G Protein Regulation of Phospholipase A_2

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Contents

Abstract
Introduction
G Proteins
G Protein Regulation of Phospholipase A_2
  Inhibitory G Proteins
  Which G Protein is Coupled to PLA_2?
  Mechanism
Reconstitution
Summary
Acknowledgments
References

Abstract

Many neurotransmitters and hormones activate receptors that are known to be coupled to their effectors by GTP-binding regulatory proteins, G proteins. Activation of many of these same receptors elicits arachidonate release and metabolism. During the past few years, novel experimental techniques have revealed that in many cells arachidonate release is independent of generation of other second messengers, including inositol phosphates, diacylglycerols, and elevation in free intracellular calcium. Much evidence has accumulated to implicate phospholipase A_2 as the enzyme catalyzing arachidonate release, and suggesting that this effector enzyme, too, is activated by G proteins. In neural tissues as well as epithelium, endothelium, contractile and connective tissues, and blood cells, G proteins coupled to receptors for a variety of peptide and nonpeptide neurotransmitters and hormones have been shown to directly activate phospholipase A_2. In retinal rod outer segments, transducin is the coupling G protein, but the G proteins coupling receptor activation to phospholipase A_2 in other cell types is less clear. Some are pertussis toxin-sensitive, whereas others are not, and evidence exists that the *ras* gene product G protein may also be coupled to and regulate phospholipase A_2.

Index Entries: Phospholipase A_2; G protein; signal transduction; receptor-effector coupling; phospholipase C; arachidonic acid; *ras*.

Introduction

The rate-limiting step in stimulation of eicosanoid synthesis by neurotransmitters, hormones, and growth factors is thought to be release of free arachidonate from phospholipids (Irvine, 1982). The mechanism for this release has been debated for many years. Many early studies concluded that phospholipase A_2 (PLA_2) is the enzyme responsible, releasing arachidonic acid from the *sn*-2 position of phospholipids (Irvine, 1982). This mechanism is consistent with the finding that in mammalian cells the *sn*-2 position of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol is enriched in arachidonic acid (e.g., Prescott and Majerus, 1981). However, activation of PLA_2 was difficult to reconcile in most tissues, because demonstration of significant PLA_2 activity required nonphysiological calcium concentrations (m*M*) and pH (8–9). During the late 1970s and early 1980s, phosphatidylinositol-specific phospholipase C became accepted as the enzyme initiating arachidonate release (Majerus et al., 1986) (Fig. 1A). This mechanism was favored by several findings.

1. Many of the receptors that release arahidonate also enhance phosphatidylinositol turn over;
2. The diacylglycerol released by phosphatidylinositol-specific phosphalolipase C is enriched in arachidonate (Prescott and Majerus, 1981); and
3. Pathways were elucidated in which diacylglycerol can be deacylated to release free archidonate (Bell et al., 1979).

In a variation of this pathway, diacylglycerol is phosphorylated to phosphatidic acid from which arachidonate is secondarily released by action of a phosphatidate-specific PLA_2 (Billah et al., 1981).

Recently, it has become clear that in many cells arachidonate release can be readily separated from phosphatidylinositol metabolism (e.g., Burch et al., 1986, 1987; Slivka and Insel, 1987, 1988; Welsh et al., 1988). In these cells, PLA_2 may play the dominant role in arachidonate release. The mechanism by which receptor occupation can activate PLA_2 has been unclear. Several potential mechanisms have been put forward (Irvine, 1982; Axelrod et al., 1988; Burch, 1989). Commonly, it is suggested that

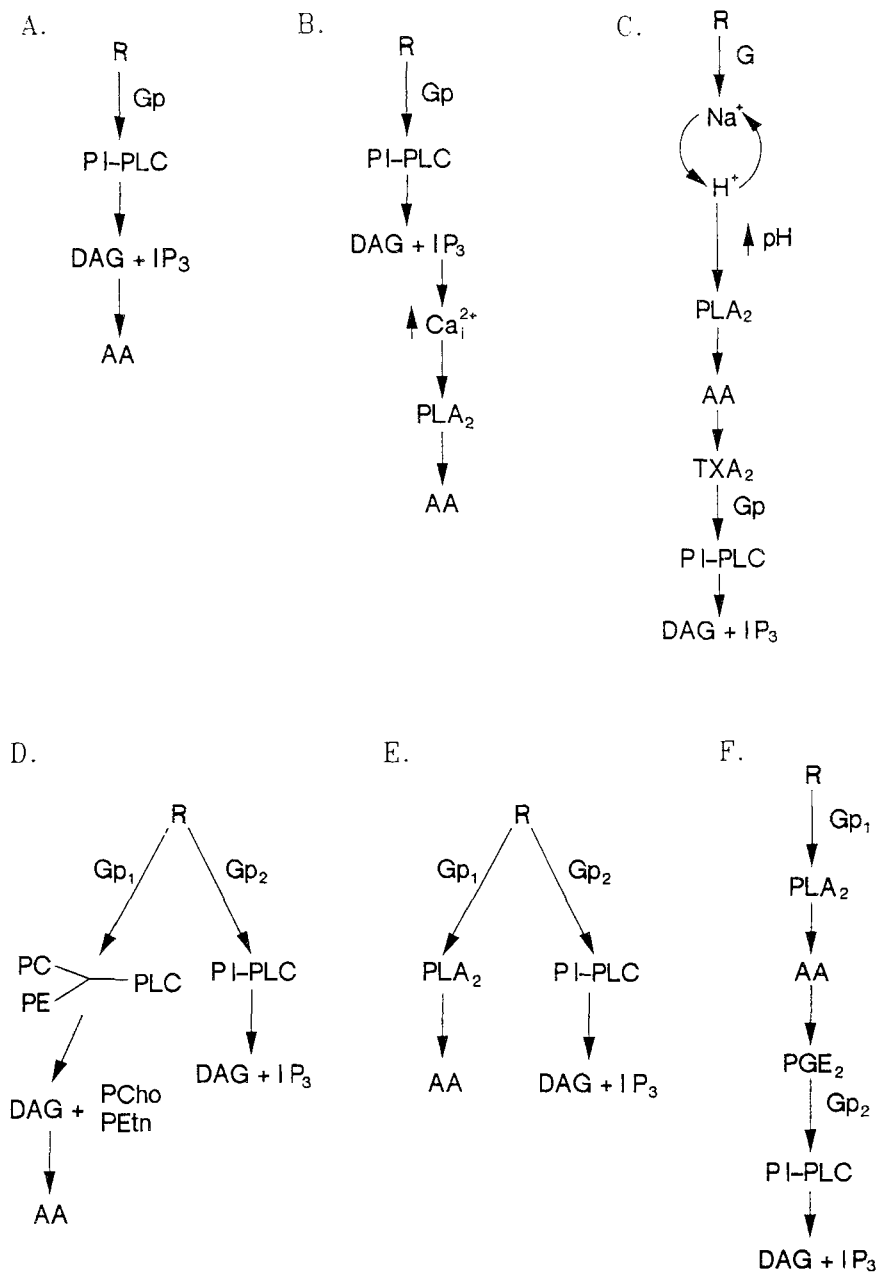

Fig. 1. Multiple pathways to arachidonate and phosphatidylinositol release. A Receptor (R) occupation activates G_p that couples to phosphatidylinositol-specific phospholipase C (PI-PLC). Arachidonate (AA) is released from diacylglycerol (DAG) by diacylglycerol lipase. B In this model AA is not derived from DAG; instead, IP_3 increases intracellular free calcium that activates PLA_2 that in turn releases AA. C R occupation activates Na^+/H^+ exchange, causing intracellular alkalinization. PLA_2 is activated by the increased pH and releases AA. In platelets, AA is metabolized to thromboxane A_2 (TXA_2), which binds its own receptor to activate PI-PLC and release IP_3 and DAG. D Receptor occupation results in activation of a PLC specific for either PC or PE. AA is released from DAG. E R occupation activates 2 distinct G proteins, each of which couples to a separate phospholipase. PLA_2 releases AA, whereas PI-PLC releases IP_3 and DAG. F R activates PLA_2 to release AA. AA is metabolized to prostaglandin E_2 (PGE_2), for example, which acts through its own receptor to release DAG and IP_3.

receptor occupation causes a rise in intracellular free calcium. This may be provided by calcium entering from outside the cell through calcium channels or may arise from the release of calcium from intracellular stores by inositol phosphates (Fig. 1B). The increased calcium concentration results in activation of PLA_2. Arguing against this mechanism is the observation that many purified PLA_2s require m*M* calcium to exhibit significant activity, whereas intracellular free calcium rises only to hundreds of n*M* or low μ*M* concentration. However, in certain cells, influx of calcium may play a role since calcium channel blockers inhibit arachidonate release in response to receptor occupation (e.g., Satoh et al., 1985).

Many purified PLA_2s require high pH, and it has been proposed that activation of Na^+/H^+ exchange may cause alkalinization of the cytoplasm sufficient to initiate PLA_2 activity (Fig. 1C). This mechanism may provide arachidonate in human platelets activated with α_2 adrenergic agonists (Sweatt et al., 1986; Banga et al., 1986), but does not seem to be widespread. For instance, in Swiss 3T3 fibroblasts, bradykinin stimulates arachidonate release with equal efficacy when extracellular pH ranges from 6.8 to 8.0. Moreover, replacement of NaCl with *N*-methyl-D-glucamine or choline-Cl to block the exchanger has no effect on bradykinin stimulation of arachidonate release (Burch, unpublished observations).

The problem of PLA_2 activation has been further compounded by the findings of multiple PLA_2s in any given cell. These enzymes prefer various substrates, including phosphatidylcholine, phosphatidylethanolamine, phosphatidyinositol (e.g., Ballou et al., 1986), or the same phospholipids with ether-linked fatty acids (Loeb and Gross, 1986); different pH values, from 5 to 9 (e. g., Wightman et al., 1981), and different calcium concentrations, from 100 n*M* to 10 m*M* (Wightman et al., 1981; Loeb and Gross, 1986). Thus, the observation that a calcium ionophore stimulates arachidonate release in a cell that never physiologically experiences intracellular free calcium concentrations greater than 1 μ*M*, suggests that ionophores may stimulate PLA_2s that are not involved in release of arachidonate by receptor agonists.

Further complicating studies of the pathways mediating arachidonate release is new evidence implicating hormone-sensitive phospholipase C catalyzing release of diacylglycerol from phosphatidylcholine (Besterman et al., 1986) or phosphatidylethanolamine (Kester et al., 1989) (Fig. 1D). Evidence that such pathways can actually result in release of arachidonate has been presented by Clark and coworkers (1986).

Many of the receptors that cause arachidonate release are thought to couple to GTP-binding regulatory proteins (G proteins). These G proteins couple receptors to a variety of effector proteins, including adenylate cyclase, ion channels, and phospholipases C (Casey and Gilman, 1988). Since arachidonate release in response to these agonists often occurs as rapidly as phospholipase C activation and can be readily separated from inositol phosphate formation, it seemed possible, even likely, that PLA_2 coupled to these receptors should also be coupled through G proteins. In this review we discuss the evidence for such coupling.

G Proteins

G proteins serve to couple numerous receptors to their effector proteins. Coupling may increase or decrease effector activity, depending on the class of G protein coupled to it. Certain effectors, in particular adenylate cyclase, are dually regulated by stimulatory (G_s) and inhibitory (G_i) G proteins (Casey and Gilman, 1988) through separate receptors. Effectors proven to be regulated by G proteins include adenylate cyclase and cGMP-phosphodiesterase in retina (G_t). Other effectors that most likely are coupled

Table 1
Selected G Proteins[a]

G protein	Mass[b]	Toxin sensitivity
G_s	45,52	Cholera
G_i	41	Pertussis
G_o	39	Pertussis
G_t	39	Cholera, pertussis
G_p[c]	?	Pertussis, cholera, none
ras	21	none

[a]Data are from Casey and Gilman (1988).
[b]kDa, of α subunit.
[c]The "p" indicates "phospholipase."

to G proteins include phosphatidylinositol-specific phospholipase C (Majerus et al., 1986; Fain et al., 1988; Casey and Gilman, 1988), and certain ion channels (Lewis et al., 1986; Bourne, 1989).

The most studied G proteins belong to a genetic family of closely related members (Table 1). These proteins are heterotrimeric, composed of α, β, and γ subunits (Casey and Gilman, 1988) (Fig. 2). The α subunits contain the binding site for guanine nucleotides and exhibit GTPase activity. The α subunit also contains the sites for NAD-dependent ADP-ribosylation by cholera toxin and/or pertussis toxin. The 4 $G_{s\alpha}$s can be ADP-ribosylated by chlolera toxin, the 3 $G_{i\alpha}$s and $G_{o\alpha}$ can be ADP-ribosylated by pertussis toxin, and the 2 $G_{t\alpha}$s by both. Cholera toxin activates G_s, whereas pertussis toxin often inactivates G proteins. There appear to be numerous other G proteins in this family that are only beginning to be characterized, some with α subunits with significantly lower masses, perhaps 21 kDa (e.g., Evans et al., 1986).

The βγ subunits are tightly associated and can be substituted among the various G_α subunits in *in vitro* assays. The βγ complex has been proposed to anchor the α subunit to the plasma membrane (although evidence is accumulating that the βγ subunit has biological activity [Neer and Clapham, 1988] and *see below*). There are 2 types of β subunits. G has only a single 36 kDa form, whereas other G proteins contain a mixture of 35 and 36 kDa forms. The γ subunits have been studied least, but evidence suggests that at least 3 forms exist (Casey and Gilman, 1988). Their mass is less than 10 kDa.

The mechanism underlying receptor–effector coupling by G_s has been characterized in some detail. In the inactive state G proteins exist as αβγ complexes with GDP bound to the α subunit (Gilman, 1987; Casey and Gilman, 1989) (Fig. 2). Upon agonist binding, the agonist–receptor complex interacts with the G_α to cause dissociation of GDP. GTP then binds to the empty guanine nucleotide site, decreasing affinity of the agonist for the receptor, resulting in dissociation of the agonist and the receptor from the G–GTP complex. The G–GTP complex binds to the effector, and the activated G_α–GTP probably dissociates from the βγ subunits (Gilman, 1987).

Deactivation of the G_α–GTP is caused by the endogenous GTPase of the α subunit. This results in G_α–GDP reassociating with βγ (Fig. 2). The rate constant of hydrolysis of GTP is 2–3/min (Gilman, 1987). Therefore, a given G_α–GTP may last many seconds.

Several agents are available that allow study of G protein activation in the absence of receptor activation. GTPγS and other poorly hydrolyzed GTP analogs can "irreversibly" activate G_αs. Activation is slow compared to receptor activation, often requiring several minutes. GDPβS

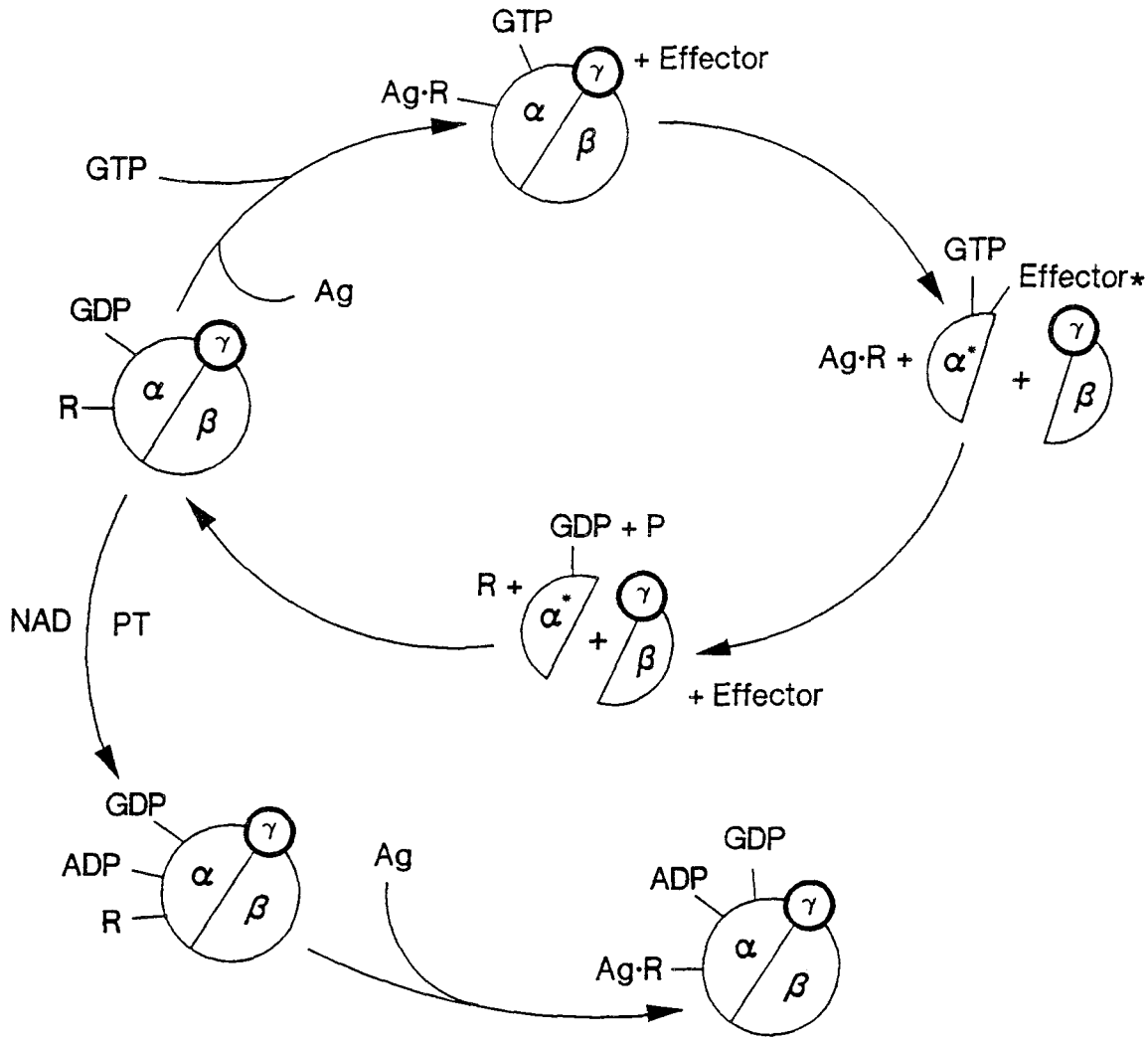

Fig. 2. Regulation of G protein activity. The inactive G protein heterotrimer exists with GDP bound to the α subunit, and a receptor (R) may be coupled. Upon agonist (Ag) binding to R, GTP is exchanged for GDP. The α subunit becomes activated (*) and the Ag·R complex and βγ dissociate from α*, whereas an effector becomes associated. The endogenous GTPase of the α subunit cleaves GTP to GDP + P, resulting in dissociation of the effector and reassociation of the α and βγ subunits. Pertussis toxin (PT) inactivates G proteins by catalyzing ADP-ribosylation of the α subunit using NAD as substrate. In this condition, Ag binding to R does not result in dissociation of the subunits.

can inhibit G protein activation. These agents cannot usually cross the cell membrane, and thus must be used in permeabilized cells or *in vitro*. AlF_4^- also activates G proteins, presumably by mimicking the terminal phosphate grouping of GTP (Sternweis and Gilman, 1982).

The *ras* gene products are a family of 21 kDa plasma membrane-bound guanine nucleotide-binding proteins (Barbacid, 1987). They also have GTPase activity and have certain sequence homology with G protein α subunits (Gilman, 1987). There is no evidence that they can interact with βγ subunits, and their activity is not affected by cholera toxin or pertussis toxin (Broek

et al., 1985). Upon mutation, these proteins cause neoplastic transformation of cells (Barbacid, 1987). They have been suggested as coupling proteins for as of yet unidentified receptors. The effectors they regulate have yet to be clearly defined; in yeast they appear to serve much the same function as G_s in mammalian cells (Broek et al., 1985).

There is evidence that they can affect coupling of receptors to phosphatidylinositol-specific phospholipase C (Fleischman et al., 1986; Wakelam et al., 1986) and they have also been implicated in regulation of PLA_2 (*see below*).

G Protein Regulation of Phospholipase A_2

Evidence suggesting that G proteins may couple receptors to ultimate arachidonate release and metabolism was first reported in 1984. Pertussis toxin was found to block arachidonate release from neutrophils in response to the calcium ionophore A23187 (Bokach and Gilman, 1984) and the chemotactic peptide f-Met-Leu-Phe (Okjima and Ui, 1984). Pertussis toxin was also found to block arachidonate release from mast cells in response to agent 48/80 (Nakamura and Ui, 1984,1985). These findings were soon followed by reports that pertussis toxin blocks arachidonate release in response to a variety of agonists in several different cell types (Table 2).

The first study to include a direct assessment of GTP analogs on PLA_2 activity appeared in 1986 (Burch et al., 1986). The α_1 adrenergic receptor in the FRTL5 thyroid cell is coupled to a pertussis toxin-sensitive G protein to stimulate PLA_2 and a pertussis toxin insensitive G protein to stimulate phosphatidylinositol-specific phospholipase C. FRTL5 cells were transiently permeabilized to incorporate GTPγS. The GTP analog stimulated release of both arachidonate and inositol phosphates. These two effects were dissociated using neomycin. Neomycin blocks the effect of phosphatidylinositol-specific phospholipase C by binding to and sequestering its substrate (Schacht, 1978). In the presence of neomycin, both norepinephrine and GTPγS still stimulated arachidonate release but neither enhanced inositol phosphate formation. Using membranes derived from FRTL5 cells and exogenous radiolabeled phosphatidylcholine substrate, GTPγS was found to activate PLA_2 and it potentiated the effect of α_1 adrenergic agonists (Burch et al., 1986).

The study providing the most convincing evidence that PLA_2 can be directly activated by G proteins was that of Jelsema (1987), which found that rhodopsin is directly coupled to PLA_2 in bovine rod outer segments by the G protein transducin (G_t). In the rod outer segment, rhodopsin serves as the "receptor" for light. Stimulation of rhodopsin causes activation of G_t, which is in turn coupled to cGMP-phosphodiesterase (Stryer, 1987). Jelsema found that light shone onto dark-adapted rod outer segments caused activation of PLA_2. G_t mediation was suggested by the observation that GTPγS mimicked light in activating PLA_2. Evidence presented then for G_t being the G protein specifically involved was provided by the finding that both pertussis toxin and cholera toxin blocked light-induced activation of PLA_2 as well as G_t-mediated, light-induced activation of cGMP-phosphodiesterase activity (*see below* for evidence that the effect of cholera toxin may not have been at the level of G_t).

Rod outer segments are a useful system in which to study the role of G proteins in the mediation of biological responses, since G_t is readily removed from the membranes by hypotonic washing of dark-adapted rod outer segments. Taking advantage of this property, Jelsema (1987) found that both light- and GTPγS-stimulated PLA_2 activity was lost coincident with removal of G_t. Ruling against a simple loss of PLA_2 with the washing was her observation that readdition of purified G_t to the depleted membranes at least partly restored light- and GTPγS-stimulated PLA_2 activity (Jelsema, 1987). Further,

mellitin, a PLA_2-activating protein, stimulated PLA_2 activity equally in G_t replete and G_t-depleted membranes (Jelsema et al., 1989).

Inhibitory G Proteins

Evidence has also been obtained suggesting that PLA_2 can be negatively modulated by G proteins. This is analogous to adenylate cyclase that is under dual regulation by G proteins (*see above, G Proteins*).

Recall that GTPγS mimicked G_t in stimulating PLA_2 activity in dark-adapted rod outer segments. If, instead, rod outer segments were exposed to light to activate PLA_2, then GTPγS added, the effect of GTPγS was to *inhibit* light-stimulated PLA_2 activity (Jelsema, 1987). Addition of GTPγS to G_t-depleted dark-adapted rod outer segments also uncovers the existence of a PLA_2 inhibitory G protein, since in this condition, GTPγS also inhibited basal PLA_2 (Jelsema, 1987).

PLA_2 inhibitory G proteins in the rod outer segment include G_s or G_o. In G_t-containing, light-activated rod outer segments, cholera toxin as well as GTPγS inhibit PLA_2 (Jelsema et al., 1989). The effect of cholera toxin was independent of G_t, since cholera toxin treatment of exogenous G_t had no effect on its ability to restore light activation of PLA_2 (Jelsema et al., 1989). In addition, $G_{s\alpha}$ inhibited $G_{t\beta\gamma}$-stimulated PLA_2.

In RAW264.7 macrophages, too, indirect evidence exists for dual regulation of PLA_2 (Burch et al., 1987). In these cells, either cholera toxin or pertussis toxin stimulated PLA_2 activity. Addition of both synergistically increased PLA_2 activity. Again, the situation is reminiscent of dual G protein regulation of adenylate cyclase. Addition of either toxin activates adenylate cyclase, either by directly stimulating (cholera toxin on G_s) or by removing inhibition (pertussis toxin on G_i). Thus, although other models may be postulated for the effects of cholera toxin and pertussis toxin on PLA_2 in RAW264.7 macrophages (Burch et al., 1988), dual regulation by stimulatory and inhibitory G proteins seems likely (Fig. 3).

Which G Protein Is Coupled to PLA_2?

The identities of the G proteins that regulate PLA_2 are unknown. That different G proteins are coupled to PLA_2 in various tissues seems clear, since in some tissues the protein is pertussis toxin-sensitive, whereas in others it is not (Table 2). In rod outer segments, the G protein naturally coupled to PLA_2 seems to be G_t. However, even in that simple system several G proteins can reconstitute PLA_2 activation (*see below*). Several authors have called any G protein coupled to PLA_2 or phospholipase C "G_p" (Joseph, 1985; Lo and Hughes, 1987). Unfortunately, a G protein isolated from placenta has also been termed G_p (Evans et al., 1986).

Ras protooncogene-derived GTP-binding proteins may also be coupled to PLA_2. Microinjection of the H-*ras* oncogene into fibroblasts activated PLA_2 (Bar-Sagi and Feramisco, 1986). Supporting a functional coupling of the *ras* G protein to PLA_2 was the finding that 85% of PLA_2 in the fibroblast ruffle membrane was within 100–500Å of *ras* (Bar-Sagi et al., 1988). In contrast, another membrane-bound protein, Na^+, K^+-ATPase, was not colocalized with the *ras* protein. In considering *ras* products as regulators of PLA_2, it should be born in mind that arguments against this hypothesis have been presented (Stacey et al., 1988).

Mechanism

The mechanism by which G proteins activate PLA_2 is unknown. The mechanism for G protein activation of another phospholipase, phosphatidylinositol-specific phospholipase C, appears to be a reduction in the enzyme's requirement for free calcium (Smith et al., 1986). We did not

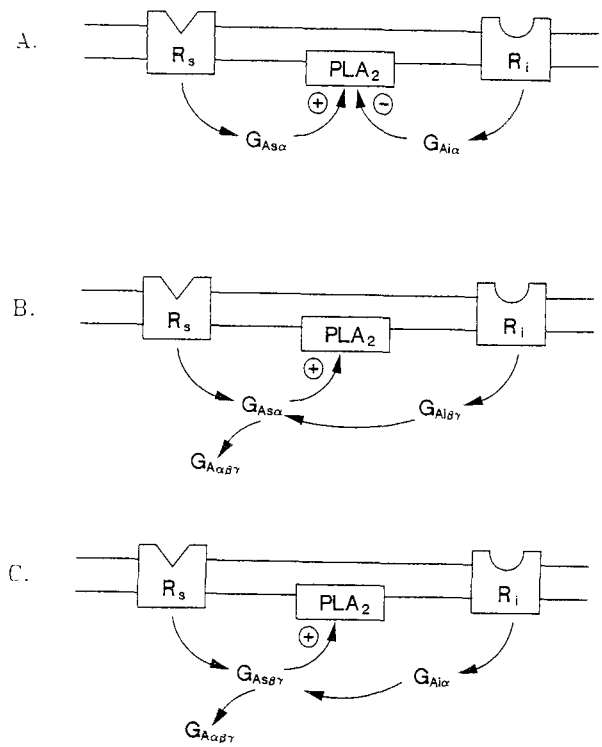

Fig. 3. Dual regulation of PLA_2 by stimulatory (G_{As}) and inhibitory (G_{Ai}) G proteins. **A** Activation of stimulatory or inhibitory receptors release the respective Gα subunits that interact with PLA_2. **B** Stimulatory Gα is removed by excess Gβγ released by an inhibitory receptor (analogous to regulation of adenylate cyclase). **C** Stimulatory Gβγ is removed by excess Gα released by an inhibitory receptor (identical to **B** but assumes the Jelsema model in which Gβγ is the activator).

examine this point in our earlier studies of PLA_2 (Burch et al., 1986). However, such a mechanism has been suggested by a study in which GTPγS lowered the calcium requirement for the chemotactic peptide f-Met-Leu-Phe to stimulate arachidonate release from saponin-permeabilized neutrophils (Nakashima et al, 1988). Further examination of this possible mechanism is presented below.

Evidence is accumulating that in at least some systems it is the βγ subunit of the G protein that activates PLA_2. βγ subunits purified from G_t, when added to dark-adapted, G_t-depleted rod outer segments, caused marked increase in PLA_2 activity, whereas addition of an equivalent amount of α subunit caused only a slight increase (Jelsema and Axelrod, 1987). Addition of equimolar amounts of α with βγ subunits inhibited βγ-induced PLA_2, presumably as a result of reassociation of the αβγ trimer. GTPγS, which prevents subunit reassociation, abolished the inhibition of βγ-stimulated PLA_2 by the α subunit. Pertussis toxin treatment of the combined subunits to prevent α subunit interaction with the receptor, blocked PLA_2 stimulation, even in the presence of GTPγS. βγ subunits prepared from G_o also stimulated rod outer segment PLA_2 (Jelsema et al., 1989). Both $G_{t\beta\gamma}$ and $G_{o\beta\gamma}$ share a common β subunit but have different γ subunit (Roof et al., 1985). Therefore, it is probably thes

Table 2
Tissue Distribution of G Protein-Coupled Phospholipases A_2

Tissue	Agonist	Toxin sens[a]	Ref[b]
Some Direct Evidence			
Thyroid	α_1 adrenergic	PT	1
Fibroblast	bradykinin	none, PT	2
	thrombin	PT	3
	ras gene product		4
Neutrophil	fMLP	PT	5
Mast cells	48/80, GTP	PT	6
Platelets	GTP	none	7
	thrombin	PT	8
Rod outer segments	Light	PT, CT	9
Cardiac myocytes	$\beta\gamma$	none	10
Macrophages	IgG_{2b}	none	11
	LPS		12
Mesangial cells	LPS	PT	13
Kidney proximal	angiotensin II	PT	14
tubule	bradykinin	PT	
Candidates			
Neuron, Aplysia	FRMFamide	PT	15
Kidney	α_1 adrenergic	none	16
Epithelium	bradykinin	none	17
Endothelium	LTC_4, LTD_4	PT	18
	TNF[c]	PT	19
Pineal gland	α_1 adrenergic	?	20

[a]PT, pertussis toxin; CT, cholera toxin.

[b]1. Burch et al., 1986; 2. Burch and Axelrod, 1987; Murayama and Ui, 1985; 3. Murayama and Ui, 1985; 4. Bar-Sagi and Feramisco, 1986; Bar-Sagi et al., 1988; 5. Bokach and Gilman, 1984; Okajima and Ui, 1984; 6. Nakamura and Ui, 1984, 1985; 7. Nakashima et al., 1987b,1988; Fuse et al., 1987; 8. Nakashima et al., 1987a; 9. Jelsema, 1987; Jelsema and Axelrod, 1987; 10. Kim et al., 1989; 11. Burch, 1987; 12. Coffee et al., 1988; 13. Wang et al., 1988; 14. Welsh et al., 1988; 15. Piomelli et al., 1987; Volterra et al., 1988; 16. Slivka and Insel, 1987; 17. Slivka and Insel, 1988; 18. Clark et al., 1986; 19. Clark et al., 1988; 20. Ho and Klein, 1987.

[c]TNF, tumor necrosis factor.

β subunit that is responsible for stimulating PLA_2. If true, this would implicate most G proteins in activation of PLA_2 (*see below*).

In addition to $\beta\gamma$ subunits, α subunits may also stimulate PLA_2 in rod outer segments. For example, $G_{o\alpha}$ stimulates PLA_2 when added with GTPγS (Jelsema et al., 1989), and this stimulation was additive with stimulation of $G_{t\beta\gamma}$ but not $G_{o\beta\gamma}$. $G_{i\alpha}$ also stimulated PLA_2 (Jelsema et al., 1989).

In another tissue, $\beta\gamma$ added to inside-out patches from cultured neonatal rat atrial myocytes activated a 40 pS potassium channel termed $I_{K.ACh}$, apparently by stimulating synthesis of a 5-lipoxygenase metabolite (Kim et al., 1989). Preincubation of the patches with anti-PLA_2,

blocked activation of the channel by βγ. Anti-PLA_2 did not block activation of the channel by GTPγS or GTP plus acetylcholine, suggesting that the channel can also be activated directly or indirectly by an α subunit. To rule out a PLA_2 contaminant in the βγ preparation, the subunit preparation was incubated with anti-PLA_2, which did not block activation of the channel. Since in these experiments free calcium was buffered to about 10 n*M*, the PLA_2 being activated does not require high calcium concentration.

Neither anti-PLA_2 nor lipoxygenase inhibitors affected acetylcholine-induced opening of the channel, suggesting that it can also be activated by the α subunit. The physiological significance of the βγ-PLA_2-5-lipoxygenase activation of $I_{K.ACh}$ will await confirmation by the finding in heart of an agonist that increases arachidonate release and formation of 5-lipoxygenase metabolites.

Since βγ activation of PLA_2 occurs in the presence of GTPγS, which should preclude reassociation of α with βγ subunits, an effect of these subunits to bind to and remove an inhibitory α subunit is precluded. A direct effect of the βγ subunit on PLA_2 has yet to be demonstrated (and has yet to be rigorously attempted). Calpactin or lipocortin is a PLA_2 inhibitory protein (Huang et al., 1986; Davidson et al., 1987) with a high affinity for calcium. In certain purification systems, calpactin copurifies with G proteins, suggesting the possibility of tight association of the proteins. Association of βγ subunits with another calcium-binding protein, calmodulin, has been reported (Katada et al., 1987). Because calpactin is found in rod outer segments, an experiment was performed in which soluble pancreatic PLA_2 was assayed in the presence of placental calpactin (Jelsema et al., 1989). In the assay, calpactin inhibited PLA_2 40%. G_t or G_o βγ subunits blocked calpactin-induced inhibition. Thus, βγ subunits may stimulate PLA_2 not directly, but by blocking a PLA_2 inhibitor. Actin is another protein that binds calpactin tightly (Glenney, 1986). Like βγ subunits, actin also blocked the ability of calpactin to inhibit PLA_2 (Jelsema et al., 1989). Curiously, in the same *in vitro* system, $G_{t\alpha}$, $G_{o\alpha}$, and $G_{s\alpha}$ all further augmented calpactin inhibition of PLA_2 (Jelsema et al., 1989).

The finding that βγ subunits can activate PLA_2 whereas the α subunit activates another effector provides further possibility for diversity in neurotransmitter/hormone action, even when a receptor couples to only a single G protein (Fig. 4). It also suggests that activation of any G protein should release βγ subunits and stimulate PLA_2. Clearly, this does not occur. We have not been able to find in any system evidence that activation of G_s results in PLA_2 activation with a time course consistent with a βγ mechanism. Of course, the existence of multiple β and γ subunits allows for many potential combinations of βγ. Thus, the physiological significance of dual activation of effectors by a single G protein remains to be determined. Because in some cells a single receptor activated two distinct G proteins (Fig. 4) (the example of pertussis toxin sensitivity of PLA_2 and insensitivity of phospholipase C in FRTL5 cells) either α subunits can activate PLA_2 as well, or, the two G proteins are compartmentalized in the cell.

Reconstitution

We have reported that bradykinin stimulated arachidonate release and inositol phosphate formation in Swiss 3T3 cells by coupling to PLA_2 and phosphatidylinositol-specific phospholipase C, respectively (Burch and Axelrod, 1987). In this cell line, pertussis toxin did not block either second messenger pathway. However, evidence was presented to suggest that both enzymes are coupled to the receptor by G proteins. If GTPγS was introduced into the cytosol by transient permeabilization with hypoosmotic shock, both arachidonate release and inositol phosphate formation were stimulated. As with other cells, addition of neomycin blocked

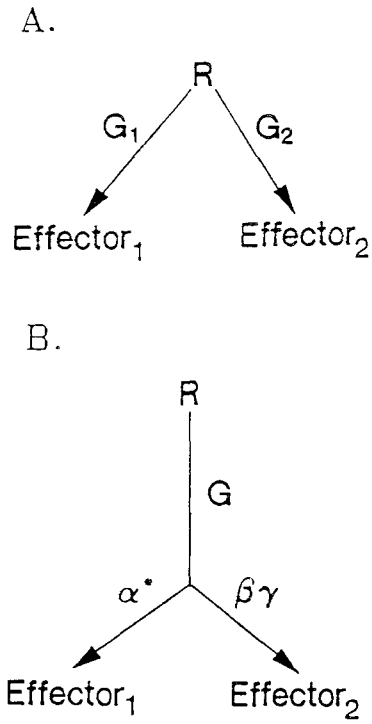

Fig. 4. Multiple effector activation by G proteins. **A** In this model, a single receptor subtype couples to two distinct G proteins (e. g., Ashkenzi et al., 1987). **B** In this model, a receptor activates a single G protein. The activated α subunit couples to one effector, whereas the βγ subunit couples to a second effector (e. g., Jelsema, 1987).

inositol phosphate formation but not arachidonate release. In membranes prepared from these cells, bradykinin alone did not significantly increase PLA_2 activity whereas GTPγS did. However, stimulation was small (Burch and Axelrod, 1987).

Other studies have shown that another murine fibroblast line, the SV-40 transformed Balb/c 3T3 line, SV-T_2, has many more bradykinin receptors per cell (about 3000) than Swiss 3T3 fibroblasts (Burch et al., 1988). In these cells, the bradykinin receptor is coupled to a G protein, as demonstrated by the stimulation of GTPase activity upon addition of bradykinin to a cell membrane preparation (Burch et al., 1988). We have found that in SV-T_2 cells, bradykinin stimulated PLA_2 and phosphatidylinositol-specific phospholipase C through pertussis toxin-insensitive G proteins, similar to Swiss 3T3 cells (unpublished).

In an effort to take advantage of the increased density of bradykinin receptors on SV-T_2 cells, we worked to develop a more useful method to permeabilize the cells than the hypoosmotic shock method we used in the past. Digitonin has allowed us to observe GTPγS-stimulated PLA_2 activity in stably-permeabilized cells. Addition of digitonin in a high K^+"intracellular" medium to stably permeabilize the cells, allowed us to add exogenous radiolabeled substrates and GTP analogs to the cells. When calcium was buffered to 10 n*M* by EGTA, GTPγS stimulated PLA_2 (Fig. 5). Bradykinin was ineffective in activating PLA_2 in the absence of GTPγS, but synergized with GTPγS to activate PLA_2. Increasing free calcium also activated PLA_2 (Fig. 6), and GTPγS shifted the dose-response to lower calcium concentrations, consistent with an effect to lower the calcium requirement of PLA_2.

The permeabilized cell system may allow us to reconstitute the receptor-G protein–effector

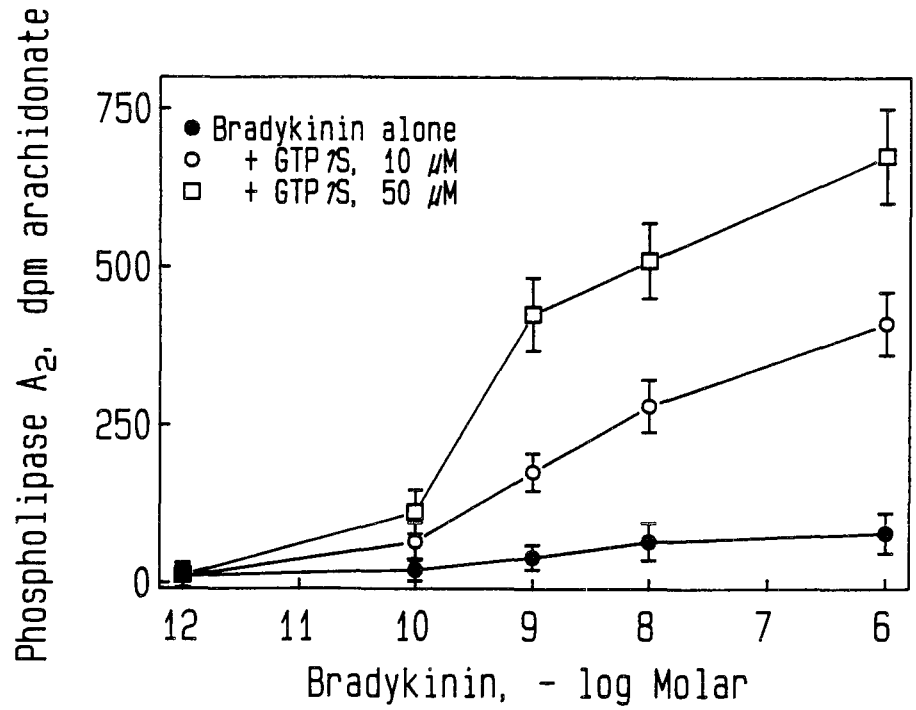

Fig. 5. Bradykinin and GTPγS stimulate PLA_2 in permeabilized fibroblasts. SV-T_2 fibroblasts grown as described (Burch et al., 1988) were dissociated from culture dishes by the addition of 2 m*M* EDTA to Ca^{2+}, Mg^{2+}-free Hanks balanced salt solution. Cells were pelleted then resuspended at 2 x 10^5 cells/mL in KCl (145 m*M*), Hepes (10 m*M*), MgCl2 (5 m*M*), ATP (5 m*M*), EGTA (1 m*M*), and free Ca^{2+} of 10 n*M* (assessed with a calcium electrode, and digitonin (50 μg/mL) added for 10 min at 37°C (resulting in >90% of cells taking up trypan blue). 1-Stearoyl-2-[3H] arachidonyl-phosphatidylcholine (100,000 dpm, 100 μM) was added along with bradykinin or GTPγS and the suspensions incubated at 37°C for 15 min. Reactions were terminated with the addition of 2 mL isopropanol:heptane:acetic acid (40:10:1), vortexing, addition of 1 mL heptane, vortexing, and centrifugation to separate phases. The upper layer was collected, 100 mg silica gel (200 mesh) was added, vortexed, and centrifuged. An aliquot of the supernatant was collected and counted by liquid scintillation spectrometry.

system. Toward this end we have successfully covalently inactivated the endogenous PLA_2 using *p*-bromophenacylbromide. This treatment did not affect bradykinin-induced GTPase activity (Burch, unpublished). We shall next attempt to add back to the system a purified PLA_2. If we identify a cell line with a pertussis toxin-sensitive G protein coupled to PLA_2, we may also be able to reconstitute the coupler.

Summary

In this report, I have presented some of the data supporting a role for G proteins in coupling receptors to PLA_2. At this time, indirect evidence suggests that G_α subunits can activate PLA_2 in a "conventional" manner, whereas at least two studies have provided direct evidence that βγ subunits can activate PLA_2. Whether βγ subunits interact with the PLA_2 enzyme or prevent an inhibitor from interacting with it is unclear. If βγ subunits are physiologically relevant, then the control mechanisms for prevention of activation of PLA_2 by activation of all G proteins are still completely unidentified. The diversity of receptors and tissues in which such coupling occurs is only beginning to be appreciated. Given the variety of potential substrates and competing neurotransmitter and hormone-regulated pathways that may give rise to arachidonate, some time will be required to fully

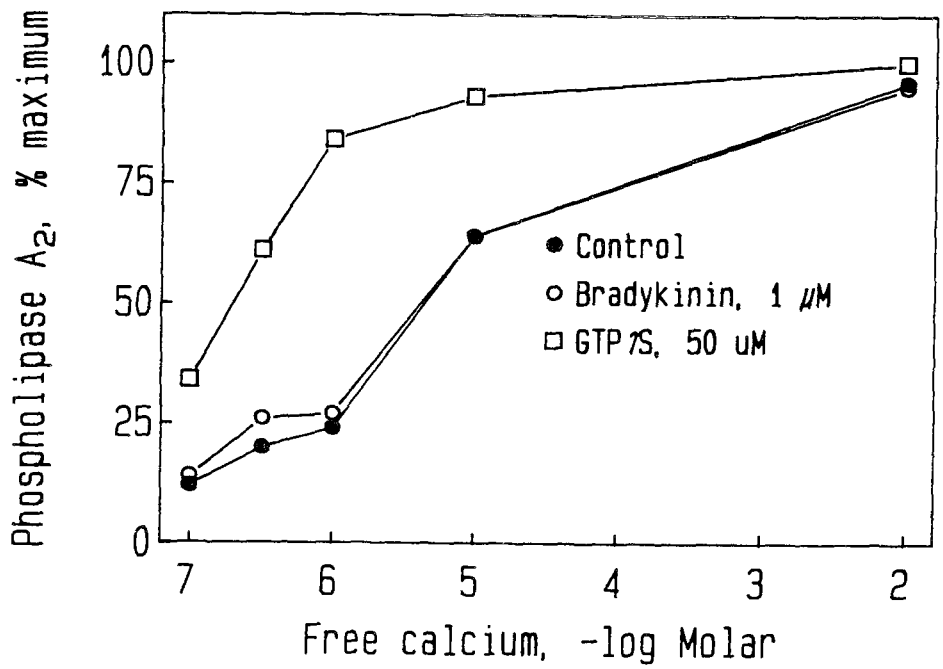

Fig. 6. GTPγS decreases calcium requirement for PLA_2 activity in permeabilized fibroblasts. Cells were permeabilized in the presence of the indicated concentrations of Ca^{2+}, then incubated as decribed in the legend for Fig. 5.

understand the system. Finally, it must be emphasized that until simple reconstitution systems become available, it will remain undemonstrated that the coupling G protein interacts with PLA_2 *directly*.

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