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Biochemical and Biophysical Research Communications 330 (2005) 714–721
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μ-Calpain is functionally required for α-processing of Alzheimer's β-amyloid precursor protein

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Received 22 February 2005
Available online 17 March 2005

Abstract

Alzheimer's β-amyloid precursor protein (APP) is normally processed by an unidentified α-secretase. A unique feature of this protease is its high sensitivity to phorbol esters, yet the mechanism involved is unclear. We have previously reported that phorbol 12,13-dibutyrate (PDBu) activates calpain, a Ca^{2+}-dependent protease, and PDBu-induced release of APPs (secreted APP) is sensitive to calpain inhibitors, suggesting that calpain is involved in APP α-processing. In the present study, we found that PDBu markedly promoted the expression of both μ- and m-calpains in cultured fibroblasts. Dose–response and time course studies revealed that μ-calpain was more sensitive to PDBu than m-calpain and the temporal course of the μ-calpain change coincides better with that of APPs release. Moreover, the stimulatory effect of PDBu on μ-calpain was selectively blocked by μ-calpain-specific siRNA (small interference RNA) and the blockage was accompanied by a concomitant decrease in APPs release. In contrast, m-calpain siRNA did not affect APPs release significantly. Measurement of amyloid β protein (Aβ) release in the μ-calpain siRNA-treated cells indicated that Aβ40 and Aβ42 levels inversely changed in relation to APPs, and the changes in Aβ42 were more prominent than in Aβ40. Together, these data suggest that calpain, particularly μ-calpain, is a potential candidate for α-secretase in the regulated APP α-processing, and that changes in this protease can affect the outcome of the overall APP processing.
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Keywords: Alzheimer; Amyloid; Calpain; siRNA

The mechanism underlying amyloid plaque formation in Alzheimer's disease (AD) remains to be understood. Amyloid plaques primarily consist of β-amyloid protein (Aβ), an undigested remnant of β-amyloid precursor protein (APP). Aβ is generated by the sequential actions of two proteases, β- and γ-secretases, yet normal processing of APP holoprotein is mediated by α-secretase which cleaves APP within the Aβ domain between residues 16 and 17 [1,2]. This latter cleavage results in secretion of a large (100–120 kDa) amino-terminal fragment of APP, i.e., secreted APP (sAPPα or APPs). Since this pathway precludes the formation of Aβ and its activation has been shown to reduce Aβ production [3], it is evident that studies on α-secretase are of critical importance for potential therapeutic intervention of AD. Although considerable efforts have been devoted in this research area and several candidate proteases have been suggested [4,5], the identity of α-secretase has not been established. This gap in our knowledge has limited progress in understanding the mechanism of amyloid plaque formation, a hallmark event in the aged brain.

We have previously observed that a protease with characteristics of α-secretase in human platelets is

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0006-291X/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.bbrc.2005.03.029

indistinguishable from calpain, a well-known Ca^{2+}-dependent neutral protease [6]. Subsequent studies reveal that the remarkable stimulation of α-secretase by phorbol esters, a unique feature of the enzyme, may also be mediated by calpain [7]. These findings have led us to propose calpain as a potential candidate for α-secretase for further studies [6,7].

Calpain is a key mediator in Ca^{2+} signal transduction and is essential for basic life processes such as cell division, differentiation, and growth [8]. In the calpain family there are 14 subtypes known today, but only two of them are ubiquitous, i.e., μ- and m-calpains (also called calpain I and II). The latter two calpains differ in the concentrations of Ca^{2+} required for their activation when tested in vitro and they both contain a small Ca^{2+}-binding subunit and a large catalytic subunit (about 80 kDa). The cDNAs of the catalytic subunits of human μ- and m-calpains are comprised of 714 and 700 amino acid residues, respectively, and they share 62% sequence similarities [9,10]. In the present study, we took advantage of RNA interference, a highly stringent method [11], to knock down calpain expression and to further explore the role of calpain in APP processing. Our data suggest that μ-calpain, but not m-calpain, is functionally required for APP α-processing.

Materials and methods

Materials. Human skin fibroblasts were purchased from ATCC (Gaithersburg, MD). Dulbecco's modified Eagel's medium (DMEM), phorbol 12,13-dibutylester (PDBu), fluorogenic peptide Suc-LLVY-AMC, calpeptin, and a protease inhibitor cocktail were from Sigma (St. Louis, MO). The siRNA duplexes specific to the catalytic subunits of μ- and m-calpains (Catalog No. M-005799-00 and M-005804-00) were from Dharmacon RNA Technologies (Boulder, CO). The siRNA for each calpain contained four RNA sequences in a pool SmartPool selected from the NCBI RefSeq Database by a proprietary algorithm. ELISA kits for measuring Aβ40 and Aβ42 were from Signet Laboratories (Dedham, MA).

Cell culture and calpain expression assay. Fibroblast cells were maintained at 37 °C with 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. To test the effects of PDBu on calpain expression, the cells were cultured in six-well plates until 80–90% confluence was reached. At that point, medium was replaced with fresh serum-free DMEM. For dose–response studies, varying concentrations of PDBu were added and incubated at 37 °C for 2 h. For time course studies, 1 μM of PDBu was added to the media and incubated for various times. At the end of incubation, the conditioned media were removed, assayed for APPs and fibronectin (see below), and cells were trypsinized, collected by low-speed, centrifugation and lysed in a Triton buffer (20 mM Hepes, pH 7.2; 145 mM NaCl, 5 mM KCl, EGTA 10 mM, 1% Triton X-100, and 1× protease inhibitor cocktail). After standing on ice for 10 min, cell lysates were centrifuged at 16,000*g* for 10 min and the supernatant fraction was processed for SDS-gel electrophoresis and Western blotting analyses as previously described [12,13]. A monoclonal antibody to human μ-calpain (dilution 1:1000) (CalBiochem, CA) and a polyclonal antibody to human m-calpain (1:500) (Triple Point Biologics, WA) were used. Actin was detected by a monoclonal antibody to human α-actin (1:5000) (Simga, MO).

APP and fibronectin secretion assays. Conditioned medium from PDBu-treated cells was collected and cleared by centrifugation at 16,000*g* for 10 min. Proteins were precipitated and processed for Western blotting analyses as we described [7]. APPs was determined with a monoclonal antibody to the N-terminus of APP, 22C11 (1:1000) (Chemicon, Temecula, CA), whereas fibronectin was detected in the same conditioned medium with a monoclonal antibody to fibronectin (1:5000) from Sigma (MO). The immunoreactive proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham, IL). The protein density was measured in a Kodak Imaging Station CF440.

siRNA-mediated calpain gene silencing. The protocol followed the manufacturer's instructions. The siRNAs specific to the catalytic subunits of μ- or m-calpains were solubilized and formed complexes separately with a lipid-based transfectant, LipofectAMINE (Invitrogen, CA). The siRNA-LipofectAMINE complexes were transfected into the cultured cells at various concentrations in a 6-well plate at 70–80% of cell confluence and incubated for 48 h (with medium change at 24 h). In mock transfection, all vehicles were used except for the siRNA. At the end of incubation, cells were washed, placed in fresh DMSM and stimulated by the addition of 1 μM PDBu for 2 h. Calpain enzymatic activity and protein levels were measured in separate experiments. Throughout the experiments, the health of the cells was monitored continuously. There was a visible shrinkage of the cells after siRNA treatments, but cells were otherwise healthy as determined by MTT cell viability test [7].

Enzymatic activity of calpain. Calpain activity in situ was measured using a fluorogenic, membrane-permeable peptide, Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC), as reported earlier [7]. Cells were treated with siRNA for 48 h. After washing, 50 μM Suc-LLVY-AMC was added and followed by stimulation with 1 μM PDBu for 2 h in the serum-free DMEM incubated at 37 °C. Fluorescence generated was measured in a Synergy HT microplate reader (BioTek Instruments, Winooski, VT) using 360 nm excitation and 460 nm emission filters [7].

Measurement of Aβ1–40 and Aβ1–42. Chromogenic sandwich ELISA kits (Signet Laboratory, Dedham, MA) were used according to the manufacturer's instructions. Cells were treated with siRNA as described above, followed by stimulation with PDBu for 8 h at 37 °C. Some cells were treated with calpeptin together with PDBu as previously described [7] but with extended duration (8 h). Conditioned media were collected and cleared by centrifugation at 16,000*g* for 15 min. Aliquots (200 μl each) of the samples were placed in a 96-well ELISA plate that had been pre-coated with a capture antibody (to the N-terminus of human Aβ protein) and incubated for 16 h at 4 °C. After washing, a reporting antibody that binds to the C-terminus of either Aβ1–40 or Aβ1–42 were added. After incubation for 2 h and extensive washing, a horseradish-conjugated secondary antibody was added. Color developed with substrate *o*-phenylenediamine was measured at OD_{492} in a BioKinetic EL3 microtiter spectrophotometer. The assay sensitivity in this method was found in the pM range and the cross-reactivity between Aβ1–40 and Aβ1–42 was insignificant.

Data analysis. Data obtained from Western blotting and ELISA were subjected to Student's *t* test analyses to assess the significance of differences between experimental and control values. Protein data were calculated as band density multiplied by the area occupied by the reactive proteins. The results were expressed as means + SEM.

Results

PDBu stimulated calpain expression

We recently reported that phorbol 12,13-dibutylester (PDBu), a widely used phorbol ester, stimulated calpain