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Hormone Signalling via G-Protein: Regulation of Phosphatidylinositol 4,5-bisphosphate Hydrolysis by G<latex>$_{\text{q}}$</latex> [and Discussion]

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Hormone signalling via G-protein: regulation of phosphatidylinositol 4,5-bisphosphate hydrolysis by G_q

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SUMMARY

Heterotrimeric GTP-dependent regulatory proteins (G-proteins) mediate modulation by many cell surface receptors. Activation of the G-proteins promotes dissociation of their α and βγ subunits. The similarity of behaviour of βγ subunits derived from a variety of G-proteins has led to their use as affinity reagents for the analysis of the more unique α subunits. The evolution of these uses is presented. One of the more insightful results was the isolation of a new class of G-protein α subunits (the α_q subfamily) which have been shown to regulate the activity of a phospholipase C (PLC) specific for phosphatidylinositols. The experimental evidence leading to this conclusion is discussed. The activation by α_q increases the apparent V_{max} of the β isoform of phosphatidylinositol-specific phospholipase C (PLCβ) and can be modulated by βγ subunits.

1. INTRODUCTION

The regulation of intracellular functions by extracellular stimuli frequently employs cell surface receptors which specifically recognize the stimuli, span the plasma membrane, and transduce the extracellular stimuli into regulation of cytoplasmic second messenger pathways. The action of many of these receptors is mediated through GTP-dependent proteins (G-proteins) which, in turn, regulate the activities of adenylyl cyclase, a cGMP specific phosphodiesterase in mammalian retina, various phospholipases, and several ion channels. This has been reviewed extensively (Gilman 1987; Ross 1989; Birnbaumer 1990; Sternweis 1990; Brown 1990; Simon *et al.* 1991; Kazir *et al.* 1991).

The G-protein-dependent pathways for hormone signalling have similar structural motifs. The receptors that have been characterized most extensively consist of single polypeptides with amino acid sequences that are predicted to span the plasma membrane seven times. Thus, these proteins seem highly suited to transduce extracellular signals into cytoplasmic regulation. The receptor-regulated G-proteins are a family of highly homologous proteins consisting of three subunits (α, β, and γ). They are predicted to lie on the cytosolic surface of the membrane. The α subunits of the G-proteins are most diverse, contain the binding site for guanine nucleotides, and so far define the species of heterotrimer. The β and γ subunits appear less diverse and are isolated as a stable dimer; that is, βγ subunits have not been separated in a functional, undenatured form. Some of the properties of the G-proteins are summarized in table 1.

The Effector proteins, the targets of regulation by the G-proteins, have proven to be structurally diverse. Adenylyl cyclase, which is stimulated by the G_s-proteins, is a family of single polypeptides that are intrinsic to the membrane (Krupinski *et al.* 1989; Bakalyar & Reed 1990; Gao & Gilman 1991) as are G-protein-regulated ion channels. In contrast, the cGMP-dependent phosphodiesterase of the mammalian visual system is an extrinsic membrane protein which contains three polypeptides (α, β, and γ); the smaller γ subunit acts as an inhibitor of enzymatic activity. The β isoform of phosphatidylinositol-specific phospholipase C (PLCβ), which is regulated by G-proteins (see below), is a single polypeptide which is both cytoplasmic as well as loosely associated with membranes (Rhee *et al.* 1989; Crooke & Bennett 1989). Thus there is little relationship among the functionally diverse effectors.

The mechanism for hormone regulation involves a GTP cycle and decreased subunit affinity. The G-protein with bound GDP is inactive. Receptors, when occupied by agonists, interact more efficiently with G-proteins to promote dissociation of GDP and subsequent binding of GTP. The G-protein with bound GTP is active and regulates effector molecules. An intrinsic GTPase activity completes the cycle by restoring the protein to the basal state with bound GDP. This GTP cycle induces a concurrent subunit dissociation cycle. Binding of GTP and Mg^{2+} promotes dissociation of the α and βγ subunits; association is promoted by GDP. In the activated state (GTP), the subunits are more likely to be dissociated giving rise to two potential regulatory molecules, the α(GTP) and βγ subunits. In the resting state (GDP), the formation of heterotrimer is favoured. The heterotrimeric form appears to interact most efficiently with

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Table 1. *The G-protein family as defined by α subunits.*[a]

G protein α subunit[b]	toxin[c]	post-trans modifications[d]	effector proteins[e]	second messenger[f]
α_s, α_{olf}	CTX	unknown	↑ ad. cyclase ↑ Ca^{2+} channels	↑ cAMP ↑ Ca^{2+}
$\alpha_{i1}, \alpha_{i2}, \alpha_{i3}$	PTX	*N*-myristoylation	↓ ad. cyclase ↑ K^+ channel ↓ Ca^{2+} current	↓ cAMP hyperpolarize ↓ Ca^{2+}
		(?)	↑ PI-PLC	↑ IP_3, DAG
$\alpha_{tr(rod)}$	CTX &	unknown	↑ cGMP-PDE	↓ cGMP
$\alpha_{tc(conc)}$	PTX			
α_{oA} &	PTX	*N*-myristoylation	↑ K^+ channel	Hyperpolarize
α_{oB}			↓ Ca^{2+} current	↓ Ca^{2+}
		(?)	↑ PI-PLC	↑ IP_3, DAG
α_z	—	unknown	?	?
α_q, α_{11}	—	unknown	↑ PI-PLC	↑ IP_3, DAG
α_{12}–α_{16}	—	unknown	?	?

[a] All the α subunits have around 340–360 amino acids and run on SDS gels in the 39–52 kDa range. Some of the subscripts are derived from original activities (s and i, stimulation or inhibition of cyclase), historical designations (t, transducin) or location. Other subscripts are more arbitrary (o, other; q, perhaps quintessential; numbers, lack of imagination or despair).
[b] The α subunits designated α_{12}, α_{13}, α_{14}, α_{15}, and α_{16} have been observed as cDNAs and mRNA; olf = olfactory equivalent of α_s.
[c] The bacterial toxins from *Cholera vibrio* (CTX) and *Bordatella pertussis* (PTX) modify some of the G-protein α subunits by ADP-ribosylation.
[d] The purified proteins have been blocked at their *N*-terminus. Those with the appropriate *N*-terminal glycine (except α_s) have been shown to be myristoylated.
[e] PI-PLC = phosphatidyl inositol specific phospholipase C; PDE = cGMP-specific phosphodiesterase.
[f] Ca^{2+} refers to intracellular free calcium; IP_3 = inositol 1,4,5-trisphosphate; DAG = diacylglycerol.

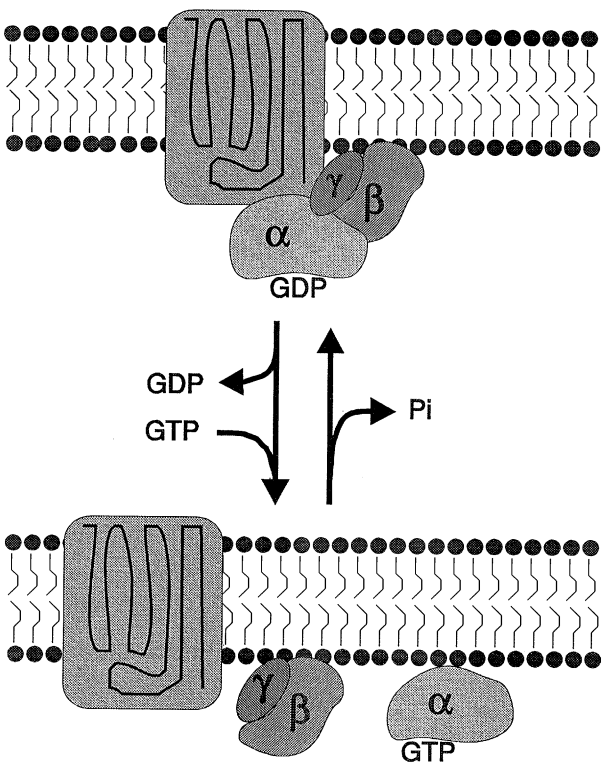


Figure 1. Schematic representation of the GTP and Subunit cycles of G-proteins.

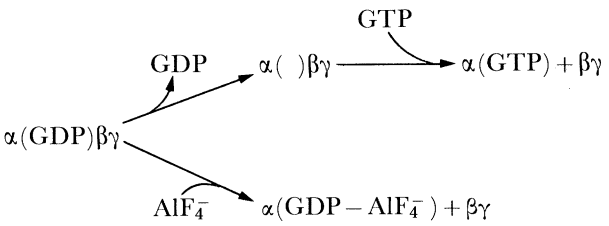
receptors. These cycles are presented schematically in figure 1.

The diversity of specialized α subunits (17 and counting) and β and γ subunits (four and more than four, respectively) raises the question of heterotrimeric structure. Early experiments indicated that βγ subunits derived from preparations of different G-proteins could interact with a variety of α subunits and behaved similarly in functional experiments. This led to the speculation that the βγ subunits were, basically, a common subunit that could act as a means of communication among the more specialized α subunits. The more recent expansion of the β and γ subunit families by cDNA cloning has rekindled the speculation that individual βγ subunits will display some specialized properties. Nevertheless, the observation that species of βγ interact with all known α subunits has been exploited by the construction of affinity matrices of immobilized βγ subunits which can be used for analysis of the known multiple α subunits. Some of the utilities of such a matrix and the information realized are summarized below.

2. AFFINITY CHROMATOGRAPHY WITH G-PROTEIN SUBUNITS

The βγ subunits from bovine brain are relatively insensitive to sulphydryl reagents. Therefore, func-

tional βγ subunits could be successfully immobilized to an agarose resin through coupling with cysteines in the protein (Pang & Sternweis 1989). Association or dissociation of α subunits with the immobilized βγ could then be reversibly controlled with the addition of GDP and EDTA or Mg^{2+} and AlF_4^- (Sternweis & Gilman 1982), respectively. The use of AlF_4^- bypasses the need for guanine nucleotide exchange as it apparently acts in concert with GDP (Bigay *et al.* 1985) to mimic activation of the protein observed physiologically with GTP.



One of the first things that could be demonstrated was the binding of the α_o subunit to the βγ matrix in the presence of GDP and its elution with either AlF_4^- or the GTP analogue, GTPγS. Competition experiments demonstrated that the affinity of the immobilized βγ for α subunits was similar to that of free βγ subunits (Pang & Sternweis 1989). One of the uses of the matrix was the separation of mixtures of α subunits. Thus α_i subunits could be separated from the abundant α_o subunits obtained in brain preparations by virtue of their higher affinity for the βγ subunits (Pang & Sternweis 1989).

A second use of the matrix was the isolation of α subunits from crude extracts of membranes derived from cells or tissues (Pang & Sternweis 1989). This was a rapid way to assess roughly the spectrum of G-protein α subunits present in the source. One dimensional SDS gels give an initial separation of the isolated α subunits that can suggest which are present or absent. However, the similarity of mobility among many of the α subunits makes identification with subunit specific antisera necessary. An advantage of examining the isolated subunits over membranes is an increased sensitivity due to concentration of the subunits and the removal of potential non-specific proteins with similar mobilities. The potential use of this method was indicated by the recovery of 50–100% of the α subunits (α_o, α_{i1}, α_{i2} and α_s tested) from bovine brain.

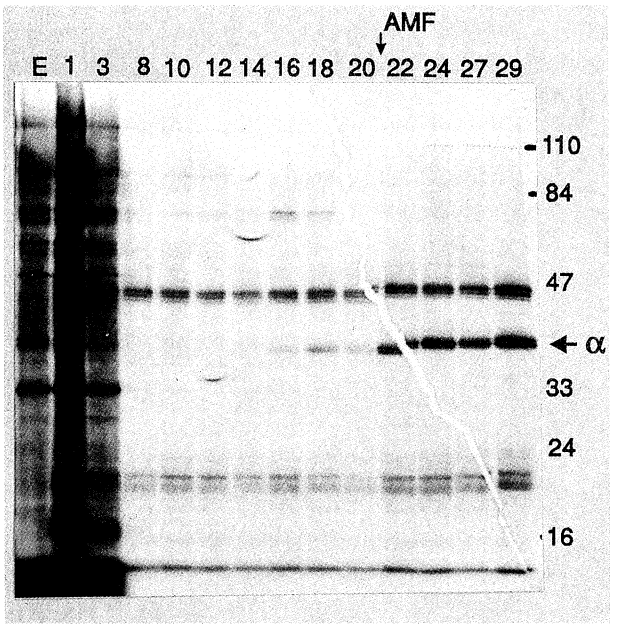


Figure 2. Isolation of myristoylated α subunits by affinity chromatography with βγ-agarose.

NIH-3T3 cells (60 mm dish at near confluence) were labelled with 1 mCi of [^{3}H]-myristic acid for 20 h. Cells were collected, washed, and homogenized in hypotonic medium with a Dounce homogenizer (Mumby 1990). The particulate fraction was collected by centrifugation, suspended in 1 ml of Solution A (10 mM NaHepes, pH 8.0, 0.1 mM EDTA, 1 mM Dithiothreitol and 5 μM GDP), and extracted for 1 h at 0°C by the addition of 100 μl of 10% (by volume) Lubrol PX (Sigma). Particulate material was removed by centrifugation and the extract applied to 1.5 ml of βγ-agarose as described (Pang 1989).

Aliquots (about 200 μl) of eluted fractions were processed, separated on SDS-polyacrylamide gel electrophoresis, and tritiated proteins were visualized with ENHANCE and fluorography for 30 days (Mumby 1990). Elutions were at 4°C for 5 min as follows: fraction 1, flow through; fractions 2–7, 1.5 ml Solution A containing 0.1% Lubrol and 400 mM NaCl; fractions 8–13, 0.5 ml Solution A containing 0.1% Lubrol, 400 mM NaCl, and 1 mM $MgCl_2$; fractions 14–19, 0.5 ml Solution A without GDP but containing 0.1% Lubrol, 400 mM NaCl, 1 mM $MgCl_2$, and 5 μM ATP; fractions 20–29, 0.5 ml Solution A containing 0.1% Lubrol, 300 mM NaCl, and AMF (30 μM $AlCl_3$, 10 mM $MgCl_2$, 10 mM NaF). AMF indicates the start of fractions containing this elution solution. The position of molecular size standards (kDa) and the α_o and α_i subunits is indicated at the right.

3. LIPID MODIFICATIONS OF G PROTEIN SUBUNITS

A second use of the βγ matrix was as a tool for the examination of subunit modification. Myristoylation of certain α subunits could be examined by labelling cells with a precursor ([^{3}H]-myristate) and isolation of the α subunits by affinity chromatography (figure 2). The matrix can retain two apparent modified α_i subunits from extracts of labelled 3T3 cells and these can be eluted specifically with AlF_4^-. No specific signal was observed in the α_s region (slower migration than α_i).

The potential importance of this lipid modification of the α_o and α_i subunits was indicated by the failure of unmodified subunits to bind tightly with the βγ matrix. Thus, α_o subunits which were expressed in *Escherichia coli* were not modified and flowed through or were only mildly retarded by immobilized βγ. When α_o and an *N*-myristoyl transferase were coexpressed in the same system, the α_o which was produced was modified and now bound to the βγ matrix like the native protein (Linder *et al.* 1991). This decreased affinity for βγ by the unmodified α subunits was confirmed by other techniques (Linder *et al.* 1990, 1991). One role for myristoylation of the α_o and α_i subunits may then be to target the α subunits to

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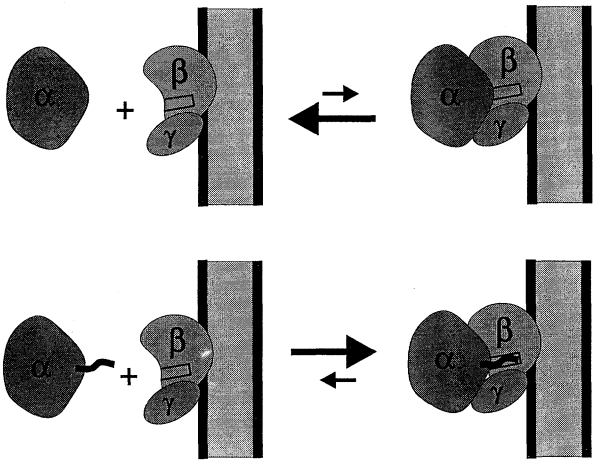

Figure 3. Schematic representation of the proposed effect of *N*-terminal myristoylation on G-protein subunit affinity.

membranes by increasing their association with the membrane-associated βγ subunits (figure 3). Indeed, mutagenesis of the N-terminus to prevent myristoylation results in the appearance of the α subunit in cytoplasmic fractions (Mumby *et al.* 1990; Jones *et al.* 1990).

A matrix of immobilized α subunit has also been constructed in order to isolate βγ subunits. This matrix was used to isolate βγ subunits from PC-12 cells that had been labelled with [^{3}H]-mevalonate, a precursor of isoprenyl groups (Mumby *et al.* 1990). The carboxyl terminal sequence of γ subunits had indicated the potential for modification by isoprenyl groups and this was confirmed for several of the γ subunits isolated by this technique. The modification was determined to be a geranylgeranyl group. Isoprenylation of γ subunits was also found by others (Yamane *et al.* 1990; Maltese & Robinshaw 1990; Fukada *et al.* 1990; Lai *et al.* 1990).

While a role for myristate on α subunits is suggested, the function of the isoprenyl group on βγ is not known. The βγ subunits are not intrinsic membrane proteins, but are hydrophobic and localize to particulate fractions. The isoprenoid may help anchor the βγ subunits to membranes; however, the presence of other isoprenylated proteins in cytosol suggest that there is much more to the membrane localization of these subunits.

4. PURIFICATION OF α_q

A utility of the βγ matrix was the rapid isolation of α subunits from crude preparations. This could be accomplished with known α subunits (§ 2) but it remains to be determined if all α subunits will interact with the βγ subunits used in the preparation of this affinity matrix. A suggestion that this might be the case was the isolation of a new G-protein α subunit from extracts of bovine brain (Pang & Sternweis 1989). This α subunit had a slower rate of migration on SDS gels than the α_i and α_o subunits normally isolated from brain by conventional chromatography. It was also distinguished by its lack of modification by pertussis toxin and its poor ability to exchange guanine nucleotides after solubilization. The latter property proved advantageous. The other α subunits could be easily activated with GTPγS which prevented tight binding to the βγ matrix. The new α subunit did not get activated, bound to the matrix and could be specifically eluted with Mg^{2+} and AlF_4^- (which does not require nucleotide exchange for activation and dissociation).

Subsequent analysis of the protein yielded primary amino acid sequences that were homologous but distinct from known G-protein α subunits. About the same time, Strathmann & Simon (1990) were characterizing new α subunit cDNAs. Primary sequences matched those of two highly homologous cDNAs and have been called α_q and α_{11} (Pang & Sternweis 1989; Strathmann & Simon 1990). The preparation from brain will subsequently be referred to as α_q (i.e. taken to indicate that it contains members of the α_q family). Although α_q was found to be expressed most abundantly in brain and lung tissue, both it and α_{11} appear to be ubiquitous among various tissues and cell lines tested (Pang & Sternweis 1989; Strathmann & Simon 1990; Gutowski *et al.* 1991).

5. G_q REGULATES THE ACTIVITY OF PHOSPHOLIPASE C

One of the best characterized pertussis toxin insensitive pathways for hormones is the regulation of hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) in a variety of systems. This reaction produces the second messengers, inositol-1,4,5-trisphosphate (IP_3) which stimulates release of intracellular Ca^{2+}, and diacylglycerol (DAG), a regulator of protein kinase C. When combined, the purified α_q stimulated the activity of phospholipase C isolated from bovine brain (Smrcka *et al.* 1991). This was largely dependent on the activator, AlF_4^-. Under the conditions reported, the stimulated phospholipase activity was specific for polyphosphorylated phosphatidylinositols (PI) and was more sensitive to concentrations of Ca^{2+}.

The validity of these experiments was clear from the simultaneous isolation of similar G-proteins from liver (Taylor *et al.* 1990) and turkey erythrocytes (Waldo *et al.* 1991). These proteins were isolated by their ability to stimulate a phospholipase C activity. The liver preparation contains two polypeptides, one of which can be identified immunologically as α_q (Wange *et al.* 1991); the turkey preparation does not react with an antiserum specific for α_q but does react with antibodies that recognize the carboxyl termini of the α_q subfamily (Waldo *et al.* 1991; Gutowski *et al.* 1991).

An example of α_q-dependent stimulation of bovine brain PLCβ1 is shown in figure 4. Here an attempt was made to examine the mechanism of this stimulation. Analysis of activities at various substrate concentrations indicates that activated α_q increased the V_{max} of the enzyme from 7 to 23 μmol min^{-1} mg^{-1} PLC at this concentration of α_q and Ca^{2+}. In several experiments, the apparent V_{max} was always increased by α_q while the apparent K_m varied between 5 and 20 μM in either the presence or absence of α_q.

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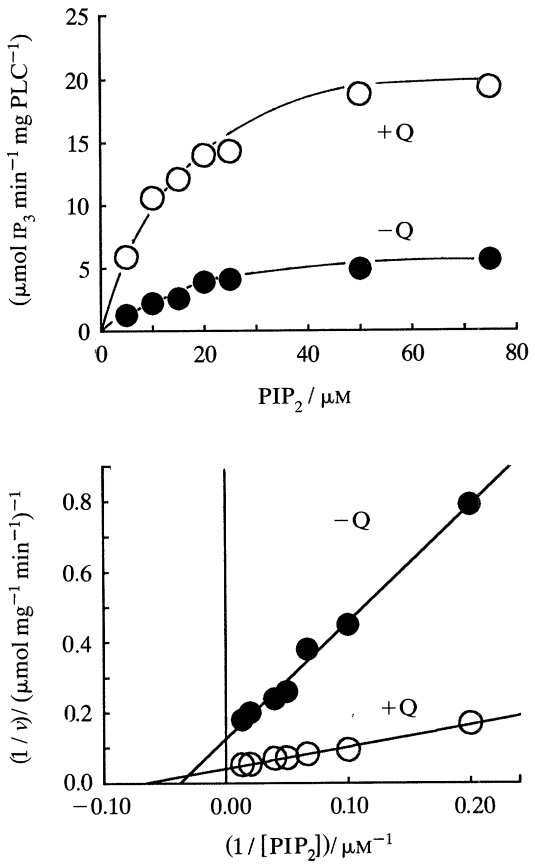

Figure 4. $G_q\alpha$ activates PLCβ by increasing its V_{max}. The activity of purified PLCβ (1 ng) from bovine brain either in the presence or absence of purified α_q (Pang 1990; Smrcka 1991) was assayed using the indicated concentrations of [^{3}H-inositol]PIP_2 presented as vesicles with a constant ratio of PIP_2:PE of 1:10 basically as described (Smrcka 1991). The assay (60 μl) contained 20 mM NaHepes, pH 7.2, 0.08% NaCholate, 0.8 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EDTA, 30 mM KCl, 20 mM NaCl, 3 mM EGTA, and 2 mM $CaCl_2$ (about 300 nM free). The bottom panel is a double reciprocal plot of the data in the top panel.

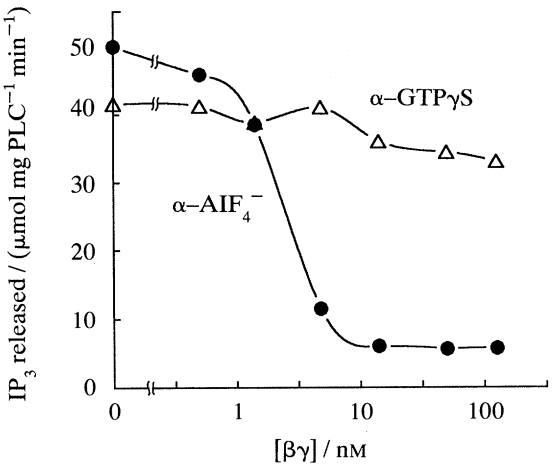

Figure 5. Activation of PLCβ by α_q with AlF_4^- but not by GTPγS-activated α_q is inhibited by βγ subunits. $G_q\alpha$ (14 ng) was preactivated with either AlF_4^- (30 μM $AlCl_3$ and 10 mM NaF) or 100 μM GTPγS followed by incubation with increasing amounts of βγ for 30 min at 20°C. Samples were diluted sixfold into the assay conditions described in figure 4 except that NaCholate was 0.016% and free Ca^{2+} was about 150 nM. Assays contained 0.17 nM PLCβ, 5 nM α_q (by mass), and the indicated concentrations of βγ.

These and previous experiments designate the α subunit of G_q as the activator of PLC. The effect of βγ on this process is shown in figure 5. Added βγ subunits inhibit α_q subunits that have been preactivated with AlF_4^- but not after α_q has been activated with GTPγS. This follows the pattern established with α_s and the activation of adenylyl cyclase. Recently, βγ has been shown to either enhance, have no effect, or inhibit the stimulation of adenylyl cyclase by GTPγS-activated G_s, depending on the subtype of cyclase (Tang & Gilman 1991). In this experiment, the βγ subunits do not appear to affect markedly the stimulation of PLCβ by GTPγS-activated α_q. The ability of βγ to inhibit activation of α_q-like preparations by AlF_4^- has been shown elsewhere (Waldo *et al.* 1991). In contrast to the turkey erythrocyte protein (Waldo *et al.* 1991), the inhibition here is very potent. The steep slope of the inhibition probably reflects stoichiometric association of the added subunits with the α_q. The βγ subunits can inhibit activation of α_q by GTPγS (data not shown); this was also shown for the G_q-preparation from liver (Blank *et al.* 1991). Although the activation of α_q by GTPγS is poor, it can be achieved with much higher concentrations of the nucleotide and longer times of incubation than with other G-proteins. This has also been recently documented for the liver preparation (Blank *et al.* 1991).

Several PI-specific PLC enzymes have been identified. It has now become clear that the β isoform (at least β1) of PLC is responsive to G_q (Taylor *et al.* 1991). The same enzyme was used here and in previous experiments (Smrcka *et al.* 1991). Both the purified PLCγ and PLCδ isoforms were not activated by the G-proteins (Taylor *et al.* 1991). The apparent regulation of PLCγ by phosphorylation by growth receptors (Wahl *et al.* 1988; Meisenhelder *et al.* 1989; Wahl *et al.* 1989; Kim *et al.* 1990) indicates that this crucial second messenger pathway is regulated differentially by several inputs. Yet, the G protein pathways may not be limited to PLCβ isoforms (or subtypes of the PLCβ family). There is evidence that other PI-specific phospholipases are responsive to GTP-dependent proteins (Thomas *et al.* 1991). A need for a second G-protein responsive phospholipase C is clear from the existence of a pertussis toxin sensitive pathway but the failure of G_o or G_i to stimulate PLCβ1.

Although G_q stimulated phospholipase C activity in an isolated system, the question of its role in hormone regulation remained. To test this, a membrane system was developed to measure hormone regulation of PLC activity. The probe for the role of G_q in such a system was an antibody (X384) developed to a peptide representing the carboxyl terminus of α_q and α_{11}. This antibody could immunoprecipitate α_q from crude extracts and could thus act on the native protein (Gutowski *et al.* 1991). When membranes from NG108

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cells were incubated with bradykinin, degradation of PIP_2 by phospholipase C was increased up to fivefold. This bradykinin-dependent stimulation could be completely blocked by the carboxyl antibody, X384 (Gutowski *et al.* 1991). Higher concentrations of GTPγS stimulated PLC activity in these membranes in the absence of hormone. This was also blocked by the antibody. Therefore, both hormone and GTPγS activation of PLC activity in these membranes appears to act through the same family of G proteins, the G_q family. Subsequent experiments demonstrated that the stimulation of PLC activity in plasma membranes of liver or 1321N1 cells by angiotensin and vasopressin or histamine, respectively, were also largely blocked by the antibodies. These results indicate that the G_q family plays a major role in a broad spectrum of hormonal regulation of PIP_2 hydrolysis.

6. CONCLUDING REMARKS

The use of subunit affinity matrices has confirmed the general interaction of βγ subunits with a broad range of known G-protein α subunits. The technique has supported separation of subunits by differences in affinity, examination of posttranslational modification of subunits, analysis of α subunit content in cells and tissues, and the isolation of new unique α subunits. In the latter case, new pertussis toxin insensitive G-proteins from mammalian brain were isolated and identified as members of a new G_q class of G-proteins. Extensive evidence indicates that this class of G-proteins plays a major role in the regulation of hydrolysis of PIP_2 in cells. This observation leads to several ancillary questions. The enzyme regulated by G_q is specific for hydrolysis of phosphatidylinositol polyphosphates. What regulates the phospholipase activity that generates diacylglycerol from other phospholipids, the generation of phosphatidic acid by the action of phospholipase D, or the hormone regulation of arachidonic acid production? Is G_q the mediator in such systems where the involvement of G-proteins is indicated and the pathways appear insensitive to pertussis toxin? If not, which new G-proteins will prove effective?

Furthermore, how is the hydrolysis of PIP_2 regulated by hormones in pertussis toxin sensitive systems? The stimulation of PLCβ with α_q is a direct and simple mechanism. Yet, attempts to reconstitute stimulation of PLC activity with activated subunits of pertussis toxin sensitive G-proteins have failed. Is there a G_o or G_i responsive phospholipase, is there a new pertussis toxin sensitive G-protein that needs to be found, or is the mechanism simply different? With the growing identification and purification of new phospholipases and the identification of new G-proteins, the answers to some of these questions may not be far away.

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Discussion

F. McCormick (*Department of Molecular Biology, Cetus Corporation, Emeryville, California, U.S.A.*). In the experiment in which Dr Sternweis was isolating subunits from brain extracts using his βα subunit matrix, did he dissociate the endogenous β and γ subunits from the α subunits first, or is he implying there is a lot of free α subunits in the brain extract?

P. C. Sternweis. No, we think there are probably excess βγ subunits. Actually, that experiment was done with an activation cycle, where you activate with tetrafluoroaluminate (AlF_4^-), and use EDTA to stop the activation. But in fact, AlF_4^- is unnecessary and after mixing, the α subunits will eventually dissociate and have a chance to bind to the fixed βγ subunits. I probably gave the impression of static heterotrimers, but they are dynamic: they can dissociate and reassociate. GDP favours the associated form, a heterotrimer, whereas if there is GTP present it is more likely to dissociate, so the equilibrium is shifted. This probably has relevance to the way the system works in the membrane.

F. McCormick. I think there is some evidence from Marc Charbre that the transducin α subunit GTPase is accelerated by the phosphodiesterase which is the effector in that system. Does Dr Sternweis know of any other evidence for acceleration of intrinsic GTPase activity of these G-proteins by effectors?

P. C. Sternweis. That is an experiment we would like to do in our system and probably have the capability to do it once we learn how to turn it over fast enough to get GTP bound. So far as I know, the transducin system is the only one where the activation has been shown so far. It is a modest activation but it is hard to get the system going fast enough to see a big effect. One of the problems we face is that we start with a faster GTPase activity already. When ras goes from the non-activated to the activated form, there is an approximately 10^5-fold change in GTPase activity to rates of approximately $10\ s^{-1}$, whereas we get at best $0.1–0.2\ s^{-1}$. So maybe our G-protein should be faster, and we should be looking for something that speeds it up.

K. C. Holmes (*Max Planck Institut für medizinishe Forschung, Heidelberg, FRG*). I have always associated myristoylation with anchoring in the membrane. Is that not always the case?

P. C. Sternweis. When myristate was first found on α_o and α_i, that was the interpretation: the fatty acid anchors the protein in the membrane. From a thermodynamic point of view, I think it is unlikely that the energy derived from sticking 13 methylene groups with a methyl at the end into a membrane is sufficient to keep the α subunit immobilized there. There may be two effects. We think that myristate increases the affinity of α subunits for βγ subunits, and that effect probably plays a role in getting the α subunits to the

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membrane in the first place. I do not know if myristate helps keep the α subunits in the membrane after dissociation. When G-proteins are activated, the α subunits do not all dissociate from the membrane, which is what one might expect to happen. I do not know whether they are bound to cytoskeleton or anchored in the membrane phospholipid bilayer. Nobody knows what holds the βγ subunits in the membrane. They do not have any transmembrane spanning regions in their protein sequences. The γ subunits are geranylgeranylated at their C terminus. That could be one factor that helps keep the βγ subunits in the particulate or membrane fractions.

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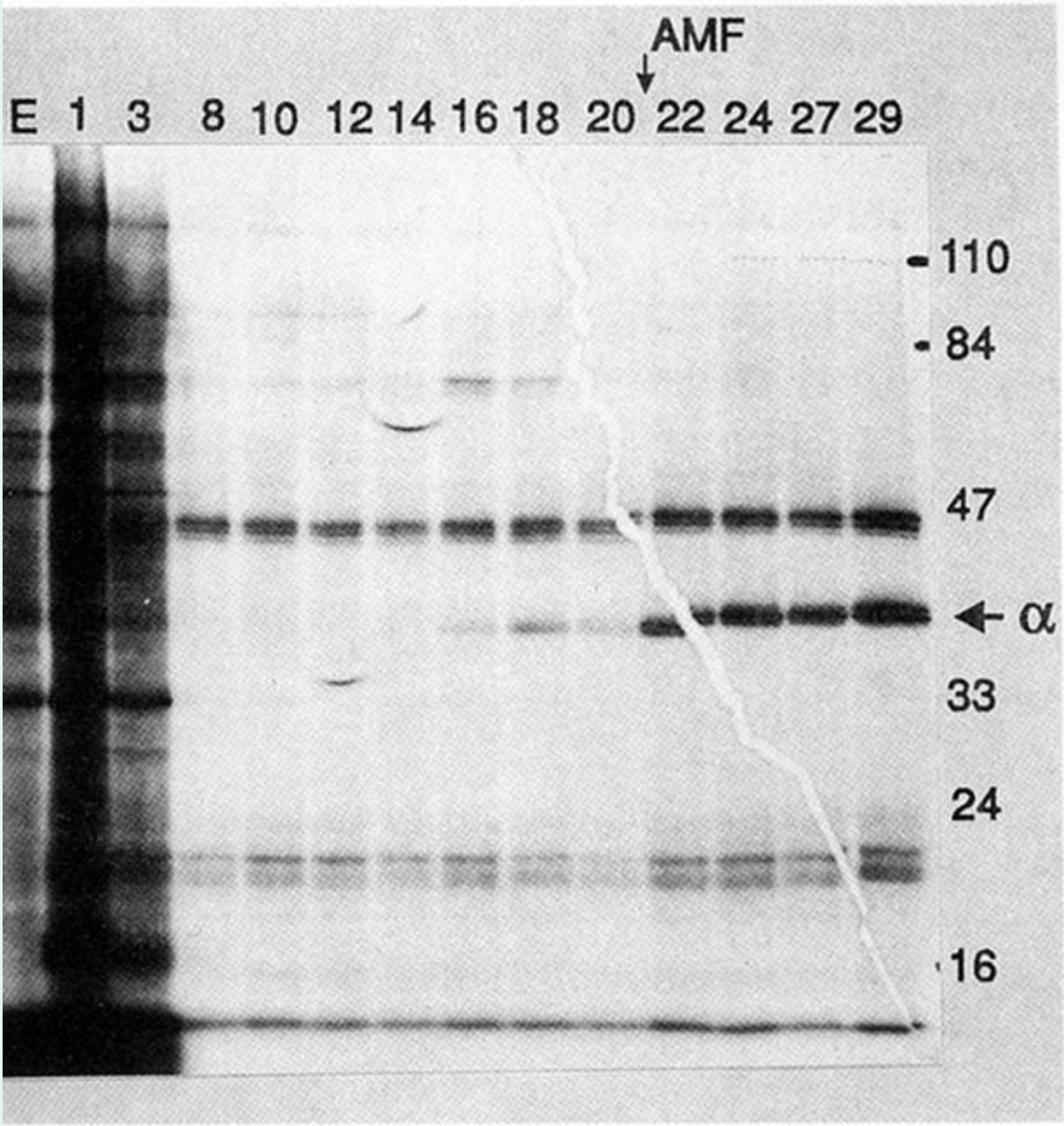

gure 2. Isolation of myristoylated α subunits by affinity romatography with βγ-agarose.

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