Heterotrimeric G Proteins Control Diverse Pathways of Transmembrane Signaling, a Base for Drug Discovery

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Abstract: Heptahelical receptors are coupled to heterotrimeric GTP-binding proteins (G proteins) which transduce most signals through their α and βγ subunits to effectors including adenylylcyclases, ion channels, phospholipases Cβ, and phosphoinositide 3-kinases. The diversity of G proteins, their effectors and regulators (RGS proteins), supports the interest of these protein families as potential drug targets.

1-HEPTAHELICAL RECEPTORS AND HETERO-TRIMERIC G PROTEINS

Heptahelical receptors, coded by about 1000 genes, constitute the largest group of plasma membrane receptors to endogenous and exogenous stimuli, neurotransmitters, hormones, autacoids, calcium ions, photons, taste and odorant molecules. Heterotrimeric GTP-binding proteins (G proteins), located on the inner side of the plasma membrane, transduce most signals delivered to heptahelical receptors following the dissociation of Gα subunits from Gβγ dimers. Both Gα and Gβγ interact with membranous or cytosolic effector proteins which initiate signaling pathways leading to cell responses (Fig. **1**).

The involvement of G proteins in transmembrane signaling was first suggested by the observations of Rodbell *et al.* in 1971 concerning the effect of GTP on glucagon binding [1] and on glucagon-stimulated adenylylcyclase activity [2]. Guanyl nucleotides were studied as analogs of ATP, the substrate of adenylylcyclase. It was concluded that guanyl nucleotides do not act competitively with glucagon or ATP but play a specific role in the activation of adenylylcyclase by an allosteric type of action. The binding sites of glucagon, ATP and GTP were considered to be located on a single molecular entity. Orly and Schramm's demonstrations [3] that components of the adenylylcyclase system could be mixed and exchanged by cell fusion, presaged their reconstitution *in vitro* [4-6]. These observations allowed to clearly distinguishing the three molecular entities, receptor, G protein and effector (adenylylcyclase in this case). Affinity chromatography techniques facilitated purification of these proteins and molecular biological approaches allowed to determine their structure and confirmed, or revealed, their extensive diversity.

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The heterogeneity of receptors to a given mediator had been suggested from pharmacological observations, beginning at the end of the 19th century for acetylcholine with the clear proposal of its muscarinic and nicotinic effects, in 1914 by Dale [7]. Following studies indicated that several nicotinic and muscarinic acetylcholine receptors might occur leading to the definition of the structure of five heptahelical muscarinic receptors around 1985. Similarly, most mediators can selectively bind to several heptahelical receptors. Each receptor subtype may be coupled to different G proteins, controlling selective cell responses. The cellular expression of a given receptor gene varies from one tissue to the other. These properties offer the opportunity to develop selective receptor ligands, agonists, antagonists, and, more recently, inverse agonists [8], with largely established therapeutic interest.

Thus, in current pharmacotherapy, the input to G protein-dependent signaling is typically manipulated by targeting the receptor with appropriate ligands. G proteins and effectors are also heterogeneous and might offer the opportunity to develop selective drugs, even if the heterogeneity of G proteins and effectors is more restricted than that of receptors. To date, the example of selective cyclic nucleotide phosphodiesterases inhibitors suggests that this view is of value to lead the drug discovery.

2-DIVERSITY OF G PROTEIN SUBUNITS

The diversity of heterotrimeric G proteins has been demonstrated, around 1980 [9, 10, for reviews], with the purification of Gs (s = stimulatory for adenylylcyclase), Gi (i = inhibitory for adenylylcyclase), and Gt (t = transducine which activate cGMP phosphodiesterase in retinal cells). G protein subunits are highly homologuous in both primary sequence and tertiary structure. To date, at least 20 Gα (39 to 52 kDa in size), 5 Gβ (36 kDa) and 13 Gγ (7- 8 kDa) subunits have been identified in mammalian system. The usual classification of G proteins remains, based on their α subunits with four subfamilies : Gs including α_s and α_{olf} (olf = olfactive); Gi including α_{i1}, α_{i2}, α_{i3}, α_{o1}, α_{o2}, α_{t1},

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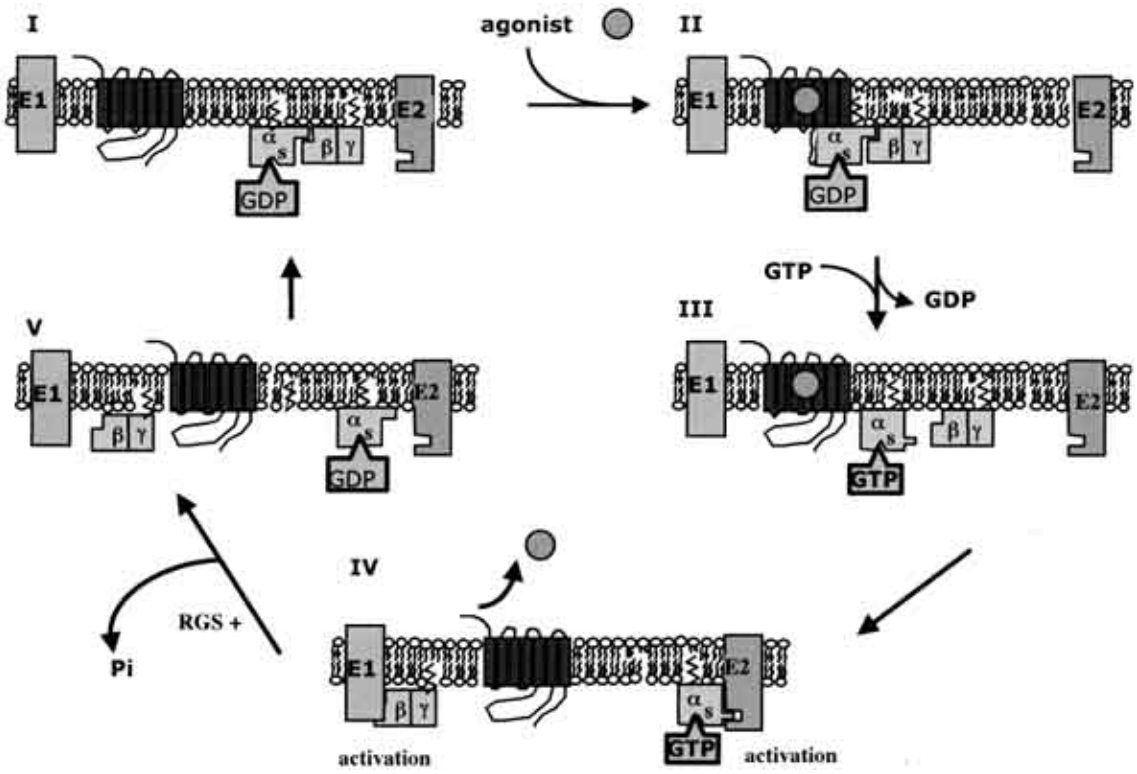

Fig. (1). Schematic model of G-protein signaling cycle. Interaction of the alpha-subunit with the agonist-stimulated receptor (I-II) causes the exchange of the bound GDP with GTP (II-III). The alpha-GTP complex and the beta-gamma dimer dissociate. The alpha-GTP complex interacts with an effector (E2) and the beta-gamma dimer with an other effector (E1) (IV). The alpha-subunit catalyses hydrolysis of the bound GTP to GDP (V) and reassociates with the beta-gamma dimer (I). This deactivation of signaling can be accelerated by proteins termed regulators of G-protein signaling (RGS) which directly bind to the alpha-subunit of G-proteins (see § 6).

α_{t2}, α_z and α_{gust} (gust = gustducin); Gq including α_q, α_{11}, α_{14}, α_{15} and α_{16}; G12 including α_{12} and α_{13} .

Gα subunits alone were thought to act on effectors, but newer evidences indicated from 1990 that the Gβγ dimers also play a major part in signal transduction [11, for review]. Thus, each trimeric G protein can initiate at least two signaling pathways from its Gα and Gβγ subunits. Selective assembly of G protein heterotrimers from the three separate components can produce a large number of unique complexes that may differ in their interactions with receptors and effectors. For instance, the trimeric complex $G\alpha_{gust}$, $\beta_3\gamma_{13}$ transduces the taste message from the bitter receptor T2R, to cAMP phosphosdiesterase and phospholipase Cβ2 through $G\alpha_{gust}$ and $G\beta_3\gamma_{13}$ dimer, respectively [12]. However, in most signaling pathways, the trimer remains characterized only by its α subunit, while the βγ dimer is usually poorly identified.

In term of tissue-specific expression, mammalian G protein subunits fall into three categories [13]. A small subset of genes is expressed in a specialized cell type, $G\alpha_{t1}$ and $G\gamma_1$ in retinal rod cells, $G\alpha_{t2}$ and $G\gamma_c$ in retinal cone cells, and $G\alpha_{gust}$ and $G\gamma_{13}$ in taste buds. Some genes are expressed mainly in one tissue: $G\alpha_{olf}$ in the olfactory neuroepithelium with low level in brain, $G\alpha_{15}$ in hematopoietic cells, $G\beta_5$, $G\gamma_3$ and $G\gamma_4$ in the nervous system. Other genes are expressed ubiquitously ($G\alpha_{s,\ q,\ 11,\ 12}$, $G\beta_1$, $G\gamma_{5,\ 10,\ 12}$), or in many tissues.

3-ALTERATION OF G PROTEINS

Some diseases are associated with defects in Gα subunits, due to alterations introduced by exogenous agents such as cholera and pertussis toxins, and to somatic or heritable mutations. Some drugs are known to bind to G proteins *in vitro* with a few correlates *in vivo*.

Cholera toxin and pertussis toxin are two microbiological enzymes that ADP ribosylate certain Gα subunits and modify their function. Cholera toxin from *Vibrio cholere* transfers the ADP ribosyl group from NAD to $G\alpha_{s,\ olf,\ t,\ gust}$. The binding of the ADP ribosyl group to the amino acyl residue of Gα (Arg201 in $G\alpha_s$) inhibit the GTPase activity of Gα leading to a permanent activation of adenylylcyclase with the extrusion of chloride ions and water from intestinal cell. Pertussis toxin from *Bordetella pertussis*, the agent responsible of whooping cough, ADP ribosylates a Cys residue at the C terminus of $G\alpha_{i,\ o,\ t,\ gust}$, preventing receptor activation of G protein. Both toxins are widely used in experiments to perturb signaling and to label G proteins.

Not surprisingly, somatic mutations that alter Arg201 in $G\alpha_s$ lead to diseases. Some adenoma of the thyroid and pituitary glands contain substitutions at this position of $G\alpha_s$ [14]. Mutants are incapable to hydrolyse GTP and are constitutively active with cell hyperproliferation. Also, McCune-Albright syndrome is characterized by a variety of abnormalities and is associated with mutations at the same position of $G\alpha_s$ [15]. The best characterized and most convincing example of a heritable disorder resulting from mutation in the gene of a G protein subunit is pseudo-hypoparathyroidism Type 1 (Albright hereditary osteodystrophy), with $G\alpha_s$ gene paternally imprinted [16-17].

Many cationic drugs can bind to G protein *in vitro*. Most of them, such as amphiphilic neuropeptides and peptidic hormones (substance P, VIP, bradykinin...) and venom peptides (mastoparan, MCD, melittin...) act as receptor mimetics, stimulating G proteins with some selectivity [18-20]. Mastoparan was the first characterized direct G protein activator [21]. Mastoparan activates $G\alpha_i$ and $G\alpha_o$ but not $G\alpha_s$ and $G\alpha_t$. If Ala10 of mastoparan is replaced by α-amino-isobutyric acid, the resulting peptide selectively activate $G\alpha_s$ [22]. Melittin stimulates $G\alpha_i$ and $G\alpha_o$ [23] and $G\alpha_{11}$ [24] activities, whereas it inhibits $G\alpha_s$ activity [24]. Among non-peptidic compounds, the natural polyamine spermine stimulates $G\alpha_o$ but inhibits $G\alpha_i$ [25], benzalkonium chloride inhibits the mastoparan-induced activation of $G\alpha_i$ but stimulates $G\alpha_s$ [23, 26]. Also, suramin analogues have been proposed as subtype-selective G protein inhibitors [27, 28].

The *in vivo* interest of selective G protein ligands is limited by the availability of drugs with membrane permeability [29]. However, numerous cationic drugs stimulate mast cells through their interaction with $G\alpha_{i2}$ and $G\alpha_{i3}$, with histamine and arachidonate metabolites release and might be involved in some diseases such as neurogenic inflammation [19]. Adverse effects of clinically used drugs might also be relevant to their direct effect on G proteins [30].

4-MAIN DIRECT EFFECTORS OF G PROTEINS

Gα and Gβγ directly interact with some of the members of enzymes or ion channels families increasing or decreasing their products or ionic currents. These products or ions are considered as "second messengers", cyclic nucléotides (cAMP, cGMP), phosphatidylinositides metabolites (IP3,

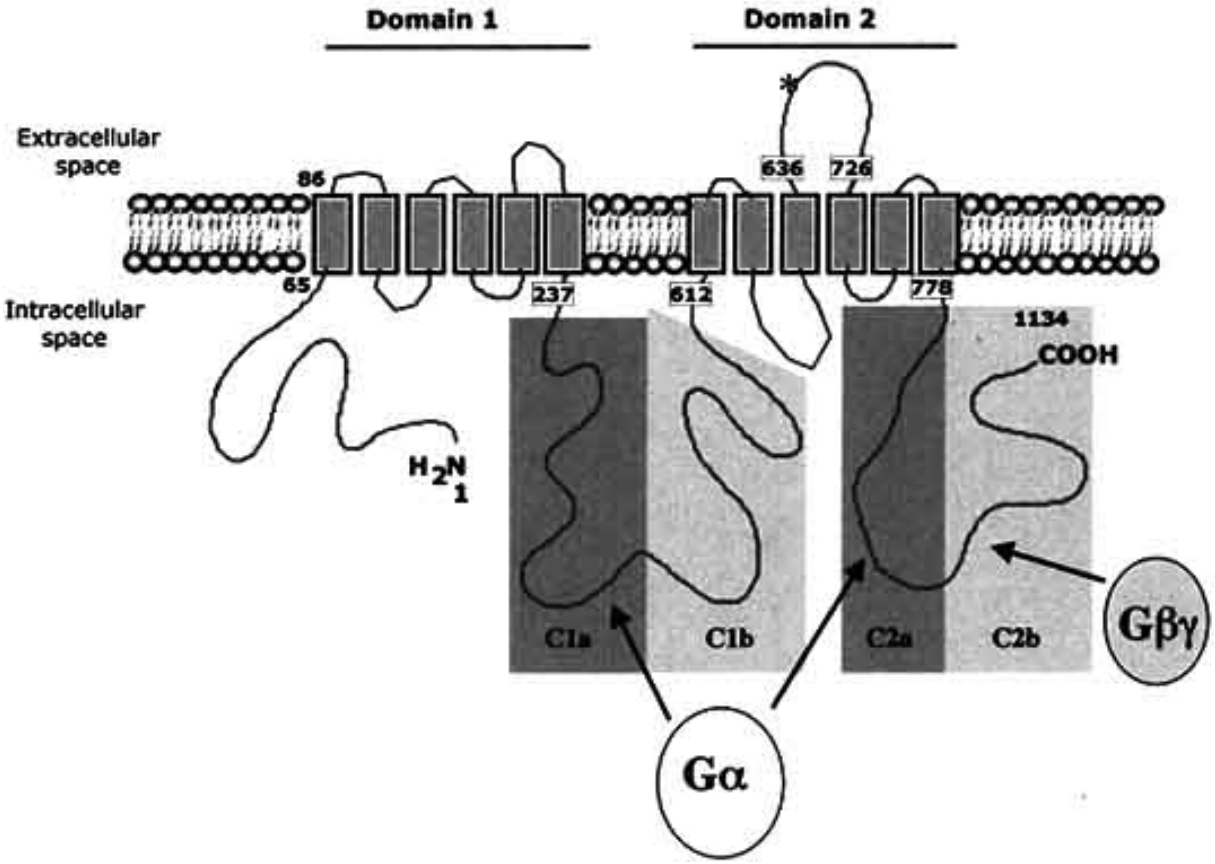

Fig. (2). Schematic representation of mammalian adenylycyclases. The C1a and C2a domains must come into physical contact to form the catalytic core with the substrate binding site. These interactions are facilitated by $G\alpha_S$ and by forskolin, a drug widely used *in vitro* to activate adenylylcyclases.

DAG, PIP3), and ions (Ca^{2+}, K^+). We describe below the main direct effectors of G proteins. Some *in vitro* evidence have suggested that other proteins with effector properties (non-receptor tyrosine kinases such as Bruton's tyrosine kinase, small G proteins, phopholipase A2, calcium ATPase pump…)[11] might interact directly with G proteins, but indirect involvement has not always been excluded, for instance through PI3K-PIP3 pathways. We will describe in §6 the direct effector role of some RGS proteins which allow to activate downstream small G proteins, and the direct or indirect effector role of the MAP kinase c-Src.

Adenylylcyclases and Cyclic Nucleotides Phosphodiesterases

G protein subunits directly modulate all mammalian adenylylcyclases, increasing or decreasing cAMP synthesis [31-33], and activate some types of cyclic nucleotide phosphodiesterase (PDE) decreasing cAMP and/or cGMP levels [33, 34].

The nine cloned isoforms of mammalian adenylylcyclase (I to IX) share a primary structure consisting of two transmembrane and two cytoplasmic regions (Fig. **2**). This is a first example of effector regulated in a membrane-delimited manner. Each transmembrane regions each contains six predicted membrane-spanning helices. $G\alpha_s$ is capable of gluing together the two cytoplamic regions (C1 and C2) of all adenylylcyclases isoforms, increasing cAMP synthesis. $G\alpha_i$ selectively inhibits adenylylcyclase types V and VI. Mutational analysis indicated that C1 is the primary site for binding of $G\alpha_i$ to type V. The inhibitory mechanism postulates a rotation of C1 in the opposite sense as that induced by $G\alpha_s$ [35].

$G\beta\gamma$ may inhibit adenylylcyclase I, but stimulates types II, IV and possibly VII. Importantly, the stimulatory effect of $G\beta\gamma$ is only substantial in the presence of $G\alpha_s$. Subnanomolar purified $G\alpha_s$ is sufficient to fully activate adenylylcyclase, but several nM of $G\beta\gamma$ is required for co-stimulation with $G\alpha_s$. The more plausible donors of $G\beta\gamma$ are G_i and G_o, both of which are close to 10-fold more abundant in cells than G_s. Thus, co-activation of adenylylcyclase by $G\alpha_s$ and $G\beta\gamma$ appears to be a mechanism for coincidence detection of the simultaneous activation of G_s and G_i/G_o either via separate receptors or a single receptor that may couple to the requisite G proteins.

The phosphodiesterases (PDE) superfamily currently includes 20 different genes subgrouped into 11 different PDE families, and it is likely that more will be added in the coming years [34]. PDE5, 6, 9 and 11 are selective for the hydrolysis of cGMP. Other families are selective for cAMP.

$G\alpha_t$ directly activates PDE 6, the photoreceptor phosphodiesterase. The subunit composition of PDE6 is unique among PDE isoforms, with four subunits, two catalytic subunits, which are slightly different in rods (α and β) but are identical in cones (α'_2), and two inhibitory γ subunits. When exposed to light, the heptahelical receptor rhodopsin activates the retinal G protein, transducin (G_t), leading to the interaction of $G\alpha_t$ with the γ subunit of PDE6 whose inhibitory action is released. Transducin-activated PDE6 exhibits an exceptionally high catalytic rate of cGMP hydrolysis ensuring high signal amplification [36].

$G\alpha_{gust}$ directly activates one or more type of PDE, resulting in the rapid breakdown of both cAMP and cGMP [13]. This pathway is stimulated by bitter taste compounds acting on T2R/TRB heptahelical receptors. Similarly, umami is another taste quality stimulated by L-glutamate through metabotropic (heptahelical) receptors (taste-mGluR4) coupled to G proteins whose α subunits activate PDE activity [37, 38].

Ca^{2+} and K^+ Channels

G protein $\beta\gamma$ dimers may directly inhibit the opening of some voltage-gated calcium channels [39, 40] and stimulate some potassium channels [41, 42]. To date, the involvement of $G\alpha$ subunits have not been clearly demonstrated in the direct control of ion channels. The superfamily of G protein-gated ion channels belongs to the effectors regulated in a membrane-delimited manner.

Electrophysiological and pharmacological studies distinguish at least six classes of Ca^{2+} currents designated L-, N-, P-, Q-, R-, and T- types. Ca^{2+} channels consists of complexes of a pore-forming α_1 subunit with auxiliary $\alpha_2\delta$, β and γ subunits. Each α_1 subunit consist of four repeats (I-IV), each of which is composed of six transmembrane segments (Fig. **3**). Ca^{2+} channels containing $Ca_v2.2$ α_1 subunits (formely α_{1B}) are responsive for N-type currents, and Ca^{2+} channels containing $Ca_v2.1$ α_1 subunits (formely α_{1A}) are responsive for P/Q-type Ca^{2+} currents. These two channel types are inhibited by agonists of receptors coupled to pertussis toxin-sensitive G proteins. $G\beta\gamma$ interacts with the intracellular I-II loop. Inhibition is caused by a shift from an easily activated "willing" (W) state to a more-difficult-to-activate "reluctant" (R) state. This inhibition can be reversed by strong depolarization, or by protein kinase C phosphorylation.

The $Ca_v2.1$ and $Ca_v2.2$ channels are located in presynaptic nerve terminal and are responsive for the Ca^{2+} entry that triggers neurotransmitter release at most signals. Recent interest in the molecular mechanism of voltage-gated calcium entry has been stimulated by the discovery that disorders such as familial hemiplegic migraine, ataxia type 6 and epilepsy are caused by spontaneous mutations in the pore-forming α_1 subunits of neuronal $Ca_v2.1$ calcium channels [43]. Note that L-type Ca^{2+} channels may be activated by phosphorylation through proteine kinase A (PKA) and protein kinase C (PKC) in response to receptors coupled to Gs, Gi or Gq and involving both α and $\beta\gamma$ subunits.

Potassium channels are highly heterogeneous (Fig. **4**). A limited subset of potassium channels (K_G) are gated by G protein and involved in the rapid inhibition of membrane excitability, such as in the slowing of heart rate by the vagus nerve. Stimulation of M2 muscarinic receptors by acetylcholine in pacemaker cells (sinoatrial, atrioventricular and atrial) activates a K^+ current (I_{KAch}.). Heart rate is thus

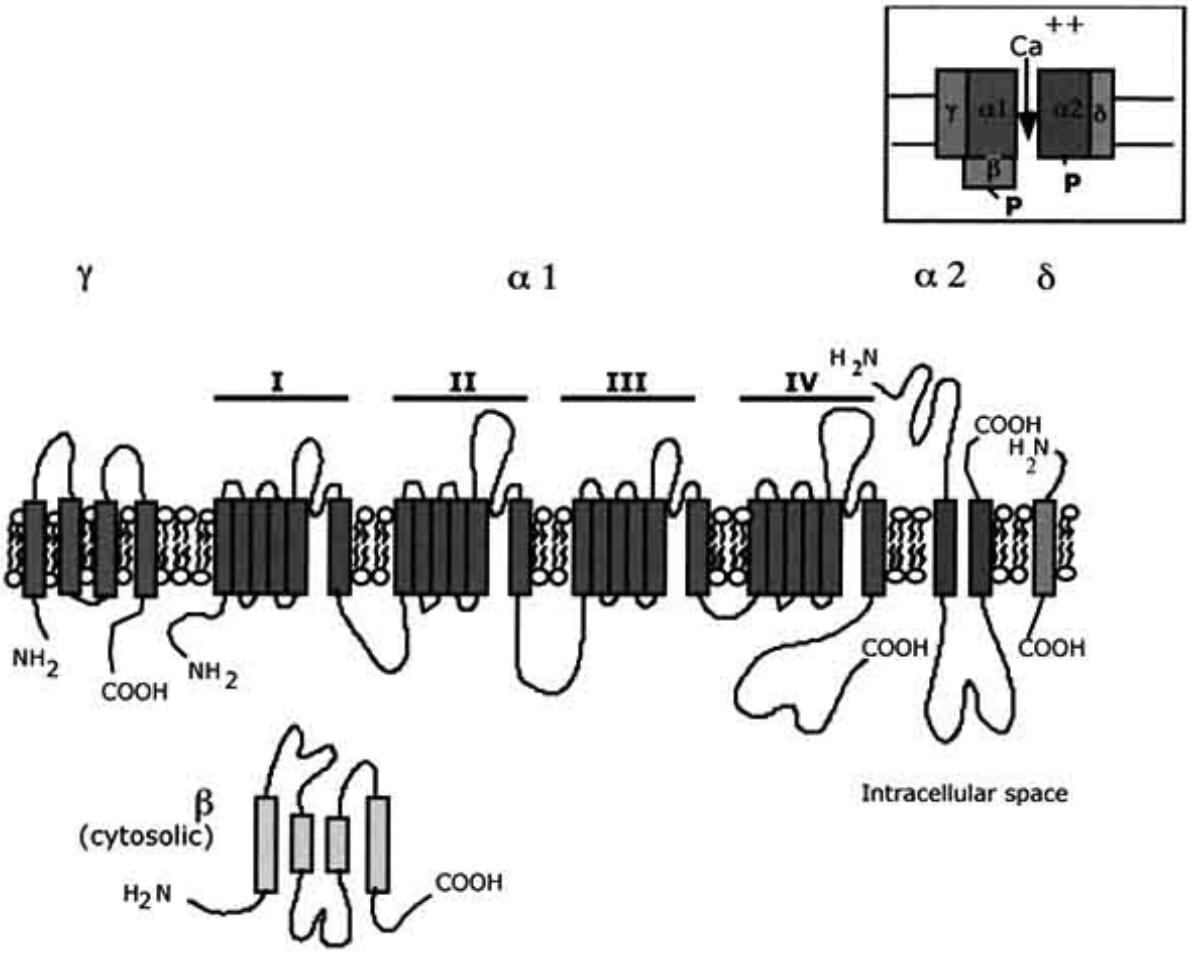

Fig. (3). Topology of voltage-gated Ca^{2+} channels. The alpha subunits of calcium channels share presumptive six-transmembrane structure, repeated four times. Calcium channels also require auxiliary proteins, alpha-2, beta, gamma and delta.

slowed by hyperpolarization of the pacemaker depolarization potential as well as by block of tonic β-adrenergic stimulation of depolarising pacemaking channels. Although usually referred to as acetylcholine-gated channels, I_{Kach} activity is also stimulated by several other neurotransmitters, including somatostatin and α-2 adrenergic agonists. The observation that Gβγ subunits could directly activate the channel when applied to the inner face of atrial membranes, initiated the demonstration of the active role of the βγ dimer of G proteins [11, 44]. In brain, activation of G protein-coupled channels is thought to underlie suppression of firing. Five unique cDNA coding for G protein-gated K^+ channels (GIRK1-5) have been isolated from heart, pancreas and brain. They belong to the larger inwardly rectifying family (K_{IR}) and have been classified as the $K_{IR}3.0$ channels. These channels include two transmembrane alpha helices, with both intracellular N- and C- terminus (Fig. 4). Gβγ interacts with multiple sites of these termini [41].

Phospholipase C and Phosphoinositide 3- Kinase

Both Gα and Gβγ subunits are involved in the direct control of phosphoinositide metabolism. Two enzyme families are directly activated by G proteins, phosphoinositide-specific phospholipase C (PLC), and phosphoinositide 3-kinase (PI3K).

PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2), generating inositol 1, 4, 5 trisphosphate (IP3), a universal calcium-mobilizing second messenger, and diacylglycerol (DAG) (Fig. 5), which remains included in membranes but activates protein kinase C. Three mammalian PLC subtypes, β, γ and δ, have been isolated and their corresponding cDNA sequences determined [45, for review]. Four β-, two γ-, four δ-isoforms, and multiple spliced variants have been described in mammals. Only the PLCβ family appears to be regulated by G proteins.

Members of $G\alpha_q$ (q, 11, 14, 15, 16) activate PLCβ isoenzymes in a pertussis toxin-insensitive manner. Gβγ from pertussis toxin-sensitive, or-insensitive, G proteins strongly activate $PLC\beta_2$ and β_3. $PLC\beta_1$ is weakly stimulated, whereas $PLC\beta_4$ is completely insensitive. The potency of Gβγ is reduced compared to that of $G\alpha_q$ as activators of PLCβ. This may provide selectivity in receptor regulation of PLCβ since presumably only activation of abundant G protein heterotrimer (for example the members

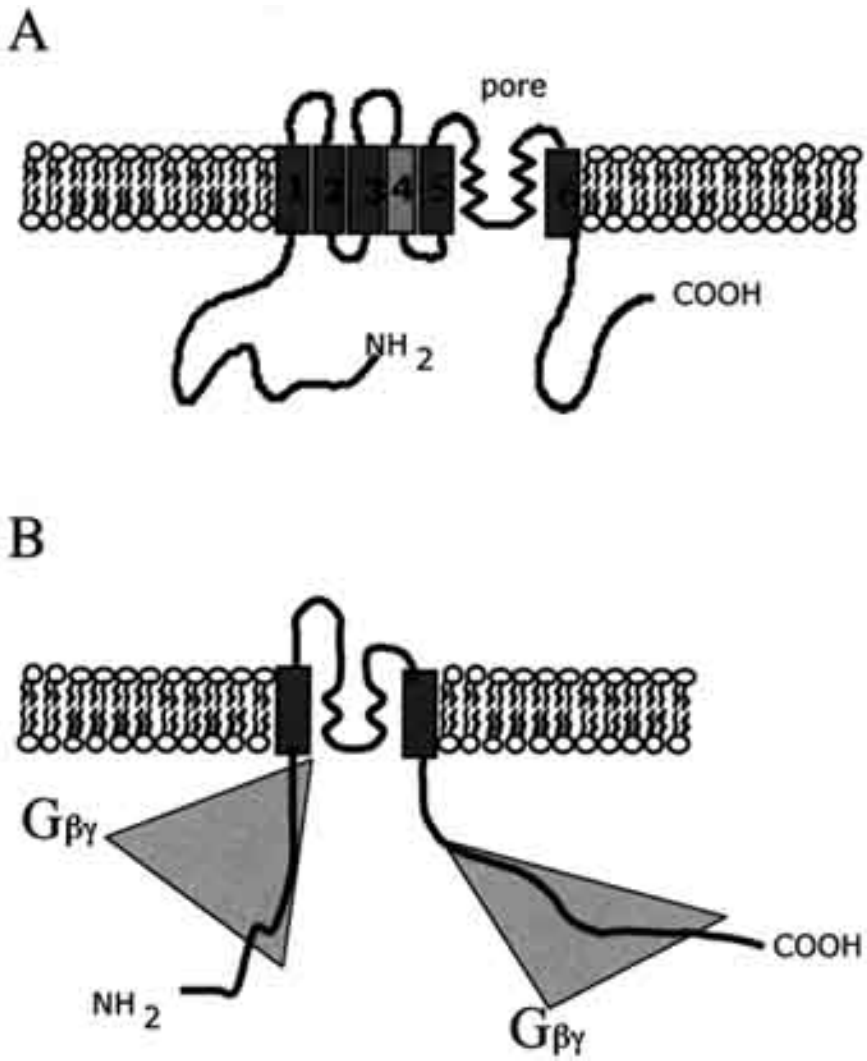

Fig. (4). A, alpha-subunit of voltage-sensitive potassium channels (Kv) and rapidly activated potassium channel (Ka). B, alpha-subunit of inwardly rectifying potassium channels (GIRK) with the identified regions responsible for Gβγ interaction (modified from ref. 42).

of the pertussis toxin-sensitive G_o and G_i families) would produce sufficient βγ-subunits for PLCβ activation [46]. The possibility that specific βγ combinations are required to activate particular PLC-isoforms has been investigated. These subunits seem interchangeable with a few exceptions such as retinal Gβγ which is less effective than other subunit combinations in stimulating PLC [46, for review].

$G\alpha_q$ interacts with the C-terminus of PLCβ isoforms. Accordingly, a recently identified PLCβ4-variant, which is missing a portion of C-terminus, is insensitive to $G\alpha_q$ stimulation [47]. Gβγ interacts with a plekstrin (PH, platelet and leukocyte C kinase substrate) domain of the N-terminus. The PH domain was originally described as a novel protein motif of about 100-amino acid residues, repeated twice in the protein plekstrin. These motifs have now been identified in more than 100 proteins. PH domains function as adaptors or tethers linking their host proteins to the plasma membrane inner surface. Principal membrane's binding partners are Gβγ and phosphoinositides such as PIP3.

PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2), generating phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Fig. 5). P13K also phosphorylates the 3-OH position of the inositol ring of other inositides producing phosphatidylinositol 3-phosphate phosphatidyl-inositol 3,4-bisphosphate and phosphatidylinositol 3,5-bisphosphate. Three classes of P13K are distinguished with multiple isoforms [51]. P13Ks of class 1B are directly activated by Gβγ. This class appears to be present only in mammals where it shows a restricted tissue distribution, being only abundant in white blood cells. This may explain why receptors coupled to G proteins do not induce PI3K activity in all cell types. Indirect involvement of other

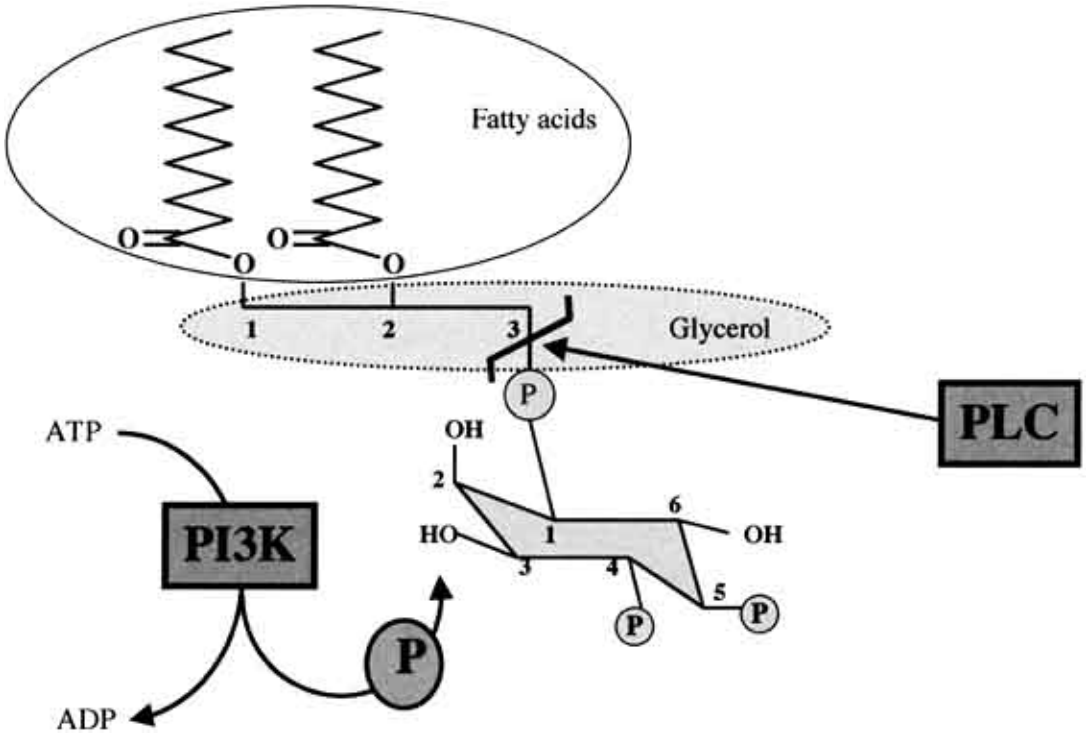

Fig. (5). Catalytic property of PLC and PI3K. PLC hydrolyses PIP2, generating IP3 and DAG. P13K phosphorylates the 3-OH position of the inositol ring of phosphoinositides generating PIP3 from PIP2.

classes of PI3K in G protein-initiated pathways has been proposed [48]. PI3Ks of class 1A are present in all types of mammalian cells and belong to tyrosine kinase-dependent pathways. The only class IB PI3K identified to date is the p110γ catalytic subunit complexed with a 101-kDa regulatory protein (p101), which has no sequence homology to any other known protein and is indispensable for the Gβγ responsiveness.

P13K lipid products are not substrates for PLC, and thus initiate original signal pathways. PIP3 remains associated to membranes and is recognized by PH domains of various proteins leading to their recruitment to the plasma membrane and to their activation.

5- MAIN SIGNALING PATHWAYS INITIATED BY SECOND MESSENGERS OF G PROTEIN DIRECT EFFECTORS

Signals arising from G protein-coupled receptors are nether unique. Several parallel pathways may be activated in response to agonist stimulation of a unique receptor, from Gα and Gβγ subunits, or from the activation of two different G proteins. Also, similar pathways may be activated through G protein-independent receptors. Crosstalk between these diverse pathways occur, with many possibilities depending on the molecular diversity of effectors and on their expression in each cell type. Note that most pathways lead to protein phosphorylations and/or to calcium-sensitive protein activation which tightly controls final cell responses such as secretion, contraction, general metabolism, and protein synthesis through gene transcription.

Cyclic Nucleotides-dependent Pathways

Cyclic AMP and cyclic GMP may bind to two types of targets, serine/threonine protein kinases (PKA and PKG, respectively), and ion channels in a limited number of cell types such as rod and cone retinal cells for cGMP and possibly in taste cells for cAMP.

PKA and PKG phosphorylate a large number of cellular proteins (including transcription factors as detailed above), increasing or decreasing their activity. G protein- and PKA-dependent pathways are present in all mammalian cells, corresponding to the diversity of heptahelical receptors coupled to $G\alpha_s$ and stimulating adenylylcyclase [33]. Adenylylcyclase typeVI, present in the liver, is under feedback inhibition by cAMP through PKA, corresponding to a homologous regulation. A heterologous inhibition of adenylylcyclase has also been observed by Ca^{2+}/DAG through PLC and PKC. Feedback regulations and heterologous regulations have also been observed through phosphorylation of G protein-coupled receptors. Oppositely, $G\alpha_s$-independent activation of adenylylcyclase may be achieved by phosphorylation and according to cytosolic calcium level. Noteworthy, the activity of guanylylcyclase is

not directly regulated by G protein subunits, but required NO synthesis, through the upstream activation of NO-synthase by Ca^{2+}-calmodulin and IP3-dependent process as given in detailed below.

PIP3-dependent Pathways

PIP3, arising from Gβγ-activated class IB PI3K, may recruit to the membrane various protein kinases (Fig. **6**), phosphoinositide-dependent kinase-1 (PDK-1) [49, 50], protein kinase B (PKB, or c-Akt) [49], protein kinase Cξ (PKCξ) [50], PLCγ [51] and tyrosine kinases such as the Bruton's tyrosine kinase (Btk) [52]. This G protein/PI3K/PIP3-dependent pathway has been observed in a limited mammalian cell types. However, in most cell types, class IA and class II PI3K activity is stimulated in response to hormones and growth factor increasing receptor or non-receptor tyrosine kinases. The p110 catalytic subunit of class IA PI3K exists in complex with an adaptor that has two Src-homology-2 (SH2) domains allowing the interaction with proteins phosphorylated on Tyr residues. Class II PI3K are large molecules (>170 kDa) which bind to membrane phospholipides in a calcium-dependent manner [48].

DAG-, IP3- and Ca^{2+}- dependent Pathways

IP3 and DAG are products of PLCβ directly activated by Gβγ, and of PLCγ, indirectly activated by Gβγ via PI3K and PIP3. DAG, as PIP3, remains among lipids of the inner face of the plasma membrane. DAG participates to the activation of the various isoforms of protein kinase C, some of which also requiring other membrane lipids and an increase of cytosolic Ca^{2+}.

IP3 freely migrates from the plasma membrane inner face to endoplasmic reticulum. IP3 bind to its receptor whose ion channel property allows a Ca^{2+} current from the inner compartment of endoplasmic reticulum to cytosol. At this point Ca^{2+} can be considered as a "third messenger", following the receptor agonist (first messenger) and IP3 (second messenger). In contrast, Ca^{2+} originating from the extracellular compartment through voltage-gated channels (inhibited by Gβγ in the case of N and P/Q types), has the properties of a second messenger.

The increase of cytosolic Ca^{2+} (from 10^{-7} to 10^{-6}-10^{-5} M) allows its binding to enzymes (Ca^{2+} dependent PKC, phospholipase A2…) and to calciproteins (calmodulin, troponine C, annexins also called lipocortins). In turn, calciproteins complexed to Ca^{2+} may bind to various membrane and cytosolic proteins to modulate their activity. For instance, the Ca^{2+}-calmodulin complex activates PDE1, decreasing cAMP, which corresponds to a crosstalk between two G protein-initiated pathway. NO-synthase is also a major target for calmodulin, leading to the activation of guanylylcyclase, with increased cGMP level. Interestingly, this pathway can be amplified by selective inhibitors of the cGMP-selective phosphodiesterase PDE5, such as sildenafil used to potentiate penile erection elicited by vagal muscarinic stimulation [34]. Another group of important targets for calmodulin is the family of calcium-calmodulin-dependent kinases (CaM-kinases) such as the myosin-light-chain-kinase (MLCK) which regulates smooth muscles contractility.

6- RECENT ADVANCES IN G PROTEIN REGULATION AND G PROTEIN-ELICITED SIGNALING PATHWAYS

Endogenous Regulation of G Proteins by RGS Proteins

The regulator of G protein signaling (RGS) proteins, largely described since 1996, modulate the activity of G

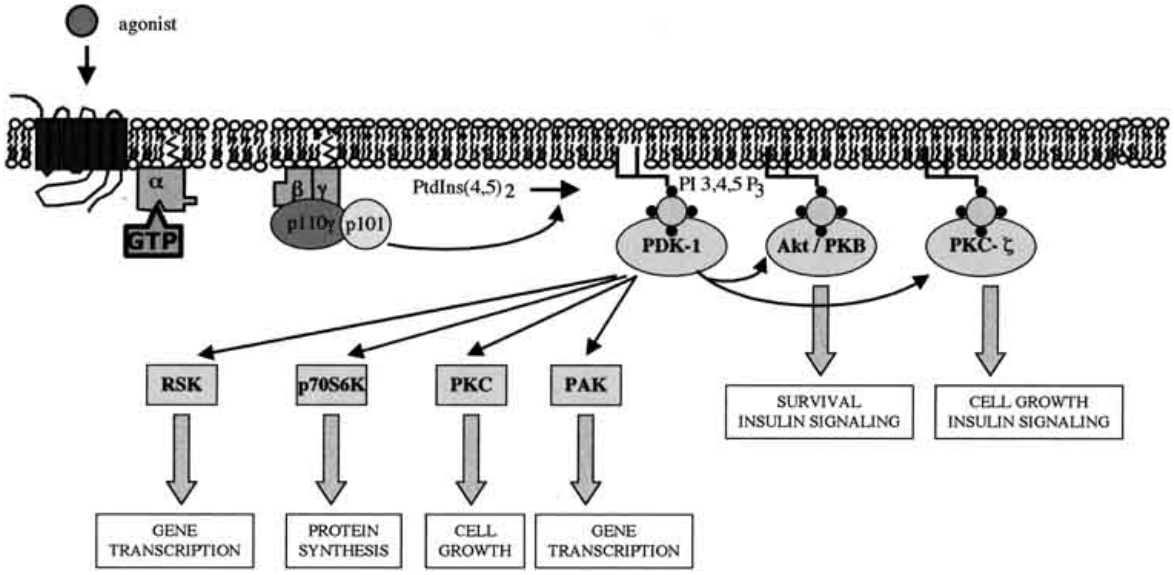

Fig. (6). Activation of protein kinases by class 1B PI3K. Activation of PI3K 1B (shown by its p110γ and p101 subunits) induces PIP3 synthesis. PIP3 recruits PDK-1, Akt/PKB and PKC-ζ, which activate their substrates such as RSK, p70S6K, PAK and PKC. Once activated these kinases have different effects on cell functions like gene transcription, cell survival and insulin-mediated metabolism. (adapted from ref. 50).

protein *in vitro*, and evidence is beginning to emerge on their role *in vivo* as well [53-56, for reviews]. The best known functions of RGS is to inhibit G protein signaling by accelerating GTP hydrolysis and turning off G protein signals. Thus, RGS are GTPase accelerating proteins (GAPs). Other GAPs stimulate small G proteins, Ras, Rho, Rac, Cdc42, Rab, Ran [57, for review]. The GAP activity explains the paradox that some signals, visual responses and cardiac potassium channels, turn off much faster than expected given the slow hydrolysis of GTP by purified Gα proteins.

RGS are a highly diverse protein family with unique tissue distributions. Evidence is emerging that besides G protein inhibition, some of them can enhance G protein activation, serve as effectors, and act as scaffold proteins to gather receptors, G proteins, effectors, and other regulatory proteins together. Several RGS, play active role in transmitting receptor signal to downstream effectors. For instance, p115-RhoGEF serve as a direct effector for $G\alpha_{12}$ and $G\alpha_{13}$, transferring the signal from G protein-coupled receptor (thromboxane A2 and endothelin receptors, thrombin receptor PAR2, lysophosphatidic acid receptors edg2 and 4, sphingosine-1-phosphate receptors edg3 and 5) to the small G protein Rho [58].

The knowledge on the role of RGS in mammalian cells is rapidly increasing and possible therapeutic uses of RGS-targeted drugs are being considered [56]. For instance, RGS inhibitors might be used as potentiators of endogenous or exogenously administrated agonists of G protein-coupled agonists, or as blockers.

G Proteins, an Access to Phospholipase A2 (PLA2) Activation

PLA2 hydrolyses cellular phospholipids to form lysophospholipids that lead to the production of platelet-activating factor and liberation of polyinsaturated fatty acids including arachidonic acid that are the precursors for prostaglandins, thromboxanes, leukotriens and a variety of other eicosanoids. Ten different groups of PLA2 enzymes have been described, based on nucleotide gene sequences [59]. Considering biological properties, the classification of PLA2 is simplified in three main types : the secretory PLA2 (sPLA2), the intracellular Ca^{2+}-independent PLA2 (iPLA2), and the cytosolic Ca^{2+}-dependent PLA2 (cPLA2).

The sPLA2s are all low molecular mass enzymes (13-15 kDa). Five sPLA2s exist in mammalian cells and some others in bees, snakes and marine snail venoms. Besides the enzyme role, a mediator property has been proposed for sPLA2, with membrane receptors [60]. The iPLA2s are the most recently identified PLA2s and are considered as important in phospholipid fatty acid remodeling. iPlA2 might be involved in some G protein-dependent signaling events, such as in α2 adrenergic control of preadipocyte actin cytoskeleton [61].

The cPLA2, or group IV PLA2 (85 kDa), is found in all cell types and is largely involved in receptor-activated signaling cascades. cPLA2 requires Ca^{2+} to interact with membranes (plasma membrane, nuclear envelop) where its substrate is localized. cPLA2 is able to translocate to membranes in response to the increases of intracellular Ca^{2+} via a calcium-lipid binding domain within the protein. The cPLA2 has been recently shown to possess a PH domain through which the enzyme is thought to strongly interact with phosphatidylinositol 4,5-bisphosphate. This interaction may help facilitate enzyme activation [59]. Moreover, cPLA2 may be phosphorylated by several protein kinases, mitogen-activated kinases (MAP kinases), stress-activated protein kinases (SAP kinases), and proteine kinases C (PKC).

cPLA2 activation is observed following stimulation of most heptahelical receptors. *In vitro* experiments have suggested its direct interaction with retinal Gβγ [62], but this proposal has not been confirmed. Alternatively, the indirect activation of cPLA2 may be proposed through $G\alpha_q$- or Gβγ-PLC-PKC/CaMK, Gβγ-PI3K-PIP3-PLCγ-PKC/CaMK, or in Gβγ-dependent pathways through MAP-kinases (see below). Also, cPLA2 activation may result from receptor-dependent or -independent tyrosine kinase activation, also involving MAP kinases.

G Proteins, an Access to MAP-kinases (MAPKs) Activation

Current understanding of the mechanism by which cell surface receptors activate MAPKs, especially the extracellular signal regulated kinases (ERKs), is founded upon studies of receptor tyrosine kinases. These receptors contain a single transmembrane spanning domain, which following agonist stimulation, dimerize and transphophorylate tyrosyl residues within their cytoplasmic domain. These phosphorylations lead to the recruitement of various proteins, including the adaptor protein SHC which associates with the protein Grb2, itself constitutively bound to Sos1, which catalyzes the exchange of GTP for GDP on the small G protein Ras. Ras, in turn, initiates a phosphorylation cascade consisting of a MAKK kinase (MAPKKK) (c-Raf), a MAPK kinase (MAPKK) (MEK) and a MAPK (ERK1/2, also termed p42/p44). Activation of Ras-ERK pathway can lead to the activation of cytosolic enzymes such as cPLA2 and to nuclear translocation of MAPK followed by gene transcription and cell cycle progression (Fig. **7**).

Evidence has emerged since 1993 that, like the receptor tyosine kinases, stimulation of many heptahelical receptors leads to the activation of the Ras-MAPK pathway [63, 64, for reviews]. Several links between G protein-coupled receptors and MAPK can be distinguished with various degrees of complexity (Fig. **7**).

A link between trimeric G proteins and Ras has been first proposed considering that the heptahelical receptor agonist phosphatidic acid (LPA) and α2 adrenergic agonists mimic EGF in rapidly stimulating Ras-GTP accumulation in fibroblasts [64]. Ras activation was fully inhibited by pertussis toxin, yet not secondary to Gi-mediated inhibition of adenylylcyclase or stimulation of PLC. Attractive candidates for linking Gi to Ras activation are Gβγ, PI3K, c-Src and related tyrosine kinase. To date underlying

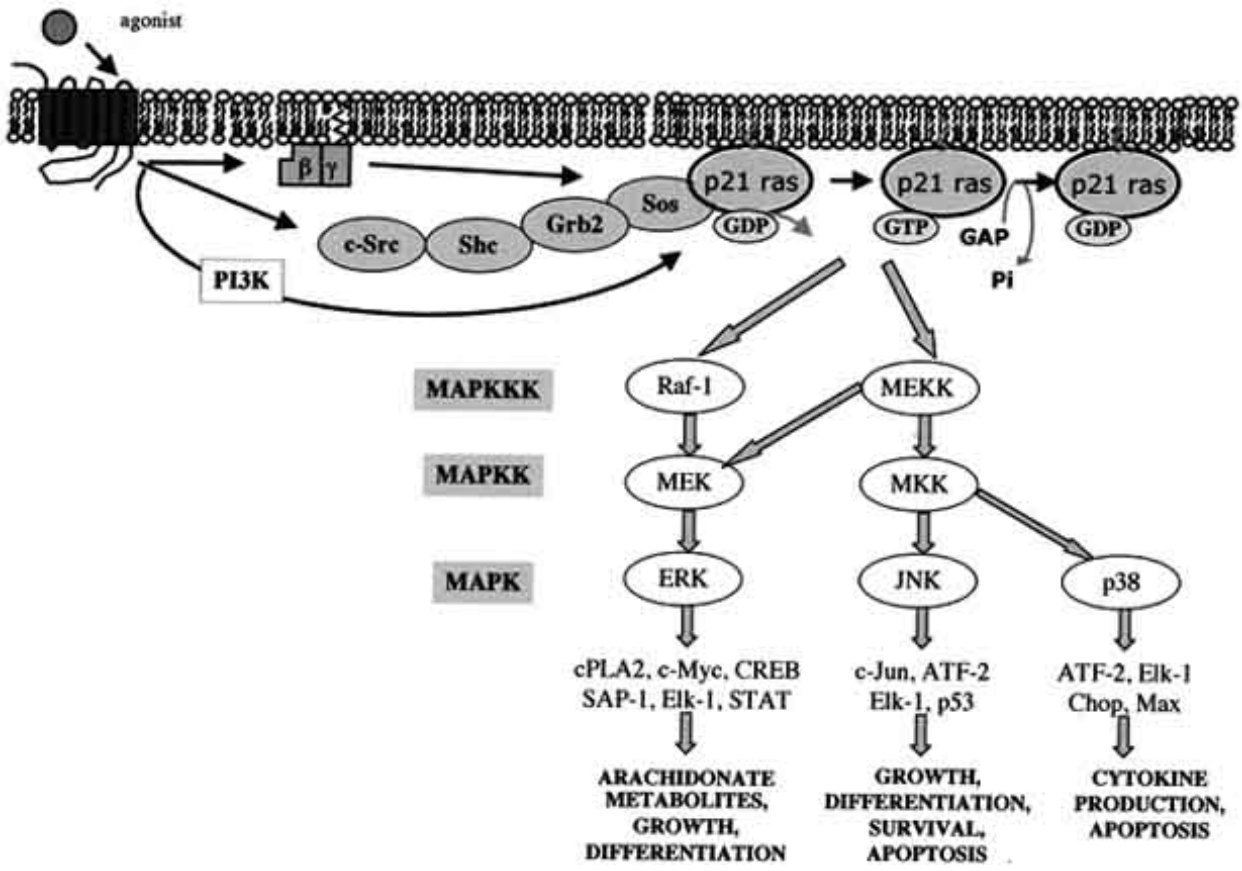

Fig. (7). Mitogen-activated protein kinase by heptahelical receptors. Gβγ, PI3K, c-Src are candidates for ras-activated MAPKs activation. MAPK cascades consist of MAPKKK, MAPKK, and MAPK; Once activated, MAPK phosphorylate transcription factors (c-Jun, c-Myc, ATF-2, …), other kinases and other regulatory enzymes such as cPLA2. These transduction pathways control cellular responses including secretion of inflammatory mediators such as arachidonate metabolites, cell growth, differentiation and apoptosis.

mechanisms remain controversial [64, for review]. The classic Gβγ-or $G\alpha_q$-PLC-Ca^{2+}/DAG pathway can also mediate MAPK activation, either via Ras or independently of Ras. For instance, in vascular smooth muscle the increase of cytosolic Ca^{2+} activates CaM kinase II which phosphorylates MEK leading to ERK activation. Ca^{2+} increase also allows the translocation of cPLA2 to the nuclear membrane. CaM kinase and ERK can both phosphorylate cPLA2 to produce arachidonate leading in these cells to prostacycline (PGI2) secretion [65].

Several other pathways can link heptahelical receptors to MAPKs. Two quite indirect pathways involve either the transactivation of receptor tyrosine kinase from the activation of G protein-coupled receptors or the involvement of β-arrestin and clathrin-dependent endocytosis processes [63, for review]. Interestingly, two direct pathways are emerging to link heptahelical receptors to MAPK. First, a direct interaction of β3 adrenergic receptors, characterized by proline rich intracellular sequences, with the SH3 domain of c-Src [66]. This direct receptor c-Src interaction requires the proline sequences, not found in most heptahelical receptors, and thus does not involve G proteins and Ras. Also, recent a evidence shows that $G\alpha_i$ and $G\alpha_s$ can directly interact with and activate c-Src via the catalytic domain of ERK [67]. Thus c-Src appears as a novel direct effector of G proteins, but the physiological correlates remain to be established.

The main concequences of ERK activation is the phophorylation of cPLA2 in the cytosol and of transcription factors (CREB, Elk-1, SAP-1, c-Myk) in the nucleus. Two other MAPK cascades leading to the MAPKKKs, JNK and p38, might also be activated, besides the ERK pathway, through the activation of heptahelical receptors. Note that the main G protein-dependent signaling pathways described above can also lead to phosphorylation and activation of transcription factors. This has first been established for the cAMP response element binding protein (CREB) activated by PKA [68, for review].

In conclusion, the large diversity of proteins involved in G protein-dependent signaling cascades offer the opportunity to conceive selective drugs as adding to the possibilities offered by heptahelical receptor ligands.

REFERENCES

[1] Rodbell, M.; Krans, H.M.J.; Pohl, S.L.; Birnbaumer, L. *J. Biol. Chem.,* **1971**, *246*, 1872.

[2] Rodbell, M.; Birnbaumer, L.; Pohl, S.L.; Krans, H.M.J. *J. Biol. Chem.,* **1971**, *246*, 1877.

[3] Orly, J.; Schramm, M. *Proc. Natl. Acad. Sci. USA*, **1976**, *73*, 4410.

[4] Ross, E.M.; Gilman, A.G. Proc. *Natl. Acad. Sci. USA*, **1977**, *74*, 3715.

[5] Ross, E.M.; Gilman, A.G. *J. Biol. Chem.,* **1977**, *252*, 6966.

[6] Pfeuffer, T. *J. Biol. Chem.,* **1977**, *252*, 7224.

[7] Dale, H.H. *J. Pharmacol. Exp. Ther.,* **1914**, *6*, 147.

[8] Daeffler, L.; Landry, Y. *Fundam. Clin. Pharmacol.,* **2000**, *14*, 73.

[9] Gilman, A.G. *Annu. Rev. Biochem.,* **1987**, *56*, 615.

[10] Sprang, S. R. *Annu. Rev. Biochem.,* **1997**, *66*, 639.

[11] Clapham, D.E.; Neer, E.J. *Annu. Rev. Pharmacol. Toxicol.,* **1997**, *37*, 167.

[12] Yan, W.; Sunalava, G.; Rosenzweg, S.; Brand, J.G.; Spielman, A.I. *Am. J. Physiol. Cell Physiol.,* **2001**, *280*, C742.

[13] Downes, G.D.; Gautam, N. *Genomics,* **1999**, *62*, 544.

[14] Farfel, Z.; Bourne, H.R.; Iiri, T. *N. Engl. J. Med.,* **1999**, *340*, 1012.

[15] Weinstein, L.S.; Shenker, A.; Gejman, P.V.; Merino, M.J.; Friedman, E.; Spiegel, A.M. *N. Engl. J. Med.,* **1991**, *325*, 1688.

[16] Carter, A.; Bardin, C.; Collins, R.; Simons, C.; Bray, P.; Spiegel, A. *Proc. Natl. Acad Sci. USA,* **1987**, *84*, 7266.

[17] Davies, D.J.; Hughes, H. E. *J. Med. Genet.,* **1993**, *30*, 101.

[18] Mousli, M.; Bueb, J.L.; Bronner, C.; Rouot, B.; Landry, Y. *Trends Pharmacol. Sci.,* **1990**, *11*, 358.

[19] Mousli, M.; Hugli, T.; Landry, Y.; Bronner, C. *Immunopharmacology*, **1994**, *27*, 1.

[20] Chahdi, A.; Daeffler, L.; Gies, J.P.; Landry, Y. *Fundam. Clin. Pharmacol.,* **1998**, *12*, 121.

[21] Higashijima, T.; Uzu, S., Nakajima, T.; Ross, E.M. *J. Biol. Chem.,* **1988**, *263*, 649l.

[22] Sukumar, M.; Ross, E.M.; Higashijima, T. *Biochemistry*, **1997**, *36*, 3632.

[23] Higashijima, T.; Burnier, J.; Ross, E.M. *J. Biol. Chem.,* **1990**, *265*, 14176.

[24] Fukushima, N.; Kohno, M; Kato, T. Kawamoto, S.; Okuda, K.; Misu, Y.; Ueda, H. *Peptides,* **l999**, *19*, 811.

[25] Daeffler, L.; Chahdi, A.; Gies, J.P.; Landry, Y. *Br. J. Pharmacol.,* **1999**, *127*, 1021.

[26] Fischer, T.; Bronner, C.; Landry, Y.; Mousli, M. *Biochim. Biophys. Acta,* **1993**, *1176*, 305.

[27] Freissmuth, M.; Boehm, S.; Beindl, W.; Nickel, P.; Ijzerman, A.P.; Hohenegger, M.; Nanoff, C. *Molec. Pharmacol.,* **1996**, *49*, 602.

[28] Hohenegger, M.; Waldhoer, M.; Beindl, W.; Boing, B.; Kreimeyer, A.; Nickel, P.; Nanoff, C.; Freissmuth, M. *Proc. Natl. Acad. Sci. USA,* **1998,** *95*, 346.

[29] Freissmuth, M.; Waldhoer, M.; Bofill-Cardona, E.; Nanoff, C. *Trends Pharmacol. Sci.,* **1999**, *20*, 237.

[30] Klinker, J.F.; Hageluken, A.; Grunbaum, L.; Selfert, R. *Exp. Dermatol.,* **1995**, *4*, 231.

[31] Hurley, J.H. *J. Biol. Chem.,* **1999**, *274*, 7599.

[32] Chern, Y. *Cell. Signal*, **2000**, *12*, 195.

[33] Antoni, F. A. *Frontiers Neuroendocrinol.,* **2000**, *21*, 103.

[34] Soderling, S. H.; Beavo, J. A. *Curr. Opin. Cell Biol.,* **2000**, *12*, 174.

[35] Dessauer, C.W.; Tesmer, J.J.; Sprang, S.R.; Gilman, A.G. *J. Biol. Chem.,* **1998**, *273*, 25831.

[36] Granovsky, A. E.; Artemyev, N.O. *J. Biol. Chem.,* **2001**, *276*, 21698.

[37] Lindemann, B. *Nat. Neurosci.,* **2000**, *3*, 99.

[38] Gilbertson, T.A.; Damak, S.; Margolskee, R. F. *Curr. Opin. Neurobiol.,* **2000**, *10*, 519.

[39] Hering, S.; Berjukow, S.; Sokalov, S.; Marksteiner, R.; Weiss, R.G.; Krauss, R.; Timin, E.N. *J. Physiol.,* **2000**, *528*, 237.

[40] Herlitze, S.; Zhong, H.; Scheur, T.; Catterall, W.A. *Proc. Natl. Acad. Sci. USA,* **2001**, *98*, 4699.

[41] Sui, J.L.; Chan, K.; Langan, M.N.; Vivandou, M.; Logothetis, D.E. *Adv. Second Messenger Phosphoprotein Res.,* **l999**, *33*, 179.

[42] Mark, M.D.; Herlitze, S. *Eur. J. Biochem.,* **2000**, *267*, 5830.

[43] Jen, J. *Curr. Opin. Neurobiol.,* **1999**, *9*, 274.

[44] Logothetis, D.E.; Kurachi, Y.; Galper, J.; Neer, E.J.; Clapham, D.E. *Nature*, **1987**, *325*, 321.

[45] Rebecchi, M.J.; Pentyala, S.N. *Physiol. Rev.,* **2000**, *80*, 1291.

[46] Morris, A.J.; Scarlata, S. *Biochem. Pharmacol.,* **1997**, *54*, 429.

[47] Kim, M.J.; Min, D.S.; Ryu, S.H.; Suh, P.G. *J. Biol. Chem.,* **1998**, *273*, 3618.

[48] Vanhaesebroeck, B.; Waterfield, M.D. *Exp. Cell Res.,* **1999**, *253*, 239.

[49] Vanhaesebrocck, B.; Alessi, D. *Biochem. J.,* **2000**, 346, 561.

[50] Toker, A. *Molec. Pharmacol.*, **2000**, *57*, 652.

[51] Falasca, M.; Logan, S.K.; Lehto, V.P.; Baccante, G.; Lemmon, M.A.; Schlessinger, J. *EMBO J.*, **1998**, *17*, 414.

[52] Cvejic, S.; Jiang, Y.; Huang, X.Y. *Trends Cardiovasc. Med.,* **2000**, *4*, 160.

[53] Hepler, J.R. *Trends Pharmacol. Sci.,* **1999**, *20*, 376.

[54] De Vries, L.; Zheng, B.; Fischer, T.; Elenko, E.; Farquhar, M.G. *Annu. Rev. Pharmacol. Toxicol.,* **2000**, *40*, 235.

[55] Ross, E.M.; Wilkie, T.M. *Annu. Rev. Biochem.*, **2000**, *69*, 795.

[56] Zhong, H.; Neubig, R.R. *J. Phamacol. Exp. Ther.,* **2001**, *297*, 837.

[57] Takai, Y.; Sasaki, T.; Matozaki, T. *Physiol. Rev.,* **2001**, *81*, 153.

[58] Sah, V.P.; Seasholtz, T.M.; Sagi, S.A.; Brown J.H. *Annu. Rev. Pharmacol. Toxicol.,* **2000**, *40*, 459.

[59] Balsinde, J.; Balboa, M.A.; Insel, P.A.; Dennis, E.A. *Annu. Rev. Pharmacol. Toxicol.,* **1999**, *39*, 175.

[60] Lambeau, G.; Lazdunski, M. *Trends Pharmacol. Sci.*, **1999**, *20*, 162.

[61] Pages, C.; Rey, A.; Lafontan, M.; Valet, P.; Saulnier-Blache, J.S. *Biochem. Biophys. Res. Commun.,* **1999**, *265*, 572.

[62] Axelrod, J. *Trends Neurosci.,* **1995**, *18*, 64.

[63] Pierce, K.L.; Luttrell, L.M.; Lefkowitz, R.J. *Oncogene*, **2001**, *20*, 1532.

[64] Kranenburg, O.; Moolenaar, W.H. *Oncogene*, **2001**, *20*, 1540.

[65] Muthalif, M.M.; Benter, I.F.; Uddin, M.R.; Malik, K.U. *J. Biol. Chem.*, **1996**, *271*, 30157.

[66] Cao, W.; Luttrell, L.M.; Medvedev, A.V.; Pierce, K.L.; Daniel, K.W.; Dixon, T.M.; Lefkowitz, R.J.; Collins, S. *J. Biol. Chem.*, **2000**, *275*, 38131.

[67] Ma, Y.C.; Huang, J.; Ali, S.; Lowry, W.; Huang, X.Y. *Cell*, **2000**, *102*, 635.

[68] Shaywitz, A.J.; Greenberg, M.E. *Annu. Rev. Biochem.*, **1999**, *68*, 821.