The effect of age on phosphatidylinositol kinase, phosphatidylinositol phosphate kinase and diacylglycerol kinase activities in rat brain cortex

The Effect of Age on Phosphatidylinositol Kinase, Phosphatidylinositol Phosphate Kinase and Diacylglycerol Kinase Activities in Rat Brain Cortex

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ABSTRACT: A previous study, in which a lysed fraction was used with endogenous phospholipids as substrate, revealed age-related changes in PA and PIP_2 formation but not in PIP formation (Bothmer *et al.*, Neurochem. Int. 21, 223-228, 1992). To rule out the influence of substrate availability in the present study, the effect of age on PI kinase, PIP kinase and DAG kinase activities was studied with exogenous phospholipids as substrate in the cerebral cortex from 8-month-old, 14-month-old and 26-month-old Brown Norway rats. PI kinase activity was predominantly located in a tight membrane-bound protein fraction, DAG kinase activity in cytosolic and loosely membrane-bound protein fractions, and PIP kinase activity was present in all three protein preparations. The effects of age were limited to a small increase in kinase activity in the tight membrane-bound protein fraction in 14-month-old and 26-month-old rats compared to 8-month-old rats, and a 10% decrease in PIP kinase activity in the cytosolic protein fraction 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 (Jolles *et al.*, J. Neurochem. 58, 2326-2329, 1992). 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, although further experimental evidence is needed to confirm this conclusion.

KEY WORDS: Phosphatidylinositol, Phosphatidylinositol phosphate, Diacylglycerol, Kinase, Aging, Rat brain.

INTRODUCTION

Normal aging is associated with numerous functional and morphological alterations of the central nervous system (CNS) in animals as well as humans. Through the past decade, interest was raised in membrane-phosphoinositides in relation to age. Phosphoinositides are present in relatively high concentrations in the CNS and 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). 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. A great number of other biochemical processes are dependent upon such changes. For instance, 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).

Up till now, various membrane-associated processes have been studied as a function of age. For instance, changes in receptor-stimulated inositol phosphate release have been noted (Burnett *et al.*, 1990; Mundy *et al.*, 1991; Nalepa *et al.*, 1989; Pietrzak *et al.*, 1990; Surichamorn *et al.*, 1989; Tandon *et al.*, 1991). Similarly, the effectiveness of the inositol phosphate response, such as IP_3 receptor density and affinity has been studied (Li *et al.*, 1991), as well as the maintenance of calcium homeostasis (Barritt, 1987; Martinez-Serrano *et al.*, 1992) and protein kinase activity (Magnoni *et al.*, 1991; Battaini *et al.*, 1990). However, the production of the IP_3 precursor phosphatidylinositol bisphosphate (PIP2) 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 turnover processes (Carpenter and Cantley, 1990).

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.* (1993) 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 disease, and Stokes and Hawthorne (1987) reported a lower concentration of phosphoinositides in Alzheimer brain as compared to brains from age-matched controls.

The present study investigates the effect of age on PI kinase, PIP kinase and DAG kinase activities in the rat brain cortex. Exogenous phospholipids are used as substrate to rule out the influence of substrate availability. 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 protein preparation because the enzymes consist of different subtypes and have their own characteristic subcellular localisation (Carpenter and Cantley, 1990; Stubbs *et al.*, 1988).

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 (eight animals per age group). 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 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 frontal cortex and a remaining part, which 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 frontal cortex, cerebral cortex, entorhinal cortex and pyriformal cortex were pooled, and the remaining parts were discarded.

Subcellular fractionation

Cellular proteins were fractionated according to Yamakawa and Takenawa (1988) with minor modifications (Bothmer *et al.*, 1994). Briefly, the cortex of individual rats 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 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 2 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-^{32}P] ATP (approx. 3000 Ci/mmol, Amersham U.K.) 20 mM Tris-HCl, 10 mM $MgCl^2$, 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 ^{32}P incorporated into PIP, PIP_2 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).

RESULTS

In the present study, the effect of age on PI kinase, PIP kinase and DAG kinase activities was measured (each rat individually) in three protein preparations of the rat brain cortex: 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.1 shows also 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 mem-

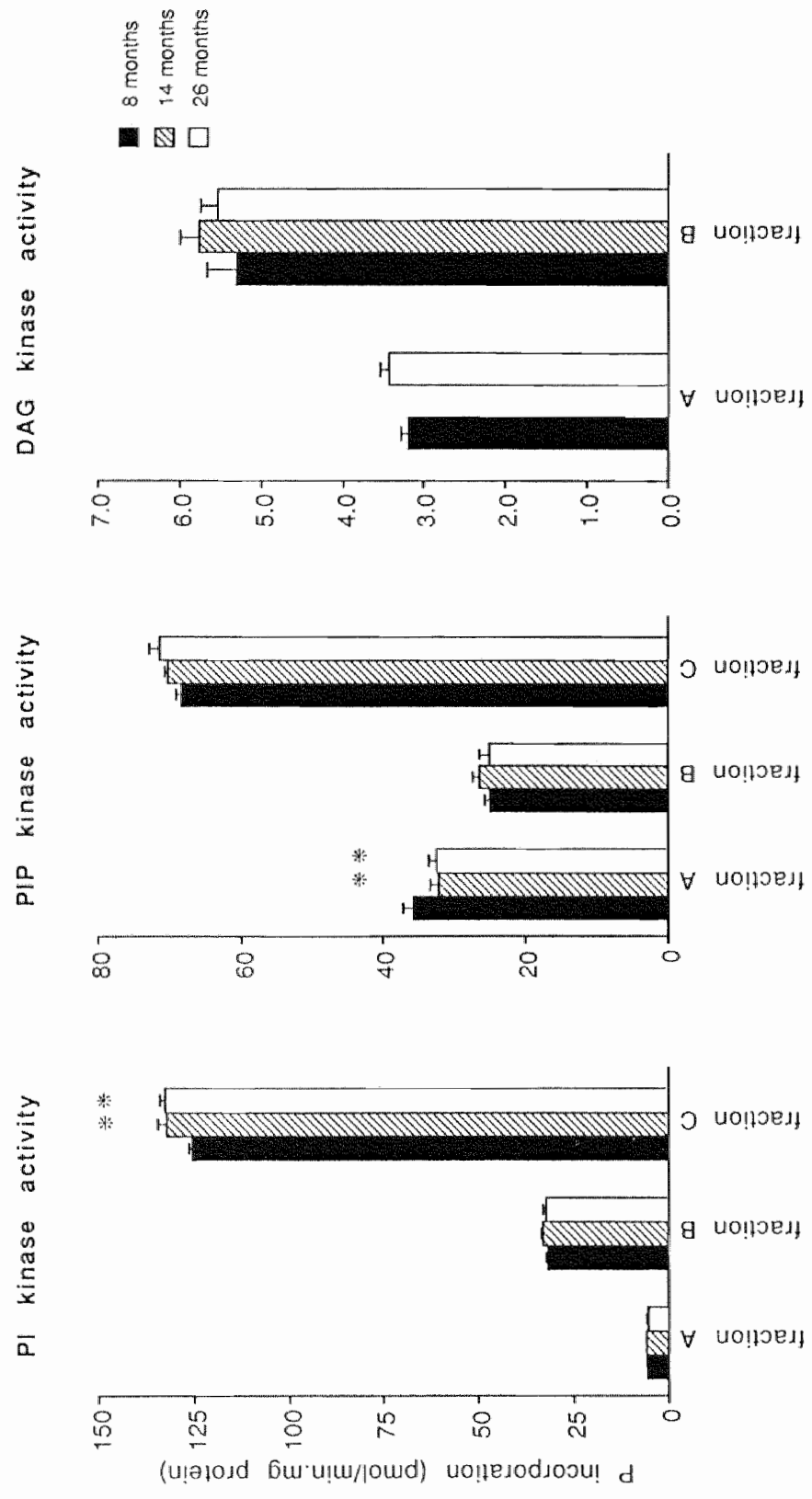

Figure 1: Distribution of, and effect of age on phosphatidylinositol (PI) kinase, phosphatidylinositol phosphate (PIP) kinase and diacylglycerol (DAG) kinase activities (pmol phosphate (P) incorporation / min.mg protein) in cytosolic preparations (fraction A), salt solubilized protein preparations (fraction B), and Triton X-100-solubilized protein preparations (fraction C) of rat brain cortex. Eight-month-old, 14-month-old and 26-month-old Brown Norway rats (eight rats per group) were used. Values shown are means (± SEM). Statistical analysis was done with Duncan's Multiple Range Test. *: $p<0.05$

brane (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.*, 1994), 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.*, 1992). 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 could be caused by changes in the availability of DAG. This is in agreement with the conclusion of Strosnajder 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, 1992), 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.*, 1993). 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 live events (BLE) than young people (Houx *et. al.*, 1991), and than rats. These rats are kept under standarized conditions and are well-cared for. BLE are factors other than severily 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. Therefore, the changes in PA formation with advancing age (Bothmer *et al.*, 1990b, 1992) found when using endogenous phospholipids as substrate, seem to be caused by age-related changes in the availability of DAG, the substrate for PA formation. However, further experimental data are needed to strengthen this

conclusion. 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.,* 1993) and shows that care should be taken when using rats, which are kept under standarized conditions and which are not exposed to environmental factors that are known to damage optimal brain functioning, as a model for human aging.

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