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The role of G-proteins in coupling cell surface receptors to phospholipase C: Studies in *Xenopus* oocytes

Moriarty, Thomas Michael, Ph.D.

City University of New York, 1990

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THE ROLE OF G-PROTEINS IN COUPLING CELL SURFACE RECEPTORS TO PHOSPHOLIPASE C: STUDIES IN *XENOPUS* OOCYTES

by

Thomas M. Moriarty

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment for the degree of Doctor of Philosophy, The City University of New York.

1990

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

THE ROLE OF G-PROTEINS IN COUPLING CELL SURFACE RECEPTORS TO PHOSPHOLIPASE C: STUDIES IN *XENOPUS* OOCYTES

by

Thomas M. Moriarty

Adviser: Emmanuel M. Landau, M.D., Ph.D.

G-proteins are a family of guanine nucleotide-binding regulatory proteins that serve to transduce extracellular signals from cell surface receptors across the plasma membrane to intracellular effector systems. The G-proteins are cytoplasmic, membrane-associated heterotrimers composed of α, β and γ subunits. Many G-proteins have been purified and cloned, but only two have undisputed functions: G_s for activation of adenylyl cyclase and G_t for activation of the retinal cGMP phosphodiesterase.

Phospholipase C is the effector enzyme of the inositol phospholipid second messenger system. It is found in all cell types and can be stimulated by a number of extracellular signals, including neurotransmitters, hormones and growth factors. Phospholipase C controls various cellular processes, including excitation, contraction, secretion, metabolism and cell growth and differentiation, through the bifurcating second messenger cascades, IP_3 and diacylglycerol. Recent evidence has implicated a role for G-proteins

in coupling receptors to phospholipase C. The evidence also suggests there exists two independent pathways: one sensitive to pertussis toxin and one insensitive. The studies described in this thesis help to elucidate the role of G-proteins in stimulation of phospholipase C.

The receptor evoked IP_3-dependent Cl^- conductance of the *Xenopus* oocyte was used as a model system of the receptor regulated phospholipase C. Receptor stimulation was by either the native oocyte muscarinic receptor or by various receptors expressed in the oocyte after injection of exogenous mRNA. Using pertussis toxin and purified G-protein subunits, the muscarinic receptor stimulated phospholipase C was found to be dependent on a pertussis toxin-sensitive G-protein and to be activated by the subunit dissociation mechanism proposed for the adenylyl cyclase system. Using purified G-protein heterotrimers and GTPγS-activated α-subunits, the 39 kD G-protein G_o was found to be uniquely capable of serving as the signal transducer of the pertussis toxin-sensitive receptor stimulated phospholipase C. Studies with receptors from liver and brain expressed in the oocyte demonstrate the capability of some receptors to couple to both the pertussis toxin-sensitive and toxin-insensitive pathways, whereas others do not have this ability to crosstalk. This also suggests that the primary structure of the G-protein serving the pertussis toxin insensitive pathway is probably quite similar to G_o, possibly differing by only one amino acid.

FORMAT OF THESIS

This thesis is prepared according to the new guidelines of the City University of New York which permit the direct incorporation of published research articles as chapters. The thesis has a general introduction, several papers as chapters and a general discussion. Each chapter contains specific introductions, materials and methods, discussions and references. The references for the introduction and discussion follow the discussion. Copyright permission for each chapter has been obtained from the respective publisher.

DEDICATION

This, as with everything I do,
is dedicated to my wife, Erin, and my father and mother.

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This thesis is the result of fruitful collaborative research.
I owe a great deal of gratitude to many individuals for many things, especially:

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INTRODUCTION

The immature oocyte of the African clawed frog *Xenopus laevis* has emerged as a major tool for the elucidation of the molecular basis of cellular communication (Dascal, 1987a, Lester, 1988, Levitan, 1988, Snutch, 1988). Gurdon's observations that the oocyte can be used as a high-fidelity *in vivo* translation system (Gurdon *et al.*, 1971, Lane *et al.*, 1971) has led to the development of a variety of uses for the oocyte. Perhaps the most powerful is the use of the oocyte as a membrane source that is functionally coupled to cellular effectors. Structurally defined foreign proteins can be introduced into the oocyte, by translation of mRNA or by direct injection of pure protein, then analyzed for function.

The oocyte is particularly suited to the study of signal transduction. Endogenous receptors which couple to the two primary second messenger systems, adenylyl cyclase and phospholipase C, have been identified and characterized (Kusano *et al.*, 1977, Dascal and Landau, 1980, Lotan *et al.*, 1982, VanRhenterghem *et al.*, 1984, for review see Dascal, 1987a, Lester, 1988). Foreign receptors which act on both of these systems can be "transplanted" into the oocyte by the expression of exogenous mRNA (Gundersen *et al.*, 1984). Most receptor types have been functionally demonstrated in the oocyte including voltage-activated channels, ligand-gated ion channels, and the receptors which couple to guanine nucleotide binding regulatory proteins (G-proteins) (Dascal, 1987a, Lester, 1988). Second messenger mediated pathways can be studied using

conventional biochemical techniques, however, the function of ligand-gated channels, such as $GABA_A$, or the receptor/G-protein regulated channels, such as the muscarinic regulated K^+ channels, can only be studied using sensitive electrophysiological methods. In that the oocyte can be readily analyzed biochemically *in vitro* and can be studied as an isolated *in vivo* electrophysiological preparation, it is uniquely suited to the study of direct and second messenger coupled receptor-effector pathways as well as the complex interactions of multiple receptor systems.

The pathways from receptor to activation of distal effectors via cAMP or inositol 1,4,5 -trisphosphate (IP_3), have been well characterized in the oocyte. This is especially true for the phosphatidyl inositol system. Activation of the oocyte's endogenous muscarinic receptors evokes a complex depolarization of the plasma membrane. A number of expressed exogenous receptors of the G-protein linked family have been demonstrated to activate this same depolarization event. Using both electrophysiology and biochemistry, it has been shown that the depolarization is due to an agonist induced turnover of phosphatidyl inositol 4,5 -bisphosphate to IP_3. This evoked release of IP_3 may be mediated by a G-protein(s). The IP_3 formed causes a release of Ca^{++} from intracellular stores. Ca^{++} sensitive Cl^- channels respond to this surge in intracellular Ca^{++} by opening. It has been shown that the magnitude of the macroscopic Cl^- current (the sum of the individual channel conductances) is proportional to the amount of IP_3 released. Therefore, in a single cell under voltage clamp, the Cl^- current can be used as a sensitive and easily monitored measure of IP_3

production and phospholipase C activity. Given that the oocyte 1) has an intact IP_3 mediated receptor pathway, 2) has the ability to express various other receptors which utilize this pathway, and 3) supports a variety of powerful experimental interventions (such as direct injection of substances into the cell), the oocyte affords a prime opportunity to discover the role of G-proteins in activation of phospholipase C.

This thesis describes studies aimed at understanding the role of G-proteins in coupling cell surface receptors to phospholipase C, the effector enzyme of a ubiquitous second messenger system. Chapter 1, the overview, reviews the current knowledge of G-proteins, the phosphatidyl inositol second messenger system and the use of oocytes to study signal transduction. Chapter 2 and 3 are preliminary studies which address basic properties and principles of electrophysiological research using oocytes. Chapter 2 documents the discovery of receptors for the peptidergic transmitters corticotropin releasing factor (CRF), arginine vasopressin (AVP) and cholecystokinin (CCK) in the native oocyte follicle. The nature of the evoked ionic conductances and the probable second messengers serving the responses are also considered. The techniques for studying coupling properties of receptors from various tissues by expression of exogenous mRNA in oocytes are described in Chapter 3. The CCK and bombesin (BBS) receptors from rat brain were studied. The ionic mechanism and the role of IP_3-mediated Ca^{++} release were also examined.

The role of G-proteins in signal transduction through phospholipase C was studied by taking advantage of the oocytes'

receptor-activated IP_3-mediated Cl^- conductance. The mechanism of signal transduction and the nature of the G-protein involved in the phosphatidyl inositol system were studied by using the native muscarinic receptor evoked Cl^- current, purified G-protein subunits and pertussis toxin. These studies are described in Chapter 4. G_o was identified as the putative signal transducer in the pertussis toxin-sensitive receptor-stimulated phospholipase C as described in Chapter 5. Chapter 6 considers the functional interaction of the two distinct G-protein coupled pathways for stimulation of phospholipase C, the pertussis toxin sensitive and toxin insensitive pathways, and provides data that allow for initial conclusions on the molecular nature of the signal transducer of the pertussis toxin-insensitive pathway. General conclusions and implications from these studies are described in Chapter 7.

CHAPTER 1

Overview

Parts of this chapter were originally published in the monograph *G proteins* (eds. R. Iyengar and L. Birnbaumer, 1990) and are reprinted with the permission of Academic Press, Inc.

A. G-proteins

Cells communicate by a variety of extracellular signal molecules. The signal is detected by cell membrane receptors and characteristic responses of the cell result. One class of receptors requires the intercession of a signal transducing protein to couple the receptor to intracellular effectors. These proteins are members of a family of membrane proteins termed the guanine nucleotide binding regulatory proteins: G-proteins (Stryer and Bourne, 1986; Iyengar and Birnbaumer, 1987; Gilman, 1987; Graziano and Gilman, 1987; Dolphin, 1987; Dunlap, *et al.*, 1987; Neer and Clapham, 1988; Freissmuth *et al.*, 1989).

A.1. Discovery

In 1971, Rodbell *et al.*, (1971b) working with the glucagon sensitive adenylyl cyclase of rat liver plasma membranes, demonstrated an absolute requirement of GTP for hormonal activation of adenylyl cyclase. Moreover, they showed that non-hydrolyzable analogs of GTP produce persistent activation of adenylyl cyclase. They also showed that "extraordinarily" low concentrations of guanyl nucleotides altered the binding of ^{125}I-glucagon (Rodbell *et al.*, 1971a). Studies by Gilman and co-workers on the β-adrenergic receptor showed that the effects of guanyl nucleotides on binding of hormone was specific for isoproterenol but not for propranolol (Macquire *et al.*, 1976); the guanyl nucleotides had no effect on binding of antagonists. This

specificity of action for the biologically active agent led to speculations on a functional role of guanine nucleotides in receptor activation of adenylyl cyclase.

Studies by Schramm and co-workers (Orly and Schramm, 1976, and Schramm *et al.*, 1977) using cell fusion techniques demonstrated that receptors were separate entities from the effector enzyme and could functionally couple to the effector systems from other cells. In the first experiments, catecholamine sensitive turkey erythrocytes, treated with N-ethylmaleimide to inactivate adenylyl cyclase, were fused by sendai virus with catecholamine insensitive Friend erythroleukaemia cells. The resulting "cell" had a catecholamine sensitive adenylyl cyclase. Subsequent studies showed that this transfer of receptors from one cell's cyclase to another's was not specific to cells of erythropoietic origin and also not specific to catecholamine receptors.

Two crucial points came from the laboratory of Cassel and Selinger. First, they showed catecholaminergic activation of adenylyl cyclase in turkey erythrocyte membranes was attended by concurrent GTPase activity (Cassel and Selinger,1976, Cassel and Selinger,1977). Next, they demonstrated that binding of agonist to hormone receptors stimulates GTP-GDP exchange (Cassel and Selinger, 1978).

In 1976, Pfeuffer and co-workers, knowing that guanine nucleotides were involved in certain receptor modulated activities, tried to obtain direct evidence for a GTP binding protein (Pfeuffer and Eckstein, 1976). They used detergent

extracts of pidgeon erythrocytes and GTP immobilized columns. Examining the flow-through effluent for adenylyl cyclase activity, they saw that a certain percentage of maximal activity was lost. They surmised that a critical ingredient required for activation of adenylyl cyclase was bound to the column: the presumed GTP binding protein.

It was not until after Henry Bourne *et al.* discovered a mutant of S49 lymphoma cells, the so-called cyc$^-$ mutant (Bourne, *et al.*, 1975), that it was possible to demonstrate more directly the existence of a GTP binding protein. The cyc$^-$ mutant has functional β-adrenergic receptors and adenylyl cyclase activity, but is not sensitive to hormone or guanine nucleotides. Ross and Gilman took advantage of this "natural laboratory". Using wild type S49 membranes heat treated to destroy adenylyl cyclase activity, they were able to functionally reconstitute hormone and guanine nucleotide sensitive cyclase in membranes from cyc- cells (Ross and Gilman, 1977). Shortly thereafter, the G-protein associated with adenylyl cyclase was purified (Northup, *et al.*, 1980).

A.2. Structure and function

In 1983, a separate G-protein necessary for inhibition of adenylyl cyclase was purified (Bokoch *et al.*, 1983). It was not until Sternweis purified yet another G-protein from brain in very large quantities (Sternweis and Robishaw, 1984) that the possibility of multiple G-proteins and multiple G-protein

regulated functions was considered. Research over the past ten years has allowed a definition of a family of G-proteins. The G-protein is an information transducer which carries extracellular information across the plasma membrane from receptors to effectors. The members of the class of receptors that require a G-protein intermediary is quite large and includes receptors for hormones, classical neurotransmitters, neuropeptides, light and odorants. The effector systems known to be modulated by G-proteins are adenylyl cyclase and retinal cGMP phosphodiesterase. There is strong evidence that G-proteins directly modulate certain ion channels and phospholipase C. However, these findings are still somewhat controversial and more work will be necessary to establish firmly the role of G-proteins in these systems (Gilman, 1987; Casey and Gilman, 1988; Brown *et al.*, 1989).

A G-protein is a plasma membrane associated heterotrimeric protein with α (36-52 kDa), β (35-36 kDa) and γ (8-10 kDa) subunits. Six classes of G-proteins have been described: G_s, G_i, G_o, G_t, G_z and G_{olf}. Protein purification has led to the identification of four α_s-subunits, three α_i, two α_o, two α_t and one each α_{43}, α_{olf}, α_z. Two β-subunits have been identified as well as a number of γ-subunits. Molecular cloning has led to the identification of the primary structure of four α_s, three α_i, the α_o, α_z, α_{olf}, both α_t-subunits, two β-subunits and one γ-subunit. All known α-subunits have a high-affinity binding site for guanine nucleotides, intrinsic GTPase activity and a site for ADP-ribosylation by cholera toxin and/or pertussis toxin (except α_z).

The α-subunit is hydrophilic after detergent solubilization. The β-subunit is generally considered to be part of a βγ complex in that the β-subunit can be separated from the γ-subunit only under harsh denaturing conditions. It is not thought to dissociate from the γ-subunit under physiological conditions. The βγ-subunits are generally hydrophobic. In that the known cDNAs of the β–subunit do not predict a hydrophobic molecule, it is thought that the γ-subunit confers this characteristic on the βγ-subunit. Whereas subtle differences in the α-subunits define unique identity for the G-protein, the βγ-subunits appear to have remarkable similarity among all known G-proteins. βγ-subunits from different G-proteins have even been shown to be functionally interchangeable in the reconstituted adenylyl cyclase system.

The criteria for involvement of a G-protein in a signal transduction system has been summarized by Gilman (Gilman, 1987) and Stryer (Stryer and Bourne, 1986):

1. agonist requires GTP for activation of the response in question
2. response can be initiated independent of agonist by nonhydrolyzable analogs of GTP (GTPγS or Gpp[NH]p)
3. there is a negative heterotropic interaction between binding of agonist and binding of GTP in a G-protein linked receptor
4. G-proteins are ADP-ribosylated by cholera toxin and/or pertussis toxin
5. "purification and reconstitution of individual components of a pathway is the ultimate criterion. This has been achieved

with the adenylyl cyclase complex and the retinal phosphodiesterase system" (Gilman, 1987).

A model has evolved which accounts for G-protein functions in receptor activation of adenylyl cyclase. It is analogous to the activation of retinal cGMP phosphodiesterase. (see Fig. 1, beginning at the top left). Agonist binds to a receptor. The receptor associates with a specific holo G-protein probably by binding to the α-subunit. The receptor-bound G-protein exchanges GDP for GTP and induces a conformational change in the associated receptor such that the affinity of the G-protein for the receptor is reduced. The GTP bound G-protein dissociates into the free Gα-GTP and the free βγ-subunit. The Gα-GTP subunit interacts directly with the effector (adenylyl cyclase). Intrinsic GTPase activity of the α-subunits hydrolyzes the GTP to GDP thereby terminating the reaction with the effector. The Gα-GDP binds to the free βγ and ends the cycle.

Despite the aforementioned model for the adenylyl cyclase system, the role of G-protein subunits is still unknown. When examined in solution, the G-protein dissociates upon activation into α- and βγ-subunits, but this has not been proven to occur *in vivo* in the cell (Gilman, 1987). Subunit dissociation has been observed, however, in liver membrane preparations (Iyengar *et al.*, 1988). The question of which subunit interacts with the effector has been the subject of much controversy (Birnbaumer, 1987; Bourne, 1987; Codina *et al.*, 1987; Logothetis *et al.*, 1987; Bourne, 1989). It is well known that $G\alpha_s$ will stimulate adenylyl cyclase

(Smigel *et al.*, 1984; Gilman, 1987) and that $G\alpha_t$ will stimulate retinal cGMP phosphodiesterase (Stryer and Bourne, 1986; Gilman, 1987). It has been shown that $G\alpha_i$ can inhibit the catalytic unit of adenylyl cyclase (Smigel *et al.*, 1984; Gilman, 1987). The $\beta\gamma$-subunit has been shown (Smigel *et al.*, 1984; Gilman, 1987), and confirmed (Neer *et al.*, 1987; Cerione *et al.*, 1987), to attenuate $G\alpha_s$ activation of cyclase by favoring re-association of α and $\beta\gamma$-subunits and by increasing the affinity of the α-subunit for GDP. The $\beta\gamma$-subunit has also been demonstrated to directly activate retinal phospholipase A_2 (Jelsema and Axelrod, 1987). Logthesis *et al.*, in two very convincing papers (Logothetis *et al.*, 1987, and see Neer and Clapham, 1988), documented direct activation of the muscarinic-gated K^+ channel ($I_{K\ ACh}$) by $\beta\gamma$-subunits. On the other hand, Codina *et al.* have shown that $I_{K\ ACh}$ is activated by resolved α-subunits at concentrations much lower than that needed with $\beta\gamma$-subunits (Codina *et al.*, 1987), that recombinant α_i proteins (ie., no contaminants) open $I_{K\ ACh}$ (Yatani *et al.*, 1988a) and that a monoclonal anti-α_i antibody blocks G-protein activation of $I_{K\ ACh}$ (Yatani, *et al*, 1988b). The most recent work by the proponents of $\beta\gamma$-subunit activation of $I_{K\ ACh}$ demonstrates that $\beta\gamma$-subunits indirectly activate $I_{K\ ACh}$ by direct stimulation of phospholipase A_2 and subsequent generation of arachidonic acid and metabolites of the 5-lipoxygenase pathway (Kim *et al.*, 1989). The issue is not entirely settled, however by the "orthodox" view of G-protein action, it is currently agreed that the α-subunit directly interacts with the effector (Bourne, 1989).

B. The phosphatidyl inositol second messenger system

Signal transduction is accomplished by a very limited number of intracellular messenger systems. The phosphatidyl inositol system has emerged as one of the more important mechanisms of cell-cell communication in the brain (Berridge and Irvine, 1984; Berridge, 1987; Berridge and Irvine, 1989). The list of receptor types known to act via polyphosphoinositide breakdown is extensive and includes: muscarinic, α_1-adrenergic, glutamate, thrombin, serotonin, TRH, substance P, CCK, neurotensin, V_1-AVP and bombesin. The "turn key" enzyme of the system, phospholipase C, has been found in all cell types (Shukla, 1982).

Inositol phospholipids constitute about one percent of the cellular phospholipid pool. Polyphosphoinositides, which are just a few percent of the inositol phospholipid pool, exist in the brain as the phosphorylated phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4, 5-diphosphate (PIP_2). The polyphosphoinositides exist in two conditions: a metabolically stabile pool associated with myelin and a metabolically labile pool found in the neuronal cell membranes. The latter pool is compartmentalized into a hormone-sensitive pool and a hormone-insensitive pool. There appears to be some cycling between the hormone sensitive and insensitive pools (Berridge, 1987).

The diphosphorylated PIP_2 is a substrate for phospholipase C (Fig. 2). Activation of phospholipase C generates two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol

(DAG) (Berridge, 1987). IP_3 is released into the cytosol, binds to receptors on endoplasmic reticulum and causes the release of stored Ca^{++}. DAG remains membrane-bound and activates protein kinase C in the presence of Ca^{++}. IP_3 is metabolized by seqential dephosphorylations. DAG is metabolized either by phosphorylation to phosphatidic acid or by sequential hydrolysis reactions to monoacylglycerol and then to arachidonic acid.

C. G-proteins and phospholipase C

Recent evidence has suggested that receptors acting through the phosphatidyl inositol system may utilize a G-protein (for review see Gilman 1987; Cockroft, 1987; Berridge, 1987; Taylor & Merritt, 1987; Casey and Gilman, 1988; Berridge and Irvine, 1989). The evidence comes from a variety of sources. The earliest evidence came from Gomperts (1983) who showed that the non-hydrolyzable analog of GTP, GTPγS, could initiate exocytosis of histamine in mast cells, a well-defined Ca^{++} dependent process. Halsam and Davidson (1984) showed that GTPγS promoted the formation of DAG in permeabilized platelets which suggested that guanine nucleotides had a role in activation of phospholipase C. Direct evidence for guanine nucleotide regulation of phospholipase C came from two groups. Litosch *et al.* (1985) measured an increased production of IP_3 from blowfly salivary gland plasma membranes in the presence of 5-HT and GTP. 5-HT alone did not produce any IP_3. The non-hydrolyzable analogs of GTP stimulated IP_3 production alone and potentiated

the stimulation due to 5-HT. Cockroft and Gomperts (1985) demonstrated that the guanine nucleotide effect on mast cell degranulation they had reported in 1983 was inhibited by neomycin, a compound known to inhibit phospholipase C. Furthermore, they showed that the phospholipase C of human neutrophil plasma membranes could be directly activated by guanine nucleotides in the presence of physiological Ca^{++}. The effects of guanine nucleotides on phospholipase C have been confirmed in a number of other tissues including cerebral cortex, GH_3 cells, liver, pancreas and various hematopoietic cells (Gilman 1987; Cockroft, 1987; Berridge, 1987; Taylor & Merritt, 1987).

The effect of fluoride on G-proteins has been well-defined in the adenylyl cyclase system (Gilman, 1987). The active form of fluoride is believed to be a complex with Al^{3+}: $(AlF_4)^-$. $(AlF_4)^-$ acts as a surrogate phosphate on GDP, which masquerades as GTP, and fraudulently activates the G-protein. The effects of fluoride on receptors coupled to phospholipase C is analogous to that of the adenylyl cyclase system. Fluoride stimulates IP_3 formation in hepatocytes (Blackmore *et al.*, 1985) and activates phospholipase C in the pituitary GH_3 cell line (Martin *et al.*, 1986) and liver plasma membranes (Cockroft and Taylor, 1987). The effect in liver membranes is inhibited by GDPβS which further supports the contention that a "classical" G-protein is involved.

A major criteria for involvement of G-proteins in transmembrane signalling reactions is a negative heterotropic interaction between binding of guanine nucleotides and binding of agonist to the G-protein linked receptor. This was observed long

before it could be appreciated in Ca^{++} mobilization (ie., phospholipase C coupled) systems (for review see Taylor and Merritt, 1987). The observed data suggested the presence of two receptor binding sites: a low affinity site in the presence of GTP or analogs, and a high affinity site in their absence. More recently, in light of the existence of the G-protein family, it has been demonstrated that there is such a negative heterotropic interaction in receptor systems known to act through phospholipase C (Taylor and Merritt, 1987; Harden *et al.*, 1986; and Lynch *et al.*,1985).

The bacterial toxins from *Vibrio cholerae* and *Bordatella pertussis* have been very useful in providing further important information about the family of G-proteins. Cholera toxin has no effect on receptors coupled to phospholipase C which suggests that G_s, a substrate of cholera toxin, is not involved. Pertussis toxin, on the other hand, has yielded contradictory results. Pertussis toxin appears to inhibit hydrolysis of PIP_2 in human polymorphonuclear leukocytes (Ohta, 1985), mast cells (Nakamura and Ui, 1985) adipocytes (Moreno *et al.*, 1983) and neutrophils (Verghese *et al.*, 1985). It does not have any effect in hepatocytes (Uhing *et al.*, 1986), fibroblasts (Murayama and Ui, 1985) and astrocytoma cells (Masters *et al.*, 1986). These seemingly contrary results can be reconciled if there is more than one G-protein involved in signal transduction through phospholipase C.

In spite of this evidence for involvement of a G-protein in phospholipase C activation, the identity of the G-protein is

unknown and considerable doubt has been expressed by some as to whether a G-protein is involved in signal transduction through phospholipase C at all (Rhee *et al.*, 1989). If such a G-protein did exist, it should be possible to reconstitute a functional system using one of the known G-proteins with one of the purified phospholipase C's. This has not been reported to date (Gilman, 1987; Casey and Gilman, 1988; Rhee *et al.*, 1989). There is one report of a reconstituted f-Met-Leu-Phe sensitive phospholipase C in HL-60 cells using G_i and G_o after pertussis toxin inactivation of the native G-protein (Kikuchi *et al.*, 1986), however, this result has not yet been repeated. The bulk of the evidence for involvement of a G-proteins actually only supports the fact that there is a GTP dependent step somewhere along the pathway of phospholipase C activation from receptor to effector. There are many processes in the cell that require GTP, any one of which may impinge on this complex activation pathway. The evidence does not directly settle the issue of a "classical" G-protein of signal transduction.

D. *Xenopus* ooctes: electrophysiology and signal transducton

D.1. Oocyte morphology, membrane properties and electrophysiology

The oocytes used in these studies are harvested surgically from mature females under tricaine anesthesia. Each of the ovarian

lobes bears numerous oocytes arrested in first meiotic prophase (Maller and Krebs, 1980). These oocytes are in various stages of growth termed stage one through six by the convention of Dumont (1972). Only the largest, stage V and VI, are used for experiments. These cells have an average diameter of 1.2 - 1.3 mm and a volume of approximately 1 μl.

Fully grown oocytes are surrounded by a number a cellular and connective tissue layers (Dumont and Brummett, 1978). Closest to the oocyte surface is the fibrous vitelline membrane. This is surrounded by a layer of follicle cells. The follicle cells and the oocyte are connected by numerous gap junctions forming a functional syncytium (Brown *et al.*, 1979). The gap junctions allow for the inter-cellular transmission of small molecular weight constituents (< 1000 Da) and the direct electrical coupling of the oocyte and follicle cells. The follicular layer is surrounded by the theca, which contains blood vessels, nerve fibers and smooth muscle cells. An epithelial cell layer, which is a continuation of the surface of the ovary, forms the outermost layer. The oocyte with the external cells intact is known as a "follicular cell" or "follicle". These layers, except the vitelline, can be removed by treatment with collagenase. Such collagenase treated oocytes are known as "denuded oocytes" or simply "collagenase treated oocytes". Most electrophysiological studies are performed in either follicles or denuded oocytes. If multiple penetrations by microelectrodes and injector pipettes are anticipated, denuded oocytes are typically used. The vitelline layer can be removed with microforceps after incubating denuded oocytes in a hyperosmolar medium. This

treatment is necessary for patch clamp studies, but is not necessary for the more usual whole-cell voltage clamping.

Fully grown oocytes are remarkable for their bipolar coloration with one hemisphere nearly black and the other light yellow or beige. This pigment separation demarks a morphological and functional polarization (for review see Dascal, 1987a). The dark pole, the animal pole, is highest in melanin containing granules, Ca^{++} dependent Cl^- channels and specialized plasma membrane to endoplasmic reticulum membrane junctions. It also demonstrates a higher sensitivity to some iontophoretically applied transmitter substances (Kusano *et al.*, 1982; Oron *et al.*, 1988a). The light colored hemisphere, the vegetal pole, has the highest concentrations of yolk proteins and RNA.

To perform single cell electrophysiological experiments, it is necessary to know the concentrations and activities of relevant ions inside and outside of the cell. The constitution of the extracellular fluid is always known because the various bathing media are defined in the laboratory and applied to the cell in a manifold controlled perfusion apparatus. Several examples of physiological bathing solutions are summarized in Table 1. The activities of ions inside the cell are known from the literature (for review see Dascal, 1987a). Dascal summarizes the approximate values as: Ca^{++}, 0.1-0.4 μM; Na^+, 1-6 mM; K^+, 80-120 mM; and Cl^-, 44-62 mM.

The experiments described in this thesis utilize the two electrode voltage clamp technique. The other commonly used electrophysiological tool is the patch clamp technique. Both methods are based on the same principle: the function of the

membrane can be analyzed by uncoupling the mutually dependent current and voltage properties of the membrane. The voltage clamp monitors macroscopic currents which are the sum of the unit conductances through all open ion channels. The patch clamp isolates a patch of membrane and allows for the monitoring of the unit conductances of one, or just a few, ion channels.

The oocyte is an ideal cell for voltage clamp experiments (Fig. 3). Its spherical shape simplifies the mathematical assumptions of the theoretical circuit. Space clamping is easily achieved. It is large enough for the two electrode voltage clamp configuration. Its size also allows for experimental control of the intracellular environment by direct injection of substances into the cell. The extracellular environment, the bathing medium, is easily manipulated. The powerful tool of patch clamping can also be used with oocytes. Both the intracellular and extracellular faces of the patch are under direct experimental control. The advantage of the whole cell voltage clamp is that one may study multiple-component pathways in an intact living cell. Direct injection of carefully chosen biologicals into oocytes allows one to dissect and understand such complex pathways. The power of patch clamping is in analyzing the function of single ion channels.

The literature on the properties of the oocyte membrane at rest are also reviewed by Dascal (1987a). Resting potentials from -20 to -90 mV have been reported, however several sources of error contribute to this wide range. Each electrode penetration introduces a depolarization which partially recovers over several minutes. Any potential recorded by electrode will underestimate the actual

resting potential. Manual or enzymatic defolliculation is associated with a hyperpolarizing drop in potential which will recover partially over several hours. Therefore, healthy defolliculated oocytes hold a resting membrane potential of approximately -45 to -65 mV. Healthy follicles resting potentials are somewhat more positive. The input resistance, also underestimated by electrode penetration, is about 0.1 - 3 MΩ. The total capacitance of the oocyte is 230 nF with a specific capacitance of 4-7 $\mu F/cm^2$. The membrane selectivity of the oocyte at rest has also been investigated. At rest, the major monovalent ions are not at equilibrium. The approximate equilibrium potentials for these are: K^+, -100 mV; Na^+, +80 mV; and Cl^-, -25 mV.

D.2. Receptor activated-IP_3 mediated Cl^- conductance in *Xenopus* oocyte: native muscarinic receptor, transplanted receptors and general pathway

In 1977, Miledi and co-workers, in a study on the neurotransmitter sensitivity of undifferentiated progenitor cells, described an electrophysiological responsiveness of oocytes to acetylcholine (ACh) (Kusano *et al.*, 1977). They found that bath application of ACh to individual voltage-clamped oocyte follicles resulted in a complex depolarization of the membrane (Fig. 4A). In this brief seminal report, the authors addressed several key issues. They showed that the response was sensitive to ACh in a dose dependent manner, was evoked by muscarinic and mixed cholinergic agents and was antagonized by atropine, but not curare, α-

bungarotoxin or tetrodotoxin. They concluded that the receptors involved are muscarinic in nature. Treatment of the oocyte follicle with collagenase, which removes the enveloping follicular layers, did not abolish the response to ACh in most cells, suggesting that the cholinergic receptors are located in the oocyte membrane itself. Morphological examination of ACh sensitive oocytes after collagenase indeed showed that the follicle cells had been removed completely. Voltage clamp analysis of the ionic basis of the depolarization revealed that ACh caused a fall in membrane resistance and an inward flux of current. Ion substitution experiments and reversal potential determinations suggested that the current flux was carried primarily by Cl^- ions. Iontophoretic application of ACh showed a marked delay in activation of the response suggesting that either a number of receptors must bind ligand and interact in some fashion to initiate the response, or that the depolarization is mediated by an intracellular second messenger.

Oocytes injected with exogenous RNA will express sensitivity to various transmitter substances. The oocyte readily expresses receptors which activate a Cl^- conductance similar to that seen for the native muscarinic receptor (Fig. 4). These receptors include 5-HT_{1C}, (Lübbert *et al.*, 1987; Julius *et al.*, 1988) muscarinic cholinergic (Kubo *et al.*, 1986), glutamate (Sugiyama *et al.*, 1987), and several peptidergic transmitters (Hirono *et al.*, 1987; Masu *et al.*, 1987; McIntosh and Catt, 1987; Oron *et al.*, 1987; Myerhoff *et al.*, 1988; Williams *et al.*, 1988) . The accumulated evidence strongly favors a theory that such "transplanted" receptors couple to native Cl^- channels via the same biochemical pathway as the native

muscarinic receptor. The remainder of this review will treat the native muscarinic response and the response to transmitters seen in RNA injected cells as two manifestations of the same phenomenon. Examples will be drawn from the literature on both native and expressed receptors such that a general mechanism can be outlined.

Further studies on the muscarinic receptor evoked depolarization were aimed at understanding the underlying ionic and biochemical mediators of the response. The complex waveform was reduced to constituent components by Dascal and Landau (1980). They reported that not all components were seen in all oocytes, however the response could be generally broken down into four components (Fig. 4A). Application of agonist will evoke, after a few seconds delay, a fast transient inward current (D_1), followed by, or perhaps concurrent with, a slower inward conductance (D_2), which is succeeded in turn by a slow outward hyperpolarizing current (H). These three components may by superimposed with rapid fluctuational currents (F) of variable magnitude and duration. The slow outward H current is often overshadowed by the more substantial inward D_2 response, and therefore not readily apparent. There is an interesting seasonal variation in the presence of the various components. The D_1 current may disappear for the entire winter. However, with proper care of the frogs, including careful monitoring of light/dark cycle, water temperature and composition, and feed, these seasonal variations can be reduced (Dascal *et al.*, 1984). If the oocytes have been treated with collagenase, the response to ACh will most likely disappear, but can reappear after two or three days rest. The reasons for this are unclear, but it may

be due to non-specific proteolysis of existing muscarinic receptors during incubation with impure collagenase. The H response is always permanently abolished by defolliculation suggesting that either the intracellular or membrane mediators of the response lie in the follicular cells. Iontophoretic application of ACh to various parts of the ooctye has revealed that these cells exhibit a marked polarity with either the animal pole (Kusano *et al.,* 1982) or the vegetal pole (Oron *et al.*, 1988a) being considerably more sensitive to ACh than the opposite hemisphere.

In their original study, Kusano *et al.* (1977) suggested that Cl^- was the ion primarily responsible for the depolarization. Dascal and Landau demonstrated that the reversal potential of the D_1-component was about -22 mV (Dascal and Landau, 1982) which was very close to the equilibrium potential of Cl^- in the oocyte (Barish, 1983). They also showed that this reversal potential depends on $[Cl^-]_{out}$ in a Nernstian fashion (Dascal *et al.*, 1984). Varying $[K^+]_{out}$ or $[Na^+]_{out}$ did not effect the reversal potential. Taken together, these data strongly suggest that the D_1-component is carried almost exclusively by Cl^- ions. The D_2 component was shown to have a somewhat more negative reversal potential and a partial dependence on $[K^+]_{out}$. (Dascal *et al.*, 1984). This is consistent with a simultaneously increased conductance to Cl^- and K^+ ions due to an overlapping of the D_2 and H components. Intracellular injection of TEA or removal of the surrounding follicular cells will eliminate the K^+ conductance "contamination" of the D_2 response. Therefore, in collagenase treated cells, both the D_1 and D_2 components are strictly carried by Cl^-. The H component has been shown to be

primarily an outward K^+ current (Dascal *et al.*, 1984). The fluctuational F component disappears if the cell is voltage clamped at the Cl^- equilibrium potential which indicates that these fluctuations are Cl^- currents. In summary, the response to ACh is a complex, time restricted increase in conductance to Cl^- and K^+ions. If collagenase treated cells are used, the entire waveform is due to the opening and closing of Cl^- channels.

The receptors responsible for the ACh response are muscarinic cholinergic (Kusano *et al.*, 1977; Dascal and Landau, 1982). Muscarinic and mixed muscarinic agents will evoke the response; atropine will antagonist the response. VanWezenbeek *et al.* (1988) show that the endogenous muscarinic response is antagonized by muscarinic antagonists in the following rank order of potency: 4-DAMP > pirenzipine > AF-DX 116 which identifies the receptor as of the M_3 subtype by the convention of Doods *et al.* (1987).

Dascal and Landau (1982) analyzed the dose-response characteristics of the D_1-component and found that either a cooperativity of binding, or a cooperation of binding to a downstream biochemical event, was necessary. They demonstrated two categories of binding site which they called the low sensitivity site, with an approximate K_d of about 0.2 μM, and the high sensitivity with approximate K_d of 0.03 μM. Individual cells expressed either/or both receptor type with the low sensitivity being most common. The existence of two apparent binding affinities is similar to that seen in the "superhigh" and "high" affinity muscarinic cholinergic binding sites in brain (Birdsall *et al.*, 1980). Later studies by Dascal and Cohen (1987b) on the muscarinic response found that the apparent K_d

is also approximately 0.4 μM for the D_2 and H components. They concluded that all major components of the complex response are a result of ACh binding to the same receptor, but are mediated by different post receptor effectors.

In 1977, Miledi and co-workers suggested, on the basis of the time course after iontophoresis, that the muscarinic response might be mediated by a second messenger (Kusano *et al.*, 1977). Since then, the biochemical steps between binding of agonist and the activation of current have been examined in detail. The following pathway is presently understood for both the native muscarinic receptor and certain expressed receptors (Fig. 5). After binding agonist, the receptor couples to a G-protein to activate phospholipase C. The lipase breaks down phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. The liberated IP_3 binds to receptors on endoplasmic reticulum to cause a release of Ca^{++} into the cytosol. The coordinated release of Ca^{++} opens populations of Ca^{++} sensitive Cl^- channels in the plasma membrane and gives rise to the complex macroscopic current seen in voltage clamp. A possible role for calmodulin has also been suggested. Each step in this pathway will now be considered in detail. The role of G-proteins in this response will be discussed in a separate section.

The function of phospholipase C in the muscarinic response was first addressed by Oron *et al.* (1985) in which they demonstrated a direct link between phosphoinositide metabolism and a transmitter induced physiological response. Oocytes prelabeled with ^{3}H-inositol were used to demonstrated cholinergic

stimulation of phosphatidylinositol 4,5 -bisphosphate breakdown to inositol 1,4,5-trisphosphate, inositol 1,4-bisphosphate and inositol 1-phosphate, within 30-120 s after challenge with agonist. Similar hydrolysis of phosphatidylinositol 4,5 -bisphosphate was demonstrated in RNA injected cells in response to ACh (Nomura *et al.*, 1987), 5-HT (Nomura *et al.*, 1987), thyrotropin-releasing hormone (McIntosh and Catt, 1987; Oron *et al.*, 1988b), and angiotensin II (McIntosh and Catt, 1987).

Direct injection of IP_3 into the oocyte evokes a depolarization with the characteristic waveform and current-voltage properties of the native muscarinic response (Oron *et al.,* 1985; Nadler *et al.*, 1986; Parker and Miledi, 1986; Gillo *et al.,* 1987) (Fig. 6). Studies on injection of IP_3 show that the site of injection can alter the response (Gillo *et al.*, 1987). Shallow injections of IP_3 give a response with prominent D_1 and D_2 components. Deeper injections give a response with a diminished D_1 and a more marked D_2. A study by Gillo *et al.* (1987), with careful control of the location of the injector pipette, demonstrated that the response to injected IP_3 is dose dependent. It has also been shown that the animal hemisphere is more sensitive to IP_3 injections (Berridge, 1988).

IP_3 activates the depolarization by mobilizing intracellular stores of Ca^{++}. Application of ACh to native oocytes or injection of IP_3 will evoke a release of $^{45}Ca^{++}$ from preloaded cells that has a time course similar to the membrane electrical response (Nadler *et al.*, 1986, Dascal *et al.*, 1985a). This is also true for expressed receptors (Oron *et al.*, 1988b, Williams *et al.,* 1988). A rise in intracellular Ca^{++} has been demonstrated after injection of IP_3 with

Ca^{++} sensitive electrodes (Busa *et al.*, 1985) and after application of agonist by fura-2 fluorescence (Takahashi *et al.*, 1987). Depletion of stored Ca^{++} with ionophore A23187 prevents transmitter and IP_3 evoked membrane responses (Gillo *et al.*, 1987). The responses to ACh or IP_3 are not abolished by depleting the extracellular fluid of Ca^{++} which indicates that the response is not due exclusively to an influx of Ca^{++} (Dascal *et al.*, 1985a; Parker and Miledi, 1986; Gillo *et al.*, 1987). Some reduction in amplitude of both the D_1 and D_2 components has been observed in Ca^{++} free media with the D_2 component being more sensitive or even abolished (Dascal *et al.*, 1984; Dascal *et al.*, 1985a; Snyder *et al.*, 1988). Intracellular injection of the Ca^{++} chelator EGTA will block the response to agonist (Dascal *et al.*, 1985a) or IP_3. Injection of Ca^{++} directly into the cell evokes either a single fast D_1-like response or a biphasic (D_1 and D_2) response carried by Cl^- ions (Miledi and Parker, 1984; Dascal *et al.*, 1985a; Gillo *et al.*, 1987). The magnitude of the Ca^{++} evoked current is dose dependent (Dascal *et al.*, 1985a). A role for calmodulin in mediating the Ca^{++} evoked Cl^- current has been suggested by the observed inhibition of both the muscarinic response and the response to injected Ca^{++} by the calmodulin inhibitor trifluoperazine. (Dascal *et al.*, 1985a; Ito *et al.*, 1988).

Dascal *et al.* (1985a) propose that there are different pools of Ca^{++} which subserve the two depolarizing components (D_1 and D_2) of the response. The F component may be due to a Ca^{++} dependent Ca^{++} release phenomenon (Gillo *et al.*, 1987; Berridge, 1988) similar to that described in other cell types (Fabiato and Fabiato, 1975). Low concentrations of agonist will induce primarily the oscillatory

response as will low doses of injected IP_3. Berridge proposes that an IP_3 sensitive pool of Ca^{++} releases Ca^{++} in response to IP_3. This increase in cytosolic Ca^{++} leads to increased cellular uptake of Ca^{++} by an IP_3 insensitive pool. The overloading of this latter pool is the trigger which causes it to release Ca^{++} back into the cytosol. Ca^{++} will again be sequestered and again released until some equilibrium prevails. The complex electrical response of the membrane is produced by Ca^{++} sensitive Cl^- channels passively responding to the changing tides of intracellular Ca^{++} concentration.

Ca^{++} sensitive Cl^- channels which carry the depolarizing response were first seen separately by Miledi (1982) and Barish (1983). They observed a rapidly inactivating transient outward ("T_{out}") Cl^- current in response to a voltage step from rest to more positive than -20 mV. This current is dependent on the influx of extracellular Ca^{++} and is sensitive to the concentration of $[Ca^{++}]_{out}$. It can be blocked by replacing external Ca^{++} with either Ba^{++}, Sr^{++}, Mg^{++} or Mn^{++}, or by injecting cells with EGTA (Miledi, 82; Barish, 1983; Miledi and Parker, 1984). The most likely explanation of this transient outward current is a voltage sensitive Ca^{++} channel in the plasma membrane which allows the entry of a sufficient quantity of Ca^{++} into the cytosol to activate the Ca^{++} dependent Cl^- channels.

From these observations, the Ca^{++} injection studies and the proposed role of Ca^{++} in receptor activation of Cl^- current, it was assumed that there existed a Ca^{++} sensitive Cl^- channel similar to those seen in other systems (for example Marty *et al.*,1984 or Owen *et al.*, 1984). The existence of this channel in oocyte was demonstrated directly by Takahashi *et al.* (1987) with the patch

clamp technique. Using brain RNA injected oocytes which expressed serotonin receptors, they looked at serotonin receptor activation of the IP_3 mediated Cl^- current. In patch clamp, they showed that application of agonist to the extracellular surface of the oocyte activates unitary conductances which are coincident with the macroscopic current seen in voltage clamp. Analysis of these unitary conductances show them have a slope conductance of 3 pS, a reversal potential of -29 mV and a lifetime of 100 ms. Various ion substitution experiments in the patch suggest that these are Cl^- selective channels. In addition, Takahashi *et al.* (1987) confirmed that the activation of the Cl^- channels was by way of intracellular messengers by demonstrating that iontophoretic application of serotonin to the outside the oocyte in the cell-attached patch configuration could activated the single channel currents. In this configuration, the agonist could not access the extracellular face of the membrane patch. The role of Ca^{++} in activating these channels was confirmed in experiments using inside-out patches where the inner surface of the plasma membrane was exposed to various concentrations of Ca^{++}. 10 μM Ca^{++} activated 3 pS unitary conductances of approximately 100 ms duration which are essentially identical to that of the receptor activated channels. Reduction of Ca^{++} to less than 10 nM caused a cessation of the Cl^- channel activity. A recent study by Oosawa and Yamagishi (1989) reports essentially the same results using oocytes injected with rat brain mRNA and glutamate receptors. In addition they showed that injection of IP_3 into cells evoked the 3pS unit conductances. This study adds further credence to the generality of the receptor evoked

Cl^- current in the oocyte. Receptors as different as those for serotonin and glutamate can feed directly into the same post receptor pathway.

Receptor activation of phospholipase C also generates the second messenger diacylglycerol (DAG). There are several examples of DAG effects in the oocyte. Dascal *et al.* (1985b) showed that activation of the native muscarinic response will inhibit cAMP dependent K^+ currents in oocyte follicles. This inhibition is mimicked by phorbol esters, however intracellular injections of IP_3 or Ca^{++} are without effect. They conclude that this inhibition is due to protein kinase C activation.

Another suspected function of DAG in the oocyte involves homologous and heterologous desensitization. Examination of two or more expressed receptors in RNA injected oocytes has shown that the receptors which couple to the IP_3 mediated Cl^- conductance exhibit marked self- and cross-desensitization (Dascal *et al.*, 1986; Hirono *et al.*, 1987; Nomura *et al.*, 1987). Kato *et al.* (1988) demonstrate that this desensitization is a result of phospholipase C liberating DAG which activates protein kinase C. They showed that an eight minute pretreatment with nanomolar concentrations of phorbol esters can inhibit the native muscarinic response as well as the expressed ACh and 5-HT responses in cells injected with rat brain RNA. Phorbol ester treatment of oocytes *in vivo* results in a marked increase in phosphorylation of 33 and 45 kDa proteins in native as well as RNA injected oocytes. Incubation of RNA injected oocytes with 5-HT, ACh or phorbol esters results in an increase in phosphoproteins as measured in washed membranes *in vitro*. This

increase is greatest at ten seconds after addition of transmitter or phorbol ester and is diminished at 5 minutes after treatment. This pattern of increase in membrane phosphorylation parallels the time course of receptor activated depolarization. Treatment of oocytes with phorbol esters, however, does not inhibit direct activation of the Cl^- channels by Ca^{++} or GTPγS (*vide infra*). Taken together, these studies suggest that ligand binding to receptors leads to activation of phospholipase C. The enzyme liberates IP_3, which initiates the short term depolarization events, and DAG, which activates protein kinase C and enables a negative feedback inhibition via phosphorylation.

A study by Sigel and Baur (1988) demonstrated multiple effects of DAG on expressed receptors in brain RNA injected oocytes. Activation of phospholipase C by quisqualate (see Sugiyama *et al.*, 1987) resulted in differential modulation of voltage-gated Na^+ and Ca^{++} channels and $GABA_A$ receptors. This modulation was mimicked by phorbol esters and 1,2-oleoylacetylglycerol, and was prevented by the protein kinase C inhibitor tamoxifen.

D.3. *Xenopus* oocytes and G-proteins

To explore the possible role of G-proteins in the receptor activated-IP_3 mediated Cl^- current in *Xenopus* oocyte, investigators have resorted to the "classical" tools of signal transduction research: bacterial toxins and guanine nucleotide analogs. Early studies on the function of adenylyl cyclase in oocytes used bacterial toxins to identify endogenous G-proteins. Sadler *et al.* (1984)

showed that pertussis toxin ADP-ribosylated a 41 kDa protein in oocyte membranes prepared from manually defolliculated oocytes. They also showed that cholera toxin ADP-ribosylated a number of proteins in the G_s size range. Olate *et al.* (1984) demonstrated pertussis toxin catalyzed ADP-ribosylation of a single 40 kDa protein in membranes from collagenase treated oocytes which co-migrated with purified G_i from human erythrocytes on SDS-polyacrylamide gel electrophoresis. They also showed that cholera toxin labeled a single 42 kDa protein. Both groups demonstrated the likely existence of another G-protein, responsible for the guanine nucleotide sensitive inhibition of adenylyl cyclase by progesterone, which was not pertussis toxin sensitive. Subsequent studies have confirmed the existence of 40 kDa (Dascal *et al.*, 1986), 41 kDa (Kaneko *et al.*, 1987) and 39 kDa (Kaneko *et al.*, 1987) pertussis toxin substrates in native oocytes, and have demonstrated the ability of oocytes to express G-proteins after injection of foreign mRNA (Kaneko *et al.*, 1987). Taken together, these studies suggest that the native oocyte contains signal transduction G-proteins of G_s, G_o and G_i sizes. Recent studies by Olate *et al.* (1989a; 1989b) demonstrated, by molecular cloning techniques, that the α-subunits of G_o, G_s and G_{i3}, are found in *Xenopus* oocytes.

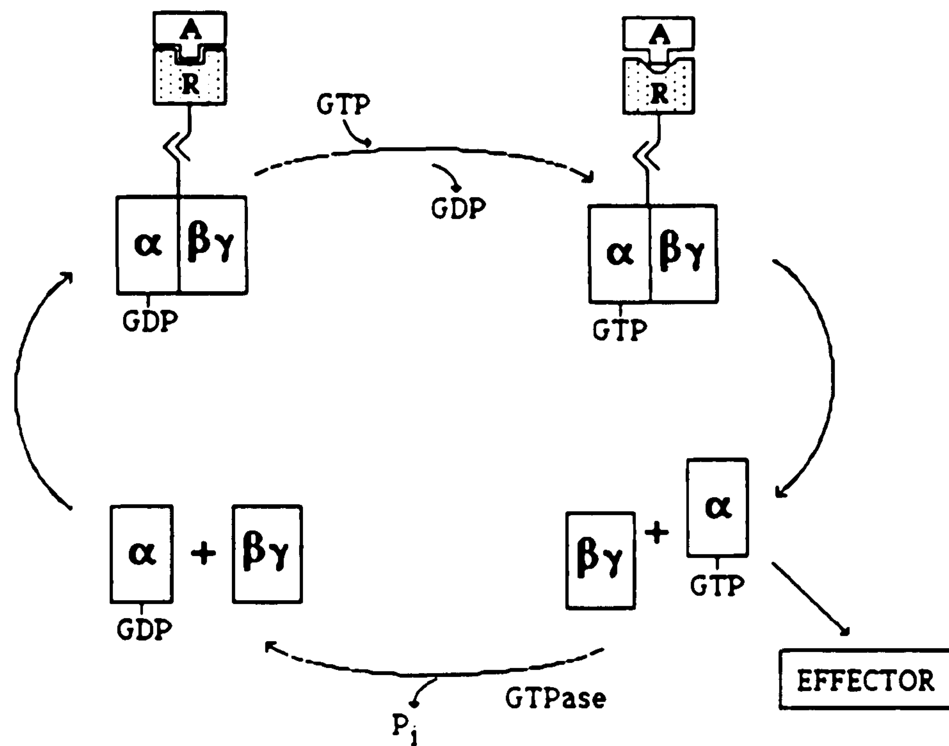
The potential role of G-proteins in coupling receptors to phospholipase C in the oocyte is further supported by functional studies utilizing pertussis toxin. Some receptors expressed from exogenous RNA are uncoupled from phospholipase C by pertussis toxin. Dascal *et al.* (1986) showed that the expressed rat brain 5-HT or ACh receptor activation of the Cl^- current is inhibited

approximately 50% by a 4 hr treatment of cells with 0.5 μg/ml pertussis toxin. Others have reported inhibitory effects of pertussis toxin on various expressed receptor responses in the oocyte (Table 2). The fact that the response to intracellularly injected IP_3 is not inhibited by pertussis toxin (Hirono *et al.*, 1987) supports the belief that the pertussis toxin effect is localized between the receptor and the formation of IP_3. Cholera toxin apparently has no effect on activation of the Cl^- current (Table 2). Taken together, these data strongly suggest that a pertussis toxin substrate is coupling some receptors to phospholipase C in the oocyte.

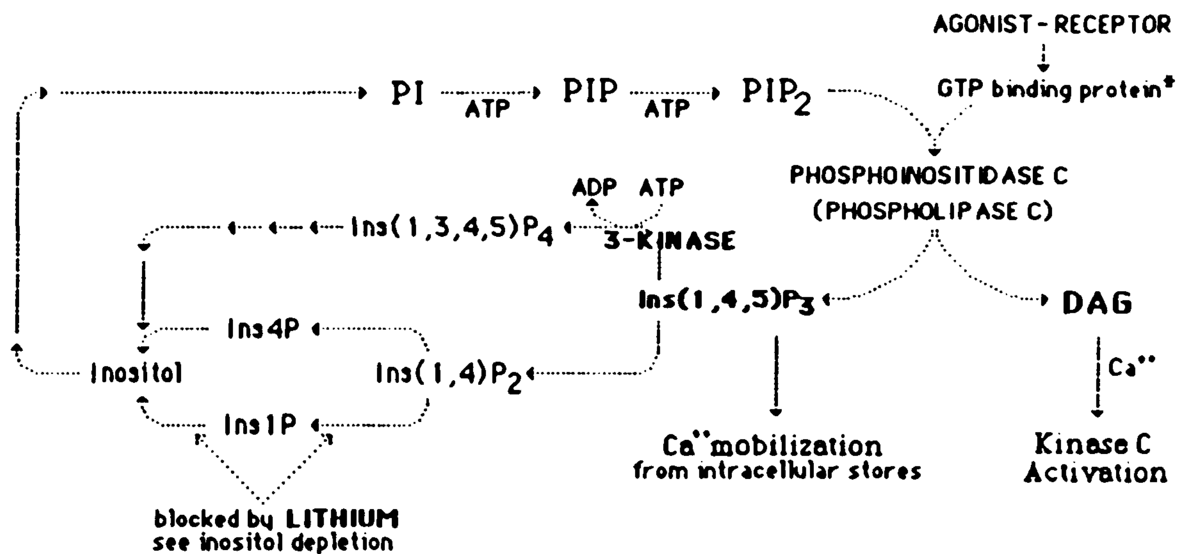
The effects of guanine nucleotides on the receptor activated Cl^- current have been investigated using the native muscarinic receptor and receptors for ACh and 5-HT expressed in the oocyte from brain RNA (Dascal *et al.*, 86; Nomura *et al.*, 87). It has been demonstrated that the Cl^- current can be elicited directly by injecting GTPγS into the cell in the absence of agonist (Dascal *et al.*, 1986; Kaneko *et al.*, 1987). The GTPγS evoked current is much more persistent, sometimes lasting for tens of minutes, than that seen with physiological activation, which can be quite brief (< 5 minutes). This persistence may be due to the asynchronous activation of other G-proteins over time as the GTPγS diffuses throughout the cell, as well as to the the inability of the cell to remove the GTPγS from the activated G-protein. Cells injected with GTPγS are unresponsive to subsequent exposure to agonist suggesting that the necessary G-proteins are disabled by binding GTPγS. Intracellular injection of GDPβS does not directly evoke a response, however, injection of GDPβS, as well as GDP, does reduce the ability of agonist to evoke

the Cl^- current. Cells injected with GTP display a reduced response to low concentrations of agonist. This may be due to a reduced affinity of receptor for agonist which is known to occur in receptor systems coupled to G-proteins (Gilman, 1987).

Figure 1



Subunit dissociation model of G-protein action in signal transduction.



key: PI=phosphatidyl inositol
PIP=phosphatidyl inositol 4-phosphate
PIP2=phosphatidyl inositol 4,5-bisphosphate
Ins(1,4,5)P3=inositol 1,4,5-trisphosphate
Ins(1,3,4,5)P4=inositol 1,3,4,5-tetrakisphosphate
DAG=diacylglycerol

Figure 2

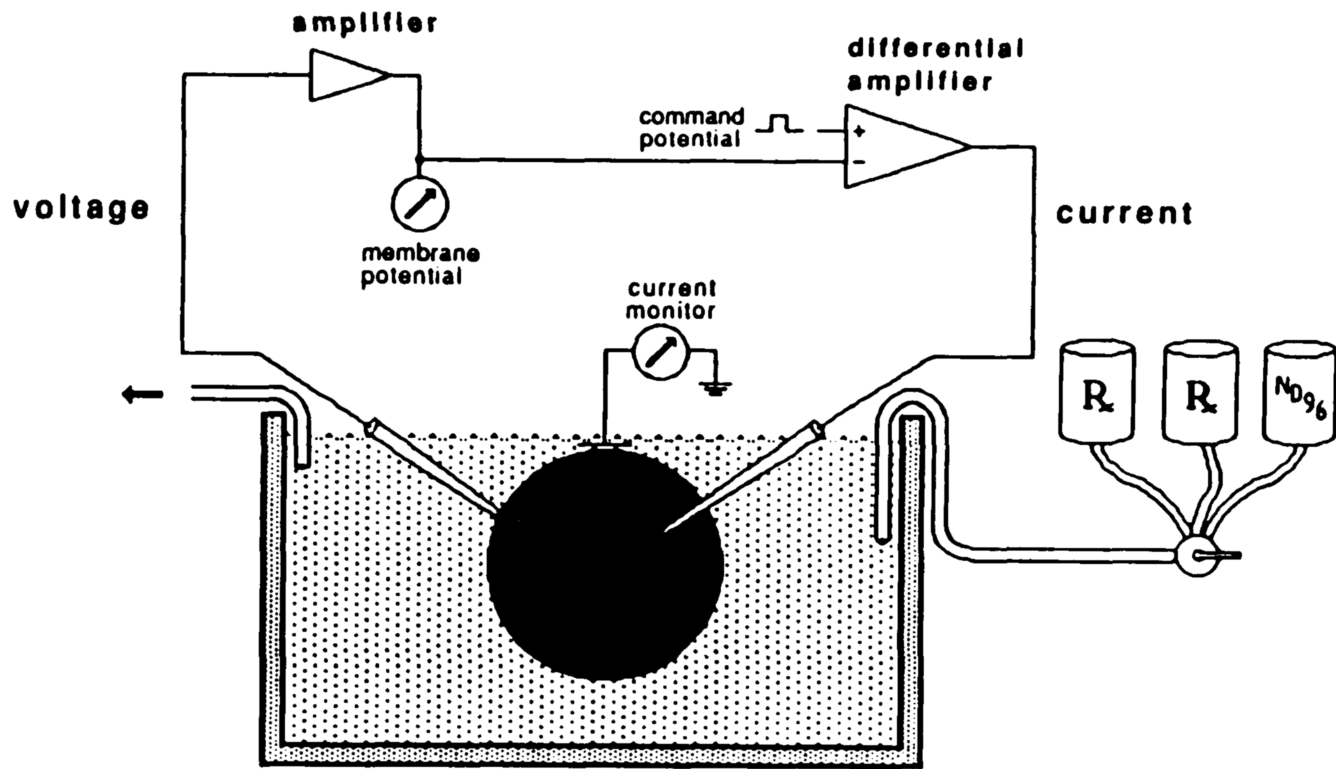


Figure 3

3. Electrophysiology experimental apparatus. The experiments described in these studies use the two electrode voltage clamp technique. Single oocytes are placed in a small chamber which is perfused with frog Ringer's solution (ND96). The content of the bathing medium is controlled using a stopcock and various reservoirs. Two glass microelectrodes are pulled to very fine points and filled with 3 M KCl (1-5 MΩ resistance). These electrodes are mounted on micromanipulators and impaled into the oocyte. One electrode (voltage) is connected to an amplifier and records the membrane potential. This potential is fed into one terminal of a differential feedback amplifier. The experimenter sets the membrane potential at which the experiment will be performed. This "command potential" is fed into the other pole of the differential amplifier. Any difference between the recorded membrane potential and the command potential will be pumped as current into the cell via the other (current) electrode to "clamp" the membrane at the desired potential. The magnitude of this current is measured at an ammeter (current monitor).

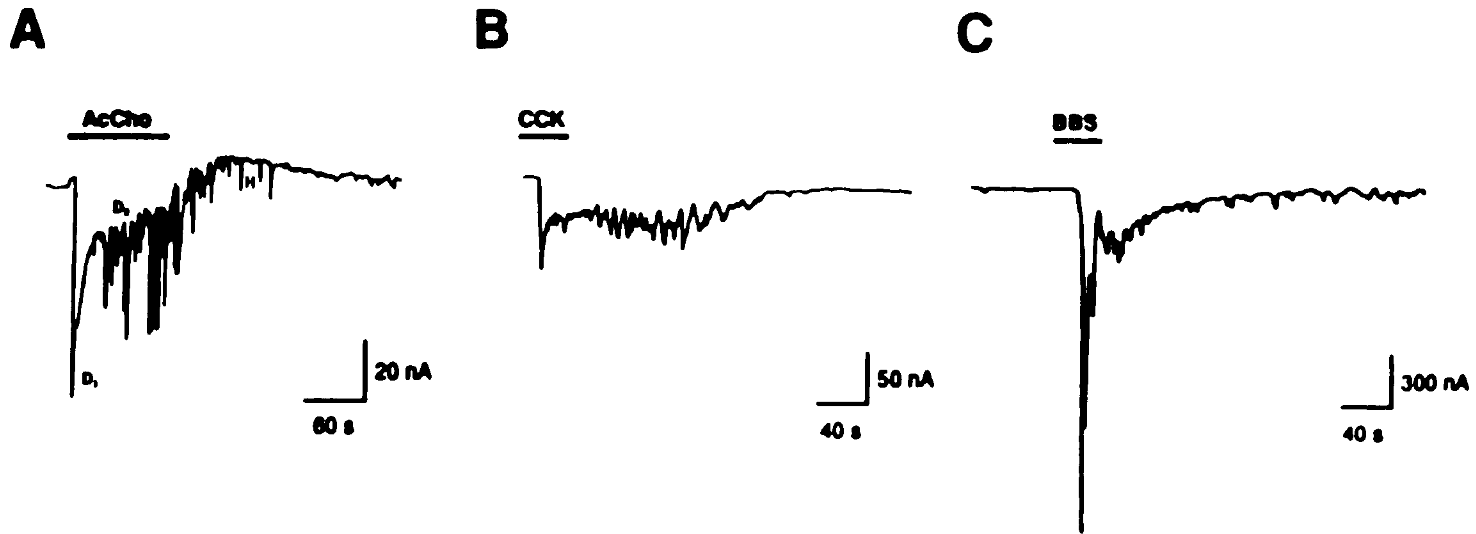


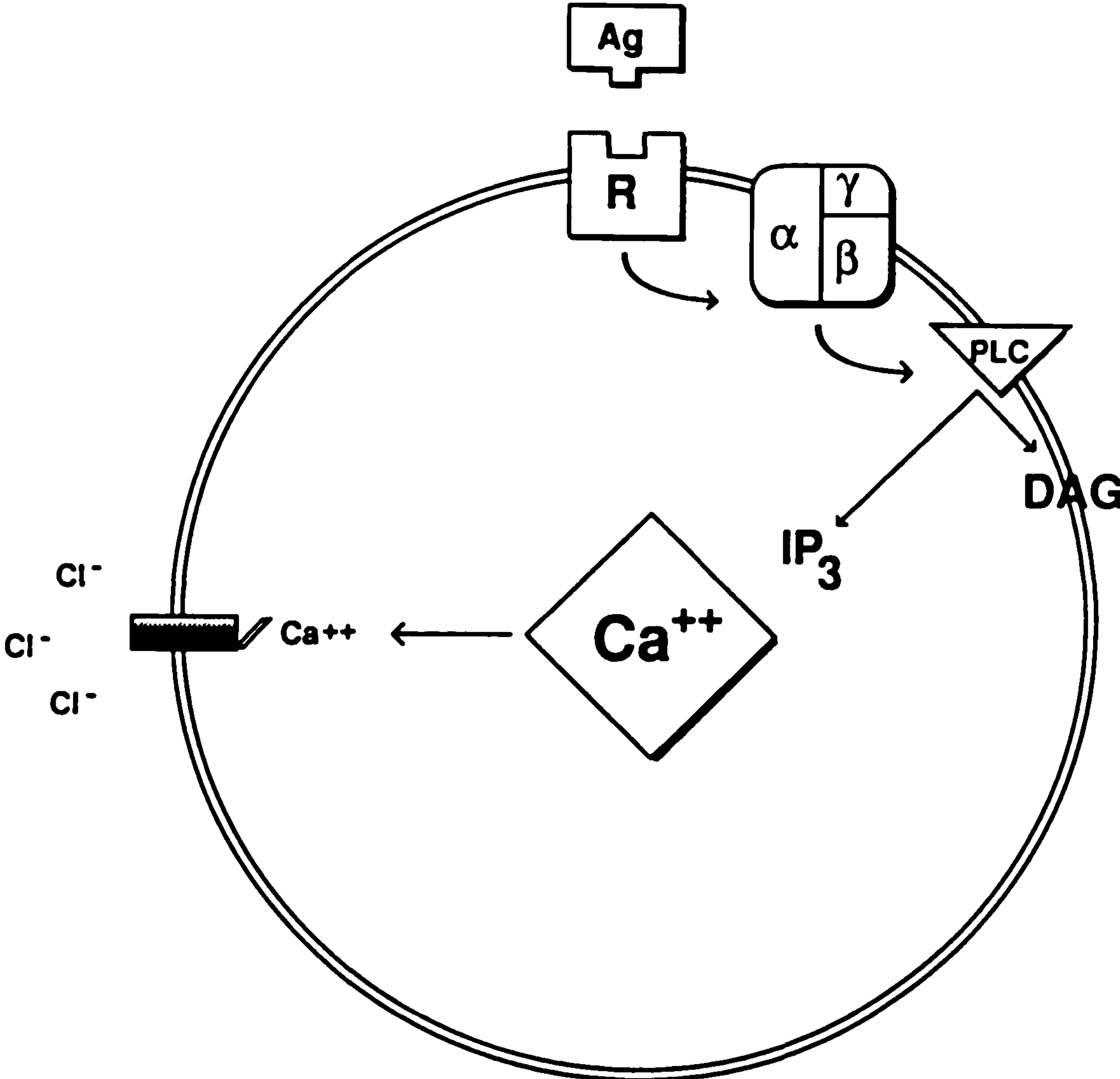
Figure 4

4. The receptor activated-IP_3 mediated Cl^- conductance: representative current traces from voltage clamped oocytes. **A)** The native muscarinic response in a follicle enclosed oocyte. The cell was clamped at -60 mV. ACh was at 0.1 μM. Note the 4 components of the response: D_1, D_2, H and the rapid, superimposed fluctuations (F). **B)** The response to cholecystokinin (0.1 μM) and **C)** to bombesin (0.1 μM) in two different RNA injected oocytes clamped at -70 mV. These oocytes had been defolliculated with collagenase then injected with 50 nl of rat brain RNA (5 mg/ml) three days prior to assay. Note that the D_1, D_2 and F components are present, but the H component is not seen in these collagenase treated oocytes.

The bar above each response represents the duration of application of transmitter.

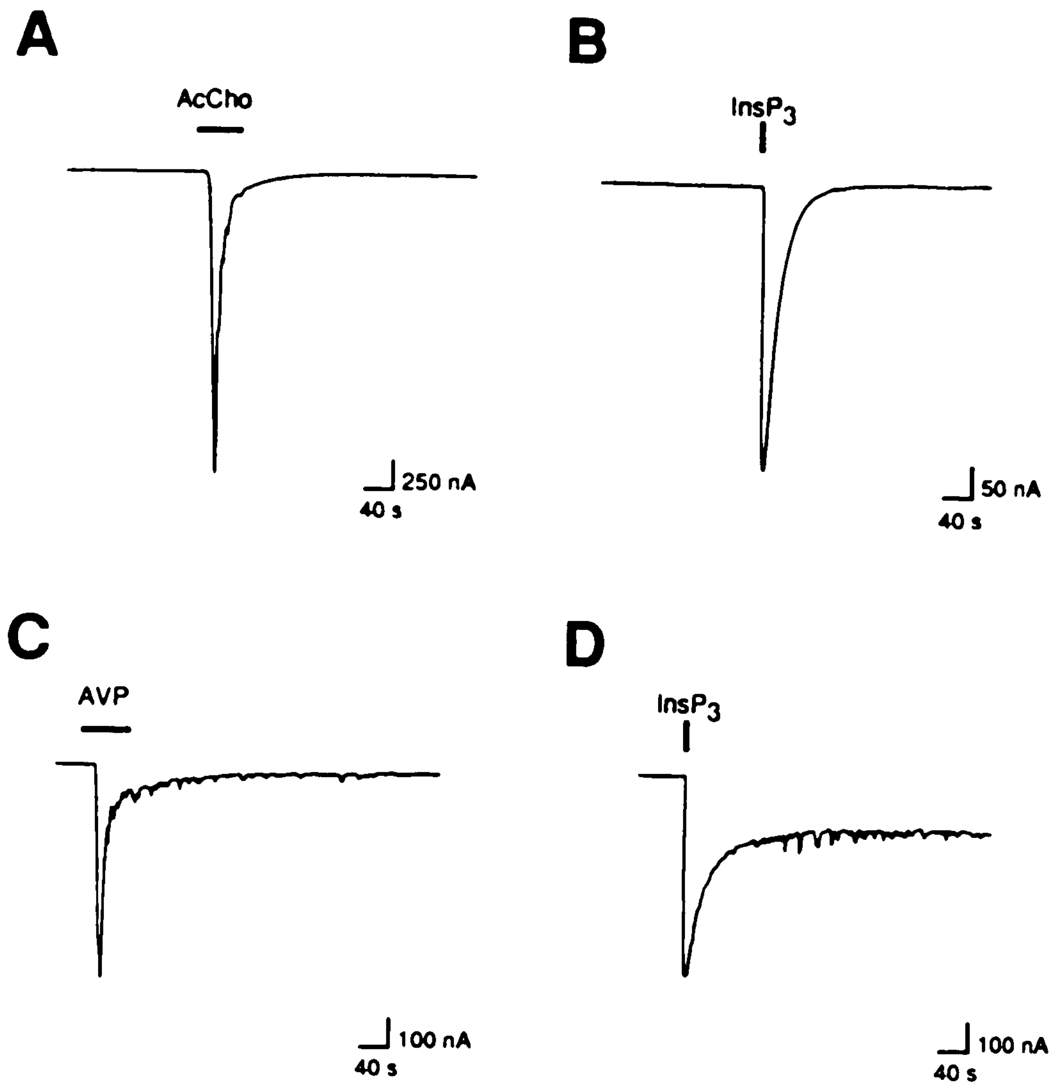
(CCK = cholecystokinin-8 (sulfated); BBS = bombesin)

Figure 5



5. The receptor activated-IP_3 mediated Cl^- conductance in *Xenopus* oocyte: general pathway. This schematic represents a single oocyte. The agonist (Ag) bound receptor (R) (either the native muscarinic receptor or a transplanted receptor) will couple to phospholipase C (PLC) via an heterotrimeric G-protein ($\alpha\beta\gamma$). Phospholipase C will break down phosphatidyl-inositol 4,5 -bisphosphate to inositol 1,4,5 -trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 will bind to receptors on endoplasmic reticulum and evoke a release of Ca^{++} into the cytosol. Ca^{++} sensitive Cl^- channels in the plasma membrane will open in response to the increased cytosolic Ca^{++}. The coordinated opening and closing of Cl^- channels is responsible for the complex electrical response of the membrane.

Figure 6



6. IP_3 injections into native oocytes and RNA injected oocytes. A) The native muscarinic response in a collagenase treated oocyte. ACh is at 1.0 μM. **B)** The response of a collagenase treated oocyte to intracellular injection of IP_3 (1.2 pmol). The oocytes in part A and B are from the same donor frog. Note the lack of the F components (compare to Fig. 3). This is a manifestation of the variability seen between animals. **C)** The response to 1.0 μM arginine vasopressin (AVP) in a liver RNA (50 nl of 2.3 mg/ml total RNA solution) injected cell. **D)** The response of an oocyte, from the same group of liver RNA injected cells, to intracellular injection of IP_3 (1.2 pmol).

The bars above the responses indicate the duration of transmitter application. The vertical bars indicate the moment of injection of IP_3.

Table 1:

Representative extracellular bathing media (mM)

	ND96[1]	Modified Barth's Solution[2]	(Berridge)[3]	(Miledi)[4]	(Barish)[5]
NaCl	96	88	115	115.6	81
KCl	2	1	2	2	2.5
$CaCl_2$	1.8	0.41	1.8	1.8	1
$MgCl_2$	1	-	1	-	1
$Ca(NO_3)_2$	-	0.33	-	-	-
$MgSO_4$	-	0.82	-	-	-
$NaHCO_3$	-	2.4	-	2.4	2.5
Hepes	5	-	-	-	5
Tris	-	7.5	7.5	-	-
pH	7.5	7.6	7.6	7.2	7.4

1 (Dascal *et al.*, 1986)
2 (Kaneko *et al.*, 1987)
3 (Berridge, 1988)
4 (Kusano *et al.*, 1982)
5 (Barish, 1983)

Table 2:

Effects of bacterial toxins on the receptor activated-IP_3 mediate Cl^- conductance in RNA injected oocytes

transmitter	inhibition	pertussis toxin concentration	
AcCho	50%	0.5 μg/ml for4 hr	(Dascal *et al.*, 1986)
5-HT	50%	0.05 μg/ml for 4 hr	(Dascal *et al.*, 1986)
5-HT	50%	0.5 μg/ml for4 hr	(Dascal *et al.*, 1986)
5-HT	50%	2 μg/ml for4 hr	(Dascal *et al.*, 1986)
AcCho	60%	4 μg/ml for 48 hr	(Sugiyama *et al.*, 1985)
AcCho	40%	2 μg/ml for 18 hr	(Nomura *et al.*, 1987)
5-HT	60%	2 μg/ml for 18 hr	(Nomura *et al.*, 1987)
glutamate	75%	2 μg/ml for 20 hr	(Sugiyama *et al.*, 1987)
AcCho	60%	2 μg/ml for 20 hr	(Hirono *et al.*, 1987)
neurotensin	85%	2 μg/ml for 20 hr	(Hirono *et al.*, 1987)

transmitter	inhibition	cholera toxin concentration	
5-HT	0%	0.2 nM for 8 hr	(Dascal *et al.*, 1986)
5-HT	0%	10 nM for 8 hr	(Dascal *et al.*, 1986)
AcCho	0%	2 μg/ml for 18 hr	(Nomura *et al.*, 1987)
5-HT	0%	2 μg/ml for 18 hr	(Nomura *et al.*, 1987)

CHAPTER 2

Molecular Brain Research

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ACTIVATION OF IONIC CURRENTS IN *XENOPUS* OOCYTES BY CORTICOTROPIN RELEASING PEPTIDES

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SUMMARY

Oocytes of the African frog *Xenopus laevis* are shown by electrophysiological methods to possess receptors for corticotropin releasing factor (CRF), arginine vasopressin (AVP) and cholecystokinin (CCK). Oocytes surrounded by their follicular cell envelope responded to CRF or AVP with an outward hyperpolarizing current. This current was mediated by an increased conductance of K^+ ions. Pretreatment with the adenylate cyclase activator forskolin or with the cAMP phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) potentiated the responses to these peptides indicating that the cAMP second messenger system may mediate the responses. Oocytes stripped of the follicular envelope, which cannot generate cAMP dependent K^+ currents, did not respond to either CRF or AVP. Oocytes exposed to CCK responded with an inward depolarizing current. This current was carried by an increased conductance to Cl^- ions. Removal of the follicular cell layer did not effect the response to CCK. The shape, time course, and reversal potential of the Cl^- current suggest that CCK acts through the phosphatidylinositol pathway.

INTRODUCTION

Oocytes of the African frog *Xenopus laevis* have been used to study the mechanism of action of neurotransmitter and hormone receptors. The oocyte has some receptors in its cell membrane, and has the ability to functionally express receptors from intracellularly injected exogenous mRNA (for review see reference 7). This latter capability has led to the adoption of the oocyte as an assay system for putative neurotransmitter receptor cloned cDNAs and for functional studies on mutated or partial positive receptor clones [8, 11, 12, 22, 23, 24]. The oocyte expression system is also used in cloning strategies for neurotransmitter receptor cDNAs which are difficult to attempt by traditional protein purification methods [18, 20]. In view of the current interest in the molecular biology of hypothalamal factors controlling the pituitary-adrenal axis (HPA), we thought it prudent to characterize the basic biology of the oocyte for the primary neuropeptides controlling ACTH: CRF, AVP and CCK [26].

A number of studies have been conducted on the native oocyte with its mantle of follicular cells intact. This follicle preparation shows electrophysiological responsiveness for agents which act at muscarinic, catecholaminergic, purinergic and gonadotropic receptors [3, 4, 5, 13, 16, 17, 29, 30]. The biological implications of the native receptors are not completely understood. It is reasonable to expect that follicles may have receptors for gonadotropins since these substances are involved in the regulation of the reproductive system and direct follicular maturation. In this study we report the

surprising finding that native oocyte follicles also contain receptors for the major hypothalamic corticotropin releasing peptides: CRF, AVP and CCK.

MATERIALS AND METHODS

Stage V and VI oocytes [9] were surgically removed from mature female *Xenopus laevis* (Xenopus One, Ann Arbor, MI) under tricaine anesthesia. Some oocytes were stripped of follicular cells by agitation for 2 hours in a Ca^{++}-free collagenase solution of the following composition (mM): NaCl, 82.5; KCl, 2.0; $MgCl_2$, 1.0; HEPES, 5.0; pyruvate, 2.5; penicillin (100 U/ml); streptomycin (1µg/ml) and collagenase (Sigma Type 1A, 2mg/ml). All oocytes were incubated at 20° C for 3 to 5 days in frog ringers (ND96) of the following composition (mM): NaCl, 96.0; KCl, 2.0; $CaCl_2$, 1.8 and Hepes, 5.0. Pyruvate (2.5 mM), penicillin (100 U/ml) and streptomycin (1µg/ml) were added to the incubation medium. Oocyte responsiveness to neuropeptides was assayed in a superfusion apparatus. Individual oocytes were placed in a 0.5 ml bath constantly perfused with ND96 at room temperature. The oocyte was voltage clamped between -50 and -70 mV with 3 M KCl microelectrodes (1-2 MΩ) using the conventional two-electrode technique. The voltage clamp circuit was connected directly to a chart recorder and an X-Y plotter. Arginine vasopressin and cholecystokinin-8 (sulfated) were from Bachem (Torrance, CA). Corticotropin releasing factor (rat) was from Peninsula Laboratories (Belmont, CA). All other chemicals and agents were from Sigma. All solutions were at pH=7.50±2. All reported values are mean ± standard error of the mean.

RESULTS

Application of 0.1 - 2 μM CRF to oocytes with the follicular cell layer intact (follicles) induced an outward hyperpolarizing current (Fig. 1a) with mean peak amplitude of 36 ± 4 nA (n=25). Application of 0.1 - 2 μM AVP to follicles induced a similar outward current (Fig. 2a) with mean peak amplitude of 77 ± 12 nA (n=23). Not all donor frogs were responsive to these peptides. In general, all oocytes from a responsive donor were sensitive to the tested peptide. The response was variable between oocytes from a single donor as evidenced in the SEM above. The percentage of responsive donors in our population of frogs declined over the course of the study from winter (60%) to summer (10%) indicating, as is true for the endogenous acetylcholine receptor [3,7], that there may be a seasonal variation in expression of the peptide receptors. Repetitive application of peptide showed a desensitization in the response. However, this desensitization was minimal after a 20-30 minute washout period.

The responses to CRF and AVP are similar to the currents elicited by activation of purinergic or b-adrenergic receptors [13,16,17,29]. To rule out cross activation of either of these native receptors, oocytes were tested for responsiveness to peptides in the presence of antagonists at concentrations that are known to completely block activation of these receptors [30]. Three cells tested in 0.1 mM propranolol and 0.1 mM theophylline were still sensitive to CRF. Another three cells were tested and found responsive to AVP in the presence of the antagonists.

The CRF or AVP induced current is due to an increased membrane conductance. We investigated the ionic composition of each current using the voltage ramp method [4]. A 800 msec. ramp-like change in holding potential was superimposed over the steady holding potential before and during peptide application. The current and voltage leads of the clamp were connected directly to an X-Y plotter such that a current voltage relationship (I-V) was plotted automatically. The cross-over point of an I-V curve before and an I-V curve during the response gave the reversal potential of the response. A series of ramp studies on the CRF and the AVP responses indicated reversal potentials of 101 ± 3 mV for CRF (n=8) and 97 ± 3 for AVP (n=7). These values are consistent with a currents carried by K^+ ions [7, 13, 17, 29] (Fig. 1c and 2c).

The β-adrenergic and purinergic currents have been shown to be mediated by increased intracellular cAMP [17,29]. The similarity of the CRF and AVP responses to these well studied systems led us to suspect that cAMP probably played a role here as well. The response to these peptides could be mimicked by bath application of 10 mM of the permeable analog 8-Br-cAMP (n = 5). A five minute pretreatment of follicles with the adenylate cyclase activator forskolin (0.2 -0.4 μM) potentiated the response to CRF (Fig. 1b). The mean peak amplitude was 20 ± 3 nA before forskolin and was 103 ± 9 nA after treatment (n=9). Forskolin also potentiated the response to AVP from a mean peak amplitude of 43 ± 14 nA before treatment to 145 ± 26 nA after treatment (Fig. 2b). The effect of forskolin on the CRF and AVP responses was shown to be reversible on washout. The potentiation by forskolin was shown by two tailed t-test to be

significant at the 0.005 level. Pretreatment with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) also potentiated the responses. Oocytes that have been stripped of the follicular cell layer have been shown to be unable to conduct cAMP dependent K^+ currents [7,30]. Oocytes which had the follicular envelope removed by collagenase treatment did not respond to AVP or CRF. These data suggest that the current induced by activation of the receptors to CRF and AVP is mediated via the cAMP second messenger system.

Application of 0.1 to 1.0 μM CCK to oocyte follicles induced an inward depolarizing current (Fig. 3a). The mean peak amplitude of this inward response was 108 ± 15 nA (n=30). Five of our twelve frogs gave oocytes that were sensitive to CCK. As summer approached, however, the number of responsive donors dropped to zero. The data reported here were collected in the winter. Repetitive application of CCK showed desensitization, but if a 20-30 minute washout period preceded subsequent applications, there was relatively no change between responses.

We examined the response to CCK using the voltage ramp method described above. Ramp studies showed a reversal potential of 22 ± 1 mV which is close to the equilibrium potential for Cl^- in oocytes [1, 4, 5, 7, 13]. This suggests that the increase in membrane conductance is carried primarily by Cl^- ions (Fig. 3b).

The response to CCK is similar in shape, time course and reversal potential to the response evoked by activation of native acetylcholine receptors [3,4,5] or activation of the expressed brain 5-HT receptors [10, 18]. These responses have been shown to be

mediated by IP_3 [25]. We could mimic the response to CCK by intracellular injection of IP_3 (0.4-0.2 pmol) (n=8). Taken together, these two findings suggest that the receptors to CCK in oocytes are linked to Cl^- channels via the phosphatidyl inositol second messenger system.

DISCUSSION

Our results indicate that *Xenopus* oocytes bear receptors specific for the three major hypothalamic modulators of the HPA axis: CRF, AVP and CCK. The receptors for CRF and AVP appear to be linked to K^+ channel via the cAMP second messenger system. The location of these receptors is not clear. They may reside in the follicular cells surrounding the oocyte, as has been suggested for the receptors for the catecholaminergic, purinergic and gonadotropic receptors, or they may be in the oocyte membrane with the K^+ channels being located in the follicular cells. The latter idea is supported by the finding that forskolin [27] and cholera toxin [28] increase intracellular cAMP in defolliculated oocytes, even though no hyperpolarizing current is possible. If this is the case, the receptors communicate with the K^+ channels via the movement of cAMP through the gap junctions between the oocyte and the follicular cells [2]. Removing the follicular cells results in loss of response because of the K^+ channels are removed.

CCK initiates the well known electrophysiological signature of phospholipase C activation. The CCK receptors in oocyte appear to be linked to Cl^- channels via an IP_3 mechanism. Defolliculation has no effect on the CCK response which suggests that the receptors are in the oocyte cell membrane.

The biological role of these peptide receptors is not clear. These receptors may be involved in the regulation of oocyte function since in some species ovaries contain AVP, POMC derivatives and CCK [14,15,21]. It is known that an elevated level of cAMP is needed to

maintain the *Xenopus* oocyte arrested in first meiotic prophase [19]. It is also known that muscarinic agonists, which act via IP_3, promote progesterone-induced maturation [6]. CRF and AVP, in concert with the other cAMP dependent transmitters, may serve to regulate this arrest and to modulate the rate of maturation against the contrary input from the IP_3 transmitters, acetylcholine and CCK.

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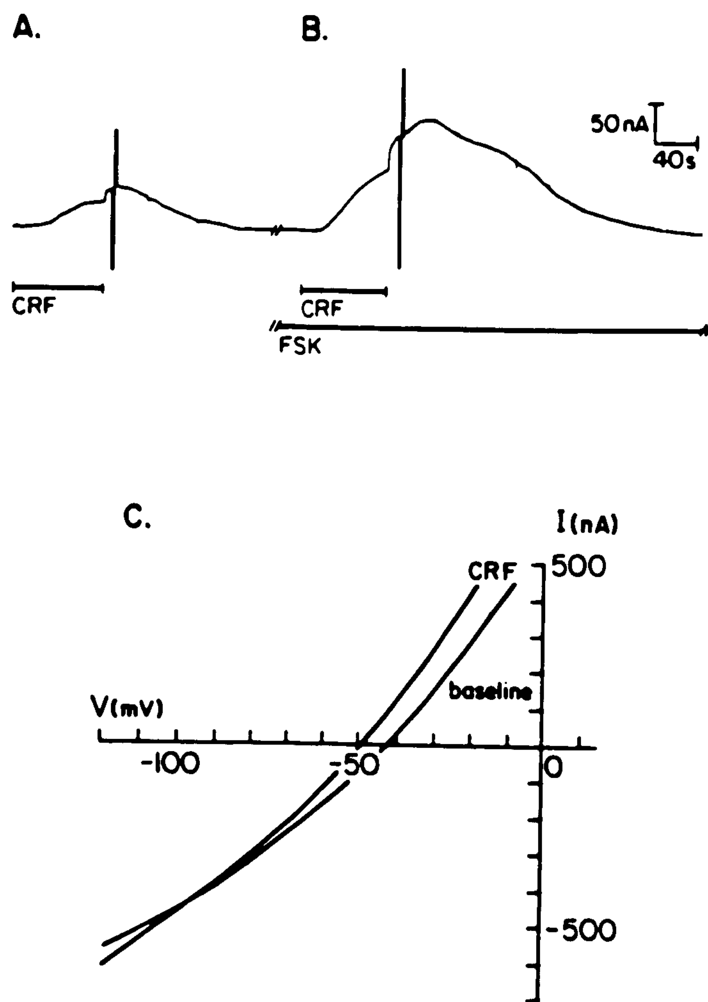
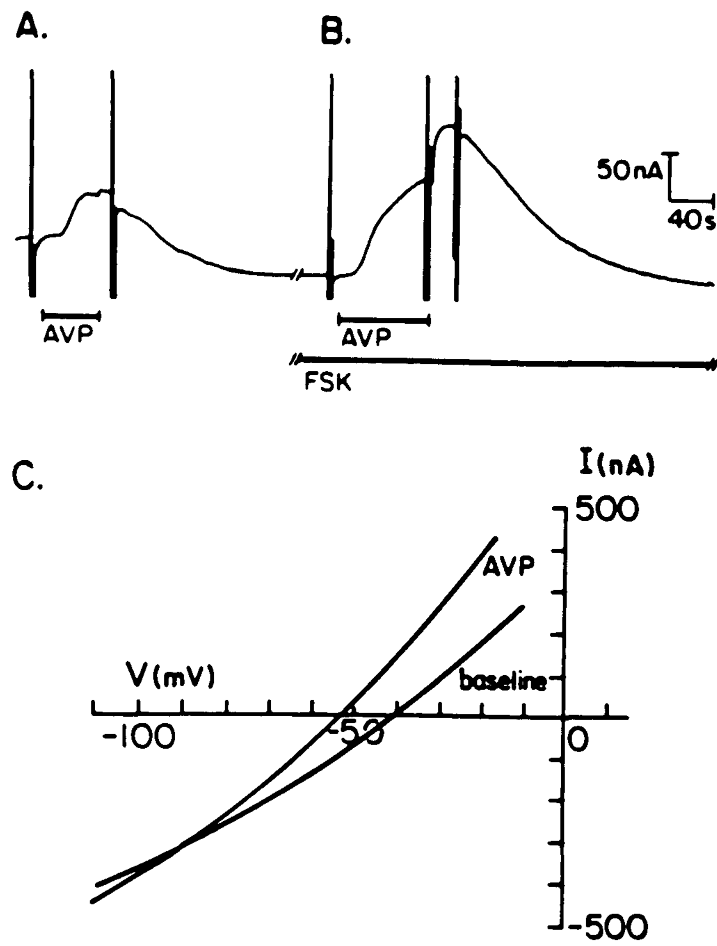
Figure 1



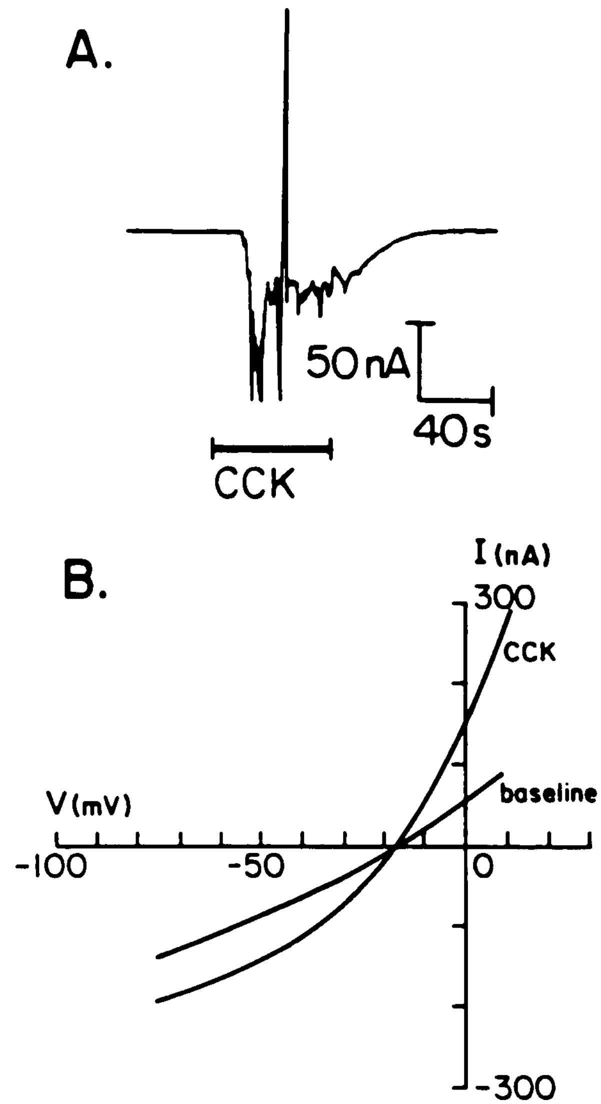
1. (a) Typical response of oocyte follicle to application of CRF (0.5 μM). Bar shows duration of peptide application. The vertical line at the peak of the response is the change in current caused by a rapid ramp change in voltage. (b) The response to CRF (0.5 μM) in the same cell after five minute pretreatment with forskolin (0.2 μM). There was a thirty minute washout period before this second application. (c) Current-voltage (I-V) characteristics of the response to CRF shown in a single cell by the ramp method. The baseline curve was made 30 seconds before application of CRF. The CRF curve was made during the peak outward current of the response.

Figure 2



2. (a) Typical response of oocyte follicle to application of AVP (0.5 μM). The vertical lines are due to the ramp protocol. (b) The response to the same concentration of AVP (0.5 μM) in the same cell after a five minute pretreatment with forskolin (0.2 μM). There was a thirty minute washout period before this second application. (c) Current-voltage (I-V) characteristics of the response to AVP shown in a single cell by the ramp method. The baseline curve was made five seconds prior to application of the peptide. The AVP curve was made during the peak outward current of the response.

Figure 3



3. (a) Typical response of oocyte follicle to application of CCK (0.5 μM). The vertical line is due to the ramp protocol. (b) Current-voltage (I-V) characteristics of the response to CCK shown in a single cell by the ramp method. The baseline curve was made one minute prior to application of the peptide. The CCK curve was made during the slow phase of the inward current of the response.

CHAPTER 3

Molecular Brain Research

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FUNCTIONAL EXPRESSION OF BRAIN CHOLECYSTOKININ AND BOMBESIN RECEPTORS IN *XENOPUS* OOCYTES

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SUMMARY

Total RNA was extracted from 15 day old whole rat brains. Microinjection of the RNA into *Xenopus laevis* oocytes induced electrophysiological responsiveness to cholecystokinin-8 (CCK) and bombesin (BBS) but not to corticotropin releasing factor (CRF) or somatostatin. The responses to CCK and BBS were similar in shape, time course, and reversal potential to that induced by receptor mediated phospholipid breakdown and that which is induced by intracellular injection of IP_3. These responses were not blocked by atropine or by mianserin, did not require extracellular Ca^{++} and were completely suppressed by intracellular injection of EGTA.

INTRODUCTION

Neuropeptides in the brain are thought to be neurotransmitters or neuromodulators [14,29]. There is a growing body of evidence that neuroactive peptides may figure prominently in brain dysfunction. This is particularly true of psychiatric disorders. Somatostatin and CRF appear to be altered in Alzheimer's disease [2, 6, 7]. There is considerable data which suggests that CCK may play a central role in the pathophysiology of schizophrenia [3, 10, 11, 19, 27]. There is also some evidence implicating BBS and somatostatin in schizophrenia [10, 11, 27, 28]. The studies by Gold and Nemeroff strongly support a role for CRF in depression [12, 20]. Arginine vasopressin (AVP) has been reported to function in memory consolidation [8]. CRF, CCK and AVP have been shown to be major modulators of the molecular mechanisms of stress [25,26].

Very little is known about the cellular action of brain peptidergic transmitters because of the complexity of neural tissue. Scrutiny of the cellular action of these neuropeptides may lead to a better understanding of the pathophysiology of their associated disorders.

We have chosen to begin our study of the cellular and molecular properties of neuropeptides using the oocyte electrophysiological assay. Oocytes of the African frog *Xenopus laevis* were first used to study neurotransmitter receptors because the oocyte was found to have receptors in its cell membrane [5]. Later it was shown that the oocyte could be used as an mRNA translation system whereby mammalian brain receptors could be

transplanted from the brain into a simple single cell preparation [5]. This system has since been used effectively to study the electrophysiology, biochemistry, pharmacology and molecular biology of brain receptors [5, 16, 18, 30, 31]. We report here our findings on a survey of brain RNA injected oocytes of five neuropeptides of potential clinical importance: CCK, BBS, somatostatin, AVP and CRF.

MATERIALS AND METHODS

Total RNA was isolated from whole brains of 15 day old rats by a modified version of the LiCl/urea method [9] and stored in H_2O at -80° C. Oocytes were surgically removed from mature female *Xenopus laevis* (NASCO, Fort Atkinson, WI) under tricaine anesthesia. The oocytes were stripped of follicular cells by agitation for 2 hours in a Ca^{++}-free collagenase solution of the following composition (mM): NaCl, 82.5; KCl, 2.0; $MgCl_2$, 1.0; HEPES, 5.0; pyruvate, 2.5; penicillin (100 U/ml); streptomycin (1μg/ml) and collagenase (Sigma Type 1A, 2mg/ml). Stage V and VI oocytes were injected with 50 nl of a 250 ng/ml RNA/H_2O solution using a Drummond microinjector. Non-injected oocytes served as controls. Oocytes were incubated at 20° C for 3 to 5 days in frog Ringer (ND96) of the following composition (mM): NaCl, 96.0; KCl, 2.0; $CaCl_2$, 1.8 and Hepes, 5.0. Pyruvate (2.5 mM), penicillin (100 U/ml) and streptomycin (1μg/ml) were added to the incubation medium. Oocyte responsiveness to neuropeptides was assayed in a superfusion apparatus. Individual oocytes were placed in a 0.5 ml bath constantly perfused with ND96 at room temperature. The oocyte was voltage clamped between -50 and -70 mV with 3 M KCl microelectrodes (1-5 MΩ) using the conventional two-electrode technique. Voltage clamp output was connected directly to a chart recorder and an X-Y plotter. Arginine vasopressin, bombesin, cholecystokinin-8 (sulfated), and somatostatin (cyclic) were from Bachem (Torrance, CA). Corticotropin releasing factor (rat) was from Peninsular Laboratories (Belmont, CA). Mianserin was from

Research Biochemicals Inc. (Wayland, MA). All other chemicals and agents were from Sigma. All solutions were at pH=7.50± 0.02. The reported responses are the peak amplitude of the depolarization ± the standard error of the mean.

RESULTS AND CONCLUSIONS

Oocytes injected with the RNA solution responded to bath application of CCK and BBS (Fig. 1). The average peak amplitude of the response to CCK (10^{-6} - 10^{-8} M) was 265.03 ± 60.88 nA (n=26). The response to BBS (10^{-6} - 10^{-8} M) was 2065.38 ± 272.54 nA (n= 13). The threshold for activation of the CCK response was between 10^{-9} and 10^{-8} M. The threshold for activation of the BBS response was between 10^{-10} and 10^{-9} M. Injected oocytes showed no electrophysiological response to somatostatin (10^{-5} M) or CRF (10^{-5} M) (n=10). The negative result for somatostatin confirms the original observation of Miledi and co-workers [23] that RNA injected oocytes do not acquire sensitivity to somatostatin. Non-injected oocytes from the same donor frogs showed no response to bath application of AVP, BBS, CCK, CRF or somatostatin (10^{-5} M) (n=10). This indicates that the oocyte responsiveness to CCK and BBS is not due to native receptors, but is likely a result of translation of the exogenous brain mRNA for the receptor proteins for these peptides. Some oocytes did show a small depolarizing response to AVP, but there was no consistent responsiveness in any batch of RNA injected oocytes. The AVP response was not characterized further.

The induced responses to CCK and BBS are similar: the general form is a slow inward current with fast oscillatory currents superimposed (Fig. 1). The responses showed minimal desensitization on the second application of peptide. An equimolar application of peptide after a 10 minute washout could elicit essentially the same response. This suggests that the CCK and BBS

receptors are to some degree different from the previously reported neuropeptide receptors: substance P and neurotensin [13, 23]. Though the amplitude of the responses varied somewhat from cell to cell, it was of the form that is characteristic of the native acetylcholine receptor [4] and the expressed brain serotonin receptor (5-HT_{1c})[16]. The similarity to these well documented receptor responses prompted us to rule out cross-activation of these receptors by CCK or BBS. Several cells were examined for responsiveness to CCK in the presence of 0.1 μM atropine [4] and 1.0 μM mianserin [24] (142.5 ± 34.79 nA; n=4). These were found not to differ significantly from control responses (112.5 ± 14.03 nA; n=10) by unpaired t-test. One group of cells was tested for BBS induced currents in the presence of 0.1 μM atropine (1862.5 ± 247.80 nA; n=4). Another group of cells was examined for BBS responsiveness in the presence of 1.0 μM mianserin (2300 ± 801.04 nA; n=4). Neither group was significantly different from control (1920 ± 251.79 nA; n=5) as compared by unpaired t-test.

We investigated the ionic composition of the induced current using the voltage ramp method [4]. A rapid ramp-like change in holding potential was superimposed over the steady holding potential before and during peptide application. The current and voltage leads of the clamp were connected directly to an X-Y plotter such that a current voltage relationship (I-V) was plotted automatically. The cross-over point of an I-V curve before and an I-V curve during the response gave the reversal potential of the response. A series of ramp studies on the initial fast phase of the CCK (n=6) and the BBS (n=5) responses indicated a reversal potential

between -18 to -25 mV which is consistent with a current carried by Cl^- ions [1, 4, 5, 15] (Fig. 2a). The ionic basis of the slow smooth phase was examined by the ramp method and by application of the neuropeptides to cells clamped at voltages more positive that the Cl^- equilibrium potential. Both methods showed that the slow phase of the CCK and BBS responses is mediated by an increased Cl^- conductance (Fig. 2b,c,d).

It has been shown that an influx of Ca^{++} into the oocyte transiently opens Cl^- channels [1,17]. To see if such an influx of Ca^{++} was responsible for the response to CCK and BBS, we examined two series of cells in Ca^{++}-free Ringer (with 0.2 mM EGTA and 10 mM Mg^{++} added). The responsiveness of RNA injected cells in Ca^{++}-free Ringer to CCK (132 ± 25.62 nA; n=5) or BBS (1400 ± 122.47 nA; n=4) was not significantly different from control CCK responses (133 ± 28.53 nA; n=5) or control BBS responses (1920 ± 251.79 nA; n=5), respectively, as compared by t-test. These results suggest that external Ca^{++} does not play a role in these responses.

The waveform of the response to CCK or BBS is characteristic of those receptors which act through phospholipase C [5, 13, 21, 22, 23] and is similar to the waveform induced by intracellular injection of IP_3 [22]. Two series of cells were studied after intracellular pressure injection of EGTA (final concentration 10^{-4} M) to see if the release of Ca^{++} from intracellular stores by IP_3 played a role in the response to CCK or BBS. Neither CCK (n=12) nor BBS (n=6) could induce a response in any cell after injection of EGTA (Fig. 3).

Xenopus oocytes injected with RNA extracted from whole rat brain functionally express the receptor proteins for CCK and BBS,

but not for CRF and somatostatin. RNA injected oocytes respond to bath application of CCK or BBS by a depolarization of the cell membrane which is characteristic of receptor mediated polyphosphoinositde breakdown. The depolarization is carried by an influx of Cl^- ions. The response is effected by mobilized intracellular Ca^{++} ions; external Ca^{++} plays no role. For these reasons, we suggest that the responses to CCK or BBS are mediated by the phosphatidyl inositol 2nd messenger system.

Having the receptors for these neuropeptides expressed in the oocyte will allow for future study on the intramembrane and intracellular processes of neuropeptide activated ionic currents. It also opens the possibility of using the oocyte as a method of cloning these receptors using the strategy of Lübbert *et al.*[16].

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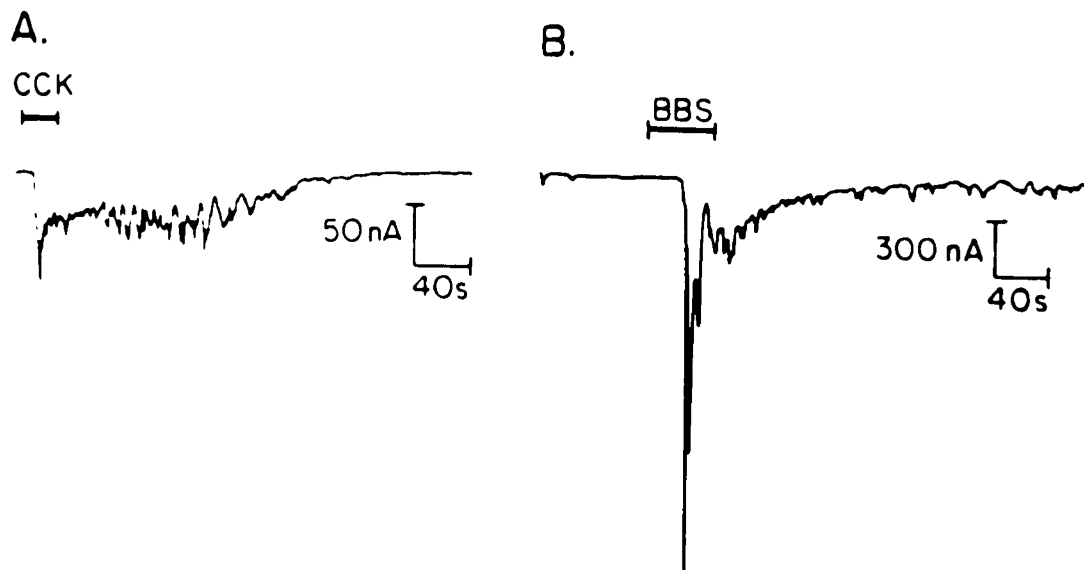
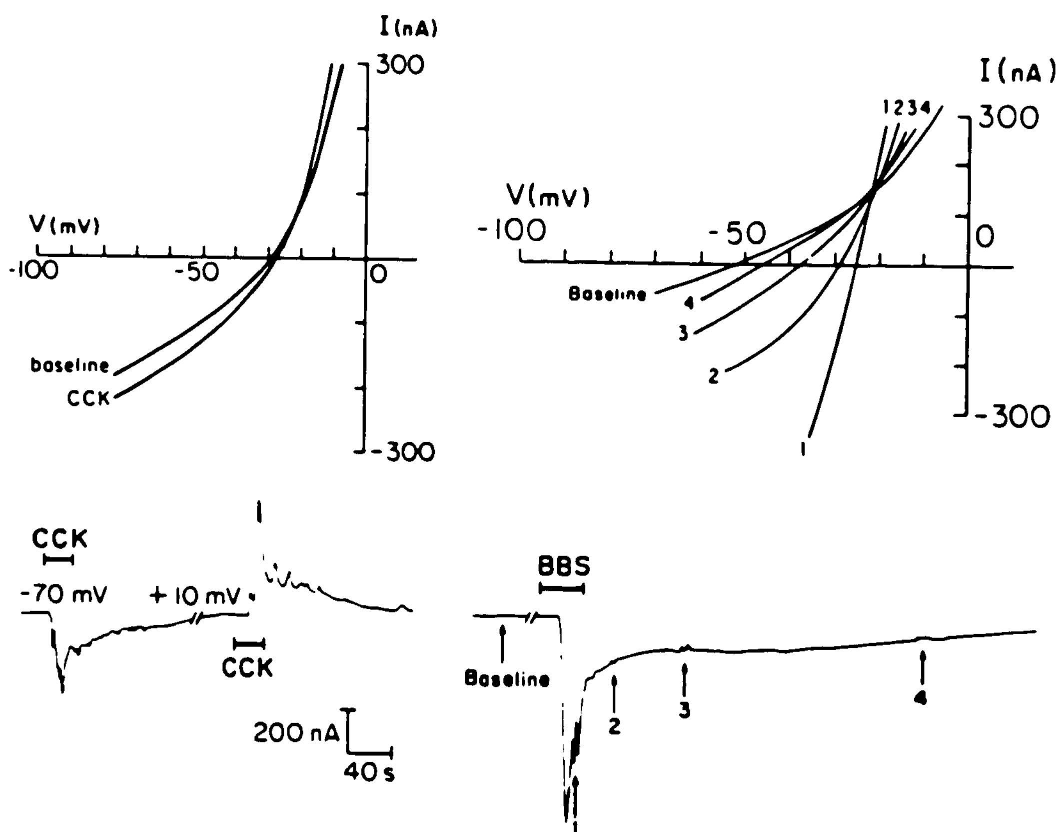
Figure 1



1. Typical responses of RNA injected oocytes to CCK (A) and BBS (B). These representative responses were obtained from different oocytes under the same conditions. CCK and BBS were at 0.1 μM. Bars show duration of peptide application. The responsiveness to CCK was shown in 26 oocytes from 5 frogs. The response to BBS was seen in 13 oocytes from 4 frogs.

Figure 2



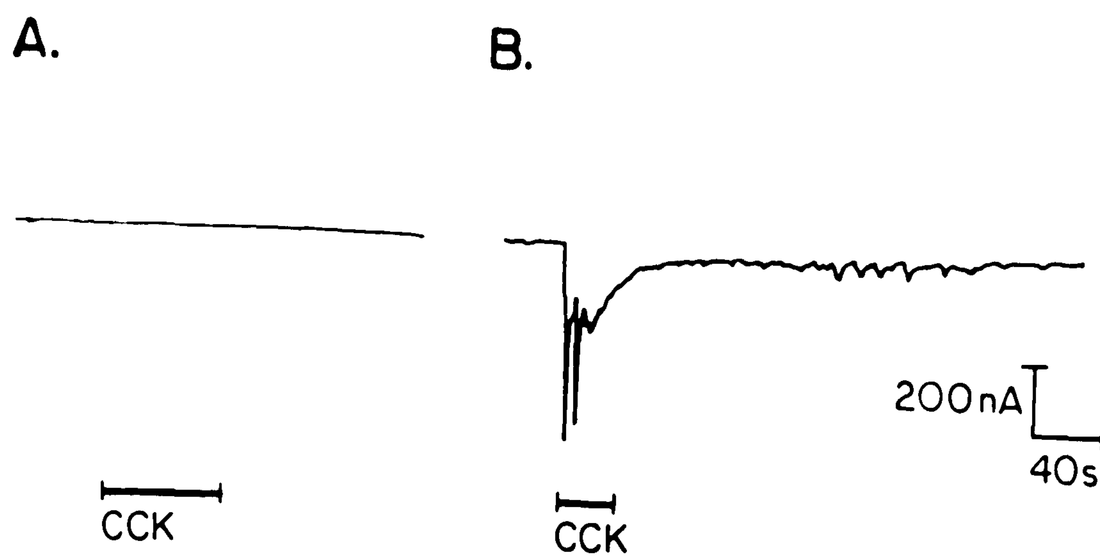
2. A) Current-voltage characteristics (I-V) of the response to CCK shown in a single cell by the ramp method. I-V curves were recorded automatically on an X-Y plotter. The baseline curve was made one minute before application of the peptide. The CCK curve was made at the peak of the fast response to the peptide. The reversal potential is the point of intersection of the two curves. Similar results were obtained for BBS.

B) A voltage ramp study of the response to BBS. Voltage ramps similar to the ones shown in part A of this figure were obtained at baseline and at various times after application of BBS (see part D). Note that all the voltage ramps cross near the same voltage (-23 mV). Similar results were obtained from multiple ramp analysis of the CCK response.

C) Application of CCK in a different cell voltage clamped at -70 mV and then at +10 mV after a 15 minute washout period. Note that both the early peak and the later part of the response were inverted at +10 mV indicating that both have a similar reversal potential.

D) The time course of the current induced by the BBS application in the same cell as in part B. The times when the ramps were applied are shown by arrows. The actual ramps were removed from the record for clarity. Note that ramps 3 and 4 were obtained at later stages of the response to BBS.

Figure 3



3. Intracellular injection of EGTA abolishes the CCK induced depolarizing current (n=12). (A) An RNA injected cell injected with EGTA (10^{-4} M) 20 minutes before application of CCK compared to an RNA injected cell which was not injected with EGTA (B). CCK was at 0.1 μM. Bars indicate duration of application of peptide. Similar results were obtained for BBS at 0.1 μM (n=6).

CHAPTER 4

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$\beta\gamma$-SUBUNITS OF GTP-BINDING PROTEINS INHIBIT MUSCARINIC RECEPTOR STIMULATION OF PHOSPHOLIPASE C

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ABSTRACT

This study examines the mechanism of G-protein coupling of receptors to phospholipase C. The *Xenopus* oocyte has a muscarinic receptor activated Cl^- current which is mediated by inositol 1,4,5-trisphosphate. Modulation of the muscarinic receptor evoked Cl^- current was examined under voltage clamp in oocytes injected with resolved G-protein subunits.

The presence of an α-subunit of G-proteins in oocytes was shown by pertussis toxin labeling of a 41 kDa band in oocyte membranes. The presence of the β-subunit of G-proteins was demonstrated by immunoblotting experiments using an antiserum (U-49) which is specific for the β-subunit. Pertussis toxin treatment of oocytes resulted in the uncoupling of muscarinic receptors from activation of the Cl^- current.

Cells microinjected with 1.5 ng of human erythrocyte βγ-subunits or 1.0 ng of bovine brain βγ-subunits showed approximately a 95% reduction in the evoked Cl^- current. Cells injected with equal volumes of protein storage vehicle showed no change in response. Cells injected with boiled βγ-subunits, bovine serum albumin or resolved α-subunits also showed no reduction in response. Cells injected with varying concentrations of βγ-subunits showed a concentration dependence with half-maximal inhibition of the muscarinic activated Cl^- current at about 10 nM. Cells injected with 1.0 ng of bovine brain βγ-subunits could not respond to bath applied agonist, but could generate the Cl^- current on intracellular injection of inositol 1,4,5-trisphosphate. These observations

suggest that there is a G-protein responsible for muscarinic receptor mediated signal transduction through phospholipase C and that it is an $\alpha\beta\gamma$ heterotrimer. It appears that the mode of action of the G-protein in the phospholipase C system may be similar to that of the hormone activated adenylyl cyclase.

INTRODUCTION

Receptor mediated stimulation of phosphatidylinositol 4,5-bisphosphate breakdown to inositol 1,4,5-trisphosphate (IP_3) is thought to be mediated through G-proteins (1-3) . Evidence that supports this line of reasoning include: a) the GTP requirement of coupling of receptors to phospholipase C, b) the stimulatory effects of nonhydrolyzable analogs of GTP on IP_3 production, and c) the guanine nucleotide sensitivity of agonist interactions with receptors known to stimulate IP_3 production. However the lack of reports on successful reconstitution of purified G-proteins with the various purified phospholipase C and the lack of a reproducible reconstitution of receptor coupling to IP_3 production by purified G-proteins is not consistent with the hypotheses of G-protein involvement. Hence, we reasoned that studies examining the role of G-protein subunits in signal transduction through phospholipase C would be informative.

The *Xenopus* oocyte has muscarinic acetylcholine receptors (4-6) that stimulate IP_3 production (7) and activate Cl^- channels (5-9). Intracellular injection of IP_3 mimics the muscarinic activation of the Cl^- conductance (8), and both the muscarinic and IP_3 mediated effects are dependent upon the increase of intracellular Ca^{2+} (8). Our present understanding of the pathway is: muscarinic receptor occupancy increases cellular IP_3 levels, this in turn increases cellular Ca^{2+} concentration, which results in opening of the Cl^- channels. It has been shown that the magnitude of the Cl^- current is proportional to the intracellular concentration of IP_3 (8). Thus

measurement of the Cl^- current can be used to monitor IP_3 production and phospholipase C activity.

Studies on both the hormone stimulated adenylyl cyclase and the light sensitive cGMP phosphodiesterase have shown that the α-subunits of G-proteins, which have the guanine nucleotide binding site, interact with the effector system and modulate its activity. The $\beta\gamma$-subunit complex, which consists of a 35 or 36 kDa β-subunit and the 5-8 kDa γ-subunit, inhibits signal transduction by associating with the α-subunit (1). We reasoned that if the coupling mechanisms of the phosphoinositide breakdown system are similar to that in the adenylyl cyclase system, then injection of excess $\beta\gamma$-subunits should inhibit muscarinic stimulation of IP_3 production and hence the chloride current. In this report we demonstrate that intracellular injection of $\beta\gamma$-subunits of G-proteins into *Xenopus* oocytes results in attenuation of the muscarinic stimulation of the Cl^- current and that this blockade is prior to IP_3 production.

MATERIALS AND METHODS

Materials: Mature female *Xenopus laevis* were obtained from NASCO (Ft. Atkinson, WI). [^{32}P] NAD^+ was synthesized according to the procedure of Cassell and Pfeuffer (10). Anti-serum U-49 was the kind gift of Drs. S. Mumby and A. Gilman. IP_3 was purchased from Boehringer Mannheim. Pertussis toxin was purchased from List Biological laboratories. Fresh bovine brains were obtained from a local slaughterhouse. Sources of all other materials have been previously described (11).

Oocyte preparation: Oocytes were surgically removed from mature *Xenopus laevis* under tricaine anesthesia. Oocytes were separated either manually with microforceps or by treatment with collagenase (agitation for 2 hours in the following solution (mM): NaCl, 82.5; KCl, 2.0; $MgCl_2$, 1.0; $CaCl_2$, 0; HEPES, 5.0; pyruvate, 2.5; penicillin (100 U/ml); streptomycin (100 μg/ml) and collagenase (Sigma Type 1A, 2 mg/ml)). Cells were maintained at 20° C for 3 to 4 days in frog Ringer (ND96) of the following composition (mM): NaCl, 96.0; KCl, 2.0; $CaCl_2$, 1.8 and HEPES, 5.0 supplemented with pyruvate (2.5 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). All solutions were at pH = 7.5 ± 0.02.

Intracellular injections: Resolved G-protein subunits, bovine serum albumin (Armour Biochemicals) or buffer were injected into oocytes using a Drummond microinjector prior to voltage clamping. All cells were injected in the animal pole. IP_3 was injected into cells using a

Picospritzer pressure injection system while recording from the cell under voltage clamp, as previously described (8).

Electrophysiological measurements: Oocytes were assayed one to four days post operatively using the two-electrode voltage clamp technique. Individual oocytes were placed in a 0.5 ml bath constantly perfused with ND96 at room temperature. The oocyte was voltage clamped between -50 and -70 mV with 3 M KCl microelectrodes (0.5 - 2.0 MΩ). Acetylcholine (ACh) was applied by superfusion. T_{out}, a Cl^- conductance which is activated by a voltage gated Ca^{++} channel (11), was evoked by a microcomputer controlled voltage step protocol. Cells were injected with the Drummond microinjector in groups of at least five and allowed to recover for five to twenty minutes. The cells were then assayed at a rate of about one every five minutes by the following protocol: cell voltage clamped for one minute, resting potential and input resistance recorded, T_{out} evoked and recorded, three minute pause and then ACh applied. Data are reported as mean peak amplitude of induced Cl^- current ± standard error of the mean (SEM).

G-protein subunits: G-proteins were purified from human erythrocytes or bovine brain as previously described (12). βγ-subunits were obtained during the second DEAE-Sephacel purification. The βγ-subunits were concentrated to 50 μg/ml and stored at -70°C. α-subunits of brain G-proteins consisting mainly of G_i and (15-20%) G_o was obtained by heptylamine-Sepharose chromatography of G protein that had been treated with 10 mM NaF,

10 μM $AlCl_3$, and 25 mM $MgCl_2$ for one hour at room temperature (22-24°C). The α-subunits peak on the heptylamine Sepharose was identified by Commassie blue staining of SDS-polyacrylamide gels. The samples containing the protein were pooled and loaded on to a DEAE- Toyopearl, washed free of NaF, $MgCl_2$, and $AlCl_3$ and eluted with 200 mM NaCl. The α-subunits were concentrated to a final concentration of 100 μg/ml. The proteins were stored in 10 mM NaHEPES, 1 mM EDTA, 20 mM β– mercaptoethanol, 30% ethylene glycol and 0.1% purified Lubrol-Px at pH 8.0.

Analysis of G-protein subunits of *Xenopus* oocytes: Oocyte membranes were prepared according to the procedure of Kobilka et.al.(13). Pertussis toxin labeling and immunoblotting analysis of the β-subunit of the *Xenopus* oocyte was performed as previously described (14). SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (15). Proteins were measured by the fluorescamine method (12).

RESULTS

ACh (1 μM) elicited an inward Cl^- current in almost all oocytes (Fig. 1, inset) which was similar to the well known IP_3 mediated muscarinic cholinergic response of the oocyte (4, 5, 7, 8, 9). Most oocytes were harvested from a frog known to yield cells which have a collagenase insensitive muscarinic response. This was done to avoid the complications of using follicle enclosed oocytes and to facilitate the injection of proteins and IP_3 into the cells (8). Some experiments were performed in follicle enclosed cells. Each frog gave oocytes with a distinct mean amplitude. The range of responses across all cells was 500 - 3000 nA.

One of the *Bordatella pertussis* toxins is known to ADP-ribosylate certain G-proteins thereby uncoupling receptors from G-proteins and thus from their effector systems (1). By treating one group of cells with PTX and comparing it to a non-treated control group, it was shown that the ACh stimulated Cl^- current is sensitive to pertussis toxin treatment (Fig. 1). The toxin treated group had a mean response of 237 ± 82 nA (n=15) whereas the control group mean response was 1683 ± 163 nA (n=15). This result suggested that the ACh response in the native oocyte was probably mediated by a PTX sensitive G-protein.

We then analyzed the oocyte membranes for the presence of G-protein subunits. Incubation of oocyte membranes with $[^{32}P]$-NAD^+ and activated pertussis toxin resulted in the labeling of a 41 kDa protein (Fig. 2a) as previously reported (16). The size of this protein is indicative of the α-subunit of the G_i family of proteins

(1). U-49 is an antiserum which was raised against a synthetic peptide encoding a sequence from the β_{36}-subunit of G-proteins (17). Immunoblotting experiments using the U-49 antiserum showed that the oocyte membrane contains the β_{36}-subunit (Fig. 2b).

These data indicate that the native *Xenopus* oocyte contains both the α and β subunits of G-proteins and that the muscarinic receptor is coupled to phospholipase C through a pertussis toxin sensitive substrate. The amount of IP_3 produced is measurable as the amplitude of receptor activated Cl- current (7,8). Hence, we used this system for analyzing the role of G-protein subunits in modulating signal transduction through phospholipase C.

First we tested the effect of $\beta\gamma$ subunits on the ACh elicited Cl^- current. One group of cells was injected intracellularly with 1.5 ng human erythrocyte $\beta\gamma$ per cell in a 40 nl volume. (The oocyte has an approximate volume of 1.0 µl.) These cells were then analyzed under voltage clamp for responsiveness to ACh. Each cell's resting membrane potential and input resistance were also recorded. These results were compared to a control non-injected group of cells. Figure 3a shows that the erythrocyte $\beta\gamma$ reduces the ACh elicited Cl^- current by about 95%. The injected cells did not show a difference in resting potential (47.3 ± 1.5 mV) and input resistance (0.43 ± 0.1 MΩ) as compared to non-injected controls (resting potential = 49.5 ±1.5 mV and input resistance = 0.73 ±0.2 MΩ). This experiment was repeated using 1.0 ng of bovine brain $\beta\gamma$ per cell in a 40 nl volume (Fig 3b). The brain $\beta\gamma$ caused approximately a 94% reduction in the receptor activated Cl^- current. These injected cells also did not show a change in resting potential and input resistance.

The native oocyte has a voltage dependent Cl^- conductance known as T_{out} (11). This current is elicited by pre-pulsing the cell from the resting potential to -100 mV for 5 s and then stepping the potential to +10 mV for 5 s. Presently there is no evidence that T_{out} is mediated by a G-protein. Therefore, we used T_{out} as a same-cell control for the specificity of the injected $\beta\gamma$ subunits. Fig. 3a and 3b show that the injected $\beta\gamma$ had no effect on T_{out}. This suggests that the injected proteins specifically affected only the receptor mediated Cl^-current.

The specificity of the material injected into the cells was examined (Table 1). Injection of the $\beta\gamma$ storage vehicle did not have an effect on the ACh response or T_{out}. Injection of bovine serum albumin (1.0 ng/cell), boiled $\beta\gamma$ subunits (1.0 ng/cell), or resolved α subunits (1.0 ng/cell) each in 40 nl of buffer did not inhibit the muscarinic stimulated Cl^- current. We also tested the effect of varying concentrations of $\beta\gamma$ subunits injected into oocytes in a fixed volume (Fig. 4). It was found that the $\beta\gamma$ subunits half-maximally inhibited the muscarinic stimulated Cl^- current at about 0.4 ng/cell. At a cell volume of 1.0 μl, this is approximately equal to 10 nM. This is consistent with the findings of Cerione et. al. (18) who showed $\beta\gamma$ inhibition of G_s coupling of β-adrenergic receptors to adenylyl cyclase to be in the 10-100 nM range in a completely reconstituted system. Under the same conditions, T_{out} was not affected by any of the tested concentrations of $\beta\gamma$-subunits.

Since we are measuring the muscarinic stimulated Cl^- current, the locus at which the $\beta\gamma$-subunits act cannot be ascertained from the experiments described above. While it is likely that the $\beta\gamma$-

subunits act to attenuate IP_3 production stimulated by muscarinic receptor activation, it is also possible that the βγ-subunits act at other sites. The experiment in Fig. 5. was designed to determine the locus of the βγ-subunit effects. In this experiment we tested the response of control cells and cells injected with 1 ng βγ-subunits to intracellular injection of IP_3. Bath application of ACh to a control oocyte elicits the inward Cl^- current (Fig. 5a). Injection of IP_3 (2.6 pmol) into the same cell 20 minutes later causes a similar Cl^- current (Fig. 5b). A cell that has been injected with 1 ng of βγ-subunits does not respond appreciably to bath application of ACh (Fig. 5c). However, injection of IP_3 (2.6 pmol) into the same cell 15 minutes later results in activation of the Cl^- current (Fig. 5d). This experiment indicates that the βγ-subunits do not appear to have a direct effect on the Cl^- channels or the release of Ca^{++} from endoplasmic reticulum, rather βγ-subunits interfere with the muscarinic receptor activated Cl^- current at a site prior to the generation of IP_3.

DISCUSSION

G-proteins play a pivotal role in signal transduction at the cell surface. The overwhelming evidence at this stage indicates that G-protein coupled receptors never directly interact with their effector systems, rather they communicate with appropriate G-proteins which in turn modulate the activity of the effector system (19). Currently, at least six G-proteins whose α-subunits are substrates of pertussis toxin are known. These are G_t, G_o, $G_{i\,1,2,3}$ and G_{43}. Several cellular signal transduction systems are also affected by pertussis toxin. In addition to inhibition of adenylyl cyclase, these include stimulation of IP_3 production (20), stimulation of phospholipase A_2 activity (21), stimulation of hyperpolarizing K^+ channels (22), and both inhibition (23) and stimulation (24, 25) of Ca^{++} channels. However, at the present time several individual G-proteins have not been uniquely identified with a specified effector function as has G_s with stimulation of adenylyl cyclase and G_t with activation of the cGMP phosphodiesterase. Furthermore, recent data indicate that there may be differences in the mechanisms involved in the signal transduction process in the various systems. Thus, while in both the adenylyl cyclase and cGMP-phosphodiesterase systems excess $\beta\gamma$-subunits inhibit the activity of the GTP liganded α-subunit, it has been reported that in atrial cells, K^+ channels can be activated by $\beta\gamma$-subunits (26). In contrast, Birnbaumer and co-workers have shown that the α-subunit of purified human erythrocyte G_i protein(s) can open K^+ channels (27). The $\beta\gamma$-

subunits have also been reported to stimulate phospholipase A_2 activity in retinal rod cells (28).

In light of the diversity of data that exist it is reasonable to examine the role of individual subunits in each signal transduction system. This was of particular interest in the phospholipase C system, where in spite of widespread reports on the effects of guanine nucleotides, no reproducible effects of G-proteins have been reported. From our initial experiments it became obvious that injection of βγ-subunits vastly attenuated muscarinic stimulation of the Cl^- current. The data presented here demonstrate that this effect is specifically observed only when βγ-subunits are injected, not when unrelated proteins such as bovine serum albumin or α-subunits are injected. Injection of the α-subunits of brain G-proteins generally gave small but significant stimulation of the muscarinic response. The basis for this stimulation is currently being explored in our laboratories. In contrast, βγ-subunits inhibited signal transduction extensively. Since this inhibition is relieved by the intracellular injection of IP_3, it is reasonable to conclude that βγ-subunits have their effects by disrupting communication between the agonist occupied receptor and phospholipase C. However, our data do not allow us to unequovically rule out direct effects of βγ subunits on phospholipase C or the muscarinic receptor, though this would appear unlikely. Since the βγ-subunits of G-proteins interact only with the α-subunit of signal transducing G-proteins, but not with other GTP-binding proteins such as the ras gene product p_{21} (29), it appears most likely that a G-protein which is an αβγ heterotrimer interfaces between the muscarinic receptor and

phospholipase C. Further, it appears likely in the phospholipase C system as well that an α-subunit activates the effector function. An immediate challenge for us is to determine the identity of this G-protein.

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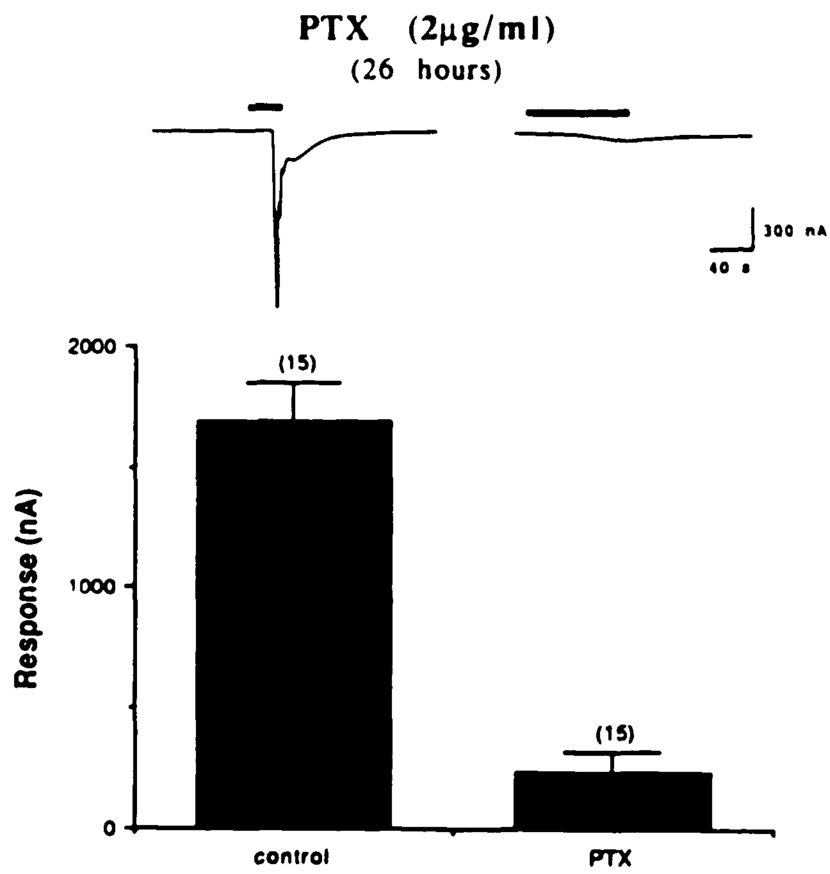
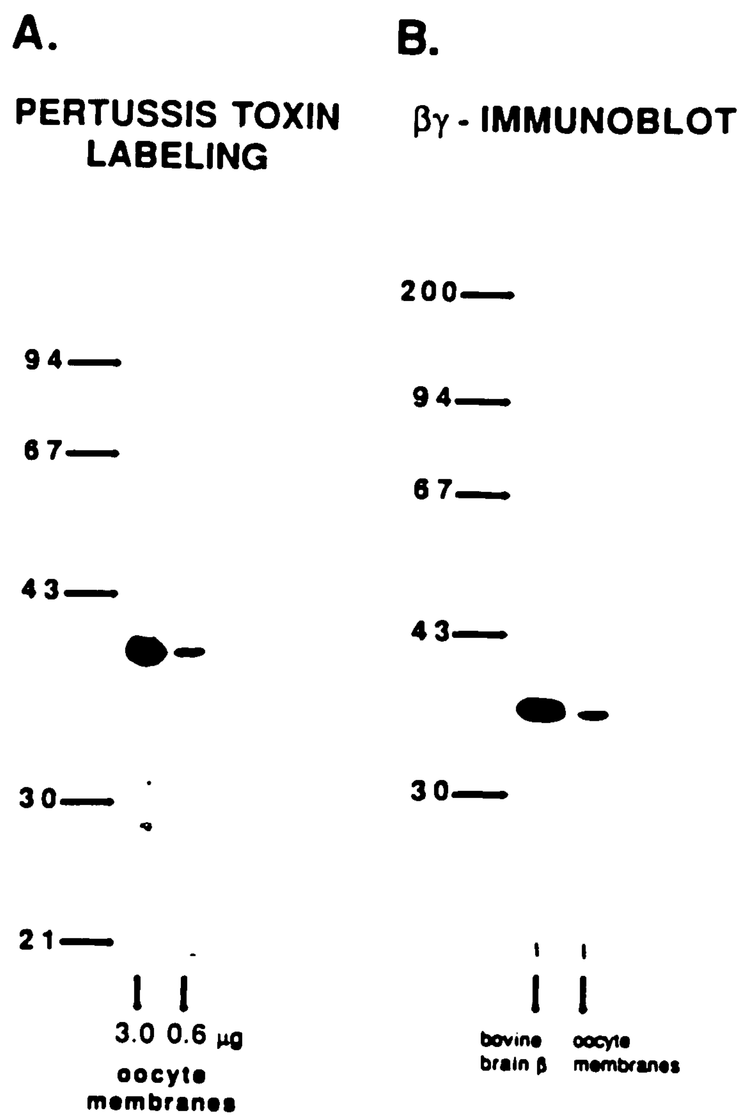
Figure 1



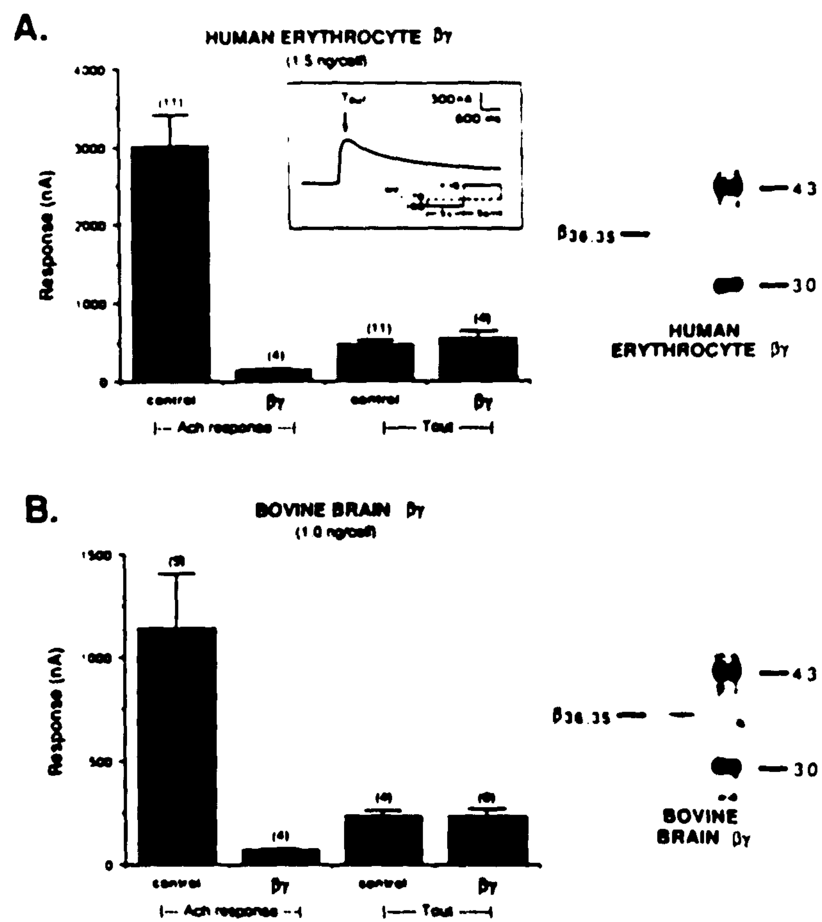
1. Effect of pertussis toxin (PTX) treatment on the muscarinic receptor evoked Cl^- current in *Xenopus* oocytes. Fifteen cells were treated with toxin (2μg/ml) for 26 hours then examined under voltage clamp for responsiveness to ACh (1 μM). The bar graph shows the mean ± SEM of the amplitude of the ACh stimulated Cl^- current in nA. The inset above the bar graph shows the characteristic waveform of the ACh evoked Cl^- current in a control cell (upper left panel) compared to a representative response in a toxin treated cell (upper right panel). Currents were recorded with two-electrode voltage clamp at a holding potential of -70 mV. Bar above the current trace indicates duration of ACh application.

Figure 2



2. A. Pertussis toxin catalyzed ADP-ribosylation of *Xenopus* oocyte membranes. B. Immunoblotting analysis of oocyte membranes with antiserum U-49 which is specific for the β 36 subunit of G-proteins. Indicated concentrations of oocyte membranes were incubated with activated pertussis toxin and [^{32}P] NAD^{+} (10^7 cpm; 1μM) in the presence of other additives as previously described [14]. Immunoblotting with U-49 antiserum (1:10,000 dilution) was performed as previously described [14]. 250 ng of purified bovine brain βγ and 50 μg of oocyte membranes were used. For detailed methodologies please see ref. 14. The pertussis toxin labeling autoradiogram is on Kodak XAR-5 film exposed for 48 hours. The immunoblotting autoradiogram is on Dupont Cronex film exposed for 48 hours.

Figure 3



3. Effect of intracellular injection of βγ - subunits on the muscarinic receptor evoked Cl^- current in *Xenopus* oocytes. (A) Each column of the bargraph represents the mean ± SEM amplitude of the activated current for a group of cells (number of cells is indicated above the column). The two columns at the left compare non-injected control cells to cells injected with 1.5 ng in 40 nl of human erythrocyte βγ-subunits. The two columns on the right compare the voltage activated T_{out} currents of the same cells. The inset shows a representative T_{out} current trace and the voltage step protocol used to elicit the T_{out}. A cell is voltage clamped at a holding potential of -70 mV. When it is prepulsed to -100 mV for 5s then stepped rapidly to +10 mV, the T_{out} Cl^- current will result. βγ-subunits inhibit the muscarinic response by about 95%, but do not effect the T_{out} Cl^- current in the same cell. Inset to right shows Commassie blue staining profile of the βγ-subunits used in the experiment. 0.5 μg of βγ-subunits was loaded on to the gel and electrophoresed, then fixed, stained and destained. (B) Experiments in B are the same as in A, except bovine brain βγ-subunits were used.

Figure 4

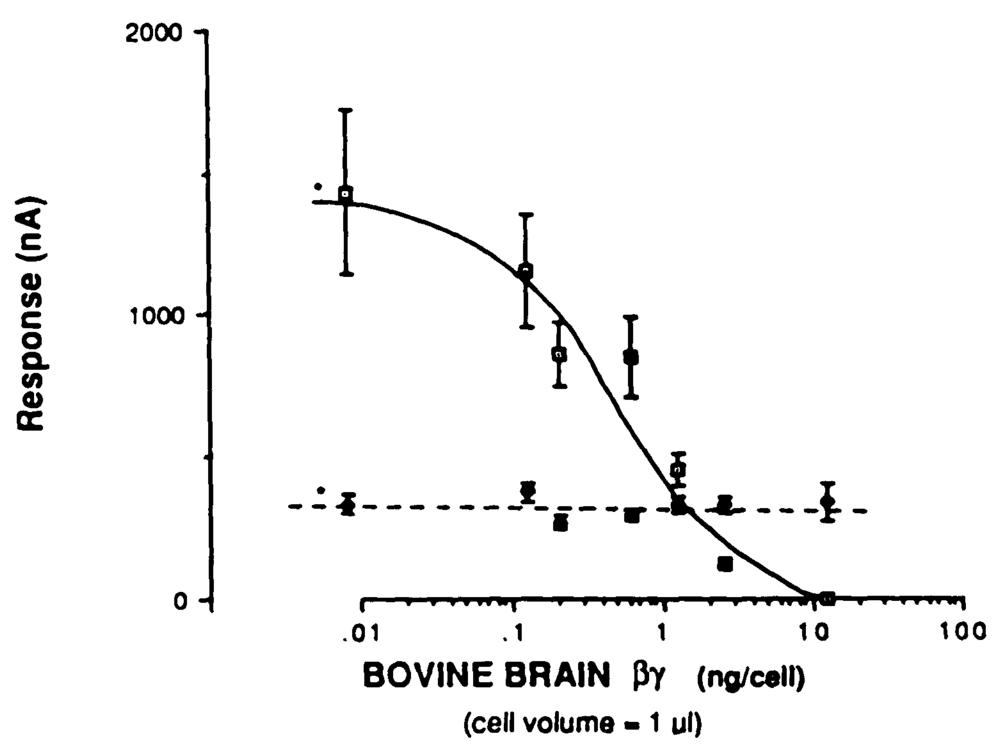
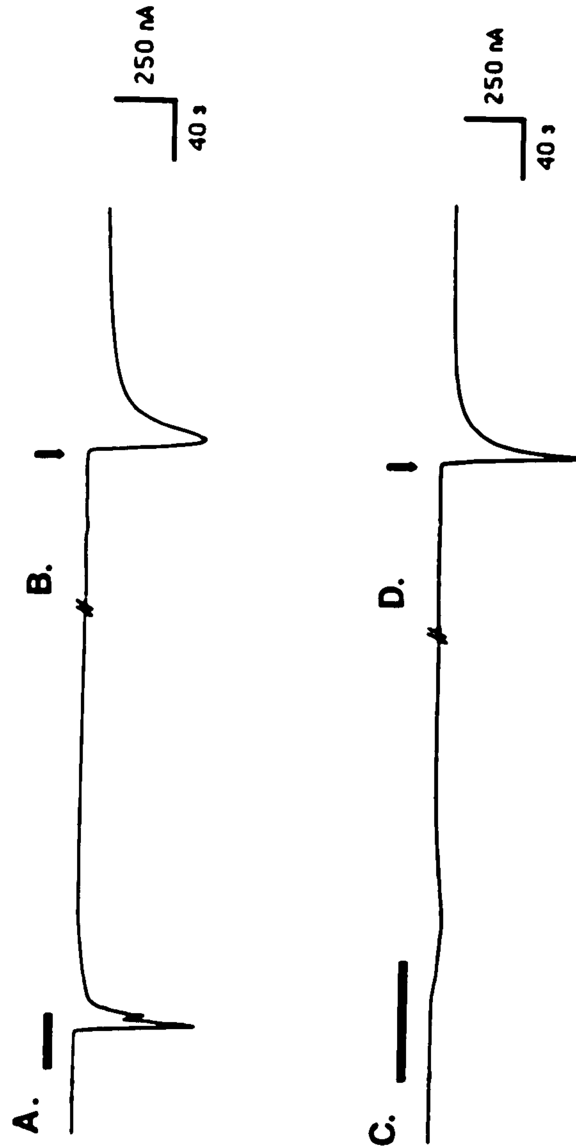


Figure 4. Effect of varying concentrations of bovine brain βγ-subunits on the muscarinic receptor evoked Cl^- current and on the voltage activated T_{out}. Each open square represents the mean ± SEM of the peak ACh evoked Cl^- current in at least five cells. The closed diamonds represent the mean ± SEM of the peak T_{out} current in the same cells. Cells were injected with varying amounts of bovine brain βγ at a fixed volume (40 nl). The asterisks (*) denote the group of cells injected with only vehicle. The oocyte has an average volume of 1 μl. The half-maximal inhibition was at approximately 0.4 ng/cell.

Figure 5



5. Effect of intracellular injections of βγ-subunits on application of ACh and intracellular injection of IP_3. Panel A shows the response of a control cell to 1 μM ACh. The bar denotes length of application. Panel B shows the response of the same cell twenty minutes later to intracellular injection of IP_3 (2.6 pmol in 2.6 nl). Arrow indicates the point of injection. Panel C shows the response of a βγ injected cell (1.0 ng of bovine brain βγ) to 1 μM ACh. Panel D shows the response of the same cell fifteen minutes later to intracellular injection of IP_3 (2.6 pmol). Intracellularly injected IP_3 is able to activate the Cl^- current. Cells were voltage-clamped at a holding potential of -70 mV. This procedure was performed on eight βγ injected cells.

Table 1: Effects of intracellular injection of various proteins and vehicle on the ACh stimulated Cl^- current and T_{out}.

MATERIAL INJECTED		CHLORIDE CURRENT (nA)	
	(n)	ACh (1 μM)	T_{out}
Experiment A			
None	(3)	1216 ± 148	---
vehicle	(3)	1151 ± 174	---
βγ-subunits	(3)	166 ± 52	---
Experiment B			
vehicle	(7)	1436 ± 287	334 ± 31
βγ-subunits	(7)	153 ± 16	333 ± 26
Experiment C			
none	(5)	1035 ± 175	492 ±59
BSA	(5)	1095 ± 134	465 ± 34
βγ (boiled)	(3)	925 ± 175	392 ± 26
βγ-subunits	(4)	68 ± 12	---
Experiment D			
none	(11)	1559 ± 174	301 ±16
α-subunits	(6)	2675 ± 384	229 ± 23

Cells were injected with either βγ-subunits (1.0 ng/cell), boiled βγ-subunits (1.0 ng/cell), α-subunits (1.0 ng/cell) or BSA (1.0 ng/cell) in the vehicle or with vehicle alone. Vehicle is the protein storage buffer with the following composition: 10 mM NaHEPES, 1mM EDTA, 20 mM β-mercaptoethanol, 30% ethylene glycol and 0.1% purified Lubrol-Px, pH 8.0. All injections were in a 40 nl volume.

CHAPTER 5

Nature

(in press)

The G-protein G_o can serve as the signal transducer in the pertussis-toxin-sensitive phosphatidylinositol pathway in *Xenopus* oocytes

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SUMMARY

The *Xenopus* oocyte has a Ca^{2+}-dependent chloride current, that can be evoked by receptors that stimulate inositol trisphosphate production or by intracellular injection of IP_3. The activated α-subunit of the G-protein G_o directly evokes this Cl^- current using Ca^{2+} from IP_3-regulated Ca^{2+} stores. The activated α-subunits of the three G_i proteins were ineffective. G_o, but not the three G_i proteins, can also increase the amplitude of the muscarinic-receptor-evoked Cl^- current. Hence we conclude that G_o can serve as the stimulatory transducer in the pertussis-toxin-sensitive phospholipase C pathway in *Xenopus* oocytes.

INTRODUCTION

Receptor regulation of phospholipase C is a major mode of signal transduction at the cell surface and is found in essentially all cell types (1-3). Stimulation of phospholipase C results in the production of two intracellular messengers: inositol 1,4,5-trisphosphate (IP_3) which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C (2-4). Although a large number of neuronal, hormonal and humoral signals are known to work through phospholipase C, the mechanism of receptor coupling to phospholipase C has remained uncertain. The accumulated evidence suggests that a GTP-binding protein (G-protein) may be involved in coupling receptors to phospholipase C, however the identity of such a G-protein, and the putative G-protein's mechanism of action, have not been elucidated (1, 3, 5).

The *Xenopus* oocyte has endogenous muscarinic receptors (6, 7) (type M3 (8)) which couple to phospholipase C (9-11). Activation of these receptors is known to stimulate the production of IP_3 with resulting mobilization of Ca^{2+} from intracellular stores (9-13). The increased cytosolic Ca^{2+} in turn opens Ca^{2+}-sensitive Cl^- channels (14-17). The concerted opening and closing of these Cl^- channels can be observed as a characteristic, large depolarizing current in a voltage-clamped oocyte. The magnitude of the evoked Cl^- current has been shown to be proportional to IP_3 as measured by direct injection of IP_3 (13). Therefore the Cl^- current can be used as a sensitive measure of stimulated phospholipase C activity.

Recent experiments using pertussis toxin and resolved G-protein $\beta\gamma$-subunits have indicated that the coupling of muscarinic receptors to phospholipase C in *Xenopus* oocytes is mediated by a pertussis-toxin-sensitive heterotrimeric G-protein (18). In this article we show that the activated α-subunit of the GTP-binding protein G_o evokes the IP_3 mediated Cl^- current, whereas the three α_i's do not. Further, G_o, but not the three G_i's, increase muscarinic stimulation of the IP_3-dependent Cl^- current. These data suggest that G_o, but not the three G_i's, can serve as a signal transducer in the phosphatidylinositol pathway in *Xenopus* oocytes.

Activated α_o specifically stimulates the IP_3 mediated Cl^- current.

To test which pertussis-toxin-sensitive α-subunit could evoke the IP_3-dependent Cl^- current, we purified the four major pertussis-toxin-sensitive G-proteins. G_o and G_{i1} were purified from bovine brain. G_{i2} and G_{i3} were prepared from human erythrocytes. The identity of the purified G-proteins used were verified by quantitative immunoblotting using known protein concentrations and recombinant α-subunits as standards. G_o, G_{i1}, G_{i2} and G_{i3} were found to be at least 97% pure using 2 µg of α-subunits for immunoblotting with a sensitivity of cross contamination of 60 ng for all of the α-subunits tested. Each of the proteins used was free of any detectable contamination of the other G-proteins α-subunits (Fig. 1C).

These G proteins were then activated with GTPγS and Mg^{2+}. The $\alpha_{GTP\gamma S}$ -subunits were purified free of $\beta\gamma$-subunits and unliganded GTPγS on hydroxylapatite columns (α_o and α_{i1}) or DEAE Sephacel (α_{i2} and α_{i3}). When these activated α-subunits were injected into *Xenopus* oocytes, it was found that the activated α_o (α_o*) evoked a rapid response. The three activated α_i subunits did not have any effect. Injection of unliganded GTPγS in concentrations equivalent to that of the activated α-subunits (in 50-75 nM) did not evoke a response (Fig. 1). It has been previously shown that injection of much larger concentrations of GTPγS can activate a depolarizing current in *Xenopus* oocytes (2). A silver-staining profile of the α_o preparation is shown (Fig. 1).

When the α_o* was injected directly into an oocyte, a very rapid and robust current was observed (Fig. 2). To eliminate the possibility that α_o* was modulating plasma membrane Ca^{2+} channels (19, 20), all direct injection experiments were performed in a Ca^{2+}-free bathing medium. The α_o*-evoked response resembled that seen with application of acetylcholine (compare Fig. 2 with Fig. 4), injection of Ca^{2+} (15) or submembranal injection of IP_3 (Ref. 13 and Fig. 3), however, the duration of the response was shorter than that typically seen with deep IP_3 injections (13) or injections of GTPγS (21). In addition, the D_2 phase (7) of the response was usually blunted due to the Ca^{2+}-free bathing medium (22). The onset of the response to α_o* was quite rapid. Typical delays were in the 200-400 msec range. Injection of heat-inactivated aliquots of the same protein or of the protein vehicle alone did not evoke a significant response (Fig. 2).

We then determined the ionic basis of the $\alpha_o{}^*$-evoked depolarization. Current-voltage analysis of the response using the voltage-ramp method (7) indicated that Cl^- ions were primarily responsible for carrying the current (Fig. 2). The resemblance of the $\alpha_o{}^*$-evoked current to the receptor-evoked, IP_3-mediated, Cl^- current suggests that $\alpha_o{}^*$-evoked current may also be dependent on intracellular Ca^{2+} as is the IP_3 dependent Cl^- current in oocytes (reviewed in Ref. 10). Hence the next experiment was designed to determine the role of in intracellular Ca^{2+} in the $\alpha_o{}^*$-evoked response. Intracellular injection of the Ca^{2+}-chelator EGTA is known to block the receptor- and IP_3-evoked Cl^- currents (14, 15, 23, 24). We found that injection of EGTA blocked the $\alpha_o{}^*$ response as well (Figure 2D).

We then determined the role of IP_3 in the $\alpha_o{}^*$-evoked response. The basis for this experiment was as follows: if $\alpha_o{}^*$ stimulates phospholipase C to produce IP_3, then the $\alpha_o{}^*$-evoked response should depend on the IP_3-sensitive Ca^{2+} stores (2). It has been previously demonstrated that injection of a large dose of IP_3 into an oocyte can render the cell refractory to subsequent injections of IP_3 or bath application of agonist (25). If $\alpha_o{}^*$ worked through the production of IP_3, then its effect should also be attenuated by preinjection of IP_3. Figure 3A shows an example of an experiment in which, after a large depolarizing response to injection of IP_3, the oocyte is unresponsive to a subsequent injection of IP_3. Similarly, if $\alpha_o{}^*$ is injected into a cell after an IP_3 injection, it, too, is unable to evoke a response (Fig. 3A). The bar graph in Figure 3B summarizes several such experiments. We have also determined that this ineffec-

tiveness is not due to inactivation of the Cl^- channels since repeated injection of Ca^{2+} evokes the Cl^- current repeatedly (data not shown). These experiments indicate that $\alpha_o{}^*$ uses the IP_3-sensitive Ca^{2+} stores. The most reasonable explanation for this phenomenon is stimulation of IP_3 production.

G_o specifically increases the amplitude of the muscarinic evoked Ca^{2+}-dependent Cl^- current.

The studies on the direct injection of activated α-subunits indicates that G_o could stimulate IP_3 production. This would suggest that G_o could couple muscarinic receptors to inositol-phosphate turnover. Hence we tested which holo-G-protein, when added to the oocyte, could increase muscarinic stimulation of the IP_3-mediated Cl^- current. This assay is based on observations in other G-protein-coupled systems such as the G_s-regulated adenylyl cyclase where it has been known for some time that addition of exogenous G-proteins to membranes that contain a "normal" complement of G-protein results in enhanced stimulated activity. Sternweis and Gilman have shown that addition of G_s results in enhanced G_s-dependent stimulation of adenylyl cyclase in membranes that contain the normal amount of G_s (26). This is also true for receptor stimulation where, for example, addition of purified G_s to liver membranes results in enhanced glucagon stimulation of adenylyl cyclase activity (27).

We used a similar assay to test which G-protein might increase muscarinic stimulation of phospholipase C activity. For

this purpose, the four major pertussis-toxin-sensitive G-proteins were tested for their ability to augment the muscarinic-stimulated Cl^- current in oocytes. Purified holo-G-proteins were injected into groups of oocytes. After 30 minutes, individual cells were then voltage-clamped and tested for responsiveness to acetylcholine. Figure 4A shows representative current traces from a control cell (*left*) and a cell injected with G_o (*right*) from the same experiment. Figure 4B is a composite of three experiments. It can be readily seen that injection of G_o results in a substantial increase in the muscarinic response while injection of the three G_i proteins does not result in any significant increase as compared to non-injected controls. To prevent observer bias, an initial injection comparing G_o and G_{i1} (the two major brain G-proteins) was performed in a blind fashion using letter-coded samples. In five experiments, the G_i proteins never showed any facilitatory effect. The G_o protein was tested nine times using two different preparations of G_o. Significant augmentation was observed in eight of these nine experiments. The extent of augmentation was variable (35 - 130%) ($72 \pm 11\%$; $n=8$). The waveform of the evoked Cl^- current was not altered by augmentation; only the amplitude of the current was increased (Fig. 4A). Augmentation by G_o was significant as assessed by the student's t-test comparing G_o-injected cells to G_i-injected cells and control cells.

DISCUSSION

The data presented here indicate that the GTP-binding protein G_o is specifically capable of coupling muscarinic receptors to IP_3 production, and that the activated α-subunit of G_o can directly stimulate the IP_3 second messenger pathway. The three G_i proteins or their α-subunits did not have any effects. In contrast to the ineffectiveness of α_i-subunits in the phosphatidylinositol pathway in *Xenopus* oocytes, all three activated α_i -subunits were capable of opening the atrial K^+ channels (28). Indeed the activated α_{i1}-subunit used in this study were from the same G_{i1}-preparation as those used in the atrial K^+ channel study (28). Taken together, the direct injection studies and the observation that G_o specifically increases muscarinic stimulation indicate that G_o and its α-subunit play a central and unique role in the IP_3 mediated pathway in the oocyte. The most reasonable locus of G_o action is between the receptor and the effector as in other G-protein-mediated signal transduction systems. This conclusion is strengthened by our finding that the onset of the response to α_o^* is much more rapid than the response to receptor activation. This would indicate that α_o^* can directly activate IP_3 production while the receptor effect is mediated via G-protein activation, which is recognized to be the slow step in G-protein mediated signal transduction pathways (5). Thus we conclude that G_o serves as the signal-transducing element in the pertussis-toxin-sensitive receptor-regulated phospholipase C system in *Xenopus* oocytes. Further the data shown here and our previous observations on the inhibitory role of G-protein $\beta\gamma$-subunits

in *Xenopus* oocyte phospholipase C activation (18) indicate that G_o functions in the phospholipase C pathway in a manner similar to that of G_s in the adenylyl cyclase system and G_t in the retinal phosphodiesterase system (5). A summary of the proposed mechanism is given in Fig. 5.

It has been assumed for some time that there exist(s) phospholipase C specific G-protein(s) (1-3, 5, 29). .However attempts to reconstitute the G-protein stimulated phospholipase C *in vitro* have been unsuccessful (3). Our ability to observe a measure of G_o stimulation of IP_3 production is due to the experimental system used: a large and easily manipulated whole cell, with a well-understood IP_3-mediated response. The voltage-clamped oocyte preparation allowed us to readily observe stimulation of phospholipase C as a Cl^- current, and to discretely study the pathway by injecting pure components of the various elements of the pathway. In this way we were able to study only the activity of the G-protein-stimulated phospholipase C. The background activity of the various other phospholipase C's (3), which may be a major impediment to accurate biochemical measurements of G-protein-dependent IP_3 production *in vitro* , was essentially zero. It is possible that G_o stimulates phospholipase C by some novel mechanism such as enhancing substrate availability which may not be easily reconstituted *in vitro.* The oocyte assay system does not appear to be constrained by these considerations.

Although G_o is generally thought of as a brain specific G-protein, since it occurs there most abundantly (30), G_o has been found in non-neuronal tissues as well. The cDNA for the G_o

α-subunit has been isolated from a *Xenopus* oocyte cDNA library (31). G_o has also been demonstrated by mRNA hybridization and immunohistochemistry in such diverse tissues as islets of Langerhans (32), heart and testis (33) and adipocytes (34). Wide occurrence of G_o as a trace protein could support the notion that it could be one of the transducers for the pertussis-toxin-sensitive phospholipase C. In the brain and neuronal cells indirect evidence from immunocytochemical (35) as well as biochemical/fluorescence techniques (36) have allowed others to conclude that G_o may play a major role in the IP_3 mediated pathway. However it ought to be emphasized that not all receptor-regulated phospholipase C responses are pertussis toxin sensitive. A recent review (1) lists equal number of pertussis-toxin-sensitive and -insensitive phospholipase C systems. Our studies are only applicable to the pertussis-toxin-sensitive pathway. The pertussis-toxin-insensitive pathway is not yet well understood. It is possible that there are two distinct G-protein-sensitive phospholipase C. However a simpler assumption is a single G-protein-regulated phospholipase C modulated by both pertussis-toxin-sensitive and -insensitive G-protein. The pertussis-toxin-insensitive pathway also involves a heterotrimeric G-protein, since it is attenuated by the injection of excess $\beta\gamma$-subunits (24). Receptors that utilize the pertussis-toxin-insensitive pathway in one cell type can utilize the pertussis-toxin-sensitive pathway in other cells (24, 37), indicating the possibility of a close relationship between the two G-proteins (38) Our data would predict that there is a close homolog of G_o which is

pertussis-toxin-insensitive that functions in the pertussis-toxin-insensitive pathway.

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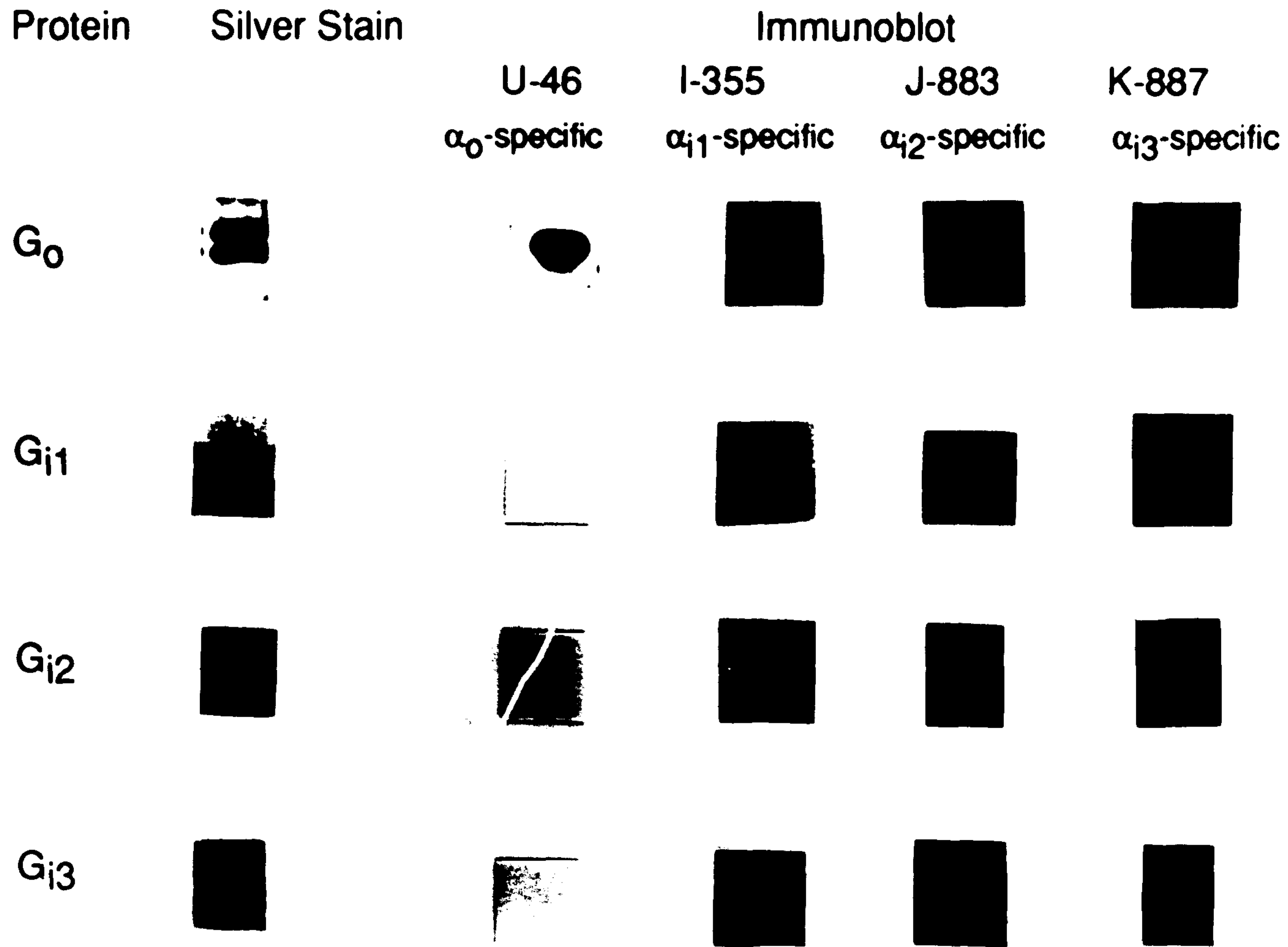


Figure 1A

Figure 1B

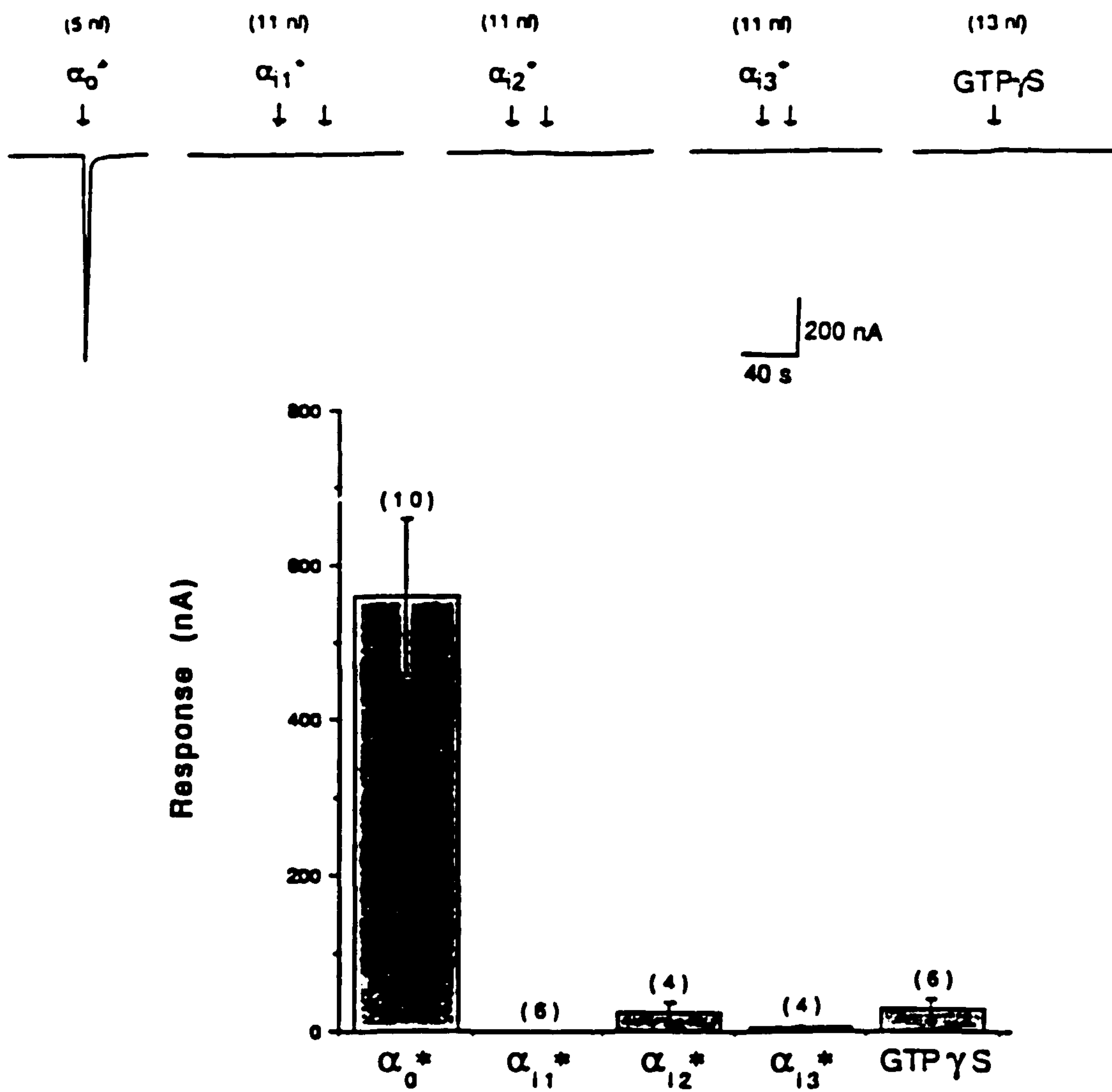
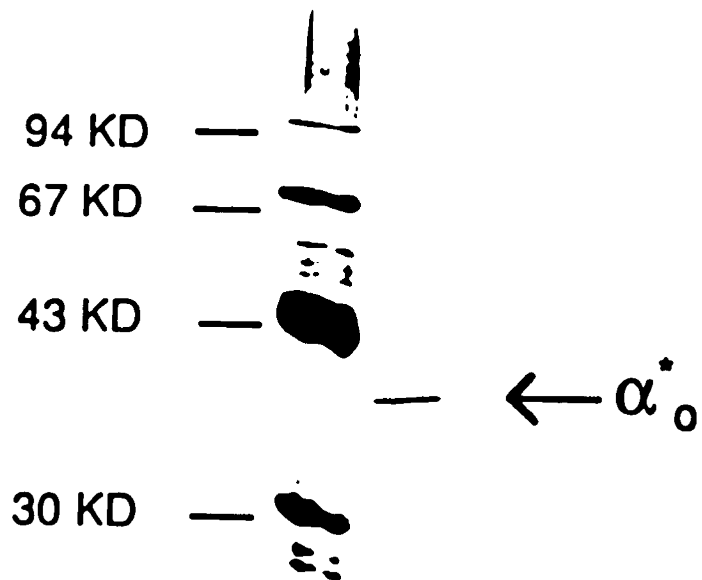


Figure 1C



1. Direct injection of the GTPγS-activated α-subunit of G_o evokes a depolarizing current in *Xenopus* oocytes, whereas the activated α-subunits of G_{i1}, G_{i2} or G_{i3} are without effect. **A**. Silver staining and immunoblotting profile of the G-proteins used in this study. **B**. The activated α-subunits of the four major pertussis-toxin-sensitive G-proteins were pressure-injected directly into single voltage-clamped oocytes. The volume of solutions injected is given in the Figure. The protein concentrations of the solutions injected were: α_o-58nM, α_{i1}-60nM, α_{i2}-50nM, α_{i3}-50 nM. The bar graph summarizes the results from several cells. The current traces above the bar graph show representative responses to each of the activated α-subunits tested. Thirteen nL of 50 nM GTPγS, in the same buffer as the α-subunits was also injected into a separate group of oocytes. This was done to rule out the possibility that the positive result seen with α_o^* might have been due to unliganded GTPγS in the injector pipette. **C**. Silver staining profile of the GTPγS activated α_o^* after purification on hydroxylapitite column and concentration on a Centricon-concentrator.

Methods.

G-protein purification. G_o and G_{i1} were purified from bovine brain and G_{i2} and G_{i3} were purified from human erythrocytes according to the procedure of Codina et al. (39) with the following modifications. The mixed G-proteins obtained after the second DEAE-sephacel were diluted and loaded onto a Mono-Q column connected to a Pharmacia FPLC column. The column was eluted with a shallow salt gradient (100-300 mM NaCl over 60 min) in a buffer containing 10 mM NaHEPES, 1mM EDTA, 20 mM β-mercaptoethanol,11

mM CHAPS and 0.1% Lubrol at pH 7.5. The peak tubes containing the resolved G-proteins were identified by Commassie-Blue staining of the electrophoretically-resolved proteins on SDS-polyacrylamide gels. The identity of the individual G-proteins were obtained by immunoblotting with α-subunit sequence-specific antisera which were the kind gift of Drs. S. Mumby and A. G. Gilman. Repeated chromatography (2-4 times) of the peak tubes over Mono-Q was required to obtain apparently homogenous individual G-proteins as assessed by immunoblotting. The purified G-proteins were stored in a buffer containing 25 mM NaHEPES, 1mM EDTA, 20 mM β-mercapto-ethanol, 30% (v/v) ethyleneglycol, 0.1% Lubrol and 100 mM NaCl in a final concentration of 50 - 60 μg/ml. G-proteins were diluted in 100 mM KCl before injecting into oocytes. For the determination of protein profile, approximately 200-500 ng of each G-protein were loaded on SDS-polyacrylamide gel for silver staining. After electrophoresis the gels were washed as previously described (40) and stained with silver according to the procedure of Wray *et al.* (41). Immunoblotting was performed with four sequence-specific antisera that are specific for α_o, α_{i1}, α_{i2} and α_{i3}. Specificity of these antisera was verified by studying cross reactivity with recombinant α-subunit fusion proteins expressed in *E. coli.* For immunoblotting purposes, 2-4 μg of purifed holo-G-proteins were resolved by SDS-gel electrophoresis, transferred to nitrocellulose paper and analyzed with the appropriate antisera. For detailed immunoblotting protocol used in our laboratory please see Ref. 36.

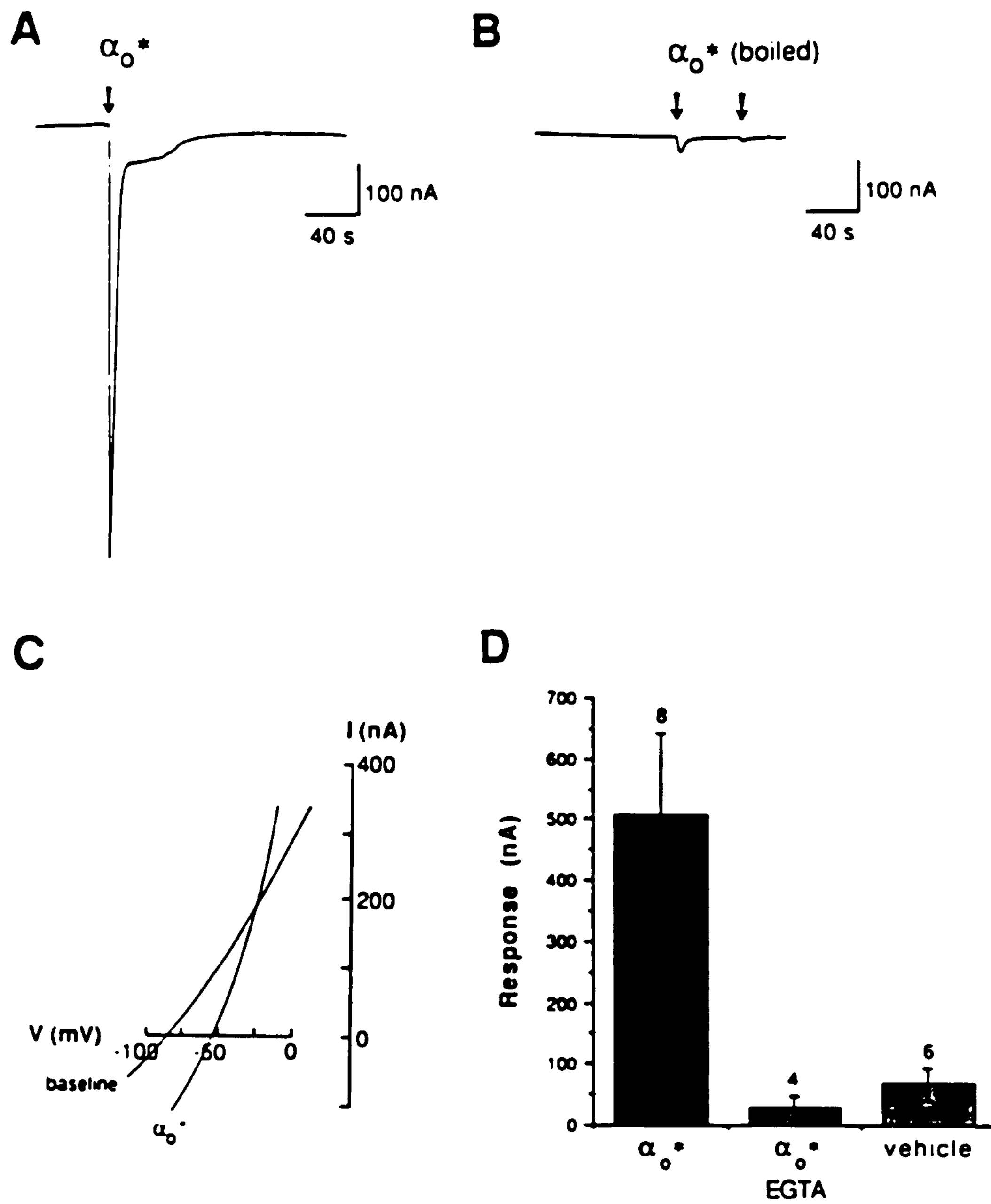
Electrophysiology. Stage V and VI oocytes from mature female *Xenopus laevis* were removed surgically under anesthesia (0.15%

tricaine immersion) and placed in ND96 frog Ringer's solution (96.0 mM NaCl/2.0 mM KCl/1.8 mM $CaCl_2$/5.0 mM NaHepes with pH = 7.50 ± 0.02). The oocytes' surrounding follicular tissues were enzymatically removed by gentle aggitation in collagenase (2 mg/ml) containing Ca^{2+}-free ND96. The oocytes were incubated in ND96 with pyruvate (2.5 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) added. Oocytes were assayed 3-7 days after harvesting using a standard two-electrode voltage clamp technique as previously described (24). Individual oocytes were placed in a 0.5 ml bath constantly perfused with ND96 at room temperature. The oocyte was voltage-clamped at -70 mV with two KCl (3 M) filled electrodes (0.5 - 2.0 MΩ). Amplifier outputs were recorded directly on chart paper and by an X-Y plotter. Direct injection experiment was done using a picospritzer injector apparatus as previously described (24). Each injection consisted of several very brief pulses. The site of injections was less than 100 µm from the inner surface of the plasma membrane as measured when pulling the injector pipette out. All experiments were performed in a Ca^{2+}-free external bathing medium (Ca^{2+}-free ND96 with 10 mM Mg^{2+} and 0.1 mM EGTA added).

G-protein α-subunit activation. For G_o and G_{i1}, 20 µg of the G-protein was incubated in a solution containing 25 mM NaHEPES, 1mM EDTA, 20 mM β-mercaptoethanol, 30% (v/v) ethyleneglycol, 0.1% Lubrol and 100 mM NaCl, 10 µM [^{35}S]-GTPγS (~3 x 10^6 cpm) and 25 mM $MgCl_2$ (pH 8.0) for 60 min at 32° C (final volume of 500 µl). At the end of incubation the protein was loaded onto a 100 µl hydroxylapitite column. The column was washed extensively (~500

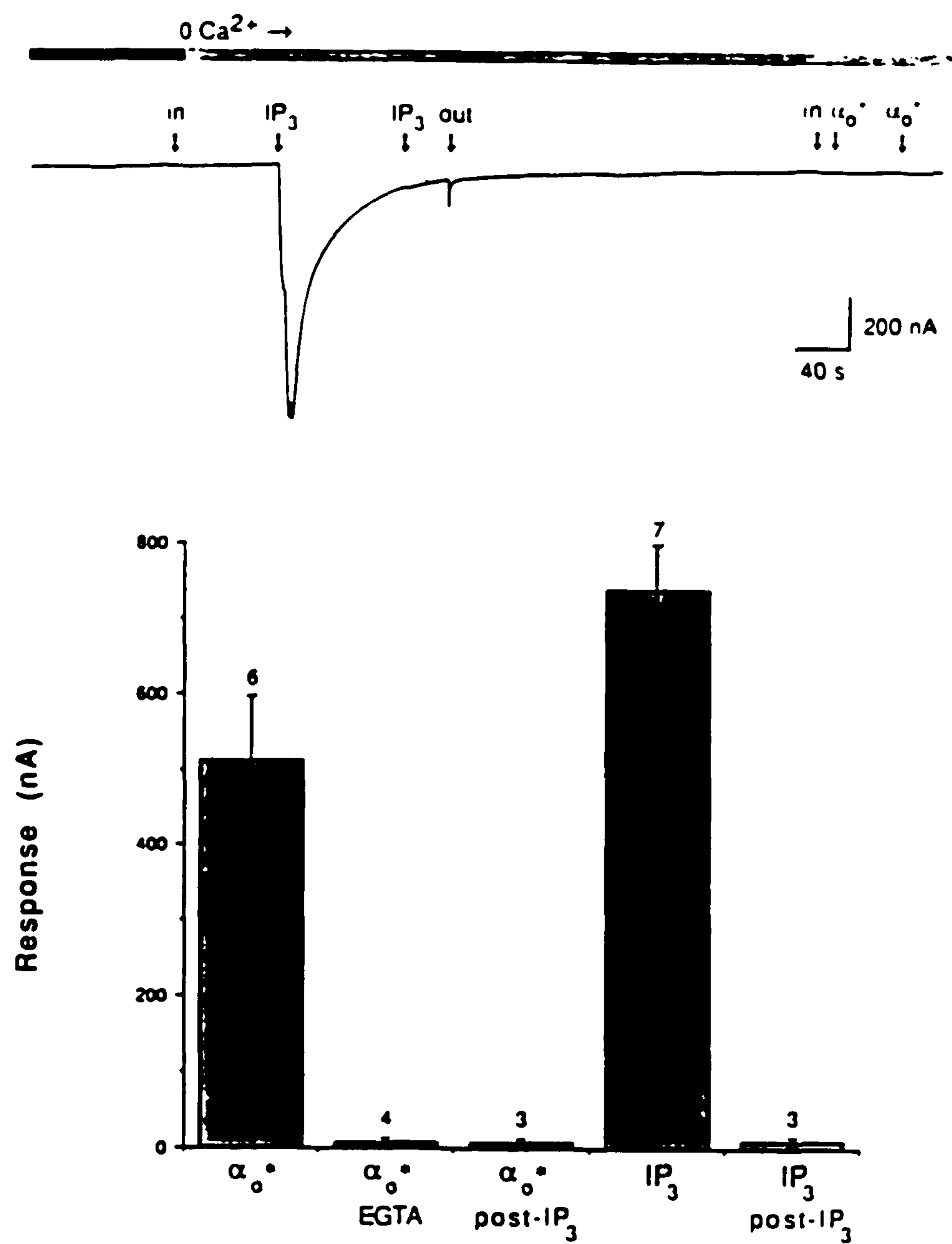
ml) with 25 mM NaHEPES, 20 mM β-mercaptoethanol, 100 mM NaCl and 2 mM $MgCl_2$ at pH 8.0. The protein was eluted in the same buffer containing 200 mM potassium phosphate. The samples containing the protein were identified by counting, then pooled, diluted tenfold and concentrated to 200 μl using a Centricon concentrator. The protein concentration was found to be the same by both silver staining and [^{35}S]-GTPγS counting indicating that all of the GTPγS was in the liganded state. The concentration of the protein was calculated from counting bound [^{35}S]-GTPγS. Activated α_{i2} and α_{i3} were the kind gift of Drs. Juan Codina and Lutz Birnbaumer and were prepared as previously described (42).

Figure 2



2. Direct injection of GTPγS-activated G_o α-subunit (α_o*) evokes a Ca^{2+} dependent Cl^- current. **A**. Direct pressure injection of α_o* into a voltage-clamped oocyte resulted in a rapid depolarization which resembled the acetylcholine-evoked response. A representative response is shown (6 nl of 50 nM α_o*). **B**. Direct injection of a boiled aliquot of the same α_o* as in Fig. 2A (9 nl and 7 nl). For experiments such as those show in Fig. 2A and 2B, the experimenter was blind to the identity of the injected substances. **C**. The ionic nature of the α_o* evoked-conductance was examined using the ramp method (7). Briefly, a ramp change in voltage applied to a voltage-clamped oocyte will give the current-voltage (I-V) properties of the membrane at that moment. The I-V curve is plotted directly on an X-Y plotter. The crossover point of a I-V curve applied at rest (**baseline**) and during an evoked current (**α_o***) will give the reversal potential of the response. Voltage ramp analysis of the α_o* evoked response shows a reversal potential of -23 ± 2 mV (n=3). Since the equilibrium potential for Cl^- in *Xenopus* oocytes is approximately -25 mV (10, 43, 44), these results indicate that the current evoked by α_o* is carried primarily by Cl^- ions. A single ramp experiment is shown. **D**. The Ca^{2+} dependence of the α_o* evoked Cl^- current was addressed by pre-injecting cells with the Ca^{2+} chelator EGTA. The bar graph compares three groups of cells: control cells in response to α_o*, cells pre-injected with EGTA (10^{-4} M) in response to α_o* and a group of cells injected with the protein storage vehicle. The α_o* injections were approximately 4-7 nl of 50 nM α_o* per oocyte. The vehicle injections were 5-10 nl of storage buffer per oocyte.

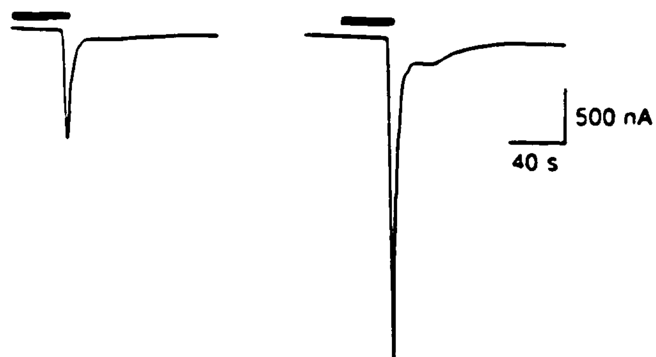
Figure 3



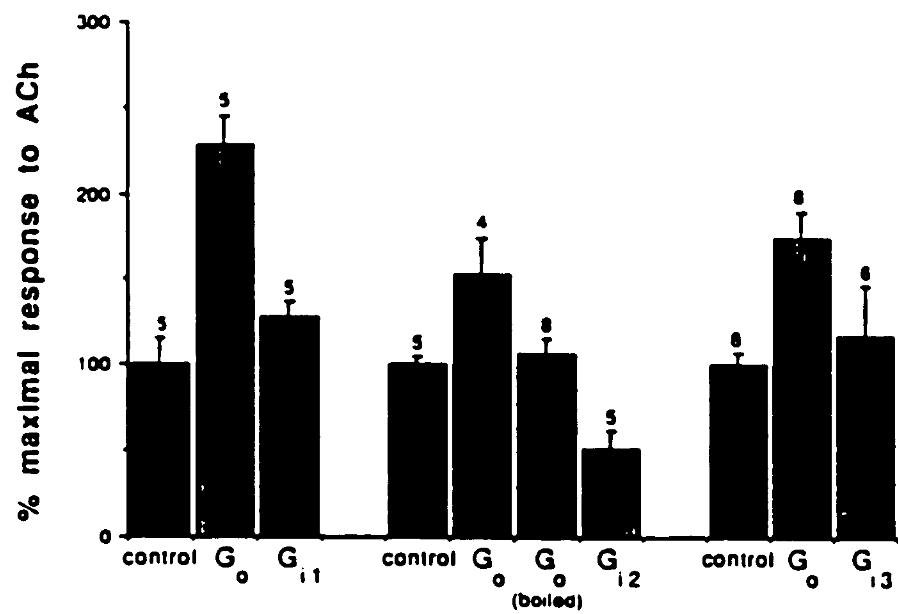
3. Direct injection of α_o* and IP_3 evoke the Ca^{2+}-dependent Cl^- current through the same mechanism. A voltage-clamped oocyte was impaled with an IP_3-containing injector pipette (**in**). After switching the external medium from ND96 to Ca^{2+}-free ND96 (bar above trace), IP_3 (2.6 pmol) was injected (**IP_3**) and a large depolarization resulted. Subsequent injection of IP_3 (2.2 pmol) (**IP_3**) did not yield a response. The injector pipette was then removed (**out**), and a new pipette containing α_o* (50 nM) was inserted (**in**). Injection of α_o* failed to elicit a response (**α_o***) (18 nl and 7 nl). The α_o* containing injector pipette was impaled into a subsequent cell to confirm that the sample of α_o* was active. In general, each aliquot of α_o* was tested in at least one cell either prior to, or subsequent to, the cell used in this experiment. The results for a series of experiments are summarized in the bar chart. The numbers above the bars indicate number of cells tested for each condition.

Figure 4

A

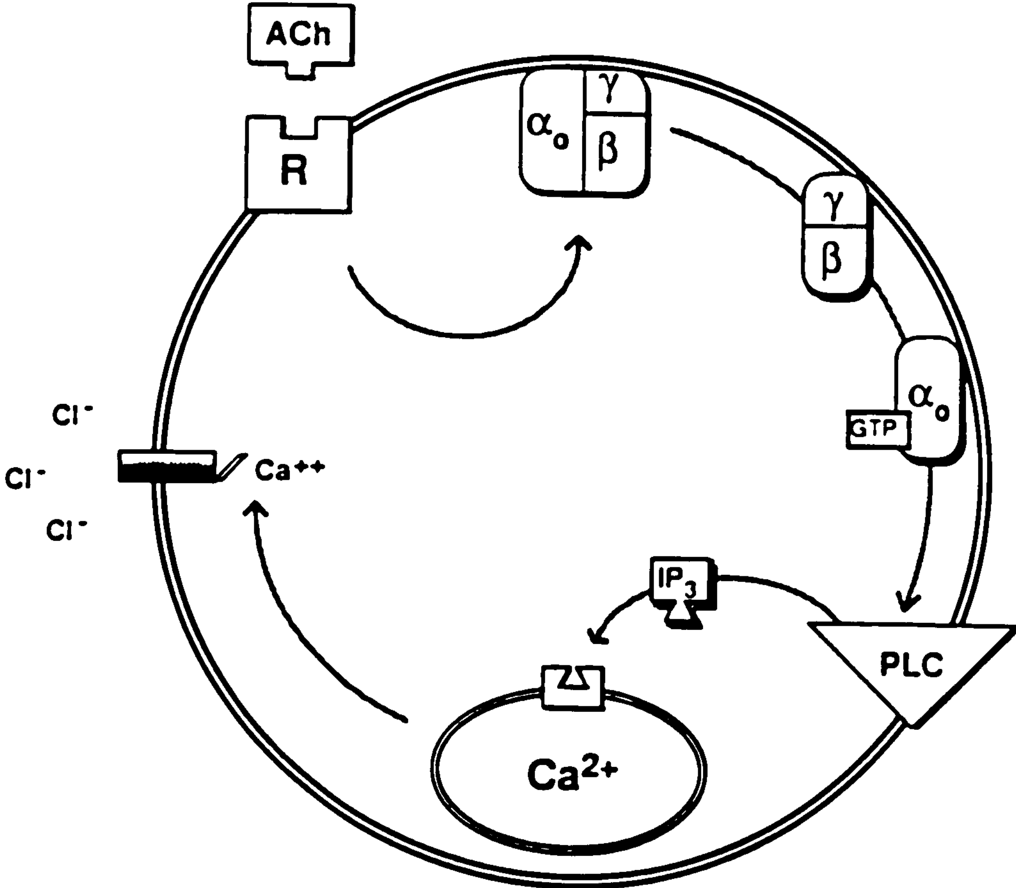


B



4. G_o, but not G_{i1}, G_{i2} or G_{i3}, increases the amplitude of the muscarinic-receptor-stimulated chloride current.
A. Representative current traces from a voltage-clamped control cell (*left*) and a cell injected with holo-G_o (*right*) in response to acetylcholine (1 μM). The small bar above each trace indicates the duration of acetylcholine application. Acetylcholine was applied by superfusion. Holo-G-proteins were injected using a Drummond microinjector as previous described (24). The G-proteins in storage buffer were diluted seven fold in 100 mM KCl, so as to give a 50-60 nM G_i-protein solution. 40 nl of this diluted solution was injected into an oocyte 30 minutes prior to the electrophysiological measurements. The amplitude of the Cl^- current was recorded at the peak of depolarization. **B**. Four pertussis-toxin-sensitive G-proteins were studied. Three experiments are shown comparing the acetylcholine responses of groups of oocytes that have been injected with either holo-G_o, G_{i1}, G_{i2} or G_{i3}. The control cells have not been injected. A group of cells injected with a heat-inactivated G_o (*center*) is included for comparison to non-injected controls. (Significant augmentation by t-test: control vs. G_o for the three experments: $p < .005$; $p < .025$; $p < .005$; control vs. Go(boiled): $p < .4$ (not significant); control vs. G_{i1}: $p < .1$ (not significant); control vs G_{i2}: $p < .005$ (significant reduction); control vs G_{i3}: $p < .4$ (not significant).)

Figure 5



5. The receptor-evoked, IP_3-mediated, Ca^{2+} dependent Cl^- current in *Xenopus* oocytes. This schematic represents the pathway of receptor activation of the phospholipase-C-dependent Cl^- current. Binding of acetylcholine to cell surface receptors permits the dissociation of $\alpha_{o\text{-}GTP}$ from $\beta\gamma$ and the activation of phospholipase C by $\alpha_{o\text{-}GTP}$. Phospholipase C generates IP_3 which releases stored intracellular Ca^{2+}. The released Ca^{2+} opens Ca^{2+}-sensitive Cl^- channels. The sum of individual Cl^- conductances gives the macroscopic Cl^- current seen in a voltage-clamped whole cell.

CHAPTER 6

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Coupling of exogenous receptors to phospholipase C in *Xenopus* oocytes through pertussis toxin-sensitive and -insensitive pathways: crosstalk through heterotrimeric G-proteins

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SUMMARY

Heterotrimeric G-proteins can be categorized into molecularly divergent groups by their differential sensitivity to pertussis toxin. Receptors specifically use either pertussis toxin sensitive or insensitive G-proteins to couple to specific effectors. Receptor stimulation of phospholipase C, however, is pertussis toxin sensitive in some systems and pertussis toxin insensitive in others. We studied the coupling of receptors to phospholipase C by expresssing receptors from both systems into a single cell: the *Xenopus* oocyte. AVP receptors from liver and CCK receptors from brain were expressed in oocytes by intracellular injection of RNA. Both receptors stimulated a Ca^{2+} dependent Cl^- current which can also be evoked by intracellular injection of IP_3. Hence, receptor stimulation of phospholipase C was measured as the evoked Ca^{2+} dependent Cl^- current. The liver AVP receptor, which is known to stimulate phospholipase C in a pertussis toxin insensitive manner (Lynch, C.J., Prpic, V., Blackmore, P.F. and Exton, J.H. (1986) *Mol. Pharmacol.* **29**, 196-203), was found to stimulate phospholipase C through a pertussis toxin sensitive pathway in the *Xenopus* oocyte. The CCK receptor from brain stimulated phospholipase C through a pertussis toxin insensitive pathway. Both AVP and CCK stimulation of phospholipase C were attenuated by the intracellular injection of excess G-protein $\beta\gamma$ subunits. Neither pertussis toxin treatment nor intracellular injection of $\beta\gamma$ subunits affected any steps subsequent to IP_3 production. From these data we conclude that both the pertussis toxin sensitive and insensitive pathways for receptor

coupling to phospholipase C are transduced by heterotrimeric G-proteins. We also find that there is a lack of coupling fidelity of receptors to G proteins in stimulation of phospholipase C which can be influenced by the membrane environment.

INTRODUCTION

The role of G-proteins in coupling receptors to effectors is well established (1,2). This coupling is characterized by either a sensitivity or lack of sensitivity to pertussis toxin. For example, receptor mediated stimulation of adenylyl cyclase is pertussis toxin insensitive in all systems whereas receptor mediated stimulation of K^+ channels is pertussis toxin sensitive in all systems (3-7). One exception to this clear distinction is the receptor stimulated phospholipase C which shows pertussis toxin sensitivity in some system, but not in others. It has been proposed that two distinct pathways operate in these systems (8,9). An interesting and obvious question is whether these two pathways are always distinct or whether under appropriate conditions receptors that use one pathway can use the other.

To address this question we have utilized the receptor regulated phospholipase C in *Xenopus* oocytes. The oocyte exhibits a receptor evoked Cl^- current that can be measured electrophysiologically under voltage clamp (10,11). This current can be evoked by native muscarinic receptors and various exogenous receptors expressed in the oocyte by intracellular injection of RNA (12,13). It has been demonstrated that such receptors act by stimulating phospholipase C and liberating inositol 1,4,5-trisphosphate (IP_3) into the cytosol (14-17). IP_3 evokes the Cl^- current by releasing Ca^{2+} from intracellular stores (14,18,19). The released Ca^{2+} opens Ca^{2+} sensitive Cl^- channels in the plasma membrane (20). The amplitude of the macroscopic Cl^- current has

been demonstrated to be directly proportional to the intracellular levels of injected IP_3. (21). Therefore the receptor evoked Cl^- current is an amplified and sensitive electrophysiological measure of receptor-stimulated phospholipase C activity.

We have recently shown that the native muscarinic receptor is coupled to phospholipase C through a heterotrimeric G-protein that is pertussis toxin sensitive (22). To test the nature of exogenous receptor coupling to phospholipase C, we expressed receptors from brain and liver. We tested if these foreign receptors could couple to phospholipase C through heterotrimeric G-proteins that can be regulated by G-protein βγ subunits and if such regulation occurred for both the pertussis toxin sensitive and insensitive pathways.

In this article we show that receptors that use both pertussis toxin insensitive and sensitive pathways couple to phospholipase C through heterotrimeric G-proteins, since in both pathways excess βγ-subunits attenuate the receptor signal. Further we show that "crosstalk" between the pertussis toxin sensitive and insensitive pathways is possible since the liver V_1-receptor, which utilizes a pertussis toxin insensitive G-protein in its native environment (23,24,25), utilizes a pertussis toxin sensitive pathway in the *Xenopus* oocyte.

EXPERIMENTAL PROCEDURES

Materials: [Arg[8]]-vasopressin, cholecystokinin-$8_{(sulfated)}$, cholecystokinin-4 and the V_1-receptor antagonist $d(CH_2)_5Tyr(Me)AVP$ (Manning compound) were obtained from Bachem (Torrance, CA) . Female *Xenopus laevis* were from Xenopus One (Ann Arbor, MI) or NASCO (Ft. Atkinson, WI). Inositol 1,4,5-trisphosphate was from Boeheringer Manneheim (Indianapolis, IN). Pertussis toxin was from List Biological Laboratories (Campbell, CA). Sources of all other materials have been previously described (22). ND96 frog Ringer's solution of the following composition was used: 96.0 mM NaCl/2.0 mM KCl/1.8 mM $CaCl_2$/5.0 mM NaHepes.

RNA and G-protein subunit preparation: Total RNA was prepared from 15 day old female rat livers or brains by the LiCl/urea method (26) and stored at -80° C in H_2O. G-protein $\beta\gamma$ subunits were prepared from bovine brain G-proteins as previously described (22,27) and stored in 10 mM NaHEPES/1 mM EDTA/20 mM β-mercaptoethanol/0.1% Lubrol/30% v/v ethylene glycol.

Injection of oocytes: Stage V and VI oocytes from mature female *Xenopus laevis* were removed surgically under tricaine anesthesia (immersion for 30 minutes in 0.15% tricaine solution). The oocytes were enzymatically defolliculated (2 mg/ml Sigma, Type 1A-S collagenase/Ca^{2+}-free ND96) then injected with RNA using a Drummond (Broomall, PA) microinjector (60 or 70 nl/oocyte total liver RNA (2.3 mg/ml) or 50 nl/oocyte total brain RNA (1 mg/ml)). Non-injected and water injected oocytes served as controls. The oocytes were incubated for 3-5 days in ND96 with pyruvate (2.5

mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) added. βγ subunits and storage-vehicle controls were injected using the Drummond microinjector 20-60 minutes before assay. IP_3 was injected using a Picospritzer injection system (General Valve, Fairfield, NJ). Briefly, glass capillaries (World Precision Instruments, New Haven, CT) were pulled to fine points on an electrode puller (Narishigi, USA, Greenvale, NY) then broken back to a point diameter of 2-3 μm under 100X magnification. IP_3 was back filled into each capillary which was then mounted on a Narisigi micromanipulator and connected to a N_2 gas source. Known volumes were ejected from the capillary by pulsing N_2 at preset pressure and duration.

Measurement of evoked Cl^- currents: Oocytes were assayed 3-5 days after injection of RNA using a standard two-electrode voltage clamp technique. Individual oocytes were placed in a 0.5 ml bath constantly perfused with ND96 at room temperature. The oocyte was voltage-clamped at -70 mV with two KCl (3 M) filled electrodes (0.5 - 2.0 MΩ) using a Dagan 8100 voltage-clamp amplifier (Minneapolis, MN). Amplifier outputs were recorded directly on chart paper (Gould 2200S, Cleveland, OH) and by an X-Y plotter (Hewlett Packard, Palo Alto, CA). Agonists and antagonists were applied by superfusion. The amplitude of the Cl^- current was recorded at the peak of depolarization.

Replication of results: All experiments were repeated at least twice. The number of oocytes used for each experiment is indicated in the individual experiment. Typical experiments are shown.

RESULTS

Oocytes injected with total liver RNA showed a substantial depolarizing response to bath application of 1.0 μM [Arg^8]vasopressin (AVP) (Fig. 1a). The average peak amplitude of the response to 1 μM AVP varied from frog to frog (range of means: 160 - 1200 nA), however, the variability among cells from a single frog was generally less that 30% of the mean maximal response. Non-injected oocytes from the same frogs showed no response to AVP (assayed in at least 10 cells from six different frogs). Water injected oocytes were also unresponsive to bath application of AVP. The response to AVP was detected at concentrations between 1 nM and 1 μM which is consistent with previous reports of successful expression of V_1-receptors in oocyte (28,29). The response to 1 μM AVP could be blocked by the co-addition of 5 μM V_1-receptor specific antagonist $d(CH_2)_5$Tyr(Me)AVP (30) to the bath (Fig. 1a). Oocytes injected with total brain RNA showed a robust depolarizing current in response to cholecystokinin-8(sulfated) (CCK) as has been previously described by us (31). The mean amplitude of the CCK (μM) evoked current ranged from 130-1300 nA among all experiments, however the variability on a single experiment was generally less than 25% of the mean maximal response. Non-injected oocytes from the four frogs used in the experiments described here never displayed a CCK response (Fig. 1b). While bath application of 0.5 μM CCK-8(s) evoked a depolarization, application of 50 μM CCK-4 did not evoke any response. This indicates that the expressed CCK receptors are

"peripheral" type which have been observed in several regions of the mammalian brain (32-35).

The responses to both AVP and CCK displayed the characteristic waveform of receptors which activate phospholipase C through G proteins (14-17,22,36). Such receptors mobilize Ca^{2+} from intracellular stores and thereby activate a depolarizing current mediated by Ca^{2+} sensitive Cl^- channels. To confirm that we were observing receptor stimulation of phospholipase C and to rule out the possibility that we were seeing some other form of depolarizing current (11,12,13), two experiments were performed. First, the nature of the ionic conductance was addressed by current-voltage (I-V) analysis of the responses (Fig. 2). The reversal potentials for both the AVP and CCK response were determined to be 20 ± 1 mV (n=9) and 23 ± 3 mV (n=6), respectively, by the voltage ramp method (37). These reversal potentials indicate that the depolarizing current evoked by AVP and CCK are carried by Cl^- ions (12,37,38,39). Second, if the response to AVP and CCK were due to phosphatidylinositol 4,5-bisphosphate hydrolysis and subsequent Ca^{2+} mobilization, then buffering the released Ca^{2+} with the Ca^{2+} chelator, EGTA, should abolish both responses. Liver RNA injected oocytes and whole brain RNA oocytes injected with EGTA (10^{-4} M) were unresponsive to AVP or CCK, respectively, whereas RNA injected cells not injected with EGTA displayed robust stimulation of the Cl^- current (Fig. 3). From these experiments, and previously published reports of the role of IP_3 in activating this Ca^{2+} sensitive Cl^- current, we conclude that the responses of liver RNA injected

oocytes to AVP, and whole brain RNA injected oocytes to CCK, are mediated by stimulation of phospholipase C.

We tested for the pertussis toxin sensitivity in the coupling of these exogenous receptors to phospholipase C (Fig. 4). For this purpose RNA injected oocytes were treated with pertussis toxin (2 μg/ml) for 24 hours. These conditions were used since it has been widely reported to result in maximal reduction of pertussis toxin sensitive phenomena in the *Xenopus* oocyte (22,36,40,41). Typical experiments are shown in Figure 4. Toxin treatment of liver RNA injected cells resulted in near abolition (~83% reduction) of the AVP response. Since in the liver it has been well established that the V_1-receptor, as well as other receptors, couples to phospholipase C through a toxin insensitive pathway (23,24,25), we confirmed our finding in groups of cells from two other frogs in subsequent weeks (Table 1). From these experiments it appears that the liver V_1-receptor in the *Xenopus* oocyte can utilize the pertussis toxin sensitive G-protein to couple to phospholipase C.

Oocytes injected with brain RNA and then treated with pertussis toxin displayed mixed sensitivity to pertussis toxin. In the experiment shown in Figure 4b it was found that the CCK response was insensitive to pertussis toxin. Another experiment performed in the same time period also demonstrated that the CCK response was pertussis toxin insensitive (control: 1138 ± 607 nA (n=5) vs. pertussis toxin treated: 1306 ± 267 nA (n=6)). In order to determine that the lack of pertussis toxin sensitivity of the CCK responses in brain RNA injected cells was not due to the lack of effect of pertussis toxin on the cells, we studied the effect of

pertussis toxin on another receptor regulated Cl^- current in the same batch of oocytes. We have previously shown that brain RNA injection also results in the expression of bombesin receptors that can evoke a Cl^- current (31). We tested if the bombesin response in brain RNA injected cells, from the same batch of cells as Fig. 4b, was sensitive to pertussis toxin. As shown in Fig. 4c, the bombesin response was substantially attenuated by pertussis toxin. Thus the lack of pertussis toxin effect on the CCK response was not due to a lack of pertussis toxin action on the oocytes.

While in this series of experiments we found that pertussis toxin did not inhibit the CCK response, in one subsequent experiment we have found some (~52 %; mean maximal amplitude 1320 ± 278 nA) inhibition of the CCK response by pertussis toxin. This variability in pertussis toxin sensitivity over a 12 month period is, however, not confined to the expressed CCK response. It is also found in the native muscarinic response. In the experiment in Fig. 4d, the native muscarinic response is inhibited by about 70% by pertussis toxin treatment. In contrast, previous experiments have shown 85-95% inhibition of the muscarinic signal. At the present time we do not understand the basis for this variability, however all of the CCK experiments reported here were performed during a time period when the CCK response was pertussis toxin insensitive.

In light of the finding that the liver V_1-receptor couples to phospholipase C through a pertussis toxin sensitive pathway in the *Xenopus* oocyte, we sought to determine if we had uncovered a pertussis toxin-sensitive step subsequent to IP_3 production. It is well established that IP_3 releases Ca^{2+} from intracellular stores

(14,18,19,42), and that Ca^{2+} is responsible for the opening of the Cl^- channels (20). Therefore, we compared the capacity of intracellular IP_3 injection to evoke the Cl^- current in control and pertussis toxin treated cells. The experiment in Figure 5 shows that there is no measurable difference in the Cl^- current evoked by IP_3 in control and pertussis toxin treated cells. Further, prior intracellular injection of EGTA blocks the stimulation of the Cl^- current by IP_3 indicating that the IP_3 evoked Cl^- current is Ca^{2+} mediated as has been described (14,19,21,42).

We then tested if both the pertussis toxin and toxin insensitive coupling pathways were mediated by heterotrimeric G-proteins. We have previously shown that the oocyte's native muscarinic response is transduced via a heterotrimeric G-protein by virtue of the regulation of this response by exogenous G-protein $\beta\gamma$ subunits. Here we studied the effect of intracellular injection of excess $\beta\gamma$ subunits on the AVP and the CCK evoked Cl^- currents. Previous work indicated that a 1 ng/oocyte injection (final intracellular concentration of 25 nM) should inhibit agonist evoked currents by approximately 80-90%. It was found that injection of $\beta\gamma$ subunits substantially inhibited both the AVP and CCK evoked responses. Injection of the $\beta\gamma$ storage vehicle did not cause any measurable effect on either AVP or CCK stimulation of the Cl^- current (Figure 6). As we have shown previously (22), injection of boiled $\beta\gamma$ subunits or other proteins does not cause this attenuation of receptor mediated stimulation.

We then determined if the $\beta\gamma$ subunits affected steps subsequent to IP_3 production. For this purpose we measured the

capacity of intracellular injection of IP_3 to evoke the Cl^- current in $\beta\gamma$ subunit injected cells. As can be seen in the experiment in Figure 7, intracellular injection of IP_3 in control and $\beta\gamma$-subunit injected cells evokes Cl^- currents of similar magnitude. Figure 7b(1) shows the response of a $\beta\gamma$ subunit injected oocyte in response to injection of IP_3. Figure 7b(2), the response of an oocyte to application of AcCho, is included for comparison. These observations indicate that G-protein $\beta\gamma$ subunits do not affect steps subsequent to IP_3 production.

DISCUSSION

The capability of *Xenopus* oocytes to faithfully express foreign messenger RNAs is now well established (12,13,43,44). Numerous receptors that couple to phospholipase C have been functionally expressed. This ability of the oocyte to express functional receptors from foreign messages, and the experimental capacity to inject both proteins and intracellular second messengers into the oocyte, allowed us to uniquely use this system to address several issues involved in the coupling of receptors to phospholipase C.

The first question addressed was whether the pertussis toxin sensitive and toxin insensitive coupling pathways are always distinct, or whether receptors can utilize both pathways depending on the environment. We found that liver V_1-receptors coupled to the *Xenopus* oocyte phospholipase C through a pertussis toxin sensitive G-protein. This finding was unexpected since total liver RNA was injected. It would be expected that, in addition to the receptor, the liver G-proteins are also being synthesized in the oocyte. It is known that oocytes will synthesize foreign G proteins (45). Our data do not offer any clues as to whether the liver G-proteins are being synthesized or not, but our data do indicate is that the liver V_1-receptors couple almost exclusively through the pertussis toxin sensitive G-protein in the oocyte since 85% of the V_1-receptor evoked Cl^- current is inhibited by pertussis toxin. This would suggest that the liver V_1-receptor can utilize both the pertussis toxin sensitive and insensitive G-proteins with similar efficiency to couple to phospholipase C. In contrast, in the brain RNA injected

cells, the CCK response was insensitive to pertussis toxin indicating that a pertussis toxin insensitive pathway also functions in the oocyte. It is not yet certain whether the oocyte has an endogenous pertussis toxin insensitive G-protein or if the protein is derived from the injection of brain mRNA. A noteworthy aspect of the CCK response is its total lack of sensitivity to pertussis toxin in these experiments which suggests that, at least in these situations, it preferentially utilizes the pertussis toxin insensitive G-protein in spite of the presence of the pertussis toxin sensitive G-protein as evidenced by the attenuation of the bombesin evoked Cl^- current. The CCK receptor expressed from brain mRNA appears to be of the "peripheral" type, since CCK-8(s) is an agonist while CCK-4 is not effective. "Peripheral" CCK receptors have been found in several brain regions including nucleus accumbens, area postrema, nucleus tractus solitarius, and the interpeduncular nuclei (33,34,35) and in a neural cell line (46). It has been reported that the peripheral type CCK-receptors couple to phospholipase C through a pertussis toxin insensitive G-protein (46,47,48). Thus in the case of CCK-receptors, the fidelity of coupling is retained even after expression in the oocyte. These observations suggest that not all receptors are promiscuous in their interactions with G-proteins. At this time we are unable to determine why some receptors do, and others do not, display specificity in coupling to phospholipase C.

The second question we addressed was whether both the toxin sensitive and insensitive pathways utilized heterotrimeric G-proteins. We approached this issue by the injection of G-protein $\beta\gamma$ subunits since in all heterotrimeric systems $\beta\gamma$ subunits appear to

attenuate α subunit mediated stimulation of effectors. This question was of particular relevance to us since it has been reported that oncogene products such as the *ras* gene product that are monomeric GTP-binding proteins may be involved in the regulation of phospholipase C (49). We found that both the pertussis toxin insensitive and sensitive pathways are attenuated by $\beta\gamma$ subunits at a step prior to IP_3 production, strongly suggesting that heterotrimeric G-proteins operate in both pathways.

The involvement of heterotrimeric G-proteins in both the pertussis toxin sensitive and insensitive pathways, and the observation that the liver V_1-receptor can utilize both G-proteins, suggests that the structural divergence between the α subunits of the two G-proteins cannot be great, at least in the C-terminus region, which is known to be involved in interaction with the receptor, since single amino acid substitutions or modifications in this region leads to uncoupling of the G-protein from the receptor (50, 51, 52). Future experiments in our laboratories will be geared toward determining how receptor coupling is maintained by these G-proteins in spite of the loss of pertussis toxin sensitivity.

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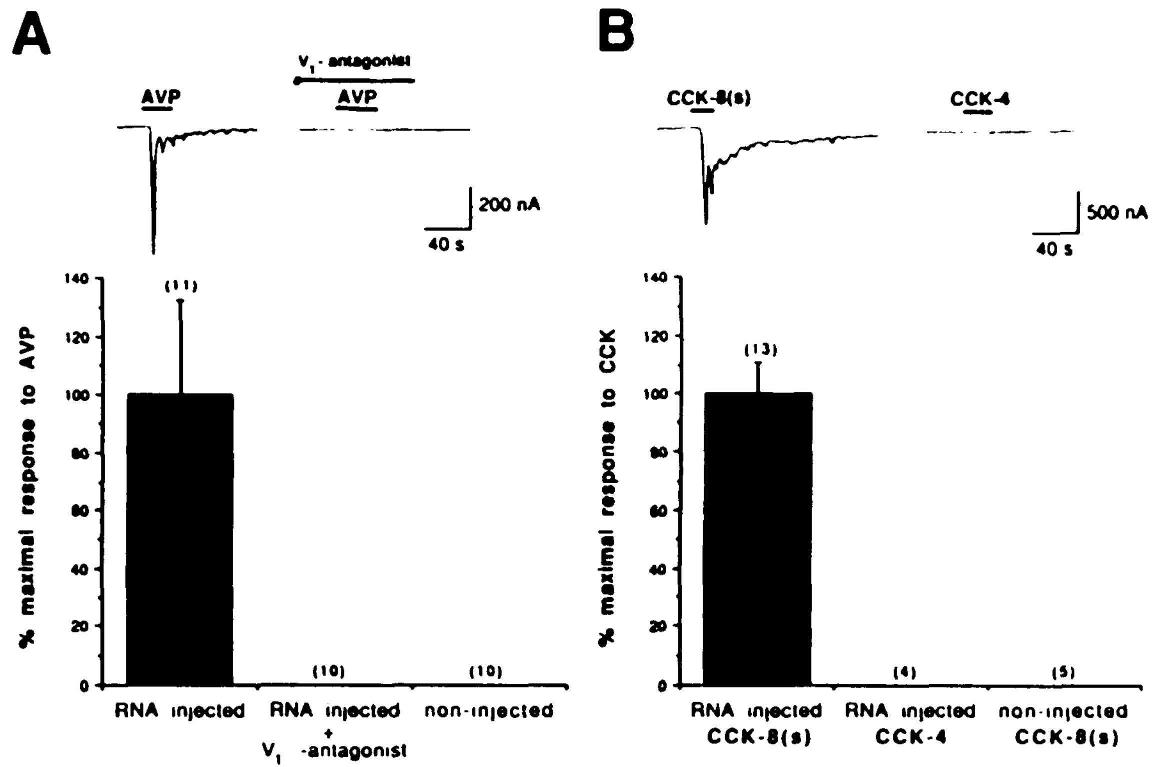
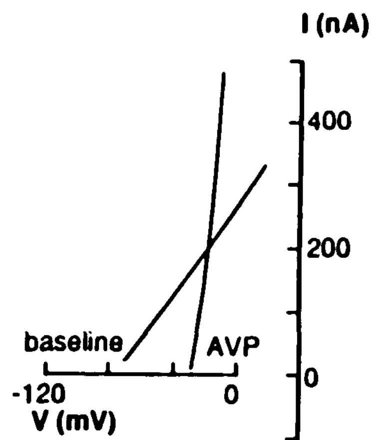


Figure 1

1. Oocytes injected with liver RNA express functional V_1 AVP receptors, and oocytes injected with whole brain RNA express functional peripheral (type A) CCK receptors. RNA injected oocytes were analyzed 3-5 days after injection using two-electrode voltage clamp at a holding potential of -70 mV . Bath application of AVP (Fig. 1a; inset, left) or CCK-8(s) (Fig. 1b; inset, left) resulted in activation of a characteristic depolarizing current. The amplitude of this current served as a measure of stimulation of phospholipase C. The bar graphs show the mean ± SEM of the amplitude of the agonist stimulated current as percent of maximal response from groups of cells. The number of cells in each group is indicated above the bar. **A)** Activation of the current by AVP is antagonized by a specific V_1-receptor antagonist. The bar graph compares the relative response of a group of liver RNA injected cells to bath application of AVP (1 μM) compared to a group of cells tested for AVP responsiveness in the presence of the specific V_1 antagonist $d(CH_2)_5Tyr(Me)AVP$ (5 μM) and a group of non-injected control cells. The inset above the graph compares representative current traces of oocytes in the presence of AVP (left) and AVP + $d(CH_2)_5Tyr(Me)AVP$ (right). The small bar above each current trace indicates the duration of AVP application. The V_1 antagonist was applied for 60 seconds before application of the AVP + V_1 antagonist solution. The mean maximal response to AVP was 399 ± 127 nA (n=11). **B.** 0.5 μM CCK-8(s) activates a similar depolarizing current whereas 50 μM CCK-4 is ineffective. The bar graph compares the % mean maximal amplitude of response to agonists in three groups of cells: whole brain RNA injected oocytes in response to CCK-8(s), whole brain RNA

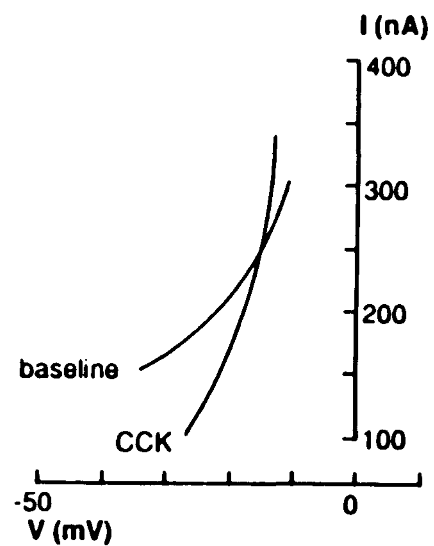
injected cells in the presence of CCK-4 and non-injected control cells. The inset shows representative traces from brain RNA injected cells in response to CCK-8(s) (left) and CCK-4 (right). The bars above each trace indicate duration of agonist application. The mean maximal response was 1181 ± 133 nA (n=13).

A



AVP

B



CCK

Figure 2

2. The current-voltage (I-V) characteristics of the response to AVP (1 μM) and CCK (1 μM) assessed by multiple trails in single oocytes using the voltage ramp method previously described (37). A computer driven ramp-change in voltage (from +10 mV to -100 mV) is applied to the voltage-clamped oocyte. The instantaneous current for each change in voltage is plotted on an X-Y plotter. The crossover point of a ramp before application of the agonist (baseline) and a ramp during the peak of the response to an agonist (AVP or CCK) will give the reversal potential for that current. The reversal potential for the response to AVP was determined to be 20 ± 1 mV (n=9). The reversal potential for the response to CCK was found to be 23 ± 3 mV (n=6). These results indicate that Cl^- is the primary ion carrying the depolarizing response to AVP and CCK. Representative ramp experiments are shown for AVP (**A**) and CCK (**B**). (Traced from original recordings for clarity.)

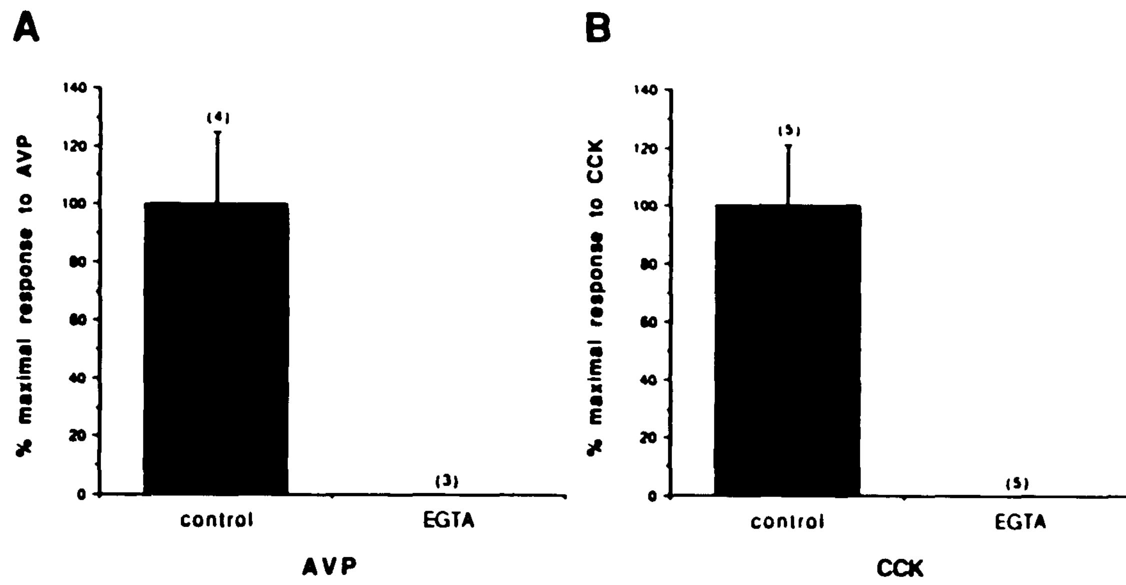
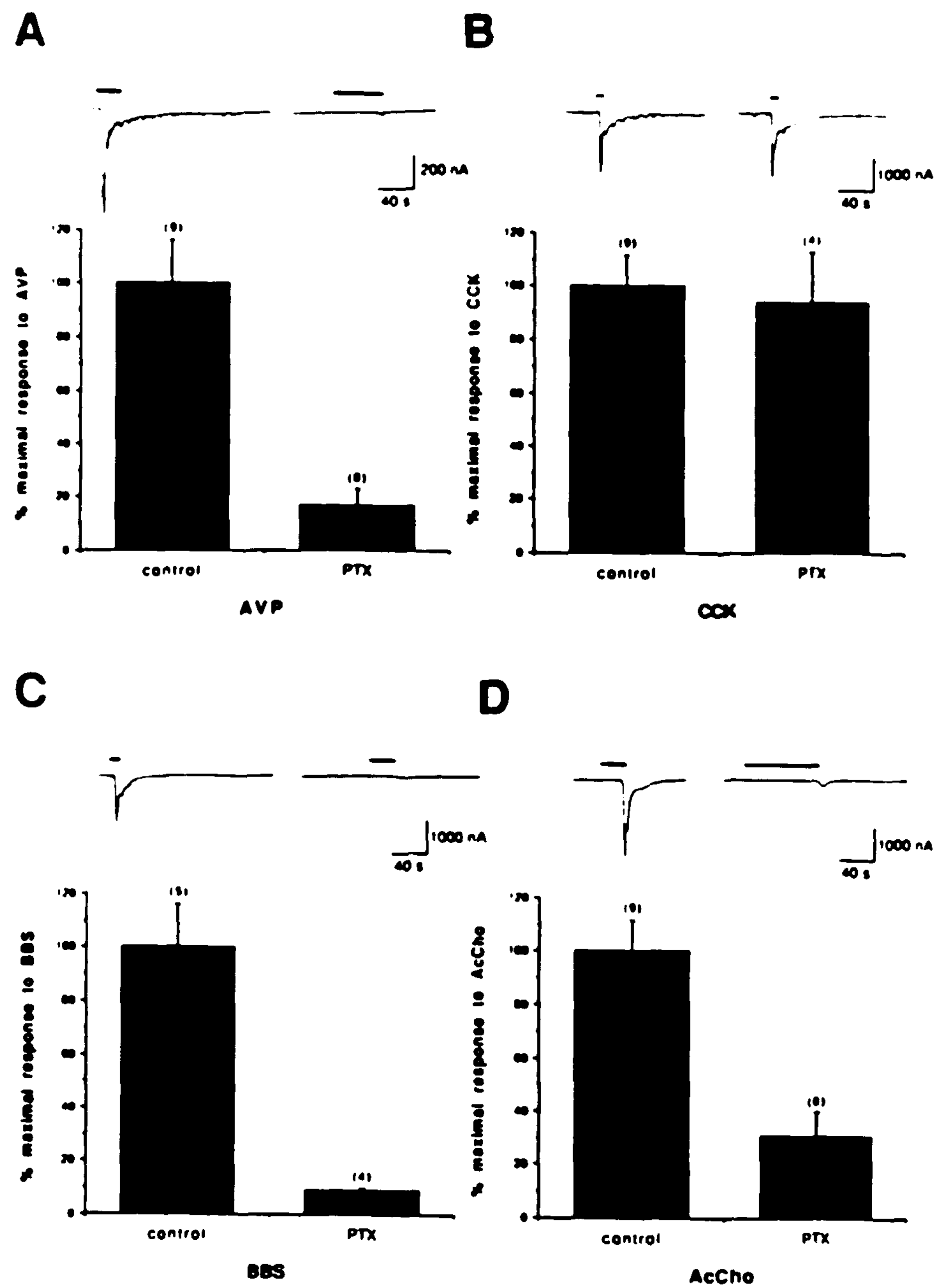


Figure 3

3. Effect of intracellular injection of EGTA on the response to AVP and CCK. **A.** A group of liver RNA injected oocytes were injected with the Ca^{2+} chelator EGTA to a final concentration of 10^{-4} M one half hour prior to assay for responsiveness to AVP (1 μM). The bar graph compares the % mean maximal response to AVP in a group of RNA injected cells (control) and a group of RNA injected cells also injected with EGTA. The mean maximal response to AVP was 1200 ± 305 nA (n=4). **B.** A similar experiment comparing the % maximal response to CCK (1 μM) of whole brain RNA injected oocytes (control) and a group of RNA injected cells injected with EGTA. The mean maximal response to CCK was 133 ± 28 nA (n=5).

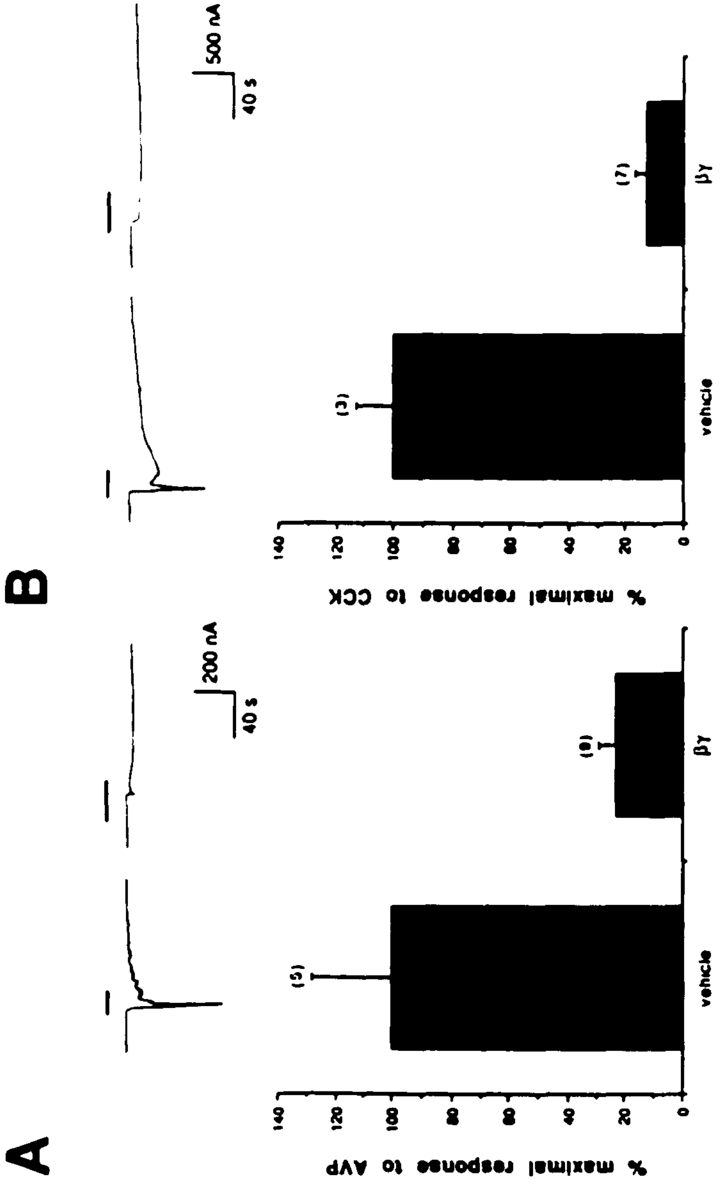
Figure 4



4. Effects of pertussis toxin treatment on the exogenous and native receptor activated Cl^- current in *Xenopus* oocytes. Groups of cells were incubated in PTX (2 μg /ml) for 24 hours. These cells were examined for responsiveness to various agonists under voltage clamp and compared to cells that were not treated with PTX. The bar graphs show the mean ± SEM of the percent maximal response to agonist. The inset above each graph compares representative current traces from a control cell (left) and a PTX treated cell (right). The small bar above each trace indicates the duration of agonist application. **A.** Effect of pertussis toxin treatment on V_1-receptor stimulation of Cl^- current in liver RNA injected oocytes. The mean maximal response to 1 μM AVP was 686 ± 108 nA (n=9). **B.** Effect of pertussis toxin treatment on CCK receptor stimulation of Cl^- current in whole brain RNA injected oocytes. The mean maximal response to 1 μM CCK was 1329 ± 156 nA (n=9). To rule out the possibility that the lack of effect here is due to either the toxin being not functional or that the oocytes were not receptive to pertussis toxin, we attempted to observe a known pertussis toxin sensitive response in cells from the same batch of whole brain RNA injected oocytes. **C.** Two groups of cells from the same batch as those in 4b were assayed for responsiveness to 1 μM bombesin (BBS). Pertussis toxin substantially inhibits the coupling of bombesin receptors to activation of the Cl^- current. The mean maximal response to BBS was 1010 ± 163 nA (n=5). **D.** Effect of pertussis toxin treatment on muscarinic receptor stimulation of Cl^- current in native oocytes. The mean maximal response to 1 μM AcCho was

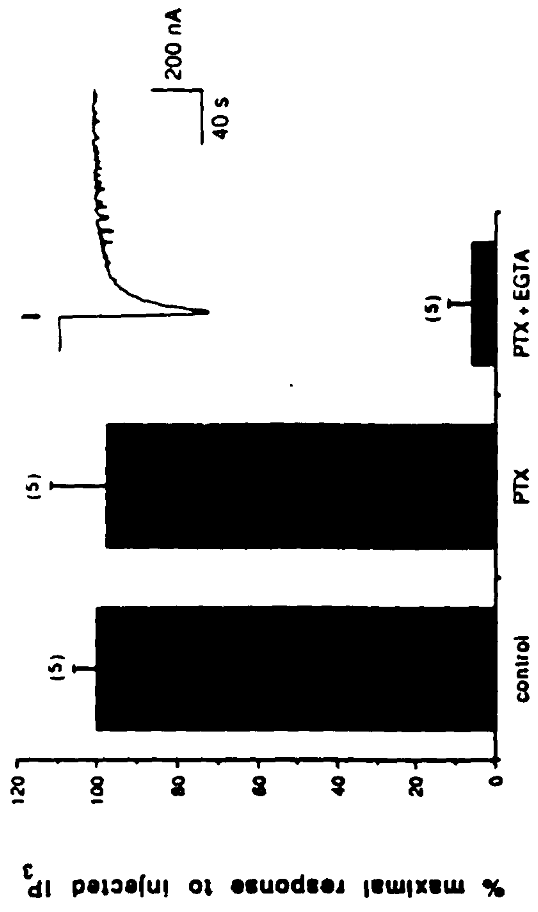
1700 ± 207 nA (n=9). The inhibition seen in this experiment was not as substantial as that observed in other experiments.

Figure 5



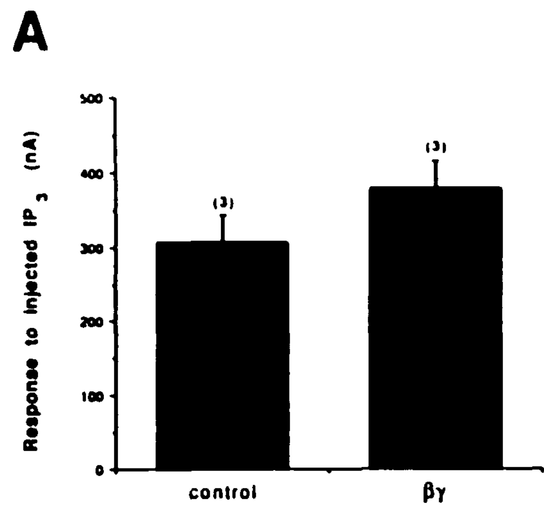
5. Effects of intracellular injection of excess G-protein βγ subunits on the pertussis toxin sensitive (AVP) and toxin insensitive (CCK) signal transduction pathways. **A.** The bar graph compares the % maximal response to 1 μM AVP of a group of liver RNA injected cells injected with the protein storage buffer (vehicle) and a group of cells injected with the same volume (40 nl/cell) of bovine brain βγ subunits (1 ng/oocyte). The oocyte has an approximate volume of 1 μl. The inset above the graph compares representative current traces from a vehicle injected cell (left) to a cell injected with βγ subunits. The small bars above the traces indicate the duration of agonist application. The mean maximal response was 160 ± 45 (n=5). **B.** A similar experiment comparing whole brain RNA injected cells in response to 1 μM CCK. Cells were injected with either 40 nl of storage buffer (vehicle) or 40 nl of βγ subunits. The mean maximal response to CCK was 818 ± 109 (n=3).

Figure 6



6. Effects of pertussis toxin on the cellular response to injected IP_3. The bar graph compare the responses of groups of RNA injected oocytes to injection of IP_3 (1.2 pmol): liver RNA injected cells (control), liver RNA injected cells that have been treated with PTX (2 μg/ml for 26 hours), and liver RNA injected cells that have been treated with PTX and pre-injected with EGTA (final concentration 10^{-4} M). The mean maximal response was 438 ± 28 nA (n=5). The inset is a current trace from an oocyte during injection of IP_3 (1.2 pmol). Arrow indicates moment of injection. Intracellular injection of IP_3 evokes essentially the same response as receptor activation of phospholipase C (compare to insets in other figures).

A



B

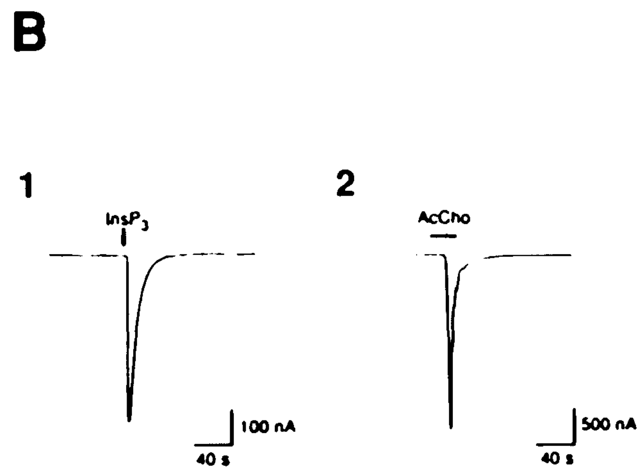


Figure 7

7. Effects of G-protein $\beta\gamma$ subunits on the cellular response to injected IP_3. **A.** The bar graph compares two groups of native oocytes (no exogenous RNA) in response to direct injection of IP_3 (0.52 pmol/cell). One group of cells has been injected with 40 nl of bovine brain $\beta\gamma$ subunits (1 ng/cell) 30 minutes prior to assay. The cells' responses to injection of IP_3 are not inhibited by excess $\beta\gamma$ subunits. **B.** (1) A current trace showing the response induced by direct injection of IP_3 in a $\beta\gamma$ injected oocyte. (2) The response of a native oocyte (from the same frog as in (1)) to application of AcCho (μM) for comparison.

Table 1: Effects of pertussis toxin on AVP evoked Cl^- current. Three experiments from different weeks and different frogs are shown. The mean maximal responses ± SEM of groups of cells in response to 1 μM AVP are given. The number of cells tested is indicated for each group.

expt.	control	n	pertussis toxin	n
1	686 ± 108	9	120 ± 44	8
2	1200 ± 305	4	154 ± 81	5
3	400 ± 161	3	61 ± 39	4

CHAPTER 7

Discussion

In 1953, Hokin and Hokin discovered that phospholipid turnover could be regulated by receptor stimulation (1953). Two decades later, Michell proposed that stimulated turnover of inositol phospholipids was in some way linked to intracellular Ca^{++} regulation (1975). Work by Berridge and collaborators linked these two observations by demonstrating that a metabolite of phosphatidylinositol 4,5 -bisphosphate hydrolysis, inositol 1,4,5 -trisphosphate (IP_3), was capable of mobilizing an extra-mitochondrial Ca^{++} pool. (Streb *et al.*, 1983, Berridge and Irvine, 1984). Since then, the details, ubiquity and significance of the phosphatidyl inositol second messenger system have become appreciated: it is found in essentially all cell types and is the central mechanism in controlling numerous physiological functions.

As early as 1984 it was postulated that a G-protein may have some role in coupling receptors to inositol phosphate metabolism (Berridge and Irvine, 1984). In light of very strong evidence suggesting that G-proteins are an intrinsic part of signal transduction through the phosphatidyl inositol system, by 1986 the supposed G-protein was casually referred to as G_p (following the suggestion of Cockroft and Gomperts, 1985). The implicit assumption was that G_p would be soon identified by protein purification and molecular cloning. However, contradictory results with pertussis toxin, suggestive results with p21 $^{N\text{-}ras}$ (Wakelam *et al.*, 1986), unsuccessful attempts at functional reconstitutions of pure receptor, G-protein and phospholipase C (Gilman, 1987, Rhee *et*

al., 1989) and the lack of any demonstrated direct effect of a G-protein on phospholipase C (Casey and Gilman, 1988) raised substantial doubt as to the role of G-proteins in stimulation of phospholipase C. The questions surrounding the subject at the outset of this thesis work included: is the involved G-protein heterotrimeric or monomeric? Is it one of the known GTP-binding proteins? What is the mechanism of signal transduction? Are there other mechanisms for receptor stimulation phospholipase C? The studies presented in this thesis have hopefully resolved some of these questions.

Since biochemical reconstitution efforts were unsuccessful in demonstrating that any G-protein directly stimulated the effector enzyme of the phosphatidyl inositol second messenger pathway, we used a different approach to address the question. Conceivably, the reconstitution experiments failed because some crucial element was omitted or the proteins and lipids could not orient properly in the supplied lipid environments. To avoid such problems, we attempted to study the mechanisms of signal transduction in a normal cellular environment. The *Xenopus* oocyte electrophysiological preparation allowed us to experimentally intervene in a single, functioning receptor-stimulated phospholipase C pathway while monitoring an unambiguous end-product, the Cl^- current. The comprehensive literature on the mechanism of the receptor-stimulated IP_3 mediated Cl^- current in the oocyte, the size of the cell and the availability of pure, functional G-proteins allowed us to experiment at almost every level of the cascade. Stimulation of the pathway could be achieved physiologically, by various receptors (native and

expressed from mRNA), and by direct addition of the activated intermediates of the pathway (α-GTPγS, IP_3 or Ca^{++}). Inhibition could be accomplished by very specific inhibitors at each level of the cascade using receptor antagonists, pertussis toxin, $\beta\gamma$-subunits and EGTA.

In the preliminary studies (Chapters 2 and 3) we explored the use of the oocyte electrophysiological preparation to study signal transduction. Chapter 2 describes the characteristic hyperpolarization in response to receptor stimulation of the adenylyl cyclase system and the depolarizing Cl^- current characteristic of stimulation of the phosphatidyl inositol second messenger system. In chapter 3 we demonstrated that the brain receptor proteins for CCK and bombesin could be expressed in the oocyte and functionally couple to the IP_3-mediated Cl^- current, whereas somatostatin, which is well known to couple to the adenylyl cyclase system, could not stimulate the Cl^- current. There is a growing list of reports where injection of RNA into oocytes has conferred sensitivity to various transmitter substances known to stimulate phospholipase C in native tissues (eg., Masu *et al.*, 1987, Hirono *et al.*, 1987, Julius *et al.*, 1988, Myerhof *et al.*, 1988, Oron *et al.*, 1988b). Each evokes the characteristic depolarizing Cl^- current in the oocyte. Patch clamp studies of two dissimilar receptors, the brain 5-HT_{1c} receptor (Takahashi *et al.*, 1987) and the brain glutamate receptor (Oosawa and Yamagishi, 1989), show that both activate the same Cl^- channel when expressed in oocytes. Adrenergic agents will evoke the Cl^- current (unpublished observation) in oocytes injected with RNA prepared from the cDNA

of the smooth muscle α_1-adrenergic receptor (Cotecchia *et al.*, 1988). Taken together, the picture which emerges is that the native oocyte has all the components of a phosphatidyl inositol second messenger system, and receptors from varied tissues can "plug in" to this pathway to elicit the same biological end-product. Whereas the α_1-receptor of smooth muscle mobilizes Ca^{++} to contract the cell, when expressed in the oocyte, it serves to depolarize the cell.

Since so many receptors can utilize this pathway, an understanding of the G-protein component should have general implications. In the study in Chapter 4 we confirmed that oocyte membranes have a G-protein α-subunit by virtue of pertussis toxin ADP-ribosylation of a 41 kDa protein. We also demonstrated the presence of the β subunit of heterotrimeric G-proteins by immunoblotting. Our observations that G-protein $\beta\gamma$-subunits inhibited the muscarinic stimulated Cl^- current in a dose dependent fashion suggested that receptor stimulation of phospholipase C proceeds by a mechanism similar to that of the well known adenylyl cyclase or cGMP phosphodiesterase. This result is of particular significance in that it provides the first evidence that receptor stimulation of phospholipase proceeds via an "orthodox" (Bourne, 1989) signal transduction pathway where the α-subunit interacts directly with the effector.

It is possible that the observed $\beta\gamma$-subunit effect is due to some direct action of $\beta\gamma$ on another system, for example phospholipase A_2 (Kim *et al.*, 1989), which then feeds back on the phosphatidyl inositol system. This possibility was ruled out by the following study. Using GTPγS activated α-subunits of the known

pertussis toxin sensitive G-proteins, we showed that $G_{o\alpha}$ could directly activate the Ca^{++} sensitive Cl^- current. Moreover, the addition of excess heterotrimeric G_o to oocytes resulted in an increased sensitivity to ACh. Neither the activated $G_{\alpha i}$'s nor the holo-G_i's were effective. This is the first demonstration of a direct effect of a G-protein on a phospholipase C dependent process. The conclusion is that, of the known heterotrimeric G-proteins, G_o may serve as the signal transducer in the pertussis toxin-sensitive phosphatidyl inositol second messenger pathway.

The next major problem faced in understanding G-protein stimulation of phospholipase C is the contradictory results with pertussis toxin wherein receptor stimulated phospholipase C activity is pertussis toxin sensitive in some cell types and is not sensitive in others. The most obvious explanation is that two distinct pathways exist. What is not clear is how similar or different these mechanisms may be. The study in Chapter 6 address this question.

There is little doubt that the pertussis toxin insensitive pathway is mediated by a G-protein (Lo and Hughes, 1987). The ability of the liver AVP receptor to utilize a pertussis toxin sensitive pathway in the oocyte, whereas it only uses a pertussis toxin-insensitive mechanism in the native tissue, indicates that the G-proteins serving both pathways must be quite similar. Both pathways were demonstrated to be modulated by G-protein $\beta\gamma$-subunits in the oocyte. Work from Bourne's group using single amino acid substitutions and chimeric proteins indicates that even small changes in the receptor domain of a G-protein prohibits receptor/G-

protein interaction. Taken together, the conclusion is that the G-protein serving the pertussis toxin insensitive pathway is heterotrimeric, acts through "orthodox" stimulation of phospholipase C and must by very similar to G_o. The primary structure may be different by as little as the one amino acid which is the pertussis toxin ADP-ribosylation site.

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