Journal of Neuroscience Research 81:244–252 (2005)

Phosphatidic Acid and Diacylglycerol Generation Is Regulated by Insulin in Cerebral Cortex Synaptosomes From Adult and Aged Rats

Gabriela A. Salvador, Monica G. Ilincheta de Boschero, Susana J. Pasquaré, and Norma M. Giusto*

Instituto de Investigaciones Bioquímicas de Bahía Blanca, Universidad Nacional del Sur-CONICET, Bahía Blanca, Argentina

Insulin receptor associated with the cerebral cortex (CC) has been shown to be involved in brain cognitive functions. Furthermore, deterioration of insulin signaling has been associated with age-related brain degeneration. We have reported previously that aging stimulates phospholipase D/phosphatidate phosphohydrolase 2 (PLD/PAP2) pathway in CC synaptosomes from aged rats, generating a differential availability of their reaction products: diacylglycerol (DAG) and phosphatidic acid (PA). The aim of this work was to determine the effect of aging on DAG kinase (DAGK), as an alternative pathway for PA generation, and to evaluate the effect of insulin on PLD/PAP2 pathway and DAGK. PLD, PAP2, and DAGK activities were measured using specific radiolabeled substrates in CC synaptosomes from adult (4 months old) and aged rats (28 months old). In adult animals, in the presence of the tyrosine phosphatase inhibitor (sodium *o*-vanadate), insulin stimulated PLD activity at 5 min incubation. DAGK activity was also increased at the same time of incubation and PAP2 was inhibited. In aged animals, PLD activity was not modified by the presence of insulin plus vanadate, PAP2 was inhibited, and DAGK was stimulated by the hormone. Insulin, vanadate, and the combination of both induced protein tyrosine phosphorylation in adult CC synaptosomes. Aged rats showed a lower level of protein phosphorylation with respect to adult rats. Our results show that insulin modulates PA and DAG availability through the regulation of PLD/PAP2 and DAGK pathways in adult rat CC synaptosomes. Additionally, we demonstrated that PA and DAG generation is regulated differentially by insulin during aging.

Key words: phosphatidic acid; diacylglycerol; insulin; synaptosomes; aging

Insulin fulfills many functions in the central nervous system (CNS), which are different from its classical role as a peripheral hormone. Insulin (I) and insulin receptors (IRs) are both present in the brain. Insulin is transported actively across the blood–brain barrier and it may be produced locally in the brain (Unger et al., 1991; Schulingkamp et al., 2000). These receptor-mediated functions regulate cognition and memory processes (Zhao and Alkon, 2001). IRs are located at the synapse where they regulate neurotransmitter release and receptor recruitment, indicating a potential involvement of insulin in synaptic plasticity (Abbott et al., 1999). Aged-related changes in hormone levels have a key role in a variety of human diseases. Clinical studies have demonstrated links between Alzheimer's disease (AD), insulin resistance, and diabetes mellitus, indicating that the insulin signaling pathway might directly influence the cellular and molecular events that underlie AD pathology (Gasparini et al., 2002). IRs expression is increased in Alzheimer brains whereas tyrosine kinase activity coupled to IRs is reduced (Frolich et al., 1998). Recent findings provide clear evidence that the function of the neuronal insulin/insulin receptor signal transduction cascade is of pivotal significance to maintain normal cerebral blood flow and oxidative energy metabolism. It has become evident that normal metabolism of both amyloid precursor protein and tau-protein are part of interactive processes controlled by the neuronal I/IR signal transduction cascade. In physiologic brain aging, the function of this cascade starts to fail with respect to normal adult brain. These processes as a whole may increase the vul-

Contract grant sponsor: Secretaria General de Ciencia y Tecnología, Universidad Nacional del Sur; Contract grant sponsor: Consejo Nacional de Investigaciones Cientificas y Técnicas.

*Correspondence to: Norma M. Giusto, Instituto de Investigaciones Bioquímicas de Bahía Blanca, Universidad Nacional del Sur and CONICET, C.C. 857, B8000FWB Bahía Blanca, Argentina. E-mail: ngiusto@criba.edu.ar

Received 2 February 2005; Revised 5 April 2005; Accepted 14 April 2005

Published online 9 June 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20565

nerability of the aging brain and may facilitate the generation of late-onset sporadic AD.

In the past years, strong evidence has been reported about the involvement of insulin in phospholipid signal transduction processes in nonneuronal tissues. Phospholipase D (PLD) has been implicated in insulin signaling in several cellular types (Donchenko et al., 1994; Slaaby et al., 2000; Li et al., 2003). PLD generates phosphatidic acid (PA) by phosphatidylcholine (PC) hydrolysis. PA can be hydrolyzed further by PA hydrolase (PAP) to produce diacylglycerol (DAG; Jamal et al., 1992). PLD isoforms PLD1 and PLD2 are coupled mainly to PAP2 isoforms and they generate the bioactive PA and DAG in a coupled mechanism that has been involved in multiple signal transduction processes (Massenburg et al., 1994; Liscovitch et al., 1999; Sciorra and Morris, 1999).

We have reported previously that PAP and PLD activities were regulated differentially in cerebral cortex (CC) and cerebellar subcellular fractions by the effect of aging (Pasquaré et al., 2001, 2004; Salvador et al., 2002). We also observed that aging stimulates the PLD/PAP2 pathway in cerebral cortical synaptosomes (Salvador et al., 2002). Concomitantly, DAG lipase activity has been shown to remain unchanged as age function (Salvador et al., 2002). The increase in PLD/PAP2 pathway in aged brain may provoke an increased DAG availability that could not be removed efficiently by DAG lipases (Salvador et al., 2002). Another important pathway for DAG removal is diacylglycerol kinase (DAGK) activity (Topham and Prescott, 1999). Because DAGK plays a key role in balancing cellular DAG and PA levels, its regulation represents a critical target for lipid signal transduction mechanisms.

In view of the previous data, the aim of the present work was to determine the effect of aging on DAGK activity and to evaluate whether insulin has any effect on PLD/PAP2/DAGK pathway in CC synaptosomes from adult and aged rats.

MATERIALS AND METHODS

Wistar rats were kept under constant environmental conditions and fed on a standard pellet diet. [2-^{3}H]Glycerol (200 mCi/mmol), 1,2-dipalmitoyl-sn-glycero-3-phospho[methyl-^{3}H]-choline (DPPC; 43 Ci/mmol), 1-palmitoil-2[^{14}C]arachidonoyl-sn-glycero-3-phosphocholine (55 mCi/mmol), [γ-^{32}P]ATP (3,000 Ci/mmol), and Omnifluor were obtained from New England Nuclear-Dupont (Boston, MA). All other chemicals were from Sigma-Aldrich (St. Louis, MO). Monoclonal anti-phosphotyrosine antibody PY99 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Preparation of Subcellular Fractions

Homogenates were prepared from the CC of 4-month-old (adult) and 28-month-old (aged) rats. Rats were killed by decapitation and cerebral cortices were dissected immediately (2–4 min after decapitation). Synaptosomal fraction was obtained as described previously by Cotman (1974) with slight modifications (Salvador et al., 2002).

Determination of Phosphatidylinositol-4,5-Bisphosphate-Dependent PLD Activity

For the determination of phosphatidylinositol-4,5-bisphosphate (PIP_2) dependent PLD activity, PC hydrolysis was assayed as described previously (Salvador et al., 2002). Briefly, 50 µl of mixed lipid vesicles (phosphatidylethanolamine/PIP_2/PC, molar ratio 16:1.4:1) with [choline-methyl-^{3}H]DPPC to yield 200,000 cpm per assay were added to 100 µl of synaptosomal membranes (200 µg of protein) in a total volume of 200 µl containing 50 mM HEPES (pH 7.5), 3 mM EGTA, 80 mM KCl, and 2.5 mM $MgCl_2$. The reaction was incubated at 37°C for 2, 5, or 30 min and stopped by the addition of 1 ml of chloroform/methanol/concentrated HCl (50:50:0.3 by volume) and 0.35 ml of 1 M HCl/5 mM EGTA. The aqueous phase was obtained after centrifugation for 5 min at 3,000 rpm and choline was separated from the other water-soluble products using thin-layer chromatography (TLC) on silica gel G plates and a mobile phase made up of methanol/0.5% NaCl/NH_3 (50:50:1 by volume; Salvador et al., 2002). Choline was scraped off the plate and quantified by liquid scintillation spectroscopy. For measuring the transphosphatidylation reaction catalyzed by PLD, assays were conducted in the presence of phosphatidylcholine, 1-palmitoyl-2 [^{14}C]arachidonoyl-sn-glycero-3-phosphocholine and 2% ethanol. Reactions were stopped by the addition of chloroform/methanol (2:1, by volume), mixing, and centrifugation (Folch et al., 1957). The aqueous phase was removed and the organic phase was dried under a nitrogen stream. Phosphatidylethanol (Peth) was separated by one-dimensional TLC on silica gel H and developed with chloroform/methanol/acetone/acetic acid/water (50:15:15:10:5 by volume), up to 70% of the plate. To separate the neutral lipids, the plate was rechromatographed up to the top of the plate using hexane/diethyl ether/acetic acid (70:30:2.6 by volume) as the developing solvent and the radioactivity of the lipid spots were determined as described previously (Salvador and Giusto, 1998).

Preparation of Radioactive 1,2-Diacyl-sn-Glycerol-3-Phosphate

Radioactive PA was obtained from [2-^{3}H]phosphatidylcholine, which was synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as described previously (Pasquaré de García and Giusto, 1986). Lipids were extracted from the tissue as described in Folch et al. 1957. [2-^{3}H]Phosphatidylcholine was isolated by one-dimensional TLC and eluted therefrom (Arvidson, 1968). [2-^{3}H]PC was hydrolyzed with cabbage PLD, in order to obtain [2-^{3}H]phosphatidic acid (PA). PA was purified by one-dimensional TLC on silica gel H developed with chloroform/methanol/acetic acid/acetone/water (9:3:3:12:1.5 by volume). The substrate was eluted from silica gel with neutral solvents to avoid the formation of lysophosphatidic acid and was converted subsequently into free acid by washing it twice using an upper phase containing 0.1 M sulfuric acid and then an upper phase containing water. Radioactivity and phosphorous content (Rouser et al., 1970) were measured to determine specific radioactivity. [2-^{3}H]PA had a specific radioactivity of 0.1–0.2 µCi/µmol. To determine PAP2 activity, the substrate was prepared by sonicating 0.6 mM

[2-^{3}H]PA (0.1–0.2 μCi/μmol) in 5.56 mM EGTA and 5.56 mM EDTA (Jamal et al., 1991).

Determination of PAP Activity

For the determination of NEM-insensitive PAP activity (PAP2), each assay contained 50 mM Tris-maleate buffer, pH 6.5, 1 mM DTT, 1 mM EDTA plus 1 mM EGTA, 4.2 mM NEM, and 100 μg of membrane or cytosolic protein in a volume of 200 μl. The reaction was started by adding 0.6 mM of [2-^{3}H]phosphatidate (Jamal et al., 1991).

For the determination of PAP2 activities, all assays were conducted at 37°C as a function of time (2, 5, or 30 min). The enzyme assays were stopped by adding chloroform/methanol (2:1 by volume). Blanks were prepared identically, except that membrane fractions were boiled for 5 min before being used. Lipids were extracted with chloroform/methanol (2:1 by volume) and washed with a 0.2 volume of $CaCl_2$ (0.05%; Folch et al., 1957). Neutral lipids were separated by gradient-thickness TLC on silica gel G (Giusto and Bazán, 1979) and developed with hexane/diethyl ether/acetic acid (35:65:2.6 by volume). To separate monoacylglycerol (MAG) from phospholipids, the chromatogram was rechromatographed up to the middle of the plate by using hexane/diethyl ether/acetic acid (20:80:2.3 by volume) as developing solvent. Once the chromatogram was developed, [2-^{3}H]PA and phospholipids were retained at the spotting site. Lipids were visualized by exposure of the chromatograms to iodine vapors and scraped off for counting by liquid scintillation as described previously (Pasquaré de Garcia and Giusto, 1986).

Determination of DAGK Activity

DAGK activity was determined in the synaptosomal membranes either measuring radioactive phosphate incorporation into PA using [γ-^{32}P]ATP and synaptosomal DAG as substrates or determining PA synthesis from [^{3}H]DAG as radioactive substrate and ATP. The standard assay contained in a volume of 150 μl, 50 mM HEPES, pH 7.4, 20 mM NaF, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. When radioactive DAG was added to the assay, it was resuspended in 1% dimethylsulfoxide (DMSO) and detergent was at a final assay concentration of 0.3%. Reactions were carried out at 37°C during 2, 5, and 30 min. Reactions were stopped by adding chloroform/methanol/1 N HCl (2:1:0.2 by volume) and lipids were extracted as described by Folch et al. (1957). Blanks were prepared identically, except that membrane fractions were boiled for 5 min before being used. Lipids were separated by TLC on silica gel G (Giusto and Bazán, 1979) and developed with hexane/diethyl ether/acetic acid (30:70:1 by volume). When [^{3}H]DAG was used as precursor, for separating [^{3}H]MAG, the plate was rechromatographed up to the middle by using hexane/diethyl ether/acetic acid (20:80:2.3 by volume) as developing solvent. Lipids were visualized by exposure of the chromatograms to iodine vapors and scraped off for counting by liquid scintillation spectroscopy as described bellow.

Preparation of Radioactive 1,2-Diacyl-sn-Glycerol

Radioactive DAG was obtained from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as described previously (Pasquaré de García and Giusto, 1986). Lipids were extracted from the tissue as described in Folch et al. (1957). [2-^{3}H]DAG plus cholesterol was isolated by one-dimensional TLC in silica gel G plates and with a solvent system of hexane/ether/acetic acid (60:40:2.3 by volume). Lipids were eluted (Arvidson, 1968) and DAG was purified by one-dimensional TLC on silica gel G developed with chloroform/methanol/acetic acid (98:2:1 by volume). The substrate was eluted as above from the silica gel and stored in chloroform solution to avoid the production of 1,3-diacyl-sn-glycerol.

SDS-PAGE and Immunoblot Analysis

SDS-PAGE was carried out using 7.5 or 10% gels according to Laemmli (1970). Resolved proteins were transferred to plastic-backed nitrocellulose sheets (0.2 μm) or Immobilon P membranes using a Mini Trans-Blot cell electroblotter (Bio-Rad, Hercules, CA) for 1 hr. Membranes were blocked overnight with Tris-buffered saline (20 mM Tris-HCl and 300 mM NaCl), pH 7.5, containing 0.1% Tween 20 and 5% crystalline grade bovine serum albumin (BSA). Incubations with primary antibody (monoclonal anti-phosphotyrosine antibody, PY99; Santa Cruz Biotechnology) were carried out for 2–3 hr at room temperature. Immunoreactions were detected either with horseradish peroxidase conjugated to goat anti-rabbit or goat anti-mouse IgG, followed by enhanced chemiluminescence substrates (ECL; Amersham Biosciences, Inc.). In some experiments, immunoblots were stripped by incubation in 200 mM Tris-HCl buffer, pH 6.7, containing 100 mM β-mercaptoethanol and 2% SDS for 1 hr at 50°C with gentle agitation. Blots were reblocked with 5% BSA in Tris-buffered saline and probed as described above.

Other Methods

Protein and lipid phosphorus were determined according to Bradford (1976) and Rouser et al. (1970), respectively.

Statistical Analysis

Statistical analysis was carried out using Student's *t*-test, with the values representing the mean ± standard deviation (SD) of six individual samples per condition. Each sample was obtained from a different adult or aged animal.

RESULTS

Effect of Insulin on PLD Activity in CC Synaptosomes From Adult and Aged Rats

To determine the effect of insulin on synaptosomal PIP_2-dependent PLD, enzyme activity was evaluated as a time function (2, 5, and 30 min) in the presence and absence of the hormone. PLD activity was measured using metabolically active synaptosomes from adult and aged rats as an enzyme source. Radiolabeled exogenous PC prepared as a mixed micelle with PIP_2 (4.5 μM) was used as substrate. Using choline-labeled PC, choline generation was measured as the enzyme product, whereas when PC radiolabeled in fatty acid moiety was used as substrate, Peth was quantitated as PLD product. The time-course of insulin (100 nM) effect on PLD activity in CC synaptosomes from adult rats is shown in Figure 1. At all times assayed, insulin alone (100 nM)

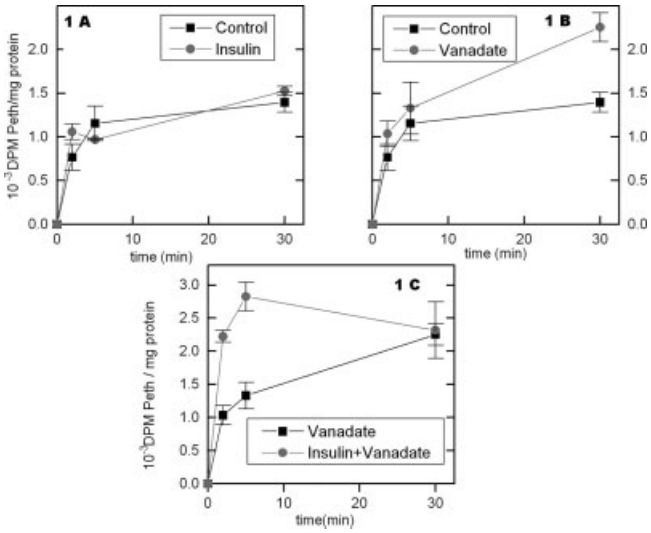

Fig. 1. Effect of insulin and vanadate on cerebral cortex synaptosomal phospholipase D (PLD) activity from adult rats. PLD activity was determined using a mixed lipid vesicle formed by phosphatidylethanolamine (PE)/phosphatidylinositol-4,5-bisphosphate (PIP2)/1,2-dipalmitoyl-sn-glycero-3-phospho[methyl-^{3}H]choline (DPPC) as substrate in a molar ratio 16:1.4:1 with [^{3}H]DPPC (200,000 cpm). PLD activity was measured as a time function in the presence of vehicle (control), insulin (**A**), vanadate (**B**), or insulin plus vanadate (**C**). The enzyme activity was expressed as DPM per mg protein of [^{3}H]choline or [^{14}C]phosphatidylethanol ([^{14}C]Peth). Each value is the mean ± standard deviation corresponding to six individual samples per condition. Each sample was obtained from a different adult animal.

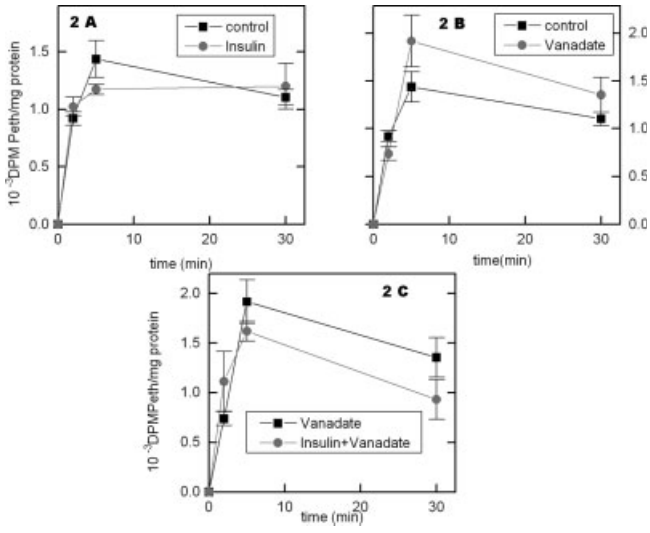

Fig. 2. Effect of insulin and vanadate on cerebral cortex synaptosomal Phospholipase D (PLD) activity from aged rats. PLD activity was determined using a mixed lipid vesicle formed by phosphatidylethanolamine (PE)/phosphatidylinositol-4,5-bisphosphate (PIP2)/1,2-dipalmitoyl-sn-glycero-3-phospho[methyl-^{3}H]choline (DPPC) as substrate in a molar ratio 16:1.4:1 with [^{3}H]DPPC (200,000 cpm). PLD activity was measured as a time function in the presence of vehicle (control), insulin (100 nM; **A**), vanadate (0.2 mM; **B**), or insulin plus vanadate (**C**). The enzyme activity was expressed as DPM per mg protein of [^{3}H]choline or [^{14}C]phosphatidylethanol ([^{14}C]Peth). Each value is the mean ± standard deviation corresponding to six individual samples per condition. Each sample was obtained from a different aged animal.

did not modify PIP_2-dependent PLD activity in adult rats with respect to control conditions (Fig. 1A). In the presence of the tyrosine phosphatase inhibitor sodium vanadate (Na_3VO_4; 0.1 mM), PLD activity was stimulated at all times assayed and was maximal at 30 min of incubation (60% increase with respect to control conditions; Fig. 1B). Insulin plus vanadate caused significant Peth production with a maximum at 5 min incubation, stimulating PLD activity by 100% with respect to the activity levels found in the presence of vanadate alone (Fig. 1C). Figure 2 shows the same experiment as that in Figure 1 except that CC synaptosomes were used from aged rats as enzyme source. As it occurred in adult animals, the presence of insulin produced no significant changes in synaptosomal PLD activity from aged animals (Fig. 2A). Vanadate slightly stimulated synaptosomal PLD activity from aged animals by 34% at 5 min of incubation, after which activity levels returned to control values at 30 min incubation (Fig. 2B). The presence of insulin plus vanadate produced no significant changes in synaptosomal PLD activity from aged animals (Fig. 2C).

Effect of Insulin on PAP2 Activity in Adult and Aged Rat Synaptosomes

PA, the product of PLD activity, can be additionally hydrolyzed to DAG by PAP2 action. One of the queries in the present research was whether insulin had any effect on DAG generation from exogenously added PA. We demonstrated previously that the only PAP isoform present in CC synaptosomes was PAP2 (Salvador et al., 2002). Figure 3 shows the effect of insulin, vanadate, and insulin plus vanadate on CC synaptosomal PAP2 activity from adult animals. Insulin alone did not produce any effect on PAP2 activity whereas vanadate inhibited PAP2 activity at all times assayed (Fig. 3A,B). Insulin plus vanadate inhibited PAP2 activity by 20% at 5 min incubation (Fig. 3C). Figure 4 shows PAP2 activity in aged rats under the same experimental conditions depicted in Figure 3. PAP2 activity was not modified by insulin nor by vanadate at any time of incubation compared to control conditions (Fig. 4A,B). Figure 4C shows that insulin plus vanadate inhibits the enzymatic activity to the same extent as that observed in adult animals at 2 min of incubation.

DAGK Activity in Synaptosomes From Adult and Aged Rats

To evaluate the levels of DAGK activity in CC synaptosomes from adult and aged rats, two experimental approaches were followed. In the presence of [γ-^{32}P]ATP, we could measure DAGK activity using synaptosomal endogenous DAG. This type of assay depends on the DAG availability in the system because

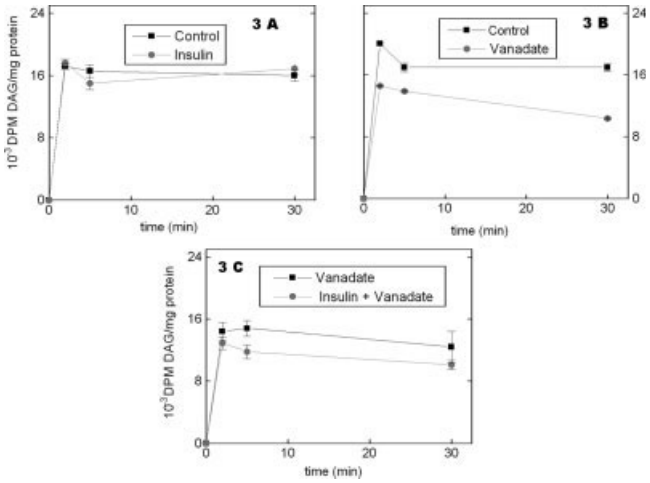

Fig. 3. Effect of insulin and vanadate on cerebral cortex synaptosomal phosphatidic acid (PA) hydrolase (PAP)2 activity from adult animals. For the determination of PAP2 activity, the assay was carried out by preincubating the membranes with 4.2 mM NEM for 10 min. The reaction was started by adding 0.6 mM [2-^{3}H]PA. PAP2 activity was measured as a time function in the presence of vehicle (control), insulin (100 nM; **A**), vanadate (0.2 mM; **B**) or insulin plus vanadate (**C**).The enzyme activity was expressed as DPM per mg protein of [2-^{3}H]diacylglycerol (DAG) plus [2-^{3}H]monoacylglycerol (MAG). Each value is the mean ± standard deviation corresponding to six individual samples per condition. Each sample was obtained from six different adult animals.

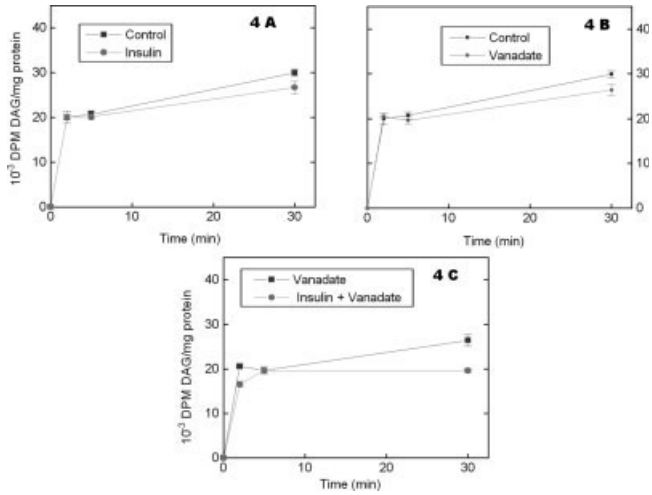

Fig. 4. Effect of insulin and vanadate on cerebral cortex synaptosomal phosphatidic acid (PA) hydrolase (PAP)2 activity from aged animals. For the determination of PAP2 activity, the assay was carried out by preincubating the membranes with 4.2 mM NEM for 10 min. The reaction was started by adding 0.6 mM [2-^{3}H]PA. PAP2 activity was measured as a time function in the presence of vehicle (control), insulin (100 nM; **A**), vanadate (0.2 mM; **B**) or insulin plus vanadate (**C**). The enzyme activity was expressed as DPM per mg protein of [2-^{3}H]diacylglycerol (DAG) plus [2-^{3}H]monoacylglycerol (MAG). Each value is the mean ± standard deviation corresponding to six individual samples per condition. Each sample was obtained from six different aged animals.

DAG generated by different enzyme pathways (PLD/PAP, PC-PLC, and PIP_2-PLC) could be DAGK substrate. In the micellar assay, exogenously added DAG in DMSO was used. In this case, PA generation is independent of the endogenous DAG levels. Figure 5 shows DAGK activity in CC synaptosomes from adult and aged rats, using the two DAGK assays. In the presence of [γ-^{32}P]ATP, synaptosomal PA synthesis from aged rats was decreased 40% with respect to values found in adult animals (Fig. 5A). Using the micellar assay, DAGK inhibition by aging was more pronounced. Under these assay conditions, PA generation decreased by 60% with respect to the values found in adult animals (Fig. 5B).

Insulin Action on DAGK Activity in Synaptosomes From Adult and Aged Rats

Figure 6 shows insulin action in DAGK activity in adult rats under experimental conditions similar to those described previously for PLD and PAP2. To evaluate insulin effect on DAGK activity, the micellar assay containing DMSO was used as detergent. The enzyme activity was stimulated significantly by insulin at 5 min incubation (46%), as compared to that with control conditions (Fig. 6A). The presence of vanadate alone in the incubation medium also stimulated DAGK activity with respect to control conditions (Fig. 6B). Vanadate effect on DAGK was also evaluated through [^{32}P] incorporation from [γ-^{32}P]ATP into PA. Under this experimental condition, DAGK activity was stimulated strongly (by 100%) with respect to control conditions (Fig. 7D). The percentage of DAGK stimulation by insulin and vanadate was the same; however, insulin plus vanadate exerted an effect similar to that exerted in the presence of vanadate alone (Fig. 6C). The same experiment carried out in aged rats is depicted in Figure 7. Insulin stimulated DAGK activity by 50% with respect to control conditions at 5 min incubation (Fig. 7A). As it was shown in Figure 5, however, DAGK activity levels in aged animals were lower than were those in adult animals. Vanadate strongly stimulated DAGK activity at short incubation times (2 and 5 min; Fig. 7B). In the presence of [γ-^{32}P]ATP, vanadate did not activate synaptosomal DAGK from aged animals, whereas when [^{3}H]DAG was used as substrate, the tyrosine phosphatase inhibitor stimulated DAGK from aged animals to the same extent as that in adult animals (Fig. 7D). Insulin slightly increased the vanadate-stimulated DAGK activity at 30 min incubation (Fig. 7C).

Effect of Insulin and Vanadate on Synaptosomal Protein Tyrosine Phosphorylation From Adult and Aged Rats

To evaluate the effect of insulin and vanadate on synaptosomal protein tyrosine phosphorylation, synaptosomes from adult and aged rats were incubated under the above-described conditions. After incubation, samples were lysed in Laemmli buffer and subjected to SDS-PAGE. Levels of tyrosine phosphorylation were eval-

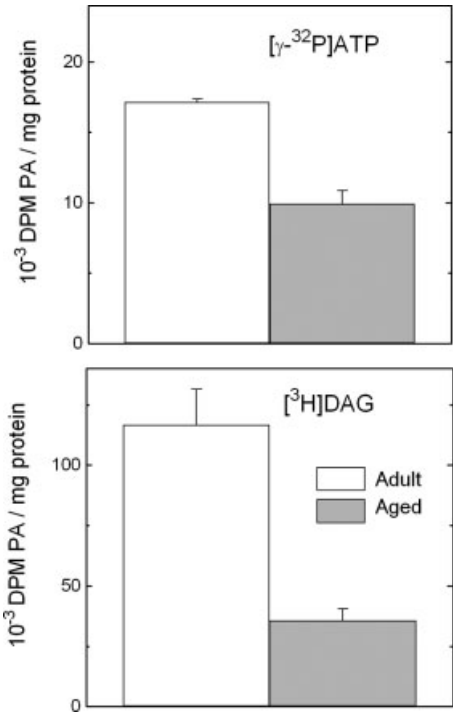

Fig. 5. Diacylglycerol (DAG) kinase (DAGK) activity in cerebral cortex synaptosomes from adult and aged rats. DAGK activity was determined in synaptosomal membranes from cerebral cortex. The standard assay contained in a volume of 150 μl, 200 μg of synaptosomal protein resuspended in 50 mM HEPES, pH 7.4; 20 mM NaF; 10 mM $MgCl_2$; 1 mM DTT, as follows: enzymatic activity was measuring [^{32}P] incorporation into phosphatidic acid (PA) through [γ-^{32}P]ATP (60 μM ATP) and synaptosomal DAG as substrates (**A**) or PA synthesized from [^{3}H]DAG as radioactive substrate (20 μM) and 1 mM ATP (**B**). Data were the mean ± standard deviation from three individual samples and are expressed as DPM per mg synaptosomal protein.

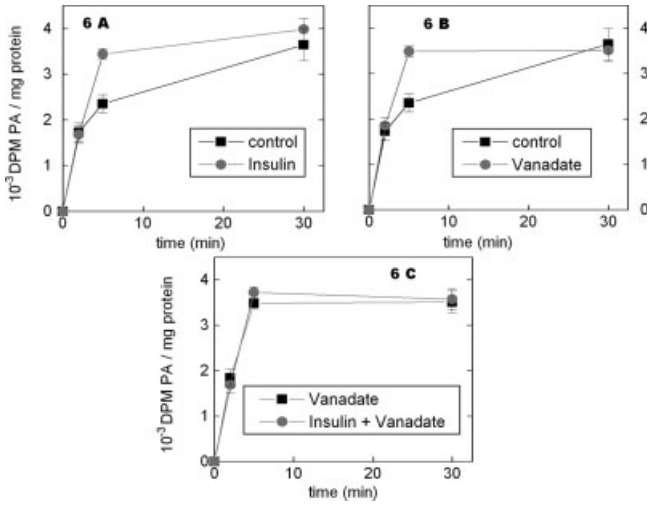

Fig. 6. Effect of insulin and vanadate on cerebral cortex synaptosomal diacylglycerol (DAG) kinase (DAGK) activity from adult rats. DAGK activity was measured as specified in Figure 5B, using a micellar assay containing DMSO (final concentration: 0.3%) [^{3}H]DAG (30,000 DPM/sample and specific activity A: 5,000 DPM/nmol) and 1 mM ATP. Enzyme activity was measured as a time function in the presence of vehicle (control), insulin (100 nM; **A**), vanadate (0.2 mM; **B**), or insulin plus vanadate (**C**). Data were the mean ± standard deviation from three individual adult animals and they are expressed as DPM per mg protein.

uated by Western blot using an anti-phosphotyrosine antibody (PY99).

Insulin (100 nM) alone induces tyrosine phosphorylation of different molecular weight proteins in synaptosomes from adult rats. The presence of vanadate alone also produced an increased level in protein tyrosine phosphorylation. Insulin plus vanadate enhanced protein tyrosine phosphorylation. This effect was more pronounced at early times of incubation (2 and 5 min; Fig. 8). When tyrosine phosphorylation was evaluated in aged membranes under basal conditions, levels of protein phosphorylation were lower than were those observed in adult animals. Vanadate and insulin stimulated tyrosine protein phosphorylation as a function of time, in agreement with the results obtained for adult animals. Under all conditions assayed, however, aged rats showed a lower level of protein phosphorylation than adult rats did (Fig. 8).

DISCUSSION

We present evidence indicating that insulin regulates PA and DAG generation by different enzymatic pathways in CC synaptosomes from adult rats. Additionally, we demonstrate a different insulin effect on PLD, PAP2, and DAGK activities in aged rat synaptosomes.

The insulin effect on PA production in synaptosomal membranes was evaluated by measuring PLD and DAGK activities. Peth formation by PLD activity was stimulated by insulin in the presence of vanadate at 2 and 5 min incubation. The maximal levels of PA production by DAGK in the presence of insulin were observed at the same time as described by PLD. These data are in accordance with previous findings that demonstrate an insulin-targeted first wave of PA production through PLD activation in rat hepatocytes (Donchenko et al., 1994). Additionally, insulin plus vanadate inhibited PAP2 activity at all incubation times. The analysis of the results observed in adult rat synaptosomes led us to infer that in the presence of insulin, the principal event is PA formation. This occurred by the stimulation of two different pathways: (1) PC breakdown by PC-PLD; and (2) DAG phosphorylation by DAGK activity. It is therefore possible either that DAGK operates downstream PAP2 for the ''recovery'' of PA generated by PLD/PAP2 pathway or that insulin initiates several signaling pathways mediated by different PA species through PLD and DAGK activation.

PA itself is an intracellular lipid second messenger that mediates multiple biological effects. It induces DNA synthesis, myc/fos expression, and hormone secretion (Yu et al., 1988; Van Corven et al., 1992). Different cellular targets for PA include PI-4-kinase, PKCz, MAP kinase, protein tyrosine phosphatases, and raf-1 (Moritz

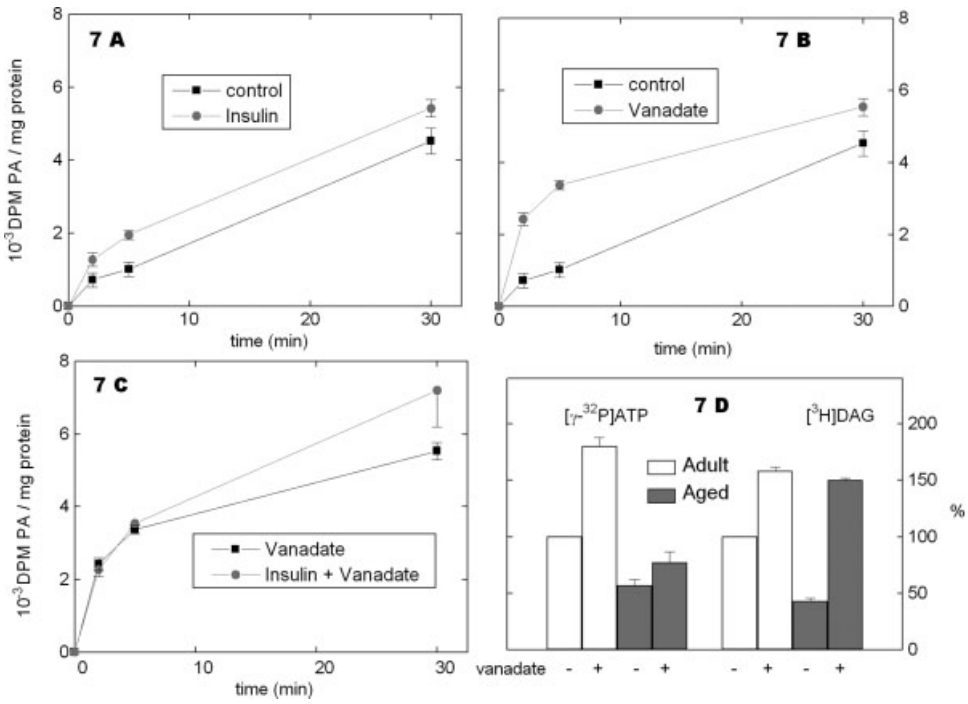

Fig. 7. Effect of insulin and vanadate on cerebral cortex synaptosomal diacylglycerol (DAG) kinase (DAGK) activity from aged rats. DAGK activity was measured as specified in Figure 5B using a micellar assay containing DMSO (final concentration 0.3%), [^{3}H]DAG (30,000 DPM/sample and specific activity A: 5,000 DPM/nmol) and 1 mM ATP. Enzyme activity was measured as a time function in the presence of vehicle (control), insulin (100 nM; **A**), vanadate (0.2 mM; **B**), or insulin plus vanadate (**C**). Data were the mean ± standard deviation from three individual aged animals and are expressed as DPM per mg protein. **D:** Effect of vanadate on DAGK activity from adult and aged animals using the two experimental assays as specified in Figure 5. Data are presented as percentages with respect to controls.

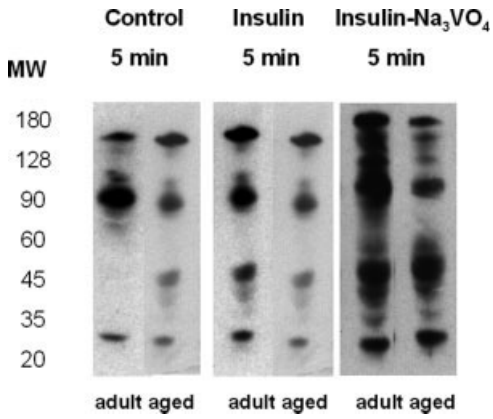

Fig. 8. Effect of insulin on protein tyrosine phosphorylation in cerebral cortex synaptosomes from adult and aged rats. Synaptosomal fraction was incubated in the presence of vehicle (control), insulin (100 nM), and vanadate (0.2 mM). After incubation, synaptosomes were boiled in Laemmli buffer and proteins were resolved by SDS-PAGE and transferred to Immobilon membranes. Adult: Immunoblot of synaptosomal fraction from adult rats using anti-phosphotyrosine antibody PY99 (1:500; Santa Cruz Biotechnology). Each line contained 50 μg protein. Aged: Immunoblot of synaptosomal fraction from aged rats using anti-phosphotyrosine antibody PY99. Each line contained 50 μg protein.

et al., 1992; Zhao et al., 1993; Jenkins et al., 1994; Limatola et al., 1994; Ghosh et al., 1996). It has been reported that the acyl groups composition of PA produced from PC contains mainly saturated and monounsaturated species whereas PA produced by DAGK activity may be saturated or unsaturated (Pettitt et al., 2001). The potency of PA in cell systems in which species containing different fatty acyl chains have been tested generally demonstrates a marked effect for species containing longer fatty acids with higher degrees of unsaturation (Kraback et al., 1991; Pearce et al., 1994; El Bawab et al., 1995).

We found that basal DAGK activity is inhibited strongly in CC synaptosomes from aged rats. This activity measured through PA synthesis from [γ^{32}P]ATP represented 60% of values from adult rats. As mentioned above, this experiment led us to evaluate DAGK activity with the endogenous DAG available. In addition, we have reported that PLD/PAP2 pathway is increased in synaptosomes from aged rats, thus suggesting increased DAG availability (Salvador et al., 2002). DAGK inhibition in aged rats may indicate: (1) that DAGs from PC are not DAGK substrates; (2) that DAGK activity is properly decreased; or (3) DAG production and DAG phosphorylation occur in different compartments. Taking into account that only DAGKε presents substrate selectivity (DAG containing stearoyl-arachidonoyl species), option 1 may be ruled out. PA synthesis measured by using exogenous DAG showed a more pronounced inhibition than did that found with [γ-^{32}P]ATP and endogenous DAG. This may indicate that DAGs produced by PLD/PAP pathway are DAGK substrates. The basal increase in DAG production by the PLD/PAP2 pathway along with the inhibition of DAGK activity pointed to a higher availability of DAG during aging.

It has been reported recently that endogenous DAG inhibits the nicotine-induced current in neuronal nicotinic acetylcholine receptor (nAChR; Andoh et al., 2004). This finding was reported as a novel, PKC-independent nAChR regulatory pathway. The previously

described DAG/PA imbalance through PLD/PAP2 pathway during aging (Salvador et al., 2002) as well as the DAGK inhibition reported in the present study could be related to the modulatory mechanism exerted by DAG in the nAChR function.

Despite DAGK stimulation by insulin, in aged animals this hormone modified neither PLD nor PAP2 activities. The absence of insulin effects on PLD activity in aged rat synaptosomes and the basal DAGK inhibition suggest a strong alteration in the DAG/PA balance during aging. These findings suggest that PA signaling pathways are affected strongly by aging in CNS. It has been reported recently that PA produced by PLD is implicated in the exocytosis regulation in various secretory cell types (Humeau et al., 2001; Vitale et al., 2001). More precisely, PLD1 accumulates in neuronal regions that are known to exhibit high levels of exo-endocytosis activities, such as varicosities that release neurotransmitter and growth cones that sprout or retract by membrane incorporation or retrieval (Humeau et al., 2001).

Insulin, vanadate, or the combination of both induced tyrosine phosphorylation of different molecular weight proteins in adult membranes whereas a lower degree in tyrosine phosphorylation occurred in aged synaptosomes. PLD activity was stimulated by the presence of vanadate in adult animals; however, the enzyme activity was inhibited in synaptosomes from aged animals. Interestingly, vanadate inhibited PAP2 and stimulated DAGK activity in aged animals. It can therefore be concluded that non-receptor-mediated tyrosine phosphorylation promotes different pathways for PA generation as an age function. The results presented here suggest that in adult rats, synaptosomal PA comes from PLD, whereas in aged animals synaptosomal PA is generated through DAGK activity.

PA inhibits the insulin receptor tyrosine kinase activity in detergent lipid micelles or reconstituted membranes (Arnold and Newton, 1996). This selective sensitization suggests that the receptor-mediated generation of PA in the plasma membrane is a negative modulator of insulin receptor function (Arnold and Newton, 1996). Taking into account this fact and the results reported in the present study, the insulin modulation of PA generation by PLD and DAGK seems to have a key role in insulin-mediated signaling in CNS.

REFERENCES

Abbott MA, Wells DG, Fallon JR. 1999. The insulin receptor tyrosine kinase substrate p58/53 and the insulin receptor are components of CNS synapses. J Neurosci 19:7300–7308.

Andoh T, Itoh H, Higashi T, Saito Y, Ishiwa D, Kamiya Y, Yamada Y. 2004. PKC-independent inhibition of neuronal nicotinic acetylcholine receptors by diacylglycerol. Brain Res 1013:125–133.

Arnold RS, Newton AC. 1996. Inhibition of the insulin receptor tyrosine kinase by phosphatidic acid. J Cell Biochem 62:516–528.

Arvidson GA. 1968. Structural and metabolic heterogeneity of rat liver glycerophosphatides. Eur J Biochem 4:478–486.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Cotman CW. 1974. Isolation of synaptosomal and synaptic plasma membrane fractions. Methods Enzymol 31:445–452.

Donchenko V, Zannetti A, Baldini PM. 1994. Insulin-stimulated hydrolysis of phosphatidylcholine by phospholipase C and phospholipase D in cultured rat hepatocytes. Biochim Biophys Acta 1222:492–500.

El Bawab S, Macovschi O, Lagarde M, Prigent AF. 1995. Time-course changes in content and fatty acid composition of phosphatidic acid from rat thymocytes during concanavalin A stimulation. Biochem J 308:113–118.

Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509.

Frolich L, Blum-Degen D, Bernstein HG, Engelsberger S, Humrich J, Laufer S, Muschner D, Thalheimer A, Turk A, Hoyer S, Zochling R, Boissl KW, Jellinger K, Riederer P. 1998. Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease. J Neural Transm 105:423–438.

Gasparini L, Netzer W, Greengard P, Xu H. 2002. Does insulin dysfunction play a role in Alzheimer's disease? Trends Pharmacol Sci 23:288–292.

Ghosh S, Strum JC, Sciorra VA, Daniel L, Bell RM. 1996. Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. J Biol Chem 271:8472–8480.

Giusto NM, Bazán NG. 1979. Phospholipids and acylglycerols biosynthesis and ^{14}C production from [^{14}C]glycerol in the bovine retina: the effects of incubation time, oxygen and glucose. Exp Eye Res 29:155–168.

Humeau Y, Vitale N, Chasserot-Golaz S, Dupont JL, Du G, Frohman MA, Bader MF, Poulain B. 2001. A role for phospholipase D1 in neurotransmitter release. Proc Natl Acad Sci USA 98:15300–15305.

Jamal Z, Martin A, Gomez-Muñoz A, Brindley DN. 1991. Plasma membrane fractions from rat liver contain a phosphatidate phosphohydrolase distinct from that in the endoplasmic reticulum and cytosol. J Biol Chem 266:2988–2996.

Jamal Z, Martin A, Gomez-Muñoz A, Hales P, Chang E, Russell JC, Brindley DN. 1992. Phosphatidate phosphohydrolases in liver, heart and adipose tissue of the JCR:LA corpulent rat and lean genotypes: implications for glycerolipid synthesis and signal transduction. Int J Obes Relat Metab Disord 16:789–799.

Jenkins GH, Fisette PL, Anderson RA. 1994. Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. J Biol Chem 269:11547–11554.

Li HS, Shome K, Rojas R, Rizzo MA, Vasudevan C, Fluharty E, Santy LC, Casanova JE, Romero G. 2003. The guanine nucleotide exchange factor ARNO mediates the activation of ARF and phospholipase D by insulin. BMC Cell Biol 4:13.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Limatola C, Schaap D, Moolenaar W H, van Blitterswijk 1994. Phosphatidic acid activation of protein kinase C-ζ overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. Biochem J 304:1001–1008.

Liscovitch M, Czarny M, Fiucci G, Lavie Y, Tang X. 1999. Localization and possible functions of phospholipase D isozymes. Biochim Biophys Acta 1439:245–263.

Massenburg D, Han JS, Liyanage M, Patton WA, Rhee SG, Moss J, Vaughan M. 1994. Activation of rat brain phospholipase D by ADP-ribosylation factors 1, 5, and 6: separation of ADP ribosylation factor-dependent and oleate-dependent enzymes. Proc Natl Acad Sci USA 91:11718–11722.

Moritz A, DeGraan PN, Gispen WH, Wirtz KW. 1992. Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase. J Biol Chem 267:7207–7210.

Pasquaré de García SJ, Giusto NM. 1986. Phosphatidate phosphatase activity in isolated rod outer segment from bovine retina. Biochim Biophys Acta 875:195–202.

Pasquaré SJ, Ilincheta de Boschero MG, Giusto NM. 2001. Aging promotes a different phosphatidic acid utilization in cytosolic and microsomal fractions from brain and Liver. Exp Gerontol 36:1387–1401.

Pasquaré SJ, Salvador GA, Giusto NM. 2004. Phospholipase D and phosphatidate phosphohydrolase activities in rat cerebellum during aging. Lipids 39:553–560.

Pearce B, Jakobson K, Morrow C, Murphy S. 1994. Phosphatidic acid promotes phosphoinositide metabolism and DNA synthesis in cultured cortical astrocytes. Neurochem Int 24:165–171.

Pettitt TR, McDermott M, Saqib KM, Shimwell N, Wakelam MJ. 2001. Phospholipase D1b and D2a generate structurally identical phosphatidic acid species in mammalian cells. Biochem J 360:707–715.

Rouser G, Fleischer S, Yamamoto A. 1970. Two dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5:494–496.

Salvador GA, Giusto NM. 1998. Characterization of phospholipase D activity in bovine photoreceptor membranes. Lipids 33:853–860.

Salvador GA, Pasquaré SJ, Ilincheta de Boschero MG, Giusto NM. 2002. Differential modulation of phospholipase D and phosphatidate phosphohydrolase during aging in rat cerebral cortex synaptosomes. Exp Gerontol 37:543–552.

Schulingkamp RJ, Pagano TC, Hung D, Raffa RB. 2000. Insulin receptors and insulin action in the brain: review and clinical implications. Neurosci Biobehav Rev 24:855–872.

Sciorra VA, Morris AJ. 1999. Sequential actions of phospholipase D and phosphatidic acid phosphohydrolase 2b generate diglyceride in mammalian cells. Mol Biol Cell 10:3863–3876.

Slaaby R, Du G, Altshuller YM, Frohman MA, Seedorf K. 2000. Insulin-induced phospholipase D1 and phospholipase D2 activity in human embryonic kidney-293 cells mediated by the phospholipase C gamma and protein kinase C alpha signalling cascade. Biochem J 3:613–619.

Topham MK, Prescott SM. 1999. Mammalian diacylglycerol kinases with signaling functions. J Biol Chem 274:11447–11450.

Unger JW, Livingston JN, Moss AM. 1991. Insulin receptors in the central nervous system: localization, signalling mechanisms and functional aspects. Prog Neurobiol 36:343–362.

Van Corven EJ, Van Rijswik A, Jalink K, Van Der Bend RL, Van Blitterswijk WJ, Moolenaar W H. 1992. Mitogenic action of lysophosphatidic acid phosphatidic acid on fibroblasts. Dependence on acyl-chain length and inhibition by suramin. Biochem J 281:163–169.

Vitale N, Caumont AS, Chasserot-Golaz S, Du G, Wu S, Sciorra VA, Morris AJ, Frohman MA, Bader MF. 2001. Phospholipase D1: a key factor for the exocytotic machinery in neuroendocrine cells. EMBO J 20:2424–2434.

Yu CL, Tsai MH, Stacey DW. 1988. Cellular ras activity and phospholipid metabolism. Cell 52:63–71.

Zhao WQ, Alkon DL. 2001. Role of insulin and insulin receptor in learning and memory. Mol Cell Endocrinol 177:125–134.

Zhao Z, Shen SH, Fischer EH. 1993. Stimulation by phospholipids of a protein-tyrosine-phosphatase containing two src homology 2 domains. Proc Natl Acad USA 90:4251–4255.