Title

A novel transduction pathway associated with receptors coupled to the inhibitory guanine nucleotide binding progein Gi that amplifies ATP-stimulated arachidonic acid release

Author

Williams, Holly,

Publication Date

1993

Peer reviewed|Thesis/dissertation

A Novel Transduction Pathway Associated With Receptors Coupled To The Inhibitory Guanine Nucleotide Binding Protein G_i That Amplifies ATP-Stimulated Arachidonic Acid Release

by

Holly L. Williams

Advisor: Julius Axelrod, Ph.D.
Laboratory of Cell Biology
National Institute of Mental Health
Bethesda, MD 20892

A Thesis submitted to the Faculty of the School of Medicine of the University of California at San Francisco in partial fulfillment of requirements for the degree of Medical Doctor with Thesis

1993

ABSTRACT

Adenosine triphosphate (ATP) is copackaged and coreleased with norepinephrine, serotonin, and acetylcholine suggesting a possible interaction between the signalling pathways for ATP and these neurotransmitters. Chinese hamster ovary (CHO) cells possessing endogenous ATP (P_2 purinergic) receptors coupled to arachidonic acid release were individually transfected with genes for m_2- and m_4-muscarinic, α_2-adrenergic, D_2-dopaminergic, 5-HT_{1a}-serotonergic, and A_1 adenosine receptors which have in common their ability to inhibit adenylate cyclase through the inhibitory guanine nucleotide binding protein G_i. These cells were then used as models to examine the interaction between inhibitory G_i-coupled receptors and ATP in the production of the second messenger arachidonic acid. Normal functional coupling of each of the transfected G_i-coupled receptors was demonstrated by their ability to inhibit forskolin-stimulated cAMP accumulation with dose-response activities consistent with previous reports. Stimulation of m_2, m_4, α_2, D_2, 5-HT_{1a}, and A_1 receptors resulted in an augmentation of ATP-stimulated arachidonic acid release (as well as augmentation of thrombin-, calcium ionophore-, melittin-, and phorbol ester-stimulated arachidonic acid release). With the exception of the m_4 receptor, none of the receptors tested were able to stimulate arachidonic acid release in the absence of ATP. Potentiation of ATP-stimulated arachidonic acid release was independent of changes in cAMP. The augmentation of ATP-stimulated arachidonic acid release and the inhibition of cAMP accumulation were both blocked by pertussis toxin, an inhibitor of G_i, but with different dose-response characteristics. Inhibition

of protein kinase C with staurosporine or long-term pretreatment of the cells with the phorbol ester PMA (phorbol 12-myristate 13-acetate) blocked the augmentation response. Only the A_1 and 5-HT_{1a} receptors were linked to inositolphospholipid turnover as a possible mechanism for protein kinase C activation. These findings demonstrate that G_i-coupled inhibitory receptors can amplify ATP receptor-stimulated arachidonic acid release in addition to their classical role in the inhibition of adenylate cyclase activity. The mechanism of this novel transduction pathway appears to involve a pertussis-toxin-sensitive G-protein and protein kinase C, but is independent of adenylate cyclase inhibition.

TABLE OF CONTENTS

Introduction 1

Materials and Methods 8

Results

A. Muscarinic m_2 and m_4, D_2-dopaminergic, α_2-adrenergic, 5-HT_{1a}-serotonergic, and A_1 adenosine receptors transfected and stably expressed in CHO cells inhibit cAMP accumulation. 12

B. The transfected inhibitory G_i-coupled receptors augment ATP-stimulated arachidonic acid release. 14

C. Potentiation of ATP-stimulated arachidonic acid release is independent of changes in cAMP. 20

D. Potentiation of ATP-stimulated arachidonic acid release is sensitive to pertussis toxin. 22

E. Inhibitors of protein kinase C block the potentiation of ATP-stimulated arachidonic acid release. 25

F. The transfected A_1 adenosine and 5-HT_{1a}-serotonergic receptors weakly couple to inositolphospholipid turnover. 28

Discussion 32

Bibliography 35

LIST OF FIGURES

1. Receptors may couple to many different effector systems via G-proteins. 3

2. Arachidonic acid is liberated from membrane phospholipids by the action of phospholipases. 5

3. Stimulation of the A_1 adenosine receptor inhibits cAMP accumulation. 13

4. Stimulation of the m_2-muscarinic receptor augments ATP-stimulated arachidonic acid release. 15

5. Stimulation of D_2-dopaminergic, m_4-muscarinic, α_2-adrenergic, 5-HT_{1a}-serotonergic, and A_1 adenosine receptors augments ATP-stimulated arachidonic acid release. 16

6. Maximal stimulation of D_2, α_2, 5-HT_{1a}, and A_1 receptors has no effect on arachidonic acid release in the absence of ATP and the m_4 receptor caused a modest increase in arachidonic acid release. 18

7. The transfected A_1 adenosine receptor potentiates the release of arachidonic acid stimulated by thrombin, A23187, melittin, and PMA. 19

8. Pertussis toxin inhibits A_1-receptor-mediated augmentation of ATP-stimulated arachidonic acid release. 23

9. Pertussis toxin inhibits m_2-receptor augmentation of ATP-stimulated arachidonic acid release and reverses m_2-receptor-inhibited cAMP generation. 24

10. Inhibitors of protein kinase C block A_1-receptor-mediated potentiation of ATP-stimulated arachidonic acid release. 26

11. Staurosporine, an inhibitor of protein kinase C, blocked m_2, D_2, and α_2 receptor augmentation of ATP-stimulated arachidonic acid release. 28

12. Stimulation of the A_1 adenosine receptor results in a modest increase in inositolphospholipid turnover. 29

13. Stimulation of the A_1 adenosine receptor increases inositol monophosphate accumulation in a dose-dependent manner. 30

14. Stimulation of the 5-HT_{1a} receptor, but not the m_2 or D_2 receptor, causes a modest increase in inositolphospholipid turnover. 31

LIST OF TABLES

1. Changes in cAMP levels do not effect ATP- or m_2-receptor-mediated augmentation of arachidonic acid release. 21

2. Changes in cAMP levels do not effect ATP- or A_1-receptor-mediated augmentation of arachidonic acid release. 21

INTRODUCTION

Neurotransmitters, hormones, cytokines, growth factors and other water-soluble signaling molecules transmit their specific messages by binding to receptor proteins on the surface of the target cells they influence. Binding of the ligand causes the receptor to undergo a conformational change enabling the receptor to act directly or indirectly as a transducer, converting the extracellular signal into an intracellular message. Most cell-surface receptor proteins belong to one of three classes, which are defined by the transduction mechanism used: channel-linked, catalytic, and G-protein-linked (1). Channel-linked receptors are transmitter-gated ion channels involved mainly in rapid synaptic signaling between electrically excitable cells. Catalytic receptors, when activated by their ligand, operate directly as enzymes such as tyrosine-specific protein kinases. The third class of cell-surface receptors includes a large family of homologous, seven-pass transmembrane glycoproteins which couple to guanine-nucleotide-binding proteins (G-proteins) in order to indirectly stimulate or inhibit separate membrane-associated ion channels and enzymes such as adenylate cyclase and phospholipases. The G-proteins are heterotrimers that respond to a ligand-activated receptor by disassociating into their component α- and $\beta\gamma$-subunits upon exchange of GTP for the GDP bound to the α-subunit. Specific α-subunits are associated with the activation and inhibition of different effector systems. The α-subunits have endogenous GTPase activity which hydrolyzes the bound GTP to GDP, resulting in reassociation of the inactive $\alpha\beta\gamma$-heterotrimer and subsequent termination of the signal (2). The sequential

stimulation of receptor, G-protein, and effector enzyme or ion channel alters the concentration of intracellular second messengers such as Ca^{++}, cAMP, arachidonic acid, and inositol phosphates (Figure 1). These second messengers act in turn to elicit the biological responses characteristic of a given cell often through activation of protein kinases and phosphatases.

Arachidonic acid and many of its eicosanoid metabolites have received much attention as important second messengers in both neural and nonneural cells (3,4). Arachidonic acid may be metabolized by cyclooxygenases to prostaglandins and thromboxanes; by lipoxygenases to leukotrienes and hydroxyeicosatetraenoic acids (HETEs), and by epoxygenases to epoxides. Unlike other second messengers, arachidonic acid and its metabolites are membrane permeable and so can mediate both intra- and extracellular signalling. Arachidonic acid is known to affect the activities of neuronal ion channels and protein kinases and may play a role in longterm potentiation and synaptic plasticity, both models for mammalian memory, by acting as a retrograde messenger to regulate neurotransmitter release (4,5). Arachidonic acid itself can mobilize cytosolic calcium, independent of inositol triphosphate formation (6). Arachidonic acid and its metabolites can also amplify other second messenger systems such as cAMP (7).

Arachidonic acid is liberated from cell membrane phospholipids by the action of phospholipases. The brain is particularly rich in arachidonate-containing phospholipids and various CNS stimuli such as ischemia, anoxia, and seizure activity cause increased release of arachidonic acid (8). Receptor-generated arachidonic acid

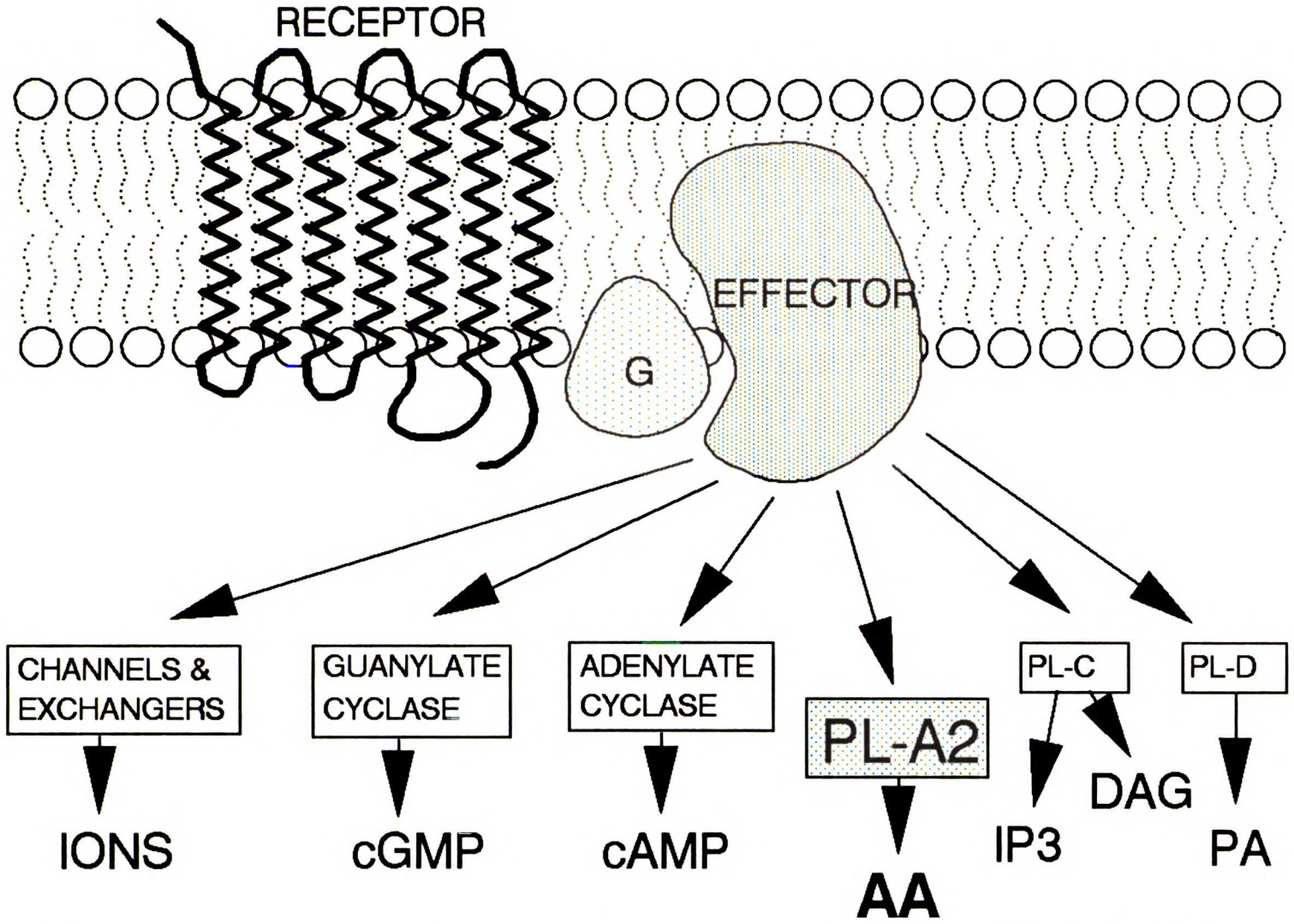


Figure 1: Receptors couple to many different effector systems via G-proteins. PL-A2, phospholipase A2. AA, arachidonic acid. PL-C, phospholipase C. IP3, inositol triphosphate. DAG, diacylglycerol. PL-D, phospholipase D. PA, phosphatidic acid.

release has been demonstrated in a variety of primary neuronal cultures and associated with the α_1-adrenergic, 5-HT_2-serotonergic, m_1-muscarinic, and N-methyl-D-aspartate (NMDA) receptors (9-12). Several pathways exist for receptor-mediated arachidonic acid release. The most direct involves coupling of the receptor protein to activation of the effector enzyme phospholipase A_2 via a G-protein (13). Phospholipase A_2 hydrolyzes arachidonic acid-containing phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositides, to generate free arachidonic acid and lysophospholipids. Indirect routes have been demonstrated involving the release of arachidonic acid from diacylglycerol by diglyceride lipase (14). Diacylglycerol can be generated from the action of phospholipase C on several phospholipids such as phosphatidylcholine and phosphoinositides. Alternatively, arachidonic acid can be released from phosphatidic acid produced from the hydrolysis of phospholipids by phospholipase D (15) (Figure 2). In many studies, receptor-mediated release of arachidonic acid was found to be calcium dependent, while phospholipase C and D are partially dependent on or insensitive to calcium (16,17).

Following neural stimulation, neurotransmitters are released from storage vesicles and bind to cell surface receptors to initiate transmembrane signalling. Adenosine triphosphate (ATP) is copackaged with several neurotransmitters, including norepinephrine, serotonin, and acetylcholine, in storage vesicles and coreleased during the process of nerve stimulation (18). ATP, in addition to its role in intermediary metabolism and cellular energy, can itself act as a neurotransmitter

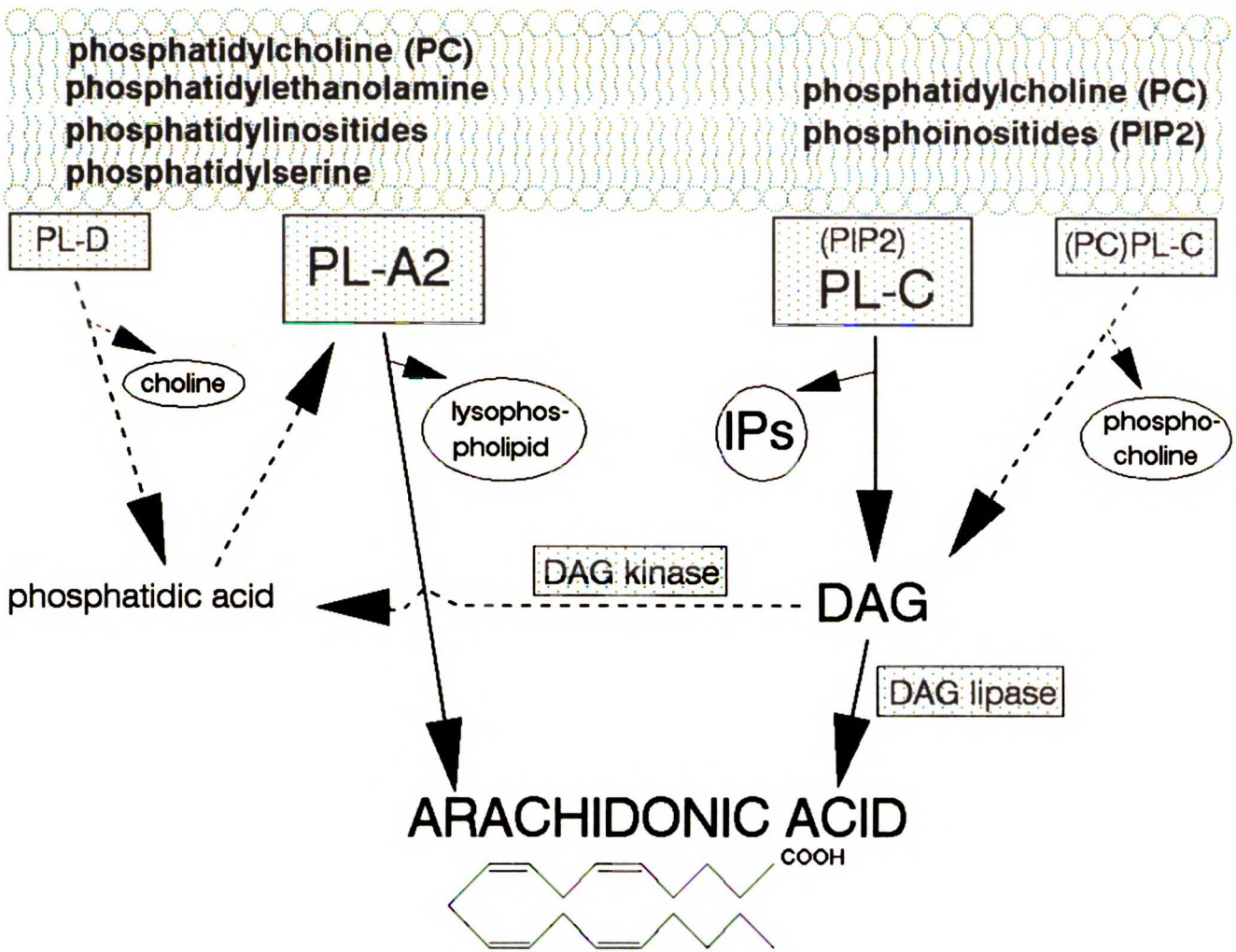


Figure 2: Arachidonic acid is liberated from membrane phospholipids by the action of phospholipases. The most direct path involves PL-A2, phospholipase A2. PL-D, phospholipase D. PL-C, phospholipase C. DAG, diacylglycerol. IPs, inositol phosphates.

by binding with high affinity to cell surface purinergic receptors. The P_2-purinergic receptor is coupled via G-proteins to the stimulation of phospholipase A_2 and phospholipase C resulting in the liberation of arachidonic acid and inositol phosphates, respectively (19). The corelease of ATP with neurotransmitters suggests a possible interaction between their signalling pathways. A recent observation was made that calcium ionophore-stimulated arachidonic acid release can be amplified by transfected inhibitory D_2 receptors in Chinese hamster ovary (CHO) cells (20). This suggested that an interaction might exist between receptor-mediated arachidonic acid release and inhibitory G_i-coupled receptors (where G_i is the inhibitory G-protein). To examine this possibility CHO cells were selected that contain endogenous ATP receptors coupled to arachidonic acid release (21). CHO cells are devoid of most neurotransmitter receptors, yet contain the appropriate G-proteins and effector enzymes to allow arachidonic acid, inositol phosphate, and cAMP second messenger production. Neurotransmitter receptors have been expressed in these cells through DNA transfection techniques permitting the study of mechanisms of signal transduction associated with individual receptor subtypes (22,23). In this study, CHO cells were transfected with genes for muscarinic m_2 and m_4, α_2-adrenergic, D_2-dopaminergic, 5-HT_{1a}-serotonergic, and A_1 adenosine receptors which have been previously characterized (24-31) and have in common their coupling to the inhibition of adenylate cyclase through a G_i protein. All six of these inhibitory G_i-coupled receptors were shown to augment ATP-stimulated arachidonic acid release, thus demonstrating a novel transduction pathway linking this class of receptors to

MATERIALS AND METHODS

Materials:

Pertussis toxin, staurosporine, phorbol 12-myristate 13-acetate (PMA), and 4-α phorbol were purchased from Calbiochem (La Jolla, CA). [5,6,8,9,11,12,14,15-^{3}H(N)] arachidonic acid was purchased from New England Nuclear (Boston, MA) and *myo*-[2-^{3}H(N)]inositol from American Radiolabeled Chemicals (St. Louis, MO). Clonidine, quinpirole, R(-)N^{6}-(2-phenylisopropyl)adenosine (R-PIA) and xanthine amine congener (XAC) were purchased from Research Biochemicals (Natick, MA). (Rp)-Adenosine cyclic 3',5'-phosphorothioate {(Rp)-cAMP[S]} was purchased from Biolog Life Sciences Institute (La Jolla, CA). RO 20-1724, a phosphodiesterase inhibitor, was purchased from Biomol (Plymouth Meeting, PA). Reagents used in the radioimmunoassay of cAMP were purchased from Gary Brooker (Dept. of Biochemistry, Georgetown University School of Medicine, Washington DC). Cell culture media, reagents, and fetal bovine serum were obtained from GIBCO Laboratories (Grand Island, NY). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Cell Culture and Stable Expression of Receptor Clones in Chinese Hamster Ovary Cells (CHO):

CHO cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's Modified Eagle's Media containing high glucose (4,500 mg/L), sodium pyruvate (1 mM), and 10% fetal bovine serum,

in a humidified atmosphere of 5% CO_2 in air at 37°C. CHO cells that expressed physiologic levels of the m_2 receptor (84 fmol/mg protein) and the m_4 receptor (202 fmol/mg protein) (24) were generously provided by M.R. Brann, N.J. Buckley, and T.I. Bonner. CHO cells expressing the α_2 receptor (64 fmol/mg protein) have been described (25). CHO cells expressing physiologic levels of the D_2 receptor (1-2 pmol/mg protein) (26,27) were provided by D.R. Sibley and L.C. Mahan. CHO cells expressing physiologic levels of the 5-HT_{1a} receptor (900 fmol/mg protein) (28,29) were provided by J.R. Raymond. CHO cells expressing the recently cloned A_1 adenosine receptor (1 pmol/mg protein) (30,31) were provided by L.C. Mahan. CHO cells do not express endogenous muscarinic, adrenergic, dopaminergic, serotonergic, or adenosine receptors as determined by radioligand binding (24-31).

Measurement of cAMP Accumulation in CHO cells:

CHO cells were grown to confluence in 24-well Costar plastic culture plates (Becton Dickinson, Oxnard, CA) or placed in suspension at a density of 3-4 x 10^6 cells/mL. The growth medium was replaced with 250 μl of Eagles #2 Minimal Essential Media containing 28 μM of the phosphodiesterase inhibitor RO 20-1724. Following 10 minutes of preincubation at 37°C, experimental agents were added. The reaction was stopped after 5 minutes with 250 μL of an ice-cold solution containing 0.1 N HCl and 0.1 mM $CaCl_2$. The accumulation of cAMP was measured by radioimmunoassay as described (22).

Measurement of [^{3}H]arachidonic acid release:

CHO cells were grown to 50-75% confluency in 24-well culture plates and labeled overnight with [^{3}H]arachidonic acid (0.20 μCi/mL/well; 1 Ci = 37 GBq). Before the addition of experimental agents, the cells were washed twice with 500 μL of Eagles #2 Minimal Essentials Media containing 2.0 mM $CaCl_2$, 0.2 mM $MgCl_2$, and 0.2% fatty acid-free bovine serum albumin (BSA). The BSA was used to trap free [^{3}H]arachidonic acid in the extracellular medium. The experimental agents were added in a final volume of 500 μL and the reaction allowed to proceed for 10 minutes at 37^{oC}. The reaction was stopped by removal of the incubation medium, which was then centrifuged at 12,000 x G for 1 minute to remove nonadherent cells. An aliquot of the supernatant was removed and released [^{3}H]arachidonic acid was measured with a liquid scintillation counter. High performance liquid chromatography analysis revealed that greater than 99% of released radioactivity in the presence of 0.2% BSA was attributable to arachidonic acid (data not shown).

Measurement of [^{3}H]inositol phosphate release:

CHO cells were grown to 80% confluency in 24-well culture plates and labeled overnight with [^{3}H]inositol (1 μCi/mL/well). Prior to the addition of experimental agents, the cells were washed twice with 250 μL of Eagles #2 Minimal Essentials Media containing 10 mM $LiCl_2$. The experimental agents were added in a final volume of 250 μL and the reaction allowed to proceed for 15 minutes at 37^{oC}. The reaction was stopped by the addition of 250 μL of an ice-cold solution containing 1

M KOH, 18 mM $Na_2B_4O_7$, 3.8 mM EDTA, and 7.6 mM NaOH. The stop solution was immediately neutralized with the addition of 250 μL of a solution containing 7.5% HCl. Released inositol phosphates were separated by anion exchange chromatography as described (22).

Data analysis:

All experiments were done in triplicate, with data being presented as the means of at least three experiments $\pm$ standard error of the mean (SEM). EC_{50} values were estimated by nonlinear regression of primary data using InPlot 3.0 (GraphPAD Software, San Diego, CA).

RESULTS

(A) Muscarinic m_2 and m_4, D_2-dopaminergic, α_2-adrenergic, 5-HT_{1a}-serotonergic, and A_1 adenosine receptors transfected and stably expressed in CHO cells inhibit cAMP accumulation.

To verify the functional association between the transfected G_i-coupled receptors and adenylate cyclase in CHO cells, forskolin-stimulated cAMP accumulation was measured after the addition of receptor agonists. In the case of CHO cells transfected and stably expressing the A_1 adenosine receptor, addition of R-PIA, a selective A_1 receptor agonist, inhibited forskolin-stimulated cAMP accumulation in a concentration-dependent manner (Figure 3). The inhibition was similar to unpublished radioligand binding data (K_d 8.8 nM) (31) and studies using cells expressing native A_1 adenosine receptors (32,33). Addition of R-PIA to untransfected CHO cells or CHO cells transfected with the D_2-dopaminergic receptor failed to inhibit forskolin-stimulated cAMP accumulation (data not shown), demonstrating that the R-PIA-mediated inhibition of adenylate cyclase was neither a native function of the CHO cells nor a byproduct of the transfection procedure. The R-PIA-mediated inhibition of forskolin-stimulated cAMP accumulation in CHO cells transfected with the A_1 receptor was completely blocked by adding 10 μM of a selective A_1 antagonist, XAC (Figure 3). These data demonstrate normal functional coupling of the transfected A_1 adenosine receptor to the inhibition of adenylate cyclase. Inhibition of forskolin-stimulated cAMP accumulation by m_2- and m_4-muscarinic, D_2-dopaminergic, α_2-adrenergic, and 5-HT_{1a}-serotonergic receptors

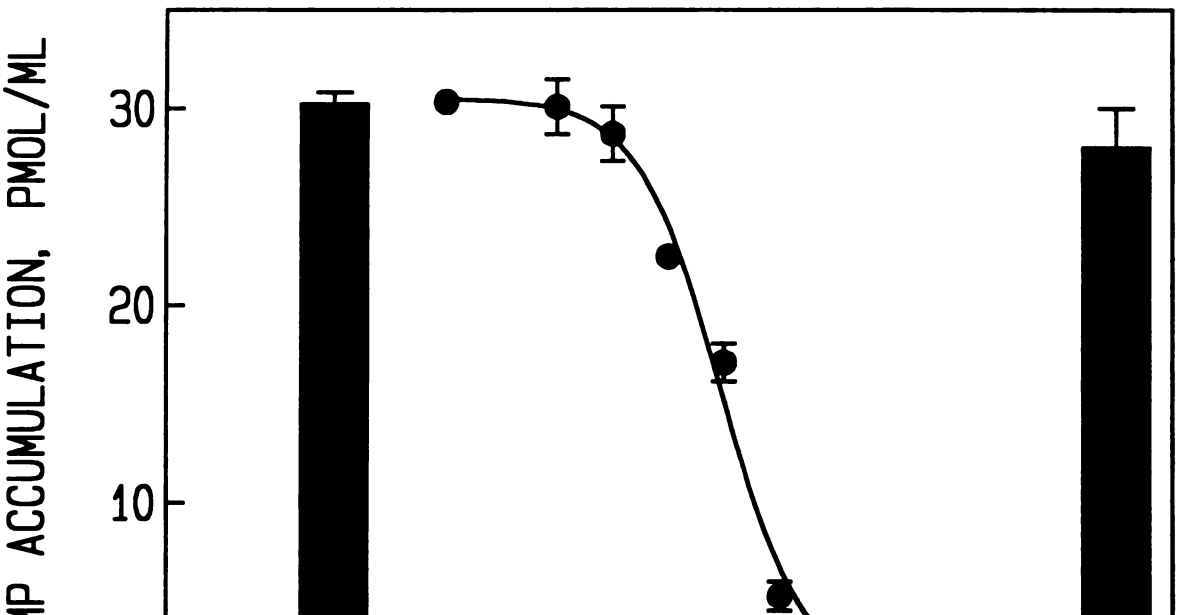


Figure 3: Stimulation of the A_1 adenosine receptor inhibits cAMP accumulation. Clonal CHO cells transfected with and expressing the A_1 adenosine receptor were incubated in suspension with 500 nM forskolin (FSK) for 5 minutes and cAMP accumulation above basal (BSL) levels was measured. R-PIA, a selective A_1 adenosine receptor agonist, inhibited forskolin-stimulated cAMP accumulation with an estimated EC_{50} value of 2.94 $\pm$ 0.12 nM. Maximal inhibition of forskolin-stimulated cAMP accumulation by 1 μM R-PIA was completely reversed by 10 μM XAC, an A_1 receptor antagonist.

CHO cells expressing the m_2, m_4, α_2, D_2, and 5-HT_{1a} receptors also tested for coupling to adenylate cyclase (not shown on graph). The EC_{50} values for the inhibition of forskolin-stimulated cAMP generation using the selective agonists carbachol (m_2,m_4), quinpirole (D_2), clonidine (α_2), and serotonin (5-HT_{1a}) are: m_2, 537 $\pm$ 24nM; m_4, 34 $\pm$ 1.1nM; D_2, 2.0 $\pm$ 0.01nM; α_2, 2.6 $\pm$ 0.1nM; and 5-HT_{1a}, 65 $\pm$ 9nM (24-26,29).

transfected and stably expressed in CHO cells was also demonstrated (Figure 3 legend) (24-26,29).

(B) The transfected inhibitory G_i-coupled receptors augment ATP-stimulated arachidonic acid release.

Potentiation of ionophore-stimulated arachidonic acid release was recently observed in CHO cells transfected with an inhibitory dopamine D_2 receptor (20). The following studies were undertaken to determine if an interaction might exist between ATP receptor-stimulated arachidonic acid release and transfected inhibitory receptors. Transfected m_2 muscarinic acetylcholine receptors were first examined for an effect on the generation of arachidonic acid release after stimulation by ATP. ATP stimulated arachidonic acid release in the CHO cell, but the stimulation of expressed m_2 receptors with the muscarinic agonist carbachol, in the absence of ATP, failed to generate arachidonic acid. Stimulation of the m_2 receptor after the addition of ATP markedly potentiated ATP-stimulated release of arachidonic acid (Figure 4). Addition of the muscarinic antagonist atropine, blocked the carbachol-mediated augmentation of arachidonic acid release, but had no effect on ATP-stimulated arachidonic acid release suggesting a muscarinic-receptor-mediated process (data not shown). Other receptors associated with the inhibition of adenylate cyclase were tested for their ability to augment ATP-stimulated release of arachidonic acid. CHO cells expressing m_4-muscarinic, D_2-dopaminergic, α_2-adrenergic, 5-HT_{1a}-serotonergic, and A_1 adenosine receptors also augmented ATP-stimulated release of arachidonic

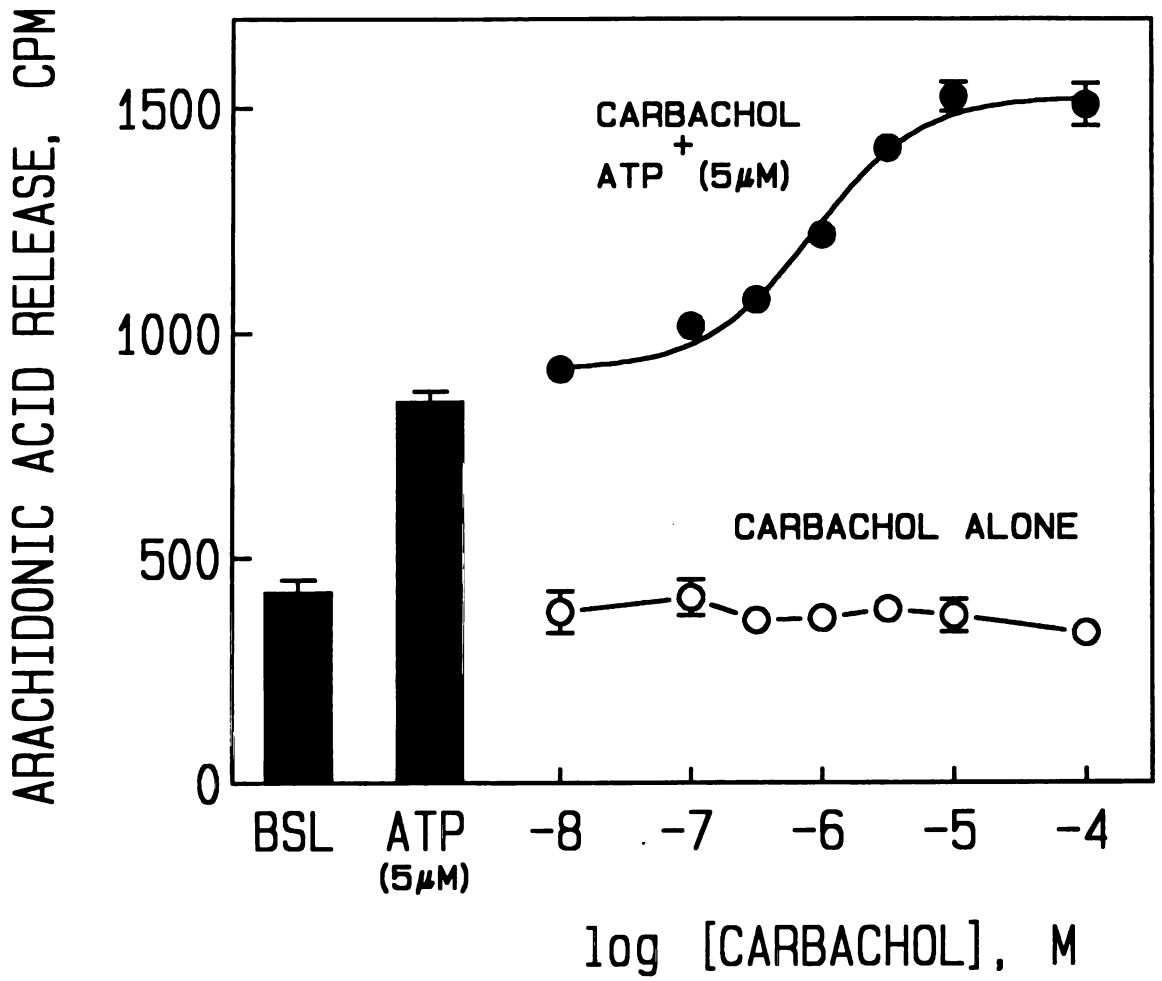


Figure 4: Stimulation of the m_2-muscarinic receptor augments ATP-stimulated arachidonic acid release. Clonal CHO cells transfected with and expressing the m_2-muscarinic receptor were prelabeled overnight with [^{3}H]arachidonic acid and arachidonic acid release was measured after 15 minutes. Maximal ATP (5μM)-stimulated arachidonic acid release is shown (EC_{50}: 958 $\pm$ 4nM). Carbachol, a muscarinic agonist, augmented ATP-stimulated arachidonic acid release with an estimated EC_{50} of 871 $\pm$ 24nM. BSL, basal level.

acid (Figure 5) suggesting a commonality of this response among receptors that inhibit adenylate cyclase activity. Maximal stimulation of D_2, α_2, 5-HT_{1a}, and A_1 receptors had no effect on arachidonic acid release in the absence of ATP (Figure 6) and the m_4 receptor caused a modest increase in arachidonic acid release (38% over basal) (Figure 6). Therefore, receptors that normally inhibit adenylate cyclase can amplify purinergic receptor-stimulated arachidonic acid release.

Other methods of stimulating arachidonic acid release were tested for G_i-coupled-receptor-mediated augmentation. In addition to ATP receptors, CHO cells possess endogenous thrombin receptors coupled to arachidonic acid release (21). Several nonreceptor-mediated mechanisms of phospholipase A_2 activation have also been described: ionophore A23187 (which stimulates phospholipase A_2 by elevating intracellular calcium), melittin (which stimulates phospholipase A_2 independent of calcium), and the phorbol ester PMA (which stimulates phospholipase A_2 through protein kinase C activation) (34-37). Activation of the transfected A_1 receptor with R-PIA resulted in the potentiation of thrombin-, A23187-, melittin-, and PMA-stimulated arachidonic acid release (Figure 7). Similar results were seen for the D_2 and 5-HT_{1a} receptors (data not shown). Thus, G_i-coupled receptors which normally inhibit adenylate cyclase can amplify arachidonic acid release stimulated by several agents that activate phospholipase A_2 in CHO cells.

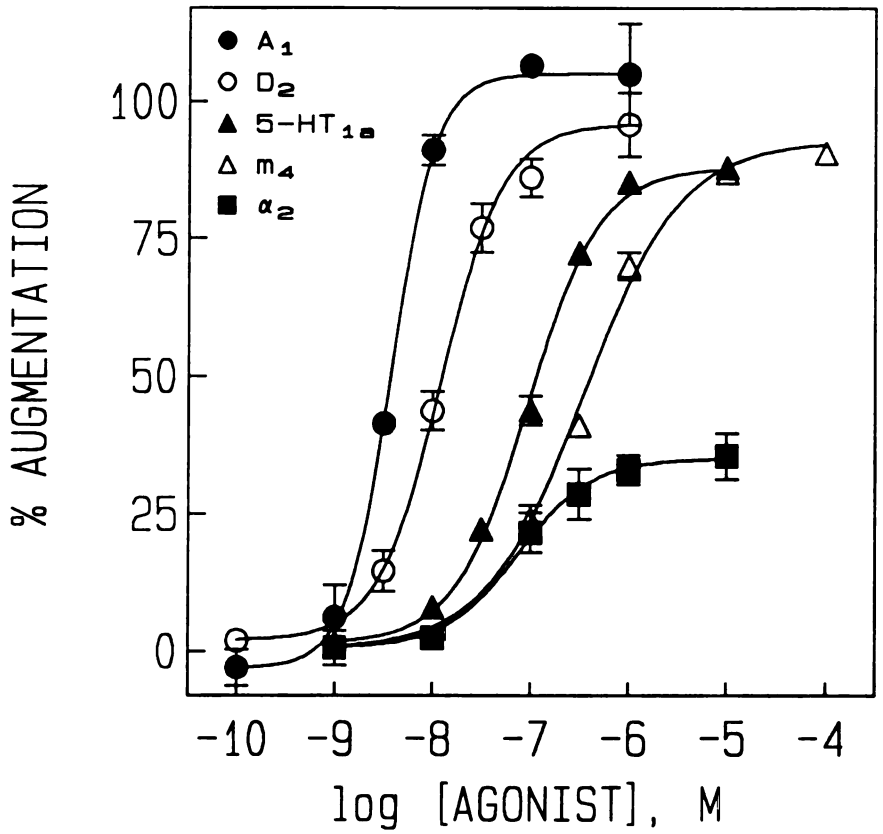


Figure 5: Stimulation of D_2-dopaminergic, m_4-muscarinic, α_2-adrenergic, 5-HT_{1a}-serotonergic, and A_1 adenosine receptors augments ATP-stimulated arachidonic acid release. Clonal CHO cell lines individually transfected with and expressing the D_2, m_4, α_2, 5-HT_{1a}, and A_1 receptors were stimulated with receptor agonists quinpirole, carbachol, clonidine, serotonin, and R-PIA, respectively. Arachidonic acid release was measured after 15 minutes. Data represents the percent augmentation of arachidonic acid release over maximal ATP stimulation (5μM). Estimated EC_{50} values for percent augmentation are as follows: D_2, 11 $\pm$ 0.1nM; m_4, 338 $\pm$ 3nM; α_2, 129 $\pm$ 0.1nM; 5-HT_{1a}, 93 $\pm$ 3.5nM; and A_1, 4.3 $\pm$ 0.2nM.

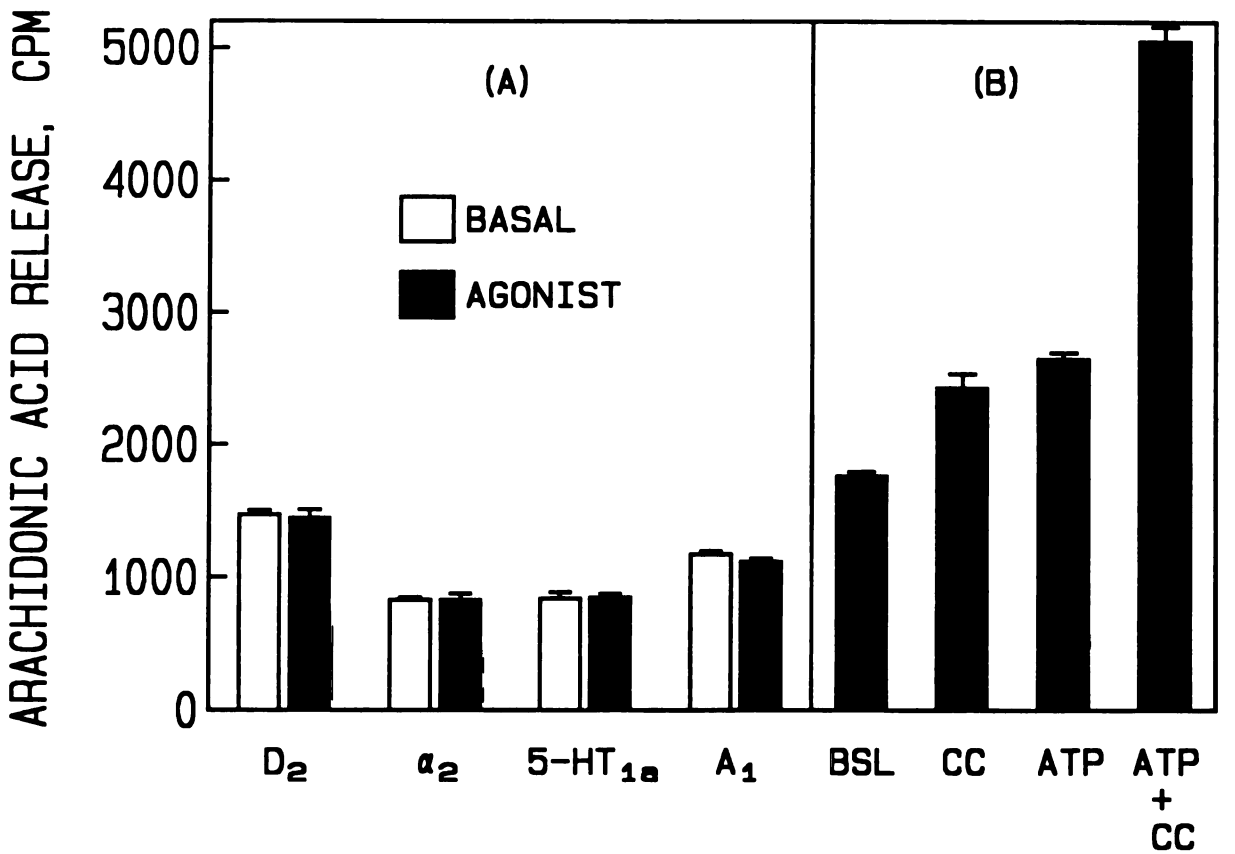


Figure 6: Maximal stimulation of D_2, α_2, 5-HT_{1a}, and A_1 receptors has no effect on arachidonic acid release in the absence of ATP and the m_4 receptor caused a modest increase in arachidonic acid release. (A) Clonal CHO cell lines expressing the D_2, α_2, 5-HT_{1a}, and A_1 receptors were stimulated with receptor agonists quinpirole (1 μM), clonidine (10 μM), serotonin (10 μM), and R-PIA (1 μM), respectively. Arachidonic acid release was measured over basal after 15 minutes. (B) Maximal stimulation of the m_4 receptor with carbachol (CC) (100 μM) in the absence of ATP increased arachidonic acid release by 38.46 $\pm$ 5.98 percent over basal (BSL). Maximal stimulation of the m_4 receptor in the presence of ATP augmented arachidonic acid by 90.71 $\pm$ 2.22 percent over maximal ATP stimulation (5 μM).

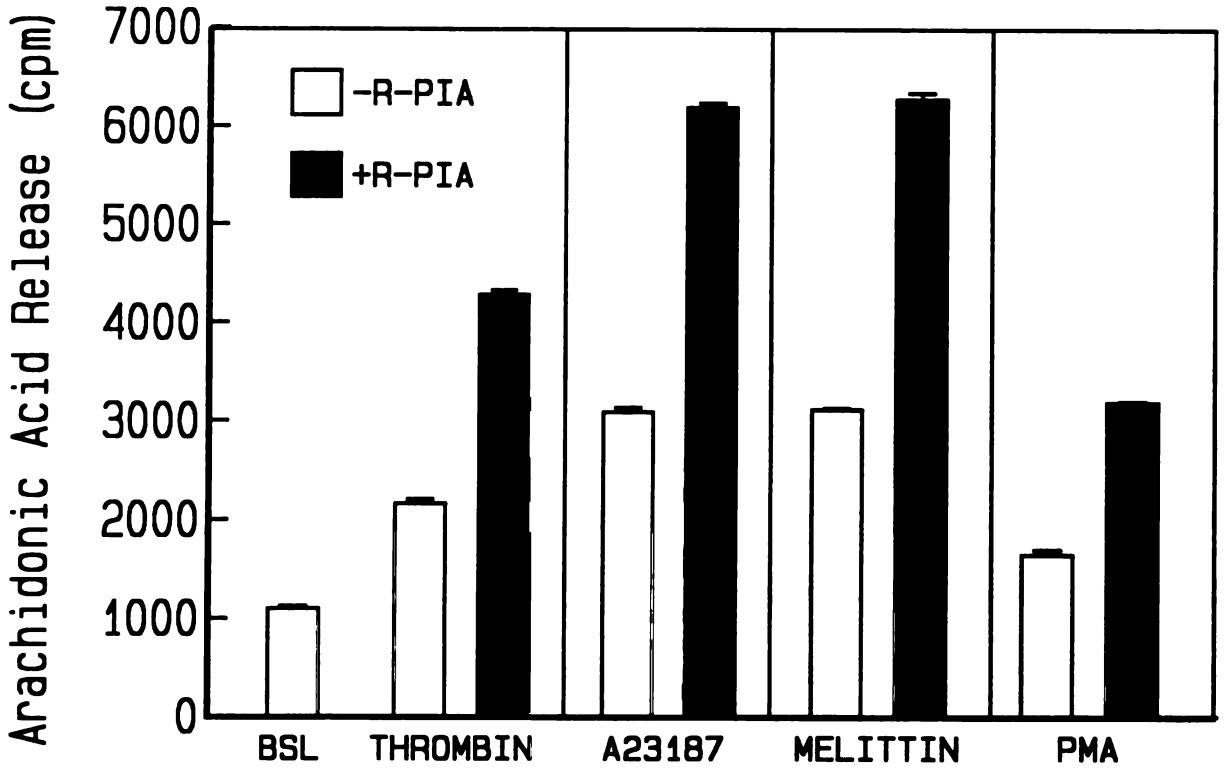


Figure 7: The transfected A_1 adenosine receptor potentiates the release of arachidonic acid stimulated by thrombin, A23187, melittin, and PMA. [^{3}H]arachidonic acid release in clonal CHO cells expressing the A_1 receptor was stimulated with thrombin (0.1 U/mL, 10 minutes), A23187 (10 μM, 10 minutes), melittin (1 μg/mL, 10 minutes), or PMA (100 nM, 30 minutes) and amplified with R-PIA (1 μM).

(C) Potentiation of ATP-stimulated arachidonic acid release is independent of changes in cAMP.

Since the known second messenger pathway common among the transfected G_i-coupled receptors tested is the inhibition of adenylate cyclase, it is possible that decreases in cAMP levels may be required for amplification of ATP-stimulated arachidonic acid release. It would follow that increases in cAMP should reverse the augmentation effect. Stable analogs of cAMP or agents that stimulate cAMP production were added to CHO cells expressing the m_2 receptor in an attempt to overcome the augmentation of arachidonic acid release (Table 1). Addition of the non-hydrolyzable cAMP analogs 8-(4-chlorophenylthio)-cAMP and 8-bromo-cAMP had no effect on the inhibitory receptor-mediated amplification of arachidonic acid. Similar results were seen for forskolin, which directly activates adenylate cyclase, and for PGE_2, which stimulates cAMP generation through an endogenous receptor coupled to the G protein G_s. Decreases in cAMP levels within the cell may result in a decrease in cAMP-dependent protein kinase A activity, which may play a role in the amplification response. (R_p)-cAMP[S], a membrane-permeable inhibitor of cAMP-dependent protein kinase A (38), was added to mimic the augmentation of ATP-stimulated arachidonic acid release. (R_p)-cAMP[S] had no effect on the augmentation. Thus changes in cAMP had no influence on m2-muscarinic-receptor-mediated amplification of the ATP-stimulated arachidonic acid release. Similar results were seen for the A_1 receptor (Table 2) and D_2, α_2, and 5-HT_{1a} receptors (data not shown). The EC_{50} values for inhibition of forskolin-stimulated cyclase were

Table 1: Changes in cAMP levels do not effect ATP- or m_2-receptor-mediated augmentation of arachidonic acid release.

	Arachidonic Acid Release, cpm	
Addition	ATP	ATP + Carbachol
None (control)	1639 ± 8	2465 ± 18
Prostaglandin E_2 (1μM)	1682 ± 4	2321 ± 52
Forskolin (500nM)	1565 ± 28	2387 ± 30
CPT-cAMP (10μM)	1697 ± 36	2448 ± 48
8-Bromo-cAMP (10μM)	1590 ± 43	2477 ± 40
(R_p)-cAMP[S] (1μM)	1568 ± 18	2283 ± 33

Maximal ATP (5μM)-stimulated arachidonic acid release or carbachol (10μM)-mediated augmentation of maximal ATP (5μM)-stimulated arachidonic acid release was assayed 15 minutes following a 10-minute preincubation with various agents as indicated. Prostaglandin E_2 (1μM) stimulated cAMP generation (basal = 2.1 $\pm$ 0.1, PGE_2 = 12.3 $\pm$ 0.9 pmol/mL) through endogenous receptors. Forskolin (500nM) stimulated cAMP generation (basal = 1.6 $\pm$ 0.1, forskolin = 12.6 $\pm$ 0.9 pmol/mL) through direct activation of adenylate cyclase. Basal arachidonic acid release was 668 $\pm$ 11. CPT-cAMP, 8-(4-chlorophenylthio)-cAMP. (Rp)-cAMP[S], (Rp)-adenosine cyclic 3',5'-phosphorothioate.

Table 2: Changes in cAMP levels do not effect ATP- or A_1-receptor-mediated augmentation of arachidonic acid release.

	Arachidonic Acid Release, cpm	
Addition	ATP	ATP + R-PIA
None (control)	2687 $\pm$ 28	5709 $\pm$ 124
Prostaglandin E_2 (1μM)	2694 $\pm$ 56	5715 $\pm$ 188
Forskolin (500nM)	2655 $\pm$ 22	5889 $\pm$ 290
CPT-cAMP (10μM)	2704 $\pm$ 39	5659 $\pm$ 318
8-bromo-cAMP (10μM)	2721 $\pm$ 65	5956 $\pm$ 245
(Rp)-cAMP[S] (1μM)	2693 $\pm$ 5	5767 $\pm$ 231

Maximal ATP (10μM)-stimulated arachidonic acid release or R-PIA (1μM)-mediated augmentation of maximal ATP (10μM)-stimulated arachidonic acid release was assayed 15 minutes following a 10-minute preincubation with various agents as indicated. Basal arachidonic release was 1028 $\pm$ 35.

lower than EC_{50} values for augmentation of ATP-stimulated arachidonic acid release for all inhibitory receptors tested (Figure legends 3-5), further dissociating the augmentation response from changes in cAMP.

(D) Potentiation of ATP-stimulated arachidonic acid release is sensitive to pertussis toxin.

The transfected receptors used in this study inhibit adenylate cyclase activity through the G-protein G_i. Pertussis toxin blocks the action of G_i by inducing ADP-ribosylation and persistent association of the G_i α- and $\beta\gamma$-subunit heterotrimer, thereby preventing the G_i α-subunit from transducing the inhibitory signal to adenylate cyclase (39). Pertussis toxin has also been shown to inhibit receptor-stimulated arachidonic acid release, suggesting the involvement of G_i like proteins in this signalling pathway (21,40). After 12 hours of preincubation, pertussis toxin completely inhibited A_1-receptor-mediated potentiation of arachidonic acid release but had no effect on basal or ATP-stimulated arachidonic acid release (Figure 8). This suggests the involvement of a pertussis-sensitive-G_i-like protein in the amplification response. Pertussis toxin also inhibited carbachol-mediated potentiation of ATP-stimulated arachidonic acid release in CHO cells expressing m_2 receptors (Figure 9) and reversed carbachol-mediated inhibition of forskolin-stimulated adenylate cyclase consistent with a previous report (41). The m_2-receptor-mediated inhibition of forskolin-stimulated adenylate cyclase was more sensitive to inhibition by pertussis toxin than was augmentation of arachidonic acid release, as

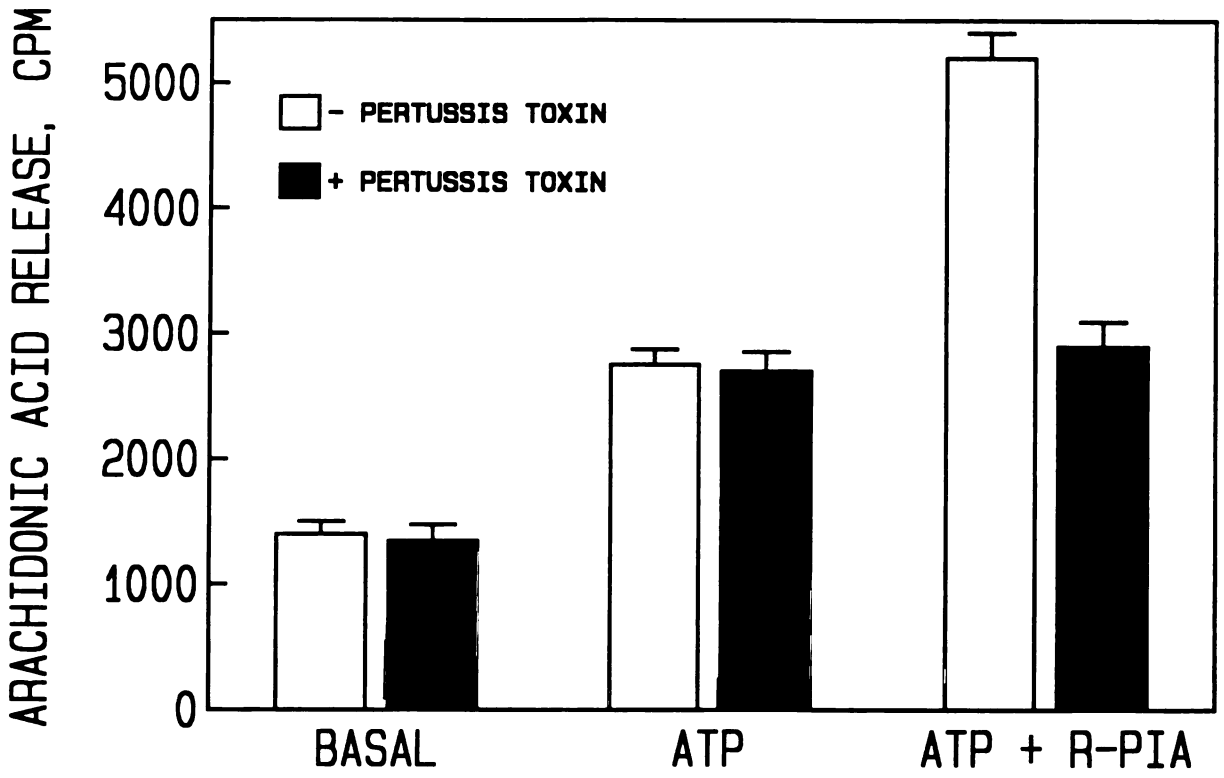


Figure 8: Pertussis toxin inhibits A_1-receptor-mediated augmentation of ATP-stimulated arachidonic acid release. Clonal CHO cells expressing the A_1 receptor were treated for 12 hours with pertussis toxin (1 ng/mL). ATP (10μM)-stimulated and R-PIA (1μM)-potentiated arachidonic acid release were measured and compared to cells which had not been treated with pertussis toxin.

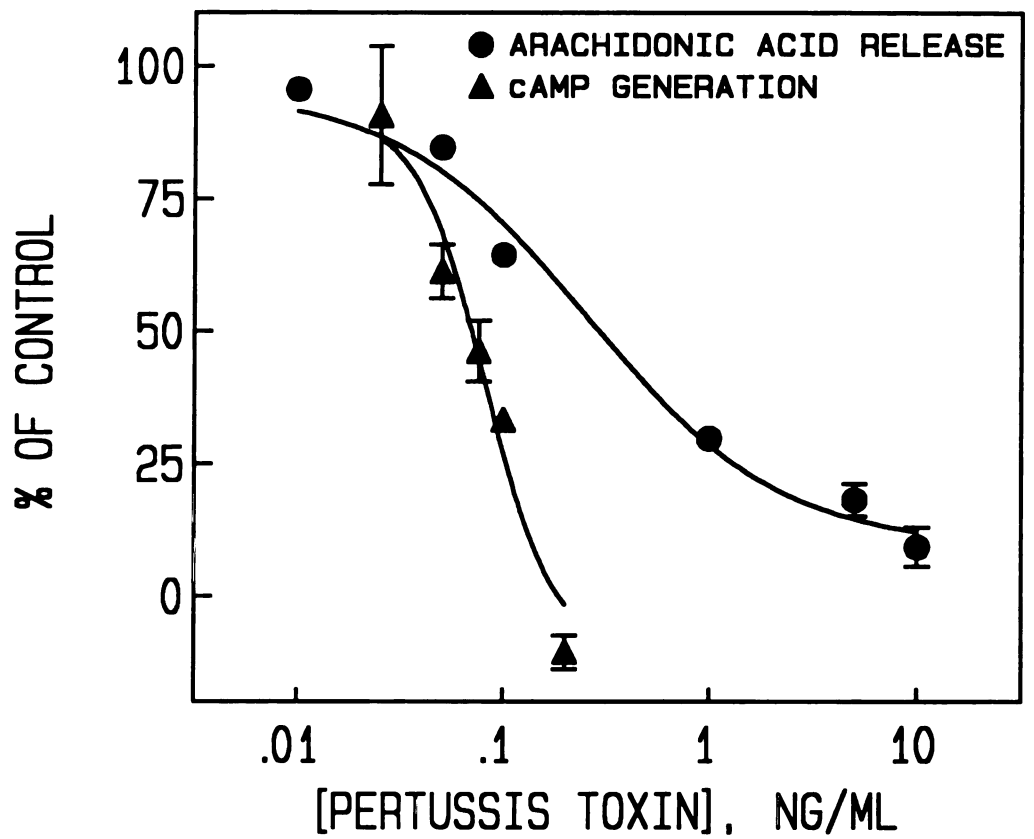


Figure 9: Pertussis toxin inhibits m_2-receptor augmentation of ATP-stimulated arachidonic acid release and reverses m_2-receptor-inhibited cAMP generation. Clonal CHO cells expressing the m2 muscarinic receptor were treated for 12 hours with increasing concentrations of pertussis toxin. CHO cells expressing the m_2 receptor were then assayed for either carbachol (100 μM)-mediated inhibition of forskolin (500nM)-stimulated cAMP accumulation or carbachol (10μM)-mediated augmentation of maximal ATP (5μM)-stimulated release of arachidonic acid.

shown by the left shift of the dose-response curve (Figure 9). Although both responses are sensitive to pertussis toxin, the differential sensitivities suggest either that distinct G_i-like proteins are involved or that the augmentation response is a more complex multistep process.

(E) Inhibitors of protein kinase C block the potentiation of ATP-stimulated arachidonic acid release.

Receptor-mediated stimulation of phospholipase A_2 activity in CHO cells has been shown to involve the activation of protein kinase C (23,42). Staurosporine, a selective inhibitor of protein kinase C (43), blocked A_1-receptor-mediated amplification of arachidonic acid release but had no effect on basal or ATP-stimulated arachidonic acid release (Figure 10 A). Further evidence of protein kinase C involvement in the A_1-mediated augmentation response was shown by protein kinase C desensitization after prolonged treatment with the phorbol ester PMA (22) (Figure 10 B). After 4 hours of preincubation, PMA inhibited the A_1-mediated augmentation of arachidonic acid release. Under the same conditions, the inactive phorbol ester, 4-α phorbol, had no effect on the augmentation response (data not shown). Similar results were seen for the m_2, D_2, and α_2 receptors (Figure 11). These data suggest a role for protein kinase C in the augmentation of arachidonic acid release mediated by inhibitory G_i-coupled receptors.

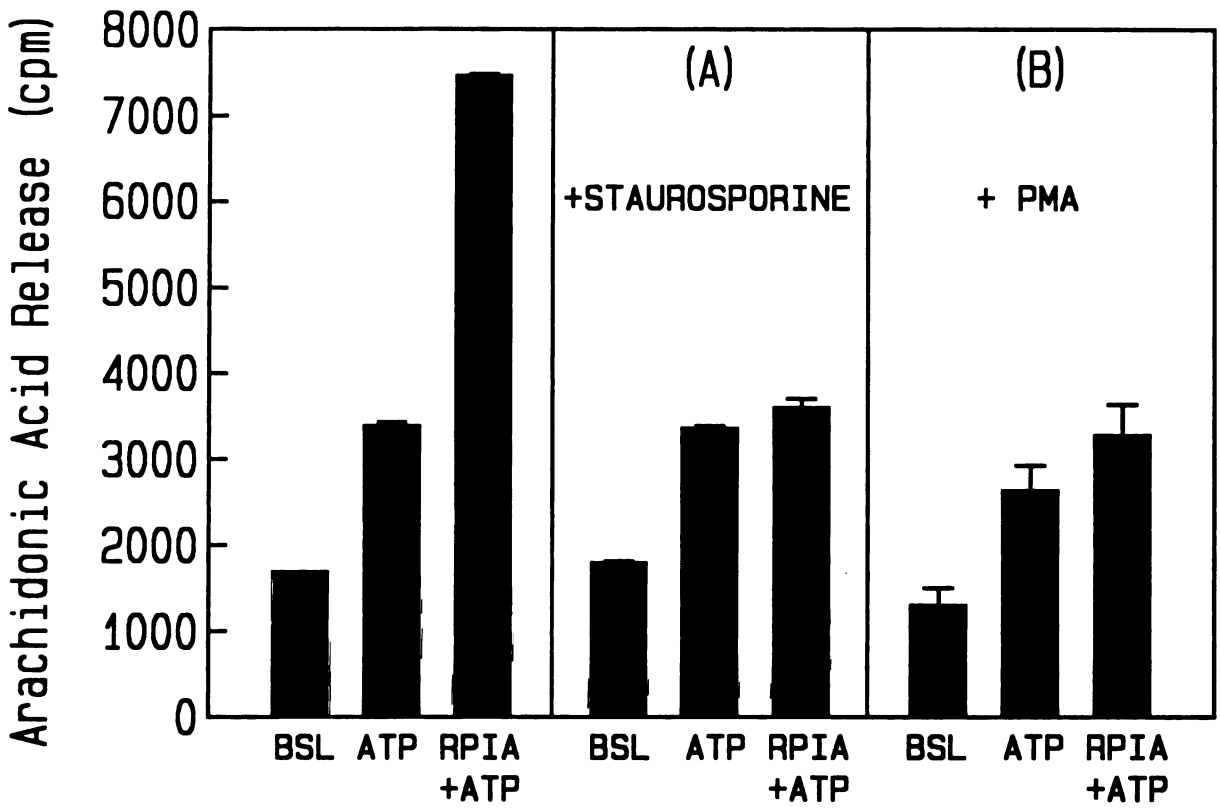


Figure 10: Inhibitors of protein kinase C block A_1-receptor-mediated potentiation of ATP-stimulated arachidonic acid release. Clonal CHO cells expressing the A_1 receptor were stimulated with ATP (10 μM) alone or with ATP and R-PIA (1 μM) and arachidonic acid release was measured after 10 minutes. **(A)** Cells were preincubated with staurosporine (1 μM, 10 minutes), an inhibitor of protein kinase C. **(B)** Cells were pretreated with the phorbol ester PMA (100 nM, 4 hours) to desensitize protein kinase C.

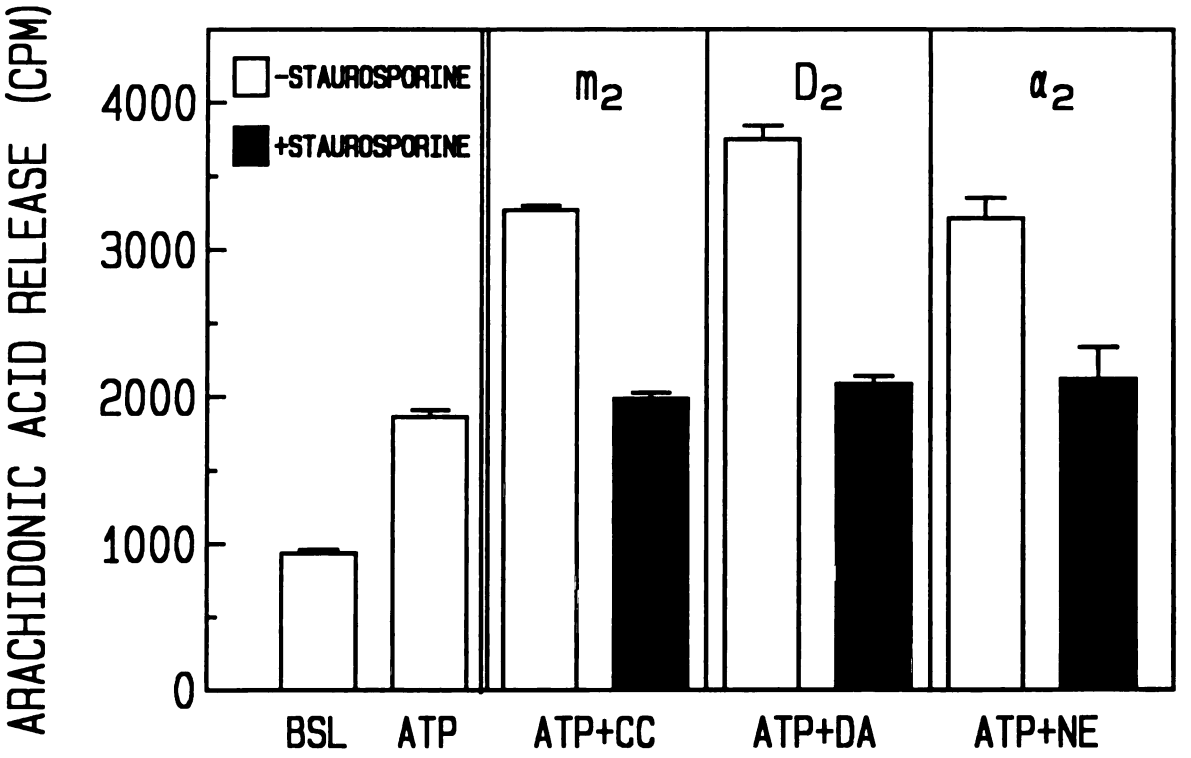


Figure 11: Staurosporine, an inhibitor of protein kinase C, blocked m_2, D_2, and α_2 receptor augmentation of ATP-stimulated arachidonic acid release. Clonal CHO cells expressing m_2, D_2, or α_2 receptors were stimulated with ATP (5μM) alone or with ATP in combination with either 10μM carbachol (CC), 100nM dopamine (DA), or 10μM norepinephrine (NE) and arachidonic acid release was measured after 15 minutes. Data represented by the solid bars were preincubated with 1μM staurosporine for 10 minutes. Staurosporine inhibited the augmentation response with an IC_{50} of 50 nM and the complete inhibition at 1 μM is shown.

(F) The transfected A_1 adenosine and 5-HT_{1a}-serotonergic receptors weakly couple to inositolphospholipid turnover.

Protein kinase C activation requires both diacylglycerol and calcium (44). Receptor-generated diacylglycerol could arise from activation of inositolphospholipid- or phosphatidylcholine-specific phospholipase C (Figure 2). Calcium could arise from opening of ion channels or inositol triphosphate-induced release of intracellular calcium stores subsequent to activation of phospholipase C (Figure 1). Stimulation of the transfected A_1 adenosine receptor in CHO cells resulted in a modest generation of inositolphospholipid turnover, especially inositol monophosphate (Figure 12 A-C). As in the case for A_1 receptor-mediated inhibition of adenylate cyclase, the estimated EC_{50} for A_1-generated inositol monophosphate was lower than the EC_{50} for A_1-mediated augmentation of arachidonic acid release (Figure 13). Maximal stimulation of CHO cells expressing 5-HT_{1a} receptors also resulted in a modest (26% over basal) generation of total inositol phosphates after 30 minutes, but maximal stimulation of m_2 and D_2 receptors failed to affect inositolphospholipid turnover for the expression levels tested (Figure 14). These data suggest that activation of inositolphospholipid-specific phospholipase C is not the mechanism by which all of the G_i-coupled receptors in this study augment ATP-stimulated arachidonic acid release.

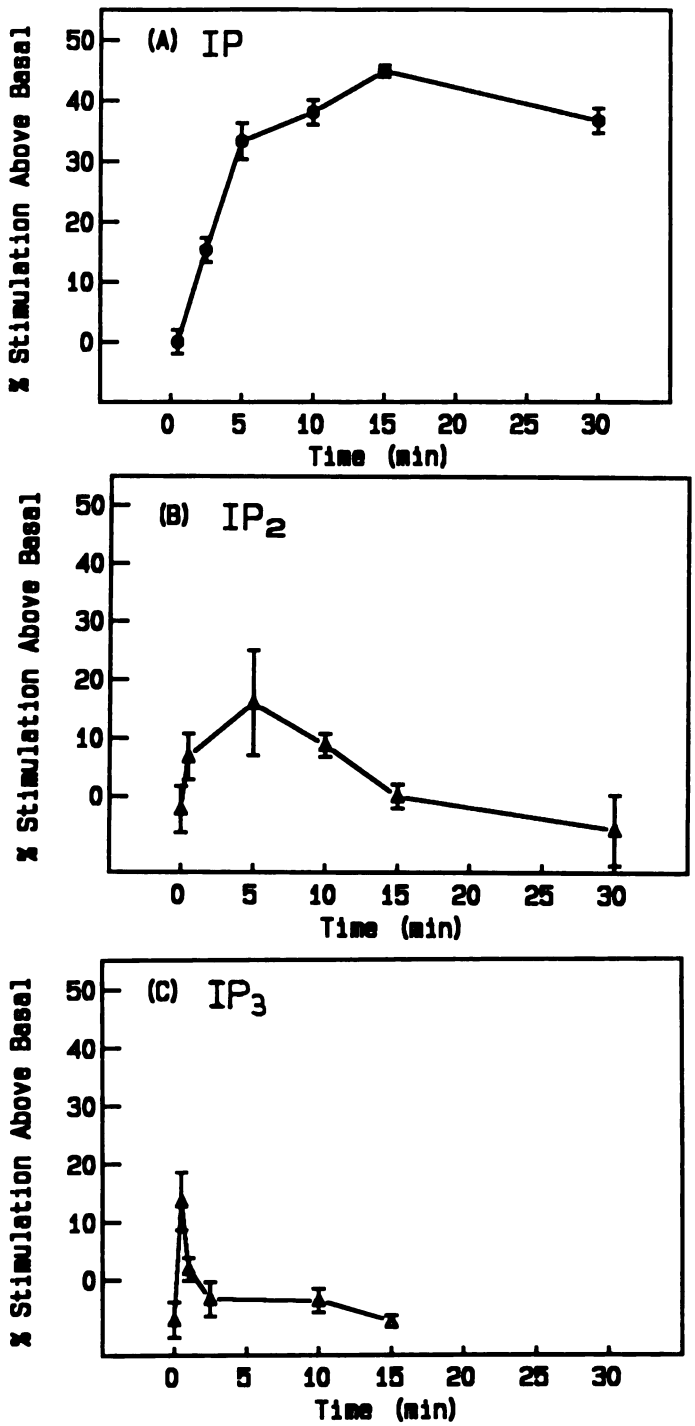


Figure 12: Stimulation of the A_1 adenosine receptor results in a modest increase in inositolphospholipid turnover. Clonal CHO cells expressing A_1 adenosine receptors were prelabeled overnight with [^{3}H]inositol and monitored for generation of inositol phosphates over 30 minutes following stimulation by 1 μM R-PIA. Accumulated inositol phosphates were separated by anion exchange chromatography and are shown separately as percent stimulation of above basal release: (A) inositol monophosphate, IP; (B) inositol diphosphate, IP_2; and (C) inositol triphosphate, IP_3.

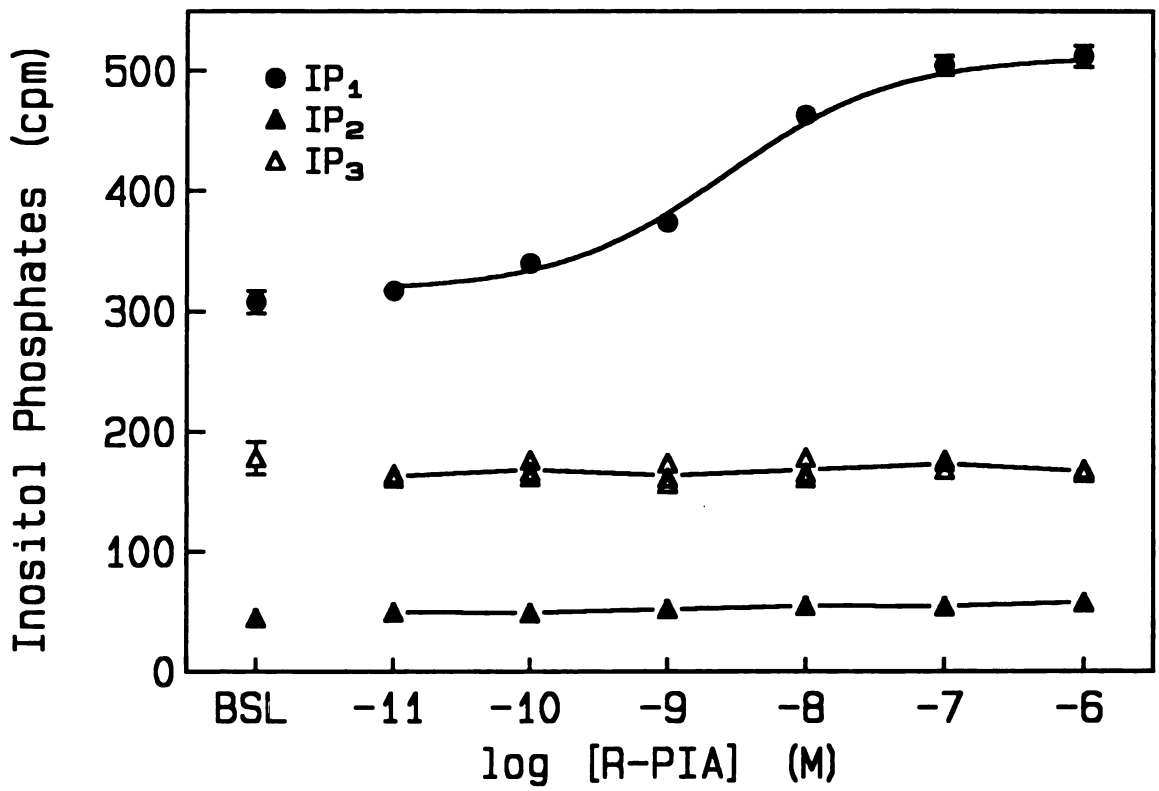


Figure 13: Stimulation of the A_1 adenosine receptor increases inositol monophosphate accumulation in a dose-dependent manner. Clonal CHO cells transfected with and expressing A_1 adenosine receptors were stimulated with increasing concentrations of R-PIA and accumulation of inositol phosphates measured after 30 minutes. R-PIA stimulated inositol monophosphate (IP) accumulation with an estimated EC_{50} of 2.37 $\pm$ 0.11 nM. IP_2, inositol diphosphate. IP_3, inositol triphosphate. BSL, basal.

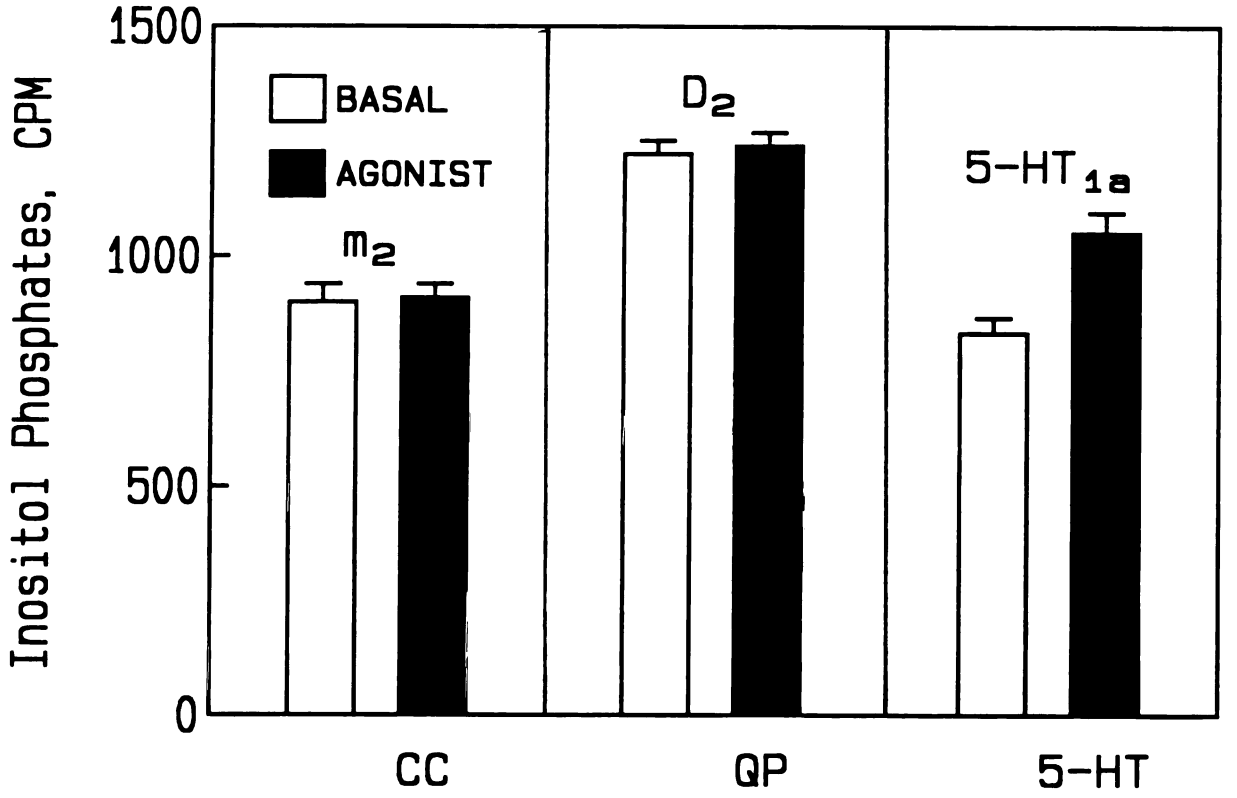


Figure 14: Stimulation of the 5-HT_{1a} receptor, but not the m_2 or D_2 receptor, causes a modest increase in inositolphospholipid turnover. Clonal CHO cells individually transfected with and expressing m_2, D_2, and 5-HT_{1a} receptors were stimulated with the receptor agonists carbachol (CC) (100 μM), quinpirole (QP) (1 μM), and serotonin (5-HT) (10 μM), respectively. Total accumulation of inositol phosphates was measured after 30 minutes.

DISCUSSION

These results demonstrate that inhibitory G_i-coupled receptors can amplify ATP-receptor-mediated release of arachidonic acid, as well as arachidonic acid release stimulated by thrombin, A23187, melittin, and PMA. Except for a modest increase seen for the m_4 receptor, the receptors tested were unable to stimulate arachidonic acid release in the absence of ATP. The potentiation of arachidonic acid was independent of changes in cAMP, suggesting that an alternative signalling pathway might be operating to induce this response. The amplification of arachidonic acid release was dependent on a pertussis-toxin-sensitive G-protein which was less sensitive to the toxin than was the inhibition of cAMP. These results further suggest that an alternate G_i protein might be involved in the potentiation response. Similar conclusions were suggested in a previous study using CHO cells, in which low concentrations of pertussis toxin differentially inhibited muscarinic-receptor-mediated inositol phosphate release and cAMP inhibition, whereas at higher concentrations, complete ADP-ribosylation of pertussis sensitive substrates was observed (45).

Inhibition of the potentiation response by two inhibitors of protein kinase C, staurosporine and long term preincubation with PMA, suggests that protein kinase C may be activated by inhibitory G_i-coupled receptors, although evidence of direct activation by these receptors has yet to be demonstrated. Two of the six G_i-coupled receptors were able to stimulate inositolphospholipid-specific phospholipase C which would presumably liberate diacylglycerol to activate protein kinase C indirectly. This specific pathway to protein kinase C activation is not likely to explain the

augmentation of arachidonic acid release in these studies since not all of the receptors demonstrated coupling to inositolphospholipid turnover. However, release of diacylglycerol by phosphatidylcholine-specific phospholipase C, independent of inositol phosphate release, was not studied. Protein kinase C, as well as phospholipase A_2, requires calcium for activation and coupling of G_i receptors to calcium channels could be envisioned in addition to inositol triphosphate-induced release of calcium from intracellular stores (23,46). Logical next steps in exploring the mechanism behind G_i-coupled-receptor-augmentation of arachidonic acid release might include direct examination of protein kinase C translocation, diacylglycerol formation, and calcium mobilization.

The manner in which protein kinase C augments release of arachidonic acid was not investigated in this study. Protein kinase C may stimulate phospholipase A_2 activity by increasing Na^+/H^+ exchange (47) or disinhibition of Na^+/H^+ exchange (48), for instance. It is possible that protein kinase C may play a role in reducing the calcium requirement of phospholipase A_2, reminiscent of the action of diacylglycerol on protein kinase C (49).

Further studies are needed to explore G-protein involvement in the augmentation of arachidonic acid by receptors which couple to the inhibition of adenylate cyclase. For example, co-purification of activated-receptor-G-protein complexes using affinity columns and identification of complexes using specific antibodies might be attempted along with studying the effects of mutant G_i-proteins and antisense phosphorothioate oligonucleotides directed at the 5'prime initiating

methionine of the G_i message.

Future studies should also be undertaken to look for G_i-receptor-mediated augmentation of arachidonic acid release in neural systems. For instance, hippocampal preparations could be utilized to study the role of augmented arachidonic acid release in long-term potentiation. G_i-receptor-mediated augmentation of arachidonic acid release may also be relevant to platelet activation since serotonin and ATP are coreleased by platelets and augmentation of thrombin-stimulated arachidonic acid release was demonstrated in this study.

It is becoming apparent that activation of multiple receptors is involved in inducing (50), enhancing (51), and inhibiting (52) receptor mediated signalling processes. The results in this study demonstrate that the inhibitory m_2- and m_4-muscarinic, α_2- adrenergic, D_2 dopaminergic, 5-HT_{1a}-serotonergic, and A_1 adenosine receptors augment ATP-stimulated arachidonic acid release. This association between signalling pathways for neurotransmitters which are copackaged and coreleased with ATP would result in the enhanced release of arachidonic acid and its many bioactive eicosanoid metabolites. Modulation of ATP-stimulated arachidonic acid release may be a common feature of G_i coupled inhibitory receptors.

BIBLIOGRAPHY

1. Berridge, M. The molecular basis of communication within the cell. (1985) Sci. Am. 253, 142-152.

2. Gilman, A. G. G proteins: transducers of receptor-generated signals. (1987) Annu. Rev. Biochem. 56, 615-649.

3. Axelrod, J., Burch, R. M., & C. L. Jelsma. Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. (1988) Trends Neurosci. 11, 117-123.

4. Piomelli, D., & P. Greengard. Lipoxygenase metabolites of arachidonic acid in neuronal transmembrane signalling. (1990) Trends Pharmacol. Sci. 11, 367-373.

5. Williams, J. H., Errington, M. L., Lynch, M. A., et al. Arachidonic acid induces a long-term activity-dependent enhancement of synaptic transmission in the hippocampus. (1989) Nature 341, 739-742.

6. Wolf, B. A., Turk, J., Sherman, W. R., et al. Intracellular Ca^{2+} mobilization by arachidonic acid. Comparison with myo-inositol 1,4,5-triphosphate in isolated pancreatic islets. (1986) J. Biol. Chem. 261, 3501-3511.

7. Bitonti, A. J., Moss, J., Tandon, N. N., et al. Prostaglandins increase GTP hydrolysis by membranes from human mononuclear cells. (1980) J. Biol. Chem. 255, 2026-2029.

8. Bazan, N. G., Birkle, D. L., Tang, W., et al. (1986) in Basic Mechanisms of the Epilepsies: Molecular and Cellular Approaches (Advances in Neurology, Vol. 44) (Delgado-Escueta, A. V., Ward, A. A., Woodbury, D. M., et al, eds.), Raven Press, 879-902.

9. Kanterman, R. Y., Axelrod, J. & C. C. Felder. Alpha-1 adrenergic receptor mediates the release of arachidonic acid in spinal cord neurons independent of phosphatidylinositol-specific phospholipase C. (1990) J. Neurochem. 54, 1225-1232.

10. Felder, C. C., Kanterman, R. Y., Ma, A. L., et al. Serotonin stimulates phospholipase A_2 and the release of arachidonic acid in hippocampal neurons by a type 2 serotonin receptor that is independent of inositolphospholipid hydrolysis. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2187-2192.

11. Kanterman, R. Y., Ma, A. L., Briley, E. M., et al. Muscarinic receptors mediate the release of arachidonic acid from spinal cord and hippocampal neurons in primary culture. (1990) Neurosci. Lett. 118, 235-237.

12. Dumuis, A., Sebben, M., Haynes, L., et al. NMDA receptors activate the arachidonic acid cascade system in striatal neurons. (1988) Nature 336, 68-70.

13. Burch, R. M. G protein regulation of phospholipase A_2. (1989) Mol. Neurobiol. 3, 155-171.

14. Bell, R. L., Kennerly, D. A., Stanford, N., et al. Diglyceride lipase: A pathway for arachidonate release from human platelets. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3238-3241.

15. Exton, J. H. Signaling through phosphatidylcholine breakdown. (1989) J. Biol. Chem. 265, 1-4.

16. Taki, T. & J. N. Kanfer. Partial purification and properties of rat brain phospholipase D. (1979) J. Biol. Chem. 24, 9761-9765.

17. Wolf, R. A. & R. W. Gross. Identification of neutral active phospholipase C which hydrolyses choline glycerophospholipids and plasmalogen-selective phospholipase A_2 in canine myocardium. (1985) J. Biol. Chem. 260, 7295-7303.

18. Gordon, J. L. Extracellular ATP: effects, sources and fate. (1986) Biochem. J. 233, 309-319.

19. Cockcroft, S. & J. Stutchfield. The receptors for ATP and fMetLeuPhe are independently coupled to phospholipase C and A_2 via G-protein(s). (1989) Biochem. J. 263, 715-723.

20. Kanterman, R. Y., Mahan, L. C., Briley, E. M., et al. Transfected D_2 dopamine receptors mediate the potentiation of arachidonic acid release in Chinese hamster ovary cells. (1991) Mol. Pharmacol. 39, 364-369.

21. Gupta, S. K., Diez, E., Heasley, L. E., et al. A G protein mutant that inhibits thrombin and purinergic receptor activation of phospholipase A_2. (1990) Science 249, 662-665.

22. Felder, C. C., Kanterman, R. Y., Ma, A. L., et al. A transfected m_1 muscarinic acetylcholine receptor stimulates adenylate cyclase via phosphatidylinositol hydrolysis. (1989) J. Biol. Chem. 264, 20356-20362.

23. Felder, C. C., Dieter, P., Kinsella, J., et al. A transfected m_5 muscarinic acetylcholine receptor stimulates phospholipase A_2 by inducing both calcium influx and activation of protein kinase C. (1990) J. Pharmacol. Exp. Therap. 255, 1140-1147.

24. Bonner, T. I., Buckley, N. J., Young, A. C., et al. Identification of a family of muscarinic acetylcholine receptor genes. (1987) Science 237, 527-532.

25. Voigt, M. M., McCune, S. K., Kanterman, R. Y., et al. The rat α_2-C4-adrenergic receptor gene encodes a novel pharmacological subtype. (1991) FEBS Lett. 278, 45-50.

26. Monsma, F. J., McVittie, L. D., Gerfen, C. R., et al. Multiple D2 dopamine receptors produced by alternative RNA splicing. (1989) Nature 342, 926-929.

27. Rinaudo, M. S., Monsma Jr., F. J., Black, L. E., et al. (1991) Neuroscience 16, ab#94.12.

28. Fargin, A., Raymond, J. R., Regan, J. W., et al. Effector coupling mechanisms of the cloned 5-HT_{1a} receptor. (1989) J. Biol. Chem. 264, 14848-14852.

29. Raymond, J. R. of Duke University Medical Center. Personal communication regarding 5-HT_{1a} receptor expression in CHO cells (data not yet published).

30. Mahan, L. C., McVittie, L. D., Smyk-Randall, E. M., et al. Cloning and expression of an A_1 adenosine receptor from rat brain. (1991) Mol. Pharmacol. 40, 1-7.

31. Mahan, L. C. of the National Institute of Mental Health. Personal communication regarding expression of A_1 receptors in CHO cells (data not yet published).

32. Stiles, G. L. Adenosine receptors: structure, function, and regulation. (1986) Trends Pharmacol. Sci. 7, 486-490.

33. Williams, M. Purine receptors in mammalian tissues: pharmacology and functional significance. (1987) Annu. Rev. Pharmacol. Toxicol. 27, 315-345.

34. Bradford, P. G., Marinetti, G. V. & L. G. Abood. Stimulation of phospholipase A_2 and secretion of catecholamines from brain synaptosomes by potassium and A23187. (1983) J. Neurochem. 41, 1684-1693.

35. Irvine, R. How is the level of free arachidonic acid controlled in mammalian cells? (1982) Biochem. J. 204, 3-16.

36. Ho, A. K. and D. C. Klein. Activation of alpha$_1$-adrenoreceptors, protein kinase C, or treatment with intracellular free Ca^{2+}-elevating agents increases pineal phospholipase A_2 activity. (1987) J. Biol. Chem. 262, 11764-11770.

37. Clark, M. A., Conway, T. M., Shorr, R. G. L., et al. Identification and isolation of a mammalian protein which is antigenically and functionally related to the phospholipase A_2 stimulatory peptide melittin. (1987) J. Biol. Chem. 262, 4402-4406.

38. Botelho, L. H. P., Rothermal, J. D., Coombs, R. V., et al. cAMP analog antagonists of cAMP action. (1988) Meth. Enzymol. 159, 159-172.

39. Casey, P. J., & A. G. Gilman. G protein involvement in receptor effector coupling. (1988) J. Biol. Chem. 263, 2577-2580.

40. Burch, R. M., Luini, A., & J. Axelrod. Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α_1-adrenergic stimulation in FRTL5 thyroid cells. (1986) Proc. Natl. Acad. Sci. USA 83, 7201-7205.

41. Nathanson, N. M. Molecular properties of the muscarinic acetylcholine receptor. (1987) Ann. Rev. Neurosci. 10, 195-236.

42. Godson, C., Weiss, B. A., & P. A. Insel. Differential activation of protein kinase C is associated with arachidonate release in Madin-Darby canine kidney cells. (1990) J. Biol. Chem. 265, 8369-8372.

43. Tamaoki, T., Nomoto, H., Takahashi, I., et al. Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. (1986) Biochem. Biophys. Res. Comm. 135, 397-402.

44. Kikkawa, U. & Y. Nishizuka. The role of protein kinase C in transmembrane signalling. (1986) Annu. Rev. Cell Biol. 2, 149-178.

45. Ashkenazi, A., Winslow, J. W., Peralta, E. G., et al. An M2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. (1987) Science 238, 672-675.

46. Brooks, R. B., McCarthy, K. D., Lapetina, E. G., et al. Receptor-stimulated phospholipase A_2 activation is coupled to influx of external calcium and not to mobilization of intracellular calcium in C62B glioma cells. (1989) J. Biol. Chem. 264, 20147-20153.

47. Besterman, J. M., May, W. S., Jr., LeVine, H. III., et al. Amiloride inhibits phorbol ester-stimulated Na^+/H^+ exchange and protein kinase C: An amiloride analog selectively inhibits Na^+/H^+ exchange. (1985) J. Biol. Chem. 260, 1155-1159.

48. Sweatt, J. D., Connolly, T. M., Cragoe, E. J., et al. Evidence that Na^+/H^+ exchange regulates receptor-mediated phospholipase A_2 activation in human platelets. (1986) J. Biol. Chem. 261, 8667-8673.

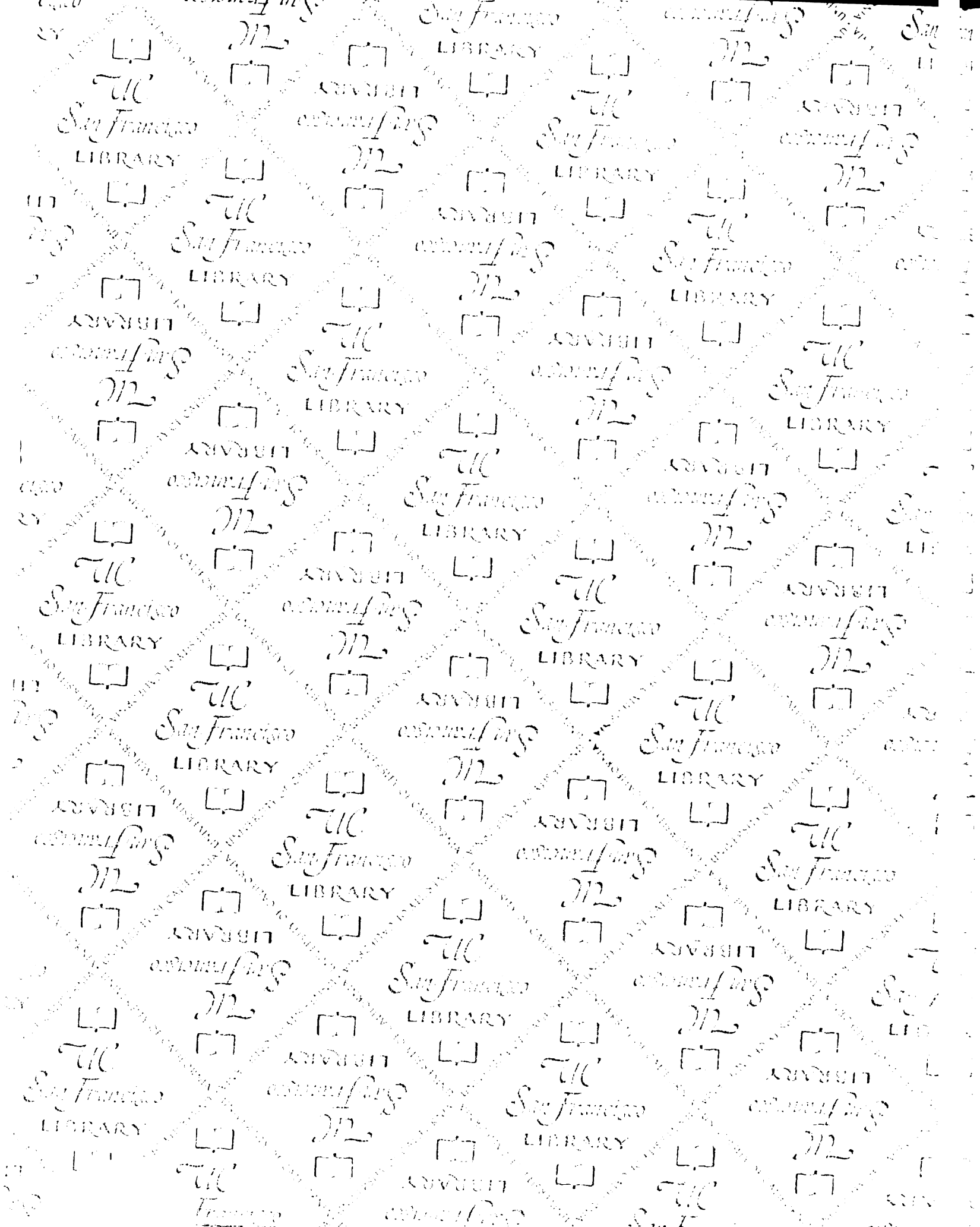
49. Kishimoto, A., Takai, Y., Mori, T., et al. Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. (1980) J. Biol. Chem. 255, 2273-2276.

50. Dumuis, A., Pin, J. P., Oomagari, K., et al. Arachidonic acid released from striatal neurons by joint stimulation of ionotropic and metabotropic quisqualate receptors. (1990) Nature 347, 182-183.

51. Sugden, L., Sugden, D., & D. C. Klein. Alpha 1-adrenoreceptor activation elevates cytosolic calcium in rat pinealocytes by increasing net influx. (1986) J. Biol. Chem. 261, 11608-11612.

52. Bertorello, A. M., Hopfield, J. F., Aperia, A., et al. Inhibition by dopamine of (Na^+ + K^+) ATPase activity in neostriatal neurons through D_1 and D_2 dopamine receptor synergism. (1990) Nature 347, 386-388.

Acknowledgements: Funding for this project was obtained from the United States Department of Health and Human Services Intramural Research Budget for the Laboratory of Cell Biology within the National Institute of Mental Health in Bethesda, MD. A stipend was provided by the Howard Hughes Medical Institute Research Scholars Program.


San Francisco
LIBRARY