Insulin modifies aging-related inhibition of 1-stearoyl, 2-arachidonoylglycerol phosphorylation in rat synaptic terminals

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ABSTRACT

The purpose of the present study was to analyze diacylglycerol kinase (DAGK) activity in synaptic terminals from cerebral cortex (CC) and hippocampus (Hp) from adult (3–4 month-old) and aged (26–28 month-old) rats. The effect of insulin through DAGK activity on synaptosomes from adult and aged rats was also analyzed under conditions favoring saturated or unsaturated phosphatidic acid (PA) formation, using exogenous di-palmitoil glycerol (DPG) or 1-stearoyl-2-arachidonoylglycerol (SAG) as substrates. Results showed that the enzymatic activity preferentially uses SAG as substrate, thus indicating the presence of ε-type DAGK. A significant decrease in DAGK activity transforming SAG into PA was also observed in both tissues from aged rats. Western blot detection of DAGKε showed that enzyme content undergoes no changes with aging.

[3H] inositol incorporation into phosphoinosites was also analyzed to evaluate the role of DAGKε in their synthesis. Data obtained from 3H-inositol incorporation into phosphoinositides revealed that in synaptosomes from aged rats phosphatidylinositol (PI) synthesis is lower than in adult animals. Interestingly, in the presence of SAG, PI synthesis was restored to adult values.

DAGK activity over SAG was more highly stimulated by insulin in CC and Hp synaptosomes of aged rats with respect to adult rats. On the other hand, insulin exerted a stimulatory effect on PI and phosphatidylinositol 4 phosphate (PI(4)P) synthesis in synaptosomal CC from aged rats. Taken together, our findings indicate that in aged rats insulin triggers a stimulatory mechanism that reverts the diminished synaptosomal ability to synthesize arachidonoyl phosphatidic acid (20:4 PA). The recovery of this PA species indicates that insulin positively regulates phosphoinositide synthesis.

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1. Introduction

In contrast to early onset familial Alzheimer disease, aging is the main risk factor for late-onset sporadic Alzheimer disease. Brain aging is associated with a multitude of inherent changes in cerebral glucose/energy metabolism, its control and related pathways at cellular, molecular and genetic levels. Brain insulin receptors (IRs) are present in particularly high concentrations in neurons and abundant IRs are found in cell bodies and synapses (Abbott et al., 1999). Several studies have drawn links between insulin signaling and intracellular trafficking and plasma membrane expression of ion channels and neurotransmitter receptors at the central nervous system, mainly cerebral cortex and hippocampus synapses (Wan et al., 1997; Zhao et al., 1999). At the synapse, IR regulates neurotransmitter release and receptor recruitment, thus indicating a potential involvement of insulin in synaptic plasticity (Abbott et al., 1999). However, the molecular mechanism by which insulin exerts these beneficial effects has not been elucidated to date.

Both density of insulin receptor and brain insulin level decrease with aging (Laron, 2009; Stolk et al., 1997). A statistically significant decrease in the number of insulin high-affinity binding sites in whole brain of old BALB:c-nu mice has also been reported (Zaia and Piantanelli, 2000). In view of the above, the purpose of the present research was to study insulin transduction mechanisms in brain aging and their relation to PA generation through DAGK activity.

PA is an intracellular lipid second messenger that mediates multiple biological effects. It induces DNA synthesis, myc/fos expression, and hormone secretion (van Corven et al., 1992; Yu et al., 1988). Different cellular targets for PA include PI-4-kinase, PKCζ, mitogen-activated protein kinase (MAP kinase), protein tyrosine phosphatases, and raf-1 (Ghosh et al., 1996; Jenkins et al.,

1994; Limatola et al., 1994; Moritz et al., 1992; Zhao et al., 1993). It has been reported that the acyl group composition of PA produced from phosphatidylcholine (PC) contains mainly saturated and monounsaturated species whereas PA derived from DAGK activity can be either saturated or polyunsaturated (Pettitt et al., 2001). The potency of PA in cell systems in which species containing different fatty acyl chains have been tested is, in general, more marked for species containing longer fatty acids with higher degrees of unsaturation (el Bawab et al., 1995; Krabak and Hui, 1991; Pearce et al., 1994). In addition, based on the substrate preference and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) regulatory properties of cytidine diphosphate diacylglycerol synthase (CDS) cloned from rat brain cDNA library, previous research has suggested a significant contribution of PA species with arachidonate to CDP-DAG and phosphoinositide synthesis (Saito et al., 1997). DAGKε is fully committed to the PI turnover cycle in the enrichment of its lipids with arachidonate through multiple iterations of this cycle (Lung et al., 2009).

Based on the action of insulin on different enzymes for PA and DAG processing in CC synaptosomes from adult rats, we have previously reported that after insulin exposure PA formation is stimulated in CC synaptosomes and that PLD and DAGK activities are strongly stimulated by insulin (Salvador et al., 2005). The stimulatory effect of insulin on DAGK activity depends on tyrosine kinase activity (Zulian et al., 2009). Our results with phospholipase inhibitors indicate that DAGK acts in response to increased DAG generated by PIP2-specific-phospholipase C (PIP2-PLC) and phospholipase D-phosphatidate phosphohydrolase type 2 (PLD-PAP2) pathways, this action being reinforced by an additional insulin-induced DAGK activation mechanism (Zulian et al., 2006).

In view of the above, we carried out studies on CC and Hp synaptic endings to analyze the effect of aging and insulin action on PA synthesis from exogenous saturated (1,2-dipalmitoylglycerol, DPG) and unsaturated (1-stearoyl-2-arachidonoylglycerol, SAG) diacylglycerol. Our experimental model represents conditions under which DAG from either PC-PLD or PIP2-PLC was present, enabling us to study insulin action on PA formation independently of its action on DAG production. Based on the potential role of 20:4-PA on PI synthesis, phosphoinositide labeling through [3H] inositol was also analyzed in this experimental model.

2. Materials and methods

2.1. Animals and tissue processing

Four-month-old (adult) and 24–28-month-old (aged) Wistar-strain rats were used. They were housed under controlled conditions (constant room temperature, 12-h light/12-h dark cycle), bred in our colony and fed on a standard rat chow diet with free access to water. All procedures were in strict accordance with the guidelines published in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

The rats were killed by decapitation and CC and Hp were immediately dissected from rat brain hemispheres (2–4 min after decapitation). Synaptosomal fraction was obtained following Cotman (1974) with slight modifications (Salvador et al., 2002; Uranga et al., 2007).

2.2. Materials

Antibodies for DAGK were generously provided by Dr. Matthew K. Topham from Huntsman Cancer Institute, University of Utah. The radioactive substrates [γ-32P] ATP (3000 Ci/mmol) and [3H] inositol and the Omnifluor were obtained from New England Nuclear-Dupont (Boston, MA). All the other chemicals used in the present research were from Sigma Aldrich (St. Luis, MO).

2.3. Determination of DAGK activity

DAGK activity was determined in purified synaptosomal membranes measuring radioactive PA by using 3 μCi [γ-32P] ATP and either endogenous DAG or DPG or SAG as exogenous substrates. The standard assay contained 0.1% dimethyl sulfoxide (DMSO) or 50 mM octyl-β-glucopyranoside (OG), 50 mM Hepes, pH 7.4, 10 mM MgCl2, 1 mM DTT and 500 μM ATP in a volume of 200 μl. When DAG acyl chain

selectivity of DAGK was measured, ATP concentration was 4 mM and 10 μCi [γ-32P] ATP to ensure saturating concentration of this co-substrate. When exogenous DAG was added to the assay, an appropriate volume of DAG stock solution was evaporated under a stream of nitrogen in a glass test tube. It was then re-suspended in DMSO or in OG and the detergent–lipid mixture was sonicated in a test tube placed in a Cup Horn system (Branson Digital Sonifier, model 450). Prior to incubation, synaptosomes (300 μg protein) were sonicated during 15 s with radioactive ATP and detergent–lipid suspension in a water bath (Branson Sonifier). Reactions were performed at 37 °C during 5 min. Blanks were prepared identically, except that membrane fractions were boiled for 5 min before use.

For a standard assay, when [γ-32P] ATP was used as radioactive substrate, reactions were stopped by adding chloroform/methanol/1 N HCl (2:1:0.2, by volume) and lipids were extracted following Folch et al. (1957). Five additional washes of the lipid extract for [γ-32P] ATP elimination were carried out using theoretical upper phase. PA, PI(4)P and PI(4,5)P2 were separated by TLC on 1% potassium oxalate in silica gel H developed with chloroform/acetone/methanol/ acetic acid/water (40:15:13:12:7.5, by vol) (Ilincheta de Boschero and Giusto, 1992). Lipids were visualized by exposure of the chromatograms to iodine vapors, identified with appropriate lipid standards and scraped off for counting by liquid scintillation spectroscopy.

Proteins were determined according to Bradford's method (1976).

PA mass units (pmol) were calculated on the basis of the specific activity of ATPγ32P (X dpm/pmol P equivalent to X dpm/pmol PA). A scintillation counter program (Wallac Oy, model RackBetas 1214), which calculates real dpm taking into account the reference date and half life of the radioisotope (14.29 d), permitted us to obtain dpm of 32P. In our assay, ATP (4 mM or 0.5 mM) was used to dilute radioactive ATPγ32P and this concentration was used to calculate the specific activity of ATPγ32P (expressed as dpm/pmol ATP). ATP endogenous concentration was not taken into account because it was too low with respect to exogenous concentration. Phospholipid mass (expressed as pmol of PA) was equivalent to pmol of P from PA.

2.4. Inositol lipid synthesis

To evaluate inositol lipid synthesis, reactions were carried out in the presence of 7 μCi of [3H] inositol (AE 16.5 Ci/mmol) as radioactive substrate and 500 μM of cytidine-5′- triphosphate (CTP) as cofactor. The standard assay contained 0.1% DMSO, 50 mM Hepes, pH 7.4, 10 mM MgCl2, 1 mM DTT and 0.5 mM ATP in a volume of 200 μl. The assay was performed in the presence of either endogenous or exogenous DAG (DPG or SAG).

Detergent–lipid mixtures (DPG or SAG conditions) were carried out as described above. Prior to incubation, synaptosomes (300 μg protein) were sonicated during 15 s with ATP and detergent–lipid suspension in a water bath (Branson Sonifier). Reactions were performed at 37 °C during 5 min. Blanks were prepared identically, except that membrane fractions were boiled for 5 min before use.

[3H]PI, [3H]PI(4)P and [3H]PI(4,5)P2 were separated as described above using TLC. In the case of PIP2, PIP and PI labeling (Figs. 6 and 7) with 3H inositol, specific activity was not calculated because endogenous inositol concentration was not determined. Incorporation into inositol lipids was expressed as dpm/mg of protein.

2.5. Synaptosomal integrity and DAG inclusion

Synaptosomal fraction purity was routinely controlled by transmission electron microscopy. The integrity of incubated synaptosomes under 0.1% DMSO (endogenous DAG condition) or DPG–DMSO or SAG–DMSO conditions was also evaluated by transmission electron microscopy. Nile red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, is an intensely fluorescent dye with unique properties making it an ideal stain for the fluorescence detection of lipids (Fowler and Greenspan, 1985). Nile red fluorescence varies depending on the relative hydrophobicity of the surrounding environment. When it is dissolved in hydrocarbon solvents such as heptane or in neutral lipids such as triacylglycerol, diacylglycerol or cholesteryl ester droplets, Nile red fluoresces yellow-gold. When it is dissolved in polar solvents such as ethanol or in phospholipids such as phospholipids from synaptic terminal membranes, it fluoresces red. In aqueous media, however, Nile red fluorescence is totally quenched. It has been demonstrated that by taking advantage of the variation in fluorescence colors at 488 nm of wavelength, Nile red can be used as a selective stain to detect cytoplasmic lipid droplets in living cells by fluorescence microscopy and flow cytofluorometry (Greenspan et al., 1985). The fluorescent hydrophobic probe Nile red was used to demonstrate intrasynaptosomal micelle DAG–detergent incorporation. Synaptosomes were treated with 0.1% DMSO or DAG–DMSO micelle and sonicated for 15 s. Synaptosomes were then fixed with paraformaldehyde (2%) at room temperature for 30 min. After washing three times at room temperature with PBS, synaptosomes were treated with Nile red (1 μl/ml). An aliquot of the suspension was analyzed by fluorescence microscopy at 488 nm of wavelength.

2.6. DAGKε detection into sealed synaptosomes from CC

For the analysis of purified synaptosomes from cerebral cortices of adult rats they were resuspended in Hepes buffer saline (HBS) containing 10 mM HEPES,

142 mM NaCl, 2.4 mM KCl, 1.2 mM K$_2$HPO$_4$, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM D-glucose, pH 7.4. An equal suspension was fixed with paraformaldehyde (2%) for 30 min at room temperature and permeabilized with 0.1% of Triton X-100 at room temperature for 15 min. Synaptosomes were subsequently washed three times at room temperature for 2 min with HBS and blocked with 2% dry non-fat milk. Rabbit polyclonal DAGKε antibody (anti-DAGKε) diluted in HBS/2% dry non-fat milk (1:100) was added and incubated overnight at 4 °C. After washing four times with HBS, goat anti-rabbit secondary antibody coupled to FITC (1:200) was added and incubation was again carried out at room temperature, this time for 2 h. Finally, after washing three times with HBS, synaptosomes were suspended in 30 μl of HBS. Confocal images from 5 μl of the suspension were obtained. Controls were performed by omitting one or both antibodies from the procedure.

2.7. Western blotting

Synaptosomal proteins from Hp and CC were heat denatured at 95 °C in 4× loading buffer (250 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 30% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 2% (v/v) bromophenol blue). In a volume ratio 3:1 (sample:buffer), 10 μl aliquot (50 μg protein) of each sample were resolved in a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blot was blocked for 2 h at room temperature with 5% non-fat milk in TBST (5% (w/v) non-fat dried milk in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween 20) and then incubated overnight at 4 °C in blocking buffer containing rabbit polyclonal DAGKε antibody (anti-DAGKε) (1:750). After four washes in TBST, it was incubated for 2 h at room temperature with polyclonal horseradish peroxidase conjugated to total anti-rabbit IgG antibody diluted 1:2000 in blocking buffer and washed as above. Bands were visualized by the ECL method. The blot was subsequently stripped and reprobed with polyclonal anti-alpha tubulin antibody (1:5000) to ensure equal protein loading. Quantification analyses of protein bands detected by Western blot were performed using ImageJ 1.38 software by working in a linear range (Abramoff et al., 2004; Rasband, 1997).

DAGKε levels in each sample were normalized to the amounts of alpha-tubulin present in each band. Molecular weight determination was performed with high molecular weight range marker (M3788) from Sigma.

2.8. Statistical analysis

Results are expressed as means ± SD. Values for insulin effect on synaptosomal phosphoinositides synthesis from adult and aged rats were compared using the unpaired Student's t test. Aging effects were analyzed by two-way ANOVA. A p value < 0.05 was considered significant.

GraphPad Prism software was used for the graphical representation of our results. This software package includes statistical analysis of data. One way ANOVA compares three or more groups defined by one factor. For example, DAGK activity from endogenous DAG group can be compared with DPG and SAG groups. However, in our case it was also necessary to compare substrate utilization in aged and adult animals. A two-way ANOVA was thus used to analyze more than one factor. Two-way ANOVA determined how DAGK activity is affected by two factors, namely substrate and aging, GraphPad Prism performs post-tests following two-way ANOVA using the Bonferroni method. This post-test was carried out to statistically evaluate significance of variation in each factor. The asterisks, which denote significance in the figures, correspond to p values that were calculated with the post-test. Curve fit (Fig. 2) was also performed using the Graphpad Prism software package. A non-linear regression analysis fitting an enzyme kinetic curve (Michaelis–Menten graph) was used.

3. Results

3.1. Exogenous SAG was preferentially used for PA synthesis in CC synaptosomes from adult rats

PA synthesis from exogenous DAG of different composition was analyzed. As mentioned in Sections 2 and 2.5, synaptosomal integrity and inclusion of exogenous DAG into sealed structures (after DMSO and 15 s sonication treatment) were determined independently by means of electron microscopy (Fig. 1A) and Nile red staining and fluorescence microscopy (Fig. 1B1 and B2). A great magnification was chosen in order to show intrasynaptosomal content after treatment (Fig. 1A).

When Nile red is dissolved in more polar solvents such as ethanol or in phospholipids such as PC vesicles, or in the phospholipids present in synaptosomes, it fluoresces red. When synaptosomal preparation was exposed to Nile red in the absence of SAG or DPG but in the presence of DMSO (control condition), the dye also fluoresced red (Fig. 1B1). When the dye was incubated after synaptosomal exposure to SAG or DPG resuspended in DMSO, it fluoresced yellow-gold (Fig. 1B2). Intrasynaptosomal content of exogenous diacylglycerol and the integrity of synaptosomal preparations (shape and dimension) after addition of SAG or DPG together with DMSO were confirmed. Under our experimental conditions, PA labeling with DPG or SAG as substrate increased as a function of increasing ATP concentrations (data not shown).

Taking into account that in our experimental model DAGK activity rapidly phosphorylated DAGs containing an arachidonoyl group, we investigated whether or not this selectivity is a consequence of the effects of the arachidonoyl group on non-specific physical properties. Experiments were then carried out to study DAGK activity using 1,2-dioleoylglycerol (DOG) as substrate. Comparative data at saturating ATP concentration (4 mM) and different concentrations of DAGs (DPG, SAG and DOG) are shown in Fig. 2.

It should be noted that specificity of DAGK activity for SAG is relative rather than absolute because DOG is also phosphorylated at about 40% of the rate of SAG. However, the preferential use of SAG in synaptosomal preparations of CC appears to be due to a DAGKε synaptosomal enzymatic source that is detected mainly under our assay conditions.
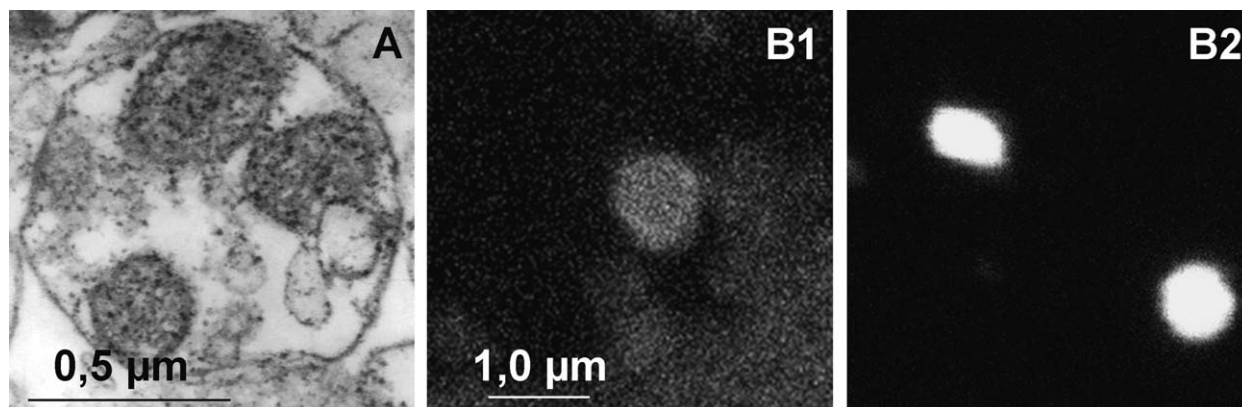
Fig. 1. Synaptosomal integrity and exogenous DAG inclusion into sealed synaptosomes: (A) synaptosomal integrity study by electron microscopy after micelle detergent treatment: synaptosomes from rat CC were treated with 0.1% DMSO and sonicated for 15 s. They were subsequently fixed with 2.5% glutaraldehyde and postfixed with 2% osmium tetroxide. After several washes synaptosomes were dehydrated using increasing concentrations of acetone and included in resin to be further cut. (B) Exogenous DAG inclusion into sealed synaptosomes: synaptosomes from rat CC were treated with 0.1% DMSO (B1) or DAG–DMSO micelle (B2) and sonicated for 15 s. They were subsequently fixed with 2% paraformaldehyde at room temperature for 30 min. After being washed for three times, they were treated with Nile red (1 μl/ml). An aliquot of the suspension was analyzed by fluorescence microscopy using 488 nm wavelength.
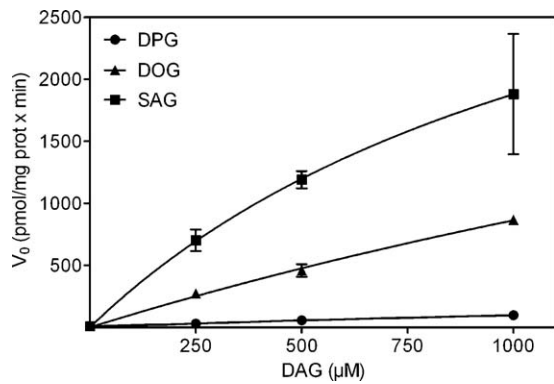
Fig. 2. DAG acyl chain selectivity of DAGK from rat CC synaptosomes: 1,2-dipalmitoylglycerol (DPG), 1,2-dioleoylglycerol (DOG) and 1-stearoyl, 2-arachidonoylglycerol (SAG) were used as lipid substrate and 4 mM of ATP as co-substrate (10 μCi of [γ-32P] ATP per assay). DAG concentrations were added in the presence of 50 mM OG (lipid–detergent micelles) as described in Section 2. Data shown are averages of three individual samples. Curve fit was performed using the Graphpad Prism software package. A non-linear regression analysis fitting an enzyme kinetic curve (Michaelis–Menten graph) was used. Error bars indicate the standard deviation (SD).

3.2. PA synthesis from exogenous SAG was diminished in CC and Hp synaptosomes from aged rats

To evaluate PA formation through DAGK activity in synaptosomes from CC or Hp of aged rats, endogenous DAG or exogenous DAG (DPG or SAG) were used in a micellar assay with 50 mM OG. As shown in Fig. 3, a similar SAG preference over DPG as DAGK substrate was also observed in synaptosomes from CC and Hp. The interaction factor calculated by two-way ANOVA shows that DAGK activity in CC and Hp is affected by two factors: substrate and aging ($p < 0.0028$ in CC and $p < 0.0024$ in Hp). Furthermore, the statistical post-test analysis reveals that although DAGK activity from CC and Hp synaptic preparations of aged rats utilizes DPG for PA synthesis in a similar manner as in adult rats, the transformation of SAG is significantly lower (20%).

To evaluate DAGKε content in synaptic terminals from CC and Hp, Western blot analysis using anti-DAGKε antibody and alpha
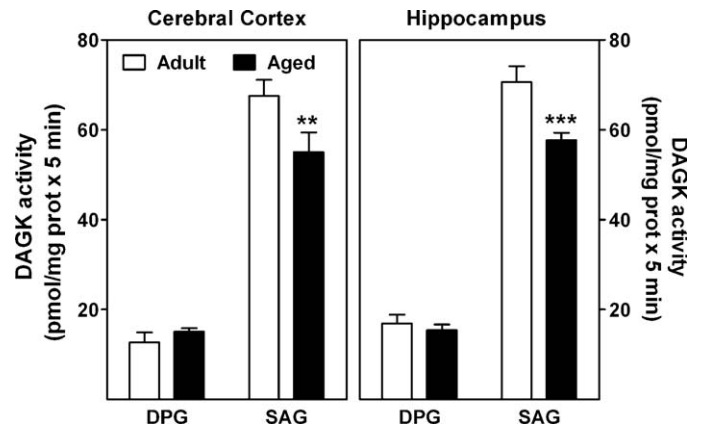

Fig. 3. Effect of aging on DAGK activity from rat cerebral cortex and hippocampus synaptosomes: DAGK activity was measured in cerebral cortex or hippocampus synaptosomes isolated from adult (4 months old) or aged (24–28 months old) rats. Synaptosomal membranes were incubated during 5 min under the DAGK assay condition as described in Sections 2 and 2.3, by measuring radioactive phosphate incorporation into DAG through [γ-^{32}P] ATP as radioactive substrate (3 μCi of [γ-32P] ATP per assay) in the presence of either 250 μM DPG or 250 μM SAG (DMSO as detergent). DAGK activity was expressed in pmol of PA per mg protein. Results derived from aged animals (black bars) were compared to those derived from adult ones (white bars) and were analyzed by two-way ANOVA followed by a Bonferroni test. Values are the mean ± SD of six individual samples. **$p < 0.01$ and ***$p < 0.001$.

tubulin detection to ensure equal protein loading was carried out. A similar DAGKε content, normalized with alpha tubulin detection was found in Hp and CC. A non-significant, decreased level of protein DAGKε expression in synaptosomes from CC with respect to Hp was also observed (Fig. 4).

3.3. Insulin differentially stimulates PA synthesis from exogenous SAG in CC and Hp synaptosomes from adult and aged rats

Insulin action on DAGK activity was measured in synaptosomes from CC and Hp of 26–28 month-old rats using endogenous or exogenous DAG (DPG or SAG). Comparative data of insulin stimulatory effects on DPG or SAG phosphorylation in synaptosomes from CC or Hp of adult and aged rats are shown in Fig. 5. Data
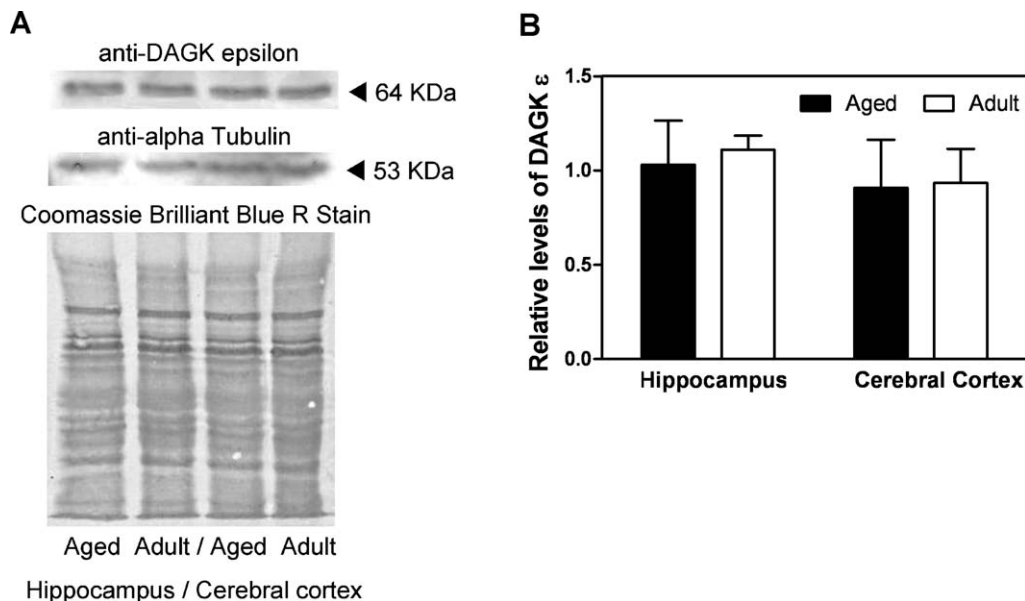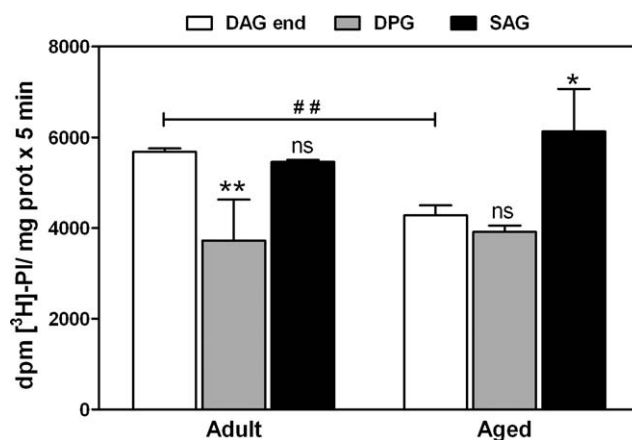

Fig. 4. Detection of synaptosomal DAGKε by Western blot assay: (A) detection of synaptosomal DAGKε was evaluated with rabbit polyclonal DAGKε antibody (anti-DAGKε). The blot was subsequently stripped and exposed to rabbit polyclonal anti-alpha tubulin antibody (1:5000) to ensure equal protein loading. Coomassie brilliant blue (R-250) was used to stain total proteins on the blot. Molecular weight determination was performed with high molecular weight range marker (M3788) from Sigma. (B) Relative levels of DAGKε in each sample were expressed as a ratio of the densitometric values of DAGKε to alpha-tubulin present in each band. Coomassie blue was used as control protein loading.
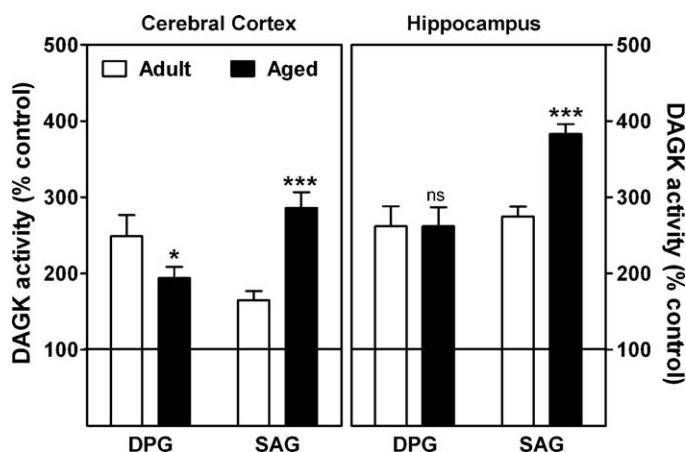
Fig. 5. Effect of aging on the activation of synaptosomal DAGK from cerebral cortex or hippocampus by insulin: DAGK activity was measured in cerebral cortex or hippocampus synaptosomes isolated from adult or aged rats. Synaptosomal membranes were incubated during 5 min under DAGK assay conditions as described in Sections 2 and 2.3, by measuring [32P]-PA in the presence of either 250 μM DPG or 250 μM SAG (DMSO as detergent). Incubation time was started with insulin (200 nM) addition in the presence of vanadate (200 μM). Results derived from aged animals (black bars) were compared to those derived from adult animals (white bars) and were analyzed by two-way ANOVA followed by a Bonferroni test. Values are expressed as percentage of incorporation values with respect to the conditions without insulin (set at 100) in each case (adult or aged) and they are the mean ± SD of six individual samples. $^{*}p < 0.05$, $^{***}p < 0.001$.

Fig. 6. Effect of aging on PI synthesis in CC synaptosomes: synaptosomes from adult and aged rats CC were incubated during 5 min in the presence of 7 μCi of [3H]-inositol (AE 16.5 Ci/mmol) as radioactive substrate and 500 μM of cytidine-5′-triphosphate (CTP) as cofactor. 500 μM of ATP was also present as cofactor to ensure kinase activities. Reactions were carried out under the same conditions as described for DAGK assay and by measuring [3H]-PI synthesis in the absence (endogenous DAG) or in the presence of either 250 μM DPG or 250 μM SAG (DMSO as detergent). Results derived from different treatments in aged and adult animals (endogenous-DPG and SAG) were analyzed by two-way ANOVA followed by a Bonferroni test. Results are expressed as dpm of [3H]-inositol incorporation into PI per mg protein in 5 min. Values are the mean ± SD of three individual samples. $^{*}p < 0.05$, $^{**}p < 0.01$. A t-test was performed to evaluate differences between aged and adult rats (endogenous DAG). $^{##}p < 0.0017$.

are presented as a percentage of control without insulin set as 100%. In CC from aged rats, insulin was found to slightly decrease PA synthesis from DPG with respect to adult rats whereas it strongly stimulated PA synthesis from SAG. The interaction factor was, in fact, highly significant ($p < 0.0001$). In Hp from aged rats, insulin increased PA synthesis from DPG in a similar manner as in adults and it strongly increased PA synthesis from SAG. The interaction factor was also highly significant ($p < 0.0017$). Although insulin preferentially increased SAG over DPG phosphorylation in both tissues from aged rats, a higher stimulation effect was observed in Hp than in CC synaptic terminals.

3.4. Phosphatidylinositol (PI), phosphatidylinositol phosphate (PI(4)P) and phosphatidylinositol bisphosphate (PI(4,5)P2) synthesis in CC synaptosomes

We carried out experiments in synaptic terminals from CC to evaluate PI, PI(4)P and PI(4,5)P2 synthesis through [3H] inositol incorporation. SAG inclusion into sealed synaptosomes was chosen as the experimental approach to follow in order to favor DAGKε activity as well as to evaluate the role of this isozyme in PI synthesis. Fig. 6 shows a significant inhibition (28%) in PI synthesis as a result of aging in the absence of exogenous DAG (white bars, t-
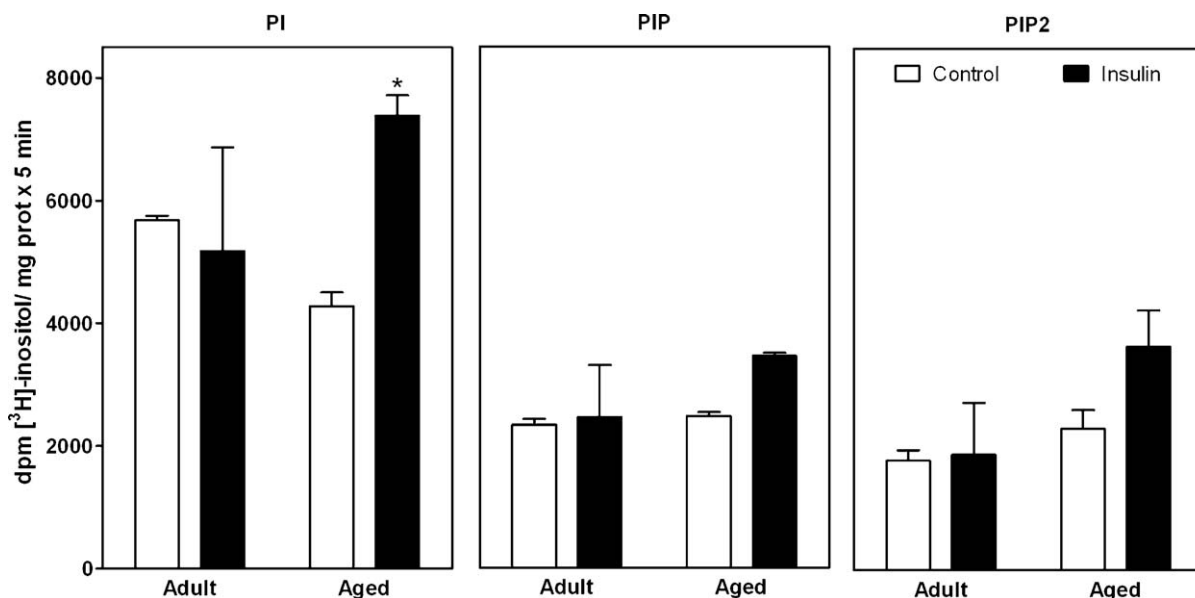
Fig. 7. Insulin effect on synaptosomal phosphoinositides synthesis from adult and aged rats CC: synaptosomes were incubated as described in Fig. 6 and [3H]PI, [3H]PIP and [3H]PIP2 synthesis were measured in the absence of exogenous DAG. Incubation time was started with insulin (200 nM) addition in the presence of vanadate (200 μM). Results are expressed as dpm of [3H]-inositol incorporation into phosphoinositides (PIs) per mg protein in 5 min and values are the mean ± SD of three individual samples. In adult and aged animals results derived from insulin condition (black bars) were compared to those derived from control condition (white bars) and were analyzed by two-way ANOVA followed by a Bonferroni test. $^{*}p < 0.05$.

test analysis, $p < 0.0017$). In the presence of DPG an inhibition in PI synthesis was observed in adult rats (Bonferroni post-test after two-way ANOVA, $p < 0.01$). However, in the presence of SAG PI synthesis underwent no changes. Interestingly, whereas in the presence of SAG a recovery of [3H]PI synthesis was observed in aged rats, this was not observed in the presence of DPG (Bonferroni post-test after two-way ANOVA, $p < 0.05$).

The incorporation of [3H] inositol into PI, PI(4)P and PI(4,5)P2 was analyzed in the presence of insulin in order to study insulin-dependent DAGKε activity stimulation on phosphoinositide synthesis. Fig. 7 shows that in the presence of insulin phosphoinositide synthesis (PI, PI(4)P and PI(4,5)P2) underwent no changes in adult animals. In contrast, in aged rats insulin increased PI synthesis significantly (Bonferroni post-test after two-way ANOVA, $p < 0.05$).

4. Discussion

It has been reported that, compared to other diacylglycerols, DAGKε exerts greater activity in phosphorylating arachidonoyl–DAG. With isolated DAGKζ, an increase in PA formation from DOG as precursor with respect to SAG was also reported (Thirugnanam et al., 2001).

As shown in Fig. 2, an efficient utilization of SAG over DOG or DPG was observed and PA labeling with DPG, DOG or SAG as DAGK substrates increased as a function of increasing DAG concentrations under ATP saturating conditions.

PA formation in synaptosomes from CC, through a DAGK assay, showed preferences for OG and Triton X100 (unpublished observations) as well as DAGK insensitivity to R59022 and R59949 (DAGK inhibitors) in the presence and absence of calcium ions (Zulian et al., 2009). R59022 and R59949 are potent inhibitors of calcium-dependent isoforms of DAGK. However, neither R59022 nor R59949 were found to inhibit arachidonoyl–DAG specific DAGK from testis (Jiang et al., 2000). All these data indirectly indicate the preferential formation of PA through DAGKε activity under our assay conditions (micelle detergent assay).

DAGK activity over endogenous DAG was found to be lower in synaptosomes from aged rats (28 months old) than from 3–4 month-old rats (Salvador et al., 2005). In accordance with this finding, a 20% inhibition in DAGK activity over endogenous DAG was found in 24–28 month-old rats (Fig. 3). Although DAG content was increased, an arachidonate reduction in DAG acyl chain composition was also detected (unpublished results).

To evaluate DAGK activity in synaptic terminals from aged rats, independently of endogenous DAG, either DPG or SAG was incorporated into synaptosomes from CC and Hp. Whereas DPG utilization for PA synthesis was similar to that found in adult rats, SAG utilization was significantly lower as it was also observed in synaptic terminal preparations from CC and Hp (Fig. 3). However, similar DAGKε content in synaptic terminals from both tissues from adult and aged rats was observed by WB determinations (Fig. 4).

On account of the fact that DAGK undergoes no changes with aging, it could be hypothesized that an aging-associated change in the enzyme induces a low SAG phosphorylation. Further experiments are thus necessary to explain this phenomenon. Alternatively it could be hypothesized that changes occur either in the enzyme localization or in some regulatory component.

Although DAGKε has the ability to phosphorylate either DPG or other saturated DAGs, it is highly efficient in 2-arachidonoyl DAGs phosphorylation (Milne et al., 2008; Thirugnanam et al., 2001). Previous research has demonstrated that DAGKε contributes significantly to the arachidonoyl enrichment of PI(4,5)P2 on account of the fact that cells from KO mice have less arachidonoyl-PI(4,5)P2 (Milne et al., 2008).

Experimental evidence derived from 3H-inositol incorporation into synaptosomal CC phosphoinositides reveals that PI synthesis was lower in aged rats than in adult rats (Fig. 6). This figure also shows that synaptosomal PI synthesis in adult rats was inhibited in the presence of DPG which favors saturated PA synthesis. A displacement of a preferential PA substrate for CDP-DAG synthesis could thus be suggested. In contrast, PI synthesis in aged rats seemed to be insensitive to DPG presence.

Based on DAGK activity (Fig. 3), it could therefore be hypothesized that preferential PA substrate for CDP-DAG synthesis in synaptic terminals from aged rats is diminished. The inhibition of DAGKε, as evidenced through decreased SAG utilization in aged brain, could be mainly related to 2-arachidonoyl PA synthesis and PI re-synthesis and could also play a regulatory role in PI(4,5)P2 formation.

Aging effects on the acyl composition of lipids from CC synaptosomes and from synaptosomal DRMs (detergent-resistant membranes) have been recently reported (Mateos et al., 2010). Their results indicate that aged animals underwent a dramatic decrease in PUFA content in Syn and DRM fractions and that changes occurred in 20:4n-6 content in PC and PE, the most significant changes being detected mainly in PI. Furthermore, although PI(4)P and PI(4,5)P2 were not measured, Mateos et al. (2010) results from PI agree with our hypothesis.

Controversial results on the aging effect on phosphoinositide synthesis and/or phosphatidylinositol kinase activities were previously reported (Bothmer et al., 1992; Jolles et al., 1992; Stokes et al., 1983). PI(4)P and PI(4,5)P2 formation from endogenous precursors has been recently measured as a function of age. In parallel, PI(4)P and PI(4,5)P2 production by PI4K and PIP5K activities, respectively, was assayed with exogenous substrates. It was demonstrated that aging affects the incorporation of [32P] from [γ-32P] ATP into endogenous PI(4)P and subsequently decreases the level of radioactivity in PI(4,5)P2 with no changes in PI4K and PIP5K activity. This finding reveals that aging does not affect phosphoinositide kinases and that factors, other than PI4K and PIP5K changes, must be responsible for the low PI(4,5)P2 formation in aged brain (Zambrzycka, 2004). Interestingly, the 30% inhibition in PI synthesis recorded in aged rats in our study (Section 3.4, Fig. 6) could be the cause of the low PI(4,5)P2 formation observed by Zambrzycka (2004).

DAGKε activity was also found to be stimulated by insulin in synaptic terminals from Hp and CC. In addition, insulin was found to preferentially stimulate PA formation from SAG in Hp synaptosomes. It is thus interesting to note that insulin also preferentially stimulates PA formation from SAG in CC and mainly in Hp synaptosomes from aged rats rather than from adult animals (Section 3.3, Fig. 5).

Although insulin stimulates phosphatidylinositol synthesis, stimulation of arachidonoyl-containing phosphatidic acid species by insulin has not yet been determined. Further experiments should be carried out to answer this interesting query.

Preferential DAGKε activation by insulin in aged brain may therefore be a compensatory mechanism for phosphoinositide supply. Taking into account that PI(4,5)P2 inhibits DAGKε activity (Walsh et al., 1995; Thirugnanam et al., 2001), the stimulation of IR-related PI(4,5)P2 hydrolysis as previously reported in adult rats (Zulian et al., 2006) could remove PI(4,5)P2 as a DAGKε-negative regulator.

It has been reported that DAGKε $-/-$ mice displayed attenuated LTP in perforant path–dentate granular cell synapses, 20:4-inositol lipid signaling being involved in hippocampal synaptic plasticity (Rodriguez de Turco et al., 2001). On the other hand, insulin has been shown to exert a memory-enhancing effect on both humans and experimental animals (Zhao et al., 2004). Insulin-induced DAGKε activity in aged brain could thus participate in a final compensatory PI(4,5)P2 resynthesis mechanism, thus suggesting a beneficial insulin role in brain function.

5. Conclusions

In synaptic terminals from CC and Hp of aged rats, SAG utilization by DAGK activity, which was postulated to be indicative of DAGKε activity, was significantly lower than in adults. PI synthesis measured from [3H] inositol incorporation was also lower in aged rats than in adult rats.

DAGKε activity was found to be stimulated by insulin in synaptic terminals from Hp and CC in adults and insulin was found to preferentially stimulate PA formation from SAG in Hp. Insulin was also found to preferentially stimulate PA formation from SAG in CC and mainly in Hp synaptosomes from aged rats rather than from adult animals. In synaptosomes from aged rats the inhibition of PI synthesis was found to be reverted by insulin. In view of this, preferential DAGKε activation by insulin in aged brain could therefore be interpreted as a compensatory mechanism for phosphoinositide supply.

Disclosure statement

All the authors declare that there are no actual or potential conflicts of interests involving them or the institutions with which they are affiliated.

Acknowledgments

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