Acetylcholine muscarinic receptors and phosphatidylinositol turnover in the locust CNS

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ACETYLCHOLINE MUSCARINIC RECEPTORS AND PHOSPHATIDYLINOSITOL TURNOVER IN THE LOCUST CNS.

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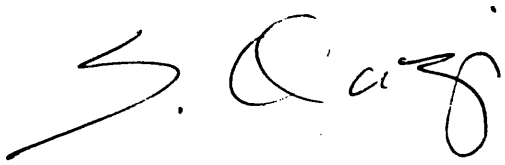

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ABSTRACTS.

1). Qazi, S. and Lunt, G.G., (1989). Inositol phosphates in locust ganglia: effects of muscarinic stimulation. The Biochemical society, 32nd Harden Conference, Wye college, England.

2). Qazi, S. and Lunt, G.G., (1989). Inositol phosphates in locust ganglia: effects of muscarinic stimulation. Proc. Int. Symp. Molec. Insect Sci. Tuscon, Arizona.

3). Qazi, S., Lunt, G.G., Blythe, J.L. and Earley, F.G.P., (1991). Phosphatidylinositol metabolism in insect nervous system. Neurotox '91, an international symposium on the molecular basis of drug and pesticide action, University of Southampton. 13th ISN Meeting, Sydney, Australia.

4). Qazi, S. and Lunt, G.G., (1991). Muscarinic receptors are coupled to phosphatidylinositol turnover and to adenylate cyclase. 21st Meeting of the Society of Neuroscience, New Orleans, USA.

PUBLICATIONS.

1). Qazi, S. and Lunt, G.G., (1989). Inositol phosphates in locust ganglia: effects of muscarinic stimulation. Proc. Int. Symp. Molec. Insect Sci. Editors Hildebrand, J. and Hagedorn, H.H., Plenum Press (1991).

2). Qazi, S., Lunt, G.G., Blythe J.L. and Earley, F.G.P., (1991). Phosphatidylinositol metabolism in insect nervous system. Extended Summaries Neurotox '91, Pesticide Science, 33, 249-252.

REFEREED PAPERS.

1).Qazi, S., Lunt, G.G., Blythe, J.L. and Earley, F.G.P.. Phosphatidylinositol metabolism in insect nervous system: Potential new target sites for insecticides. Pesticide Science, (in press).

SUMMARY.

The inositol-lipid system has not been extensively characterised in insect central nervous system. Such systems with their multiple sequential enzyme cascades offer the potential of a number of key modulatory sites at which exogenous agents could act to disrupt the system.

The results presented here show that phosphatidylinositol metabolism in locust (*Schistocerca gregaria*) ganglia is affected by treatment with muscarinic agonists. Incorporation of [^{3}H]-inositol and [^{32}P]-Pi into the phospholipids and into the acid soluble products of the inositol-lipid pathway was used to examine these effects. To show carbachol stimulation of phosphatidylinositol turnover, by measuring the accumulation of [^{3}H]-inositol phosphates, required a preincubation period and the presence of calcium, lithium and [^{3}H]-inositol in the incubation buffer. Atropine inhibited this effect, suggesting that these muscarinic like receptors are coupled to the activation of phospholipase C. The phosphatidylinositol pool could be highly labelled with [^{3}H]-inositol. It was found that there is a dose dependent breakdown of [^{3}H]-phosphatidylinositol to give [^{3}H]-inositol phosphates in the presence of carbachol and pilocarpine. H.p.l.c. analysis of the inositol phosphates showed the presence of at least two isomers of inositol trisphosphates: inositol 1,3,4-trisphosphate, which predominated, and inositol

1,4,5-trisphosphate.

Phosphorylation of phosphatidylinositol, phosphatidylinositol phosphate and diacylglycerol resulted in the rapid incorporation of [^{32}P]-Pi into phosphatidylinositol phosphate, phosphatidylinositol 4,5-bisphosphate and phosphatidic acid. Stimulation of phospholipase C activity in the presence of carbachol increased the rate of phosphatidic acid labelling and decreased the amount of label associated with the polyphosphoinositides. This being consistent with the production of inositol phosphates, seen in [^{3}H]-inositol labelling experiments, and diacylglycerol from the cleavage of polyphosphoinositides.

The muscarinic-like receptors in the locust ganglia were more fully characterised by inhibiting the binding of [^{3}H]-quinuclidinyl benzilate using various muscarinic agonists and antagonists. [^{3}H]-QNB bound to at least two classes of binding sites. The most potent of the muscarinic selective ligands for the [^{3}H]-QNB labelled sites was 4-DAMP, an M_3 selective muscarinic antagonist, which also recognised two sites (Ki's of 24 and 120nM).

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Chapter 3. [^{3}H]-INOSITOL LABELLING OF PI.

1.1). BACKGROUND.

Although at least five million chemicals have been tested for useful effects on various pest species, only a few target sites have emerged to be of practical and economic value. Neuroactive insecticides provide the most reliable, effective and economic solutions to insect pest problems and account for upto 90% of the insect control market. The cholinergic system in insects has been and still is a major target site for pesticides. This is not altogether surprising as acetylcholine is the major excitatory transmitter in insect CNS; its concentration ranges from 7.6 nmol/mg protein in housefly brain to 50 nmol/mg protein in locust thoracic ganglion (Clarke and Donnellan, 1982). Some elements of the cholinergic system have been well characterised and are known to be important targets for pesticide action, thus the nicotinic receptor is a target site for the nitromethylenes and cartap (Eldefrawi *et al.*, 1982; Satelle, 1985) and the enzyme acetylcholinesterase has long been known to be the site of action of the organophosphates and carbamates. Problems such as multiresistance and insecticide effects on non-target organisms require the development of new more selective target sites. Muscarinic-like receptors have been demonstrated to be present in the insect CNS (Knipper and Breer, 1988; Knipper and Breer, 1989) but their potential as an insecticide target site has not been exploited to date. The insect muscarinic receptor, like its mammalian counterpart appears to be a member of the G-

protein coupled, second messenger class of receptors. Such systems with their multiple sequential enzyme cascades offer the potential of a number of key modulatory sites at which exogenous agents could act to perturb the system.

1.2). THE CNS OF THE LOCUST.

In insect embryos the body is segmented and each segment contains a bilaterally symmetrical ganglion. These ganglia fuse, to some degree, during development to form the central nervous system (CNS). The most anterior ganglion in the insect after hatching is the supraoesophageal ganglion (also known as the cerebral ganglion or brain), it includes ganglia fused together with the primitive, presegmental archecerebrum. The suboesophageal ganglion is connected, via the circumoesophageal connectives, either side of the oesophagus, to the brain. This ganglion is the first ganglion in the ventral nerve cord. The chain of ganglia, running the length of the body are linked to each other longitudinally by connectives made up only of axons and supporting cells, while extending from each ganglion to the peripheral sense organs and effectors are the peripheral nerves (fig. 1).

The ganglia all have the same general structure. The mass is surrounded by an acellular structure called the neural lammella over a cellular layer, the perineurium.

Under the perineurium are found the cell bodies of the neurones, supported by glial cells. The neuropile, a complex of axons and dendrites which is where all synapses are thought to occur, occupies the centre of the ganglion. In insects the axons are not myelinated and there are few glia in the neuropile. This localisation of function and the lack of axo-somatic synapses is in contrast with the vertebrates where cell bodies recieve many synaptic connections.

Structurally the supraoesophageal ganglion is composed of three parts, the protocerebrum, deutocerebrum and the tritocerebrum. The protocerebrum is the largest structure, and may be the seat of behavioural organisation (Chapman, 1982). The deutocerebrum contains the antennal lobes, containing the the cell bodies of the sensory neurones, and the tritocerebrum is the point of origin for the connectives to the suboesophageal and frontal ganglia (fig. 2).

Figure 1.

The brain and the ventral nerve chain in the locust.

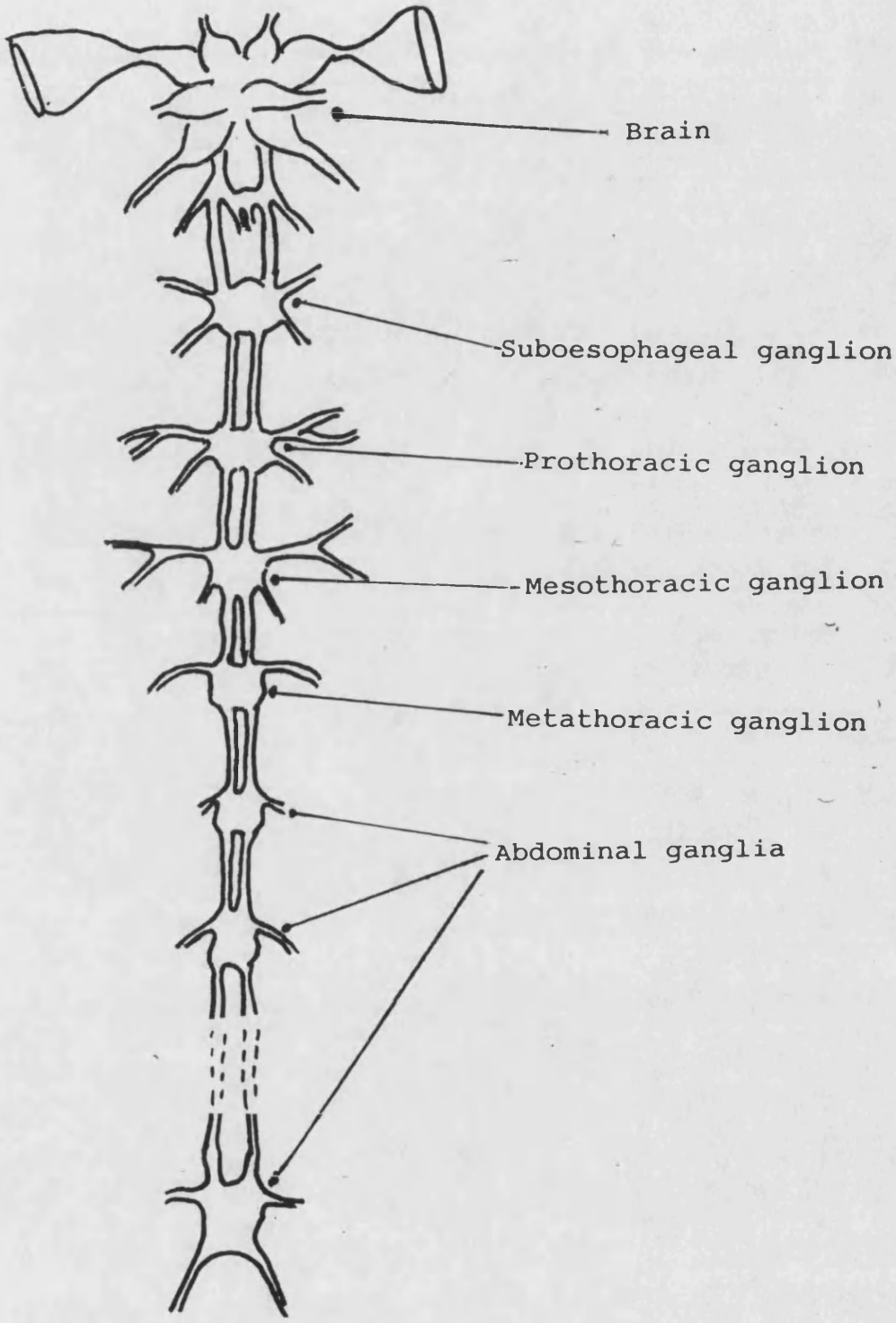
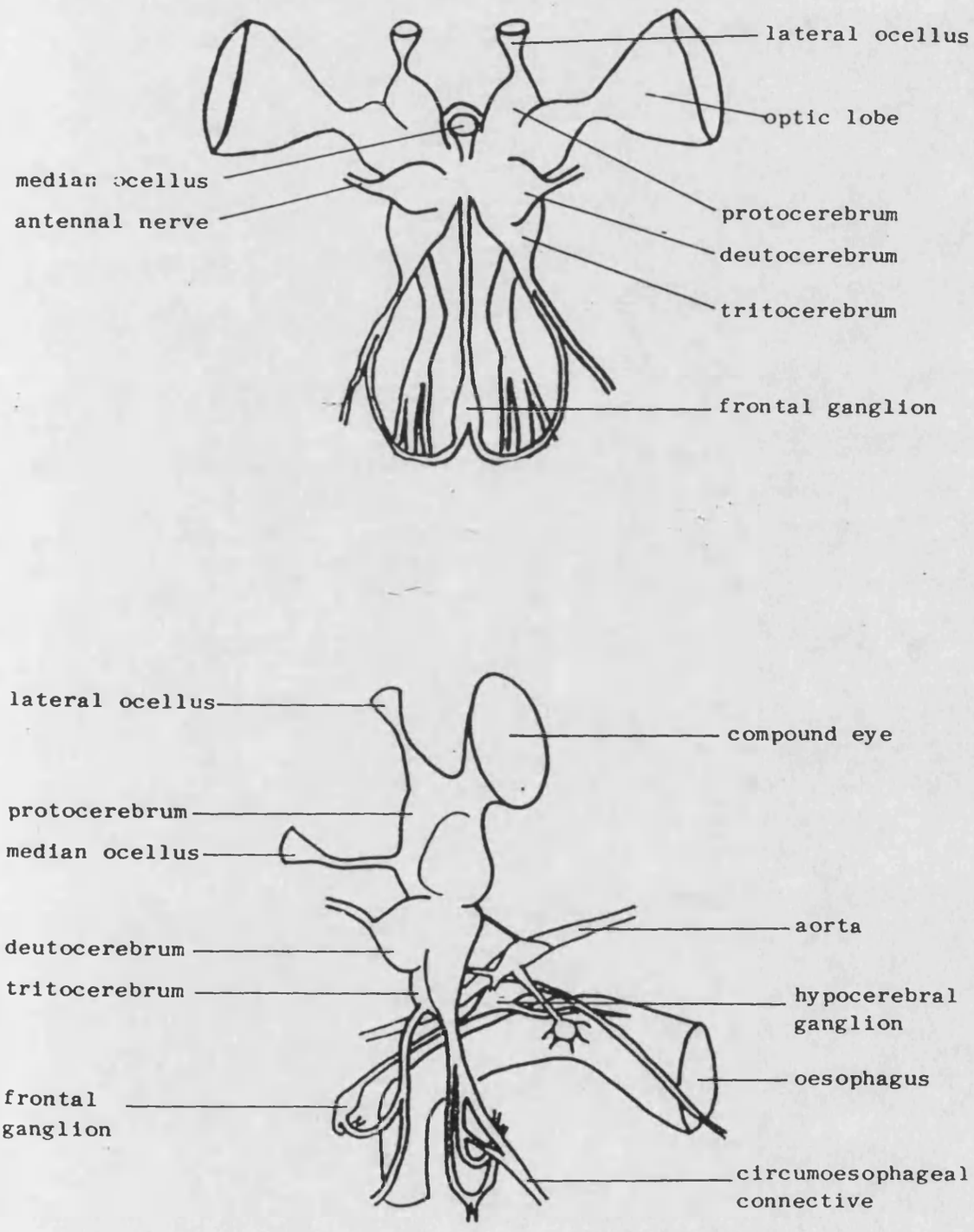


Figure 2.

The supraoesophageal ganglion of the locust (modified from Duggan, 1987).



1.3). THE MUSCARINIC RECEPTOR.

Cells in a multicellular organism need to be able to communicate, so that each cell population can call on the services of others, and respond to their requirements. This coordination is achieved by chemical signals. The central nervous system consists of highly specialised cells which can rapidly receive and respond to internal and external stimuli. Communication is achieved by propagation of electrical impulses along membranes of axons, which are elongated processes arising from cell bodies. Where nerve cells impinge on one another a synapse is formed. In cholinergic synapses , acetylcholine (ACh) is the chemical transmitter that is released from the synaptic terminal. Acetylcholine receptors (AChR), present on the post-synaptic membrane, receive the ACh. Binding of the ligand activates receptors which can open channels to allow potassium, sodium or calcium ions to pass through, or activate G-proteins. Existence of two superfamilies of receptors is confirmed from the genetic sequences of the ligand gated ion channels (nAChR, GABA, glutamate, 5-HT_3) and the metabotropic receptors (mAChR, adrenergic receptors). Acetylcholine can activate receptors belonging to both superfamilies, but the receptors can be pharmacologically distinguished by the action of nicotine and muscarine. The nicotinic acetylcholine receptors open cation channels to cause excitatory post-synaptic potentials, whilst the muscarinic

receptors via second messengers (cyclic GMP, cyclic AMP, inositol lipid pathway) activate protein kinases.

In mammals muscarinic receptors are found in all the effector cells stimulated by the postganglionic neurons of the parasympathetic nervous system, as well as those stimulated by the postganglionic cholinergic neurones of the sympathetic system (sweat glands, arterioles of the muscle), and also in the CNS (see Hoss and Ellis, 1985 for review). Muscarinic receptors can also be found on the presynaptic membrane. These receptors can alter the reactivity of the synapse to incoming signals, and hence are called synaptic modulators. One mechanism by which transmitter release can be modulated is by the level of calcium in the presynaptic terminal, which can activate calcium dependent potassium channels to hyperpolarise the presynaptic membrane. This can be mimicked by a second messenger in the inositol lipid pathway, inositol trisphosphate (Higashida and Brown, 1986). Thus, presynaptic activation of muscarinic receptors may regulate calcium levels through the production of second messengers, and hence the membrane resting potential. Hyperpolarisation would increase, and a depolarisation would decrease neurotransmitter release on arrival of an action potential at the presynaptic membrane.

1.3.1). MUSCARINIC ANTAGONIST BINDING.

The extensive distribution of muscarinic receptors in the central nervous system of mammals led to the attempt to pharmacologically characterise these receptors. Development of high specific activity, high affinity radioligands such as [^{3}H]-quinuclidinylbenzilate (Yamamura and Snyder, 1974 a,b) and N-methylscopolamine for use in binding assays resulted in the discovery of the complexity of the muscarinic receptor system.

Earlier studies on the binding of antagonists suggested that the muscarinic receptor populations in different tissues were homogenous (Hulme *et al.*, 1978). Binding capacities of various muscarinic [^{3}H] antagonists; [^{3}H]-QNB, [^{3}H]-NMS, [^{3}H] N-Methyl Atropine, [^{3}H]-PrBch, in the rat cerebral cortex (Hulme *et al*, 1978) was shown to be equal. In competition experiments with [^{3}H]PrBch (N-(2,3 [$^3H_2$] propyl) N, N-dimethyl-2-aminoethyl benzilate, a reversible muscarinic antagonist) , the Hill coefficients of all the antagonists tested were found to be close to unity, suggesting the reactions followed mass action principles at equilibrium.

The first real indication that there were subtypes of muscarinic receptors (table 1) came from competition studies against [^{3}H]-NMS using a novel antagonist, pirenzepine (Hammer *et al.*, 1980), which showed selectivity for the receptors in the cortex. These were termed M_1 muscarinic

receptors. As well as binding selectivity, pirenzepine exhibited functional selectivity (Hammer and Giachetti, 1982) in blocking responses at the atria and the sympathetic ganglia. Use of [^{3}H]-pirenzepine has shown high affinity binding in the rat cerebral cortex (Watson *et al.*, 1982), whilst only a few [^{3}H] -pirenzepine binding sites were demonstrated in cerebellar and heart homogenates. These high affinity binding sites for pirenzepine have been shown to be a subset of the available receptor binding sites as determined by [^{3}H]-QNB (Watson *et al.*, 1986). An analogue of pirenzepine, AFDX116, has been shown to have higher affinity for receptors in the heart, thought to be predominantly of the M_2 subtype (Hammer *et al.*, 1986). Other M_2 selective antagonists such as the polymethylene tetramines (Melchiorre, 1988) can distinguish between the glandular and cardiac types of muscarinic receptors. The glandular type of muscarinic receptors are termed M_3. 4-DAMP and hexahydrosiladifenidol have been shown to be selective for the M_3 subtype (Waelbroeck *et al*, 1989). More recently, tritiated analogues of pirenzepine have been developed which show better selection for the muscarinic subtypes. These being telenzepine (Eveleigh *et al.*, 1989) which has higher affinity for the M_1 receptor than pirenzepine, and AFDX384 which shows higher affinity for the M_2 than the M_3 muscarinic receptor (Entzeroth and Mayer, 1990).

Heterogeneity of muscarinic binding sites can also be

demonstrated by kinetic studies using [^{3}H]-NMS binding to rat CNS (Waelbroeck *et al.*, 1986). First order dissociation kinetics of [^{3}H]-NMS from striatum, cortex, hippocampus, cerebellum and heart differed markedly both in the extent of deviation from linearity and magnitude. These results suggested the existence of at least three classes of muscarinic receptors; type A having high affinity for pirenzepine and high affinity for NMS, type B having low affinity for pirenzepine and high affinity for NMS and type C having low affinity for both pirenzepine and NMS. A and B receptors were found in the hippocampus, cortex and striatum, whilst C receptors were localised in the heart. The deviations from linearity of the first order dissociation rate kinetics in the cerebral cortex were explained by the presence of at least three classes of receptors. This was postulated from the observation that [^{3}H]-NMS dissociation was occupancy dependent. In these studies the proportion of rapidly dissociating receptors increased with receptor occupancy. Furthermore, the affinity of the unlabeled ligand, pirenzepine, for each receptor class could be deduced, from competition assays, by allowing the tracer to dissociate from the most rapidly dissociating receptors. The nature of selectivity of antagonists to various muscarinic receptors has not been conclusively demonstrated. This is due to small differences in antagonist affinity for each receptor subtype (10 fold selectivity = 1.4Kcal/mole = Van der Waals energy for one methyl group)

(Hulme *et al.*, 1990).

However, some experimental evidence is provided by the studies of Kubo *et al.* (1988). Chimaeric receptors made from transmembrane regions 1-5 of the m1 receptor and transmembrane regions 6-7 of m2 expressed in *Xenopus* oocytes, showed that TM6-7 contributes to m1 selectivity, whilst m2 selectivity resided in TM1-5.

Birdsall *et al.* (1989) have shown that the binding of antagonists can be inhibited by the protonation of groups in free receptors with pK's in the range of 5.5-6.8. Binding of non-selective antagonists to rat cardiac receptors can be inhibited at pK values 5.5-6.1. However, inhibition of binding of an m2 selective antagonist, AFDX116, requires the protonation of an ionizable group which has a pK of 6.5-6.8. Thus ligands may have a choice of negatively charged residues with which to interact. An interesting finding was that methoctramine, a cardiac selective antagonist, could bind simultaneously to two ionisable groups which can be allosterically regulated by yet another titratable group with a pK of approximately 7.5.

Binding of [^{3}H]-atropine and [^{3}H]-4NMPB (N-methyl-4-piperidyl benzilate) to different regions of the mouse brain (Kloog *et al.*, 1979) indicated interaction of the ligand with a homogenous population of binding sites in each

region. There were small but consistent differences between the affinity constants in various regions. However, large differences in the kinetics of association and dissociation were demonstrated between the five regions of the brain investigated. The apparent half times of dissociation of [^{3}H]-4NMPB from the binding sites in the cerebellum and medulla were 3 to 4 times faster than from the hippocampus, caudate and cortex. Determination of the association rate constants at different [^{3}H]-NMPB concentrations showed biphasic behaviour at low ligand concentrations. This was indicative of receptor isomerisation. A binding mechanism involving receptor isomerisation was also suggested from the binding of 3-quinuclidinyl benzilate and N-methyl-4-piperidinyl benzilate to rat smooth muscle membranes from the small intestine (Jarv *et al.*, 1979). Equilibrium studies showed binding to a single high affinity binding site, while the pseudo-first order rate constant of association showed a hyperbolic dependence on the concentration of antagonist.

Further complexities in the binding of muscarinic antagonists have been demonstrated by detailed analysis of the effects of a competing ligand, whose binding pattern is known, on the binding pattern of the primary labeled ligand. Such detailed kinetic analyses have been used to identify negative cooperativity in the rat adenohypophysis, to which the binding of antagonists ((-) 3QNB, 4NMPB, NMS) yields curvilinear Scatchard plots (Henis and Sokolovsky, 1983). On

the assumption that these antagonists bind to two sites, the affinity constants of the high and low affinity sites can be determined. From this, the effect of a constant concentration of the unlabeled ligand on the binding pattern of the primary labeled ligand can be calculated for site heterogeneity without interactions. The inhibition of the binding of the primary ligand by the competing ligand showed significant deviations from that expected assuming heterogeneity, suggesting site-site interactions. The presence of multiple allosteric regulatory sites associated with the muscarinic receptor has been postulated from the actions of gallamine, which has nicotinic blocking properties, on the dissociation kinetics of [^{3}H]-N-methylscopolamine (NMS) (Stockton *et al.*, 1983) and [^{3}H]-quinuclidinyl benzilate (QNB) (Ellis and Siedenberg, 1989). Both association and dissociation of [^{3}H]-NMS showed a rapid then a slower phase of binding, and gallamine decreased the rates of these phases. The extent of decrease was greater for association than for dissociation. Gallamine had a biphasic effect on the dissociation of [^{3}H]-QNB. At low concentrations (0.1μM to 100μM), the dissociation rate of [^{3}H]-QNB was accelerated. This effect was most pronounced in the rat cardiac membranes. Above 100μM gallamine the disssociation rate was decelerated.

1.3.2). MUSCARINIC AGONIST BINDING.

The diversity of muscarinic subtypes has come from antagonist binding. The agonist binding properties of muscarinic receptors have not turned out to be simple. Complications arose from the fact that agonist binding is mirrored by function.

Agonist binding on crude synaptosomal fractions from rat cerebral cortex (Birdsall *et al.*, 1978) was found to deviate significantly from that predicted for a simple 1:1 interaction with a uniform population of sites. Hill plots of [^{3}H]-antagonist/agonist competition curves were found to be less than one suggesting binding site heterogeneity, negative cooperativity or receptor desensitisation. Exposure of membrane preparations to carbachol for up to 1000 sec did not result in any change in the inhibition of [^{3}H]-PrBCh binding by carbachol. This suggested that the receptor did not desensitise.

The possibility that there were negatively cooperating sites was investigated by irreversibly blocking some of the receptors using propylbenzilylcholine mustard (PrBCM), and then competing [^{3}H]-PrBCh with carbachol. This was based on the supposition that the receptors are organised as dimers, therefore by randomly alkylating a high proportion of the receptors the associations would be disrupted. The agonist inhibition curve would then be indistinguishable from a mass action curve. This was not found to be the case. Thus, the

low Hill coefficients of the agonists used (carbachol 0.33, acetylcholine 0.45, oxotremorine-M 0.25) suggested heterogeneity of binding sites. Also, by analysing the gradient of the occupying curve assessed by the inhibition of the specific binding of [^{3}H]-PrBCh three sites were seen; super high (SH), high (H), low (L) (Birdsall and Hulme, 1983). From the binding of [^{3}H] labelled agonists such as [^{3}H]-oxo-M the proportions of these sites were determined (Birdsall *et al.*, 1980). Examination of the character of muscarinic receptors in the different areas of the rat brain has shown the binding curves for the agonists to be different, whilst the binding curves for the antagonists were shown to be similar. These differences in agonist binding were attributed to the different abundances of the three receptor subtypes distinguished by agonists. The binding parameters of the super high state were determined by the use of [^{3}H]-oxotremorine-M, which almost exclusively binds to the highest affinity states. There was only a small variation in the absolute concentration of the SH sites between the six brain regions, whereas there were major differences in the absolute abundance of the low affinity states.

Comparisons of carbachol-stimulated turnover of phosphatidylinositol to the occupancy curve for carbachol, inferred from competition versus [^{3}H]-NMS (Hoss and Ellis, 1985), suggested that the phosphatidylinositol response of

the rat brain slices may be associated with a single population of muscarinic receptors. Analysis of the binding data showed the presence of at least two sites, while the dose response curve was based on a one site model (EC_{50} = 37μM). This single site for activation had an affinity between the values for the high and low affinity binding sites, and was tentatively postulated to be related to the H site of the three site fit of Birdsall *et al.*, (1980).

Agonist binding properties can be modulated by divalent ions and nucleotides. In the presence of GTP and magnesium ions the IC_{50} for carbachol, in competition experiments against [^{3}H]-NMS, is increased and most of the binding sites are converted to the low affinity type. The effect of magnesium ions alone was to decrease the IC_{50} by converting high affinity sites to super high affinity sites (Birdsall *et al*, 1984). Divalent ions are thought to promote receptor G-protein coupling in the absence of GTP, in which the receptor attains a very high affinity state. When GTP is present, the coupling is disrupted and the G-protein activated. Micromolar concentrations of Gpp(NH)p, a stable analogue of GTP, will not reduce the affinity of agonist binding unless there are super high and high affinity sites present, and there is a sufficiently high concentration of magnesium ions to allow nucleotide binding (Hulme *et al*, 1983). Some studies have shown that populations of binding sites can be locked into the super high affinity state and

cannot be affected by the addition of magnesium ions and GTP (Hulme *et al*, 1983; Gillard *et al*, 1987).

1.3.3). MUSCARINIC RECEPTOR GENES.

Molecular cloning studies have led to the identification of at least five muscarinic receptor clones (table 1). Screening of cDNA libraries from porcine cerebral and cardiac tissues with oligonucleotide probes, based upon the the partial amino acid sequences from purified receptors, led to the discovery of the m1 (cerebral) and m2 (heart) receptors (Kubo *et al.*, 1986a and b). A different strategy led to the identification of the next two muscarinic receptor clones (m3 and m4). A region of sequence homology between the rat m1 receptor and the hamster ß2 adrenergic receptor was used to construct the oligonucleotide, and was used to screen the rat cortex cDNA library (Bonner *et al.*, 1987) to identify m3 and m4. As all these four clones lacked introns, screening of the genomic library led to the cloning and sequencing of the fifth receptor m5 (Bonner *et al.*, 1988).

The presence of muscarinic receptors in insects has also been established from molecular cloning studies (Shapiro *et al.*, 1988; Onai *et al.*, 1989). Genomic and cDNA clones encoding a muscarinic receptor was isolated from *Drosophila melanogaster* in both studies. The receptor

displayed greater than 60% homology with the mammalian muscarinic receptors. High level of identity to the m1, m3, and m5 sub-types in the membrane proximal portions of the third cytoplasmic loop was shown (Shapiro *et al.*, 1989). Expression of the receptor into mouse Y1 adrenal cells, showed that it could stimulate phospholipase C activity. Thus, this gene was structurally and functionally most similar to the mammalian m1, m3, m5 subtypes. The insect receptor contains a longer intracellular loop between putative transmembrane segments V and VI. It has also been found that the locust muscarinic receptor is more susceptible to the action of disulphide reducing agents and arsenicals (Fonseca *et al.*, 1991). This could arise from weaker hydrophobic interactions between the transmembrane segments III and IV, which are thought to contain the residues involved in ligand binding. The receptor cloned from *Drosophila melanogaster* was found to be less hydrophobic in transmembrane region IV than the mammalian receptor. Assuming this is found to be the case in the locust, it could explain the differences observed in thermal stability and sensitivity to the action of reducing agents by Fonseca *et al.* (1991).

These muscarinic clones can be stably transfected into the chinese hamster ovary cell line (CHO-K1). Thus, the characteristics of pure receptor subtypes can be assessed (Buckley *et al.*, 1989). Pharmacological characterisation

using pirenzepine, methoctramine, AFDX116, hexocyclium and silahexocyclium showed that the binding profiles derived from displacement curves against competitions with [^{3}H]-NMS, of m1, m2 and m3 were similar to that of M_1, cardiac M_2 and glandular M_3. That is, pirenzepine had a high affinity for the m1 receptor, AFDX116 and methoctramine expressed a graded profile with the highest affinity for the m2, and hexocyclium was the most selective agent for the m3 receptor. A number of binding isotherms exhibited low hill numbers suggesting heterogeneity or negative cooperativity. This question of whether the sites are interacting in these pure populations of cells is currently being investigated (Buckley *et al.*, 1989).

A report that the the M_4 sites in rabbit lung, chicken heart and NG108-15 cells can be pharmacologically characterised has appeared in the literature (Lazareno *et al.*, 1990). This study measured the inhibitory potency, against [^{3}H]-NMS labelled sites, of 28 antagonists, and found that the binding profile was unlike that of M_1, M_2 and M_3 sites. These receptors had a moderate to high affinity for [^{3}H]-pirenzepine, but they were not M_1 sites, as the cardioselective antagonist himbacine had a 10 fold higher affinity at these sites than at the M_1 sites in rat rabbit cortex. The chicken heart receptor has been cloned and sequenced (Tietje *et al.*, 1990), and shows greatest homology with the mammalian m4 receptor (hence designated cm4). The

pharmacological characterisation of M_4 may be related to the cm4 molecular characterisation.

The differences in structure of the binding sites for the antagonists must be very subtle as no antagonist exhibits more than five fold selectivity for one subtype over all the other subtypes. This is expected, due to strong conservation of the relevant amino acids.

1.3.4). RESIDUES INVOLVED IN LIGAND BINDING.

Propylbenzilylcholine mustard (PrBCM) being a muscarinic selective covalent affinity label (Burgen and Hiley, 1974; Burgen *et al.*, 1974), has been used to identify the residues involved in the ground state binding of antagonists (Kurtenbach *et al.*, 1990). The covalently labelled receptors from the rat forebrain were cleaved with a specific protease, lys C, yielding two glycosylated peptides of 34 and 28KDa. This difference was sufficient to differentially enrich these two peptides using high resolution FPLC gel filtration. Subcleavage of the 28KDa [^{3}H]-PrBCM labelled peptide, with cyanogen bromide (CNBr), yielding primarily a 2.4KDa labelled product. Fractions enriched in the 34KDa peptide yielded a 3.9KDa labelled product after digestion with CNBr. The populations of polypeptides could be explained by the presence of more than one subtype of muscarinic receptor, as cleavage of a

preparation of cloned m1 mAChR purified from CHO-K1 cells gave primarily a 2.4KDa labelled peptide. This shorter peptide contains 26 residues which arise from m1, m3 and m5 sequences that contain an additional methionine residue (Met 88 between Met 79 and Met 114).

An Edman degradation of the [^{3}H]-PrBCM labelled CNBr peptides identified a conserved aspartate residue at position 105 (on the m1 sequence) to be the binding site. A PrBCM binding site also exists in the head and thoracic ganglia of locusts (Knipper and Breer, 1988). Incubation of neuronal membrane preparations with [^{3}H]-PrBCM, followed by SDS-polyacrylamide gel electrophoresis and autoradiography showed that a polypeptide with a molecular mass of 75KDa was labelled.

A considerable advance in the understanding of receptor structure-function relationships has occurred from the use of immortalised cell lines to express pure receptors. Site directed mutagenesis of these receptors has been performed to test which residues are important for function and binding. There are a number of highly conserved aspartate residues in the second and third segments of all the muscarinic receptors (Hulme *et al.*, 1990). This conservation extends to other members of this multigene family including the ß-adrenergic receptors (Venter *et al.*, 1989). There are four conserved aspartate residues in the muscarinic

receptors occurring at positions 71, 99, 105 and 122 in the m1 sequence. Point mutations of these residues to neutral asparagine (Asn) in the m1 receptor have been used to determine their importance to binding and function (Fraser *et al.*, 1989). Mutation of Asp105 abolished binding of QNB, thus characterisation of the Asn105 mutant could not be performed. Mutation of the corresponding residue in the ß-receptor reduced the level of binding for iodocyanopindolol to very low levels (Strader *et al*, 1987). Thus the aspartate 113 in the ß-receptor and the aspartate 105 in the m1 sequence suggests that this residue forms a part of the binding site.

The Asn99 mutant of the m1 receptor showed reduced affinity for the muscarinic antagonist QNB, atropine, pirenzepine and PrBCM and the agonists carbachol and oxotremorine. This mutant showed maximal carbachol stimulated PI hydrolysis, but higher concentrations of agonist were required for the response, reflected in the lower affinity for the agonists. Submaximal levels of covalent receptor labelling with PrBCM were observed, which implied that this Asp99 residue could also form part of the binding site. Thus both Asp105 and Asp99 may be required for stable ligand binding conformation.

Substitution of Asp71 or Asp122 with Asn produced mutant receptors that displayed high affinity for carbachol

(in fact the Asn71 mutant showed a higher affinity than the wild type receptor for carbachol), but decreased the efficacy and the potency of activation of PI hydrolysis. Asn71 and Asn122 mutants showed maximal PrBCM incorporation, thus suggesting that the Asp71 and Asp122 residues were not involved in antagonist binding. However, Asn122 reduced the affinity of the receptor to PrBCM indicating some interaction between Asp105 and Asp122 in the native receptor. Again, these findings were found to be similar for the ß-adrenergic receptor in which the mutations of the corresponding aspartate residues effected agonist binding but not antagonist binding (see Venter *et al.*, 1989 for review).

Determination of the residues involved in agonist binding may be made possible from the advent of alkylating acetylcholine analogues (Hulme *et al.*, 1991). Acetyl-choline mustard, acetylethylcholine mustard, butyltrimethylammonium mustard and methylfurmethide mustard were all capable of blocking mAChR's in a manner which deviated significantly from the simple Langmuir isotherm. Binding of these mustards clearly showed the presence of low and high affinity sites typical of agonists.

There is also a tyrosine residue on TM7 (position 408 in the m1 sequence) which is specific to the cationic amine receptor sequences and thought to be involved in the

formation of a hydrogen bond with the aspartate in TM3 (Hulme *et al*, 1989). This tyrosine residue is precisely homologous to the lysine residue which forms the site of covalent attachment of the cis-retinal chromophore in the rhodopsin family of proteins (Findlay and Pappin, 1986). Site directed mutagenesis of this residue will help to determine the importance of tyrosine 408 in ligand binding.

1.3.5). STRUCTURE OF THE MUSCARINIC RECEPTOR.

A major goal in receptor research is to determine the relationship between the function of a receptor and its three dimensional structure. Progress in obtaining the crystal structure for the large membrane bound proteins has been hampered by the difficulty in generating crystals of sufficient quantity and quality to refract for X-ray analysis. This may be remedied by high density expression of cloned receptor genes. Fortunately, the existence of bacteriorhodopsin in high density in a periodic lattice in the bacterial purple membrane has enabled electron microscopy and low dose electron diffraction analysis (Henderson *et al.*, 1990), which resolved the structure of the 7 transmembrane helical bundle to a resolution of 0.35-0.6nM.

Hydropathy analysis of bacteriorhodopsin, rhodopsin, muscarinic receptors and adrenergic receptors show a

strikingly similar pattern of seven strongly hydrophobic regions which are thought to correspond to the seven transmembrane helices observed in the bacteriorhodopsin diffraction maps (Venter *et al.*, 1989). Analysis of the primary sequences of these proteins exhibits significant similarity in these membrane spanning regions. These regions can be thought of as an everted version of the structure of water soluble proteins, in that the core residues tend to have polar side chains and the surface residues apolar ones. The surface residues are subject to fewer bonding constraints than the core residues, and are consequently more susceptible to genetic drift. Therefore, the helices should exhibit a conserved inward pointing face, whilst the outward pointing face should be less conserved. After alignment of the transmembrane regions of the twelve muscarinic receptors the number of different amino acid types occurring at each position in the aligned sequences can be determined, thus giving an index of variation at each position (Vj) (Hulme *et al.*, 1991). The fourier transform power spectrum (P(w)) was calculated given Vj, the number of residues in the transmembrane sequence and the angular rotation per residue around the helix axis. In the case of a standard α-helix with a conserved face and a non-conserved face the P(w) is expected to peak at 100^{o}. The results for the transmembrane sequences were found to be consistent with the predicted α-helical conformation, even when the calculations were repeated to derive the power spectrum for

amino acid polarity at each position. However, some variation in the power spectrum peaks were observed, and was thought to be due to tilting of the α-helix within the membrane, or to the dislocations in the helical wheel structure caused by the presence of proline residues.

In conclusion, by combining the three dimensional structure of bacteriorhodopsin, as a guide to protein folding, with fourier transform analysis of the residues, to search for periodicity, a working model of the intramembrane portion of receptor structure can be obtained (fig. 3). As this region contains the ligand binding site, the model allows interpretation of known features of the muscarinic receptor. More constraints for the model will come from studies in which the important residues involved in ligand binding are identified, to enable correlation between structure and function.

1.4). MUSCARINIC RECEPTORS COUPLED TO PI TURNOVER.

Cells in a multicellular organism need to be able to communicate, so that each cell population can call on the services of some cell populations and respond to the requirements of others (Berridge, 1984). This coordination is achieved by chemical signals such as peptide hormones and neurotransmitters, the primary messengers. Thus,

transduction of this signal occurs in the plasma membrane. On the outer surface of the membrane there are receptors which detect incoming signals. These activate proteins, by inducing conformational changes, to produce second messengers, whose diffusion enables a signal to propagate rapidly throughout the cell. These internal signals ultimately regulate cellular processes such as secretion, contraction, metabolism and growth..

Inositol compounds play a key role as regulators of calcium dependent metabolic processes. Phosphoinositides and inositol phosphates are involved in cellular signal transduction mechanisms. Agonists which stimulate phosphoinositide turnover also increase intracellular calcium (Michell, 1975), which binds to a family of proteins including calmodulin and troponin which then activate other kinases (Berridge, 1987 for review). More recent studies have shown that carbachol stimulation of m3 muscarinic receptors expressed in SY-SY5Y human neuroblastoma cells, and m1 receptors expressed in CHO cells leads to the biphasic production of inositol 1,4,5-tris phosphate, inositol 1,3,4,5-tetrakisphosphate and an increase in internal calcium ion concentration (Lambert *et al.*, 1992). As there are many steps in the cellular response, amplification and regulation can occur at many points.

The muscarinic receptor subtypes couple to multiple G-

proteins (fig. 4) to modulate many signal transduction pathways including stimulation of phospholipases A_2, C and D, attenuation of cAMP synthesis, stimulation of cAMP degradation, stimulation of cGMP production and regulation of several ion channels (see Hosey, 1992 for review). From the expression of cloned receptors in cell lines it has been possible to determine which receptor subtype couples to which effector protein. Generally, m1, m3 and m5 are coupled to PI turnover, whilst m2 and m4 are linked to adenylate cyclase inhibition (see Hulme et al., 1990 for review).

Analysis of the sequence data from m1-m5, revealed that a 16-17 amino acid segment at the amino terminal end of the third cytoplasmic loop (i3) is highly conserved between m1, m3, m5, but is different in m2 and m4 (Wess *et al.*, 1989). Construction of cDNA encoding chimaeric m2/m3 receptors, in which the small fragment or the whole i3 loop have been exchanged between the human m2 and the rat m3 receptor, have showed that the wild type m2 with the i3 loop or the small fragment from the m3 can strongly couple PI turnover. The wild type m3 with the i3 loop or the small fragment from the m2 receptor reduced PI turnover. Hence, PI specificity has been localised more accurately to the N-terminal 17 amino acids of i3 of the m3 muscarinic receptor. Further research will be required to determine whether the discrimination between G-proteins is exclusively in the cytoplasmic domains, or whether other, as yet unknown factors are

involved in functional selectivity.

Hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C yields sn1,2-diacylglycerol and inositol 1,4,5-trisphosphate. Diacylglycerol is required for the activation of protein kinase C (Nishizuka, 1986), and inositol 1,4,5-trisphosphate mobilises calcium from internal stores in the endoplasmic reticulum (see Berridge and Irvine, 1989 for review).

1.5). IMPORTANCE OF INOSITOL PHOSPHATES IN CALCIUM HOMEOSTASIS.

Agonists which function through the inositol lipid pathway have been shown to mobilise intracellular calcium and to stimulate extracellular calcium entry. This process involves both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (see Downes and MacPhee, 1990 for review). The dual action of these inositol phosphates provides an important mechanism for the control of the intracellular calcium ion concentration. The regulation of Ins(1,4,5)P_3 3-kinase by calcium ions may, in turn, regulate the relative levels of the two inositol phosphates, and thus control the calcium signal generated by the activation of the receptor.

One of the features of calcium entry and mobilisation is that it occurs in localised regions in the cell. This

permits the cell to use calcium for different purposes in different places at the same time. (Harary and Brown, 1984). As inositol phosphates are freely diffusible through the cytoplasm and act at sites remote from the agonist receptor, they may serve as a second messenger for the calcium response. Rapid formation of inositol phosphates, and their ability to control calcium levels in the cytosol provide an important mechanism by which short and long-term regulatory functions of the cell can be controlled.

1.5.1). THE INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR.

Inositol 1,4,5-trisphosphate (Ins (1,4,5)P_3) injected into permeabilized cell preparations stimulates calcium release from non-mitchondrial stores thought to be part of the endoplasmic reticulum (Berridge & Irvine, 1989).

Spat *et al.* (1987) demonstrated high affinity binding of radiolabelled Ins (1,4,5)P_3 in liver cells and neutrophils. Furthermore, this binding correlated with release of $^{45}Ca^{2+}$ from permeabilized cells. The binding site displays strict stereo selectivity with the D isomer of Ins (1,4,5)P_3 being greater than 3000 fold more potent than the L-isomer in the rat cerebellum, adrenal cortex and liver (Nunn *et al.*, 1990). The receptor is not thought to densensitize as the inositol 1,4,5 trisphosphorothioate (Nahorski and Potter, 1989), shows a sustained release of

calcium in human SY5Y neuroblastoma cells.

The abundance of the Ins(1,4,5)P_3 receptor in rat cerebellar purkinje neurons has enabled purification of the receptor and the raising of an antibody to it (Ross *et al.*, 1989). This antibody recognises a single polypeptide of relative molecular mass of 260 KDa. Reconstitution of the IP_3 binding protein into lipid vesicles (Ferris *et al.*, 1989), shows the receptor to be functional, in that Ins(1,4,5)P_3 stimulated calcium flux. The IP_3 receptor binds with high affinity to heparin and concanavalin A, thus enabling the development of an effective purification scheme (Suppattapone *et al.*, 1988). This protein is a major substrate for cyclic AMP-dependent phosphorylation, which inhibits Ins(1,4,5)P_3 activated release of calcium (Suppattapone *et al.*, 1988).

Availability of three monoclonal antibodies to the purified receptor IP3 protein (termed P400) enabled Furuichi *et al* (1989) to identify complementary DNA's within mouse cerebellum cDNA libraries that expressed the protein. The entire cDNA sequence, had an open reading frame encoding a protein of 2749 amino acids of theoretical molecular mass of 313KDa. From hydropathy profiles, the protein may contain up to nine membrane spanning domains, but immunolocalisation studies showed the presence of a large N-terminal region on the cytoplasmic face of the endoplasmic reticulum. Hence, a

cluster of up to seven transmembrane regions were proposed close to the C-terminus.

A similar clone was obtained by Mignery *et al.* (1989). It was noticed that a partial sequence of a major purkinje cell transcript named PCD6 (500 amino acids) was for a protein containing several transmembrane domains. With the antibodies to the c-terminal peptide, the sequence of the part of a heparin binding protein was identified. The 500 amino acid sequence differed by only one amino acid from the c-terminal sequence obtained by Furuichi *et al.*, (1989). A region of 134 amino acids in the c-terminal of the IP_3 receptor closely resembled the c-terminal of the ryanodine receptor in skeletal muscle (64% homology including conservative changes). Both of these receptors mediate calcium release into the cytosol and they form multimeric structures possibly in a square configuration (Furuichi *et al.*, 1989). There is evidence that the IP_3 receptor may function cooperatively, in which the opening of the calcium channel requires occupancy of three or more binding sites (Meyer *et al.*, 1988).

Ins(1,4,5)P_3 sensitive calcium stores were thought to exist within discrete calcium storage organelles, calsiosomes, based on observations by Volpe *et al.*, (1988), who showed that calsequestrin-like protein in non muscle cells copurified with markers of the IP_3 sensitive calcium

store. However, immunohistochemical studies showed that the receptor is located in the endoplasmic reticulum and related membranes such as golgi and nuclear envelope (Furuichi *et al.*, 1989; Mignery *et al.*, 1989).

1.5.2). CALCIUM MOBILISATION AND ENTRY STIMULATED BY INOSITOL 1,4,5-TRIPHOSPHATE AND INOSITOL 1,3,4,5-TETRAKISPHOSPHATE

Inositol 1,4,5-trisphosphate (IP_3) induced calcium release from internal stores into the cytoplasm shows complexity in the way it occurs (Muallem *et al.*, 1989; Taylor and Potter, 1989). A maximum concentration of IP_3 is expected to rapidly mobilise all the calcium from the internal pool, further mobilisation will not occur until the pool is replenished. One might expect submaximal concentrations of IP_3 to cause a submaximal opening of calcium channels, the result being a slower efflux of calcium, but on prolonged stimulation leading ultimately to the same net release. Studies on permeabilised pancreatic acinar cells (Muallem *et al.*, 1989) and permeabilised hepatocytes (Taylor and Potter, 1989) have shown this type of continuous release using a calcium ionophore, ionomycin. This contrasts with the addition of IP_3, in which submaximal concentrations of IP_3 release a small fraction of the calcium stored. Increasing the IP_3 concentration increased the fraction of calcium mobilised. Prolonged stimulation

with a calcium mobilising hormone or maintained levels of IP_3 did not fully deplete the calcium store. This rapid release of a fraction of releasable calcium was termed "quantal release" (Muallem *et al.*, 1989). Taylor and Potter (1989) argued that the quantal release observed by Muallem *et al.*, (1989) was probably due to IP_3 regulating the size of the IP_3-sensitive calcium pool, not to partial depletion of the total pool. This being an extension of the idea that GTP could augment calcium release by increasing the size of the IP_3 sensitive pool through translocation of calcium ions between intacellular organelles (Ghosh *et al.*, 1989). A mechanism to explain both of the above observations has been proposed by Irvine (1990). This hypothesis suggests a role for a calcium ion binding site on the intraluminal side of the IP_3 receptor and also, that the binding of calcium to this site increases the affinity of the receptor for IP_3; consequently binding of IP_3 increases the affinity of the calcium site.

As well as calcium mobilisation, agonists which function through the inositol lipid pathway are able to stimulate influx of external calcium. This regulation of calcium entry is poorly understood (Putney *et al.*, 1989). Direct stimulation of a plasma membrane calcium channel by ATP in smooth muscle cells has been shown (Benham and Tsien, 1987). However, in most cells studied, calcium entry is triggered by a fall in the levels of internal stores

(Takemura *et al.*, 1989). The two components of calcium mobilisation and calcium entry can be resolved by comparing the calcium signals when the cells are stimulated in calcium free or calcium containing media (Merritt and Rink, 1987). In the absence of calcium the response is transient, whilst in the presence of extracellular calcium the levels of intracellular calcium remain elevated. Lag times for influx and release seem to be indistinguishable, hence as soon as calcium is released more enters through the plasma membrane. The capacitance model, proposed by Putney (1986), argues that the empty state of the IP_3 sensitive calcium store activates an influx pathway that refills the store.

A possible role for inositol 1,3,4,5-tetrakisphosphate (IP_4) in regulating the calcium influx phase has been postulated. Irvine *et al.* (1988) proposed a mechanism analogous to that of GTP stimulated channels linking IP_3 dependent and IP_3 independent calcium stores. The requirement for IP_3 and IP_4 for prolonged stimulation of calcium-dependent outward potassium current in lachrymal acinar cells (Morris *et al.*, 1987) and activation of sea urchin oocytes (Irvine and Moor, 1986) suggests that IP_4 might serve some specific cellular function. IP_4 binding sites have been shown in calf adrenal cortex (Enyedi and Williams, 1988) and Scatchard analysis of the binding data indicated that the population was heterogenous. This receptor was distinct from the IP_3 receptor as IP_3 was

unable to displace [^{32}P]-IP_4 from its binding site.

Irvine (1990) has suggested a mechanism by which the IP_3 receptor and the IP_4 receptor may interact to stimulate calcium entry. In this proposal, the calcium levels in the endoplasmic reticulum lumen influence calcium influx through the plasma membrane via the association with the IP_3 receptor, with an intraluminal calcium binding site, and a plasma membrane associated IP_4 receptor. In this model, low intraluminal calcium would reduce calcium release into the cytosol by reducing the affinity of the IP_3 receptor, and on binding of IP_3 and IP_4 to their respective receptors would promote calcium entry. The role of IP_4 binding would be to dissociate the IP_3 and IP_4 receptors to allow the entry of calcium.

1.5.3). METABOLISM OF INOSITOL PHOSPHATES.

Cells posess an ever increasing number of phosphatases and kinases which interconvert a bewildering array of inositol phosphates (Shears, 1989). This complexity is more apparent than real, as the metabolism of inositol phosphates need to fulfill three basic functions:

1). The rapid metabolism and hence control of cellular concentrations of Ins(1,4,5)P_3.

2). Recycling of inositol phosphates back to inositol, which is used for the regeneration of phosphatidylinositol.

3). The synthesis of higher inositol phosphates such as inositol tetrakisphosphates, inositol pentaphosphates and phytic acid. These may have distinctive functions in the cell.

Levels of inositol phosphates which change in response to agonist stimulation, and hence may be related to intracellular signalling will be considered. Inositol-1,4,5 trisphosphate ($Ins(1,4,5)P_3$ and inositol-1,3,4,5 tetrakisphosphate ($Ins(1,3,4,5)P_4$) are the principle second messengers. The enzymes involved in the interconversions between the two above mentioned inositol phosphates, and their subsequent degradation back to inositol are $Ins(1,4,5)P_3$ 3-kinase, inositol polyphosphate 3-phosphatase, inositol polyphosphate 5-phosphatase, inositol polyphosphatase 4-phosphatase, inositol polyphosphate 1-phosphatase and inositol monophosphate phosphatase (see Shears, 1989 for review).

Inositol 1,4,5-trisphosphate is metabolised in cells via two routes. The first involving a 5-phosphatase cleavage to yield inositol 1,4-bisphosphate. Two distinct 5-phosphatases have been isolated from human platelets (Mitchell *et al*, 1989) and bovine brain (Erneux *etal*, 1989). The second involves a 3-kinase phosphorylation to yield a potential second messenger molecule inositol 1,3,4,5-tetrakisphosphate (Irvine *etal*, 1988). Calcium ions at

physiological concentrations (Johanson *etal*, 1988), and protein kinase C (King and Rittenhouse, 1989) stimulate the activity of the 3-kinase.

The metabolism of inositol phosphates is known to be affected by lithium ions, which non-competitively block inositol monophosphate phosphatase with an apparent Km of 0.8mM (Hallcher and Sherman, 1980). This noncompetitive action of lithium is very important in selectively operating on receptors that are overactive, whilst the receptors that are working normally are less affected (Nahorski *et al*, 1991). In its action lithium is thought to bind to the enzyme-substrate complex and not to the free enzyme. Administration of lithium ions has been used for the treatment of manic depression. Research into the effects of lithium ions on inositol polyphosphate hydrolysis led to the inositol depletion hypothesis to explain the neural and developmental effects of lithium, on the basis that it inhibits signal transduction indirectly by slowing down the supply of PI. Evidence for this hypothesis has come from studies using blowfly salivary glands (Fain and Berridge, 1979; Berridge and Fain, 1979), in which inositol depletion and the consequent PI loss could be associated with receptor desensitisation. The replenishment of inositol simultaneously restores the lipid and the response. Carbachol or electrical stimulation of CA1 hippocampal cells block the inhibitory effect of adenosine. Preincubation of

the hippocampal slices with lithium resulted in the restoration of the inhibitory effect of adenosine. On addition of phorbol 12,13 -diacetate, the inhibitory effect of adenosine is blocked, and the population spike from electrical stimulation of schaffer collaterals is restored (Worley *et al*, 1988). This suggests that the phosphoinositide derived diacylglycerol release was attenuated by lithium treatment.

1.6). PHOSPHOINOSITIDE SPECIFIC PHOSPHOLIPASE C.

1.6.1). SUBTYPES OF PLC.

Several distinct isoenzymes of phosphoinositide specific PLC have been identified which differ from one another with respect to their molecular mass, charge, divalent cation requirements, substrate specificity and the form of their released products (Rhee *et al*, 1989). Two distinct enzymes have been shown to be present in sheep seminal vesicles (Hofmann and Magerus, 1982), and antibodies to each of these isoenzymes did not react with the other. Three more PLC isoenzymes purified from bovine brain (Ryu *et al*., 1987), and a series of monoclonals prepared against these showed that these enzymes were immunologically distinct. Isolation of the cDNA clones encoding the structural genes of four distinct forms of PLC showed low overall homology, except for two regions in three of the

forms; one of 150 amino acids and another of 120 amino acids (Suh *et al.*, 1988). These regions designated X and Y, may constitute domains which are responsible for catalytic properties, such as recognition of polyphosphoinositides, hydrolysis of the diester bond, or interaction with G-proteins. The five isoenzymes of PI-PLC have been termed, PLCα, PLCβ, PLCγ, PLCδ, PLCϵ (Rhee *et al.*, 1989).

1.6.2). REGULATION OF PLC BY G-PROTEINS.

The Gq class of G-proteins are thought to stimulate the membrane bound form of phospholipase C (Shaw and Exton, 1992). This class of GTP-binding α-subunits lack the cysteine residue in the fourth position from the carboxyl terminus identified to be the ADP-ribosylation site for the pertussis toxin (Nakamura et al., 1991). Purification of phospholipase C isozymes from bovine liver, and their subsequent activation by GTPγS activated Gαq/Gα11 in reconstitution assays resulted in the specific activation of the β isoform of phospholipase C (Shaw and Exton, 1992). Other members of the phospholipase C family; PLCγ and PLCδ, identified immunologically by the use of antisera against purified bovine brain PLCβ1, PLCγ1 and PLCδ1, could also be resolved but not activated. There were two forms of activatable phospholipase C (150kDa PLC M1 and 140kDa PLC M2) identified by the anti-PLCβ1 immunoreactive protein. The 150kDa enzyme was postulated to be the liver homologue of

the brain PLCβ1 because of its comigration. This enzyme was found to preferentially hydrolyse phosphatidylinositol bisphosphate , and its calcium sensitivity was shown to increase in the presence of Gαq/11.

Transient transfections of various G-protein α-subunits into Cos 7 cells showed Gαq/11 to be localised in the plasma membrane (Wu et al, 1992). After prelabelling the cells with [^{3}H]-inositol, the formation of inositol phosphates was shown to increase in cells transfected with Gαq/11 and stimulated with aluminium fluoride. Further stimulation was observed when the cells were co-transfected PLCβ1, but there was no activity when the cells were transfected with PLCδ3, GαoA and Gαz. Moreover, membranes derived from Gαq and Gα11 and not GαoA transfected cells showed GTPγS-stimulated phosphatidylinositol bisphosphate hydrolysis when added to purified PLCβ1 in a cell free system.

A second phospholipase C-β subtype (PLCβ2) has been cloned from cDNA library of HL-60 cells (Park et al., 1992). Its expression in HeLa cells and subsequent purification showed that this PLC isozyme could not be stimulated in the presence of aluminium fluoride or GTPγS when reconstituted with Gαq. Lee et al. (1992) determined the relative specificities of the Gαq's in their ability to stimulate PLCβ1 or PLCβ2. Cos-7 cells were transfected with Gαq, Gα11, Gα14 or Gα16. The membrane preparations were reconstituted

with purified PLCß1 or PLCß2 enzymes. All members of the Gq family stimulated GTPγS-dependent enzyme activity of PLCß1, with Gαq and Gα11 being the most active. However, Gα16 most stimulated PLCß2, whereas the other α-subunits showed little activation. Thus, there may be differences in the relative interactions of the Gαq subunits with specific members of the PLCß subfamily.

1.7). RECYCLING OF PHOSPHATIDYLINOSITOL.

Upon stimulation of the phosphoinositide-cycle diacylglycerol kinase (DAG-kinase) rapidly converts diacylglycerol released to phosphatidic acid (PA).In resting cells the activity of this enzyme must be suppressed, as the supply of DAG is required for the synthesis of major glycerolipids. A very important functional implication of this enzyme is that it controls the intracellular concentration of the second messenger, DAG, which as a regulator of protein kinase C (Nishizuka, 1986).

To date only one form of the enzyme has been purified from animal sources, that being a 80kDa protein found in pig brain and thymus cytosol. Rabbit antibodies have been raised to the 80kDa enzyme from pig brain. Using this antibody, the enzyme was found to be distributed in both the membrane and cytosol fractions. Enzymological studies have shown the enzyme to be relatively inactive without the addition of

various activators such as deoxycholate, phosphatidyl choline , phosphatidylserine or sphingosine (see Kanoh *et al.*, 1990 for review). The cytosolic 80kDa enzyme is thought to become membrane associated upon phosphorylation, possibly by a protein kinase C (Maroney and Macara, 1989). This enzyme translocation is analogous to that of protein kinase C (Nishizuka, 1986), thus suggesting a functional interrelationship between DAG kinase and protein kinase C. Phosphorylation of DAG kinase by PKC does not alter its activity.

Phosphatidic acid is converted to phosphatidylinositol, via CDP-diacylglycerol, by a CTP-dependent inositol transferase (phosphatidylinositol synthetase), whose activity has been found in both the plasma membrane and the endoplasmic reticulum of the rat GH3 pituitary cell (Imai and Gershengorn, 1987). Phosphatidylinositol is the precursor for the synthesis of the polyphosphoinositides and comprises between 5 and 20% of the total phospholipids in eukaryotic cells (Maccallum *et al*, 1989). It is present in several of the membrane systems of the cell. The highest concentrations of PI are found in the endoplasmic reticulum (Paulus and Kennedy, 1960). Given this and the differential distribution of the polyphosphoinositides, cells face a potential problem in regulating the local supply of PI to support phospholipase C mediated transmembrane signalling. All of the PI in the cell might serve as a substrate for

polyphosphoinositide synthesis (Maccallum *et al.*, 1989), in which case PI from the endoplasmic reticulum would have to be efficiently delivered to the plasma membrane when needed. Alternatively, there might be a metabolically discrete pool of PI used for receptor mediated hydrolysis, which remains associated with the plasma membrane over long periods and is phosphorylated to PIP_2 on demand (Imai and Gershengorn, 1987).

The phosphorylation of PI to phosphatidylinositol 4-phosphate (PI(4)P) is catalysed by phosphatidylinositol 4-kinase. Several isozymes of PI 4-kinase are known to exist, although the possible roles of each remain to be elucidated, there are at least two distinct forms in bovine brain (Endemann *et al*, 1987). PI 4-kinase activity has been found in the nuclear envelope of hepatocytes (Smith and Wells, 1984), the plasma membrane (Imai *et al.*, 1986) and low density membranes (Lundberg and Jergil, 1988). From a functional point of view this kinase should reside in the plasma membrane, but Lundberg and Jergil (1988) found that in the rat hepatocytes synthesis of PI takes place in the endoplasmic reticulum, and its phosphorylation to PI(4)P occurs intracellularly in the low density membranes before translocation to the plasma membrane. PI(4)P-5 kinase phosphorylates PI(4)P to $PI(4,5)P_2$. Its activity is found in a membrane bound form (Imai *et al.*,1986), and is located in the plasma membrane where it needs to be in order to

contribute to receptor mediated PI metabolism (Jergil and Sundler, 1983; Seyfred and Wells, 1984) (see fig. 5 for PI cycle.).

Discovery of the phosphatidylinositol 3-kinase (Whitman *et al.*, 1985), which phosphorylates the D-3 positiion of the myoinositol moiety, indicated the existence of a new signalling pathway not involving the inositol phosphates. The 3-kinase phosphorylates PI, PI(4)P, PI(4,5)P_2 to yield PI(3)P, PI(3,4P_2 and PI(3,4,5)P_3. Although these novel phosphoinositides were discovered in transformed cells, 3-phosphate containing phospholipids have been found in normal cells and in growing cells such as neutrophils (Stephens, 1991), platelets, and brain cells. Thus these lipids may serve a function other than or in addition to stimulating cell proliferation (Bansal and Majerus, 1990). PI(3)P is not hydrolysed by phospholipase C, but by a 3-phosphatase (Lips *et al.*, 1989).

Duggan (1987) showed that both adenylate cyclase and phosphatidylinositol turnover were coupled to a muscarinic - like receptor in locust supraoesophageal ganglia. We have attempted to more fully investigate the coupling of muscarinic-like receptors to PI turnover in these ganglia. There are many enzymic steps involved in the inositol-lipid pathway, the characterisation of which in the insect may reveal important differences from the mammalian system, and thus provide potential species-selective target sites for new control agents.

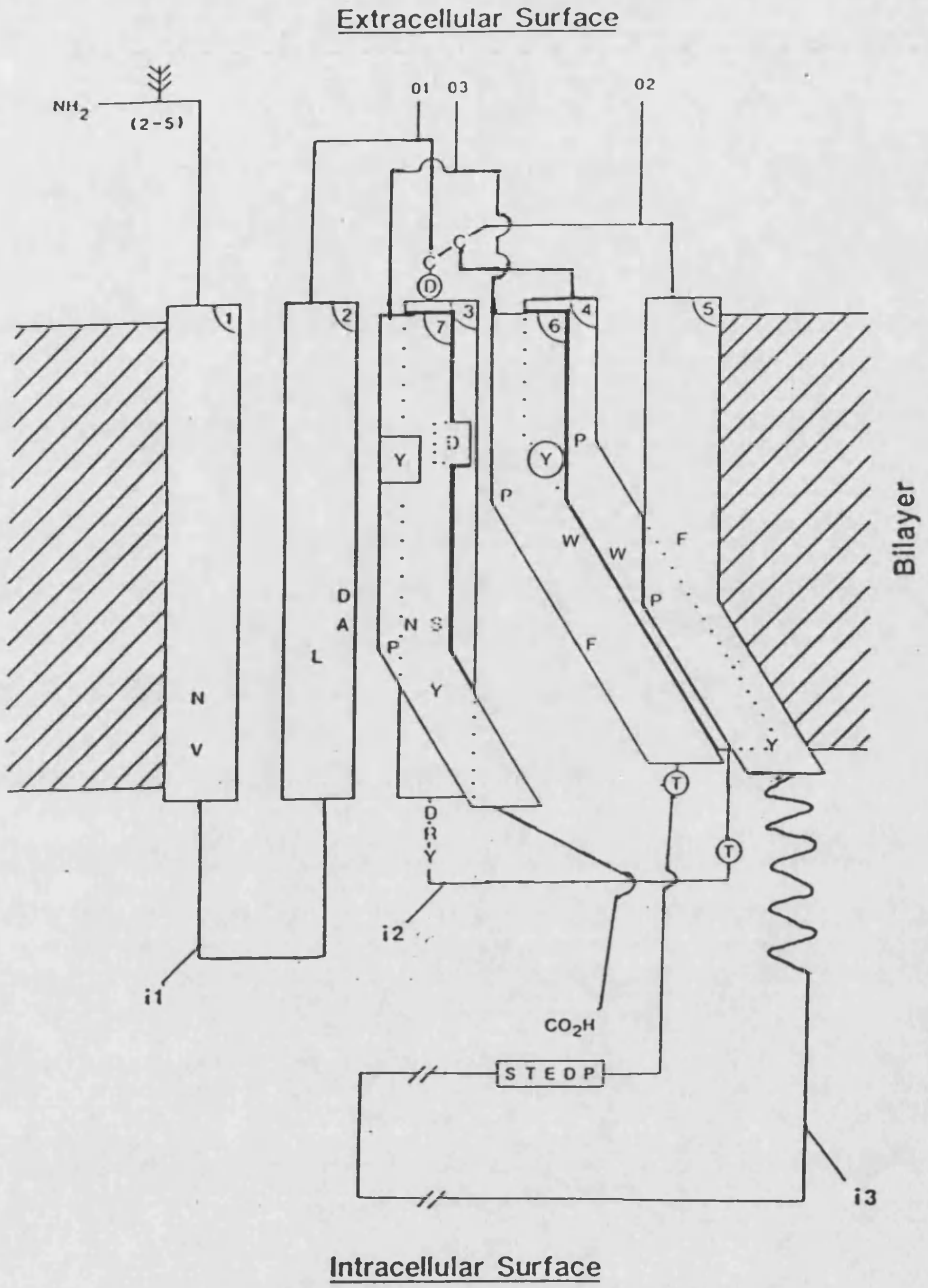


Figure 3.

Model of the muscarinic receptor.

The positions of the seven transmembrane helices (1-7), the extracellular loops (o1-3) and the intracellular loops (i1-3) are shown. Residues conserved across the G-protein coupled receptor family are shown as capitals, and the receptor specific residues are shown in boxes (in TM3 and 7) (modified from Hulme *et al.*, 1990).

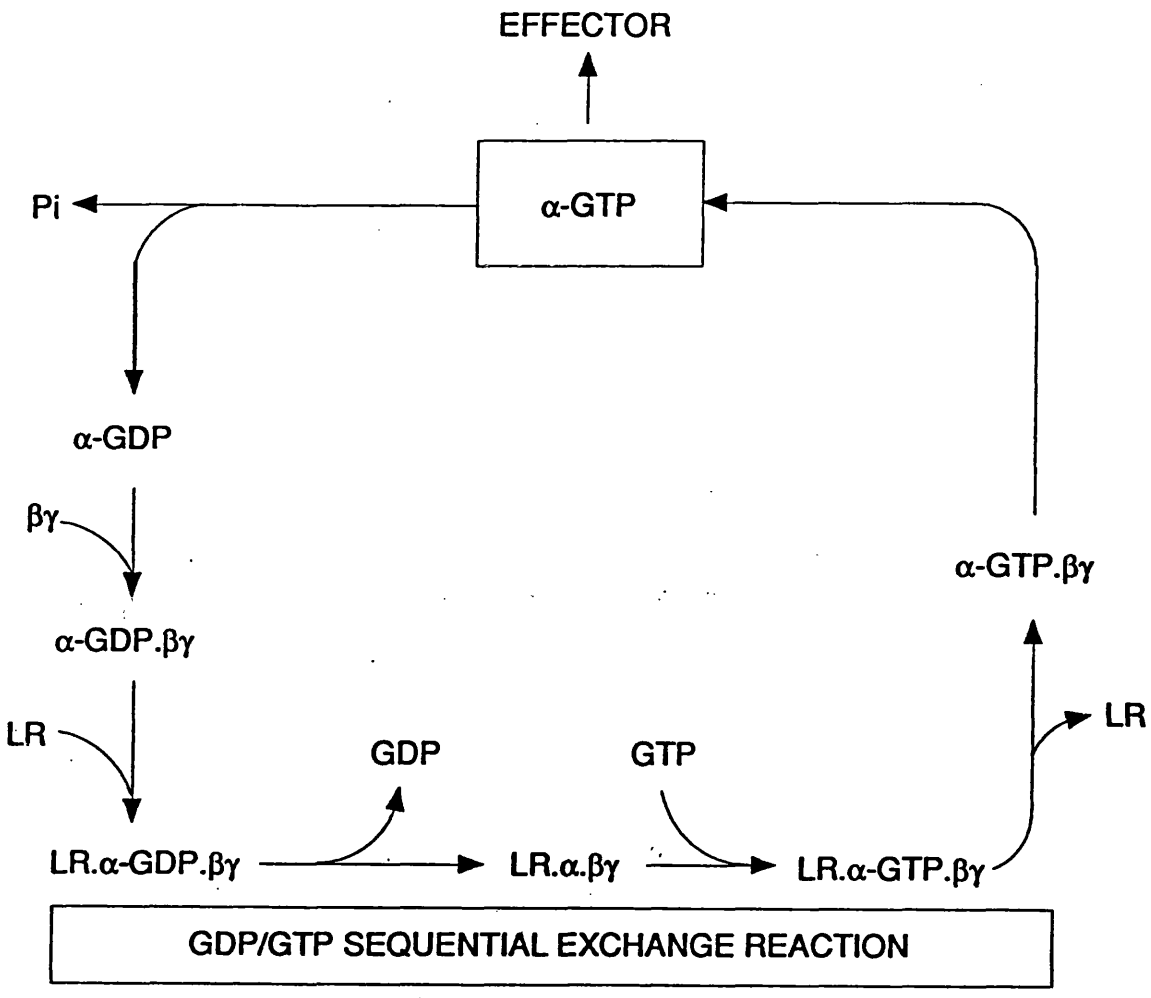


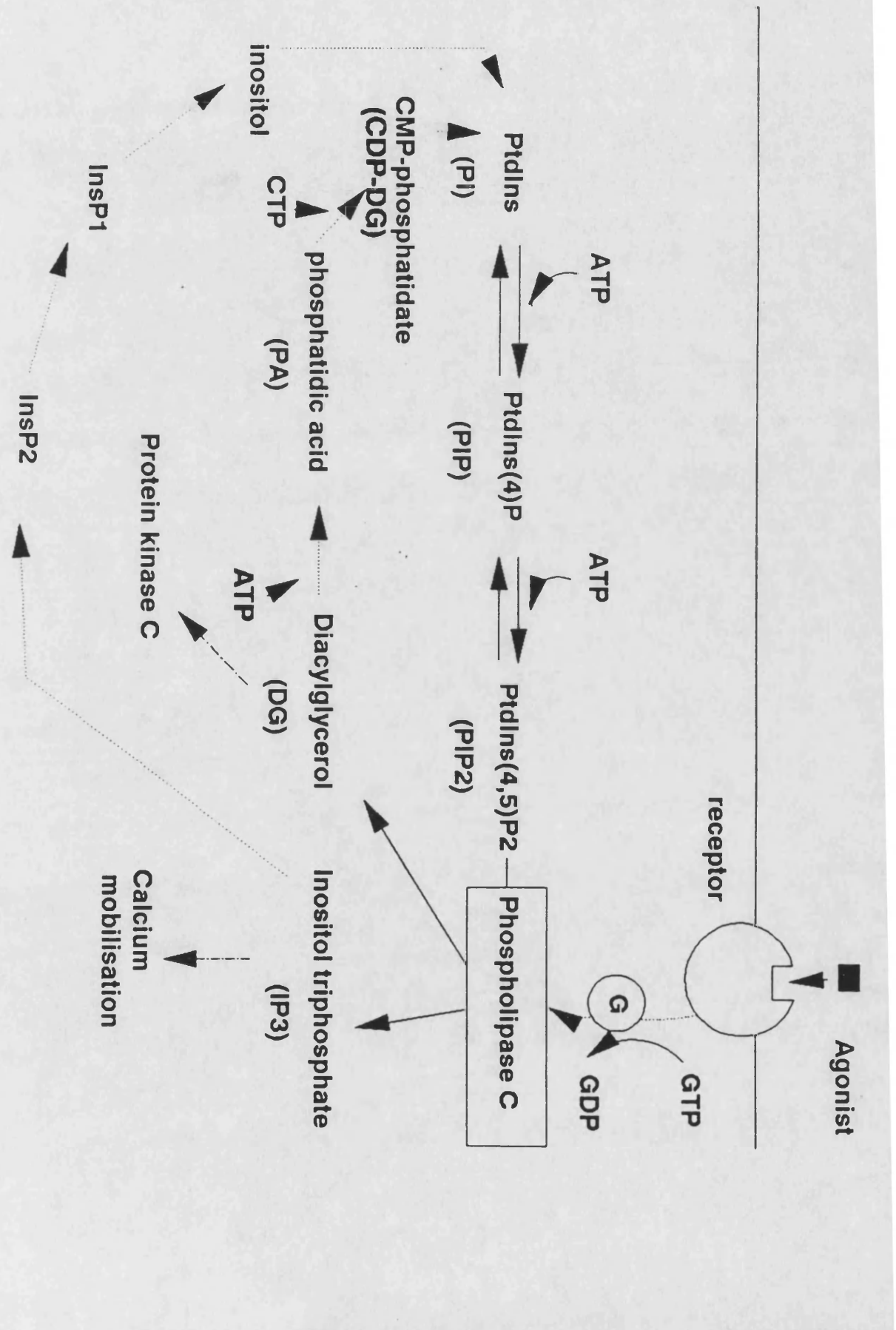
Figure 4.

The GTPase cycle.

Agonist binding to the receptor forms an agonist-receptor complex (LR). The complex catalyses the release of bound GDP from the G-protein α-subunit. The LR complex is displaced on binding of GTP to the α-subunit, and the α-GTP complex forms the activated state of the G-protein. Activity is terminated by the intrinsic GTPase activity of the G-protein.

Figure 5.

The inositol lipid cycle.



PHARMACOLOGICAL CHARACTERISATION	SELECTIVE ANTAGONISTS	MOLECULAR CHARACTERISATION	NO. AMINO ACIDS
M_1	Pirenzepine Telenzepine	m1	460
M_2	AFDXII6 Methoctramine AFDX384	m2	466
M_3	HHSiD 4-DAMP	m3	589/590
-		m4	478/479
-		m5	531/532

Table 1. Muscarinic receptor nomenclature as recommended by the proceedings of the fourth international symposium on subtypes of muscarinic receptors.

HHHSiD - Hexahydrosiladifenidol. AFDX116 - (11-2((-((diethylamino)methyl)-1-piperidinyl)-acetyl)-5,11-dihydro-6H-pyrido(2,3-b) (1,4)-benzodiazepine-6-one). AFDX384 - (5,11-dihydro-11-(((2-(2-((dipropylamino)methyl)-1-piperidinyl)ethyl)amino)carbonyl)-6H-pyrido(2,3-b)(1,4)-benzodiazepin-6-one methansulfonate). 4-DAMP - 4-diphenylacetoxy-N-methylpiperidine methiodide.

2.1). INTRODUCTION

In insects PI metabolism has been shown to be important in peripheral tissues (Fain and Berridge, 1979); the addition of 5-hydroxytryptamine to isolated blowfly salivary glands (*Calliphora erythrocephala*) stimulates the breakdown of radiolabelled PI, transepithelial calcium transport and fluid secretion. More recent studies on the isolated tick (*Amlyomma americanum*) salivary glands have shown that calcium is mobilised from microsomal intracellular stores in response to agonists which increase inositol trisphosphate levels (Roddy *et al.*, 1989). Interaction between the cAMP pathway and the inositol lipid pathway has been suggested in the tick salivary gland during changes in feeding stages (McSwain *et al.*, 1989). Later stages of feeding produce higher levels of inositol phosphates mediated by an unknown brain factor. However, the inositol-lipid pathway has not been extensively characterised in the insect CNS. It has been shown that muscarinic-like acetylcholine receptors are linked to both the cAMP pathway and the inositol lipid pathway in locust (*Schistocerca gregaria*) supraoesophageal ganglia (Duggan, 1987). Carbachol caused an increase in PI turnover which was blocked by atropine and a selective M_1 muscarinic antagonist, pirenzepine. Other studies using locust metathoracic ganglia (Trimmer and Berridge, 1985) could not establish an increase in the levels of inositol phosphates in the presence of many cholinergic agonists, including carbachol. A muscarinic antagonist, atropine,

however, decreased the levels of inositol phosphates suggesting that the ganglia were already maximally stimulated before the addition of agonist, probably due to the trauma of dissection. One of the primary aims of this study was to reduce these control values by experimenting with incubation conditions. Here we show the effects of carbachol on [^{32}P]-Pi incorporation into the polyphosphoinositides, and the effect of several doses of pilocarpine and carbachol on the incorporation of [^{3}H]-Inositol into PI and inositol phosphates.

2.2). GENERAL METHODS

2.2.1). DISSECTION.

Adult locusts (*Shistocerca gregaria*) supplied by Blades Chemicals were used in all experiments. The insects were anaesthetised by cooling and gassing with carbon dioxide, and then decapitated. On shaving the cuticle from the front of the head, the supraoesophageal ganglion was exposed and removed using metal forceps. The optic lobes were routinely removed. Dissection of 100 ganglia provided approximately 300mg wet weight of ganglia.

2.2.2). INCUBATION OF LOCUST GANGLIA IN [^{3}H]-INOSITOL

Initial experiments were performed under varying conditions, both with regards to time of incubation and the saline used. It was found that low basal levels of incorporation were obtained when a preincubation was introduced under calcium free conditions. Then the effects of reintroducing calcium in the incubation saline could be determined. The final procedure adopted was as follows.

2.2.3). PREINCUBATION

Freshly dissected ganglia were immediately rinsed in ice cold buffer A (see below). Groups of 10 to 20 ganglia were placed in 5mls of buffer A previously gassed with 95%O_2/5%CO_2, in 10 ml beakers covered with parafilm. To this buffer, 5μCi of [^{3}H]-inositol (84Ci/mmol, Amersham) was added to preload the ganglia with radiolabelled lipids during the 90min preincubation period at room temperature.

Buffer A:

NaCl	150mM
KCl	10mM
$MgCl_2$	7mM
$NaHCO_3$	8mM
KH_2PO_4	6mM
Glucose	10mM

pH = 6.7

2.2.4). INCUBATION

Groups of 20 ganglia were subdivided into groups of 10. The level of [^{3}H]-inositol incorporation into the inositol phosphates during the preincubation period was determined.

The 10 ganglia were equally divided into 5 Eppendorf tubes (1.5ml), each of which contained 0.5 mls of buffer and 1μCi of [^{3}H]-inositol. All these tubes were incubated for 20min at 25^{o}C in a water bath which was gently agitated. The incubation conditions were manipulated to investigate the effects of calcium, lithium and muscarinic drugs. In agonist treated experiments, the agonist was added 4min after the addition of lithium chloride.

2.2.5). EXTRACTION OF ACID SOLUBLE INOSITOL PHOSPHATES.

Incubations were terminated by placing the ganglia on dry ice after which they were homogenised in acidified chloroform/methanol (1ml chloroform/methanol (1:2 by vol.), 0.2ml 0.5M hydrochloric acid). On addition of 0.2ml distilled water and 0.2ml chloroform the phases were separated by centrifugation (2000g, 20min). The upper phase contained the acid soluble inositol phosphates and the lower phase contained the phospholipids.

2.2.6.1). SEPARATION OF INOSITOL PHOSPHATES USING ANION EXCHANGE

Anion exchange columns were prepared using Dowex-1 chloride resin (8% crosslinked, dry mesh 200-400, Sigma) converted to formate form. Approximately 1g of Dowex resin was washed 4 times with 4ml of 1M sodium hydroxide in a 5ml beaker. The resin was neutralised by washing with distilled water, and converted to formate form by adding 1M ammonium formate to the resin. After mixing the large particles were allowed to settle, and the remaining small particles discarded. The Dowex-1 formate resin was neutralised with distilled water and packed by pouring the slurry into a pasteur pipette.

2.2.6.2). CHARACTERISATION OF DOWEX-1 FORMATE COLUMNS USING INOSITOL PHOSPHATE STANDARDS.

2.2.6.2.1). METHOD

A mixture of inositol polyphosphates (inositol 1-phosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate, 0.37 KBq/μl, obtained from Amersham International) was added to Dowex-1 formate columns (see above for preparation). The inositol mono-, bis- and tris phosphates were separated by sequential batch elution with 0.2M ammonium formate/0.1M formic acid, 0.5M ammonium formate/0.1M formic acid and 1M ammonium formate/0.1M formic acid. The recovery of each of the inositol phosphates was determined.

2.2.6.2.2). RESULTS

The elution profile of the inositol phosphates (fig. 6) showed that the inositol mono-, bis- and tris phosphates were well resolved using the elution protocol (81.1% +/- 3.4(s.d), n=3, 20 437 d.p.m. added to the column).

2.2.6.3). SEPARATION OF [^{3}H]-INOSITOL LABELLED WATER SOLUBLE PRODUCTS.

The upper phase of the chloroform/methanol extraction was added to 1ml of resin in a pasteur pipette. The inositol phosphates could then be sequentially eluted with distilled water (inositol), 5mM disodium tetraborate/ 60mM ammonium formate (glycerophosphoinositol); 0.1M formic acid/ 0.2M ammonium formate (inositol phosphates); 0.1M formic acid/ 0.5M ammonium formate (inositol bisphosphates); 0.1M formic acid/ 1.0M ammonium formate (inositol trisphosphates). The eluted fractions were collected in scintillation vials, to which 8mls of Optiphase Safe scintillant were added (LKB scintillation products). Radioactivity from each fraction could be determined using a scintillation counter (counting efficiency 35-40%). For each solvent mixture 10mls of eluant were collected to be counted (see fig. 7 for the separation of inositol phosphates).

2.3). RESULTS

2.3.1). EFFECT OF SINGLE INCUBATIONS.

Groups of 10 ganglia were incubated for 20 min at room temperature in 2 ml calcium free buffer A, to which 3μCi [^{3}H]-inositol was added. To measure the effects of agonist stimulation on the accumulation of radiolabelled inositol phosphates, lithium (10mM) and carbachol (1mM) were added to the treated incubation saline. Incorporation of [^{3}H]-inositol into the inositol phosphates was determined as described in general methods. The percentage of each inositol phosphate fraction was expressed as a function of total radioactivity recovered (table 2). This showed that carbachol increased the proportion of inositol incorporated into the inositol trisphosphate fraction. But there was no indication of any increase in total count.

On addition of 1mM EGTA (ethyleneglycol-bis(ß-amino ethylene ether)N,N,N',N'-tetra acetic acid), a calcium ion chelator, to the incubation buffer, the incorporation of [^{3}H]-inositol into the inositol phosphates was reduced. Both counts recovered and percentage of incorporation showed low standard errors (S.E.M.). Low calcium levels in the incubation buffer may have either reduced phospholipase C activity or decreased the amount of endogenous neurotransmitter release to have this effect (see table 3).

2.3.2). INTRODUCTION OF A PREINCUBATION.

A preincubation period of 90 min at room temperature in calcium free, lithium free buffer A was introduced to investigate whether a carbachol enhanced accumulation of inositol phosphates could be obtained. Experiments were performed to determine the effects of adding 2mM EGTA, no calcium, 0.3mM calcium and 3mM calcium to the incubation buffer. At each of these concentrations of calcium, the effect of 10mM carbachol in the presence or absence of 10mM lithium on the incorporation of [^{3}H]-inositol into the inositol phosphates was measured.

2.3.2.1). PREINCUBATIONS IN THE PRESENCE OF EGTA.

The requirement for calcium was highlighted by performing experiments in the presence of 2mM EGTA in both the preincubation and incubation conditions.

Groups of 10 ganglia were preincubated for 60min in 10mls of buffer (calcium free, lithium free, 2mM EGTA) at room temperature. In control experiments (n=6) the ganglia were placed in buffer A containing 1μCi [^{3}H]-inositol, 2mM EGTA and 10mM lithium, whilst in agonist treated experiments (n=6) 10mM carbachol was added. Incubations were performed at 25^{o}C for 30mins. Carbachol had no effect on the incorporation of [^{3}H]-inositol into the inositol phosphates in these conditions (fig. 8).

2.3.2.2). EFFECT OF CALCIUM, LITHIUM AND CARBACHOL ON THE INCORPORATION OF [^{3}H]-INOSITOL INTO THE INOSITOL PHOSPHATES.

2.3.2.2.1). CONTROL INCUBATIONS IN THE ABSENCE OF CALCIUM.

In the preincubation buffer, groups of 20 ganglia were incubated in the presence of 5μCi [^{3}H]-inositol in buffer A. Incorporation of the radiolabel into the inositol phosphates was determined immediately after this 90 min.preincubation period (fig. 9), and was taken to be the basal level of activity for 10 ganglia. The level of [^{3}H]-inositol incorporation into the inositol phosphates after 110 min incubation in buffer A is also shown in fig. 9. This shows that the radioactivity recovered after the 110 min preincubation period was not significantly greater than the 90 min preincubation period. The remaining 10 ganglia were transferred into incubation buffer (no added calcium or lithium), after being split into 5 groups of 2. Pooling the counts from 10 ganglia after the 20 min incubation period, showed significant increases in the inositol phosphate fraction ($p< 0.01$), the inositol bisphosphate fraction ($p<0.01$), and the total counts ($p<0.005$) when compared to levels of incorporation after the preincubation period alone.

2.3.2.2.2). THE EFFECT OF LITHIUM AND CARBACHOL IN THE PRESENCE OF 0.3mM CALCIUM.

Experiments were performed in which 0.3mM calcium was added to the incubation buffer. The effect of 10mM carbachol was investigated in the presence or absence of 10mM lithium. figs. 10 and 11). In the absence of lithium, carbachol had no effect on the incorporation of [^{3}H]-inositol into the inositol phosphates (fig. 10). However, in the presence of 10mM lithium and 10mM carbachol there was an agonist induced accumulation of radiolabelled inositol phosphates. Significant increases were observed in the inositol phosphate fraction ($p<0.02$), inositol bisphosphate fraction ($p<0.05$) and total counts ($p<0.05$) between control (n=9) and treated (n=9) experiments (fig. 11). When carbachol treated ganglia in the absence of lithium were compared with carbachol treated ganglia in the presence of lithium , there were significant increases in the inositol phosphate fraction ($p<0.01$), inositol bisphosphate fraction ($p<0.01$) and total counts ($p<0.05$). Thus, in the presence of lithium carbachol enhanced the accumulation of radiolabelled inositol phosphates (1.6 fold increase in the total amount of radioactivity recovered (see fig. 11)).

2.3.2.2.3). THE EFFECT OF CARBACHOL AND LITHIUM IN THE PRESENCE OF 3mM CALCIUM.

Similar experiments were performed in the presence of higher concentrations of calcium (3mM) in the incubation buffer. Both in the presence and absence of 10mM lithium

(figs. 12 and 13), carbachol stimulated the incorporation of [^{3}H]-inositol into the inositol phosphates. There was a 2.2 fold increase in the presence of 3mM calcium ($p< 0.01$), and a 1.73 fold increase in the presence of 3mM calcium and 10mM lithium ($p< 0.01$). Results from these experiments showed that at this increased concentration of calcium, in the absence of carbachol and presence of 10mM lithium, there was an increase in the incorporation of [^{3}H]-inositol into the inositol phosphates (significant differences ($p<0.01$) were shown between control values in the presence of 0.3mM calcium (fig. 11) and 3mM calcium (fig. 13)).

To show that this carbachol induced increase in phosphatidylinositol turnover was due to a muscarinic-like receptor, experiments were performed in the presence of a muscarinic antagonist, atropine. Groups of 10 ganglia were incubated in the presence of 3mM calcium, 10mM lithium, 10mM carbachol and 0.1mM atropine ($n=5$). When these results were compared with agonist treated experiments in the absence of atropine (fig. 13), there were significant decreases in [^{3}H]-inositol incorporation into the inositol bisphosphate fraction ($p<0.05$) and total inositol phosphates ($p<0.05$).

2.4). DISCUSSION

The inositol phosphate standards were well resolved using anion exchange chromatography on Dowex-1 formate

columns (fig. 6). The elution profile of the [^{3}H]-inositol labelled water soluble products is shown in fig. 7. Initial experiments showed that the peak corresponding to inositol trisphosphates was the highest fraction recovered in both control and treated experiments (fig. 7). This implied phospholipase C activity even in the absence of the agonist, carbachol. This indication of phosphatidylinositol turnover in the control conditions could have been due to damage of the cells containing the relevent transmitters which activate phospholipase C, or it may have been the result of calcium-dependent release of endogenous transmitters triggered by the conditions of the experiments (Duggan, 1987).

The single incubation experiments (table 2) showed the immediate effects of removing the ganglia and placing them in buffer containing the label. To limit endogenous activity, the incubations were performed in the absence of calcium in the buffer. The results show that the appearance of label in the inositol phosphates was very variable (see total counts in table 2), and that carbachol had no detectable effect on total counts recovered. This suggested high endogenous activity, perhaps caused by the trauma of dissection. When the proportion of [^{3}H]-inositol incorporated into the inositol phosphates was examined (table 2), there were indications that in the presence of carbachol the labelling of inositol trisphosphate increased, whilst that of inositol monophosphate decreased. This suggested that the production of inositol

trisphosphate increased relative to its dephosphorylation to inositol phosphate. Adding a calcium chelator, EGTA, to the incubation buffer dramatically reduced the extent and variability of [^{3}H]-inositol incorporation into the inositol phosphates, especially into the inositol trisphosphate fraction (table 3). The level of calcium in the incubation buffer profoundly affected phosphatidylinositol turnover.

In an attempt to reduce the level of endogenous activity, experiments were performed in which a preincubation was introduced, and in which 2mM EGTA was added to both the incubation and preincubation buffers. There were no significant differences when the ganglia were incubated in the presence of carbachol and lithium. This lack of agonist induced enhancement of phosphatidylinositol turnover may have been due to the fact that calcium is required for phospholiase C activity (Rhee *et al.*, 1989). The exposure of locust ganglia to EGTA may have sufficiently reduced the content of calcium in the cells to inactivate the phospholipase C.

Experiments were performed in which the EGTA was removed from buffers. Counts recovered after the 90 min preincubation showed low activity, much of it was accounted for as background (each scintillation vial with 10 mls of scintillation fluid had an average count of 20 c.p.m.). Transferring the ganglia into buffer A (no added calcium or lithium) and incubating for 20 min nearly

doubled the incorporation of [^{3}H]-inositol into the inositol phosphates. Hence, the act of placing the ganglia in the incubation buffer caused stimulation (fig. 9).

The effect of re-introducing calcium into the incubation buffer was determined. On addition of 0.3mM calcium (figs. 10 and 11), carbachol enhancement of phosphatidylinositol turnover was only observed in the presence of 10mM lithium (fig. 11) . The inhibition of myo-inositol 1-phosphatase by lithium (Sherman *et al.*, 1981) is commonly used to identify receptors that function through the inositol lipid pathway. Increasing the calcium to 3mM (figs 12 and 13), in control conditions, also increased the incorporation of [^{3}H]-inositol into the inositol phosphates (there was a significant difference between control+0.3mM calcium and control+3mM calcium ($p< 0.01$) in the presence of lithium). This suggests that calcium enhances endogenous activity, probably by initiating transmitter release which activates receptors linked to the inositol lipid pathway. At this concentration of calcium (3mM), carbachol (10mM) enhanced the accumulation of inositol phosphates both in the presence and absence of 10mM lithium (figs 12 and 13).

The effects of intracellular calcium and the receptor-G-protein activation on the modulation of phospholipase C may be interrelated. The ability of muscarinic agonists to stimulate polyphosphoinositide turnover can be enhanced by a calcium ionophore in

synaptosomes (Fisher and Agranoff, 1981). Synergy between calcium and guanine nucleotides in the activation of phospholipase C has been demonstrated in permeabilised cells (Valler *et al.*, 1987). An increase in cytosolic calcium may, therefore, increase the sensitivity of phospholipase C to receptor activation. Thus, in neuronal cells, the entry of calcium through voltage gated or ligand gated channels may be involved in a positive feedback loop with inositol trisphosphate production. The results from these experiments showed that in general the levels of inositol phosphates were considerably higher in 3mM calcium than 0.3mM calcium.

The action of inositol phosphates involve many biochemical steps, which makes it unlikely that they are responsible for rapid changes in calcium levels observed during depolarisations. The pathway is more likely to be involved in regulating cytosolic calcium in the presynaptic terminal, and therefore serve as a modulatory function for incoming signals. Many neurons respond to neurotransmitters that stimulate inositol phosphate turnover with a transient hyperpolarisation, which can be mimicked by the addition of inositol 1,4,5-trisphosphate (Higashida and Brown, 1986). This response has been shown to result from the opening of calcium gated potassium channels. Hyperpolarisations of the presynaptic terminals would modulate the amount of neurotransmitter released on arrival of a depolarisation signal. A physiological role for the muscarinic-like receptor in the insect central

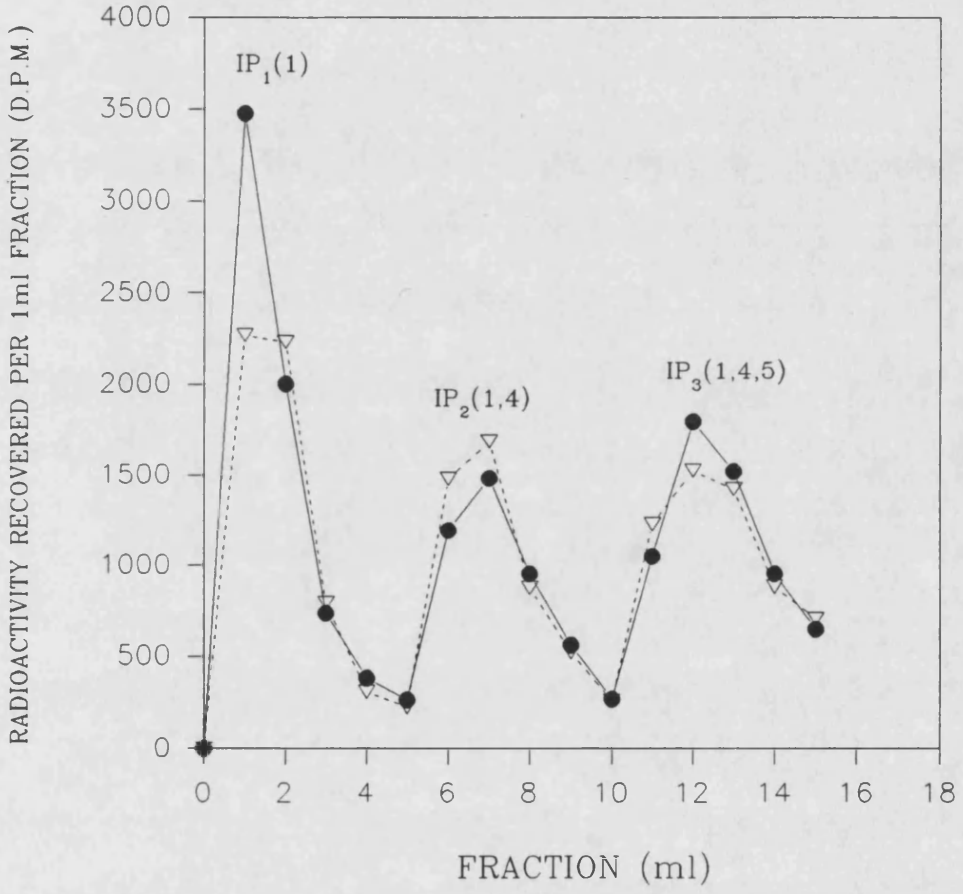
nervous system has been more clearly established in an identified motoneurone of the principle planata retractor muscle in the proleg of the tobacco hornworm, *Manduca sexta* (Trimmer and Weeks, 1991). this motoneurone forms a monosynaptic connection with a mechanosensory afferent at the tip of the proleg. The muscarinic-like receptor appears to regulate excitibility of the motoneurone. Microinjection of a muscarinic agonist, oxotremorine-M, into the neuropile increases the excitibility of the motoneurone by a slow depolarisation and by lowering its spike threshold. The muscarinic current underlying these effects is voltage sensitive and carried by sodium. The current requires the entry of extracellular calcium, and is affected by agents that stimulate G-protein turnover, such as GTPγS (Trimmer and Weeks, 1991).

The effect of carbachol can be blocked by atropine, a muscarinic antagonist (fig. 13). This suggests that the locust supraoesophageal ganglia contain receptors similar to the vertebrate muscarinic receptors, which can function through the inositol lipid pathway.

Figure 7.

Separation of [^{3}H]-inositol labelled water soluble products.

Twenty ganglia were preincubated for 90min in 10ml of buffer A containing 3mM calcium. After which they were transferred to buffer A containing 2mM calcium and 5μCi [^{3}H]-inositol, and incubated for 30min in presence (open inverted triangles) or absence of 1mM carbachol (closed circles). The twenty ganglia were homogenised in acidified chloroform/methanol, and the water soluble metabolites were separated on Dowex-1 formate columns using sequential elution. The free inositol was washed from the column by eluting with 10 x 2ml distilled water. The inositol phosphates were separated by eluting with 5mM disodium tetraborate/60mM ammonium formate (5 x 2ml fractions, glycerophosphatidylinositol), 0.2M ammonium formate/0.1M formic acid (10 x 1ml fractions, inositol monophosphates (IP_1)), 0.5M ammonium formate/0.1M formic acid (10 x 1ml fractions, inositol bisphosphates (IP_2)) and 0.8M ammonium formate/0.1M formic acid (10 x 1ml fractions, inositol trisphosphates (IP_3)).


RADIOACTIVITY RECOVERED PER 1ml FRACTION (D.P.M.)
4000
3500
3000
2500
2000
1500
1000
500
0
IP1(1)
IP2(1,4)
IP3(1,4,5)
0
2
4
6
8
10
12
14
16
18
FRACTION (ml)

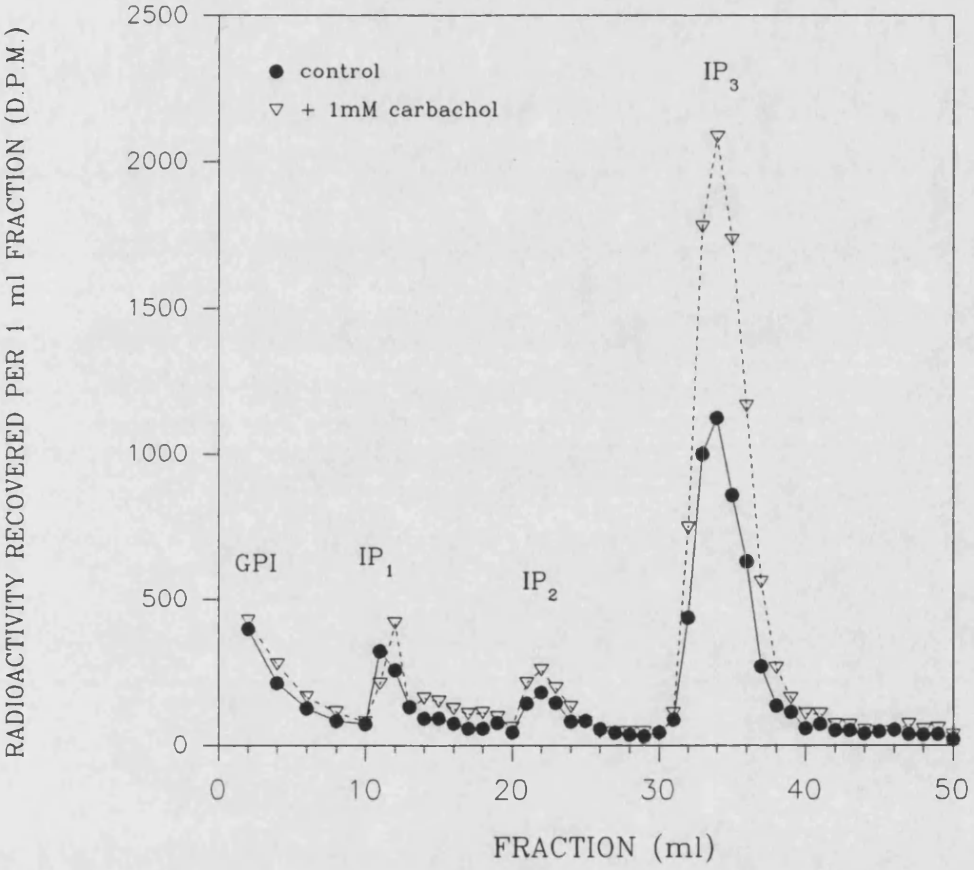

RADIOACTIVITY RECOVERED PER 1 ml FRACTION (D.P.M.)
2500
2000
1500
1000
500
0
control
+ 1mM carbachol
IP3
GPI
IP1
IP2
0
10
20
30
40
50
FRACTION (ml)

Figure 8.

The effect of carbachol on the incorporation of [^{3}H]-inositol into the inositol phosphates in the presence of EGTA.

Ten ganglia were preincubated for 60min in 10ml of buffer A containing 2mM EGTA. The ganglia were split into 5 groups of 2. In control experiments (n=6) each group was incubated in buffer A containing 1μCi [^{3}H]-inositol, 2mM EGTA and 10mM lithium (hatched rising to the right) for 30min at 25^{0}C. In treated experiments (n=5) 10mM carbachol was added (hatched rising to the left). Results from 10 ganglia were pooled, and the incorporation of [^{3}H]-inositol into inositol monophosphate (IP1), inositol bisphosphate (IP2), inositol trisphosphate (IP3) and total inositol phosphates (TOTAL) were determined.

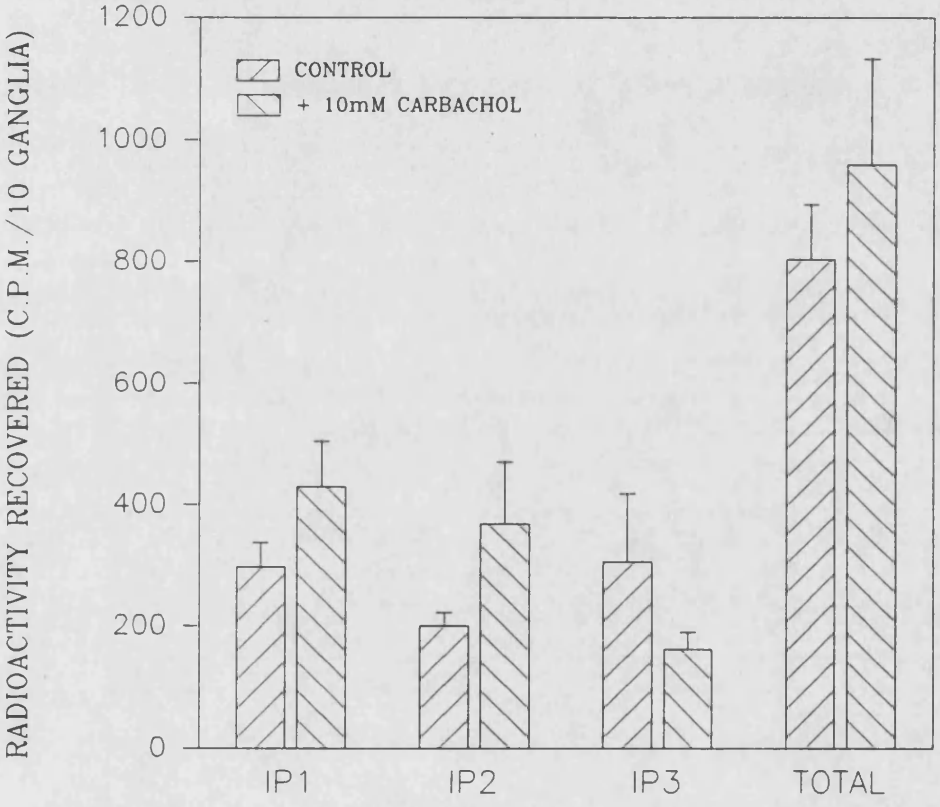

RADIOACTIVITY RECOVERED (C.P.M./10 GANGLIA)
1200
1000
800
600
400
200
0
CONTROL
+ 10mM CARBACHOL
IP1
IP2
IP3
TOTAL

Figure 9.

Incubations in the absence of calcium, lithium and carbachol.

Twenty ganglia were preincubated for 90 min in buffer A containing 5μCi [^{3}H]-inositol. Counts for 10 ganglia (n=8) were taken immediately after this preincubation period (hatched rising to the right). The remaining 10 ganglia were transferred into buffer A after being split into 5 groups of 2, and incubated for 20 min at 25^{0}C (cross hatched n=6). Groups of 10 ganglia were also incubated for 110min in the preincubation conditions (hatched rising to the left n=5). Each data point represents the mean and standard error of mean for n replicates. Comparing radioactivities recovered per 10 ganglia after the 90min preincubation period and after the subsequent 20min incubation period showed significant increases in the inositol monophosphate fraction (IP), inositol bisphosphate fraction (IP2) and total counts (TOTAL).

* $p<0.05$, *** $p<0.01$, **** $p<0.005$.

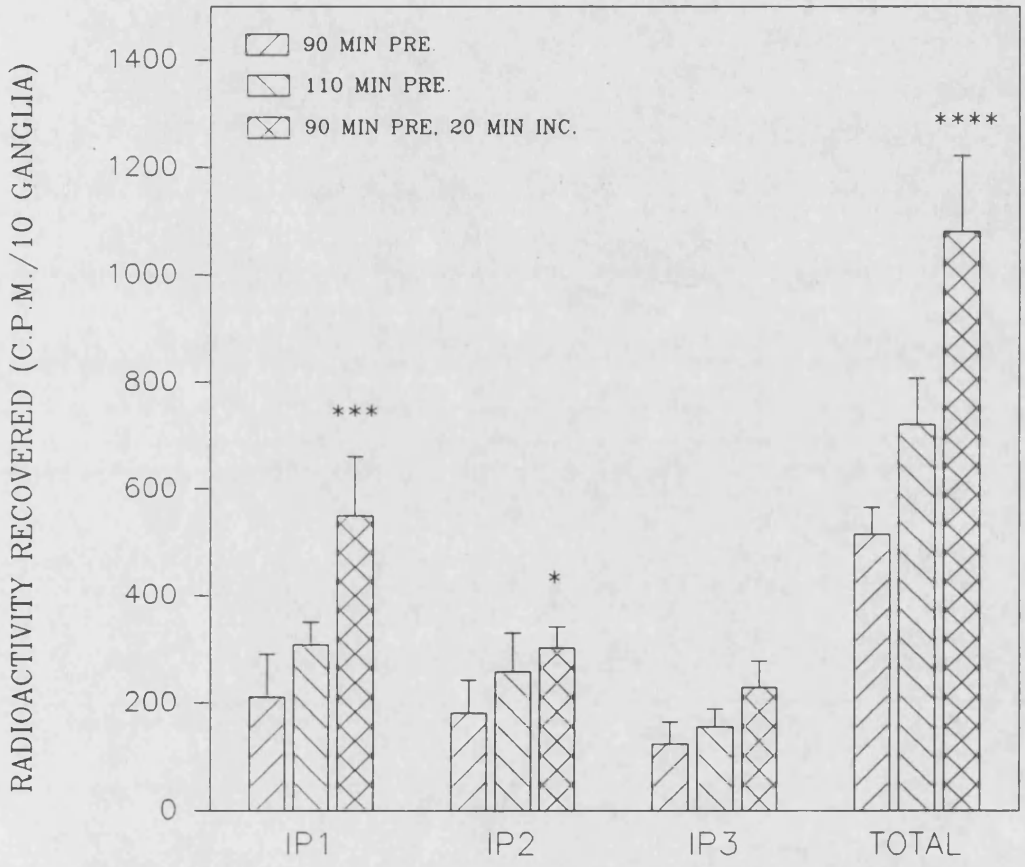

RADIOACTIVITY RECOVERED (C.P.M./10 GANGLIA)
90 MIN PRE.
110 MIN PRE.
90 MIN PRE. 20 MIN INC.
1400
1200
1000
800
600
400
200
0
***
*
****
IP1
IP2
IP3
TOTAL

Figure 10

The effect of carbachol in the presence of 0.3mM calcium.

Groups of 20 ganlia were preincubated as described in fig. 9. In control conditions, 0.3mM calcium was added to buffer A during the 20min incubation period (hatched rising to the left, n=4). To the agonist treated ganglia, 10mM carbachol was added to the incubation buffer (cross hatched, n=5). Mean incorporation of [^{3}H]-inositol into inositol monophosphate (IP), inositol bisphosphate (IP2), inositol trisphosphate (IP3) and total inositol phosphates (TOTAL) were determined for n replicates (results obtained from pooling of 10 ganglia). The standard error of mean is shown for each data point. There were no significant differences observed.

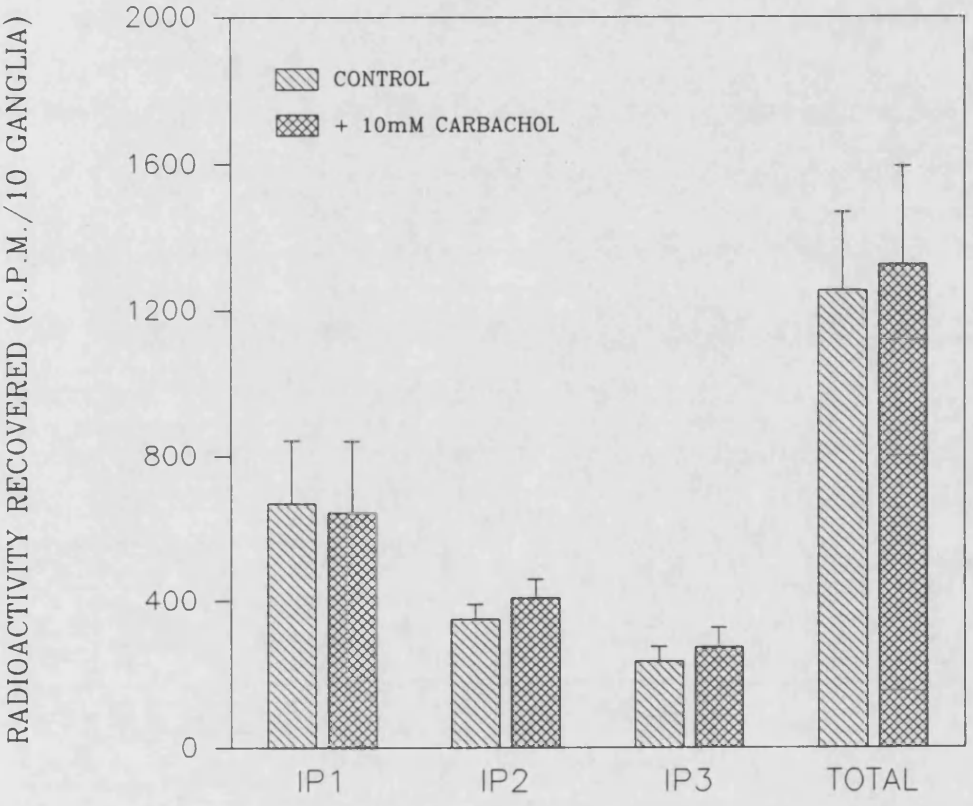

RADIOACTIVITY RECOVERED (C.P.M./10 GANGLIA)
2000
1600
1200
800
400
0
CONTROL
+ 10mM CARBACHOL
IP1
IP2
IP3
TOTAL

Figure 11

The effect of carbachol in the presence of 0.3mM calcium and 10mM lithium.

Groups of 20 ganglia were preincubated for 90 min (fig. 9). In control conditions 0.3mM calcium and 10mM lithium were added to buffer A during the 20min incubation period (hatched rising to the left, n=9). Carbachol (10mM) was added to agonist treated ganglia (cross hatched, n=9). Each data point represents mean incorporation of [^{3}H]-inositol into the inositol phosphates (results obtained from pooling of 10 ganglia) for n replicates and the standard error of the mean. There were significant increases in the IP fraction, IP2 fraction, and TOTAL counts recovered. When carbachol treated ganglia without lithium (fig. 10) are compared with carbachol treated ganglia in the presence of 10mM lithium there were significant increases in the IP1 $p<0.01$), IP2 ($p<0.01$) and total counts ($p<0.05$).

* $p<0.05$, ** $p<0.02$

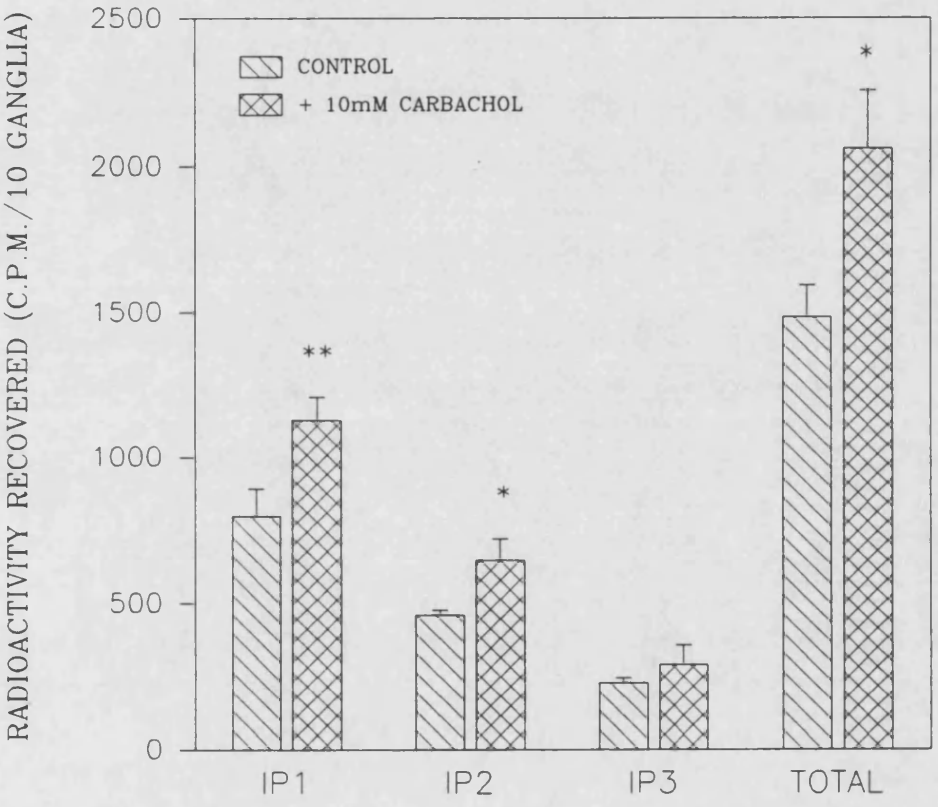

RADIOACTIVITY RECOVERED (C.P.M./10 GANGLIA)
2500
2000
1500
1000
500
0
CONTROL
+ 10mM CARBACHOL
**
*
*
IP1
IP2
IP3
TOTAL

Figure 12

The effect of carbachol in the presence of 3mM calcium.

Groups of 20 ganglia were preincubated for 90min. They were then incubated in buffer A containing 3mM calcium for 20min in the presence (cross hatched, n=5) or absence (hatched rising to the left, n=5) of 10mM carbachol. Comparing mean incorporation of [^{3}H]-inositol into the inositol phosphates (results obtained from pooling of 10 ganglia for n replicates) showed significant increases in the IP1, IP3 and total inositol phosphates.

* $p<0.05$, *** $p<0.01$

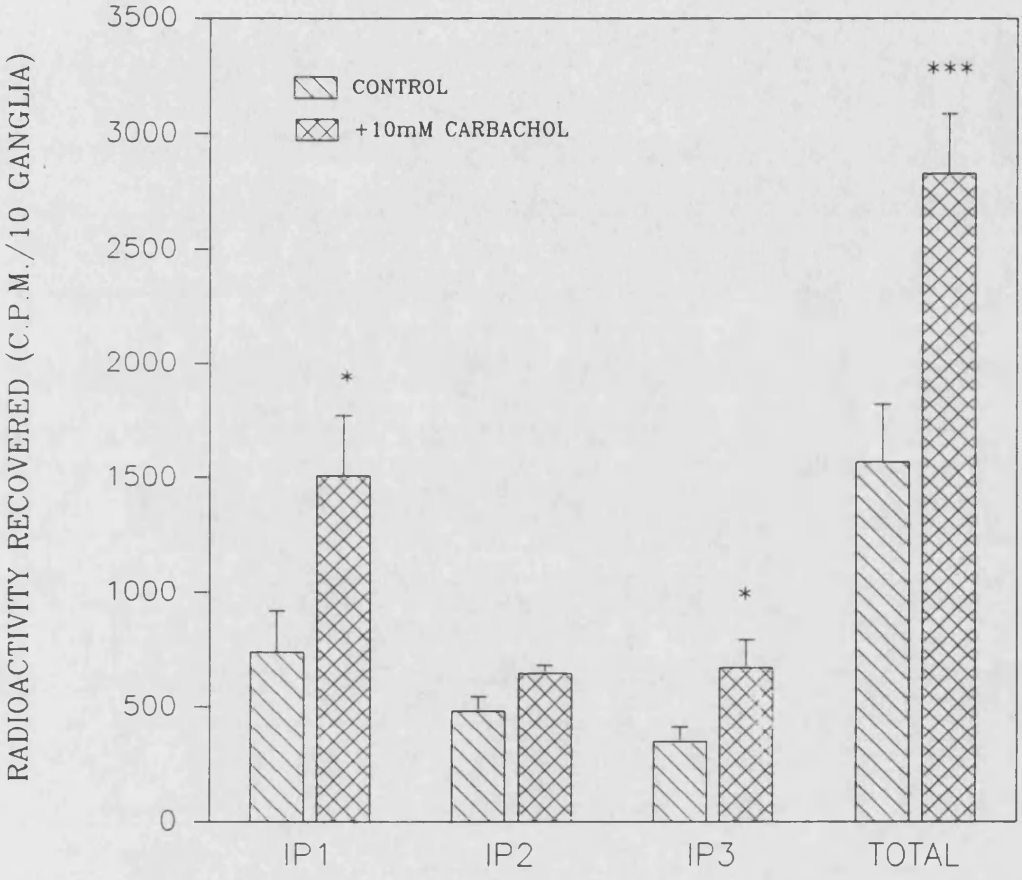
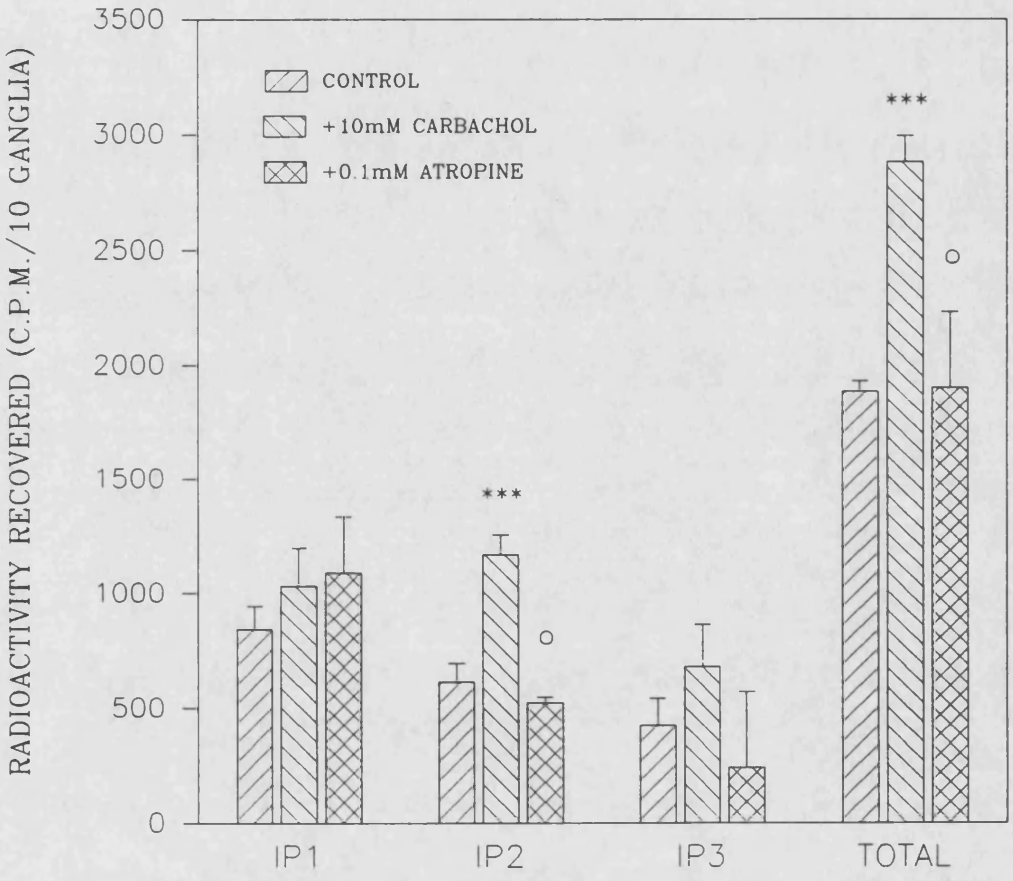

RADIOACTIVITY RECOVERED (C.P.M./10 GANGLIA)
3500
3000
2500
2000
1500
1000
500
0
CONTROL
+10mM CARBACHOL
*
*
***
IP1
IP2
IP3
TOTAL

Figure 13

The effect of carbachol and atropine in the presence of 3mM calcium and 10mM lithium.

After a preincubation of 90min, the ganglia were then incubated in buffer A containing 3mM calcium and 10mM lithium (control results represented by hatched bars rising to the right, n=4). The ganglia were treated with 10mM carbachol (hatched rising to the left, n=4) or 10mM carbachol and 0.1mM atropine (cross hatched, n=5). The mean incorporation of [^{3}H]-inositol from n replicates (results obtained from pooling of 10 ganglia per replicate). The error bars represent the standard error of mean. Significant increases were observed when comparing controls and carbachol treated ganglia in the IP2 fraction and total inositol phosphates. Atropine inhibited the effect of carbachol. There were significant decreases in the IP2 fraction and total inositol phosohates.

* $p<0.05$, *** $p<0.001$
0 $p<0.05$ compared with carbachol treated ganglia.


RADIOACTIVITY RECOVERED (C.P.M./10 GANGLIA)
3500
3000
2500
2000
1500
1000
500
0
CONTROL
+10mM CARBACHOL
+0.1mM ATROPINE
***
***
o
o
IP1
IP2
IP3
TOTAL

	% OF TOTAL INCORPORATION			TOTAL COUNT
	IP	IP2	IP3	(C.P.M.)
CONTROL1	65.8	24.0	10.2	3951
CONTROL2	56.9	27.7	15.3	2020
TREATED1	45.9	28.5	25.6	2201
TREATED2	45.7	28.5	26.1	2114

Table 2

The effect of carbachol in single incubations.

Locust ganglia (10) were incubated for 20min in buffer A containing 3μCi of [^{3}H]-inositol (CONTROLS 1 and 2). To the agonist treated ganglia (TREATED 1 and 2) carbachol (10mM) and lithium (10mM) were added. The percentage of each inositol phosphate fraction as a function of total radioactivity recovered was determined. This showed that carbachol increased the proportion of inositol incorporated into the inositol trisphosphate (IP3) fraction.

	RADIOACTIVITY RECOVERED (C.P.M./10 GANGLIA)		
	IP1	IP2	IP3
MEAN	1135	278	152
S.E.M.	85	27	42
	% OF TOTAL INCORPORATION		
MEAN	72.7	18.1	9.2
S.E.M.	2.1	2.2	1.7

Table 3.

The effect of EGTA, a calcium chelator, on control values.

Groups of 10 ganglia were incubated in buffer A containing 1mM EGTA and 3μCi [^{3}H]-inositol. The results were obtained from 5 separate experiments. The mean incorporation of [^{3}H]-inositol into the inositol phosphate fractions and the standard error of mean are shown, expressed both as the radioactivity recovered from 10 ganlia and the percentage of total incorporation into the inositol phosphates.

3.1). INTRODUCTION

Having shown that a muscarinic cholinergic drug, carbachol, increased the levels of inositol phosphates in the locust ganglia, work could begin on a more rigorous characterisation of the muscarinic receptor linked to the inositol lipid pathway.

However, many problems existed with the assay system employed to determine inositol phosphate turnover. Many replicates needed to be performed in order to show an increase, due to the variability and the size of the response, which required a high agonist concentration to be measurable.

Variability could have arisen at many points of the assay procedure: handling of the ganglia; losses of inositol phosphates in the extraction; diffusion of inositol into the ganglia which could effect its availability for incorporation into phosphatidylinositol; presence of [^{3}H]-inositol radiolytic products, which adhere to the dowex resin and elute with the inositol phosphates to give high blanks. A protocol was developed to minimise the causes of the variability described. It was essential to improve the signal further to characterise the receptor by performing dose response curves for the muscarinic ligands.

Under conditions in which the inositol-lipid cycle is turning over rapidly, the phosphatidylinositol pool will be labelled first as this is the entry point for the [^{3}H]-inositol into the cycle. Thus, for there to be a sufficient accumulation of inositol phosphates the phosphatidylinositol pool must be adequately labelled; the incorporation of [^{3}H]-inositol into phosphatidylinositol was assessed by using thin layer chromatography to separate the phospholipids and then examining the phosphatidylinositol fraction.

3.2). GENERAL METHODS.

3.2.1). THIN LAYER CHROMATOGRAPHY OF PHOSPHOLIPIDS

The incorporation of a radiolabelled tracer into phosphatidylinositol was assessed by using t.l.c. to separate the phospholipids and examining the PI fraction.

In t.l.c. separations, different components of a phospholipid mixture can be resolved, by utilising the different degrees to which the lipids are adsorbed onto a solid support coating a glass plate, and then eluted by the passage of a suitable solvent system. After separation the lipids can be easily recovered.

T.l.c. plates (20 X 20cm) were prepared by spreading a slurry of a highly porous amorphous silicic acid, silica gel G60, onto a glass support using a plate spreading

apparatus. This gel contained a binder, calcium sulphate, to facilitate adhesion. The plates were impregnated with 1% w/v potassium oxalate to sequester any calcium ions which may interfere with the running of the phospholipids.

Slurry mixture:

20g silica gel G60

40mls water + 1% w/v potassium oxolate

20mls acetone.

With the above mixture up to 5 plates, 0.25mm thick could be prepared. Before use the plates were activated in an oven at 100^{o}C for 60mins.

The phopholipid extractions were spotted or streaked on to the t.l.c. plates using capillary tubes, and dried with a cool hair dryer. After equilibrating the tank with the solvent mixture for 2 h, the chromatographic plates were developed by placing in the tank and letting the mobile phase ascend for the required length of time (Simpson *et al.*, 1987). To keep the tank saturated it was lined with filter paper.

Mobile phase:

Chloroform 40

Acetone 15

Methanol 13

Acetic acid 12

Water 7 (by vol.)

Initially, the polar solvent mixture tended to strip off the silica gel at the base causing uneven solvent runs. By washing the glass plates with 0.5M HCl and ensuring all the grease was removed by wiping with acetone prior to the spreading of the slurry better binding of the silica gel was achieved. Also the run time was shortened by heating the solvent tank on a hot plate to 45^{o}C during the run. The solvent tank was placed on a gauze to dissipate the heat.

3.2.2). IDENTIFICATION OF PI IN LOCUST GANGLIA.

Experiments were performed in which the phosphatidylinositol in the locust ganglia (*Schistocerca gregaria*) was labelled with [3H]-inositol. The ganglia were dissected and incubated as described above.The phospholipids were extracted using acidified chloroform/ methanol. The lower chloroform phase was washed with 2M KCl. High salt solutions prevent the phosphoinositides solubilising in the upper aqueous phase.The lipid samples were concentrated under vacuum in a freeze drier, aliquots of which were streaked on t.l.c. plates and developed as described (see above section.)Visualisation of the phospholipid spots was achieved by exposure to iodine vapour in another tank.

Various phospholipid standards were run with the samples.

After developing, 5mm bands of silica gel were scraped off the glass plate, and the radioactivity in each band was determined. It was found that the appearance of radioactivity corresponded with the Rf value of the PI standard.

Rf value = distance moved by the spot/ distance from the origin to the solvent front

When the chromatographs were ran at different temperatures and times, the Rf values changed but the order in which the phospholipids migrated remained constant: phosphatidic acid> phosphatidyl-ethanolamine> phosphatidyl-serine> phosphatidyl-choline> phosphatidyl-inositol> lysophosphatidyl-choline. Phosphatidyl-choline and phosphatidylinositol ran very close to each other, and sometimes did not separate (tables 4a and 4b).

3.2.3) QUANTIFICATION OF PHOSPHOLIPIDS.

By oxidising phospholipids with perchloric acid, inorganic phosphates are released, which react with ammonium molybdate to form phosphomolybdic acid. Reduction of the latter with L-ascorbic acid yields a blue complex which was measured spectroscopically (Chen *et al.*, 1956)

3.2.3.1). ASHING PROCEDURE.

After extracting the phospholipids using acidified

chloroform/ methanol, the chloroform phase was washed with 2M KCl and then dried down using a freeze drier under vacuum. The lipids were then resuspended in 200µl of chloroform/ methanol (2:1 by vol.). A 50µl aliquot of this sample was oxidised with 300µl of perchloric acid, first by heating over a bunsen flame until the solution turned black, then by placing in an oven at 120°C for 4-5 hours until the solution went clear.

With the phospholipid samples which were separated on thin layer chromatographic plates, the perchloric acid oxidation was carried out in the presence of silica after being scraped off. But, prior to the absorbance reading (820nm) the silica suspension was centrifuged and the supernatant decanted.

3.2.3.2). ANALYSIS OF THE INORGANIC PHOSPHATE CONTENT.

The ashed sample was made up to a volume of 6mls, and then divided into two 3ml portions. To each portion 3mls of the assay reagent was added (Chen *et al*, 1956). The tubes containing the reagent and the sample were mixed and incubated in a shaking water bath for 90mins at 37°C. The absorbance was measured at 820nm, and the phosphate concentration calculated by comparision with a standard curve prepared from inorganic phosphate (fig. 14).

3.3). MANIPULATION OF RESULTS.

3.3.1). [^{3}H]-INOSITOL INCORPORATION INTO THE INOSITOL PHOSPHATES.

3.3.1.1). DETERMINATION OF RADIOLYTIC DECOMPOSITION PRODUCTS.

[^{3}H]-Inositol breaks down upon storage to release ionised decomposition products, which elute from ion exchange columns. The stock solution was precleaned on Dowex-1 formate before use and stored with approximately 5mg of the resin in the stock bottle.

Even after being precleaned the [^{3}H]-inositol still contained some contamination, the levels of which were determined. A known amount of [^{3}H]-inositol was added to a Dowex formate column containing 0.2g of wet resin in a pasteur pipette. [^{3}H]-Inositol was eluted with distilled water, and the decomposition products were eluted with 2M ammonium formate/ 0.1M formic acid. All the 2ml fractions that were collected were counted, and the amount of radiolytic contamination was expressed as a percentage of total tritium recovered. Typically, 0.5 to 1% of tritium was present as radiolytic products.

To calculate the levels of inositol phosphates recovered in the experiments, the [^{3}H]-inositol was eluted with distilled water and counted. From this the level of contamination was determined and subtracted from the total

radioactivity eluted with the formate solutions.

3.3.1.2). SEPARATION OF [^{3}H]-INOSITOL LABELLED WATER SOLUBLE PRODUCTS.

The aqueous phase of the extraction was added to Dowex-1 resin in formate form. Free inositol was eluted with 10mls of distilled water, and the radioactivity was determined. The glycerophosphoinositides were eluted with 10mls of 60mM ammonium formate/ 0.1M formic acid and discarded.All the [^{3}H]-inositol products were eluted with 5x2mls of 2M ammonium formate/ 0.1M formic acid. The fractions were collected and counted.

Total radioactivity = Inositol phosphates + Radiolytic decomposition products

Level of radiolytic decomposition products = [^{3}H]-Inositol x % decomposition/100

A known fraction of the chloroform phase (1/4) was taken and ashed for lipid phosphorus determination. The results were expressed as the amount of [3H]-inositol incorporated into the inositol phosphates per microgram of total lipid phosphorus (d.p.m./μg P).

3.3.2). [^{3}H]-INOSITOL INCORPORATION INTO PI

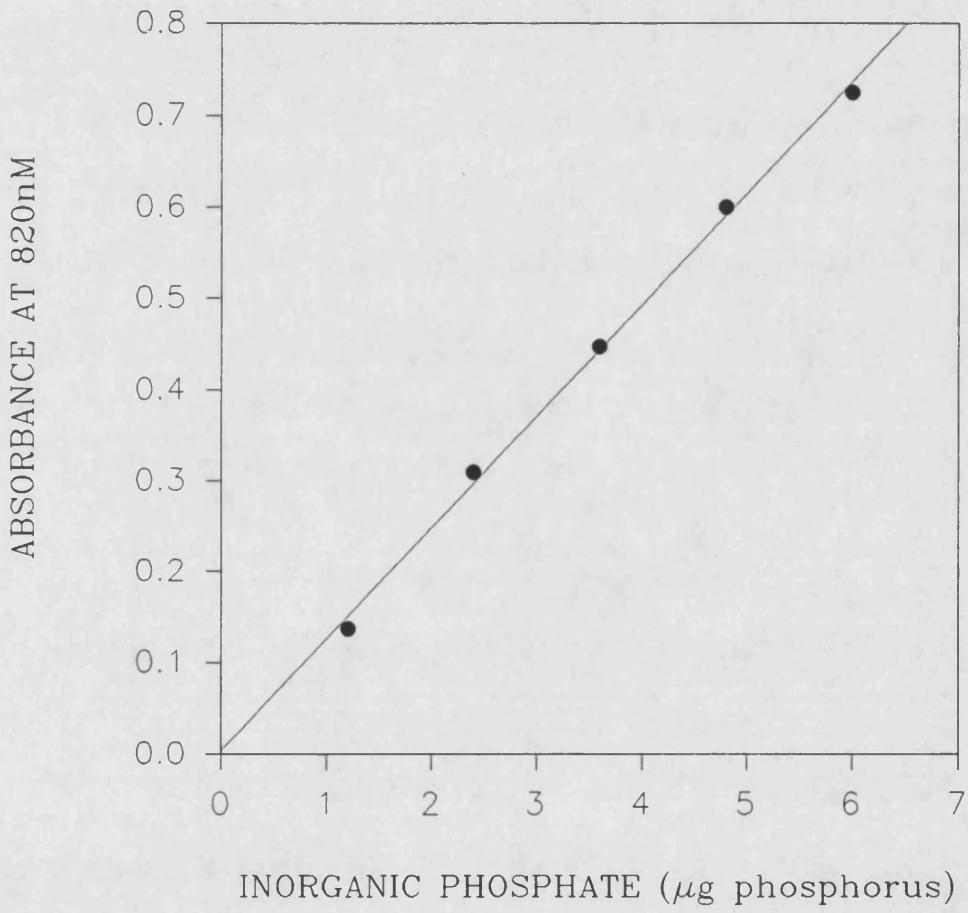
An aliquot (100μl from a total of 200μl) of the chloroform phase was streaked 2cm from the base of the 20x20cm t.l.c. plate, and dried before developing in a solvent tank. The phospholipids separated into four major bands as visualised with iodine vapour.

Phosphatidylethanolamine (PE) ascended furthest up the plate, well clear of the region in which there was [^{3}H]-inositol incorporation. The silica gel in this region, corresponding to the Rf value of the PI standard, was scraped off. On addition of scintillant the radioactivity was determined. Silica gel from other regions on the t.l.c. plate were also scraped off and counted. It was found that almost all the radioactivity recovered was in the PI fraction (fig. 15). Some radioactivity was found near the origin of the plate and may be explained as [^{3}H]-inositol incorporation into the polyphosphoinositides. PE separated completely from PS, PC, PI, and thus this lipid could be scraped off and used as an internal standard. The results were expressed as the amount of [^{3}H]-inositol incorporated into PI per microgram of phosphorus in PE (d.p.m./ μg P in PE).

Figure 14.

Standard curve for inorganic phosphate assay.

Optical densities were measured (820nm)against reagent blanks for known phosphate standards (a standard solution of potassium 26mg dihydrogen orthophosphate in 100ml distilled water contained 6μg of phosphorus per 0.1ml). The results shown are expressed as mean of triplicate determinations for each phosphate concentration (from regression analysis, OD_{820} = 0.122μgP +/- 0.004.(r = 0.999)).


ABSORBANCE AT 820nM
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
INORGANIC PHOSPHATE (μg phosphorus)

PHOSPHOLIPID	Rf VALUE
LYSOPHOSPHATIDYLINOSITOL	0.18
PHOSPHATIDYLINOSITOL	0.35
PHOSPHATIDYLCHOLINE	0.37
PHOSPHATIDYLSERINE	0.41
PHOSPHATIDYLETHANOLAMINE	0.62
PHOSPHATIDIC ACID	0.76

Table 4a.

PHOSPHOLIPID	Rf VALUE
PHOSPHATIDYLCHOLINE	0.57
PHOSPHATIDYLINOSITOL	0.59
PHOSPHATIDYLSERINE	0.61
PHOSPHATIDYLETHANOLAMINE	0.91
PHOSPHATIDIC ACID	0.93

Table 4b.

Table 4.

Separation of known phospholipids on t.l.c. plates.

The Rf values for known phospholipids, visualised by iodine vapour, are shown for chromatographic plates developed at 60^0C for 60 min (table 4a, solvent front = 135 mm) and at 25^0C for 115 min (table 4b, solvent front = 115 mm).

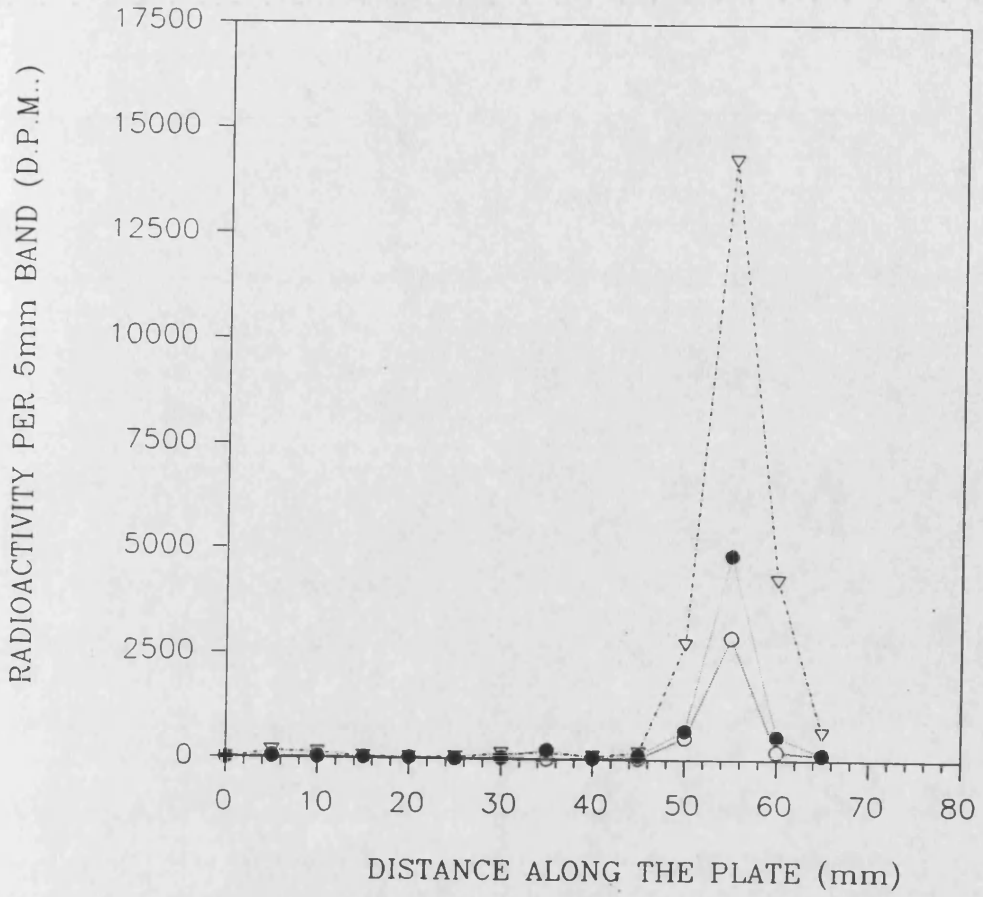


Figure 15.

[^{3}H]-Inositol incorporation into phosphatidylinositol.

The phospholipid samples from incubation of locust ganglia with [^{3}H]-inositol were prepared as described (3.2.2). Increasing aliquots of the chloroform phase (5µl (open circles), 10µl (closed circles) and 50µl (inverted open triangles)). The chromatogram was developed at 45^{0}C for 1hr. The solvent front ascended 130mm from the origin. The t.l.c. plate was divided into 5mm bands, and the silica gel was scraped off and counted. The radioactivity recovered in each band was determined. Identification of phosphatidylinositol was achieved by co-chromatography with a known standard (Rf value of PI=0.41 (53mm from the origin), PE=0.69, PS=0.50, PC=0.38).

3.4). EFFECT OF CARBACHOL AND PILOCARPINE ON THE INOSITOL LIPID PATHWAY.

3.4.1) METHOD

Supraoesophageal ganglia from adult locusts were incubated in [^{3}H]-inositol as already described. Twenty ganglia were preincubated for 90mins at room temperature in 5mls of lithium free, calcium free buffer A containing 2μCi of [^{3}H]-inositol. The 20 ganglia were split into two groups of 10; 5 ganglia were placed in an eppendorf tube containing 1ml of buffer A (+10mM lithium, +3mM calcium) and 2μCi of [^{3}H]-inositol, then incubated for 20mins at 25^{o}C. For treated experiments, 10μl of the agonist was added to the buffer to give the required concentration. Ten ganglia were pooled to determine the amount of [^{3}H]-inositol incorporation into inositol phosphates and PI, by using ion exchange chromatography and thin layer chromatography respectively.

For each agonist used, pilocarpine or carbachol, the results were collected over three separate experiments. In each experiment 10 groups of 10 ganglia were used, in which the controls and the dose response were performed together The mean [^{3}H]-inositol incorporation, into the inositol phosphates and phosphatidylinositol, for control and ganglia treated with agonist were determined by pooling the results from three experiments. From inorganic

phosphate determinations, total lipid phosphorus and the percentage of PE of total lipid phosphorus were calculated.

3.4.2). RESULTS.

3.4.2.1). Dose response for pilocarpine.

In the presence of pilocarpine the incorporation of [^{3}H]-inositol into the inositol phosphates increased significantly at 1mM pilocarpine ($p<0.002$) and 0.1mM pilocarpine ($p<0.05$) when compared to control values (fig. 16). The level of [^{3}H]-inositol incorporation into the phosphoinositides tends to decrease with increasing agonist concentration. At 0.1mM pilocarpine the phosphatidylinositol that is labelled is significantly lower than the control ($p<0.02$) (fig. 17).

3.4.2.2). Dose response for carbachol.

As with pilocarpine, carbachol also increased [^{3}H]-inositol incorporation into the insitol phosphates, but higher concentrations were required (significant result obtained at 10mM carbachol ($p<0.05$), see fig. 18). [^{3}H]-inositol incorporation into phosphatidylinositol tended to decrease with increasing agonist concentration (fig. 19), significant results were obtained at 0.5mM carbachol ($p<0.02$).

3.4.3). DISCUSSION.

A more rigorous characterisation of the suggested muscarinic receptor linked to the inositol lipid pathway required development of an assay system which could define agonist potency. Then application of various antagonists to the response elicited by the agonist would give an indication of the subclass of the receptor involved.

A dose-effect study was carried out on two muscarinic agonists: pilocarpine and carbachol. Pilocarpine has been claimed to possess M_1 selectivity for the muscarinic receptor (Caulfield and Stabley, 1982). As it is postulated that the muscarinic receptor linked to the inositol lipid cycle in the locust ganglia is M_1 (Duggan, 1987), the potency of pilocarpine was compared with carbachol in stimulating inositol phosphate turnover.

One has to exercise caution in comparing agonist potencies in dose-effect studies, which measure the physiological response that is the the final outcome of a sequence of events , amongst which the binding of the agonist to the receptor is the first. Agonist potency is determined by two drug dependent parameters: affinity and intrinsic efficacy (Kenakin, 1984). Affinity is the ability of the agonist to bind to the receptor, and is reflected in the dissociation constant of the agonist-receptor complex. Intrinsic efficacy relates to the ability of the agonist-receptor complex to elicit a

cellular response, and is determined by the occupancy of the receptor for a given response. Many tissues have a receptor reserve for muscarinic agonist, that is, only a proportion of the total number of receptors need to be occupied for a full response. This is because the primary signal is amplified to give the final response. Thus, the apparent affinity constants obtained from dose-response studies are poor indicators of agonist affinity (Ringdahl, 1984). In tissues which have a high receptor reserve, a full cellular response can still occur with an agonist with low affinity and high efficacy.

In these studies all the experiments were performed on the same tissue, the locust supraoesophageal ganglia. Thus, if the agonist were assumed to be acting on the same receptor population in this tissue, then the potencies can be compared and related to affinity. The response investigated was the effect on [^{3}H]-inositol incorporation into the inositol phosphates and phosphatidylinositol.

There was a dose dependent stimulation of phosphatidylinositol turnover in the presence of pilocarpine (fig. 16). In the stimulated state, above 10^{-5}M pilocarpine, the variability of the results increases (see S.E.M.) significantly. Thus, before any statistical comparisions could be performed, the data had to be normalised by performing a $\log_{10}$ x transformation, and then analysed using a students t-test. In comparision with the dose-response curve obtained in carbachol treated

experiments (fig. 18) it was shown that the maximum response occurred at 10^{-3}M carbachol. Therefore, the ganglia responded at a lower concentration of pilocarpine suggesting higher potency. The response itself is relatively small, in the stimulated state [^{3}H]-inositol incorporation into the inositol phosphates increases approximately two fold, this along with the variation observed in the responses masked any stimulated states between no response and a full response.

In both the carbachol and pilocarpine dose-effect curves the [^{3}H]-inositol incorporation into phosphatidylinositol showed large variations (S.E.M was over 30% of the mean at 10^{-3}M pilocarpine (fig. 17)). In the presence of both agonists there was a trend of decreasing labelled phosphatidylinositol recovered with increasing agonist concentration (figs. 17 and 19). One could postulate that the rate at which the phosphatidylinositol pool is replenished is slower than the loss during receptor activation and inositol phosphate accumulation. There are many intermediates between formation of the second messengers and their recycling into the phosphatidylinositol pool. The kinetics of the cycle are therefore very complicated, as there are many enzymes involved in the interconversions.

It was interesting to note that the incorporation of [^{3}H]-inositol into the phosphatidylinositol pool was greater in the experiments performed with carbachol than

with pilocarpine (see controls in figs. 17 and 19). This may have accounted for the greater accumulation of inositol phosphates observed in the carbachol dose response experiments (compare figs. 16 and 18). These results suggest that the labelling of the phosphatidylinositol pool can vary significantly from one set of experiments to the next,

This perhaps is not altogether surprising, as the phosphatidylinositol pool gets labelled initially from the traumatised state of the ganglia during the dissection and the period spent in the preincubation buffer. One would expect the ganglia to be in different states of trauma because dissection of 20 ganglia takes 15 min., hence the last ganglion to be dissected has a shorter preincubation period than the first ganglion. Variability in the labelling of the phosphatidylinositol pool would result in variability of the response, as it is from this pool that the accumulation of inositol phosphates is obtained.

The stimulation of inositol phosphate turnover in the presence of pilocarpine lends further evidence to the suggestion that a muscarinic like receptor is present in insects which is linked to the inositol lipid cycle. Even though pilocarpine seems to be more potent than carbachol (the ganglia respond at 10^{-4}M pilocarpine, but not at the same concentration of carbachol), high concentration of this agonist is required for phosphatidylinositol hydrolysis. Other doseresponse studies (Fisher and Snider,

1987) also show high concentration requirements for the phosphoinositide response (EC_{50} for carbachol in the guinea pig cerebral cortex is 200μM). These results suggest that a high degree of receptor occupancy may be needed to maximally activate the inositol lipid cycle. Also, as the experiments were performed using whole ganglia, high concentrations of agonist may have been required to overcome diffusion barriers.

The receptor number can be reduced in any tissue by irreversible alkylation. After which, the changes in the functional response can be assessed. If the muscarinic receptors in chick atrial cells are alkylated using propylbenzilycholine mustard, Brown and Goldstein (1986) demonstrated that carbachol failed to give a maximal response, and by increasing the alkylation the magnitude of the response decreased progressively. Thus for the phosphatidylinositol system there seems to be little receptor reserve. In comparison the dose response for the inhibition of adenylate cyclase shifts to the right, but there is no reduction in the response until up to 95 % of the muscarinic receptors are alkylated. Therefore, there is a far greater receptor reserve for adenylate cyclase inhibition.

Figure 16.

Effect of increasing doses of pilocarpine on the incorporation of [^{3}H]-inositol into the inositol phosphates.

Twenty ganglia were preincubated for 90min. In control experiments the ganglia were transferred into buffer A containing 10mM lithium and 3mM calcium for 20min (n=9). For agonist treated ganglia, 5 doses of pilocarpine were used (10^{-7} (n=3), 10^{-6} (n=3), 10^{-5} (n=4), 10^{-4} (n=3) and 10^{-3} (n=3)). The upper water soluble phase of the acidified chloroform/methanol mixture was pooled from 10 ganglia and added to Dowex-1 formate columns. The results were obtained from four separate experiments, and the mean incorporation of [^{3}H]-inositol into the inositol phosphates from n replicates were determined (d.p.m. radioactivity recovered per μg of total lipid phosphorus, total lipid phosphorus in 10 ganglia = 20.2 μg P +/- 2.5 (s.d.), n=10). Control value is represented by the dashed line (114 d.p.m./μg P +/- 14.3 (S.E.M.). Agonist treated ganglia are represented by the filled circles. Fmax test for the largest and smallest variances showed that they were significantly different ($p<0.05$, two tailed). The results were compared using students t-test after a $\log_{10}x$ transformation. There were significant increases at 0.1mM and 1mM pilocarpine.

* $p<0.05$, *** $p<0.002$.

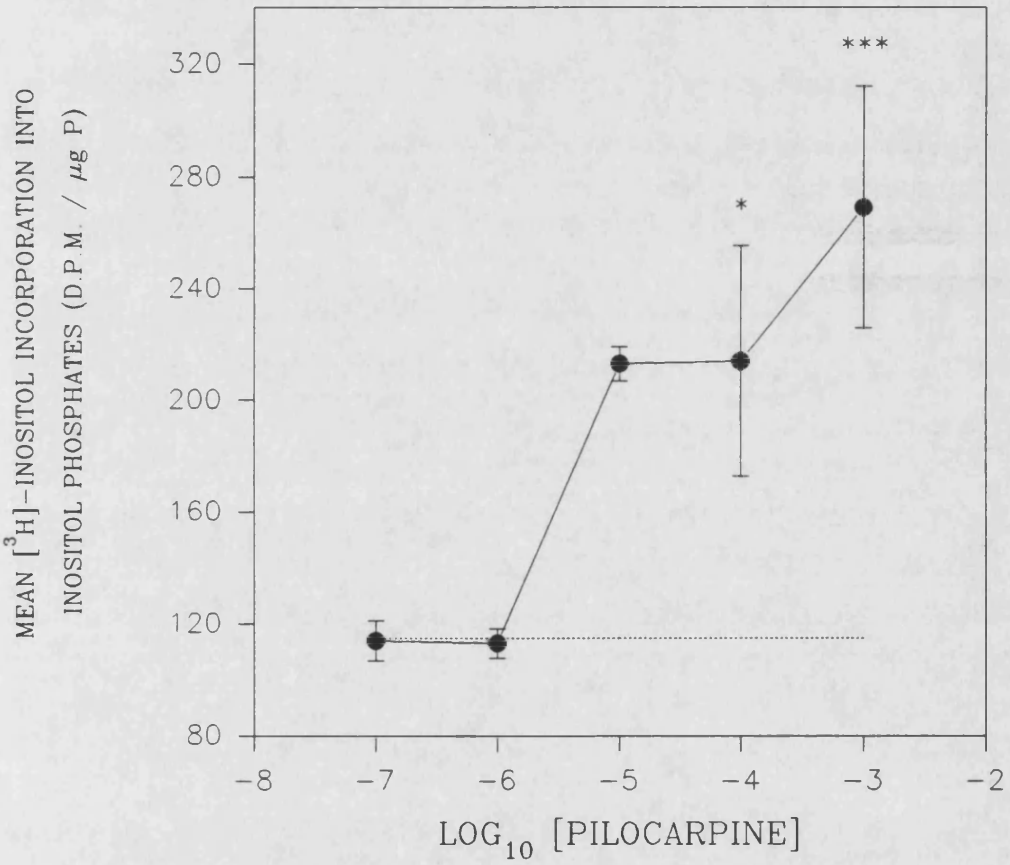

MEAN [3H]-INOSITOL INCORPORATION INTO
INOSITOL PHOSPHATES (D.P.M. / μg P)
320
280
240
200
160
120
80
*
***
−8
−7
−6
−5
−4
−3
−2
LOG10 [PILOCARPINE]

Figure 17.

The effect of increasing doses of pilocarpine on the incorporation of [^{3}H]-inositol into phosphatidylinositol (PI).

The ganglia were preincubated and incubated as described in fig. 16. The phospholipids were prepared from the lower chloroform phase of the acidified chloroform / methanol mixture, and separated by t.l.c.. Radioactivity recovered from the phosphatidylinositol fraction (see fig.15) of the ganglia (10) incubated in control conditions (n=5) and in the presence of pilocarpine (n=3 for 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M) was measured. The results were pooled from three separate experiments, and the mean incorporation of [^{3}H]-inositol into PI for n replicates were determined (radioactivity in PI (d.p.m.) per μg phosphorus in phosphatidylethanolamine (PE), % PE of total lipid phosphorus = 16.7% +/- 3.9 (s.d.), n=10). The control value is represented by a dashed line (3860 d.p.m. / μg P in PE +/- 483 (S.E.M.). There was a significant decrease in the presence of 0.1mM pilocarpine (open circles with associated S.E.M.).

** $p<0.02$.

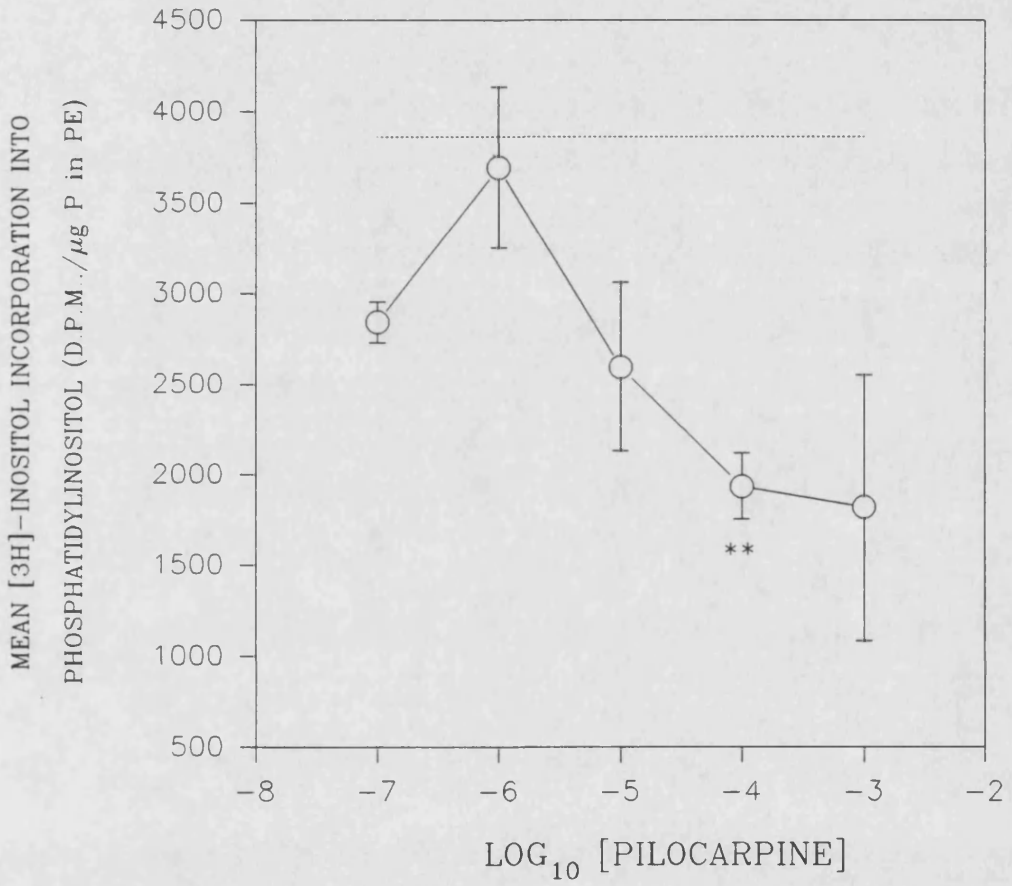

MEAN [3H]-INOSITOL INCORPORATION INTO
PHOSPHATIDYLINOSITOL (D.P.M./μg P in PE)
4500
4000
3500
3000
2500
2000
1500
1000
500
**
−8
−7
−6
−5
−4
−3
−2
LOG10 [PILOCARPINE]

Figure 18.

The effect of increasing doses of carbachol on the incorporation of [^{3}H]-inositol into the inositol phosphates.

For preincubation and incubation conditions see fig. 16. The upper water soluble phase of the acidified chloroform /methanol mixture was pooled from 10 ganglia Radioactivity recovered in [^{3}H]-inositol labelled inositol phosphates was determined using anion exchange chromatography for control (n=3) and carbachol treated ganglia (n=3 for 10^{-5}, 10^{-4}, $5x10^{-4}$, 10^{-3} and 10^{-2} M carbachol). The results were pooled from three separate experiments, and the mean incorporation of [^{3}H]-inositol is shown for n replicates (d.p.m. of total radiolabelled inositol phosphates per μg of total lipid phosphorus, total lipid phosphorus per 10 ganglia = 18μg P +/- 1.6 (s.d.), n=10). Control value is represented by a dashed line (214 d.p.m./μg P +/- 16 (S.E.M.), n=3). There was a significant increase at 10mM carbachol (filled inverted triangle with associated S.E.M.).

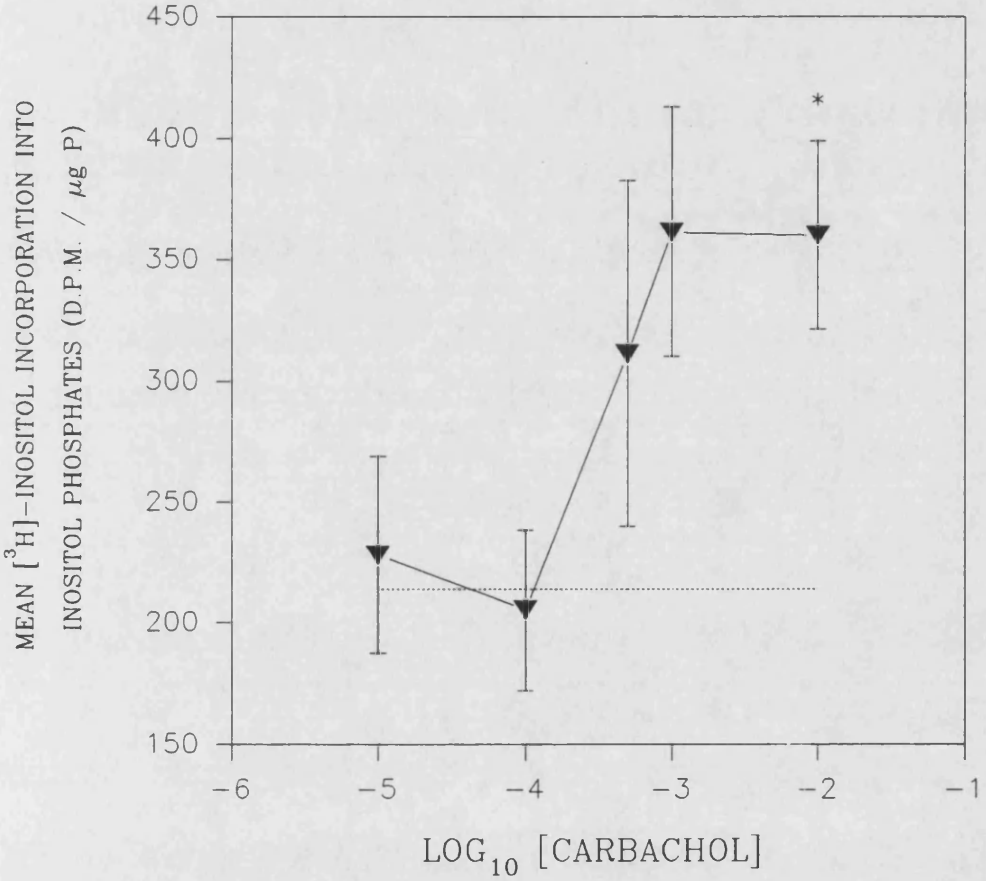
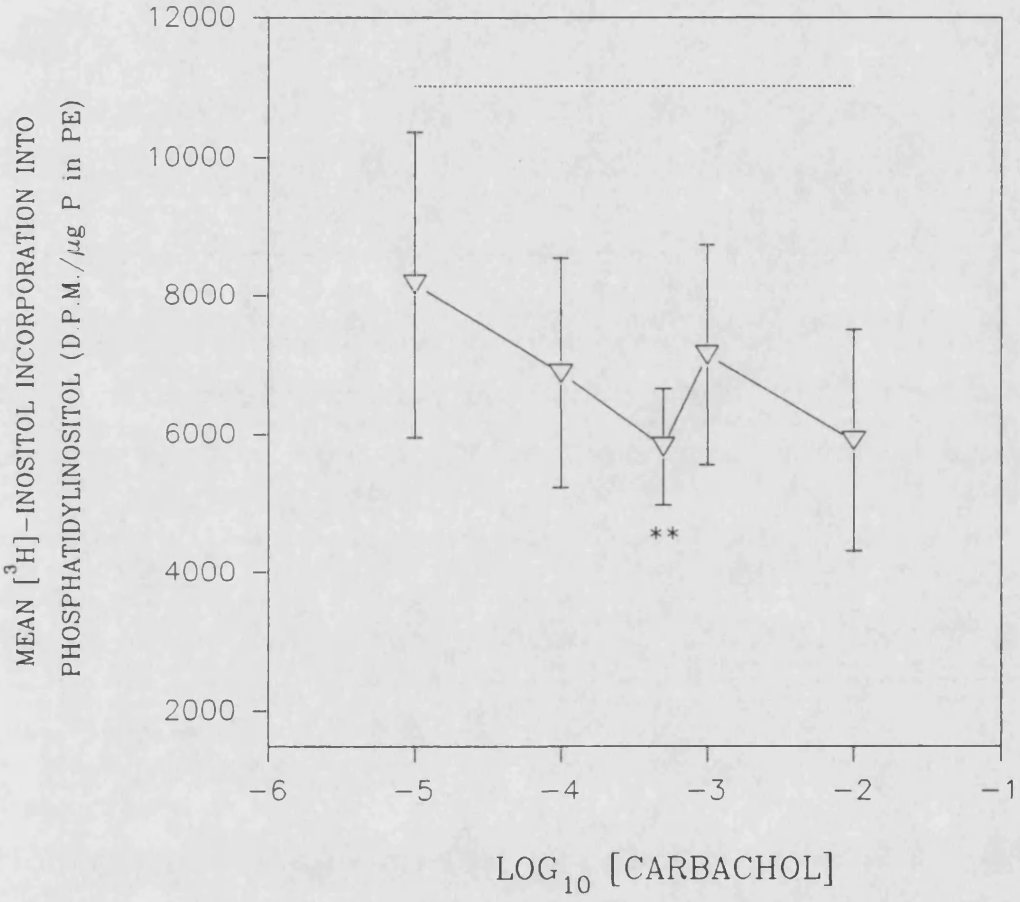

MEAN [3H]-INOSITOL INCORPORATION INTO
INOSITOL PHOSPHATES (D.P.M. / μg P)
450
400
350
300
250
200
150
*
−6
−5
−4
−3
−2
−1
LOG10 [CARBACHOL]

Figure 19.

The effect of increasing doses of carbachol on the incorporation of [^{3}H]-inositol into phosphatidylinositol (PI).

For preincubation and incubation conditions see fig. 16. The phospholipids were prepared the chloroform soluble phase of the acidified chloroform / methanol extraction mixture, and separated by t.l.c.. Radioactivity recovered from [^{3}H]-inositol labelled phosphatidylinositol was determined from ganglia incubated in control conditions (n=3) and those treated with carbachol (10^{-5} (n=3), 10^{-4} (n=4), 5 x 10^{-4} (n=3), 10^{-3} (n=3), 10^{-2} (n=3) M). The results were pooled from three separate experiments, and the mean incorporation of [^{3}H]-inositol is shown for n replicates (d.p.m. of labelled phosphatidylinositol / μg of lipid phosphorus in phosphatidylethanolamine (PE), % PE of total lipid phosphorus = 16.7% +/- 3.9 (s.d.), n=10). The control value is represented by the dashed line (11 020 d.p.m. / μg of P in PE). There was a significant decrease at 0.5mM carbachol (open inverted triangles with associated S.E.M.).

** $p<0.02$


MEAN [3H]-INOSITOL INCORPORATION INTO
PHOSPHATIDYLINOSITOL (D.P.M./μg P in PE)
12000
10000
8000
6000
4000
2000
**
−6
−5
−4
−3
−2
−1
LOG10 [CARBACHOL]

3.5). EFFECT OF REMOVING [^{3}H]-INOSITOL FROM THE INCUBATION BUFFER.

Experiments were performed in which the radiolabel was only present in the preincubation buffer. This would determine whether or not the PI pool labelled during the preincubation period is utilised to show an increase in inositol phosphate turnover. A muscarinic cholinergic agonist pilocarpine, was used in the incubation buffer not containing the radiolabel.

3.5.1). METHOD.

See general methods for detailed descriptions. Twenty ganglia were preincubated for 90mins at room temperature in 5mls of buffer A containing 5μCi of [^{3}H]-inositol. Groups of 10 ganglia were taken for determination of [^{3}H]-inositol incorporation into PI and inositol phosphates (preincubation).

In control experiments, 5 ganglia were placed in 1ml of buffer (+10mM lithium, 3mM calcium) without radiolabel and incubated for 20mins at 25^{o}C. For agonist treated experiments, 10μl of 0.1M pilocarpine was added to 1ml of incubation buffer (to give final concentration of 1mM).

The results were obtained for two separate experiments. The total lipid phosphorus and the mean

percentage of phosphatidylethanolamine were determined.

3.5.2). RESULTS

When the ganglia were treated with 1mM pilocarpine there was no effect on [^{3}H]-inositol incorporation into phosphatidylinositol and inositol phosphates in the absence of [^{3}H]-inositol in the incubation buffer (figs. 20a and 20b).

3.5.3). DISCUSSION.

Results obtained from experiments in which the radiolabel was removed from the incubation buffer (fig. 20), showed that the incorporation of [^{3}H]-inositol into the phosphatidylinositol pool was much less than that obtained from the dose response studies. Thus, the presence of the radiolabel further labels the phosphatidylinositol pool after the preincubation period. In experiments with carbachol the appearance of the label into the phosphatidylinositol fraction increases by nearly ten fold (1256 d.p.m./μg in PE (fig. 20a) to 11020 d.p.m./μg in PE (fig. 19)), if one compares the activity after the preincubation period and that obtained from control experiments, which included a 20 min incubation period in the presence of [^{3}H]-inositol.

There was a small but insignificant increase in the accumulation of [^{3}H]-inositol phosphates from the labelled

phosphatidylinositol pool when incubated in the presence of 1mM pilocarpine (fig. 20b). Thus, in inositol free conditions during the incubation period, the label could not be chased out of the phosphatidylinositol pool. This suggested the presence of an agonist insensitive phosphatidylinositol pool. Koreh and Monaco (1986) have demonstrated that rat WRK-1 rat mammary tumour cells contain at least two distinct pools of phosphatidylinositol, only one of which is sensitive to hormonal action.

Since a pilocarpine induced response was observed in experiments in which [^{3}H]-inositol was added to the incubation buffer (fig. 16), one can conclude that much of the turnover of the inositol lipid cycle occurs during the incubation period. There has to be a large excess of [^{3}H]-inositol in the buffer to enable rapid incorporation into the phosphatidylinositol pool, in order to observe the appearance of the label in the inositol phosphates. Studies in the blow fly salivary gland investigating the hormonal activation of phosphatidylinositol hydrolysis (Fain and Berridge, 1979) indicated that newly synthesised [^{3}H]-phosphatidylinositol appears to be preferentially hydrolysed following stimulation by the agonist, 5-hydroxytryptamine. Results from these experiments on locust ganglia show that the phosphatidylinositol pool can be further labelled and acted upon by an agonist within the 20 min incubation period. Even though the phosphatidylinositol pool is labelled during the

preincubation period, the absence of accumulation of [^{3}H]-inositol phosphates in the presence of a muscarinic agonist, suggests that this pool is inaccessible to agonist action. A small sub-pool has to be constantly replenished with labelled phosphatidylinositol to observe an accumulation of [^{3}H]-inositol phosphates.

Figure 20.

The effect of removing [^{3}H]-inositol from the incubation buffer.

Groups of 20 ganglia were prelabelled for 90min in 5ml of buffer A containing 5μCi [^{3}H]-inositol. The radioactivity incorporated into the inositol phosphates and phosphatidylinositol were determined immediately following this preincubation period (represented by P in tables 20a and b (hatched rising to the right) n=7)). Incubations were carried out in the absence of the radiolabel (buffer A + 10mM lithium and 3mM calcium). Radioactivity recovered from the inositol phosphates and PI after the 20 min incubation period were determined for groups of 10 ganglia in control conditions ('C' (hatched rising to the left), n=6)and from those treated with 1mM pilocarpine ('T' (cross hatched), n=6). The results were pooled from 2 separate experiments, and the mean (+/- S.E.M.) incorporation into the inositol phosphates (d.p.m./μg total lipid phosphorus, total lipid phosphorus from 10 ganglia = 16.4μg P +/- 2.3 (s.d.), n=10), and PI (d.p.m./μg P in PE, % of PE of total lipid phosphorus = 24.5% +/- 5.2 (s.d.), n=10).

FIG 20a

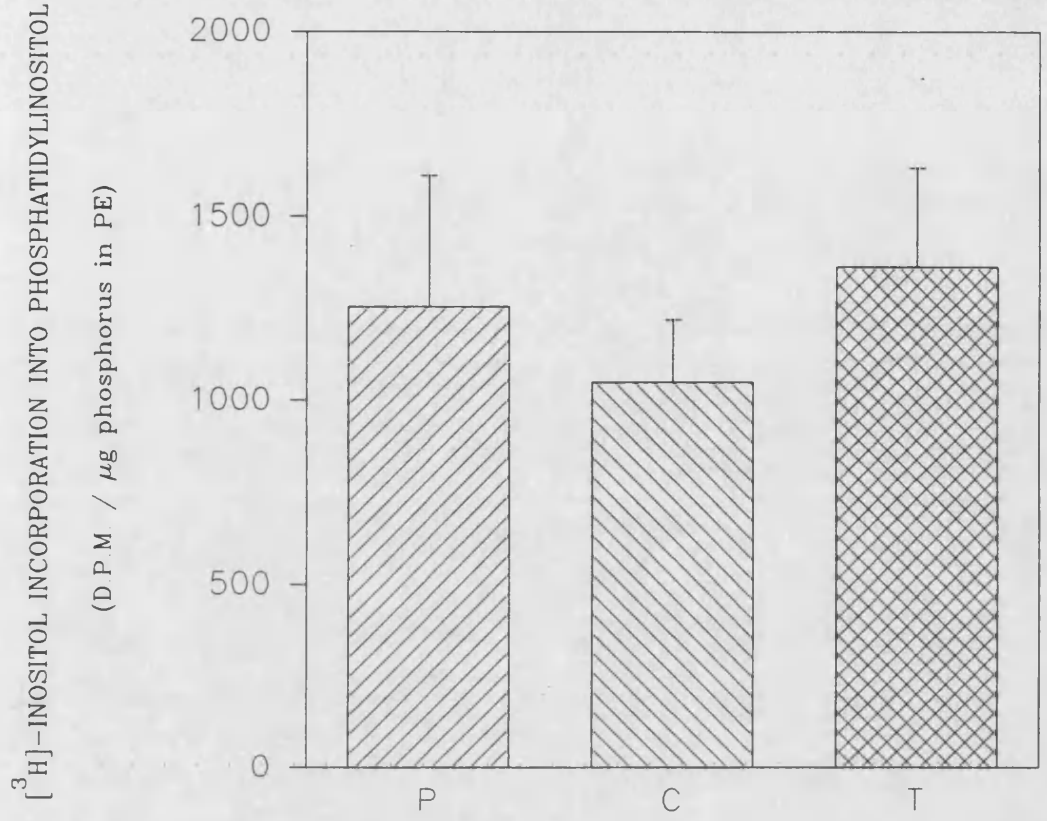
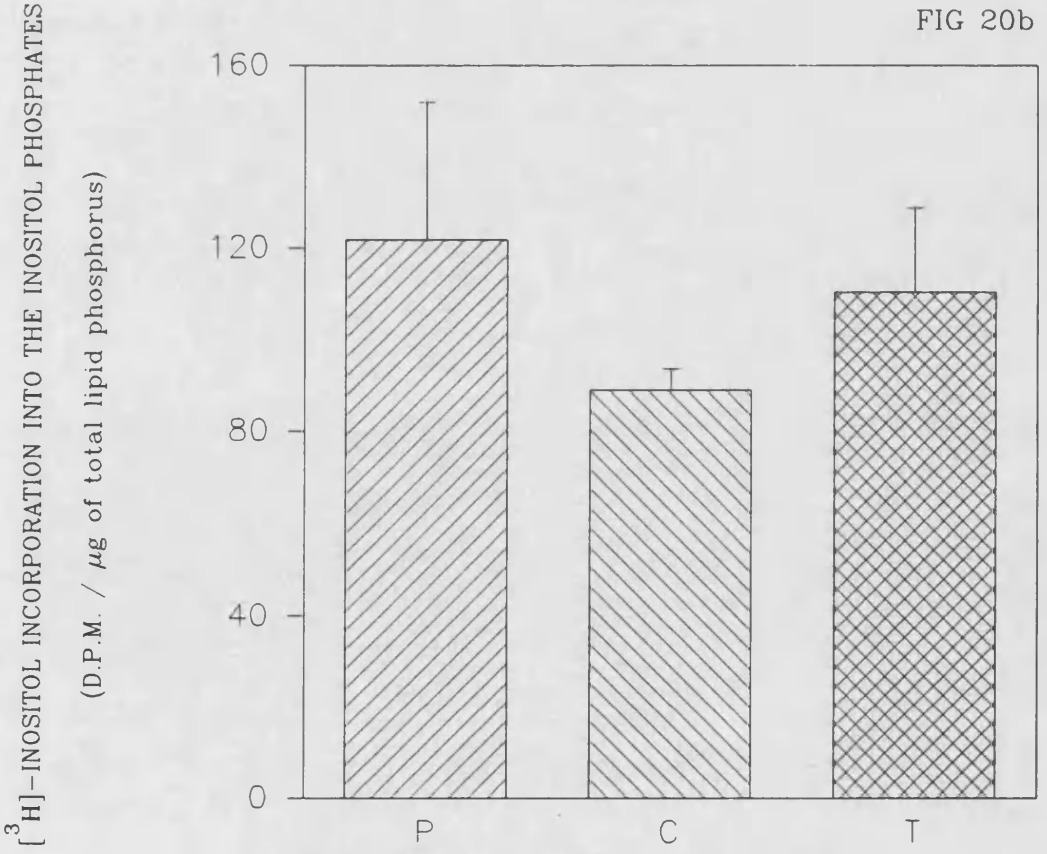


FIG 20b



3.6). THE EFFECT OF ADDING PHYTATE HYDROSYLATE IN THE EXTRACTION PROCEDURE.

3.6.1). INTRODUCTION.

The experiments were repeated to investigate the effects of pilocarpine, atropine and lithium on the incorporation of [^{3}H]-inositol into the inositol phosphates and PI. In these experiments the phytate hydrosylate was added in the extraction procedure as described below. The effect of phytate hydrosylate on the recovery of inositol phosphate standards (inositol 1-phosphate, inositol 1,4-bisphosphate, inositol 1,4,5-trisphosphate) from the extraction procedure and Dowex columns was also determined.

3.6.2) PREPARATION OF PHYTATE HYDROSYLATE.

Losses of trace amounts of labelled inositol phosphates in the extraction procedure can be reduced by the addition of a random mixture of cold inositol phosphates (Wreggett *et al.*, 1987). This was prepared by dissolving 1g of sodium phytate in 5mls of 0.2M sodium acetate/ acetic acid, pH 4.0, in a glass stoppered tube and heated in a boiling water bath for 8 hours. After cooling the solution was desalted by passage through 4ml of Amberlite resin (IR-120(H), BDH Chemicals) in a pasteur pipette (Wreggett and Irvine, 1987).

To the stock solutions of 0.5M HCl and distilled water, the phytate prepared above was added in the ratio 1:100 v/v. Thus, the acidified chloroform/ methanol contained 1ml of 1:2 chloroform: methanol, 0.2ml 0.5M HCl and 2μl of phytate hydrosylate. The water which was used to separate the phases (0.2mls) also contained 2μl of phytate hydrosylate.

3.6.3). THE EFFECT OF ADDING PHYTATE HYDROSYLATE TO THE EXTRACTION MIXTURE ON THE RECOVERY OF INOSITOL PHOSPHATE STANDARDS IN THE PRESENCE OF LOCUST GANGLIA.

As the losses of inositol phosphates on the Dowex-1 formate columns were limited (up to 85% recovery), the whole acidified choloform/methanol extraction procedure was performed in the presence of the ganglia in order to determine whether or not the tissue itself binds to the inositol phosphates. The effect of adding phytate hydrosylate on the recovery was also determined.

3.6.3.1). METHOD

Groups of 10 ganglia were homogenised in acidified chloroform/methanol (see above) containing a known amount of radiolabelled inositol phosphates (approximately 20 000

d.p.m. of the mixture). This was performed in the presence or absence of phytate hydrosylate, which was added to the 0.5M HCl and distilled water. The upper phase of the extraction mixture was added to the Dowex-1 formate columns and eluted with distilled water and 2M ammonium formate/0.1M formic acid. The total inositol phosphates recovered was determined by scintillation counting of the fractions eluted (see above).

3.6.3.2). RESULTS

The recovery of inositol phosphates was increased in the presence of phytate hydrosylate (from 32.5% to 52.5%, n=5), but losses of up to 50% were incurred when the extraction was peformed in the presence of locust ganglia (c.f. 81% recovery in the absence of ganglionic tissue).

	% RECOVERY (S.D)	
GANGLIA	32.5 (3.7)	
GANGLIA +PHYTATE	52.5 (5.4)	n=5

3.7). THE EFFECT OF PILOCARPINE, ATROPINE AND LITHIUM ON [^{3}H]-INOSITOL INCORPORATION INTO THE INOSITOL PHOSPHATES AND PHOSPHATIDYLINOSITOL

3.7.1). METHOD

Groups of 20 ganglia were preincubated for 90 min at room temperature in 5mls of buffer containing 5μCi of [^{3}H]-inositol. Groups of 5 ganglia were then placed in 1 ml of incubation buffer, which included 3mM calcium and 2μCi of [^{3}H]-inositol for 20min at 25^{o}C. The reaction was stopped on dry ice, and [^{3}H]-inositol incorporation into the inositol phosphates and phosphatidylinositol was determined for 10 ganglia (2 groups of 5 pooled). The mean radioactivity recovered in the inositol phosphate fractions (d.p.m./μg total lipid phosphorus) and phosphatidylinositol (d.p.m./μg lipid phosphorus in PE) were determined from pooling the results obtained from three separate experiments.

The incubations were treated to investigate the effects of 0.1mM pilocarpine, 0.1mM atropine and 10mM lithium. Control experiments were performed in the presence or absence of 10mM lithium in the incubation buffer. To determine the effect of a muscarinic agonist, the ganglia were treated with 0.1mM pilocarpine in the presence or absence of 10mM lithium. The effect of adding an antagonist, 0.1mM atropine, to ganglia treated with

0.1mM pilocarpine was also measured.

3.7.2). RESULTS.

The results show that there was an increase in the recovery of inositol phosphates in the presence of phytate hydrosylate in the extraction procedure (compare the radioactivity recovered when stimulated with 0.1mM pilocarpine in the absence of phytate hydrosylate (fig 16), and that obtained from experiments in which phytate hydrosylate was added to the extraction mixture (fig. 21a)). Figure 21a shows an increase in [^{3}H]-inositol incorporation into the inositol phosphates in experiments treated with 0.1mM pilocarpine. On addition of 10mM lithium, the accumulation of inositol phosphates. was enhanced (fig. 21a, $p<0.01$ when compared with control+lithium). However, no significant effect of the antagonist, atropine, was observed on pilocarpine (0.1mM) and lithium (10mM) treated ganglia. Comparing the effects of pilocarpine on the incorporation of [^{3}H]-inositol into phosphatidylinositol showed no significant changes (fig. 21b). When the ganglia were treated with 0.1mM pilocarpine, 0.1 mM atropine and 10mM lithium there was a significant increase (fig. 21b, $p<0.02$) in the recovery of radiolabelled phosphatidylinositol.

Whereas, enhancement of [^{3}H]-inositol phosphate accumulation was not observed in the presence of 10mM lithium, 3mM calcium and 10mM carbachol, these results

show that by improving the recovery of inositol phosphates and stimulating with a more potent agonist, pilocarpine, lithium enhances the effect of pilocarpine. However, atropine did not antagonise the effect of the agonist. The relative potencies of pilocarpine and atropine require elucidation.

Figure 21

The effect of adding phytate hydrosylate in the extraction procedure.

After 90min preincubation, groups of 10 ganglia were incubated for 20 min in buffer A containing 3mM calcium and 5μCi [^{3}H]-inositol. Phytate hydrosylate was added to the acidified chloroform / methanol extraction mixture to increase the recovery of inositol phosphates. Control incubations are represented by 'C' (hatched rising to the right, n=6) and control incubations in the presence of 10mM lithium are represented by 'C+LI' (hatched rising to the left, n=6). Experiments were performed in which the ganglia treated with 0.1mM pilocarpine in the absence ('PIL' (cross hatched), n=4) or presence of 10mM lithium ('PIL+LI' (vertical lines), n=4). The effect of an antagonist, atropine, was measured by treating the ganglia with 0.1mM pilocarpine, 10mM lithium and 0.1mM atropine ('ANT' (horizontal lines), n=4). The results were pooled from three separate experiments, and the mean incorporation of [^{3}H]-inositol incorporation into the inositol phosphates (fig. 21a) and PI (fig. 21b) were determined. In the presence of pilocarpine and lithium, there was an increase in the recovery of radiolabelled inositol phosphates, when compared with control+lithium. Atropine did not antagonise the effect of pilocarpine in the presence of lithium (fig. 21a). There was a significant increase in the labelling of the PI pool in the presence of atropine (fig. 21b).

** $p<0.02$, *** $p<0.01$.

fig. 20A

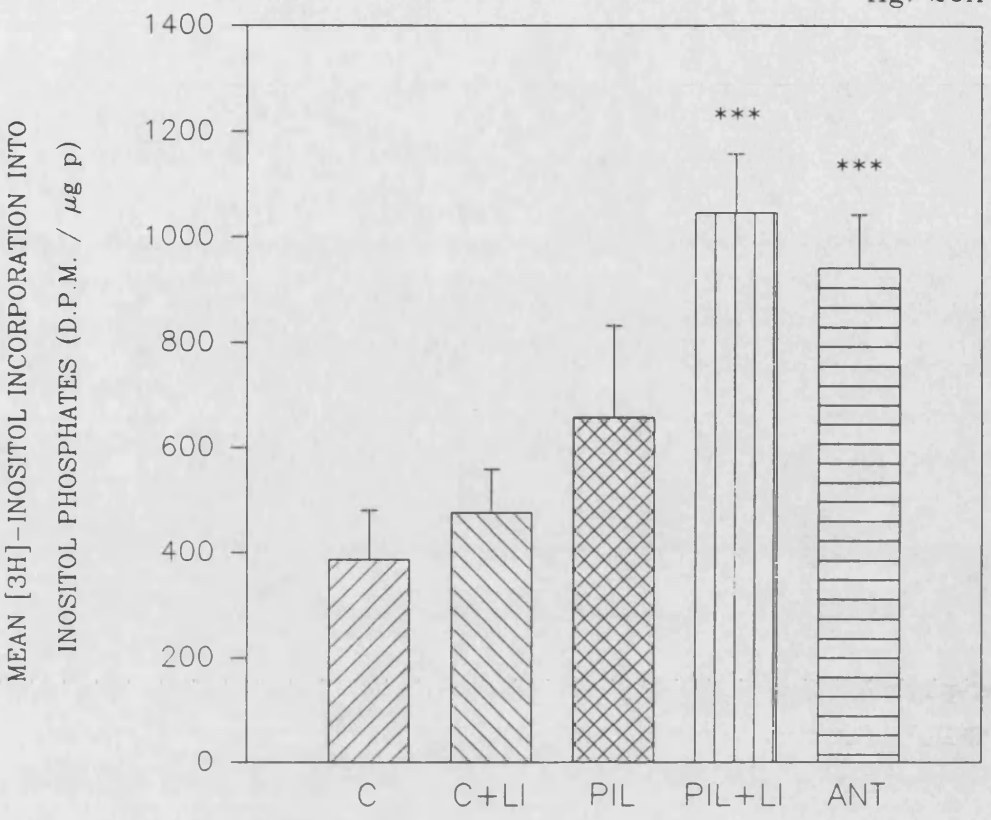
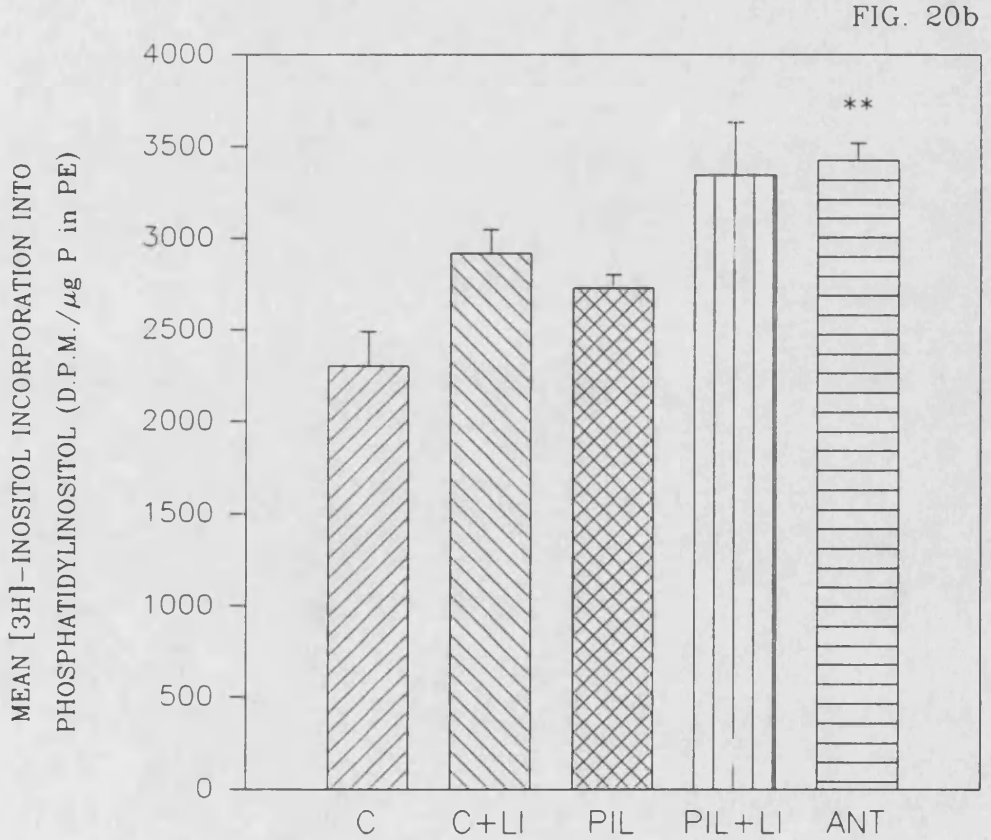


FIG. 20b



3.8) FURTHER WORK.

A more definitive demonstration that phosphatidylinositol breakdown is linked to the muscarinic-like receptor activation in the locust ganglia may be obtained from using cell free systems. The 5-hydroxytrptamine receptor in the blow fly salivary gland was first characterised using whole glands, which then led to the development of a cell free system to further ascertain the effects of receptor activation on G-protein and phospholipase C activation.

5-HT receptors in intact blowfly salivary glands linked to both cyclic adenosine 3', 5'-monophosphate formation and calcium mobilisation (Berridge and Heslop, 1981) could be distinguished pharmacologically using both agonists: 5-methyltryptamine was more potent in stimulating hydrolysis of phosphatidylinositol and the transport of calcium, whist 4- and 5-fluoro-α-methyltryptamine was capable of raising cAMP levels and stimulating fluid secretion, and antagonists: methysergide was a potent inhibitor of phosphatidylinositol hydrolysis, whilst cinanserin was far more selective in blocking the stimulatory effect of 5-HT on cAMP formation. Such pharmacological characterisations of blow fly salivary glands were made possible because of the well defined and consistent responses to the agonists, both with respect to second messenger generation and the associated

physiological responses. This was not found to be the case in our studies using locust ganglia, in which the formation of inositol phosphates in response to the addition of agonists was variable and dependent on other factors such as calcium ion concentrations. Using whole tissue preparations the indirect effect of drug action remain indetermined. The agonist may act on other receptors or second messenger systems which indirectly affect the system being studied.

A cell free system to study the effects of receptor activation enables characterisation of the G-protein link to the effector: adenylate cyclase, guanylate cyclase and phospholipase C. Evidence for the presence of G-proteins in insects has come from studying the effects of GTP on the binding of muscarinic agonists (Whyte and Lunt, 1986), the cloning of a *Drosophila melanogaster* guanine nucleotide regulatory protein ß-subunit gene (Yarfitz *et al.*, 1988), and the effects of guanine nucleotide analogues in stimulating inositol phosphate formation in cell free blow fly salivary glands (Litosch and Fain, 1985; Litosch *et al.*, 1985).

To demonstrate phospholipase C activity in cell free systems the agonist sensitive phosphatidylinositol pool must be adequately prelabelled. Initial studies on the blowfly salivary gland prelabelled with [^{3}H]-inositol (Fain *et al.*, 1983) found that on homogenisation there was rapid degradation of labelled phosphatidylinositol. This

problem was circumvented by performing the studies in the presence of EGTA, a calcium ion chelator. Experiments by Litosch *et al.* (1985) on the cell free system, provided direct evidence that guanine nucleotides were required for the expression of the hormone effect on phosphoinositide breakdown. Stable GTP analogues, Gpp(NH)p and GTPγS could directly activate inositol phosphate formation, and could act synergistically with 5-methyltryptamine, a PI specific 5-HT receptor agonist. A linear rate of inositol phosphate formation was not maintained for more than 15 sec., and a decline in the enzymatic rate was readily apparent after 30sec. This departure from the first order kinetics suggested rapid depletion of hormone sensitive lipid substrate during incubation of the homogenate at levels where the Vmax can no longer be maintained.

In these studies using locust ganglia, the phosphatidylinositol pool labelled with [^{3}H]-inositol during the preincubation period was found not to be sensitive to the action of agonist (fig. 20a and b). This would suggest that development of a cell free preparation to investigate phospholipase C activity may be impracticable. Labelling of the agonist sensitive phosphatidylinositol pool required the presence of the agonist: carbachol or pilocarpine, and the extent of labelling with [^{3}H]-inositol was found to be very variable (see figs. 16-19). A better approach may be to add exogenous labelled phosphoinositides to the homogenate, similar to the studies by Litosch and Fain (1985) on the

blowfly salivary gland cell free experiments. The contribution of the endogenous substrate must be considered in such studies, as the exogenous lipid substrate may compete poorly with the endogenous lipid substrate (Litosch and Fain, 1985). Accessibility of the substrate to the catalytic site would require optimization.

Cell free systems provide a valuable approach to the study of receptor/G-protein/phospholipase C relationships. Prelabelling of the agonist sensitive phosphoinositide pools prior to experimentation may prove to be problematic. Availibility of assays which measure mass changes of inositol 1,4,5-trisphosphate in stimulated conditions may help to counter these problems, as this would alleviate the need to prelabel the phosphoinositides.

4). HIGH PRESSURE LIQUID CHROMATOGRAPHY OF INOSITOL PHOSPHATE ISOMERS.

4.1). INTRODUCTION

Separation and purification of the various inositol phosphate isomers can be achieved by the use of an h.p.l.c. separation procedure (Batty *et al*, 1989). Although the batch technique using Dowex chromatography can be used to separate inositol mono-, bis-, and tris phosphates, it does not separate different isomers such as inositol 1,3,4-trisphosphate and inositol 1,4,5-trisphosphate. Experiments were performed to determine whether [^{3}H]-inositol was incorporated into inositol 1,4,5-trisphosphate in the locust ganglia.

Before separation of the inositol phosphates using h.p.l.c. a purification procedure was employed using Accell QMA ion exchange SEP-PAK columns (Wreggett and Irvine, 1987). This offered a rapid, high recovery and reproducible method for the separation of the total inositol phosphates.

4.2). METHODS.

The dissections, preincubation and incubation conditions were essentially the same as already described

in general methods (see 2.2).

4.2.1). PREINCUBATION.

Groups of 10/20 ganglia were preincubated in 5 mls of buffer A (calcium free, lithium free) containing 3μCi of [^{3}H]-inositol, for 90 min at room temperature.

4.2.2). INCUBATION.

The ganglia were split into groups of 5 and placed in Eppendorf tubes containing 1 ml of buffer A, 3mM calcium and 4μCi [^{3}H]-inositol. Incubations were performed in the presence or absence of 0.5mM carbachol. The agonist treated experiments were performed in the presence or absence of 10mM lithium. Incorporation of [^{3}H]-inositol into the inositol phosphates was determined over a 1 hour period at 30^{o}C.

4.2.3). SAMPLE PREPARATION.

The acidified chloroform/methanol extraction procedure was applied to groups of 10 ganglia. Further purification of the inositol phosphates was achieved by loading the upper phase onto Accell QMA SEP-PAK cartridges (Waters Associates) in formate form. The free [^{3}H]-inositol was eluted with distilled water (2 x 2mls) and counted. Radiolabeled inositol phosphates were eluted with 3mls of 0.5M ammonium formate (see characterisation of

Accell QMA SEP-PAK columns (see 4.2.4.2). A fraction of this eluate (0.5 mls) was taken to be counted, and the remainder (2.5 mls) was freeze dried. The inositol phosphates were then redissolved in 0.5 mls of distilled water, filtered and then freeze dried. The samples were then prepared for h.p.l.c. analysis by redissolving in a solution containing nucleotides and phytate hydrosylate. Nucleotides could then be detected by continuous u.v. monitoring of the column eluate at 254nm. Thus, variations in retention times between runs or column ageing could be routinely measured. These nucleotides were not used as absolute markers for inositol phosphates. Inositol monophosphates were expected to be eluted at retention times intermediate to cyclic adenosine monophosphate (cAMP) and guanine monophosphate (GMP), inositol bisphosphates between adenosine diphosphate (ADP) and guanine diphosphate (GDP), and inositol trisphosphates between adenosine triphosphate (ATP) and guanine triphosphate (GTP), thus providing a means for standardising each run (Batty *et al*, 1989).

STOCK SOLUTIONS:

Nucleotide mixture: 0.2ml of 5mM cAMP
0.2ml of 5mM GMP
0.2ml of 5mM GDP
0.2ml of 5mM ADP
0.2ml of 5mM ATP
40μl of Phytate hydrosylate.

H.p.l.c. sample: 100μl of the nucleotide mixture added to the freeze dried sample of radiolabelled inositol phosphates.

Standards: 20μl of [^{3}H]-inositol phosphates (inositol mono-, bis-, tris-phosphate (0.37KBq/μl)).
0.5mls nucleotide mixture.
20μl phytate hydrosylate.

The h.p.l.c. sample (50μl) and the inositol phosphate standards (40μl) were injected and separated on a Partisil 10 SAX anion exchange column (see 4.2.5).

4.2.4). SEP-PAK CHROMATOGRAPHY.

4.2.4.1). COLUMN PREPARATION.

Accell QMA anion exchange SEP-PAK cartridges (Waters Associates) were converted to formate form by washing first with 10 mls of a solution of 2M ammonium formate in 0.1M formic acid (pH 4.75) followed by 20 mls of distilled water.

Sample loading and solution delivery were performed manually using disposable syringes (with Luer fitting); an approximate flow rate of 10-15 ml/min was typically maintained. The inositol phosphates could then be eluted

with increasing concentrations of ammonium formate.

4.2.4.2). CHARACTERISATION OF SEP-PAK CARTRIDGES.

4.2.4.2.1). GRADIENT ELUTION.

The inositol phosphate standard mixture was prepared as described and loaded (40μl) on to SEP-PAK cartridges converted into formate form . The inositol phosphates were eluted with 1ml of 10mM increments of ammonium formate/ formic acid (pH 4.75)/ 5mM disodium tetraborate (0 to 300mM). The recovery of inositol 1-phosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate per fraction was determined. Radioactivity was measured by liquid scintillation counting (Beckman LS6000 TA, 35-40% efficiency for ^{3}H).

4.2.4.2.2). BATCH ELUTION.

The data obtained from the gradient elution were used to determine suitable concentrations of ammonium formate for batchwise separation of inositol phosphates.

4.2.5). H.P.L.C. OF INOSITOL PHOSPHATES.

Separation of the [^{3}H]-inositol phosphate isomers was achieved with a Partisil (10μM) SAX anion exchange column (HPLC Technology Ltd) (25cm x 4.6cm) equipped with a guard column packed with the same resin.

The samples prepared from the [^{3}H]-inositol incorporation experiments were injected (50μl of the 100μl sample prepared from the pooling of 20 ganglia) using an auto injector (Shimadzu SIL-6B). After sample injection the isomers of inositol phosphates were separated by gradient elution from the column. The output volume was regulated by two separate pumps (Shimadzu LC-6A) feeding into a mixing chamber: pump A contained distilled water, and pump B contained 1.7M ammonium formate adjusted to pH 3.7 with phosphoric acid. The flow rate (1.2 ml/min) and the gradient were programmed in and controlled by a system controller (Shimadzu SCL-6B).

Time program.

TIME	%B.
0	0
10	0
20	60
30	70
35	100
40	100
42	0
60	0

The elution of guanine and adenine nucleotides was monitored using a u.v. spectrophotometric detector

(Shimadzu SPD-6AV) set at 254nm. Fractions were collected (Gilson fraction controller (0.5 min/fraction, from 10 to 45 min)) and counted by liquid scintillation using a Packard Tri-Carb 460 scintillation counter (background approximately 20 d.p.m., 35-40% efficiency for tritium). Identification of the inositol phosphates was achieved by co-elution with known [^{3}H]-inositol labelled standards (Inositol polyphosphate marker set obtained from Amersham (0.37KBq/μl), which contained [^{3}H]-inositol 1-phosphate, [^{3}H]-inositol 1,4-bisphosphate, [^{3}H]-inositol 1,4,5-trisphosphate).

4.2.6). SEPARATION OF INOSITOL 1,4,5-TRISPHOSPHATE, INOSITOL 1,3,4-TRISPHOSPHATE AND INOSITOL 1,3,4,5-TETRAKISPHOSPHATE.

4.2.6.1). SEPARATION OF STANDARDS.

From previous experiments it was shown that at least two peaks of [^{3}H]-inositol trisphosphates were resolved using high pressure liquid chromatography. The major peak did not co-elute with the inositol 1,4,5-trisphosphate standard, and was postulated to be the inositol 1,3,4-trisphosphate isomer.

Inositol 1,4,5-trisphosphate, inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate (obtained from NEN Research Products, 0.37 KBq/μl each) were resolved using the Partisil 10 SAX ion exchange

column.

Nucleotide mixture: 100μl of 5mM cyclic guanine monophosphate, guanine diphosphate, guanine trisphosphate, adenosine diphosphate, adenosine triphosphate, adenosine tetraphosphate.
10μl of phytate hydrosylate.

Inositol phosphate standards:

300μl of the nucleotide mixture.
10μl each of the inositol phosphate standards (0.37KBq/μl).

The inositol phosphate mixture (40μl) was injected onto the h.p.l.c. column and separated by gradient elution (1 ml/min., output volume controlled by two pumps: pump A contained distilled water and pump B contained 1.5M ammonium phosphate (pH 3.7 with phosphoric acid)).

Time program:

TIME	% B
0	5
30	45
40	100
45	100

47	0
52	0

The intermediate components between the pump and the fraction collector/u.v. detector have a volume of several millilitres. At a flow rate of 1 ml/min an appropriate compensation of 8 min was made when superimposing gradients on the elution profiles obtained. Fractions were collected (0.5 min/fraction) from 30 to 50 min, and the radioactivity in each fraction was determined by liquid scintillation counting.

4.2.6.2). SEPARATION OF [^{3}H]-INOSITOL LABELLED INOSITOL PHOSPHATES IN LOCUST GANGLIA

Groups of 24 ganglia were preincubated for 90 min in 5 mls of buffer A containing 4μCi of [^{3}H]-inositol. These were split into groups of 6 ganglia, and incubated for 60 min at 25^{o}C in 1 ml of buffer A containing 4μCi of [^{3}H]-inositol, 3mM calcium, 10mM lithium and 0.5mM carbachol. .

The incubation was terminated by placing the ganglia on dry ice. The upper phase of the acidified chloroform/methanol extraction mixture from the pooling of 24 ganglia was added to SEP-PAK columns. Radiolabelled inositol phosphates were eluted and prepared for h.p.l.c. analysis as described above (see sample preparation). The freeze dried samples were resuspended in 100μl of the nucleotide mixture, and 50μl was injected onto the

h.p.l.c. column. The elution protocol was as described for the separation of the standards. Fractions were collected (0.5min/fraction) from 8 min to 48 min, and the radioactivity in each fraction determined by liquid scintillation.

4.2.7). SEP-PAK SEPARATION OF INOSITOL TRIS- AND TETRAKIS PHOSPHATE.

Gradient elution of inositol 1,4,5-trisphosphate (IP_3) and inositol 1,3,4,5-tetrakisphosphate (IP_4) on SEP-PAK cartridges in formate form was performed.

4.2.7.1) RECOVERY OF IP_4 FROM SEP-PAK COLUMNS.

The inositol 1,3,4,5-tetrakisphosphate standard (10μl from a mixture containing 10μl of the standard (0.37 KBq/μl, NEN Research Products), 10μl phytate hydrosylate and 100μl of distilled water) was added to SEP-PAK columns in formate form, and eluted with 1 ml of ammonium formate (pH 4.75 with formic acid) in steps of 10mM (from 150mM to 350mM). Radioactivity per fraction was determined by liquid scintillation counting.

4.2.7.2) RESOLUTION OF IP_3 AND IP_4.

The inositol phosphate mixture (10μl of a mixture containing 10μl IP_4, 10μl IP_3, 10μl phytate hydrosylate, 100μl distilled water) was added to the SEP-PAK column and

eluted with ammonium formate (pH 4.75) at 10mM increments (1 ml). Fractions (1 ml) were collected, and the radioactivity determined by liquid scintillation counting.

4.2.8). TIME COURSE FOR THE PRODUCTION OF INOSITOL PHOSPHATES

Groups of 20 ganglia were dissected and preincubated for 90 min in 5 mls of buffer A containing 5μCi of [^{3}H]-inositol. After which, the ganglia were transferred into the incubation buffer containing 4μCi [^{3}H]-inositol, 3mM calcium, 10mM lithium in the presence or absence of 0.5mM carbachol. The ganglia were incubated at 25^{o}C in groups of 5 for 10, 20, 40, 60 and 120 min in control and carbachol treated conditions. Thus, 50 ganglia were used in each experiment, and four separate experiments were performed.

The radiolabelled inositol phosphates were recovered from the ganglia using the acidified chloroform/methanol extraction procedure in the presence of phytate hydrosylate. The upper water soluble phase was taken for analysis on SEP-PAK columns. Free [^{3}H]-inositol and the glycerophosphatidylinositol were eluted with 2x2 mls distilled water and 1x2 mls 5mM disodium tetraborate respectively. The radiolabelled inositol phosphates were eluted with 0.4M ammonium formate (pH 4.75).

The amount of [^{3}H]-inositol incorporated into the inositol phosphates per 5 ganglia was determined at each

incubation time point for control and carbachol treated experiments (n=4).

4.3). RESULTS.

The inositol phosphate standards were well resolved by gradient elution from SEP-PAK ACELL QMA cartridges (fig. 22). The inositol 1-phosphate, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate peaks occurred at 40mM, 140mM and 190mM ammonium formate respectively. Thus, by elution with 0.5M ammonium formate, total radiolabelled inositol phosphates can be determined. From the gradient elution profile, a batch elution protocol for each of the inositol polyphosphates was determined. Inositol mono-, bis- and trisphosphates may be resolved by sequential batch elution with 80mM, 150mM and 240mM ammonium formate respectively. A mixture of inositol polyphosphate standards was separated by batch elution as shown in figure 23 (% recovery of inositol mono-, bis-, tris phosphate and total inositol phosphates were 80.8% +/- 4.8 (S.E.M.), 103% +/- 10.9 (S.E.M.), 82.7% +/- 6.7 (S.E.M.) and 89.1% +/- 3.5 (S.E.M.), n=4). Using this protocol upto 90% of the total inositol phosphates could be recovered.

The mixture of inositol phosphate standards was also separated using h.p.l.c. (fig. 24a). However, using this method the recoveries of the inositol trisphospates were reduced, especially that of inositol trisphosphate (%

recovery of inositol (1)-phosphate, inositol (1,4)-bis phosphate, inositol (1,4,5)-trisphosphate and total inositol phosphates were 82.1% +/- 6.6 (S.E.M.), 68.0 +/- 1.5 (S.E.M.), 26.1% +/- 4.8 (S.E.M.) and 58.8 +/- 1.0 (S.E.M.) respectively, n=4). Stimulation of locust ganglia with carbachol in the presence or the absence of 10mM lithium resulted in the incorporation of [^{3}H]-inositol into at least two isomers of inositol trisphosphate (fig. 24b). Highly consistent retention times of the nucleotides between different runs (cGMP = 17.4-17.8 min, ADP = 22.7-22.8 min, GDP = 25.4-25.5 min, ATP = 30.9-31.0 min, GTP = 36.9-37.2 min, for 4 separate runs), enabled identification of one of the inositol tris phosphate isomers to be inositol 1,4,5-trisphosphate (compare fig 24a and b). Separation of the higher inositol polyphosphoinositides (inositol 1,3,4-trisphosphate 36.5min), inositol 1,4,5-trisphosphate (38.5min) and inositol 1,3,4,5-tetrakisphosphate (42.5 min)) was achieved using a modified gradient elution with ammonium phosphate (fig. 25a). The incorporation of [^{3}H]-inositol into the inositol phosphates was very much reduced in these experiments, and only one radiolabelled inositol tris phosphate peak was resolved (fig. 25b, retention time = 36.5 min), which corresponded with the retention time of the inositol 1,3,4-trisphosphate standard (fig. 25a). As the retention times of the nucleotides were very consistent between the 4 succesive runs (cGMP = 7.6-10.1 min, ADP = 18.4-18.6 min, GDP = 22.6-22.8 min, ATP = 34.5-34.6 min, GTP = 40.3-40.5 min, adenosine tetraphosphate =

45.4-45.5 min.), this inositol trisphosphate peak was postulated to be inositol 1,3,4-trisphosphate.

Elution of inositol 1,3,4,5-tetrakisphosphate from SEP-PAK cartridges (fig. 26) showed that the IP_4 peak occurred at the 290mM fraction and 92% of the radioactivity added was recovered (compare large losses in the h.p.l.c. column). The separation of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate by gradient elution is shown in figure 27. The inositol trisphosphate peak, at 200mM, and the inositol tetrakisphosphate peak at 260mM ammonium formate were resolved. However, there was too much overlap in the tails of the IP_3 and the IP_4 peaks to allow for the development of a batch elution protocol. Substantial amounts of IP_3 and IP_4 were eluted together between 200 and 260mM ammonium formate.

The time course for the incorporation of [^{3}H]-inositol into the inositol phosphates approached equilibrium after approximately 60 min in both control and carbachol treated ganglia (fig. 28). The incorporation of the radiolabel became more variable at 60 and 120 min incubation times. In the presence of carbachol there were insignificant increases in labelling of inositol phosphates at early time points (10-60 min) (fig, 29, control=100).

4.4). DISCUSSION.

Locust ganglia were stimulated with carbachol and the water soluble [^{3}H]-inositol labelled products analysed using high pressure liquid chromatography. Metabolism of the various inositol phosphate isomers, such as inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate (Irvine *et al.*, 1985), can only be determined if these isomers are resolved. Results presented here show that [^{3}H]-inositol was incorporated into at least two isomers of inositol trisphosphates. The method used to separate the inositol phosphates was developed from that routinely used in investigating mammalian systems (Batty *et al.*, 1989; Safrany *et al.*, 1991; Wregget and Irvine, 1987).

In the first series of studies (figs. 24a anb b), the major inositol phosphate peak did not co-elute with the inositol 1,4,5-trisphosphate standard. Experiments were performed in which the two isomers of inositol trisphosphates were resolved using standards (fig. 25a). The incorporation of [^{3}H]-inositol into the inositol phosphates in these experiments was lower than in the previous experiments (compare recovery in figs. 24b and 25b), and only one inositol trisphosphate peak was observed (fig. 25b), which was identified to be the inositol 1,3,4-trisphosphate isomer.

The rate of appearance and disappearance of the two isomers of insitol phosphates has been followed in the rat parotid gland (Irvine *et al.*, 1985). Inositol 1,4,5-trisphosphate was more rapidly metabolised than inositol

1,3,4-trisphosphate. This is consistent with the proposed second messenger role of inositol 1,4,5-trisphosphate. The levels of inositol 1,3,4-trisphosphate rose for several minutes after the disappearance of inositol 1,4,5-trisphosphate. The kinetics of the 1,3,4-isomer corresponded with the kinetics of the inositol trisphosphate in studies by Downes and Wusteman (1983). These results were consistent with the high levels of inositol 1,3,4-trisphosphate found in the locust ganglia after the 1 hour incubation period (fig. 24b), during which the levels of inositol 1,4,5-trisphosphate would have declined. Kinetic studies in SH-SY5Y human blastoma cells (Safrany *et al.*, 1991) showed that inositol 1,4,5-trisphosphate was metabolised to inositol 1,3,4-trisphosphate via inositol 1,3,4,5-tetrakisphosphate. There was no conclusive demonstration of the presence of inositol 1,3,4,5-tetrakisphosphate in the locust ganglia, this being the probable precursor for the production of inositol 1,3,4-trisphosphate.

One of the major reasons for the absence of inositol 1,3,4,5-tetrakisphosphate in our preparation may have been the large losses of the higher inositol phosphates incurred in the 10 SAX h.p.l.c. column (figs. 24a and 25a). Whereas, up to 90% of the inositol phosphates were recovered from SEP-PAK columns (figs. 23, 26 and 27), only 66% of the inositol 1,4-bisphosphate (fig. 24a), 21% of inositol 1,3,4-trisphosphate, 16% of inositol 1,4,5-trisphosphate and 2% of the inositol 1,3,4,5-

tetrakisphosphate (fig. 25a) were recovered from the h.p.l.c. column.

Another possibility was that all the inositol 1,3,4,5-tetrakisphosphate was converted to inositol 1,3,4-trisphosphate during the 1 hour incubation period. A time course for the production of inositol phosphates was performed in order to investigate this possibility. However, the loss of inositol phosphates in the h.p.l.c. column and the reduced incorporation of inositol phosphates made identification of the isomers impracticable.

The results presented here, using SEP-PAK columns to separate the total inositol phosphates (fig 28), showed that phosphatidylinositol turnover occurred even in control conditions. The labelling of inositol phosphates approached a plateau after 60 min. In the presence of carbachol stimulation occurred between 10 and at60 min, after which the labelling was approximately equivalent to control values. This relatively rapid approach to equilibrium was similar to that observed in the [^{32}P]-Pi labelling of the polyphosphoinositides (chap 4). This suggested that the polyphosphoinositides derived from the activities of the 4- and 5-kinases were metabolised to produce the inositol phosphates. In the stimulated conditions the labelling of the inositol phosphates was not above that in control conditions when approaching the plateau, suggesting that the supply of

polyphosphoinositides became rate limiting for phospholipase C activity. Experiments performed to investigate [^{3}H]-inositol (chap.2) and [^{32}P]-Pi (chap.4) labelling of the polyphosphoinositide pool have showed that this may be a possibility. There was a depletion of [^{32}P]-Pi labelled polyphosphoinositides when stimulated with carbachol, and very little labelling was observed with [^{3}H]-inositol. Between 10 and 60 min carbachol increased the amount of radiolabelled inositol phosphates suggesting the activation of phospholipase C (fig. 29).

Inositol mono-, bis-, and trisphosphates were well resolved using SEP-PAK columns (figs. 22 and 23). Gradient elution of inositol tris- and tetrakisphosphates showed a substantial overlap between 200 and 260mM ammonium formate (fig. 27). Thus a batch elution protocol could not be developed to determine the levels of radiolabelled inositol 1,3,4,5-tetrakisphosphate.

The presence of the two isomers of inositol trisphosphate was demonstrated in these studies, Elucidation of their eventual conversion to inositol will require further studies. Development of inositol 1,4,5-trisphosphate 5-phosphatase and 3-kinase inhibitors (Wojcikiewicz *et al.*, 1990; Safrany *et al.*, 1991) will greatly facilitate studies aimed at understanding the interconversions involved. However, the inhibitors of 5-phosphatase and 3-kinase are analogues of inositol 1,4,5-trisphosphate (DL-inositol 1,4,5-trisphosphorothioate and

DL-inositol 1,4-bisphosphate 5-phosphorothioate), and hence require preparation of permeabilised cells to study their effects. Thus, demonstration of the inositol lipid pathway will be required in an insect neuronal cell line to enable studies to be made using these inhibitors. Alternatively, the enzymes may be isolated in cell free preparations and characterised by examining the metabolic breakdown of radiolabelled substrates (Safrany *et al.*, 1991).

Figure 22.

Gradient elution of [^{3}H]-inositol phosphates from SEP-PAK cartridges.

Radiolabelled inositol phosphate standards were loaded onto SEP-PAK cartridges (40μl from a stock containing 20μl of a mixture of inositol phosphates (0.37 KBq/μl) : [^{3}H]-inositol 1-phosphate (IP), [^{3}H]-inositol 1,4-bisphosphate (IP2) and [^{3}H]-inositol 1,4,5-trisphosphate (IP3), 20μl phytate hydrosylate and 0.5 ml nucleotide mixture. The inositol phosphates were separated by gradient elution with 1 ml 10mM increments of ammonium formate (pH 4.75) from 10 to 300mM. Inositol mono-, bis- and tris phosphate peaks occurred at 40mM (14 393 d.p.m.), 130mM (15 571 d.p.m.) and 190mM (16 426 d.p.m.) respectively.

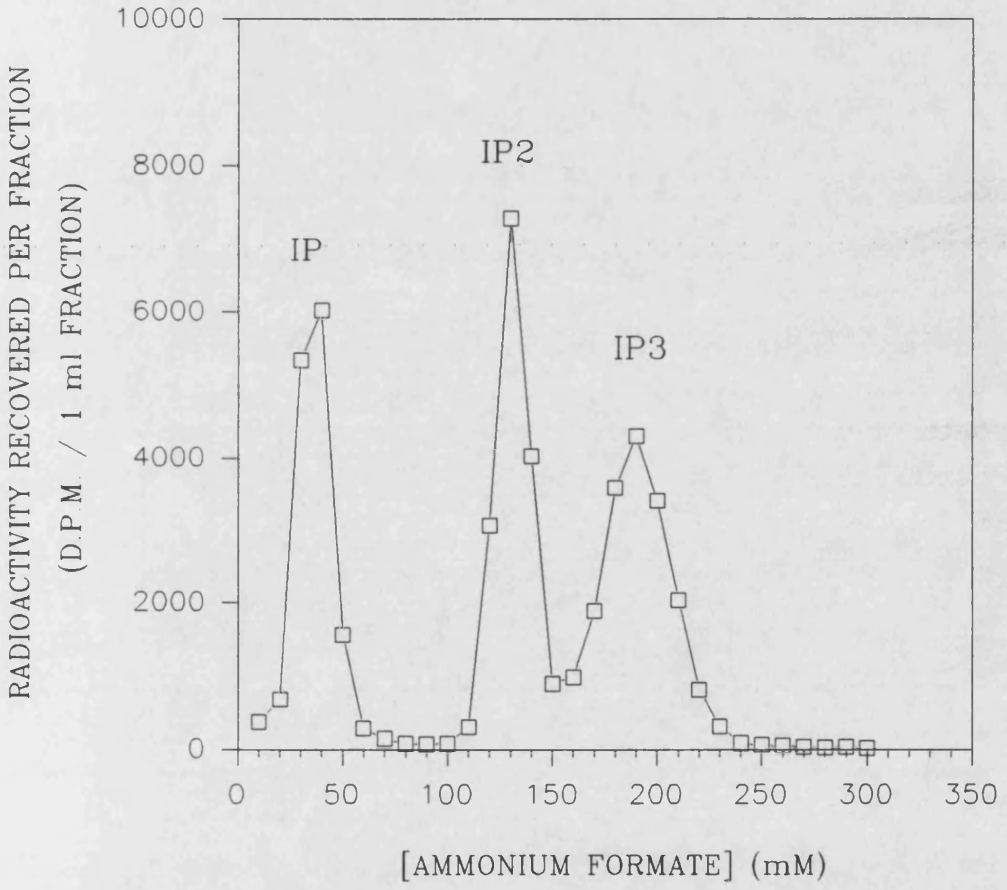

RADIOACTIVITY RECOVERED PER FRACTION
(D.P.M. / 1 ml FRACTION)
10000
8000
6000
4000
2000
0
IP
IP2
IP3
0
50
100
150
200
250
300
350
[AMMONIUM FORMATE] (mM)

Figure 23.

Batch elution of [^{3}H]-inositol phosphates from SEP-PAK cartriges.

A known amount of a mixture of inositol phosphates (approximately 40 000 d.p.m. (0.37 KBq/μl) of [^{3}H]-inositol 1-phosphate (IP), [^{3}H]-inositol 1,4-bisphosphate (IP2) and [^{3}H]-inositol 1,4,5-trisphosphate (IP3) were loaded onto SEP-PAK cartridges. Inositol mono-, bis- and trisphosphates were eluted by sequential batch elution with 4 x 1 ml fractions of 80mM (fractions 1-4), 150mM (fractions 5-8) and 240mM ammonium formate (pH 4.75) respectively. The elution profiles from two SEP-PAK columns are shown (% recovery for IP = 80.8% +/- 4.8 (S.E.M.), IP2 = 103.7 +/- 10.9 (S.E.M.), IP3 = 82.7 +/- 6.7 (S.E.M.) and total inositol phosphates = 89.1 +/- 3.51 (S.E.M.), n=4).

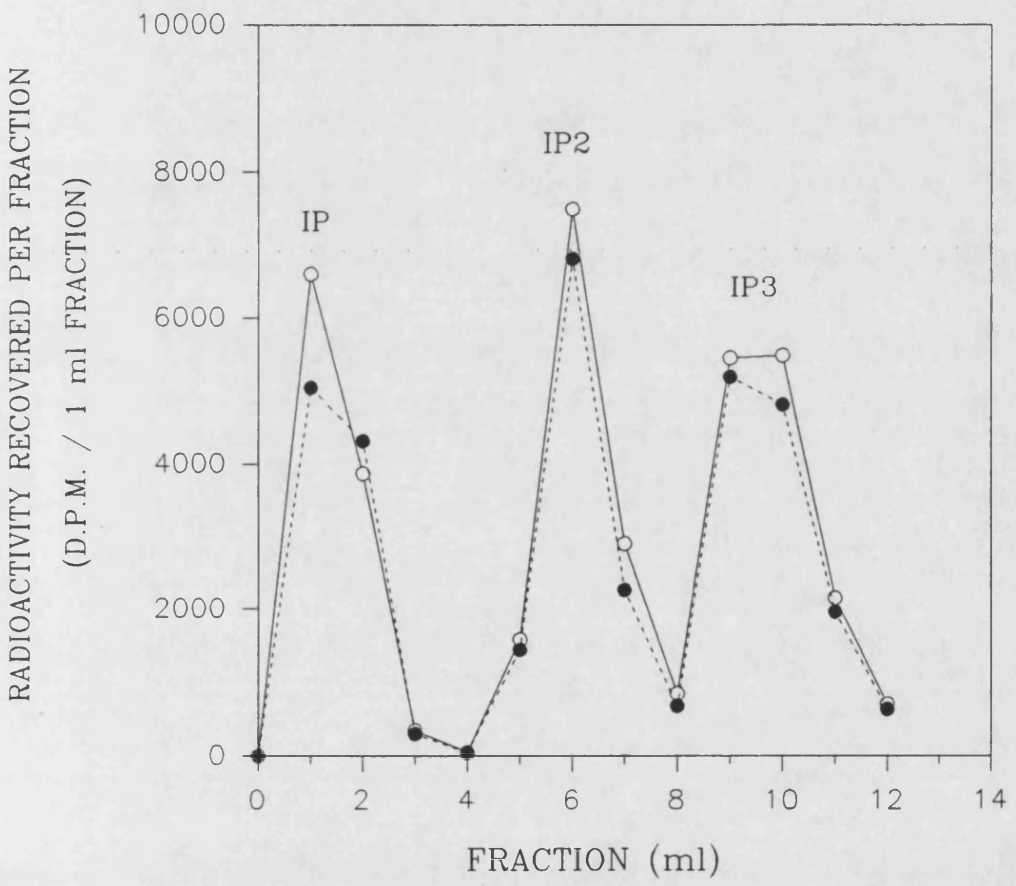
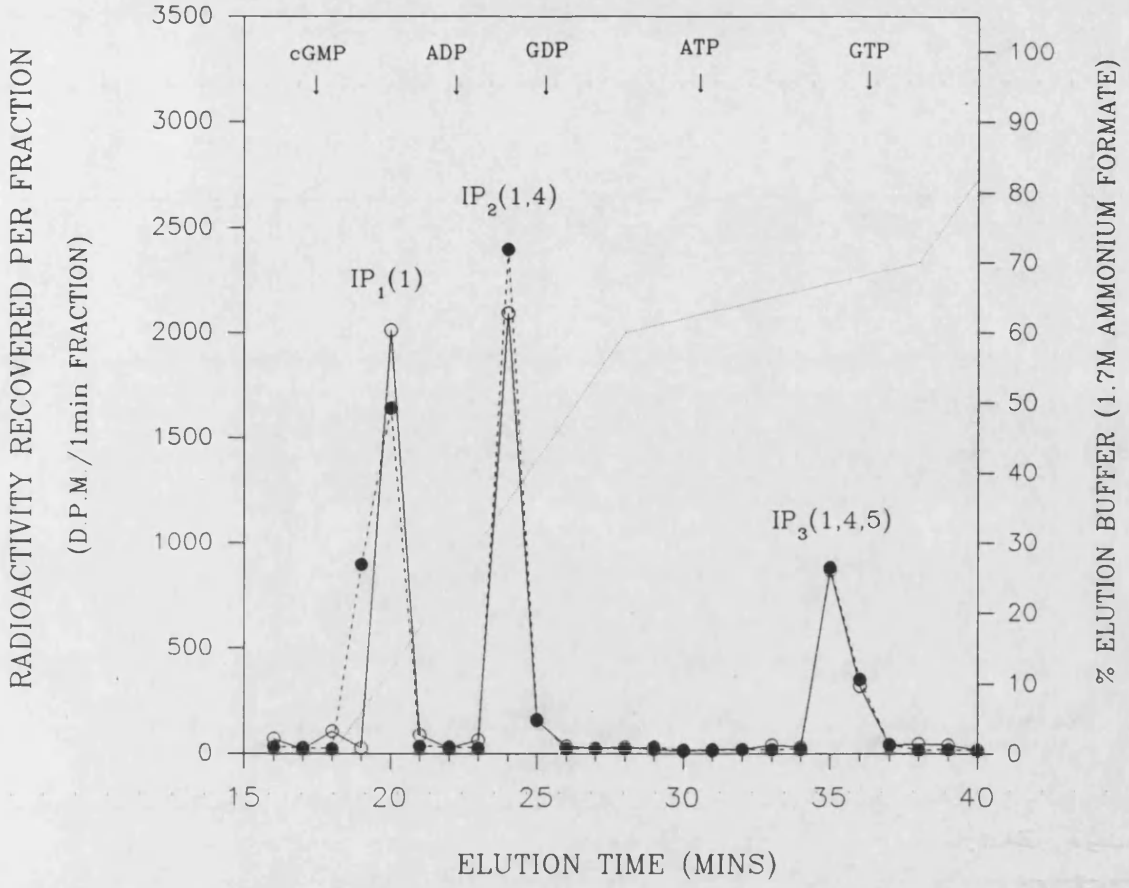

RADIOACTIVITY RECOVERED PER FRACTION
(D.P.M. / 1 ml FRACTION)
10000
8000
6000
4000
2000
0
IP
IP2
IP3
0
2
4
6
8
10
12
14
FRACTION (ml)

Figure 24.

Separation of inositol phosphates using high pressure liquid chromatography.

Radiolabelled inositol phosphate standards ([^{3}H]-inositol 1-phosphate ($IP_1(1)$, [^{3}H]-inositol 1,4-bisphosphate ($IP_2(1,4)$, and [^{3}H]-inositol 1,4,5-trisphosphate ($IP_3(1,4,5)$, (0.37KBq/μl)) spiked with nucleotides (cyclic guanine monophosphate (cGMP), adenosine diphosphate (ADP), guanine diphosphate (GDP), adenosine trisphosphate (ATP) and guanine trisphosphate (GTP)) were loaded onto a Partisil 10 SAX column (40μl of stock). Separation was achieved by gradient elution with ammonium formate (100% elution buffer= 1.7M (pH 3.7), flow rate = 1.2 ml/min) (fig. 24a). Radioactivity collected from 1 min (1.2ml) fractions were determined. The elution profiles for two successive separations are shown. Retention times for $IP_1(1)$, $IP_2(1,4)$ and $IP_3(1,4,5)$ were 20 min, 24 min and 35 min respectively (% recovery for $IP_1(1)$ = 82.1% +/- 6.6 (S.E.M.), $IP_2(1,4)$ = 68.0% +/- 1.5 (S.E.M.), $IP_3(1,4,5)$ = 26.1% +/- 4.8 (S.E.M.) and total inositol phosphates = 58.8% +/- 1.0 (S.E.M.), n=4). The samples prepared from the [^{3}H]-inositol incorporation experiments: ganglia treated with 0.5mM carbachol in the presence (inverted triangles) or the absence of 10mM lithium (filled circles), were injected (50μl of the 100μl sample prepared from the pooling of 20 ganglia) onto the h.p.l.c. column, and separated using the same protocol as for the standards (fig 24b). Radioactivity per 0.5 min (0.6ml) fraction was determined. Inositol 1,4,5-trisphosphate was identified by co-elution with the standard. Sucessive runs could be directly compared because of very consistent retention times obtained from the separation of the nucleotides (cGMP = 17.6 min, ADP = 22.7 min, GDP = 25.4 min, ATP = 30.9 min and GTP = 37 min.).


RADIOACTIVITY RECOVERED PER FRACTION
(D.P.M./1min FRACTION)
% ELUTION BUFFER (1.7M AMMONIUM FORMATE)
cGMP
ADP
GDP
ATP
GTP
IP1(1)
IP2(1,4)
IP3(1,4,5)
ELUTION TIME (MINS)

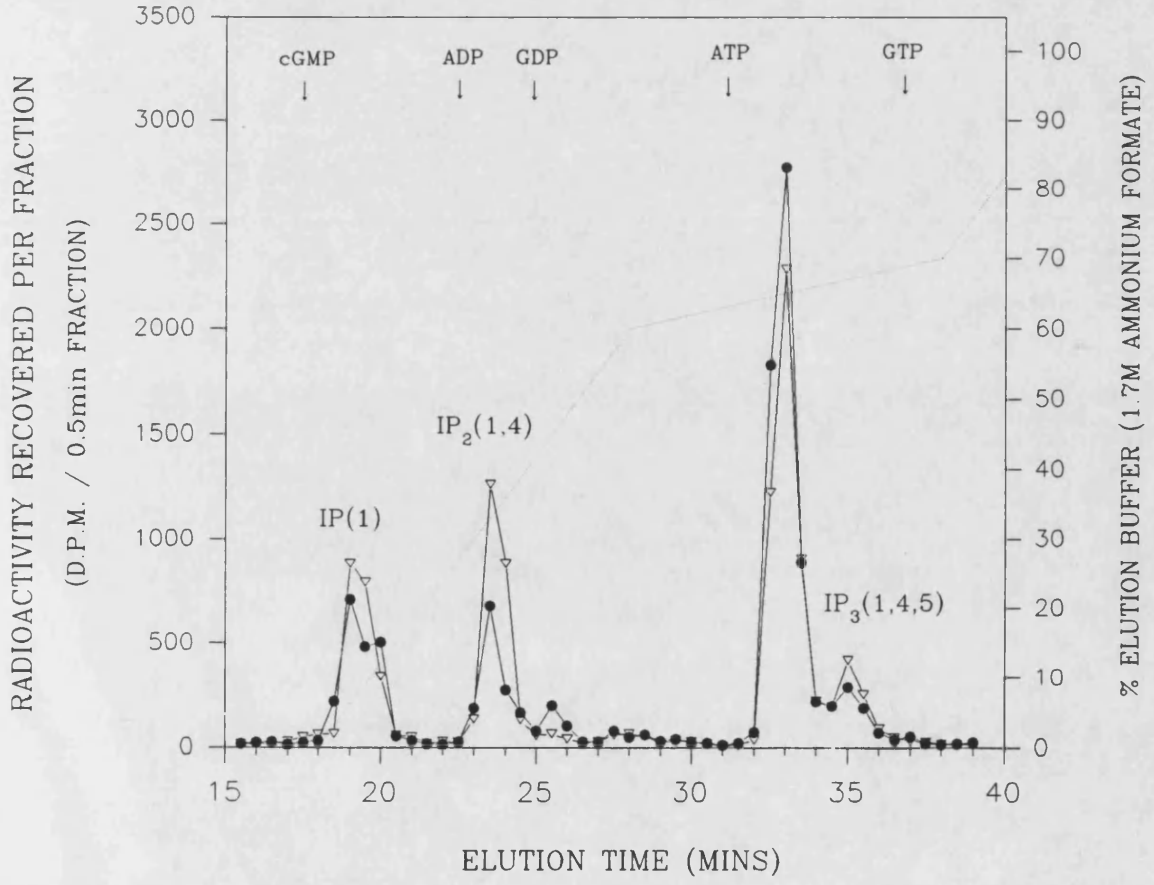
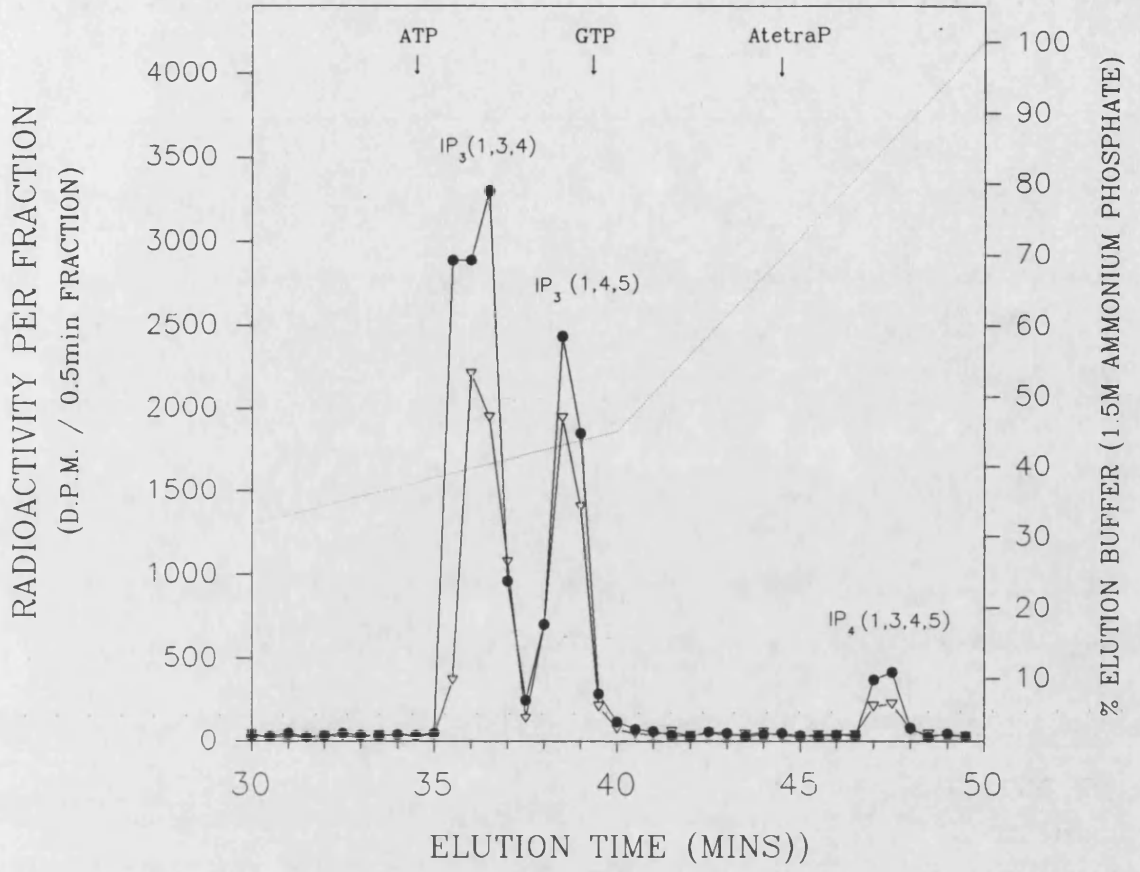

RADIOACTIVITY RECOVERED PER FRACTION
(D.P.M. / 0.5min FRACTION)
% ELUTION BUFFER (1.7M AMMONIUM FORMATE)
cGMP
ADP
GDP
ATP
GTP
IP(1)
IP2(1,4)
IP3(1,4,5)
ELUTION TIME (MINS)

Figure 25.

Separation of inositol trisphosphate isomers using h.p.l.c.

Inositol 1,4,5-trisphosphate ($IP_3(1,4,5)$, inositol 1,3,4-trisphosophate ($IP_3(1,3,4)$) and inositol 1,3,4,5 tetrakisphosphate (IP_4) (0.37 KBq/μl, approximately 80 000 d.p.m. added) spiked with nucleotides (cyclic monophosphate (cGMP), guanine diphosphate (GDP), guanine triphosphate (GTP), adenosine diphosphate (ADP), adenosine triphosphate (ATP) and adenosine tetraphosphate (AtetraP)) were injected onto a 10SAX column (40μl from stock). Separation was achieved by gradient elution with ammonium phosphate (100% elution buffer = 1.5M ammonium phosphate (pH 3.7), flow rate = 1 ml/min). Fractions were collected from 30-50 min (0.5 min/fraction (0.5ml/fraction)), and the radioactivity per fraction determined. The elution profiles for successive runs are shown (fig. 25a). Retention times for $IP_3(1,3,4)$, $IP_3(1,4,5)$ and $IP_4(1,3,4,5)$ were 36.5min, 38.5min and 47.5min respectively (% recovery for $IP_3(1,3,4)$ = 21.2% +/- 1.6 (S.E.M.), IP3(1,4,5) = 16.0% +/- 1.0 (S.E.M.) and $IP_4(1,3,4,5)$ = 2.1 +/- 0.2 (S.E.M.), n=6). Samples prepared from [^{3}H]-inositol incorporation experiments: ganglia treated with 10mM lithium and 0.5mM carbachol, were separated after injection onto the h.p.l.c. column (50μl of the 100μl sample prepared from the pooling of 24 ganglia). Fractions were collected from 8min to 48min (0.5min/fraction, 1ml/min). The elution profiles from two separate experiments are shown (from 30-50 min). Inositol 1,3,4-trisphosphate was identified by co-elution with a known standard (retention time = 36min). Retention times for cGMP = 9.3min, ADP = 18.6min, GDP = 22.8min, ATP = 34.6min, GTP = 40.4min and AtetraP = 45.5min.


ATP
GTP
AtetraP
IP3(1,3,4)
IP3 (1,4,5)
IP4 (1,3,4,5)
RADIOACTIVITY PER FRACTION
(D.P.M. / 0.5min FRACTION)
% ELUTION BUFFER (1.5M AMMONIUM PHOSPHATE)
ELUTION TIME (MINS))

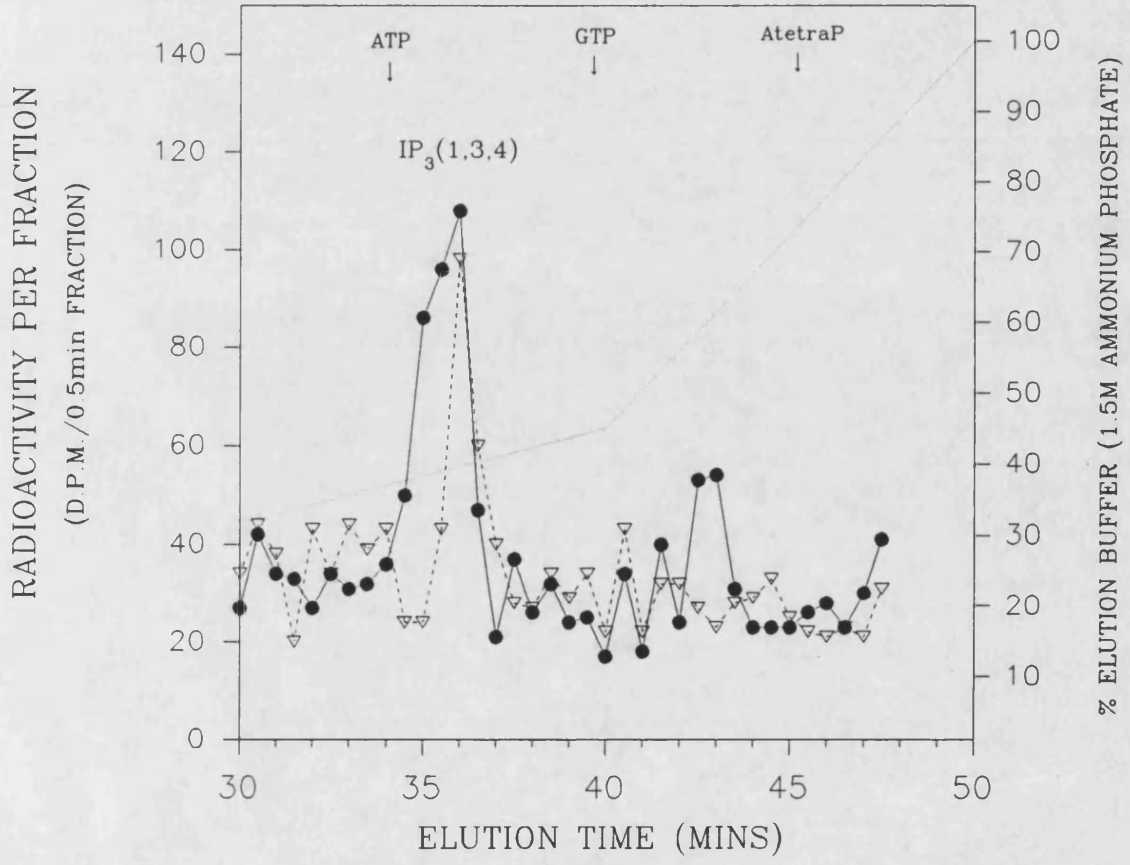

ATP
GTP
AtetraP
IP3(1,3,4)
RADIOACTIVITY PER FRACTION
(D.P.M./0.5min FRACTION)
% ELUTION BUFFER (1.5M AMMONIUM PHOSPHATE)
ELUTION TIME (MINS)

Figure 26.

Gradient elution of inositol tetrakisphosphate from SEP-PAK cartridges.

The inositol 1,3,4,5-tetrakisphosphate standard (25 776 d.p.m., 0.37KBq/μl) was loaded onto a SEP-PAK column in formate form. The elution profile with increasing concentrations of ammonium formate (pH 4.75), from 150mM to 350mM in steps of 10mM per fraction, is shown. Radioactivity per 1 ml fraction collected was determined (92% of the inositol phosphate added was recovered). The inositol tetrakisphosphate peak occurred at 290mM ammonium formate.

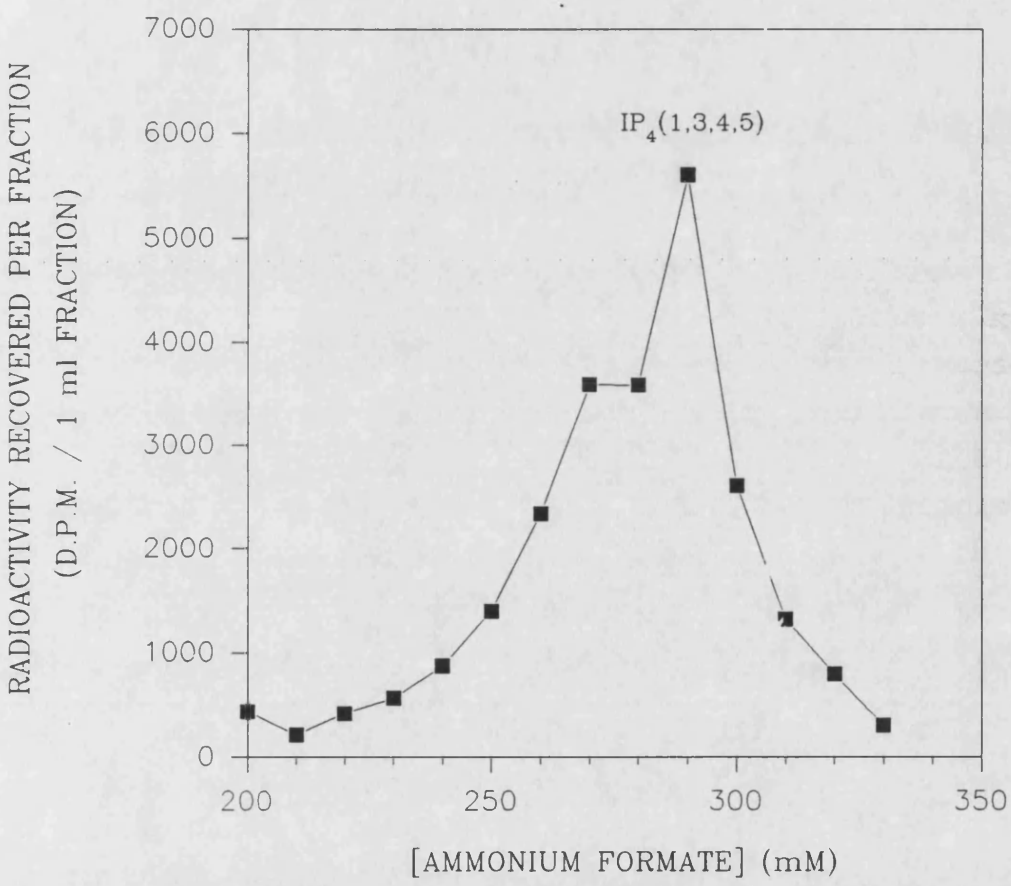

RADIOACTIVITY RECOVERED PER FRACTION
(D.P.M. / 1 ml FRACTION)
7000
6000
5000
4000
3000
2000
1000
0
IP4(1,3,4,5)
200
250
300
350
[AMMONIUM FORMATE] (mM)

Figure 27.

Separation of inositol tris- and tetrakis phosphate using gradient elution from SEP-PAK columns.

A mixture of inositol 1,4,5-trisphosphate ($IP_3(1,4,5)$) and inositol 1,3,4,5-tetrakisphosphate ($IP_4(1,3,4,5)$) (34 604 d.p.m. added, 0.37KBq/μl) were loaded onto a SEP-PAK cartridge, and eluted with increasing concentrations of ammonium formate (pH 4.75) (from 150mM to 350mM in steps of 10mM). Radioactivity per 1 ml fraction collected was determined (86.5% of the radioactivity added was recovered). The inositol tris- and tetrakisphosphate peaks occurred at 200 and 260mM ammonium formate respectively.

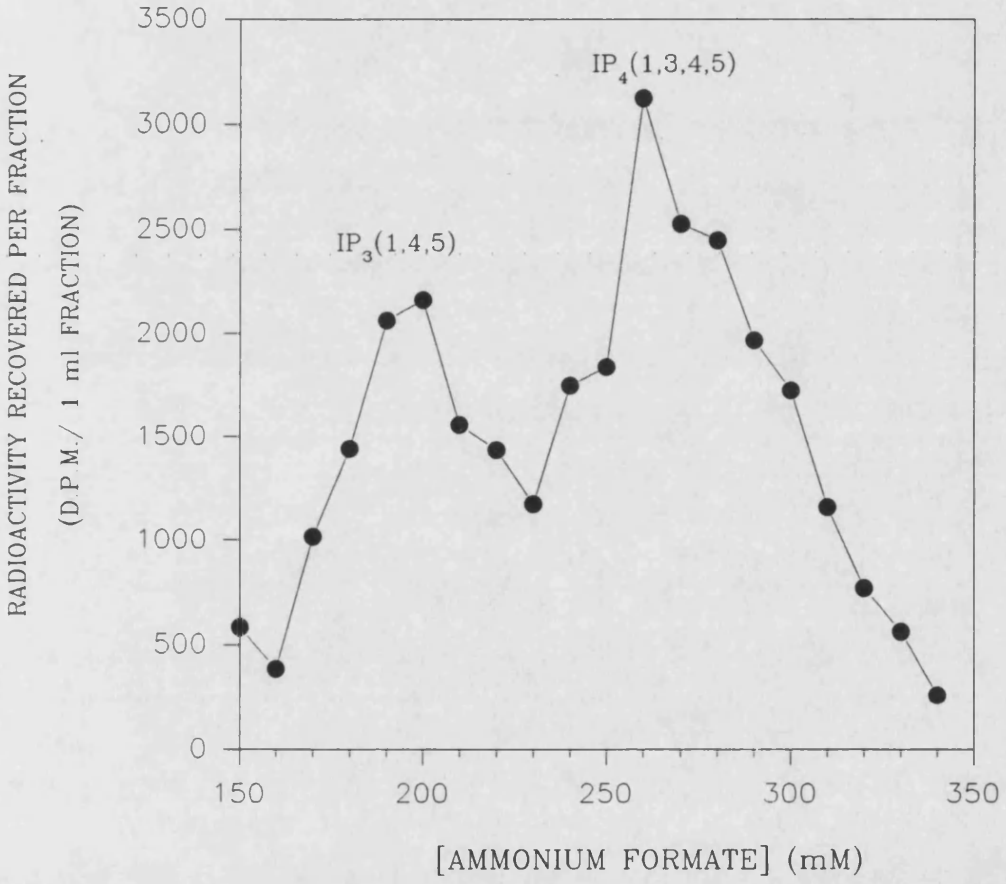

RADIOACTIVITY RECOVERED PER FRACTION
(D P M./ 1 ml FRACTION)
3500
3000
2500
2000
1500
1000
500
0
IP4(1,3,4,5)
IP3(1,4,5)
150
200
250
300
350
[AMMONIUM FORMATE] (mM)

Figure 28.

Time course for the production of inositol phosphates.

After a preincubation period of 90 min, groups of 5 ganglia were incubated in buffer A containing [^{3}H]-inositol, 3mM calcium and 10mM lithium in the presence (filled circles) or absence (open circles) of 0.5mM carbachol. The ganglia were incubated for 10, 20, 40, 60 and 120 min. On extraction of the water soluble metabolites (upper phase of the chloroform/methanol extraction mixture), the total inositol phosphates were separated by SEP-PAK chromatography (free inositol was eluted with distilled water and the inositol phosphates were eluted with 0.4M ammonium formate). The results obtained from 2 separate experiments were pooled, and the mean incorporation of [^{3}H]-inositol into the inositol phosphates (+/- S.E.M.) in control and carbachol treated ganglia determined.

Figure 29.

Enhancement of PI turnover in the time course experiments.

The experiments were performed as described in fig. 28. The results were pooled from 4 separate experiments, and the incorporation of [^{3}H]-inositol into the inositol phosphates determined. For each experiment, the percentage enhancement at each incubation time point was determined (treated/control multiplied by 100), and the mean percentage enhancement (+/- S.E.M.) for each time point from 4 separate experiments was deduced (filled circles). The control is shown as a dotted line (100).

FIG. 28

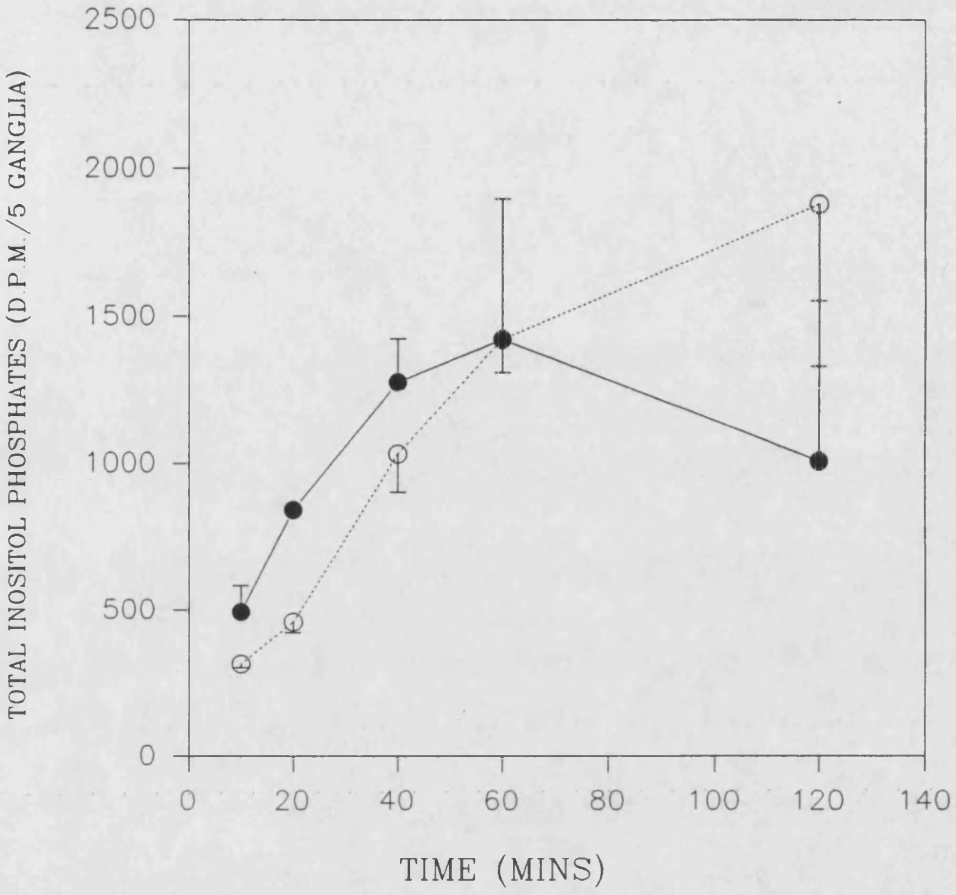
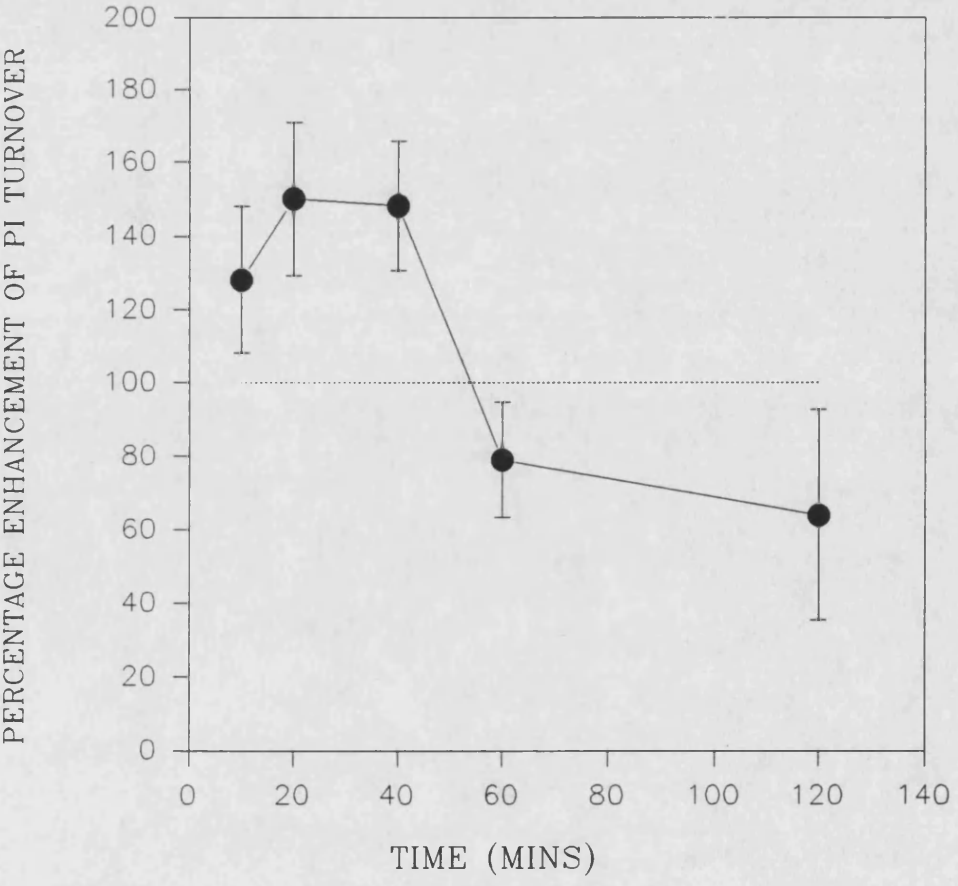

TOTAL INOSITOL PHOSPHATES (D.P.M./5 GANGLIA)
2500
2000
1500
1000
500
0
0
20
40
60
80
100
120
140
TIME (MINS)

FIG. 29


PERCENTAGE ENHANCEMENT OF PI TURNOVER
200
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
TIME (MINS)

5). [^{32}P]-INORGANIC PHOSPHATE INCORPORATION INTO THE PHOSPHOLIPIDS.

5.1). INTRODUCTION

The principal substrate in the intact cell for the membrane bound phospholipase C is PI(4,5)P_2 (Drummond *et al.*, 1984). This has some very interesting consequences for a receptor transducing mechanism. First of all, the polyphosphoinositides represent a very small pool that must be constantly replenished (Berridge, 1983), to ensure adequate sustrate supply under receptor activation of the phospholipase C. Subtle alterations of the kinases or the phophomonoesterases will shift the position of equilibrium that exists between the polyphosphoinositides, resulting in the change of the PIP_2 pool. Any reduction in the levels of PIP_2 will reduce the effectiveness of the receptor mechanism (Downes *et al.*, 1983) and could account for the phenomenon of desensitisation.

Since the formation of polyphosphoinositides requires ATP, the agonist dependent breakdown of PI is sensitive to metabolic inhibitors (Downes & Wustemann, 1983). As the metabolism of PIP_2 is linked to the onset of calcium signal (Berridge and Irvine, 1989), it appears that both entry and extrusion of calcium from the cytosol require energy. The consequence of this would be to prevent the cell from overloading itself with calcium when low levels of ATP prevent calcium extrusion (Berridge, 1983).

In mammalian cells, the kinases and the phosphomonoesterases play key roles in the regulation of substrate supply. The size of the PIP_2 pool will determine the characteristics of the IP_3/Ca^{2+} response on stimulation of phospholipase C by the G/proteins via receptor activation. Phospholipase C may hydrolyse other phosphoinositides to produce DG without IP_3. Thus the relative sizes of the phosphoinositide pools could determine which of the signalling pathways predominate in a cellular response. Hydrolysis of PIP_2 releases inositol -1,4,5 trisphosphate (IP_3) into the cytosol, whilst leaving diacylglycerol within the plane of the membrane. Together these two limbs of the bifurcating signalling pathway provide an exceptionally versatile signalling mechanism (Berridge, 1987). Short term cellular responses such as contraction, secretion and metabolism are controlled by the IP_3/Ca^{2+} limb of the pathway. The other limb controlled through DG, stimulates protein kinase C which may play a role in long term events such as growth and differentiation (Nishizuka, 1986). Hydrolysis of PI with phospholipase C would provide a mechanism by which 1,2sn-diacylglycerol is produced without simultaneously producing IP_3. Imai and Gershengorn (1986) have shown that the hydrolysis of PIP_2 is transient, but there is direct and persistent hydrolysis of PI in the rat pituitary cells stimulated with thyrotrophin releasing hormone.

To accommodate enormous changes in the demand for

polyphosphoinositides imposed by the stimulation of phospholipase C, two types of mechanisms may operate to give net synthesis of PIP and PIP_2 during receptor activation. The first is the rise in the rate of one or both of the kinases (PI 4-kinase, PI(4)P 5-kinase). The second is the drop in the activity of the phosphomonoesterases. This second mechanism assumes that substrate cycling is occurring at basal levels, at which the activities of the 4-kinase, 5-kinase and the phosphomonoesterases are being expressed. Studies using human erythrocytes to determine [^{32}P]-Pi flux into the monoester phosphates of the polyphosphoinositides most clearly demonstrate substrate cycling (Muller *et al.*, 1986). As these cells lack a hormone linked phospholipase C system, the turnover observed can be reasonably attributed to the phosphomonoesterases.

An increase in the net synthesis of PIP_2 can occur during stimulation. The term 'net synthesis' refers to the activity of PIP_2 kinase minus phosphomonoesterase. Cultured hepatocytes stimulated with vasopressin demonstrate this phenomenon (Thomas *et al.*,1983). After attaining isotopic equilibrium of the phosphoinositides with added [^{32}P]-Pi, the addition of vasopressin causes a transient decrease in the levels of radiolabelled PIP_2, reaching a minimum after 30 sec. If there was no increase in net synthesis, the levels of [^{32}P]-PIP_2 should reach a new steady state, due to the activity of the phospholipase C. A mechanism by which this may occur is through the

activation of PI(4)P -5kinase. Imai and Gershengorn (1986) have observed a hormone induced increase in the [^{32}P}-specific radioactivity of PIP_2, on exposure of GH_3 cells to TRH.

The mechanism by which the kinases are activated is uncertain. PI(4)P 5-kinase shows product inhibition (Downes and MacPhee, 1990), thus the removal of PIP_2 could raise the kinase activity. Interactions with protein kinase C may occur as phorbol esters markedly increase the levels of PIP and PIP_2 (Taylor *et al.*, 1984). It has also been postulated that calcium may stimulate synthesis of polyphosphoinositides (Whipps *et al.*, 1987), but the mechanism of this action was unresolved.

Substrate supply for the phospholipase C is determined by the activities of the kinases, the regulation of which will have important consequences for the production of second messengers. It seems that substrate supply is indeed under active control during receptor activation, but how this is achieved is unclear. Kinetic analysis performed on a system in which there is a rapid equilibration of the gamma labelled phosphate of ATP with the tracer, and the design of inhibitors of the kinases will help to elucidate the mechanism.

In view of the importance of regulation of polyphosphoinositide supply realised in mammalian cells, we have performed preliminary experiments to investigate

the metabolism of the polyphosphoinositides in the insect CNS. Previous studies on the locust supraoesophageal ganglia (Duggan, 1987) could not demonstrate any effects of an acetylcholine receptor agonist, carbachol, on the labelling of the polyphosphoinositides with [^{32}P]-Pi. Thus, one of the major aims was to show an effect with carbachol, in the locust (*Shistocerca gregaria*) supraoesophageal ganglia, by using a [^{32}P]-Pi chase-in protocol over a 150 min time course.

5.2). METHODS.

5.2.1) PREINCUBATION.

Adult female locusts (*Schistocerca gregaria*) in the fifth instar were used in all experiments. The insects were anaesthetised by cooling and gassing with carbon dioxide and then decapitated. The supraoesophageal ganglion was exposed by shaving off the cuticle from the front of the head. To allow the tissue to recover from the trauma of dissection, the ganglia were preincubated for 90 min, at room temperature in buffer containing 208mM sodium chloride, 3.1mM potassium chloride, 5.4mM calcium chloride, 2mM sodium hydrogencarbonate, 10mM HEPES, 10mM glucose.

5,2.2). INCUBATION.

Batches of 5 ganglia were labelled with carrier free

[^{32}P]-inorganic phosphate (2MBq) (Amersham International). The incubations were performed at room temperature for 0-150 mins in the preincubation buffer. In the case of experiments involving treatment with agonist, carbachol and the [^{32}]P-inorganic phosphate were added at the same time. Incorporation at zero time was determined by the addition of 0.2 mls of trichloroacetic acid prior to addition of the label. The incubations were terminated by the removal of medium and rinsing of ganglia with ice cold buffer (preincubation buffer containing 3mM $CaCl_2$, 8mM K_2HPO_4, 2mM EGTA but without glucose).

5.2.3). EXTRACTION OF PHOSPHOLIPIDS.

The groups of 5 ganglia were homogenised in 0.5 ml acidified chloroform/methanol (1:2 v/v $CHCl_3$:CH_3OH, 0.1 ml 4M HCl). After addition of 0.5 ml 4M KCl/chloroform (1:1 v/v) the phases were separated by centrifugation (500g, 5min). Carrier phospholipids (200μg of a mixture of phosphatidyl-serine, phosphatidyl-inositol, phosphatidyl-inositol 4-phosphate, phosphatidyl-inositol 4,5-bisphosphate (Sigma Chemical Co.) were added to the extraction mixture to reduce the loss of [^{32}P]-labelled phospholipids.

5.2.4) T.L.C. SEPARATION OF PHOSPHOLIPIDS.

The lower chloroform phase of the acidified chloroform/methanol extraction mixture contained all the

labelled phospholipids. This phase was dried by evaporation under nitrogen, and then resuspended in 200μl of chloroform/methanol (2:1 by vol.).

The phospholipids were analysed by two-step one-dimensional thin layer chromatography on silica gel according to the method of Medh and Weigel (1989). The chromatograms were developed on aluminium-backed thin layer chromatography plates (20 x 20 cm, No.5554, Kieselgel F_{254}, 0.2mm thickness obtained from Merck Chemical Company). The plates were activated by heating at 110°C for 15 min.The phospholipid extracts were then spotted as 8mm wide bands on different lanes 1.5cm from the bottom of the plate using a capillary tube. The plates were developed by ascending chromatography successively in two different solvent systems, which were equilibrated in solvent tanks. The first acidic solvent system was composed of $CHCl_3$-CH_3OH-CH_3COOH-H_2O (55:43:3:4. by vol.). The development was stopped when the solvent front reached about 0.5 cm from the top of the plate. The plates were allowed to air dry before development in a second basic solvent composed of $CHCl_3$-CH_3OH-NH_4OH-H_2O (40:70:10:20. by vol.). The development in the second solvent system was stopped when the solvent front migrated a distance equivalent to 25 % of the first solvent.

Identification of the phospholipids was achieved by developing the chromatograms with known phospholipid standards (phosphatidic acid, phosphatidylethanolamine,

phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate (Sigma Chemical Co.)). The phospholipids were visualised by charring at 160^{o}C after spraying with 10 % H_2SO_4, in 50% ethanol. The radiolabelled phospholipids were located using an AMBIS radioanalytic imaging system (Ray Test Instruments, Sheffield), which detects ß emmisions. Identification of the phospholipids was based on co-chromatography with known standards.

Quantification of [^{32}P]-Pi incorporation into the phospholipids was achieved by determining the radioactivity over the required area of the chromatogram. Autoradiographs of the chromatograms taken from the AMBIS scanner were developed for 3 hours, during which the disintegration in counts per min was determined (c.p.m./5 ganglia). Results from all the incubation times (0, 15, 60, 150 min), in both control and carbachol treated experiments were obtained from scanning of a single t.l.c. plate. Thus, incorporation of [^{32}P]-Pi into the phospholipids could be directly compared.

5.3). RESULTS.

An autoradiograph of a chromatogram taken from the AMBIS scanner is shown in figure 30. The migration of known standards on the t.l.c. plates is reflected in their Rf values (fig.30). Identification of the radiolabelled

phospholipids was based on co-chromatography with known standards. This showed the presence of two unidentified phospholipids (X, Y). Phosphatidylinositol bisphosphate was not adequately resolved from lipid X to allow for it to be quantified. The levels of [^{32}P]-Pi incorporation into phosphatidylinositol (PIP), phosphatidylinositol 4-phosphate (PIP) and phosphatidic acid (PA) were plotted against incubation time (fig 31). This showed that the levels of [^{32}P] associated with PA increased in the presence of 0.5mM carbachol, throughout the incubation period, while that in PIP decreased. The results were expressed as the ratio of radioactivity in PIP relative to that in PA (table 5). The extent of labelling of PIP relative to PA increased over the 150 min incubation period in control conditions. This was not found to be the case in carbachol treated experiments, in which the label associated with PIP was approximately half that of PA at all time points.

5.4). DISCUSSION.

The metabolism of polyphosphoinositides has been extensively studied by using radioisotope labelling techniques. As with phosphatidylinositol, the polyphosphoinositides may be labelled to isotopic equilibrium using [^{3}H]-inositol or [^{32}P]-Pi, but this can take upto 3 days in GH3 pituitary tumour cells (MacPhee and Drummond, 1984). Hence, stable cultured cell lines are essential for such studies to allow for equilibration of

the diester phosphate of phosphatidylinositol with the labelled precursors. However, the polyphosphoinositide monoester phosphates attain isotopic equilibrium with the γ-phosphate of adenosine trisphosphate within 70 minutes in hepatocytes, when there is very little radioactivity in the diester phosphate groups (Palmer *et al*, 1986; Thomas *et al.*, 1983).

The results presented here show that when the locust ganglia were incubated with [^{32}P]-Pi there was a rapid appearance of label in the phospholipids involved in the inositol lipid signalling cycle (fig. 30). This labelling is dependent on the activities of the 4-kinase, 5-kinase and diacylglycerol kinase, which phosphorylate phosphatidylinositol, phosphatidylinositol 4-phosphate and diacylglycerol respectively using [^{32}P]-labelled adenosine trisphosphate. There was a rapid appearance of label into phosphatidylinositol 4-phosphate and phosphatidic acid within the first 15 minutes, which tended to plateau by 150 minutes (fig. 31). This [^{32}P]-Pi was postulated to be associated with the 4 and 5 positions on the inositol ring of the polyphosphoinositides. The labelling of the monoester phosphate groups from added [^{32}P]-Pi in unstimulated hepatocytes was slower than the labelling of the γ-phosphate of adenosine trisphosphate, the immediate metabolic precursor (Palmer *et al.*, 1986). However, the time lag which separated the labelling of the adenosine trisphosphate and of the phospholipids was only of a few minutes, suggesting that the metabolic renewal of the 4-

and 5-phosphate groups of the hepatic polyphosphoinositides at near steady state was no more than 5 minutes. Thus, the rapid labelling observed in these experiments also suggests that 4- and 5-kinase activities are present in the locust ganglia.

Under control conditions the levels of phosphatidylinositol 4-phosphate labelling increased throughout the 150 min incubation period (fig. 31). The ratio of labelled phosphatidylinositol 4-phosphate to phosphatidic acid also increased with time (table 5). Thus, the labelling of phosphatidylinositol 4-phosphate due to 4-kinase was greater than the rate of labelling of phosphatidic acid from the activity of diacylglycerol kinase. This suggested that under these conditions the amount of diacylglycerol was rate limiting. In GH3 pituitary tumour cells (MacPhee and Drummond, 1984) the addition of thyrotrophin-releasing hormone resulted in an increase in the levels of both diacylglycerol and phosphatidic acid, also suggesting that the amount of diacylglycerol was rate limiting in resting conditions. Hormone stimulated rat pituitary cells (Imai and Gershengorn, 1986) and hepatocytes (Thomas *et al.*, 1983) showed increases in phosphatidic acid levels presumably from the activity of diacylglycerol kinase.

In the presence of 0.5mM carbachol, the level of [^{32}P]-Pi associated with phosphatidylinositol 4-phosphate decreased while that in phosphatidic acid increased (fig.

31). The decrease in [^{32}P]-Pi labelled phosphatidylinositol 4-phosphate occurred at the earliest time point (15 min), hence recently labelled inositol lipids can become susceptible to agonist action. Work on blow fly salivary glands (Fain and Berridge, 1979) showed that there was a preferential breakdown of recently labelled [^{3}H]-phosphatidylinositol, which occurred within 6 min. Longer labelling periods of up to 187 min produced only a slight increase in [^{3}H]-phosphatidylinositol breakdown. Results from our experiments suggests that phospholipase C is activated by the agonist, and that the polyphosphoinositides are broken down at a faster rate than their formation by phosphorylation. The increase in diacylglycerol production will account for the increase in the rate of labelling observed in phosphatidic acid. The incorporation of [^{32}P]-Pi into phosphatidylinositol occurred at a slower rate than into the other phospholipids involved in the pathway, and the breakdown of [^{32}P]-phosphatidylinositol in the presence of carbachol was not detected (fig. 31). Labelling of phosphatidylinositol with [^{32}P]-Pi in WRK-1 cells (Koreh & Monaco, 1986) rat pituitary cells (Imai & Gershengorn, 1986) and GH3 pituitary tumour cells (MacPhee & Drummond, 1983) have shown sharp increases in the rate of phosphatidylinositol and phosphatidic acid labelling in the presence of agonists. It can be postulated that in our system the labelled phosphatidic acid serves mainly for the production of the large agonist-insensitive phosphatidylinositol pool. Experimental support for

receptors triggering the hydrolysis of a small metabolically active inositol lipid has come from studies using WRK-1 rat mammary tumour cells lines (Koreh and Monaco, 1986. These experiments demonstrated that [^{32}P]-Pi incorporation into the 1-phosphate position of the hormone sensitive pool of phosphoinositides require, either a long term incubation period (8 hours), or the presence of an agonist during a short term incubation (2 hours). After equilibrium labelling with [^{32}P]-Pi only 15% of the phosphatidylinositol pool was hormone sensitive (Monaco, 1982; Monaco and Woods, 1983). Also, the phosphatidic acid pool could be sufficiently large to highly dilute the labelled phosphatidic acid. This would lead to the production of an agonist sensitive phosphatidylinositol pool which is of low specific activity. Any changes in the size of this pool will not be detected.

Radiolabel ([^{32}P]-Pi) flux into the monoester phosphates of the polyphosphoinositides does not conclusively demonstrate phospholipase C activity. Studies using human erythrocytes, which lack a hormone linked phospholipase C system, have shown that polyphosphoinositides undergo a rapid turnover of their phosphomonoester groups (Muller *et al.*, 1986). This substrate cycling can be reasonably attributed to the activities of the 4- and 5-kinases, and the phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate phosphomonoesterases. Thus, any changes observed in [^{32}P]-Pi incorporation into the

polyphosphoinositides may be due to the changes in the activities of the kinases and the phosphomonoesterases.

Phospholipase C activity can be demonstrated by measuring the flux of [^{3}H]-inositol through the phosphoinositides into the inositol phosphates (Downes and Wusteman, 1983). Incubation of the ganglia in the presence of [^{3}H]-inositol showed that the label was incorporated predominantly into phosphatidylinositol. There was little or no incorporation into the polyphosphoinositides, as they constitute a small fraction of the total phosphatidylinositol pool (Thomas *et al.*, 1983). The phosphatidylinositol pool that is rapidly turning over would be labelled to high specific activity ([^{3}H]-inositol added 84 Ci/mmol), enabling detection of any changes in pool size. Any breakdown of phosphatidylinositol would result in the formation of inositol phosphates, thus giving a measure of phosphatidylinositol turnover.

All the experiments were performed in the presence of [^{3}H]-inositol in the preincubation buffer. It was found that the phosphatidylinositol fraction was labelled, but could not be chased out by subsequent addition of agonist in inositol free conditions (fig. 20). This suggests that the phosphatidylinositol fraction labelled during the preincubation is agonist insensitive. The presence of such a pool is consistent with findings from the [^{32}P]-Pi labelling experiments (fig. 31).

However, with the label present in the incubation buffer, there was an increase in the labelling of the phosphatidylinositol pool. This can be explained by the presence of 3mM calcium, a higher concentration of [^{3}H]-inositol and the fact that the incubations were performed at 25^{o}C.

In the presence of pilocarpine and carbachol there was an agonist dependent accumulation of [^{3}H]-inositol phosphates (figs. 16 and 18). The inositol phosphate isomers were identified by co-elution with known standards from the h.p.l.c. column. The presence of inositol 1,4,5-trisphosphate suggested that phospholipase C activity was present in the locust ganglia. There was also a corresponding decrease in the [^{3}H]-inositol labelling of the phosphatidylinositol pool. A rapid withdrawal of lipid from the polyphosphoinositide pool in stimulated ganglia could result in the depletion of the phosphatidylinositol pool , which serves to replenish phosphatidylinositol 4,5,-bisphosphate required for phospholipase C activity. The production of inositol phosphates would account for the loss in [^{32}P]-Pi label in the polyphosphoinositides observed (fig. 31). This also suggests the production of diacylglycerol which is phosphorylated to phosphatidic acid. Preliminary experiments (Chapter 2) have shown that the carbachol enhancement of the incorporation of [^{3}H]-inositol into the inositol phosphates can be inhibited by atropine and augmented by lithium. Myo-inositol 1-phosphatase is known to be inhibited by lithium ions

(Allison *et al*, 1976; Sherman *et al*, 1981). Thus, this action of lithium can be used as a method for identifying receptors that function through the inositol lipid pathway, by observing an accumulation of labelled inositol phosphates in the presence of an agonist.

The results presented here show that locust ganglia will take up and metabolise [^{3}H]-inositol and [^{32}P]-labelled inorganic phosphate into acid soluble and lipid soluble products. Analysis of these products has established the presence of a functional phosphatidylinositol signalling cascade. The key enzymes involved in the inositol lipid cycle may be exploited as potential target sites for the development of pest control agents. Both the activation and inhibition of the enzymes will disrupt the signalling cascade. Purification of phospholipase C , diacylglycerol kinase, polyphosphoinositide kinases, phosphatidylinositol synthetase and inositol polyphosphatases from mammalian tissues show that each of these key enzymes is present in different isoforms (Kanoh *et al.*, 1990; Downes and MacPhee, 1990; Rhee *et al.*, 1989). There are at least five distinct isoenzymes of phosphoinositide specific phospholipase C's (Rhee *et al*, 1989), which differ from one another with respect to their molecular mass, charge, divalent cation requirement, substrate specificity and form of their released products. Similar purification and biochemical characterisation studies could be performed on the insect forms of these enzymes. Once these enzymes are

isolated, potential pesticides may be screened to elucidate their mode of action, from which structure-activity relationships can be determined and new lead structures obtained. *In vitro* screens are a good indicator of bioactivity on a particular system. However, this type of data has to be used in conjunction with primary screens on whole organisms, as toxicity of compounds also depends on other factors such as accessibility. It is also possible to increase the selectivity of potential pesticides by running parallel *in vitro* studies based on isolated mammalian enzymes. One of the attractions of disrupting second messenger systems is that the effect of the drug can be amplified if it acts early in the cascade. The inositol lipid cycle provides the investigator with many potential target enzymes which regulate the supply of second messengers such as diacylglycerol and inositol 1,4,5-trisphosphate. This has important implications in the development of resistance, as targeting many sites reduces the chance of the insect becoming totally resistant.

Figure 30.

The effect of carbachol on the incorporation of [^{32}P]-Pi into the phospholipids.

After a preincubation period of 90 min, groups of 5 ganglia were incubated for the required length of time (0-150 min.) in the presence (filled circles) or the absence (open circles) of 0.5mM carbachol. Phospholipid samples were prepared after extraction and separated by two-step one-dimensional thin layer chromatography. The radiolabelled \lipids were located and quantified using the AMBIS scanner. The results presented here were obtained from one of three experiments. Identification was achieved by co-chromatography with known standards (O = origin, Rf values for unknown X = 0.05, phosphatidylinositol4,5-bisphosphate (PIP2) = 0.10, phosphatidylinositol4-phosphate (PIP) = 0.17, sphingomyelin = 0.24, unknown Y = 0.26, phosphatidylcholine (PC) = 0.29, phosphatidylserine (PS) = 0.55, phosphatidylinositol (PI) = 0.63, phosphatidylethanolamine (PE) = 0.79 and phosphatidic acid (PA) = 0.80).

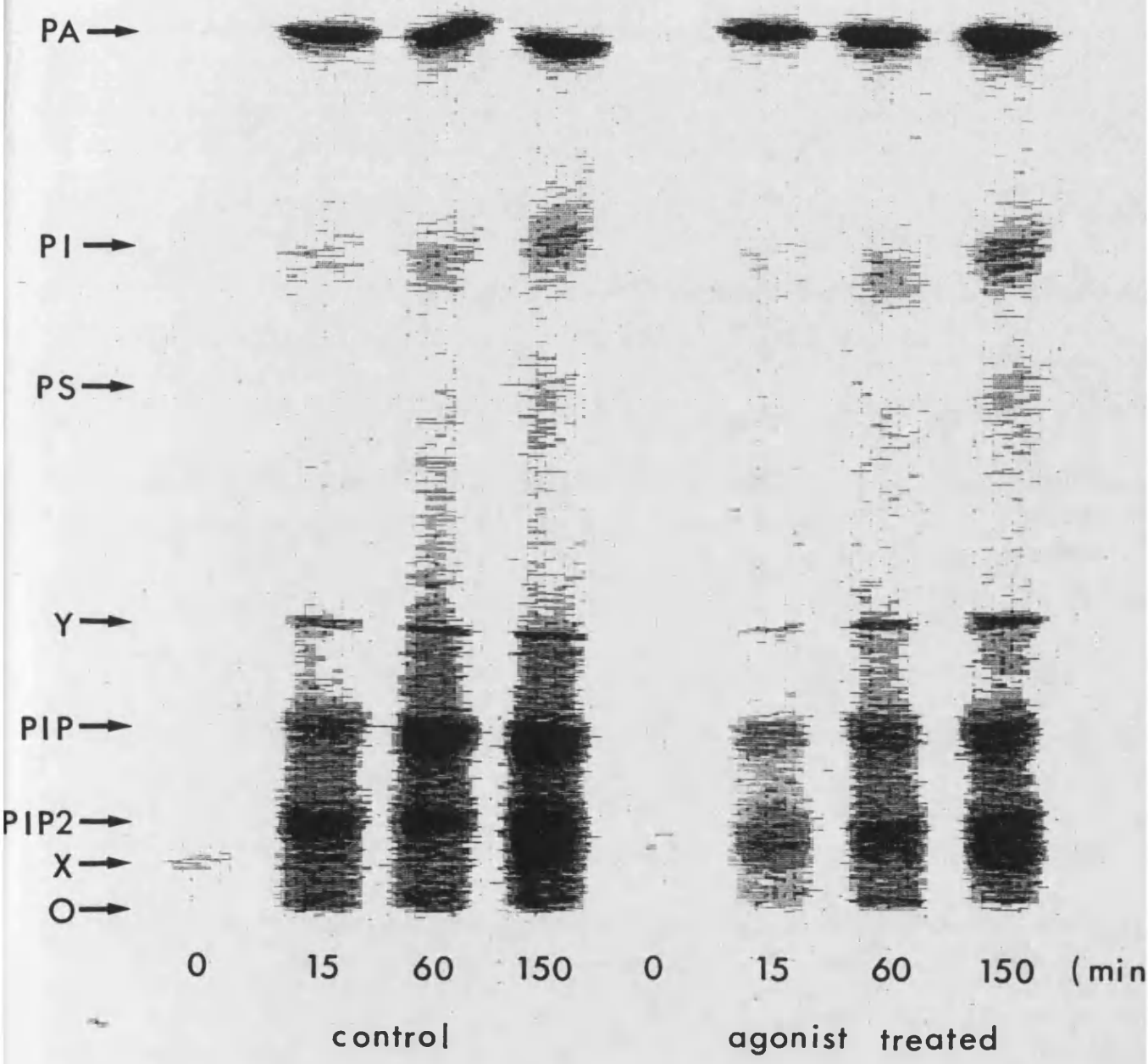
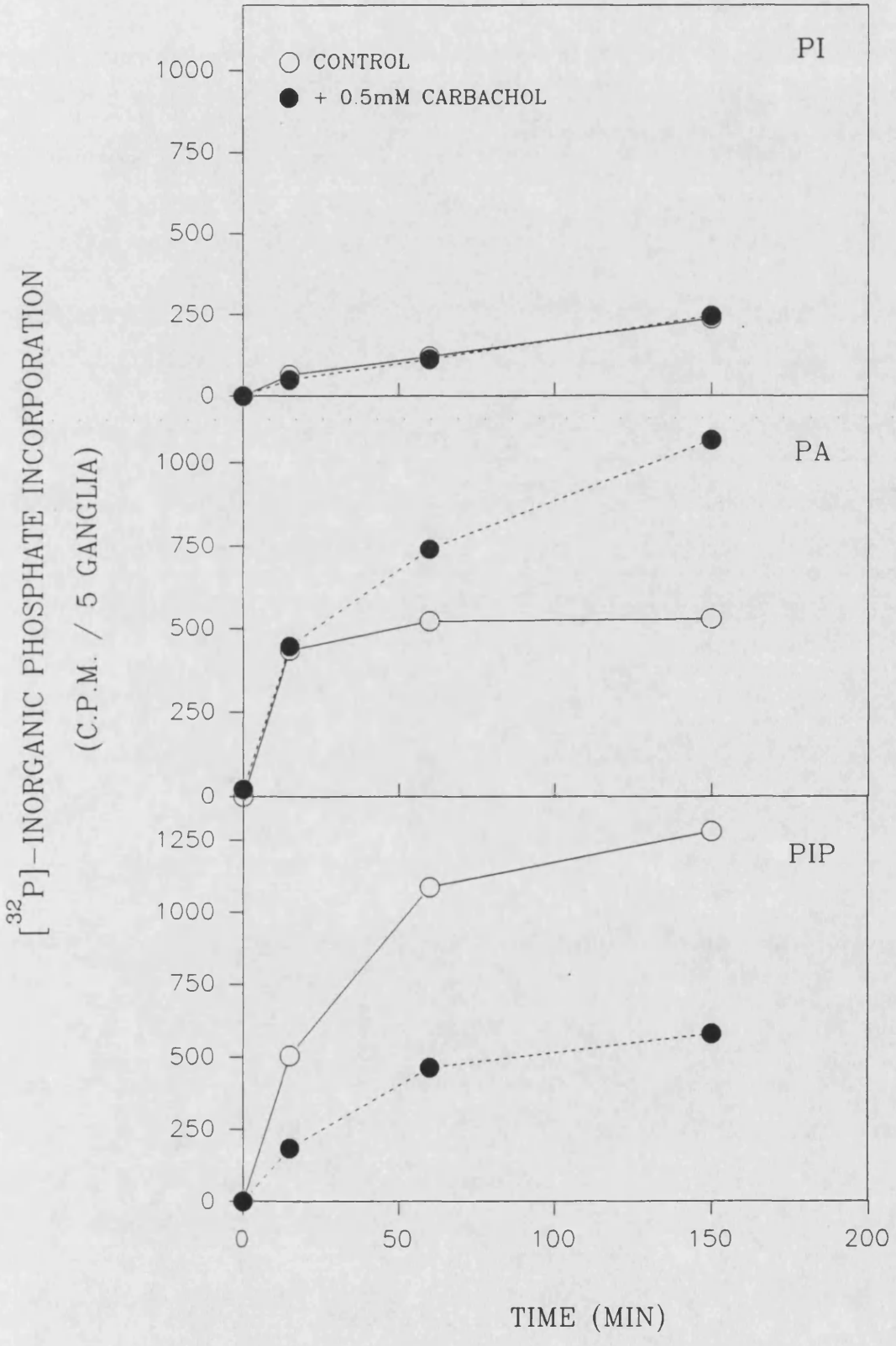

PA
PI
PS
Y
PIP
PIP2
X
O
0
15
60
150
0
15
60
150
(min
control
agonist treated

Figure 31.

Quantification of [^{32}P]-Pi incorporation into PI, PIP and PA.

Total radioactivity in each phospholipid band was determined (c.p.m. per 5 ganglia). The radiolabelled phospholipids from all the incubations were spotted and counted on a single t.l.c. plate, so that the incorporation of the label could be directly compared.


PI
CONTROL
+ 0.5mM CARBACHOL
PA
PIP
1000
750
500
250
0
1250
0
50
100
150
200
TIME (MIN)
[32P]-INORGANIC PHOSPHATE INCORPORATION
(C.P.M. / 5 GANGLIA)

	RATIO [32P]-Pi IN PIP v. PA	
TIME (min)	CONTROL	CARBACHOL
15	1.2	0.4
60	2.0	0.6
150	2.4	0.5

Table 5.

Results presented as the ratio of radioactivity in PIP to that in PA.

6). MUSCARINIC RECEPTORS IN INSECT NERVOUS TISSUES

6.1). INTRODUCTION.

6.1.1). ELECTROPHYSIOLOGICAL EVIDENCE FOR MUSCARINIC RECEPTORS.

One source of evidence for the presence of muscarinic-like receptors in insect nervous tissue has come from electrophysiological assay systems. Nicotinic and muscarinic responses could be distinguished pharmacologically in isolated somata of locust (*Locusta migratoria*) thoracic neurones, maintained under voltage clamp (Benson, 1988). Muscarinic evoked responses were blocked by muscarinic antagonists; QNB > scopolamine > atropine > 4-DAMP, pirenzepine, HHSiD, (Benson, 1992). QNB, scopolamine and atropine are not known to be selective for any subtype of receptor (Buckley *et al*, 1989). Gallamine, methoctramine and AFDX116 are all thought to be M_2 selective muscarinic antagonists but are inactive at the locust somata. These results suggest that these receptors have a pharmacological profile unlike that in vertebrates, in which pirenzepine is expected to show M_1 selectivity and HHSiD is expected to show M_3 selectivity. Muscarinic drugs were also able to antagonise nicotinic responses ($EC_{50}<1\mu M$). This is not a mixed receptor as alpha-bungarotoxin, a nicotinic antagonist,

has no effect. Other muscarinic agonist such as oxotremorine, muscarine, pilocarpine and arecoline could evoke an electrophysiological response. McN-A-343, thought to be an M_1 selective agonist (Hammer and Giachetti, 1982) was found to be inactive.

Other studies using dorsal unpaired median neurones (DUM) in adult cockroaches, *Periplanata americana*, for patch clamp recordings indicated the presence of mixed nicotinic/muscarinic receptors (Lapied *et al.*, 1990). In these cells there was a nicotine induced biphasic response. This was composed of an initial fast depolarisation followed by a slow depolarising second phase. The fast response was not affected by alpha-bungarotoxin or atropine, however the slow depolarising response could be blocked by both muscarinic (gallamine and pirenzepine) and nicotinic antagonists (d-tubocurare and alpha-bungarotoxin). Application of a muscarinic agonist, McN-A-343 (Lapied *et al.*, 1992), produced a biphasic response. The fast hyperpolarisation was suppressed by 10nM gallamine. The slow depolarising response was antagonised by 10nM pirenzepine, which is a M_1 muscarinic antagonist. These results suggest that the pharmacological properties of the DUM neurone ACh receptors are complex, and the functional significance of this is yet to be elucidated.

It has been suggested that the muscarinic-like receptors in insects may be involved in the negative

feedback regulation of acetylcholine release (Breer and Knipper, 1984; Knipper and Breer, 1989). In these studies it was shown that muscarinic agonists; muscarine (10μM) and oxotremorine (1μM), reduced tritiated aetylcholine release from insect synaptosomes from adult locusts (*Locusta migratoria migratorioides*). A muscarinic antagonist, atropine, enhanced ACh release. Activation of these muscarinic-like autoreceptors was found to decrease cAMP levels leading to a decrease in ACh release. Stable analogues of cAMP (8-bromoadenosine cAMP, butrylyladenine cAMP) facilitated synaptosomal release and antagonised the inhibitory effects of agonists. Binding studies in subcellular fractions showed striking differences in affinities for pirenzepine for the synaptosomes and cell bodies (Knipper and Breer, 1988). These observatons suggested that the cell bodies contained M_1-like receptors (high affinity for pirenzepine) and the nerve terminals contained M_2-like receptors; on the basis that oxotremorine, which has a high preference for M_2 receptors, induced a reduction in cAMP levels. A more definitive characterisation of this presynaptic muscarinic-like autoreceptor would require the use of other selective ligands such as methoctramine (M_2), AFDX116 (M_2), AFDX384 (M_2), hexahydrosilafenidol (M_3) and 4-DAMP (M_3). The differences in the pharmacological profile of the locust receptor and the vertebrate receptor can then be determined.

Electrophysiological studies have also suggested that

the insect muscarinic-like receptor may be involved in negative modulation of ACh release (Trimmer and Weeks, 1989; LeCorronc and Hue, 1991). The sixth abdominal (A6) ganglion of the cockroach has provided a suitable preparation for the characterisation of the presynaptic muscarinic-like receptor (LeCorronc and Hue, 1991). This preparation included a cercus, the cercal nerve XI and the isolated giant interneurone 2 for stimulation and single fibre recordings. It was found that an excitatory post synaptic potential (EPSP) could be evoked either by elecrical stimulation of the cercal nerve XI or by micropressure injection of 1μM carbachol. Bath application of 1μM arecoline, a muscarinic agonist, had no effect on carbachol induced potentials. Hence, arecoline does not act postsynaptically. However, muscarinic agonists were able to inhibit EPSP's evoked by presynaptic stimulation of the cercal nerve XI. This suggests the presence of muscarinic-like autoreceptors which can regulate ACh release, as the EPSP's evoked by the stimulation of the cercal nerve is thought to occur by the action of ACh on postsynaptic nicotinic receptors. Muscarininc M_2 selective antagonists (100nM AFDX116 and 100nM methoctramine) antagonised the inhibition of the EPSP's induced by arecoline. 4-DAMP and pirenzepine had no effect. Thus, this muscarinic autoreceptor shares the pharmacological properties of the vertebrate M_2 subtype of receptor (LeCorronc *et al.*, 1991).

6.1.2). RADIOLIGAND BINDING ASSAYS IN INSECT TISSUES

Binding of radiolabelled ligands has been used extensively to pharmacologically characterise receptors. The binding of high affinity, specific, saturable radiolabelled drugs such as quinuclidinylbenzilate (QNB) and N-methyl scopolamine (NMS) has been used to characterise muscarinic receptors in the mammalian nervous system. Binding of [^{3}H]-QNB has been shown to occur in the particulate fractions of many insects, including fruitfly heads (Dudai and Benbarak, 1977), housefly heads (Shaker and Eldefrawi, 1981), cockroach ventral nerve cords (Lummis and Satelle, 1985) and supraoesophageal ganglia from two different species of locust (Breer, 1981; Aguilar and Lunt 1984).

Locust mAChR is found to be different from the rat muscarinic receptor in pharmacological properties, thermal stability, hydrophobic properties (Aguilar and Lunt, 1984; Aguilar *et al*, 1984; Duggan, 1987) and sensitivity to disulphide reducing agents (Fonseca *et al.*, 1991). further investigation into the differences of the muscarinic receptors in insects and mammals with respect to their biochemical properties, may prove the receptor to be a potential target site for the development of pest control agents.

Binding of [^{3}H]-QNB to the supraoesophageal ganglia of locusts, *Schistocerca gregaria*, has been used to

further characterise the muscarinic-like receptor. The kinetic analysis of [^{3}H]-QNB binding revealed at least two binding sites (Aguilar and Lunt, 1984). Duggan (1987) attempted to more fully characterise the muscarinic receptor in the locust ganglia, by performing similar studies to those of Aguilar and Lunt (1984), using known muscarinic antagonists in competition assays. Pirenzepine, an M_1 selective antagonist, and AFDX116, an M_2 selective antagonist showed low affinity (Ki's in the micromolar range) in competition with [^{3}H]-QNB. The assays were performed at 1nM [^{3}H]-QNB, a concentration at which both the high affinity and low affinity sites would be occupied (Aguilar and Lunt, 1984). This may explain the low Hill numbers for the antagonists used in these studies.

Recent studies (Abdullah *et al*, 1991) have found consistent differences between the affinities of pirenzepine, AFDX116 and 4-DAMP in various insect brains and mammalian brains. Binding of [^{3}H]-QNB to the heads of honey bee (*Apis mellifera*), housefly (*Musca domestica*) and the american cockroach (*Periplanata americana*) showed a single high affinity binding site (0.47nM in honey bees, 0.17nM in houseflies and 0.13nM in cockroach). However, the saturation curve was obtained using a narrow concentration range of [^{3}H]-QNB (0-6nM in the cockroach brain, compared with 0-30nM used in locust brain (Aguilar and Lunt, 1984)), and hence Abdullah *et al* (1991) may not have shown binding of [^{3}H]-QNB to the second low affinity component. The three insect brain receptors showed lower

affinity for pirenzepine and 4-DAMP, an M_3 selective muscarinic receptor antagonist, than did the mammalian brain receptors (27-50 fold lower affinity for pirenzepine, and 9-27 fold lower affinity for 4-DAMP). It had been postulated that the cloned muscarinic receptor from *Drosophila* was the M_1 subtype, based on its activation of phosphoinositide turnover (Shapiro *et al*, 1989). The studies on the insect brains including that in *Drosophila* suggested that there was a predominance of this subtype of muscarinic-like receptors, and that it is unlike the mammalian M_1 subtype (mammalian M_1 has a Ki of 16nM (Buckley *et al*, 1989), and insects have Ki's ranging from 484-900nM). The higher affinities of the insect muscarinic receptors for 4-DAMP than pirenzepine, suggested that the this subtype is more like the mammalian M_3 than M_1.

We have used the supraoesophageal ganglia of the locust *Schistocerca gregaria* for studies that re-examined [^{3}H]-QNB binding. In these studies competition assays were performed against [^{3}H]-QNB using subtype selective muscarinic antagonists; 4-DAMP, AFDX116, pirenzepine and methoctramine. The experiments performed were similar to that of Aguilar and Lunt (1984) and Duggan (1987), but the competition assays were peformed at a concentration of [^{3}H]-QNB which was approximately equivalent to the Kd of the high affinity site. This was to ensure that the binding of [^{3}H]-QNB was predominantly to this site. Assuming that the competing ligand binds to a single class

of sites, and that the radiolabelled ligand binds to two classes of sites, then at high concentrations of [^{3}H]-QNB the competing ligand will appear to recognise two sites, but at low concentrations of [^{3}H]-QNB (where binding of the radiolabelled ligand occurs predominantly to the high affinity site) the competing ligand will appear to recognise just one site. Duggan (1987) found that at 1nM [^{3}H]-QNB, pirenzepine gave a statistically better two site fit. In our experiments, we tested whether at lower concentrations of [^{3}H]-QNB, inhibition of binding with pirenzepine showed a higher Hill number, suggesting recognition of a single site. If the non-linear Scatchard plotfor [^{3}H]-QNB binding (Aguilar and Lunt, 1984) was due to negative cooperativity, then pirenzepine would recognise two sites irrespective of the [^{3}H]-QNB concentration at which the competition assay is performed.

6.2). METHODS FOR RECEPTOR BINDING STUDIES.

6.2.1). TISSUE PREPARATION.

Supraoesophageal ganglia were used in all experiments. Typically one hundred ganglia were dissected to obtain enough tissue for binding experiments. On removing the optic lobes and rinsing the ganglia with cold phosphate buffer (50mM disodium hydrogen orthophosphate, 2mM EDTA, pH 7.2), the ganglia could be stored at -20^{o}C.

6.2.2). MEMBRANE PREPARATION.

Purified membrane fractions (P2) were prepared from the 100 ganglia for binding experiments. Crude supernatant (S1) was obtained by homogenisation of the ganglia in approximately 5mls of 50mM phosphate buffer using a hand held homogeniser, and then in a Teflon-glass motorised Potter-Elvejhem homogeniser (radial clearance of 0.15mm, 20 strokes at 500revs/min). After low speed centrifugation (500g, 10mins, the S1 was decanted and the P1 fraction was washed three times with ice cold buffer. The combined S1 fractions were made upto 20ml with phosphate buffer. The P2 fraction was prepared by high speed centrifugation of the S1 fraction by centrifigation (Beckman L5-50B, SW 50.1 rotor) at 80 000g for 60mins. This P2 pellet was resuspended in 10mls of buffer, and could be stored at -20^{o}C. 100 ganglia provided approximately 10-12mg of protein, as determined by the method of Lowry *et al.*(1951).

6.2.3). BINDING ASSAYS.

1-Quinuclidinyl[phenyl-4-^{3}H]benzilate ([^{3}H]-QNB) obtained from Amersham International was used to characterise the muscarinic like receptors in the locust ganglia. Samples of membrane preparations (1ml, 100-250μg protein) in phosphate buffer were incubated in the presence of different concentrations of [^{3}H]-QNB for 2 hours at 25^{o}C. Enough protein was obtained from 100 ganglia to perform 48 incubations. Thus, a range of 12

concentrations of [^{3}H]-QNB could be performed. At each concentration, duplicates of non-specific and total binding were determined. The non-specific binding was taken as the amount of [^{3}H]-QNB bound in the presence of 1mM atropine. Binding was terminated by the addition of cold PBS (10mM phosphate, 140mM sodium chloride, pH 7.4). The samples were filtered under reduced pressure through glass fibre filters (type A/E, Gelman Sciences) in a Brandel Cell Harvester. After washing 3 times with 5mls of buffer, the filters were placed in scintillation vials. Radioactivity was determined by the addition of 5mls of scintillant (optiphase safe) and counting in a Packard scintillation spectrometer (counting efficiency 30-35%).

In competition experiments binding of [^{3}H]-QNB to locust ganglia was inhibited by various muscarinic agonists; carbachol, muscarine, pilocarpine and methacholine (Sigma Chemical Co.), and antagonists; N-methyl scopolamine, AFDX116, methoctramine, 4-DAMP, pirenzepine. The radioligand concentration was kept constant and competed with increasing concentrations of unlabeled drug. As with the saturation experiments, a P2 membrane preparation was used. The binding assays were performed in 1ml of buffer A containing the resuspended membrane preparation (100-250 μg of protein) and 0.4nM [^{3}H]-QNB. Total binding was determined in the absence of the competing ligand. Non-specific binding was determined in the presence of 0.1mM atropine. Incubations were performed at 30^{o}C for 2 hours in a water bath. Binding was

terminated by the addition of 5mls of cold PBS, and a filtration assay was performed (see above). Dissection of 100 ganglia provided enough tissue for duplicate determinations of total binding, non-specific binding and binding at 22 concentrations of competing drugs (2-4 drugs) per experiment.

6.2.4). DATA ANALYSIS.

6.2.4.1). SATURATION STUDIES.

The binding data were analysed using a computer assisted non-linear curve-fitting technique. The raw data were formatted using an EBDA program (developed by McPherson (1985)). Initial estimates for dissociation constants (Kd's), inhibition constants (Ki's) and receptor concentrations (Bmax's) were obtained for analysis using LIGAND (Munson and Rodbard, 1980). Estimates for multiple sites were made from Hill plots and Eadie-Hoftsee plots by a linear interpolation method.

All the curves were fitted using an iterative method. After the first set of estimates for the multiple sites is made, the actual amount of binding predicted for each independent site is calculated. The sets of figures are then added for each concentration of free ligand. Finally, the resultant calculated curve is compared with the actual data for "goodness of fit".

In equilibrium binding experiments a model was used that described the binding of a single ligand to a heterogenous population of sites given by the equation:

$$Bt = (Bm1*F)/(F+Kd1) + (Bm2*F)/(F+Kd2) .....+ N*F$$

Bt = Total bound
Bm = Total number of binding sites
F = Free ligand concentration
Kd = Dissociation constant.
N = Non-specific binding.

As EBDA produces two SCAFIT files: one in which the non-specific binding is subtracted from the total binding; and one in which only the total binding is used, the theoretical non-specific binding can be determined using LIGAND and compared with that obtained experimentally. The estimate of N is based on the formal definition that N is the ratio of Bound/Free at infinite free concentration of ligand. This can be derived from expressing total binding as a function of the free ligand concentration, and then determining the non-specific binding component from the asymptote of the curved plot using the Scatchard transformation. If the non-specific binding is determined correctly, then the parameters estimated with and without the empirical non-specific bound should not differ significantly.

LIGAND allows for the simultaneous analysis of multiple experiments, by calculation of a correction factor (C) for each experiment relative to the first. This method of calculation is used when insufficient information from any one experiment is obtained for determination of parameters for multiple sites: if only 10 points are obtained to fit 5 parameters of a two classes of sites model, then the estimates of the parameters are essentially statistically indeterminate. However, if the Scatchard plots obtained from multiple experiments differ only in terms of their intercepts with both the horizontal and vertical axis, then the correction factor can be used. This pattern of congruent curves would be expected if K1, K2 and the ratios of R1:R2:N are the same for all experiments, suggesting that these experiments are qualitatively similar, but only differ in the total amount of receptor present.

6.2.4.2). COMPETITION STUDIES.

For competition analysis, the free ligand concentration is usually held constant while the concentration of the unlabeled competitor becomes the independent variable.

For a given concentration of the competitive inhibitor (I) and the free ligand (F), the occupancy equation is simply the extension B = (Bm*F)/(F+Kd), where the Kd of the ligand is modified by the concentration of

the competitor and its particular affinity for the particular binding site.

(1) $Bt = (Bm*F)/(F+Kd(1+I/Ki))$.

For more than one independent site,

(2) $Bt = (Bm1*F)/(F+Kd1(1+I/Ki1)) + (Bm2*F)/(F+Kd2(1+I/Kd2))$

Bt = Amount bound at [F]
F = free ligand concentration.
Bm1 = Total number of binding sites (site 1)
Bm2 = Total number of binding sites (site 2)
Kd1, Kd2 = Dissociation constants for sites 1 and 2.
Ki1, Ki2 = Inhibition constants for sites 1 and 2.

The occupancy equation allows for the testing of various binding models. The simplest case assumes that both the nonlabelled and labelled drug bind to a single independent site, and the parameters for this model (Ki, Kd. Bmax and non-specific binding) can be calculated by goodness of fit to binding predicted in equation (1).

In all curve fits the saturation data for [^{3}H]-QNB (HOT experiments) were co-analysed with the competition data from each unlabeled drug (DRUG experiments). This multiexperiment analysis required determination of additional correction factors, which reduced the degrees

of freedom but increased the data set from which better determinations of the parameters could be obtained. The residual sum of squares for each binding model was obtained and tested for significance using the F-test. More complicated models used equation (2) to determine the parameters.

Binding models:

1) The labelled drug and the unlabeled drug bound to an independent single site.

2) The labelled drug bound to two classes of sites, whilst the unlabeled drug bound to a single class of sites (K21 and K22 were shared parameters).

3) Both the labelled and unlabeled drugs bound to two classes of sites.

6.2.4.3). TESTING FOR SIGNIFICANCE OF EXTRA PARAMETERS (F-TEST).

When testing between single and multiple sites, additional parameters are added to the model and the goodness of fit will tend to improve. The sum of squares will decrease simply because of the added flexibility of the model. Ultimately if there are as many parameters as there are data points, then the curve can theoretically run exactly through each point.

There is a statistical test of whether the increase in the goodness of fit for a model with additional parameters is significantly more than expected by chance alone. It is based on the "extra sum of squares principle".

$$F = ((SS1-SS2)/(df1-df2))/(SS2/df2)$$

SS1 and SS2 are residual sum of squares of the deviations of the points to the fitted curve and df1, df2 are the associated degrees of freedom (data points - parameters) for the original model and the model with additional parameters, respectively. The calculated F ratio is then compared to the tabulated value for the F statistic with (df1 - df2) and df2 degrees of freedom.

6.3). RESULTS.

6.3.1). [^{3}H]-QNB SATURATION.

The specific binding isotherm at equilibrium for the P2 membrane fractions (fig 32) showed a consistent tendency to reach saturation in the range 2-5 nM [^{3}H]-QNB. At higher concentrations of the radioligand (upto 50nM) a second binding site was observed, the saturation of which was not achieved, probably due to the limitations of the filtration assay (Bennett and Yamamura, 1985).

The equilibrium binding data were then analysed using models for a single and two class population of non-interacting binding sites using LIGAND (fig. 33a and b). Results were co-analysed from four separate experiments, each with twelve data points (see methods for the use of correction factors). There was a statistically significant better fit of the experimental data for the two site model Data obtained from the saturation curve (fig. 33a) were transformed and presented as a Scatchard plot (fig. 33b). The dissociation constants were 0.36nM for the high affinity site and 39.8nM for the low affinity site. The estimation for the low affinity site was found to be more variable.

The saturation binding data from three experiments, which had the lowest sum of squares, were used for co-analysis with the competition data. Statistical analysis of the five binding models are shown in table 6. One and two site fits with non-specific binding set to zero (N1), as SCAFIT files with non-specific binding subtracted (models 1 and 2) were analysed, again showed a highly significant difference ($p<0.0001$). To test whether the low affinity site was obtained from an incorrect determination of non-specific binding, a one site fit with a floating N1 parameter (model 3) was compared with the two site model with N1 fixed to zero (model 2). This showed that model 2 was significantly different from model 3 ($p<0.001$), hence the second site was not due to non-specific binding. Further evidence that the non-specific binding was

correctly determined came from comparing the theoretical value of N1 by analysis of SCAFIT files without non-specific binding subtracted (fig. 34, theoretical value = 4% +/- 0.36 (S.E.M.), n=3), with the experimental empirical value (fig. 32, empirical value = 3% +/- 0.80 (S.E.M.), n=4). The theoretical value for non-specific binding was obtained for a one site fit for [^{3}H]-QNB binding with a dissociation constant of 0.25nM, a receptor concentration of 233 fmol/mg protein, this suggested that the second site and the non-specific component could not be distinguished in this Scatchard analysis of toal binding (fig. 34). A two site model with a floating N1 (model 4) did not significantly differ from the two site model with N1 fixed to zero (model 2), but was significantly different from the one site model with a floating N1 (model 3) ($p<0.002$).

The use of correction factors was justified as shown in table 6. In the fifth binding model (2 sites), the correction factors; C1, C2 and C3, for the three experiments were set to 1. When compared to model 2 (two site model with floating parameters C1, C2 and C3, the F-test showed a highly significant difference ($p<0.0001$).

6.3.2). COMPETITION ASSAYS.

The pharmacological characteristics of the binding sites were investigated in experiments in which the effects of muscarinic ligands on the inhibition of [^{3}H]-

QNB binding at equilibrium were measured (tables 7 and 8). A typical inhibition curve is shown in figure 35. For each competitive ligand, three binding models were tested (see methods), and the fitted curve in this example (fig. 35) was obtained from co-analysis of seven experiments for a two site model for [^{3}H]-QNB binding (3 experiments) and a two site model for 4-DAMP binding (4 experiments) (model 3). The results shown were obtained from one of the four 4-DAMP competition experiments co-analysed.

Displacement of [^{3}H]-QNB binding to locust ganglia by five muscarinic cholinergic antagonists gave a rank order of potency of N-methylscopolamine > 4-DAMP > pirenzepine > methoctramine > AFDX116 (table 7). For all the ligands tested, including the agonists (tables 7 and 8), model 2 (two sites for QNB binding, one site for the competing ligand) was significantly different from the first binding model. The third binding model was not tested for N-methylscopolamine and methoctramine, as the iteration process failed using these data for such a complex model. However, the two site model for the unlabelled ligand, 4-DAMP, was shown to be significantly different from the one site model (Ki for the low affinity site was 120nM and Ki for the high affinity site was 24nM). Two site binding models (model 3) for pirenzepine and AFDX116 were not significantly different from the one site binding models (model 2). The Hill coefficient for pirenzepine was close to one (0.92 +/- 0.105 (s.e.m.), n=6), suggesting a single binding site for this ligand.

The inhibition constants for the four muscarinic agonists showed lower affinities than the antagonists, the rank order of potency being pilocarpine > muscarine > carbachol > methacholine. The two site fit for muscarine (model 3) was significantly different from the one site fit (model 2, p=0.015, Ki for the low affinity site = 500μM, Ki for the high affinity site = 37μM). The fitted curve for the two sites for muscarine binding is shown in figure 36. The data points were obtained from one of three experiments.

6.4). DISCUSSION.

Insect muscarinic-like receptor subtypes have been identified by their specific ligand binding properties. Results presented here show that [^{3}H]-QNB binds with high affinity to membranes from the supraoesophageal ganglia of the locust.

The data analysis was performed using a curve fitting technique, as this method has many advantages over graphical methods. Scatchard plots and Eadie-Hoftsee plots are useful in that transformation of data from single non-interacting sites are linear, and hence useful for initial estimates of the binding parameters (Munson and Rodbard, 1980). However, using these graphical methods, small errors in the amount bound are propagated to both the x and y-axis (Bylund, 1986). Also, the magnitude of the

error increases with increasing free radioligand concentration (Scatchard plot) and decreasing competing ligand concentration (Eadie-Hoftsee plot), which makes statistical analysis difficult.

Evidence for muscarinic receptor heterogeneity in insect tissues has come from both binding (Aguilar and Lunt, 1984; Duggan, 1987; Abdullah *et al.*, 1991) and functional studies (Knipper and Breer, 1988; Duggan 1987). Functional characterisation using electrophysiological studies in DUM neurones of the cockroach, *Periplanata americana*, (Hue et al., 1989; LeCorronc and Hue, 1991), has shown the receptor to be similar to the mammalian M_2 subtype. Duggan (1987) showed that carbachol enhanced phosphatidylinositol turnover was inhibited by an M_1 subtype selective antagonist, pirenzepine. A cloned Drosophila receptor expressed in Y1 cells (Shapiro *et al.*, 1989) stimulates phospholipase C activity, this and its sequence similarity to the mammalian m1, m3 and m5 receptor subtypes suggested the existence of a functional muscarinic-like receptor in insects.

Similar results were found in [^{3}H]-QNB binding studies (fig. 33) to those obtained by Aguilar and Lunt (1984), in that [^{3}H]-QNB recognised at least two classes of binding sites. Heterogeneity of binding sites was also suggested by findings that Hill coefficients of the various cholinergic ligands were less than one (scopolamine = 0.38, gallamine = 0.76, carbachol = 0.46,

acetylcholine = 0.43 from Aguilar and Lunt, 1984). The data obtained from dissociation kinetics were also in keeping with the two populations of sites model. As the competition experiments were performed at 1.5nM [^{3}H]-QNB, one would have expected low Hill numbers as at this concentration of radioligand both the low and high affinity sites would be occupied. Duggan (1987) showed that pirenzepine recognised at least two sites with inhibition constants of 0.15μM and 7.5μM, when competition was performed at 1nM [^{3}H]-QNB (from the Hill plot Ki = 1.9 +/- 1.1 μM, Hill coefficient = 0.5 +/- 0.04). In our studies performed at 0.4nM [^{3}H]-QNB, where a higher proportion of high affinity sites are expected to be labelled, pirenzepine only recognised one site (two site model did not give a better fit, see table 7) of 2.8μM (Hill coefficient = 0.92 +/- 0.105).

Muscarinic receptor heterogeneity could also explain the discrepancies between Hill numbers obtained from studies by Abdullah *et al.* (1991) and those of Duggan (1987). In these studies Abdullah *et al.* 1991 argued that [^{3}H]-QNB bound to one site. The competition experiments were performed at concentrations of the radioligand approximately corresponding to its Kd value, which was consistent with the dissociation constant of our high affinity site. Furthermore, the similarity of the inhibition constants obtained from our experiments and those from studies by Abdullah *et al.* (1991), suggest that the high affinity site in the locust ganglia is similar to

the site characterised in the brains of honey bees, houseflies and american cockroaches (Ki's for AFDX116 range from 1.6 to 5μM in the insect species, table 7 showed that the locust ganglia had a Ki value of 14μM. Ki's for pirenzepine range from 0.48μM to 0.9μM in the insect brains whilst in the locust ganglia was 2.84μM). Studies on the locust brain (fig. 32; Aguilar and Lunt, 1984) showed there was a tendency for the high affinity site to saturate between 2-5nM [^{3}H]-QNB. Higher concentrations of radioligand were required to show the existence of the second site. As Abdullah *et al.* (1991) obtained the binding parameters from 0-6nM [^{3}H]-QNB, the establishment of the second site in these insect species will require further saturation at higher radioligand concentrations. The low affinities for pirenzepine, an M_1 antagonist, methoctramine and AFDX116, M_2 antagonists, suggested that the insect muscarinic receptor was unlike the mammalian M_1 subtype. There seems to be a predominance of this characteristic in insects, as similar results were also found by Shapiro *et al.* (1989) in a cloned *Drosophila* receptor expressed in Y1 cells (Ki for pirenzepine = 570nM).

The most potent of the muscarinic subtype selective ligands for the [^{3}H]-QNB labelled site was shown to be 4-DAMP, an M_3 muscarinic antagonist (Doods *et al.*, 1987; Lazareno *et al.*, 1990). This ligand recognised two sites (Ki of the high affinity site = 24nM, Ki of the low affinity site = 120nM). The high affinity site was similar

to that described by Abdullah *et al.* (1991) in the three insect brains tested (Ki's ranged from 19-57nM). In the rat sub-maxillary gland, which has a predominance of M3 receptors, 4-DAMP binds with higher affinity (Ki 0.37nM). Thus, the insect muscarinic receptor appears to bind with lower affinities to all the ligands tested when compared to the mammalian muscarinic subtypes, but the order of potency suggests that the insect receptor is more like the mammalian M_3 than the M_1 subtype. Unlike the studies by Abdullah *et al.* (1991), our studies have shown the presence of another 4-DAMP binding site with a Ki of 120nM (fig. 35), as well as the presence of another [^{3}H]-QNB binding site (fig. 33). Therefore it seems reasonable to postulate that the insect species may include more than one muscarinic subtype, similar to the multiple subtypes found in the mammalian brains.

An alternative explanation for the curvi-linear Scatchard plots for [^{3}H]-QNB binding is that there is a single population of binding sites which induce negative cooperative interactions. More extensive binding studies, such as those performed on the rat adenohypophysis (Henis and Sokolovsky, 1983), can help to elucidate the nature of the interaction. In this approach, the binding of the labelled ligand in the presence or absence of the unlabeled competing ligand can be predicted if the sites show heterogeneity. The binding parameters of the both the labelled and unlabeled ligands have to accurately determined. As 4-DAMP and QNB bind to the locust ganglia

with high affinity to heterogenous sites, the above approach to test this may be used if the binding parameters of labelled 4-DAMP are elucidated in the absence of any competing ligand. Other experiments which investigate the effect on dissociation kinetics (Waelbroeck *et al.*, 1986), and the effects of irreversibly alkylating the receptors to disrupt cooperative interactions (Hulme *et al.*, 1978) can also be used. Dissociation kinetics from the locust ganglia suggest site heterogeneity of [^{3}H]-QNB binding sites Aguilar and Lunt, 1984).

There have been reports that [^{3}H]-QNB, a hydrophobic ligand, may label more sites than [^{3}H]-N-methylscopolamine (NMS), a hydrophillic ligand (Brown and Goldstein, 1986). [^{3}H]-N-methylscopolamine bound to a uniform number of sites with affinities agreeing with those observed in functional studies. However, [^{3}H]-QNB bound to approximately 80% more sites, and labelled the same sites as [^{3}H]-NMS sites but also labelled additional sites. The possibility in our preparation that [^{3}H]-QNB may label additional non-functional sites has to be investigated by comparing binding affinities using [^{3}H]-NMS.

One of the main aims of binding studies is to compare affinities with functional assays. Correlations between structure and function may prove to be useful in rationally designing drugs which selectively act with high efficacy on target effectors (cAMP pathway, inositol lipid

pathway, ion channels etc.). Our attempts to functionally characterise the receptor by stimulating phosphatidylinositol turnover were fraught with difficulties. High concentrations of agonists, pilocarpine and carbachol, were required to show a response and the variability and the size of the response made a complete pharmacological study impracticable.

Advantages of radioligand binding assays over *in vivo* assays include minimisation of the problems of access of drugs to their binding site, and their removal by uptake or chemical/enzymic degradation. Since the event being examined is the initial interaction of the ligand with the receptor, effects of the drug distal to the receptor are eliminated. In addition, compensatory effects mediated through other receptors or cellular processes do not occur in radioligand binding assays. Furthermore, quantitative comparisons of the affinities of various drugs in different tissues is relatively straightforward. Radioligand binding assays can be useful in determining classification of receptor subtypes. On the other hand, it is important to recognise that most organs contain a variety of cell types, and that different subtypes can mediate the same physiological response.

Inhibition of [^{3}H]-QNB binding by muscarinic agonists showed low affinities relative to the antagonists (table 8). This is consistent with findings from other studies using insects (Dudai and Ben-Barak, 1977; Aguilar and

Lunt, 1984; Abdullah *et al.*, 1991). Binding of agonists to the muscarinic receptor shows complexity in their interactions (Birdsall *et al.*, 1978; Birdsall *et al.*, 1980). The receptor can exist in three interconvertible states, the proportions of which depends upon nucleotide and magnesium ion concentrations (Birdsall *et al.*, 1984). The dissociation constants for the super high, high and low binding sites for carbachol in the rat cerebral cortex were 77nM, 1.7μM and 125μM respectively (Birdsall *et al.*, 1980). Of the agonists tested only muscarine showed recognition of two sites (fig. 36, Ki low affinity = 500μM, Ki high affinity = 37.5μM). Pilocarpine showed the highest potency in inhibiting [^{3}H]-QNB binding (Ki 19.1μM). This was consistent with the finding from functional studies, in which pilocarpine was more potent than carbachol in stimulating phosphatidylinositol turnover. Thus, the receptor characterised using the ligand binding studies may be the same receptor which stimulated phosphatidylinositol turnover (see chapter 3). The low affinities of the agonists for the receptor may be due to the absence of any super high or high affinity states of the receptor in the conditions employed in these assays. In the rat cortex (Birdsall *et al.*, 1980), [^{3}H]-oxotremorine-M was found to almost exclusively bind to the highest affinity states of the muscarinic receptor. Measurement of its displacement by unlabeled oxotremorine-M gave the affinity and the abundance of the superhigh sites. Moreover, by the use of [^{3}H]-oxotremorine-M to label the high affinity state of the

cerebral cortex muscarinic receptors and [^{3}H]-N-methylscopolamine to label predominantly the low affinity state, it is possible to get an approximate affinity of any ligand for either state of the receptor. The ratio of affinities of these compounds in these assays appears to correlate with the ability of compounds to stimulate phosphatidylinositol turnover (Freedman *et al.*, 1990). This has led to the development of highly efficacious and potent muscarinic agonists such as L-670,207, a novel non-quaternary oxadiazole (EC_{50} in stimulating phosphatidylinositol turnover = 0.18μM, carbachol = 220μM, pilocarpine = 13μM). Similar studies could be undertaken with the receptor in the locust ganglia.

Figure 32.

[^{3}H]-QNB binding to locust ganglia.

Locust membranes (P2) were suspended in 1 ml of buffer (protein = 0.224 gm/ml) and incubated for 2hr at 25^{0}C with [^{3}H]-QNB (duplicates from 0-50nM). Binding was determined by filteration using a Brandell Cell Harvester. Total binding (filled inverted triangles) and non-specific binding in the presence of 0.1mM atropine (open circles) at each [^{3}H]-QNB concentration are shown. The percentage of non-specific binding was determined by regression analysis (4.8% (r=0. 98)). The data were obtained from one of four experiments (mean non-specific binding = 3.0% +/- 0.8 (S.E.M.), (r > 0.980), n = 4).

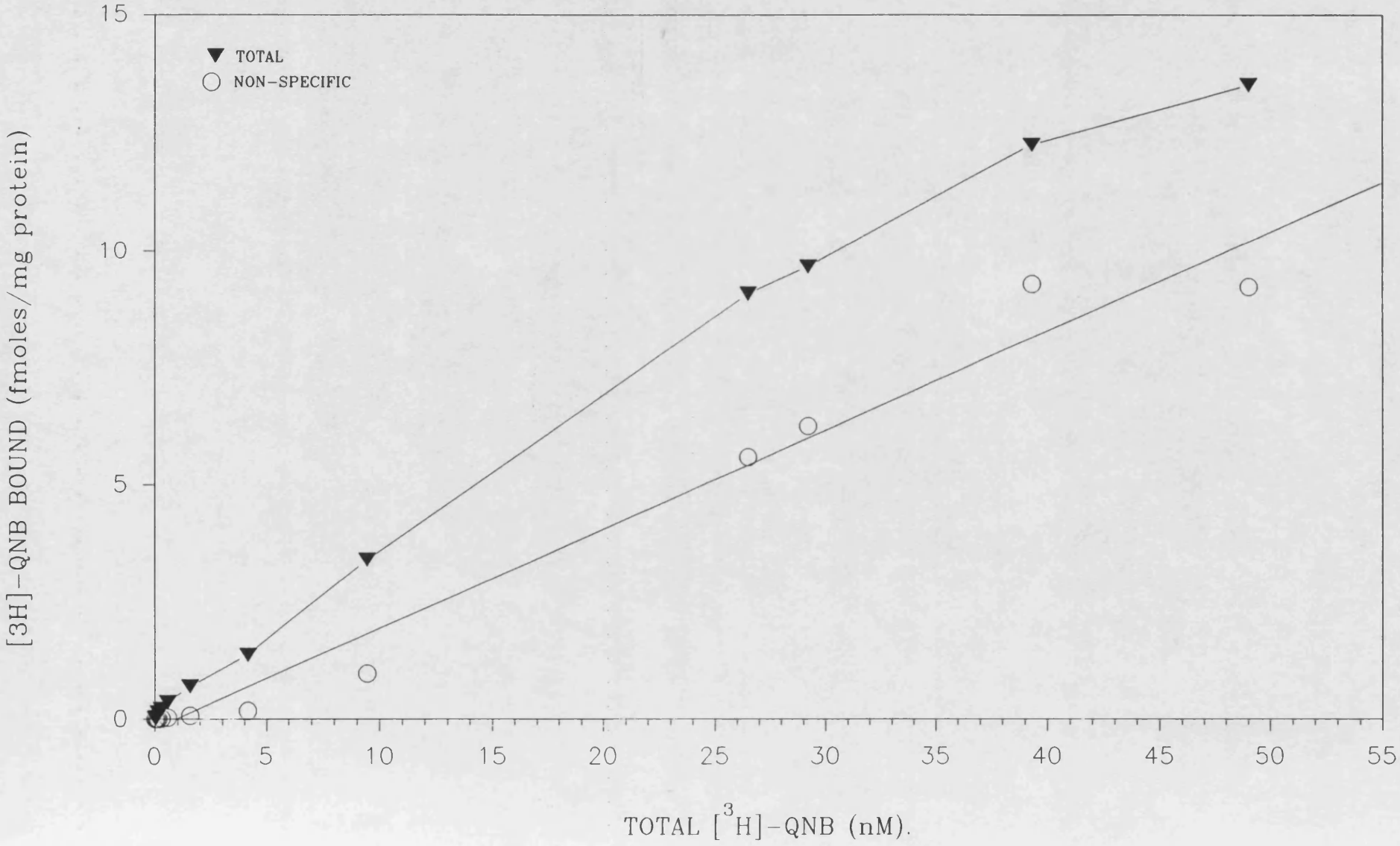
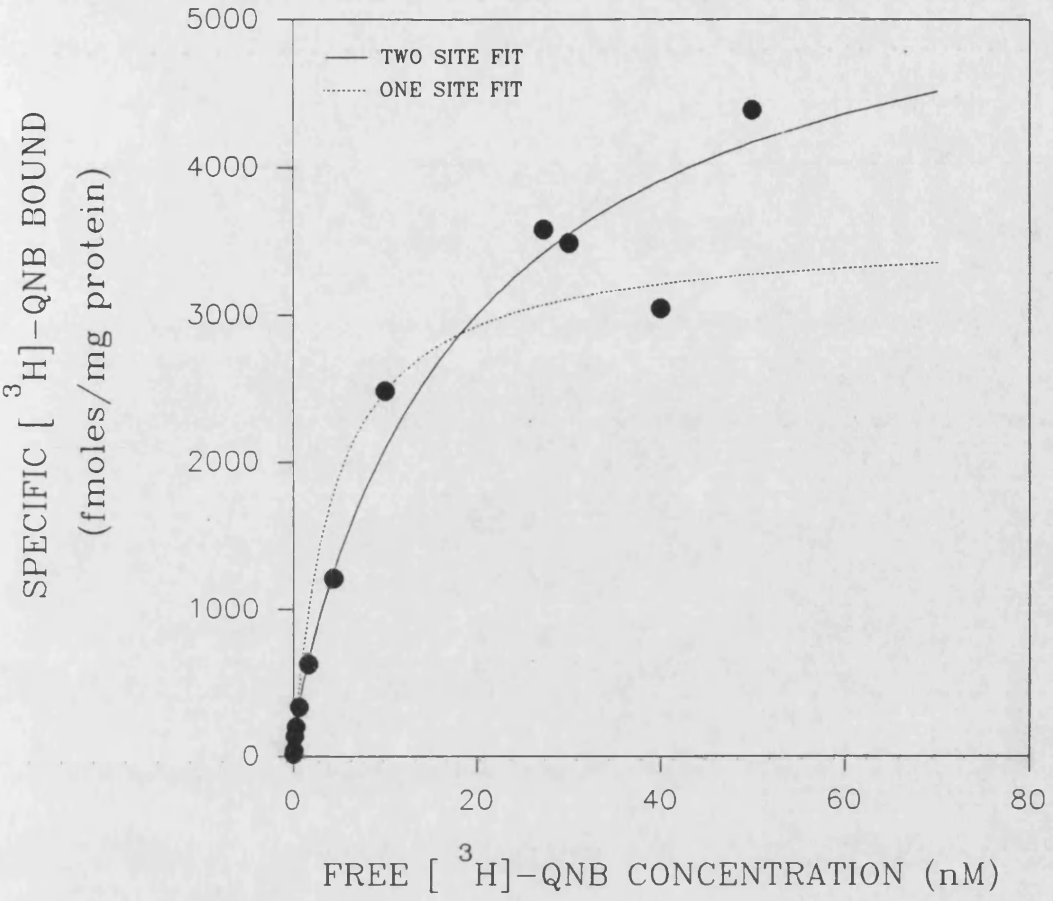

TOTAL
NON-SPECIFIC
[3H]-QNB BOUND (fmoles/mg protein)
TOTAL [3H]-QNB (nM).
0
5
10
15
20
25
30
35
40
45
50
55

Figure 33.

Specific binding of [^{3}H]-QNB to locust ganglia.

The saturation curve for specific binding (total minus non-specific from fig. 32) is shown in figure 33a. The data, from one of four experiments, were tranformed and represented as a Scatchard plot (fig. 33b). Results obtained from these four experiments were co-analysed using a computer assisted non-linear least-squares curve-fitting technique (LIGAND). A two site fit (Kd1 = 0.36nM, Kd2 = 39.8nM, Bmax1 = 300 fmoles/mg, Bmax2 = 5411 fmoles/mg) gave a significantly better fit ($p < 0.001$, using the F-test) than a one site fit (Kd 4.3nM, Bmax = 3550 fmoles/mg.). Two site and one site curve fits are represented by a solid and dashed lines respectively.


TWO SITE FIT
ONE SITE FIT
SPECIFIC [3H]-QNB BOUND
(fmoles/mg protein)
FREE [3H]-QNB CONCENTRATION (nM)
0
1000
2000
3000
4000
5000
0
20
40
60
80

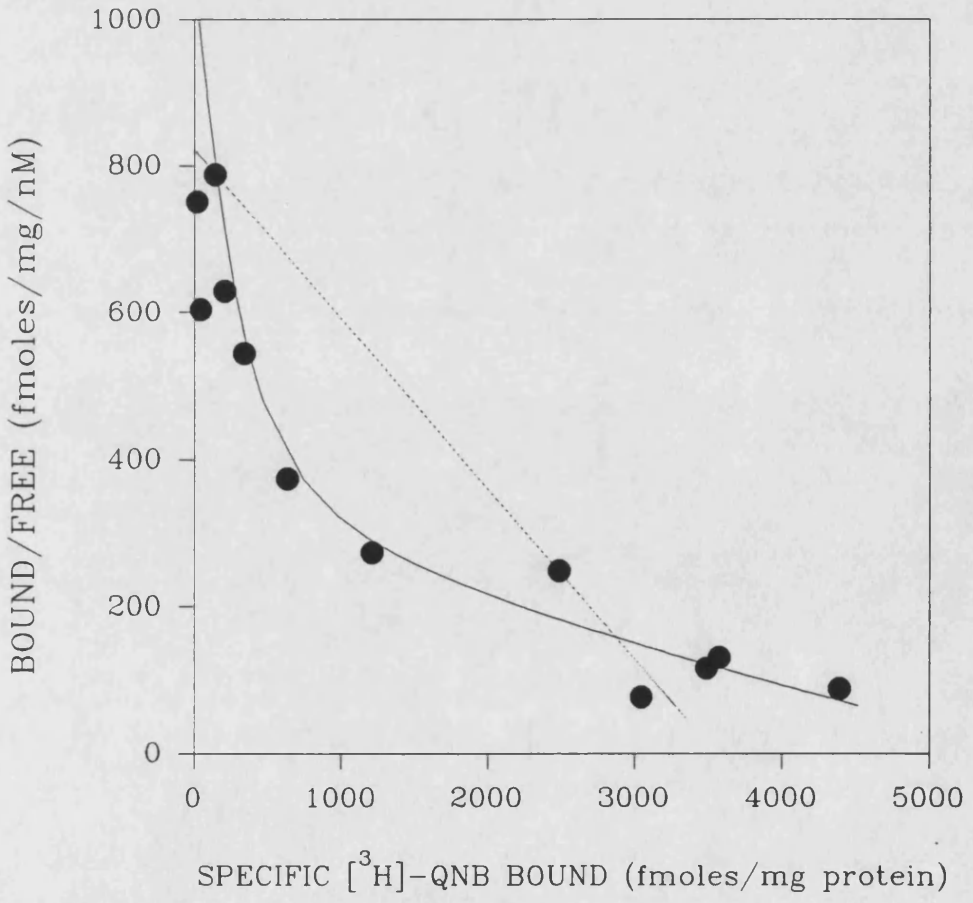

BOUND/FREE (fmoles/mg/nM)
SPECIFIC [3H]-QNB BOUND (fmoles/mg protein)
0
200
400
600
800
1000
0
1000
2000
3000
4000
5000

Figure 34

Theoretical determination of non-specific binding.

A Scatchard transformation of the total binding of [^{3}H]-QNB to locust P2 (from fig. 32) is shown. The non-specific binding component was determined from the asymptote of the curved plot. The dashed line represents the curve fit for one of the experiments (Kd = 0.25nM, Bmax = 233 fmoles/mg, % non-specific binding = 6.4%), and the solid line represents the curve obtained from the co-analysis of three experiments (Kd = 0.25nM, Bmax = 233 fmoles/mg, % non-specific = 4.0 +/- 0.4 (S.E.M.)).

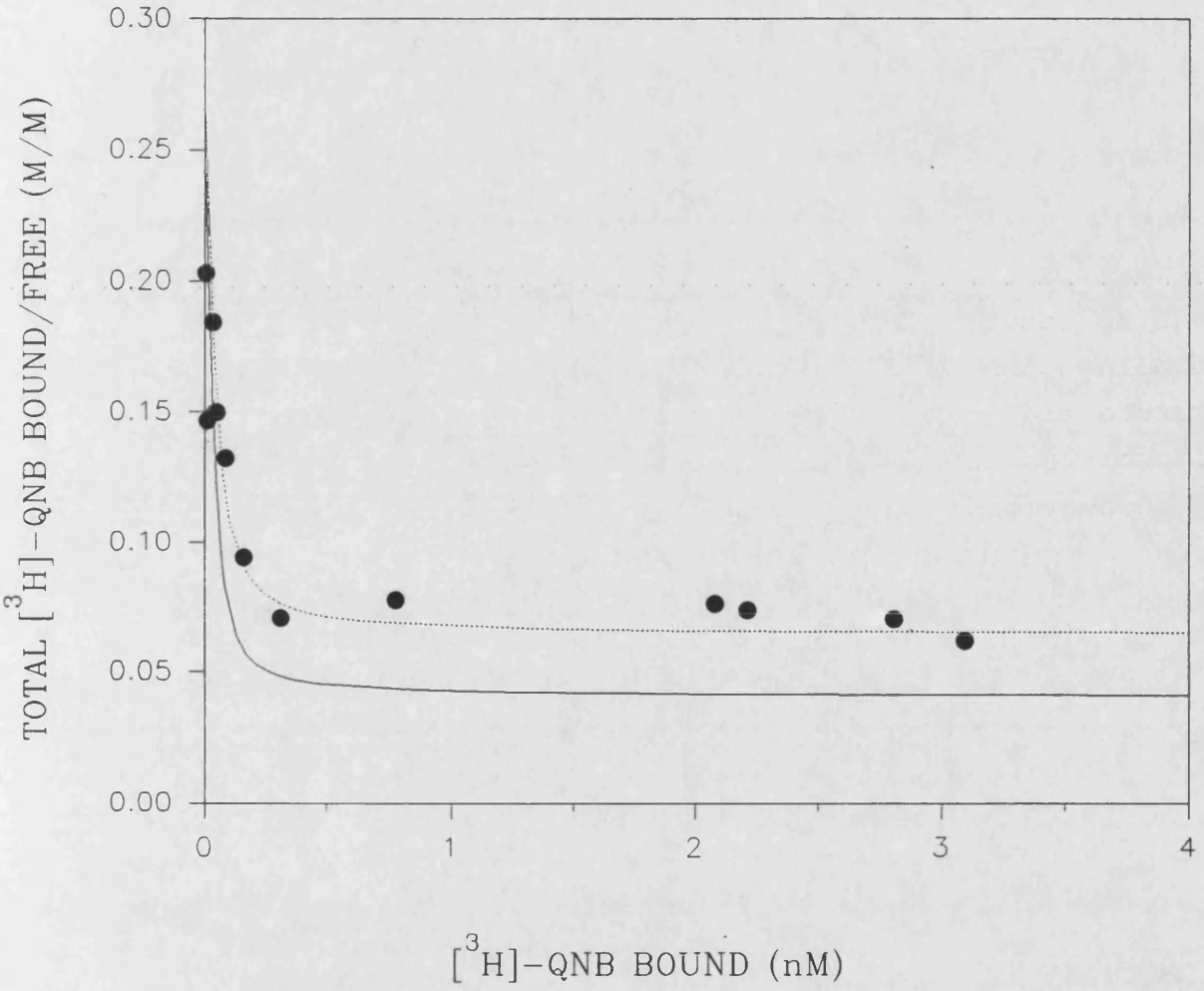

0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4
TOTAL [3H]-QNB BOUND/FREE (M/M)
[3H]-QNB BOUND (nM)

Figure 35.

Inhibition by 4-DAMP of total [^{3}H]-QNB binding.

Binding of 0.4nM [^{3}H]-QNB to locust purified P2 membranes (0.143 mg of protein/ml incubation buffer) was displaced by increasing concentrations of 4-DAMP (0.1nM to 1μM). The binding (duplicates) from one of four experiments is shown. The fitted curve was obtained from co-analysis of three [^{3}H]-QNB saturation and four 4-DAMP competition assays (two sites for [^{3}H]-QNB (Kd1 = 0.28nM, Kd2 = 13.3nM, Bmax1 = 145 fmol/mg, Bmax2 = 2905 fmol/mg) and two sites for 4-DAMP (Ki1 = 120nM, Ki2 = 24nM)).

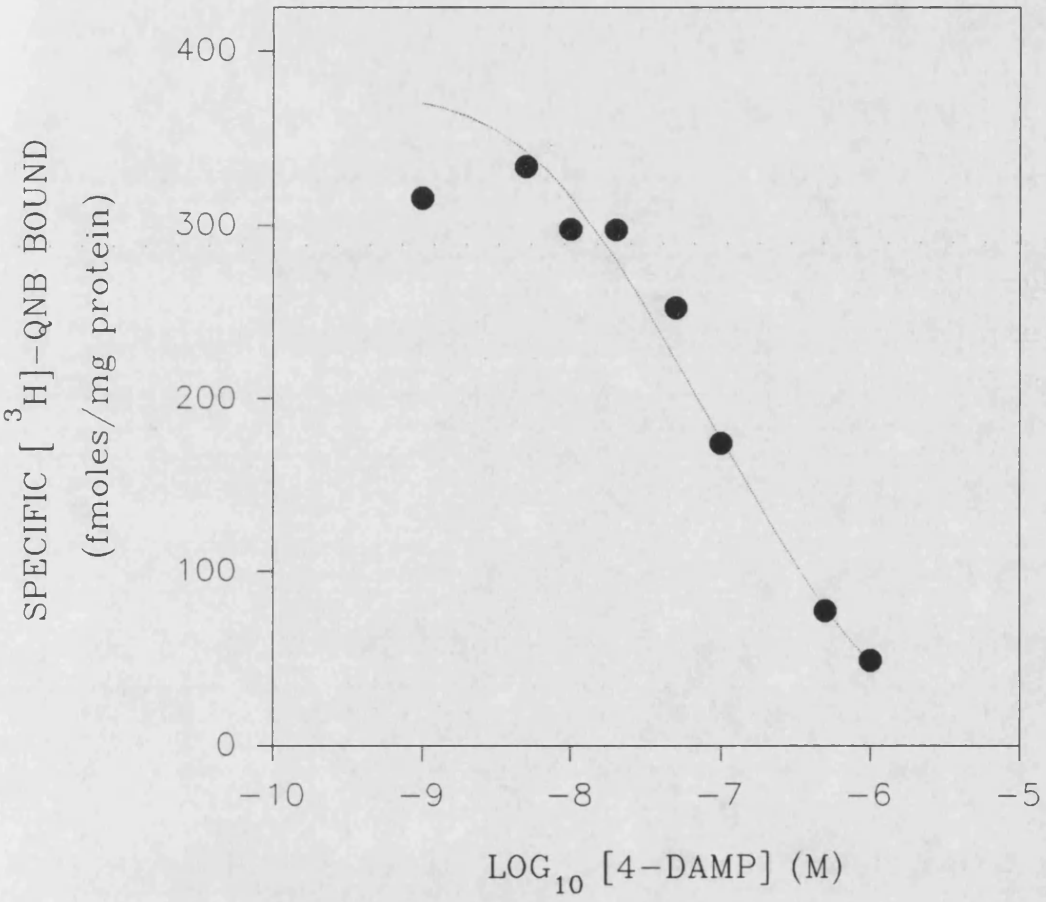

SPECIFIC [3H]-QNB BOUND
(fmoles/mg protein)
400
300
200
100
0
−10
−9
−8
−7
−6
−5
LOG10 [4−DAMP] (M)

Figure 36.

Inhibition by muscarine of total [^{3}H]-QNB binding.

Binding of 0.4nM [^{3}H]-QNB to P2 membranes (0.181 mg protein/ml incubation buffer) was displaced with increasing concentrations of muscarine (1μM to 1mM). The fitted curve was obtained from co-analysis of three [^{3}H]-QNB saturation and three competition assays (two sites for [^{3}H]-QNB (Kd1 = 0.23nM, Kd2 = 8.8nM, Bmax1 = 73 fmol/mg, Bmax2 = 1326 fmol/mg) and two sites for muscarine (Ki1 = 500μM, Ki2 = 37.5μM).

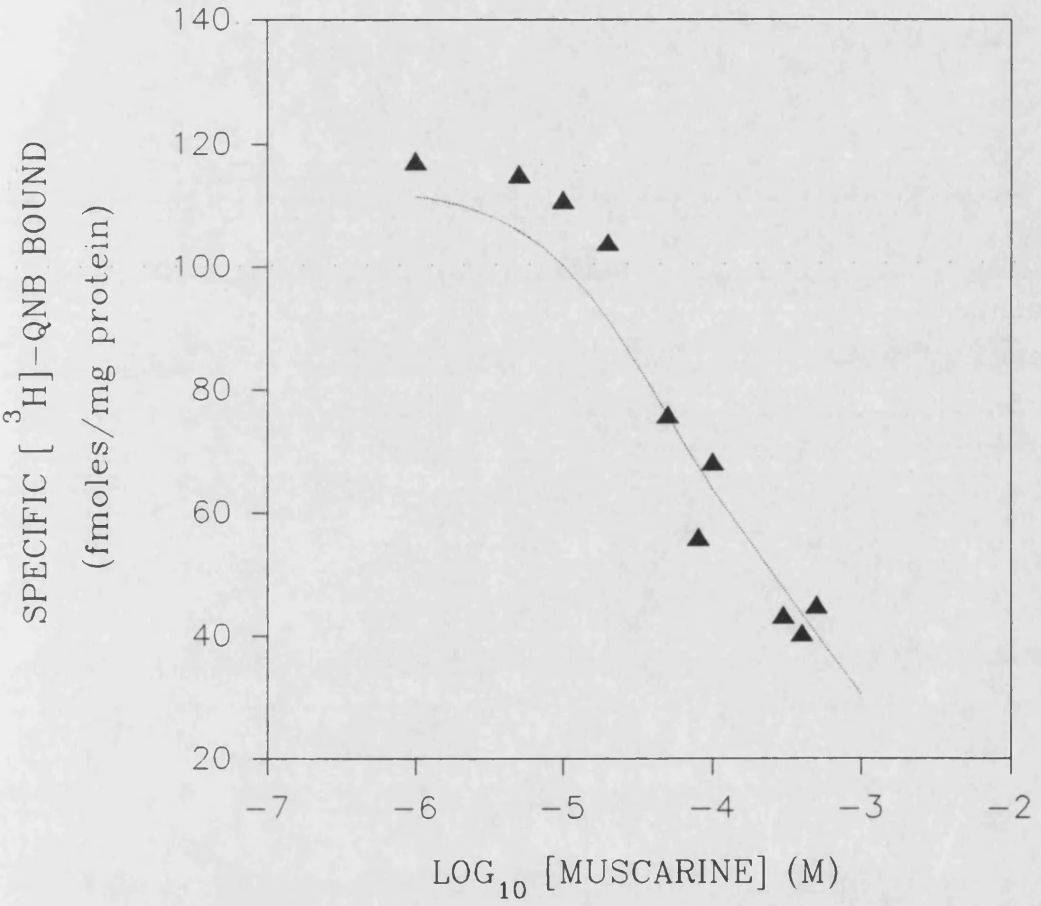

SPECIFIC [^{3}H]-QNB BOUND
(fmoles/mg protein)
140
120
100
80
60
40
20
−7
−6
−5
−4
−3
−2
LOG$_{10}$ [MUSCARINE] (M)

Table 6.

LIGAND analysis of [^{3}H]-QNB binding to locust P2.

Specific binding of [^{3}H]-QNB obtained from three experiments were tested using 5 binding models:
One site for the ligand, non-specific binding (N1) fixed at 0 (model 1).
Two sites for the ligand, N1 fixed at 0 (model 2).
One site for the ligand, N1 floated (model3).
Two sites for the ligand, N1 floated (model4)
Two sites for the ligand, the correction factors (C2 and C3) and N1 fixed (model 5).
The residual sum of squares for each binding model was determined. Significantly better fits were obtained for the two site models ($p < 0.0001$ between models 1 and 2 and $p < 0.002$ between models 3 and 4). There were no significant differences between models 1 and 3 and between models 2 and 4. This suggested that the second site was not part of the non-specific component. Model 5 was significantly different from model 2 ($p < 0.0001$), thus justifying the use of correction factors for Bmax (C1 = 1).

	Kd (H) nM	Kd (L) nM	Bmax(H) fmol/mg	Bmax(L) fmol/mg	N1 %oftotal	C2	C3	SUM OF SQUARES
MODEL1	4.40	----	1707	----	0	2.18	1.35	26428
MODEL2	0.38	20.0	173	3124	0	1.82	1.09	12660
MODEL3	1.40	----	657	----	0.90	1.87	1.13	18813
MODEL4	0.37	17.0	168	2746	0.16	1.82	1.09	12400
MODEL5	0.39	69.4	229	11417	0	1	1	34350

Table 7.

Inhibition of total [^{3}H]-QNB binding by muscarinic antagonists.

Binding of 0.4nM [^{3}H]-QNB to locust P2 membranes (approximately 0.2 mg protein per 1 ml incubation buffer) was displaced by N-methyl scopolamine (NMS), 4-DAMP, pirenzepine (PIREN), AFDX116 and methoctramine (METHOC) for 'n' experiments. For each drug the competition data were co-analysed with three [^{3}H]-QNB saturation curves (dissociation constant and receptor concentration for the high affinity site (Kd(H), Bmax(H), and the low affinity site (Kd(L), Bmax(L) are shown). From using a curve fitting technique to obtain the least residual sum of squares for each binding model, the inhibition constants were determined for one and two sites (Ki(L), Ki(H)). Significance was tested using the F-test (F-ratio obtained against a defined model '****'). Significantly better curve fits were obtained for two [3H]-QNB sites (compare models 1 and 2 for all drugs ($p = 0.006-0.00001$)). 4-DAMP recognised two sites (compare models 2 and 3 (F-ratio = 5.8, $p = 0.019$).

DRUG	MODEL	QNB SITES Kd(H) nM	Kd(L) nM	COMPETITIVE DRUG Ki(L) µM	Ki(H) µM	RECEPTOR CONC. Bmax(H) fmol/mg	Bmax(L) fmol/mg	F-TEST RATIO
NMS	1	1.30	----	0.007	----	570	----	****
(n=2)	2	0.003	2.2	0.006	0.006	5	774	5.8
	3	----	----	----	----	----	----	----
4-DAMP	1	2.50	----	0.094	----	875	----	12.6
(n=4)	2	0.25	12.3	0.076	0.076	93	1898	5.8
	3	0.28	13.3	0.120	0.024	102	2015	****
PIREN	1	2.79	----	3.80	----	1079	----	10.9
(n=6)	2	0.30	13.3	2.84	2.84	122	2145	2.1
	3	0.34	14.5	3.97	1.25	152	2422	****
AFDXII6	1	1.30	----	12.60	----	596	----	7.0
(n=3)	2	0.29	9.9	14.1	14.1	113	1640	0
	3	0.38	22.6	32.0	4.3	159	3380	****
METHOC	1	1.90	----	2.79	----	613	----	42.4
(n=5)	2	0.25	247	3.87	3.87	108	32190	****
	3	----	----	----	----	----	----	----

Table 8.

Inhibition of total [^{3}H]-QNB binding by muscarinic agonists.

Binding of 0.4nM [^{3}H]-QNB was inhibited by muscarine (MUSC), carbachol (CARB), pilocarpine (PILO) and methacholine (METHA). The results were analysed using LIGAND as for the antagonists (see table 7). Two site fits for [3H]-QNB (model 2) were significantly better than one site fits (model 1) (p ranged from 0.005 to 0.00001). When comparing models 2 and 3, only muscarine recognised two sites (F-ratio = 6.4, p = 0.015).

DRUG	MODEL	QNB SITES Kd(H) nM	Kd(L) nM	COMPETITIVE DRUG Ki(L) µM	Ki(H) µM	RECEPTOR CONC. Bmax(H) fmol/mg	Bmax(L) fmol/mg	F-TEST RATIO
MUSC	1	1.26	----	160	----	712	----	8.02
(n=3)	2	0.24	7.89	155	155	121	1766	****
	3	0.23	8.77	500	37.5	113	2064	6.4
CARB	1	1.07	----	565	----	640	----	****
(n=2)	2	0.24	7.4	834	834	124	1719	6.1
	3	----	----	----	----	----	----	----
PILO	1	2.71	----	21.7	----	1125	----	****
(n=3)	2	0.47	22.1	19.1	19.1	198	3098	26.6
	3	----	----	----	----	----	----	----
METHA	1	1.4	----	725	----	644	----	****
(n=3)	2	0.32	10.2	770	770	133	1620	5.5
	3	0.33	10.6	870	618	136	1687	0

REFERENCES

Abdullah, E.A.M., Eldefrawi, M.E., Eldefrawi, A.T. (1991). Pharmacological characterization of muscarinic receptors of insect brains. *Archives of Insect Biochemistry and Physiology*, **17**, No.2-3, 107-118.

Aguilar, J.S. and Lunt, G.G (1984). Cholinergic binding sites with muscarinic properties on membranes from the supraoesophageal ganglion of the locust (*Schistocerca gregaria*). *Neurochem. Int.*, **6**, 501-507.

Aguilar, O, Fonseca, M. and Lunt, G.G. (1984). Susceptibility of a muscarinic acetylcholine receptor from locust (*Shistocerca gregaria*) central nervous system to perturbation by ethanol and by sulphydryl reagents. *Biochemical Society Transactions*, **12**, 809-810.

Allison, J.H., Blisner, M.E., Holland, W.H., Hipps, P.P. and Sherman, W.R. (1976). Increased brain myo-inositol 1-phosphate in lithium treated rats. *Biochem. Biophys. Res. Commun.*, **71**, 664-670.

Bansal, V.S. and Majerus, P.W. (1990). Phosphatidylinositol derived precursors and signals. *Annu. Rev. Cell Biol.*, **6**, 41-67.

Batty, I.H., Letcher, A.J. and Nahorski, S.R. (1989). Accumulation of inositol polyphosphate isomers in agonist stimulated cerebral cortex slices. *Biochemical Journal*, **258**, 23-32.

Benham, C.D. and Tsien, R.W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature*, **328**, 275-278.

Bennett, J.P. and Yamamura, H.I. (1985). Neurotransmitter, hormone, or drug receptor binding methods. Yamamura, H.I., Enna, S.J. and Kuhar, M.J., editors. *Neurotransmitter receptor binding*, 2nd edition. New York: Raven Press, 61-89

Benson, J.A. (1988). Transmitter receptors on insect neuronal somata: GABAergic and cholinergic pharmacology. In *The Molecular Basis of Drug and Pesticide Action-Neurotox '88*, ed. Lunt, G.G., Elsevier Biomedical, Amsterdam, 193-206.

Benson, J.A. (1992). Natural and synthetic toxins at insect receptors and ion channels: the search for insecticide leads and target sites. In *The Molecular Basis for Drug and Pesticide Action-Neurotox '91*, ed. Duce I.R., Elsevier Applied Science, London and New York, 57-76.

Berridge, M.J. (1983). Rapid accumulation of inositol trisphosphates reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochemical Journal*, **212**, 849-858.

Berridge, M.J. (1984). Inositol trisphosphate and diacylglycerol as second messengers. *Biochemical Journal*, **220**, 345-360.

Berridge, M.J. (1988). Inositol trisphosphate induced membrane potential oscillations in *Xenopus* oocytes. *Journal of Physiology* (Lon), **403** (Sep), 589-599.

Berridge, M.J. and Fain, J.N. (1979). Inhibition of phosphatidylinositol synthesis and the inactivation of calcium entry after prolonged exposure of the blowfly salivary gland to 5-hydroxytryptamine. *Biochemical Journal*, **178**, 59-69.

Berridge, M.J. and Heslop, J.P. (1981). Separate 5-

Hydroxytryptamine receptors on the salivary gland of the blowfly are linked to the generation of cyclic adenosine 3, 5-monophosphate or calcium signals. *British Journal of Pharmacology*, **73**, 729-738

.

Berridge, M.J. and Irvine, R.F. (1989). Inositol phosphate and cell signalling. *Nature*, **341**, 197-205.

Birdsall, N.J.M. and Hulme, E.C. (1983). Muscarinic receptor subclasses. *TIPS*, **4**, 459-463.

Birdsall, N.J.M., Burgen, A.S.V., Hulme, E.C. (1978). The binding of agonist to brain muscarinic receptors. *Molecular Pharmacology*, **14**, 723-736.

Birdsall, N.J.M., Hulme, E.C. and Burgen Sir, A. (1980). The character of the muscarinic receptors in different regions of the rat brain. *Proc. R. Soc. Lond B.*, **207**, 1-12.

Birdsall, N.J.M., Hulme, E.C. and Stockton, J.M. (1984). Muscarinic receptor heterogeneity. *Trends in Pharmacological Sciences*, **Suppl. I**, 4-8.

Birdsall, N.J.M., Chan, S, Eveleigh, P. Hulme, E.C. and Miller, K.W. (1989). The modes of binding of ligands to cardiac muscarinic receptors. *TIPS Supplement: Subtypes of Muscarinic Receptors IV*, 31-34.

Bonner, T.I., Buckley, N.J., Young, A.C. and Brann, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science*, **237**, 527-532.

Bonner, T.I., Young, A.C., Brann, M.R. and Buckley, N.J. (1988). Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron*, **1**, 403-410.

Breer, H. (1981). Properties of putative nicotinic and muscarinic cholinergic receptors in the central nervous system of *Locust migratoria*. *Neurochem. Int.*, **3**, 43-52.

Breer, H. and Knipper, M. (1984). Characterisation of acetylcholine release from insect synaptosomes. *Insect Biochemistry*, **14**, 337-344.

Brown, J.H. and Goldstein, D. (1986). Differences in muscarinic receptor reserve for inhibition of adenylate cyclase and stimulation of phosphoinositide hydrolysis in chick heart cells. *Molecular Pharmacology*, **30**, 566-570.

Buckley, N.J., Bonner, N.J., Buckley, C.M. and Brann, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors, expressed in CHO-K1 cells. *Molecular Pharmacology*, **35**, 469-476.

Burgen, A.S.V. Hiley, C.R. and Young, J.M. (1974a). The binding of [^{3}H]-propylbenzilylcholine mustard by longitudinal muscle strips from guinea-pig small intestine. Br. J. Pharmac., 50, 145-151.

Burgen, A.S.V. Hiley, C.R. and Young, J.M. (1974b). The properties of muscarinic receptors in mammalian cerebral cortex. *Br. J. Pharmac.*, **51**, 279-285.

Bylund, D.B. (1986). Graphic presentation and analysis of inhibition data from ligand binding experiments. *Analytical Biochemistry*, **159**, 50-57.

Caufield, M.P. and Stubley, J.K. (1982). Pilocarpine selectivity stimulates muscarinic receptors in rat sympathetic ganglia. *British Journal of Pharmacology*, **76**, 216P.

Chapman, R.F. (1982). The insects, 3rd edition, Hodder and Stoughton, Hong Kong.

Chen, P.S. Jr., Tombara, T.Y. and Warner, H. (1956). Microdetermination of phosphorus. *Analytical Chemistry*, **28**, 1756-1758.

Clarke, B.S. and Donnellan, J.F. (1982). Concentrations of some putative neurotransmitters in the CNS of quick frozen insects. *Insect Biochem.*, **12**, 623-638.

Doods, H.N., Mathy, M., Davidesko D., Charldorp, K.J., Jonge, A. and Zwieten, P.A. (1987). Selectivity of muscarinic antagonists in radioligand and in vivo experiments for putative M_1, M_2 and M_3 receptors. *The Journal of Pharmacology and Experimental Therapeutics*, **242**, 257-262.

Downes, C.P. and MacPhee, C.H. (1990). Myo-inositol metabolites as cellular signals. *Eur. J. Biochem.*, **193**, 1-18.

Downes, C.P., Dibner, M.D. and Hanley, M.R. (1983). Sympathetic denervation impairs agonist-stimulated phosphatidylinositol metabolism in rat parotid glands. *Biochemical Journal*, **214**, 865-870.

Downes, C.P. and Wusteman, M.M. (1983). Breakdown of polyphosphoinositides and not phosphatidylinositol accounts for muscarinic agonist-stimulated inositol phospholipid metabolism in rat parotid gland. *Biochemical Journal*, **216**, 633-640.

Drummond, A.H., Bushfield, M and MacPhee, C.H. (1984). Thyrotrophin-releasing hormone-stimulated [^{3}H]-inositol metabolism in GH_3 pituitary tumor cells: studies with lithium. *Molecular Pharmacology*, **25**, 201-208.

Dudai, Y. and Ben-Barak, J. (1977). Muscarinic receptor in Drosophila melanogaster demonstrated by binding of [^{3}H]-

quinuclidinyl benzilate. *FEBS Letters*, **81**, No. 1, 134-136.

Duggan, M.J. (1987). Studies on the muscarinic acetylcholine receptors of the locust. Ph.D. thesis (Bath Uni.).

Eldefrawi, A.T., Mansour, N.A. and Eldefrawi, M.E. (1982). Insecticides affecting acetylcholine receptor interactions. *Pharmac. Ther.*, **16**, 45-65.

Ellis, J. and Seidenberg, M. (1989). Gallamine exerts biphasic allosteric effects at muscarinic receptors. *Molecular Pharmacology*, **35**, 173-176.

Entzeroth, M. and Mayer, N. (1990). Labeling of rat heart muscarinic receptors using the new M_2 selective antagonist [^{3}H]-AFDX384. *Biochemical Pharmacology*, **40**, No. 7, 1674-1676.

Endemann, G., Dunn, S.N. and Canley, L.C. (1987). Bovine brain contains two types of phosphatidylinositol kinase. *Biochemistry*, **26**, 6845-6852.

Enyedi, P. and Williams, G.H. (1988). Heterogenous inositol tetrakisphosphate binding sites in the adrenal cortex. *The Journal of Biological Chemistry*. **263**, 7940-7942.

Erneux, C., Lemos, M., Verjans, B., Vanderhaegan, P., Delvaux, A. and Dumont, J.E. (1989). Soluble and particulate Ins(1,4,5)P_3/ Ins(1,3,4,5)P_4 -5 phosphatase in bovine brain. *Eur. J. Biochem.*, **181**, 317-322.

Eveleigh, P., Hulme, E.C., Schudt, C. and Birdsall, N.J.M. (1989). The existence of stable enantiomers of telenzepine and their stereoselective interaction with muscarinic receptor subtypes. *Molecular Pharmacology*, **35**, 477-483.

Fain, J.N. and Berridge, M.J. (1979). Relationship between hormone activation of phosphatidylinositol hydrolysis, fluid secretion and calcium flux in the blowfly salivary gland. *Biochemical Journal*, **178**, 45-58.

Fain, J.N., Lin, Sue-Hwa, Litosch, I., and Wallace, M. (1983). Hormonal regulation of phosphatidylinositol breakdown. *Life Sciences*, **32**, 2055-2067.

Ferris, C.D. Supattapone, S., Huganir, R.L. and Snyder, S.H. (1989). Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. *Nature*, **342**, 87-89.

Findlay, J.B.C. and Pappin, D.J.C. (1986). The opsin family of proteins. *Biochemical Journal*, **238**, 625-642.

Fisher, S.K. and Agranoff, B.W. (1981). Enhancement of the muscarinic synaptosomal phospholipid labelling effect by the ionophore A23187. *Journal of Neurochemistry*, **37(4)**, 968-977.

Fisher, S.K. ans Snider, R.M. (1987). Differential receptor occupancy requirements for muscarinic cholinergic stimulation of inositol lipid hydrolysis in brain and neuroblastomas. *Molecular Pharmacology*, **32**, 81-90.

Fonseca, M.T., Lunt, G.G. and Aguilar, J.S. (1991). Inhibition of muscarinic cholinergic receptors by disulfide reducing agents and arsenicals: Differential effects on locust and rat. *Biochemical Pharmacology*, **41**, No. 5, 735-742.

Fraser, C.M., Wang, C., Robinson,D.A., Gocayne, J.D. and Venter, J.C. (1989). Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: Conserved aspartic acids play important roles in receptor function. *Molecular Pharmacology*, **36**, 840-847.

Freedman, S.B., Harley, E.A., Patel, S., Newberry, N.R., Gilbert, M.J., McKnight, A.T., Tang, J.K., Maguire, J.J., Mudunkotuwa, N.T. Baker, R., Street, L.J., MacLeod, A.M., Saunders, J. and Iversen, L.L. (1990). A novel series of non-quaternary oxadiazoles acting as full agonists at muscarinic receptors. *British Journal of Pharmacology*, **101**, 575-580.

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989). Primary structure and functional expression of inositol 1,4,5-trisphosphate-binding protein P400. *Nature*, **342**, 32-38.

Ghosh, T.K., Mullaney, J.M., Tarazi, F.I. and Gill, D.L. (1989). GTP-activated communication between distinct inositol 1,4,5-trisphosphate-sensitive and -insensitive calcium pools. *Nature*, **340**, 236-239.

Gillard, M., Waelbroeck, M. and Christophe, J. (1987). Muscarinic receptor heterogeneity in rat central nervous system II. Brain receptors labelled by [^{3}H]-oxotremorine-M correspond to heterogenous M_2 receptors with very high affinity for agonists. *Molecular Pharmacology*, **32**, 100-108.

Hallcher, L.M. and Sherman, W.R. (1980). The effects of lithium ion and other agents on the activity of myo-inositol 1-phosphatase from bovine brain. *The Journal of Biological Chemistry*, **255**, 10896-10901.

Hammer, R. and Giachetti, A. (1982). Muscarinic receptor subtypes: M_1 and M_2 biochemical and functional characterisation. *Life Sciences*, **31**, 2991-2998.

Hammer, R., Berrie, C.P., Birdsall, N.J.M., Burgen, A.S.V. and Hulme, E.C. (1980). Pirenzepine distinguishes between subclasses of muscarinic receptor. **Nature**, **283**, 90-92.

Hammer, R., Giraldo, E., Schiavi, G.B., Monferini, E. and Ladinsky, H. (1986). Binding profile of a novel cardioselective muscarine receptor antagonist, AFDX116, to membranes of peripheral tissues and brain in the rat. *Life Sciences*, **38**, 1653-1662.

Harary, H.H. and Brown, J.E. (1984). Spatially non-uniform changes in intracellular calcium ion concentrations. *Science (NY)*, **224**, 292-294.

Henis, Y.I. and Sokolovski, M. (1983). Muscarinic antagonists induce different receptor conformation in rat adenohypophysis. *Molecular Pharmacology*, **24**, 357-365.

Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E. and Downing, K.H. (1990). Model for the structure of bacteriorhodopsin based on high-resolution electron cryomicroscopy. *The Journal of Biological Chemistry*, **213**, 899-929.

Higashida, H. and Brown, D.A. (1986). Two polyphosphatidyl-inositide metabolites control two K^+ currents in a neuronal cell. *Nature*, **323**, 333-335.

Hofmann, S.L. and Majerus, P.W. (1982). Identification and properties of two distinct phosphatidylinositol specific phospholipase C enzymes from sheep seminal vesicular glands. *The Journal of Biological Chemistry*, **257**, 6461-6469.

Hosey, M.M. (1992). Diversity of structure, signalling and regulation within the family of muscarinic cholinergic receptors. *FASEB Journal*, **6**, No. 3, 845-852.

Hoss, W. and Ellis, J. (1985). Muscarinic receptor subtypes in the central nervous system. *International Review of Neurobiology*, **26**, 151-199.

Hulme E.C., Birdsall, N.J.M., Burgen, A.S.V. and Mehta, P. (1978). The binding of antagonists to brain muscarinic receptors. *Molecular Pharmacology*, **14**, 737-750.

Hulme, E.C., Berrie, C.P., Birdsall, N.J.M., Jameson, M. and Stockton, J.M. (1983). Regulation of muscarinic agonist binding by cations and guanine nucleotides. *Eur. J. Pharmacol.*, **94**, 59-72.

Hulme, E.C., Curtis, C.A.M., Wheatley, M., Aitken, A. and Harris, A.C. (1989). Localization and structure of the muscarinic receptor ligand-binding site. *Trends in Pharmacological Sciences*, **Suppl. Subtypes of Muscarinic Receptors IV**, 22-25.

Hulme, E.C., Birdsall, N.J.M. and Buckley, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633-673.

Hulme, E.C., Kurtenbach, E. and Curtis, C.A.M. (1991). Muscarinic acetylcholine receptors: Structure and function. *Biochemical Society Transactions*, **19**, No. 1, 133-138.

Imai, A. and Gershengorn, M.C. (1986). Phosphatidylinositol 4,5-bisphosphate turnover is transient while phosphatidylinositol is persistent in thyrotrophin releasing hormone-stimulated rat pituitary cells. *Proc. Natl. Acad. Sci. USA*, **83**, 8540-8544.

Imai, A. and Gershengorn, M.C. (1987). Independent phosphatidylinositol synthesis in pituitary plasma membrane and endoplasmic reticulum. *Nature*, **325**, 726-730.

Irvine, R.F. (1990). Quantal Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates - a possible mechanism. *FEBS letters*, **263**, No.1, 5-9.

Irvine, R.F. and Moor, R.M. (1986). Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. *Biochemical Journal*, **240**, 917-920.

Irvine, R.F., Anggard, E.E., Letcher, A.J. and Downes, C.P. (1985). Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate in rat parotid glands. *Biochemical Journal*, **229**, 505-511.

Irvine, R.F., Moor, R.M., Pollock, W.K., Smith, P.M. and Wreggett, K.A. (1988). Inositol phosphates: Proliferation, metabolism and function. *Phil. Trans. R. Soc. Lond. B.*, **320**, 281-298.

Jarv, J., Hedlund, B. and Bartfai, T. (1979). Isomerisation of the muscarinic receptor-antagonist complex, *The Journal of Biological Chemistry*, **254**, 5595-5598.

Jergil, B. and Sundler, R. (1983). Phosphorylation of phosphatidylinositol in rat liver golgi. *The Journal of Biological Chemistry*, **258**, No.13, 7968-7973.

Johanson, R.A., Hanson, C.A. and Williamson, J.R. (1988). Purification of D-myo-inositol 1,4,5-trisphosphate 3-kinase from rat brain. *The Journal of Biological Chemistry*, **263**, No. 16. 7465-7471.

Kanoh, H., Yamada, K. and Sakane, F. (1990). Diacylglycerol kinase: A key modulator of signal transduction? TIBS, 47-50.

Kenakin, T.P. (1984). The classification of drugs and drug receptors in isolated tissues, *Pharmacol. Rev.*, **36**, 165-222.

King, W.G. and Rittenhouse, S.E. (1989). Inhibition of protein kinase C by staurosporine promotes elevated accumulations of inositol trisphosphates and tetrakisphosphates in Human platelets exposed to thrombin. *The Journal of Biological Chemistry*, **264**, 6070-6074.

Kloog, Y., Egozi, Y. and Sokolovsky, M. (1979). Characterisation of muscarinic acetylcholine receptors from mouse brain: Evidence for regional heterogeneity and isomerisation. *Molecular Pharmacology*, **15**, 545-558.

Knipper, M. and Breer, H. (1988). Subtypes of muscarinic receptors in insect nervous system. *Comp. Biochem. Physiol.*, **90C**, No. 1, 275-280.

Knipper, M. and Breer, H, (1989). Muscarinic receptors modulating acetylcholine release from insect synaptosomes. *Comp. Biochem. Physiol.* **93C**, No.2, 287-292.

Koreh, K. and Monaco, M.E. (1986). The relationship of hormone-sensitive and hormone-insensitive phosphatidylinositol to phosphatidylinositol 4,5-bisphosphate in the WRK-1 cell. *The Journal of Biological Chemistry*, **261**, No. 1, 88-91.

Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, Matsuo, H., Hirose, T. and Numa, S. (1986a). Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature*, **323**, 411-416.

Kubo, T., Maeda, A., Sugimoto, K., Akiba, I., Mikami, A., Takahashi, H., Haga, T., Haga, K., Ichiyama, A., Kangawa, M., Matsuo, H., Hirose, T. and Numa, S. (1986b). Primary structure of porcine cardiac muscarinic receptor deduced from the cDNA sequence. *FEBS Letters*, **209**, 367-372.

Kubo, T., Bujo, H., Akiba, I., Nakai, J., Mishina, M. and

Numa, S. (1988). Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling. *FEBS Letters*, **241**, 119-125.

Kurtenbach, E., Curtis, C.A.M., Pedder, E.K., Aitken, A., Harris, A.C.M. and Hulme, E.C. (1990). Muscarinic acetylcholine receptors. Peptide sequencing identifies residues involved in antagonist binding and disulfide bond formation. *The Journal of Biological Chemistry*, **265**, No. 23, 13702-13708.

Lambert, D.G., Burford, N.T. and Nahorski, S.R. (1992). Muscarinic receptor subtypes-inositol phosphates and intracellular calcium. *Biochemical Society Transactions*, **20**, No. 1, 130-135.

Lapied, B., LeCorronc, H. and Hue, B. (1990). Sensitive nicotinic and mixed nicotinic-muscarinic receptors in insect neurosecretory cells. *Brain Research*, **533**, 132-136.

Lapied, B., Tribut, F. and Hue, B. (1992). Effects of McN-A-343 on insect neurosecretory cells-Evidence for muscarinic-like receptor subtypes. *Neuroscience Letters*, **139**, No. 2, 165-168.

Lazareno, S., Buckley, N.J. and Roberts, F.F. (1990). Characterisation of muscarinic M_4 binding sites in rabbit lung, chicken heart and NG108-15 cells. *Molecular Pharmacology*, **38**, 805-815.

LeCorronc, H. and Hue, B. (1991). Pharmacological properties of presynaptic muscarinic receptors in the 6th abdominal ganglion of the cockroach, *Periplaneta americana*. *Pesticide Science*, **33**, No. 2, 205-211.

LeCorronc, H, Lapied, B. and Hue, B. (1991). M_2-like presynaptic receptors modulate acetylcholine release in the cockroach (*Periplaneta americana*) central nervous

system. *Journal of Insect Physiology*, **37**, No. 9, 647-652.

Lee, C.H., Park, D., Wu, D., Rhee, S.G. and Simon, M.I. (1992). Members of the Gqα subunit gene family activate phospholipase C ß isozymes. *The Journal of Biological Chemistry*, **267**, 16044-16047.

Lips, D.L., Majerus, P.W., Gorga, F.R., Young, A.T. and Benjamin, T.L. (1989). Phosphatidylinositol 3-phosphate is present in normal and transformed fibroblasts and is resistant to hydrolysis by bovine brain phospholipase C II. *The Journal of Biological Chemistry*, **264**, 8759-8763.

Litosch, I. and Fain, J.N. (1985). 5-Methyltryptamine stimulates phospholipase C-mediated breakdown of exogenous phosphoinositides by blowfly salivary gland membranes, *The Journal of Biological Chemistry*, **260**, No. 30, 16052-16055.

Litosch, I., Wallis, C. and Fain, J.N. (1985). 5-Hydroxytryptamine stimulates inositol phosphate production in a cell free system from blowfly salivary glands. *The Journal of Biological Chemistry*, **260**, 5464-5471.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, **193**, 265-275.

Lummis, S.C.R. and Satelle, D.B. (1985). Binding of N-[propionyl-^{3}H]-propionylated α-bungarotoxin and L-[benzilic-4,4'-^{3}H]-quinuclidinyl benzilate to CNS extracts of the cockroach *Periplaneta americana*. *Comp. Biochem. Physiol.*, **80C**, 75-83.

Lundberg, G.A. and Jergil, B. (1988). Generation of phosphatidylinositol 4,5-bisphosphate proceeds through an intracellular route in rat hepatocytes. *FEBS letters*, **240**, 171-176.

Maccallum, S.H., Barker, C.J., Hunt, P.A., Wong, N.S., Kirk, C.J. and Michell, R.H. (1989). The use of cells doubly labelled with [^{14}C]-inositol and [^{3}H]-inositol to search for a hormone-sensitive inositol lipid pool with atypically rapid metabolic turnover, *Journal of Endocrinology*, **122**, 379-389.

MacPhee, C.H. and Drummond, A.H. (1984). Thyrotrophin-releasing hormone stimulates breakdown of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate in GH_3 pituitary tumour cells. *Molecular Pharmacology*, **25**, 193-200.

Maroney, A.C. and Macara, I.G. (1989). Phorbol ester induced translocation of diacylglycerol kinase from the cytosol to the membrane in Swiss 3T3 cells. *The Journal of Biological Chemistry*, **264**, 2537-2544.

McSwain, J.L., Tucker, J.S., Essenberg, R.C. and Sauer, J.R. (1989). Brain factor induced formation of inositol phosphates in tick salivary glands. *Insect Biochemistry*, **19**, No. 4, 343-349.

Medh J.D. and Weigel, P.H. (1989). Separation of phosphatidylinositols and other phospholipids by two step one dimensional thin layer chromatography. *J.Lipid Res.*, **30**, 761-764.

Melchiorre, C. (1988). Polymethylene tetraamines: A new generation of selective muscarinic antagonists, *Trends in Pharmacological Sciences*, **9**, 216-220.

Merritt, J.E. and Rink, T.J. (1987). Rapid increases in cytosolic free calcium in responses to muscarinic agonists. *The Journal of Biological Chemistry*, **262**, 4958-4960.

Meyer, M.R. and Reddy, G.R. (1985). Muscarinic and nicotinic cholinergic binding sites in the terminal abdominal ganglion of the cricket (*Acheta domesticus*). *Journal of Neurochemistry*, **45**, 1102-1112.

Meyer, T., Holowka, D. and Stryer, L. (1988). Highly co-operative opening of calcium channels by inositol 1,4,5-trisphosphate. *Science (NY)*, **240**, 653-656.

Michell, R.H. (1975). Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta.*, **415**, 81-147.

Mignery, G.A., Sudhof, T.C., Takei, K. and DeCamilli, P. (1989). Putative receptor for inositol 1,4,5-trisphosphate similar to the ryanodine receptor. *Nature*, **342**, 192-195.

Mitchell, C.A., Connolly, T.M. and Majerus, P.W. (1989). Identification and isolation of a 75-kDa inositol polyphosphate 5-phosphatase from human platelets. *The Journal of Biochemistry*, **264**, 8873-8877.

Monaco, M.E. (1982). The phosphatidylinositol cycle in WRK-1 cells. Evidence for a separate hormone sensitive phosphatidylinositol pool. *The Journal of Biological Chemistry*, **257**, No. 5, 2137-2139.

Monaco, M.E. and Woods, D. (1983). Characterisation of the hormone sensitive phosphatidylinositol pool in WRK-1 cells. *The Journal of Biological Chemistry*, **258**, 15125-15129.

Morris, A.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987). synergism of inositol trisphosphate and tetrakisphosphate in activating Ca^{2+}-dependent K^{+} channels. *Nature*, **330**, 653-655.

Muallem, S., Pandol, S.J. and Beeker. T.G. (1989).

Hormone-evoked calcium release from intracellular stores is a quantal process. *The Journal of Biological Chemistry*, **264**, 205-212.

Muller. E., Hegewald, H., Jaroszewicz, K., Cumme, G.A., Hoppe, H. and Frunder, H. (1986). Turnover of phosphomonoester groups and compartmentation of polyphosphoinositides in human erythrocytes. *Biochemical Journal*, **235**, 775-783.

Munson, P.J. and Rodbard, D. (1980). Ligand: A versatile approach for characterisation of ligand binding systems. *Analytical Biochemisrty*, **107**, 220-239.

Nahorski, S.R. and Potter, V.L. (1989). Molecular recognition of inositol polyphosphates by intracellular receptors and metabolic enzymes. *Trends in Pharmacological Sciences*, **10.**, 139-144.

Nahorski, S.R., Ragan, I. and Challiss, R.A. (1991). Lithium and the phosphoinositide cycle: An example of uncompetitive inhibition and its pharmacological consequences. *Trends in Pharmacological Sciences*, **12**, 297-303.

Nakamura, F., Ogata, K., Shiozaki, K., Kameyama, K., Ohara, K., Haga, T. and Nukada, T. (1991). Identification of two novel GTP-binding α-subunits that lack apparent ADP-ribosylation sites for pertussis toxin. *The Journal of Biological Chemistry*, **266**, 12676-12681.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science*, **233**, 305-312.

Nunn, D.L., Potter, B.V.L. and Taylor, C.W. (1990). Molecular target sizes of inositol 1,4,5-trisphosphate receptors in liver and cerebellum. *Biochemical Journal*, **265**, 393-398.

Onai, T., FitzGerald, M.G., Arakawa, S., Gocayne, J.D., Urquhart, D.A., Hall, L.M., Fraser, C.M., McCombie, R.W. and Venter, J.C. (1989). Cloning, sequence analysis and chromosome localisation of a *Drosophila* muscarinic acetylcholine receptor. *FEBS letters*, **255**, No. 2, 219-225.

Palmer, S., Hawkins, P.T., Michell, R.H. and Kirk, C.J. (1986). The labelling of polyphosphoinositides with [^{32}P]-Pi and the accumulation of inositol phosphates in vasopressin-stimulated hepatocytes. *Biochemical Journal*, **238**, 491-499.

Park, D., Jhon, Deok-Young, Kriz, R., Knopf, J. and Rhee, S.G. (1992). Cloning, sequencing, expression, and Gq-independent activation of phospholipase C-ß2. *The Journal of Biological Chemistry*, **267**, 16048-16055.

Paulus, H. and Kennedy, E.P. (1960). The enzymic synthesis of inositol monophosphatide. *The Journal of Biological Chemistry*, **235**, 1303-1311.

Putney, Jr., J.W. (1986). A model for receptor-regulated calcium entry. *Cell Calcium*, **7**, 1-12.

Putney, Jr., J.W., Takemura, H., Hughes, A.R., Horstman, D.A. and Thastrup. O. (1989). How do inositol phosphates regulate calcium signaling ?. *FASEB J.*, **3**, 1899-1905.

Rhee, S.G., Suh, P.G., Ryu, S.H. and Lee, S.Y. (1989). Studies of inositol phospholipid-specific phospholipase C. Science, 244, 546-550.

Ringdahl, B. (1984). Determination of dissociation constants and relative efficacies of oxotremorine analogues at muscarinic receptors in guinea pig ileum by pharmacological procedures. *J. Pharmacol. Exp. Ther.*, **229**, 199-206.

Roddy, C.W., McSwain, J.L., Kocan, K.M., Essenberg, R.C. and Saver, J.R. (1990). The role of inositol 1,4,5-trisphosphate in mobilising calcium from intracellular stores in the salivary glands of *Amblyomna americanum* (L.). *Insect Biochemistry*, **20(1)**, 83-90.

Ross, C.A., Meldolesi, J., Milner, T.A., Satoh, T., Supattapone, S. and Snyder, S.H. (1989). Inositol 1,4,5-trisphosphate receptor localised to endoplasmic reticulum in cerebellar purkinje neurons. *Nature*, **339**, 468-470.

Ryu, S.H., Suh, P.G., Cho, K.S., Lee, K.Y. and Rhee, S.G. (1989). Bovine brain cytosol contains 3 immunologically distinct forms of inositol phopholipid specific phospholipase C. *Proc. Natl. Acad. Sci. USA*, **84**, 6649-6653.

Safrany, S.T., Wojcikiewicz, R.J.H., Strupish, J., McBain, J., Cooke, A.M., Potter, B.V.L. and Nahorski, S.R. (1991). Synthetic phosphorothioate-containing analogues of inositol 1,4,5-trisphosphate mobilize intracellular stores and interact differentially with inositol 1,4,5-triphosphate 5-phosphatase and 3-kinase. *Molecular Pharmacology*, **39**, 754-761.

Satelle, D.B. (1985). Acetylcholine receptors. Kerkut, G.A. and Gilbert, L.I. (editors), *Comprehensive Insect Physiol. Biochem. and Pharmacol.*, 2, 395-434, Pergamon Press, Oxford.

Seyfred, M.A. and Wells, W.W. (1984). Subcellular incorporation of ^{32}P into phosphoinositides and other phospholipids in isolated hepatocytes. *The Journal of Biological Chemistry*, **259**, No. 12, 7659-7665.

Shapiro, R.A., Wakimoto, B.T., Subers, E.M. and Nathanson, N.M. (1989). Characterisation and functional expression in

mammalian cells of genomic and cDNA clones encoding a Drosophila muscarinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA*, **86**, 9039-9043.

Shaker, N. and Eldefrawi, A. (1981). Muscarinic receptor in housefly brain and its interaction with chlorobenzilate. *Pest. Biochem. Physiol.*, **15**, 14-20.

Shaw, K. and Exton, J.H. (1992). Identification in bovine liver plasma membranes of a Gq-activatable phosphoinositide phospholipase C. *Biochemistry*, **31**, 6347-6354.

Shears, S.B. (1989). Review article. Metabolism of the inositol phosphates produced upon receptor activation. *Biochemical Journal*, **260**, 313-324.

Sherman, W.R., Leavitt, A.L., Honchar, M.P., Haacher, L.M. and Phillips, B.E. (1981). Evidence that lithium alters phosphoinositide metabolism: Chronic administration elevates primarily D-myo-inositol 1-phosphate in cerebral cortex of the rat. *J. Neurochem.*, **36**, 1947-1951.

Simpson, C.M.F., Batty, I.H. and Hawthorne, J.N. (1987). Physiological responses to receptor activation: phosphoinositide turnover. *Neurochemistry: A practical approach*. Ed. Turner, A.J. and Bachelard, H.S. IRL Press, Oxford.Washington D.C., pp 193-224.

Smith, C.D. and Wells, W.W. (1984). Solubilisation and reconstitution of a nuclear envelope associated ATPase. *The Journal of Biological Chemistry*, **259**, No. 19, 11890-11894.

Spat, A., Lukacs, G.L., Eberhardt, I., Kiesel, L. and Runnebaum, B. (1987). Binding of inositol phosphates and induction of Ca^{2+} release from pituitary microsomal fractions. *Biochemical Journal*, **244**, 493-496.

Stephens, L.R., Hughes, K.T. and Irvine, R.F. (1991). Pathway of phosphatidylinositol (3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature*, **351**, 33-39.

Stockton, J.M., Birdsall, N.J.M., Burgen, A.S.V. and Hulme, E.C. (1983). Modification of the binding properties of muscarinic receptors by gallamine. *Molecular Pharmacology*, **23**, 551-557.

Suh, P.G., Ryu, S.H., Moon, K.H., Suh. H.W. and Rhee, S.G. (1988). Cloning and sequence of multiple forms of phospholipase C. *Cell*, **54**, 161-169.

Supattapone, S., Worley, P.F., Baraban, J.M. and Snyder, S.H. (1988). Solubilization, purification and characterisation of an inositol triphosphate receptor. *The Journal of Biological Chemistry*, **263**, No.3, 1530-1534.

Supattapone, S., Danoff, S.K., Theibert, A., Joseph, S.K., Steiner, J. and Snyder, S.H. (1988). Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. *Proc. Natl. Acad. Sci. USA*, **85**, 8747-8750.

Takemura, H., Hughes, A,R., Thastrup, O. and Putney, Jr., J.W. (1989). Activation of calcium entry by the tumour promoter thapsigargin in parotid acinar cells: Evidence that an intracellular calcium pool, and not an inositol phosphate regulates calcium fluxes at the plasma membrane. *The Journal of Biological Chemistry*, **264**, No. 21, 12266-12271.

Taylor, C.W. and Potter, B.V.L. (1990). The size of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores depends on inositol 1,4,5-trisphosphate concentration. *Biochemical Journal*, **266**, 189-194.

Taylor, M.V., Metcalfe, J.C., Hesketh, T.R., Smith, G.A. and Moore, J.P. (1984). Mitogens increase phosphorylation of phosphoinositides in thymocytes. *Nature*, **312**, 462-465.

Thomas, A.P., Marks, J.S., Coll, K.E. and Williamson, J.R. (1983). Quantitation and early kinetics of inositol lipid changes induced by vasopressin in isolated and cultured hepatocytes. *The Journal of Biological Chemistry*, **258**, No. 9, 5716-5725.

Tietje, K.M., Goldman, P.S. and Nathanson, N.M. (1990). Cloning and functional analysis of a gene encoding a novel muscarinic acetylcholine receptor expressed in chick heart and brain. *The Journal of Biological Chemistry*, **265**, 2828-2834.

Trimmer, B.A. and Berridge, M.J. (1985). Inositol phosphates in the insect nervous system. *Insect Biochemistry*, **15**, No. 6, 811-815.

Trimmer, B.A. and Weeks, J.C. (1989). Effects of nicotinic and muscarinic agents on an identified motoneurone and its direct afferent inputs in larval *Manduca sexta*. *The Journal of Experimental Biology*, **144**, 303-337.

Trimmer, B.A. and Weeks, J.C. (1991). The mechanism of an excitibility change induced by muscarinic receptors in an identified motoneurone. *Soc. Neurosci. Abstr.*, **17(1)**, 199.

Vallar, L., Biden, T.J. and Wollheim, C.B. (1987). Guanine nucleotides induce Ca^{2+}-independent insulin secretion from permeabilised RINm5F cells. *The Journal of Biological Chemistry*, **262**, 5049-5056.

Venter, J.G., Fraser, C.M., Kerlavage, A.R. and Buck, M.A. (1989). Molecular biology of adrenergic and muscarinic cholinergic receptors. *Biochemical Pharmacology*, **38**, 1197-1208.

Volpe, P., Krause, Karl-Heinz, Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J., Lew, D.P. (1988). "Calciosome," a cytoplasmic organelle: The inositol 1.4.5-trisphosphate-sensitive Ca^{2+} store of non-muscle cells? *Proc. Natl. Acad. Sci. USA*, **85**, 1091-1095.

Waelbroeck, M., Gillard, M., Robberecht, P. and Christophe, J. (1986). Kinetic studies of [^{3}H]-N-methylscopolamine binding to muscarinic receptors in the rat central nervous system: Evidence for the existence of three classes of binding sites. *Molecular Pharmacology*, **30**, 305-314.

Waelbroeck, M., Tastenoy, M., Camus, J., Christophe, J., Strohmann, C., Linoh, H., Zilch, H., Tacke, R., Mutschler, E., and Lambrecht, G. (1989). Binding and fuctional properties of antimuscarinics of the hexocyclium/sila-hexocylium and hexahydro-diphenidol/hexahydro-sila-difenidol type to muscarinic receptor subtypes. *British Journal of Pharmacology*, **98**, 197-205.

Watson, M., Roeske, W.R. and Yamamura, H.I. (1982). [^{3}H]-Pirenzepine selectively identifies a high affinity population of muscarinic cholinergic receptors in the rat cerebral cortex. *Life Sciences*, **31**, 2019-2023.

Watson, M., Roeske, W.R. and Yamamura, H.I. (1986). [^{3}H]-Pirenzepine and (-)-[^{3}H]-quinuclidinyl benzilate binding ti rat cerebral cortical and cardiac muscarinic cholinergic sites. Characterisation and regulation of antagonist binding to putative muscarinic subtypes. *The Journal of Pharmacology and Experimental Therapeutics*, **237**, No. 2, 419-427.

Wess, J., Brann, M.R. and Bonner, T.I. (1989). Identification of a small intracellular region of the muscarinic M_3 receptor as a determinant of selective

coupling to PI turnover. *FEBS Letters*, **258**, 133-136.

Whipps, D.E., Armstom, A.E., Pryor, H.J. and Halestrap, A.P. (1987). Effects of glucagon and Ca^{2+} on the metabolism of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in isolated rat hepatocytes and plasma membranes.

Whitman, M., Kaplan, D.R., Schaffhausen, B., Cantley, L. and Roberts, T.M. (1985). Association of phosphatidylinostol kinase activity with polyoma middle-T competent for transformation. *Nature*, **315**, 239-242.

Whyte, J. and Lunt, G.G. (1986). The influence of guanine nucleotides on muscarinic receptor binding in the locust supraoesophageal ganglion. *Biochemical Society Transactions*, **14**, 690-691.

Wojcikiewicz, R.J.H., Cooke, A.M., Potter, B.V.L. and Nahorski, S.R. (1990). Inhibition of inositol 1,4,5-trisphosphate metabolism in permeabilised Sh-SY5Y human neuroblastoma cells by phophorothioate-containing analogues of inositol 1,4,5-trisphosphate. *Eur. J. Biochem.*, **192**, 459-467.

Worley, P.F., Heller, W.A., Snyder, S.H. and Baraban, J.M. (1988). Lithium blocks a phosphoinositide-mediated cholinergic response in hippocampal slices. *Science (NY)*, **239**, 1428-1429.

Wreggett, K.A. and Irvine, R.F. (1987). A rapid separation method for inositol phosphates and their isomers. *Biochemical Journal*, **245**, 655-660.

Wu, D., Lee, C.H., Rhee, S.G. and Simon, M.I. (1992). Activation of phospholipase C by the α-subunits of the Gq and G11 proteins in transfected Cos-7 cells. *The Journal of Biological Chemistry*, **267**, 1811-1817.

Yamamura, H.I. and Snyder, S.H. (1974a). Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. USA*, **71**, 1725-1729.

Yamamura, H.I. and Snyder, S.H. (1974b). Muscarinic cholinergic receptor binding in the longitudinal muscle of the guinea pig ileum with [^{3}H]-quinuclidinyl benzilate. *Molecular Pharmacology*, **10**, 861-867.

Yarfitz, S., Provost, N.M. and Hurley, J.B. (1988). Cloning of Drosophila melanogaster guanine nucleotide regulatory protein ß-subunit and characterisation of its expression during development. *Proc. Natl. Acad. Sci. USA*, **85**, 7134-7138.