to the second messengers diacylglycerol (DAG) and

25   inositol trisphosphate (IP_3). DAG activates protein kinase C (PKC), and the mitogen-activated protein

26   kinase (MAPK) signalling pathway. IP_3 binds to IP_3 receptors at the endoplasmic reticulum (ER),

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27   which mobilise intracellular calcium (Ca^{2+}_i) release into the cytoplasm. This increase in Ca^{2+}_i can

28   further activate MAPK signalling. The CaSR also activates $G_{i/o}$ signalling pathways, which inhibits

29   adenylate cyclase (AC), resulting in reductions in cAMP and protein kinase A (PKA) activity. This

30   reduction in PKA relieves the inhibition on MAPK signalling and therefore provides another

31   activation pathway for MAPK signalling. The net effect of all these signalling pathways is a change in

32   gene transcription.

33   **Figure 3   Disruption of an Arg680-Glu767 salt-bridge within CaSR mediates signalling**

34   **bias by activation of a G-protein independent, β-arrestin pathway**

35   (**A**) Schematic diagram of a CaSR monomer at the plasma membrane (PM) showing the seven

36   transmembrane domains (TM1-7) with extracellular loops 1–3 (ECL1–3) and intracellular loops 1–3

37   (ICL1–3). Arg680 is located at the extracellular end of TM3 and is predicted to form a salt-bridge

38   with either a Glu767 residue on ECL2 or a Glu837 residue on TM7. (**B**) Homology model of the

39   CaSR TM3, TM7 and ECL2 region, reproduced with permission from Gorvin *et al*, 2018, Science

40   Signaling (Gorvin et al. 2018a). The homology model is based on the published structure of mGluR1

41   (Wu, et al. 2014). The Arg680 residue is shown projecting from TM3 and is predicted to form a salt

42   bridge with Glu767 on ECL2. The Glu837 residue on TM7 lies at a 5.7Å distance from the Arg680

43   residue, and therefore formation of a salt bridge between Arg680 and Glu837 is less likely, but was

44   tested as the homology model may not reflect the true state of CaSR.

45

46   **Figure 4   Mechanisms by which calcium-sensing receptor (CaSR) expression is regulated**

47   **at plasma membranes**

48   Schematic showing trafficking pathways regulating the expression of CaSR at the plasma membrane.

49   The CaSR is continuously synthesised at the endoplasmic reticulum (ER) and undergoes post-

50   translational modifications at the ER and Golgi, before export to the plasma membrane surface. In

51   addition to this constitutive anterograde trafficking of the CaSR, an additional export pathway for the

52   CaSR has been described. This pathway is activated by high $[Ca^{2+}]_e$ and has been named the agonist-

2

53   driven insertional signalling, or ADIS, pathway. Removal of the CaSR from the cell surface is

54   regulated by clathrin-mediated endocytosis that requires the heterotetrameric adaptor protein-2 (AP2).

55   AP2 binds directly to transmembrane proteins using its μ-subunit or σ-subunit. The CaSR has a

56   putative dileucine motif within its C-terminus with which it is predicted to bind to AP2σ. The α-

57   subunit and β-subunit have large appendages that are important for binding to clathrin coat proteins,

58   plasma membrane phospholipids, and endocytic accessory proteins. Once internalised within clathrin-

59   coated vesicles, CaSR is targeted to the endosomal-lysosomal system. Experimental evidence

60   suggests CaSR is degraded at the lysosome, and very little, if any, recycling of the receptor occurs.

61   **Figure 5   The calcium-sensing receptor (CaSR) can activate plasma membrane and**

62   **endosomal signalling pathways**

63   Schematic diagram showing the signalling pathways that occur downstream of the CaSR. As

64   described in Figure 2, CaSR predominantly signals via: the $G_{q/11}$ pathway, leading to activation of the

65   intracellular calcium (Ca^{2+}_i) and mitogen-activated protein kinase (MAPK) pathways; and the $G_{i/o}$

66   pathway leading to activation of MAPK and reductions in the cAMP pathways. These pathways are

67   activated rapidly (within 2-5 minutes), and originate at the plasma membrane. The CaSR can also

68   signal from an internal location using a $G_{q/11}$ pathway. This sustained signal that occurs later than the

69   plasma membrane signal (from 30 minutes), is sensitive to: global clathrin-mediated endocytosis

70   blockade (shown experimentally using dyngo); inhibition of CaSR-mediated internalisation

71   (demonstrated in cells expressing FHH3-associated AP2σ mutants); and maturation of internalised

72   vesicles to the early endosome (demonstrated experimentally using a dominant-negative (DN) form of

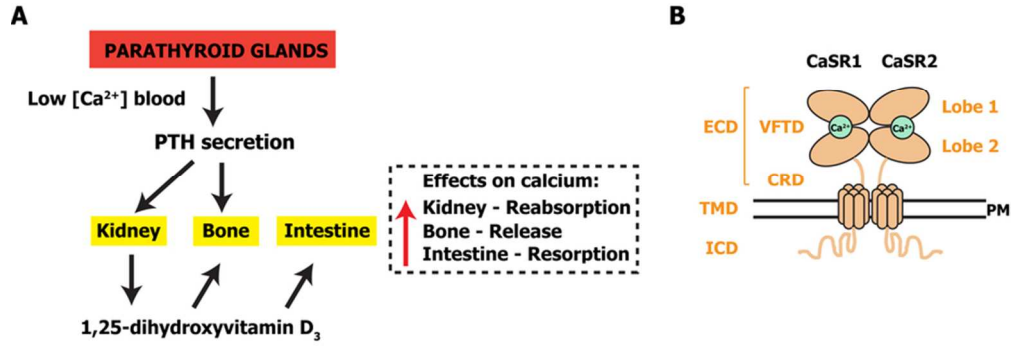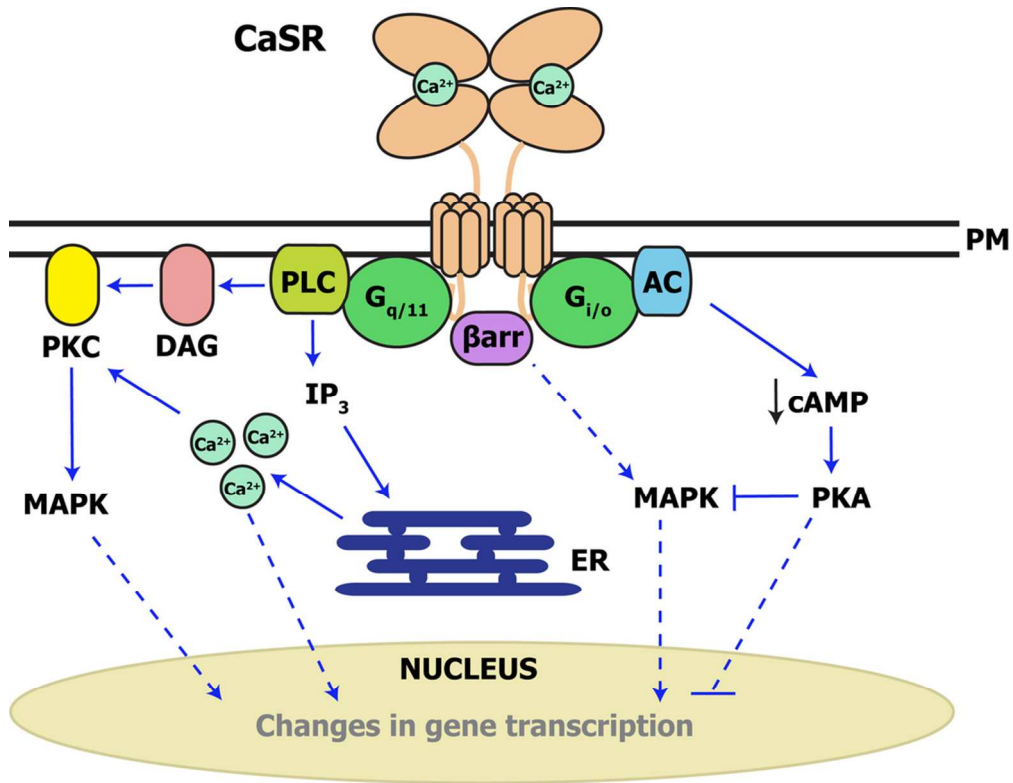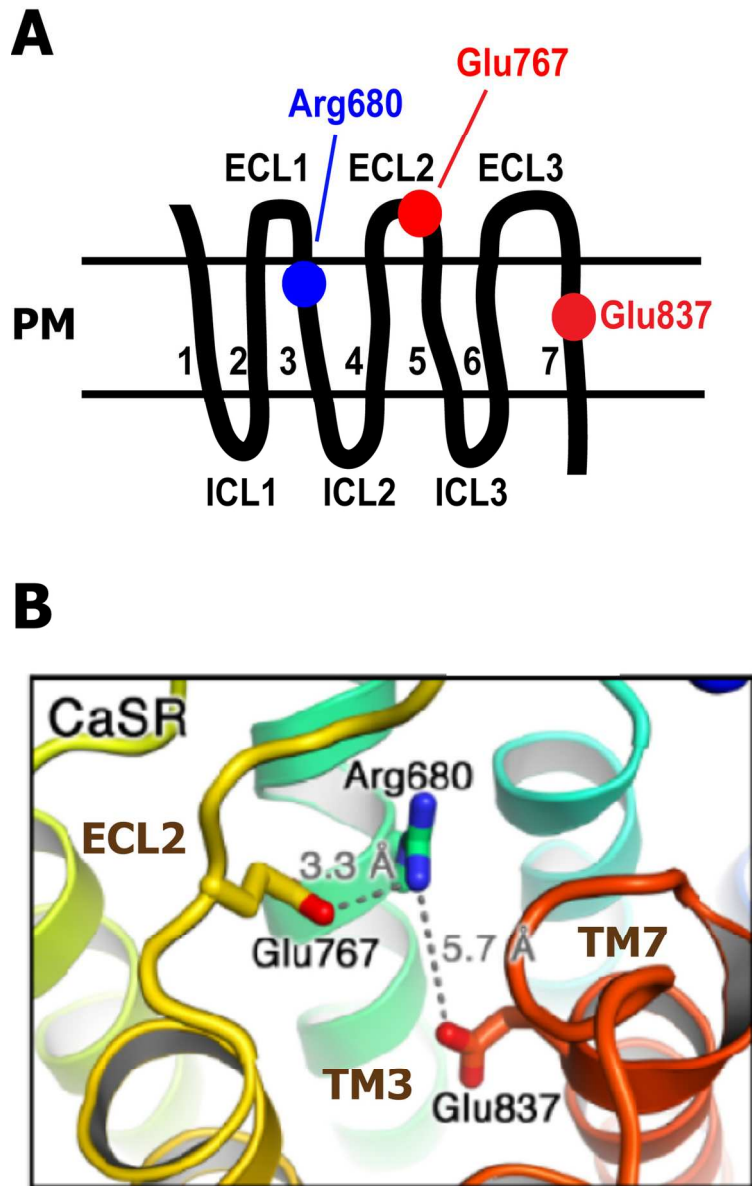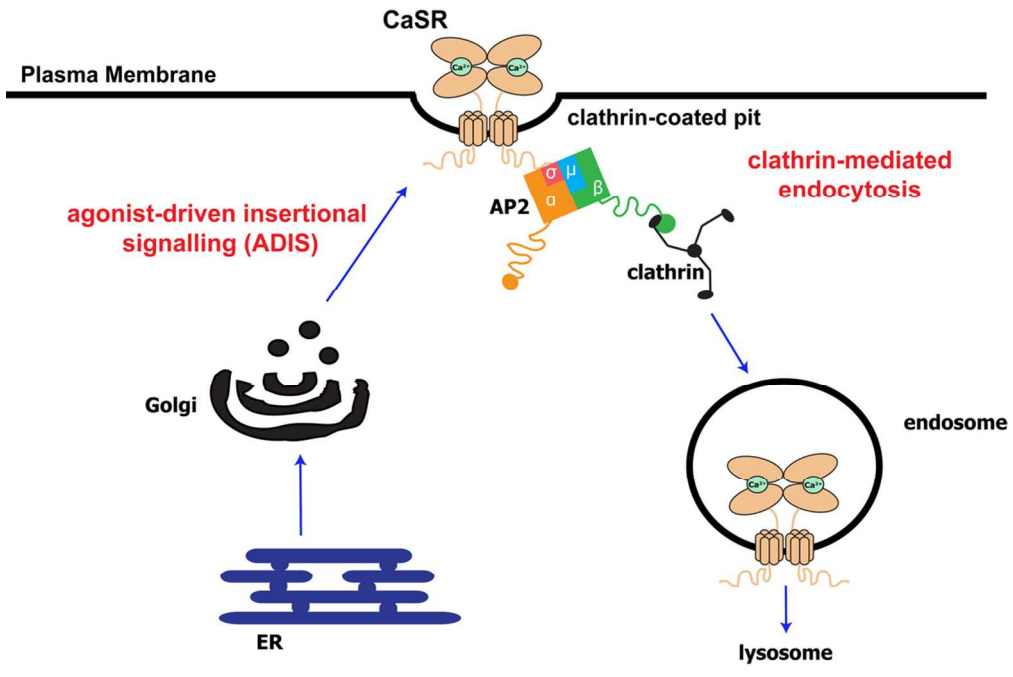73   Rab5). Thus, this sustained signal is likely to arise within endosomes.

74

3

Figure 1

78x27mm (300 x 300 DPI)

94x73mm (300 x 300 DPI)

A

B

Figure 3
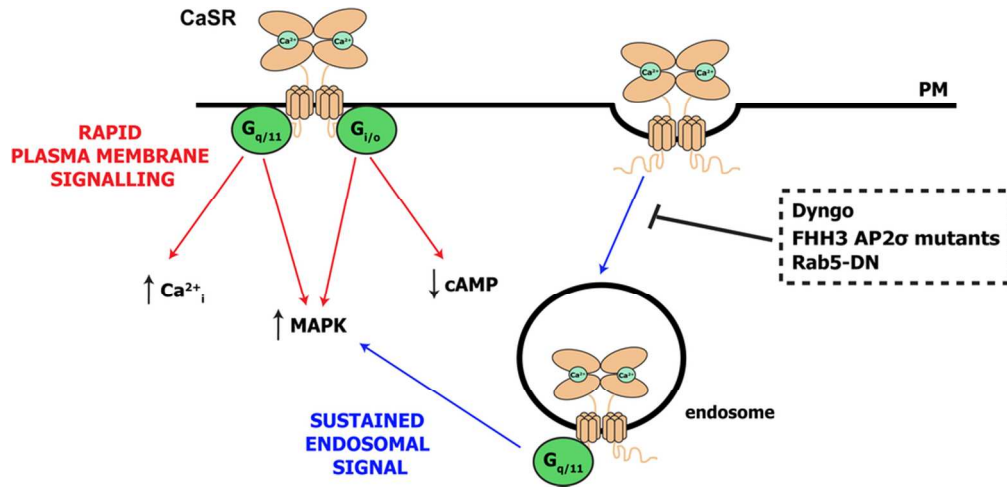
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104x68mm (300 x 300 DPI)

87x42mm (300 x 300 DPI)