BRAIN PHOSPHATIDIC ACID AND POLYPHOSPHOINOSITIDE FORMATION IN A BROKEN CELL PREPARATION: REGIONAL DISTRIBUTION AND THE EFFECT OF AGE

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(Received 13 November 1991; accepted 10 January 1992)

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.

Recent research suggests that receptor-stimulated phosphoinositide hydrolysis plays an important role in signal transduction. PIP2 is 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 (Herschkowitz 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 endogenous 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 adrenocorticotropic (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, 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 s (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 pyriform cortex were removed from the caudal part and separated from each other. The entorhinal cortex and pyriform 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 (P₁) 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 g to remove unlysed structures. The supernatant was taken as the enzyme fraction referred to as the membrane–cytosol preparation which contains P₁, PIP₂, and DAG kinase activity, but also endogenous lipid substrates.

**Phosphorylation assay**

Endogenous 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 μM ATP, 2–3 μCi [γ-32P]ATP (approx 3000 Ci/mmol, Amersham, U.K.), 50 mM Na-acetate, 10 mM Mg₂⁺-acetate, pH 6.5, and 15 μg enzyme fraction (10 μg protein) in a final vol of 25 μl at 30°C. After 10 s, 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). 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. (1957). Briefly, 2 ml chloroform: methanol (2:1 (v/v)) and 310 μl water were added to 90 μl 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 μl) 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
Brain phosphatidic acid and polyphosphoinositide formation

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/pyriform 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, Student’s 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/pyriform 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/pyriform 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/pyriform 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.

**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
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 (± SEM) from four rats per group. Statistical analysis of the age differences was performed according to Student's t-test (two tailed).

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Total protein (µg/mg wet weight ± SEM)</th>
<th>Total phospholipid phosphorus (ng/mg wet weight ± SEM)</th>
<th>Correlation (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 month</td>
<td>32 month</td>
<td>Diff. (%)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.13 ± 0.05</td>
<td>1.06 ± 0.03</td>
<td>-6</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>1.30 ± 0.05</td>
<td>1.23 ± 0.07</td>
<td>-6</td>
</tr>
<tr>
<td>Entorhinal/pyriform cortex</td>
<td>1.67 ± 0.04</td>
<td>1.45 ± 0.06</td>
<td>-13*</td>
</tr>
<tr>
<td>Occipital/parietal cortex</td>
<td>1.24 ± 0.04</td>
<td>1.08 ± 0.03</td>
<td>-13*</td>
</tr>
<tr>
<td>Striatum/septum</td>
<td>1.00 ± 0.06</td>
<td>0.85 ± 0.05</td>
<td>-15</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1.06 ± 0.01</td>
<td>0.94 ± 0.02</td>
<td>-11*</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.97 ± 0.03</td>
<td>0.85 ± 0.04</td>
<td>-12*</td>
</tr>
</tbody>
</table>

* P < 0.05.
† P < 0.005.
‡ Pearson correlation (r) between protein and phospholipid phosphorus content.

a source of enzyme and of endogenous 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 endogenous 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 5-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. (1983) 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/parietal 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 (DeKosky 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 DeKosky and Scheff (1990) and of Geinisman et al. (1986). Lower mental status scores appear to be associated with greater loss of synapses (DeKosky 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.

REFERENCES


