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From the Department of Pharmacology
University of Umeå, Umeå, Sweden

Signal transduction in the brain

Modulation of receptor-mediated inositol phospholipid breakdown by potassium and fluoride ions

by

Gunnar Tiger

University of Umeå
Umeå 1990

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Gunnar Tiger

Umeå 1990

Abstract

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In recent years, the receptors in the brain coupled to PLC and modulation of such receptor-mediated PtdIns breakdown have been characterised. One such modulation is the "potassium effect", whereby an increase in the assay [K^+] from 6 to 18 mM potentiates the PtdIns breakdown response to the muscarinic agonist carbachol in the rat brain. It has been speculated that this effect is one way of enhancing the signal:noise ratio of muscarinic neurotransmission. The mechanisms responsible for the potassium effect have been studied in this thesis.

Initial methodological studies indicated that the temperature of the Krebs buffer used after tissue dissection was an important factor regulating the PtdIns response to receptor stimulation. Expressing the PtdIns breakdown response as a fraction of the total labelled phosphoinositides was more useful than other ways of expressing the data. Acid extraction of the Lipid fraction was also superior to neutral extraction.

Miniprisms prepared from pig striatum and hippocampus showed qualitative (but not quantitative) similarities with the rat with respect to stimulation by carbachol, noradrenaline and the potassium effect. Dopamine also stimulated PtdIns breakdown, though probably via a noradrenergic mechanism.

The enhancing actions of potassium appeared to be selective for muscarinic M1-type receptors. Thus glutamate, quisqualate and NaF-stimulated PtdIns breakdown are not affected by raised [K^+].

The potassium effect is brought about by two mechanisms. In calcium-free Krebs buffer, the effect could be mimicked by the calcium channel agonist BAY K-8644 and partially antagonised by verapamil. At an assay [Ca^{2+}] of 2.52 mM, however, modulation of calcium uptake had little effect on carbachol-stimulated PtdIns breakdown at either normal or raised [K^+]. The synergy between potassium and carbachol at 2.52 mM Ca^{2+} is not dependent upon tissue depolarisation per se, since other ways of depolarising the tissue did not enhance the response to carbachol. It is suggested that potassium might have a direct effect on the muscarinic M1-type receptor - G protein - PLC complex.

In order to investigate this possibility, the effect of fluoride ions (which activate G proteins via formation of AlF_4^-) on basal and carbachol-stimulated PtdIns breakdown was investigated. Fluoride ions inhibited the enhanced breakdown response to carbachol found at raised [K^+]. However, this effect is secondary to effects of fluoride on PLC substrate availibility rather than on G protein function.

Key words: Inositol phospholipid breakdown, muscarinic receptors, potassium, fluoride, rat brain, pig brain

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To my friends
and my surprise

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LIST OF PAPERS

The thesis is based on the following papers which will be referred to by their Roman numerals.

I. Fowler CJ, Court JA, Tiger G, Björklund P-E and Candy JM. Stimulation of inositol phospholipid breakdown in rat cortical and hippocampal miniprisms by noradrenaline, 5-hydroxytryptamine and carbachol: some methodological aspects. Pharmacol Toxicol 1987; 60: 274-9.

II. Tiger G, Björklund P-E and Fowler CJ. Stimulation of inositol phospholipid breakdown in pig brain by carbachol and monoamines: effect of K^+. Int J Biochem 1989; 21: 157-63.

III. Tiger G, Björklund P-E, Cowburn R and Fowler CJ. Enhancement by potassium of carbachol-stimulated inositol phospholipid breakdown in rat cortical miniprisms: comparison with other depolarising agents. J Neurochem 1989; 52: 1843-53.

IV. Tiger G, Björklund P-E, Cowburn R, Garlind A, O´Neill C, Wiehager B and Fowler CJ. Effect of monovalent ions upon G-proteins coupling muscarinic receptors to phosphoinositide hydrolysis in the rat cerebral cortex. Eur J Pharmacol [Mol Pharmacol Sect] 1990; 188: 51-62.

V. Tiger G, Björklund P-E and Fowler CJ. Differential enhancement by potassium ions of M1-type and M2-type muscarinic receptor-mediated phosphoinositide breakdown in the rat brain. Neurosci Letts. In press 1990.

VI. Tiger G, Björklund P-E and Fowler CJ. Dual mechanism of enhancement by potassium ions of carbachol-stimulated phosphoinositide breakdown in the rat cerebral cortex. Submitted 1990.

VII. Tiger G, Björklund P-E, Brännström G and Fowler CJ. Multiple actions of fluoride ions upon the phosphoinositide cycle in the rat brain. Submitted 1990.

ABBREVIATIONS

AC	Adenylate cyclase
ACh	Acetylcholine
ATP	Adenosine 5'-triphosphate
B_{max}	Total number of binding sites
cAMP	Cyclic adenosine 3',5'-monophosphate
5'AMP	Adenosine 5'-monophosphate
Cb	Carbachol (carbamoylcholine chloride)
CNS	Central nervous system
DA	Dopamine
DAG	1,2-diacylglycerol
d.p.m.	Disintegrations per minute
EAA	Excitatory amino acids
EC_{50}	Concentration of a drug causing half-maximal effect
GABA	γ-aminobutyric acid
GDP	Guanosine 5'-diphosphate
G_i	G protein(s) inhibitorily coupled to adenylate cyclase
G_o, $G_{(other)}$	G proteins other than G_i and G_s
Gpp[NH]p	Guanosine 5-[$\beta\gamma$-imido]triphosphate
G protein (G_p)	Guanine nucleotide binding protein
G_s	G protein(s) stimulatorily coupled to adenylate cyclase
GTP	Guanosine 5'-triphosphate
GTP[γ]S	Guanosine 5-[γ-thio]triphosphate
5-HT	5-Hydroxytryptamine
IC_{50}	Concentration of a drug causing half-maximal inhibition
InsP	Inositol phosphates
$Ins(1,4,5)P_3$	Inositol 1,4,5-trisphosphate
$Ins(1,3,4,5)P_4$	Inositol 1,3,4,5-tetrakisphosphate
K_D	Dissociation constant
mAChR	Muscarinic acetylcholine receptor
NA	Noradrenaline
QNB	Quinuclidinyl benzilate
PDE	3',5'-cyclic nucleotide phosphodiesterase
PKC	Protein kinase C
PLC	Phosphoinositide specific phospholipase C
PZ	Pirenzepine
$PtdIns(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate

1 INTRODUCTION

In 1905 Langley introduced the notion "receptive substance" to describe the part of the cell with which hormones, neurotransmitters and neuromodulators combine. In 1926 Clark suggested that a reversible monomolar reaction occurs between acetylcholine and its receptive substance, and that the latter was a very minor constituent of the cell. In general, cell surface receptors (such as those for acetylcholine) fall into two types: ligand gated ion channels (such as the nicotinic receptor) and G protein coupled receptor systems (such as for the muscarinic receptor). In the latter, receptors are coupled via GTP binding (G) proteins to a number of effector systems such as cyclic guanosine monophosphate (cGMP)-phosphodiesterase (rhodopsin receptor), phospholipase A_2 (e.g α_1-adrenoceptor), adenylate cyclase (AC) (e.g β-adrenoceptors and muscarinic receptors) and phosphoinositide specific phospholipase C (PLC) (e.g. α_1-adrenoceptor and muscarinic receptors) (for reviews, see Michell 1988, Axelrod et al 1988, Linden & Delahunty 1989).

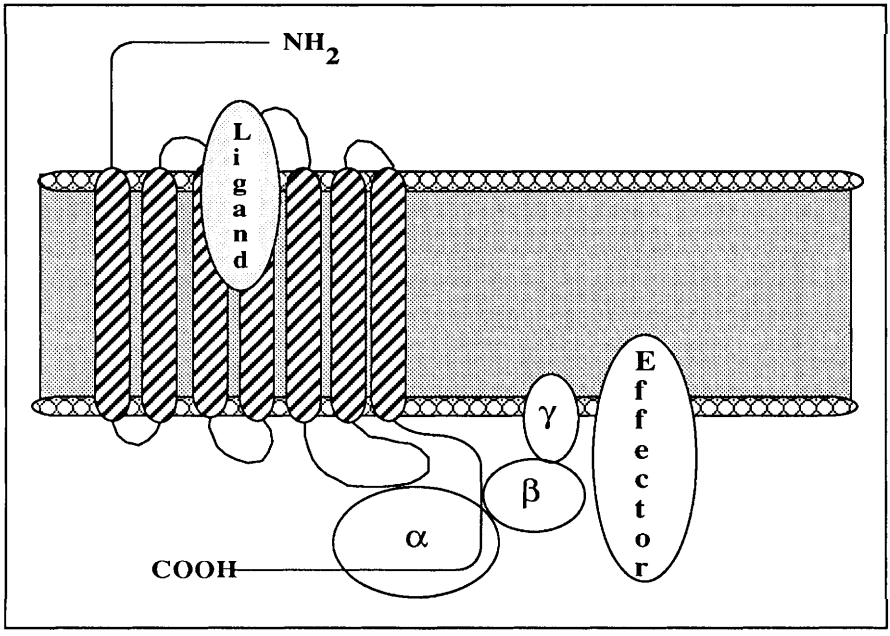

Fig 1. Structure of the "serpentine receptors" (e.g. rhodopsin, β-adrenoceptors and muscarinic receptors) showing the ligand-binding domain situated within the 7 transmembrane α-helical sequences (Dixon et al 1987) and the G protein binding domain on the inner surface (Neer & Clapham 1988).

The complexity of these transmembrane signalling systems has become appearent in recent years. Thus, a transmitter such as acetylcholine can mediate its cellular effects via

several transmembrane pathways. Nerve cells can in addition release both a transmitter and a neuropeptide, which can act as a modulatory agent. Finally, transmembrane signalling sytems can influence each other by various "cross-talk" mechanisms (Nishizuka 1986) (fig 2).

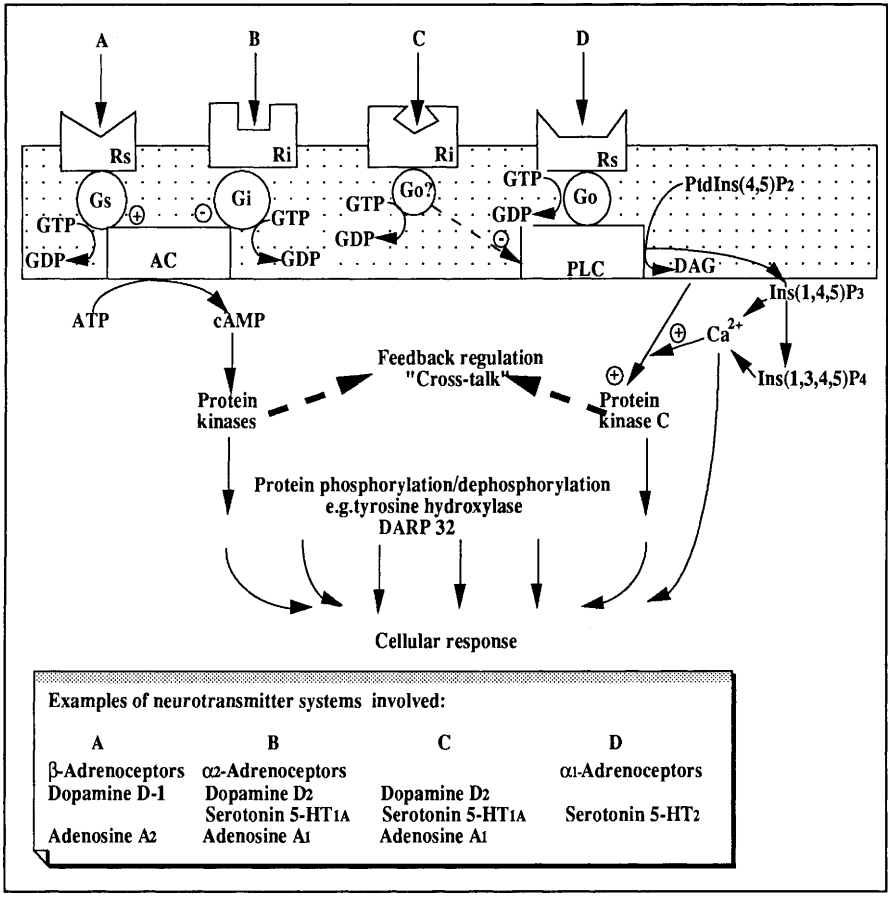

Fig 2. Two of the G protein coupled signal transduction pathways; the adenenylate cyclase and phosphoinositide specific phospholipase C system.

The above mentioned transmembrane signalling events are transmitted via guanosine nucleotide binding (G) proteins and therefore these receptors are called G protein coupled receptors. The G protein coupled receptors so far cloned have the serpentine structure as illustrated in fig 1. The G proteins themselves are heterotrimeric in nature, constituting of α, β and γ subunits, situated on the cytoplasmic face of the cell membrane. There are a number of different G protein families, such as the G_s proteins stimulatorily coupled to AC, G_i proteins inhibitorily coupled to AC and G_o proteins coupled, among

others, to PLC.

For a G protein coupled receptor, stimulation by an agonist produces a conformational change in the G protein binding domain, which in turn promotes the exchange of bound GDP with GTP on the G protein α-subunit. This activates the G protein leading to subunit dissociation of the heterotrimer into α and βγ subunits. Although the separate roles of α and βγ subunits have been a matter of some dispute for inhibitorily coupled G proteins, it is generally believed that for stimulatory coupled G proteins the released α subunit activates the effector systems (Reviews by Gilman 1987, Neer & Clapham 1988, Cockroft & Stutchfield 1988 and Friessmuth et al 1989). The G protein also has intrinsic GTPase activity and thereby hydrolyses the bound GTP to GDP, allowing reassociation of the heterotrimer and completing the cycle of activation. The G protein can also be activated by non-hydrolysable GTP analogues such as GTP[γ]S and Gpp[NH]p. In addition, fluoride ions activate G proteins, by combining with trace amounts of aluminium to form fluoroaluminate, (AlF_x see Chabre 1990).These fluoroaluminate complexes have been shown in the case of transducin (G_t) to interact with the GDP bound α subunit in a manner mimicking the γ-phosphate group of GTP (Bigay et al 1985).

2 BACKGROUND

2.1 G protein-mediated signal transduction in the CNS

In the present thesis, two G protein signal transduction systems have been studied, namely adenylate cyclase and phosphoinositide specific phospholipase C. These are shown schematically in fig 2, and discussed in detail below.

2.1.1 Adenylate cylase

The discovery of the existence cAMP in animal cells was made by Sutherland and Rall (1958) and its physiological role has been extensively studied since then. It was also shown by Sutherland and collegues that the synthesis of cAMP from ATP is catalyzed by the enzyme AC (Sutherland et al 1962) and that the degradation of cAMP to 5'AMP is catalyzed by the enzyme 3',5'-cyclic nucleotide phosphodiesterase (PDE) (Butcher & Sutherland 1962). These authors demonstrated further that AC was present in almost all animal cells. In mammalian tissue, the highest activity of AC is found in the grey matter of the brain (Sutherland et al 1962). Cyclic AMP is responsible for a variety of biological effects in the cell, due to its ability to activate protein kinases, which in turn phosphorylate different target proteins (Krebs & Beavo 1979). Receptors can couple both

stimulatorily (via G_s) and inhibitorily (via G_i) to AC. Examples of such receptor systems are shown in fig 2. (see also Michell 1988, Levitzki 1988).

Inhibitory effects upon AC are best seen when the system has been primed. One such primer often used is forskolin, which stimulates directly the catalytic unit of AC (Seamon & Daly 1981). This method has been used in the present thesis (**IV**) to study the effect of K^+ ions on carbachol (Cb)-inhibition of forskolin-stimulated AC which is mediated by M2-type muscarinic receptors (Michell 1988). In routine AC assays, levels of cAMP are increased by the inclusion of PDE-inhibitors such as caffeine, theophylline and 3-isobutyl-1-methylxanthine (IBMX) (Rall 1982).

2.1.2 Phosphoinositide-specific phospholipase C (PLC)

2.1.2.1 The phosphoinositide cycle

In the scientific literature, the word inositol was first used in 1850 by Scherer to name a cyclohexanehexol ($C_6H_{12}O_6$) discovered in muscle tissue. There are nine possible isomers of inositol (six naturally found and three synthetic). One of these, D-*myo*-inositol, (structure see fig 3) is present in biological membranes and acts as the precurser of phosphatidylinositol (see below). D-*myo*-inositol deprivation in diets for mice and rats leads to characteristic symptoms, inluding alopecia, inadeqate growth and "fatty liver", which are relieved when inositol is included in the diet again. Thus inositol was considered to be a vitamin (the mouse anti-alopecia factor) belonging to the group B (see Michell 1986, Sherman 1989). In 1930, it was discovered that inositol was a component of the phospholipids of mycobacteria and in 1949 Folch detected a brain lipid with a greater than 1:1 ratio of phosphate:inositol (review see Michell 1986).

The first report of receptor-mediated inositol phospholipid metabolism came in 1953 when Hokin and Hokin reported a striking increase in ^{32}P labelling of phospholipids when brain slices were treated with acetylcholine. In 1975, Michell proposed that receptor-stimulated inositol lipid hydrolysis in some way was connected with the rise of cytosolic Ca^{2+} (Michell 1975).

The realisation that the hydrolysation of a quantatively minor (<10%) (see Rana & Hokin 1990) membrane inositol phospholipid is an essential reaction in the transmembrane signalling mechanism for different cellular processes as metabolism, secretion, contraction, neural activity and cell growth, came in the late 1970's - early 1980's. Thus, in 1981, it was discovered that the lipid which is hydrolysed upon receptor stimulation is phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2, structure fig 3) (Michell et al 1981). In 1979, it was demonstrated that protein kinase C (PKC) is activated by 1,2-diacylglycerol (DAG, structure fig 3) which is formed from PtdIns(4,5)P_2 (Takai et al 1979).

Finally in 1983, it was shown that inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$, structure fig 3), the head group of $PtdIns(4,5)P_2$, released Ca^{2+} from intracellular stores (Streb et al 1983).

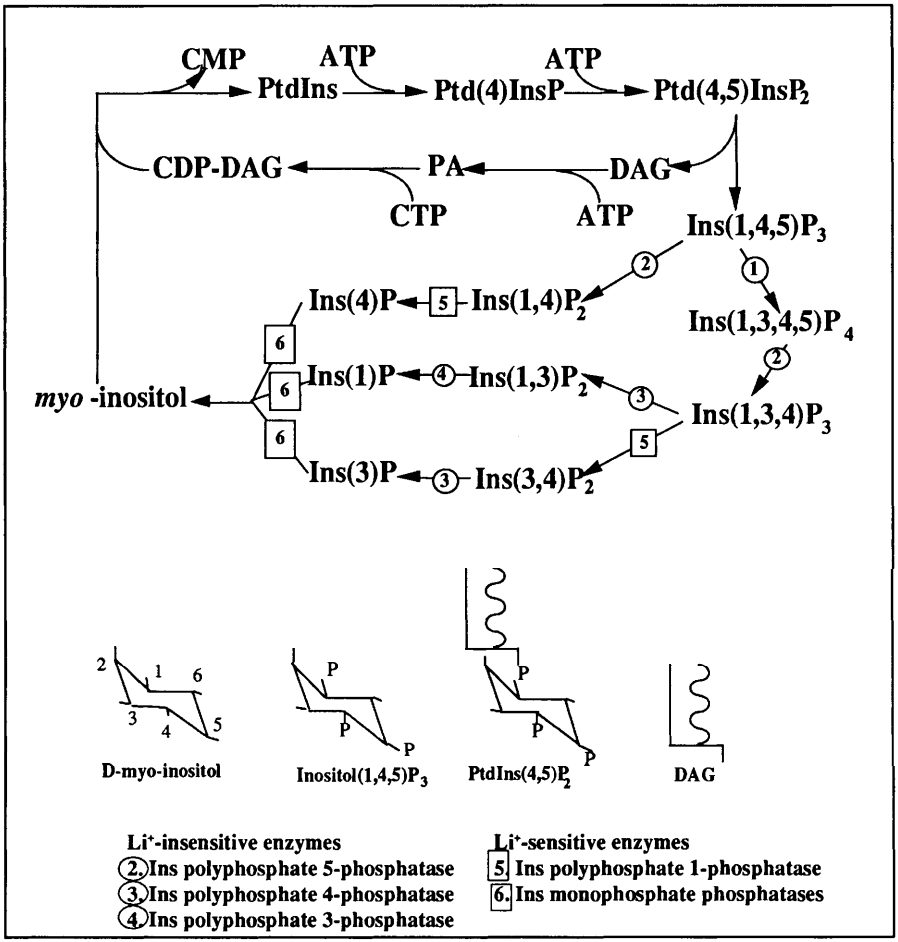

Fig 3. The phosphatidylinositol cycle.

2.1.2.2 Phosphoinositide-derived second messengers

It is now well established that stimulatation of cell-surface receptors by a number of different neurotransmitters or hormones leads to hydrolysation of $PtdIns(4,5)P_2$ and the formation of at least two second messengers, DAG and $Ins(1,4,5)P_3$. The generation of these messengers are formed by the actions of a receptor, a coupling G protein and PLC. There is considerable multiplicity in the systems. Thus, both pertussis toxin-sensitive and -insensitive G proteins have been shown to couple to PLC (review see Cockcroft

1987), sometimes within the same cell (Brass et al 1988). In addition, there are multiple forms of PLC, as many as five (Rhee et al 1989) to seven (Crooke & Bennet 1989) distinct isoenzymes having been reported. There are in addition at least seven subspecies of the DAG target enzyme PKC (Shearman et al 1989), which in turn phosphorylates a range of cellular proteins (see fig 2). PKC-mediated processes have been shown involved in secretion and exocytosis, modulation of ion conductance, smooth muscle contraction, gene expression and cell proliferation (review see Nishizuka 1986). At a molecular level PKC interacts both with components of the phosphoinositide signal transduction apparatus (as for example the negative feedback control that PKC exerts on the $Ins(1,4,5)P_3$ induced elevation of intracellular Ca^{2+}, Nishizuka 1988), and by "cross-talk" on other signal transduction systems (see e.g. Nordstedt 1990).

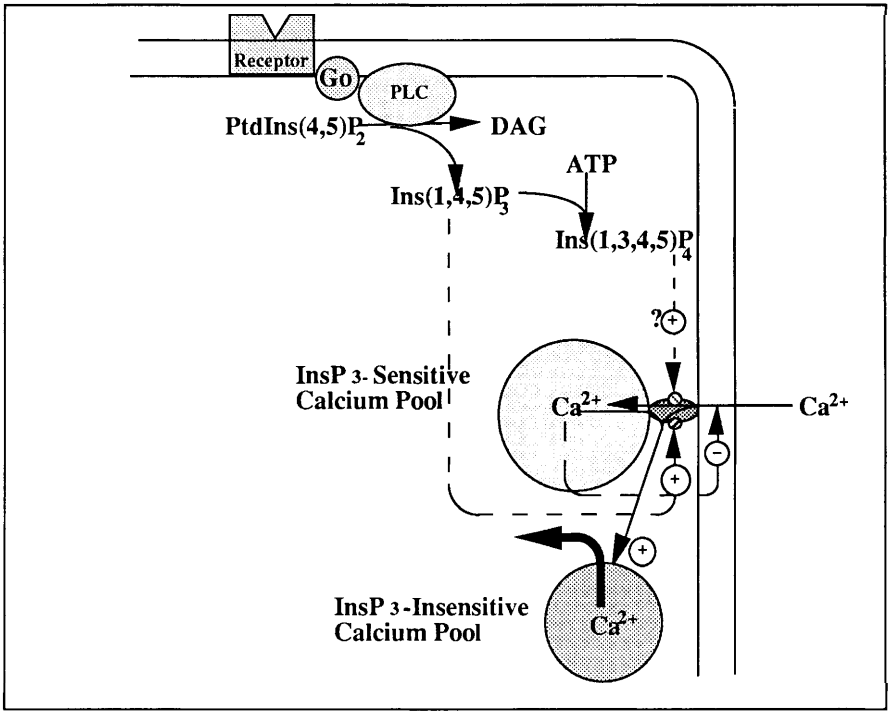

Fig 4. Model for $Ins(1,4,5)P_3$ mediated calcium mobilization (adapted from Irvine 1989).

The other second messenger generated by inositol phospholipid breakdown, $Ins(1,4,5)P_3$, releases intracellular Ca^{2+} by acting upon a population of intracellular calcium stores, in the endoplasmatic reticulum (Ghosh et al 1989, see fig 4). The $Ins(1,4,5)P_3$-sensitive releasable Ca^{2+} pool has different pharmalogical properties from the the $Ins(1,4,5)P_3$-insensitive release (see Schulz et al 1989; Palade et al 1989b). Recent

studies (Volpe et al 1988; Krause et al 1989) have suggested a specific organelle, termed 'calciosome' to be the Ins(1,4,5)P_3-sensitive Ca^{2+}-pool. The binding of [^{3}H]Ins(1,4,5)P_3 to its recognition site has been well characterised. In membranes from autopsied human cerebral cortices [^{3}H]Ins(1,4,5)P_3 binds with a K_D of 27±8 nM and B_{max} of 1.09±18 pmol/mg protein (Young et al 1988) and in rat brain, cerebellum shows the greatest binding density (40 pmol/mg protein) whereas lower binding levels are found for hippocampus, corpus striatum and cerebral cortex (Worley et al 1987). Compounds such as heparin and tetraethylammonium inhibit both the binding of [^{3}H]Ins(1,4,5)P_3 to its receptor and the mobilization of Ins(1,4,5)P_3-induced cellular Ca^{2+} (Hill et al 1987; Supattapone et al 1988; Palade et al 1989a). Functions of Ins(1,4,5)P_3 have been difficult to study due to its rapid dephosphorylation. However, the use of metabolically stable analogues of Ins(1,4,5)P_3 have indicated that K^+-conductances in pyramidal cells in the hippocampus may be regulated by this second messenger pathway (McCarren et al 1989). The purified Ins(1,4,5)P_3 receptor has been shown to mediate calcium flux in reconstitution studies (Ferris et al 1989), and the cloned receptor has a structural similarity to the skeletal muscle ryanodine receptor, which is also involved in the gating of intracellular calcium release (Furuichi et 1989; Mignery et al 1989). Even here receptor heterogenity is apparent as high-affinty (K_D= 0.052 nM) and low-affinty (K_D= 2.53 nM) [^{3}H]Ins(1,4,5)P_3 binding has been found in bovine adrenal cortical membranes (Challiss et al 1990). Ins(1,4,5)P_3 is a substrate for a specific 3-kinase, producing Ins(1,3,4,5)P_4 (Batty et al 1985). Recent studies have suggested that Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 act synergistically in controlling intracellular mobilization of Ca^{2+}. One possible model for this is shown schematically in fig 4. Other authors have, however questioned the role played by Ins(1,3,4,5)P_4, and suggested that a small molecular weight G protein is involved in transferring calcium from Ins(1,4,5)P_3-insensitive to Ins(1,4,5)P_3-sensitive pools (Ghosh et al 1989).

The metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 is shown schematically in fig 3. In addition to these, cyclic inositol phosphate products are found as result of PtdIns(4,5)P_2 hydrolysis. These compounds, however, are present in lower amounts, and their exact cellular role is uncertain (review, see Putney et al 1989). The different phosphatases have been charactericed in some detail (for review see Shears 1989).

A number of the phosphatase enzymes are inhibited by lithium (see fig 3). The inhibition that Li^+ exerts on the monophosphatases is uncompetetive in nature, whereby the ion binds to the enzyme-substrate complex. This type of inhibition has the interesting property that an increased InsP concentration enhances the inhibition produced by lithium. This means that Li^+ more efficiently inhibits the monophosphatases in cells whose PLC coupled receptors are more actively stimulated. These inhibitory properties of Li^+ have been suggested to be involved in the relievement of the symptoms of mania

that Li^+ exerts in bipolar depressive illness. In the brain, the blood-brain barrier prevents free access to external inositol. By preventing the breakdown of inositol monophosphates, lithium treatment results in the depletion of cellular inositol in the brain. This depletion in turn will lead to a reduced synthesis of PtdIns(4,5)P_2, and hence a partial shut-down of this signal transduction pathway (for review see Berridge et al 1989). Lithium, however has a multitude of actions, with a corresponding multitude of hypothesis to explain its clinical efficacy. Avissar et al (1988), for example, suggested that effects of Li^+ on G protein function may be involved. The inhibition by Li^+ of inositol monophosphatases forms the cornerstone of the inositol phospholipid breakdown assay used in the present thesis (see below).

2.1.3 Inositol phospholipid breakdown methodolgy

A number of methods have been used to measure inositol phospholipid breakdown. These include measuring the rate of disappearance of ^{32}P-labelled phosphoinositides, direct measurements of PLC activity, mass measurements of endogenous Ins(1,4,5)P_3 production and by entrapment of [^{3}H]inositol monophosphates (for review, see Dean & Beaven 1989). In this latter method, polyphosphoinositides are prelabelled by incubation with *myo*[2-^{3}H]-inositol and the rate of inositol phospholipid breakdown is followed in the presence of Li^+, which blocks the dephosphorylation of inositol monophosphates (Berridge et al 1982). This method can be used after the removal (by washing) of excess *myo*[2-^{3}H]-inositol after the incubation phase ("pulse label", e.g. Court et al 1986) or by allowing the labelling of the phosphoinositides to continue after the addition of the agonist ("continuous label", see e.g Batty & Nahorski 1987). Such assays require that lithium ions do not interfere with the rate of inositol phospholipid breakdown, for example by affecting G protein function. In this regard, Li^+ inhibits both agonist- and forskolin-stimulated AC activity (Ebstein et al 1978, Newman & Belmaker 1987), completely blocks the increase in [^{3}H]GTP binding induced by isoprenaline and Cb, and also abolishes guanine nucleotide modulation of agonist binding, suggesting effects on both G_s and G_i (Avissar et al 1988).

When using brain cross-chopped slices ("miniprisms"), a number of factors may be of importance in determing the inositol phospholipid breakdown response to stimulation. Johnson and Minneman (1985) found that miniprism size was of importance, optimum results being obtained with sizes in the range of 0.175 x 0.175 to 0.700 x 0.700 mm. Our own studies have indicated that similar responses to Cb (and synergy with K^+) are found in Krebs-Henseleit and Krebs-HEPES buffers, and that variations of the assay pH from 6.9 to 7.8 has relatively minor effects on the degree of K^+-Cb synergy observed (G. Tiger, P-E Björklund & CJ Fowler, unpublished results). The importance of buffer

temperature immediately after dissection, however, has not been investigated.

When studying inositol phospholipid breakdown, the amount of [^{3}H]labelled inositol phosphates (InsP) produced have been expressed as InsP d.p.m. (e.g. Berridge et al 1982), InsP d.p.m./mg protein (e.g. Schoepp et al 1984) and % increase over basal InsP d.p.m. (Watson & Downes 1983, Brown et al 1984). All of these ways of expressing the results have the disadvantage of not taking account of differences in the labelling of the phospholipids and/or variations in the number of miniprisms beeing pipetted. Other authors, using the pulse label assay, have used the unit % of total tritium incorporated (here termed InsP/(Lipid+InsP)) (Gonzales & Crews 1985, Court et al 1986). The relative merits of these methods, however, have not been compared.

2.1.4 Neurotransmitter stimulated inositol phospholipid hydrolysis

Since the discovery of the receptor-mediated breakdown of inositol phospholipid, at least twenty classes of biologically and pharmacologically active substances have been proposed to activate the release of labeled inositol phosphates in intact and broken cell preparations. In the brain, serotoninergic, α_1-adrenergic, histaminergic, excitatory amino acid, peptidergic and muscarinic cholinergic receptors coupled to PLC mediated breakdown of PtdIns(4,5)P_2 have been demonstrated (review by Chuang 1989). In the case of quisqualate-stimulated breakdown, the receptors involved appears pharmacologically different from those defined by electrophysiological studies (review, see Smart 1989). Recently, receptors inhibitorily coupled to PLC have also been described (Linden & Delahunty 1989, see fig 2) although it is not clear whether this inhibition is directly, or indirectly mediated. Stimuli such as raised [K^+] are also capable of increasing inositol phospholipid breakdown (see below). Most of the brain studies, however, have been undertaken on rats, mice and guinea-pigs, and little is known about these systems in the brains of higher species.

2.1.5 G protein involvement in the receptor-mediated inositol phospholipid breakdown in brain tissue

While the G proteins coupling receptors to PLC have been investigated in detail in intact and permeabilized cells in culture (review, see Cockroft 1987), studies in brain tissue are more difficult to perform due to the limited accessibility of these proteins. However, high concentration of the GTP analogues GTP[γ]S and Gpp[NH]p have been shown to stimulate inositol phospholipid breakdown in rat brain miniprisms (Li et al 1989). Investigation of PLC activation in brain membranes (either after prelabelling with *myo*[2-^{3}H]-inositol or by the use of the substrate [^{3}H]PtdInsP_2 itself) has also demonstra-

ted a GTP requirement of the response to Cb (Claro et al 1989a,b; Chiu et al 1988). In slices, however, most of the studies on G protein function coupled to PLC have utilized fluoride ions. Thus Jope (1988) found that NaF stimulates inositol phospholipid breakdown and that addition of aluminium ions was not necessary for the response, indicating that the presence of sufficient quantities of aluminium in the assays to build fluoroaluminate. Godfrey and Watson (1988) demonstrated further that NaF inhibits the inositol phospholipid breakdown response to Cb, which they interpreted as demonstrating the G protein involvement in this response. The actions of fluoride on G proteins coupled to PLC may, however, be more complicated than was originally thought, since Li et al (1990) have shown fluoride ions to act additively with GTP[γ]S in stimulating the inositol phospholipid breakdown in prelabelled membranes from rat cerebral cortex.

2.2 Muscarinic cholinergic receptor subtypes in the brain

Dale (1914) defined muscarinic acetylcholine receptors (mAChR) as those receptors that are stimulated by acetylcholine (ACh) and muscarine and blocked by atropine. The mAChR:s are widely distributed in the brain (Palacios et al 1986) and are involved in a variety of physiological functions, such as extrapyramidal motor function, learning and memory (review, see Lippa et al 1986). The existence of subtypes of mAChR in the brain was proposed on the basis of different affinity states for muscarinic agonists and antagonists (Birdsall et al 1978, see table 1). Thus the M_1-type muscarinic receptors have a high affinity for pirenzepine (PZ) whereas the M_2-type (both M_2(cardiac) and M_2(glandular), see table 1) receptors have a lower affinity for this compound. The reverse is true for their affinity for the agonist Cb (Birdsall et al 1978, see also table 1).

The regional localization of M_1- and M_2-type receptors in human brain by the use of quantitative autoradiography shows that the M_1-type dominates in basal ganglia, hippocampus, substantia nigra and the upper layers of neocortex. M_2-type receptors are found in highest densities in deeper layers of neocortex, thalamus, cerebellum and brainstem and they are equally distributed in hypothalamus (Palacios et al 1986).

2.3 Muscarinic cholinergic receptor-mediated phosphoinositide breakdown

mAChR:s are coupled to PLC in the brain, and there is a strong correlation between the density of the receptors and the magnitude of the inositol phospholipid breakdown (Downes 1982, 1983). Muscarininc agonists, however, differ in their ability to stimulate inositol phospholipid breakdown. In the cerebral cortex ACh, Cb and oxotremorine-M are more effective than pilocarpine, bethanecol and oxotremorine, which act as partial agonists in this system (Fisher et al 1983, 1984; Gonzales & Crews 1984; Jacobson et

Pharmacological characterization					
Subtype	M_1-type	M_2-type		-	-
Other names used previously	$M_{1\alpha}$, A	$M_{2\alpha}$, cardiac M_2, C	M_3, $M_{2\beta}$,glandular M_2, B	M_2	-
Selective antagonists	PZ, (+)-telenzepine	AF-DX 116, himbacine, methoctramine, gallamine	*p*-fluorohexahydrosiladifenidol, hexahydrosiladifenidol	-	-
Molecular characterization					
Sequences	m1	m2	m3	m4	m5
Other names used previously	mAChRI, M1	mAChRII, M2	mAChRIII, M4	mAChRIV, M3	
Stimulation of PLC	yes	no	yes	no	yes
Inhibition of AC	no	yes	no	yes	no
PZ K_i (nM)	16	906	180	561	628

Table 1. Muscarinic receptor nomenclature (modified from Birdsall et al 1989, Buckley et al 1989 and Bonner 1989).

al 1985),unlike the situation for AC inhibition, where they act as full agonists (Olianas et al 1983; Brown & Brown 1984). Using PZ to discriminate between M_1-type and M_2-type receptors, it was initially suggested that M_1-type receptors were coupled to inositol phospholipid breakdown whereas M_2-type receptors were inhibitorily coupled to adenylate cyclase (Watson et al 1985, Gil & Wolfe 1985). However, M_2-type receptor-mediated inositol phospholipid breakdown was soon after demonstrated in guinea-pig neocortex (Fisher & Bartus 1985). More recently, muscarinic receptor genes have been cloned. So far, five mAChR:s from rat and human (Bonner 1989), have been cloned (see table 1). There has been some confusion in the terminology of the receptor gene products, since two groups who cloned the genes at the same time adopted a slightly different nomenclature (see e.g table 1). The nomenclature of Peralta et al (1988) is used in **IV** and that of Bonner et al (1987) in **V**.

It has been shown in mammalian cells that m1, m3, and m5 receptor gene products are efficiently coupled, generally through a pertussis toxin-insensitive G protein (Bonner 1989), to inositol phospholipid breakdown (Peralta et al 1988, Fukuda et al 1988) and do not inhibit AC (Peralta et al 1988). On the other hand m2 and m4 strongly inhibit AC, through a pertussis toxin-sensitive G protein (Bonner 1989), and have at best minor

stimulatory effects on inositol phospholipid breakdown (Peralta et al 1988). Ashkenazi at al (1989) reported, in chinese hamster ovary cells, that m1 and m3receptors not only coupled strongly to inositol phospholipid breakdown through pertussis toxin-insensitive G proteins, but also coupled weakly to inositol phospholipid breakdown through the pertussis toxin-sensitive G proteins. Positive coupling of m1 receptors to AC has also been reported (Ashkenazi at al 1989), although this may be secondary to PLC activation (Felder et al 1989). Some of these anomalies, however, may result from a non-specific choice of G proteins secondary to receptor overexpression in the cells (see e.g Kenakin & Morgan 1989).

In the rat brain, mRNA:s for the m1, m3 and m4 genes are abundantly and broadly expressed, whereas that for m2 is expressed widely, but at much lower levels. The mRNA for m5 is also expresed in the rat brain, but its abundance is rather low (Bonner 1989). The m1 gene product appears to correspond to the M_1-type receptors, whereas the m3 may be the M_2-glandular type coupled to inositol phospholipid breakdown. The m4 gene product appears to correspond to the M_2-type inhibitorily coupled to AC in the rat striatum (McKinney et al 1989)

Recent work from Bonner's group (Wess et al 1989) has shown that the third cytoplasmic loop (see fig 1) of the receptors m1, m3 and m5 have a 16-17 amino acid segment that is highly conserved, but different from the corresponding sequence in m2 and m4. They suggest, consistent with results from Lefkowitz' group (Kobilka et al 1988) that this region determines the coupling selectivity with G proteins and thereby how efficiently different mAChR:s are coupled to inositol phospholipid breakdown.

2.4 The physiological role of ion channels in nervous tissue

Across nerve cell plasma membranes, there are concentration gradients of potassium, sodium and chloride ions. Thus, in its resting state the concentrations inside and outside the nerve cells, respectively, are approximately as follows: potassium 140 and 4 mM; sodium 14 and 142 mM; chloride 8 and 107 mM. Thus, in the resting state, there is a potential across the cell membrane determined mainly by the resting potential for potassium. Changes in the membrane potential can rapidly (msec) be obtained by opening of different ion channels. These ion channels are regulated by the membrane voltage, second messengers, or directly operated by receptors. Opening of the channels result in depolarisation (Na^+- and Ca^{2+}-channels) and hyperpolarisation (Cl^-- and K^+-channels) of the cell membrane (reviews, see Reichardt & Kelly 1983). Activation of calcium channels allows Ca^{2+} ions to enter the cell, increasing the $[Ca^{2+}]_i$ from a resting level of ≈ 0.1 μM and leading to depolarisation. The intracellular Ca^{2+} ions have a central role in the functioning of enzymes, gating of ion channels, transmitter release, metabo-

lism, gene expression and neurite outgrowth or retraction (Tsien et al 1988).

Membrane depolarisation of brain slices *in vitro* can be accomplished by altering the balance of these ions. Veratrine for example activates Na^+-channels, while monensin and ouabain increase the $[Na^+]_i$ by their actions as sodium ionophore and Na^+-K^+-ATPase inhibitor, respectively. Action of an agonist acting on L-type calcium channels e.g. BAY K-8644 will also depolarise the tissue. Depolarisation, secondary to activation of voltage-dependent calcium channels, can also be brought about by raising the extracellular $[K^+]$. Membrane depolarisation can even be obtained by actions of the excitatory amino acids glutamate and N-methyl-D-aspartatic acid (NMDA) again secondary to changes in the ion-flux. All these approaches have been used in the present thesis.

It has been shown that some of these ion channels are regulated by G proteins. Thus, in the heart, for example, muscarinic cholinergic and adenosine receptors open an inward rectifying potassium channel by a G protein (Cook 1988; Castle et al 1989) and the outward K^+ channel activation by muscarinic cholinergic receptors is transmitted by the $\beta\gamma$ subunits (Logothetis et al 1987). In hippocampal neurons, $GABA_B$-, $5\text{-}HT_{1A}$- and adenosine A_1 receptors stimulate an outward potassium channel by a pertussis toxin-sensitive G protein (Nicoll 1988). G proteins are present in the functioning of voltage dependent Ca^{2+} channels of all three types (T, L and N) (Glossman & Striessnig 1988).

2.5 Depolarising stimuli and inositol phospholipid breakdown

A number of studies have demonstrated that depolarising stimuli will result in an increased inositol phospholipid breakdown in brain slices and in synaptosomes (see e.g. Gusovsky et al 1987). With respect to potassium ions, Brossard and Quastel (1963) found that 100 mM concentrations increased the ^{32}P labelling of phospholipids in rat brain cortical slices. Later studies using *myo*[2-^{3}H]-inositol have confirmed the increased breakdown of inositol phospholipids elicited by raised $[K^+]$ (for dose-response curves see Court et al 1986 and Kendall & Nahorski 1987). In general, in the "pulse label" assay, 18 mM K^+ produces only a relatively small increase, whereas 35-50 mM produce large increases in breakdown. At an extracellular $[Ca^{2+}]$ of 1.3 mM, the effects of raised $[K^+]$ on basal inositol phospholipid breakdown appear secondary to activation of voltage-sensitive calcium channels. Thus, the response can be reduced by L-type calcium channel antagonists and mimicked by agonists (Kendall & Nahorski 1985a; Zernig et al 1986). The degree of stimulation by K^+ ions across the brain mirrors the regional distribution of L-type calcium channel binding sites (Rooney & Nahorski 1986). The role played by calcium channels at higher extracellular $[Ca^{2+}]$, have not been investigated, however.

2.6 Interaction between potassium ions and neurotransmitter-stimulated inositol phospholipid breakdown

Court et al (1986) found that raising the assay [K^+] to 18 mM greatly enhanced the inositol phospholipid breakdown response to stimulation by Cb. A simular result was found by Eva and Costa (1986), who further demonstrated that this was unaccompanied by changes in the binding characteristics of the muscarinic receptor recognition site. The response appeared selective for muscarinic receptors, and neither NA-, histamine- or 5-HT-stimulated inositol phospholipid breakdown were enhanced to the same degree (Court et al 1986, see also Conn & Sanders-Bush 1986). The effect is due to raised [K^+] rather than a reduction of [Na^+], since replacement of Na^+ ions with $Tris^+$ ions did not alter the effect on basal or Cb-stimulated inositol phospholipid breakdown (Court et al 1986).

In their original article Court et al (1986) speculated that the enhancement may represent a way for the amplification of a cellular response to a cholinergic stimulus under transient depolarising conditions. Baird and Nahorski (1986, 1989) demonstrated that the inositol polyphosphate most affected by K^+ in the presence of Cb is Ins(1,3,4,5)P_4, and speculated that potassium ions, by activating voltage-sensitive calcium channels, activate the 3-kinase enzyme, which is sensitive to calcium. More recently, however, these authors have found that the pattern of stimulation of the inositol polyphosphates with K^+ and Cb differs from that seen by the combination of the calcium ionophore ionomycin and Cb (Baird & Nahorski 1990), thus questioning this model. In addition Eva and Costa (1986) found no effect of the L-type calcium channel antagonist nifedepine upon Cb-stimulated inositol phospholipid breakdown at raised [K^+]. Voltage-sensitive Na^+-channels do not seem to be involved in the Cb-K^+ synergy, since the response to Cb and raised [K^+] is not altered by tetrodotoxin, a blocker of these channels (Eva & Costa 1986; Gurwitz & Sokolovsky 1987). Based on these experiments, Eva and Costa (1986) suggested that K^+ exerts its effects by changing the coupling efficiency of the recognition site with its transducer.

Control experiments have indicated that this "potassium effect" is unlikely to be an artifact of the assay conditions used. The K^+ incubation (which was mimicked by Rb^+ and Cs^+ ions) did not effect the ATP concentration of the slices (Court et al 1986). Experiments with inulin have shown that the effect of K^+ (replacing Na^+ in the media) is not simply due to osmotic changes in the medium (G Tiger, P-E Björklund and CJ Fowler, unpublished results).

A number of questions remain, however, concerning the nature of the K^+-Cb synergy, namely the selectively of the response within the muscarinic system (i.e. PLC vs AC, M_1-type vs M_2-type muscarinic receptors); the role played by modulatory factors released by K^+ on the Cb-response (adenosine, for example, can modulate a number of receptor-

mediated inositol phospholipid breakdown responses, Delahunty et al 1988; Kendall & Hill 1988; Petcoff & Cooper 1987; El-Etr et al 1989); whether other depolarising agents can mimic the response to K^+; and to determine further the role played by calcium in the response.

3 THE PURPOSES OF THE PRESENT INVESTIGATION

The purposes of this investigation were as follows:

1. To improve the inositol phospholipid breakdown methodology to give reliable measures not dependent upon labelling efficiency or pipetting variations.

2. To investigate the effects of neurotransmitters (or their agonists) and K^+ ions on inositol phospholipid breakdown in the brain of a non-rodent species.

3. To determine in more detail the selectivity of potassium ions upon Cb-stimulated inositol phospholipid breakdown in brain tissue, and to characterize the muscarinic receptor subtypes involved.

4. To determine the roles played by tissue depolarisation, calcium and adenosine in the K^+-Cb synergy.

5. To investigate further the effect of other ions (fluoride and lithium) on the phosphoinositide system, both in terms of inositol phospholipid breakdown and inositol phosphate metabolism.

4 MATERIALS AND METHODS

4.1 Animals

The experiments were undertaken using brains from either rats (Strain R, University of Umeå, Sweden, Body weight 150-350 g) (**I - VII**) or 4-8 month-old pigs (Swedish Landrace) (**II**).

4.2 Chemicals

4.2.1 Radiochemicals

In the case of *myo*[2-^{3}H]-inositol, three preparations were used: in the initial studies (**I-III**) D-*myo*-inositol (specific activity 10-20 Ci/mmol) (NEN GmbH, Dreieich, West Germany) was used. For the lipid analyses (**III** table 2), D-*myo*-inositol of higher specific activity (40 Ci/mmol, NEN) was used. One difficulty associated with *myo*[2-^{3}H]-inositol is its instability: the breakdown products are adsorbed and eluated from Dowex columns as if they were [^{3}H]-Inositol phosphates (Amersham 1988). However, this has been circumvented by the use in **IV-VII** of *myo*[2-^{3}H]-inositol (specific activity 10-20 Ci/mmol), where the solution contains PT6-271, a polymer that adsorbes the breakdown products and preserves the low blanks (Amersham International plc, Amersham, England).

D-*myo*[2-^{3}H]-inositol-1,4,5-trisphosphate (specific activity 1 Ci/mmol) and D-*myo* [2-^{3}H]phosphatidylinositol-4,5-diphosphate (1-5 Ci/mmol) were obtained from Amersham and NEN, respectively.

4.2.2 Other compounds

Compounds in the studies were obtained from the Sigma Chemical Co St. Louis. Mo, USA, with the following exceptions: Amprep SAX mini columns and nonradioactive Inositol-1,4,5-trisphosphate (Amersham); BAY K-8644 (Bayer AG, Leverkusen, West Germany); BRL 34915 (Beecham Pharmaceuticals, Harlow, England); ω-conotoxin (Cambridge Research Biochemicals Ltd, Cambridge, England); Dopamine hydrochloride, ionomycin, staurosporine (Fluka AG, Buchs, Switzerland). Dowex-1 X8(chloride form) was obtained from both Sigma and Fluka.

4.3 Preparation of miniprisms

The rats were killed by decapitation (**I-III**) or with O_2/CO_2 (1/5 l/min) followed by decapitation (**IV-VII**) and the brains were dissected at room temperature (20 °C). In some experiments in (**II** fig 1) the brains were left in situ at room temperature (20 °C) for up to 120 min before dissection. Experiments were undertaken using cerebral cortex (**I - IV, VI, VII**) or one or more of the frontal cortex, striatum, hippocampus, hypothalamus, basal forebrain and cerebellum (**I, III-V, VII**). For the regional distribution studies, brain samples were pooled from 2-4 rats. Samples were placed immediatly in room-temperature Krebs-Henseleit bicarbonate (KHB) buffer, unless otherwise stated (**I**).

The brains from the pigs (**II**), obtained from the local slaughterhouse, were removed from the skull after the Vetrinary officer's examination, which was undertaken 10-30 min after slaughter, and placed in a thermos flask containing KHB buffer at room temperature (20 °C). Ten minutes later the brain regions were dissected.

Miniprisms were made by flattening the brain tissue on a plastic plate whereafter it was cut perpendicularly with a McIlwain tissue chopper giving slices of 0.35 x 0.35 mm. In the experiments performed in **I-IV**, the miniprisms were dispersed and washed several times in ice-cold (**I**) or room temperature (20 °C) (**I-IV**) KHB buffer, equilibrated with 95% O_2 : 5% CO_2. In the experiments in **V-VII**, the miniprisms were transferred to bottles (250 ml) containing KHB buffer (37 °C) and the slices were continuously gassed with 95% O_2 : 5% CO_2 and gently agitated at 37 °C in a shaking water bath for 1 hour with two intermediate changes of buffer (Brown et al 1984).

4.4 Assay of inositol phospholipid breakdown

4.4.1 "Pulse label" assay

Inositol phospholipid breakdown was measured as described by Berridge et al (1982) and Watson and Downes (1983), with slight modifications. Miniprisms were incubated for 60 min at 37 °C with *myo*[2-^{3}H]-inositol (10 μCi/ml of suspension) under an atmosphere of 95% O_2 : 5% CO_2 in a volume of 1.5 - 3 ml KHB buffer in 1 - 6 bottles depending on the tissue volume used. As much as possible of the excess *myo*[2-^{3}H]-inositol was removed by washing the samples with warm (37 °C) KHB buffer and reincubating them for ten min at 37 °C under an atmosphere of 95% O_2 : 5% CO_2. The samples were then washed three times, regassed and reincubated for ten minutes in the absence or presence (**I** and **II**) of 50 μM pargyline. Pargyline present when low (i.e.<2 μM) NA concentrations were used, since it is only at these low concentrations that metabolism of NA by monoamine oxidase in the miniprisms during the inositol phospholipid breakdown assay is significant (Fowler et al 1986). The miniprisms were then washed three times (KHB buffer 37 °C). When Mg^{2+}-free KHB buffer was used for the washing procedures (**III**), the $MgSO_4$ was replaced by Na_2SO_4. The [K^+] and [Li^+] in these initial stages were in all cases 6 and 0 mM respectively. When Ca^{2+}-free medium was used (**VI**), $CaCl_2$ was excluded from the buffer troughout. Other variations of the KHB buffer are indicated in the results and discussion section.

Aliquots (40 μl in the pig and 20 - 30 μl in the rat experiments) of the miniprisms were added to 15 ml glass centrifuge tubes containing a modified KHB buffer and the test compounds in a final volume of 300 μl. The modified KHB buffer contained assay concentrations of: Li^+ 8.8 mM (0 - 35 mM in **IV**), K^+ 1-80 mM as indicated, and in **III**

0-37 mM Rb^+ or 0 mM Mg^{2+}, with the [Na^+] changed by the corresponding amount. In **I** and **II** 0,1 mg/ml ascorbic acid was included as antioxidant. The samples were then gassed with 95% O_2 : 5% CO_2 and incubated for 25 min at 37 °C unless otherwise stated. When a preincubation step was used, samples were incubated in the absence of agonist but with test substance (such as PZ, **IV** and **V**) for 10-30 min. Agonist was added, the samples regassed and incubated for another 0-25 min.

The reactions were stopped by addition of 0.94 ml chloroform:methanol (1:2 v/v) followed by vortex mixing for five min. Separation of the phases was accomplished by the addition of 0.31 ml H_2O and 0.31 ml chloroform followed by vortex mixing (1 min) and centrifugation (3000 v/min) for 5 min after which aliquots (750 µl) of the aqueous phase were taken and placed in centrifugation tubes (15 ml). In **I-III** aliquots (300 µl) from the organic layer were taken and placed in scintillation vials. The solvent was allowed to evaporate before addition of scintillant and determination of radioactivity (here termed "Lipid dpm") by liquid scintillation spectroscopy with quench correction. In **III-VII**, the mixture was acidified by addition of concentrated HCl (63-75 µl) after removal of the aqueous phase aliquots. The samples were vortex mixed, centrifuged and the organic layer aliquots removed.

The radioactivity labelled inositol phosphates ("InsP") in the aqueous phase aliquots were separated from the *myo*[2-^{3}H]-inositol by adsorbtion to, and elution from Dowex-1x8-formate anion exchange resin, based on the method described by Berridge et al (1982). Briefly, the Dowex was suspended (1:3) in water. The suspension was gently stirred and two ml aliquots were added to the aqueous layer aliquots. The tubes were vortex mixed for two min after which 800 µl aliquots were removed when appropriate (**I, VII**) and the radioactivity corresponding to unadsorbed *myo*[2-^{3}H]-inositol determined. The Dowex-1x8 resins were washed with two ml water, vortex mixed for 20 sec and left to sediment for one min. The excess water was removed with a Pasteur pipette connected to a water vacuum pump. This washing procedure was repeated three times. The radioactivity labelled inositol phosphates ("InsP") were eluted from the resin by the addition of one ml 1 M ammonium formate in 0·1 M formic acid to the centrifugation tubes followed by vortex mixing for five min. The resin was allowed to settle, and 800 µl aliquots were removed and added to scintillation vials for determination of their tritium content by liquid scintillation spectroscopy with quench correction.

4.4.2 "Continuous label" assay

In (**VII** table 3) the "continuous label" assay was used. After the pregassing phase, the minprisms were added to a vial containing 3 ml KHB buffer with *myo*[2-^{3}H]-inositol (10 µCi/ml of suspension), gently mixed and gassed with 95% O_2 : 5% CO_2. Aliquots (30 µl)

of the miniprisms were added to 15 ml glass centrifuge tubes containing a modified KHB buffer, incubated with Li^+, raised [K^+] and NaF for 45 min at 37 °C under an atmosphere of 95% O_2 : 5% CO_2. Cb was then added, the samples regassed and incubated for a further 25 min at 37 °C. Reactions were stopped and worked up as decribed in **4.4.1** for the "pulse label" assay.

4.5 Preparation of Dowex-1x8 formate resin

Dowex-1x8 resin is commercially available (Sigma, Fluka) in the chloride form. The resin was treated with an excess of 1 M NaOH and washed three times with distilled water. Formic acid (0·1 M) was added in excess. The resin was then washed with formic acid solution followed by three washes with distilled water, and was then ready to use. After use the resin was collected and regenerated by using the same protocol.

4.6 Separation of labelled phosphoinositides (paper III)

The labelled phosphoinositides in the organic phase described in **4.4** were separated from one another by the methods of Jolles et al (1981) and Van Roijen et al (1983). Briefly, a further 200-µl aliquot was taken from the organic phase, placed in Eppendorf tubes, dried over night and redissolved in 30 µl chloroform/methanol/water (75:25:2, by volume). Twenty microliter samples were spotted on TLC plates (silica gel 60; 20 x 20 cm, 0.25 mm thickness; Merck, Darmstadt, F.R.G.) that had previously been treated in a solution containing 1.2 g potassium oxalate in 100 ml methanol/water (40:60 vol/vol), dried at room temperature and then activated at 110 °C. Chromatograms were eluted in a paper-lined chromatographic chamber with chloroform/acetone/ methanol/acetic acid/ water (40:15:13:12:8 by volume) as the solvent system and then dried at room temperature. The plates were then exposed to iodine vapour and the bands corresponding to PtdIns, PtdInsP and $PtdInsP_2$ (with unlabelled standards, including phosphatidic acid, assayed in parallel) were scraped off and counted for radioactivity by liquid scintillation spectroscopy with quench correction.

4.7 Adenylate cyclase assay (paper IV)

The activity in adenylate cyclase was measured by the method described by Kelly and Nahorski (1986) with minor modifications. Briefly, miniprisms (0.3 x 0.3 mm) were made as described above (**4.3**). The miniprisms were resuspended in 5 ml modified KHB buffer (containing 1mM theophylline and with a [$CaCl_2$] of 1.25 mM). The 5 ml suspension was then incubated at 37 °C for 90 min under an atmosphere of 95% O_2 : 5%

CO_2. Aliquots of the miniprisms were then gassed with 95% O_2 : 5% CO_2 and incubated with forskolin and/or Cb at different $[Li^+]$ and $[K^+]$ for 20 min at 37 °C.

Cyclic AMP was extracted from the samples essentially as described by Khym (1975). Incubations were terminated by addition of 50 µl of ice-cold 2·1M trichloroacetic acid. After centrifugation the supernatant (300 µl) was removed and 330 µl of ice-cold 0·5 M alamine in freon was added. After separation of phases, duplicate aliquots (50 µl) of the aqueous phase were removed and assayed for cAMP levels using a standard cAMP assay kit (TRK 437, Amersham International plc).

4.8 Phosphatidylinositol-4,5-bisphosphate ($PtdInsP_2$)-phospholipase C assay (paper IV)

Membranes from cerebral cortices were assayed essentially as described by Litosch (1987). Briefly, small pieces of the cortices were incubated for 30 min at 37 °C in a modified KHB buffer. After incubation, the samples were homogenized 1:15 (w/v) at 4 °C in 280 mM sucrose containing 0·5 mM EDTA (disodium salt), 60 µg soyabean trypsin inhibitor/ml and 10 mM Tris-HCl, pH 7.5. Cell debris was removed by centrifugation at 500 g x 10 min, and the supernatant was centrifugated at 32000 g for 10 min. The resulting pellet was washed and resuspended in assay buffer (50 mM Tris-HCl, pH 7.0, 45 µM $CaCl_2$, 100 mM NaCl, 1 mM sodium deoxycholate) at 4 °C. $[^3H]PtdInsP_2$ was prepared for assay as described by Wallace et al (1988). Assay incubation was initiated by the addition of membrane aliquots to assay buffer containing $[^3H]PtdInsP_2$. After incubation with/without Gpp[NH]p for 60 sec, the assay tubes were placed on ice and chloroform was added. The phases were separated and two aliquots of the aqueous phase (containing the $[^3H]PtdInsP_2$ hydrolysis products) were removed and counted for tritium content by liquid scintillation spectroscopy with quench correction.

4.9 $[^3H]$Inositol-(1,4,5)-trisphosphate ($[^3H]Ins(1,4,5)P_3$) dephosphorylation assay (paper VII)

The assay used was a modification of that described by Dean and Moyer (1988). Briefly, homogenates (1:10 (w/v), in 20 mM Tris-HCl + 0·2 mM EDTA, pH 7.6) were made from rat cerebral cortices. After centrifugation at 1000 g for 6.5 min to remove cell debris followed by centrifugation of the supernatants at 100 000 g for 60 min, the pellets ("Particulate fractions") were resuspended in homogenising buffer. Both pellets and supernatants ("Soluble fractions") were frozen until further use.

Aliquots from respective fractions were preincubated for 10 min at 37 °C in assay buffer (10 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, pH 7.2 in the presence

of LiCl and NaF±$AlCl_3$, as appropriate). [^{3}H]Ins(1,4,5)P_3 (10 μM final concentration) was added, and the samples incubated for 10 min at 37 ^{0}C. Reactions were stopped by addition of ice-cold chloroform:methanol (1:2 v/v). After mixing and centrifugation, phases were separated.

The [^{3}H]-labelled insositol phosphates were separated on Amprep SAX minicolumns by modification of the method of Minchin and Wood (1986).

5 STATISTICS

In general, unless otherwise stated, two-tailed paired *t*-test was used for parametric comparisons and Wilcoxon matched-pairs signed ranks test for nonparametric comparisons.

6 RESULTS AND DISCUSSION

6.1 Methodological considerations

6.1.1 Dissection time and temperature

When several brain samples are dissected there will be a considerable time between death and the start of an assay. In order to avoid sample desiccation, which greatly affects the degree of phosphoinositide labelling (CJ Fowler, personal communication) samples are usually placed in ice-cold KHB buffers immediatly upon dissection. Such a procedure, however, subjects the tissues to a temperature "shock" before they are used in the inositol phospholipid breakdown assay.

The effect of the temperature of the KHB buffer used directly after the dissection and during the washes of the miniprisms prior to the phosphoinositide labelling phase of the assay was investigated. The inositol phospholipid breakdown response to NA at an assay [K^+] of 5.88 mM was significantly lower when the miniprisms were prepared at 4 °C than at 20 °C (**I** fig 3). For Cb and 5-HT there was a much smaller (if any) temperature dependence (**I** table 3). When the assay [K^+] was raised to 18.2 mM, no temperature dependence for NA was observed (**I** fig 3). In the remaining experiments in this thesis, the miniprisms were prepared at 20 °C.

In order to examine the sensivity of post-mortal time on inositol phospholipid breakdown, rat brains were dissected and left *in situ* at room temperature (20 °C) for 0, 40, 80 and 120 min. Although the Lipid d.p.m. were reduced as the post-mortem time was increased, there was a very good post-mortal stability of the basal, NA- and Cb-stimulated inositol phospholipid breakdown responses at [K^+] of 6 and 18 mM up to 120

min (**II** fig 1). This is in good agreement with a previous study by Candy et al (1984), who used Cb, 6 mm K^+ and a post-mortem interval of 4 hours. The good post-mortem stability of the phosphoinositide system thus does not explain the variable labelling of pig brain miniprisms (20 to 40 min post-mortem time), where one third of the experiments had to be discarded because of low d.p.m. (**II**).

In later studies (**V-VII**) a miniprism pregassing phase was included prior to incubation with *myo*[2-^{3}H]-inositol (see **4.3**). This was found to increase the phosphoinositide labelling and the responsiveness of the PLC to stimulation by Cb at 6 mM K^+ (data not shown). Relevant to this, Whittingham (1987) has shown that for hippocampal miniprisms it takes at least 1 hour from the start of incubation, for ATP and energy charge values to reach 80 % of their *in situ* steady-state levels and that the slices continue to improve metabolically during an 8-hour incubation.

6.1.2 Freezing of tissue

It would be very convenient if the tissues used for inositol phospholipid breakdown studies could be frozen and stored prior to use. The "slow freeze - fast thaw" tissue storage method described by Hardy et al (1983), has proved to be useful for neurotransmitter uptake and release measurements in human post-mortem samples (for review see Fowler 1986). To examine the cryostability of the phosphosinositide system, the "slow freeze-fast thaw" method was used for hippocampal samples. Storage of tissue for 24-48 hours at -70 °C led to both a large reduction of the incorporation of the radiolabel into the Lipid and InsP fractions and a reduced inositol phospholipid breakdown response to stimulation by NA (**I** table 2). Thus, it seems that this storage method is not suitable when studying NA-stimulated inositol phospholipid breakdown, unlike the situation for Cb-stimulated inositol phospholipid breakdown (Candy et al 1984).

6.1.3 Expression of units and incorporation time with *myo*[2-^{3}H]-inositol

As mentioned in section **2.1.3**, several different ways of expressing the data from inositol phospholipid breakdown assays are used in the literature. Three of these units, InsP, InsP/Lipid, and InsP/(Lipid+InsP) were investigated in **I**. The latter units can be used in the "pulse label" assay since there is no further labelling of the lipids after the removal of the *myo*[2-^{3}H]-inositol (**I** table 1, **III** table 2, **VII** table 2). In the "continuous label" assay, on the other hand, lipid synthesis continues, particularly at high degrees of receptor stimulation (**VII** table 3) rendering these units inapplicable.

In the "pulse label" assay, InsP/(Lipid+InsP) was found to be a better unit than InsP d.p.m., since a) it is not dependent upon the number of miniprisms pipetted (**I** fig 1) or

b) upon the degree of phosphoinositide labelling (**I** fig 2). Both these factors affect InsP d.p.m., and since there is variability of (a) within and (b) between experiments, it is not surprising that the relative S.D. values were higher with InsP d.p.m. than with InsP/(Lipid+InsP). InsP/Lipid was found to be a poor unit, since at high rates of stimulation, there is significant lipid depletion (**I** table 1).

6.1.4 The use of lithium in the inositol phospholipid breakdown assay

Accurate measurement of the inositol phospholipid breakdown by the "pulse label" assay requires that lithium, at concentrations inhibiting the inositol monophosphatases, does not affect the basal or agonist-stimulated PLC activity, either by direct effects on the enzyme or upon the receptor-G protein-effector complex. The effects of different [Li^+] on miniprism AC activity, membrane PLC activity (with [^{3}H]PtdInsP_2 as substrate) and upon miniprism inositol phospholipid breakdown ("pulse label" assay) was examined in **IV**. Forskolin (10 μM)-stimulated AC activity was reduced by 28, 43, 56 and 61 % with 2.5, 5, 10 and 20 mM Li^+, a finding essentially in agreement with the study of Newman and Belmaker (1987). On the other hand, Li^+ (0-40 mM) was without significant effect upon the hydrolysis of PtdIns(4,5)P_2 in membrane preparations (**IV** table 1), indicating that it does not affect the catalytic unit of PLC *per se*.

In prelabelled miniprisms incubated with Cb or NA in the absence of lithium, there was very little accumulation of InsP due to its rapid metabolism. However, InsP could be trapped by 2.3 mM Li^+ at both 6 and 18 mM K^+ (**IV** fig 2). Over a concentration range of 2.3 - 35 mM, Li^+ acted additively with Cb- and NA-stimulated inositol phospholipid breakdown (**IV** fig 2). Additivity of Li^+ with NaF-stimulated inositol phospholipid breakdown was also seen (**IV** fig 3). Interestingly, a response to NaF was seen in the absence of Li^+, indicating an inhibition by NaF of inositol polyphosphate dephosphorylation (see **6.5**). Thus in the brain miniprism preparations, Li^+ ions does not interfere with the responses to Cb or NA as a result of effects on the receptor-G protein-PLC complex. Repeated treatment of animals with lithium, however, has been shown to down-regulate the system, though again not as a result of changes in the catalytic activity of PLC or of the PLC coupled G proteins (Casebolt & Jope 1989).

6.1.5 Extraction of the polyphosphoinositides

In the method described by Berridge et al (1982) and in **I** and **II** neutral chloroform/methanol was used to extract the phosphoinositides. However, PtdInsP and PtdInsP_2 are not extracted from the miniprisms unless acidified chloroform/methanol is used (Hauser et al 1971; see **III** table 2). Neutral extraction thus leads to an overestimation (albeit small

since the relative labelling of PtdInsP and PtdInsP$_2$ are low with respect to PtdIns, see **III** table 2) in the inositol phospholipid breakdown rate expressed as InsP/(Lipid+InsP). However, it was found that this overestimation was constant regardless of the level of stimulation of breakdown (**III** table 2). Acid extraction of the polyphosphoinositides was used in the remaing studies (**IV-VII**).

6.2 Neurotransmitter- and agonist-stimulated inositol phospholipid breakdown in rat and pig brain miniprisms

6.2.1 Carbachol

Cb (0-5 mM) dose-dependently stimulated inositol phospholipid breakdown in rat cortical and striatal miniprisms with EC_{50} values (at 6 mM K^+) of 79 and 23 μM, respectively (**V** fig 1). In pig striatum the EC_{50} for Cb was approximately 1 μM (**II** fig 3). Regional distribution studies showed larger responses to Cb (at 18 mM K^+) in the cortex, hippocampus and striatum than in the hypothalamus and cerebellum (**III** table 4) in agreement with the literature (Rooney & Nahorski 1986). The EC_{50} value for rat cortical miniprisms is in agreement with the findings of others (Brown et al 1984; Gonzales & Crews 1984; Jacobson et al 1985; Court et al 1986) and the rat striatal value is close to the values reported by Rooney and Nahorski (46 μM) (1986). The value for pig striatum is quite similar to that found in guinea-pig (7-8 μM, Fisher & Bartus 1985; Fisher & Snider 1987). These authors found higher EC_{50} values in guinea-pig cortex and hippocampus than in striatum, due to regional differences in receptor occupancy requirements.

The muscarinic antagonist PZ inhibited Cb-stimulated inositol phospholipid breakdown in cortical (**IV** table 3, **V**) and striatal (**V**) miniprisms. In the cortex, the IC_{50} value and Hill coefficients were 45 nM and 0.8, respectively at 18 mM K^+, in line with the literature (e.g. Gil & Wolfe 1985) at 6 mM K^+, and suggesting predominance of M_1-type receptors. In the striatum, however, the IC_{50} value was higher (275 nM) and the Hill coefficient lower 0.4 (at 6 mM K^+), in agreement with the study of Monsma et al (1988) and suggesting the involvement of both M_1 and M_2-type receptors (presumably corresponding to the m1 and m3 receptor gene products, respectively).

6.2.2 Noradrenaline

In rat brain miniprisms, NA is known to stimulate inositol phospholipid breakdown via α_1-adrenoceptors and with an EC_{50} value of 2-3 μM (see Fowler et al 1986). Such stimulatory effects of NA in rat hippocampal and cortical miniprisms were confirmed in

the present studies (**I, IV, VII**). In pig hippocampal miniprisms, NA stimulated inositol phospholipid breakdown with an EC_{50} value at 18 mM K^+ of approx 25 μM (**II** fig 3).

6.2.3 5-hydroxytryptamine

5-HT stimulation of inositol phospholipid breakdown is mediated in cortical regions via 5-HT_2-receptors, and in the choroid plexus by 5-HT_{1C} receptors (Conn & Sanders-Bush 1985). The stimulation by 5-HT is generally lower than that found with Cb and NA (see e.g. Kendall & Nahorski 1985b), particularly when the present assay conditions are used (Court et al 1986). Thus, weak stimulation of breakdown was seen in the rat cortex (**I** table 2) and only at raised [K^+] in the hippocampus (**I** table 1).

In pig brain miniprisms, 5-HT did not stimulate inositol phospholipid breakdown in either the striatum (**II** fig 5) or the hippocampus (**II** fig 6). The lack of stimulation in pig hippocampus is somewhat surprising given the presence of 5-HT_2 receptors in this region (Pazos et al 1985). However, the presence of binding sites does not always mean that there is a corresponding functional response. Thus, for example, in the rat cerebellum, α_1-adrenoceptor binding sites are present, but there is only a very small inositol phospholipid breakdown response to NA (Fowler et al 1986).

6.2.4 Dopamine

The literature as regards the effects of DA on inositol phospholipid breakdown in different tissues are contradictory. In rat anteroir pituitary cells, DA has been reported to inhibit inositol phospholipid breakdown *per se* (Journot et al 1987) or to inhibit angiotensin II-stimulated inositol phospholipid breakdown (Enjalbert et al 1986, for review see Enjalbert 1989), although both these findings have been disputed (Canonico et al 1986). In the rat striatum, Kelly et al (1989) found no effect of DA on inositol phospholipid breakdown. DA was found to stimulate inositol phospholipid breakdown in pig striatal miniprisms, although high (mM) concentrations were needed (**II** fig 5). Stimulatory effects were also seen in hippocampal slices (**II** fig 6), making it unlikely that dopaminergic receptors are involved in this effect. Since DA stimulates noradrenergic receptors at high concentrations (see e.g. Thoenen 1969), the most probable explanation is that the inositol phospholipid breakdown response is elicited by a noradrenergic mechanism. This is supported by the results shown in (**II** fig 7) where addition of 1mM DA reduced the response produced by NA (1mM) to the levels found in presence of DA alone, whereas the response to Cb was not affected.

6.2.5 Excitatory amino acids

The excitatory amino acids (EAA) glutamate, quisqualate, kainate and N-methyl-D-aspartate (NMDA) are known to stimulate inositol phospholipid breakdown in brain tissues (for reviews, see Chuang 1989 and Smart 1989). In the case of quisqualate response, this appears to mediated by a receptor different from the quisqualate receptor defined electrophysiologically (the AMPA receptor) and it is at present referred to as the metabotropic receptor (see Receptor Nomenclature suppl, 1990). The effects of NMDA upon inositol phospholipid breakdown are in general much smaller (when present) than the quisqualate response and may represent indirect rather than direct effects on PLC. With the "pulse label" assay, quisqualate (and glutamate) stimulated inositol phospholipid breakdown, the best response to quisqualate being found in the cerebellum (**III** fig 4). The response levels were much lower, however, than found with Cb and NA. On the other hand, no response to NMDA or quinolinate was found (**III** fig 4, table 3). The lower range of stimulation by the EAAs found in **III** are due in part to the age of the animals used. Thus, the greatest responses to EAA stimulation are found just after birth, and the response then decline as the animal grows older (Nicoletti et al 1986, see also Récasens et al 1987).

6.3 Effects of depolarising stimuli on inositol phospholipid breakdown in rat and pig

As mentioned in section **2.6** potassium enhances inositol phospholipid breakdown in rat brain miniprisms. Consistent with the study of Court et al (1986), we found that increasing the $[K^+]$ (or $[Rb^+]$) from 6 to 18 mM only had minor effects on basal inositol phospholipid breakdown in rat (see **III** fig 1 for dose-response curve) and pig (see e.g. **II** fig 2) brain miniprisms, whereas considerable stimulation was seen at ≈50 mM K^+ (**III** table 1; **VII** fig 3; **II** fig 4). The muscarinic antagonist PZ did not affect the inositol phospholipid breakdown stimulated by raised $[K^+]$ (**V** fig 2 and **IV** table 3), indicating that the effect of raised $[K^+]$ on basal breakdown is not secondarily to ACh release. Stimulation secondary to ACh release can, however, be seen in the presence of an ACh-esterase inhibitor (Kendall & Nahorski 1987).

Potassium exerts its tissue depolarising effects by activating voltage-sensitive calcium channels. This may not, however, explain the stimulatory effects of 50 mM K^+ upon basal inositol phospholipid breakdown, since at an assay $[Ca^{2+}]$ of 2.52 mM the L-type Ca^{2+}-channel agonist BAY K-8644 and the calcium ionophore ionomycin had, under the conditions used, rather minor effects on basal inositol phospholipid breakdown (**VI** fig 1, table 1). The L-type and N-type Ca^{2+}-channels antagonists verapamil and ω-

conotoxin, respectively, have also only minor effects upon the stimulation of breakdown produced by raised [K^+] at 2.52 mM Ca^{2+} (**VI** fig 1, table 1). These results are surprising, since there is a regional correlation between the degree of stimulation produced by K^+ and the density of L-type Ca^{2+}-channels in the rat brain (Rooney & Nahorski 1986), although these authors used different assay conditions (1.3 mM extracellular [Ca^{2+}], "continuous label" assay). On the other hand, voltage-sensitive Na^+-channels may be involved in the inositol phospholipid breakdown response to raised K^+, since veratrine (a sodium channel activator) strongly stimulated inositol phospholipid breakdown at 5.7 mM [K^+] in agreement with other results (Maier & Rutledge 1986; Benuck et al 1989) and weakly at 17.8 mM (**III** table 1). A stimulation was also found with the sodium ionophore monensin (G Tiger, P-E Björklund and CJ Fowler, unpublished results) in agreement with the study of Gusovsky et al (1986) who used synaptosomes.

6.4 Interaction between potassium ions and neurotransmitter-stimulated inositol phospholipid breakdown

6.4.1 Species and receptor selectivity

Using cortical and hippocampal miniprisms, Cb-stimulated inositol phospholipid breakdown is greatly enhanced by varying the assay [K^+] (for dose-response curves at 6-18 mM [K^+] on Cb (100 μM) -stimulated inositol phospholipid breakdown, see **III** fig 1) in agreement with the literature (Court et al 1986). 18 mM K^+ appears to be optimal, higher [K^+] increasing basal breakdown without producing further enhancement of the response to Cb (**III** table 1). The combination of K^+ and Cb does not radically alter the relative labelling of the individual polyphosphoinositides (**III** table 2) indicating that the synergy is not simply a reflection of an increased specific labelling of [^{3}H]PtdInsP_2.

The effects of K^+ were mimicked by Rb^+ (**III** table 1). The selectivity of the response was investigated in **I-V**. NA- and 5-HT-stimulated breakdown was enhanced by raised K^+, but to a much smaller extent than Cb-stimulated breakdown (**I** table 1 and 3, **IV** fig 2), in agreement with the study of Court et al (1986). Quisqualate- and glutamate-stimulated breakdown showed no synergy with raised [K^+] (**III** fig 3, table 3). In the pig, selectivity was also found, since raised [K^+] enhanced the response to Cb but not to NA (and DA) (**II**). Selectivity was also obtained within muscarinic receptors in the rat brain: M_2-type receptors coupled stimulatorily to PLC and inhibitorily to AC (corresponding to the m3 and m4 gene products, respectively) did not appear to be as sensitive to raised [K^+] as M_1-type receptors coupled to PLC (**IV,V**). Consistent with this Gurwitz and Sokolovksy (1987) reported that in rat atrial slices the synergy between M_2-type muscarinic receptor-mediated inositol phospholipid breakdown and K^+ was much lower

and only seen at lower calcium concentrations. On the other hand SH-SY5Y, IMR-32 and SK-N-MC human neuroblastoma cells, which express M_1-type muscarinic receptors coupled to inositol phospholipid breakdown, do not show synergistic effects with raised [K^+] (Fowler et al 1989; CJ Fowler and CP Ahlgren, unpublished results). The reasons for this discrepancy are at present not clear. Similarly surprising is the lack of effect of raised [K^+] upon Cb-stimulated inositol phospholipid breakdown (in the presence of GTP[γ]S) in rat brain membrane preparations (Claro et al 1989b). However, a direct effect of K^+ on the receptor-G protein-PLC system, beyond the level of the acceptor site (see below), might not be measurable in the membranes with the assay system used.

6.4.2 Comparison with other depolarising agents

The dependence of calcium in receptor-mediated inositol phospholipid breakdown responses has been demonstrated in several reports using an extracellular [Ca^{2+}] ≤ 1.3 mM (see e.g. Kendall & Nahorski 1984, 1985a; Baird & Nahorski 1986; Zernig et al 1986; Gurwitz & Sokolovsky 1987; Baird et al 1989, see also EGTA dependence of the response in Ca^{2+}-free KHB buffer in **VI** fig 1). Baird and Nahorski (1986) suggested that the synergy between K^+ and Cb was secondary to stimulation of voltage-sensitive Ca^{2+}-channels. The role of these channels have been investigated in **VI**. In Ca^{2+}-free KHB buffer, the response to Cb at 6 and 18 mM K^+ is greatly inhibited by the L-type Ca^{2+}-channel antagonist verapamil, and there is some synergy at 6 mM K^+ between the agonist of the same channel, BAY K-8644 and Cb (**VI** fig1), consistent with this notion. However, at an extracellular [Ca^{2+}] of 2.52 mM these compounds have marginal effects on Cb-stimulated inositol phospholipid breakdown at either 6 or 18 mM K^+ (**VI** fig1, table 1), suggesting that at this extracellular [Ca^{2+}] potassium ions exert the synergistic effects independently of actions upon voltage-sensitive calcium channels. Such a conclusion would explain why depolarisation produced by high concentrations of EAA:s (leading to increased $[Ca^{2+}]_i$) do not produce synergistic effects on Cb-stimulated inositol phospholipid breakdown at an extracellular [Ca^{2+}] of 2.52 mM (**III** fig 3 and 4, table 3).

Other tissue depolarising agents were investigated at an assay [Ca^{2+}] of 2.52 mM. Veratrine was found to inhibit, rather than enhance the breakdown response to Cb (**III** table 1, see fig 5), a result also found with the sodium ionophore monensin (G Tiger, P-E Björklund and CJ Fowler, unpublished observations) and the K^+-channel antagonists 4-aminopyridine and tetraethylammonium (**III**). However, veratrine, 4-aminopyridine and tetraethylammonium were found potently to inhibit the binding of [^{3}H]QNB to muscarinic receptors (**III**), suggesting that the effects of these compounds on Cb-stimulated inositol phospholipid breakdown are secondary to muscarinic receptor

antagonism rather than to modulation of ion channels. With respect to this, alaproclate (which modulates K^+-ion currents but which does not affect muscarinic receptors) does not affect Cb-stimulated inositol phospholipid breakdown (Fowler et al 1987).

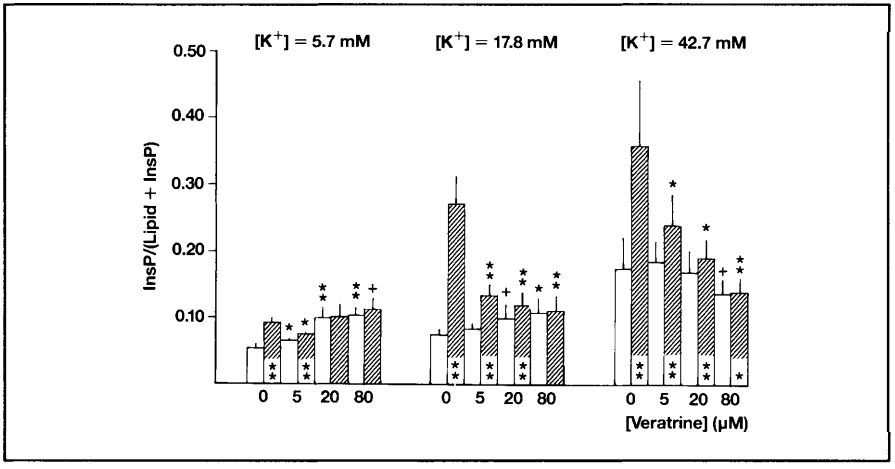

Fig 5. Effect of veratrine on basal and Cb-stimulated inositol phospholipid breakdown in rat cerebral cortical miniprisms. Miniprisms were incubated with veratrine in the absence (open columns) or presence (shaded columns) of 100 μM Cb for 25 min at 37°C at the assay [K^+] shown. Data are means ±SD, n=4. The asterisks enclosed within the columns indicate significance levels for differences between the basal and Cb-stimulated inositol phospholipid breakdown values at the same [veratrine]; asterisks above the columns indicate significance levels for differences between inositol phospholipid breakdown values in the absence or presence of the appropriate [veratrine] at the same [Cb]: $^+0.10>p>0.05$, $^*p<0.05$, $^{**}p<0.01$, two-tailed paired t-test. Data included in the text in ***III****.*

Lithium ions at a concentration of 20 mM are reported to depolarise rat cortical slices to about the same extent as that found with 20 mM K^+ (Adam-Vizi et al 1987). However, raising the [Li^+] from 2.3 to 35 mM was additive rather than synergistic with the Cb- (and NA-) stimulated inositol phospholipid breakdown (**IV** fig 2A). Depolarisation secondary to inhibtion of Na^+-K^+-ATPase activity produced by 10 μM ouabain did not enhance the Cb-stimulated inositol phospholipid breakdown at 6 mM K^+ (G Tiger, P-E Björklund and CJ Fowler, unpublished results). Finally, the K^+-channel agonist BRL 34915 (cromakalim), which produces hyperpolarization in hippocampal neurons (Alzheimer et al 1989), did not reduce the enhancement by raised K^+ on Cb-stimulated inositol phospholipid breakdown (**III**, see fig 6). Thus, in rat brain miniprisms at an extracellular [Ca^{2+}] of 2.52 mM the synergistic effects of K^+ and Cb do not appear to be related to the tissue depolarising actions of this ion *per se*.

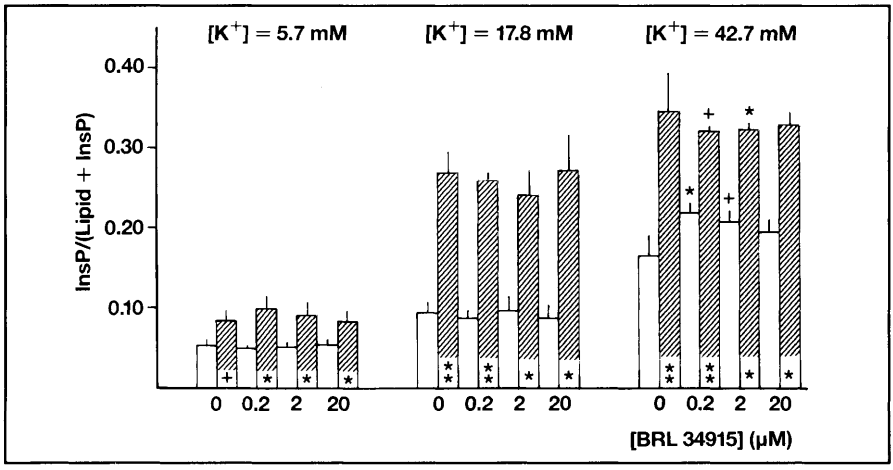

Fig 6. Effect of BRL 34915 on basal and Cb-stimulated inositol phospholipid breakdown in rat cerebral cortical miniprisms. Miniprisms were incubated with BRL 34915 for 30 min at 37 °C at the assay [K+] shown, after which Cb was added to give final concentrations of either 0 (open columns) or 100 μM (shaded columns) and the miniprisms were incubated for a further 25 min at 37 °C. Data are means ±SD, n=3. Significance levels are as described in fig 5. Data included in the text in III.

6.4.3 K^+-induced release of modulatory substances

The conclusions made in **6.4.2** above would tend to rule out *per se* the suggestion that K^+ acts synergisticaly with Cb secondary to the K^+-induced release of modulatory agents. Independent evidence supports this contention. K^+-evoked release of ACh, for example, would have been expected to affect the EC_{50} for the response to Cb without affecting the maximal response, whereas the reverse is true in the frontal cortex (V fig 1). K^+-induced release of adenosine (which can modulate the inositol phospholipid breakdown response to Cb in striatal slices, see El-Etr et al 1989), does not appear to involved at 2.52 mM Ca^{2+} in the cortex, since 2-chloroadenosine did not affect the response to Cb at either 6 or 18 mM K^+ for either untreated or adenosine deaminase-treated miniprisms (**VI**). K^+-induced release of peptides (which have also been shown to modulate Cb-stimulated inositol phospholipid breakdown Raiteri et al 1987; Palazzi et al 1988) is also unlikely, since depletion of their intracellular stores induced by raising the $[K^+]$ to 50 mM during the first, but not second, washing phase of the assay, has been found not to reduce the enhancement of the Cb-response by raised assay K^+ (G Tiger, P-E Björklund and CJ Fowler, unpublished results). These results, together with the reports that Cb-K^+ synergy is also found in synaptosomes and synaptoneurosomes (Brammer et al 1988; Guiramand et al 1989), suggests that release of a modulatory factor by K^+ is not a likely explanation

for the synergy.

6.4.4 K^+-Cb synergy, "cross-talk" and feed-back mechanisms

As mentioned in section **1** of this introduction (see also fig 2) the different effector systems influence each other, an effect called "cross-talk". In order to determine whether "cross-talk" and/or feed-back mechanisms could be responsible for the K^+-Cb synergy at 2.52 mM Ca^{2+}, forskolin (a stimulator of AC), 8BrcGMP (a stable analogue of cGMP), sodium nitroprusside (an activator of guanylate cyclase) as well as staurosporine (a potent PKC inhibitor) were studied in **VII**. These compounds were found to have at best minor effects on inositol phospholipid breakdown and they did not affect the Cb-stimulated response at either 6 or 18 mM K^+ (**VII** table 1). Although forskolin has been reported to inhibit Cb-stimulated inositol phospholipid breakdown in human SK-N-SH neuroblastoma cells (Akil & Fisher 1989), the lack of effect of forskolin (at the concentrations affecting AC, **IV**) on the Cb-response in rat brain is in agreement with a previous study at 6 mM K^+ (Godfrey & Watson 1988) and suggests that the effect of raised K^+ on Cb-stimulated inositol phospholipid breakdown is not secondary to activation of AC (**VII** table 1). The lack of effect of nitroprusside (with either "pulse" or "continuous" label assays) or 8BrcGMP (**VII**) is at variance with the finding of Ormandy and Jope (1989), who reported that these compounds inhibited Cb-stimulated inositol phospholipid breakdown at 20, but not 6, mM K^+ in rat cortical miniprisms using the "continuous label" assay. The reasons for this discrepancy are not clear. Changes in the activity of PKC have been reported to affect the muscarinic receptor-mediated inositol phospholipid breakdown (Labarca et al 1984), but the lack of effect of staurosporine upon Cb-stimulated inositol phospholipid breakdown indicates that modulation of a tonic PKC-mediated feed-back mechanism is not involved in the Cb-K^+ synergy (**VII** table 1).

6.5 Effects of GTP analogues and fluoride ions on Cb-stimulated inositol phospholipid breakdown

From the above discussion, it is clear that at an extracellular [Ca^{2+}] of 2.52 mM, potassium ions do not enhance Cb-stimulated inositol phospholipid breakdown secondary to tissue depolarisation, release of modulatory substances or activation of "cross-talk" mechanisms. These results are consistent with the speculation of Eva and Costa (1986) that K^+ ions increase the efficiency of signal transduction via the muscarinic receptor-G protein-PLC complex. Since K^+ ions do not affect the antagonist binding (or agonist displacement of antagonist binding) to the muscarinic receptor, it is reasonable

to assume an action of K^+ distal to the neurotransmitter acceptor site. In order to investigate the role by G proteins, the effects of the GTP analogues GTP[γ]S and Gpp[NH]p, and fluoride ions, have been studied in **IV** and **VII**. All three compounds were found to stimulate inositol phospholipid breakdown in the miniprisms (**IV** and **VII**), consistent with the literature (see **2.1.5**). In the case of fluoride, addition of $AlCl_3$ did not increase the stimulation, indicating that there are sufficient aluminium present to build the AlF_4^- complex (**IV**), a result in agreement with the study of Jope (1988). NaF has been reported to inhibit Cb-stimulated inositol phospholipid breakdown (Godfrey & Watson 1988). In our first study without the pregassing phase, NaF was found to act additively with Cb at 6 mM K^+ (**IV**), whereas with the pregassing phase at 6 mM K^+ (**VII**) and with both conditions at 18 mMK^+, inhibitory actions of NaF on Cb-stimulated inositol phospholipid breakdown was seen (**IV, VII**). Cb stimulates inositol phospholipid breakdown by a tetrodoxin-sensitive and a tetrodotoxin-insensitive pathway (Gurwitz & Sokolovsky 1987). At 18 mM K^+, the tetrodotoxin-insensitive pathway dominates (Gurwitz & Sokolovsky 1987), whereas at 6 mM K^+, the relative components may be dependent upon the assay conditions used. Assuming that NaF inhibits the G protein coupled responses (i.e. the tetrodotoxin-insensitive component), than an increase in the relative amount of this component will influence the observed additivity of the response.

Initial experiments suggested that NaF (19 mM) produced a complete antagonism of the enhancement by K^+ ions of Cb-stimulated inositol phospholipid breakdown (**VII** fig 2). Further experiments in **VII**, however, suggest that the inhibitory effects of fluoride ions on Cb-stimulated inositol phospholipid breakdown or upon the K^+-Cb synergy cannot simply be ascribed to effects on G proteins coupled to PLC. Thus Gpp[NH]p and GTP[γ]S do not inhibit the Cb response or affect the K^+-Cb synergy (**VII** table 3). It is possible that NaF produces its effects secondary to activation of all (i.e. not only those coupled to PLC) GDP-bound $G_{\alpha\beta\gamma}$ heterotrimeric proteins within the preparation. This action would lead to the release of large amounts of βγ subunits, which have been shown to inhibit inositol phospholipid breakdown responses of Cb and other stimulatory agents (Boyer et al 1989; Moriarty et al 1988, 1989). However, NaF not only interacts with G proteins coupled to PLC, but also inhibit the PI kinase responsible for the synthesis of PtdInsP from PtdIns (Kai et al 1966; Biffen & Martin 1989). Such an inhibitory action would have been expected to limit availability of $[^3H]PtdInsP_2$ at high rates of stimulation and thereby produce a response "ceiling". The time course study in (**VII** fig 4) and also the data in (**VII** fig 3) are consistent with this suggestion, as in the presence of NaF, stimulation above an InsP/(Lipid+InsP) value of about 0.15-0.20 (a value expected from the relative proportions of the polyphosphoinositides in the lipid phase, see **III** table 2) is not possible, regardless of the stimulatory agonist used (see also **VII** fig 3). Such an effect is exacerbated by the fluoride-induced hydrolysis of the nonphosphorylated

phosphatidylinositol (**VII** tables 2 and 3) further reducing substrate availibility.

NaF not only affects PLC substrate availibility, but also prevents the breakdown of the inositol polyphosphates produced. This is demonstrated in (**IV** fig3 and table 3), where the response to NaF (and Cb in the presence of NaF) can be seen even in the absence of Li^+. NaF has been shown to inhibit inositol monophosphatases (Hallcher & Sherman 1980; Creba et al 1989), as well as other phosphatase enzymes (Igarashi & Hollander 1968; Swanson 1950). NaF also inhibits the breakdown of inositol 1,4-bisphosphate produced from addition of [^{3}H]Ins(1,4,5)P_3 to soluble rat brain preparations (**VII** fig 5). Thus, the multiplicity of actions of NaF upon the phosphoinositide cycle severly limit its usefulness in the study of G proteins coupled to PLC.

7. GENERAL CONCLUSIONS

1. For measurement of receptor-mediated inositol phospholipid breakdown in brain miniprisms, a dissection and miniprisms preparation temperature of 20 ^{0}C is more suitable than 4 ^{0}C. There is good post-mortal tissue stability of the response for up to 2 hours, but the cryostability of the response is poor.

2. The unit InsP/(Lipid+InsP) is a useful measure of inositol phospholipid breakdown, assayed by the "pulse label" method, since it is not dependent upon the lipid labelling of the tissue and the number of miniprisms pipetted. The use of lithium to "trap" the labelled inositol phosphates is not contraindicated, since lithium does not interfere with the receptor-G protein-PLC complex.

3. In the pig, carbachol, noradrenaline, dopamine and raised [K^+] stimulate inositol phospholipid breakdown. Dopamine, however, probably exerts its action through a noradrenergic mechanism. As in rat brain miniprisms, the response to carbachol is enhanced by raised [K^+] .

4. The synergy found between carbachol and potassium ions in rat brain miniprisms is selective for M_1-type muscarinic receptors coupled to PLC, with respect either to M_2-type receptors coupled to AC or PLC, or to other receptor systems coupled to PLC.

5. The K^+- Cb synergy is dependent upon the assay [Ca^{2+}] used. In Ca^{2+} -free buffer, the synergy may result in part from activation of voltage-sensitive calcium channels. At an extracellular calcium ion concentration of 2.52 mM, however, the synergy between potassium ions and carbachol is not due to the depolarising actions of K^+ *per se*, since other ways of depolarising the tissue do not produce such synergy. Furthermore, the synergy is not due to the K^+-induced release of modulatory substances, or to the modulation of tonic feed-back or "cross-talk" mechanisms. It is concluded that potassium ions exert their synergistic effects on the muscarinic M_1-type receptor-G protein-PLC complex to improve the efficiency of signal transduction mediated by this pathway.

6. Fluoride ions have a number of effects on the phosphoinositide cycle, in addition to their effects on G proteins. Fluoride reduces PLC substrate availability, presumably secondary to inhibition of phosphoinositide kinase and inhibits inositol bis- and monophosphate dephosphorylation, thus severly limiting its usefulness for study of G proteins coupled to PLC.

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