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Recruitment of Casein Kinase 2 is Involved in AβPP Processing Following Cholinergic Stimulation

Silvia C. Lenzken[1], Serena Stanga, Cristina Lanni, Fabio De Leonardis[2], Stefano Govoni and Marco Racchi*
Department of Experimental and Applied Pharmacology, Centre of Excellence in Applied Biology, University of Pavia, Pavia, Italy

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Abstract. The amyloid-β protein precursor (AβPP) is an integral membrane protein subjected to constitutive and regulated proteolytic processing. We have previously demonstrated that protein kinase C ε (PKCε) plays a key role in the regulation of AβPP metabolism via cholinergic receptors. The purpose of the present work is to clarify whether other putative signaling systems are involved in the same pharmacological pathway. We focused particularly on casein kinase 2 (CK2), demonstrating a direct interaction between PKCε and CK2 following cholinergic stimulation. Treatment of human neuroblastoma SH-SY5Y cells with a selective inhibitor of CK2 reduced the effect of carbachol on the release of sAβPPα. This treatment did not influence the activation and translocation of PKCε suggesting that the latter is located upstream of CK2. On the basis of our results, we add another player to the complex cellular mechanisms regulating non-amyloidogenic processing of AβPP.

Keywords: Alzheimer's disease, amyloid-β protein precursor, casein kinase 2, cholingergic, neuroblastoma, protein kinase C, signal transduction

INTRODUCTION

Alzheimer's disease (AD) is characterized by deposition in the brain of fibrillar aggregates of a peptide named amyloid-β (Aβ), derived from proteolytic processing of a larger precursor called the amyloid-β protein precursor (AβPP) [1].

AβPP is an integral membrane protein with a complex proteolytic metabolism that can be simplified in a so called "non amyloidogenic" pathway, based on the action of α-secretases cleaving AβPP inside the Aβ region producing a soluble fragment sAβPPα, thus precluding Aβ production, and in the "amyloidogenic" pathway, based on the activities known as β- and γ-secretase, which generates Aβ [2].

The most studied proteases involved in α-AβPP processing are TNFα converting enzyme (TACE; ADAM-17) [3] and ADAM10 [4,5]. Interestingly, while TACE seems to be involved in the phorbol esters-mediated release of sAβPPα, ADAM10 has been shown to be involved in both the regulated and constitutive secretion of AβPP [6].

AβPP processing by α-secretase occurs via a constitutive pathway and by receptor mediated activation of multiple signal transduction pathways among which protein kinase C (PKC) is a major player [7,8]. Among others, cholinergic receptors (mAChR) conduce to an increase in the release of soluble N-terminal AβPP

[1]Present affiliation: Department of Biotechnology and Biosciences, University of Milano Bicocca, Milano, Italy.
[2]Present affiliation: Department of Biochemistry "A. Castellani", University of Pavia, Pavia, Italy.
*Correspondence to: Marco Racchi, Dept. of Experimental and Applied Pharmacology, University of Pavia, Viale Taramelli 14, 27100 Pavia, Italy. Tel.: +39 0382 987738; Fax: +39 0382 987405; E-mail: racchi@unipv.it.

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derivates [9], and this has been the basis of numerous studies that demonstrated that acetylcholinesterase inhibitors may influence the non amyloidogenic processing of AβPP [10–12].

We have previously demonstrated that PKCε, among other kinase isoforms, is specifically involved in cholinergic-mediated AβPP processing [13], however, the exact target of PKC phosphorylation related to AβPP processing is still elusive. Several observations in the literature suggest that activation of AβPP cleavage/release by PKC involves phosphorylation/interaction with some still unknown components of the processing pathway.

We turned our attention to casein kinase 2 (CK2), an ubiquitous and essential serine/threonine protein kinase, found in eukaryotic cells, with a list of more than 300 known protein substrates [14]. It has traditionally been classified as a stable tetrameric complex consisting of two catalytic subunits, α or α', and two regulatory subunits β. The actual occurrence of the three predicted tetramers, α2β2, αα'β2, and α'2β2, has been well documented [15]. Interestingly, some reports have suggested that CK2 could be activated by PKC [16–18]. Consequently, we inquired the possibility of a CK2 participation in the sAβPPα release downstream of cholinergic stimulation.

MATERIALS AND METHODS

Materials and chemicals

All culture media, supplements, and fetal calf serum (FCS) were obtained from Euroclone (Life Science Division, Milan, Italy). Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA, USA), PULSin™ was purchased from Polyplus-transfection SA (Ilkirch, France) and the inhibitor peptide of PKCε translocation was purchased from Calbiochem (EMD Chemical Inc., San Diego, CA, USA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Carbachol was dissolved in PBS and then diluted in serum free medium to working concentrations at the time of use; inhibitor peptide (IP) of PKCε translocation was dissolved in PBS and stored at −20°C and 4,5,6,7-tetrabromobenzotriazole (TBB) was dissolved in DMSO and stored at 4°C. Stocks were diluted in serum-free medium to working concentration at the moment of use.

Cell cultures

The human neuroblastoma SH-SY5Y cell line from European Collection of Cell Cultures (ECACC No. 94030304) was used for these experiments. SH-SY5Y cells were cultured in medium with equal amount of Eagle's minimum essential medium and Nutrient Mixture Ham's F-12, supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μM streptomycin, 1% non-essential aminoacids at 37°C in 5% CO_2. Cells were plated at a density of 1×10^5 cells/cm^2, and treatments were performed after 24 h from seeding. Overexpressing PKCε cells were obtained by transfection of the plasmid encoding HA-tagged PKCε-[pcDNA3-HA- PKCε] (a gift from Dr. Jae-Won Soh, Inha University Korea) [19]. SH-SY5Y cells were transfected with 400 ng of plasmid with Lipofectamine 2000, according to the manufacturer's instructions and positive clones were selected with 400 μg/ml G418 (Geneticin). PKCε overexpression was detected in western blots with either anti-HA antibody or anti-PKCε antibody.

Experimental treatments

Confluent monolayers of cells were washed twice with PBS and treated at 37°C in serum free medium in the presence of vehicle alone or in the presence of the compound of interest. Before treatment, cells were incubated for 20 min with or without inhibitors; at the end of treatment, the conditioned medium was collected for the analysis of sAβPPα release by western blot. When the inhibitor peptide of PKCε translocation was used, the cells were pretreated for 2 h with the inhibitor peptide in presence of PULSin™ in serum free medium and following rinsing twice with PBS and subject to treatment.

Immunodetection of sAβPPα, PKCε, and tubulin

Conditioned medium was collected after treatment and centrifuged at 13.000×g for 5 min to remove detached cells and debris. Proteins in the medium were quantitatively precipitated by the deoxycholate/trichloroacetic acid procedure as previously described [12]. Cell monolayers were washed twice with ice cold PBS and lysed on the tissue culture dish by addition of ice-cold lysis buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 20 μg/ml leupeptin, 25 μg/ml aprotinin, 0.5 μg/ml pepstatin A and 0.5% Triton X-100). An aliquot

of the cell lysate was used for protein analysis with the Pierce Bicinchoninic Acid kit, for protein quantification. Normalization of protein loading on each blot was obtained by loading a volume of sample of conditioned medium standardized to total cell lysate protein concentration. Proteins were subjected to SDS-PAGE (10%) and then transferred onto PVDF membrane 0.45 μm (Immobilon, Millipore Corp, Bedford, MA, USA). The membrane was blocked for 1 h with 5% non-fat dry milk in Tris-buffered saline containing 1% Tween 20 (TBST). Membranes were immunoblotted with the antibody 6E10 (1:1000 dilution in 5% non fat dry milk, from Chemicon-Prodotti Gianni, Milano, Italy). The detection was carried out by incubation with horseradish peroxidase conjugated goat anti-mouse IgG (1:10000 dilution in 5% non fat dry milk, from Pierce, Rockford, IL, USA) for 1 h. The blots were then washed extensively and sAβPPα visualized using enhanced chemiluminescent methods (Pierce, Rockford, IL, USA). For the detection of PKCε and tubulin, proteins were measured as described earlier, diluted in sample buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% Bromophenol blue) and subjected to Western blot analysis with the method indicated previously using the mouse anti-human PKCε monoclonal antibody (1:10000 dilution in 5% non fat dry milk, from BD biosciences, Erembodegem, Belgium) or the mouse anti α-tubulin, clone DM1A, monoclonal antibody (at 1:5000 dilution in 5% non fat dry milk, from Sigma Chemical Co., St. Louis, MO,USA).

Co-immunoprecipitation

The cells were washed twice with PBS, and treated with 100 μM CCh in serum free medium, or serum free medium alone, for 10 and 20 min at 37 °C. Cells were washed twice with cold PBS and lysed in immunoprecipitation buffer (10 mM Tris-HCl (pH 7.6), 138 mM NaCl, 0.5% Nonidet P40, 0.2 mM AEBSF, 20 μg/ml leupeptin, 25 μg/ml aprotinin, 0.5 μg/ml pepstatin A, 1 mM NaF). Lysed cells were sonicated twice at 40 Hz for 10 s, and centrifuged at 12.000 $\times$ g at 4 °C for 10 min. The detergent-soluble material (supernatant) was precleared by incubation with 20 μl of protein AG Plus agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. The samples were centrifuged at 3.000 $\times$ g at 4 °C for 2 min, and protein quantity was determined using BCA reagent (Pierce). Immunoprecipitation was performed with 1 μg of mouse anti-human CK2α monoclonal antibody (Chemicon, Prodotti Gianni, Milano, Italy), together with approximately 0.5–1 mg of protein diluted in the immunoprecipitation buffer to a total volume of 500 μl. After overnight incubation at 4 °C, immunocomplexes were collected by using protein AG Plus agarose, incubated at 4 °C for 1 h and subsequently washed three times with immunoprecipitation buffer. Immunoprecipitated CK2 was recovered by resuspending the pellets in sample buffer. PKCε was detected using the mouse anti-human PKCε monoclonal antibody at a 1:1000 dilution (BD bioscences, Erembodegem, Belgium).

Immunocytochemical analysis of PKC and CK2 co-localization

SH-SY5Y neuroblastoma cells were seeded on glass coverslips at a density of 1×10^5 viable cells per well in a 24-well plate. Cells on coverslips were treated with carbachol 100 μM in serum free medium for 20 min, whereas control cells were incubated with medium alone for 20 min; when indicated TBB was added 20 min prior to the treatment. After treatment, cells were fixed in ethanol 70% at -20 °C, washed with PBS and permeabilized for 15 min at room temperature with 0.01% Triton X-100 in PBS. Nonspecific bindings with PKCε and CK2 were blocked by incubation for 30 min with PBS containing 1% BSA. Cells were incubated for 1 h with the rabbit antibody specific for PKCε (Santa Cruz Biotechnology Inc., CA, USA) diluted 1:50 in PBS/1% BSA solution. After rinsing in PBS, a FITC conjugated rabbit anti-IgG antibody (Invitrogen, CA, USA) was diluted at 1:500 in PBS/1% BSA to detect PKCε. Cells were then subjected to a further incubation for 1 h with a monoclonal antibody specific for CK2 (Millipore, MA, USA) diluted 1:20 in PBS/1% BSA solution. To detect CK2 cells were incubated for 1 h at room temperature with an anti-mouse IgG antibody conjugated with ALEXA 633 (Invitrogen, CA, USA) diluted 1:50 in PBS/1% BSA. After the fluorescent labeling procedures, cells were finally counterstained for DNA for 5 min with a 0.1 μg/ml HOECST 33342 solution in PBS, and mounted upside down on glass slides, in a drop of Mowiol (Calbiochem). Images were obtained with a confocal microscope Leica DM IRBE with Leica TCS SP software.

Immunodetection of ADAM10 and TACE/ADAM17

Following 2 h of 100 μM CCh treatment in the presence or absence of 16 μM TBB, cell monolayers were washed twice with ice cold PBS and lysed on the tissue

culture dish by addition of ice-cold lysis buffer 20 mM Tris/HCl pH 7.4, 0.32 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 50 mM β-mercaptoethanol, 0.2 mM AEBSF, 20 μg/ml leupeptin, 25 μg/ml aprotinin and 0.5 μg/ml pepstatin A. Cell lysis was achieved using a Dounce homogenizer (15–20 strokes were performed for each sample). Cell lysates were centrifuged at 3,000 $\times$ g, for 5 min at 4°C, and the surnatants were separated from the pellets (nuclei), and were further pelleted by centrifugation for 60 min at 4°C and 100,000 $\times$ g. The resulting pellet (referred to here as the particulate fraction) was resuspended in lysis buffer 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.2 mM AEBSF, 20 μg/ml leupeptin, 25 μg/ml aprotinin, 0.5 μg/ml pepstatin A, and 0.5% Triton X-100 and an aliquot was used for protein analysis with the Pierce Bicincronic Acid kit, for protein quantification. Subsequently it was diluted in sample buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% Bromophenol blue) and subjected to Western blot analysis with the method indicated previously, using the mouse anti-ADAM10 monoclonal antibody (1:1000) from Abcam Inc., Cambridge, MA, USA or the goat anti-TACE/ADAM17 polyclonal antibody (1:200), able to detect the C-terminal region of both the precursor and mature TACE/ADAM17, from Santa Cruz Biotechnology Inc., Santa Cruz, California, USA; in both cases tubulin was also performed as a normal control of proteins.

Densitometry and statistics

Analysis of Western blot images was performed by calculating the relative intensity of the immunoreactive bands after acquisition of the blot image through a Nikon CCD video camera module and analysis by means of the Image 1.47 program (Wayne Rasband, NIH, Research Service Branch, NIMH, Bethesda, MD, USA). The relative densities of the bands were expressed as arbitrary units and normalized to data obtained from control sample run under the same conditions. Controls were processed in parallel with stimulated samples and always included in the same blot. Preliminary experiments with serial dilutions of secreted protein allowed determination of optimal linear range for chemiluminescence reaction. Results are reported as means of three to four independent experiments $\pm$ S.E.M. Statistical analysis was made by one-way analysis of variance followed, when significant, by the multiple comparison Bonferroni test; $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

PKCε and the processing of AβPP

We produced a stable clone of SH-SY5Y neuroblastoma cells overexpressing PKCε. The clone (W3) expresses higher levels of PKCε determined by western blot (Fig. 1A) and real time PCR (not shown). The overexpressing cells were subjected to a treatment with 100 μM of CCh in serum free medium for 2 h and sAβPPα release was detected by western blot in the conditioned medium. PKCε overexpressing cells, as expected, produce a 2-fold increase in sAβPPα release compared to stimulated wild type cells (Fig. 1B). These data are all in agreement and sustain our previous findings obtained in PKCε antisense downregulated cells [13], indicating that the main PKC isoform involved in the downstream events following carbachol stimulation is PKCε. Other authors also suggested that PKCε participates in AβPP α-secretase processing [20–22], and the fact that some PKC isoforms, namely PKCα, are involved mostly in constitutive AβPP processing [23], indicate that the multiple isoforms of PKC may be selectively involved in different aspects of AβPP processing.

We further explored the involvement of PKCε using a specific inhibitor, namely an inhibitor peptide mimicking the V1 region of PKCε, which interacts with the Receptor for Activated C Kinase 2 (RACK2) [24,25], in order to block the translocation of PKCε following cholinergic stimulation. The peptide is not cell permeable [25], therefore the cells were pretreated for 2 h with 5 μM of inhibitor peptide using PULSin as delivering agent; following incubation, the cells were washed with phosphate buffer and subjected to the treatment, adding medium without serum or with 100 μM CCh in serum free medium for 2 h. The effect of the addition of PKCε inhibitor peptide was a decrease of about 40% in sAβPPα release compared to untreated cells (Fig. 1C) suggesting that PKCε translocation is necessary for sAβPPα release. Since we used a membrane permeating agent, the proper controls were included in our protocol, however, no significant effect on sAβPPα release was detected (data not shown). Overall these data reinforce and confirm the involvement of PKCε in carbachol stimulated AβPP processing [13]. In addition since the sequence of the inhibitor peptide is identical to the V1 region of PKCε, which is the domain of interaction with RACK2 [24,25], the data suggest that the anchoring protein is involved in PKCε translocation following cholinergic stimulation. The observation that

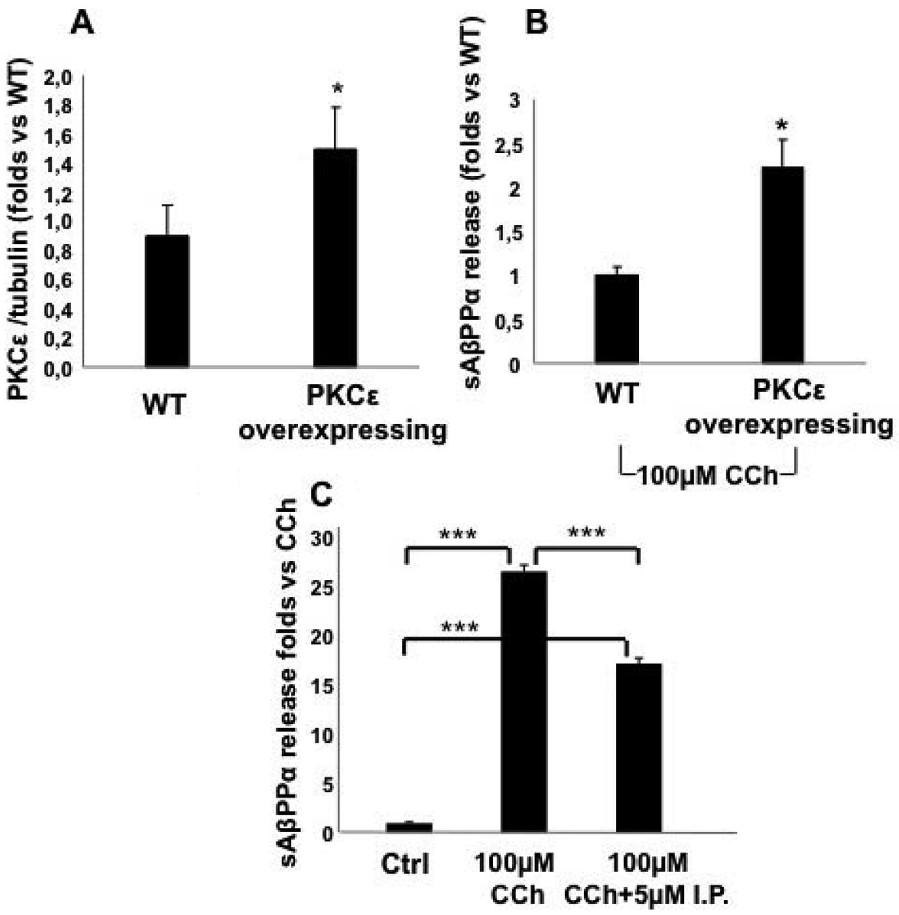

Fig. 1. A) Stably transfected SH-SY5Y cells show PKCε expression level about 1.6 fold higher than corresponding wild type cells. The clone characterization was achieved by western blotting (quantitation shown in figure) and RT-PCR (not shown) evaluating the presence of both the endogenous and esogenous form of PKCε. B) The overexpression of PKCε in the SH-SY5Y cells induces an increase in sAβPPα release about 2-fold higher than the corresponding wild type cells, in cholinergic stimulus condition upon treatment with 100 μM CCh for 2 h. C) Blocking the translocation of PKCε with the inhibitor peptide (IP) reduces sAβPPα release after carbachol treatment. Cells have been treated with 100 μM CCh for 2 h in presence or absence of 5 μM IP (added 2 h before). $*p < 0.05$; $***p < 0.001$ (Bonferroni Multiple Comparison Test).

the decrease in sAβPPα release was greatly reduced by the peptide inhibitor but did not fully return to basal secretion suggests that other additional mechanisms of signal transduction may be involved.

CK2 participation in AβPP processing

As suggested by some authors [16–18], we addressed the issue of a close relationship between the activity of CK2 and PKCε. First of all we performed a set of experiments using TBB (4,5,6,7-tetrabromobenzotriazole), a specific inhibitor of CK2 which has been recommended in cell-based assays [26] and observed that treatment of SH-SY5Y cells with 16 μM TBB prior to treatment with 100 μM CCh, reduces stimulated sAβPPα release by 60% (Fig. 2). Interestingly, treatment with TBB also induced an inhibition in the basal release of sAβPPα (Fig. 2). Various TBB concentrations were assayed, leading to the choice of the 16 μM concentration, showing consistent effect on sAβPPα release without significant effects on cell viability (MTT assays–not shown).

These initial results suggested an involvement of CK2 in the pathway leading to α-secretase activation following PKCε stimulation by cholinergic receptor agonists.

To test the hypothesis of a direct interaction between CK2 and PKCε, we set up an experiment in which, following a treatment with 100 μM CCh for 10 and 20 min, cell lysates were subjected to immunoprecipitation with anti-CK2 antibody. Using an immunoprecipitating antibody mouse anti-human alpha CK2 subunit, followed by western blot for PKCε, revealed that

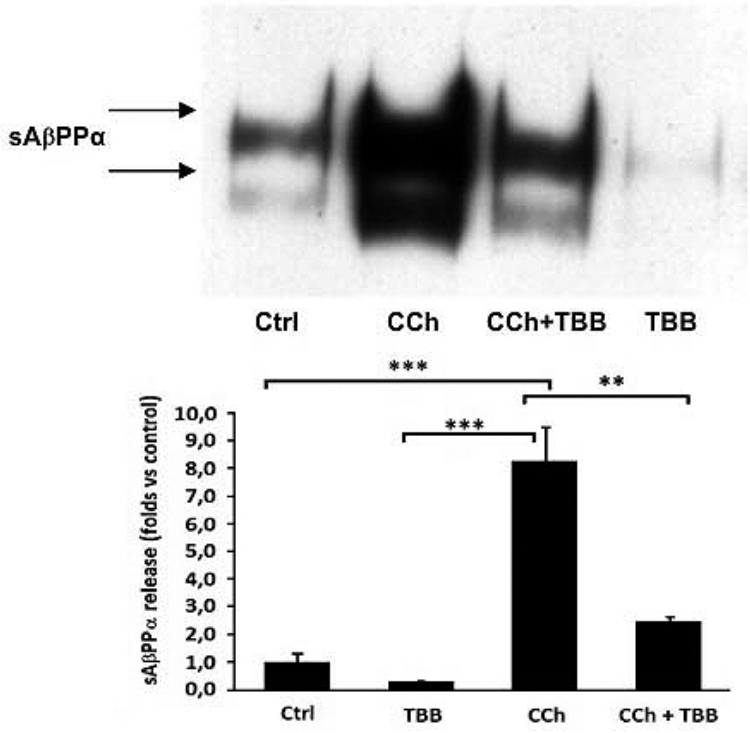

Fig. 2. Inhibition of CK2 with TBB reduces the cholinergic stimulated release of sAβPPα. SH-SY5Y cells release sAβPPα as a doublet corresponding to the AβPP isoforms expressed by the cells [12]. Cells have been pretreated with 16 μM TBB (see methods for details) for 30 min and then treated with 100 μM CCh for 2 h. The effect of TBB is an almost complete inhibition of stimulated sAβPPα release, returning levels of secretion almost to control levels. We have observed also a reduction of basal sAβPPα secretion which however does not reach statistical significance. $^{**}p < 0.01$; $^{***}p < 0.001$ (Bonferroni Multiple Comparison Test).

the two proteins are co-immunoprecipitated with a significant PKCε band present already at 10 min and increasing further after 20 min of CCh treatment (Fig. 3). The data is suggestive of a direct interaction between CK2 and PKCε that takes place immediately after the application of CCh. The interaction between PKCε and CK2 was also confirmed by immunocytochemical experiments. As shown in Fig. 4, in basal conditions (Ctrl) CK2 and PKCε are diffusely distributed at a cellular level, whereas after treatment with 100 μM CCh for 20 min, the two proteins are both localized near the nucleus. This is further supports several reports that suggest a clear functional relationship between PKCε and CK2; for example, Sanghera and coworkers found that PKCs from a brain homogenate were able to increase the CK2 activity *in vitro* [16], whilst Boehring and colleagues recently reported a similar increase on CK2 activity after phorbol ester stimulation in hippocampal neurons [18].

We wanted to investigate whether the activity of CK2 related to AβPP processing was located upstream or downstream of PKC in our cellular model. Silva-Neto

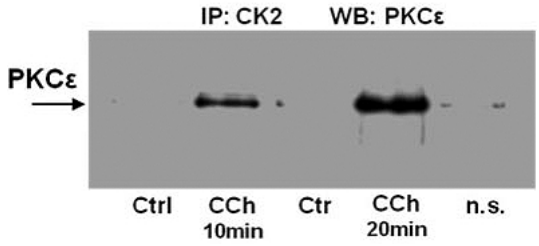

Fig. 3. Co-immunoprecipitation analysis. In order to establish if the two kinases directly interact, a co-immunoprecipitation assay has been performed using as immunoprecipitating antibody mouse anti CK2α, then mouse anti PKCε for western blot.

and coworkers suggested that CK2 activity could be located downstream of PKC [17]. Previously, our group showed that after a cholinergic stimulus, PKCε translocates from the cytosol to Golgi-like structure [13]. We treated cells with 100 μM CCh for 20 min in presence or absence of 16 μM TBB. After treatment, the cells were fixed and immunolocalization of PKCε was performed, in order to observe the re-localization of this kinase after treatment with CCh. Accordingly with previous results, in unstimulated conditions PKCε was distribut-

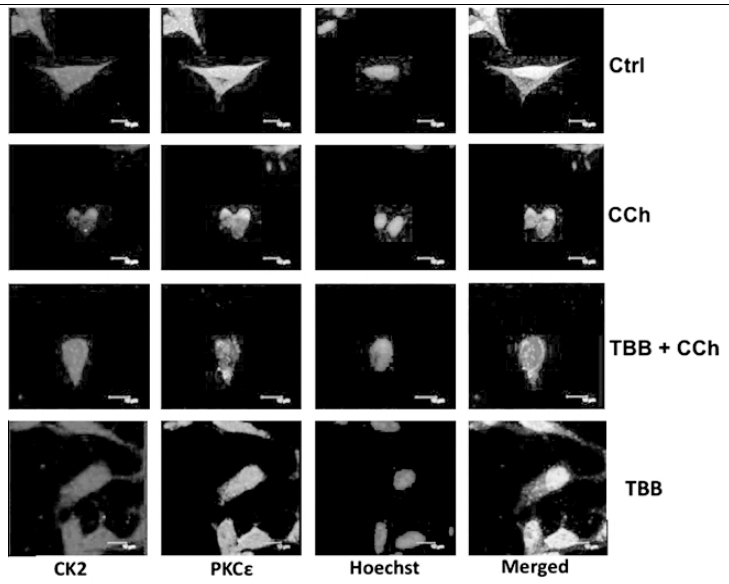

Fig. 4. Immunolocalization of PKCε and CK2. In basal conditions PKCε, marked in green by FITC conjugated polyclonal antibody, and CK2, marked in red by ALEXA 633 conjugated monoclonal antibody, are localized diffusely at a cellular level (panel CTRL). After 100 μM CCh treatment for 20 min, PKCε and CK2 colocalize in Golgi-like structures. Pretreatment with 16 μM TBB for 20 min followed by 100 μM CCh specifically inhibited CK2 translocation, without affecting PCKε subcellular localization in Golgi-like structure (panel TBB+CCh). Treatment with only 16 μM TBB for 20 min does not alter the diffuse distribution of both PKCε and CK2 similar to basal condition (panel TBB). Confocal image, magnification: 63x. (Colours are visible in the electronic version of the article at www.iospress.nl.)

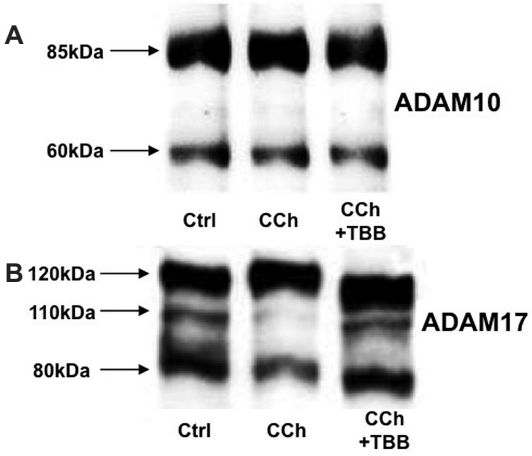

Fig. 5. ADAM10 and ADAM17 profiles after cholinergic stimulation. Western blots have been performed to detect and evaluate the pattern of expression of the two principal putative α-secretase, ADAM10, and ADAM17 (TACE). A) The western blot with anti ADAM10 antibodies does not reveal changes in the pattern of the mature and immature form of the protease during CCh challenge both in the presence or absence of TBB. B) Western blots of ADAM17 show that the addition of CCh induces a significant modification of the pattern of proteins which is abolished by the treatment with TBB.

ed rather uniformly in the cytosol (Ctrl); whereas, following cholinergic stimulation, the kinase translocated to perinuclear Golgi-like structures. Pretreatment with the specific inhibitor of CK2, TBB, did not affect PKCε activation and translocation, thus suggesting that CK2 could be involved downstream of PKCε (Fig. 4) in the CCh stimulated AβPP processing pathway.

ADAM10 and ADAM17 profiles after cholinergic stimulation

The proteases ADAM10 and ADAM 17 (TACE) are the most important and best characterized activities known as α-secretases [3–5]. While TACE/ADAM17 seems to be involved only in the regulated release of sAβPPα [3], ADAM10 appears to be affecting both the regulated and constitutive processing of AβPP [4, 5]. We investigated whether the pattern of maturation and distribution of ADAM 10 and ADAM 17 was affected during CCh challenge. Consistent with previous reports [27], we did not observe modification of the protein pattern of ADAM 10 after CCh treatment (Fig. 5A). We have, however, observed a modification of the protein profile of ADAM17 after challenge with CCh (Fig. 5B). Similarly to the pattern described by Alfa Cissè et al. [28], CCh decreased the appearance of a band that may be ascribed to an active state of ADAM17 as suggested to occur following cholinergic stimulation. Pretreatment with TBB, the specific in-

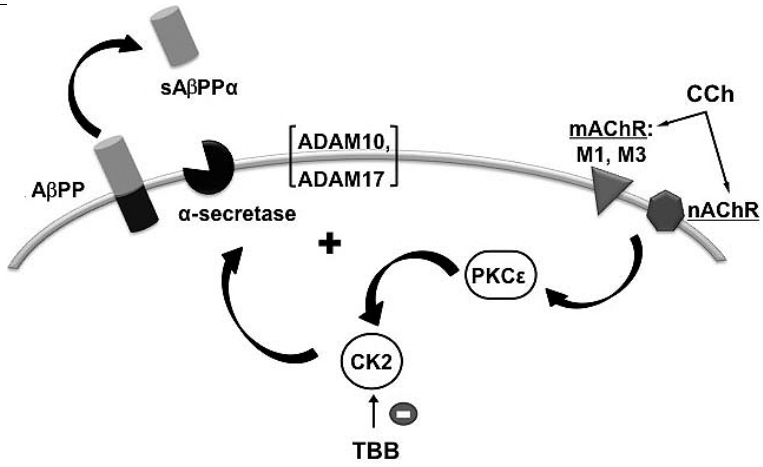

Fig. 6. Schematic pathway of CK2 involvement in AβPP processing downstream of cholinergic stimulation. Carbachol stimulates M1 and M3 muscarinic receptors (mAChR) and the nicotinic receptor (nAChR) activating PKCε which in turn interacts with CK2. This signaling cascade results in sAβPPα release after the AβPP proteolityc cleavage by ADAM10 and especially by ADAM17 as a consequence of their activation by CK2.

hibitor of CK2, abolished the capability of CCh to modulate the profile of ADAM17 (Fig. 5B). It is possible to speculate that the pattern expressed by ADAM17 is derived from the involvement of pro-protein convertates such as furin, which has been suggested to be one of the proteolytic activator of ADAMs [29]. Moreover other authors identified in the structure of furin a consensus sequence for CK2 phosphorylation [30], an event that can occur *in vivo* [31]. We cannot rule out the possibility that the evidence described for the involvement of CK2 in the regulated processing of AβPP depends on one or more of these mechanisms.

Overall, our data suggest that the stimulation of cholinergic receptors by carbachol leads to regulated AβPP processing and release of sAβPPα, through the activation of a signaling cascade that involves specifically PKCε which in turn interacts with CK2 and leads to activation of ADAM17 (as the main α secretase involved) (Fig. 6).

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=320).

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