EFFECT OF MUSCARINIC RECEPTOR STIMULATION ON PHOSPHATIDYLINOSITOL TURNOVER IN RAT HEART AND GUINEA PIG SMOOTH MUSCLE

by

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Abstract

Stimulation of various cell surface receptors produces an enhanced turnover of phosphatidylinositol (PI). This phenomenon, referred to as the PI response, has been suggested to be an obligatory response of muscarinic receptor stimulation and to play a role in calcium mobilization mediated by muscarinic and other receptor systems. In this study, this hypothesis has been investigated by studying PI response in rat atria and longitudinal smooth muscle of the guinea pig ileum.

It was observed that even though muscarinic receptor density is known to be similar in rat left and right atria, an increase (35%, p < 0.05) in phosphate incorporation into PI was observed in rat left atrium but not in right atrium. By contrast to the small effect of muscarinic receptor stimulation, alpha-adrenergic receptor stimulation by 0.1 mM methoxamine produced a two-fold increase in phosphate incorporation into PI in both rat left and right atria, despite the lower density of these receptors compared to the muscarinic receptors in atria. These results, along with studies showing a lack of effect of muscarinic receptor stimulation on inositol lipid breakdown, suggested that only a small population (approximately 15%) of muscarinic receptors in rat atria is coupled to PI turnover. These are termed m₁ receptors, by analogy to a₁ adrenergic receptors which enhance PI turnover and Ca²⁺ mobilization. The remaining population of muscarinic receptors, termed m₂, most probably is coupled in an inhibitory manner to adenylate cyclase. While these findings support the postulate that PI
response may accompany only the stimulation of those receptors whose response is mediated through calcium mobilization, the results also suggest the presence of a muscarinic receptor population whose stimulation is not coupled to PI turnover.

The role of PI response in calcium mobilization was investigated in longitudinal smooth muscle of guinea pig ileum. Consistent with previous findings, muscarinic receptor stimulation of guinea pig ileum was accompanied by a PI response. Carbachol (0.1 mM) produced an atropine-sensitive incorporation of $[^{32}\text{P}]$phosphate and $[^{3}\text{H}]$inositol into PI. The effect of carbachol on the breakdown of inositol lipids was studied in the presence of 10 mM lithium, which causes accumulation of inositol phosphate by inhibiting inositol phosphatase. Carbachol produced a 20-fold increase in the accumulation of inositol phosphates in the presence of lithium. Analysis of the inositol phosphates by ion-exchange chromatography indicated that, unlike many other tissues, in guinea pig ileum less than 20% of the $[^{3}\text{H}]$inositol label occurred in inositol monophosphate, while the remainder was found in the inositol polyphosphate fraction. This indicated that similar to findings in other tissues reported recently, the primary event of muscarinic receptor stimulation in guinea pig ileum may be the enhanced breakdown of polyphosphoinositides, rather than PI.

The effect of phenylmethylsulfonylfluoride (PMSF), a putative inhibitor of PI-specific phospholipase C, on carbachol and potassium-stimulated PI turnover and contraction in
longitudinal smooth muscle of guinea pig ileum was studied. PMSF almost completely inhibited carbachol-stimulated inositol incorporation into PI, while it had no effect on potassium-stimulated inositol incorporation. This suggests that the two stimuli produce "PI responses" by different mechanisms. In contrast to its specific inhibition of carbachol-stimulated PI turnover, PMSF produced a nonspecific, transient inhibition of contraction of guinea pig ileum by both carbachol and potassium. The nonspecific PMSF effect on contraction suggests that it is not the result of its inhibitory effect on PI turnover. The suggestion of Walenga et al. (1980) that PMSF inhibition of PI turnover may be mediated through its inhibition of PI-specific phospholipase C was based on indirect findings. PMSF (2 mM) produced only a 16% inhibition of the carbachol-stimulated inositol phosphate accumulation in the presence of lithium, indicating that the PMSF effect on PI-specific phospholipase C cannot fully account for the observed inhibition by PMSF of carbachol-stimulated inositol incorporation. The results obtained do not contradict the possibility that PI breakdown is involved in calcium mobilization.
Table of Contents

Abstract .................................................................................................................. ii
List of Tables ........................................................................................................... viii
List of Figures .......................................................................................................... ix
Acknowledgement .................................................................................................... xiii

LIST OF ABBREVIATIONS ...................................................................................... xi

Chapter I
INTRODUCTION ...................................................................................................... 1
1.1 DISTRIBUTION OF PI ..................................................................................... 3
1.2 PI RESPONSE ................................................................................................... 4
1.2.1 Closed Cycle ................................................................................................ 4
1.2.2 The Primary Event Of PI Response ............................................................... 6
1.2.3 Alternate Mechanisms For Label Incorporation ............................................. 7
1.2.4 PI-specific Phospholipase C ........................................................................ 9
1.3 ROLE OF PI TURNOVER ............................................................................... 12
1.4 PI AS A CALCIUM GATE ............................................................................... 13
1.4.1 Calcium Requirement Of PI Response ......................................................... 16
1.4.2 Phosphatidic Acid As A Calcium Ionophore ............................................... 19
1.4.3 Is PI Degraded From Plasma Membrane? .................................................... 21
1.5 DIACYLGLYCEROL AS AN ACTIVATOR OF PROTEIN KINASE C ............ 22
1.6 PI AS A SOURCE OF ARACHIDONIC ACID .................................................. 24
1.7 INHIBITORS OF PI RESPONSE ..................................................................... 27
1.8 MUSCARINIC CHOLINERGIC RECEPTORS ............................................... 28
1.8.1 Cyclic Guanosine Monophosphate ............................................................... 29
1.8.2 Potassium Efflux .......................................................................................... 31
1.8.3 Muscarinic Acetylcholine Receptor And PI Response ............................... 32
1.8.4 Are There Subpopulations Of Muscarinic Acetylcholine Receptors? .......... 34
1.8.5 Subclassification Based On Antagonist Binding ......................................... 35
1.8.6 Receptor-Effecter Coupling ..................................................................... 37
1.9 MECHANISM OF THE NEGATIVE INOTROPIC EFFECT ........................... 39
1.10 ALPHA-ADRENERGIC RECEPTORS IN HEART ........................................ 43
1.10.1 Mechanism Of The Alpha- And Beta-Adrenergic Receptor-mediated Positive Inotropic Effect ............................................................... 45
1.11 MUSCARINIC RECEPTORS IN LONGITUDINAL SMOOTH MUSCLE OF GUINEA PIG ILEUM ................................................................. 47
1.12 OBJECTIVE ................................................................................................ 50

Chapter II
METHODS ............................................................................................................... 51
2.1 Choice Of Animal ............................................................................................ 51
2.2 Krebs-bicarbonate Buffer ............................................................................... 52
2.3 Phosphate Incorporation In Atria ................................................................... 52
2.4 Isolation Of Longitudinal Smooth Muscle Of Guinea Pig Ileum .................. 53
2.5 Label-incorporation In Guinea Pig Ileum ....................................................... 53
2.6 Extraction Of Lipid ......................................................................................... 54
2.7 Phospholipid Separation ................................................................................ 55
## Chapter III
### RESULTS

- 3.1 SEPARATION OF PHOSPHOLIPIDS
  - 3.1.1 Separation And Identification Of PI
  - 3.1.2 Phosphorus Determination
- 3.2 PHOSPHATE INCORPORATION IN RAT HEART
  - 3.2.1 $[^{32}P]$Phosphate Incorporation In Rat Atria Versus Ventricle
  - 3.2.2 Effect Of Muscarinic And Alpha-adrenergic Receptor Stimulation On $[^{32}P]$phosphate Incorporation In Combined Atria
  - 3.2.3 Effect Of Muscarinic And Alpha-adrenergic Receptor Stimulation On $[^{32}P]$phosphate Incorporation In Separate Left And Right Atria
- 3.3 PI BREAKDOWN IN HEART
  - 3.3.1 Breakdown Of Phospholipids Prelabeled With Radiolabel Phosphate
  - 3.3.2 Breakdown Of PI Prelabeled With Arachidonate
  - 3.3.3 Diacylglycerol Accumulation
  - 3.3.4 Fatty Acid Composition Of The Polar Lipid Fraction In Atria And Ventricles
- 3.4 PI RESPONSE IN GUINEA PIG ILEUM
  - 3.4.1 $[^{32}P]$Phosphate Incorporation
  - 3.4.2 $[^{3}H]$lnositol Incorporation
  - 3.4.3 Lithium-amplified Accumulation Of Inositol Phosphates
  - 3.4.4 Identification Of Inositol Phosphates
- 3.5 EFFECT OF PMSF ON PI TURNOVER AND CONTRACTION IN GUINEA PIG ILEUM
  - 3.5.1 Effect Of PMSF On PI Turnover
  - 3.5.2 Effect Of PMSF On Contraction
  - 3.5.3 Effect Of PMSF On Inositol-Phosphate Accumulation

## Chapter IV
### DISCUSSION

- 4.1 IS MUSCARINIC RECEPTOR STIMULATION IN THE HEART ACCOMPANIED BY A PI RESPONSE?
  - 4.1.1 Phospholipid Separation And Technique Verification
  - 4.1.2 Basal $[^{32}P]$phosphate Incorporation In Heart
4.1.3 Effect Of Muscarinic And Alpha-Adrenergic Receptor Stimulation On PI Turnover In Rat Atria ..........126
4.2 PI BREAKDOWN STUDIES ........................................127
4.2.1 Breakdown Of Prelabeled PI .................................127
4.2.2 Arachidonyl-Enriched Diacylglycerol .........................129
4.2.3 $[^{32}P]$Phosphate Incorporation In Rat Left And Right Atria ........................................132
4.2.4 Evidence For Two Subpopulations Of Muscarinic Receptors ........................................133
4.3 ROLE OF PI TURNOVER IN MUSCARINIC RECEPTOR STIMULATED CALCIUM MOBILIZATION IN GUINEA PIG ILEUM ........................................138
4.3.1 Incorporation Of $[^{3}H]$inositol Into PI ......................139
4.3.2 Effect Of PMSF On Carbachol And $K^+$-stimulated PI Response And Contraction ......................139
4.3.3 Is PMSF Inhibition Of Carbachol-stimulated PI Response Mediated By Inhibition Of PI-specific Phospholipase C ? ......................141
4.3.4 Lithium-Amplification Of PI Response In Guinea Pig Ileum ........................................143
4.3.5 Analysis Of Inositol Phosphates Accumulated During Lithium Amplification Of Carbachol-stimulated PI Response In Guinea Pig Ileum ..............145
4.3.6 Effect Of PMSF On Carbachol-stimulated Inositol Phosphate Accumulation ......................147

Chapter V
SUMMARY ............................................................150

BIBLIOGRAPHY ......................................................154
**List of Tables**

I. Phospholipid distribution and $[^{32}\text{P}]$phosphate label incorporation into phospholipids of rat atria ...... 82

II. Effect of carbachol stimulation on arachidonyl content of diacylglycerol. ........................................ 101

III. Effect of carbachol on $[^{3}\text{H}]$inositol incorporation into phospholipid. .................................. 108

IV. Carbachol-stimulated accumulation of inositol phosphate. ....................................................... 109

V. Composition of inositol phosphates accumulated in guinea pig ileum. ........................................... 116

VI. Effect of PMSF on the accumulation of carbachol-stimulated inositol phosphates in guinea pig ileum. ................................................................. 123
List of Figures

1. PI turnover ...............................................................5
2. PI as a calcium gate ..................................................15
3. Pirenzepine Structure ................................................36
4. Phospholipid separation .............................................80
5. Standard curve for phosphorus. ..............................83
6. Comparison of the basal phosphate incorporation into various phospholipids in atria and ventricles of rat heart. .......................................................85
7. Effect of carbachol and methoxamine on $[^{32}P]$ incorporation into PI in combined rat atria. ........87
8. Effect of methoxamine stimulation on $[^{32}P]$ phosphate incorporation into various phospholipids in combined rat atria. .......................................89
9. Effect of methoxamine stimulation on $[^{32}P]$phosphate incorporation into phosphatidylinositol of separate rat left and right atria. .................................91
10. Effect of carbachol on $[^{32}P]$phosphate incorporation into phosphatidylinositol of separate rat left and right atria. ......................................93
11. Effect of carbachol on phospholipids prelabeled with $[^{32}P]$phosphate in combined rat atria. ........95
12. Effect of carbachol on phosphatidylinositol prelabeled with arachidonic acid in rat atria. ............97
13. Separation of diacylglycerol. ....................................99
14. Gas chromatogram of fatty acid methyl esters of diacylglycerol. ........................................102
15. Fatty acid composition of the polar lipid fraction of rat atria and ventricles. .........................104
16. Effect of carbachol on $[^{32}P]$phosphate incorporation into phosphatidylinositol of longitudinal smooth muscle of guinea pig ileum. .............................106
17. Dose-response curve for carbachol-stimulated inositol phosphate accumulation in guinea pig ileum. ....110
18. Elution profile of standard inositol mono-phosphate. 112
19. Elution profile of the accumulated inositol phosphates in guinea pig ileum. 114
20. Time course of the effect of PMSF on basal $[^3H]$inositol incorporation into phospholipid in guinea pig ileum. 117
22. Effect of PMSF on carbachol- and $K^+$-stimulated contraction of guinea pig ileum 121
List of Abbreviations

ACh  acetylcholine
nAChR  nicotinic acetylcholine receptor
mAChr  muscarinic acetylcholine receptor
ACS  aqueous counting scintillant
cAMP  cyclic adenosine 3',5'-monophosphate
°C  degree centigrade
Ci  Curie
mCi  millicurie
μCi  microcurie
cm  centimeter
cpm  counts per minute
DAG  1-oleyl-2-acetyl-diacylglycerol
D-600  methoxy verapamil
dpm  disintegrations per minute
fmole  femto mole
cGMP  cyclic guanosine 3',5'-monophosphate
GTP  guanosine triphosphate
g  gravitational force
g  gram
mg  milligram
μg  microgram
cGMP  cyclic guanosine 3',5'-monophosphate
GTP  guanosine triphosphate
h  hour
IP  inositol mono-phosphate
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>IP₂</td>
<td>inositol di-phosphate</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol tri-phosphate</td>
</tr>
<tr>
<td>K</td>
<td>thousand</td>
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<td>ml</td>
<td>milliliter</td>
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<td>μl</td>
<td>microliter</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>QNB</td>
<td>quinuclidinyl benzilate</td>
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<tr>
<td>msec</td>
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</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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To my parents
I. INTRODUCTION

It is now well established that receptors at the cell surface enable the cell to recognize incoming signals, but less is known about how this information is transferred to the inside of the cell. Two important secondary messengers, which assist to transform the incoming message into cellular language, are cAMP (Sutherland and Rall, 1960) and calcium (Rasmussen, 1970).

According to the Singer and Nicolson model, the biological membrane is composed of proteins "floating" in a lipid bilayer (Singer and Nicolson, 1972). Phospholipids constitute the major lipid fraction of the plasma membrane in various cells. Both the phospholipid head group and the fatty acid chains have been shown to play an important role in various membrane characteristics (Singer, 1974; McMurchie and Raison, 1979; Sandermann, 1978), such as permeability of ions, fluidity of the membrane and activity of membrane bound enzymes. Therefore, the suggestion that membrane components (including lipid) may play a role in transmitting signals across the biological membrane appears quite rational.

Receptors which employ cAMP as a second messenger have been shown to be coupled to adenylate cyclase present on the cytoplasmic side of the membrane, through a GTP binding protein (Levitzki and Helmreich, 1979; Rodbell, 1980). Recently, a possible involvement of phospholipid in the coupling between receptor and adenylate cyclase has also been postulated (Hirata and Axelrod, 1980).

One may ask if phospholipids also play a role in
translating the incoming information in systems which do not employ cAMP as second messenger. One possible candidate for such a role is phosphatidylinositol (PI) (Michell, 1975; Jones et al., 1982). PI contains myo-inositol, an essential growth factor, in the head group, and the β fatty acyl chain of mammalian PI is usually rich in arachidonic acid, a rate-limiting precursor in the synthesis of prostaglandins. PI, along with its two phosphorylated derivatives, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-bis phosphate (PIP$_2$), constitute about 2-12% of the total phospholipid content in various tissues (Rouser et al., 1968; Galliard, 1973; White, 1973), and in most tissues the concentration of PI is much greater than its phosphorylated derivatives. Even though they constitute a very small fraction of the total phospholipid, all three inositol phospholipids undergo a very rapid metabolic turnover (Griffin et al., 1979).

Hokin and Hokin first observed in 1953 that stimulation of pigeon pancreas by pancreozymin or carbamylcholine produced an enhanced incorporation of labeled phosphate into PI, in addition to causing an increased amylase secretion (Hokin and Hokin, 1953). In the following years various other stimuli in different tissues were found to cause an enhanced incorporation of labeled phosphate into PI (Hokin, 1968, 1969; Michell, 1975), similar to that observed by Hokin and Hokin (1953) in pancreas. In the early seventies, a trend began to evolve in the long list of stimuli showing PI turnover. Firstly, the response seemed to be associated only with those ligands that interact with cell
surface receptors and secondly, only with those receptors that did not employ cAMP as their second messenger (Michell, 1975). In the past decade, many groups have been investigating the possibility that PI turnover may play a role in receptor-mediated signal transmission.

1.1 DISTRIBUTION OF PI

If inositol lipids have a role in receptor-mediated signal transmission they must be located in the plasma membrane. PI, the major component of inositol lipid, makes up about 2-10% of the total phospholipid content in the cell. The majority of this PI is present in various intracellular organelles, including endoplasmic reticulum, outer mitochondrial membrane and nuclear membrane, and a small amount has been shown to be present in the plasma membrane (Hokin-Neaverson, 1977; Kirk et al., 1981).

In contrast to PI, PIP and PIP₂ are considered to be present mostly in the plasma membrane, based principally on studies done on myelin (Soukup et al., 1978) and erythrocyte membranes (Buckley and Hawthorne, 1972). The labile nature of these polyphosphoinositides makes their quantitative estimation quite difficult. The most reliable values were obtained from the studies using microwave irradiation techniques (Soukup et al., 1978). In three different regions of brain, Soukup et al. (1978) reported values of 0.1 - 0.13 μmole PIP and 0.29 - 0.48 μmole of PIP₂ per gram of tissue. The phospholipid acting as a link between the receptor and the inside of the cell should
preferably be located in the cytoplasmic side of the plasma membrane bilayer. There is no direct evidence to suggest that inositol lipid in the plasma membrane is located in the cytoplasmic side, but the indirect evidence indicating that the ATP pool of cytosol can provide phosphate for phosphorylation of PI, suggests that inositol lipids may be located on the cytoplasmic side of the membrane. Downes and Michell (1982) have calculated that even when PIP and PIP₂ constitute only trace quantities of the total lipid fraction, the polyphosphoinositide concentration in the inner leaflet of the myelin bilayer could be as high as 5 mM.

1.2 **PI RESPONSE**

1.2.1 **Closed Cycle**

The increased incorporation of labeled phosphate into PI observed on stimulation of various receptors (PI response) could occur either by an increase in de novo synthesis of PI or by increased turnover of the PI head group. Evidence for the latter mechanism was first provided by Hokin and Hokin (1958); they found that in cerebral cortex enhanced incorporation of labeled phosphate and inositol was not accompanied by an increased incorporation of labeled glycerol. This indicated that the diacylglycerol moiety may be reutilised while the phosphoinositol head group was renewed. Direct measurement of PI content also did not show an increase in the stimulated tissue, further supporting the above mechanism. This mechanism for PI response was reconfirmed in many other tissues by the
Hokins, including sympathetic ganglia (Hokin, 1969), pancreas (Hokin, 1968), adrenal medulla (Hokin et al., 1958), salivary gland (Hokin and Sherwin, 1957), and later verified by many other groups. One well known exception to the above mechanism of phosphate incorporation is the adrenal cortex, where ACTH has been shown to provoke a two-fold increase in the adrenal contents of phosphatidic acid, PI, PIP, PIP$_2$, phosphatidylglycerol and diacylglycerol (Farese et al., 1979, 1980). This effect of ACTH on the phospholipid metabolism of adrenal cortex is quite different from the classical PI response.

The general mechanism for PI turnover is shown in Fig 1. Receptor stimulation by agonists causes the activation of PI-specific phospholipase C, resulting in the breakdown of PI to diacylglycerol and inositol phosphate. Diacylglycerol is phosphorylated by ATP to give phosphatidic acid, which further reacts with cytidine triphosphate and inositol to resynthesise PI.

Figure 1 - PI turnover
Reproduced from Berridge, 1980
1.2.2 The Primary Event Of PI Response

Usually measured as an increase in the incorporation of radioactivity into phosphatidic acid and PI, it was apparent later that the primary event of PI response was an increased rate of breakdown of PI (or its phosphorylated derivatives). The first report of the lowering of PI concentration was by Hokin and Hokin (1964) in avian salt gland. But the generality of the above mechanism for PI response became apparent only a decade later, with the reports of Durell et al. (1969), Hokin-Neaverson, 1974; Jones and Michell (1974). Though still to be proven in many tissues where a PI response has been studied, work based on platelets (Bell and Majerus, 1980), pancreas (Bansback et al., 1974), blowfly salivary gland, brain and parotid gland (Berridge et al., 1982) suggested that the primary event of PI response was the increased breakdown of PI by phospholipase C, giving rise to diacylglycerol and inositol phosphate. Studies with iris muscle (Abdel-Latif et al., 1977, 1978; Akhtar and Abdel-Latif, 1980) and more recently with platelets (Agranoff et al., 1983; Bell and Majerus, 1980; Billah and Lapetina, 1982b; Imai et al., 1983) and hepatocytes (Michell et al., 1981) have suggested that polyphosphoinositide breakdown rather than PI breakdown may constitute the primary event (Downes and Michell, 1982), and the former may be the target of phosphodiesterase action.
Activation of Phospholipase D as a mechanism of PI breakdown on acetylcholine stimulation of pancreas was once proposed by Hokin-Neaverson et al., (1975). Their conclusions were based on the finding that they could detect only $[^3]$H]-inositol as the water-soluble product in acetylcholine (ACh)-stimulated tissue. It now appears that the above result could have been obtained as a result of hydrolysis of inositol phosphate(s) by inositol phosphatase. When lithium, an inhibitor of inositol phosphatase, was present during stimulation of a PI response, it resulted in a large accumulation of inositol monophosphate in insect salivary gland, rat brain and parotid gland (Berridge et al., 1982).

1.2.3 Alternate Mechanisms For Label Incorporation

Incorporation of label into PI can occur, and has been reported by mechanisms other than PI response. They are described briefly below. In studies employing labeled fatty acid, incorporation of label into PI can be affected by the action of phospholipase $A_2$. Deacylation followed by rapid reacylation with arachidonic acid has been observed in adrenocortical cells (Schrey and Rubin, 1979) and rabbit neutrophils (Rubin et al., 1981). Phospholipase $A_2$ action on PI can also decrease the PI content substantially (Rittenhouse-Simmons, 1981), but the above effect can be distinguished from phospholipase C action by measuring the level of lysophosphatidylinositol.

A second mechanism of increasing label into PI involves the
increased availability of diacylglycerol. It has been shown in certain tissues, such as platelets (Lapetina et al., 1981b; Prescott and Majerus, 1983) that the increase in diacylglycerol observed in the PI response was due to PI breakdown. Diacylglycerol can also be obtained from other sources e.g. triacylglycerol and other phospholipids. This can then enter the PI cycle, resulting in increased incorporation of label into PI. Only 20% of the increase in diacylglycerol level, obtained during the pilocarpine stimulation of pancreas, could be accounted for by increased breakdown of PI, while the rest was considered to arise from other sources (Bansback et al., 1974).

The third possible pathway for increasing label incorporation into PI involves increased de novo synthesis of phosphatidic acid from glycerol-3-phosphate and fatty acyl CoA, with subsequent conversion to PI. This may be distinguished from the cyclic PI turnover in a number of ways. Firstly, unlike the majority of the classical PI response, this mechanism will also show an increased incorporation of labeled glycerol into PI. Secondly, since phosphatidic acid is a key intermediate of phospholipid metabolism, de novo increase in the phosphatidic acid level will be reflected by increased incorporation of label into other phospholipids, as in ACTH action on adrenocortical cells (Farese et al., 1980).

The fourth possible, but highly unlikely, way of increasing inositol incorporation into PI could be the result of phospholipase D action on PI. There is no convincing evidence for the widespread occurrence of phospholipase D in mammalian
tissue.

In spite of its limitations, label incorporation into the PI head group is a convenient and sensitive indicator of PI response. In all tissues (except blowfly salivary gland (Berridge and Fain, 1979)) where PI response is now known to occur, enhanced incorporation of label into the phosphoinositol moiety of PI has been the first step in establishing the response.

1.2.4 PI-specific Phospholipase C

As mentioned in a previous section, the primary event of PI response is considered to be the enhanced breakdown of PI (Hokin and Hokin, 1964; Durell et al., 1969; Hokin-Neaverson 1974; Jones and Michell, 1974; Michell, 1975) or its phosphorylated derivatives, PIP and PIP$_2$ (Abdel Latif et al., 1977, 1978; Akhtar and Abdel-Latif, 1980; Agranoff et al., 1983; Billah and Lapetina, 1982b; Imai et al., 1983; Michell et al., 1981) by the action of phospholipase C or polyphosphoinositide phosphodiesterase, respectively, which hydrolys the glycerophosphate bond of the phospholipid. PI-specific phospholipase C has been shown to be present in various animal tissues such as pancreas (Dawson, 1959), liver (Kemp et al., 1961), guinea pig intestinal mucosa (Atherton and Hawthorne, 1968), among others, as well as in certain microorganisms and plants (for a complete list of sources from which the enzyme has been characterized see Shukla, 1982). Most of the PI-specific phospholipase C exists in the soluble form in the cytoplasm of
various tissues, and hydrolyses PI in the presence of calcium (Allan and Michell, 1974; Atherton and Hawthorne, 1968; Dawson, 1959; Kemp et al., 1961; Thompson, 1967). A membrane bound form of the enzyme, that could play an important role in PI turnover, and which differed from soluble cytoplasmic phospholipase C, was described by Friedel et al. (1969) and Lapetina and Michell (1973). However, Irvine and Dawson (1978) later provided evidence, based on pH dependence of deoxycholate activation and the use of lactate dehydrogenase as a cytoplasmic marker, that the membrane bound activity described in brain (Friedel et al., 1969; Lapetina and Michell, 1973) could be accounted for by cytoplasmic contamination of the plasma membrane. Most of the available evidence so far indicates the presence of a Ca$^{2+}$-dependent soluble cytoplasmic enzyme and a Ca$^{2+}$-independent lysosomal phospholipase C.

The PI-specific phospholipase C enzyme from different sources showed some variation in its degree of specificity towards PI. The enzyme from guinea pig intestinal mucosa (Atherton and Hawthorne, 1968) and iris smooth muscle (Abdel-Latif et al., 1980) hydrolysed both PIP and PIP$_2$, whereas the enzyme from S.aureus hydrolyzed only PI and not PIP or PIP$_2$ (Shukla, 1982). Most of the specificity studies in vitro employ the optimal conditions for the hydrolysis of PI and therefore do not reflect the in vivo specificity of the enzyme. In rat liver and kidney, five different forms of the enzyme, differing in their isoelectric points, have been separated by column electrophoresis (Hirasawa et al., 1982). This heterogeneity of
the cytoplasmic enzyme raises new questions regarding the importance and function of these various forms of enzyme. It is quite possible that the assay conditions and isolation techniques used previously for characterization of this enzyme could have selectively favoured certain forms of the enzyme over others, and therefore caution should be exercised when comparing enzyme characteristics from different laboratories.

Hydrolysis of PI by this enzyme is increased by the presence of an optimal concentration of deoxycholate (Hofmann and Majerus, 1982; Irvine and Dawson, 1978). PI hydrolysis by phospholipase C, obtained from rat brain (Dawson et al., 1980) and sheep seminal vesicular glands (Hofmann and Majerus, 1982) was inhibited by phosphatidylcholine and positively charged proteins, which may account for the inability of phospholipase C to rapidly hydrolyse membrane bound PI in unstimulated preparations. Unsaturated phosphatidic acid and unsaturated fatty acids such as arachidonic acid (produced from diacylglycerol during the PI response), were found to be capable of stimulating the enzyme activity. This provides a possible self-amplification mechanism for the PI response (Dawson et al., 1980).

The hydrolysis of PIP$_2$ by extracts of rat brain was described as early as 1964 (Thompson and Dawson, 1964a), but PIP$_2$ phosphodiesterase has become more important with respect to PI response after recent suggestions that polyphosphoinositide hydrolysis, rather than PI hydrolysis, may be the triggering event for the PI response (Michell et al., 1981; Downes and
Michell, 1982). Data on the brain (Thompson and Dawson, 1964b) and erythrocyte (Downes and Michell, 1981) are consistent with one phosphodiesterase activity hydrolysing both PIP and PIP$_2$, without any effect on PI. Like PI-specific phospholipase C, this enzyme required calcium for its activity, and under certain assay conditions there seemed to be two levels of calcium requirement (Irvine, 1982).

1.3 ROLE OF PI TURNOVER

The widespread occurrence of the PI response has led to much speculation regarding its role. The first hypothesis regarding its involvement in exocytosis in exocrine gland (Hokin and Sherwin, 1957; Hokin et al., 1958) was found to be limited in scope, as PI response was later found to occur in both secretory and non-secretory cells. In the late sixties and early seventies, a role for polyphosphoinositides in the generation and propagation of action potentials in stimulated neurons was proposed (Dawson, 1966; Hendrickson and Reinertsen, 1971; Torda, 1972). The main feature of all the above proposals had been that PIP$_2$ has a much higher affinity for calcium than PIP (Hendrickson and Reinertsen, 1969) and therefore, their interconversion by phosphorylation-dephosphorylation might affect the amount of calcium bound to the membrane. Recognition that phospholipase C mediated breakdown of PI, rather than phosphorylation-dephosphorylation of polyphosphoinositides, was the primary event of PI response in many different systems led to reevaluation of the role of PI. Michell in 1975 (Michell,
1975), after reviewing the existing literature on PI response, concluded that the available evidence suggested a role for PI turnover in "calcium gating". In the past eight years, evidence has been presented both in favour (Berridge, 1980; Fain and Garcia-Sainz, 1980; Michell and Kirk, 1981; Putney, 1981, 1982) and against (Cockcroft, 1981; Hawthorne, 1982) the above hypothesis.

In the following pages this and two other hypotheses, which have been suggested for the physiological role of PI turnover will be analyzed. The other hypotheses are that PI turnover is a source of arachidonic acid and that PI turnover is a source of diacylglycerol, which is capable of transmitting the hormonal or neurotransmitter message across the cell membrane through activation of protein kinase C (PKC).

1.4 PI AS A CALCIUM GATE

The initial support for the hypothesis that PI functioned as a calcium gate was heavily dependent on the following observations (Michell, 1975):

a) Receptors whose stimulation was accompanied by a PI response mediated their response through calcium mobilization;
b) PI response was independent of extracellular calcium.

The correlation between stimuli producing a PI response and mediating their response through calcium mobilization has indeed been very good (Michell, 1975, 1979). If PI breakdown is only and universally involved in receptor mediated calcium gating, then a) it should be evoked in all cells and all tissues where
receptor stimulation leads to calcium mobilization and b) those receptors which employ other second messengers, such as cAMP, should not produce PI breakdown (Jones et al., 1982; Michell, 1975; 1979). Cell surface receptors, such as muscarinic cholinergic receptors in pancreas (Hokin and Hokin, 1953), parotid gland (Jones and Michell, 1974), vas deferens (Egawa et al., 1981), 5-hydroxytryptamine in blowfly salivary gland (Pain and Berridge, 1979b), alpha-adrenergic receptors in parotid gland (Michell and Jones, 1974), and heart (Gaut and Huggins, 1966) are just a few of many different cell systems where PI response is accompanied by Ca\(^{2+}\) mobilization (for a more comprehensive list see Michell 1975, Michell et al., 1981). One exception to this rule appears to be the PI response which accompanies the stimulation of pineal gland and sympathetic ganglia exposed to nerve growth factor (NGF) preparations (Lakshmanan, 1978, 1979). However, Michell et al. (1981) have provided arguments indicating that as the role of Ca\(^{2+}\) as a second messenger in the action of NGF is still not clear (Schubert et al., 1978; Landreth et al., 1980), the above exception should be treated with reservation. Another exception is adrenal cortex, where the nicotinic receptor mobilizes calcium but where it is muscarinic stimulation which produces the PI response. Only two stimuli, glucose and calcium ionophore (described in greater detail in the following section), enhance lipid labelling without acting at a cell surface receptor (Michell et al., 1981).
Figure 2 - PI as a calcium gate

Reproduced from Berridge, 1980

Three possible models for linking the PI response to calcium gating. The main components of the system (shown on the left) are a receptor (R), phospholipase C (Ph. C), a calcium gate and phosphatidylinositol (PI). The diagrams on the right illustrate three ways in which agonists might act to enhance calcium permeability. (a) The agonist occupies the receptor (R) which somehow activates phospholipase C (Ph. C) to hydrolyse PI to diglyceride (DG) and the gate opens. (b) The activated receptor acts directly on the gate. As the gate opens there are conformational changes in the surrounding bilayer which allows Ph. C to hydrolyse PI as part of a closing mechanism. (c) The activated receptor stimulates Ph. C to hydrolyse PI to DG. The latter is rapidly phosphorylated to phosphatidic acid (PA) which functions as a calcium ionophore.
1.4.1 Calcium Requirement Of PI Response

PI-specific phospholipase C requires micromolar calcium concentrations for its activity. The question is whether activation of PI-specific phospholipase C, which causes PI breakdown, occurs before or after calcium enters the cell. Many of the reported studies (Oron et al., 1975; Griffin et al., 1979; Trifaro, 1969b) have used labeled phosphate incorporation into phosphatidic acid and PI as a measure of PI response. Conclusions regarding the calcium requirement of PI response based on such studies (Griffin et al., 1979; Lennon and Steinberg, 1973) are difficult to interpret, as they do not indicate whether the primary event of PI breakdown is calcium-dependent or not.

A new dimension of complexity to the whole problem has been added by recent reports suggesting that there may be both calcium-dependent and calcium independent PI turnover (Egawa et al., 1981; Farese et al., 1982), as well as an additional mechanism for the loss of PI (Billah and Lapetina, 1982a). Two methods usually used to study the calcium dependence of PI breakdown are: a) addition of an ionophore and b) regulation of the intracellular calcium by means of calcium chelating agents. 

**Effect of ionophore**: Ionophores, such as A23187, increase the permeability of the cell to calcium, thereby increasing the cytosolic calcium concentration. The rationale behind such an experiment is that if PI breakdown is the cause rather than effect of calcium mobilization, the increase in cytosolic calcium with ionophore should not cause the PI breakdown. In
many tissues ionophore-mediated calcium increase failed to produce PI breakdown. Some of the examples are parotid gland (Jones and Michell, 1975), blowfly salivary gland (Fain and Berridge, 1977a) and hepatocytes (Billah and Michell, 1979). In pancreas and platelets, evidence has been presented both for (platelets - Billah and Lapetina, 1982a; pancreas - Hokin-Neaverson, 1977) and against (platelets - Imai and Nazawa, 1982; pancreas - Farese et al., 1980) a Ca\(^{2+}\) ionophore-mediated breakdown of PI, whereas in polymorphonuclear leukocytes (Cockcroft et al., 1981) and vas deferens (Egawa et al., 1981) a calcium-dependent PI breakdown has been reported. An ionophore-mediated PI breakdown has to satisfy the following requirements before a PI response can be considered as a calcium-mediated phenomenon; a) that the increase in cytosolic calcium concentration must be in the range obtained during receptor stimulation and b) the observed PI loss must occur by the same mechanism as suggested for the PI response. At present such data are available only for neutrophils (Cockcroft et al., 1981). Stimulation of neutrophils with fMet-Leu-Phe produced a half maximal secretion and PI breakdown (half maximal at 0.3 nM), which was calcium-dependent. Neutrophils stimulated with 1 nM fMet-Leu-Phe required 100 μM calcium to produce half maximal effects in both secretion and PI breakdown. The ionophore, ionomycin, produced neutrophil secretion and PI breakdown over a similar calcium concentration range, and ionomycin-stimulated PI breakdown was accompanied by increased phosphate incorporation into phosphatidic acid and PI, suggesting that the PI breakdown
was at least partially through activation of phospholipase C. However, the result does not completely negate the possibility of a receptor-mediated calcium-independent PI breakdown in neutrophils. Recent reports that in two tissues, rat submaxillary gland, (Farese et al., 1982) and rabbit vas deferens (Egawa et al., 1981), there is both calcium-dependent and calcium independent receptor-mediated PI turnover suggests that demonstration of calcium-dependent PI breakdown in a tissue is not enough to eliminate the occurrence of a receptor mediated calcium independent PI breakdown.

In contrast to PI, where receptor-mediated breakdown was found to be calcium independent in a large number of tissues (Billah and Michell, 1979; Fain and Berridge, 1979a; Jones and Michell, 1979), the breakdown of polyphosphoinositide, specifically PIP$_2$, by acetylcholine (ACh) was abolished in iris smooth muscle in a calcium deficient medium (Abdel-Latif et al., 1978) and enhanced by calcium ionophore in iris smooth muscle (Abdel-Latif et al., 1978), erythrocyte (Lang et al., 1977), synaptosomes (Griffin and Hawthorne, 1978) and brain (Jolles et al., 1981). The only exception to this was the rapid breakdown of PIP$_2$ observed in hepatocytes (Michell et al., 1981) and parotid gland (Weiss et al., 1982b), which occurred over a period of a few seconds and was unaffected (or slightly decreased in parotid gland) by calcium deprivation, and was also not observed with ionophore. Weiss et al. (1982b) have recently presented some very interesting and convincing data to suggest that polyphosphoinositide breakdown may be responsible
for calcium mobilization in the phasic response of parotid cells.

1.4.2 Phosphatidic Acid As A Calcium Ionophore

Diacylglycerol, obtained during the breakdown of inositol lipids (Igarashi and Kondo, 1980; Billah and Lapetina, 1982a), is rapidly converted to phosphatidic acid by diglyceride kinase, an enzyme also located in the plasma membrane (Lapetina and Hawthorne, 1971). Phosphatidic acid has been found to accumulate during PI response in many different systems, including salt gland, pancreas and platelets. After Michell's initial proposal (Michell, 1975) that the PI response may be involved in calcium gating, it was very natural to ask whether phosphatidic acid (accumulated during PI response) could act as a calcium gate. The initial hypothesis that phosphatidic acid accumulation may alter the physical properties of the membrane, thereby altering the hypothetical calcium gate (Keryer et al., 1979; Michell et al., 1977), was too undefined to be of practical use.

The second hypothesis, which was first proposed and rejected by Michell et al. (1977) based on their studies in red blood cells, was that phosphatidic acid functions as a calcium ionophore. Many different lines of available evidence tend to support such a role for phosphatidic acid. Phosphatidic acid has been shown to act as a calcium ionophore in the Pressman chamber (Tyson et al., 1976) and in liposomes (Serhan et al., 1981). It is interesting to note that while PI and arachidonic
acid failed to enhance calcium transport into liposomes, phosphatidic acid and 8,11,14-eicosatrienoic acid, two intermediates obtained during PI response, increased calcium uptake into the liposomes (Serhan et al., 1981). Phosphatidic acid translocated divalent cations with the following order of selectivity; Mn > Ca > Sr >> Mg. Liposomes with a phosphatidic acid concentration of 1-5 mole % of total lipid translocated calcium but not magnesium (Serhan et al., 1981). Non-bilayer formation in model membrane containing phosphatidic acid in the presence of calcium and other divalent cations (Verkleij et al., 1982) could explain the ionophoric property of phosphatidic acid (Cullis et al., 1980).

In parotid gland, a different line of evidence has been presented to support the role of phosphatidic acid as a calcium gate (Putney et al., 1980). Dissociation constants of various calcium antagonists (e.g. La³⁺, Tm³⁺, neomycin, Co²⁺, Ni²⁺, Mg²⁺), which inhibit receptor-stimulated calcium uptake by competing with calcium for the calcium channel during PI response, correlated very well with the inhibitory constants obtained for the same substances during the inhibition of phosphatidic acid-induced partitioning of ⁴⁵Ca from water into an organic phase (Putney et al., 1980).

The amphiphilic nature of phosphatidic acid makes the incorporation of exogenously added phosphatidic acid into membrane somewhat difficult. Even then, there have been some studies indicating that exogenously added phosphatidic acid was able to mimic the effect of calcium-mobilizing agonists. In
neuroblastoma cells (Ohsako and Deguchi, 1981) addition of as little as 1 µg/ml of phosphatidic acid exogenously, or production of phosphatidic acid in situ by phospholipase C treatment, caused an increase in the level of cGMP by activation of (calcium-stimulated) guanylate cyclase. Other systems include smooth muscle, platelet microsomes (Gerrard et al., 1978) and synaptosomes (Harris et al., 1981). On the other hand, exogeneously added phosphatidic acid has no effect on platelet aggregation (Gerrard et al., 1978) or calcium-dependent potassium release (Putney, 1981).

1.4.3 Is PI Degraded From Plasma Membrane?

One of the problems in accepting PI breakdown as a calcium gating hypothesis is the lack of evidence to suggest that the PI being degraded comes from the plasma membrane. Attempts to determine the site of loss of PI in stimulated cells have given variable results. In pancreas and sympathetic ganglia, based on differential centrifugation (Redman and Hokin, 1959; Gerber et al., 1973) and autoradiography (Hokin and Huebner, 1967; Hokin 1965), the site of PI turnover was found to be endoplasmic reticulum. The loss appeared to be from secretory vesicle membranes in electrically stimulated synaptosomes (Pickard and Hawthorne, 1978) and glucose-stimulated islet of Langerhans (Clements et al., 1977), from plasma membrane in acetylcholine-stimulated salt gland (Hokin-Neaverson, 1977) and fMeth-Leu-Phe-stimulated neutrophils (Bennett et al., 1982). By contrast, the site of PI loss could not be located in vasopressin-stimulated
hepatocytes and this was attributed to the rapid equilibration of PI among various subcellular pools (Kirk et al., 1981).

If the primary event of the PI response is the breakdown of polyphosphoinositides, as suggested by Michell and coworkers recently (Michell et al., 1981; Downes and Michell, 1982), then most probably the primary event of inositol lipid breakdown does occur at the plasma membrane, as polyphosphoinositides have been shown to be present only in the plasma membrane.

1.5 DIACYLGLYCEROL AS AN ACTIVATOR OF PROTEIN KINASE C

Phospholipid-dependent calcium-activated protein kinase C (PKC), which was first described by Nishizuka's group (Takai et al., 1979a) has now been shown to occur in neutrophils (Helfman et al., 1983), smooth muscle (Endo et al., 1982), lymphocytes (Ku et al., 1981), rat pancreas (Tanigawa et al., 1982) and many other tissues (Minakuchi et al., 1981). An involvement of PI turnover in protein kinase C activation was first postulated when it was observed that the presence of unsaturated diacylglycerol in the incubation medium shifted the affinity of the enzyme for calcium from the mM to the μM range (Kishimoto et al., 1980; Takai et al., 1979b). This shift in calcium affinity was greatest with diacylglycerol containing unsaturated fatty acid in the β carbon (the β carbon of PI is also enriched in polyunsaturated fatty acid), whereas the saturated diacylglycerols were found to be completely ineffective (Kishimoto et al., 1980). Although phosphatidylserine was essential for the PKC activity, other phospholipids were capable
of further modulation of its activity in the presence of phosphatidylserine (Kaibuchi et al., 1981). While phosphatidylethanolamine (20 μg/ml) increased the PKC activity by 50%, PI and phosphatidic acid (two other intermediates of PI response) had no effect, and phosphatidylcholine and sphingomyelin markedly diminished the enzyme activity (Kaibuchi et al., 1981).

Further evidence for the involvement of PI turnover in PKC activation was provided recently in platelets (Ieyasu et al., 1982; Kaibuchi et al., 1982b). Activation of platelets with thrombin, collagen or platelet activating factor (all three produced a PI response), caused a transient increase in diacylglycerol level, followed by a simultaneous phosphorylation of a 40,000 molecular weight (40K) protein and serotonin release (Ieyasu et al., 1982; Kaibuchi et al., 1982b). The same 40K protein was also phosphorylated during direct activation of platelets with 1-oleyl-2-acetyldiacylglycerol (DAG) (Mori et al., 1982; Kaibuchi et al., 1983). This phosphorylation of the 40K protein by DAG was not accompanied by PI breakdown or serotonin release, and therefore was considered to occur by direct activation of PKC by the synthetic diacylglycerol (Mori et al., 1982). Initially it was proposed that as diacylglycerol can cause a shift in the affinity of PKC to the micromolar level of calcium, diacylglycerol formed during PI response could mediate physiological responses without causing calcium influx (Limas, 1980; Kishimoto et al., 1980). Recent experiments clearly indicate, however, that at least in platelets, both
calcium mobilization and diacylglycerol formation are required for complete physiological response (i.e. serotonin release) (Kaibuchi et al., 1982b, 1983)

In model systems containing phosphatidylcholine/phosphatidylserine mixtures, diacylglycerol (from yeast) shifted the phase separation curve to a lower calcium concentration, causing 75% phase separation at 10 μM calcium. In comparison, PI from yeast produced only 7% phase separation at the same calcium concentration (Ohki et al., 1981). Diacylglycerol-mediated phase separation of phosphatidylserine may indicate a possible mechanism for the activation of PKC (Ohki et al., 1981). Most of the work done in this field is from Nishizuka's group, and the observations are consistent with the hypothesis that PI turnover may be involved in the activation of PKC.

1.6 PI AS A SOURCE OF ARACHIDONIC ACID

The β carbon of PI from various mammalian tissues is rich in arachidonic acid (5,8,11,14-icosatetraenoic acid; White, 1973), and it is now well established that arachidonic acid is the rate limiting factor in the formation of eicosanoids (Bergstrom et al., 1964; van Dorp et al., 1964). Therefore, it is logical to postulate that PI turnover may be involved in the production of "free" arachidonic acid, which can then mediate the physiological response through its conversion to eicosanoids (Lapetina et al., 1981a; Marshall et al., 1980, 1982). PI can generate free arachidonic acid by two mechanisms: a) by the direct action of phospholipase A₂ on PI (Hong and Deykin, 1981;
Rubin et al., 1981; Walsh et al., 1981), b) during PI response, by the action of diacylglycerol lipase on diacylglycerol; monoacylglycerol lipase on monoacylglycerol (Prescott and Majerus, 1983) or phospholipase A₂ action on phosphatidic acid (Lapetina et al., 1981b). If the arachidonyl group is hydrolyzed during PI turnover, then it would not be conserved during PI response, as was originally proposed. Initial observations, based on the incorporation of labeled glycerol, phosphate and inositol during PI response concluded that while the phosphoinositol moiety underwent renewal the diacylglycerol moiety was conserved. At least in platelets, the fatty acid acyl chain was not conserved during thrombin-stimulated PI turnover (Prescott and Majerus, 1981). Comparison of the fatty acid composition of PI in human platelets before and after thrombin stimulation (and even after recovery of PI to original levels) indicated that the latter contained a greater amount of oleate and linoleate, and a lower amount of arachidonate (Prescott and Majerus, 1981). This suggested that a) arachidonic acid was not being conserved during thrombin-induced PI turnover and b) PI was not being resynthesized with the characteristic of 1-stearoyl-2-arachidonyl fatty acid composition, which may finally be produced by a deacylation-reatcylation cycle.

Stimulation of arachidonyl-prelabeled thyroid follicles with thyrotropin produced a rapid and transient accumulation of labeled diacylglycerol (Igarashi and Kondo, 1980). It was further proposed (but no evidence was presented), that this
diacylglycerol could be acted upon by diacylglycerol lipase present in the thyroid plasma membrane (Igarshi and Kondo, 1980) to liberate free arachidonic acid, thereby resulting in transient synthesis of prostaglandin. In mouse pancreas, Marshall et al. (1980, 1982) have presented evidence to show that prostaglandins derived from arachidonyl residues during PI breakdown can cause amylase release. Only half of the radioactivity in arachidonyl-prelabeled PI lost during carbachol stimulation was recovered in phosphatidic acid and the other half was found in arachidonic acid metabolites. This suggested only that the arachidonic acid used in prostaglandin synthesis was derived from PI, but does not necessarily indicate that it was obtained during PI turnover (i.e. from diacylglycerol or phosphatidic acid), as implied by the authors. An alternate possibility which will give a similar result is that of a direct action of phospholipase A$_2$ on PI, recently suggested to occur in many tissues (Billah and Lapetina, 1982a; Hong and Deykin, 1981; Schwartzman et al., 1981; Walsh et al., 1981).

On the other hand, Litosch et al. (1982) have made the probably incorrect assumption that the decrease of arachidonyl-prelabeled PI on 5-hydroxytryptamine stimulation of blowfly salivary gland should necessarily result in release of free arachidonic acid. 5-Hydroxytryptamine stimulation of blowfly salivary gland caused a 54% decrease in both arachidonyl or phosphate-prelabeled PI, accompanied by salivary gland secretion (Litosch et al., 1982). The decrease in arachidonyl-prelabeled PI can be simply explained by its conversion to diacylglycerol,
as phosphate-prelabeled PI decreased by the same extent, rather than by the release of free arachidonic acid. It is possible that arachidonic acid was being conserved and not released during 5-hydroxytryptamine-stimulated PI turnover in blowfly salivary gland, and in that case it would not be surprising that the authors could not see any of the 5-hydroxytryptamine responses, such as calcium uptake or salivary gland secretion, with exogeneously added arachidonic acid.

1.7 INHIBITORS OF PI RESPONSE

One main hurdle in solving the PI "puzzle" is the lack of a specific inhibitor of PI turnover. Walenga et al. (1980) found that serine protease inhibitors, such as dansyl fluoride, phenylmethanesulfonyl fluoride (PMSF), 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate and p-nitrophenylantranilic acid, inhibited stimulus-induced mobilization of arachidonic acid. Walenga et al. (1980) suggested that this effect of serine esterase inhibitor was at least partly due to the inhibition of PI-specific phospholipase C, which would result in a decrease in the level of diacylglycerol, the substrate for diacylglycerol lipase and a proposed source of arachidonic acid (Igarashi and Kondo, 1980; Marshall et al., 1980, 1982). Serine esterase inhibitors (mentioned above) inhibited the production of malondialdehyde, a metabolic product of prostaglandin in platelets, in the presence of various stimuli i.e. thrombin, collagen and papain; but they had no effect on malondialdehyde production obtained with exogeneously added arachidonic acid.
(Walenga et al., 1980). Both PMSF and 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate inhibited the collagen- or papain-stimulated mobilization of arachidonic acid from PI and phosphatidylcholine, as well as formation of phosphatidic acid from PI, and showed a dose-dependent inhibition of PI-specific phospholipase C from platelets (Walenga et al., 1980). In contrast to the above findings, Downes and Michell (1981) failed to observe any effect of PMSF on polyphosphoinositide phosphodiesterase in erythrocytes.

1.8 MUSCARINIC CHOLINERGIC RECEPTORS

Cholinergic receptors have long been classified into two major categories, nicotinic and muscarinic. Nicotinic cholinergic responses can be mimicked by nicotine, are inhibited by d-tubocurarine and have a very fast onset (latency < msec) and short duration of response (30-100 msec)(for review see Adams, 1981). Nicotinic acetylcholine receptor (nAChR) stimulation brings about this response by a nonselective increase in permeability to monovalent cations, resulting in membrane depolarization. The single molecule of nAChR has been suggested to contain both the acetylcholine (ACh) binding site and the ionic channel (Raftery et al., 1980), because the ionic permeability of artificial membrane bilayers containing nAChR could be regulated by ACh (Nelso et al., 1980). In contrast, muscarinic acetylcholine receptor (mAChR)(which will be dealt with in greater detail) can be stimulated specifically with muscarine or acetyl-β-methacholine, and inhibited by the
classical muscarinic antagonist, atropine. Muscarinic responses are slow in onset (latency > 100 msec) and longer in duration (0.5 sec) (for review - see Heilbronn and Bartfai, 1978; Wastek and Yamamura, 1981).

The slow onset of the muscarinic response is explained by the following two hypotheses: First, a subunit model proposed by Kehoe and Marty (1980), postulates that several ACh receptor complexes have to interact before opening of the channel can take place. Second, the second messenger model hypothesizes that muscarinic activation leads to the formation of an intracellular messenger (i.e. cGMP, PI turnover), which is somehow responsible for the channel opening.

The following three biochemical responses usually accompany the stimulation of muscarinic receptors in whole cells:

i) increase in intracellular levels of cGMP

ii) efflux of potassium

iii) increased turnover of PI

All the above responses, in various systems, have been suggested to play a role in the muscarinic stimulus-response coupling.

1.8.1 Cyclic Guanosine Monophosphate

Stimulation of muscarinic receptors in many tissues leads to the formation of cGMP (for a list of tissues showing increases in cGMP levels see Heilbronn and Bartfai, 1978). A very large increase (200-fold) in the level of cGMP on muscarinic stimulation in neuroblastoma cells (Matsuzawa and Nirenberg, 1975; Richelson et al., 1978) makes this an
appropriate system to characterize various aspects of this muscarinic response. In the neuroblastoma cell, the cGMP response has been shown to be rapid and transient (Richelson, 1977) (peaks at 30 sec and returns to basal level in 2-3 min) and calcium- (Richelson et al., 1978) and temperature-dependent (El-Fakahany and Richelson, 1980). In these cells, exogeneously added cGMP produced hyperpolarization (Wastek et al., 1981) similar to that obtained with muscarinic stimulation. Though still highly debatable, this along with some other evidence in the literature (see also the section on "cGMP in negative inotropy") suggested a role for cGMP in the muscarinic receptor-mediated response. However, the calcium requirement for guanylate cyclase activation in various systems (Richelson et al., 1978; Study et al., 1978; Murad et al., 1979) clearly indicates that this is the result, rather than the cause, of calcium mobilization.

Mostly present in soluble form, some guanylate cyclase has also been shown to be present in the particulate fraction (Garbers et al., 1978; Sulakhe et al., 1976). All attempts, with a few exceptions, to show a direct coupling of mAChR to guanylate cyclase similar to that of hormone sensitive adenylate cyclase in broken cell preparations have been unsuccessful (Limbird and Lefkowitz, 1975; Murad et al., 1979). One exception is rat liver plasma membrane, where stimulation with $10^{-9}$ M carbachol or $10^{-7}$ M ACh caused a 2-4 fold increase in cGMP, which was atropine-sensitive (deBecemberg et al., 1982). Unlike cGMP responses in intact cells, the increase in cGMP
levels with the rat liver plasma membrane was observed over a very narrow range of carbachol and ACh concentration. Of various agents which can cause an increase in cGMP, only activation of guanylate cyclase by choline esters in cell free preparations (deBecemberg et al., 1982; George et al., 1975; Howell and Montague, 1974) are not readily explained by the presence of other guanylate cyclase activators. Other regulators of this enzyme activity, which could be physiologically important, are: Ca^{2+}-dependent proteases (Lacombe et al., 1980), unsaturated fatty acids (Glass et al., 1977; Garbers et al., 1978; Spies et al., 1980), fatty acid hydroperoxides and prostaglandins (Goldberg et al., 1978; Hidaka and Asano, 1977) and lysolecithin (Shier et al., 1976; Zwiller et al., 1976). It is interesting to note that at least two of the guanylate cyclase activators, fatty acids in platelets (Lapetina et al., 1981a, 1981b; Prescott and Majerus, 1983) and prostaglandins in pancreas (Marshall et al., 1980, 1982) are generated during PI turnover.

1.8.2 Potassium Efflux

Stimulation of muscarinic receptors in tissues preloaded with K^+ caused an increased efflux of K^+ from smooth muscle fragments (Burgen and Spero, 1968), cardiac muscle cells (Galper et al., 1982), and rat parotid cells (Putney et al., 1980), among others. In tissues such as heart, enhanced K^+ efflux caused rapid hyperpolarization, thereby reducing the duration of the action potential and causing inhibition of the slow inward
calcium current (Ten Eick et al., 1976). This suggests a role for K⁺ efflux in the muscarinic response. How muscarinic stimulation alters K⁺ permeability (as the K⁺ channel is still not considered to be a part of the mAChR) or the function of K⁺ efflux in tissues where muscarinic stimulation mediates calcium mobilization, is still not clear.

1.8.3 Muscarinic Acetylcholine Receptor And PI Response

The first observation on PI turnover was made following stimulation of the muscarinic receptors in pigeon pancreas (Hokin and Hokin, 1953). Muscarinic cholinergic receptors are the most widely studied with respect to PI response (Michell, 1975, 1979), and the stimulation of muscarinic receptor in many diverse tissues such as cerebral cortex (Hokin, 1969; Lapetina and Michell, 1974), adrenal medulla (Hokin et al., 1958; Trifaro, 1969a, 1969b), guinea pig ileum (Jafferji and Michell, 1976a), among many others (for a comprehensive list see Michell, 1975), are found to be associated with enhanced PI turnover. Almost all of the muscarinic responses investigated until the present study have at least one thing in common; they are all mediated by calcium mobilization. The correlation between muscarinic stimulation and PI response was so good that it led to the proposal that PI breakdown may be intrinsic to muscarinic receptor stimulation (Michell et al., 1976). Presynaptic mAChR of synaptosomes was the only exception, since muscarinic stimulation produced a PI response (Griffin et al., 1979), whereas calcium mobilization was not considered to mediate the
receptor response. The mAChR in heart (see the section on "mechanism of negative inotropy") and vascular smooth muscle i.e. femoral artery (DeMey and Vanhoutte, 1980) are other examples where receptor stimulation does not lead to calcium mobilization. One way to test whether PI breakdown (Michell et al., 1976) accompanying mAChR stimulation is an intrinsic characteristic of the receptor whose function is unknown, or whether it accompanies only those muscarinic receptor stimulations which mediate their effect by calcium mobilization, is to study the effect of muscarinic stimulation in a tissue where the final effect is not mediated through calcium mobilization. Such a study is presented in this thesis.

Various characteristics of PI turnover in response to stimulation of receptors have been discussed in detail in the earlier sections. PI response accompanying mAChR stimulation follows the same general pattern as for the other receptors. The concentration of cholinergic agonist required to produce the PI response in most tissues appeared to be several orders of magnitude higher than that required to produce the physiological response (Jafferji and Michell, 1976a; Michell et al., 1976). Therefore, the PI response was initially considered to be of no physiological significance. Jafferji and Michell (1976a) observed that the dose-response curve of PI response, obtained during carbachol stimulation of mAChR in longitudinal smooth muscle of guinea pig ileum, was similar to the receptor occupation curve for carbachol. The lack of spare receptors for PI response (while the presence of spare receptors has been
shown for the physiological response i.e. contraction (Yoshida et al., 1979), suggested that in the chain of events leading from receptor occupation to physiological response, PI breakdown may lie closer to the receptor occupation (Jafferji and Michell, 1976a). It was recently reported that in neutrophils, where there are no spare receptors for fMet-Leu-Phe-induced lysosomal enzyme secretion, the half maximal concentration for the PI response and the physiological response were similar (Cockcroft et al., 1981).

1.8.4 Are There Subpopulations Of Muscarinic Acetylcholine Receptors?

Based on the nature of the criterion used, either single (Birdsall, 1977; Venter, 1983; Wells et al., 1977) or multiple subtypes (Ehlert et al., 1982; Hammer et al., 1980; Rossini, 1981) of muscarinic receptors have been proposed. In solutions of physiological ionic strength, muscarinic antagonists bind to a uniform population of receptor sites on the membrane (Birdsall, 1977; Wells et al., 1977), except in pituitary cells where two affinity sites for antagonist binding sites have been reported (Mukherjee et al., 1980). By contrast, under similar conditions muscarinic agonists exhibit a "heterogeneity" in binding to the same population of receptors (Burgen et al., 1974; Strange et al., 1977; Yamamura and Snyder, 1974). This difference between the agonist and antagonist binding to muscarinic receptors is explained by the presence of three classes of binding sites having different affinity (super high,
high and low) for agonist but equal affinity for the antagonist (Birdsall et al., 1980). The three different affinity states for agonist may not represent three different macromolecules, as SDS-gel electrophoresis and radiation inactivation analysis indicate the presence of a single polypeptide with 80K molecular weight from five different tissues (Venter, 1983). Yamamura and others (Birdsall et al., 1980; Ehlert et al., 1982) have postulated that the different affinities of agonist binding sites represent different environmental or coupling states of the same receptor macromolecule. Guanine nucleotides may somehow alter the environment, as they convert the heterogeneous population of receptors to a predominantly low affinity form (Wei and Sulakhe, 1979; Rosenberger et al., 1980). Occupation of the low affinity form was correlated with the formation of cGMP in brain slices and neuroblastoma cells (Birdsall, 1977), and with inhibition of GTP-stimulated adenylate cyclase activity in cardiac homogenates (Birdsall et al., 1980b). PI response, however, was associated to both high and low affinity binding sites in guinea pig ileum (Jafferji and Michell, 1976a).

### 1.8.5 Subclassification Based On Antagonist Binding

Three subclasses of muscarinic receptors have been suggested, from pirenzepine binding studies (Hammer et al., 1980). The IC$_{50}$ for the pirenzepine inhibition of propylbenzilylcholine or N-methyl scopolamine binding differed by a factor of 30 in the three subclasses, with atria and ileum showing a homogeneous population of low affinity receptors.
In contrast, cerebral cortex and sublingual gland had an IC₅₀ of $5 \times 10^{-8}$ M and $1.1 \times 10^{-7}$ M, respectively, and also showed the presence of multiple affinity binding sites. Another type of heterogeneity, based on choline/Tris sensitivity was reported in myocardial muscarinic receptors (Sastre et al., 1982). The exposure of canine atrial homogenates to Tris (10-100 mM) or choline (0.1-1 mM) caused a 45% loss in quinuclidinylbenzilate (QNB) binding sites, while the remaining sites maintained their affinity. This led the authors to conclude that choline and Tris unmasked a heterogeneity in QNB binding, which was not apparent in their absence.

Figure 3 - Pirenzipine Structure
Three subpopulations of muscarinic receptors have been detected in cultured heart cells based on desensitization studies (Galper and Smith, 1980). 26% of the receptor sites were rapidly lost during incubation with carbachol and were insensitive to microtubule inhibitors such as colchicine. A second subclass consisted of 44% of the receptors, which underwent a slower, colchicine-sensitive desensitization on exposure to carbachol. The rest (30%) of the receptors comprised a "stable" population, which were not lost even after 3 hour exposure to carbachol. Whether there is any relationship between these three subpopulations of muscarinic receptors, classified based on agonist-induced desensitization and the three different affinity sites for agonist binding is still not known.

1.8.6 Receptor-Effecter Coupling

Just as many different types of receptors (e.g. β-adrenergic, H₂-histaminic) are capable of coupling to a single effector (e.g. adenylate cyclase) the possibility that a single receptor can be coupled to different effectors is now being recognized (Richelson and El-Fakahany, 1981). Muscarinic receptors in heart (Hartzell, 1982) and sympathetic neurones (Brown and Adams, 1980; Horn and Dodd, 1981; Weight et al., 1979) have been proposed to be coupled to two different types of ionic channels in the same tissue. Possible subclassification of the muscarinic receptor can be made, based on the nature of the effector system coupled to the receptors. A large
proportion of mAChRs's belong to a family of receptors which mediate their response through calcium mobilization and also produce a PI response on receptor stimulation (see Michell, 1975, 1979). The responses of a second, much smaller, proportion of mAChRs are mediated by inhibition of calcium influx, e.g. the negative inotropic effect in heart and the inhibition of transmitter release by presynaptic mAChR stimulation. Recently Michelson et al., (1979) have suggested that presynaptic mAChR stimulation may not inhibit calcium uptake, but rather a phosphorylation step following calcium mobilization. Stimulation of some receptors belonging to this class has been shown to cause inhibition of adenylate cyclase (Murad et al., 1962; Watanabe et al., 1978), thereby decreasing the level of cAMP (Beigon and Pappano, 1980; George et al., 1973). Cholinergic agonists may regulate both the affinity of receptors coupled to adenylate cyclase and adenylate cyclase activity by modulating the effects of GTP (Watanabe et al., 1978), possibly through its conversion to cGMP.

By analogy to the alpha-adrenergic system, Jones et al. (1982) proposed that there may be two populations of mAChR. One population of mAChR was suggested to be coupled to PI turnover and its response is mediated by calcium mobilization, while the second population was suggested to mediate its response through inhibition of adenylate cyclase. Unlike the alpha-adrenergic system, it is still not known whether the population of mAChR inhibiting adenylate cyclase is coupled to PI turnover or not (Michell et al., 1981).
1.9 MECHANISM OF THE NEGATIVE INOTROPIC EFFECT

Ligand binding studies suggest the presence of mACHRs in all regions of the heart (Hartzell, 1980; Wei and Sulakhe, 1978). In rat and rabbit, mACHr density in the atrium was two-fold higher than that of the ventricle, while guinea pig and dog showed almost equal density in these two regions (Wei and Sulakhe, 1978). A decrease in the basal contractile force is usually observed upon stimulation of mACHR in isolated atrium of various species (Furchgott et al., 1960; Ravens and Ziegler, 1980; for review see Higgins et al., 1973), but the decrease in ventricle (Endoh and Motomura, 1979; Josephson and Sperelakis, 1982) and whole heart preparations (Ingerbretsen et al., 1980) was small and variable. The difference in the mACHR density between atria and ventricle is not enough to account for the observed variation in the response to mACHR stimulation in these two regions.

The direct negative inotropic effect (decrease in basal contractility) observed on muscarinic stimulation of atrium (and sometimes ventricle), was attributed to the inhibition of the slow inward calcium current (Grossman and Furchgott, 1964; Ravens and Ziegler, 1980; Ten Eick et al., 1976). Concentrations of ACh which produced a 30-40% decrease in the force of contraction were shown to cause an indirect inhibition of the slow inward calcium current by decreasing the duration of the action potential (Beigon and Pappano, 1980; Ten Eick et al., 1976). At higher concentrations of ACh, in addition to the above mentioned indirect mechanism a direct effect was suggested to be involved
in inhibiting the slow inward calcium current (Ten Eick et al., 1976). The decreased duration of the action potential can not be solely responsible for the negative inotropic effect observed with ACh, because while there was a very good correlation between decreased duration of the action potential and the negative inotropy with ACh alone (Furchgott et al., 1960; Ten Eick et al., 1976), it was possible to neutralize the negative inotropic effect of ACh with epinephrine without producing a return of the duration of the action potential to control levels (Furchgott et al., 1960).

Other possible mechanisms for the ACh-mediated negative inotropic effects include alterations in the level of cyclic nucleotides i.e. cAMP and cGMP (George et al., 1973; Nawrath, 1976; Lee et al., 1972; for review see Drummond and Severson, 1979). In perfused rat heart, ACh stimulation produced both an increase in the level of cGMP and a decrease in the level of cAMP (George et al., 1973), but the negative inotropic effect accompanying ACh stimulation showed a much better correlation with the former (George et al., 1973). Other evidence which suggested a role for cGMP in ACh-mediated negative inotropy includes - a concentration dependent inotropic effect of 8-bromo cGMP in paced or spontaneously beating atria (Nawrath, 1976), and the ability of dibutyryl cGMP to antagonize the positive inotropic effect of isoproterenol in guinea pig heart (Watanabe and Besch, 1975). cGMP may mediate its effect through activation of cGMP-dependent protein kinase (Gill et al., 1977), which was found to cause the phosphorylation of cardiac
troponin-1 (Blumenthal et al., 1978) and a 70K endogenous protein present in the rat heart (Wrenn and Kuo, 1981). The functional significance of these phosphorylations and whether they occur under physiological conditions is still not clear (Blumenthal et al., 1978; Drummond and Severson, 1979; Wrenn and Kuo, 1981).

Under certain conditions, a dissociation between the elevation in cGMP level and negative inotropy has also been observed (Diamond et al., 1977; Linden and Brooker, 1979). At low concentrations of ACh (0.05 μM), cat atrial tissue showed a decrease in twitch tension without any change in cGMP (Diamond et al., 1977). On the other hand, increase in cGMP levels with sodium nitroprusside in cat atria (Diamond et al., 1977) and ACh in guinea pig ventricles (Watanabe and Besch, 1975) was not accompanied by a decrease in tension. Recent observations (Endoh and Yamashita, 1981) that a negative inotropic effect could be produced by more than one mechanism may help to explain some of the above discrepancies. Sodium nitroprusside increased cGMP levels in both canine atrium and ventricle, but was accompanied by a decrease in tension only in the atria. A similar result was obtained with dibutyryl cGMP (Endoh and Yamashita, 1981).

When contractile force was elevated first by sympathetic stimulation or isoproterenol administration, subsequent treatment with ACh produced a greater fractional decrease in tension than the control in both left atria and ventricle ("accentuated antagonism") (Dempsey and Cooper, 1969; Levy and
 remained unchanged.

The greater negative inotropic effect obtained with ACh in the presence of sympathetic tone or increased level of catecholamine was referred to as "accentuated antagonism" (Levy and Zieske, 1969). As accentuated antagonism with ACh was observed only with agents whose responses are mediated by cAMP, such as catecholamines and histamine H₂ agonists, but not with agonists whose responses are independent of cAMP (Levy et al., 1966; Watanabe and Besch, 1975), the results suggested that ACh produced accentuated antagonism by interfering with cAMP generation or its response.

Most of the available evidence suggests that the catecholamine-stimulated positive inotropic effects are mediated through cAMP (for a review see Drummond and Severson, 1979). Rinaldi et al. (1981) have recently shown that cAMP-dependent phosphorylation of a 23K molecular weight cardiac sarcolemmal protein, calciductin, leads to an increased calcium uptake. This may suggest a possible mechanism by which isoproterenol-stimulated cAMP-dependent protein kinase (Keely et al., 1978) may cause an increase in the slow inward calcium current (Josephson and Speralakis, 1982). ACh, by decreasing the level of isoproterenol-stimulated cAMP, may inhibit the activation of cAMP-dependent protein kinase, thereby reducing the slow inward calcium current. In cardiac membrane preparations, ACh-mediated inhibition of adenylate cyclase was brought about by an effect on the GTP-dependent regulatory component of the β-receptor/adenylate cyclase system (Watanabe et al., 1978).
Other proposed mechanisms for ACh-mediated negative inotropy include: a) a decrease in the pool of exchangeable calcium (Grossman and Furchgott, 1964) and b) increased calcium efflux (Prolopezuk et al., 1981).

1.10 ALPHA-ADRENERGIC RECEPTORS IN HEART

Alpha-adrenergic receptors have now been shown to be present in the hearts of rat, guinea pig, rabbit and cat (Wagner and Brodde, 1978; Schumann, 1980; for review see Benfey, 1980). Alpha-adrenoceptors are divided into two categories - \( \alpha_1 \) and \( \alpha_2 \), based mainly on the specificity of antagonist binding (Exton, 1982). Alpha- and \( \alpha_2 \)-adrenoceptors were previously considered to be specifically located in the post and presynaptic membrane respectively, but now the presence of postsynaptic \( \alpha_2 \) adrenoceptors has been shown in many tissues (McGrath, 1982; for a list of tissues where postsynaptic \( \alpha_2 \) receptors have been detected see Exton, 1982).

Yohimbine, a \( \alpha_2 \) blocking agent, was 20 times less potent than phentolamine in displacing \( [^{3}H] \)dihydroergocryptine-binding in rabbit (Schumann and Brodde, 1979) and rat (Williams and Lefkowitz, 1978) cardiac homogenates, suggesting that the alpha-receptors being labeled are postsynaptic. In rat heart, the presence of a single class of \( [^{3}H] \)dihydroergocryptine binding sites with a receptor density of 41 fmole/mg protein (Williams and Lefkowitz, 1978) and 307 fmole/mg of protein (Ciaraldi and Marinetii, 1977) and 100 fmole/mg protein based on \( [^{3}H] \)prazosin binding (Karliner et al., 1982) has been reported, suggesting
the presence of a homogeneous population of alpha-adrenoceptors. On the other hand, in pharmacological studies a multiphasic response obtained with stimulation of rat papillary muscle (Skomedal et al., 1980), rat atria (Osnes, et al., 1978) and guinea pig atria (Hattori and Kanno, 1981) argues against the presence of a single class of alpha-adrenergic receptors. Further, the selective blockade of the positive inotropic phase by prazosin (an $\alpha_1$ antagonist), and a complete blockade (both positive and negative inotropic response) by phentolamine in rat (Skomedal et al., 1980), as well as in guinea pig (Hattori and Kanno, 1982), led to the suggestion that there may be stimulatory and inhibitory populations of postsynaptic alpha-adrenoceptors (Skomedal et al., 1980; Hattori and Kanno, 1982).

Alpha$_2$-adrenergic responses are considered to be mediated by inhibition of adenylate cyclase. This is suggested to occur by activation of an inhibitory-GTP binding protein, which may directly interact with the catalytic subunit of adenylate cyclase or interfere with the interaction between the stimulatory and catalytic subunits of adenylate cyclase (Levitzki, 1982). A decrease in the level of cAMP on cardiac alpha-adrenergic stimulation (probably through activation of $\alpha_2$ receptors) has been reported by Watanabe et al. (1977). In isolated rat myocytes and perfused rat heart, alpha-receptor stimulation with phenylephrine or adrenaline in the presence of propranolol (a beta blocker) caused a decrease in the level of cAMP (Watanabe et al., 1977). In perfused rat heart this was accompanied by a negative inotropic effect.
A monophasic positive inotropic response devoid of the transient inhibitory effect (Hattori and Kanno, 1981, 1982; Osnes et al., 1978) has been observed on stimulation of alpha receptors in rat left atria (Martinez and McNeill, 1977; Shibata et al., 1980) and ventricles (Shibata et al., 1980; Wagner and Brodde, 1978; Wenzel and Su, 1966). Among the myocardial preparations studied, rat left and right atria showed the greatest phentolamine-sensitive positive inotropic response to methoxamine (Shibata et al., 1980).

1.10.1 Mechanism Of The Alpha- And Beta-Adrenergic Receptor-mediated Positive Inotropic Effect

Many different lines of evidence clearly indicate that alpha and beta-adrenergic receptor induced positive inotropic responses are mediated by different mechanisms (Endoh et al., 1975; Endoh and Yamashita, 1980; Skomedal et al., 1982; Wagner and Schumann, 1979). The β-adrenergic-stimulated positive inotropic effects in various tissues of the heart are accompanied by increased levels of cAMP (Kelly et al., 1978; Robinson et al., 1965; Martinez and McNeill, 1977), decreased time to peak tension and a shortened relaxation time (Ledda et al., 1975; Rabinowitz et al., 1975; Skomedal et al., 1982). In contrast, the alpha-adrenoceptor-stimulated positive inotropic effect was accompanied neither by an increase in cAMP levels (Martinez and McNeill, 1977), nor by a decrease in the time to peak tension (Skomedal et al., 1982).

Most workers agree that the alpha-adrenoceptor-stimulated
positive inotropic effect is mediated through enhanced mobilization of calcium (Handa et al., 1982; Inui et al., 1981; Ledda et al., 1980; Miura et al., 1978), but the source of calcium in this mediation is still being debated. In general, both mitochondrial and extracellular Ca pools have been implicated in alpha-adrenergic-mediated responses in various tissues (Exton, 1982). Endoh et al. (1975) found that the alpha-receptor-mediated positive inotropic response was more sensitive to D-600 (a calcium channel antagonist) than the beta-receptor response, and the greater temperature sensitivity of the alpha response in the above study led them to conclude that alpha stimulation mainly exerted its effect by increasing transmembrane calcium influx. These conclusions were also supported by the observation that conditions which favoured an enhanced influx of calcium, such as elevation of the gradient of calcium, shortening of the plateau of the action potential with carbachol, and increased efflux of calcium with dinitrophenol, were all able to increase the affinity of phenylephrine for cardiac alpha-adrenoceptors. Inui et al. (1981) and Miura et al. (1978) found that in K+-depolarized rabbit papillary muscle, alpha-adrenoceptor stimulation increased the inward calcium current, thereby supporting the Endoh et al. (1975) hypothesis. Some of the more recent observations have challenged the validity of this hypothesis. Verapamil and its methoxy derivative (D-600) not only block the calcium channel but were shown to compete for the alpha receptor binding sites (Karliner et al., 1982). This may explain the higher
sensitivity of alpha-adrenoceptor responses to D-600 observed in Endoh's studies (Endoh et al., 1975). In K⁺-depolarized guinea pig ventricular muscle, methoxamine stimulation produced a dose-dependent contractile response which was not accompanied by a slow inward calcium current (Ledda et al., 1980). In K⁺-depolarized rabbit papillary muscle, when the maximum rate of rise of the action potential was used as a measure of the inward calcium current, the alpha stimulated response was much smaller than that of the beta (Handa et al., 1982). Alpha stimulation increased the duration of the action potential, probably by suppressing the time-dependent outward K⁺ current (Handa et al., 1982). The increased duration of the action potential even with an unaltered inward calcium current could still result in an increased mobilization of calcium. In conclusion, compared to the beta-adrenergic system, little is known about how alpha-mediated positive inotropy is produced, and if calcium is involved, what its source is.

1.11 MUSCARINIC RECEPTORS IN LONGITUDINAL SMOOTH MUSCLE OF GUINEA PIG ILEUM

In longitudinal smooth muscle of guinea pig ileum, based on [³H]QNB binding studies, receptor density has been estimated to be 190 fmol/mg tissue (Yamamura and Snyder, 1974). Stimulation of muscarinic receptors of longitudinal smooth muscle of guinea pig ileum produced a biphasic contraction, consisting of a rapid phasic component and a sustained tonic component (Chang and Triggle, 1973; James-Kracke and Roufogalis, 1981). Both phases
of contraction are the result of calcium mobilization, but the pools of calcium mobilized during these two phases are considered to be different (Brading and Sneddon, 1980; Chang and Triggle, 1973; Rangachari et al., 1983). Chang and Triggle (1973) have suggested that a pool of extracellular free calcium is associated with the sustained tonic phase of contraction, while a superficially bound calcium pool is used in the transient and rapid phasic contraction.

Jafferji and Michell (1976a) were the first to observe that stimulation of muscarinic receptors in guinea pig ileum produced an enhanced incorporation of phosphate into PI, and also suggested that the primary step of this response may be an enhanced breakdown of PI. Longitudinal smooth muscle of guinea pig ileum has been shown to respond to muscarinic agonists with a receptor reserve (Taylor et al., 1975; Yoshida et al., 1979), as only 10% of the receptors are required to be occupied by the muscarinic agonist oxotremorine to produce a maximal contraction (Yoshida et al., 1979). On the contrary, the dose-response curve for carbachol-stimulated phosphate incorporation into PI was similar to the dose-response curve for receptor occupancy (Jafferji and Michell, 1976a). This provided additional evidence to support the hypothesis that in the chain of events between receptor stimulation and the physiological response, PI breakdown may occur nearer to receptor occupation and may play a role in calcium mobilization (Michell, 1975; Jafferji and Michell, 1976a). In cultured smooth muscle cells (Salmon and Honeyman, 1980) evidence was provided to suggest that the time
course of formation of phosphatidic acid during carbachol-stimulated PI turnover was rapid enough to play a role in calcium mobilization. In a later communication, Jafferji and Michell (1976b) reported that K+-induced depolarization, which also produces a calcium-mediated biphasic contraction, showed an enhanced incorporation of $[^{32}\text{P}]$phosphate into PI. Based on the inhibition of contraction by various calcium antagonists, Rosenberger and Triggle (1979) proposed that both carbachol and K+-stimulation mobilize the same pool of calcium.
1.12 OBJECTIVE

The overall objective of the present thesis was to test the following two hypotheses:

a) that PI breakdown is intrinsic to muscarinic receptor stimulation.

b) that the PI response is involved in calcium mobilization.

These objectives were sought by studying:

1) the effect of muscarinic and alpha-adrenergic stimulation on PI response in rat heart.

2) the primary event of carbachol-stimulated PI response in guinea pig ileum.

3) the effect of the putative phospholipase C inhibitor, PMSF, on carbachol-stimulated PI response and contraction.
II. METHODS

2.1 Choice Of Animal

Among the small laboratory animals, the rat has been shown to contain the largest number of both muscarinic (Wei and Sulakhe, 1978) and alpha adrenergic receptors in their atria (Schumann, 1980). Pharmacological responses following stimulation of both these receptors have also been quite well characterized in the rat (Josephson and Sperelakis, 1982; Osnes et al., 1978; Ravens and Ziegler, 1980; Skomedal et al., 1980; Ten Eick et al., 1976). In longitudinal smooth muscle of the guinea pig ileum the effect of muscarinic receptor stimulation on calcium mobilization (Chang and Triggle, 1973; Rosenberger and Triggle, 1979) and phosphatidylinositol turnover (Jafferji and Michell, 1976a) has been previously reported. Female, rather than male guinea pigs were used in order to compare the results with those of Jafferji and Michell (1976a). Male Wistar rats (300-350 g) and female guinea pigs (350-400 g) were anaesthetized in ether saturated tank before the isolation of tissue. In the case of rats, 0.4 ml of 1600 units/ml of heparin in normal saline was injected over 5-10 sec period, 10 min before the rats were anaesthetized.
2.2 Krebs-bicarbonate Buffer

All incubations were carried out in Krebs-bicarbonate buffer of the following composition (mM): sodium chloride - 113, potassium chloride - 4.7, calcium chloride - 2.5, potassium dihydrogen phosphate - 1.2, magnesium sulphate - 0.6, sodium bicarbonate - 25, and dextrose - 11.5, and the pH adjusted to approximately 7.4 with O$_2$/CO$_2$ (95/5).

2.3 Phosphate Incorporation In Atria

The atria were removed after perfusion of the isolated rat heart with Krebs buffer to free them of blood and placed in ice cold Krebs buffer saturated with O$_2$/CO$_2$ (95/5). Atria were chopped into smaller fragments, either together (in mixed atria studies) or after separation of the left and right atria (in later studies) and suspended in 5.3 ml of Krebs buffer. The atria were incubated in the presence or absence of drugs in Krebs buffer with 40 μCi of $[^{32}P]$sodium orthophosphate, in a final incubation volume of 6 ml. After 60 min incubation, the reaction was stopped with 2 ml of 20% trichloroacetic acid. Tissue fragments were separated by vacuum filtration on Whatman #1 paper and washed with ice-cold Krebs buffer. Extraction of lipid, separation of phospholipid and determination of radioactivity were done as described in later sections.
2.4 Isolation Of Longitudinal Smooth Muscle Of Guinea Pig Ileum

For each experiment, ileum was obtained from 1 or 2 female guinea pigs. Longitudinal smooth muscle was obtained by the method of Rang (1964). Ileum was cut into 2-3 cm long fragments and stored in Krebs buffer at 4°C or at 37°C for contraction studies. Each piece was mounted on a smooth glass rod and longitudinal smooth muscle was separated by careful tangential scraping with a cotton swab (Q-tip). Isolated longitudinal smooth muscles were usually stored in ice cold Krebs buffer (except for functional studies, in which case they were stored at 37°C), and aerated with O₂/CO₂ (95/5).

2.5 Label-incorporation In Guinea Pig Ileum

Studies of phosphate incorporation in guinea pig ileum were done in a manner similar to that described for phosphate incorporation in rat atrium. For [³H]inositol incorporation, ileum pieces (15-25 mg wet weight) were combined and preincubated for 30 min with 30 μCi of [³H]inositol in 15 ml Krebs buffer under an O₂/CO₂ (95/5) atmosphere. Individual pieces were then separated and incubated separately for a further 30 min period with 1 μCi of [³H]inositol in the presence of various agents, in a total volume of 4 ml. The reaction was stopped by adding 2 ml of 20% trichloroacetic acid. The tissue was separated and washed with buffer. Lipid was extracted as described in a later section. The lower organic phase obtained during extraction of the lipid was separated by centrifugation at 2100xg for 15 min, washed twice with methanol/0.1 N
hydrochloric acid/chloroform (48:41:3 v/v), and the solvent finally removed by evaporation. The residue was dissolved in chloroform (2 ml) and phosphorus was determined in two 75 μl aliquots as described in a later section. The radioactivity was determined in the remaining portion, after removal of the solvent and addition of 10 ml Econofluor. Incorporation was calculated as dpm/μg phosphorus and expressed as a percentage of the control value.

2.6 Extraction Of Lipid

Throughout the study lipids were extracted by the method of Bligh and Dyer (1959), as described below. Homogenization and extraction were all carried out on ice. Tissue (usually less than 25 mg) was suspended in 1.5 ml of 2 M KCl and homogenized at speed 3 with a teflon-coated Potter-Elvehjem homogenizer. After homogenization, the homogenate was transferred to a 15 ml capped tube, 5.2 ml of chloroform/methanol (1:2 v/v) was added and the tube mixed by vortex for 20 sec (a nonacidified chloroform/methanol mixture was intentionally used to avoid the extraction of polyphosphoinositides) Chloroform (1.8 ml) was then added and the tube mixed again on a vortex mixer for 20 sec. Finally, 1.8 ml of water was added and the suspension mixed by vortex for 5 sec. After 20-30 min, the chloroform phase was separated by centrifugation at 2100xg for 15 min at 4°C.
2.7 Phospholipid Separation

For initial experiments Supelco Redi Coat-2D plates were used for the separation of phospholipids. New plates gave very good separation, but unsatisfactory separation was obtained with plates "stored" for a longer period of time. Therefore, precoated TLC plates from Merck, silica gel 60, layer thickness 0.25 mm, were used in later studies, which gave consistently good separations. Merck thin layer chromatography plates separated all the phospholipid spots previously separated with plates from Supelco, but the relative mobilities of the phospholipid spots were slightly different in the two types of plates.

Plates were activated at 105°C for 20 min and divided into four equal quadrants. A 20 to 30 μl sample was spotted by 10 μl micropipette at each of the four corners, 2 cm from the edges. In experiments involving arachidonic acid determination or incorporation, only nitrogen was used for the drying of the spot. Phospholipid separation was achieved by two-dimensional thin layer chromatography. Chromatographic tanks were lined with Whatman #1 filter paper and presaturated with the desired solvent system for 30 min. Each plate was developed with 60 ml of solvent, and new solvent was used with each run. Between each run plates were air dried for 5 min, and for 10-15 min between runs in the first and second dimension, until the smell of ammonia was completely lost. After development of the plate in the second dimension and removal of the solvent, spots were detected by exposure to iodine vapor, which gave a yellow
colour. After the spots were marked with a pencil, the stain was allowed to fade away by storage overnight. The required spots were scraped off the plate after addition of a drop of methanol. The silica gel was transferred to a test tube and phospholipids extracted.

2.8 Extraction Of Phospholipid Spots

A mixture of chloroform/methanol/water/acetic acid (50:30:10:1 v/v) was allowed to stand for 5 min. The upper aqueous phase was discarded and 2 ml of the lower acid saturated chloroform/methanol phase was used to extract phospholipids. In some of the initial experiments spots were extracted with 2 ml methanol only.

2.9 Determination Of Radioactivity

The phospholipid extract was divided into two equal portions (0.8-0.9 ml). One portion was used for phosphorus determination. In another portion, radioactivity was determined after removal of the solvent and addition of 10 ml scintillation fluid. ACS was used as the scintillation fluid for phosphate label and aqueous extract, while Econofluor was used for determination of $[^3]$H-label in nonaqueous media.
2.10 Phosphorus Determination

All the glassware used in the phosphorus determination was acid washed. Phosphorus was determined by a slight modification of the method of Duck-Chong (1979). To a sample in a test tube, 30 µl of 10% magnesium nitrate in methanol was added and the mixture evaporated to dryness by heating it in a water bath at 85-100°C for 5 to 10 min. Digestion was achieved by heating the sample for 15 sec at the top of the flame and 10-15 sec at the tip of the blue cone of the flame. After tubes reached room temperature, 0.3 ml of 1 M HCl was added. The tubes were covered with marbles and heated at 90-95°C for 15 min. After cooling to room temperature, 0.7 ml 1 M HCl was added, followed by 2 ml of reagent C (see below). After 5 min the absorbance was measured at 620 nm in a disposable cuvette. With each experiment a standard phosphorus curve was obtained by adding a known sample of sodium dihydrogen monophosphate to a tube in which 30 µl of magnesium nitrate had been evaporated and the assay was carried out as described above.

Reagent C was prepared by mixing 1 volume of 4.2% (w/v) ammonium molybdate in 4.5 M HCl with 3 volume of 0.05% (w/v) malachite green. Just before use, Triton X-100 was added to this mixture to give a final concentration of 0.075% (w/v). It should be mentioned here that while malachite green from BDH dissolved freely, malachite green from other sources did not.
2.11 Inositol Phosphate Accumulation In The Presence Of Lithium

In preliminary experiments it was observed that blank values could be reduced drastically by prior treatment of the labeled inositol, as obtained from the manufacturer, with anion-exchange resin. This may be due to labeled anionic contaminants in the [³H]inositol, which were being removed during this step. 200 µl of Dowex-1 X8-formate resin (50% w/v) was added to 30 µCi of [³H]inositol in 800 µl water. The mixture was vortex mixed, centrifuged and the supernatant added to 14 ml of Krebs buffer saturated with O₂/CO₂ (95/5). Guinea pig ileum pieces were incubated with 1 µCi of [³H]inositol in a total volume of 1 ml Krebs-Ringer bicarbonate buffer in disposable plastic tubes, in the presence of, wherever required, 0.1 mM carbachol, 10 mM lithium chloride, 60 mM KCl, the required amount of PMSF in 1% dimethylsulfoxide or dimethylsulfoxide alone and 6.4 µM atropine sulphate. At the beginning of the incubation O₂/CO₂ (95/5) was introduced above the surface of the mixture. Then the tubes were closed tightly and incubation continued for a period of one hour. Incubation was terminated by removing the smooth muscle pieces and transferring them into 1 ml chloroform/methanol (1:2). The tissues were homogenized and the lipids extracted as described above. The aqueous phase of the extraction medium was retained and inositol phosphate determined as follows.
2.12 Determination Of Total Labeled Inositol Phosphate

The method was similar to the simplified assay procedure described by Berridge et al. (1982). A 1 ml slurry of 50% (w/v) Dowex-1 X8 (100-200 mesh) in the formate form was added to the aqueous phase. The supernatant was discarded after the resin was allowed to settle. After the resin was washed five times with 4 ml of 5 mM inositol, inositol phosphates were eluted with 0.8 ml of 0.1 M formic acid plus 1 M ammonium formate. Half of this eluate (0.4 ml) was used for the determination of radioactivity after the addition of 10 ml ACS.

2.13 Separation Of Inositol Phosphates

Washed resin containing adsorbed inositol phosphates was eluted successively in a test tube with four 2 ml portions of 5 mM borax plus 60 mM ammonium formate, 5 mM borax plus 150 mM ammonium formate, 0.1 M formic acid plus 0.4 M ammonium formate and finally 0.1 M formic acid plus 1 M ammonium formate. In the column separation of inositol phosphates, 2 ml of Dowex-1 X8-formate resin (50% w/v) was packed in a 1x4 cm column. Standard inositol mono-phosphate, or an aqueous extract of inositol phosphate accumulated in the tissue, was passed through the column. This was followed by washing of the column with 5 mM inositol until no more radioactivity was eluted. Inositol phosphates were eluted from the column with increasing ionic strengths of borax, ammonium formate and formic acid, as described above. 1 ml fractions were collected and phosphorous or radioactivity determined.
2.14 Contraction Studies

Guinea pig ileum strips were looped and suspended at 37°C under 0.35 g tension in Krebs bicarbonate buffer and aerated with O2/CO2 (95/5). Alteration in tone was monitored isometrically using force transducers after a 30 min equilibration period. The effect of PMSF on carbachol- and potassium-mediated contraction was studied by adding PMSF dissolved in dimethylsulfoxide to a final concentration of 1% dimethylsulfoxide in the bath. The hyperosmolarity of the 60 mM KCl solution was not compensated by reduction of the NaCl concentration.

2.15 PI Degradation Studies

PI degradation studies on atrial fragments prelabeled with [32P]phosphate were performed as follows. Atria from 5 rats were pooled and phospholipids labeled in vitro by incubating small fragments for 2 h at 37°C with 0.2 mCi of [32P]sodium orthophosphate in 40 ml of buffer. At the end of the incubation period fragments were separated by filtration. After the fragments were washed with Krebs buffer, they were divided into six approximately equal portions and suspended in 5.9 ml of Krebs buffer. Further incubation for 1 h at 37°C was carried out in the presence or absence of 0.1 mM carbachol. At the end of this incubation period, further reaction was stopped with 2 ml of 20% trichloroacetic acid and tissue fragments were separated by filtration. Extraction of lipids, separation of phospholipids and determination of radioactivity were done as
described in the earlier sections.

In degradation studies employing \([^{14}C]\)-labeled arachidonic acid, whole atria from 12 rats were incubated with 10 \(\mu\)Ci of \([^{14}C]\)arachidonic acid in 30 ml of Krebs buffer, for 2 h at 37°C. At the end of this period, atria were separated, washed with Krebs buffer containing 1% bovine serum albumin and incubated for a further 15 min period in the presence or absence of the drug. The reaction was stopped with 2 ml of 20% trichloroacetic acid, atria were separated, weighed and lipid and phospholipid isolated as described in an earlier section. Radioactivity in each of the PI spots was determined and results calculated as amount of radioactivity in PI per g of tissue and expressed as percent control.

2.16 Fatty Acid Composition Of Diacylglycerol

The fatty acid composition of diacylglycerol fractions was determined by methods similar to those described by Bansback et al. (1974). In these experiments, whole atria from rat were incubated in 5.3 ml of Krebs buffer at 37°C, under an \(O_2/CO_2\) (95/5) atmosphere. After 15 min a stock carbachol solution was added to give a final carbachol concentration of 0.1 mM. After a further 15 min incubation, the reaction was stopped with 2 ml of 20% trichloroacetic acid. Control tissues were treated identically in the absence of carbachol. Tissues were rapidly frozen in dry ice/methylbutane mixture, and stored overnight at -20°C. Frozen tissues were weighed, chopped into smaller fragments and lipid extracted as described in an earlier
Neutral lipids were separated by thin layer chromatography of total lipid extract with benzene:chloroform:methanol (80:15:5 v/v), and visualized with 0.01% dichlorofluorescein spray. Diacylglycerol was identified by comparison with 1,2-dipalmitoylglycerol. Diacylglycerol was extracted from the silica gel plates with diethyl ether/ethanol (9:1 v/v) containing 0.1% (v/v) 2-mercaptoethanol. Diacylglycerol was taken to dryness under N₂ and heneicosanoic acid was added as an internal standard. Fatty acids were obtained from lipids by hydrolysis with 15% methanolic potassium hydroxide at 70°C for 1 h. The fatty acids were extracted with pentane and converted to their methyl esters with 10-15% boron trifluoride in methanol. The methyl esters were extracted with pentane and further purified by thin layer chromatography in benzene. Silica gel portions corresponding to a standard, methyl stearate, were scraped from the plates, extracted twice with hexane and concentrated under N₂. The methyl ester mixture was resuspended in 20 µl hexane and injected into a gas chromatograph (Hewlett Packard 5830A) equipped with a flame ionization detector and Hewlett Packard 18850A integrator. Separation of individual methyl esters was achieved on a 10% diethylene glycol succinate on an 80/100 mesh Supelcoport column. The column conditions were as follows: column temperature - 190°C; injection port - 225°C; detector temperature - 225°C; carrier gas-helium at 56-58 ml/min. The flame ionization detector was found to have a similar sensitivity for all the fatty acids examined. Fatty acid
quantities were estimated based on the area ratio with respect to the internal standard.

2.17 Materials

Carbamylcholine chloride, atropine sulphate, inositol-2-monophosphate dl-monocyclohexylamine, myo-inositol, fatty acid methyl ester mixture and lithium chloride were obtained from Sigma chemical company, St. Louis, Mo. $[^{32}\text{P}]$sodium orthophosphate (200 mCi/mmol), $[^{1-\text{C}^1}]$-arachidonic acid (55.8 mCi/mmol), myo-$[^{3}\text{H}]$inositol (15.8 Ci/mmol) and Econofluor were obtained from New England Nuclear, Lachine, Quebec. Phentolamine mesylate was obtained from CIBA Canada, Dorval, Quebec. Methoxamine hydrochloride was from Burroughs Wellcome Co., Research Triangle Park, N.C. Dowex-1 X8-formate resin was from Bio-Rad laboratories, Mississauga, Ontario.

Malachite green (C.I. 42000) and magnesium nitrate were from BDH chemicals, ACS and ammonium molybdate were from Amersham, Seattle, Wa. Precoated Redi-coat 2D thin layer chromatography plates were obtained from Supelco Inc., Bellanfonte, Pa. and precoated silica gel 60 thin layer chromatography plates and benzene were obtained from E.Merck, Darmstadt, Germany. Glass distilled chloroform, methanol, hexane and acetone were purchased from Caledon, Ontario. All inorganic salts used in the preparation of buffers were of analytical grade.
III. RESULTS

3.1 SEPARATION OF PHOSPHOLIPIDS

3.1.1 Separation And Identification Of PI

Phospholipids were separated by two dimensional thin layer chromatography on a silica gel plate, as shown in Fig 4. Based on comparison with standard phospholipids, the following spots were identified - spot a- phosphatidylinositol, spot b- phosphatidylserine, spot c- phosphatidylcholine, spot d- phosphatidylethanolamine and spot f- phosphatidic acid. The identity of PI was further confirmed by the specific incorporation of $[^3]H$inositol into spot (a). The relative distribution of the four phospholipids determined in studies on rat atria is shown in Table I. As the objective was to determine the specific radioactivity incorporated into PI rather than the quantitative determination of all phospholipids, extraction efficiency of individual phospholipids was not determined and therefore, the percentage distribution of the four major phospholipids estimated by phosphorus determination, as shown in Table I differs from that reported by White (1973).

In a similar experiment, where phosphatidylinositol and phosphatidylcholine, as well as total lipid phosphorus, were determined, it was calculated that 16.3 µg of phosphatidylinositol and 126.6 µg of phosphatidylcholine are present per g tissue weight (one g tissue of rat heart has been
reported to contain 470 μg of lipid phosphorus (White, 1973).

The reported values for these two phospholipids in rat heart are 17.4 and 169.2 μg per g tissue, respectively (White, 1973).

3.1.2 Phosphorus Determination

Phosphorus was determined by the method of Duck-Chong (1979). An example of a standard curve obtained by that method is shown in Fig 5. This phosphorus determination method was sensitive and the standard curve was reproducible. A plot of absorbance versus phosphorus gave a straight line between 0.1 μg to 0.6 μg, with a slope around 1 and correlation coefficient of greater than 0.99. The variation in duplicate observations in each experiment were less than 0.01 absorbance unit.

3.2 PHOSPHATE INCORPORATION IN RAT HEART

3.2.1 [32P]Phosphate Incorporation In Rat Atria Versus Ventricle

Despite its low quantity in cells, PI has been found to contain a large fraction of the total phosphate label incorporated into phospholipids (Michell, 1975). We obtained a similar result in rat heart (Table I). The basal phosphate incorporation into various phospholipids in rat atria and ventricles is shown in Fig 6. Both in atria and ventricles PI incorporated labeled phosphate with a higher specific activity than that of the other phospholipids. It was also observed that phosphate-label incorporation into the various phospholipids of atria was about 5-6 fold higher than that of label incorporation
into the corresponding phospholipids in ventricles.

3.2.2 Effect Of Muscarinic And Alpha-adrenergic Receptor Stimulation On $[^{32}P]$phosphate Incorporation In Combined Atria

Enhanced incorporation of phosphate into PI is usually the first step in identifying a PI response. Both muscarinic (Wei and Sulakhe, 1978) and alpha-adrenergic (Schumann, 1980) receptors have been shown to be present in rat atria, but unlike most other tissues stimulation of these two receptors in rat atria produces opposite pharmacological responses. As muscarinic receptor stimulation in rat atria is considered not to be accompanied by calcium mobilization, it was of interest to determine whether PI response accompanies its stimulation.

Stimulation of muscarinic receptors in combined rat atrial pieces by 0.1 mM carbachol caused a 35% increase in $[^{32}P]$phosphate incorporation into PI ($p<0.05$) (Fig 7). In order to verify that the small degree of incorporation of labeled phosphate into PI on muscarinic receptor stimulation in combined rat atrial pieces was not the result of an inability to measure $[^{32}P]$phosphate incorporation into that particular preparation, the effect of methoxamine, an $\alpha$-adrenergic agonist, on $[^{32}P]$phosphate incorporation into PI of combined rat atria was investigated. In contrast to the muscarinic response, stimulation of the $\alpha$-adrenergic receptor of rat atria with methoxamine (0.1 mM) enhanced the incorporation of $[^{32}P]$phosphate into PI by 88% (Fig 7), even though the $\alpha$-adrenergic receptor density is less than 50% of the muscarinic
receptor density. This stimulation by methoxamine was blocked by the α-adrenergic antagonist, phentolamine (20 μM). In a similar experiment, it was further shown that the methoxamine-stimulated increase in phosphate incorporation was specific to PI and did not occur in other phospholipids, including phosphatidylserine, phosphatidylcholine or phosphatidylethanolamine (Fig 8).

3.2.3 Effect Of Muscarinic And Alpha-adrenergic Receptor Stimulation On $[^{32}\text{P}]$ phosphate Incorporation In Separate Left And Right Atria

Quist (1982) reported that muscarinic receptor stimulation of canine right atrium, but not left atrium or left or right ventricle, showed enhanced incorporation of phosphate into PI, even though all regions of the canine heart have been shown to contain muscarinic receptors (Wei and Sulakhe, 1978). This led us to test whether a similar situation might exist in rat heart, so that only one of the rat atria may be responsible for the small $[^{32}\text{P}]$ phosphate incorporation observed in combined atrial preparations. It was found that stimulation of the alpha-adrenergic receptor, which usually shows a smaller PI response than muscarinic receptor stimulation in other tissues (Jones and Michell, 1975), produced a 110 and 175% increase in phosphate incorporation into PI in left and right atrium, respectively (Fig 9). Muscarinic receptor stimulation by carbachol, on the other hand, produced a 35% increase in phosphate incorporation into PI in rat left atrium ($p<0.05$), but was completely without
effect in rat right atrium (Fig 10). The relative basal incorporation into rat left and right atrium was quite variable (compare Fig 9 and 10), but the comparison of results from various experiments suggests that the magnitude of the basal incorporation did not influence the sensitivity to agonists.

These results suggested that while the majority of muscarinic receptors in rat atria do not appear to be coupled to PI turnover, a small population of muscarinic receptors in rat left atrium may be coupled to PI turnover.

3.3 PI BREAKDOWN IN HEART

The primary event of PI response has been considered for a long time to be the enhanced breakdown of inositol lipid (Hokin and Hokin, 1964; Michell, 1975). At least in one tissue, blowfly salivary gland, it has been observed (Fain and Berridge, 1979a) that the primary event, inositol lipid breakdown, was not accompanied by rapid resynthesis of PI. As only a small increase in phosphate incorporation into PI during muscarinic receptor stimulation of combined rat atria was observed (Fig 7), it was decided to investigate the effect of muscarinic receptor stimulation on PI breakdown in combined rat atria (these studies were done before the differences in PI response between rat left and right atrium became apparent).
3.3.1 Breakdown Of Phospholipids Prelabeled With Radiolabel Phosphate

Muscarinic receptor stimulation of rat parotid gland has been shown to cause an enhanced breakdown of PI previously labeled with radiolabeled phosphate (Jones and Michell, 1974). In the present study stimulation of combined rat atria with 0.1 mM carbachol for 45 min did not enhance the breakdown of PI or any other phospholipids significantly (Fig 11).

3.3.2 Breakdown Of PI Prelabeled With Arachidonate

In the past few years, evidence has been presented to suggest that receptor stimulation may cause release of arachidonic acid from PI, either through activation of phospholipase A\textsubscript{2} (Billah and Lapetina, 1982a) or during PI turnover (Marshall et al., 1980, 1982). Therefore, in order to detect any loss of arachidonic acid from PI, as well as the breakdown of PI, the effect of muscarinic receptor stimulation on arachidonyl-prelabeled PI in combined rat atria was examined. A carbachol incubation period of 15 min was chosen for these experiments for the following reasons. A detectable decrease in the level of PI after a 30 min receptor stimulation has been observed in parotid gland (Jones and Michell, 1975, 1976) and guinea pig ileum (Jones et al., 1979) and after receptor stimulation for 15 min in hepatocytes (Billah and Michell, 1979). Similarly, a maximum increase in the diacylglycerol level during acetylcholine receptor stimulation of pancreas was observed after a 15 min incubation period (Bansback et al.,
1974). As the diacylglycerol moiety is considered to be conserved during the PI response, the results in arachidonate-prelabeled PI studies were expressed as the amount of radioactivity present in PI per g of tissue, rather than as specific radioactivity.

When arachidonyl-prelabeled atria were stimulated with 0.1 mM carbachol for 15 min, the amount of radioactivity in the PI fraction of unstimulated and stimulated tissue was found to be 175 and 194 cpm/mg wet tissue weight, respectively (p>0.05) (Fig 12). This suggested that muscarinic stimulation did not cause a significant breakdown of PI in combined rat atria either through activation of phospholipase A$_2$ or phospholipase C.

3.3.3 Diacylglycerol Accumulation

Nishizuka's group (Takai et al., 1979b; Kishimoto et al., 1980) have suggested that diacylglycerol formed during PI turnover may mediate its effect through activation of phospholipid-dependent PKC. In pancreas, where ACh-stimulated PI turnover has been reported (Hokin and Hokin, 1953), an increase in diacylglycerol, as well as the arachidonyl content of diacylglycerol, has been observed by Bansback et al. (1974). As a measure of inositol lipid breakdown, the effect of muscarinic receptor stimulation on the arachidonyl content of diacylglycerol in combined rat atria was examined.

Diacylglycerol was separated from phospholipids by thin layer chromatography in benzene/chloroform/methanol (80:15:5), as shown in Fig 13. Phospholipids remained at the origin, while
diacylglycerol moved with an R value of 0.53. When 2-mercaptoethanol was present a preliminary separation of fatty acid methyl esters by thin layer chromatography in benzene was done prior to gas chromatography. Spots corresponding to standard methyl stearate were removed and extracted in hexane. Fatty acid methyl esters were then analyzed by gas chromatography. Both methyl stearate and methyl arachidonate have a similar mobility in benzene, and therefore the absence of the latter in the diacylglycerol fraction is not the result of its loss during TLC in benzene. The standard fatty acid mixture was very well separated under the column conditions used, with the following retention values (min) – palmitate 3.04, stearate 5.13; olate 5.95; arachidate 8.88; linoleate 9.88 and arachidonate 17.23. The fatty acid composition of diacylglycerol in carbachol-treated and -untreated tissue is shown in Table II. Both carbachol-treated and -untreated tissues showed only 4 fatty acids in the diacylglycerol fraction; they were palmitic, stearic, oleic and linoleic acid, while arachidonic acid was absent (Fig 14).

3.3.4 Fatty Acid Composition Of The Polar Lipid Fraction In Atria And Ventricles

In order to verify that the absence of arachidonic acid was not the result of its degradation during isolation, the fatty acid composition of the polar lipid fraction remaining at the origin after separation of the diacylglycerol was determined. In both atria and ventricles about 65% of the total fatty acid
content was saturated fatty acid and the remainder was unsaturated fatty acid. Atria and ventricles did not differ in the content of the two saturated fatty acids, but differed significantly in the content of all three unsaturated fatty acids, oleic, linoleic and arachidonic acids (Fig 15). Recently, Charnock et al. (1983) arrived at similar conclusions after a more detailed investigation of the phospholipids in rat atria and ventricles.

The studies in heart indicated that while the alpha-adrenergic receptor in rat atria is coupled to PI turnover, the majority of the muscarinic receptor stimulation in rat atria is not accompanied by enhanced PI turnover. The lack of a carbachol effect on inositol lipid breakdown also rules out the possibility that carbachol may be stimulating the primary step in PI turnover without activating the accompanying resynthesis. It is therefore suggested that only a small population of muscarinic receptors in rat atria is coupled to PI turnover and the PI breakdown accompanying muscarinic receptor stimulation is either absent or so small that it could not be detected by the methods used in this investigation.

3.4 PI RESPONSE IN GUINEA PIG ILEUM

Muscarinic receptor stimulation of guinea pig ileum produces a biphasic contraction which is shown to be mediated through calcium mobilization (Chang and Triggle, 1973). Jafferji and Michell (1976a,b) have reported that in longitudinal smooth muscle of guinea pig ileum stimulation of
muscarinic receptor or K+-depolarization produced an enhanced incorporation of radiolabeled-phosphate into PI. In the present study, the PI response in guinea pig ileum has been further investigated in order to examine its possible role in calcium mobilization.

3.4.1 $[^{32}P]$Phosphate Incorporation

The effect of muscarinic receptor stimulation on $[^{32}P]$phosphate incorporation into PI in guinea pig ileum was carried out at the beginning of this study to verify our ability to detect a PI response. In agreement with an earlier report (Jafferji and Michell, 1976a), muscarinic receptor stimulation of longitudinal smooth muscle of guinea pig ileum with 0.1 mM carbachol caused a 100% increase in the incorporation of $[^{32}P]$phosphate into PI, and this increase was blocked by the specific muscarinic antagonist, atropine (1.6 μM) (Fig 16).

3.4.2 $[^{3}H]$Inositol Incorporation

Agonists which increased labeled phosphate incorporation into PI also increased incorporation of labeled inositol into PI in several tissues (for a list of tissues examined see Michell, 1975). As in a previous report by Schellenberg and Gillespie (1980), it was observed in preliminary experiments that 98% of the $[^{3}H]$inositol label incorporated into the major phospholipid fraction was present in inositol lipids. Therefore, in all subsequent inositol incorporation studies, the incorporation of radioactivity into the total lipid fraction was used as a
measure of inositol incorporation into inositol-containing lipids. In the presence of carbachol (0.1 mM), the incorporation of \[^3\text{H}\]inositol was increased from 368 to 553 dpm/μg phosphorus (Table III). This represents a 99% increase when the radioactivity incorporated during the initial 30 min preincubation period was subtracted. The increase in \[^3\text{H}\]inositol incorporation caused by carbachol was completely blocked by 16 μM atropine (Table III).

3.4.3 Lithium-amplified Accumulation Of Inositol Phosphates

Berridge et al. (1982) have shown that lithium causes accumulation of inositol phosphate during PI response in a number of tissues. In this study the effect of carbachol on the accumulation of inositol phosphates was studied in the presence of 10 mM lithium for 60 min, conditions which were previously found to be optimal (Berridge et al., 1982).

In the presence of 10 mM lithium or 0.1 mM carbachol alone there was a very small accumulation of radioactivity (31 and 23.5 dpm/μg total phosphorus, respectively) (Table IV). In the presence of lithium, 0.1 mM carbachol produced a 20-fold increase in the accumulation of radioactivity, compared to the effect of carbachol or lithium alone (Table IV). The accumulation of inositol phosphates by carbachol in the presence of lithium was dose-dependent, as shown in Fig 17. This response to carbachol was blocked by the muscarinic antagonist, atropine (16 μM) (Fig 17), suggesting that the response results from muscarinic receptor stimulation. The half maximal response
for the carbachol response was around 10 μM. This is similar to the carbachol concentration for half maximal PI response obtained using [³²P]phosphate incorporation (Jafferji and Michell, 1976a).

3.4.4 Identification Of Inositol Phosphates

Berridge et al. (1982) have reported, based on elution from an ion-exchange resin column and high voltage electrophoresis that 80% of the radioactivity accumulated during lithium-amplified PI response in brain, parotid gland and salivary gland corresponds to inositol mono-phosphate. As in the previous report (Berridge et al., 1982), standard inositol mono-phosphate was found to be quantitatively eluted from the ion-exchange resin with 5 mM borax plus 150 mM ammonium formate (Fig 18). However, less than 20% of the radioactivity which accumulated following incubation of guinea pig ileum with carbachol and lithium, and which was then adsorbed on a Dowex-1 X8-formate resin, was eluted in a peak corresponding to inositol monophosphate (Fig 19, Table V). The remainder was eluted by higher ionic strength solutions. This result suggests that the majority of the radioactivity accumulated in the presence of carbachol and lithium was present as inositol polyphosphates. Based on their elution characteristics from the ion exchange resin (Berridge et al., 1982, Downes and Michell, 1981), 2/3 appeared to be inositol biphosphate and 1/3 inositol triphosphate.
3.5 **EFFECT OF PMSF ON PI TURNOVER AND CONTRACTION IN GUINEA PIG ILEUM**

3.5.1 **Effect Of PMSF On PI Turnover**

Recently, Walenga et al. (1980) reported that serine esterase inhibitors may also be inhibitors of PI-specific phospholipase C, based on their studies in platelets. It was decided to investigate whether serine esterase inhibitors, such as PMSF, can inhibit PI-specific phospholipase C in the ileum, and if they did, what their effect was on the calcium mediated response in ileum. The effect of PMSF on basal $[^3\text{H}]$inositol incorporation into phospholipid of the longitudinal smooth muscle of guinea pig ileum is shown in Fig 20. After an initial 30 min preincubation in the absence of PMSF, basal $[^3\text{H}]$inositol incorporation was determined and shown to remain linear with time for a further 30 min, both in the presence of 2 mM PMSF and in its absence. At all time points studied, PMSF caused a small but consistent increase in $[^3\text{H}]$inositol incorporation into PI.

Jafferji and Michell (1976b) have previously shown that stimulation of guinea pig ileum by potassium, which mobilizes calcium by opening a voltage-dependent calcium channel, also produced an enhanced incorporation of labeled phosphate into PI. The effect of 2 mM PMSF on carbachol and $K^+$-stimulated $[^3\text{H}]$inositol incorporation is shown in Fig. 21. PMSF (2 mM) alone caused a slight increase in $[^3\text{H}]$inositol incorporation, but it almost completely blocked the carbachol-stimulated
increase in inositol incorporation, to a value similar to that obtained with PMSF alone (Fig 21a). In contrast, PMSF caused a slight increase, from 167 to 187%, in K⁺-stimulated [³H]inositol incorporation into PI (Fig 21b). This suggested, first, that PMSF inhibition of carbachol-stimulated inositol incorporation is not a nonspecific effect and second, carbachol and K⁺-stimulated [³H]inositol incorporation may be occurring through different mechanisms.

3.5.2 Effect Of PMSF On Contraction

Both carbachol and K⁺ produce a biphasic contraction of guinea pig ileum, which is mediated by Ca²⁺ mobilization (Chang and Triggle, 1973; Rosenberger and Triggle, 1979). If PI turnover is involved in calcium mobilization, from the observations made above one would expect that PMSF will affect carbachol-stimulated, but not K⁺-stimulated, contraction. Addition of 0.4 mM to 2 mM PMSF to guinea pig ileum strips, contracted either by carbachol or K⁺, produced a rapid transient relaxation (Fig. 22a). The peak of relaxation was achieved within 1 min and the tension returned to control levels, even at the highest PMSF concentration, in 8 minutes. When PMSF was added prior to the addition of carbachol or K⁺, it also inhibited the initial phasic responses to both agents. However, the degree of the inhibition of the phasic response depended on the time elapsed between the addition of PMSF and the contracting agent, and no inhibition was observed after a 10 min time interval. In contrast to the transient nature of the PMSF
effect on carbachol-mediated contraction in guinea pig ileum, its inhibition of carbachol-stimulated $[^{3}\text{H}]$inositol incorporation in the same tissue was effective over a 30 min period, suggesting that these two effects of PMSF may be unrelated.

The suggestion that PMSF is capable of inhibiting PI-specific phospholipase C during PI turnover is based on indirect evidence. It was necessary to verify whether PMSF inhibition of carbachol-stimulated PI turnover in guinea pig ileum is indeed due to inhibition of PI-specific phospholipase C. In order to test this, the effect of PMSF on inositol lipid breakdown was investigated.

3.5.3 Effect Of PMSF On Inositol-Phosphate Accumulation

To determine the effects of PMSF on inositol-lipid breakdown, lithium-amplified accumulation of inositol phosphates was used as a measure of the primary response of PI turnover. In preliminary experiments PMSF was shown to cause an 18% decrease in carbachol-stimulated $[^{3}\text{H}]$inositol accumulation into inositol phosphates in the presence of lithium (Table VI). As less than 20% of the accumulated $[^{3}\text{H}]$inositol phosphates have been shown to be inositol monophosphate (Fig 19), it was examined whether PMSF specifically inhibited the accumulation of the inositol mono-phosphate fraction. Selective elution of the inositol mono- and poly-phosphate from an ion exchange resin indicated that PMSF caused a similar (14.8 and 16.4%) decrease in the accumulation of the inositol mono- and poly-phosphate
pool, but the decrease was statistically significant (p<0.05) only in the latter case. This suggested that only a small part of the PMSF inhibition of carbachol-stimulated PI turnover is mediated through inhibition of PI-specific phospholipase C.
Figure 4 - Phospholipid separation

Two dimensional thin layer chromatographic separation of the rat atrial phospholipids in Merck precoated silica gel plates. Lipids were extracted from a single rat left or right atria as described in the Methods. Lipids from four separate samples were spotted on four corners of the silica gel plate. Plates were first developed in chloroform/ methanol/ 10.5 M ammonium hydroxide (17:7:1 v/v). After removal of ammonia by air drying, plates were developed in the second dimension consisting of chloroform/ acetone/ methanol/ acetic acid/ water (3:4:1:1:0.5 v/v). Phospholipids were detected by exposure to iodine vapour. a) Photograph of a TLC plate. b) Tracing of one of the four quadrants of the TLC plate.
Table I - Phospholipid distribution and [\(^{32}\)P]phosphate label incorporation into phospholipids of rat atria

Lipids were extracted from atria following incubation with 40\(\mu\)Ci \[^{32}\)P]phosphate for 120 min. Total lipid phosphorus was determined before spotting the sample on the TLC plate. After separation of the phospholipid spots, the silica gel was extracted once with 2 ml of the extracting medium (as described in Methods), and phosphorus and radioactivity determined in the eluant. The amount of total phosphorus and total radioactivity in the four phospholipids from four atrial samples were 2.5 \(\mu\)g and 4210 cpm respectively. In B) the amount of phosphatidylinositol and phosphatidylcholine from 16 atrial samples present per 470 \(\mu\)g of total lipid phosphorus was determined. As one g wet weight of rat heart has been reported to contain 470 \(\mu\)g of lipid phosphorus (White, 1973), results are reported per g tissue weight.

<table>
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<th>Spot</th>
<th>Phospholipid</th>
<th>% phosphorus</th>
<th>% (^{32})Pi incorporation</th>
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<td>a</td>
<td>Phosphatidylinositol</td>
<td>6.3 ± 1.2</td>
<td>50.0 ± 3.0</td>
</tr>
<tr>
<td>b</td>
<td>Phosphatidylserine</td>
<td>4.2 ± 0.6</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>c</td>
<td>Phosphatidylcholine</td>
<td>46.1 ± 1.1</td>
<td>35.7 ± 0.8</td>
</tr>
<tr>
<td>d</td>
<td>Phosphatidylethanolamine</td>
<td>40.0 ± 2.2</td>
<td>10.5 ± 1.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>(\mu)g Phosphorus per 470 (\mu)g total lipid phosphorus</th>
<th>Reported White 1973</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylinositol</td>
<td>16.3 ± 0.9</td>
<td>17.4</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>126.6 ± 4.0</td>
<td>169.2</td>
</tr>
</tbody>
</table>
Figure 5 - Standard curve for phosphorus.

The standard curve for phosphorus was obtained with sodium dihydrogen phosphate. Points are the mean of duplicate observations from one of several such experiments.
Figure 6 - Comparison of the basal phosphate incorporation into various phospholipids in atria and ventricles of rat heart.

Incorporation of $[^{32}\text{P}]$phosphate was measured over a period of one hour. Results are calculated as the amount of radioactivity incorporated per microgram of phospholipid phosphorus. Histograms represent phosphatidylserine (A), phosphatidylinositol (C), phosphatidylcholine (E) and phosphatidylethanolamine (G) in rat atria. Histograms B, D, F and H represent the same series of phospholipids from rat ventricles. Bars represent the mean ($\pm$ S.E.M) of three observations. Similar results were obtained in one other experiment.
Figure 7 - Effect of carbachol and methoxamine on $[^{32}\text{P}]$ incorporation into PI in combined rat atria.

Combined rat atria were incubated with buffer (A), with 0.1 mM carbachol (B), with 0.1 mM methoxamine (C) or with 0.1 mM methoxamine plus 20 $\mu$M phentolamine (D) for 60 min in the presence of $[^{32}\text{P}]$sodium orthophosphate. The mean $[^{32}\text{P}]$ incorporation into PI in the control sample was 863 cpm/µg phosphorus. Bars represent the mean ± S.E.M. The numbers in parentheses represent the sample size. Results were obtained by pooling together data from three separate experiments. * represents statistically significant increase (p<0.05) over control.
SPECIFIC RADIOACTIVITY (% CONTROL)

A

B

C

D

(4)

(14)

(11)

(10)

50
100
150
200
Figure 8 - Effect of methoxamine stimulation on $[^{32}\text{P}]$phosphate incorporation into various phospholipids in combined rat atria.

Combined rat atria were incubated with or without 0.1 mM methoxamine or with 0.1 mM methoxamine and 20 μM phentolamine for 75 min in the presence of 54 μCi $[^{32}\text{P}]$sodium orthophosphate. Histograms represent phosphatidylserine (A), phosphatidylinositol (C), phosphatidylcholine (E) and phosphatidylethanolamine (G), respectively, in control samples, and histograms B, D, F and H represent the same series of phospholipids from methoxamine-treated samples. Histogram DP represents the phosphatidylinositol obtained from methoxamine plus phentolamine-treated sample. Bars represent the mean ± S.E.M. of four samples.
Figure 9 - Effect of methoxamine stimulation on $[^{32}\text{P}]$phosphate incorporation into phosphatidylinositol of separate rat left and right atria.

Left and right atria were incubated separately with $[^{32}\text{P}]$phosphate in the presence of buffer or 0.1 mM methoxamine for 60 min. Histograms A, B and C, D represent control and methoxamine-treated tissue of left (A,B) and right (C,D) atria, respectively. Results are expressed as percent of left atrium control. Mean incorporation into left atrium was 3550 cpm/µg phosphorus. Bars represent the mean ± S.E.M. of four samples, from a single experiment. * represents statistically significant increase (p<0.05) over control.
Figure 10 - Effect of carbachol on $[^{32}\text{P}]$phosphate incorporation into phosphatidylinositol of separate rat left and right atria.

Left and right atria were incubated separately with $[^{32}\text{P}]$sodium orthophosphate in the presence of buffer or 0.1 mM carbachol for 60 min. Histograms A, B and C, D represent control and carbachol-treated left (A,B) and right (C,D) atria, respectively. Mean incorporation into rat left atrium was 1612 cpm/µg phosphorus. Bars represent the mean ± S.E.M. Numbers in parentheses indicate the sample size. Results are pooled from two separate experiments. * represents statistically significant (p<0.05) increase over the corresponding control.
Figure 11 - Effect of carbachol on phospholipids prelabeled with $[^{32}P]$phosphate in combined rat atria.

Combined atria, preincubated with 40 μCi $[^{32}P]$sodium orthophosphate for 2 h, were incubated for a further 45 min period with or without 0.1 mM carbachol in the absence of radiolabeled-phosphate. Histograms A, C, E and G represent phosphatidylserine, phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine in the control sample, and histograms B, D, F and H represent the same series of phospholipids from carbachol-treated samples. Bars represent the mean ± S.E.M. of three samples, from a single experiment.
Figure 12 - Effect of carbachol on phosphatidylinositol prelabeled with arachidonic acid in rat atria.

Combined atrial preparations, after prelabeling with $[^{14}\text{C}]$arachidonic acid, were washed with Krebs buffer containing 1% bovine serum albumin and incubated further for a 15 min period with buffer (A) or with 0.1 mM carbachol (B). The mean incorporation in the control sample was 175 cpm/mg of wet tissue weight. Bars represent the mean ± S.E.M. Numbers in parentheses indicate the sample size. Results are pooled from two separate experiments.
(¹⁴C)-ARACHIDONIC ACID (± CONTROL)
Figure 13 - Separation of diacylglycerol.

Thin layer chromatographic separation of diacylglycerol from fatty acids and phospholipids in benzene/chloroform/methanol (80:15:5). a, b, c and d represents phosphatidylinositol, phosphatidylcholine, myristic acid and 1,2-dioleate, respectively.
Table II - Effect of carbachol stimulation on arachidonyl content of diacylglycerol.

Incubation of tissues and determination of fatty acid composition is described in the Methods. Fatty acid content is expressed in µg/g tissue. The diacylglycerol levels (µmoles/g tissue) were calculated from the fatty acid content of the diacylglycerol. Results are expressed as the mean ± S.E.M of the sample size given in parentheses. A similar absence of arachidonic acid was observed in one other experiment.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Carbamylcholine (0.1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacylglycerol content (µmoles/g tissue)</td>
<td>0.168 ± 0.029 (4)</td>
<td>0.142 ± 0.025 (5)^n.s.</td>
</tr>
<tr>
<td>Fatty acid composition of diacylglycerol (µg/g tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>41.4 ± 5.2</td>
<td>28.4 ± 3.2</td>
</tr>
<tr>
<td>C18:0</td>
<td>41.8 ± 7.6</td>
<td>22.0 ± 3.9</td>
</tr>
<tr>
<td>C18:1</td>
<td>9.16 ± 0.6</td>
<td>8.6 ± 0.71</td>
</tr>
<tr>
<td>C18:2</td>
<td>8.3 ± 1.8</td>
<td>10.2 ± 2.5</td>
</tr>
<tr>
<td>C20:4</td>
<td>nil</td>
<td>nil</td>
</tr>
</tbody>
</table>

n.s. Not significantly different from control (p > 0.05).
Figure 14 - Gas chromatogram of fatty acid methyl esters of diacylglycerol.

A gas chromatogram of fatty acid methyl esters of diacylglycerol from a carbachol-treated rat atria is shown. The peaks a, b, c and d correspond to methyl palmitate, stearate, oleate and linoleate. Peak e is the internal standard and point f represents the position where methyl arachidonate if present, would have eluted.
Figure 15 - Fatty acid composition of the polar lipid fraction of rat atria and ventricles.

The polar lipid fraction remaining at the origin after thin layer chromatography in benzene:chloroform:methanol (80:15:5 v/v) was extracted from the silica gel plate and its fatty acid composition determined by gas chromatography. Histograms A1, A2, A3, A4 and A5 represent the fatty acids palmitic, stearic, oleic, linoleic and arachidonic acids, respectively, in rat atria. Histograms V1, V2, V3, V4 and V5 represent the same series of fatty acids from rat ventricle samples. Bars represent the mean ± S.E.M of six samples. *, significantly different (p<0.05) from the corresponding fatty acid content in atria.
Figure 16 - Effect of carbachol on $[^{32}\text{P}]$phosphate incorporation into phosphatidylinositol of longitudinal smooth muscle of guinea pig ileum.

Guinea pig ileum pieces were incubated without carbachol (A), with 0.1 mM carbachol (B) or with 0.1 mM carbachol and 1.6 $\mu$M atropine (C) for 60 min in the presence of $[^{32}\text{P}]$sodium orthophosphate. The mean $[^{32}\text{P}]$phosphate incorporation in PI in the control sample was 1638 cpm/µg phosphorus. Results are the mean ± S.E.M of the sample size indicated in parentheses. Similar results were obtained in one other experiment.
Table III - Effect of carbachol on $[^3\text{H}]$inositol incorporation into phospholipid.

Guinea pig ileum pieces were incubated with $[^3\text{H}]$inositol in the presence and absence of 0.1 mM carbachol. In preparations containing atropine, 16 µM atropine was added 5 min before the addition of carbachol. Incubation was carried out as described in the Methods, following a 30 min preincubation with $[^3\text{H}]$inositol in the absence of drugs. The % increase in radioactivity in the phospholipid fraction was calculated after subtraction of the counts incorporated during the initial 30 min preincubation of the tissue with $[^3\text{H}]$inositol ("basal") from the total 60 min incubation period. The radioactivity represents mean incorporation of $[^3\text{H}]$inositol ± S.E.M. The number of guinea pig ileum pieces is shown in parentheses. Similar results were obtained in four other experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>dpm/µg total phosphorus</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>182 ± 27 (4)</td>
<td>-</td>
</tr>
<tr>
<td>no addition</td>
<td>368 ± 32 (5)</td>
<td>100</td>
</tr>
<tr>
<td>Carbachol (0.1 mM)</td>
<td>553 ± 29 (6)</td>
<td>199</td>
</tr>
<tr>
<td>Carbachol (0.1 mM) + atropine (16 µM)</td>
<td>386 ± 34 (4)</td>
<td>110</td>
</tr>
</tbody>
</table>
Table IV - Carbachol-stimulated accumulation of inositol phosphate.

Guinea pig ileum pieces were incubated for 60 min in a medium containing [$^3$H]inositol, with or without carbachol, and in the presence or absence of 10 mM lithium (see Methods). Total accumulated radioactivity was eluted from the resin with 0.1 M formic acid plus 1 M ammonium formate. Phosphorus content was determined in aliquots of the organic phase. Results are given as the mean ± S.E.M. The number of guinea pig ileum pieces is shown in the parentheses. Similar results were obtained in several other experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>dpm/μg total phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>3.21 ± 1.9 (4)</td>
</tr>
<tr>
<td>Lithium (10 mM)</td>
<td>31.0 ± 2.6 (4)</td>
</tr>
<tr>
<td>Carbachol (0.1 mM)</td>
<td>23.5 ± 2.1 (4)</td>
</tr>
<tr>
<td>Carbachol (0.1 mM) + Lithium (10mM)</td>
<td>492.0 ± 34.0 (6)</td>
</tr>
</tbody>
</table>
Figure 17 - Dose-response curve for carbachol-stimulated inositol phosphate accumulation in guinea pig ileum.

The dose-response curve for carbachol-stimulated inositol phosphate accumulation was determined in the presence of 10 mM lithium, with (■) or without (●) 6.4 μM atropine. Each point is the mean of duplicate incubations from a single experiment.
200 μl of 1 mg/ml inositol-2-phosphate dl-monocyclohexylamine salt was loaded onto a Dowex-1 X8-formate ion exchange resin column. The column was washed with water, followed by elution with 5 mM borax plus 60 mM ammonium formate at A and 5 mM borax plus 150 mM ammonium formate at B. Inositol monophosphate was identified by phosphorus determination in the eluant fractions. 14.2 μg phosphorus was eluted from the column between fractions 11 and 20, thereby suggesting a complete recovery of inositol mono-phosphate.
Inositol phosphates accumulated during incubation of guinea pig ileum pieces with carbachol in the presence of lithium were loaded onto a Dowex-1 X8-formate ion-exchange resin column. The column was washed with 5 mM inositol and eluted successively with 5 mM borax plus 60 mM ammonium formate, 5 mM borax plus 150 mM ammonium formate and 0.1 M formic acid plus 1 M ammonium formate at A, B and C, respectively. Radioactivity was determined in 1 ml fractions of the eluant. The peak radioactivity eluted with solution B and solution C were compared to determine the relative amount of two inositol phosphates. In a similar experiment control unstimulated tissue showed no radioactivity peaks on elution with increasing ionic strength solutions.
Table V - Composition of inositol phosphates accumulated in guinea pig ileum.

Guinea pig ileum fragments were incubated in a medium containing \[^3\text{H}\]inositol with lithium (10 mM) and carbachol (0.1 mM) for 60 min. Inositol phosphates were eluted from the resin as described in the Methods. The results are expressed as the mean ± S.E.M. from four tissue samples. Similar results were obtained in one other experiment.

<table>
<thead>
<tr>
<th>Elution medium</th>
<th>Percent radioactivity eluted (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM Borax + 60 mM ammonium formate</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>5 mM Borax + 150 mM ammonium formate</td>
<td>16.9 ± 1.7</td>
</tr>
<tr>
<td>0.1 M Formic acid + 0.4 M ammonium formate</td>
<td>50.8 ± 0.9</td>
</tr>
<tr>
<td>0.1 M Formic acid + 1.0 M ammonium formate</td>
<td>24.3 ± 0.7</td>
</tr>
</tbody>
</table>
Figure 20 - Time course of the effect of PMSF on basal $[^3H]$inositol incorporation into phospholipid in guinea pig ileum.

Ileum pieces were preincubated with 30 μCi of $[^3H]$inositol for 30 min. Further incubation was carried out in the presence (■) and in the absence (●) of 2 mM PMSF, as shown in the figure. Radioactivity was determined in the lipid fraction and the results are reported as dpm/μg lipid phosphorus ± S.E.M of three samples.
Figure 21 - Effect of PMSF on carbachol- and K⁺-stimulated \[^{3}\text{H}]\text{inositol} incorporation into phospholipids of guinea pig ileum.

a) After preincubation of ileum pieces with \[^{3}\text{H}]\text{inositol} the ileum pieces were further incubated for 30 min with no addition (A), 2 mM PMSF (B), 0.1 mM carbachol (C) and 0.1 mM carbachol plus 2 mM PMSF (D).

b) After preincubation of ileum pieces with \[^{3}\text{H}]\text{inositol} the ileum pieces were further incubated for 30 min with no addition (A), 60 mM potassium (B) and 60 mM potassium plus 2 mM PMSF (C).

Bars represent the mean ± S.E.M. The numbers in parentheses represent the sample size. The mean \[^{3}\text{H}]\text{inositol} incorporation in the control experiment during the last 30 min of incubation was 180 dpm/μg lipid phosphorus (Fig 21a) and 166 dpm/μg lipid phosphorus (Fig 21b). Similar results were obtained in two other experiments.
Figure 22 - Effect of PMSF on carbachol- and K⁺-stimulated contraction of guinea pig ileum

Guinea pig ileum was contracted with either 1μM carbachol or 60 mM potassium. 2 mM PMSF was added as indicated by the arrows (a). PMSF inhibition of the phasic contraction and the transient nature of this effect is shown in (b). The first tracing in each set shows the K⁺-stimulated contraction prior to PMSF addition. Following this, 0.4 mM PMSF was added as indicated at the arrow and tissue restimulated 1, 2 or 10 min later with K⁺. Dose dependent inhibition by PMSF of K⁺-stimulated phasic response is shown in (c). PMSF (0.4 mM) was added 1 min before stimulation with 60 mM K⁺. Results are expressed as fractional inhibition of the phasic response compared to the PMSF-untreated tissue.
Table VI - Effect of PMSF on the accumulation of carbachol-stimulated inositol phosphates in guinea pig ileum.

Incubation with \([^3H]\)inositol and determination of total inositol phosphates (TIP), inositol mono-phosphate (IP) and inositol bi- and tri-phosphates (IP$_2$/IP$_3$) were determined in the presence of 10mM lithium as described in the Methods. PMSF was used in a concentration of 2 mM in dimethylsulfoxide. The control without PMSF contained an equivalent amount of dimethylsulfoxide (1%). Results are reported from two separate experiments. TIP was measured in one experiment and IP$_2$ and IP$_3$ were measured in another experiment. Results are reported as the mean ± S.E.M. The number of samples is indicated in the parentheses.

<table>
<thead>
<tr>
<th>Inositol phosphate</th>
<th>Carbachol</th>
<th>Carbachol + PMSF</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/µg lipid phosphorus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIP</td>
<td>206.1 ± 8.0 (3)</td>
<td>167.8 ± 11.6 (5)</td>
<td>18.5**</td>
</tr>
<tr>
<td>IP</td>
<td>22.3 ± 1.7 (7)</td>
<td>18.9 ± 0.8 (11)</td>
<td>14.8**</td>
</tr>
<tr>
<td>IP$_2$/IP$_3$</td>
<td>111.5 ± 6.5 (7)</td>
<td>93.2 ± 3.6 (11)</td>
<td>16.4*</td>
</tr>
</tbody>
</table>

*  p < 0.05  
**  0.05 < P < 0.06
IV. DISCUSSION

4.1 IS MUSCARINIC RECEPTOR STIMULATION IN THE HEART ACCOMPANIED BY A PI RESPONSE?

4.1.1 Phospholipid Separation And Technique Verification

As mentioned earlier, even though receptor-mediated increase in phosphate incorporation into PI is a secondary response of the PI turnover cycle, it has served as a very valuable indicator for the occurrence of PI response in a wide range of tissues. In all tissues (except one) the primary step of inositol lipid breakdown is accompanied by enhanced reincorporation of phosphate into PI. Therefore, phosphate incorporation into PI was chosen as the first method to study the PI response in rat heart.

Two-dimensional thin layer chromatography (Renkonen and Luukkonen, 1976), which has been used successfully in the past by many other groups, was used for the separation of phospholipids. One such separation using two dimensional thin layer chromatography is shown in Fig 4. The results of the phosphate incorporation studies were calculated as the amount of radioactivity incorporated per microgram of the individual phospholipid phosphorus content and reported as percent of control. First, the effect of muscarinic receptor stimulation on phosphate incorporation into PI in guinea pig ileum was studied, to verify our ability to observe a PI response. In
accordance with a previous report (Jafferji and Michell, 1976a), carbachol-stimulated phosphate incorporation into PI was observed, which was blocked by the muscarinic antagonist, atropine (Fig 16). The basal incorporation of 1638 cpm/μg of PI phosphorus corresponds to 51 cpm/nmole of PI and is comparable to the value reported previously (Jafferji and Michell, 1976a,b).

4.1.2 Basal $[^{32}P]$phosphate Incorporation In Heart

Phospholipids were also separated from rat atria (see Fig 4). By comparison with standards, spots were identified as phosphatidylinositol, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid. Although PI comprised only 6% of the four phospholipids measured in this study, it incorporated almost 50% of the total radiolabeled-phosphate incorporated into the four phospholipids (Table I). Similarly, Brown and Brown (1982) have reported a 60% incorporation of radiolabeled-phosphate into PI in mouse atria. When the phosphate incorporation into various phospholipids in atria and ventricle was compared (Fig 6), it was observed that the distribution profile of radioactivity into various phospholipids in the two tissues was similar. In both tissues, PI incorporated a large fraction of the total radioactivity incorporated into the phospholipids. However, the radioactivity incorporated into phospholipids in atria was at least 4-6 fold higher than that of the corresponding phospholipids in ventricle. The cause for this difference in basal phosphate
incorporation into atria and ventricle is not known at present, but a similar difference has been observed in canine atria and ventricle (Quist, 1982). The greater extent of label incorporation, as well as the higher muscarinic receptor density in rat atria compared to ventricle (Wei and Sulakhe, 1978), led to the choice of atria for further study.

4.1.3 **Effect Of Muscarinic And Alpha-Adrenergic Receptor Stimulation On PI Turnover In Rat Atria**

In combined rat atrial pieces, muscarinic and alpha-adrenergic receptor stimulation produced a 35% and 88% increase in phosphate incorporation into PI, respectively (Fig 7). The alpha-adrenergic-mediated enhancement in phosphate incorporation was specific to PI (Fig 8), and could be blocked by the alpha-adrenergic antagonist, phentolamine, indicating that PI response was the result of alpha-adrenergic receptor stimulation. This was consistent with the previous observation that epinephrine caused an increased incorporation of $[^{32}\text{P}]$phosphate into inositol phosphatides, both *in vivo* and *in vitro* (Gaut and Huggins, 1966; Kiss and Farkas, 1975). The lack of this response in guinea pig or cat ventricle using norepinephrine was attributed to the richness of $\beta$ rather than $\alpha$-adrenergic receptors in ventricle (Lapetina et al., 1976). More recently, Uchida *et al.* (1982) have observed a similar alpha-adrenergic receptor-mediated phosphate incorporation into PI in cultured rat myocytes. They further characterized the response to be $\alpha_1$ and calcium-independent (Uchida *et al.*, 1982).
As there was almost a two-fold enhancement in phosphate incorporation into PI in combined rat atrial pieces, which have a receptor density which is less than 50% of the muscarinic receptor density in this tissue, it was clearly evident that the smaller muscarinic effect was not due to an inability to detect enhanced phosphate incorporation during a PI response.

4.2 PI BREAKDOWN STUDIES

4.2.1 Breakdown Of Prelabeled PI

As indicated earlier, even though compensatory resynthesis nearly always accompanies inositol lipid breakdown, the precise relationship between inositol lipid breakdown and its subsequent resynthesis is still not known. It was therefore decided to study the effect of muscarinic stimulation of PI breakdown, while simultaneously testing for two other probable mechanisms (discussed below).

Increased breakdown of prelabeled PI during the PI response has been reported in blowfly salivary gland (Fain and Berridge, 1979), parotid gland (Jones and Michell, 1975), and vas deferens (Egawa et al., 1981), among several other tissues. In preliminary experiments, it was observed that stimulation of combined rat atria with 0.1 mM carbachol failed to cause an enhancement in the loss of label from either $[^{32}\text{P}]$phosphate-prelabeled PI or any of the other phospholipids (Fig 11). Following this preliminary experiment with phosphate-prelabeled phospholipids, arachidonyl-prelabeled phospholipids were used,
for the following reason. Stimulation of muscarinic receptors cause an increase in cGMP in heart (George et al., 1973; Nawrath, 1976; Diamond et al., 1977) by the activation of guanylate cyclase. The mechanism for guanylate cyclase activation is still unknown. Among several proposed activators for guanylate cyclase, two, arachidonic acid (Glass et al., 1977; Gabers et al., 1978; Spies et al., 1980) and prostaglandins (Goldberg et al., 1978; Hidaka and Asano, 1977) could be derived from PI. As release of arachidonic acid has been shown to occur from PI during platelet stimulation by thrombin (Billah and Lapetina, 1982a) and linoleic acid during isoproterenol stimulation of canine sarcolemma (Franson et al., 1979), it was investigated whether a release of arachidonic acid occurred during carbachol stimulation of combined rat atria, which could then suggest a possible mechanism for the activation of guanylate cyclase.

If stimulation in the breakdown of arachidonyl-prelabeled PI by either phospholipase A₂ or phospholipase C occurred, it would cause a decrease in the level of PI, which would then be reflected by a decrease in the total radioactivity present in PI, even if the specific activity of PI remains unchanged. Stimulation of muscarinic receptors in combined rat atria with 0.1 mM carbachol did not enhance the breakdown of arachidonyl-prelabeled PI (Fig 12). This indicated that muscarinic receptor stimulation did not cause significant activation of phospholipase C or phospholipase A₂. The lack of activation of phospholipase A₂ during muscarinic stimulation was not
surprising, as in most cases, except that of isoproterenol stimulation of cardiac sarcolemma (Franson et al., 1979), where there is a receptor-mediated activation of phospholipase A₂. Phospholipase A₂ activation is the result of an increase in cytosolic calcium concentration and the available evidence does not indicate that calcium is mobilized during muscarinic receptor stimulation in the heart.

4.2.2 Arachidonyl-Enriched Diacylglycerol

PI-specific phospholipase C-mediated breakdown of PI leads to the formation of diacylglycerol (Billah and Lapetina, 1982a; Ieyasu et al., 1982; Kaibuchi et al., 1982), which is considered to be rapidly phosphorylated by diacylglycerol kinase to give phosphatidic acid (Michell, 1975; Putney, 1981). As diacylglycerol is also an intermediate for many other phospholipid metabolic pathways, it is only found in small quantities in unstimulated tissues. Receptor stimulation produced a transient accumulation of diacylglycerol within seconds in both thyroid follicles (Igarshi and Kondo, 1980) and platelets (Billah and Lapetina, 1982a). On the other hand, a slower transient increase, over a period of 30 min, was observed during carbachol stimulation of mouse pancreas (Bansback et al., 1974) and an increased accumulation of diacylglycerol over a still longer period has recently been reported by Homa et al. (1983). Accumulation of diacylglycerol over a longer period may indicate the existence of cellular compartments which are not easily accessible to other enzymes in the metabolic pathways.
Widespread occurrence of calcium-activated, phospholipid-dependent protein kinase C (PKC), and its regulation by diacylglycerol, has raised the possibility that production of diacylglycerol during breakdown of PI or any other phospholipid may cause the activation of PKC (Minakuchi et al., 1981; Takai et al., 1979b). In mouse pancreas, muscarinic stimulation has been shown to produce both a PI response (Hokin-Neaverson, 1974) and increased accumulation of diacylglycerol (Bansback et al., 1974). In this study it was observed that in combined rat atria carbachol (0.1 mM) caused only a small (but nonsignificant, p>0.05) decrease, rather than an increase, in diacylglycerol level (Table 2).

The β-position of PI of mammalian tissue is rich in arachidonic acid (White, 1973). If PI is being degraded by phospholipase C, one would expect an increase in the arachidonyl content of diacylglycerol, similar to that observed by Bansback et al. (1974). Even though as low as 0.5 nmole of arachidonyl methyl ester could be detected by gas chromatography, no arachidonic acid was detected in either unstimulated- or carbachol-stimulated tissue. The lack of increase in arachidonic acid in the diacylglycerol fraction of carbachol-stimulated combined rat atria further supported the initial observation that there may be only a very small PI response in combined rat atria. Stimulation of pancreas by 0.1 mM carbachol for 15 min produced a 15-fold increase in the arachidonyl content of diacylglycerol, giving rise to a value as high as 290 nmole/g tissue (Bansback et al., 1974). If a similar
enhancement had occurred in rat heart, it would correspond to about 6 nmole of arachidonic acid in 20 mg tissue (approximately the amount present in each incubation) and it should have been detected by the methods used (Fig 14).

The detection of arachidonic acid in the polar lipid fraction of atria and ventricle (Fig 15) also rules out the possibility that arachidonic acid was completely destroyed during various steps involved in the preparation of fatty acid methyl ester. It was observed that while the saturated fatty acid contents of the polar lipid fraction from atria and ventricles were similar, a significant difference in the contents of all three unsaturated fatty acids was observed (Fig 15). In a more comprehensive study a similar difference in the content of unsaturated fatty acids in the phospholipid fraction of atria and ventricles has been reported (Charnock et al., 1983). The significance of this difference in unsaturated fatty acid content between atria and ventricles is not known at present, but it could play a role in some of the differences in the membrane characteristics observed between the two tissues, such as lower phosphate incorporation into phospholipids of ventricle compared to atria, as observed in this study and in that of Quist (1982).
4.2.3 $[^{32}\text{P}]$Phosphate Incorporation In Rat Left And Right Atria

All the evidence obtained so far suggested that stimulation of muscarinic receptors in combined rat atria was accompanied by either no, or a very small, PI turnover. Around that time Quist (1982) reported that while canine right atrium showed a PI response on muscarinic receptor stimulation, canine left atrium or left or right ventricle, failed to show a similar response. Another interesting characteristic of his observation was that unlike the alpha-adrenergic-mediated positive inotropic effect in cultured rat heart cells, which was shown to be calcium independent (Uchida et al., 1982), the muscarinic-stimulated PI response in canine right atrium (as determined by the increased incorporation of labeled phosphate into PI) was found to be calcium-dependent (Quist, 1982). The occurrence of a PI response in only one part of canine atrium raised the possibility that a similar situation might exist in rat atria. Stimulation of rat left and right atria by 0.1 mM carbachol produced a small (35%) but significant ($p < .05$) enhancement in phosphate incorporation into PI in rat left atrium, but no effect in rat right atrium (Fig 10). In contrast to this, stimulation of alpha-adrenergic receptors by 0.1 mM methoxamine produced an approximately two fold increase in phosphate incorporation into PI in both rat left and right atria (Fig 9). It was also observed that the relative incorporation of basal radioactivity into rat left and right atrium was variable (compare Fig 9 and 10).
4.2.4 Evidence For Two Subpopulations Of Muscarinic Receptors

Results from the carbachol stimulation of separate left and right atrium, as well as those obtained by Quist (1982), suggested the interesting possibility that while the majority of the muscarinic receptors in rat left and right atrium were not coupled to PI turnover, a small population of muscarinic receptors in rat left atrium may be coupled to PI turnover. In most tissues studied so far, the PI response to alpha-adrenergic stimulation is usually weaker than the muscarinic receptor stimulation in the same tissue (Jones and Michell, 1975). Nevertheless, assuming an equal coupling efficiency between receptor and the PI turnover cycle for both alpha-adrenergic and muscarinic receptors in rat atrium and based on the relative receptor density (muscarinic = 220 pmoles/g protein, Wei and Sualkhe, 1978; alpha adrenergic = 100 pmoles/g protein, Karliner et al., 1982)) and the relative PI responses obtained (Fig 9 & 10), it was calculated that only a small population (approximately 15%) of the muscarinic receptors in rat atrium may be coupled to PI turnover. The absence of inositol lipid breakdown during muscarinic receptor stimulation of atria (Fig 11, 12 and Table II) may seem to contradict the finding that a small population of muscarinic receptors may be coupled to PI turnover. However, Farese et al. (1982) have reported that in calcium-depleted media carbachol stimulation of submaxillary gland caused a four-fold enhancement in phosphate incorporation without decreasing the level of PI, while in calcium-containing
media both an increase in phosphate incorporation and a decrease in the level of PI was observed during carbachol stimulation. Therefore, it is possible that PI breakdown and phosphate incorporation may be dissociable events or that breakdown resulting from such a small population of muscarinic receptors could not be detected in combined atrial preparations.

It is now well recognized that most receptors can be considered to be composed of two distinct functional subunits, a recognition subunit and an effector subunit. Various receptors (recognition subunits is more appropriate, if one defines a receptor as a combination of both recognition and effector subunits) can be coupled to the same effector subunit e.g. beta-adrenergic receptor, H₂, glucagon receptors, which are coupled to adenylate cyclase.

On the other hand, the possibility that the same recognition subunit may be coupled to more than one effector subunit in different tissues (Richelson and El-Fakahany, 1981) or even in the same tissue (Hartzell, 1982) has been recognized only very recently. Identification of a single recognition unit coupled to different effector systems may require further subclassification of these receptors, which have previously been classified based mainly on binding studies. The two subclasses of alpha-adrenergic receptors, α₁ and α₂, have similar affinities for the physiological neurotransmitter, norepinephrine (Exton, 1982), but they appear to be coupled to different effector systems (Garcia-Sainz and Pain, 1982). The response of α₁ is suggested to be mediated by stimulation of PI
turnover while the $a_2$-receptor is suggested to exert its effects through inhibition of adenylate cyclase (Garcia-Sainz and Fain, 1982; Levitzki, 1982). An interesting example of how a single recognition unit can be coupled to two different effector systems has been provided by Morgan et al. (1983) in hepatocytes. They observed that in hepatocytes derived from juvenile rats, the response of $a_1$-receptor stimulation is mediated only by calcium mobilization, but in hepatocytes from adult rats, $a_1$-receptor stimulation was shown to be accompanied by both cAMP elevation and calcium mobilization (Morgan et al., 1983).

Heterogeneity in muscarinic receptors based on binding studies has been postulated for some time (Burgen et al., 1974; Strange et al., 1977; Yamamura and Snyder, 1974). Recent suggestions that muscarinic receptors in heart (Hartzell, 1982) and sympathetic neurones (Brown and Adams, 1980; Horn and Dodd, 1981; Weight et al., 1979) may be coupled to more than one type of channel indicates an effector-dependent heterogeneity, which may or may not be distinguished by antagonist binding.

Jones et al. (1981) have recently speculated that there may be two subclasses of muscarinic receptors similar to alpha-adrenergic receptors. The present results, as well as those of Quist (1982), support a subclassification of the muscarinic receptor. It is further proposed that the majority of the muscarinic receptors in rat left and right atrium and canine left atrium (Quist, 1982) may belong to the muscarinic receptor subtype which is not coupled to PI turnover and which will be
called $m_2$. However, a significant population of muscarinic receptors in mouse atria (Brown and Brown, 1982), canine right atrium (Quist, 1982) and a very small population of muscarinic receptors in rat left atrium (Fig. 10) (along with muscarinic receptors in many other tissues where a PI response has been reported) may belong to the subclass $m_1$ (analogous to $a_1$ of adrenergic receptors) which is coupled to PI turnover. The ability of muscarinic receptor stimulation to decrease cAMP levels in cardiac tissue (George et al., 1973; Watanabe et al., 1978) may suggest that similar to $a_2$ adrenergic receptors (Garcia-Sainz and Fain, 1982), $m_2$ muscarinic receptors may be coupled to adenylate cyclase through an inhibitory GTP binding protein (Rodbell, 1980).

Previous suggestions for multiple cholinergic receptor subtypes in heart were presented by Buccino et al., (1968). They found that while a low concentration of Ach produced a negative inotropic effect, at higher concentrations ACh produced a positive inotropic effect. This led them to suggest that there may be two types of cholinergic receptor sites. But the inability of the ACh-mediated positive inotropic response to be blocked by either atropine or hexamethonium argued against the responses being muscarinic or nicotinic in nature.

The muscarinic- and alpha-adrenergic-stimulated PI responses in heart appear to differ in their calcium requirements. Quist (1982) reported that carbachol-stimulated PI response in canine right atrium required calcium, while alpha-adrenergic-stimulated PI response in cultured rat heart
cells was found to be calcium-independent (Uchida et al., 1982). Presently the functional significance of the m₁ receptor subtype in heart is not known, but the presence of such receptors, along with their possible calcium requirement for PI response (as in canine atrium), may account for the small positive inotropic effect observed on addition of carbachol following a prior stimulation with methoxamine in rabbit papillary muscle (Endoh and Motomura, 1979).

As this subclassification of muscarinic receptors is based only on the nature of the effector subunit coupled to the recognition subunit, the muscarinic receptor subtypes may not necessarily be differentiated by binding studies. However, it is interesting to note that Gibson et al. (1983) have recently subclassified the muscarinic receptor in ventricular muscle as m₁ and caudate/putamen as m₂ based on their affinity for various QNB derivatives.

It is even more difficult to speculate at present on the reason for the differences in the relative distribution of the two muscarinic receptor subtypes in atria of various species, except to say that there are other receptors which show a large species variation in their distribution (McNeill and Verma, 1979; Schumann, 1980), probably representing differences in regulation in these species.

Michell has previously predicted that if PI turnover played a role in calcium mobilization, then only those receptors whose response is mediated by calcium mobilization will be accompanied by a PI response (Michell, 1979). The observation in this study
that stimulation of alpha-adrenergic receptors, which produced a positive inotropic effect through calcium mobilization, was coupled to PI turnover, while the majority of the muscarinic receptors, producing an opposite pharmacological response, did not enhance PI turnover, was consistent with the above postulate. The complete absence of a PI response in rat right atrium (and canine left atrium, (Quist, 1982)) provides the first evidence to suggest that the muscarinic receptor subtype m2, which has been proposed by Jones et al. (1982) to be coupled to adenylate cyclase, is not simultaneously coupled to PI turnover.

4.3 ROLE OF PI TURNOVER IN MUSCARINIC RECEPTOR STIMULATED CALCIUM MOBILIZATION IN GUINEA PIG ILEUM

Longitudinal smooth muscle of guinea pig ileum contains a homogeneous population of muscarinic receptors (Yamamura and Snyder, 1974). Stimulation of longitudinal smooth muscle of guinea pig ileum by muscarinic agonists or by potassium depolarisation produces a biphasic contraction (Chang and Triggle, 1973; James-Kracke and Roufogalis, 1981; Rangachari et al., 1983) as well as an enhanced incorporation of phosphate into PI (Jafferji and Michell, 1976 a,b). This led to the choice of longitudinal smooth muscle of guinea pig ileum for further investigation of the role of PI turnover in calcium mobilization.
4.3.1 Incorporation Of $[^3\text{H}]\text{inositol}$ Into PI

During a complete cycle of phosphoinositol head group turnover, one "new" phosphate and an inositol group will be incorporated into PI. Stimulation of muscarinic receptors by carbachol produced a two-fold enhancement in the incorporation of both phosphate (Fig 16) and inositol into PI (Table III). This was consistent with the previous suggestion that muscarinic receptor stimulation produces a PI response in this tissue (Jafferji and Michell, 1976a). The equal enhancement of both phosphate and inositol incorporation into PI (Fig 16, Table III) suggested that both pools of label are equally accessible to the PI undergoing turnover. Similar to phosphate incorporation, carbachol-stimulated inositol incorporation was also inhibited by atropine, suggesting the this response is muscarinic in nature.

4.3.2 Effect Of PMSF On Carbachol And K$^+$-stimulated PI Response And Contraction

Even though the PI response has now been known for over three decades, one reason for our ignorance of its functional significance is the lack of a specific inhibitor. As Michell and Kirk have pointed out, discovery of a specific inhibitor of the PI response would be a major step forward in solving the "PI puzzle" (Michell and Kirk, 1981).

Recently, Walenga et al. (1980) reported that PMSF and other serine protease inhibitors may also be inhibitors of PI-specific phospholipase C. It was decided to investigate whether
PMSF is capable of inhibiting carbachol- and K⁺-stimulated PI turnover in guinea pig ileum. It was found that while both carbachol and K⁺ stimulated labeled-inositol incorporation into PI in guinea pig ileum, only the carbachol effect was blocked by PMSF (Fig. 21), suggesting that the stimulation of inositol incorporation by carbachol and K⁺ occurred by different mechanisms. It is possible that carbachol and K⁺ may enhance the turnover of PI by different pathways or alternatively, they may activate different pools or forms of PI-specific phospholipase C, so that the carbachol, but not the K⁺-stimulated activity, is sensitive to PMSF. Hirasawa et al. (1982) have separated multiple forms of PI-specific phospholipase C by electrofocusing, and at least in two different tissues evidence has been presented for both calcium-dependent and calcium-independent PI turnover (Egawa et al., 1981; Farese et al., 1982). In this context it is interesting to note that substance P-stimulated inositol phosphate accumulation was found to be calcium-independent, while K⁺-stimulated inositol phosphate accumulation was found to be calcium-dependent (Michell, 1982). Therefore, PMSF may be a useful way of separating these effects.

If PI turnover played a role in carbachol-stimulated Ca²⁺ mobilization, PMSF (which completely inhibits carbachol-stimulated PI turnover (Fig. 2)), should also inhibit carbachol mediated contraction. However, unlike its selective inhibition of carbachol-stimulated inositol incorporation, PMSF relaxed both carbachol- and K⁺-contracted tissue and inhibited the
contraction produced by both agents. Furthermore, in contrast to the effects on PI turnover, the effects on contraction were transient (Fig 22). These results indicate that the effect of PMSF on PI turnover and contraction are unlikely to be correlated. The half life for PMSF degradation in Tris and HEPES buffer at pH 8 and 25°C has been reported to be 35 min (James, 1978), but it appeared that the degradation rate of PMSF under our incubation conditions was fast enough to rapidly diminish the effects of PMSF on contraction. On the other hand, under similar conditions PMSF gave prolonged inhibition of PI turnover. This suggests a separate mechanism for the two effects of PMSF. The relaxation by PMSF may be due to transient inhibition of calcium accumulation by nonspecific interaction with the membrane or other cellular components. A recent report by Harris et al. (1983) showed that PMSF inhibited K+-depolarized Ca\(^{2+}\) uptake in the first 10 sec but had no effect after a 15 sec period. This result may also be consistent with a transient membrane or cellular effect of PMSF, rather than its transient inhibition of PI-specific phospholipase C.

4.3.3 Is PMSF Inhibition Of Carbachol-stimulated PI Response Mediated By Inhibition Of PI-specific Phospholipase C?

The apparent dissociation of PMSF inhibition of carbachol-stimulated PI turnover and Ca\(^{2+}\)-dependent carbachol-stimulated contraction might appear to argue against a role for PI turnover in calcium mobilization. For such an argument to be valid, however, one must assume that the inhibition of the carbachol-
simulated inositol incorporation into PI by PMSF also inhibits the steps involved in calcium mobilization. Presently, both the breakdown of inositol lipids (Michell, 1975; Michell, 1982) and accumulation of phosphatidic acid (Putney et al., 1980; Serhan et al., 1981) are considered to play a role in calcium mobilization. If PMSF was indeed capable of inhibiting PI-specific phospholipase C, as proposed by Walenga et al. (1982), then both of these proposed calcium mobilizing steps would be inhibited by PMSF. Walenga et al. (1982) proposed that PMSF may be an inhibitor of PI-specific phospholipase C in platelets, based principally on the following findings: PMSF inhibition of thrombin or collagen-stimulated accumulation of phosphatidic acid could occur either by inhibition of PI-specific phospholipase C or diacylglycerol kinase, or both. Similarly, the reported PMSF inhibition of arachidonic acid release during collagen stimulation could be due to inhibition of one or more of the following: PI-specific phospholipase C, diacylglycerol lipase, or PI specific phospholipase A₂. In the same study, PMSF was also shown to inhibit arachidonic acid release from phosphatidylcholine, suggesting that at least part of its effect was mediated through inhibition of phospholipase A₂. However, the only direct evidence provided to suggest that PMSF was exerting its effect by interacting with PI-specific phospholipase C was the inhibition by PMSF of hydrolysis of purified PI by PI-specific phospholipase C. Harris et al. (1983) have assumed that PMSF-mediated inhibition of K⁺-depolarized calcium uptake was the result of its inhibition of
PI-specific phospholipase C. As direct inhibition by PMSF of diacylglycerol accumulation during PI turnover has not been demonstrated, the evidence that PMSF inhibits PI-specific phospholipase C during PI turnover needs verification. The present results argue against this mechanism in guinea pig ileum.

4.3.4 Lithium-Amplification Of PI Response In Guinea Pig Ileum

A sensitive method for measuring the primary step of PI response was found with the use of lithium to "amplify" the PI response, as suggested by Berridge et al. (1982). Lithium has been shown to inhibit inositol monophosphatase (Allison and Blisner, 1976; Sherman et al., 1981). Berridge et al., (1982) used this observation to develop a novel method for studying the PI response. In three different tissues (rat brain slices, rat parotid gland and blowfly salivary gland) stimulation of PI turnover in the presence of lithium leads to accumulation of inositol monophosphate (Berridge et al., 1982). They suggested, therefore, that lithium can act as an amplifier of the PI response.

The use of lithium to study the PI response has provided some interesting insights into the mechanism of the carbachol-stimulated PI response in guinea pig ileum. Under optimum conditions, which included 10 mM lithium and a 60 min incubation period, 0.1 mM carbachol produced a 20-fold increase in the accumulation of radioactive inositol phosphate(s) in the aqueous phase (Table IV). This was consistent with the results of
Berridge et al. (1982). In unstimulated tissue, 10 mM lithium produced only a small accumulation of radioactivity in the aqueous phase. Similar observations were reported by Berridge et al. (1982). This was attributed to the very small PI turnover in unstimulated tissue (Berridge et al., 1982). If it is assumed that the specific activity of labelled inositol pool is same as the labelled inositol added to the incubation medium and that in the presence of lithium all the inositol phosphate being accumulated is obtained from the PI turnover cycle, then assuming that one molecule of inositol phosphate is released per PI turnover cycle, it was calculated that $2.52 \times 10^{17}$ molecules of inositol phospholipid undergo turnover in 60 min in the unstimulated tissue (Table IV). On the other hand, if all the inositol incorporated into inositol phospholipid in the unstimulated tissue (Table III) occurred during the PI turnover cycle, a turnover rate of $28.5 \times 10^{17}$ molecules per hour was calculated. This was 10 times higher than the rate obtained from inositol phosphate accumulation studies. As inositol incorporation into inositol phospholipids could occur by mechanisms other than the PI turnover cycle, it would appear that only about 10% of the basal inositol incorporation into inositol phospholipid is due to the PI turnover cycle.

In the presence of lithium, carbachol produced a dose-dependent accumulation of inositol phosphate in the guinea pig ileum and this was blocked by the muscarinic antagonist, atropine (Fig 17). The half maximal response was obtained at a carbachol concentration of around 10 μM (Fig 17), similar to the
value reported by Jafferji and Michell (1976a) for carbachol-stimulated phosphate incorporation into PI. This further suggests that inositol phosphate accumulation occurred during carbachol-stimulated PI turnover.

4.3.5 Analysis Of Inositol Phosphates Accumulated During Lithium Amplification Of Carbachol-stimulated PI Response In Guinea Pig Ileum

The elution profile of inositol phosphates, released following carbachol stimulation, from the Dowex-1 X8 formate ion exchange resin column, gave different results to those reported by Berridge et al. (1982) in other tissues. Inositol monophosphate, eluted from the column by 5 mM borax plus 160 mM ammonium formate (Fig 18), accounted for only 17% of the total accumulated radioactivity (Fig 19). The majority of the radioactivity eluted corresponded to inositol biphosphate and inositol triphosphate (Fig 19, Table V).

Although it was previously considered that the primary event of receptor stimulation on PI turnover was the enhanced breakdown of PI (Hokin and Hokin, 1964; Jafferji and Michell, 1976a), more recently it has been suggested that in a number of tissues the primary step of receptor stimulation is the breakdown of polyphosphoinositides (PIP and PIP$_2$) rather than of PI. The breakdown of either PIP$_2$ alone (Billah and Lapetina 1982; Weiss et al., 1982) or of both PIP and PIP$_2$ (Agranoff et al., 1983, Rhodes et al., 1983; Thomas et al., 1983) has been suggested to be the primary event. It is not known, however,
whether the same enzyme causes the breakdown of these polyphosphoinositides. When the time course of inositol phosphate released during vasopressin stimulation of superior cervical sympathetic ganglia was studied (Michell et al., 1983), it was found that while inositol triphosphate \( (IP_3) \) increased during an early period, later there was an increase of inositol bi- and mono-phosphate, respectively. This led Michell et al. (1983) to suggest that \( IP_3 \) was being converted to inositol, via inositol biphosphate \( (IP_2) \), by the following mechanism.

\[
\text{IP}_3 \longrightarrow \text{IP}_2 \longrightarrow \text{IP} \longrightarrow \text{Inositol}
\]

In lithium-amplification studies more than 80% of the accumulated radioactivity produced by various neurotransmitters in rat brain, rat parotid and blowfly salivary gland was shown to be inositol monophosphate (Berridge et al., 1982). This was attributed to the inhibition by lithium of the last step in the above sequence of events, leading to the release of inositol from \( IP_3 \).

The findings in this study suggest that carbachol causes an enhanced breakdown of polyphosphoinositides, and probably of PI. One possible explanation for the accumulation of \( IP_2 \) and \( IP_3 \) may be, in guinea pig ileum lithium may inhibit all of the enzymes involved in the sequence of reactions in the conversion of inositol triphosphate to inositol. Alternatively, lithium may block one or perhaps several inositol phosphatases responsible
for the direct conversion of inositol polyphosphate(s) to inositol and phosphate, as shown below:

\[
\begin{align*}
&\text{IP}_3 \\
&\downarrow \quad \downarrow \quad \downarrow \\
&\text{Inositol} \quad \text{Inositol} \quad \text{Inositol} \\
&\quad +3P \quad +2P \quad +P
\end{align*}
\]

In both cases, lithium will lead to the accumulation of inositol polyphosphates, as observed in this study.

4.3.6 Effect Of PMSF On Carbachol-stimulated Inositol Phosphate Accumulation

The lithium-amplification method to measure the inositol lipid breakdown was used to study the effect of PMSF on this step. In a preliminary experiment, PMSF caused an 18% inhibition of inositol phosphate accumulation, but probably due to the small sample size this difference was not significant (0.05<p<.06) (Table VI). This effect of PMSF was much smaller than its complete inhibition of inositol incorporation (Fig 24), and therefore at first it was not considered to be of much significance. Later observations that less than 20% of the total accumulated inositol phosphates corresponded to inositol monophosphate suggested that PMSF may be selectively inhibiting PI-specific phospholipase C without inhibiting polyphosphoinositide phosphodiesterase. Such a result would have been consistent with the finding of both Walenga et al.
(1980) and Downes and Michell (1981). Such was not the case, however, as PMSF also decreased (Table VI) the carbachol-stimulated accumulation of inositol polyphosphate by 17%. These results show that PMSF inhibits the breakdown of carbachol-stimulated inositol lipid, but that the effect was much smaller than its effect on carbachol-stimulated inositol incorporation. This in turn suggests that PMSF may be acting at one or more of the steps following the inositol lipid breakdown in the PI response cycle, which will account for the greater inhibition of inositol incorporation compared to PI breakdown. One possible site for PMSF inhibition might be diglyceride kinase, as Walenga et al. (1980) have shown that PMSF completely inhibits the accumulation of phosphatidic acid during stimulation of platelets by collagen and thrombin. The small effect of PMSF on the putative calcium mobilizing step (PI or PPI breakdown) may account for the lack of a prolonged effect of PMSF on carbachol-stimulated contraction. Since PMSF did not block the breakdown of PI or polyphosphoinositides by more than 15-18%, the possibility that the breakdown of one or both of these inositol lipids is involved in calcium mobilization remains open. It is interesting to note in this context that Weiss et al. (1982), using cross-receptor inactivation studies, have recently provided very convincing evidence to suggest that PIP₂ breakdown may be involved in calcium mobilization during the phasic response to substance P in parotid gland. It would be interesting to know whether PIP₂/PIP₃ breakdown plays a similar role in calcium mobilization during the phasic or tonic
responses in guinea pig ileum.
V. SUMMARY

The effect of muscarinic receptor stimulation on PI response in rat heart and guinea pig ileum have been investigated in this study, in order to understand the relationship between the PI response and calcium mobilization.

1. Comparison of the basal \( {^{32}}P \)phosphate incorporation into various phospholipids in rat atria and ventricle indicated that in both tissues PI incorporated a higher amount of radioactivity than other phospholipids. However, the incorporation of radioactivity into various phospholipids in atria was 4–6 fold higher than that in the corresponding phospholipids in ventricle. Similar differences have been recently reported in canine heart. This may represent differences in the rate of phospholipid metabolism between these two areas of the heart.

2. Stimulation of muscarinic receptors in combined rat atria by carbachol (0.1 mM) produced a small increase (\( p < 0.05 \)) in phosphate incorporation into PI. In contrast, stimulation of alpha-adrenergic receptors in combined rat atria and muscarinic receptors in longitudinal smooth muscle of guinea pig ileum were accompanied by a two-fold increase in \( {^{32}}P \)phosphate incorporation into PI.

3. Stimulation of muscarinic receptors in combined rat atria by 0.1 mM carbachol did not enhance the breakdown of PI as measured by the loss of \( {^{32}}P \)phosphate- or \( {^{14}}C \)arachidonyl-labeled PI or by the level of arachidonyl-containing diacylglycerol. This result suggested that muscarinic receptor
stimulation had a very small effect, if any, on PI turnover in rat atria.

4. Muscarinic receptor stimulation produced a different PI response in rat left and right atria. Stimulation of muscarinic receptors in rat left atria was accompanied by a small PI response (35%, p<0.05), but had no effect in rat right atria. On the other hand, stimulation of alpha-adrenergic receptors in both rat left and right atria was accompanied by an enhanced incorporation of phosphate into PI. These findings are consistent with the suggestion that PI response may accompany only the stimulation of receptors whose response is mediated by calcium mobilization. Based on the relative receptor densities and relative PI response of muscarinic and alpha-adrenergic receptor in rat heart, it is suggested that a small population (approximately 15%) of muscarinic receptors in rat atria is coupled to PI turnover. These are termed m₁ receptors, by analogy to the α₁ adrenergic receptors which enhance PI turnover and Ca²⁺ mobilization. The remaining population, termed m₂, most probably is coupled in an inhibitory manner to adenylate cyclase.

6. In addition to the enhanced incorporation of [³²P]phosphate into PI, muscarinic receptor stimulation in longitudinal smooth muscle of guinea pig ileum by carbachol (0.1 mM) produced an enhanced incorporation of [³H]inositol into PI, and also caused an increased accumulation of inositol phosphates in the presence of lithium (10 mM). The half-maximal response for carbachol-stimulated inositol phosphate accumulation was
around 10μM, similar to the value reported previously for carbachol-stimulated phosphate incorporation and in receptor binding studies. These findings provide further evidence to support the previous suggestion that muscarinic receptor stimulation in guinea pig ileum is accompanied by enhanced inositol lipid turnover and may closely accompany the receptor occupancy by the agonist.

7. PMSF, a putative inhibitor of PI response, selectively inhibited carbachol-, but not potassium-, stimulated \[^3H\]inositol incorporation into inositol lipids in guinea pig ileum. This suggested that carbachol- and potassium-stimulated PI response may occur by different mechanisms and could be differentiated on the basis of their sensitivity to PMSF.

8. In contrast to its differential effects on inositol incorporation, PMSF produced a non-specific and transient inhibition of both carbachol- and potassium-stimulated contraction of guinea pig ileum. This effect of PMSF was probably due to the nonspecific inhibition of calcium uptake rather than the result of its inhibition of PI turnover. If PMSF-mediated inhibition of carbachol-stimulated PI turnover was the result of its inhibition of PI-specific phospholipase C, the lack of an effect of PMSF on contraction (a calcium-mediated step) would argue against the role of PI turnover in calcium mobilization.

9. Analysis of the inositol phosphates accumulated during carbachol-stimulated PI turnover in the presence of lithium indicated that less than 20% of the inositol phosphate was
eluted in the peak corresponding to inositol mono-phosphate and the rest was eluted at higher ionic strength, corresponding to inositol bi- and tri-phosphate. This provided direct evidence to suggest that like some other tissues, the primary event of PI turnover in guinea pig ileum during muscarinic receptor stimulation may be the breakdown of polyphosphoinositides rather than PI.

10. Accumulation of a large proportion of inositol polyphosphates rather than inositol monophosphate in guinea pig ileum indicated either that lithium inhibited all three enzymes involved in the stepwise conversion of inositol triphosphate to inositol or it inhibited one or more enzymes involved in the direct conversion of inositol phosphates to inositol.

11. In contrast to its almost complete inhibition of carbachol-stimulated inositol incorporation, PMSF caused only a small (approximately 16%) inhibition of carbachol-stimulated inositol phosphate accumulation, indicating that inhibition by PMSF of PI-specific phospholipase C cannot completely account for the observed inhibition by PMSF of carbachol-stimulated inositol incorporation. Therefore, PMSF may inhibit other steps following the initial step of inositol-lipid breakdown. As PMSF had only a very small effect on the inositol-lipid breakdown, a putative calcium mobilizing step of PI turnover, the results obtained do not contradict the possibility that inositol-lipid breakdown is involved in calcium mobilization.


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