Phosphoinositides, aging and Alzheimer's disease

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PHOSPHOINOSITIDES, AGING AND ALZHEIMER'S DISEASE

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PHOSPHOINOSITIDES, AGING AND ALZHEIMER'S DISEASE

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, Prof. Mr. M.J. Cohen,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op donderdag, 26 november 1992 om 14.00 uur

door

JOHN BOTHMER

geboren op 26 augustus 1964 te Geleen

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The studies described in this thesis were carried out at the department of Neuro-Psychology & Psychobiology within the research programme 'Aging of Brain and Behavior' of the University of Limburg, Maastricht, The Netherlands.

Aan mijn vader

*Voor mijn moeder
Francine*

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CHAPTER 1

PHOSPHOINOSITIDES, AGING AND ALZHEIMER'S DISEASE : A SURVEY.

J. Bothmer

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1. INTRODUCTION

In the twentieth century, the aging of the population is becoming an increasing problem in economically developed western countries as a result of an increased longevity and a relatively low birth rate. Aging is a problem because it is associated with decreased functioning. The most obvious changes with age in humans are grey hair, wrinkled skin, replacement of muscles by fat, but also a decline in the ability to see, hear and taste. Furthermore, decreased motor function, sleep, behaviour and cognitive functions, which probably all result from age-related deficiencies in the brain, are also observed. The effort and the expense of caring for disabled elderly people are enormous and still growing. More important, however, is the loss of the quality of life caused by age-related deficits in the last decades of life. Aging research should therefore focus on factors that influence successful aging, which seems to be more important than trying to prolong the maximal life-span of humans, by searching for the causative factor of aging. According to Knook (1982), biochemical research on cellular aging could lead to a better insight into the disturbed physiological functions associated with aging and probably to the prevention of many serious age-related diseases, such as different types of cancer, osteoporosis, atherosclerosis and age-related brain diseases, such as dementing conditions and especially Alzheimer's disease.

A decline in the performance of some brain functions is an important factor in the loss of the quality of life in elderly people. In the study of brain functions in relation to aging, much effort has been put into histological research, in the counting of cells and synapses, and in the detection of molecules with antibodies etc. Pharmacological research has a strong tradition in studies with receptor blockers, receptor agonists, ion-channel blockers and agonists etc. Electrophysiological research aims at detecting electrical signal transduction in various regions of the heterogenous brain as a function of age. The present thesis aims at presenting more information on the neurochemical basis of brain aging and Alzheimer's disease. Three enzyme activities, important for the functioning of phosphoinositide metabolism, are studied.

The good functioning of the brain is based on the communication between neurons, which are the main building blocks of the brain. These neurons are well equipped with different means to communicate not only with neighbouring cells but also with more distant cells. These means include signal-sending as well as signal-processing systems. One of these signal-processing systems is the receptor-mediated phosphoinositide metabolism system which is essential for the activation of some cell functions. This system will be described in section 2. The most common theories of aging are described in section 3 of this chapter, followed by the more specific age-related changes directly related to phosphoinositide metabolism and its functions. Dementing diseases and especially Alzheimer's disease decreases the quality of life severely. In section 4, cellular changes occurring in Alzheimer's disease, which is the most frequently occurring type of dementia, are discussed in relation to the phosphoinositide metabolism and its functions. Section 5 describes the outline of this thesis.

2. PHOSPHOINOSITIDE METABOLISM

2.1. Introduction

The main characteristic of the brain is that its constituent cells communicate with each other and with the other parts of the body. Much of this communication is achieved by chemical signals, such as neurotransmitters, neuropeptides and hormones, which are released by one cell and create a reaction in an other cell. However, most chemical signals do not enter the target cells, so this extracellular signal has to be transformed into an intracellular signal. Phosphoinositides, a class of phospholipids which are relatively enriched in brain tissue (Hawthorne and Kai, 1970), play an important role in one of the pathways of signal transformation through the plasma membrane. Phosphoinositides are phospholipids with the C6-sugar inositol as polar head group and, predominantly, 1-stearoyl, 2-arachidonoyl as fatty acid tails attached to the glycerol backbone (Hawthorne, 1982). Phosphatidylinositol, the most abundant phosphoinositide, can be phosphorylated by addition of one or more phosphates to the inositol ring with ATP as phosphate donor, resulting in the formation of polyphosphoinositides.

The observation of Hokin and Hokin (1953) that acetylcholine increased the incorporation of labelled phosphate into phosphatidate and phosphatidylinositol of brain slices was the first report of a connection between signal transduction and the metabolism of these acidic phospholipids. Since then, many investigators have studied the metabolism of phosphoinositides in relation to receptor mediated signal transduction. Other (possible) functions of phosphoinositides have also received attention, such as regulation of growth, cytoskeletal rearrangements, and protein anchoring to the membrane. Many articles and reviews have been published on these topics. Phosphatidylinositol appears to have a rich diversity of forms and functions within cells, as do the enzymes associated with its metabolism. This part of the introduction, however, does not give a historical survey (as in Hokin, 1985) or a complete review of the whole field, but just a "state of the art" of the topics which are essential to this thesis.

2.2. Receptor-stimulated hydrolysis of polyphosphoinositides

An extracellular signal, such as a neurotransmitter, initiates signal transduction through the membrane by binding to a specific receptor on the cell surface. This receptor is coupled to a "second messenger-producing system" at the cytosolic site of the membrane, or to an ion channel. Receptors coupled to the phosphoinositide signal transduction mechanism are coupled to phospholipase C (PLC), a phosphoinositide-specific hydrolase, via an intermediate G-protein (Litosch, 1987; Strnad et al., 1986), in analogy to the adenylate cyclase messenger system. PLC activities have been purified from a wide variety of sources (Rhee et al., 1989; Baldassare et al., 1989). The G-protein requires GTP to be active in the stimulation of second messenger generation (Litosch, 1987). Besides stimulation

of phosphoinositide hydrolysis by agonist-receptor interactions, inhibition of hydrolysis has also been observed after agonist-receptor interactions (Linden and Delahunty, 1989). In analogy to the adenylate cyclase messenger system, Linden and Delahunty (1989) hypothesized a distinct G-protein for the inhibition of phosphoinositide hydrolysis. However, up to now there is no evidence to support this notion.

Because $I(1,4,5)P_3$ is the first inositol metabolite which accumulates after receptor activation (Burgess et al., 1985; Irvine et al., 1985), the main candidate for receptor-stimulated hydrolysis is the twice phosphorylated phosphatidylinositol 4,5-bisphosphate (PIP_2) (Berridge et al., 1983), formed by the consecutive phosphorylation of phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PIP) by PI 4-kinase and of $PI(4)P$ to $PI(4,5)P_2$ by $PI(4)P$ 5-kinase (reviewed by Carpenter and Cantley, 1990; Downes and MacPhee, 1990). However, the receptor-stimulated hydrolysis of PI and PIP has also been suggested (Berridge, 1987; Wilson et al., 1985). Only a small portion of the inositol lipid pool is involved in signalling (Billah and Lapetina, 1982; Fain and Berridge, 1979b; Monaco and Woods, 1983) and appears to be confined to the plasma membrane (Rana et al., 1986). Remarkably, the phosphoinositides seem to be located predominantly in the cytoplasmic leaflet of the plasma membrane (Berridge, 1984; Hokin, 1985). Even $PI(4,5)P_2$ may not be distributed homogeneously in the plasma membrane (Koreh and Monaco, 1986; Muller et al., 1986). The hydrolysis of $PI(4,5)P_2$ results in the release of the second messengers inositol-1,4,5-trisphosphate ($I(1,4,5)P_3$) and diacylglycerol (DAG) (Berridge, 1984).

Water-soluble $I(1,4,5)P_3$ enters the cytosolic compartment and either binds to an $I(1,4,5)P_3$ receptor or is metabolized (reviewed by Catt et al., 1991). Binding to the $I(1,4,5)P_3$ receptor results in the release of Ca^{2+} from intracellular stores (Streb et al., 1983), probably the endoplasmic reticulum (Streb et al., 1984), via the opening of a Ca^{2+} channel (Smith et al., 1985). This Ca^{2+} release from intracellular stores is followed by the entrance of extracellular calcium (Berridge, 1987). Increased Ca^{2+} levels result in the activation of Ca^{2+} /calmodulin-dependent protein kinase (Berridge, 1984). It has been suggested that the entry of extracellular calcium is initiated by inositol tetrakisphosphate ($I(1,3,4,5)P_4$) (Irvine and Moor, 1986; Morris et al., 1987). However, both IP_3 and IP_4 are required for a full response (Morris et al., 1987; Berridge and Irvine, 1989), in which $I(1,4,5)P_3$ causes a transient rise in Ca^{2+} and the presence of $I(1,3,4,5)P_4$ leads to a sustained Ca^{2+} level, probably by replenishing the $I(1,4,5)P_3$ sensitive Ca^{2+} pool (Irvine et al., 1988).

IP_4 is the phosphorylation product of $I(1,4,5)P_3$ in rat brain cytosol (Irvine et al., 1986) and other tissues (Berridge, 1987), and is present in the cytosol together with IP_3 after receptor stimulation (Batty et al., 1985). $I(1,3,4,5)P_4$ is dephosphorylated to $I(1,3,4)P_3$, which has no Ca^{2+} -mobilizing activity (Batty et al., 1985; Irvine et al., 1986). $I(1,3,4)P_3$ can be hydrolysed back to inositol by other routes (Berridge and Irvine, 1989). $I(1,4,5)P_3$ is rapidly dephosphorylated to $I(1,4)P_2$ by an enzyme similar, if not identical, to the enzyme which dephosphorylates IP_4 (Batty et al., 1985). $I(1,4)P_2$ is also inactive in releasing Ca^{2+} (Berridge and Irvine, 1984). $I(1,4)P_2$

Fig. 1 : Receptor-mediated phosphoinositide metabolism. PI: phosphatidylinositol, PIP: phosphatidylinositol phosphate, PIP₂: phosphatidylinositol bisphosphate, PLC: phospholipase C, G: G-protein, R: receptor, DAG: diacylglycerol, PA: phosphatidic acid, Ins-1,4,5-P₃: inositol-1,4,5-trisphosphate, Ins-1,3,4,5-P₄: inositol-1,3,4,5-tetrakisphosphate, PKC: protein kinase C.

is further dephosphorylated to myo-inositol (Storey et al., 1984), a reaction which can be inhibited by Li⁺ ions (Berridge et al., 1982; Sherman et al., 1981), resulting in a reduction in the supply of inositol. Because the inhibition by Li⁺ of signal transduction is non-competitive, Li⁺ ions are selectively active towards overactive receptors. This is the putative basis of the action of Li⁺ ions in manic depression, in contrast to the minimal effect of Li⁺ in subjects not suffering from manic depression (Berridge and Irvine, 1989). The significance of the complexity of inositol phosphate metabolism is unknown, although Berridge and Irvine (1989) hypothesized that this is simply an inevitable result of the 3-phosphorylation of I(1,4,5)P₃ to I(1,3,4,5)P₄, so more enzymes will be required to catabolize these and a complex pattern of intermediates cannot be avoided. In addition, higher phosphorylated derivatives, such as IP₅ and IP₆, have been

identified in various tissues including brain (Tilly et al., 1987) and possibly act as extracellular messengers (Vallejo et al., 1987). However, most of the inositol metabolites that accumulate in the cytosol of stimulated cells seem to be products of the metabolism of a single compound, $I(1,4,5)P_3$ (Downes, 1986).

Besides this Ca^{2+} -mobilizing second messenger, DAG is also released by the receptor-stimulated hydrolysis of PIP_2 (Berridge, 1984). DAG, composed of two fatty acid tails attached to a glycerol backbone, remains in the plasma membrane and activates protein kinase C (PKC), although phosphatidylserine and calcium are also required for PKC activation (Nishizuka, 1984). DAG increases the affinity of PKC for Ca^{2+} , so that maximal PKC activity is possible at basal cytosolic Ca^{2+} concentrations (Nishizuka, 1988). When PKC is fully activated it is tightly associated with the plasma membrane (Kaczmarek, 1987). This physical translocation of PKC from the cytosol into the membrane might be mediated by Ca^{2+} (May et al., 1985). Phospholipid-, calcium- and DAG-dependent PKC regulates many key processes in growth and development (Nishizuka, 1989), but also ion channels and neurotransmitter release (Kaczmarek, 1987). Remarkably, PKC appears to be a target for phorbol esters, as these tumour-promoting agents activate this enzyme directly both in vitro and in vivo, and there is an approximate correlation between the ability of individual phorbol esters to promote tumours and to activate the enzyme (Castagna et al., 1982; Yamanishi et al., 1983). Thus, PKC seems to be a receptor for tumour promoters, and possibly some, if not all, of the pleiotropic actions of tumour-promoting phorbol esters may be mediated through the action of PKC (Nishizuka, 1984). The level of DAG required for activation of PKC is a function of its production from phosphoinositides and its phosphorylation to PA or its degradation to its building blocks (Hokin, 1985).

Upon cell stimulation, DAG is rapidly converted by diacylglycerol kinase to phosphatidic acid (PA) (Kanoh et al., 1990), which could have two functions: first, to initiate the resynthesis of the phosphoinositides, and second, to control the concentration of the second messenger DAG, thus acting as a regulator of PKC (Nishizuka, 1986). Thus PA formed after DAG phosphorylation could be "just" an intermediate in the resynthesis of phosphoinositides as described above, but there are also indications that PA may directly mediate the inward movement of Ca^{2+} after activation of membrane receptors (Putney et al., 1980), or may mimic the action of growth factors by stimulating the hydrolysis of $PI(4,5)P_2$ (Moolenaar et al., 1986). DAG breakdown is catalysed by DAG lipase resulting in the liberation of arachidonic acid (AA). The DAG phosphorylation product PA can be broken down to release AA by the activation of specific phospholipase A_2 (Billah et al., 1981; Lapetina and Billah, 1981). AA and its metabolites are discussed in section 2.3.

The IP_3 branch and the DAG branch of the phosphoinositide signal cascade appear to act synergistically to phosphorylate proteins, the former by elevating Ca^{2+} concentrations to activate calmodulin-dependent protein phosphorylation, and the latter to activate PKC, which needs Ca^{2+} for its activity (Berridge, 1987; Hokin, 1985). These protein kinases regulate many other enzymes, ion-channels

etc. by phosphorylation, ultimately resulting in a biological response (Magnoni et al., 1991).

Different sources of inositol can be used for the resynthesis of phosphatidylinositol: Inositol can be formed *de novo*, by dephosphorylation of inositol phosphates, or by import from the extracellular fluid (Downes and MacPhee, 1990). Inositol is formed *de novo* by a myo-inositol-1-phosphate synthase, which forms IP₁ out of glucose 6-phosphate (Eisenberg, 1967), followed by dephosphorylation (Sherman et al., 1981). Phosphatidylinositol is also formed from myo-inositol and CDP-DAG (product of CTP and PA) by the action of PI synthase or CDP-DAG:myo-inositol 3-phosphatidyl transferase (Downes and MacPhee, 1990).

Receptor-stimulated inositol phospholipid hydrolysis or the so-called "PI effect" is found in various tissues of several species (listed in Abdel-Latif, 1986; Berridge, 1984; Hokin, 1985) including, of course, the central nervous system (CNS). Fisher et al. (1992) listed in their extensive review the receptors present on neurons and/or glia known to be linked to phosphoinositide turnover. They distinguish two groups of receptors: Category 1 receptors, which elicit a robust increase in inositol phospholipid turnover in tissue preparations of both the CNS and neurotumour cells, including muscarinic cholinergic, adrenergic, histaminergic, serotonergic, glutamatergic, and endothelin receptors; and Category 2 receptors, which elicit only a small increase in inositol phospholipid turnover in CNS-derived preparations, as evidenced by the use of neurotumour cells or other neural-related tissues, including purinergic, nerve growth factor, prostaglandin, bradykinin, vasopressin, oxytocin, thyrotropin-releasing hormone, and other receptors (Fisher et al., 1992).

2.3. Arachidonic acid

Receptor stimulation, which results in the release of the second messengers IP₃ and DAG, also appears to stimulate a lipase acting preferentially on species of DAG and PA which carry arachidonic acid on the 2-position (Berridge, 1984). Arachidonic acid (AA) is released from phosphoinositides and PA by different phospholipase A₂ (PLA₂) enzymes leading to the accumulation of lysophospholipids (Billah et al., 1981; Flower and Blackwell, 1976; Lapetina and Billah, 1981). The release of AA from phosphoinositides, however, requires much higher levels of Ca²⁺ and may only occur at supramaximal stimulation (Billah et al., 1980). AA can also be released from the second messenger DAG by a DAG lipase (Bell et al., 1979). AA is metabolized in the brain via three pathways (Piomelli and Greengard, 1990), namely the cyclooxygenase pathway, leading to the formation of prostaglandins, prostacyclins and thromboxane A₂, the lipoxygenase pathway, leading to the formation of leukotrienes, and the cytochrome P450 pathway, leading to the formation of an array of epoxyeicosatrienoic acids. AA appears to be involved in the induction of long-term potentiation, the modulation of signal transduction, the regulation of enzyme activities, and in intracellular Ca²⁺ homeostasis (see Strosznajder and

Samochocki, 1991, and ref.). Free AA and its metabolites raise the cytosolic Ca^{2+} level independently of IP_3 (Halushka and Burch, 1984; Wolf et al., 1986). Sekar and Hokin (1986) suggest in their review that the action of free arachidonate must be interpreted in the light of the detergent actions of this fatty acid.

2.4. D-3 phosphorylated inositol phospholipids

The metabolism of inositol phospholipids has recently become more complex with the discovery of D-3 phosphorylated inositol phospholipids. Whitman et al. (1987) divided the PI kinases into two different types. Type 1 PI kinase appears to be a PI 3-kinase (Whitman et al. 1988). The D-3 phosphorylation of the novel inositol phospholipid $\text{PI}(3)\text{P}$ was also shown by Stephens et al. (1989). The characteristics of the different PI kinases are discussed in section 2.6. Besides $\text{PI}(3)\text{P}$, $\text{PI}(3,4)\text{P}_2$ (with $\text{PI}(4)\text{P}$ as substrate) and $\text{PI}(3,4,5)\text{P}_3$ (with $\text{PI}(4,5)\text{P}_2$ as substrate) were also detected (Auger et al 1989). An inositol tetrakisphosphate containing inositol phospholipid was also found by Traynor-Kaplan et al. (1988) and Vadnal and Parthasarathy (1989), who suggested that PIP_3 could be the parent

Fig. 2 : A schematic diagram of the known phosphoinositide phosphorylation routes. The upper pathway is the "conventional" polyphosphoinositide pathway, and the lower pathways are the D-3 phosphorylation pathways.

compound for IP_4 . However, polyphosphoinositides phosphorylated in the D-3 position are poor substrates for phospholipase C from rat liver and bovine brain (Serunian et al., 1989). Probably, just one enzyme is responsible for the production of these D-3 phosphorylated compounds, as purified type 1 PI 3-kinase can use PI, $\text{PI}(4)\text{P}$ and $\text{PI}(4,5)\text{P}_2$ as substrate with almost equal kinetic

characteristics (Carpenter et al., 1990). However, Cunningham et al. (1990) reports the phosphorylation of PI(3)P to PI(3,4)P₂ and PI(3,4)P₂ to PI(3,4,5)P₃. The main feature of this enzyme activity or these enzyme activities is its (their) association with activated protein tyrosine kinases of growth factor receptors and oncogene products. These protein tyrosine kinases include pp60v-src, the polyoma middle T antigen / pp60c-src complex, the PDGF receptor, the CSF-1 receptor, the insulin receptor, and p68v-ros, the transforming protein of the avian sarcoma virus UR2, as listed by Cantley et al. (1991). Therefore, PI 3-kinase seems to be involved in cell transformation and cell division. This notion is strengthened by the results of mutation studies in which a loss of PI 3-kinase association coincides with a loss of the potency to transform cells (Chan et al., 1990; Courtneidge and Heber, 1987; Fukui and Hanafusa, 1989; Kaplan et al., 1986; Kaplan et al., 1987; Varticovski et al., 1991; Whitman et al., 1985). However, cells in a terminally differentiated form, such as neutrophils and platelets, also produce D-3 phosphorylated phosphoinositides as a result of cell activation by agonists (listed in Auger and Cantley, 1991). Auger and Cantley (1991) suggest that the novel polyphosphoinositides are involved in cytoskeletal reorganization because the effects found in neutrophils and platelets, as mentioned above, coincide with cytoskeletal changes, and a lack of association of PI 3-kinase appears to correlate with the lack of actin reorganization and mitogenic signals.

2.5. Glycosylated inositol phospholipids

Membrane-bound proteins can be attached to the membrane in various ways (Low, 1987). Until recently, this attachment was believed to be non-covalent in nature, by hydrophobic interaction of the protein with the membrane bilayer. However, in the last decade it has become clear that various membrane-bound proteins contain covalently bound lipid, in some cases a glycosylated phosphatidylinositol moiety (Low, 1987). The proteins utilizing glycosyl-phosphatidylinositol (GPI) as a membrane anchor are listed in reviews by Low (1989) and Lisanti et al. (1990). The structure of the glycan protein consists of ethanolaminephosphate, three mannose residues, and glucosamine linked to PI. The PI used for protein anchoring has a fatty acid composition rich in myristic acid, whereas the fatty acids attached to PI(4,5)P₂ are predominantly stearyl-arachidonyl in composition (Berridge and Irvine, 1989). Lisanti et al. (1990) discuss the possible functions of this strange and complicated way of membrane attachment, and suggest functions in the cellular distribution of proteins, lateral mobility of proteins, the exclusion of the anchored proteins from clathrin-coated pits, and/or, the regulation of the release of proteins (Berridge and Irvine, 1989; Lisanti et al., 1990).

In several cell types, a GPI has been described which shares the core structure of PI-glucosamine and contains additional monosaccharides, but which lacks ethanolamine and is not attached to protein (Saltiel and Cuatrecasas, 1988). These non-protein-bound GPIs located on the cytoplasmic aspect of the plasma membrane can be hydrolysed by an insulin-stimulated and phospholipase-

mediated mechanism, resulting in the release of a novel second messenger, the inositol-glycan, and DAG (Saltiel and Cuatrecasas, 1986; Saltiel et al., 1986). This novel second messenger affects particular enzymes *in vitro*, but its mechanism of action is not known, although it has marked effects on protein phosphatase and could have a direct regulatory mechanism of action (Saltiel, 1991). With respect to the many insulin-mediated effects, insulin can regulate both the phosphorylation of particular proteins by PKC (activated by DAG release), and the dephosphorylation of other proteins by protein phosphatases (activated by inositol-glycan). Besides insulin, insulin-like growth factors and a limited number of other peptides, for example NGF, also share this signal transduction mechanism. These agonists also share insulin-like biological activities such as the promotion of neurite outgrowth, enhancement of survival of certain neurons etc. (Saltiel, 1991).

2.6. Kinases in inositol phospholipid metabolism

For the generation of the second messengers IP_3 and DAG, $PI(4,5)P_2$ has to be formed out of PI by sequential phosphorylation via $PI(4)P$ to $PI(4,5)P_2$. The DAG formed after PIP_2 hydrolysis is rapidly phosphorylated resulting in the formation of PA. The kinases involved in the formation of PIP_2 out of PI and in the formation of D-3-phosphorylated phosphoinositides have been reviewed recently (Carpenter and Cantley, 1990; Downes and Macphée, 1990). DAG kinase activities have been reviewed recently by Kanoh et al. (1990). Current knowledge of the characteristics of kinases and the (possible) regulatory mechanisms are summarized below. Because only one form of DAG kinase has been purified and characterized (Kanoh et al., 1990), the information about DAG kinase will not be as lengthy as that for the inositol phospholipid kinases.

- PI kinase

There are three types of PI kinase (Carpenter and Cantley, 1990). These types differ in the position at which they phosphorylate the inositol ring, in some kinetic properties and in molecular mass. Whitman et al. (1987) identified two types of PI kinases in fibroblasts which differed in their sensitivity to adenosine and non-ionic detergents (Triton X-100).

Type 1, which appears to phosphorylate the D-3 position of the inositol ring (Whitman et al. 1988), is inhibited by detergent and is almost unaffected by adenosine (Whitman et al., 1987). This PI 3-kinase was originally described as a PI kinase that co-purified with pp60v-src, polyoma middle T antigen/pp60v-src complex and the PDGF receptor, which are all protein tyrosine kinases (Kaplan et al., 1987; Sugimoto et al., 1984; Whitman et al., 1985). PI 3-kinase is present in all eukaryotic organisms examined to date, but has not been found in bacteria (Carpenter and Cantley, 1990). This enzyme phosphorylates phosphatidylinositol, but also phosphatidylinositol-4-phosphate and phosphatidylinositol 4,5-bisphosphate at the D-3 position, with apparent K_m values for ATP of 30-60 μM

(Carpenter et al., 1990). The molecular mass of the native heterodimer enzyme appeared to be 190 kDa and is composed of a 85 kDa unit, which is the same protein shown to associate with polyoma virus middle T antigen and the PDGF receptor (Kaplan et al., 1987) and a 110 kDa unit (Carpenter et al., 1990). Both, the 110 kDa unit and the native 190 kDa protein have been purified from bovine thymus cytosol as active PI 3-kinases (Shibasaki et al., 1991), although Morgan et al. (1990) purified an active PI 3-kinase from bovine brain with a molecular mass of 85 kDa. However, further research revealed that the 85 kDa unit is a receptor tyrosine kinase substrate (Hayashi et al., 1991). Tyrosine phosphorylation of the 85 kDa subunit associated with PI 3-kinase activity results in trafficking or recruitment from the cytosol to the membrane (Cohen et al., 1990). Thus, an interaction between an agonist and a receptor which contains intrinsic tyrosine kinase activity (such as the insulin receptor) reveals an active tyrosine kinase which phosphorylates the 85 kDa subunit, resulting in the association of the PI 3-kinase with the receptor (Otsu et al., 1991). The activation of PI 3-kinase by the stimulation of receptors which contain intrinsic tyrosine kinase activity has been shown for PDGF (Auger et al., 1989), EGF (Pignatoro and Ascoli, 1990), IL-2 (Merida et al., 1991; Remillard et al., 1991), CSF-1 (Varticovski et al., 1989), insulin (Hayashi et al., 1991; Rudermann et al., 1990), and IGF-1 (Kapeller et al., 1990). However, whether the tyrosine phosphorylation of the 85 kDa unit also causes the activation of PI 3-kinase in addition to its translocation is unknown (Carpenter and Cantley, 1990). It has been suggested that a G-protein could be involved in the activation of PI 3-kinase (Traynor-Kaplan et al., 1988). The human gene encoding for PI 3-kinase associated p85 is located at chromosome region 5q12-13 (Cannizzaro et al., 1991). The 85 kDa subunit has been cloned (Escobedo et al., 1991; Skolnik et al., 1991) and appears to contain SH2 domains homologous to domains found in other receptor-associated enzymes (Escobedo et al., 1991). These domains provide a binding site for sequences that contain phosphotyrosine (Mayer and Hanafusa, 1990). It is anticipated that the 110 kDa unit, which is the PI 3-kinase itself (Shibasaki et al., 1991), will be cloned in the near future.

Type 2 PI kinase appears to phosphorylate the D-4 position of the inositol ring (Whitman et al., 1988), is activated by non-ionic detergents, and is inhibited by adenosine. However, Endemann et al. (1987) reported the existence of two PI 4-kinases in bovine brain, which differed clearly in molecular mass. The most extensively studied enzyme, the type 2 enzyme with a molecular mass of 55 kDa, appears to be an integral membrane protein as it requires detergent for its extraction from the membrane and is not present in large amounts in the soluble fraction of homogenized cells (Carpenter and Cantley, 1990; Downes and MacPhee, 1990). The type 2 PI kinase has been found in bovine brain (Endemann et al., 1991; Jenkins et al., 1991; Wetzker et al., 1991), sheep brain (Scholz et al., 1991), porcine liver microsomes (Hou et al., 1988), bovine uterus (Porter et al., 1988), and A431 cells (Walker et al., 1988). Type 2 PI kinase has a K_m value for ATP of 20-60 μM and a K_i for adenosine of 10-70 μM .

The type 3 PI 4-kinase is also membrane bound (Endemann et al., 1987),

although a type 3 PI 4-kinase seems to be present in the soluble fraction of the human placenta (Downes and MacPhee, 1990). Type 3 PI 4-kinase has a high K_m for ATP, is relatively resistant to adenosine inhibition and has an apparent molecular mass of 200 kDa (Carpenter and Cantley, 1990; Downes and MacPhee, 1990). However, the properties of the type-3-like PI kinases are not completely consistent (Li et al., 1989; Yamakawa and Takenawa, 1988) with the properties of the type 3 PI kinase reported by Endemann et al. (1987). The classification of PI kinases presented by Carpenter and Cantley (1990) is not complete, as other PI 4-kinases have been purified with properties other than those of the type 2 and type 3 PI kinases (Belunis et al., 1988; Kanoh et al., 1990; Saltiel et al., 1987).

Membrane-associated PI 4-kinase activity is located predominantly in low-density membranes possibly involved in membrane trafficking, and in the plasma membrane (Carpenter and Cantley, 1990; Lundberg and Jergil, 1988). Because PI is synthesized in the endoplasmic reticulum, this PI could be phosphorylated on its way to the plasma membrane. However, erythrocytes do not have intracellular membranes, yet contain a rather active PI 4-kinase (Downes and MacPhee, 1990). Careful analysis of type 2 versus type 3 PI 4-kinase activities in subcellular fractions has not been done (Carpenter and Cantley, 1990). The regulation of PI 4-kinase activity is poorly understood for several reasons listed by Carpenter and Cantley (1990). However, some possible mechanisms for the regulation of PI 4-kinase activity include activation by EGF (in A431 cells; Walker and Pike, 1987), activation by phorbol esters (Halenda and Feinstein, 1984; Taylor et al., 1984), activation by an elevation of cAMP (Kato et al., 1989) and activation by polyamines (Schuber, 1989).

- PIP kinase

PI(4)P 5-kinase catalyses the ultimate step in the production of the IP_3 and DAG precursor $PI(4,5)P_2$. PI(4)P 5-kinase is found in both soluble and membrane-bound forms (Carpenter and Cantley, 1990; Downes and MacPhee, 1990). The membrane-bound form is not as tightly bound to the membrane as PI 4-kinase is, as it can be solubilized by high salt concentrations in the absence of detergent (Ling et al., 1989; Moritz et al., 1990). Ling et al. (1989) have been the only group to demonstrate the phosphorylation of the 5-position of the inositol ring. PIP kinase has been purified from human red blood cells (Bazenet et al., 1990; Ling et al., 1989), human platelets (Suzuki et al., 1991), bovine brain (Moritz et al., 1990) and rat brain (Cochet and Chambaz, 1986; Van Dongen et al., 1984). The PI(4)P 5-kinase of human red blood cells has been studied in detail. Bazenet et al. (1990) found two distinct PI(4)P 5-kinases, a membrane-bound type 1, which has a more ubiquitous distribution and is responsible for most $PI(4,5)P_2$ synthesis, and a membrane-bound and cytosolic type 2, which is abundant only in cells of bone marrow origin and is identical to the enzyme purified by Ling et al. (1989). The latter type 2 PI(4)P 5-kinase has an apparent molecular mass of 53 kDa by SDS-PAGE (Bazenet et al., 1990; Ling et al., 1989) whereas the composition of the type 1 enzyme is still uncertain. The two forms of human platelet membrane PI(4)P 5-

kinase have molecular weights of 51 and 47 kDa, respectively (Suzuki et al., 1991). The PI(4)P 5-kinase of rat brain cytosol (Cochet and Chambaz, 1986) has a molecular mass of 45 kDa, whereas the membrane-bound PI(4)P 5-kinase of bovine brain has a molecular mass of 110 kDa by SDS-PAGE (Moritz et al., 1990).

PI(4)P 5-kinase could be regulated by product feedback inhibition (Van Rooijen et al., 1985), via the loss of PI(4,5)P₂, as a number of investigators have reported a stimulation of PIP₂ formation during/after receptor stimulation (Renard et al., 1987; Taylor et al., 1984). However, this is unlikely to be the only form of regulation (Imai et al., 1986). Jolles et al. (1980) reported an inverse relationship between B-50 phosphorylation by PKC and PIP₂ formation, which suggests that PKC could inhibit PIP kinase activity via the phosphorylation of a brain-specific protein (B-50). However, more recent data suggest that PKC is involved in the stimulation of PIP kinase, as phorbol esters increase PIP₂ formation in intact cells (Chaffoy de Courcelles et al., 1985; Halenda and Feinstein, 1984; Taylor et al., 1984). Other possible regulators of PI(4)P 5-kinase activity could be the previously mentioned elevation of c-AMP (Kato et al., 1989) or polyamines (Schuber, 1989), which might be the physiological regulators of PI(4,5)P₂ formation (Downes and MacPhee, 1990).

- DAG kinase

DAG kinase phosphorylates the second messenger DAG, resulting in the formation of phosphatidic acid (PA). DAG kinase is predominantly present in the cytosol, although DAG kinase activity has also been demonstrated in membrane fractions (Kano and Akesson, 1978; Kano et al., 1983; Stubbs et al., 1988). A 80 kDa DAG kinase has been purified from pig brain and thymus (Kano et al., 1983; Sakane et al., 1989). The antibody raised against this enzyme precipitated only part of the enzyme activity from the brain cytosol and failed to react with DAG kinase from pig liver cytosol, suggesting the presence of DAG kinase iso-enzymes (Kano et al., 1990). A heat stable 150 kDa DAG kinase purified from pig thymus showed no contamination with the 80 kDa enzyme (Sakane et al., 1989; Yamada and Kano, 1988). Human platelets appear to contain three DAG kinase iso-enzymes, which differ in molecular weight and sensitivity to deoxycholate, phosphatidylserine, and the DAG kinase inhibitor R59022 (Yada et al., 1990).

Very little is known about the mechanisms regulating DAG kinase. DAG kinase activity seems to be regulated by substrate (DAG) availability, as PA is directly formed after receptor-mediated hydrolysis of PI(4,5)P₂ (Agranoff et al., 1983; Litosch et al., 1983). Because of the important regulatory functions of DAG and AA, which are derived from both DAG and PA, DAG kinase seems to be a modulator of signal transduction (Kano et al., 1990). DAG kinase is thought to be activated by its translocation from the cytosol to the membrane as observed in several cell types including rat brain (Besterman et al., 1986; Kano et al., 1990). One possibility for the initiation of such a translocation is phosphorylation of DAG kinase, which has been shown *in vitro* to occur with purified DAG kinase and PKC from pig thymus cytosol (Kano et al., 1989). In addition, Jeng et al.

(1988) found an endogenous, water-soluble, macromolecular inhibitor of DAG kinase activity in rat brain homogenate, and this compound is suggested to be a possible physiological regulator.

3. NEUROCHEMICAL ASPECTS OF AGING

3.1. Theories of aging

All living things change with age. The numerous biogerontological theories which describe the possible universal cause for age-related changes have one common feature: there are both supporting and contradictory arguments for each theory. According to Hayflick (1985), one reason why so many biogerontological theories exist is that the manifestations of biological changes over time affect virtually all components of living systems, from the molecular level up to that of the whole organism. Most of these theories are aspect theories, which arise from transforming the observation of one narrow aspect of the numerous age changes into a generalized theory of universal applicability. Examples of such aspect theories are the organ theories of the immune system and of the brain/neuroendocrine system (Meites et al., 1987; Meites, 1988). The general concept of the immune system theory is that the immune system deteriorates with age and that this deterioration causes a decline in functionality with age in the whole organism. The brain/endocrine system theory has the same basic concept. However, many organisms that do age lack an immune system or brain/endocrine system. Furthermore, these systems are regulated by other factors such as hormones (reviewed by Everitt and Meites, 1989) and the genome which makes it more plausible that changes in these regulatory mechanisms cause the changes in the organ systems mentioned. Meites et al. (1987), however, argue that aging in more complex animals probably involves the critical cells of the homeostatic and integrative systems of the body. The current cellular and molecular theories of aging cannot be applied to the age-related changes in the neuroendocrine system according to Meites et al. (1987). More specific than the endocrine system theory is the cholinergic hypothesis of geriatric dysfunction (reviewed by Bartus et al., 1982). They describe biochemical, electrophysiological and pharmacological evidence supporting a role for cholinergic dysfunction in age-related memory disturbances.

There are other aspect theories that could be generalized to all cells, such as the free radical theory, the cross-linkage theory, the waste-product accumulation theory and the genome-based theories (reviewed by Hayflick, 1985). The basic concept of the free radical theory is that free radicals, formed during the reduction of O_2 to water in mammals, oxidize intracellular structures, resulting in cell damage and ultimately in loss of function (Harman, 1981). Lipofuscin, the so called age-pigment, which accumulates in non-dividing cells, is one of the products of free radical oxidation of polyunsaturated fatty acids. This makes the theory of waste-product accumulation, with lipofuscin as the predominant waste

product, just a sub-theory of the free radical theory of aging. There is no evidence that lipofuscin actually damages cells by impeding their function (Hayflick, 1985), which makes the waste-product accumulation theory unlikely to be a general and universal aging theory. Harman (1981) stated that all cells are susceptible to damage by free radicals. In vitro, this damage can be prevented by the addition of antioxidants. In vivo, feeding antioxidants to laboratory animals increases life expectation. Harman (1981) suggested that the aging process may be simply the sum of the deleterious free radical reactions that occur in cells and tissues. However, the role of the "fundamental cause of age" of the free radical theory is doubtful as it only affects life expectation and not life span. Antioxidants may, for example, simply inhibit or delay the expression of some diseases just as vaccines and antibiotics, which also increase life expectation but which are not involved with aging (Hayflick, 1985).

The free radical theory is, according to Bjorksten (1974), simply a special case of the more general cross-linkage theory because free radicals are effective cross-linkers. The basic concept of this cross-linkage theory is that with age molecular changes occur in substances within the extracellular compartment and intracellularly localized information-containing molecules such as DNA and RNA (Bjorksten, 1974), resulting in covalent or hydrogen bonds between macromolecules. These linkages cause DNA damage, mutation or cell death, impedance of intracellular transport, decreased solubility, elasticity and permeability, and decreased extracellular transport impairing the flow of nutrients and waste products. An increase in cross-linking in collagen, other proteins and DNA with time has been demonstrated (Bjorksten, 1974). However, this potentially universal theory cannot account for the great differences in the life-span of animals. It is not realistic, for example, that mice have a 30 times faster cross-linkage than man to account for their shorter life-span (Hayflick, 1985).

The genome-based theories can be subdivided into the somatic mutation theory, the error theory, and the programme theory. The central concept of the somatic mutation theory, which has much in common with the error theory, consists of the accumulation of a sufficient level of mutations in somatic cells with age, which will produce the physiological decrements characteristic of aging. However, experiments testing this theory revealed no supporting evidence as irradiated animals did not show accelerated age-changes and life-span was unaffected (Curtis and Miller, 1971). In contrast, there is evidence that DNA sequence variations accumulate during aging (reviewed by Vijg, 1990b). Furthermore, Vijg discussed the possible role of somatic mutations in aging. These mutations would most likely occur through a disturbance in the subtly balanced gene regulatory network present in each differentiated cell of a higher eukaryote.

The central concept of the programme theory is that a purposeful sequence of events written into the genome leads to age changes, in a similar way as instructions in the genetic message lead to the orderly expression of developmental sequences (Hayflick and Moorhead, 1961). Sacher (1982) believes

aging to be errors in logic. Aging is, in his view, a programme of life phases with a functional role for senescence and/or death in a specific temporal relationship with other life processes. The best evidence in support of this theory is the fundamental fact that aging occurs naturally in intact animals (Hayflick, 1985), which makes it also a potentially universal theory. A variation on this theory is a theory of Medawar (1952), who postulated the presence of deleterious genes which pile up as a result of a selection process in the post-reproductive period, when their expression would do less harm to the species. Williams (1957) postulated, as a variation on the theory of Medawar (1952), pleiotropic genes with both favourable and, in the post-reproductive phase, unfavourable or deleterious actions. These molecular-based theories are, supplemented with new data, discussed by Vijg (1990a). Vijg emphasizes the existence of a balance between favourable and unfavourable genes, in which the favourable genes code for enzymes that defend the organism against damage, for example, superoxide dismutase.

Two other theories are discussed by Swaab (1991). He promotes an aging theory for neurons called "use it or lose it" in contrast to the "wear and tear" theory. The "wear and tear" theory is a theory in which, for example, the free radical theory and the cross-linkage theory are represented. According to this theory, increased activity causes an increased formation of reactive metabolites, resulting in increased damage in the cells used. This theory does not apply to the central nervous system, according to Swaab (1991): "Activation of nerve cells within the physiological range seem to lead to maintenance of neurons during aging and in Alzheimer's disease, possibly by preferentially stimulating the action of protective mechanisms such as DNA repair". This "use it or lose it" principle might explain why certain neurons degenerate in aging and Alzheimer's disease (Swaab, 1991). Greenamyre (1991) suggests that rather than "use it or lose it", it may be a case of "use it and lose it", because glutamate, for example, normally acts as a neurotransmitter, although under energy-deficient periods glutamate is excitotoxic. The "use it or lose it" theory of Swaab is supported by the review of Raff (1992) on social controls on cell survival and cell death. Programmed death seems to occur by default unless suppressed by signals from other cells. This programmed death of cells would also be an argument supporting the programme theory of aging.

Besides the theories listed above, there is in addition the so-called membrane theory of aging. The main tenet of this theory is that the membrane shows an age-associated increase in microviscosity which correlates with the increase in the cholesterol/phospholipid ratio with age. The condition of the membrane is essential for communication, import of nutrients and export of waste-products because proteins performing these functions are embedded in this membrane, and the cytoskeleton is attached to it. A less fluid membrane could result in a decline in cell function (Naeim and Walford, 1985).

However, according to Knook (1982) the process of aging is so complex that it is doubtful that one theory could explain all aspects of aging. I will therefore, not focus on one particular theory, but on those aspects of aging that are directly

related to phosphoinositide metabolism as described before.

3.2. Phosphoinositide metabolism and aging

Phosphoinositide metabolism has many different aspects that are important for the functioning of cells, as we have seen in part 2. According to Magnoni et al. (1991), there is considerable evidence that the aging process affects this signalling cascade at different levels, from receptor availability to stimulation of second and third (protein phosphorylation) messengers. An overview of current knowledge of these different levels of the inositide signalling cascade in relation to aging is given below.

3.2.1. Phospholipids and aging

Phosphoinositides are normal components of the plasma membrane, mentioned earlier. The most remarkable change in the plasma membrane with age is the increase in the cholesterol/phospholipid ratio (Calderini et al., 1983; ref. in Naeim and Walford, 1985). This increase in cholesterol/phospholipid ratio appears to correlate with the microviscosity of the membranes involved (Rivnay et al., 1978, 1979), which means that membrane fluidity decreases with age. A diet low in calories and high in fibre appears to reduce the increase in cholesterol/phospholipid ratio or even restore it to the ratio of young animals in aged rat brains (Tacconi et al., 1991). The effects of changes in membrane fluidity on integral membrane proteins are discussed later. Other age-associated changes in plasma membrane components include an increased turnover of phospholipids in rat brain (Pettegrew et al., 1990), an increased methylation of phosphatidylethanolamine by phospholipid methyltransferase I whereas phospholipid methyl transferase II is unaffected in rat brain (Crews et al., 1981), a decreased phosphatidylethanolamine and phosphatidylcholine synthesis, which seems to involve mostly neuronal rather than glial cells (Gaiti et al., 1982), a rapid loss of membrane lipids after 90 years of age in humans (Svennerholm et al., 1991) and last, but potentially the most important for this thesis, a decrease in lipid-myo-inositol concentrations in human anterior temporal cortex (Brodman area 38) (Stokes et al., 1983). Biochemical changes in the membranes of cytoplasmic organelles of aging cells are mostly similar to those occurring in the plasma membrane (Naeim and Walford, 1985). In mitochondrial membranes of aged animals, however, lipid peroxidation is increased (Hegner, 1980). Lipofuscins, or age pigments, that are precipitated in lysosomes, are derived from the peroxidation of subcellular membranes containing polyunsaturated lipids (Naeim and Walford, 1985 and ref. therein).

3.2.2. Receptor-mediated phosphoinositide hydrolysis and aging

Receptor-mediated phosphoinositide hydrolysis is one of the signal transformation systems by which a cell transforms an extracellular signal into an

intracellular response via the release of intracellular messengers. A disruption of the phosphoinositide second messenger systems with age, could be related to the impairment of neurological responsiveness and behavioural deficits observed with aging (Li et al., 1991). Recently, the carbachol stimulated release of inositol phosphates in hippocampal slices was found to increase with age (Tandon et al., 1991). Similar findings were done with cerebral cortex slices (Mundy et al., 1991; Nalepa et al., 1989), and noradrenaline-stimulated release of inositol phosphates, also in cerebral cortical slices (Nalepa et al., 1989). These effects occur in the absence of a change in the number of binding sites (Mundy et al., 1991; Tandon et al., 1991). However, other investigators have reported no change in receptor-mediated inositol phosphate release (muscarinic receptor, Surichamorn et al., 1989). A decrease in inositol phosphate release was even reported after stimulation of cortical α -1-adrenergic receptors (Burnett et al., 1990), cortical muscarinic receptors (Pietrzak et al., 1990) and striatal muscarinic receptors (Mundy et al., 1991).

Age-related changes in receptor-stimulated inositol phosphate release may be dependent upon the receptor-effector coupling or a change in the stoichiometry of the receptor-G-protein-phospholipase C interaction (Pietrzak et al., 1990). This latter idea is supported by the finding that in cultured rat heart myocytes the inhibition of c-AMP accumulation through Gi-proteins is lost with age (Moscona-Amir et al., 1989) and that the decrease in adenylate cyclase activity in the submandibular salivary glands of aged rats seems partly due to changes in the availability of G-proteins (Ahmad et al., 1990). An age-related uncoupling of the receptor-G-protein-phospholipase C interaction could be caused by age-related changes in membrane properties, such as membrane fluidity, as described before (Calderini et al., 1983; Heron et al., 1980). Uncoupling of the "muscarinic receptor/c-AMP inhibition" interaction in aged rat heart myocytes in culture is restored by phosphatidylcholine-liposome treatment (Moscona-Amir et al., 1989). Membrane viscosity also appears to correlate with α -1-adrenergic signal transduction in the cerebral cortex of aged rats (Miyamoto et al., 1990) and, in addition, chronic treatment with phosphatidylserine restores muscarinic cholinergic receptor deficits in the brains of aged mice (Gelbmann and Muller, 1991). The effect of membrane fluidity on the "receptor-G-protein-second messenger production" interaction is accompanied by an effect of membrane fluidity on receptor binding with age (Heron et al., 1980; Hershkowitz et al., 1982); however, receptor binding as a function of age is not discussed here.

3.2.3. The cytoskeleton and aging

The cytoskeleton plays an important role in receptor-mediated phosphoinositide metabolism by keeping the receptors, G-proteins and enzymes involved together, as receptors are directly linked to the cytoskeleton (Naeim and Walford, 1985), as are inositol phospholipid kinases, diacylglycerol kinase and phospholipase C (Grondin et al., 1991; Payastre et al., 1991). Furthermore, D-3 phosphorylated inositol phospholipids seem to be involved in cytoskeleton turnover processes

(part 2).

In aging, there is a disequilibrium between polymer and monomer forms of tubulin and a disorganization of the cytoskeleton (Naeim and Walford, 1985). The microtubule-associated proteins MAP1 and MAP2, which contribute significantly to the polymerization of brain tubulin, are degraded to a greater extent in aged rat brain because of an increased proteolytic activity of cathepsin D (Matus and Green, 1987). The age-related changes in the cytoskeleton are probably partially due to age-related changes in Ca^{2+} homeostasis, as calcium/calmodulin-dependent kinase II is an important regulator of the neuronal cytoskeleton (Harris and Delorenzo, 1987).

3.2.4. Inositol phosphate action and aging

Age-related changes in the level of inositol phosphates released after receptor stimulation have been described. Inositol trisphosphate releases calcium from intracellular stores, and cytosolic free calcium is an important second messenger controlling many aspects of neuronal function and excitability. However, the cytosolic calcium concentration has to be normalized by calcium extrusion at the plasma membrane (Na/Ca exchanger, and Ca^{2+} -ATPase) and calcium sequestration in intracellular organelles (mitochondria and endoplasmic reticulum). Calcium homeostasis and its changes with age are reviewed by Gibson and Peterson (1987). The action of inositol polyphosphates in releasing Ca^{2+} from intracellular stores is suggested to be affected by the aging process (Barritt, 1987). The density of inositol trisphosphate receptors is decreased in the cerebellum, but not in the cerebral cortex of old rats (Li et al., 1991). Furthermore, the amount of calcium released from microsomes by inositol trisphosphate decreases with age in rat brain (Burnett et al., 1990) and parotid cells (Ishikawa et al., 1988), and resting levels of cytosolic free calcium are decreased in fibroblasts from aged humans (Peterson et al., 1986).

3.2.5. Protein kinases and aging

Two protein kinases are activated as a result of receptor stimulated phosphoinositide hydrolysis. First, calcium/calmodulin-dependent protein kinase is activated by the elevated cytosolic calcium concentrations caused by the action of inositol trisphosphate. Second, protein kinase C is activated by calcium and diacylglycerol, the second messenger which is released simultaneously with inositol trisphosphate. PKC has been reported to be modified during aging in various cerebral areas, as a result of either enzyme activity or substrate availability (Magnoni et al., 1991). How aging affects the activity of calcium/calmodulin-dependent protein kinase needs to be explored.

In Sprague-Dawley rats, phorbol ester binding and PKC activity are reduced in the cortex, enhanced in the hippocampus and unmodified in the cerebellum (Battaini et al., 1990a). At the level of the hypothalamus, PKC activity appears to decrease with age (Battaini et al., 1990b) in contrast to pituitary PKC, which shows

an age-related up-regulation (Battaini et al., 1990b). Fisher 344 rats also show an age-related reduction in PKC activity in the cortex, but no changes in the hippocampus and the cerebellum (Friedmann and Wang, 1989). The age-related impairment in PKC activity in rat brain cortex may affect transmitter release, because antibodies against the PKC substrate B-50 inhibit noradrenaline release, and, furthermore, B-50 phosphorylation is promoted by depolarization (Dekker et al., 1989). In addition, the stimulatory action of activated PKC on serotonin release in the cortex of adult rats is changed to an inhibition with age (Friedmann and Wang, 1989). The age-related modifications of the PKC pathway in different cerebral areas and rat strains are reviewed by Magnoni et al. (1991). Hershkowitz et al. (1982) have shown that a decrease in lipid fluidity, which occurs with age, results in a modulation of protein phosphorylation in synaptic membranes.

4. NEUROCHEMICAL ASPECTS OF ALZHEIMER'S DISEASE

4.1. Introduction

Aging as defined in part 1 also includes age-associated disorders such as dementia. About 55-75% of the cases of senile dementia are of the Alzheimer type. Alzheimer's disease (AD) generally occurs at ages greater than 65 years, but in a small number of cases it strikes people in their forties and fifties (Iqbal, 1991). The diagnosis of AD is very difficult especially in the early stages of AD because the symptoms overlap with those seen in normal aging (Jolles and Hijman, 1983). There is no certain means of diagnosis other than brain biopsy (Tanzi et al., 1991). AD is characterized clinically by a progressive deterioration in memory, personality and intellect (Advokat and Pellegrin, 1992). In addition to the clinical syndrome, AD is characterized histochemically by the occurrence of neuritic plaques and neurofibrillary tangles. These protein deposits in the brain were first described by Alois Alzheimer in 1907 (Selkoe, 1991). According to Selkoe (1991), the difference between normal brain aging and AD is quantitative rather than qualitative, because normal non-demented subjects also develop at least a few senile plaques and neurofibrillary tangles, particularly in the hippocampus and other brain regions important for memory. Tangles appear with age in the olfactory nucleus, the parahippocampal gyrus and the hippocampus, but are rare in the neurocortex except in demented persons. Conversely, plaques may develop first in the neocortex, which implies that tangles and plaques first develop in different parts of the brain. (Price et al., 1991). The neurochemical composition and probable development of the neuritic plaques and neurofibrillary tangles are outlined in the following sections.

4.2. Neuritic plaques and the role of beta-amyloid in Alzheimer's disease.

Plaques consist of a central core of amyloid fibrils surrounded by dystrophic neurites together with reactive microglia and astrocytes. (Hardy and Allsop, 1991).

With increasing severity of dementia, these mature plaques are formed out of diffuse plaques which do not have a central core and are already present in very mildly demented people. (Price et al., 1991). Down's syndrome, which shows remarkable similarities to AD, especially in the occurrence of plaques, also shows a maturation of diffuse plaques in early-stage patients to mature plaques in late-stage patients (Mann and Esiri, 1988).

Masters et al. (1985) isolated and sequenced the β -amyloid (β /A4) polypeptide from the core of these plaques. β /A4, which is the major component of plaque cores, appears to be the same protein as the meningo-vascular β protein isolated by Glenner and Wong (1984). The sequence of β /A4 is encoded by a host gene for a much longer protein, which has been termed the amyloid precursor protein (APP) (Tanzi et al., 1989). APP has been highly conserved through evolution and is expressed in a variety of tissues (Holtzman and Mobley, 1991). APP spans the membrane once and has a large extracellular amino-terminal domain with a short carboxy-terminal cytoplasmatic tail (Tanzi et al., 1989). The β /A4 sequence in APP begins close to the membrane on the extracellular side and ends part-way through the putative transmembrane region (Hardy and Allsop, 1991). Under normal conditions, the extracellular part can be released by enzyme action in the β /A4 region (Esch et al., 1990; Sisodia et al., 1990), resulting in the release of a soluble APP. A protease activity associated with acetylcholinesterase releases soluble APP from the membrane-bound form of APP (Small et al., 1992). In addition, Small et al. (1992) suggest that as acetylcholinesterase is decreased in AD, the association with this protease might explain why APP is processed abnormally in AD. In AD the β /A4 sequence is released intact, which means that there has to be an extracellular cleavage site and another site in the transmembrane region of APP. How an enzyme gains access to that transmembrane region is still unsolved.

The physiological role of a soluble, normally processed APP is not clear. Two of the three most abundant isoforms of APP (see Hardy and Allsop, 1991) contain an internal Kunitz-type protease inhibitor insert (Kitaguchi et al., 1988), suggesting that APP may act as protease inhibitor in the brain and elsewhere (Holtzman and Mobley, 1991). Holtzman and Mobley (1991) also presented results suggesting a role for APP in the modulation of cell growth and adhesion.

Several investigators hypothesize the release of β /A4 to be the primary cause of AD, and think that all other aspects of AD (some of them are discussed later) are secondary events. The fact that a mutation of the APP gene, as in familial AD, can give rise to all of the neuropathological hallmarks of AD without there being any other defect strongly suggests that amyloid mist metabolism and deposition is the seminal event in the pathogenesis of all cases of AD (Selkoe, 1991; Hardy and Allsop, 1991). This is called "the biochemical amyloid cascade theory" (Hardy, 1992). The molecular genetics of familial AD and the regulation and genetic control of brain amyloid (reviewed by Tanzi et al. (1991) and Gajdusek et al. (1991), respectively) will not be dealt with in this survey. In addition to this biochemical amyloid cascade theory, Hardy (1992) presented an "anatomical cascade hypothesis" for AD in which beta-amyloid is initially

deposited in the limbic areas for unknown reasons; this stimulates a stress response in the surrounding neuropil, which leads to up-regulation of APP and other proteins and, after APP is axonally transported (Koo et al., 1990), to increased deposition of β /A4. The reasons behind β /A4 deposition must include an altered APP metabolism, which possibly results from trisomy 21 (Down's syndrome), APP gene mutation (familial AD), other unknown mutations and/or environmental factors such as brain damage (Hardy and Allsop, 1991). Consistent with this hypothesis is that the promotor of the gene encoding APP contains both heat-shock and interleukin-responsive elements (Hardy, 1992), and that APP expression is up-regulated by heat shock in lymphocytes (Abe et al., 1991a) and by ischaemia in the brain (Abe et al., 1991b). This up-regulation of APP expression, which already occurs in an early stage of brain damage (Kawarabayashi et al., 1991), would give the enzymes which release the intact β /A4 the opportunity to act in addition to the enzymes which release the soluble APP, as hypothesized by Selkoe (1991). In vitro, β /A4 stimulates glial cells to produce growth factors which accumulate in senile plaques in AD, suggesting that in this way cascades may arise that contribute to the process of plaque development (Araujo and Cotman, 1992). Furthermore, APP is deposited in rat hippocampus lesioned by ibotenic acid injection (Nakamura et al., 1992), and needle stab injury in rat brain results in the rapid appearance of APP immunoreactivity in damaged axons and reactive glial cells (Otsuka et al., 1991). However, it is doubtful whether neurons react in a stress response against extracellular β /A4 because the β /A4 protein (parts of) has neurotrophic effects (Araki et al., 1991; Whitson et al., 1989; Whitson et al., 1990; Yankner et al., 1990). Furthermore, according to the hypothesis of Hardy and Allsop (1991) neurofibrillary tangles would develop secondary to the deposition of β /A4. However, Price et al. (1991) have shown that the first development of tangles and plaques occurs in different parts of the brain.

4.3. Neurofibrillary tangles

Another prominent feature in AD brains is the abnormal accumulation of markedly insoluble filamentous material within neuronal cell bodies and neurites (Holtzman and Mobley, 1991). These neurofibrillary tangles (NFTs) are found in several neurodegenerative diseases besides AD, such as post-encephalitic Parkinson's disease, Guam Parkinsonism dementia complex, dementia pugilistica, and Down's syndrome (Iqbal, 1991). Neurofibrillary tangles are widely distributed in AD brains, especially in the temporal and frontal cortices, hippocampus, some parts of the brain stem and hypothalamus. Microscopically, they appear as bundles of paired helical filaments (PHFs) in which microtubule-associated protein (MAP), called tau, is an important constituent (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986). Under normal conditions, tau acts as a promoting and stabilizing factor in tubule polymerization (Weingarten et al., 1975). It has been suggested that tau is abnormally phosphorylated in PHFs (Baudier and Cole, 1987; Grundke-Iqbal et al., 1986; Ihara et al., 1986), a notion that is supported by the fact that the monoclonal

antibody for normal tau recognizes tau in PHFs only after treatment with alkaline phosphatase (Bancher et al., 1989; Lee et al., 1988; Wood et al., 1986). A68 proteins in AD appears to be composed of several tau isoforms in a phosphorylated state which affects their electrophoretic mobility (Brion et al., 1991). This abnormal phosphorylation of tau has been demonstrated in AD and Down's syndrome (Brion et al., 1991; Hanger et al., 1991). PHF tau appears to contain 12 moles of phosphate per mole of tau, in contrast to normal tau from the brain of AD and control brain, which contains approximately 3 moles of phosphate per mole of tau (Ksiezak-Reding et al., 1990). Because phosphorylation by cAMP-dependent protein kinase inhibits the degradation of tau by calpain, it is suggested that phosphorylation has a role in regulating the degradation of tau. In addition, abnormal phosphorylation could result in a protease-resistant tau, which may contribute to the formation of paired helical filaments in AD (Literski and Johnson, 1992). Because of the important function of tau in promoting and stabilizing tubule polymerization, a disturbance in the phosphorylation of tau could possibly lead to destabilization of the cytoskeleton and ultimately to degeneration of axons and dendrites, which does occur in AD (Flood et al., 1987). Among other proteins, only ubiquitin has convincingly been shown to be a part of the PHFs (Kosik et al., 1989), although there are indications that tropomyosine, a microfilament protein (Galloway and Perry, 1991), neurofilament proteins (Mulvihill and Perry, 1989) and MAP 2 and 5 (Kosik et al., 1984) are also constituents of PHFs.

4.4. Theories of Alzheimer's disease

In analogy to the aging theories, there are several theories as to the possible cause of AD in addition to the widely supported β /A4 theory of Hardy and colleagues described above. These theories overlap with the aging theories, such as the free radical theory reviewed by Vollicer and Crino (1990), the "use it or lose it" theory postulated by Swaab (1991), the somatic mutation theory (in a somewhat other form proposed by Hardy and Allsop, 1991, and Mullaart et al., 1990), and the endocrine system theory. With respect to AD the endocrine system theory is divided into an excitatory amino acid theory (reviewed by Advokat and Pellegrin, 1992) and a cholinergic theory (Bartus et al., 1982). The excitatory amino acid transmitter system may be involved in mechanisms underlying learning and memory under normal conditions. However, overactivity of this system produces excitotoxic damage to neurons. Both a decline in cognitive function and damage to neurons occur in AD, which suggests that excitatory amino acids may contribute to the aetiology of AD (Advokat and Pellegrin, 1992). A sub-theory of the excitatory amino acid theory is the hypothesis that a dysfunction of glutamate, the major excitatory neurotransmitter, is involved in the pathophysiology of AD (Maragos et al., 1987). In addition, a cholinergic theory has been formulated for AD comparable to the cholinergic theory of aging, because 1.) acetylcholine synthesis in AD biopsy samples has been reported to be decreased, 2.) a severe loss of cholinergic neurons in the nucleus basalis of Meynert has been

reported, 3.) a positive correlation has been shown between the extent of cognitive loss in senile dementia, the decrease in choline acetyltransferase activity, and the incidence of major neuropathological markers (reviewed by Bartus et al., 1982).

Furthermore, the hypothesis that calcium-activated proteases (calpains) are causal agents in brain aging and, in particular, in the gradual development of abnormalities and degeneration (Lynch et al., 1986) also seems applicable as an AD theory. That is, calpain is involved in the turnover of cytoskeletal and structural elements (Lynch et al., 1986), which are affected in AD. In addition, tau, which is abnormally phosphorylated in AD, is degraded by calpain under normal conditions (Literski and Johnson, 1992), and calpain immunoreactivity is localized in senile plaques and in neurons undergoing neurofibrillary degeneration in AD (Iwamoto et al., 1991).

The overlap between aging theories and AD theories is probably not accidental because, according to Swaab (1991), present evidence indicates that AD is a precocious accelerated form of aging.

4.5. Phosphoinositide metabolism and Alzheimer's disease

As in aging, the different levels of the inositide signalling cascade are or could be involved in AD. Fowler et al. (1990) hypothesized that there is a decline in the functional integrity of receptor-mediated signal transduction mechanisms, such as cAMP release and the inositol phosphate/diacylglycerol release systems, in AD, although most studies of receptor function in AD have considered only the receptor recognition site (reviewed by Morgan et al., 1988). Interestingly, several of the neurotransmitter systems affected in AD, such as the cholinergic, serotonergic and noradrenergic systems, are coupled to these effector systems (Fowler et al., 1990). Because AD patients are also subject to aging, age-matched controls and not young controls should be used in studies with AD patients.

4.5.1. Phospholipids and Alzheimer's disease

Phospholipids are the main components of the membrane, and the condition of this membrane can influence the proteins attached to it, for instance receptors, G-proteins and enzymes, as mentioned above. ^{31}P NMR studies have demonstrated that there are increased levels of phosphomonoesters, which could be the result of higher PLC activities, early in the course of AD and increased levels of phosphodiesterases as the disease progresses (Pettegrew, 1989). The levels of PME are inversely correlated with the number of senile plaques whereas the levels of PDE are positively correlated with the numbers of senile plaques (Pettegrew and Klunk, 1990). These and additional findings resulted in the hypothesis that the earliest molecular/metabolic changes in AD result in elevated levels of PME in the neocortex and allocortex, followed by a cortical and subcortical elevation of PDE later in the course of the disease, which reflects cellular degeneration and death (Pettegrew, 1989 and ref. therein).

A physiologically relevant action of L-phosphoserine, one of the phosphomonoesters elevated in AD, has been suggested because whole brain concentrations may reach over 1 mM in AD and L-phosphoserine has close structural similarities to L-glutamate, suggesting that it could interact with the glutamate receptor (Klunk et al., 1991).

Furthermore, there appears to be an increased degradation of phospholipids in AD brains as phosphatidylcholine and phosphatidylethanolamine levels and the levels of their precursors choline and ethanolamine were decreased in contrast to the levels of their deacylation products, which were increased (Nitsch et al., 1992). These phospholipid abnormalities appear not to be an epiphenomenon of neurodegeneration but may be specific for the pathomechanism of AD (Nitsch et al., 1992). In the anterior temporal cortex of AD brains, PI levels are significantly lower than in the same area of age-matched control brains (Stokes and Hawthorne, 1987). Stokes and Hawthorne (1987) have also reported that PIP and PIP₂ levels tend to decrease in AD; however, these data may have been affected by the long and variable post-mortem delay, because polyphosphoinositides are rapidly degraded post-mortem (Dawson and Eichberg, 1965).

The composition of brain membranes is thus altered in AD, and this probably results in functionally significant alterations in the biophysical properties of brain cell membranes, as shown by Zubenko (1986).

4.5.2. Receptor-mediated phosphoinositide hydrolysis and Alzheimer's disease

As mentioned above, most studies on receptor-mediated functions in AD have considered only the receptor recognition side (Fowler et al., 1990). Receptor binding and receptor number are reviewed by Morgan et al., (1988) and will not be discussed further here. However, the "receptor-G-protein-second messenger producer" coupling in AD brain membranes had not been studied up to two years ago. Stimulated adenylate cyclase activity appears to be reduced in AD brains (Cowburn et al., 1991; Ohm et al., 1991), although in another study stimulated adenylate cyclase activity was unaffected (Bergstrom et al., 1991). In addition, Cowburn et al. (1992) report a widespread impairment of G-protein-stimulated adenylate cyclase activity in AD brains. This impairment occurs in the absence of altered enzyme catalytic activity and is unlikely to be the result of non-disease-related factors associated with the nature of the terminal illness of individuals. The coupling of the 5-hydroxytryptamine-1A receptor, which has been reported to be coupled to both adenylate cyclase and phospholipase C, to G-proteins appears to be intact in AD (O'Neill et al., 1991). In addition, the coupling of M1-muscarinic receptors, which preferentially activate phospholipase C, to G-proteins in AD is also unaffected (Pearce and Potter, 1991). According to McLaughlin et al. (1991), alterations in the amount of G-protein alpha-subunits are not a feature of AD, in contrast to the aberrant accumulation of phospholipase C-delta in AD brains. This accumulation is suggested to be a crucial event that may ultimately contribute to the formation of PHF (Shimohama et al., 1991). Because there is very little tritium labelling of

polyphosphoinositides following incubation of autopsy samples, receptor-mediated inositol phospholipid breakdown in post-mortem material is difficult to measure (Fowler et al., 1990). The bradykinin-induced formation of IP_3 and IP_2 was measured in fibroblasts from AD donors, but did not differ from that of normal aged donors (Huang et al., 1991). The enzyme PLC does not appear to be affected in AD brains (Shimohama et al., 1992), thus a possible change in inositol phosphate release can only be caused at the receptor-G-protein-PLC coupling level, at the level of receptor density, or at the level of PIP_2 supply (activity of PI and PIP kinases).

4.5.3. Calcium release, protein phosphorylation and Alzheimer's disease

It is not known whether the release of inositol phosphates is altered in AD brain, mainly because of the technical problems mentioned above. However, the number of IP_3 binding sites essential for calcium release in the temporal cortex and hippocampus of AD brain is severely reduced (50-70% loss) (Young et al., 1988). The same technical problems mentioned above for measurements of inositol phosphate release also exist for measurements of calcium concentrations and fluxes in AD brains. Cytosolic free calcium and cell spreading of calcium is decreased in fibroblasts from AD donors (Peterson et al., 1986). Furthermore, Na^+/Ca^{2+} exchange activity is increased in AD brain tissue, which is interpreted as indicating that neurons surviving in brain regions suffering the greatest degeneration due to AD have increased Na^+/Ca^{2+} exchange activity (Colvin et al., 1991). According to Mattson et al. (1991), a disturbed calcium homeostasis, resulting in higher intracellular calcium concentrations, together with a disturbed inositol phospholipid metabolism, could be involved in neurofibrillary degeneration through the activation of protein kinases, because tau, a microtubule-associated protein, is abnormally phosphorylated in PHFs.

Elevated calcium levels and activation of PKC both induce an increased PHF-tau immunoreactivity in neuronal cell cultures and addition of the PKC inhibitor H-7 blocks the increase in immune reactivity (Mattson et al., 1991). Furthermore, PKC is able to phosphorylate tau (Baudier et al., 1987; Hoshi et al., 1987) as well as neurofilament proteins (Georges et al., 1989; Sihag et al., 1988).

Protein phosphorylation and the occurrence of senile plaques also seem to be related. A synthetic peptide corresponding to a region of APP is phosphorylated by PKC, suggesting that APP could be a physiological substrate of PKC (Gandy et al., 1988). Furthermore, PKC immunoreactivity is present in mature plaques (Clark et al., 1991; Masliah et al., 1990), whereas diffuse plaques only show immunoreactivity for the PKC subtype βII . This PKC βII subtype is probably an early biochemical marker of AD, because diffuse plaques do not show immunoreactivity for senile plaque markers such as casein kinase II, tau, PHF (Masliah et al., 1991). Interestingly, this βII PKC subtype is strongly associated with the cytoskeleton and is probably responsible for the phosphorylation of the growth-associated GAP43 or B-50 protein (Tanaka et al., 1991). In AD brains, the level of PKC and the *in vitro* phosphorylation of its 86 kDa substrate are

decreased, and there is a translocation of PKC activity from the particulate pool to the soluble cytosolic pool (Cole et al., 1988).

A possible function of PKC is the regulation of protein tyrosine phosphorylation (Cochet et al., 1984; Northwood and Davis, 1989). The protein tyrosine kinase activity in the particulate fraction of AD frontal cortex appeared to decrease twofold whereas cytosolic protein tyrosine kinase activity was not affected (Shapiro et al., 1991). However, the amount of two (55 and 60 kDa) phosphotyrosine immunoreactive polypeptides appeared to be increased in AD (Shapiro et al., 1991).

4.5.4. Aluminum and Alzheimer's disease

Aluminum has been associated with Alzheimer's disease for the reason that aluminum induces neurofibrillary changes in experimental animals (Terry and Pena, 1965), although these changes do not mimic exactly the NFTs of AD (Krishnan et al., 1987). Orally administered aluminum increases the phosphorylation of rat brain cytoskeletal proteins (Johnson and Jope, 1988), which could be the mechanism underlying the aluminum-induced neurofibrillary changes. As aluminum has a high affinity for phosphate (Martin, 1986), this metal ion is found predominantly in the nuclear compartment of the cell, but even there it has a particular compartmentalization in AD, where it could have an effect on the nuclear structure (Lukiw et al., 1991). Aluminum also appears to impair cholinergic activity in rat brain *in vitro* (Johnson and Jope, 1986), and in rabbit brain tissue containing "in vivo aluminum-induced" tangles (Yates et al., 1980). Interestingly, a cholinergic deficit is a major characteristic of AD. The high affinity of aluminum for phosphate may also be the reason for the interaction of aluminum ions with polyphosphoinositide metabolism. Free aluminum ions appear to stimulate PI hydrolysis while inhibiting PI(4,5)P₂ hydrolysis in a concentration-dependent manner (McDonald and Mamrack, 1988). In contrast, aluminum salts activate inositol phospholipid hydrolysis in rat cerebral cortex membranes, probably by activating a regulatory G-protein at a location distinct from the GTP-binding site or by direct stimulation of PLC (Candura et al., 1991).

5. OUTLINE OF THIS THESIS

The metabolism of phosphoinositides and its relevance for cell functioning has been outlined in section 2 of this chapter. The membrane-associated processes involved in receptor-stimulated inositol phosphate release, the effectiveness of the inositol phosphate response, the maintenance of calcium homeostasis, and protein kinase activities as a function of age and AD have been studied extensively, as described in sections 3 and 4 of this chapter. However, the production of the inositol trisphosphate precursor, phosphatidylinositol bisphosphate, by phosphatidylinositol (PI) kinase and phosphatidylinositol

phosphate (PIP) kinase, and the phosphorylation of DAG into phosphatidic acid have not received much attention in aging research and AD research. As far as we know, there are no data on these kinase activities as a function of age and AD, with the exception of a study in rat brain which revealed a decrease in PIP₂ formation in synaptic plasma membranes of the hippocampus and whole brain with advancing age (Van Dongen et al., 1983). Changes in the activity of these enzymes could explain changes in the receptor stimulated inositol phosphate response and changes in protein kinase C activity with advancing age and possibly also in AD.

PI, PIP and DAG kinases are, at least partially, membrane bound (Stubbs et al., 1988), and therefore can be influenced by age- and AD-related changes in the physical properties of membranes (Naeim and Walford, 1985), as described in sections 3 and 4. Furthermore, these kinases could be sensitive to age- and AD-related changes in the cytoskeletal system, as described in sections 3 and 4, because PI, PIP and DAG kinase appear to be associated with the cytoskeleton (Grondin et al., 1991; Payraastre et al., 1991).

Interestingly, the function of PI kinase has become more important with the discovery of the D-3 phosphorylated inositides, which are implicated in growth, mitogenic signalling and cytoskeletal turnover processes (Carpenter and Cantley, 1990). Because AD is characterized by cytoskeletal deterioration in the brain, a change in these D-3 phosphorylating inositide kinases could be involved in the development of NFTs in AD brains.

Enzymes or other functional proteins can also lose their functionality by covalent modifications, such as mixed function oxidation, deamidation, ubiquitin conjugation and glycation, as a consequence of aging, but also in AD (Smith et al., 1991).

As described above, the enzymes PI kinase, PIP kinase and DAG kinase have not been studied as a function of age and AD. In the first part of this thesis we investigated whether PI kinase, PIP kinase and DAG kinase are affected by age in the rat brain. Because of the possible influence of the micro-environment of these kinases on activity, the effect of age was studied in a membrane-cytosol preparation of different rat brain regions (Chapter 5). The relationship between enzyme activity and endogenous substrate availability was measured and not just enzyme activity or phospholipid concentrations. The preparation of this membrane cytosol preparation is described in chapter 2. Chapter 3 describes the effect of changing the conditions of the phosphorylation assay on the different kinase activities. In chapter 4 the subcellular distribution of PI, PIP and DAG kinase activities, measured with endogenous lipids as substrate, is described, together with the detection of a probably new inositol phospholipid species in mitochondria (Chapter 4). In Chapter 6, specific PI, PIP and DAG kinase activities were measured, with exogenous lipids as substrate, as a function of age in rat brain.

The second part of this thesis deals with PI kinase and PIP kinase activities in post-mortem human brain cortex as a function of age and AD. Chapter 7 describes the specific PI and PIP kinase activities (measured with exogenous lipid substrate)

in cytoplasmic fractions prepared from brains of AD patients and of age-matched controls. In chapter 8 the effect of age on these activities was determined in the control group and the AD group, but also the effect of "age of first symptoms" in the AD group. Chapter 9 describes the search for a specific type of PI kinase activity involved in AD, because of recent reports on the different types of PI kinases and their different functions (see chapter 1). Finally, chapter 10 deals with PI and PIP kinase activities in the blood platelets of AD patients and controls because there is growing evidence that AD is a systemic disease and not just a disease of the central nervous system.

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CHAPTER 2

PHOSPHATIDIC ACID AND POLYPHOSPHO- INOSITIDE FORMATION IN RAT BRAIN IN VITRO: EFFECTS OF POST-MORTEM DELAY AND PROCEDURAL MANIPULATIONS.

J. Bothmer, M. Markerink and J. Jolles

Abstract

The effect of post mortem delay and procedural manipulations on phosphate incorporation into phosphatidylinositol phosphate (PIP), phosphatidylinositol bisphosphate (PIP₂) and phosphatidic acid (PA) in a lysed crude synaptosomal fraction from rat brain is studied in the present paper. PIP₂ formation was already decreased in vitro after a post-mortem delay of 2 min. Only 5% of activity was still present after 24 hours. PA formation increased 3-fold in this period whereas PIP formation did not change. A delay of 2 to 4 hours before the incubation resulted in a 25% increase in PIP formation whereas PIP₂ and PA formation was reduced in this period. Slow freezing of brain material after decapitation of animals had no effect on PIP and PIP₂ formation in vitro, but increased the formation of PA. Rapid freezing resulted in a 30% increase in PIP formation and a 25% decrease in PA formation. Thawing had no effect. Finally, age (8 ± 1 week) and body weight appeared to influence PIP₂ and PA but not PIP formation, which is indicative of developmental changes in this period. The results suggest that it is of utmost importance to use a strict and standardized procedure in studies in which groups of subjects with different biological variables (e.g., pretreatment in vivo, age, post-mortem delay, diagnosis in case of human material) are compared with respect to inositol phospholipids measured in vitro.

Introduction

Nervous tissue is especially rich in phosphoinositides (Michell, 1975), which are involved in important neurochemical processes related to neurotransmission (Abdel-Latif, 1986; Berridge, 1987). An important feature of phosphoinositides is their rapid interconversion. The values reported for phosphoinositide phosphorus in nervous tissue vary greatly, which is probably caused by the rapid hydrolysis of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP₂) post-mortem (Hawthorne and Pickard, 1979). Hydrolysis of PIP and PIP₂ by phospholipase C yields diacylglycerol (Avelano and Bazan, 1975; Tang and Sun, 1985), which is rapidly phosphorylated to phosphatidic acid (PA) by diacylglycerol kinase. This rapid hydrolysis places particular restrictions on the investigation of inositol phospholipids and PA in vitro and on studies which measure phospholipid levels and/or kinase activities with endogenous phospholipids as substrate in vitro in relation to biological manipulations in vivo. Earlier studies from our research group have shown that small variations in assay conditions can give rise to substantial changes in inositol phospholipids measured in an in vitro assay system. For instance, Jolles et al. (1981) used a synaptosomal fraction in which rat brain membranes were prelabeled in vivo and showed that half of the ³²P from PIP and PIP₂ was lost after two minutes of incubation at 37°C. Furthermore, Bothmer et al. (1990) have shown that PIP₂ formation in a 10-second incubation decreases substantially with preincubation periods longer than 10 seconds.

The issue of differences in the formation of inositol phospholipids and PA in relation to the time needed to prepare and use an enzyme fraction is of both

fundamental and methodological importance. There is a great variance in the values reported for phosphoinositide phosphorus in nervous tissues in animals and in humans (Stokes et al., 1983; Stokes and Hawthorne, 1987) which may be because of differences in post-mortem interval. Jolles et al. (1992) have recently shown that the brains of patients with Alzheimer's disease are characterized by a substantial decrease in PI kinase but not PIP kinase activity. The aging rodent, however, appears to be characterized by a general lowering in PA forming activity (Bothmer et al., 1992). These results show that it is essential to reduce variability between experimental subjects in studies in which the relation between biological variables (disease state, age, treatment in vivo etc.) and inositol phospholipids/PA formation in vitro is investigated. The present study was performed to establish whether the variance in the formation of PIP, PIP₂ and PA in vitro is due to any of the following four methodological parameters: the post mortem-interval, the time between enzyme preparation and incubation, the developmental stage of the animal in terms of age and brain weight, and the freeze/thaw procedure used for storage of the brain material. It was also investigated whether PIP, PIP₂ and PA producing activities show a differential susceptibility to these parameters.

In the study presented here, a crude lysed synaptosomal fraction was used under hypo-osmotic conditions and (very) short incubation periods, which enables the simultaneous measurement -and thus comparison- of the three kinase activities (Bothmer et al., 1990; Jolles et al., 1981).

Experimental procedures

Animals and brain dissection

Male rats (about 160 g and 8 weeks old) of an inbred Brown Norway strain were used. After decapitation, the head was immediately immersed in liquid nitrogen for 8 sec. All subsequent operations were performed at 0-4°C. The brain was taken out of the skull rapidly and the bulbi olfactory, the cerebellum and brainstem were removed.

Subcellular fractionation

Tissue from individual rats was homogenized in homogenization medium (0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4; Booth and Clark, 1978) in a total volume ten times the brain tissue volume, by ten up and down strokes (16 s each) of a Potter Elvehjem teflon-glass homogenizer with a radial clearance of 0.125 mm and rotating at 700 rpm. The method of Gray and Whittaker (1962) was used for the subcellular fractionation of the homogenate. Briefly, the homogenate was centrifuged for 10 min at 1000 g. After centrifugation of the supernatant at 10,000 g for 10 min, the resulting crude mitochondrial/synaptosomal pellet (P₂) was subjected to osmotic lysis: the pellet was resuspended

in 10 vol. distilled water and stirred for 20 min at 4°C. This suspension was centrifuged for 20 min at 10,000 g to remove unlysed structures. The 10,000 g supernatant of the lysed P₂ fraction was taken as the enzyme fraction, and is referred to in the text as the lysed synaptosomal fraction. The influence of delay in the various stages of preparation of the lysed synaptosomal fraction was estimated by keeping the time needed for all steps constant except for the step to be examined. Changes per experiment are described in the legends of the figures. Protein was determined according to the method of Lowry et al. (1951).

Phosphorylation assay

Endogenous phosphorylating activity was assayed as described before (Jolles et al., 1981; Bothmer et al., 1990). The following conditions were used: 7.5 µM ATP, 2-3 µCi ly³²P]-ATP (approx. 3000 Ci/mmol, Amersham, U.K.), 50 mM Na-Acetate, 10 mM Mg-Acetate, pH 6.5, and 15 µl enzyme fraction (10 µg protein) in a final volume of 25 µl at 30°C. The lipid phosphorylation reaction was terminated after 10 s by the addition of 2 ml ice-cold chloroform : methanol : 12 N HCl (200:100:0.75 , by volume; Shaikh and Palmer,1977).

Fig.1 : The effect of the post mortem delay, started by decapitation and ended by start of subcellular fractionation, on the incorporation of phosphate into PIP, PIP₂ and PA. Values shown are means (±SEM) of triplicate determinations. Data were analyzed with a T-test in which a deviation from the control value (100%) was tested. *: p<0.05.

Lipid extraction, thin-layer chromatography and determination of radioactivity

Lipid extraction and thin-layer chromatography were performed as described previously (Jolles et al., 1981). ^{32}P was determined by liquid scintillation counting in a commercially available scintillation fluid (Ready safe liquid scintillation fluid, Beckman). Two hundred microlitres of water was added to the scraped silica portions followed by 3.8 ml of scintillation fluid. The radioactivity was counted in a scintillation spectrometer (Beckman LS 1801) with an efficiency of 100%.

Results

The influence of the post mortem interval on phospholipid phosphorylation activity *in vitro* was investigated in an experiment in which the time period between decapitation and brain dissection (followed by rapid homogenization at 0°C) was varied from 0 s to 24 h. A substantial reduction in PIP_2 formation was already evident at a post-mortem interval of 2 min, and the lysed crude synaptosomal fraction had lost 95% of its PIP kinase activity after a delay of 24 hours (Fig. 1). Interestingly, there was no change in PI kinase activity whereas PA formation increased threefold with increasing post-mortem interval.

The time the enzyme fraction was allowed to stand at 4°C before the start of the incubation had a marked effect on phosphorylation. As Figure 2 shows, increasing the time from 0 to 4 hours led to an increased formation of PIP and a decreased formation of PIP_2 and PA.

The third experiment investigated whether lipid phosphorylating activities are affected by the age of the animals. Rats were used of slightly different ages (8 ± 1 weeks) and hence different body weights (weight range 120 - 200 g.). The difference in age reflected the usual variation in age and weight of animals used in experiments. PI formation was not dependent upon age/weight, whereas both PA formation and PIP_2 formation increased with increasing age and weight (Fig. 3) ($r^2=0.725$ ($p<0.05$) and $r^2=0.435$ ($p<0.05$), respectively).

The fourth experiment investigated whether the way in which brain material is stored and regenerated by freezing and thawing has an influence on the lipid phosphorylating activities. Brain material obtained from different rats of the same age and weight was subjected to the various combinations of fast or slow freezing and fast or slow thawing. Freezing had a substantial influence on PIP formation (60% increase compared to control) and PA formation (25% decrease) whereas PIP_2 formation was not affected (Fig. 4). Thawing had no effect on the formation of the lipids. With respect to storage of the lysed synaptosomal fraction, rapid freezing (5 s liquid nitrogen) and rapid thawing (contact with water at $0-4^{\circ}\text{C}$) appeared to give the highest incorporation of phosphate (results not shown). Storage at -80°C for periods up to three months did not decrease enzyme activity (results not shown).

Fig. 2 : The effect of storage time of the lysed synaptosomal fraction (at 0-4°C) on the incorporation of phosphate (pmol/mg protein) into PIP, PIP₂ and PA. The storage times were 0 (subcellular fractionation followed directly by incubation), 2 and 4 hours. Each value is the mean of nine determinations ($\pm$ SEM). Data were analyzed with a repeated measures analysis of variance. *: $p < 0.05$.

Fig. 3 : The effect of body weight of 8 ($\pm$ 1) week-old-rats on the incorporation of phosphate into PIP, PIP₂ and PA. Values shown are means of triplicate determinations. Data were analyzed with linear regression analysis.

Discussion

The present study shows that basal PIP, PIP₂ and PA formation in the lysed synaptosomal fraction of rat brain is affected by delays at different stages of preparation of the lysed synaptosomal fraction. The differential centrifugation steps were not changed so the evaluated characteristics (Whittaker, 1969) of the lysed synaptosomal fraction were not influenced.

A post-mortem delay from 2 minutes up to 24 hours caused a marked increase in PA formation and a decrease in PIP₂ formation, whereas PIP formation remained unaffected (Fig. 2). PIP₂ formation probably decreased as a result of the substantial decline, within a few minutes after death, in the content of PIP₂ and PIP, the substrate of PIP kinase, in rat brain (Dawson and Eichberg, 1965; Ikeda et

al., 1986; Sun et al., 1990) and in gerbil brain (Abe et al., 1987). This decrease in inositol phospholipids after decapitation is immediately followed by an increase in the amount of diacylglycerol (Abe et al., 1987; Ikeda et al., 1986), which is the substrate for PA formation, and is the probable cause of the increased formation of PA measured in our assay system. The concentration of PI does not change after decapitation (Ikeda et al., 1986) or shows only a small decrease (Abe et al., 1987), which explains the unaltered PIP formation in our assay system after decapitation.

Fig. 4 : The effect of different methods of freezing and thawing brain tissue on the incorporation of phosphate into PIP, PIP₂ and PA. Values shown are means of triplicate determinations. A: control; B: slow freezing/rapid thawing; C: rapid freezing/rapid thawing; D: slow freezing/slow thawing; E: rapid freezing/slow thawing. Rapid freezing, by immersion in liquid nitrogen, followed by storage at -80°C; slow freezing, placed in a polyethylene container at -80°C; rapid thawing, immersion in a water bath at 4°C; slow thawing, in a 4°C room. Data were analyzed by Duncan's multiple range test. *: different from control value, $p < 0.05$.

A delay after the subcellular fractionation / before the phosphate incorporation assay affected phosphate incorporation activities differently to the delay post-mortem. A delay after subcellular fractionation resulted in a decrease in PIP₂ formation and PA formation but an increase in PIP formation. This differential effect was not the same as the one observed with increasing post-mortem delay. These differences are probably due to the difference in the concentration of

calcium. Phospholipase C is Ca^{2+} dependent (see Abdel-Latif, 1986) and the phosphomonoesterases also require this ion (Jolles et al., 1981). Because the hypotonic lysed synaptosomal fraction contains less calcium than is present in the brain after decapitation, the enzymes responsible for inositol phospholipid degradation, such as phospholipase C and the phosphomonoesterases, will also have a different activity.

The range of the weight/age of the rats used correlated positively with the incorporation of phosphate into PIP_2 and PA (Fig.1). This positive correlation probably reflects the development of inositol phospholipid metabolism in synaptosomal plasma membranes, or the development of a factor (e.g. myelination) which influences subcellular fractionation in such a way that it affects the incorporation of phosphate into PIP_2 and PA in the lysed synaptosomal fraction. This is in accordance with the finding that both PI and PIP kinase activity increases 4-5 fold in chick brain during the period of active myelination (Shaikh and Palmer, 1977). Salway et al. (1968) observed a similar developmental pattern for PIP kinase activity in postnatal rat brain, but PI kinase activity reached a maximum immediately after birth, although clearly before the onset of the myelination in the rat. Interestingly, in our assay PI kinase activity did not change with weight / age.

We used a lysed synaptosomal fraction to assess the consequences of freezing and thawing procedures. Hardy et al. (1983) found that rapid freezing of brain tissue severely impaired the subsequent performance of synaptosomes. Our findings are consistent with this because slow freezing of brain tissue followed by storage at -80°C results in a incorporation of phosphate into lipids comparable to that measured with fresh material (Fig.3), although incorporation into PA was slightly increased. Fast freezing increased PIP formation and decreased PA formation. The way of thawing the brain tissue before subcellular fractionation did not influence the incorporation of phosphate into PIP, PIP_2 and PA measured in vitro. However, Hardy et al. (1983) found a relatively good metabolic performance of synaptosomes prepared from tissue frozen slowly and thawed rapidly in contrast to slowly thawed material.

In conclusion, this study shows that basal formation of PIP, PIP_2 and PA in a lysed synaptosomal fraction is influenced differently by delays at different stages in the preparation of a lysed synaptosomal fraction. Thus the factor time must be controlled to reduce experimental error, especially in studies in which groups of subjects (animals, human brain material) are used, such as in aging research (Bothmer et al., 1992) and in studies on human brain (e.g. Jolles et al., 1992; Stokes et al., 1983). Furthermore, one should control for variations in age and weight within groups, because of the developmental effects found in the present and other studies. Further research should be devoted to the differential effects on PIP, PIP_2 and PA formation found in the present study. Care should be taken to use a strict and standardized procedure for the preparation of the enzyme fraction as variations in the method of preparation influence phospholipid phosphorylation measured in vitro.

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CHAPTER 3

PHOSPHATIDIC ACID AND POLYPHOSPHO-INOSITIDE FORMATION IN A BROKEN CELL PREPARATION FROM RAT BRAIN : EFFECTS OF DIFFERENT INCUBATION CONDITIONS.

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Abstract

The formation of phosphatidic acid (PA) and polyphosphoinositides in rat brains *in vitro* was studied under hypotonic conditions by incubating a lysed crude synaptosomal fraction with [γ - ^{32}P]ATP for 10 sec. Only phosphatidic acid, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP₂) were labelled. The formation of PIP₂ decreased with increasing preincubation times. PIP and PA formation, however, were not affected. The rate of phosphorylation of PIP, PIP₂ and PA was linear with incubation time for at least 10 sec, indicating that the ATP/enzyme ratio was sufficiently high. The maximal incorporation of phosphate into the three phospholipids was found at 30°C. Ten millimolar Mg²⁺ and pH 6.5 were the best compromise for measuring the incorporation of phosphate into PIP, PIP₂ and PA simultaneously. A sodium acetate / magnesium acetate buffer system gave optimal kinase activities. The results obtained show that, with minor adjustments of the incubation conditions, it is possible to measure simultaneously the formation of PIP, PIP₂ and PA.

Introduction

The membrane phospholipids phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP₂) are quantitatively minor components of cell membranes but they have an important physiological role (for reviews see Abdel-Latif, 1986; Berridge, 1987). They are rapidly interconverted by phosphorylation and dephosphorylation. Furthermore, their breakdown into diacylglycerol and inositol phosphates is followed by rapid phosphorylation of the diglyceride into phosphatidic acid (PA).

The majority of the biochemical studies on the inositol phospholipid system have been carried out with intact structures, such as synaptosomes derived from rat brain (e.g. Jolles et al., 1981b; Stubbs et al., 1988), whereby the incorporation of ^3H , ^{14}C or ^{32}P labelled substrates into inositol phospholipids was studied under isotonic conditions and relatively long incubation periods (minutes). A second method for the *in vitro* investigation of inositol phospholipids has not been used so often, but is "of potential relevance" according to Fisher et al. (1984). This method involves the incubation of isolated synaptic plasma membranes in the presence of cytosolic factors under hypotonic conditions. Gamma-labelled ATP is used as the phosphate donor in very short incubations (eg 10 s). This is of importance because the enzymes involved in the interconversion of PI, PIP and PIP₂ are among the fastest acting enzymes known (Hawthorne and Pickard, 1979).

Previous studies with this method have shown phosphate incorporation into PIP₂ and PA to be sensitive to adrenocorticotropin (ACTH₁₋₂₄), and phosphate incorporation into PIP, PIP₂ and PA to be sensitive to Ca²⁺ and dependent on Mg²⁺ (Jolles et al., 1981a). Besides ACTH₁₋₂₄, c-AMP and endorphins have also been shown to affect phosphoinositide phosphorylation, albeit in a different way (Jolles et al., 1981b; Jolles et al., 1982).

Unfortunately, nothing is known about potentially important assay conditions such as incubation and preincubation times, temperature, concentration of Mg²⁺,

ATP and tissue protein, pH, and buffer systems. The present study was designed to provide more information about these variables so that future studies will be performed under optimal assay conditions.

Experimental procedures

Animals and brain dissection

Male rats (165 g) of an inbred Brown Norway strain were used. After decapitation, the head was immediately immersed in liquid nitrogen for 8 s. All subsequent operations were performed at 0–4°C. The brain was taken out of the skull rapidly and the bulbi olfactori, the cerebellum and brainstem were removed.

Subcellular fractionation

Tissue from individual rats was homogenized in homogenization medium (0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4, Booth and Clark, 1979) in a total volume ten times the brain tissue volume, by ten up and down strokes (16 s each) of a Potter Elvehjem teflon-glass homogenizer with a radial clearance of 0.125 mm and rotating at 700 rpm. The method of Gray and Whittaker (1962) was used for the subcellular fractionation of the homogenate. Briefly, the homogenate was centrifuged for 10 min at 1000 g. After centrifugation of the supernatant at 10,000 g for 10 min, the resulting crude mitochondrial/synaptosomal pellet (P_2) was subjected to osmotic lysis: the pellet was resuspended in 10 vol. aqua bidest and stirred for 20 min at 4°C. This suspension was centrifuged for 20 min at 10,000 g to remove unlysed structures. The 10,000 g supernatant of the lysed P_2 fraction was taken as the enzyme fraction. This fraction was frozen in 1 ml aliquots in liquid nitrogen for 5 s, and stored at -80°C. There was no decline in enzyme activity after at least one month of storage (results not shown).

Phosphorylation assay

Endogenous phosphorylating activity was assayed as described before. (Jolles et al., 1981a) Briefly, a routine incubation was performed under the following conditions: 7.5 μ M ATP, 2–3 μ Ci [γ - 32 P]-ATP (approx. 3,000 Ci/mmol, Amersham, U.K.), 50 mM Na-Acetate, 10 mM Mg-Acetate, pH 6.5, and 15 μ l enzyme fraction (10 μ g protein) in a final volume of 25 μ l at 30°C. Unless otherwise specified, the membrane samples were thawed on ice (1 to 1.5 hours) and preincubated for 5 min and the incubation was started by the addition of ATP. The influence of the various assay conditions was estimated by keeping the conditions constant except for the condition to be tested. The changes in the assay per experiment are described in the legends of the figures. The lipid phosphorylation reaction was terminated by the addition of 2 ml ice-cold

chloroform : methanol : 12 N HCl (200:100:0.75 , by vol., Shaikh and Palmer, 1977). Protein determination was performed according to the method of Lowry et al. (1951).

Fig. 1 : Effect of preincubation (A and B) and incubation times (C) on the incorporation of phosphate (pmol/mg protein) into phosphatidylinositol 4-phosphate (PIP ; squares), phosphatidylinositol 4,5-bisphosphate (PIP₂ ; triangles) and phosphatidic acid (PA ; circles). The start of the preincubation in the first procedure (A) was the moment when the ice cold incubation tube, containing 15 μ l membrane-cytosol fraction, was put into the incubation bath (30°C). The temperature of the content of the tube was 20°C after 15 s, 28.5°C after 1 min and 30°C after 1.5 min. The start of preincubation in the second experiment (B) was the moment when 15 μ l membrane-cytosol fraction was added to prewarmed (30°C) incubation tubes. The membrane-cytosol fraction reached a temperature of 30°C within seconds. Each point is the mean of 6 (A and B) or 3 (C) determinations ($\pm$ SEM).

Lipid extraction, thin layer chromatography and determination of radioactivity

Lipid extraction and thin layer chromatography were performed as described previously (Jolles et al., 1981a). ^{32}P was determined by liquid scintillation counting in a commercially available scintillation fluid (Ready safe liquid scintillation fluid, Beckman). Two hundred microlitres of water was added to the scraped silica portions followed by 3.8 ml of scintillation fluid. The radioactivity was counted in a scintillation spectrometer (Beckman LS 1801) with an efficiency of 100%.

Results*Effect of preincubation and incubation time*

Only radiolabelled PIP, PIP₂ and PA were detected after incubation of the lysed crude synaptosomal fraction with [gamma- ^{32}P]ATP for 10 s. The influence of the preincubation time on the incorporation of phosphate is shown in Fig. 1a. It appeared that [^{32}P]-PIP₂ formation decreased with increasing preincubation times. The difference between phosphate incorporation into PIP and PA with respect to different preincubation times was not significant. A second experiment was performed in order to gain more detailed information on shorter preincubation times. The procedure was adapted slightly in order to allow preincubation times in the range of seconds. Again, the preincubation time did not affect the incorporation of phosphate into PIP and PA whereas the incorporation into PIP₂ decreased with increasing preincubation times (Fig. 1b). It is probable that the values at 0 s are an artefact caused by the low temperature at that time. Phosphate incorporation into PIP₂ reached its highest level with a 5 s preincubation and was reduced to 30% after 3 to 5 min. Phosphate incorporation into the phospholipids did not change with preincubations of 3 to 5 min (results not shown).

The influence of the incubation time was investigated under standard incubation conditions with a 5 min preincubation. Fig. 1c shows that there was a linear incorporation of phosphate into PIP and PA over 20 to 30 s, indicating that the amount of ATP was not limiting during the standard incubation (10 sec). The curve for phosphate incorporation into PIP₂ suggests that the reaction is also linear during the first ten seconds. A second experiment showed that there was a linear increase in phosphate incorporation into PIP₂ from 5 to 15 seconds (0.77 - 1.16 - 1.57 pmol Pi/mg protein, respectively). The degradation of ATP was linear for at least 20 s after which time it levelled off.

Effect of incubation temperature

The effect of the incubation temperature (23°C, 30°C, 37°C and 44°C) was investigated under conditions of short (5 s) and long (5 min) preincubation times. The temperature optimum for PIP formation (Fig. 2a) under standard conditions

(5 min preincubation) appeared to be 37°C. The optimum for PA formation, however, was between 23°C and 30°C (Fig. 2c) whereas under standard conditions the optimum for PIP₂ formation was lower than 23°C (Fig. 2b). The temperature effect appeared to be dependent on the preincubation time as the curves levelled off at a preincubation time of 5 s. The temperature effects observed with a 5 min preincubation were thus probably caused during preincubation and not during incubation. With a 5 s preincubation, the overall incorporation of phosphate into PIP, PIP₂ and PA was greater at 30°C than at 37°C.

Fig. 2 : Effect of temperature on the incorporation of phosphate (pmol/mg protein) into phosphatidylinositol 4-phosphate (PIP ; A), phosphatidylinositol 4,5-bisphosphate (PIP₂ ; B) and phosphatidic acid (PA ; C) with 5 s (circles), 1 min (triangles) and 5 min (squares) preincubation. Each point is the mean of 6 determinations ($\pm$ SEM). The incubation temperatures were 23, 30, 37 and 44°C.

Effect of magnesium ions, ATP and tissue concentration

The incorporation of phosphate into PIP, PIP₂ and PA appeared to be dependent on Mg²⁺ (Fig. 3a). The highest values for individual phosphate incorporation were measured for PIP at 60 mM Mg²⁺ or higher and for PIP₂ and PA at 1 mM Mg²⁺. Ten millimolar Mg²⁺ appeared to be a good compromise for measuring phosphate incorporation into PIP, PIP₂ and PA in the same assay.

Fig. 3 : Effect of magnesium (A), ATP (B) and tissue (C; $\mu\text{g}/\text{incubation}$) concentration on the incorporation of phosphate (pmol/mg protein) into phosphatidylinositol 4-phosphate (PIP ; squares), phosphatidylinositol 4,5-bisphosphate (PIP₂ ; triangles) and phosphatidic acid (PA ; circles). Each point is the mean of 3 (A) or 6 (B and C) determinations ($\pm\text{SEM}$). In (A) the buffer was 60 mM Na-acetate (pH 6.5) and Mg²⁺ was added as MgCl₂ in the different concentrations. In (B) and (C) the Mg²⁺ concentration was 10 mM and the specific activity of the [γ -³²P]ATP-label was 3 μCi .

The buffer system used in this experiment was 60 mM Na-Acetate (pH 6.5), and different concentrations of Mg^{2+} were added as a solution of $MgCl_2$. With regard to the optimal ATP concentration, it appeared that the ATP concentration (7.5 μM) used in our experiments did not yield the highest rates of phosphate incorporation into PIP, PIP_2 and PA (Fig. 3b). ATP (7.5 μM) degradation was linear for at least 20 sec after which it levels off. Higher levels of ATP, up to at least 225 μM , resulted in higher phosphate incorporation rates but the addition of cold ATP made the autoradiographic detection of the labelled products difficult because there was a lower ratio of labelled ATP to cold ATP. The influence of the amount of enzyme/membrane fraction used was investigated by varying the amount of protein in the incubation mixture (Fig. 3c). The ATP concentration in this experiment was 7.5 μM . It appeared that the specific incorporation of phosphate into PIP, PIP_2 and PA was linear with the protein/tissue concentration over the range of 10 to 15 μg per incubation. Higher protein/tissue concentrations cannot be obtained with this lysed crude synaptosomal fraction. At lower protein/tissue concentrations, PA formation decreased and PIP formation increased, although PIP_2 formation was not influenced by alterations in the protein/tissue concentration.

Effect of pH and the buffer system

The effect of the pH on the incorporation of phosphate into PIP, PIP_2 and PA was tested over a pH range from 5.5 to 9.0 by using acetate, Tris-maleate and Tris-HCl buffers. Fig. 4a,b and c show that different buffer systems cannot be used indiscriminately in this assay. The acetate buffer used in this experiment resulted in the formation of more PIP (Fig. 4a) and PA (Fig. 4c) than both Tris buffers did. These Tris buffers were supplemented with 50 mM Sodium ion and 10 mM magnesium ion in order to make the three buffers comparable with regard to the possible effect of these ions. The increased ionic strength caused by the addition of 50 mM NaCl had no effect on the incorporation of phosphate into PIP and PIP_2 , but caused a 45% decrease in the incorporation of phosphate into PA (results not shown). A clear optimal pH for the formation of PIP and PA was not observed. The highest incorporation of phosphate was measured between pH 7.0 to 8.5 for PIP and between pH 6.0 and/or pH 7.5 to 8.0 for PA. The optimal pH for PIP_2 formation (Fig. 4b) in this assay system was 6.0. The values for PIP_2 formation using the different buffer systems corresponded very well. Slightly more PIP_2 was formed in the Tris-maleate buffer (pH 6.0) than in the sodium acetate buffer.

Discussion

Few investigators have validated the assays used to measure the kinase activities involved in PI metabolism. The present study examined the assay conditions for the phosphorylation of PI, PIP and DAG by kinases present in a lysed synaptosomal fraction. A short incubation time (10 sec) was used in the

assay of these activities. It appears that the assay conditions used in earlier studies (Jolles et al., 1981a and 1982) are not optimal for the activity of individual kinases to incorporate phosphate into PIP, PIP₂ or PA, but are a good compromise for measuring the kinase activities simultaneously.

Fig. 4 : Effect of pH and different buffer systems on the incorporation of phosphate (pmol/mg protein) into phosphatidylinositol 4-phosphate (PIP ; A), phosphatidylinositol 4,5-bisphosphate (PIP₂ ; B) and phosphatidic acid (PA ; C). The buffer systems used were Tris-maleate (squares, 50 mM, pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5), Tris-HCl (triangles, 50 mM, pH 7.0, 7.5, 8.0, 8.5, 9.0) and sodium acetate-magnesium acetate (circles, 50/10 mM, pH 6.0, 6.5, 7.0). All incubations contained 50 mM Na⁺ and 10 mM Mg²⁺, the other conditions were standard. Each point is the mean of 9 determinations ($\pm$ SEM).

The measurement of the three phosphate incorporation activities in one assay system is essential because these activities are present in the same membrane fraction and various agonists (eg ACTH₁₋₂₄ or endorphins) have different, sometimes opposite, effects on individual phosphorylation activities (Jolles et al., 1981a,b). Unexpectedly, the duration of the preincubation markedly influenced the formation of PIP₂. This and other findings from the present experiments are of interest:

Firstly, PIP₂ forming activity appears to be inactivated during prolonged preincubations. At 30°C the incorporation of phosphate into PIP₂ clearly decreased to a steady level during 5 min of preincubation whereas the other phosphorylating activities did not change (Fig. 1b). This effect on PIP₂ formation was more pronounced at higher temperatures (37°C and 44°C). At these temperatures, there was also a decrease in the incorporation of phosphate into PA (Fig. 2b,c). These effects could be due to enzyme inactivation or substrate depletion or both. Smith and Wells (1983), who studied the incorporation of phosphate into PIP, PIP₂ and PA in rat nuclear envelopes, suggested that PIP₂ breakdown was probably not caused by phospholipase A₂ activity because lysophospholipids were not found. If the preincubation effect on PIP₂ formation was caused by substrate breakdown, then this could only be the result of phosphomonoesterase and/or phosphodiesterase activity. However, if these enzymes were active there would be an immediate change in the activities of the other kinases at 30°C, leading for instance to an increased PA formation. This is because phosphodiesterase activity is directly correlated with DAG-kinase activity via DAG production (Agranoff et al., 1983). The decreased incorporation of phosphate into PIP₂ with increasing preincubation time is thus probably caused by inactivation of a specific enzyme. These findings concerning the effect of the preincubation time will be relevant for the design of future studies on the influence of agonists on PIP₂ formation. The choice of the preincubation time depends on the problem to be studied. For instance, a 5 min preincubation is to be recommended if reactivation is to be studied. It is possible that the stimulatory effect of ACTH₁₋₂₄ on PIP₂ formation after a 5 min preincubation (Jolles et al., 1980, 1981a) is a reactivation phenomenon. Short preincubation times on the other hand are to be recommended when an inhibition of PIP₂ formation is expected. Further studies will address this matter more specifically.

Secondly, when the amount of enzyme/membrane fraction per unit volume is low, diacylglycerol (DAG) kinase and PI kinase seem to compete for the available ATP (Fig. 3c). It also appears that the incorporation of phosphate into PIP₂ is independent of the incorporation of phosphate into PIP or PA. This may indicate that PIP kinase has a higher affinity for ATP than PI kinase and DAG kinase have. The ATP concentration in these experiments was 7.5 μ M. A maximal incorporation of phosphate was attained with a concentration of ATP in excess of 225 μ M. Similar findings have been made in studies on phosphoinositide kinases in rat brain homogenate (Kai et al., 1966) and chick brain homogenate (Shaikh and Palmer, 1977) in which millimolar concentrations of ATP yielded a maximal phosphate incorporation. Our findings suggest that it is not necessary to use

higher ATP concentrations because phosphorylation is linear with time at an ATP concentration of 7.5 μM and a short incubation time of 10 s.

Thirdly, 10 mM Mg^{2+} appears to be the best compromise for measuring the three phosphorylation activities simultaneously at an ATP concentration of 7.5 μM (Fig. 3a), although it is not the optimal concentration for measuring individual phosphorylating activities. The relevance of the simultaneous measurement of these kinases has already been mentioned. The optimal concentrations of Mg^{2+} were dependent on the concentrations of the substrates in the assay system. Therefore, it is difficult to compare the Mg^{2+} optima found in other studies with the values obtained in this study.

Lastly, the choice of the buffer system appears to be very important when phosphorylation is measured in a system such as the lysed synaptosomal plasma membrane fraction. More phosphate was incorporated into phosphoinositides in an acetate buffer system than in a Tris-maleate buffer (50 mM) of the same pH. Tris-HCl appeared to be a good buffer for measuring phosphate incorporation into PIP and PA. The difference between the Tris-HCl and acetate buffers with regard to PA formation was caused by the addition of 50 mM NaCl to the Tris-HCl incubations. However, Tris-HCl buffer is not suited for simultaneous investigation of PIP, PIP_2 and PA formation because its buffering capacity is very low at pH 6.0, the optimal pH value for PIP_2 formation. Remarkably, only the incorporation of phosphate into PA was affected by the higher ionic strength caused by the addition of 50 mM NaCl to the Tris buffer incubations. The optimal pH for phosphate incorporation into PIP_2 appeared to be pH 6.0. This contrasts with the optimum of pH 7.4 (Shaikh and Palmer, 1977) in chicken brain and pH 8.0 (Smith and Wells, 1983) in rat liver nuclear envelopes. The pH optimum for PIP formation was found to be between pH 7.0 and pH 8.5. Jergil and Sundler (1983), who studied rat liver golgi, and Collins and Wells (1983), who studied rat liver lysosomal membranes, also found a broad pH optimum in the neutral range for PI-kinase. The pH curve for phosphate incorporation into PA did not show a clear pH optimum.

In conclusion, the assay conditions described in the present paper for the simultaneous measurement of PIP, PIP_2 and PA formation can be optimized for the determination of the individual enzyme activities. Compromises have to be made for the simultaneous assay in comparison with the optimal conditions for measuring the kinase activities individually. The results indicate that the method used, which is characterized by a short incubation time, hypotonic conditions and ATP as the phosphate donor, merits consideration in studies of inositol phospholipid metabolism. The method is well suited for the determination of the direct effects of pharmacological agents on the phosphorylation of individual phospholipids as has been shown in earlier studies with neuropeptides, neurotransmitters and cyclic nucleotides (Jolles et al., 1981a, 1981b, 1982).

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CHAPTER 4

EVIDENCE FOR A NEW INOSITOL PHOSPHO- LIPID IN RAT BRAIN MITOCHONDRIA.

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Abstract

Phosphorylation of phosphatidylinositol (PI), phosphatidylinositol mono-phosphate (PIP) and diacylglycerol (DAG) was studied in rat brain cortex myelin, synaptosomal and mitochondrial fractions, with ATP as phosphate donor and endogenous phospholipids as substrate. All fractions had PI, PIP and DAG phosphorylating activity with their own characteristic subcellular distribution. However, in the mitochondrial fraction an unidentified lipid was phosphorylated, which had a slower R_f value than PIP_2 on TLC (R_f values of 0.167 and 0.222, respectively). After hydrolysis of the polar head group of the lipid and separation on anion exchange columns, it appeared to be a phosphoinositide. The elution profile showed that it was not phosphatidylinositol trisphosphate (PIP_3), or a lyso-compound. The available evidence suggests that the unknown inositol phospholipid in rat brain mitochondria is a phosphatidylinositol 4,5-bisphosphate isomer, although the possibility of it being a glycosyl-phosphoinositide cannot be excluded.

Introduction

Nervous tissue is especially rich in phosphoinositides, a class of phospholipids which are involved in important neurochemical processes related to neurotransmission (Abdel-Latif, 1986; Berridge, 1987). For instance, phosphatidylinositol 4-phosphate ($PI(4)P$) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), which are synthesized by sequential phosphorylation of phosphatidylinositol (PI), are implicated in receptor-mediated signal transduction (Berridge, 1987). In this process, $PI-4,5-P_2$ is hydrolyzed by phospholipase C into the second messenger inositol trisphosphate and diacylglycerol (Akhtar and Abdel-Latif, 1980) followed by rapid phosphorylation of the diglyceride (DAG) into phosphatidic acid.

Phosphoinositide metabolism has become more complex with the discovery of D-3 phosphorylated inositides (Carpenter and Cantley, 1990). The inositides which are phosphorylated at the D-3 position of the inositol ring ($PI(3)P$, $PI(3,4)P_2$ and phosphatidylinositol 3,4,5-trisphosphate (PIP_3)) seem to be involved in cell proliferation (Martelli et al., 1991). In addition, it is suggested that these inositides are involved in the cytoskeletal rearrangement that occurs during the mitotic cell cycle and in post mitotic cells during exocytosis of protein tyrosine kinase and growth factor receptors (Auger and Cantley, 1991; Carpenter and Cantley, 1990; Cochet et al., 1991; Martelli et al., 1991; Varticovski et al., 1989). PIP_3 has been identified in several cell types (Carpenter and Cantley, 1990) and in rat brain (Vadnal and Parthasarathy, 1989).

Inositol phospholipids were thought to be primarily located in the plasma membrane (Abdel-Latif, 1986). Recent subcellular fractionation studies with rat brain, however, have shown that PI kinase, PIP kinase and DAG kinase activities are present in all subcellular fractions (Stubbs et al., 1988). The common feature in the subcellular distribution of these three lipid kinases appears to be their presence in both membrane and cytosol.

The present paper is concerned with the phosphorylation of brain myelin, synaptosomal and mitochondrial fractions under hypotonic conditions with gamma-labeled ATP as phosphate donor and endogenous phospholipids as substrate. Our assay system has been shown to favor the phosphorylation of inositol phospholipids (Bothmer et al., 1990; Jolles et al., 1981). Our results suggest that a new phospholipid, probably an inositol phospholipid, is formed in rat brain mitochondria but not in synaptosomes.

Experimental procedures

Animals and brain dissection

Male rats (approx. 165 g) of an inbred Brown-Norway strain were used. After decapitation the head was immediately immersed in liquid nitrogen for 8 seconds. All subsequent operations were performed at 0-4°C. The brain was taken out of the skull, and the cortex was dissected rapidly.

Subcellular fractionation

The tissue was homogenized in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, in a total volume 10 times the brain tissue volume by 10 up-and-down strokes of a Potter-Elvehjem Teflon-glass homogenizer (radial clearance 0.125 mm, 700 rpm). The homogenate was spun at 1000 g for 10 minutes. After centrifugation of the supernatant at 10,000 g for 10 minutes, the resulting crude mitochondrial-synaptosomal pellet was resuspended in homogenization buffer and then applied to a discontinuous Ficoll-sucrose gradient as described by Booth and Clark (1978). The tubes were centrifuged at 100,000 g for 30 minutes. Myelin and synaptosomes banded at the first and second interphases, respectively, with the free mitochondria being pelleted at the bottom. Membranes from each layer were carefully removed and contaminating synaptosomes appearing on the top of the mitochondrial pellet were eliminated by swirling the pellet with a small amount of 0.32 M sucrose buffer. Myelin, synaptosomes and mitochondria were suspended in 0.9% NaCl/5 mM Tris-HCl, pH 7.4, and spun down at 10,000g for 10 minutes to remove the Ficoll. The pellets were subjected to osmotic lysis (the pellets were resuspended in 10 vol. aqua bidest) and stored at -80°C.

Phosphorylation assay

Endogenous phosphorylating activity was assayed as described earlier (Bothmer et al., 1990; Jolles et al., 1981). The lipid phosphorylation reaction was terminated by the addition of 2 ml ice-cold chloroform:methanol: 12 N HCl (200: 100: 0.75, by vol., Shaikh and Palmer, 1977). Protein determination was performed according to the method of Lowry et al. (1951).

Fig.1 : Distribution of phosphate incorporation (pmol phosphate/mg protein) in phosphatidylinositol monophosphate (PIP), phosphatidylinositol biphosphate (PIP₂), phosphatidic acid (PA) and the new phospholipid (X) measured in the subcellular fractions: myelin (1), synaptosomes (2) and mitochondria (3) prepared by Ficoll discontinuous gradient centrifugation.

Lipid extraction, thin-layer chromatography and determination of radioactivity

Lipid extraction and thin-layer chromatography were performed as described previously (Jolles et al., 1981). Two hundred microliters of water was added to the scraped silica proteins followed by 3.8 ml scintillation fluid (Ready Safe, Beckman). Radioactivity was counted in a scintillation spectrometer (Beckman LS 1801) with an efficiency of 100%.

Alkaline hydrolysis of inositol phospholipids and separation of inositol phosphates

The alkaline hydrolysis of PIP, PIP₂ and band "X" was performed according to Vadnal and Parthasarathy (1989). The hydrolysates were buffered with 200mM Tricine, pH 8.0. Separation of inositol phosphates was performed according to Downes and Michell (1981) on Dowex AG1x8 anion exchange columns (formate form), with the exception of the last elution step when 1.5 M HCOONH₄/0.1 M HCOOH was used.

Results

The crude mitochondrial/synaptosomal pellet (P₂) was further purified by Ficoll discontinuous gradient centrifugation resulting in myelin, synaptosomal and mitochondrial fractions. Fig.1 shows that phosphate incorporation rates into PIP, PIP₂ and PA with endogenous lipid substrate had their own unique subcellular distribution, with the highest rate of PIP formation being in the synaptosomal and mitochondrial fractions, the highest rate of PIP₂ formation being in the myelin fraction and the highest rate of PA formation being in the synaptosomal fraction.

Surprisingly, a fourth phospholipid was phosphorylated in our assay system which has a short incubation time and use endogenous lipids as substrate. This new phospholipid appeared to be enriched in rat brain mitochondria (Fig. 1). The phospholipid "X" had a R_f value on TLC of 0.167; PIP₂, PIP and PA had R_f values of 0.222, 0.300, and 0.778, respectively.

A time curve of phosphate incorporation, measured in the mitochondrial fraction, showed that phospholipid "X" was formed within seconds and that formation reached a maximum at 60 seconds, whereafter dephosphorylation took place (Fig. 2).

To identify the new phospholipid, we subjected PIP, PIP₂ and "X", extracted and concentrated from the lipid spots on the TLC plate, to alkaline hydrolysis. The hydrolyzed polar head groups of the phospholipids were separated by anion exchange chromatography (Fig. 3). The PIP hydrolysate showed one peak eluting in the 0.2 M and one in the 0.5 M ammonium formate fractions, corresponding to IP₁ and IP₂, respectively. The PIP₂ hydrolysate and the "X" hydrolysate had peaks that eluted in the 0.2 M, 0.5 M and 0.8 M ammonium formate fractions,

corresponding to IP_1 , IP_2 and normally IP_3 , respectively. The "X" hydrolysate peak eluting in the 0.8 M fraction was much broader than the corresponding peak in the elution pattern of the PIP_2 hydrolysate. No peak was found in the 1.5 M ammonium formate fraction.

Fig.2 : Time of phosphate incorporation (pmol phosphate /mg protein) in phosphatidylinositol monophosphate (PIP), phosphatidylinositol bisphosphate (PIP₂), phosphatidic acid (PA) and the new phospholipid (X) measured in rat brain mitochondria.

Discussion

The study presented reports the formation of a novel inositol phospholipid in vitro in a mitochondrial fraction of rat brain cortex after incubation with ATP under hypotonic conditions. Further subcellular differentiation of a crude mitochondrial-synaptosomal pellet by discontinuous Ficoll-sucrose gradient centrifugation resulted in myelin, synaptosomal and mitochondrial fractions. All fractions had PI, PIP and DAG phosphorylating activity as previously described by Stubbs et al. (1988), who also used endogenous lipid as substrate.

In the mitochondrial fraction, however, we found a fourth radiolabeled phospholipid which had a slower R_f value than PIP_2 on TLC (R_f values were 0.167 and 0.222, respectively). Several investigators have described an inositol phospholipid that migrates slower than PIP_2 does on TLC, and have identified this substance as phosphatidylinositol-trisphosphate (PIP_3) by revealing an

Fig.3 : Elution profiles of alkaline hydrolysis products of phosphatidyl-inositol monophosphate (PIP), phosphatidylinositol bisphosphate (PIP₂) and the new phospholipid (X) on anion exchange columns. Inositol monophosphate is expected to elute in the 0.2 M ammonium formate fractions, inositol bisphosphate in the 0.5 M ammonium formate fractions, inositol trisphosphate in the 0.8 M ammonium formate fractions and inositol tetrakisphosphate in the 1.5 M ammonium formate fractions.

inositol tetrakisphosphate after deacylation and deglyceration (Auger et al., 1989; Traynor-Kaplan et al., 1988; Vadnal and Parthasarathy, 1989). In order to identify the polar head group of the new phospholipid found in the mitochondria of rat brain cortex, we subjected this phospholipid to alkaline hydrolysis (Vadnal and Parthasarathy, 1989). Instead of an inositol tetrakisphosphate peak eluting in the 1.5 M ammonium formate fraction on Dowex AG1x8 anion exchange columns, we found a peak at the position of inositol trisphosphate (IP_3). However, the pattern of this peak was clearly different from the inositol trisphosphate after PIP_2 hydrolysis, as the peak showed partial retention on the column (tailing). This new phospholipid found in mitochondria is not PIP_3 but possibly a PIP_2 -like substance. Gumber and Lowenstein (1986) reported non-enzymic phosphorylation of polyphosphoinositides catalyzed by bivalent metal ions resulting in pyrophosphates. Phosphatidylinositol 4-pyrophosphate had a slower R_f value than the $PI(4,5)P_2$ standard. However, the new phospholipid described in the present study is probably not PI-pyrophosphate because the phosphorylation of mitochondrial lipids (mitochondria possibly contain higher concentrations of bivalent metal ions than synaptosomes, in analogy to the pyrophosphate hypothesis) would also reveal other pyrophosphates (Gumber and Lowenstein, 1986), which were not found in our experiments.

The new phospholipid is an inositol phospholipid, because IP_1 and IP_2 were also present in the mixture of products after hydrolysis of the new phospholipid. The unidentified phospholipid is not a lyso- PIP_2 which also migrates slower than PIP_2 (Traynor-Kaplan et al., 1988) because alkaline hydrolysis of a lyso- PIP_2 would produce a normal inositol trisphosphate. The polar head groups of $PI(3,4)P_2$ and $PI(3,5)P_2$ ($I(1,3,4)P_3$ and $I(1,3,5)P_3$, respectively) elute at somewhat lower salt concentrations than the polar head group of $PI(4,5)P_2$ ($I(1,4,5)P_2$) does (Auger et al., 1989), but on Dowex anion exchange columns these isomers cannot be separated. Another possibility could be a mitochondrion-specific glycosyl-phosphoinositide, phosphorylated in our assay system. Glycosyl-phosphoinositides are responsible for anchoring several proteins to the membrane (Low, 1989). Alkaline hydrolysis of a glycosyl-phosphoinositide could result in diacylglycerol and an inositolphosphate glycan which possibly coelutes with IP_3 on Dowex anion exchange columns.

In conclusion, this study reports the in vitro formation of a new inositol phospholipid in purified rat brain mitochondria. The new inositol phospholipid was not identified as phosphatidylinositol trisphosphate. The new inositol phospholipid is probably a phosphatidylinositol 4,5-bisphosphate isomer or a glycosyl-phosphoinositide.

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CHAPTER 5

BRAIN PHOSPHATIDIC ACID AND POLY-PHOSPHOINOSITIDE FORMATION IN A BROKEN CELL PREPARATION: REGIONAL DISTRIBUTION AND THE EFFECT OF AGE.

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Abstract

The effect of age on phosphate incorporation into phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidic acid (PA) was studied. Lysed crude synaptosomal fractions of different brain regions of 3-month-old and 32-month-old Brown Norway rats were used. The brain regions tested were the hippocampus, frontal cortex, occipital / parietal cortex, entorhinal / pyriformal cortex, striatum / septum, thalamus and hypothalamus. The individual specific phosphorylating activities were unevenly distributed within the brain of Brown Norway rats. Strikingly, the distribution of phosphate incorporation into PIP₂ was opposite from that of phosphate incorporation into PA. Phosphate incorporation into PA decreased (-15%) with age in almost all brain regions tested, whereas phosphate incorporation into PIP₂ decreased with age only in the frontal cortex (-20%) and in the hypothalamus (-8%). The effects of age may reflect a deterioration of phosphoinositide metabolism, with its function in signal transduction coupled to receptors via G-proteins, in the brain regions involved. In addition, there was an age related decrease in protein content and total phospholipid phosphorus content of lysed crude synaptosomal preparations of all brain regions. The high correlation between the changes in these parameters may be indicative of a decrease in the number or size of synaptosomes with age in the brain regions involved.

Introduction

Phosphatidylinositol and its phosphorylated congeners phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP₂) play an important role in signal transduction. PIP₂ is thought to be hydrolysed by phospholipase C into the second messengers inositol trisphosphate and diacylglycerol (DAG) after receptor stimulation (Abdel-Latif, 1986; Berridge, 1987).

Recently, receptor-stimulated phosphoinositide hydrolysis was found to be increased in the hippocampus of old rats after muscarinic M1 receptor stimulation (Tandon et al., 1991) and in the brain cortex of old rats after alpha-1 adrenoceptor and muscarinic M1 receptor stimulation (Nalepa et al., 1989). This increased responsiveness in senescence could be a compensatory mechanism for neuronal cell death and reduced levels of transmitters (Tandon et al., 1991). Protein phosphorylation, which is the final pathway in the action of transmitters and hormones at the neuronal level, is also affected in aging (Magnoni et al., 1991). Cyclic AMP-dependent protein kinase and protein kinase C have been reported to be modified during aging in various cerebral areas. These changes may involve either enzyme activity or substrate availability (Magnoni et al., 1991).

However, hardly anything is known about possible changes in the conversions of brain phosphoinositides, other than receptor stimulated hydrolysis, with aging. This aspect of phosphoinositide metabolism is also essential for the conversion of extracellular signals into biological responses. The DAG-kinase activity in pig brain (Kanoh et al., 1983) appears to be regulated by its

phospholipid microenvironment. PI-kinase and PIP-kinase in rat brain, which are partially attached to the plasma membrane (Stubbs et al., 1988), are probably also influenced by their phospholipid microenvironment. This phospholipid microenvironment changes during aging by way of an increased cholesterol / phospholipid ratio, which results in a more viscous membrane (Rouser et al., 1972). Changes in lipid fluidity can influence synaptic transmission processes, ligand-receptor binding properties, and the dynamics of membrane-bound enzymatic processes such as protein phosphorylation (Hershkowitz et al., 1982). A previous study on phosphoinositide metabolism and aging (Van Dongen et al., 1983) showed a decrease in PIP₂ formation in synaptic plasma membranes of the hippocampus and whole brain with advancing age. Furthermore, the concentrations of free inositol and total phospholipid inositol decrease with age in humans (Stokes et al., 1983). These findings support the idea that the metabolism of phosphoinositides changes during aging. Such changes may affect the receptor-stimulated hydrolysis of polyphosphoinositides (Downes, 1982), and may also affect mitogenic signalling when phosphatidylinositol 3-kinase is involved (Carpenter and Cantley, 1991; Downes and MacPhee, 1990). A complicating factor in studies of the central nervous system is the heterogeneous structure of the brain. Little is known about the regional distribution of enzymes involved in phosphoinositide metabolism.

The present study was designed to provide more information on the effect of age on the incorporation of phosphate into PIP, PIP₂ and PA in membrane-cytosol preparations. These were prepared from seven discrete brain regions of young adult (3-month-old) and old (32-month-old) Brown Norway rats. This preparation was used to measure age related changes in the relationship between enzyme activity and endogeneous substrate availability and not just enzyme activity or substrate concentrations. The three phosphorylation activities have been characterized previously (Bothmer et al., 1990a). Furthermore, studies with this method have shown phosphate incorporation into PIP₂ and PA to be sensitive to adrenocorticotropin (ACTH₁₋₂₄), and phosphate incorporation into PIP, PIP₂ and PA to be sensitive to Ca²⁺ and dependent on Mg²⁺ (Jolles et al., 1981b). Besides ACTH₁₋₂₄, c-AMP and endorphins have also been shown to affect phosphoinositide phosphorylation, albeit in a different way (Jolles et al., 1981a; Jolles et al., 1982).

Experimental procedures

Animals and brain dissection

Male rats (3 months and 32 months old, 4 animals per group in each of 2 experiments) of an inbred Brown Norway strain were used. After decapitation, the head was immediately immersed in liquid nitrogen for 8 sec (Jolles et al., 1981b) to decrease brain temperature quickly. All subsequent operations were performed at 0-4°C. Brains were taken out of the skull rapidly and dissected

according to Gispen et al. (1972) with slight modifications. Briefly, the cerebellum was excised by cutting the brachium pontis. The medulla/pons was removed by a cut just rostral to the pons. The forebrain was cut transversally through the optic chiasm. The frontal part was dissected into the striatum, septum and frontal cortex. The tissue ventral to the septum, anterior commissure and rhinal fissure was removed. The cerebral cortex, hippocampus, entorhinal cortex and amygdala with overlying pyriformal cortex were removed from the caudal part and separated from each other. The entorhinal cortex and pyriformal cortex (with amygdala) were pooled. The remaining part was dissected into the thalamus, hypothalamus, septum, striatum and midbrain, which was removed. Frontal and caudal parts of the septum and striatum were pooled.

Subcellular fractionation

Tissue from individual rats was homogenized in homogenization medium containing 0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) as described before (Bothmer et al., 1990a). The method of Whittaker (1969) was used to prepare a crude mitochondrial-synaptosomal pellet (P_2) which was subjected to osmotic lysis: the pellet was resuspended in 10 vol aqua bidest. This suspension was centrifuged for 20 min at 10,000 $\times$ g to remove unlysed structures. The supernatant was taken as the enzyme fraction referred to as the membrane-cytosol preparation which contains PI, PIP, and DAG kinase activity, but also endogeneous lipid substrates.

Phosphorylation assay

Endogeneous phosphorylating activity was assayed as described before (Bothmer et al., 1990a; Jolles et al., 1981b). Briefly, a routine incubation was performed under the following conditions: 7.5 μ M ATP, 2-3 μ Ci [γ - 32 P]ATP (approx. 3000 Ci/mmol, Amersham, U.K.), 50 mM Na-acetate, 10 mM Mg-acetate, pH 6.5, and 15 μ l enzyme fraction (10 μ g protein) in a final vol of 25 μ l at 30°C. After 10 sec the lipid phosphorylation reaction was terminated by the addition of 2 ml ice-cold chloroform : methanol : 12 N HCl (200 : 100 : 075, by vol.). Lipid extraction, thin layer chromatography and determination of radioactivity were performed as described previously (Bothmer et al., 1990a; Jolles et al., 1981b). Protein content was determined according to the method of Lowry et al. (1951).

Total phospholipid phosphorus determination

Phospholipids were extracted according to the method of Folch et al. (1956). Briefly, 2 ml chloroform : methanol (2 : 1 (v/v)) and 310 μ l water were added to 90 μ l membrane-cytosol preparation. The lower phase of the resulting biphasic system was removed and stored. The upper phase was washed again with chloroform : methanol (2 : 1 (v/v)). Both lower phases were pooled and dried under a stream of nitrogen at 30°C. Perchloric acid (350 μ l) was added to the dried

lower phase to digest the phospholipids (Duck-Chong, 1978). The tubes were covered with glass marbles to minimize evaporation during 90 min heating at 180°C. After brief cooling, total phospholipid phosphorus was determined according to Fiske and Subbarow (1925).

Results

The regional distribution of basal specific phosphate incorporation into PIP, PIP₂ and PA within the rat brain was uneven (Fig. 1). Strikingly, the distribution of the three individual phosphorylating activities was different. Phosphate incorporation into PIP was highest, in terms of specific activity, in the thalamus, hypothalamus and striatum / septum. The regions with the lowest rates of phosphate incorporation into PIP₂ (hippocampus, entorhinal / pyriformal cortex, thalamus and hypothalamus) appeared to be the regions with the highest rates of phosphate incorporation into PA. The correlation between the distribution of these two activities was -0.56 (Pearson correlation coefficient), which is statistically significant ($p < 0.001$).

With age, phosphate incorporation into PA tended to decrease in all brain regions tested (Fig. 1). The decrease in PA formation in the hippocampus (-17%), frontal cortex (-11%), striatum / septum (-15%), thalamus (-14%) and hypothalamus (-15%) reached statistical significance ($p < 0.05$, Students t-test). A decrease in phosphate incorporation into PIP₂ with age was found in the frontal cortex (-20%, $p < 0.05$) and hypothalamus (-8%, $p < 0.05$). Phosphate incorporation into PIP was not affected by age in any of the seven brain regions tested.

Table 1 shows the effect of age on the protein content and the phospholipid phosphorus content of the membrane cytosol preparations of different brain regions. Protein content per mg tissue (wet weight) tended to decrease with age in all brain regions tested. The differences in protein content of the membrane-cytosol preparations of the entorhinal / pyriformal cortex, occipital / parietal cortex, thalamus and hypothalamus reached statistical significance.

The effect of age on phospholipid content of the membrane-cytosol preparation was determined by measuring total phospholipid phosphorus in these fractions. The total phospholipid phosphorus content (per mg tissue used to make the membrane-cytosol preparations) also showed an overall tendency to decrease with age. The differences in total phospholipid phosphorus of the membrane-cytosol preparation of the entorhinal / pyriformal cortex, striatum / septum and hypothalamus reached statistical significance. The protein content and the phospholipid phosphorus content of the membrane-cytosol preparation of the hippocampus, frontal cortex, entorhinal / pyriformal cortex, and hypothalamus were highly correlated. There was no correlation between the protein content and phospholipid phosphorus content of the membrane-cytosol preparation of the occipital / parietal cortex, striatum / septum and thalamus.

Fig 1: Effect of age on the incorporation of phosphate (pmol/mg protein) into phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidic acid (PA) in membrane cytosol preparations of seven brain regions. Three-month-old (black bars) Brown Norway rats were compared with 32-month-old (white bars) rats. Values shown are means ($\pm$ SEM) from eight rats per group. Statistical analysis was performed according to Students t test (two tailed). *: $p < 0.05$

Table 1 : Protein content and total phospholipid phosphorus content of the membrane cytosol preparations of different brain regions from Brown Norway rats of different ages. Values shown are means ($\pm$ SEM) from four rats per group. Statistical analysis of the age differences was performed according to Students t test (two tailed).

Brain region	Total protein (μ g/mg wet weight $\pm$ SEM)			Total phospholipid phosphorus (ng/mg wet weight $\pm$ SEM)			Correlation (r) ¹
	3 month	32 month	diff. (%)	3 month	32 month	diff. (%)	
Hippocampus	1.13 $\pm$ 0.05	1.06 $\pm$ 0.03	- 6	8.47 $\pm$ 0.42	8.10 $\pm$ 0.26	- 4	0.860 *
Frontal cortex	1.30 $\pm$ 0.05	1.23 $\pm$ 0.07	- 6	9.96 $\pm$ 0.38	9.19 $\pm$ 0.66	- 8	0.941 **
Entorhinal/pyriformal cortex	1.67 $\pm$ 0.04	1.45 $\pm$ 0.06	-13 *	11.23 $\pm$ 0.54	9.31 $\pm$ 0.44	-17 *	0.894 **
Occipital/parietal cortex	1.24 $\pm$ 0.04	1.08 $\pm$ 0.03	-13 *	9.86 $\pm$ 0.71	8.90 $\pm$ 0.55	-10	0.575
Striatum/septum	1.00 $\pm$ 0.06	0.85 $\pm$ 0.05	-15	9.88 $\pm$ 0.50	8.04 $\pm$ 0.10	-19 *	0.659
Thalamus	1.06 $\pm$ 0.01	0.94 $\pm$ 0.02	-11 **	10.73 $\pm$ 0.26	9.60 $\pm$ 0.34	-12	0.748
Hypothalamus	0.97 $\pm$ 0.03	0.85 $\pm$ 0.04	-12 *	10.82 $\pm$ 0.40	9.28 $\pm$ 0.27	-14 *	0.920 **

* : $p < 0.05$

** : $p < 0.005$

¹ : Pearson correlation (r) between protein and phospholipid phosphorus content.

Discussion

This study describes the effect of age on phosphate incorporation into phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidic acid (PA). A membrane-cytosol preparation of different brain regions from 3-month-old and 32-month-old Brown Norway rats was used as a source of enzyme and of endogeneous phospholipid substrate.

Basal phosphate incorporation into PIP, PIP₂ and PA, was unevenly distributed in the rat brain, and the distribution appeared to be different for each phosphorylation product. The opposite distribution within the brain of PIP₂ and PA formation could reflect a regional distribution of basal phospholipase C (PLC) activity because PIP₂-specific PLC activity and DAG kinase activity are directly correlated (Agranoff et al., 1983). Thus, brain regions with high DAG kinase activities would be expected to have high basal PLC activities. However, studies on the regional distribution of agonist stimulated PLC activities (Fisher and Bartus, 1985; Gonzales and Crews, 1985) do not confirm this notion.

With respect to the effects of age on the basal rates of phosphate incorporation into PIP, PIP₂ and PA, our results show that phosphate incorporation into PA was decreased in the membrane-cytosol preparation of the hippocampus, frontal cortex, striatum / septum, thalamus and hypothalamus of aged rats. Phosphate incorporation into PIP₂ was decreased in the membrane-cytosol preparation of the frontal cortex and hypothalamus of aged rats, and phosphate incorporation into PIP was not affected. In a previous study on the rates of phosphate incorporation into endogeneous PIP, PIP₂ and PA in a crude synaptosomal fraction (P₂-fraction) of whole rat brain cortex of young (7 months) and old (27 months) Wistar rats, we also found a decrease in PA formation with age (Bothmer et al., 1990b). Strosznajder and Samochocki (1991) showed a decreased Ca²⁺-independent arachidonic acid (AA) release in synaptoneurosomes from brain cortex of 27-month-old rats compared with 4-month-old rats. This lower AA release was due to a modified substrate utilization which seems to be connected with lower phosphorylation of DAG to PA and higher degradation of DAG. This conclusion of Strosznajder and Samochocki (1991) is in agreement with our findings presented here.

Previous studies on phosphoinositide metabolism (Van Dongen et al., 1983) in synaptic plasma membranes, revealed a decrease in PIP₂ formation in the hippocampus and whole brain of the rat with age, but no age-related changes in PIP and PA formation. In the study presented here, we did find changes in PA formation but (almost) not in PIP₂ formation. These differences are probably caused by the fact that Van Dongen et al. used a synaptic plasma membrane preparation without cytosolic factors. Such a fraction contains little or no DAG-kinase activity because this enzyme is predominantly located in the cytosol (Lundberg and Jergil, 1988; Stubbs et al., 1988). PIP-kinase is not as tightly associated with the membrane as PI-kinase is, as it can be solubilized with low salt concentrations, whereas PI-kinase solubilization requires detergents (Saltiel et al., 1987). With age, PIP-kinase may be translocated from the particulate pool to

the soluble pool. This could be caused by the increased viscosity of the membrane in aged subjects (Rouser et al., 1972). PI-kinase, which is more tightly attached to the membrane, may not be affected by this change in the microenvironment. In our study, we did not differentiate between synaptosome membrane and synaptosome cytoplasm, so a possible change in the distribution pattern between these two pools could not be observed.

We also found that the protein content of membrane-cytosol preparations from various brain regions decreased with age. The decrease in protein content appeared to be a result of a lower number or size of nerve endings because the age-related changes in total protein content correlated highly with the age-related changes in total phospholipid content in the membrane cytosol preparation of hippocampus, frontal cortex, entorhinal / pyriformal cortex and hypothalamus. Calderini et al. (1983) also found a parallel decrease in protein and phospholipid content in synaptic plasma membrane (whole brain) of aged rats, a result which is consistent with our data. Decreased numbers of synapses have been shown in the dentate gyrus of human brain (Bertoni-Freddari et al., 1990) with age and Alzheimer's disease, and in frontal cortex biopsies from subjects with Alzheimer's disease (DeKoski and Scheff, 1990). Both studies also showed a primarily compensatory mechanism of increased synapse size with decreasing synapse number. The functional implications of these morphological processes were shown in the studies of DeKoski and Scheff (1990) and of Geinisman et al. (1986). Lower mental status scores appear to be associated with greater loss of synapses (DeKoski and Scheff, 1990) and old, spatial memory-impaired rats have a decreased number of perforated axospinous synapses as compared to old, memory-intact rats (Geinisman et al., 1986). Both old groups of rats had a diminished number of non-perforated axospinous synapses as compared to young rats.

In conclusion, it can be said that basal specific incorporation of phosphate into PIP, PIP₂ and PA, measured in a lysed synaptosomal fraction, also called a membrane-cytosol preparation, is unevenly distributed within the rat brain. Therefore, future studies on basal phosphate incorporation into PIP, PIP₂ and PA, but probably also other biochemical studies, should take these regional differences into account. Furthermore, age seems to decrease endogenous PA formation in whole brain and PIP₂ formation in the frontal cortex and hypothalamus. The effects of age may reflect a deterioration of phosphoinositide metabolism in the brain regions involved. Therefore, the function of polyphosphoinositide metabolism in, for example, signal transduction coupled to receptors via G-proteins could be affected by aging. This study also supports data of some morphological studies showing a decline in the number of synaptic nerve endings in brain with age.

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CHAPTER 6

THE EFFECT OF AGE ON PHOSPHOINOSITIDE KINASE, PHOSPHOINOSITIDE PHOSPHATE KINASE AND DIACYLGLYCEROL KINASE IN RAT BRAIN CORTEX.

J. Bothmer, M. Mommers, M. Markerink and J. Jolles

Abstract

The effect of age on PI kinase, PIP kinase and DAG kinase activities in the cerebral cortex was studied in 8-month-old, 14-month-old and 26-month-old Brown Norway rats. Three protein preparations were used: a cytosolic preparation (A), a salt-solubilized preparation (loosely membrane-bound proteins, B) and a Triton X-100-solubilized preparation (integral or tightly membrane-bound proteins, C). PI kinase activity was predominantly located in the tight membrane-bound fraction, DAG kinase activity in the cytosolic and loosely membrane-bound fraction, and PIP kinase activity was present in all three protein preparations. The effects of age were limited to a small increase in PI kinase activity in fraction C in 14-month-old and 26-month-old rats compared to 8-month-old rats, and a 10% decrease in PIP kinase activity in fraction A in 14-month-old and 26-month-old rats compared to 8-month-old rats. DAG kinase activity showed no age-related changes. In conclusion, one should take care in comparing rat aging with human aging as PI kinase activity shows an age-related decline in human brain cortex. Furthermore, previously reported decreases in PA formation rates in rat brain are probably not due to changes in DAG kinase itself but to changes in DAG availability.

Introduction

Normal aging is associated with numerous functional and morphological alterations of the central nervous system (CNS) in animals as well as humans. Phosphoinositides, which are present in relatively high concentrations in the CNS, play an important role in receptor-mediated signal transduction, resulting in the release of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1984). In the CNS, various receptors are coupled, via G-proteins, to this phosphoinositide hydrolyzing signal transduction system (Fisher et al., 1992) in which IP_3 releases calcium from intracellular stores (Streb et al., 1983). This results in the activation of Ca^{2+} /Calmodulin-dependent protein kinase (Berridge, 1984), whereas DAG activates protein kinase C directly (Nishizuka, 1984). These protein kinases regulate many other enzymes, ion-channels etc. by phosphorylation ultimately resulting in a biological response (Magnoni et al., 1991). As mentioned by Li et al. (1991), a disruption of the phosphoinositide second messenger system with aging could be related to the impairment of neurological responsiveness and behavioral deficits observed with aging.

The membrane-associated processes involved in receptor-stimulated inositol phosphate release (Burnett et al., 1990; Mundy et al., 1991; Nalepa et al., 1989; Pietrzak et al., 1990; Surichamorn et al., 1989; Tandon et al., 1991) but also the effectiveness of the inositol phosphate response, such as IP_3 receptor density and affinity (Li et al., 1991), the maintenance of calcium homeostasis (Barritt, 1987; Martinez-Serrano et al., 1992) and protein kinase activity (Magnoni et al., 1991; Battaini et al., 1990), have been studied as a function of age. However, the production of the IP_3 precursor phosphatidylinositol bisphosphate (PIP_2) by

phosphatidylinositol (PI) kinase and phosphatidylinositol phosphate (PIP) kinase, and the phosphorylation of DAG into phosphatidic acid (PA) have not received this attention in aging research. The relevance of a study of the phosphoinositide phosphorylation system is strengthened by the notion that changes in the activity of these enzymes could also explain changes in the receptor-stimulated inositol phosphate response. Interestingly, the function of PI kinase has received an additional dimension with the discovery of novel phosphoinositides which are phosphorylated at the D-3 position of the inositol ring. These substances are implicated in growth, mitogenic signalling and cytoskeletal turn-over processes (Carpenter and Cantley, 1990). PI, PIP and DAG kinases are partially membrane-bound (Stubbs et al., 1988), and can therefore be influenced by the changes in membrane fluidity associated with aging (Hershkowitz et al., 1982). In addition, enzymes or other functional proteins can lose their functionality as a result of covalent modifications such as mixed function oxidation, deamidation, ubiquitin conjugation and glycation as a particular consequence of the aging process (Smith et al., 1991).

A previous study, in which a lysed P_2 fraction was used with endogenous phospholipids as substrate, revealed that phosphatidic acid (PA) formation decreased with age in almost all brain regions tested. In contrast, PIP_2 formation was only decreased in the frontal cortex and the hypothalamus, and PIP formation was not affected at all (Bothmer et al., 1992). Furthermore, Stokes et al. (1983) reported a decrease in total lipid myo-inositol with age in human brain cortex, and Jolles et al. (submitted) showed an age-related decrease in PI kinase activity in human brain cortex. Finally, Jolles et al. (1992) found a 50% reduction in PI kinase activity in patients suffering from pathological brain aging, i.e., Alzheimer's disease.

In the present study, the effect of age on PI kinase, PIP kinase and DAG kinase activities was investigated in the rat brain cortex. Brain cortical structures of 8-month-old, 14-month-old and 26-month-old Brown Norway rats were fractionated into a cytosolic protein preparation, a salt-solubilized protein preparation and a Triton X-100-solubilized preparation.

Experimental procedures

Animals and brain dissection

Eight-month-old, 14-month-old and 26-month-old male rats of an inbred Brown Norway strain were used. After decapitation, the head was immediately immersed in liquid nitrogen for 8 s. All subsequent operations were performed at 0-4°C. The brain was rapidly taken out of the skull and dissected according to Gispén et al. (1972) with slight modifications. Briefly, the cerebellum was excised by cutting the brachium pontis. The medulla/pons was removed by a cut just rostral to the pons. The forebrain was cut transversally through the optic chiasm.

The frontal part was dissected into the frontal cortex and a remaining part, which was removed. The cerebral cortex, hippocampus, entorhinal cortex and amygdala with overlying pyriform cortex were removed from the caudal part and separated from each other. The frontal cortex, cerebral cortex, entorhinal cortex and pyriform cortex were pooled, and the remaining parts were discarded.

Subcellular fractionation

Cellular proteins were fractionated according to Yamakawa and Takenawa (1988). Briefly, tissue was homogenized (1:10, wet weight/volume) in homogenization buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM DTT, pH 7.4) by 12 up-and-down strokes of a Potter-Elvehjem Teflon glass homogenizer (radial clearance of 0.125 mm, 700 rpm), followed by homogenization in a glass-glass homogenizer with 3 up-and-down strokes (clearance 0.125 mm). The homogenate was centrifuged (100,000xg, 4°C, 45 min), and the resulting membrane-free supernatant was taken as the "cytosolic protein preparation" (fraction A). The pellet was homogenized as described above in homogenization buffer containing 1 M NaCl, and the homogenate was stirred for 60 min at 4°C. After centrifugation (100,000xg, 4°C, 45 min), the supernatant was taken as the "salt-solubilized protein preparation" (fraction B). The pellet was homogenized as described above in homogenization buffer containing 1% Triton X-100 (w/v), followed by sonication on ice for 3 x 10 s at maximal power, with 1 min pauses. breaks. The homogenate was stirred for 60 min at 4°C and centrifuged (100,000xg, 4°C, 45 min). The supernatant was taken as the "Triton-solubilized protein preparation" (fraction C). The pellet or residual fraction was discarded.

PI kinase, PIP kinase and DAG kinase assay

The phospholipid kinase activities were assayed as described earlier (Jolles et al., 1992). Briefly, the incubation volume (normally 25 µl) of the PIP kinase assay was doubled to reduce interassay variability. Before the start of the kinase assay, the "salt" and "Triton" preparations were dialysed (1:100) against 20 mM Tris-HCl, 1 mM EGTA, 1 mM DTT, pH 7.4. Protein solutions were preincubated for 20 min. Lipid precursors (20 µM PI, 20 µM PI-4-P or 100 µM 1-stearoyl-2-arachidonoyl-sn-glycerol (Sigma), solubilized in 0.1% Triton x-100, 20 mM Tris-HCl, 1 mM EGTA, 1 mM DTT, pH 7.4, were added 15 s prior to phosphorylation, which was started by the addition of ATP and lasted 1 min. Incubations were performed under the following conditions: 7.5 µM ATP, 2-3 µCi [gamma-³²P] ATP (approx. 3000 Ci/mmol, Amersham U.K.) 20 mM Tris-HCl, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT and 0.02% Triton X-100, pH 7.4. The reaction was terminated, and the extraction and further analysis of the ³²P incorporated into PIP, PIP₂ and PA were performed as described elsewhere (Bothmer et al., 1990a; Jolles et al., 1981). Protein determination was performed according to the method of Lowry et al. (1951).

Fig.1 : Distribution of phosphatidylinositol (PI) kinase, phosphatidylinositol phosphate (PIP) kinase and diacylglycerol (DAG) kinase activities in a cytosolic protein preparation (fraction A), a salt-solubilized protein preparation (fraction B), and a triton X-100-solubilized protein preparation (fraction C) of 8-month-old rat brain cortex. ND: not detected. Values shown are means ($\pm$ SEM).

Results

In the present study, the effect of age on PI kinase, PIP kinase and DAG kinase activities was measured in three rat brain cortex protein preparations: a cytosolic protein preparation (fraction A), a salt-solubilized protein preparation (fraction B) and a triton X-100-solubilized protein preparation (fraction C). The salt-solubilized preparation contains proteins which are under physiological conditions loosely attached to the membrane although with a cytoplasmic location, in contrast to the triton-solubilized preparation, which contains proteins which are, under physiological conditions integral or tightly-bound membrane proteins.

Fig.1 shows that PI kinase activity seemed to be located primarily in fraction C. The fractions with proteins of a cytoplasmic location (fraction A and B) contained only small amounts of PI kinase activity. PIP kinase activity was located in all three protein fractions, although primarily in fraction C. In contrast, DAG kinase activity was not detected in fraction C, but could only be detected in the fractions with proteins of a cytoplasmic location (fraction A and B).

Fig.2 : Effect of age on phosphatidylinositol (PI) kinase, phosphatidylinositol phosphate (PIP) kinase and diacylglycerol (DAG) kinase activities in a cytosolic preparation (fraction A), a salt-solubilized protein preparation (fraction B), and a Triton X-100-solubilized protein preparation (fraction C) of rat brain cortex. Eight-month-old, 14-month-old and 26-month-old Brown Norway rats were used. Values shown are means ($\pm$ SEM) as a % of values of 8-month-old rats. Statistical analysis was done with Duncan's Multiple Range Test. * : $p < 0.05$.

Fig.2 shows the effect of age on PI kinase, PIP kinase and DAG kinase activities in the different protein fractions of rat brain cortex, measured with exogenous lipids as substrates. PI kinase activity showed no age-related changes with the exception of fraction C, which showed a statistically significant ($p < 0.05$) but small increase in PI kinase activity with age. PIP kinase activity was decreased by 10% in fraction A in 14- and 26-month old rats, but was not affected in fractions B and C. DAG kinase activity showed no age-related changes in any of the fractions tested. The protein content of these fractions also showed no age-related changes (data not shown).

Discussion

In this study, three protein preparations were used to differentiate between cytosolic (fraction A) and membrane-bound (fraction B and C) proteins of rat

brain cortex. As shown in Fig. 1, DAG kinase was predominantly located in the cytosolic compartment (fraction A) or just loosely bound to the membrane (fraction B), which is consistent with previous findings (Lundberg and Jergil, 1988; Stubbs et al., 1988). PI kinase was located in fraction C and to a small extent in fraction B, which is consistent with the findings of Saltiel et al. (1987), who reported that PI kinase is tightly associated with the membrane because its solubilization requires detergent. PIP kinase of bovine brain is located for 40% in the cytosolic compartment and for 60% in the membrane-bound compartment (Moritz et al., 1990). Saltiel et al. (1987) also reported that PIP kinase can be solubilized with low salt concentrations. These findings are in contrast with the findings presented in this study in which the highest PIP kinase activity was found in the Triton-solubilized fraction (C). In a study with human brain cortex material (Bothmer et al., submitted), we did find high PIP kinase activity in fractions A and B, and very low PIP kinase activity in fraction C.

In a previous study, PI kinase, PIP kinase and DAG kinase activities in rat brain were measured as a function of age with endogenous lipids, present in the membrane-cytosol preparation, as substrate (Bothmer et al., in press). In that study, we could not differentiate between an effect of age on substrate availability and an effect of age on enzyme activity. In the present study, we used exogenous phospholipids (PI, PIP and DAG) as substrate for measuring PI kinase, PIP kinase and DAG kinase, respectively, in different protein fractions of the rat cerebral cortex.

DAG kinase activity did not show age-related changes when 8-month-old rats were compared with 26-month-old rats, in contrast to PA formation measured with endogenous lipids as substrate, which decreased (-15%) with age in almost all brain regions tested (Bothmer et al., 1992). This decrease in PA formation with age was also found in a crude synaptosomal fraction (P_2 -fraction) of whole rat brain cortex of old (27 months) compared to young (7 months) Wistar rats (Bothmer et al., 1990b) in experiments in which endogenous lipids were also used as substrate. The present findings combined with the findings of Bothmer et al. (1990b, 1992) show that the decrease in PA formation with age found in previous studies is not caused by changes in DAG kinase itself, but by changes in the availability of DAG. This is in agreement with the conclusion of Strosnjajder and Samochocki (1991), who found a decreased Ca^{2+} -independent arachidonic acid (AA) release in synaptoneurosomes from rat brain cortex of 27-month-old rats compared with 5-month-old rats. These authors concluded that their findings were due to a decreased availability of DAG.

The decrease in PIP kinase activity with age in rat brain cortex seems to be restricted to the cytosolic compartment. PIP_2 formation in a membrane-cytosol preparation of rat brain frontal cortex was also found to decrease (-20%, Bothmer et al., 1992) with age, which supports our present findings. Van Dongen et al. (1983) also found a decrease in PIP_2 formation with age, although they used a synaptic plasma membrane preparation of whole brain. It is possible that no decrease in PIP kinase activity was found in fraction C (tightly membrane-bound proteins) because there was no differentiation between synaptic membranes and

other membranes in this study.

With respect to PI kinase activity, we found only a small increase with age in fraction C, and no differences in fraction A and B (the cytosolic and loosely membrane-bound protein preparations, respectively). In previous studies (Bothmer et al., 1990b, in press), PI kinase activity or PIP formation rates were not changed in old rats compared to young rats. In crude cytosolic preparations of human cerebral cortex, however, PI kinase activity decreased with advancing age (Jolles et al., submitted). In addition, Stokes et al. (1983) reported a decrease in total lipid myo-inositol with age in human brain cortex. The lack (or almost lack) of age-related changes in PI kinase activity in rat brain, in contrast to the age-related decline in PI kinase activity in human brain, might be due to species differences or to the fact that old people are more likely to have been exposed to biological life events (BLE) than young people (Houx et al., 1991), and than rats. These rats are kept under standardized conditions and are well-cared for. BLE are factors other than severely impairing conditions like dementia that are known to damage optimal brain functioning. Examples of BLE are mild, closed-head injuries, repeated anesthesia and intoxication (Houx et al., 1991).

In conclusion, PI kinase, PIP kinase and DAG kinase activities show no, or only minor, age-related changes in the rat cerebral cortex. The changes in PA formation with advancing age (Bothmer et al., 1990b, 1992) found when using endogenous phospholipids as substrate are probably caused by age-related changes in the availability of DAG, the substrate for PA formation. The lack of an age-related change in PI kinase activity in rat brain cortex is in contrast to the age-related decline in PI kinase activity in human brain cortex (Jolles et al., submitted) and shows that care should be taken when using rats, which are kept under standardized conditions and which are exposed to environmental factors that are known to damage optimal brain functioning, as a model for human aging.

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CHAPTER 7

PHOSPHATIDYLINOSITOL KINASE IS REDUCED IN ALZHEIMER'S DISEASE.

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Abstract

Phosphatidylinositol kinase (PI kinase) and phosphatidylinositol phosphate kinase (PIP kinase) were measured in post-mortem samples of brain tissue from patients with Alzheimer's disease and non-demented control subjects. A membrane-free cytosolic fraction from four neocortical locations, with exogenous inositol lipids as substrate, was used. Tissue from patients with Alzheimer's disease was characterized by reduced PIP formation; the reduction was 50% in prefrontal cortex, temporal cortex and parietal cortex, and 40% in precentral gyrus. In contrast, no alterations were found in phosphatidylinositol bisphosphate (PIP₂) formation in these four neocortical locations. The specific changes in PI kinase but not PIP kinase activity suggest that the findings may have functional relevance to the involvement of brain membrane processes in Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease and the most common cause of adult onset dementia. The etiology and pathogenesis are presently not known. Interest in brain membrane phospholipids was aroused several years ago in relation to the hypothesis of a cholinergic dysfunction in AD (e.g. Bartus et al., 1982) and the notion that these neurons utilize choline for the formation of the membrane-phospholipid phosphatidylcholine (Wurtman et al., 1990). Alterations in phosphatidylcholine and its metabolites, and in other phospholipids in AD brains have now been described (e.g., Blusztajn et al., 1990; Miatto et al., 1986; Pettegrew et al., 1989). Changes in other phospholipids, notably phosphatidylinositol (PI) and its breakdown product myo-inositol have also been investigated in AD and in normal aging (Stokes et al., 1983; Stokes and Hawthorne, 1987). Such findings may have functional significance because the interconversion of PI into phosphatidylinositol phosphate (PIP) and phosphatidylinositol bisphosphate (PIP₂), and the breakdown of the latter substance into diacylglycerol and inositolphosphate, are key processes in neuronal function (see for review, Abdel-Latif, 1986; Berridge, 1987; Downes and MacPhee, 1990).

The present study was undertaken to investigate whether inositol phospholipid phosphorylating activity is different in brains from patients with AD as compared with brains from non-demented control subjects. Considering the rapid breakdown of inositol phospholipids after death (Lin et al., 1990) and after low-energy periods *in vitro* (Jolles et al., 1981b), we chose to measure enzyme activity rather than analyze phospholipid contents. Brain samples were obtained by rapid autopsy (within 4 to 6 hours after death). AD and control subjects were matched for age in order to reduce possibly confounding factors such as interaction between age and disease, and duration of disease. Four neocortical locations were compared in order to detect possible regional variations like those observed in old animals (Bothmer et al., 1990a). PI kinase and PIP kinase were studied in a membrane-free supernatant of AD and control brain, with exogenous PI and PIP as lipid substrates.

Experimental procedures

Subjects

Brain samples from 5 patients with AD (3M, 2F; mean age 65.2 yrs) and 5 control subjects (3M, 2F; mean age 69 yrs) were used in this study (see Table 1). Patients and controls were individually matched for age and post-mortem interval. Brain tissue was obtained from the Netherlands Brain Bank. The mean post-mortem interval was 5 h for AD subjects and 5 h 15 min for the controls. The patients with AD were clinically diagnosed as "probable Alzheimer's disease" (McKhann et al., 1984, APA 1987) and this was verified by post-mortem neuropathological examination. Control subjects had no history of dementia, or any other neurological or psychiatric disorder.

Table 1 : Autopsy data of subjects used in the study.

Subjects experiment number	Age (years)	Sex	Brain weight (g)	PM delay (min)	PH	Cause of Death
Controls						
C1	60	m	1350	360	6.45	Septic shock following aorta valve implantation
C2	65	m	1310	315	6.18	Heart failure
C3	66	f	1100	375	6.24	Metastases of breast cancer
C4	71	f	1240	305	6.28	Sepsis and cardiogenic shock
C5	73	m	1340	255	6.89	Cerebral infarct without evident cause
C6	73	m	1410	320	6.93	Postoperative heart failure
AD						
A1	59	f	780	305	6.59	Cardiac arrest
A2	64	m	1210	330	6.21	Collum-fracture; died after operation
A3	68	f	895	345	6.52	Bronchopneumonia
A4	70	m	1075	270	6.38	Cachexia
A5	65	m	1360	240	-	Cachexia/lungemboli/ dehydration
A6	72	m	1040	285	6.89	Pneumonia and cachexia

Crude enzyme fractions from the frontal superior gyrus and the parietal lobe were prepared from subjects C1-C5 and A1-A5, crude enzyme fractions from the precentral gyrus were prepared from subjects C1-C4 and C6 and A1-A5, and crude enzyme fractions from the medial temporal gyrus were prepared from patients C1-C5 and A2-A6.

Brain dissection

Brain specimens for analysis of inositol phospholipid kinase activity were obtained from four neocortical locations in the right hemisphere: 1) the frontal superior gyrus; 2) the precentral gyrus; 3) the medial temporal gyrus, and 4) the parietal lobe. The leptomeninges were removed and samples were excised. These samples were sealed in plastic, rapidly frozen by immersion in liquid nitrogen, and stored at -80°C until use.

Preparation of crude enzyme fraction

Pieces of approximately 0.5 g were excised from the tissue samples, and thawed in a water bath at 0°C (20 min). The tissue was homogenized in a medium consisting of 0.32 M sucrose, 1mM EGTA, 50 mM Tris. HCl, pH 7.4 in a total volume 10 times the brain tissue volume, by 12 up and down strokes of a Potter-Elvehjem Teflon-glass homogenizer (radial clearance: 0.125 mm, 700 rpm), followed by homogenization by hand in a glass-glass homogenizer with 3 up and down strokes. The homogenate was centrifuged for 60 min at $100,000\times g$, and the resulting membrane-free supernatant was used as the crude enzyme fraction. This fraction was stored at -80°C .

PI kinase and PIP kinase assay

Inositol phospholipid kinase activity was measured as described before (Moritz et al., 1990; Van Dongen et al., 1986), with some modifications. The incubation volume (normally 25 μl) was doubled for the PIP kinase assay in order to reduce interassay variability. Supernatant fractions of 15 or 30 μl (10 and 20 μg protein, respectively), were preincubated for 2 minutes. Lipid precursors (20 μM PI or 20 μM PIP (Sigma), solubilized in 0.1% Triton X-100 / 50 mM Tris-HCl / 1 mM EGTA, pH 7.4) were added 15 sec before the phosphorylation reaction was started by the addition of ATP. The reaction lasted 1 min. Incubations were performed under the following conditions: 7.5 μM ATP, 2-3 μCi [γ - ^{32}P] ATP (approximately 3000 Ci/mmol, Amersham UK) 50 mM Tris-HCl pH 7.4, 10 mM MgCl_2 , 1mM EGTA and 0.02% Triton X-100. The reaction was terminated, and the extraction and further analysis of the ^{32}P incorporated into PIP and PIP_2 were performed, as described elsewhere (Bothmer et al., 1990b; Jolles et al., 1981a). Protein was determined according to the method of Lowry et al. (1951).

Results

The results of the PI kinase assay in brain samples from patients with AD and controls are shown in Fig. 1A. A significant difference between AD and control patients was found in the four neocortical locations tested (MANOVA; $p < 0.01$). The AD group was characterized by a reduction in ^{32}P -PIP formation by 50% (prefrontal cortex, temporal cortex and parietal cortex) or 40% (precentral cortex).

Fig. 1: PI kinase (A) and PIP kinase (B) activities in crude cytoplasmic fractions from four neocortical regions of AD brains and age-matched controls, expressed as pmol Pi incorporated into phosphatidylinositol monophosphate per mg protein. Data were analyzed by MANOVA. *: $p < 0.01$.

In the control samples, there was a regional variation in PIP formation: ^{32}P -PIP formation was significantly lower in the temporal and parietal cortex than in the prefrontal and precentral cortex ($p < 0.01$; Siegel and Catellan, 1988). There were no such differences in the AD samples. The results of the PIP kinase assay with tissue from patients with AD and controls are shown in Fig. 1B. There were no differences between AD and control subjects in any of the four structures.

Discussion

The present study shows that a crude cytosolic PI kinase fraction obtained from neocortical structures taken from brains of patients with AD has about 50% less activity than similar fractions obtained from control brains. This effect appears to be quite specific as there was no decrease in PIP kinase activity. The difference between the two patients groups can not be ascribed to factors such as age and post-mortem delay because the groups had been matched for these factors. The post-mortem interval (4-6 h) was very short, which is an advantage considering the rapid breakdown of the various compounds after death. The large standard deviations in the studies of Stokes and Hawthorne (1987), in which they measured the absolute concentrations of free myo-inositol and inositol

phospholipid-bound inositol in brains from patients with AD and control subjects, are probably caused by the post-mortem interval (7-83 hrs.), as they suggested. In this study we measured enzyme activities and thus circumvented the problem that the levels of PIP and PIP₂ -and to a lesser extent PI- are dependent upon the post-mortem interval. A rapid post-mortem breakdown of the polyphosphoinositides in rat brain has been described in previous studies (Dawson and Eichberg, 1965; Jolles et al., 1981b): Jolles and coworkers showed that 50% of the labile form of PIP and PIP₂ was no longer detectable within 2 min. Likewise, Lin et al. (1990) provided evidence for a very rapid hydrolysis of rat brain PIP₂ within minutes after decapitation.

Both the present results and the findings of Stokes and Hawthorne (1987) suggest that the inositol phospholipid system may be involved in the pathogenesis of AD. It is of importance in this respect that other investigators have also found evidence for an involvement of membrane phospholipids and their metabolites in AD. Several research groups, using ³¹P NMR spectroscopy, have found elevations in the phosphomonoesters (e.g., phosphocholine) early in the course of the disease, followed by an elevation of phosphodiester (e.g., glycerol-3 phosphorylcholine; Barany et al., 1985; Blusztajn et al., 1990; Brown et al., 1989; Miatto et al., 1986). In addition, AD brain appears to be characterized by a decrease in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol (Pettegrew et al., 1986). Apart from phospholipids, phosphorylated proteins are implicated in AD as well. For example, the phosphorylation of protein tyrosine (Shapiro et al., 1991) and of microtubule-associated proteins (Hanger et al., 1991) has received much attention over the last years. It remains to be established whether there is any relation between these changes and the findings reported in the present paper.

The change in PI kinase activity is of potential relevance because of the large effect found: a reduction of 50% in enzyme activity is substantial in view of the fact that neocortical tissue can be expected to contain cell types which do not degenerate in AD. Thus if the reduced PI kinase activity is confined to (a) particular cell type(s), the effect may be still greater. Furthermore, inositol phospholipids play a key role in impulse initiation and propagation, and thus with intrinsic neuronal and brain functions (see for reviews Abdel-Latif, 1986; Berridge, 1987). A large decrease in PIP formation can therefore be expected to have a widespread influence on membrane function, because the pathway which leads to PIP₂ formation is blocked. This may result in a blockade of PIP₂ hydrolysis, and thus inhibit the formation of the second messengers inositol trisphosphate (IP₃) and diacylglycerol. It is relevant in this respect that reduced IP₃ binding sites have been found in the parietal cortex and hippocampus of patients with AD (Young et al., 1988).

With respect to the biochemical mechanisms underlying the reduced incorporation of ³²P into PIP, it is possible that the AD brain samples are characterized by a reduced quantity of PI kinase, by changes in enzyme kinetics, or by the presence or absence of cofactors. Questions as to the type of PI kinase involved are also relevant in view of the fact that two different types of PI kinase are known,

namely the kinase which phosphorylates PI at the 4 position of the inositol ring (the so-called "PI 4-kinase") and the one which phosphorylates the 3 position ("PI 3-kinase"). This difference is of importance because the two kinases are implicated in different functions in the cell (Downes and MacPhee, 1990). Involvement of the PI 3-kinase would be of great relevance because this kinase is suggested to be involved in cell growth and in the maintenance of the cytoskeleton (Carpenter and Cantley, 1990). The fact that the present findings were obtained with a membrane-free supernatant suggests that the kinase responsible for these findings might be the PI 3-kinase. Research is in progress to investigate whether the present findings are due to changes in the activity of PI 3-kinase or PI 4-kinase.

The finding that the differences between AD brains and control brains were quite similar for the four structures tested is indicative of a global degeneration of the neocortex. Indeed, the patients were in the terminal stage of the disease (i.e., stage 7 on the Global Deterioration Scale, Reisberg et al., 1983) when most neocortical tissue is known to be involved. Research is planned to compare the tertiary association zones investigated in the present study with the primary sensory cortex and limbic zones and with the cerebellum. A comparison with the latter structure is judged to be especially relevant because this region is not pathologically affected in AD. In addition, other neurological disease groups (Parkinson's disease and multi-infarct dementia) are investigated in order to ascertain the specificity of the observed changes in PI kinase activity. In conclusion, specific alterations in inositol phospholipid phosphorylation have been found in the present study. The findings may be important because of the key role played by inositol phospholipids in neuronal functioning.

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CHAPTER 8

REDUCED PI KINASE ACTIVITY IN ALZHEIMER'S DISEASE: EFFECTS OF AGE AND ONSET.

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Abstract

Phosphatidylinositol kinase (PI kinase) and phosphatidylinositol phosphate kinase (PIP kinase) were assayed in a membrane-free cytosolic fraction prepared from the medial temporal cortex of patients with Alzheimer's disease (AD) and non-demented controls, with exogenous lipids as substrate. ^{32}P -PIP formation appeared to be reduced by 21 % in patients with Alzheimer's disease who died before 80 years, compared to matched controls. In addition, there was an age-related decrease in PI kinase activity in the control group. In the AD patients group there was no age-related decrease, because PI kinase activity was increased in old AD patients (older than 80 when they died) with a short duration of the disease. No differences were found in the closely related enzyme PIP kinase. The results are indicative for heterogeneity in AD.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease and the most common cause of dementia. The etiology and pathogenesis are presently not known. Interest in brain membrane phospholipids in AD was aroused several years ago when it was found that cholinergic neurons especially are vulnerable to the degenerative process of Alzheimer's disease (e.g. Bartus et al., 1982). These neurons utilize choline both for the synthesis of acetylcholine and for the formation of the membrane-phospholipid phosphatidylcholine (PC). Alterations in PC and its metabolites, and in other phospholipids in AD brain have now been described (Blusztajn et al., 1990; Miatto et al., 1986; Pettegrew et al., 1989).

Recently, cytosolic fractions obtained from neocortical structures taken from brains of AD patients were found to have approximately 50% less activity in phosphatidylinositol (PI) kinase activity than similar fractions obtained from control brains. No such differences were found in the activity of the closely related enzyme phosphatidylinositol phosphate (PIP) kinase (Jolles et al., 1992). Stokes and Hawthorne (1987) had earlier reported that AD brain is characterized by a decreased content of phosphatidylinositol (PI). Phospholipids which have the sugar moiety inositol as their polar head group are particularly important for membrane functioning. Nervous tissue is especially rich in inositol phospholipids, which are involved in neurochemical processes related to neurotransmission (for review see Abdel-Latif, 1986; Berridge, 1987). Specifically, this class of compounds is implicated in agonist-receptor coupling, Ca^{2+} gating and Ca^{2+} mobilization from internal stores, and the formation of the intracellular second messengers diacylglycerol and inositoltrisphosphate (Downes and MacPhee, 1990).

The present study elaborates on the findings of an earlier study (Jolles et al., 1992). The focus is not on phosphoinositide levels but on the formation of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). Phosphoinositides are very labile and are rapidly broken down after death (Jolles et al., 1981b; Lin et al., 1990), which makes it difficult to study these

substances in brain material obtained hours after death. It was therefore decided to investigate the activity of the enzymes involved in the interconversion of the inositol phospholipids and not the concentration of the inositol phospholipids. PI kinase and PIP kinase were studied in a membrane-free supernatant of medial temporal cortex prepared from AD and control brains, with exogenous PI and PIP as lipid substrate. Care was taken to use brain material from rapid autopsies (between 4 and 6 hours after death). In the first experiment, we investigated the possible importance of the factor age: groups of AD patients and controls younger and older than 80 years at death were compared. This age was chosen in view of the finding of Rossor et al. (1984) that there are differences in the neurotransmitter systems of AD patients who were younger than 79 years when they died compared to patients who were older when they died. In a second experiment, we investigated the age of disease onset in patients older than 80 years, in view of increasing clinical evidence that the clinical course of AD is heterogeneous. Very old patients who develop the great symptoms at great age (e.g., above 75 years) are often characterized by depression and psychosocial changes. In this study we set the age of onset of first symptoms arbitrarily at 75 years and compared old AD patients with an onset before or after 75 years.

Experimental procedures

Subjects

Brain samples from patients with AD and non-demented controls were obtained from the Netherlands Brain Bank in Amsterdam. For the first experiment, 18 AD brains and 17 controls were used. Subgroups were composed of "aged" subjects, aged 80 or younger, and "very old" subjects, older than 80 years. Care was taken to include only patients in whom AD started when they were 75 or younger. The duration of illness did not differ between aged and very old AD subjects (see Table 1 for description). In all cases the interval between death and autopsy did not exceed 5 1/4 hrs: the mean post-mortem delay was 240 and 230 minutes for the aged and very old AD groups and 320 and 310 for the controls, respectively. The difference in post-mortem delay was not significant. In the second experiment, 15 AD patients aged 81-90 years were divided into two subgroups which differed with respect to age of onset (75 years and younger, or over 75) and compared with 6 control subjects aged 82-90 years. There was a statistically significant difference in the mean duration of illness between the two AD groups (14.3 ± 3.2 versus 8.3 ± 1.1 ; see Table 2 for description). The patients with AD were clinically diagnosed as "probable Alzheimer Disease" (McKahn et al., 1984; APA, 1987), and this was verified by post-mortem neuropathologic examination. Control subjects had no history of dementia or any other neurological or psychiatric disorder.

Brain dissection

Brain specimens for analysis of inositol phospholipid kinase activity were taken from the medial temporal gyrus in the right hemisphere. The leptomeninges were removed and samples were excised measuring approximately 1 cm³. These samples were sealed in plastic and rapidly frozen by immersion in liquid nitrogen. The frozen samples were stored at -80°C until use.

Preparation of crude enzyme fraction

Pieces of approximately 0.5 g were excised from the tissue samples and thawed in a water bath at 0°C (20 min). The tissue was homogenized in medium consisting of 0.32 M sucrose, 1mM EGTA, 50 mM Tris. HCl, (pH 7.4) in a total volume 10 times the brain tissue volume, by 12 up-and-down strokes (16 s each) of a Potter-Elvehjem Teflon-glass homogenizer (radial clearance, 0.125 mm; 700 rpm), followed by homogenization by hand in a glass-glass homogenizer with 3 up-and-down strokes (clearance of cylindrical section: 0.125 mm) in order to destroy synaptosomes and thus increase the yield of intrasynaptosomal enzymes. The homogenate was centrifuged for 60 min at 100,000xg, and the resulting membrane-free supernatant was used as the crude enzyme fraction. This fraction was frozen in 0.2 ml aliquots in liquid nitrogen for 5 s and stored at -80°C. There was no decline in enzyme activity after 1 month of storage.

PI kinase and PIP kinase assay

Inositol phospholipid kinase activity was measured, with some modifications, as described by Van Dongen et al. (1984) and Moritz et al. (1990). The incubation volume (normally 25 µl) was doubled in the case of the PIP kinase assay in order to reduce interassay variability. Supernatant fractions of 15 or 30 µl (10 and 20 µg of protein, respectively) were preincubated for 2 min. Lipid precursors (20 µM PI or 20 µM PIP; Sigma) were solubilized in 0.1% Triton X-100, 50 µM Tris-HCl, and 1mM EGTA (pH 7.4) and added to the incubation mixture 15 s before the phosphorylation reaction was started by addition of ATP. The reaction lasted 1 min. Incubations were performed under the following conditions: 7.5 µM ATP, 2-3 µCi [γ -³²P] ATP (approximately 3000 Ci/mmol, Amersham UK), 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1mM EGTA, and 0.02% Triton X-100. The reaction was terminated and the extraction and further analysis of the ³²P incorporated into PIP and PIP₂ were performed as described elsewhere (Bothmer et al., 1990; Jolles et al., 1981a). Protein content was determined according to the method of Lowry et al. (1951).

Results

In the first experiment, a comparison was made between aged AD patients (who were younger than 80 when they died) and very old AD patients (who were

older than 80 when they died), in whom the onset of disease occurred before 75 years, and age-matched controls.

Table 1 : Patient characteristics of study 1: EFFECT OF AGE.

	ALZHEIMER		CONTROL	
	Aged	Very old	Aged	Very old
N	10	8	11	6
Age mean (years)	70.4±2.4	83.2±0.8	68.1±2.2	86.0±1.4
Age range (years)	55 - 79	81 - 88	51 - 76	82 - 90
Male/female	7 M/3 F	0 M/8 F	8 M/3 F	3 M/3 F
Duration of illness (years)	10.4±1.9	14.4± 3.2	-	-
Brain weight (g)	1101±38	1010±25	1314±34	1188±48
Post mortem delay (min)	233±16	231±7	384±93	348±43
pH of the brain	6.6±0.2	6.6±0.1	6.5±0.1	6.6±0.2
Choline acetyltransferase (ChAT)	0.48±0.16	0.46±0.21	5.77±0.77	3.68±0.47

The pH of the brain, as a measure of post-mortem delay, did not differ between any of the groups. However, the groups were different with respect to the factor brain weight (see Table 1). The brains of AD patients weighed less than those of controls ($p<0.001$; Student-Newman-Keuls test). In addition, there was an effect of the factor 'age': the brains of AD patients and control subjects older than 80 weighed less than the brains of subjects younger than 80 ($p<0.01$). Remarkably, the mean brain weight of very old controls (1188 g) was not different from that of aged AD patients (1102 g). Choline acetyltransferase (ChAT) activity was reduced by 90 % in both aged and very old AD brains. Interestingly, the older controls were characterized by a significant decrease in ChAT activity compared to the younger controls.

Data on PI kinase activity were analyzed with a two-way ANOVA with factors group and age. A group effect was found and an interaction age x group. Thus, as shown in Fig. 1, there was a reduction in PI kinase activity in aged AD patients compared to aged controls ($p<0.05$). This group effect was absent in very old controls because of the reduction in PI kinase activity in very old controls compared to aged controls. A regression analysis of PI kinase activity versus age (Fig. 2) indicated that PI kinase activity decreased with age ($P<0.05$). Furthermore, in agreement with findings presented elsewhere (Jolles et al., 1992), AD patients and controls had similar PIP kinase activity (results not shown). In addition, age did not affect PIP kinase activity in either AD patients or controls.

Fig. 1 : PI kinase activity in cytosolic fractions from brain cortex of controls and AD patients with onset ≤ 75 years. Data were analyzed with a two-way ANOVA with factors group and age. * : $p < 0.05$.

Fig. 2 : Effects of age on PI kinase activity in cytosolic fractions of control brain cortex. Data were analyzed with regression analysis.

The second experiment was devised to analyze the possible influence of the factor 'onset'. Very old AD patients and controls older than 80 were compared (see Table 2). As Table 3 shows, PI kinase activity in AD patients in whom the onset of first symptoms occurred before and after 75 years were significantly different. Very old AD patients in whom AD started when they were older than 75 had increased PI kinase activity compared to AD patients with an earlier onset and controls. Remarkably, the PI kinase activity in this group was even higher than in the younger controls measured in experiment 1 (20% increase, $p < 0.01$). The incorporation of ^{32}P into PIP_2 was similar in patients in whom the onset of AD occurred after 75 years and in patients with an earlier onset (results not shown).

Table 2 : Patient characteristics of study 2: EFFECT OF ONSET

	ALZHEIMER		CONTROL
	ONSET $\leq$ 75	ONSET $>$ 76	
N	8	7	6
Age mean (years)	83.3 $\pm$ 0.8	88.3 $\pm$ 0.7	86.0 $\pm$ 1.4
Age range (years)	81 - 88	85 - 90	82 - 90
Male/female	0 M/8 F	0 M/7 F	3 M/3 F
Duration of illness (years)	14.4 $\pm$ 3.2	8.3 $\pm$ 1.1	-
Brain weight (g)	1010 $\pm$ 26	961 $\pm$ 27	1188 $\pm$ 48
Post mortem delay (min)	231 $\pm$ 7	221 $\pm$ 16	347 $\pm$ 43
pH of the brain	6.6 $\pm$ 0.1	6.4 $\pm$ 0.1	6.6 $\pm$ 0.2
Choline acetyltransferase	0.46 $\pm$ 0.21	1.01 $\pm$ 0.32	3.68 $\pm$ 0.47

Discussion

Recently, cytosolic PI kinase fractions obtained from neocortical structures taken from brains of patients with AD were found to have approximately 50% less activity than similar fractions obtained from control brains. No such differences were detected in PIP kinase activity (Jolles et al., 1992). The fractions were prepared from patients with early onset AD who died at a relatively early age (below 72 years). The present series of investigations confirms this finding for a larger group of subjects, some of whom lived to be 80. Interestingly, very old controls (older than 80, with a mean age of 85 years) had a reduced PI kinase

Table 3 : PI kinase activity in old patients: EFFECT OF ONSET

group	N	PI kinase activity (pmol/min.mg protein)
Onset $\leq$ 75 years	8	3.719 $\pm$ 0.200
Onset $>$ 76 years	7	5.523 $\pm$ 0.484
Control	6	3.125 $\pm$ 0.314

activity compared to younger controls (younger than 80, with a mean age of 67.3 years), and evidence for an age gradient in PI kinase activity was found. The decreased activity in old controls could explain the lack of difference between old controls and very old AD patients (older than 80) with onset before 75 years in the present series of investigations. The factor 'onset' -which is defined as 'age of first symptoms'- was found to be important in the second experiment in which old AD subjects with a relatively long duration of disease (mean duration 14.4 years, onset before 75) were compared with AD patients with a short duration of disease (mean duration 8.3 years; onset after 75). The results clearly show that the latter group is characterized by high PI kinase activity.

The present findings are important for both methodological and theoretical considerations. Age-related decreases in myo-inositol and inositol bound to phosphatidylinositol have been found in normal subjects aged 20-70 (Stokes et al., 1983). An age-related decrease in PI kinase activity, found in the present study, has relevance for AD research, as the lack of brain material from well-defined AD patients forces the researcher to use groups which are composed of AD patients of widely different ages (e.g., 60 through 90 years; Stokes and Hawthorne, 1987). The present data suggest that care should be taken in the selection of patient groups. The use of control and patient groups that are more homogeneous with respect to age is to be recommended in view of the age-related decline in enzyme activity.

Old AD subjects with an onset before and after 75 years had different PI kinase activity, which could be important in view of the fact that the late onset group showed an increased formation of ^{32}P -PIP compared to the early onset group, whereas the late onset group was older and thus -in view of the findings of the first study- would be expected to have a decreased formation of PIP. In addition, old AD patients with a short disease duration (onset after 75 years) had a much higher PI kinase activity than subjects with a longer disease duration. In conclusion, the present data suggest that the pathogenesis of AD in old AD patients with a short duration of disease might be different, as far as PI kinase is concerned, from that of old patients with a longer duration of disease. This is of relevance in view of the ongoing discussion on the possible heterogeneity of AD.

Until now, the question whether or not early and late onset AD differ has not been settled. An increasing number of studies favor such a distinction in order to account for clinical heterogeneity and different syndromes which are observed in early and late onset Alzheimer dementia (Chui et al., 1985; Filley et al., 1986; Mayeux et al., 1985). Likewise, there is increasing evidence that early and late onset AD also differ with respect to neurobiological parameters (Bondareff et al., 1987; 1988). Even age at death appears to be a relevant variable in the differentiation of AD patients in view of the findings reported by Rossor et al. (1984). These authors found clearcut differences in neurotransmitter systems in AD patients younger than 79 years versus AD patients dying at an older age. The present findings corroborate these observations for another enzyme system. In addition to age at death, age at onset of first symptoms also appeared to be relevant, which suggests that it might be necessary to reevaluate existing data in

order to find whether our findings on the effect of onset are paralleled by similar early/late discrepancies found in other neurobiological or neurochemical parameters.

Both the present results and those presented by Stokes and coworkers (Stokes et al., 1983; Stokes and Hawthorne, 1987) suggest that the inositol phospholipid system may be involved in the pathogenesis of AD. Stokes and Hawthorne (1987) measured absolute levels of myo-inositol and lipid-bound inositol and found a significant decrease in PI in the temporal cortex from AD brain. In addition, an age gradient for myo-inositol and lipid-bound inositol was found, although there was a large individual variation. Several other investigators have reported the involvement of membrane phospholipids and their metabolites in Alzheimer's disease, for instance, by ^{31}P NMR spectroscopic examination of AD brains. Elevated levels of phosphomonoesters such as phosphocholine early in the course of the disease have been reported, followed by elevation of phosphodiesteres such as glycerol-3 phosphocholine (Barany et al., 1985; Blusztajn et al., 1990; Brown et al., 1989; Miatto et al., 1986; Pettegrew et al., 1989). In addition, AD brain appears to be characterized by a decrease in phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol (Pettegrew et al., 1989). It remains to be established whether there is a relation between these changes and the findings reported in the present paper.

The changes in PI kinase activity seem to be relevant because of the large effect found, especially in young AD patients (Jolles et al., 1992) and because of the key role played by inositol phospholipids in impulse initiation and propagation, the most intrinsic neuronal and brain functions (Abdel-Latif, 1986; Berridge, 1987). A large decrease in PIP formation can therefore be expected to have a widespread influence on membrane function. PIP₂ hydrolysis could be blocked, and formation of the second messengers inositol phosphate and diacylglycerol inhibited. This in turn can be expected to give rise to a number of tertiary changes in intracellular mechanisms and lead to neuronal deactivation and possibly degeneration.

With respect to the biochemical mechanisms involved in the reduced incorporation of ^{32}P into PIP with age and in AD patients, changes in the absolute amount of enzymes or changes in enzyme kinetics are possible. Alternatively, the presence or absence of particular ions or other cofactors could also be causal to the decreased incorporation. Follow-up research into the various possibilities is presently being undertaken. In addition, uncertainty exists about the nature of the PI kinase involved. It is known that this enzyme exists in two forms, namely the kinase which phosphorylates PI at the 4 position of the inositol ring, and the one which phosphorylates at the 3 position. This difference is of importance because the two kinases appear to have different functions in the cell. The PI 4-kinase is involved in the pathways leading to hormone stimulated phospholipase C action, whereas the PI 3-kinase is suggested to have a function in mitogenesis, cell differentiation, and in the control of cytoskeletal rearrangements (Carpenter and Cantley, 1990; Downes and MacPhee, 1990). Various isoenzymes of PI 4-kinase exist, which are located either in the membrane as an

integral membrane protein (e.g. Porter et al., 1988), or in the cytoplasm, or both (Carpenter and Cantley, 1990).

In conclusion, the alterations in inositol phospholipid phosphorylation found in the present study may be relevant for AD research because of the key role played by inositol phospholipids in neural functioning. In addition, the results indicating an age related effect in AD patients suggest that PI kinase activity can be used to divide AD patients into subgroups characterized by the age of onset of the disease. More attention should be given to the factors age and onset in future AD research.

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CHAPTER 9

EVIDENCE FOR A SELECTIVE DECREASE IN TYPE 1 PHOSPHATIDYLINOSITOL KINASE ACTIVITY IN BRAINS OF PATIENTS WITH ALZHEIMER'S DISEASE.

J. Bothmer, M. Markerink and J. Jolles

Abstract

We have previously shown that phosphatidylinositol (PI) kinase activity is 40-50% lower in cytosolic fractions of neocortical regions from brains of patients with Alzheimer's disease (AD) than in similar fractions prepared from control brains. To investigate whether changes in the amount of inhibiting or stimulating compounds are involved, we performed chelation and combination experiments with cytosolic preparations of temporal cortex from AD brains and control brains. Chelation of ions did not increase PI kinase activity. Combination of AD and control cytosolic fractions did not result in stimulation or inhibition of PI kinase activity. These results suggest that no inhibiting or stimulating compounds are involved in the mechanism underlying the lower PI kinase activity in AD brains. The PI kinase activity in the cytosolic fraction and in the loosely membrane-bound protein preparation of the temporal cortex of AD brains was predominantly affected (70% lower PI kinase activity compared to control brain samples). PI kinase in these fractions was inhibited (-75%) by 1% Triton X-100, whereas the PI kinase in the tightly membrane-bound protein preparation was stimulated (+80%) by 1% Triton X-100. PI kinase activity in the loosely membrane-bound protein preparation was almost unaffected by adenosine in contrast to PI kinase activity in the tightly membrane-bound protein preparation, which was strongly inhibited by adenosine. These results indicate that the PI kinase that is specifically affected in AD is the PI 3-kinase, or type 1 PI kinase, because 1.) the cytosolic localization; 2.) the inhibition by the non-ionic detergent Triton X-100; and 3.) the insensitivity to adenosine inhibition are characteristic features of type 1 PI kinase. The specificity of the effect on PI kinase is strengthened by the observation that PIP kinase was not affected in any of the subcellular fractions of the temporal cortex of AD brains. The relevance of our findings namely that type 1 PI kinase seems to be specifically affected in AD brains is that this type of PI kinase is thought to be involved in the regulation of cytoskeletal turnover processes. In this context, changes in the type 1 PI kinase activity could be related to the cellular pathology of AD because neurofibrillary tangles, which are a prominent feature of AD, are partially composed of wrongly phosphorylated cytoskeletal components.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the occurrence of protein deposits in brain neurons, the neurofibrillary tangles, as well as in the extracellular space, the neuritic plaques (Holtzman and Mobley, 1991). Although these features are always found in AD they are not unique to AD. AD is also characterized by changes in the metabolism of phospholipids. The metabolism of phosphatidylcholine is altered in AD brain (Miatto et al., 1986; Pettegrew, 1989), as is the amount of the M_2 cholinergic muscarinic receptor (Bird et al., 1985) coupled to phosphoinositide specific phospholipase C (Berridge and Irvine, 1984). The concentration of phosphoinositides, a class of phospholipids enriched in neuronal membranes, is lower in AD brains than in brains from age-matched controls (Stokes and Hawthorne, 1987). Furthermore, aluminum, which probably plays a role in AD (MacDonald and Martin, 1987), influences the interconversion of phosphoinositides in a very specific way (MacDonald and Mamrack, 1988). Jolles et al. (1991, 1992) have shown that cytosolic fractions

prepared from neocortical areas of AD brains have about 50% less PI kinase activity than similar fractions from control brains. PIP kinase activity was the same in AD and control brains. The importance of these findings is suggested by the fact that phosphoinositides are involved in impulse initiation and propagation, calcium mobilization, agonist-receptor interactions, and the formation of the intracellular second messengers, diacylglycerol and inositol trisphosphate (Abdel-Latif, 1986; Berridge, 1987; Downes and MacPhee, 1990). Furthermore, PI kinase seems to be involved in cell proliferation (Martelli et al., 1991), and in the cytoskeletal rearrangement that occurs during the mitotic cell cycle and in post-mitotic cells during exocytosis (Downes and MacPhee, 1990). Interestingly, a prominent feature of AD brains is the abnormal accumulation of neurofibrillary tangles which are partially composed of multi-phosphorylated microtubule-associated proteins (Hanger et al., 1991; Kosik, 1991). Under physiological conditions these proteins stabilize the cytoskeleton (Kosik, 1991).

Different types of PI kinases have been discovered and are probably responsible for the different functions in which PI kinases seem to be involved (Carpenter and Cantley, 1990). There are two PI kinases, type 1 or PI 3-kinase and type 2 or PI 4-kinase (Whitman et al., 1987), which differ from each other in the phosphorylation position of the inositol ring of their products. Thus, PI 3-kinase phosphorylates at position D-3 and PI 4-kinase at position D-4 of the inositol ring (Whitman et al., 1988). The PI 4-kinases can be further divided into type 2 and type 3 PI kinase, which differ from each other in size (55 and 230 kDa, respectively) and in some kinetic properties (Endemann et al., 1987). Type 1 PI kinase, or PI 3-kinase, is not implicated in the pathway producing the second messenger precursor $PI(4,5)P_2$, unlike type 2 PI kinase which is (Downes and MacPhee, 1990). Type 1 PI kinase, which is located in the cytosolic compartment, is implicated in growth, mitogenic signalling, and cytoskeletal turnover, and appears to be associated with protein tyrosine kinases and growth factor receptors (Carpenter and Cantley, 1990; Cochet et al., 1991; Martelli et al., 1991; Varticovski et al., 1989). Type 1 PI kinase associates with receptors and is activated by stimulation of these receptors by agonists (e.g., insulin-receptors, platelet-derived-growth-factor receptor; Auger and Cantley, 1991).

PI and PIP kinase activities have been studied in a cytosolic fraction from AD brains (Jolles et al., 1991, 1992). However, nothing is known about PI and PIP kinase activities in the membranes of AD brains. In fact, the identity of the PI kinase measured earlier (Jolles et al., 1991, 1992) is not clear, and we do not know what biochemical mechanisms underlie the reduced incorporation of ^{32}P i into PIP. The present study was therefore, designed to provide more information about the identity of the type of PI kinase affected in AD, and on the biochemical mechanism underlying the lower level of PI kinase activity in AD brains, as PIP kinase is not affected. We therefore prepared four subcellular fractions from AD and control brains in order to measure both cytosolic and membrane-bound PI kinases. We also investigated whether changes in the amount of inhibiting (e.g., metal) or stimulating (e.g., cofactor) compounds are involved in the low PI kinase activity of AD brains.

Experimental procedures

Subjects

Brain samples from five AD patients (three males and two females; mean age 65.2 years) and five controls (three males and two females; mean age 69 years) were used in this study. The brains were individually matched for age and postmortem interval with brain tissue obtained from the Netherlands Brain Bank. The mean postmortem interval was 5 h for the AD patients and 5 h 15 min for the controls. The AD patients had been clinically diagnosed as "probable Alzheimer's Disease" and this was verified by postmortem neuropathological examination. Controls were age-matched individuals without a history of dementia, or any other neurological or psychiatric disorder.

Brain dissection

Brain specimens for analysis of inositol phospholipid kinase activity were obtained from the medial temporal gyrus. The leptomeninges were removed and samples were excised. These samples were sealed in plastic, rapidly frozen by immersion in liquid nitrogen, and stored at -80°C until use.

Preparation of crude enzyme fraction

Pieces of approximately 0.5 g were excised from the tissue samples and rapidly thawed in a water bath at 0°C (20 min). The tissue was homogenized in a medium consisting of 0.32 M sucrose, 1 mM EGTA, 50 mM Tris. HCl, pH 7.4, in a total volume 10 times the brain tissue volume, by 12 up-and-down strokes of a Potter-Elvehjem Teflon-glass homogenizer (radial clearance: 0.125 mm, 700 rpm), followed by homogenization by hand in a glass-glass homogenizer with 3 up-and-down strokes (radial clearance: 0.125 mm). The homogenate was centrifuged for 60 min at 100,000xg and the resulting membrane-free supernatant was used as the crude enzyme fraction. This fraction was stored at -80°C. There was no decline in enzyme activity after 1 month of storage.

Solubilization of membrane-bound PI and PIP kinase

Solubilization of PI and PIP kinases, loosely attached to the membrane or membrane bound, was performed according to Yamakawa and Takenawa (1988). Briefly, tissue was homogenized (1:10, wet w/v) in homogenization buffer (20 mM Tris-HCl, 1 mM EGTA, 1 mM DTT, pH 7.4) by 12 up-and-down strokes of a Potter-Elvehjem Teflon glass homogenizer (radial clearance of 0.125 mm, 700 rpm), followed by homogenization in a glass-glass homogenizer with 3 up-and-down strokes (clearance 0.125 mm). The homogenate was centrifuged (100,000xg,

4°C, 60 min), and the resulting membrane-free supernatant was taken as the "cytosolic fraction". The pellet was homogenized as described above in homogenization buffer containing 1 M NaCl, and the homogenate was stirred for 60 min at 4°C. After centrifugation (100,000xg, 4°C, 60 min), the supernatant was taken as the "salt fraction". The pellet was homogenized as described above in homogenization buffer containing 1% Triton X-100 (w/v), followed by sonication on ice for 3 x 10 s at maximal power. The homogenate was stirred for 60 min at 4°C and centrifuged (100,000xg, 4°C, 60 min). The supernatant was taken as the "Triton fraction", and the pellet, after homogenization in homogenization buffer as described above, as the "residual fraction".

PI kinase and PIP kinase assay

Inositol phospholipid kinase activity was measured as described earlier (van Dongen et al., 1986), with some modifications. The incubation volume (normally 25 µl) of the PIP kinase assay was doubled to reduce interassay variability. Before the start of the kinase assay, the "salt" and "Triton" fractions (100µl) were dialyzed for 2 h against 10 ml 20 mM Tris-HCl/1 mM EGTA/1mM DTT (pH 7.4). Protein solutions of 15 or 30 µl (10, 20 µg protein, respectively) were preincubated for 2 min. Lipid precursors (20 µM PI or 20 µM PIP (Sigma), solubilized in 0.1% Triton X-100/20 mM Tris-HCl / 1 mM EGTA/1 mM DTT (pH 7.4), were added 15 s prior to phosphorylation, which was started by the addition of ATP, and lasted 1 min. Incubations were performed under the following conditions: 7.5 µM ATP, 2-3 µCi (gamma-³²P) ATP (approximately 3000 Ci/mmol, Amersham UK) 20 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, and 0.02% Triton X-100. The reaction was terminated, and the extraction and further analysis of the ³²P incorporated into PIP and PIP₂ were performed as described elsewhere (Bothmer et al., 1990; Jolles et al., 1981). Protein determination was performed according to the method of Lowry et al. (1951).

Results

As shown previously (Jolles et al., 1991, 1992), PI kinase activity in cytosolic fractions of AD brains was lower than that of age-matched controls. In the first series of experiments we investigated whether the lower PI kinase activity in cytosolic fractions of AD brains is due to the presence of metal ions as modulating cofactors. Cytosolic fractions of AD and control tissue were treated with chelating agents. Chelation of the cytosolic fraction with EDTA and EGTA (both 5 mM) under standard conditions appeared to have had a small stimulating effect on PI kinase activity in both AD and control preparations (Fig. 1). However, this effect was accounted for by a small loss of protein, probably without loss of PI kinase, during dialysis after the chelation. Total PI kinase activity did not increase after chelation (results not shown).

Fig. 1 : Effect of chelation (5 mM EDTA and 5 mM EGTA) on PI kinase activity (pmol/mg protein) in crude cytosolic preparations of AD and control brains. Values shown are means ($\pm$ SEM) of triplicate determinations from one of two experiments that gave similar results.

Fig. 2 : Effect of combination of crude cytosolic preparations of AD and control brains on PI kinase activity (% of control brain value). C : cytosolic preparation from control brains, AD : cytosolic preparation from AD brains, C+AD : combined cytosolic preparation of control and AD cytosolic fractions, C+AD* : combined cytosolic preparation of control and denaturated (10 min, 100°C) AD cytosolic fractions. Values shown are means ($\pm$ SEM) of triplicate determinations from one of two experiments that gave similar results.

In order to investigate whether other (non-metal) cofactors are involved in the lower PI kinase activity in cytosolic fractions from AD brains, we assayed PI kinase activity in combined AD and control fractions. PI kinase activity was neither stimulated nor inhibited (Fig. 2): PI kinase activity in the combined preparation (C+AD) was equal to the sum of activities of the individual preparations (C and AD). Denaturation (10 min at 100 °C) of the cytosolic fraction from AD brains also had no effect on PI kinase activity in the combined preparation (C+AD*) compared with control (C).

The following experiments were designed to provide more information about the membrane-bound PI and PIP kinases. Solubilization experiments (Fig. 3)

Fig. 3 : PI kinase activity (pmol/mg protein), PIP kinase activity (pmol/mg protein) and protein content (mg/ml) of four subcellular fractions from AD and control brains. I : cytosolic protein preparation, II : 1 M NaCl-solubilized protein preparation, III : 1% Triton X-100-solubilized protein preparation, IV : residual protein preparation. Values shown are means ($\pm$ SEM) of five control subjects and five AD subjects. Statistical analysis was performed according to Student's t-test (two-tailed). * : $p < 0.05$, ** : $p < 0.005$.

showed that PI kinase activity (Fig. 3a) was located in the membrane-bound (=Triton) fraction as well as in the soluble fraction (cytosolic and salt fraction). Total PI kinase activity was located for 10% in the soluble fraction and for 90% in the insoluble fraction (Triton and residual fraction). PIP kinase activity (Fig. 3b) was located primarily in the soluble fraction (cytosolic and salt fraction). Total PIP kinase activity was located for 60% in the soluble fraction and for 40% in the insoluble fraction. PI kinase activity in AD brains was decreased in the cytosolic fraction as well as in the other fractions tested ("salt", "Triton", and "residual") (Fig. 3a). However, the cytosolic and salt fractions showed a 70% decrease in PI kinase activity and the Triton and residual fraction only a 25% decrease in PI kinase activity. PIP kinase activity in AD brains was not different from that of control brains in any of the fractions tested (Fig. 3b). The cytosolic fraction of AD brains contained less protein and the residual fraction more protein than similar fractions prepared from control brains (Fig. 3c).

The two types of PI kinases (type 1 and 2, or PI 3-kinase and PI 4-kinase) described by Whitman et al. (1987) have a different subcellular distribution and are affected differently by the non-ionic detergent Triton X-100 and the potentially inhibiting component adenosine. In order to investigate which type of PI kinase

Fig. 4 : Effect of 1% Triton X-100 on PI kinase activity (expressed as % of control) in the 1 M NaCl-solubilized protein preparation (salt fraction) and the 1% Triton X-100-solubilized protein preparation (Triton fraction) of control brains. Triton X-100, present in the Triton-solubilized protein preparation, was first removed with detergent-removing gel. Values shown are means ($\pm$ SEM) of triplicate determinations from one of two experiments that gave similar results.

Fig. 5 : Effect of adenosine on PI kinase activity (expressed as % of control) in the 1 M NaCl-solubilized protein preparation (salt fraction) and the 1% Triton X-100-solubilized protein preparation (Triton fraction) of control brains. Values shown are means ($\pm$ SEM) of triplicate determinations from one of two experiments that gave similar results.

was the most abundant in the different solubilized fractions, we measured the effect of 1% Triton X-100 on PI kinase activity in the salt fraction and in the Triton fraction of control brains. Triton X-100, present in the Triton-solubilized fraction, was first removed with a detergent-removing gel (Extracti-Gel D, Pierce). PI kinase activity was inhibited in the salt fraction (-75%) and stimulated in the Triton fraction (+80%) after addition of 1% (w/v) Triton X-100 (Fig. 4). Furthermore, adenosine appeared to inhibit PI kinase activity in the Triton fraction whereas PI kinase activity in the salt fraction was rather insensitive to adenosine (Fig. 5).

Discussion

In this paper we investigated the biochemical mechanism underlying the

lower levels of PI kinase activity in cytosolic fractions from AD brains than in age-matched control brains. We explored what type of PI kinase is affected in AD, and whether inhibiting or stimulating compounds are involved in the lower PI kinase activity found in AD brains.

Jolles et al. (1991, 1992) have shown a 50% decrease in PI kinase activity in cytosolic fractions of four neocortical regions from AD brains compared with control brains. Our results suggest that this difference is not caused by increased levels of inhibitory (e.g., metal ion) or decreased levels of stimulatory (e.g., cofactor) compounds. Potentially inhibitory metal ions were chelated (Fig. 1) in the first experiment, but PI kinase activity was not changed in AD and control preparations. An inhibitory compound in the cytosolic fractions of AD brains would have been expected to decrease PI kinase activity in a combined preparation to below the level of the sum of both cytosolic fractions (control and AD) assayed individually. No difference was observed (Fig. 2). If levels of a stimulatory compound were decreased in AD brains and normal in control brain, we would have expected an increase in PI kinase activity in combined preparations compared with the sum of individual activities. We observed no such increase. Our results suggest that the AD brain samples are characterized by changes in PI kinase itself or by changes in the amount of PI kinase, and probably not by changes in the level of other compounds that influence PI kinase activity.

Different types of PI kinases have been discovered recently, which differ from each other in molecular weight, enzyme kinetic properties, probable physiologic function, and subcellular localization (Carpenter and Cantley, 1990). In order to make a crude separation between these types of PI kinases, we prepared four subcellular fractions: the "cytosolic" fraction, the "salt" fraction (proteins loosely attached to the membrane), the "Triton" fraction (membrane-bound proteins), and the "residual" fraction. The decrease in PI kinase activity (Fig. 3a) was markedly greater in the cytosolic and salt fractions than in the Triton and residual fractions of AD brains.

These differences between the four fractions in control brains and the differences between control and AD brains are relevant in view of the findings of Whitman et al. (1987). These authors found two distinct PI kinases in fibroblasts. Type 1 PI kinase appeared to be a PI 3-kinase and type 2 PI kinase a PI 4-kinase (Whitman et al., 1988). The latter is located primarily in the membranes of bovine brain myelin (Saltiel et al., 1987), human platelets (Kano et al., 1990), and human cell cultures (Walker et al., 1988), whereas type 1 PI kinase is located primarily in the cytosol (Carpenter et al., 1990; Shibasaki et al., 1991). However, a significant amount of type 1 PI kinase appears to be associated with the membrane in growth factor-stimulated and/or oncogene-transformed fibroblasts (Auger and Cantley, 1991; Cohen et al., 1990a,b). Thus the PI kinase activity in the cytosolic and salt fractions, which was affected to a greater extent than in the Triton and residual fractions in AD preparations, is probably predominantly due to a type 1 or PI 3-kinase, whereas the PI kinase activity in the Triton and residual fractions is probably due to a PI 4-kinase, with some PI 3-kinase also being present. The observation that the PI kinase activity in the salt fraction was

inhibited by 1% Triton X-100 and the PI kinase activity in the Triton fraction was stimulated by this detergent (Fig. 4) also points in this direction because Whitman et al. (1988) showed that type 1 PI kinase is inhibited by Triton X-100 in contrast to type 2 and 3 PI kinase, which are stimulated by Triton X-100. Furthermore, adenosine, which is a potent inhibitor of type 2 PI 4-kinase at concentrations where type 1 PI 3-kinase remains unaffected (Carpenter and Cantley, 1990), inhibited the PI kinase activity in the Triton fraction whereas the PI kinase activity of the salt fraction was rather insensitive to adenosine inhibition. Thus, the soluble fractions (cytosolic and salt), which were affected the most in AD brains, are probably enriched in one specific type of PI kinase, namely type 1 or PI 3-kinase.

This finding is important in view of the different physiologic functions of the different PI kinases. Type 2 and probably also type 3 PI kinase are involved in the pathways leading to hormone-stimulated phospholipase C activity, which results in the release of the second messengers inositol trisphosphate and diacylglycerol (Carpenter and Cantley, 1990). Type 1 PI kinase, however, is suggested to function in mitogenesis and cytoskeletal rearrangements as this enzyme associates with tyrosine kinases and growth factor receptors (Auger and Cantley, 1991; Carpenter and Cantley, 1990; Martelli et al., 1991; Varticovski et al., 1989). A lack of association with tyrosine kinases and growth factor receptors of type 1 PI kinase correlates with a lack of actin reorganization and the mitogenic signal (Auger and Cantley, 1991). Thus the relationship between type 1 PI kinase and the turnover of the cytoskeleton could have implications for the cellular pathology of AD because this disorder is characterized by the occurrence of neurofibrillary tangles (Holtzman and Mobley, 1991). These tangles are protein deposits inside the neurons, and are partially composed of abnormally phosphorylated microtubule-associated proteins (Hanger et al., 1991; Kosik, 1991). Under physiologic conditions microtubule-associated proteins stabilize the cytoskeleton (Kosik, 1991).

The specificity of the effect on PI kinase is suggested by the lack of a difference between PIP kinase activity in control and AD preparations (Fig. 3b). Jolles et al. (1991, 1992) also found no differences in PIP kinase activity in cytosolic fractions from AD and control brains. The fact that PIP kinase, which like type 2 PI kinase is implicated in the pathways leading to hormone-stimulated phosphoinositide specific PLC activity, is not affected in AD brains suggests that this physiologic function of PI kinase is not affected in AD brains. The highest PIP kinase activity was measured in the cytosolic and salt fractions (Fig. 3b). Previous studies have revealed a cytosolic PIP kinase and a loosely membrane-bound PIP kinase in human erythrocytes (Bazenet et al., 1990; Ling et al., 1989) and bovine brain (Moritz et al., 1990).

Protein deposits in the brain are a prominent characteristic of AD. Our results are of interest in this respect because there appeared to be a shift of protein from the cytosolic fraction to the residual fraction (Fig. 3c) in AD brains compared with control brains. This shift probably represents a shift of protein from the soluble fraction to the insoluble fraction in AD brains, which would be consistent with

the cellular pathology of AD.

In conclusion, we have shown that the lower PI kinase activity in cytosolic fractions from AD brains is not caused by increased levels of an inhibitory compound or decreased levels of a stimulatory compound in the cytosol of AD brains. Furthermore, our results suggest that just one type of PI kinase is affected in AD brains, namely type 1 PI kinase. Interestingly, type 1 PI kinase seems to be involved in the turnover of the cytoskeleton, which is closely related to the cellular pathology of AD. PIP kinase, which is implicated in hormone-stimulated phosphoinositide specific phospholipase C activity and results in the release of the second messengers inositol trisphosphate and diacylglycerol, is not affected in AD brains. The present data provide new avenues for investigating the biochemical mechanism underlying the well-known cellular pathology of Alzheimer's disease.

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CHAPTER 10

PLATELET PHOSPHOINOSITIDE KINASE ACTIVITY IS NOT ALTERED IN ALZHEIMER'S DISEASE.

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Abstract

We previously reported a specific decline in phosphatidylinositol (PI) kinase activity in the neocortex of patients with Alzheimer's disease (AD) as compared to controls, whereas phosphatidylinositol phosphate (PIP) kinase activity appeared not to be affected (Jolles et al. (1992) *J.Neurochem.* 58, 2326-2329). In search for a possible systemic effect of Alzheimer's disease, in the present study we investigated phosphoinositide kinase activity in platelets from patients with AD and from control subjects. The study was based upon the notion that disease-specific abnormalities in the brain could be reflected in blood platelets. PI kinase activity was studied in platelet homogenates and in a salt-solubilized protein fraction of platelets, because of the difference in subcellular localization of the different types of PI kinases. In addition, NADH cytochrome-C reductase was measured in platelet homogenates as a marker for the endoplasmic reticulum, to detect a possible proliferation of the endoplasmic reticulum. AD patients and normal elderly controls showed no difference in PI kinase activity in either enzyme fraction. Furthermore, NADH cytochrome-C reductase activity and the protein / phospholipid ratio per 10^6 platelets were the same for AD patients and controls. This was taken as an indication that platelets in AD patients do not show proliferation of intracellular membranes.

Introduction

Alzheimer's disease is a neurodegenerative disorder characterized by large numbers of neurofibrillary tangles and neuritic plaques in the central nervous system (CNS) (Selkoe, 1991). The neuritic plaques are composed of amyloid or β /A4 protein, which is produced by abnormal proteolytic cleavage of a cell-surface protein, the amyloid precursor protein (APP; Selkoe, 1991). Studies on the expression of APP have shown that it is not restricted to the brain, but that it is almost ubiquitously present (Bush et al., 1991). The potentially amyloidogenic APP is, for example, present in human platelets (Bush et al., 1990) which also show an abnormal membrane fluidity in AD (Zubenko et al., 1987a,b), probably caused by the proliferation of internal membranes (Hajimohammadreza et al., 1990; Piletz et al., 1991; Zubenko et al., 1987a). Furthermore, abnormalities of the function and morphology of platelets in Parkinson's disease, Huntington's disease and depression indicate that disease-specific abnormalities in the brain could be reflected in human platelets (Bush et al., 1991).

The metabolism of phosphoinositides, a class of phospholipids enriched in neuronal membranes, is also affected in the CNS of AD patients. The concentration of phosphoinositides is lower in AD brains than in brains from controls (Stokes and Hawthorne, 1987). Furthermore, Jolles et al. (1992) reported a substantial decline in PI kinase activity in AD brains whereas PIP kinase activity was not affected. If the 50% lower PI kinase activity found in AD brains were also reflected in AD platelets, platelets could be used as a diagnostic tool. With respect to the type of PI kinase involved in the decrease in PI kinase activity in AD brain, there is some evidence that type 1 PI kinase or PI 3-kinase is specifically affected (Bothmer et al., submitted). This is also a reason why platelets were chosen in

this study and not erythrocytes, because platelets contain a complete phosphoinositide metabolizing system with both PI 4-kinase (Kano et al., 1990) and PI 3-kinase (Cunningham et al., 1990; Huang et al., 1991), in contrast to human erythrocytes in which PI 3-kinase has not been identified (Wetzker et al., 1991). These authors purified PI 4-kinase to near homogeneity from human erythrocytes, but found no evidence for any PI 3-kinase activity. The AD patients in the present study had an "age of first symptoms" below 75 years, which is important because AD patients with an "age of first symptoms" above 75 years did not differ from age-matched controls with respect to brain cytosolic PI kinase activity (Jolles et al., in press). Because disease-specific abnormalities in the brain can be reflected in platelets, and because platelets have a complete phosphoinositide metabolism with both PI 4-kinase and PI 3-kinase we compared PI kinase activity in platelets from AD patients with that of platelets from age-matched controls. We also measured NADH cytochrome-C reductase as a marker for the endoplasmic reticulum (Sun et al., 1988).

Experimental procedures

Patient selection

The subjects were five patients (three males and two females) with Alzheimer's disease and five normal elderly controls (three males and two females). Prior to entry in this study, all subjects underwent thorough medical, neurological and psychiatric examinations. The psychogeriatric scales for determination of the severity of dementia were Global Deterioration Scale, Blessed Dementia Scale, Mini Mental State Exam, Haschinski ischemic score and Hamilton depression scale (Verhey et al., 1992). All patients met ADRDA-NINCDS criteria for "probable Alzheimer's Disease" (McKhann et al., 1984). AD patients (mean age 69.2 years) and controls (mean age 70.6 years) were individually matched for age and sex. The mean age of first symptoms in the AD patient group was 63.6 years with a maximum of 72 years.

Preparation of platelet homogenates and cytosolic fractions

Blood was drawn into a tube containing 2 ml ACD (80 mM tri-Na-citrate, 52 mM citric acid and 183 mM glucose, pH 4.5) and centrifuged at 250 $\times$ g for 15 min (room temperature). The resulting platelet-rich plasma (4 ml) was added to 200 μ l ACD and centrifuged at 850 $\times$ g for 15 min (room temperature). The platelet pellet thus obtained was resuspended in 1 ml Hepes buffer (10 mM, pH 6.6) containing 5 mg/ml bovine serum albumin (BSA). The volume was adjusted to 14 ml with the same buffer, 1 ml ACD was added and the suspension was centrifuged for 15 min at 850 $\times$ g. The supernatant was discarded and the pellet was resuspended in 1 ml Hepes buffer (10 mM, pH 6.6) without BSA. After centrifugation at 850 $\times$ g for 15 min, the pellet was resuspended in 1 ml ice-cold Tris-buffer (20 mM, 1 mM

EGTA, 1 mM DTT, pH 7.4) and put on ice. The platelet suspension was homogenized by 10 up-and-down strokes in a Teflon-glass homogenizer rotating at 700 rpm. The homogenate was sonicated on ice at maximum power for 3 x 10 sec with 1 min pauses. The resulting homogenate was stored at -80°C. Crude cytosolic and salt-solubilized protein fractions were prepared by the addition of an equal volume of Tris-buffer (as described above) containing 2 M NaCl to the homogenate. After thorough mixing, the sample was allowed to stand for 1 hour at 4°C, and was then spun down at 15,000xg for 15 min at 4°C. The resulting supernatant was dialysed (1:100) on micro-dialysis filters (Millipore, pore size 5 µm) against Tris-buffer for 2 hours at 4°C.

Enzyme assays

Phosphatidylinositol (PI) kinase activity was measured as described before (Jolles et al., 1992). Homogenate and cytosolic fractions (15 µl corresponding to 10 µg protein) were preincubated for 2 min. Lipid precursors (20 µM PI, Sigma), solubilized in 0.1% Triton X-100 in 50 mM Tris-HCl / 1 mM EGTA / pH 7.4, were added 15 sec before the phosphorylation reaction was started by the addition of ATP. Incubations were performed under the following conditions: 7.5 µM ATP, 2-3 µCi [gamma-³²P]ATP (approx. 3000 Ci/mmol, Amersham, U.K.), 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 1 mM EGTA and 0.02% Triton X-100. The reaction was terminated after 1 min, and the extraction and further analysis of the ³²P incorporated into PIP were performed as described elsewhere (Jolles et al., 1981; Bothmer et al., 1990). NADH cytochrome-C reductase, a marker enzyme for the endoplasmic reticulum, i.e., the microsome fraction, was assayed according to the method of Sun et al. (1988). Briefly, the assay mixture contained 0.1 mM NADH, 36 µM cytochrome-C, 0.3 mM KCN and 50 mM phosphate buffer (pH 7.6) with 0.1 mM EDTA. After addition of the homogenate, the reaction was monitored spectrophotometrically at A₅₅₀ for 30 min. Protein was determined according to the method of Lowry et al. (1951) and total lipid phosphorus was determined as described before (Bothmer et al., 1992).

Results

Platelets from AD patients and normal elderly controls incorporated phosphate into PIP (Fig. 1A) in both the homogenate as well as in the cytosolic / salt-solubilized enzyme fraction. The cytosolic / salt-solubilized protein fraction had a higher specific PI kinase activity than the platelet homogenate, but there was no difference in PI kinase activity between AD patients and controls.

A second experiment was performed in order to determine whether there was proliferation of internal membranes in platelets of AD patients. NADH cytochrome-C reductase, which is a specific marker for the endoplasmic reticulum, and the protein / total phospholipid ratio, which is expected to be

Fig. 1 : PI kinase (Fig. 1A) activity (pmol/min.mg protein) and NADH cytochrome C reductase (Fig. 1B) activity (nmol/min.mg protein) in homogenates and cytosolic/salt-solubilized fractions of platelets from patients with probable Alzheimer's disease (AD) (n=5) and age-matched controls (n=5). Values shown are means ($\pm$ SEM).

decreased in the case of proliferating internal membranes, were measured. Fig.1B shows that NADH cytochrome-C reductase activity was not increased in homogenates of platelets from AD patients as compared to controls.

Proliferation of internal membranes (increasing content of phospholipids) without expansion of the cytoplasm (slight or no increase in protein) would result in a decrease in the protein / total phospholipid ratio per 10^6 platelets. However, the protein / phospholipid ratio per 10^6 platelets, the protein content per 10^6 platelets and the total phospholipid phosphorus content per 10^6 platelets were not altered in homogenates of platelets from AD patients as compared to controls (Table 1).

Discussion

We previously reported a specific decline in PI kinase activity in the neocortex of AD patients as compared to controls, whereas PIP kinase appeared not to be affected (Jolles et al., 1992). The identity of the type of PI kinase affected in AD is not yet clear, but we have experimental evidence that PI 3-kinase is involved

Table 1 : Protein content, total phospholipid phosphorus content and the ratio protein content vs total phospholipid phosphorus content in the homogenate of platelets from both controls and probable Alzheimer (AD) patients. Values shown are means ($\pm$ SEM). Data were analysed with paired student T-test (two tailed).

subjects	protein (mg/10 ⁶ platelets)	phospholipid phospho- rus (μ g/10 ⁶ platelets)	protein/phosphorus (per 10 ⁶ platelets)
Controls (n=5)	0.778 ($\pm$ 0.053)	0.323 ($\pm$ 0.020)	2.416 ($\pm$ 0.114)
AD (n=5)	0.768 ($\pm$ 0.065)	0.311 ($\pm$ 0.021)	2.461 ($\pm$ 0.044)
paired stu- dent T-test (two tailed)	p=0.87	p=0.43	p=0.76

(Bothmer et al., submitted). Because disease-specific abnormalities in the brain, such as those occurring in diseases like Parkinson's disease, Huntington's disease, and depression, can be reflected in platelets, this study focussed on the PI kinase activity in platelets from AD patients and controls.

The results presented in this study show that PI kinase activity in platelet homogenates from AD patients was not different from PI kinase activity in platelet homogenates from controls. PI kinase activity was also measured in a cytosolic / salt-solubilized fraction of platelets because in this fraction, prepared from AD brain, PI kinase activity was affected to a greater extent than in the triton-solubilized fraction and the residual fraction (Bothmer et al., submitted). However, PI kinase activity in this fraction of platelets also revealed no differences between AD patients and controls. The number of controls and AD patients in the study (n=5 for each) may seem small, this number of experimental subjects was sufficient to detect the clear difference in PI kinase activity in the brains of AD patients and controls (Jolles et al., 1992). Moreover, the patient group was very homogenous as to age, age of first symptoms, and other patient characteristics.

The membranes of platelets from AD patients appear to have an elevated platelet membrane fluidity (PMF), which correlates with the severity of dementia in patients with AD, as assessed with the Mini-Mental Status Exam (Zubenko et al., 1987c). The plasma membrane of platelets do not show an elevated PMF, but the intracellular membranes do (Piletz et al., 1991). That intracellular membranes are affected in platelets from AD patients had been shown earlier by Zubenko et al. (1987a), and was confirmed by Hajimohammadreza et al. (1990), who found proliferation of a system of trabeculated cisternae bounded by the smooth

endoplasmic reticulum (SER) in platelets from AD patients as compared to controls after electron microscopic analysis. For the determination of such a proliferation of intracellular membranes in platelets, we measured NADH cytochrome-C reductase, which is a marker enzyme for the smooth endoplasmic reticulum (Sun et al., 1988), and the protein / phospholipid ratio in platelet homogenates, which is expected to be decreased when there is proliferation of intracellular membranes without an increase in the cytoplasm. These markers for the possible increase in the amount of intracellular membranes did not differ in homogenates of platelets from AD patients and controls. However the literature provides contradictory evidence as to whether an increase or decrease in NADH cytochrome-C reductase activity can be expected as a result of proliferating intracellular platelet membranes in AD patients. Zubenko (1989) reported an increased activity of NADH cytochrome-C reductase in platelets from AD patients. In contrast, Hajimohammadreza et al. (1990) found a decreased activity of antimycin A-sensitive NADH cytochrome-C reductase in platelets from AD patients as compared to controls. This decrease in the activity of the smooth endoplasmic reticulum marker enzyme is explained as an abnormal proliferation of the smooth endoplasmic reticulum without normal levels of enzyme markers. In both studies, however, there were more control subjects and AD patient groups than in the present study, which is possibly too small for these measurements although the variation between subjects was reasonable.

Despite the indications that, firstly, specific abnormalities of some diseases in the brain can be reflected in platelets, and secondly, PI kinase activity is substantially decreased in AD brains, the present study revealed no differences in PI kinase activity in platelets from AD patients as compared to controls. Although evidence for a proliferation of intracellular membranes in platelets from AD patients has been described in the literature, no evidence for such proliferation was found in this study.

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CHAPTER 11

SUMMARY AND CONCLUDING REMARKS.

J. Bothmer

SUMMARY AND CONCLUDING REMARKS.

The aim of this thesis was to provide more information about the neurochemical changes that occur in the brain with age and Alzheimer's disease (AD). Inositol phospholipids and diacylglycerol phosphorylation were chosen as neurochemical parameters because of their involvement in various important cell functions, such as receptor-mediated signal transduction and calcium release, but also mitogenic signalling and the regulation of protein kinases. Several investigators have described age-related changes in phosphoinositide metabolism and their possible involvement in the age-related decline in cell function. Phosphoinositide metabolism also seems to be involved in AD, as AD-related changes at different levels of the phosphoinositide cascade have been reported. However, phosphatidylinositol (PI) kinase, phosphatidylinositol phosphate (PIP) kinase and diacylglycerol (DAG) kinase have not been studied with respect to aging and AD up to now, even there is enough evidence that these enzymes could be affected.

- Experiments with rat brain material

PI, PIP and DAG kinase activities were studied in cytosolic, salt-solubilized and Triton-solubilized protein fractions of rat brain cortex as a function of age. This crude purification was used to differentiate between the different enzymes and enzyme subtypes, each of which have their own subcellular localization. There appeared to be no, or only minor, age-related changes in the formation of PIP, PIP₂ and PA after addition of exogenous substrate (chapter 6).

However, when endogenous lipids were used as substrate, PIP₂ formation was decreased in the frontal cortex. PA formation was also decreased in almost all brain regions tested (chapter 5). The enzyme/endogenous substrate fraction used for this study was a membrane-cytosol preparation containing plasma membranes and cytosol obtained from a fraction enriched in synaptosomes. The kinases in this fraction are probably still associated with the membrane, as they are *in vivo*, and in direct contact with their substrates PI, PIP and DAG. Therefore, the age-related changes in PIP kinase activity and DAG kinase activity found in this fraction of rat brain, in contrast to the lack of changes in an assay system with solubilized proteins and exogenous lipid substrate, could be due to changes in the properties of the membrane. As elaborated in chapter 1, age-related changes in the physical properties of membranes can result in changes in the activity of enzymes attached to this membrane (Naeim and Walford, 1985); however, we did not measure the correlation between enzyme activity and membrane fluidity.

A decrease in endogenous substrate availability could also have caused the decrease in PIP₂ and PA formation. It is possible that basal PLC activity is decreased as a result of the age-related changes in membrane properties. This would result in a decreased hydrolysis of PIP₂ and a decreased supply of DAG.

This in turn would result in a decreased formation of PA in our phosphorylation assay. In addition, decreased PIP₂ hydrolysis would result in an increased level of PIP₂, which can inhibit PIP kinase activity by product feedback inhibition (Van Rooijen et al., 1985).

In conclusion, PI, PIP and DAG kinase activities are not affected by age in the rat brain. The age-related changes measured in the membrane cytosol preparation with endogenous lipids as substrates could be a reflection of the age-related changes in the physical properties of the membrane. Thus, the age-related changes in phospholipid phosphorylation rates reported in this thesis cannot explain the age-related changes in receptor-mediated inositol phospholipid hydrolysis reported by other investigators, as suggested in chapter 1. These data are therefore consistent with the membrane hypothesis of aging (Naeim and Walford, 1985). The main concept of this hypothesis is that the membrane shows an age-associated increase in microviscosity, which correlates with an increase in the cholesterol/phospholipid ratio. A less fluid membrane would result in a decline in the functioning of membrane-bound enzymes, receptors and ion channels, and, ultimately, in a decline in cell function.

In these aging studies, groups of Brown-Norway rats of different age were used. Young Brown-Norway rats showed no, or only minor, differences compared with Sprague-Dawley and Wistar rats with respect to PI, PIP and DAG kinase activities in a membrane-cytosol preparation (results not shown). Because of the high inter-experimental and inter-individual variation in phosphorylation (with the membrane-cytosol preparation as enzyme/lipid substrate fraction), several variables in the preparation of the enzyme/lipid substrate fraction and the phosphorylation assay were tested. After the influence of each variable on the ultimately measured kinase activities had been evaluated, a value was chosen which was kept constant in following studies. These studies on the experimental variables revealed some interesting data.

First, the effect of time variation within experiments appeared to be very important for the different kinases, which were affected in different ways. Chapter 2 shows that the effect of time variation is dependent on the phase of the subcellular fractionation procedure. After decapitation, which is regarded as a form of global cerebral ischaemic insult, PLC activity is activated in both brain tissue as well as brain homogenate, probably as a result of higher intra-cellular calcium concentrations under such conditions (Sun et al., 1990). The high levels of DAG formed under these conditions explain the increased PA formation and the decreased PIP₂ formation (chapter 2) as PIP (substrate for PIP₂ formation) is also hydrolysed with increasing post-mortem delay (Dawson and Eichberg, 1965). A delay after the subcellular fractionation or before the phosphorylation assay is characterized by low calcium concentrations. The rates of PIP₂ and PA formation appeared to be decreased and the rate of PIP formation increased. This is probably due to phosphomonoesterase activity increasing the level of PI (Jolles et al., 1981).

Chapter 3 shows that the amount of PIP₂ ultimately formed in the phosphorylation assay decreases markedly with prolonged preincubation times. PA and PIP formation were not affected by changes in preincubation time. This

could be an effect of increased phosphomonoesterase activity; however, if this were so, PIP formation would be expected to be increased. Another mechanism for this phenomenon could be a regulation by protein phosphorylation, as Jolles et al. (1980) reported a relationship between phosphoinositide phosphorylation and protein phosphorylation. A comparable protein fraction also showed an analogous decrease in the phosphorylation of some membrane proteins (Dunkley and Robinson, 1981).

Second, chapter 5 shows that there are regional differences in PI, PIP and DAG kinase activities in the rat brain. These differences probably reflect a regional distribution of PLC activity. This is because the rates of PIP₂ and PA formation, measured in seven rat brain regions, showed an inverse correlation. A high basal PLC activity would result in high levels of DAG and, consequently, in an increased formation of PA in the phosphorylation assay used. In addition, a high basal PLC activity would result in decreased levels of PIP and PIP₂ and, consequently, in lower rates of PIP₂ formation in the phosphorylation assay. However, no studies on regional differences in basal PLC activity in rat brain have been reported up to now.

Third, a study of the subcellular distribution of endogenous lipid phosphorylation activities (chapter 4) revealed an unidentified phosphorylated inositol phospholipid, specifically located in the mitochondrial fraction. The unknown inositol phospholipid was not phosphatidylinositol trisphosphate or lyso-phosphatidylinositol 4,5-bisphosphate, as judged from the elution profiles on Dowex anion-exchange columns. It could be a phosphatidylinositol bisphosphate isomer, a glycosyl-phosphoinositide or a phosphatidylinositol pyrophosphate. The formation of phosphatidylinositol pyrophosphates can be catalysed non-enzymically by bivalent metal ions (Gumber and Lowenstein, 1986). Because mitochondria contain high levels of Ca²⁺ a substance such as PI 4-pyrophosphate could be formed. PI-4-pyrophosphate has a R_f value on TLC similar to that of the unknown inositol phospholipid. HPLC analysis of the chemically hydrolysed inositol phosphate head-group or even NMR analysis is needed to identify this unknown inositol phospholipid.

- Experiments with human brain material

PI kinase activity in cytosolic preparations of normal human brain cortex appeared to decrease with age, whereas PIP kinase activity showed no age-related change (chapter 8). These enzyme activities were analysed with exogenous lipids as substrate. These findings are in contrast to the findings in rat brain cortex, where no age-related differences were found (chapter 6). This discrepancy between rats and humans can be ascribed to various factors. First, species differences may provide an explanation. Although the rat is regarded as a valid model for age-related changes in physiological parameters in man, various processes are known to be different in aging rats than in aging humans. It can be questioned whether the aging rat can be regarded as a valid model for pathological brain aging in humans (AD). Second, environmental influences

may affect the aging organism. The fact that old people are more likely to have been exposed to biological life events than young people and than rats, which are kept under standardized conditions and are well-cared for, may also underlie the difference in inositol phospholipid metabolism in aging rats and humans. Biological life events are factors other than severely impairing conditions like dementia that damage optimal brain functioning (Houx et al., 1991). Aged rats of an inbred strain kept under standardized conditions without almost any damaging influence of the environment are, therefore, possibly not an adequate model for human aging. However, mimicking human biological life events in rats as a model for human aging would probably meet with ethical problems. The use of animals that have been used in other studies, in which for example the animals were anaesthetized, should be taken into consideration.

In cytosolic preparations of brain cortex of AD patients, PI kinase activity appeared to be decreased as compared with that of age-matched control subjects. PIP kinase activity was not altered (chapter 7). No age-related decline in PI kinase activity and PIP kinase activity could be detected in the AD patient group (chapter 8). However, AD patients with an "age of first symptoms" above 75 years had a higher PI kinase activity (comparable to control values) than AD patients with an "age of first symptoms" below 75 years. These results suggest that PI kinase activity can be used to differentiate AD patients into subgroups, characterized by a difference in the age of onset of AD. This interesting finding could be interpreted as an indication for heterogeneity in Alzheimer's disease, in line with the results of an earlier study (Rossor et al., 1984) in which enzyme activities were found to differ in old and young AD patients. The present results suggest that more research should be performed into the biochemical differentiation of AD subtypes.

The decline in PI kinase activity in AD is probably not caused by the presence of an inhibitory compound in AD cytosol, for instance metal ions, or by the loss of a soluble activating compound in AD cytosol, for instance cofactors, because chelation experiments and experiments in which AD cytosol and control cytosol were combined did not reveal inhibition or stimulation of PI kinase activity (chapter 9). PI kinase can be divided into at least three subtypes (Carpenter and Cantley, 1990), each with a different subcellular localization, adenosine sensitivity, detergent sensitivity, and supposed function. The greatest decline in PI kinase activity was found in the cytosolic fractions. The PI kinase activity in these fractions was inhibited by Triton X-100 and was relatively insensitive to adenosine, in contrast to the PI kinase activity in integral membrane protein fractions, which was stimulated by Triton X-100 and inhibited by adenosine. The PI kinase in the cytosolic fraction has the characteristics of type 1 PI kinase, or PI 3-kinase, suggesting that this PI 3-kinase could be specifically affected in AD. Identification of the products of the PI kinases in AD and control brains will reveal the ultimate proof of identity. These experiments will be done in future studies.

If type 1 PI kinase, or PI 3-kinase, is specifically affected in AD, then protein tyrosine kinase activity could be involved. Several receptors which induce

cytoskeletal rearrangements in the cell after stimulation contain intrinsic protein tyrosine kinase activity (listed in chapter 1). Activation of these receptors results in stimulation of protein tyrosine activity, resulting in the coupling and activation of PI 3-kinase and cytoskeletal rearrangements. This possible involvement of PI 3-kinase and protein tyrosine kinase in the regulation of cytoskeletal rearrangements, together with the decline in PI kinase activity (chapter 7) and tyrosine kinase activity (Shapiro et al., 1991) in AD patients, suggests that these enzymes may be involved in the cellular pathology of AD. This is because neurofibrillary tangles, which are a prominent feature of AD, are composed predominantly of wrongly metabolized cytoskeletal components.

There is growing evidence that AD may be a systemic disease and not just a disease with central effects. For this reason, PI kinase activity was measured in blood platelets from AD patients and age-matched controls. Platelets were chosen because disease-specific abnormalities in the brain, such as those occurring in diseases like Parkinson's disease, Huntington's disease, and depression, are also detected in platelets (Bush et al., 1991). Furthermore, platelets contain a complete phosphoinositide-metabolizing system, including type 1 or PI 3-kinase, and are easy to obtain. However, we did not find any difference between the PI kinase activities of platelets obtained from AD patients and control subjects. More research into the PI kinase activities present in peripheral tissues and cells should be performed to be able to conclude whether the changes in the brain cortex are reflected systemically.

In conclusion, the results in this thesis provide new information about the neurochemical basis of brain aging and AD. Three enzymes of phosphoinositide metabolism, phosphatidylinositol kinase, phosphatidylinositol phosphate kinase and diacylglycerol kinase, appeared to be affected in aging and/or AD. The micro-environment of these enzymes appeared to be important for their activity. Therefore, the operationalization used in this thesis with a differentiation in measuring kinase activities as a function of age with and without their microenvironment was essential. In addition, the decrease in type 1 PI kinase activity in AD, in view of the proposed relation between this kinase activity and cytoskeletal turnover processes, could be relevant to the cellular pathology of AD.

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SAMENVATTING EN CONCLUSIES

De doelstelling van het onderzoek beschreven in dit proefschrift, was meer informatie te verkrijgen over neurochemische veranderingen die plaatsvinden in de hersenen tijdens veroudering en de ziekte van Alzheimer (AD). De fosforyleringen van inositol fosfolipiden en diacylglycerol werden gekozen als neurochemische parameters omdat deze betrokken zijn bij verschillende belangrijke celfuncties zoals receptor gemedieerde signaal transductie en het vrijmaken van calcium, maar tevens bij mitogene signaal overdracht en de regulatie van eiwit kinases. Leeftijdsafhankelijke veranderingen in het inositol fosfolipiden metabolisme en de mogelijke betrokkenheid van deze veranderingen bij de leeftijdsgelateerde afname in het functioneren van de cel zijn door meerdere onderzoekers beschreven. Het inositol fosfolipiden metabolisme lijkt ook betrokken te zijn bij de ziekte van Alzheimer, omdat op verschillende nivo's van de fosfoinositiden cascade veranderingen zijn waargenomen, die aan de ziekte van Alzheimer zijn gerelateerd. De enzymen fosfatidylinositol (PI) kinase, fosfatidylinositol fosfaat (PIP) kinase en diacylglycerol (DAG) kinase zijn echter nog niet onderzocht in relatie tot veroudering en AD, hoewel er voldoende aanwijzingen zijn dat deze enzymen veranderd zouden kunnen zijn.

- Experimenten met hersen materiaal van de rat

PI, PIP en DAG kinase activiteiten werden gemeten in relatie tot de leeftijd, in cytosolaire, zout-gesolubiliseerde en Triton-gesolubiliseerde eiwit fracties van de hersen cortex van de rat. Deze ruwe zuivering werd toegepast om onderscheid te maken tussen de verschillende enzymen en hun sub-types welke allemaal hun eigen subcellulaire localisatie hebben. In dit experiment waarbij exogeen substraat werd toegevoegd, werden geen, of maar zeer kleine, aan de leeftijd gerelateerde veranderingen gevonden in de mate van PIP, PIP₂ en PA vorming (Hoofdstuk 6).

Als endogeen aanwezige lipiden als substraat werden gebruikt, nam in de frontale cortex de vorming van PIP₂ af met de leeftijd. De vorming van fosfatide zuur (PA) uit DAG nam in dit geval in bijna alle bestudeerde hersen regio's af met de leeftijd (Hoofdstuk 5). De voor deze studie gebruikte enzym/endogeen substraat fractie bestond uit een membraan-cytosol preparaat verkregen uit een fractie verrijkt aan synaptosomen. De kinases in deze fractie zijn waarschijnlijk nog gebonden aan de membraan en staan in direct contact met hun substraten PI, PIP en DAG, vergelijkbaar met de in vivo situatie. De aan de leeftijd gerelateerde verschillen in PIP kinase en DAG kinase activiteit in deze fractie, in tegenstelling tot de assay met gesolubiliseerde eiwitten en exogeen substraat waarin geen verschillen werden gevonden, worden dus mogelijk veroorzaakt door veranderingen in de eigenschappen van de membraan. Veranderingen in de fysische eigenschappen van de membraan als functie van de leeftijd kunnen,

zoals beschreven in hoofdstuk 1, de activiteit van membraan-gebonden enzymen beïnvloeden (Naeim en Walford, 1985). Wij hebben echter geen correlatie bepaald tussen enzym activiteiten en, bijvoorbeeld, membraan vloeibaarheid.

De afname in PIP_2 en PA vorming met de leeftijd kan ook veroorzaakt worden door een afname in de hoeveelheid endogeen substraat. De niet gestimuleerde PLC activiteit kan afgenomen zijn ten gevolge van leeftijds afhankelijke veranderingen in de eigenschappen van de membraan. Dit zou dan resulteren in een verlaagde hydrolyse van PIP_2 en een verlaagd aanbod van DAG. In de in dit proefschrift beschreven fosforylerings assay leidt een verlaagd aanbod van DAG tot een verlaagde vorming van PA. Een verlaagde PIP_2 hydrolyse leidt tot een toename in de hoeveelheid PIP_2 , met als mogelijk resultaat de remming van PIP kinase door produkt inhibitie (Van Rooijen et al., 1985).

De conclusie uit het voorgaande is dat de PI, PIP en DAG kinase activiteiten in ratte hersenen niet veranderen als functie van de leeftijd. De leeftijds gerelateerde veranderingen gemeten in het membraan-cytosol preparaat met endogene fosfolipiden als substraat zijn waarschijnlijk een afspiegeling van de leeftijds gerelateerde veranderingen in de fysische eigenschappen van de membraan. Deze leeftijds gerelateerde veranderingen in fosfolipiden fosforylerings ratio's kunnen de door andere onderzoekers beschreven leeftijds gerelateerde veranderingen in receptor gemedieerde inositol fosfolipiden hydrolyse dus niet verklaren, zoals gesuggereerd werd in Hoofdstuk 1. Deze gegevens stroken met de membraan hypothese voor veroudering (Naeim en Walford, 1985). Het hoofdpunt van deze hypothese is de leeftijds gerelateerde toename in de micro-viscositeit van de membraan, welke correleert met een toename in de cholesterol/fosfolipiden ratio. Een lagere membraan vloeibaarheid zou resulteren in een afname in het functioneren van membraan gebonden enzymen, receptoren en ion kanalen, en uiteindelijk in een afname in het functioneren van de cel.

In de beschreven verouderings studies zijn Brown-Norway ratten van verschillende leeftijden gebruikt. Jonge Brown-Norway ratten vertonen geen, of nauwelijks, verschillen met Sprague-Dawley en Wistar ratten met betrekking tot PI, PIP en DAG kinase activiteiten in een membraan cytosol preparaat van de hersenen (resultaten niet getoond). Omdat de inter-experimentele en inter-individuele variatie in fosforylerings nivo (met membraan/cytosol preparaat als enzym/substraat fractie) te hoog bevonden werd, werden verschillende variabelen binnen de bereiding van de enzym/lipide substraat fractie en binnen de fosforylerings assay getest. Na meting van het effect van een variabele op de uiteindelijke kinase activiteit, werd een constante waarde gekozen voor de volgende studies. Het bestuderen van de experimentele variabelen leverde enkele interessante gegevens op.

Ten eerste blijken veranderingen in experimenteer tijd zeer belangrijk voor de verschillende kinases, die elk op een verschillende wijze hierdoor beïnvloed werden. Hoofdstuk 2 laat zien dat het effect van tijd afhankelijk is van het stadium binnen de subcellulaire fractionering. Na decapitatie, wat gezien wordt als een vorm van volledige cerebrale ischemie, neemt de PLC activiteit toe in

zowel hersenweefsel als hersenhomogenaat. Dit wordt waarschijnlijk veroorzaakt door de hoge intra-cellulaire calcium concentraties onder deze condities (Sun et al., 1990). Zowel PIP_2 als PIP worden gehydrolyseerd waarbij DAG gevormd wordt (Dawson and Eichberg, 1965). De post mortem hydrolyse van het substraat voor PIP_2 vorming (PIP) verklaard de afname in PIP_2 vorming, en de post mortem toename in DAG concentratie verklaard de toename in PA vorming in de hier gebruikte assay (Hoofdstuk 2). Een verlenging van de experimenteer tijd na de subcellulaire fractionering, of voor de fosforylerings assay wordt gekarakteriseerd door lage calcium concentraties. De PIP_2 en PA vorming zijn hierdoor toegenomen en de PIP vorming is hierdoor afgenomen. Dit wordt waarschijnlijk veroorzaakt door fosfomonoesterase activiteit waardoor de hoeveelheid PI toeneemt.

Hoofdstuk 3 laat zien dat de uiteindelijk in de fosforylerings assay gevormde hoeveelheid PIP_2 sterk afneemt bij langere preïncubatie tijden. De vorming van PA en PIP worden niet beïnvloed door veranderingen in de preïncubatie tijd. Als dit een gevolg van fosfomonoesterase activiteit zou zijn, dan mocht verwacht worden dat de PIP vorming ook zou zijn toegenomen, wat dus niet het geval is. Een andere mogelijke verklaring voor dit verschijnsel is de regulatie van PIP_2 vorming door eiwit fosforylatie. Jolles et al. (1980) laten namelijk een relatie zien tussen fosfoinositide fosforylatie en eiwit fosforylatie, en Dunkley en Robinson (1981) laten een afname in de fosforylatie van enkele membraan eiwitten zien t.g.v. langere preïncubatie tijden in een vergelijkbare subcellulaire fractie, analoog aan de afname in PIP_2 fosforylatie zoals hier beschreven.

Ten tweede laat hoofdstuk 5 zien dat er regionale verschillen in PI, PIP en DAG kinase activiteit bestaan in de hersenen van de rat. Deze verschillen zijn waarschijnlijk een afspiegeling van een regionale verdeling van PLC activiteit. De mate van PIP_2 en PA vorming, gemeten in zeven hersen gebieden van de rat, laten een negatieve correlatie zien. Een hoge basale PLC activiteit resulteert in een hoge DAG concentratie en, als gevolg hiervan, in een verhoogde PA vorming in de fosforylerings assay. Daarnaast resulteert een hoge basale PLC activiteit in verlaagde PIP en PIP_2 concentraties en, als gevolg hiervan, in een verlaagde vorming van PIP_2 in de fosforylerings assay. Tot nu toe zijn er echter nog geen gegevens bekend m.b.t. de regionale verdeling van basale PLC activiteit in de hersenen van de rat.

Ten derde leverde een onderzoek naar de subcellulaire distributie van de fosforylerings activiteiten van endogene lipiden een nog niet geïdentificeerd inositol lipide op, specifiek gelocaliseerd in de mitochondriële fractie. Na beoordeling van de elutie profielen verkregen na scheiding van de polaire kopgroep van het "nieuwe" inositol lipide op Dowex anionen wisselaar, bleek het onbekende inositol lipide in ieder geval geen fosfatidylinositol trisfosfaat of een lyso-fosfatidylinositol 4,5-bisfosfaat te zijn. Andere kandidaten zijn een fosfatidylinositol bisfosfaat isomeer, een glycosyl-fosfoinositide of een fosfatidylinositol pyrofosfaat. De vorming van fosfatidylinositol pyrofosfaten kan non-enzymatisch gekatalyseerd worden door twee-waardige metaalionen (Gumber and Lowenstein, 1986). Omdat mitochondriën relatief veel Ca^{2+}

bevatten zou een stof als fosfatidylinositol 4-pyrofosfaat mogelijk gevormd kunnen worden. Fosfatidylinositol 4-pyrofosfaat heeft een R_f waarde op TLC die vergelijkbaar is met de R_f waarde van het onbekende inositol lipide. Verdere identificatie van dit onbekende inositol lipide vereist HPLC analyse van de chemisch gehydrolyseerde kopgroep of zelfs NMR analyse.

- Experimenten met hersen materiaal van de mens.

PI kinase activiteit in cytosolaire fracties van de hersencortex van de mens neemt af met de leeftijd, in tegenstelling tot PIP kinase activiteit welke niet verandert (Hoofdstuk 8). Deze enzym activiteiten werden gemeten met exogene lipiden als substraat. De bevinding staat in contrast met de bevindingen gedaan in de hersencortex van de rat, waar geen leeftijds gerelateerde verschillen gevonden werden (Hoofdstuk 6). Deze discrepantie kan toegeschreven worden aan verschillende factoren. Ten eerste kunnen species verschillen een verklaring zijn. Ondanks het feit dat de rat wordt gezien als een valide model voor leeftijds gerelateerde veranderingen in fysiologische parameters in de mens, is het bekend dat meerdere processen in de verouderende rat verschillen t.o.v. de verouderende mens. Het blijft de vraag of de verouderende rat gezien kan worden als een valide model voor pathologische hersenveroudering bij de mens (AD). Ten tweede kunnen omgevings factoren het verouderende organisme beïnvloeden. Het feit dat oude mensen meer waarschijnlijk zijn blootgesteld aan zogenaamde 'biological life events' t.o.v. jonge mensen en t.o.v. ratten, welke onder constante condities worden gehouden en tevens goed verzorgd worden, kan ook een oorzaak zijn van het verschil in fosfoinositide metabolisme tussen verouderende ratten en mensen. 'Biological life events' zijn factoren die het optimaal functioneren van het brein aantasten, uitgezonderd de factoren die het functioneren van het brein in zeer sterke mate aantasten, zoals dementie (Houx et al., 1991). Oude ratten uit een inteelt stam, gehouden onder gestandaardiseerde condities met nauwelijks schadelijke invloeden uit hun omgeving zijn mogelijk dus geen adequaat model voor de verouderende mens. Echter, het nabootsen van 'biological life events' in de rat als model voor veroudering bij de mens zal hoogst waarschijnlijk stuiten op ethische bezwaren. Het gebruik van dieren die reeds gebruikt zijn in andere studies, waarin zij bijvoorbeeld een anaesthesie hebben ondergaan, zou daarom overwogen moeten worden.

PI kinase activiteit in cytosolaire fracties van de hersencortex van AD patienten is afgenomen vergeleken met die van op leeftijd gematchte controle personen. PIP kinase activiteit is niet veranderd (Hoofdstuk 7). In de AD patienten groep werd geen leeftijds gerelateerde afname gevonden van PI kinase en PIP kinase activiteit (Hoofdstuk 8). AD patienten met een leeftijd waarop de eerste symptomen van de ziekte werden waargenomen boven de 75 jaar hebben echter een hogere PI kinase activiteit (vergelijkbaar met controle waarden) dan AD patienten met een leeftijd van eerste symptomen beneden de 75 jaar. Deze resultaten suggereren dat PI kinase activiteit gebruikt kan worden om binnen de

AD patiënten populatie subgroepen te onderscheiden die verschillen in de leeftijd waarop de ziekte toegeslagen heeft. Deze interessante bevinding kan geïnterpreteerd worden als een indicatie voor heterogeniteit binnen de ziekte van Alzheimer, vergelijkbaar met de resultaten van een eerdere studie (Rossor et al., 1984) waarin enzym activiteiten verschillend waren in oude t.o.v. jonge AD patiënten. Deze resultaten geven aan dat meer onderzoek gedaan moet worden naar de biochemische differentiatie van AD subtypen.

De daling van PI kinase activiteit in AD wordt waarschijnlijk niet veroorzaakt door de aanwezigheid van een inhiberende component in AD cytosol, bijvoorbeeld een metaal ion, of door het verlies van een oplosbare activerende component in AD cytosol, bijvoorbeeld een cofactor. Chelatie experimenten en experimenten waarin AD cytosol en controle cytosol gecombineerd werden, gaven namelijk geen inhibitie of stimulatie te zien (Hoofdstuk 9). Van het enzym PI kinase kunnen drie subtypen onderscheiden worden (Carpenter and Cantley, 1990), elk met een andere subcellulaire localisatie, adenosine gevoeligheid, gevoeligheid voor detergentia, en veronderstelde functie. De sterkste afname in PI kinase activiteit werd aangetroffen in de cytosolaire fracties. De PI kinase activiteit in deze fracties werd geremd door Triton X-100 en was relatief ongevoelig voor adenosine, in tegenstelling tot de PI kinase activiteit in de integrale membraaneiwit fractie die gestimuleerd werd door Triton X-100 en geremd werd door adenosine. Het PI kinase in de cytosolaire fractie heeft de eigenschappen van het type I PI kinase, oftewel het PI 3-kinase, waardoor dit PI 3-kinase specifiek lijkt aangedaan in AD. Het uiteindelijke bewijs hiervoor dient te komen uit de identificatie van de produkten van de PI kinases welke actief zijn in de hersenen van AD patiënten en controles. Deze experimenten zullen deel uitmaken van vervolg studies.

Als type I PI kinase, of PI 3-kinase, specifiek is aangedaan in AD, dan kan eiwit tyrosine kinase activiteit hierbij betrokken zijn. Verschillende receptoren, die na stimulatie cytoskelet veranderingen in de cel induceren, bevatten een intrinsieke eiwit tyrosine kinase activiteit (opgesomd in Hoofdstuk 1). Stimulatie van deze receptoren resulteert in stimulatie van deze tyrosine kinase activiteit, gevolgd door de koppeling en activatie van PI 3-kinase en cytoskelet veranderingen. Deze mogelijke betrokkenheid van PI 3-kinase en tyrosine kinase activiteit bij de regulatie van cytoskelet veranderingen, samen met de daling van PI kinase activiteit (Hoofdstuk 7) en tyrosine kinase activiteit (Shapiro et al., 1991) in AD, duidt op een mogelijke betrokkenheid van deze enzymen bij de cellulaire pathologie van AD. 'Neurofibrillary tangles', welke één van de hoofd kenmerken zijn van AD, zijn namelijk hoofdzakelijk opgebouwd uit verkeerd gemetaboliseerde cytoskelet componenten.

Een toenemende hoeveelheid onderzoeks resultaten laat zien dat AD een ziekte van het gehele lichaam zou kunnen zijn, en niet een ziekte met alleen centrale effecten. Dit is de reden waarom PI kinase activiteit werd gemeten in bloed plaatjes van AD patiënten en op leeftijd gematchte controles. Voor deze studie werden bloed plaatjes gekozen omdat ziekte-specifieke abnormaliteiten in de hersenen, welke bijvoorbeeld optreden bij aandoeningen als de ziekte van

Parkinson, de ziekte van Huntington en depressie, ook worden aangetroffen in bloed plaatjes (Bush et al., 1991). Daarnaast bevatten bloed plaatjes een compleet fosfoinositide metaboliserend systeem, inclusief type 1 of PI 3-kinase, en zijn ze gemakkelijk te verkrijgen. De PI kinase activiteit in bloedplaatjes afkomstig van AD patienten en controle personen bleken echter niet van elkaar te verschillen. Om te concluderen dat veranderingen in PI kinase activiteit in de hersen cortex niet perifeer te zien zijn, moet echter meer onderzoek gedaan worden in meerdere perifere weefsels en/of cellen.

Concluderend kan gesteld worden dat de resultaten van dit onderzoek nieuwe informatie leveren over de neurochemische basis van hersenveroudering en AD. Drie enzymen, betrokken bij het fosfoinositide metabolisme, fosfatidylinositol kinase, fosfatidylinositol fosfaat kinase en diacylglycerol kinase, blijken te zijn aangedaan in hersenveroudering en/of AD. De micro-omgeving van deze enzymen blijkt heel belangrijk te zijn voor hun activiteit. De in dit proefschrift gebruikte operationalisatie, namelijk het onderscheiden van kinase activiteits metingen als functie van de leeftijd met en zonder de aanwezigheid van hun micro-omgeving, was om deze reden essentieel. Verder kan de afname in type 1 PI kinase activiteit in AD, met het oog op de veronderstelde relatie tussen dit kinase en cytoskelet turn-over processen, zeer relevant zijn voor de cellulaire pathologie van AD.

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Curriculum Vitae

John G.M. Bothmer werd geboren op 26 augustus 1964 te Geleen. In 1982 behaalde hij het eindexamen Atheneum-B aan de Albert Schweitzer Scholengemeenschap te Geleen. In september van dat jaar startte hij de studie Biologie aan de Katholieke Universiteit Nijmegen waar het propedeutisch examen werd behaald in 1983. Zijn doctoraalfase was opgebouwd uit de bijvakken Toxicologie (Prof. Dr. P. Henderson) en Bio-Organische Chemie (Prof. Dr. E. Meyer, DSM) en het hoofdvak Microbiologie (Prof. Dr. Ir. G.D. Vogels), en werd in 1988 afgesloten met het afleggen van het doctoraal examen. Vanaf juli 1988 tot september 1992 was hij werkzaam als assistent-in-opleiding binnen de afdeling Neuropsychologie en Psychobiologie (Prof. Dr. J. Jolles) van de Rijksuniversiteit Limburg, alwaar het hier beschreven promotie onderzoek werd verricht. Sinds september 1992 wordt het onderzoek binnen deze afdeling voortgezet en is hij op tijdelijke basis aangesteld als toegevoegd onderzoeker.

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