MUSCARINIC RECEPTOR MODULATION OF THE
PHOSPHOLIPID EFFECT IN CARDIAC MYOCYTES

THESIS

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By

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The muscarinic agonist carbachol stimulates a rapid increase in 32Pi incorporation into phosphatidic acid (PA) and phosphatidylinositol (PI) in calcium tolerant myocytes prepared from heart tissue. The density of muscarinic receptors, determined by [3H]-QNB binding, is greater in the atria than in the ventricles. 250 μM carbachol decreased specific [3H]-QNB binding to muscarinic receptors on myocyte membranes by fifty percent. Trifluoperazine, also a phospholipase C inhibitor, inhibited the carbachol stimulated increase in 32Pi incorporation into PA and PI and did not interfere with muscarinic receptor binding.

Therefore, isolated canine myocytes provide a suitable model system to further study the muscarinic receptor stimulated phospholipid effect, and its role in mediating biochemical processes and physiological function in the heart.
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Regulation of Cardiac Function by the Autonomic Nervous System

The heart is regulated by the autonomic nervous system which is subdivided into the sympathetic and the parasympathetic nervous systems. Generally, these two branches have opposing effects on the heart.

The sympathetic nervous system controls the heart by releasing the neurotransmitter norepinephrine, which acts primarily on the beta-one adrenergic receptors on the myocytes (Guyton, 1971). In addition, it can act on the alpha-one adrenergic receptors in the vasculature of the heart (Guyton, 1971). Stimulation of the sympathetic nervous system increases the heart rate, force of contraction and the refractory period of the action potential through activation of beta-one adrenergic receptors.

The parasympathetic nervous system controls the heart through the vagus nerve, which releases the neurotransmitter, acetylcholine. Acetylcholine stimu-
receptors associated with the vagal nerve endings or found on the plasma membrane of the cardiac myocytes, respectively. Physiological studies demonstrate that acetylcholine may also stimulate muscarinic receptors on pre-junctional sympathetic nerve terminals to decrease the release of norepinephrine. This requires further elucidation, since binding studies from different groups attempting to demonstrate the presence of pre-junctional muscarinic receptors disagree (Sharma and Banerjee, 1978; Story et al., 1979; Yamada et al., 1980). Stimulation of the parasympathetic nervous system decreases heart rate (Brown and Eccles, 1934), the force of contraction (Guyton, 1971) and the duration of the nodal refractory period of the action potential (Hoffman, 1967; Hoffman and Suckling, 1953). The parasympathetic nervous system is predominant over the sympathetic nervous system under resting conditions (Levy, 1971); whereas, during exercise the sympathetic nervous system predominates. The remainder of this discussion will be concerned primarily with parasympathetic control of the heart.

The parasympathetic nervous system elicits effects in atrial and ventricular tissue of the heart. Early investigators believed the ventricles to be devoid of cholinergic nerve terminals and vagal stimulation.
Although the vagus primarily innervates the atria, it also extends into the upper region of the ventricles. Additionally, cholinergic muscarinic receptors have been found in all regions of the heart (Fields et al., 1978; Wei and Sulakhe, 1978).

The physiological effects elicited by parasympathetic nervous system stimulation have been well documented in the canine heart. However, the biochemical interactions leading to these physiological effects are still unclear. The decrease in the heart's force of contraction or the negative inotropic effect seen with parasympathetic stimulation results from as yet incompletely defined electrophysiological and/or biochemical change elicited by the stimulation of muscarinic receptors. The electrophysiological changes that occur due to muscarinic receptor activation include: an increase in the outward potassium ion current (Fleming et al., 1981) and a decrease in the slow inward current which is carried by sodium and calcium ions (Watanabe et al., 1981). The biochemical changes include an increase in the cyclic-guanosine 5'-monophosphate (GMP) concentrations (Gardner and Allen, 1977; Watanabe et al., 1981), and/or a decrease in cyclic-adenosine 5' monophosphate (AMP) concentrations in the myocytes (Brown et al., 1980; Gardner and Allen, 1977). More
recently, stimulation of muscarinic receptors has been shown to increase phosphoinositide turnover in the heart (Quist, 1982; Brown and Brown, 1983). These changes and how they may relate to regulation of cardiac function are discussed below.

Electrophysiological Changes Resulting from Muscarinic Receptor Activation

Acetylcholine increases the outward potassium ion current in atria (Burgen and Terroux, 1953; Trautwein, 1963; Fleming et al., 1981), which can be measured as an increase in the potassium conductance of the membrane or by an increase in $^{42}K^+$ efflux (Harris and Hutter, 1956). This increase in $K^+$ permeability hyperpolarizes the atrial cells which depresses automaticity, reduces the slope of diastolic depolarization and reduces the duration of the action potential in myocardial cells (Ten Eick et al., 1976). Further study is required to determine how the potassium ion channel is coupled to or can be activated by the muscarinic receptor. The changes in potassium permeability seen with muscarinic receptor stimulation results in a reduction in heart rate and a shortened duration of the action potential, which may contribute to the negative ino-
tropic effect. In addition, acetylcholine has been shown to directly inhibit the slow inward current ($i_{si}$) carried by calcium and sodium ions (Giles and Noble, 1976; Ten Eick et al., 1976). A reduction in the $i_{si}$ by acetylcholine will reduce or inhibit the Ca$^{+2}$-dependent action potentials in the atria, ventricles and cardiac Purkinje fibers, thereby producing a negative inotropic effect (Loffelholz and Pappano, 1985). Thus, the negative inotropic effect seen with muscarinic receptor stimulation can result from either an increase in K$^+$ efflux or a decrease in the $i_{si}$. Since the K$^+$ efflux is delayed in comparison to the $i_{si}$ it has been postulated that the increase in K$^+$ efflux is a result of rather than a stimulus for the negative inotropic effect (Loffelholz and Pappano, 1985). However, Hume (1985) found when comparing K$^+$ and Ca$^{+2}$ channels in frog atrial cells that the K$^+$ efflux is not a $i_{si}$-activated K$^+$ conductance mechanism. In the ventricle, acetylcholine does not increase K$^+$ efflux (Inoue, Hachisu and Pappano, 1983; Hino and Ochi, 1980), suggesting that $i_{si}$ alone is responsible for inhibition of the ventricular action potential.

The parasympathetic nervous system suppresses the response of the sinoatrial node to norepinephrine (Carrier and Bishop, 1972; Grodner et al., 1970).
Ventricles exhibit a positive inotropic effect in the presence of catecholamines, and muscarinic receptor stimulation will antagonize this effect. However, in ventricular tissue without sympathetic tone, acetylcholine was found to have no significant effect on contractility (Watanabe and Besch, 1975) or on the action potential (Bailey et al., 1979; Levy, 1971). Thus, in ventricular tissue muscarinic stimulation is somehow coupled to sympathetic stimulation in an inhibitory fashion.

The Role Of Cyclic AMP In Muscarinic Receptor Function

The indirect muscarinic receptor-induced responses may be associated with changes in cyclic adenosine monophosphate (cyclic-AMP). In both atria and ventricles, beta-adrenergic agonists activate adenylate cyclase; and, muscarinic agonists inhibit adenylate cyclase. The muscarinic induced attenuation of cyclic AMP formation parallels the attenuation of the catecholamine induced positive inotropic effect (Brown et al., 1980; Ingebretson, 1980; Keely et al., 1978).

The ability of muscarinic agonists to inhibit adenylate cyclase is dependent on guanosine nucleotides.
Muscarinic agonists in the absence of guanosine nucleotides have no effect on basal adenylate cyclase activity in purified membrane preparations; however, in the presence of guanosine 5'-triphosphate (GTP), muscarinic agonists inhibit adenylate cyclase activity (Ross and Gilman, 1980; Josephson and Sperelakis, 1982). Ross and Gilman (1980) have proposed that adenylate cyclase is composed of three parts: the hormone receptor, the catalytic subunit and the coupler of the receptor to the catalytic subunit known as the N protein. GTP is thought to act on the N protein to couple the muscarinic receptor with adenylate cyclase in an inhibitory manner. Ehlert (1985) reports that the efficacy of a muscarinic agonist is directly proportional to its inhibition of adenylate cyclase and influence on GTP. Thus, the muscarinic predominance over the sympathetic nervous system in the heart can be attributed to the inhibition of adenylate cyclase.

The relationship between cyclic AMP and the slow inward channel and the requirement of metabolic energy for $i_{si}$ to function initiated the hypothesis that a membrane protein must undergo phosphorylation to provide voltage activation of the slow channel (Sperelakis, 1984). A positive inotropic agent could activate cyclic AMP-dependent protein kinase by elevating
the cyclic AMP levels which, will phosphorylate proteins in the presence of ATP. However, the muscarinic induced attenuation of catecholamine induced cyclic AMP levels may not be the only mechanism for the antagonism of the beta adrenergic agonist's effects. Under certain conditions muscarinic agonists can totally inhibit the positive chronotropic and inotropic response induced by catecholamines without effecting cyclic AMP levels (Brown et al., 1980; Ingebretsen, 1980; Keely et al., 1978). During these conditions, muscarinic agonists significantly reduce cyclic AMP-dependent protein kinase activity. However, in the absence of beta receptor stimulation, acetylcholine has no effect on cyclic AMP-dependent protein kinase activity (Keely et al., 1978; Ingebretsen, 1980). Thus, muscarinic agonists may inhibit or prevent phosphorylation of proteins such as: troponin I (England, 1975), phosphorylase kinase (McCullough and Walsh, 1979) and phospholamban (Lindemann et al., 1983; Watanabe et al., 1981) either by inhibition of adenylate cyclase or by inhibition of cyclic AMP-dependent protein kinase activity. Therefore, the muscarinic-induced inhibition of the $i_{si}$ may be attributed to inhibition of cyclic AMP-dependent protein kinase C in the presence of a sympathetic tone.
The Role of Cyclic GMP in Muscarinic Receptor Function

Stimulation of the muscarinic receptor results in an increase in cyclic GMP levels through the activation of guanylate cyclase (Endoh, 1980; Mirro et al., 1979). Acetylcholine concentrations which stimulate an increase in cyclic GMP levels correlate with those that elicit a negative inotropic effect in rat atria (George et al., 1970; Lincoln and Keely, 1980). Analogues of cyclic GMP have been shown to mimic certain effects of muscarinic receptor stimulation. For example, 8-bromo-cyclic GMP decreased calcium ion uptake without affecting potassium ion content in atrial muscle preparations (Nawrath, 1977). 8-Bromocyclic GMP also inhibited the atrial slow response action potential (Kohlhardt and Haap, 1978). Dibutyryl cyclic GMP decreased the spontaneous heart beat of isolated cultured heart cells (Krause et al., 1972). These observations suggest that cyclic GMP may be associated with a decrease in Ca\(^{2+}\) influx. Cyclic GMP may also mediate muscarinic anti-adrenergic effects. Cyclic GMP analogues have been found to antagonize the positive inotropic effects of catecholamines (Watanabe et al.,
1978), and the electrophysiological effects of catecholamines or cyclic AMP (Kohlhardt and Haap, 1978).

Dibutyryl cyclic GMP, like acetylcholine, antagonized the isoproterenol-induced activation of phosphorylase in heart tissue without lowering cyclic AMP levels (Watanabe et al., 1978). The biochemical mechanisms by which cyclic GMP may mediate muscarinic receptor function is still unknown. An hypothesis is that cyclic GMP stimulates a specific protein kinase which phosphorylates protein sites and alters protein function (Lincoln and Corbin, 1983). Kuo et al. (1972) identified a cyclic GMP-dependent protein kinase. Wahler and Sperelakis (1985) have found that the cyclic GMP-dependent protein kinase modulates the slow inward current, in opposition to the cyclic AMP-dependent protein kinase. Thus, there is a possibility that cyclic GMP acts as a second messenger of muscarinic receptor function through protein phosphorylation in much the same way that cyclic AMP regulates adrenergic receptor function.

There is also evidence that cyclic GMP is not involved in muscarinic receptor regulation of the negative inotropic effect (Linden and Booker, 1979). A concentration-response study showed that at low concentrations of muscarinic agonist, contractility decreased
with no change in cyclic GMP levels. Cyclic GMP levels were found to increase only in the presence of high muscarinic agonist concentrations (Brooker, 1977). Thus, cyclic GMP levels may not play a part in regulating the muscarinic stimulated negative inotropic effect. Most reports, however, support a role for cyclic GMP in muscarinic receptor function.

Myocardial Muscarinic Receptors

Myocardial muscarinic receptors have been studied by many investigators. Muscarinic receptor binding is examined with the aid of radiolabeled antagonists such as $[^3\text{H}]$-quinuclidinylbenzylate (QNB) (Fields et al., 1978), and $[^3\text{H}]$-N-methylscopolamine (Chassaing et al., 1984); or with the use of radiolabeled agonists such as $[^3\text{H}]$-cis-methylidioxolane (CD) (Roeske and Yamamura, 1980) and $[^3\text{H}]$-oxotremorine (Harden et al., 1983). The distribution of muscarinic receptors, as determined by measurement of specifically bound $[^3\text{H}]$-QNB to the receptor in various regions of the rabbit heart, was reported to be (fmoles/mg protein): left atrium (302.4), right atrium (200.0), ventricular septum
(58.1), right ventricle (52.7), and left ventricle (37.4), (Fields et al., 1978). In the dog heart, Wei and Sulakhe (1978) found the regional distribution to be (pmoles $[^3H]$-QNB/g protein): right atria (108.4), left atria (148.1), septum (87.2), right ventricular base (139.3), right ventricular apex (163.7), left ventricular base (100.7), and left ventricular apex (109.3). The distribution of muscarinic receptors in the dog and the guinea pig heart have similarities, and both species reveal similar receptor densities in atria and in ventricles. When comparing muscarinic receptor densities in other species, the frog and chick heart display a significantly greater density of muscarinic receptors in atria than in ventricles (Hartzell, 1980; Siegel and Fischbach, 1984).

There is little known about the properties of the muscarinic receptor in the canine heart. Binding studies indicate that muscarinic receptors are primarily located on plasma membranes of the heart cells (Wei and Sulakhe, 1978; Schimerlik and Searles, 1980). Although Wei and Sulakhe (1978) have studied the distribution of the muscarinic receptor in the dog heart, they did not report the affinity of the receptor for $[^3H]$-QNB or muscarinic agonists. The affinity constants for $[^3H]$-QNB and other agents reported in Table 1 are those for
TABLE 1. MUSCARINIC RECEPTOR AFFINITIES IN PORCINE, RAT AND RABBIT HEART.

<table>
<thead>
<tr>
<th>ANTAGONIST</th>
<th>PORCINE ATRIA</th>
<th>RAT HEART</th>
<th>RABBIT HEART</th>
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<tr>
<td></td>
<td>$F_1$</td>
<td>$K_1 (M)$</td>
<td>$F_2$</td>
</tr>
<tr>
<td>$^3$H-QNB</td>
<td>1.0</td>
<td>1.2 x 10^-10</td>
<td></td>
</tr>
<tr>
<td>ATROPINE</td>
<td>1.0</td>
<td>1.0 x 10^-9</td>
<td></td>
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<thead>
<tr>
<th>AGONIST</th>
<th>PORCINE ATRIA</th>
<th>RAT HEART</th>
<th>RABBIT HEART</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_1$</td>
<td>$K_1 (M)$</td>
<td>$F_2$</td>
</tr>
<tr>
<td>ACETYLCHOLINE</td>
<td>0.35</td>
<td>1 x 10^-8</td>
<td>0.65</td>
</tr>
<tr>
<td>OXOTREMORINE</td>
<td>0.35</td>
<td>1 x 10^-7</td>
<td>0.65</td>
</tr>
<tr>
<td>CARBACHOL</td>
<td>0.35</td>
<td>2 x 10^-7</td>
<td>0.65</td>
</tr>
<tr>
<td>PILOCARPINE</td>
<td>0.20</td>
<td>2.2 x 10^-7</td>
<td>0.80</td>
</tr>
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Schimerlik and Searles, Waelbrock et al. 1982
Fields et al. 1978

F - Denotes fraction of receptors at noted affinity
K - Affinity constant for agent at receptor
(Schimerlik and Searles, 1980), and rat heart (Waelbroeck et al., 1982). Antagonists bind to a single, apparently homogenous population of high affinity binding sites as depicted in Table 1. Pirenzipine, a relatively strong \( M_1 \) receptor antagonist, binds weakly to cardiac muscarinic receptors (Hammer et al., 1980). Pirenzipine does not only exhibit heterogeneity between different tissues but also within a tissue (Hammer and Giachetti, 1982). This study suggests that cardiac receptors are primarily \( M_2 \), but a small population of higher affinity \( M_1 \) receptors are also present.

In heart, there appears to be at least two agonist binding sites, as evidenced by differences in binding affinity (Table 1). Waelbroeck et al. (1982) suggest the possibility for a third subtype in heart. Heterogeneity of agonist binding has been found in most muscarinic binding studies; Birdsall and Hulme (1983) have classified the agonist binding sites as superhigh (SH), high (H), and low (L), based on their affinity for muscarinic agonists. The predominant type of receptor found in heart has low affinity, is guanine nucleotide regulated, adenylate cyclase inhibitory, and may exert an effect on the slow inward calcium ion current (Birdsall and Hulme, 1983). In contrast, Brown and Brown (1984) report the existence of two distinct
biochemical responses which distinguish the high and low agonist affinity binding sites in the chick embryo heart. A recent study by Hosey et al. (1985) found three agonist affinity states in membranes from chick embryonic hearts and two in new born hearts. Acetylcholine's affinity for oxotremorine was found to increase during development. The high affinity site ($M_1$) is believed to be associated with the inhibition of adenylate cyclase, while the low affinity ($M_2$) is associated with the stimulation of the phosphatidylinositol effect. Both sites become a single low affinity site in the presence of $10^{-4}$M of the guanine nucleotide Gpp(NH)p (Waelbroeck et al., 1982). There is evidence for another type of muscarinic receptor found in heart conduction tissue whose binding properties are not effected by guanine nucleotides (Birdsall et al., 1983). This other type of receptor may be linked to the opening of potassium ion channels (Birdsall and Hulme, 1983).

Muscarinic receptor number can be decreased by sustained activation of muscarinic receptors in vivo (Halvorsen and Nathanson, 1981; Klein, 1980). This down regulation of muscarinic receptors has been found to decrease the fractions of high and low affinity muscarinic receptors without changing receptor affinity
for carbachol (Halvorsen and Nathanson, 1981). Muscarinic receptor number is decreased in diabetes (Carrier and Aronstam, 1985), heart failure (Vatner et al., 1985) and in the presence of muscarinic agonists (Roskoski et al., 1985). These changes in receptor number may reflect changes in cholinergic regulation of heart function with these disorders.

**Properties Of Phosphoinositides**

Phosphoinositides refer to the three inositol containing phospholipids; phosphatidylinositol, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Phosphatidylinositol (PI) content is usually between two and twelve percent of the total phospholipid of the cell membrane. PI is the predominant inositol phospholipid ranging in concentration from 0.5 to 2.5 micromolar per gram tissue. PIP and PIP$_2$ content were first measured by Kfourny and Kerr (1966), and have since been reported in numerous tissues and species (Santiago-Calvo et al., 1964; Hawthorne et al., 1966; Dawson and Eichberg, 1965; Dittmer and Douglas, 1969). Due to the small quantities of PIP and PIP$_2$ in the membrane, it is difficult to accurately
assess their tissue concentration. Hauser and Eichberg (1973) report the highest concentrations for PIP (0.153 umol/g tissue) and PIP₂ (0.083 umol/g tissue) in unmyelinated brain tissue and PIP (0.219 umol/g tissue) and PIP₂ (0.280 umol/g tissue) in myelinated brain tissue. Phosphatidylinositol and the polyphosphoinositides are illustrated in Figure 1. The phosphoinositides are similar in structure all having a three carbon diacylglycerol backbone with inositol bound to the third carbon via a phosphate group. PIP has an additional phosphate at the 4' position of the inositol ring, and PIP₂ has two phosphate groups at the 4' and 5' positions of inositol. The phosphoinositides have another characteristic feature which is their fatty acid composition. The fatty acid bound to the first carbon, designated R₁ in Figure 1, is an saturated fatty acid — usually stearic acid (18:0). R₂ is primarily arachidonic acid (20:4) or another unsaturated fatty acid. The fatty acid composition of PI and the polyphosphoinositides, especially arachidonic acid content, is very important for prostaglandin, leukotriene and thromboxane synthesis. Phospholipids are the only storage site for arachidonic acid in the cell and are thus under extensive investigation.
FIGURE 1

THE PHOSPHOINOSITIDES

\[ \text{CH}_2 - O - R_1 \]
\[ \text{CH} - O - R_2 \]
\[ \text{CH}_2 - O - \text{P} - O - \text{OH} \quad \text{OH} \quad \text{OH} \]

PHOSPHATIDYLINOSITOL

\[ \text{CH}_2 - O - R_1 \]
\[ \text{CH} - O - R_2 \]
\[ \text{CH}_2 - O - \text{P} - O - \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OPO}_3^{-2} \]

PHOSPHATIDYLINOSITOL 4-PHOSPHATE

\[ \text{CH}_2 - O - R_1 \]
\[ \text{CH} - O - R_2 \]
\[ \text{CH}_2 - O - \text{P} - O - \text{OH} \quad \text{OPO}_3^{-2} \quad \text{OPO}_3^{-2} \]

PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE
The Phospholipid Effect

The phospholipid effect was first described by Hokin and Hokin (1953) in response to cholinergic receptor stimulation in pigeon pancreas slices. They found that cholinergic receptor stimulation increased the incorporation of radiolabeled inorganic phosphate ($^{32}$P) into phosphatidic acid (PA) and PI and termed this response the phospholipid effect. This increase in $^{32}$P labeled PI and PA could arise from either an increase in the de novo synthesis of these phospholipids, or it could be a result of increased turnover of these phospholipids. The Hokins showed that $^3$[H]-glycerol labeled PI did not accompany the receptor stimulated increase in $^{32}$P labeled PI. Indicating that the diacylglycerol backbone is not recycled at the same rate as the phospholipid effect (Hokin and Hokin, 1958). Putney (1985) states that the main questions regarding the phospholipid effect are: What are the biochemical pathways involved in the phospholipid effect? and What is the physiological relevance of the phospholipid effect? From recent studies by Abdel-Latif et al. (1977), $^{32}$P labeled PIP$_2$ and PIP were found to decrease in response to muscarinic receptor
stimulation in the rabbit iris smooth muscle. Other studies have found a direct correlation between the decrease in labeled PIP<sub>2</sub> and PIP and the increase in <sup>32</sup>P-labeled PA and PI (Kirk et al., 1981). Because PIP<sub>2</sub> breakdown occurred at a faster rate than cholinergic receptor stimulated PI labeling, it was suggested that the increase in PI labeling occurs secondarily to the phosphodiesteric cleavage of PIP<sub>2</sub> and PIP. The enzyme responsible for the breakdown of PIP<sub>2</sub> and PIP is a phosphatidylinositol phosphodiesterase known as phospholipase C. It is still not known how muscarinic receptor stimulation activates phospholipase C. Phospholipase C cleaves the inositol phosphate groups; inositol bisphosphate (IP<sub>2</sub>) and inositol trisphosphate (IP<sub>3</sub>) from PIP and PIP<sub>2</sub> respectively, leaving diacylglycerol (DAG). The metabolism of the polyphosphoinositides is illustrated in Figure 2 taken from Hawkins et al. (1984). DAG is rapidly phosphorylated by DAG kinase and adenosine 5' triphosphate (ATP) to PA. Phosphatidic acid and cytidine 5' triphosphate (CTP) then form CMP-phosphatidate which is further synthesized by phosphatidate cytidylyl transferase and inositol to form PI. The de novo synthesis of PI occurs at the endoplasmic reticulum (Bleasdale et al., 1979). Very little is known about where polyphosphoinositides
FIGURE 2. PI-Phosphatidylinositol, PIP-Phosphatidylinositol 4'-phosphate, PIP2-PI 4',5'-Bisphosphate, DAG-Diacylglycerol, PA-Phosphatidate, CMP-Cytidine monophosphate, IP2-Inositol trisphosphate, ATP-Adenosine triphosphate, ADP-Adenosine diphosphate, Pi-Inorganic phosphate, PPI-Inorganic pyrophosphate.
in polymorphonuclear leukocytes suggest that the synthesis of PIP and PIP$_2$ occurs at the surface membrane of the cell (Hauser and Eichberg, 1973; Wienecke and Woodin, 1967). PI can be phosphorylated by PI kinase forming PIP. PIP can also be phosphorylated by PIP kinase to yield PIP$_2$. These reactions are reversible. That is, specific monoesterases can cleave the phosphate groups of PIP and PIP$_2$ to yield PI and PIP, respectively. Since PA, PIP and PIP$_2$ syntheses occur on the cell membrane surface, these lipids may be functionally significant at the membrane surface. The phospholipid effect has been demonstrated in several tissues and with different stimuli. A common feature in the different tissues is that the stimuli act at cell surface receptors and not at sites within the cell membrane. For example, the PI effect has been demonstrated in fibroblasts stimulated by serum factors (Pasternak, 1972), in adipose tissue stimulated by insulin (Torrentegui and Berthet, 1966), in platelets stimulated by ADP, thrombin, collagen, and adrenaline (Watson and Lapetina, 1985; Seiss and Binder, 1985), in blowfly cell-free salivary gland preparations stimulated by serotonin (Fain et al., 1983), in thyroid gland stimulated by thyroid stimulating hormone (Hokin, 1969) and by muscarinic cholinergic agonists and alpha-
adrenergic agonists in various tissues (Forsberg and Pollard, 1985; Nadler et al., 1984). This great diversity in stimuli and tissues indicates that the phospholipid effect may play an important role in cell function.

There has been extensive speculation as to the purpose of phosphoinositide turnover. It has been linked to many physiological reactions; for example, platelet aggregation due to thrombin stimulation (Seiss and Binder, 1985). A number of investigators believe that one of the phosphoinositides or their breakdown products is an important second messenger. Michell (1975) proposed that the receptor-mediated phospholipid effect might be responsible for calcium mobilization, since most receptor stimuli seem to induce a phospholipid effect independent of calcium. Ca^{2+} influx was observed after receptor stimulation in these tissues, suggesting that PI turnover and calcium ion influx may be linked. Lapetina (1982) suggested that PA produced secondarily to phosphoinositide breakdown might act to mobilize calcium because of its proposed ionophoretic properties. Diacylglycerol (DAG) may also have important biological implications. For instance, Kaibuchi et al. (1982) and Nishizuka (1984) have described a DAG-dependent protein kinase (also referred to as C
kinase) which requires calcium and phospholipid. It has been proposed that DAG activated C kinase may phosphorylate and regulate important physiological substrates and affect cellular function. Berridge (1984) believes that both IP$_3$ and DAG act as second messengers: inositol trisphosphate by mobilizing intracellular calcium and DAG by stimulating protein phosphorylation. Changes in PIP$_2$ itself, may be linked to receptor desensitization and super-sensitivity (Berridge, 1983). Streb et al. (1983) first demonstrated the calcium mobilizing effects of IP$_3$ in leaky pancreatic acinar cells. IP$_3$ also mobilized calcium in saponin-permeabilized hepatocytes (Joseph et al., 1984; Williamson et al., 1985).

Recently, carbachol has been found to induce a phospholipid effect in canine atrial tissue (Quist, 1982) and in murine atria and ventricles (Brown and Brown, 1983). Conversely, Sekar and Roufogalis (1982) reported that carbachol has no effect on phospholipid turnover in the rat atrium. A more recent paper by Sekar and Roufogalis (1984) reports that a carbachol stimulated phospholipid effect occurs in the left atrium, but not in the right atrium. Quist (1982) and Brown and Brown (1983) found that the carbachol-induced phospholipid effect in heart is calcium dependent.
Quist and Sanchez (1982) report a calcium independent phospholipid effect in response to alpha-adrenergic receptor stimulation in the canine heart. Uchida et al. (1982), however, report that the alpha-adrenergic stimulated phospholipid effect in rat neonatal heart cells is calcium dependent.

There is still a great deal to learn about the phospholipid effect in heart. The heart is an extremely complex muscle under tight neural control. Muscarinic receptors mediate a number of effects, however the relationship of the phospholipid effect to heart function is presently unknown. In this study, the carbachol stimulated phospholipid effect was examined in isolated canine myocytes. This study was undertaken to ensure that the phospholipid effect occurs in cardiac myocytes rather than in the vascular or neural cells, which are also present in cardiac tissue. Calcium dependence and phospholipase C inhibition of the phospholipid effect were also investigated. Finally, the distribution of muscarinic receptors in various regions of cardiac tissue and their respective affinities and densities were examined.
MATERIALS AND METHODS

Materials

Radiolabeled inorganic orthophosphate \(^{32}\text{P}\) was obtained from ICN, Cleveland, OH. N-2-Hydroxyethyl Piperazine-N'-2-Ethanesulfonic acid (HEPES), carbamylcholine chloride, collagenase (from Clostridium histolyticum), 4-bromophenacyl bromide, quinacrine dihydrochloride, acetylcholine, atropine and phospholipid standards were obtained from Sigma Chemical Co., St. Louis, MO. Trifluoperazine was obtained from Smith, Kline & French, Philadelphia, PA. \(^{3}\text{H}\)-Quinuclidinylbenzylate (QNB) was purchased from New England Nuclear, Boston, MA.

Isolation of Canine Myocytes

A mongrel dog was anesthetized intravenously with Surital. The heart was quickly excised and the right atrium was removed and placed in cold medium composed of 124 mM NaCl, 5 mM KCl, 0.3 mM NaH\(_2\)PO\(_4\), 1.3 mM HEPES,
primarily used the right atria; however, the right ventricle, left atrium and left ventricle were also analyzed in some studies. The above medium was oxygenated for 15 minutes (min) prior to use. The right atrium was trimmed of fat, weighed and sliced into 0.5 centimeter (cm) slices using a razor blade. The atrial slices were suspended with 8 milliliters (mls) of medium and 12 milligrams (mgs) of collagenase. The mixture was incubated for 20 min at 37°C. Ten mls of medium was added to the mixture, and the suspension was filtered through a 350 micrometer (um) course nylon mesh. The tissue slices were returned to the tube and suspended with 8 mls of medium and 12 mgs collagenase and incubated and filtered as above. The above steps were repeated four more times, except the tubes were incubated 30 min; and after the last incubation the cells were separated from the tissue in the tube with a loose fitting pestle. The last four filtrates were combined and washed twice with 30 mls of physiological medium and centrifuged at 500 x gravity (g). The cells were resuspended to 5 mls in the above medium and then fractionated on 5 mls of a ten percent ficoll gradient at 500 x g to remove debris.
Viability of Myocytes

Cell viability was determined by the addition of one drop of 0.4% Trypan blue in physiological buffer to isolated cells (Kruse and Patterson, 1973). The myocytes were observed under phase contrast microscopy. The myocytes were elongated, striated cells, and initially beat spontaneously. The morphology was examined at a magnification of 200 x in an American Optical binocular microscope throughout the course of some experiments to monitor for changes in calcium tolerance and cell viability. Cells were used in our studies which excluded 80 to 90 percent of the Trypan blue. Protein was assayed by the method of Peterson (1977). Protein content ranged from 0.8 to 1.2 mgs per assay tube (volume equal to 0.5 ml).

Prelabeling of Cells with $^{32}\text{p}$

The isolated heart cells were prelabeled with $^{32}\text{p}$-inorganic orthophosphate for 60 min at 37°C, protein content was approximately 2 mg/ml. The incubation medium was composed of 124 mM NaCl, 5 mM KCl, 0.3 mM
NaH$_2$PO$_4$, 1.3 mM MgCl$_2$, 25 mM HEPES, pH 7.4, 0.5 mM -
1.5 mM CaCl$_2$ and 5 mM dextrose. The cells were sus-
pended in a total volume of 0.5 mls per tube.

**Lipid Extraction**

The incubation was stopped by the addition of 2.0
mls of CHCl$_3$/CH$_3$OH/HCl (20:40:1 by volume) to the cell
suspension. 0.75 mls CHCl$_3$ and 0.25 mls H$_2$O were added
to the tubes, vortexed thoroughly and centrifuged at
1200 x g at 5°C. The aqueous phase was aspirated and
2.0 mls of 0.1 N HCl was added. The tubes were vor-
texed and centrifuged at 1200 x g for 10 min at 5°C.
The aqueous layer was aspirated, and one milliliter of
the CHCl$_3$ layer was collected and dried under N$_2$ gas.
The lipids were resuspended in 30 uls of
CHCl$_3$/CH$_3$OH/HCl (60:30:1, by volume) vortexed, and 20
ul aliquots were spotted on silica 60 gel plates (E.
Merck). To determine $^{32}$P labeled PI, PA and PC, the
plates were developed in an equilibrated tank with
CHCl$_3$/CH$_3$OH/CH$_3$NH$_2$ (65:35:10 by volume) containing 0.1% Butylated hydroxytoluene (BHT), using a modification of
the method of Harrington et al. (1980). To determine
$^{32}$P labeled PIP and PIP$_2$ the silica gel 60 plates were
developed in an equilibrated tank in
\[ \text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_4\text{OH}/\text{H}_2\text{O} \ (45:35:2.5:7.5 \text{ by volume}). \]
Radiolabeled phospholipids were located by autoradiography using Kodak X-OMAT AR X-ray film. The phospholipids were identified in an Iodine chamber and were compared to authentic standards. Labeled phospholipids were scraped into scintillation vials containing 8 mls of toluene cocktail (toluene/triton X-100/H\(_2\)O, 6:3:1, PPO (2,5-diphenyloxazole), and POPOP (1,4-Bis(2-(5-Phenyloxazole))Benzene). The labeled phospholipids were then counted using a Packard scintillation counter, Tri Carb 460C.

\(^3\text{H}\)-Quinuclidinyl Benzyline (QNB) Binding

The distribution of muscarinic receptors was examined in various regions of the canine heart. Either heart homogenates from selected regions of the heart or heart cells prepared from these regions were analyzed. Homogenate was prepared by slicing heart tissue into less than one cm slices with a razor blade, and the slices were minced with scissors. The minced tissue was suspended to 1 ml per 0.1 gram wet tissue weight in normal physiological medium (as above) and homogenized
for 15 seconds at a setting of 5 using a Polytron R homogenizer at 5°C. The homogenate was then filtered through two layers of cheese cloth. The homogenate was frozen, then later used for binding assays. The protein content was determined to be 0.5 mg/ml physiological buffer for most binding assays, homogenates or heart cell preparations. Binding assays were incubated in 1 ml of the same physiological medium as above. [3H]-QNB binding concentration curves (0.05 - 5 nM), in the presence and absence of 10 μM atropine, using various concentrations of carbachol (5 - 1000 μM) and using various protein concentrations (0.2 - 1.6 mg), were determined by incubating [3H]-QNB with homogenate or cells with the predetermined agents. Incubation was for 60 min at 27°C. Homogenate or heart cells were then washed three times with buffer and filtered through glass fiber filters (Whatman GF/C) under mild vacuum. The filters were suspended in toluene cocktail and counted using a Packard scintillation counter. Specific binding (total [3H]-QNB cpm - [3H]-QNB cpm in presence of atropine) were plotted according to Rosenthal (1967). Protein content was determined by the method of Peterson (1977). The effects of non-specific phospholipase inhibitors; quinacrine, trifluoperazine and 4-bromophenacyl bromide on [3H]-QNB
binding and carbachol's inhibition of $[^3H]$-QNB binding, were also analyzed.
RESULTS

For this study it was important that the heart myocytes isolated be calcium tolerant. Since, the myocytes have to be tolerant to the physiological calcium concentrations found in the heart. Calcium intolerant cells have leaky surface membranes so that $\text{Ca}^{2+}$ readily crosses the membrane significantly reducing the cell's life span. Calcium tolerant myocytes were isolated from 2 to 3 grams of atrial tissue. Approximately one third of the starting was recovered as calcium tolerant myocytes. This was sufficient to perform sixty determinations on phospholipid metabolism. In the presence of 1 mM extracellular calcium, the physiological calcium concentration, isolated cells retained their elongated shapes and striations for up to ninety minutes (Figure 3). 50 uM calcium was included in the medium used to isolate myocytes during collagenase treatment to obtain calcium tolerant cells, as reported previously by Powell et al. (1980) and Kao et al. (1980). Exclusion of calcium during collagenase treatment resulted in the isolation of calcium intolerant cells, which rapidly transformed into granular
FIGURE 3. ISOLATED ATRIAL MYOCYTES

Isolated atrial myocytes as viewed through an American Optical Microscope, magnification of 200x.
Inclusion of 1 mM Ca\(^{2+}\) during collagenase treatment resulted in low yields of viable myocytes. Cell viability was confirmed by trypan blue exclusion and was determined to be 80 to 90 percent by visual cell count.

**Time Course of \(^{32}\)Pi Incorporation into Phospholipids**

After isolating the heart cells, the labeling of the phospholipids with radiolabelled inorganic phosphate (\(^{32}\)Pi) was examined. Phospholipids incubated with \(^{32}\)Pi in the presence and absence of 250 uM carbachol displayed time-dependent increases in labeling of PC, PI and PA (Figure 4), and PIP and PIP\(_2\) (Figure 5). The labeling of phosphatidylethanolamine (PE) and lyso PC was significant (not shown), but was less than with the above phospholipids. Incorporation of \(^{32}\)Pi into PA, PIP and PIP\(_2\) plateaued after 40 to 60 minute incubations; whereas, labeling of PC increased sharply after an initial lag phase of 40 minutes. Labeling of PI was linear from 20 to 80 minutes. Carbachol selectively increased \(^{32}\)Pi incorporation into PA and PI, and this increase was apparent at all times studied. Addition of 10 uM atropine blocked the carbachol stimulated increase in labeled PI and PA (not shown). The \(^{32}\)Pi
FIGURE 4. Time course of 32P incorporation into atrial myocytes phospholipids. Incorporation into PI (●), PA (■), and PC (▲) during control conditions. Incorporation into PI (○), PA (□), and PC (△) in the presence of 250 μM carbachol. Student's T-test on the data gave a p value of ≤ 0.005 difference between the control and carbachol values, exception being PC.
FIGURE 5. Time course of $^{32}$P incorporation into atrial myocyte polyphosphoinositides. Incorporation into $\text{PIP} (●)$ and $\text{PIP}_2 (■)$ during control conditions. Incorporation into $\text{PIP} (○)$ and $\text{PIP}_2 (■)$ in the presence of 250 μM carbachol. Results indicate the mean of four separate experiments with standard errors of less than five percent.
10 uM atropine was added to myocytes in the presence and absence of carbachol. Since atropine is a muscarinic antagonist, the ability of atropine to block the carbachol-induced increase $^{32}$Pi labeling demonstrates that the carbachol-induced increase in $^{32}$Pi labeling of PI and PA results from the stimulation of the cholinergic muscarinic receptor in the heart cell.

**Carbachol Concentration Curve**

Isolated myocytes were pre-incubated for 60 minutes with $^{32}$Pi and were then further incubated in the presence of varying concentrations of carbachol. Figure 6 shows that less than 10 uM carbachol is required to increase PI labeling in both right atrial and right ventricular myocytes. Carbachol stimulated increases in PI labeling, which plateaued at 250 uM. PA labeling was similarly increased by the same carbachol concentrations (not shown). However, PC labeling was not affected at any of these carbachol concentrations.
FIGURE 6. The concentration curve of the carbachol-induced phospholipid effect in myocytes. The myocytes were preincubated for 60 minutes with $^{32}$P in physiological medium, after which carbachol (10 - 1000 μM) in medium or medium was added and incubated 30 minutes. Phosphatidylinositol labeling was examined in right atrial myocytes (○) and right ventricular myocytes (□). The results represent the means of three experiments with standard errors of less than five percent.
Protein Requirement for Phospholipid Effect in Myocytes

The isolated myocytes were added at several dilutions to analyze the effects of varying protein content on carbachol induced increases in PA labeling (Figure 7). At least 0.4 mg protein/0.5 ml were required for carbachol to significantly increase PA labeling. Both basal PA labeling and carbachol stimulated PA labeling increased linearly from 0.6 to 1.6 mg protein. In the majority of the phospholipid determinations, myocyte suspensions of approximately 1.0 mg protein/0.5 ml were used.

Time Course of the Phospholipid Effect

Studies were done to determine the rate of the carbachol stimulated phospholipid effect. Myocytes were incubated for 60 minutes with $^{32}\text{P}i$ in physiological buffer in order to label the phospholipids. 250 uM carbachol, or physiological buffer, was then added and the cells were further incubated, as depicted in Figure 8. The level of labeled PA or PI did not change during the 15 minute post-incubation period in the absence of carbachol. However, 250 uM carbachol produced an in-
FIGURE 7. Isolated atrial myocytes were prelabeled for 60 minutes with $^{32}$P at several protein dilutions, carbachol (250 μM) in buffer or buffer was added and the suspensions were further incubated 30 minutes. Phosphatidate labeling was measured in the absence of carbachol (■) and in the presence (□), at protein concentrations determined by Peterson (1977). Results are the means of three experiments with standard errors.
FIGURE 8. The time course of the carbachol induced phospholipid effect in atrial myocytes. Myocytes were preincubated for 60 minutes with $^{32}$P after which carbachol (250 μM) in buffer or buffer was added. Labeling of PI (○), PA (■), and PC (▲) was examined after the addition of the buffer and PI (○), PA (■), and PC (▲) was examined after the addition of carbachol. The results represent the means of three experiments with standard errors of less than five percent.
A maximal increase in PA labeling was obtained after 2.5 to 5.0 minutes. Carbachol induced an increase in $^{32}\text{Pi}$ labeling of PI after 2.5 minutes, and this increase was maximal at 15 minutes. PC labeling increased slightly from zero to 15 minutes, but carbachol had no effect on PC labeling. Carbachol also had no effect on PE and lyso PC labeling (not shown).

**Calcium Dependence of the Phospholipid Effect**

Myocytes were incubated in media containing varying concentrations of calcium in the presence or absence of 250 uM carbachol. At zero calcium (50 uM EGTA), carbachol had no effect on PA labeling (Figure 9). At 5 uM calcium, carbachol increased PA labeling by approximately 15%, but at higher concentrations (greater than 100 uM), carbachol elicited a more significant response; for example a 37.5% increase at 100 uM and a 62.5% increase at 1 mM calcium. At zero calcium, carbachol had no effect on PI labeling. Carbachol increased PI labeling 22% at 5 uM calcium and 37% at 1 mM calcium. Basal PI labeling decreased 50% with increasing calcium concentrations from 0 - 500 uM. At 25 uM calcium there is a decrease in $^{32}\text{Pi}$ labeled
FIGURE 9. The calcium concentration dependence of the phospholipid effect. The labeling of PI (●), PA (■) and PC (▲) was examined in the absence of carbachol. The $^{32}$P labeling of PI (○), PA (□) and PC (△) was examined in the presence of 250 μM carbachol in right atrial myocytes. Results indicate the mean of four experiments with standard errors of less than five percent.
at 50 uM calcium. These changes in $^{32}$Pi labeling of PI and PA are probably the result of Ca$^{2+}$ dependent activation or inhibition of enzymes required for the synthesis and degradation of these phospholipids. PC labeling was not affected by altering the calcium concentration nor was it affected by carbachol concentration. Increasing the calcium concentration from 50 to 1000 uM also increased PE labeling 67% (not shown).

**Effect of Phospholipase Inhibitors**

Increasing the concentration of trifluoperazine from 0 to 50 uM markedly reduced PA and PC labeling by 62 and 75% respectively, and increased PI labeling by 66% (Figure 10). Carbachol (250 uM) induced increases in PI and PA labeling were completely inhibited by 20 and 50 uM trifluoperazine. 4-bromophenacyl bromide (100 uM) and quinacrine (50 uM) strongly decreased PC labeling and increased PI labeling (not shown). In controls, 250 uM carbachol increases PA and PI labeling by 46 and 42% respectively, while having no affect on PC labeling (Table 2). In the presence of 20 uM trifluoperazine carbachol increased PA and PI labeling by only 13 and 15%. In the presence of 50 uM quinacrine,
FIGURE 10. The effect of trifluoperazine on the carbachol-induced phospholipid effect. Right atrial myocytes were prelabeled with $^{32}$P and trifluoperazine (0 - 50 μM) in physiological buffer after which carbachol in buffer or buffer alone was added. Incorporation of $^{32}$P into PI (●), PA (■) and PC (▲) in the absence of carbachol. Incorporation of $^{32}$P into PI (○), PA (◇) and PC (△) in the presence of 250 μM carbachol. The results represent the mean of three experiments with standard errors of less than five percent.
**INHIBITION OF THE CARBACHOL STIMULATED PHOSPHOLIPID EFFECT BY TRIFLUOPERAZINE, QUINACRINE AND 4-BROMOPHENACYL BROMIDE**

<table>
<thead>
<tr>
<th>AGENT</th>
<th>PERCENT CHANGE IN PHOSPHOLIPID LABELING</th>
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<tbody>
<tr>
<td></td>
<td>PA</td>
</tr>
<tr>
<td>250 μM CARBACHOL</td>
<td>46 ± 11</td>
</tr>
<tr>
<td>250 μM CARBACHOL + TRIFLUOPERAZINE (20 μM)</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>250 μM CARBACHOL + QUINACRINE (50 μM)</td>
<td>-7 ± 12</td>
</tr>
<tr>
<td>250 μM CARBACHOL + 4-BROMOPHENACYLBROMIDE (100 μM)</td>
<td>0 ± 4</td>
</tr>
</tbody>
</table>

**TABLE 2.** These data represent the percent change in phospholipid labeling in the presence of carbachol versus control. Thus the agents trifluoperazine, quinacrine and 4-bromophenacyl bromide reduce the percent change in the presence of carbachol indicating that they may act as phospholipase C inhibitors.
In the presence of 100 μM 4-bromophenacyl bromide, carbachol had no effect on PA and PI labeling.

\[ ^{3}\text{H}]\text{-QNB Concentration Curve} \]

Figure 11 shows the concentration dependence of \[ ^{3}\text{H}]\text{-QNB binding in atrial and ventricular homogenates.} \]

Binding of \[ ^{3}\text{H}]\text{-QNB} is maximal at 0.4 nM and is half-maximal at 0.13 nM in atrial and ventricular homogenates. Identical \[ ^{3}\text{H}]\text{-QNB} concentration binding curves were obtained from right atrial myocyte suspensions. \[ ^{3}\text{H}]\text{-QNB} binds to ventricular and atrial muscarinic receptors in an equipotent manner. However, \[ ^{3}\text{H}]\text{-QNB} plateaus in atrial homogenate with 27\% greater cpm per mg protein than in ventricular homogenate.

\[ \text{Rosenthal Plot Of } ^{3}\text{H}]\text{-QNB Binding} \]

In order to obtain a more quantitative assessment of \[ ^{3}\text{H}]\text{-QNB affinity for muscarinic receptors and muscarinic receptor density in various regions of the heart, a Rosenthal (1967) plot was used. Figure 12 indicates the difference in QNB binding to muscarinic receptors in the auricles and in the ventricles. The affinity (Kd) determined from the negative reciprocal}
FIGURE 11. $^{3}$H-Quinuclidinylbzylate concentration curve in atrial (○) and ventricular (□) homogenates. Results represent the specific binding of $^{3}$H-QNB taken from the means of five experiments with non-specific binding of approximately 10 percent.
FIGURE 12. A Rosenthal plot of $[^3H]$-Quinuclidinylbenzylate binding to the muscarinic receptors in the right auricle (○), left auricle (●), right ventricle (■) and left ventricle (□). The results represent the mean of four separate experiments with standard errors of less than ten percent. The R values were greater than 0.985, as determined by linear regression analysis.
right auricle \((3.08 \times 10^{-10})\), left auricle \((3.76 \times 10^{-10})\), right ventricle \((1.72 \times 10^{-10})\) and left ventricle \((1.99 \times 10^{-10})\). The abscissa gives an indication of the density of muscarinic receptors, \(B_{\text{max}}\). \(B_{\text{max}}\) values obtained for the regions of the heart were: right auricle \((158.6 \, \text{pmoles/mg protein})\), left auricle \((161.1)\), right ventricle \((96.9)\) and left ventricle \((94.3)\).

**Carbachol Displacement of \[^{3}\text{H}\]-QNB Binding**

Increasing concentrations of carbachol linearly decreased \[^{3}\text{H}\]-QNB binding in homogenates prepared from different regions of the heart (Figure 13). Approximately 250 \(\mu\text{M}\) carbachol displaced 50% of 1 \(\text{nM}\) \[^{3}\text{H}\]-QNB specifically bound to the muscarinic receptors in all regions. Carbachol decreased \[^{3}\text{H}\]-QNB binding with the same affinity in the right and left auricle, the right and left atrial wall and in the right and left ventricle (not shown).
FIGURE 13. $^3$H-Quinuclidinylbenezylate (1nM) binding to the muscarinic receptors in the right auricle (●), left auricle (○), right atrial wall, (■), and left atrial wall (□) is inhibited by increasing concentrations of carbachol. The Ki for $^3$H-QNB inhibition with carbachol was 25 μM. The results represent the mean of three separate experiments with a standard error of less than ten percent.
Effect of Phospholipase Inhibitors on Muscarinic Receptor Binding

Table 3 indicates the effects of phospholipase inhibitors on specifically bound $[^3$H]$\text{-QNB}$ (1 nM) and on the ability of carbachol to decrease $[^3$H]$\text{-QNB}$ binding (1 nM). Specifically bound $[^3$H]$\text{-QNB}$ (1 nM) was decreased 21 and 35% in the presence of 10 and 50 μM carbachol, respectively. Trifluoperazine (20 μM) had no effect on $[^3$H]$\text{-QNB}$ binding or on the effect of carbachol on $[^3$H]$\text{-QNB}$ binding. Quinacrine (50 μM), however, reduced $[^3$H]$\text{-QNB}$ binding by 71% and reduced the effect of carbachol on $[^3$H]$\text{-QNB}$ binding. 4-Bromophenacyl bromide (100 μM) had no effect on $[^3$H]$\text{-QNB}$ binding, however, it prohibited carbachol's ability to decrease $[^3$H]$\text{-QNB}$ bound to muscarinic receptors.
EFFECT OF PHOSPHOLIPASE INHIBITORS

ON MUSCARINIC RECEPTOR BINDING

CPM $^3$[H]-QNB SPECIFICALLY BOUND

<table>
<thead>
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<th>CARBACHOL (μM)</th>
<th>0</th>
<th>10</th>
<th>50</th>
</tr>
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<tbody>
<tr>
<td>CONTROL</td>
<td>2121</td>
<td>1678</td>
<td>1383</td>
</tr>
<tr>
<td></td>
<td>±211</td>
<td>±198</td>
<td>±31</td>
</tr>
<tr>
<td>TRIFLUOPERAZINE (20 μM)</td>
<td>1926</td>
<td>1621</td>
<td>1359</td>
</tr>
<tr>
<td></td>
<td>±126</td>
<td>±170</td>
<td>±59</td>
</tr>
<tr>
<td>QUINACRINE (50 μM)</td>
<td>610</td>
<td>479</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>±31</td>
<td>±55</td>
<td>±38</td>
</tr>
<tr>
<td>4-BROMOPHENACYL BROMIDE (100 μM)</td>
<td>1818</td>
<td>1813</td>
<td>1716</td>
</tr>
<tr>
<td></td>
<td>±219</td>
<td>±314</td>
<td>±40</td>
</tr>
</tbody>
</table>

**TABLE 3.** Isolated myocytes were incubated with $^3$[H]-QNB, physiological buffer and the agent in the right column and 0, 10 and 50 μM concentrations of carbachol were added. The specific binding to muscarinic receptor was determined based on the addition of 1 nM $^3$[H]-QNB to each assay tube. The results indicate the mean with the standard deviation of the mean for n=3.
DISCUSSION

The results of this study show that isolated myocytes, prepared from heart tissue by collagenase treatment, remain viable and calcium tolerant. This was evidenced by their retention of morphological integrity, their ability to incorporate $^{32}\text{P}$ into PA and PI, as well as their responsiveness to muscarinic agonists.

Carbachol-Induced Phospholipid Effect

Carbachol significantly increased $^{32}\text{P}$ incorporation into PA within 30 seconds in atrial myocytes, which was followed by an increase in PI labeling within 2.5 minutes. This rate is similar to those reported in atrial slices (Quist et al., 1985) and in other tissues such as platelets (MacIntyre and Pollock, 1983; Lapatina et al., 1981; Broekman et al., 1980), pancreatic acini cells (Halenda and Rubin, 1982; Best and Malaisse, 1983), ilial smooth muscle (Jefferji and Michell, 1976) and adipocytes (Schimmel et al., 1983). Thus, collagenase treatment does not destroy the ability of the myocardial muscarinic receptors to stimulate a phospholipid effect. The antagonism of the carbachol
pretreatment, suggests that carbachol stimulates the increase of $^{32}$P incorporation into PI and PA by stimulating muscarinic receptors. Carbachol initially stimulates breakdown of the polyphosphoinositides to diacylglycerol, probably by muscarinic receptor activation of phospholipase C. The diacylglycerol produced is then rapidly phosphorylated to PA by DAG kinase, and part of the newly synthesized PA is further synthesized to PI. The polyphosphoinositides, PIP and PIP$_2$ are rapidly broken down by receptor stimulation in hepatocytes (Litosch et al., 1983; Joseph et al., 1984), iris smooth muscle (Abdel-Latif et al., 1977), pancreas (Putney et al., 1983) and platelets (Vickers et al., 1982; Billah and Lapetina, 1982). Recently, Mattern et al. (1985), reported that carbachol stimulated a rapid decrease in $^{32}$P prelabeled PIP and PIP$_2$ within 30 seconds in isolated cardiac myocytes. This suggests that the carbachol induced phospholipid effect occurs by activating phospholipase C. Brown and Masters (1984) found an increase in inositol 1-phosphate formation after 10 minutes of carbachol exposure in chick myocytes prelabeled with $[^3H]$-myoinositol. Since hydrolysis of PI to DAG and inositol 1-phosphate occurs at a slower rate than the breakdown of PIP and PIP$_2$ in cardiac myocytes; then, polyphosphoinositide breakdown,
and not PI breakdown is the primary signal leading to the phospholipid effect. Unfortunately, we have been unsuccessful in measuring carbachol stimulated increases in inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate in cardiac myocytes prelabeled with [³H]-myoinositol, nor have we been successful at measuring decreases in ³²P labeled PI following carbachol stimulation (unpublished observations).

The $K_d$ for the carbachol stimulated increase in ³²P labeled PA and PI in isolated myocytes is 10 µM. This agrees with earlier studies done in atrial tissue (Quist, 1982; Brown and Brown, 1983). Furthermore, the results of this study have shown that carbachol stimulates the phospholipid effect in both atrial and ventricular myocytes, which confirms previous studies in heart slices by Brown and Brown (1983) and Quist et al. (1985).

**Calcium Dependence of the Phospholipid Effect**

These results indicate that the muscarinic stimulated phospholipid effect in cardiac myocytes is also dependent on the presence of calcium in the extracellular medium. This finding is in agreement with previous
studies done in heart slices (Quist, 1982; Brown and Brown, 1983). However, in isolated myocytes, only 5 uM calcium was required in the extracellular medium for carbachol to stimulate a phospholipid effect; while in atrial tissue slices, calcium concentrations of 100 uM were required (Quist et al., 1985). This may indicate that the isolated cells are more sensitive to changes in extracellular calcium concentrations than intact tissue slices. In the absence of carbachol, increasing the calcium concentration had no effect on PA labeling, and decreased PI labeling half-maximally at 500 uM calcium. This diminution in $^{32}$P labeled PI in the presence of increasing concentrations of calcium may be the result of inhibition of enzymes required for PI synthesis from PA. It is unlikely that extracellular calcium stimulates the phospholipid effect by entering cells through calcium channels because the Ca$^{2+}$ channel blockers; verapamil, nifedipine and diltiazem, had no effect on the carbachol stimulated phospholipid effect (Quist et al., 1985). At the present time, the site of Ca$^{2+}$ activation of the phospholipid effect has not been determined.

The heart is not unique in requiring extracellular calcium for carbachol to induce a phospholipid effect. Akhtar and Abdel-Latif (1978) reported that the mus-
carinic receptor activated breakdown of PIP and PIP₂ in iris smooth muscle was Ca²⁺ dependent. Cockroft et al. (1980), also reported a Ca²⁺-dependent, f-MetLeuPhe-stimulated phospholipid effect in rabbit neutrophils. The phospholipid effect may not play a role in calcium mobilization, since the phospholipid effect is calcium dependent. Thus, it may not mediate Ca²⁺ dependent cell processes in these tissues, since calcium is required for carbachol to activate the phospholipid effect. The phospholipid effect, however, may require less calcium in these tissues for activation than the increase in calcium due to polyphosphoinositide breakdown. In most tissues, the phospholipid effect has been found to be either calcium independent or partially calcium dependent. Examples include: parotid acinar cells (Weiss et al., 1982), blowfly salivary glands (Litosch et al., 1984), hepatocytes (Billah and Michell, 1979) and the pancreas (Putney et al., 1983; Farese et al., 1982). Weiss et al. (1982) reported that muscarinic receptor induced PIP₂ breakdown is also independent of both calcium influx and calcium release. The calcium ionophores, A23187 and ionomycin, did not induce a phospholipid effect; implying that the phospholipid effect is not a consequence of calcium mobilization (Putney et al., 1983; Litosch et al., 1984).
Therefore, the phospholipid effect is not a result of a change in extracellular calcium concentration nor does it cause a change in the extracellular calcium concentration. This suggests that if the calcium gradient is increased it is due to mobilization of intracellular calcium stores.

Uchida et al. (1982) found that alpha-adrenergic receptor stimulation of the phospholipid effect in myocytes was not dependent on extracellular calcium, and believes that because of this the alpha-adrenergic-stimulated breakdown of phosphoinositides may lead to calcium mobilization in the heart. Exton (1981) also postulated that alpha adrenergic stimulation in the heart may produce a signal to mobilize calcium. Therefore, the calcium-dependent, muscarinic-induced phospholipid effect might not be responsible for mobilizing calcium but instead another biochemical response in the heart, such as the phosphorylation of certain proteins. In further support of this concept, it is known that alpha adrenergic and muscarinic stimulation have opposing effects on cardiac contractility.
Inhibition of the Phospholipid Effect with Phospholipase C Inhibitors

In order to investigate whether muscarinic receptor stimulation activated phospholipase C to induce a phospholipid effect, the non-specific phospholipase C inhibitors, quinacrine (Irvine et al., 1978; Best et al., 1984; Hoffman et al., 1982), 4-bromophenacyl bromide (Hoffman et al., 1982; Best et al., 1984) and trifluoperazine (Craven and DeRubertis, 1983), were employed. These agents are considered non-specific, because they affect other processes in the cell. For instance, trifluoperazine acts as a calmodulin antagonist (Weiss and Wallace, 1980; Levin and Weiss, 1977) and inhibits phospholipid-sensitive, calcium-dependent protein kinase (Shatzman et al., 1981). 4-Bromophenacyl bromide (Roberts et al., 1977) and quinacrine (Vargraftig and Dao Hai, 1972) also inhibit phospholipase A₂. Trifluoperazine, quinacrine and 4-bromophenacyl bromide were shown to block the carbachol-stimulated increases in ³²P labeled PA and PI in isolated myocytes. It is not known if this inhibition is specifically due to phospholipase C inactivation or whether it is a result of some other non-specific
Muscarinic Receptor Binding

Muscarinic receptor binding studies were done to investigate the regional distribution of muscarinic receptors in canine heart. Muscarinic receptor density was found to be greater in auricles than in ventricles. This finding disagrees with the studies by Wei and Sulakhe (1978) who found similar muscarinic receptor densities in the atria and ventricles. However, in other species such as the rabbit (Fields et al., 1977; Wei and Sulakhe, 1978) and the rat (Wei and Sulakhe, 1978; Roeske and Yamamura, 1980), the density of muscarinic receptors were found to be greater in atria than in ventricles. Wei and Sulakhe (1978) and Fields et al. (1977) also found the left atrium to be more densely populated with muscarinic receptors than the right atrium; unlike our results which demonstrated similar muscarinic receptor densities in the left and right atrium. The affinity for $[^3H]$-QNB was found to be slightly greater for muscarinic receptors in the ventricles than in the atria. However, the muscarinic agonist carbachol was found to decrease $[^3H]$-QNB bind-
ing with the same affinity in auricles as in the ventricles. The affinities of carbachol and [3H]-QNB do not coincide in the auricles and ventricles.

The distribution of muscarinic receptor subtypes in the heart may explain the different electrophysiological effects elicited by muscarinic agonists in the atria than in the ventricles. In the atria, muscarinic agonists increase potassium ion efflux; whereas in the ventricles, muscarinic agonists have no effect on potassium conductance. Thus, the distribution and comparison of muscarinic receptor subtypes in the auricles and ventricles should be further investigated.

Brown and Brown (1984), in studying low and high affinity muscarinic agonist binding sites, found PI hydrolysis associated with the low affinity muscarinic receptor binding sites and adenylate cyclase inhibition associated with the high affinity binding site of carbachol. Quist et al. (1985) also found that muscarinic agonists, which interact primarily with the low affinity form of the muscarinic receptor stimulate the phospholipid effect; whereas oxotremorine, a high affinity agonist, does not. Brown et al. (1985) found that the M1 muscarinic receptor does not regulate phosphoinositide hydrolysis in the heart. Brown et al. (1985) found predominantly M1 muscarinic receptors in chick
embryonic heart and Hammer and Giachetti (1980) reported a greater $M_2$ receptor population in heart tissue. Chaissang et al. (1985) stated that there were at least two muscarinic receptor binding sites in the atria; one which regulates heart rate and one that mediates the contractile force. Additional studies are necessary to correlate the specific muscarinic subtype with its biochemical effect and physiological function. Halvorsen and Nathanson (1981) have postulated that the negative chronotropic effect may be mediated by agonist binding to the low affinity muscarinic receptor in the atria. Since the low affinity muscarinic receptor stimulates phosphoinositide turnover, it may then mediate the negative chronotropic effect.

Phospholipase C Inhibitors: Their Effects on Muscarinic Receptor Binding Studies

Quinacrine, a non-specific phospholipase inhibitor, reduced $[^3H]QNB$ binding and carbachol's ability to decrease $[^3H]QNB$ binding. Quinacrine could therefore be considered a muscarinic antagonist, and its use as a phospholipase C inhibitor should be questioned; particularly as it relates to muscarinic receptor activated phosphoinositide turnover. 4-Bromophenacyl bro-
mide had no effect on $[^3H]-QNB$ binding; however, it prevented carbachol from decreasing $[^3H]-QNB$ bound to muscarinic receptors. 4-Bromophenacyl bromide may allosterically antagonize carbachol's ability to bind to the muscarinic receptor; however, additional studies are required to further elucidate 4-bromophenacyl bromide's antagonism of carbachol binding. Trifluoperazine was found to have no effect on $[^3H]-QNB$ binding or carbachol's ability to decrease $[^3H]-QNB$ binding. Thus, trifluoperazine's inhibition of the carbachol stimulated phospholipid effect may be due to its inhibition of phospholipase C. Trifluoperazine has been reported to inhibit carbachol-evoked secretion of catecholamines by inhibiting calcium uptake in adrenal medullary cells (Wada et al., 1983) and to inhibit thyrotropin releasing hormone stimulated TSH release and Ca$^{2+}$ influx in pituitary tissue (Fleckman et al., 1981). Thus, trifluoperazine could inhibit the carbachol stimulated increase in $^{32}P$ labeled PI and PA, by preventing calcium entry into cells or by inhibiting the phospholipase C.

In conclusion, isolated myocytes do exhibit a muscarinic receptor stimulated phospholipid effect which is calcium dependent and found in all regions of the heart. Carbachol activates phospholipase C, which
then stimulates phosphoinositide hydrolysis. Therefore, isolated myocytes are a useful model system to study the muscarinic receptor stimulated phospholipid effect. Further studies are required to elucidate the biochemical and electrophysiological consequences of the muscarinic induced phospholipid effect in the heart, and how it may effect the physiological function of the heart.
SUMMARY

Stimulation of muscarinic receptors in heart decreases heart rate, the force of contraction and conduction velocity (Higgins et al., 1973). The electrophysiological and biochemical responses elicited by muscarinic agonists to produce physiological effects include: an increase in the outward potassium current (Giles and Noble, 1976), a decrease in the slow inward current (Pappano et al., 1982; Sperelakis, 1984), a decrease in cyclic AMP formation (Endoh, 1980; Sperelakis, 1984) and an increase in cyclic GMP formation (Endoh, 1980; Mirro et al., 1979).

Recently, it has been shown that stimulation of muscarinic receptors in heart initiate "a phospholipid effect" (Quist, 1982; Brown and Brown, 1983). The phospholipid effect, first demonstrated by Hokin and Hokin (1953) in pancreas, refers to the selective increase in $^{32}$P incorporation into phosphatidic acid and phosphatidylinositol following receptor stimulation. In most tissues, in which a phospholipid effect can be demonstrated, receptor stimulation results in the activation of phospholipase C which cleaves a phospholipid, presumably a phosphoinositide to diacylglycerol and
pidly phosphorylated to phosphatidic acid and further synthesized to phosphatidylinositol. Carbachol, a non-hydrolyzable muscarinic receptor agonist, stimulates a phospholipid effect in canine atrial tissue slices (Quist, 1982) and in murine atria and ventricles (Brown and Brown, 1983). Very few studies have been done to characterize the physiological relevance of these observations in the heart. In this study the phospholipid effect was examined in isolated heart myocytes. Heart myocytes were isolated by collagenase treatment and were found to be viable and calcium tolerant. 10 μM carbachol was required to stimulate a selective increase in $^{32}$P labeling of PI and PA in isolated canine myocytes and a maximal effect was achieved at 250 μM carbachol. Carbachol selectively increased the labeling of PA within 30 seconds and the labeling of PI after 2.5 minutes. Carbachol had no effect on $^{32}$P labeling of PC. Inclusion of at least 5 μM calcium in the extracellular medium was required for carbachol to induce the phospholipid effect demonstrating that the phospholipid effect is calcium dependent.

Phospholipase C inhibitors were used to determine if carbachol initiates the phospholipid effect by activating phospholipase C. Agents known to be phospholipase C inhibitors include: trifluoperazine, quinacrine
and 4-bromophenacylbromide. These agents were found to inhibit the carbachol stimulated increases in both \(^{32}\)P-labeled PA and PI, supporting the hypothesis that carbachol stimulates phospholipase C activity.

Muscarinic receptor binding density was studied in various regions of the heart by measuring the binding of a radiolabeled muscarinic receptor antagonist \([^3H]\)-QNB. In homogenates, the affinity for QNB was found to be slightly greater for muscarinic receptors in ventricles than in atria. The density of muscarinic receptors was greater in atria than in ventricles. Carbachol decreased \([^3H]\)-QNB binding half-maximally at 250 \(\mu M\) in atrial and ventricular homogenates and in myocytes preparations. The phospholipase C inhibitors, quinacrine and 4-bromophenacylbromide were found to decrease \([^3H]\)-QNB binding, whereas trifluoperazine did not alter either the binding of \([^3H]\)-QNB or carbachol's attenuation of \([^3H]\)-QNB binding.

In conclusion carbachol stimulates a rapid phospholipid effect in canine myocytes, which is calcium dependent, is inhibited by trifluoperazine (20 \(\mu M\)) and is found in all regions of the myocardium. Thus, isolated myocytes provide a useful model system to further study molecular properties of the muscarinic receptor mediated phospholipid effect and its role in
cardiac function.
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