J. Biochem. 2012;151(3):299–316 doi:10.1093/jb/mvs001

JB THE JOURNAL OF BIOCHEMISTRY

Short peptide tools for monitoring caspase and proteasome activities in embryonal and adult rat brain lysates: an approach for the differential identification of proteases

Received August 25, 2011; accepted November 21, 2011; published online January 5, 2012

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The numerous caspase-like activities present in nervous tissue can be investigated with labelled peptides. However, the cross-reactivities of peptides with both proteasomes and caspases complicate the analysis of protease activity. The pharmacological features of substrates and inhibitors specific for either caspases or proteasome caspase-like proteases in rat brain lysates were similar or identical to the profiles of commercially purified proteasome preparations. Caspase inhibitors bind directly to active proteasome centres, thus competing with selective antagonists of proteasomes. Separation of lysates by molecular weight does not separate active caspases from proteasomes because these enzymes co-localize under native electrophoresis. The addition of ATP or its analogues is associated with the differential modulation of proteasomal activity, which also leads to ambiguity in the data. However, induced caspase activity could be successfully differentiated from proteasome activity in embryonal brain lysates with the non-selective caspase inhibitors Z-VAD-FMK and Q-VD-OPh and the proteasome inhibitor AdaAhx(3)L(3)VS that are not cross-reactive. This strategy is proposed for the simultaneous examination of caspases and proteasomes using proteolysis experiments. The present study reveals that all of the caspase-like activities in the tissue lysates of non-injured adult rat brains were related to proteasomal caspase-like activities.

Keywords: apoptosis/brain/caspase/inhibitor/proteasome/rat/substrate.

Abbreviations: AAF-CMK, Ala–Ala–Phe–chloromethylketone·TFA; Ac-DEVD-AMC or Ac-DEVD–CHO, N-acetyl-Asp–Glu–Val–Asp–AMC or –CHO; Ac-DQMD-CHO, *N*-acetyl-Asp–Gln–Met–Asp–CHO; Ac-GPLD-AMC, *N*-acetyl-Gly Pro–Leu–Asp–AMC; Ac-LEHD-AMC or Ac-LEHD-CHO, *N*-acetyl-Leu–Glu–His–Asp–AMC or –CHO; Ac-IETD-AMC or Ac-IETD-CHO, *N*-acetyl-Ile–Glu–Thr–Asp–AMC or –CHO; Ac-VDVAD-AMC or Ac-VDVAD-CHO, *N*-acetyl-Val–Asp–Val–Ala–Asp–AMC or –CHO; Ac-VEID-AMC or Ac-VEID-CHO, *N*-acetyl-Val–Glu–Ile–Asp–AMC or –CHO; Ac-YVAD-AMC or Ac-YVAD-CHO, *N*-acetyl-Tyr–Val–Ala–Asp–AMC or –CHO; AdaAhx(3)L(3)VS, adamantaneacetyl-(6-aminohexanoyl)3-(leucyl)3-vinylmethyl-sulphone; AFC, 7-amino-4-trifluoromethyl coumarin; AMC, 7-amino-4-methylcoumarin; biotinyl-AdaAhx(3)L(3)VS, Ada–Lys(biotinyl)–(Ahx)3–(Leu)3–vinyl sulphone; –CHO, aldehyde; LRR-AMC, Boc–Leu–Arg–Arg–AMC; Q-VD-OPh, Quinoline-Val–Asp–Difluorophenoxymethylketone; Suc-LLVY-AMC, Suc–Leu–Leu–Val–Tyr–AMC; Z-LLE-AMC, Z-Leu–Leu–Glu–AMC; Z-VAD-AFC, Z-Val–Ala–Asp–AFC; Z-VAD-FMK, Z-Val–Ala–Asp–fluoromethylketone.

Proteasomes are macromolecular enzyme complexes related to the N-terminal nucleophilic hydrolases (NTN hydrolases). Proteasomes are responsible for the destruction of most intracellular proteins; ~90% of the intracellular proteins in growing mammalian cells are degraded by proteasomes (*1*, *2*). Protein substrates that are targeted for destruction are polyubiquitinated and then degraded by the 26S proteasome, which is composed of more than 31 different subunits. The multi-catalytic proteasome includes a cylindrical catalytic 20S complex, which is found in all eukaryotic cells and several types of regulatory subunits (PA28R, PA28γ and PA200) that are added to form the 26S proteasome (*3*, *4*).

The 20S subunit is composed of four seven-membered rings, with the two outer rings composed of α subunits and the two central rings composed of β-subunits, as well as 19S caps composed of 6 ATPase and 11 non-ATPase subunits. In eukaryotes, three of the seven β-subunits have threonine protease catalytic centres of diverse substrate specificity, such that every proteasome has six protease centres. Subunits β1 and β2 have caspase-like and trypsin-like activities, respectively, hydrolyzing the peptide bond after negatively (β1) or positively charged (β2) residues. Subunit β5 has chymotrypsin-like activity, hydrolyzing the peptide bond after large hydrophobic residues (*5*). All of the protease centres face an internal proteolytic chamber

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formed by β-subunits. The substrate accesses these centres via a gate formed by the α-subunits. In addition to these three main types of proteinase centres, two other centres have been reported. ATP serves multiple functions in proteolysis, but the only step that absolutely requires ATP hydrolysis is the unfolding of globular proteins (*3*, *6*, *7*).

Programmed cell death (apoptosis) is a physiological mechanism that maintains organism homoeostasis. Apoptosis is caused by the activation of proteases whose substrate specificity is much higher than that of proteasomes (*8–10*). Blocking of proteasome activity with specific inhibitors often leads to apoptosis (*2*, *11*, *12*). Oxidative stress, mitochondrial disruption or an imbalance of calcium homoeostasis can all alter the functionality of proteasomes and induce apoptosis (*13*, *14*). This correlation suggests the existence of direct or indirect linkages between proteasome activity and apoptosis (*15*).

Caspases are dimeric thiol proteases with primary specificity for aspartate residues. The initiator caspases begin the process of cell death by activating the effector caspases. The effector caspases are activated through autocatalysis and can cleave another caspase or a specific substrate with high selectivity (*8*, *10*, *16*). The activation of apoptosis after the permeabilization of the mitochondrial membrane is associated with the release of apoptosis-inducing factor, which induces the mitochondria to release the apoptogenic proteins cytochrome C and procaspase-9. Apoptotic peptidase activating factor 1 (APAF-1), dATP (or ATP), procaspase-9 and cytochrome C form a multi-protein complex that activates procaspase-9, leading to apoptosome formation. Cleaved caspase-9 in the apoptosomes directly activates caspases-3 and -7 (*10*, *14*, *17–20*).

The tissue-specific localization of various caspases has been described. In particular, the induction of apoptosis in the rodent brain is associated with the activation of caspase-3 and -9 and is mediated by APAF-1 and the release of mitochondrial cytochrome C. This process can be induced in rat brain lysates, but some specific proteins necessary for apoptosis (perhaps APAF-1 and/or procaspase-9) are normally only present in embryos, although they can be induced in adult rats by trauma (*21–23*).

The proteolysis of regulatory proteins by proteasomes with the help of specific ubiquitination machinery rapidly eliminates and modifies these proteins. Ubiquitination directs target proteins for destruction by proteasomes. Protein ubiquitination can also modify protein activity through the regulatory abilities of conjugated ubiquitin chains. A family of proteins called the IAPs (Inhibitor of Apoptosis Protein) and IAP-antagonists serve as a physical bridge that mediates binding to E2 ubiquitin-conjugating enzymes, enabling the IAPs or IAP-antagonists to act as E3 ubiquitin ligases. The E3 ligases work as substrate-binding modules, thereby underlying substrate specificity. Both IAPs and IAP antagonists can regulate caspases by direct binding and ubiquitination (*9*, *24*, *25*). Protein stability controlled by ubiquitination can also be used to regulate pre-activated caspases as a way of maintaining continuously low levels of the apoptotic machinery (*9*). There is information regarding the direct interaction between proteasomes and caspases with the participation of caspase recruitment domain-containing protein, which is found in the 26S proteasome in the rat brain (*26*).

Much is known about the relationship between the proteolysis machines and their regulatory pathways. The proteolysis activities of various caspases and proteasomes have been studied using short peptides of 3–5 amino acids with an N-terminal β-naphthol or fluorochrome (*17*, *27–32*). As a rule, such studies have focused on the examination of either caspase or proteasome activity; to our knowledge, no study has examined both activities simultaneously. Furthermore, there is no detailed information about peptides selective against both proteolysis machineries.

For example, one of the three major types of proteasome activities (peptidyl-glutamyl-peptidase) can be examined with the caspase substrates Ac-DEVD-AMC and Ac-YVID-AMC (*33*). Taking into account these observations, one should consider the possibility of cross-reactivity between the machineries before using the aforementioned peptide tools. Nevertheless, numerous studies have used such short peptides while ignoring the cross-reactivities of the different proteases. Additionally, the actual specificity of the substrates and inhibitors for different types of caspases has not been confirmed (*34*, *35*).

The purpose of this study was to determine a method for the simultaneous study of two proteolytic processes in nervous tissue: processes associated with caspase activation and with proteasome caspase-like activity. Caspase inhibitors bind directly to the active centres of proteasomes, thus competing with selective proteasome antagonists. The induced caspase activity *in vitro* in the developing rat brain was on the same or higher level as the proteasomal activity. The caspase activity was successfully differentiated from the proteasomal activity with the non-selective caspase inhibitors Q-VD-OPh and Z-VAD-FMK (at concentrations specific for inhibition of caspases), which do not inhibit the proteasome caspase-like activity. These data suggest that in adult non-injured nervous tissue, the primary and possibly the only targets of peptide caspase inhibitors and substrates are proteasomes.

Materials and Methods

Materials

All substrates, standards and inhibitors were from Enzo Co. or SM Biochemicals LLC. Ferritin and the higher molecular weight (MW) standards were from Invitrogen Inc. All other chemicals were from GE Healthcare, Sigma, Aldrich, USB or Enzo and were of molecular biology grade or higher. Enzymes, substrates and inhibitors were dissolved in dimethyl sulfoxide (DMSO), aliquoted and stored in a freezer or in liquid nitrogen. All reactions (except for reactions in the on-line mode) were performed in Eppendorf Thermomixers.

The lysis buffer and reaction mixture were of the following composition: 25 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 7.4), 3 mM dithiothreitol, (2S,3S)-1,4-bis(sulfanyl)butane-2,3-diol (DTT), 5 mM $MgSO_4$, 5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 50 mM NaCl, 0.01% (w/v) Triton X-100, 10% (w/v) glycerol (not used at homogenization) and a protease inhibitor cocktail (Roche Applied Science). dATP and other dNTP/NTPs were added to the reaction mixture in a buffered form.

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Adding bovine serum albumin (BSA) (1%) to the reaction mixture did not significantly affect the reaction but was performed according to the manufacturer's instructions in experiments with proteasomal preparations.

Rats and tissue preparations

Wistar male and female adult rats (weight, 180–240 g) and embryos at E18–E22 (15–19 days of pregnancy) were used. All of the animal experiments were conducted according to locally and generally accepted rules and norms.

Rat brain tissues were washed several times with a physiological solution that had been cooled on ice. The tissues were homogenized in a glass homogenizer in lysis buffer with 3 mM DTT in the presence of a protease inhibitor cocktail and diluted according to the manufacturer's recommendations. The homogenate was centrifuged twice at 14,500*g* (4°C, 30 min). The supernatant was stored in liquid nitrogen or used fresh. Mini-columns loaded with Sephadex G-10 equilibrated with 20 volumes of a solution identical to the reaction mixture (with DTT but without protease inhibitors) were used to remove low MW components such as nucleotide di- and triphosphates.

All efforts were made to minimize the number of animals used and to minimize distress to the animals.

Determination of protein concentration

The protein concentration was determined by a modified Bradford method (*36*). The protein concentrations in the samples of lysates ranged from 72 to 96 μg ml^{-1}. In experiments with purified commercial preparations of 26S proteasomes from human erythrocytes and proteasomal fraction 2 from rabbit reticulocytes, the protein concentrations were 0.5 and 5 μg ml^{-1}, respectively.

Cytochrome C quenching of the AMC fluorophore

The cytochrome C content was determined by optical spectroscopy using an extinction coefficient $\varepsilon_{410\,nm}$ of 106.1 $mM^{-1}cm^{-1}$. The fluorescence of free AMC/AFC was quenched by cytochrome C in a dose-dependent and non-linear manner. Up to 20% of the AMC or AFC fluorescence was quenched at a cytochrome C concentration of 10 μM.

Substrates to determine enzyme activities

Chymotrypsin-like activity was determined using the substrate Suc-LLVY-AMC. Trypsin-like activity was determined using the substrate Ac-LRR-AMC. Caspase-like activity was determined using the substrates Z-LLE-AMC, Ac-GPLD-AMC, Ac-VEID-AMC, Ac-VEID-AFC, Ac-IETD-AMC, Ac-VDVAD-AMC, Ac-DEVD-AMC, Ac-LEHD-AMC, Ac-YVAD-AMC and Z-VAD-AFC and the inhibitors Z-VAD-FMK, Ac-DQMD-CHO, Ac-VEID-CHO, Ac-IETD-CHO, Ac-VDVAD-CHO, Ac-DEVD-CHO, Ac-LEHD-CHO, Q-VD-OPh and Ac-YVAD-CHO. Oligopeptide-peptidase activity was determined using the substrate AAF-CMK. Epoxomicin, biotinyl-AdaAhx(3)L(3)VS and AdaAhx(3)L(3)VS were used as selective irreversible inhibitors of proteasomes (*28*, *30*, *37*). Fluorescent substrates were used at concentrations of 50 μM (for caspase-like activity) or 100 μM (for trypsin-like or chymotrypsin-like activities). The indicated manufacturer qualities of the substrates Ac-VEID-AMC, Ac-GPLD-AMC and Z-LLE-AMC were verified by high-performance liquid chromatography followed by mass spectroscopy; it was confirmed that ≥95% of the peptides were labelled with AMC.

Spectrofluorescence

The rate of substrate consumption was determined by monitoring the absorption at 325 nm (intact substrate) and 350 nm (maximum absorption of free 7-amino-metilcumarin—AMC) for AMC and 380 nm/505 nm for AFC. The fluorescence of free AMC/AFC was determined by spectrofluorometry using a Hitachi F4000 at excitation and emission wavelengths of 350/380 nm and 440/505 nm, respectively. Calibration curves were obtained using the components of the reaction mixture, test protein and a certain amount of standard solution of AMC/AFC (BioMol). Plots were constructed for each size of excitation and emission gap. The online monitoring of the samples in a temperature-controlled cuvette for 2 h at 34°C indicated that the reaction process was linear.

Determination of enzyme activities

The amount of cleaved substrate did not exceed 10% of the total peptide concentration as determined by measuring the absorption of the sample at 325 and 350 nm at the end of the reaction. The linearity of the peptidase assays with the indicated amount of brain tissue extract and a reaction time of ~1.5 h was demonstrated in the preliminary experiments.

The background signal was determined at the zero time-point in the presence of all the ingredients of the reaction mixture, as well as thermally inactivated enzyme/lysate. The basal level of each reaction was detected for each probe with the enzyme/lysate, which had been inactivated by the inhibitor. The incubation of the fluorescent substrates in a reaction mixture without lysate or enzyme did not alter the background fluorescence levels.

Due to the presence of 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and leupeptin, molecules that inhibit trypsin-like activity, in the protease inhibitor cocktail, the trypsin-like activity of proteasomes was examined to a lesser extent than the other enzymes in the brain tissue extracts. Usually, the protease inhibitor cocktail was not used in the experiments with the commercially purified preparations of proteasomes. The addition of this cocktail will be indicated separately.

The samples were pre-incubated with the inhibitors at 4°C under shaking conditions for 30 min. In the inhibitor assays, the amount of protein used to study the effect of the inhibitors was the same as in the control and pre-incubation without inhibitors (or with 5% DMSO only) and did not affect the quantification of the peptidase activities when compared with the untreated samples. The reaction was started by heating the samples to 34°C and adding the fluorescent substrates. The reactions were stopped by cooling on ice.

Native PAGE and western blot procedure

A total of 75–85 μg of protein lysate or 0.5–5 μg of either human 26S proteasome preparations or proteasomal fraction 2 from rabbit reticulocytes per well were subjected to 8–12% SDS–PAGE. The protocols and the dilutions of the antibodies used were according to the manufacturers' recommendations.

The primary antibodies, including anti-caspase-9 (ab32539), anti-caspase-8 (ab52910 and ab25901) and anti-caspase-3 (ab13847) (Abcam) were used in the presence of 1% normal goat serum. The secondary anti-rabbit antibodies [IgG - H and L - F (ab) 2 Fragment] were also from Abcam. The streptavidin–HRP conjugate, membrane blocking agent (used at 2.5% with 1% BSA in phosphate buffered saline) and enhanced chemiluminescence (ECL) western blotting detection reagents were from GE Healthcare. The polyvinylidene fluoride membranes with supports were from Millipore and Invitrogen.

Native PAGE and two-dimensional SDS–PAGE were performed as described previously (*3*, *38*, *39*). Native PAGE (or native PAGE–agarose) 3.5–13% gradient gels were subjected to 18-h electrophoresis at 5°C. After electrophoresis, the gels were immersed for 10 min in the reaction solution with the protease inhibitor cocktail, followed by immersion in the same solution containing the investigated substrate. The protease activity was visualized after 30 min at 34°C.

Statistical analysis

Data were obtained from at least three independent measurements. The data were plotted using the Origin 4 software with a confidence interval of $P = 0.05$. The data are presented as the mean ± standard deviation (SD).

Results

Comparative investigation of the influence of inhibitors on protease activities from different sources

Protease activities in the extracts of adult rat brains. An initial screening revealed that the different caspase substrates were cleaved with different efficiencies in the adult and embryonal rat brain. The ratios between caspase substrate cleavage, peptidylglutamyl-peptide and chymotrypsin-like hydrolyzing activities of the proteasomes in the lysates are shown on Fig. 1 with

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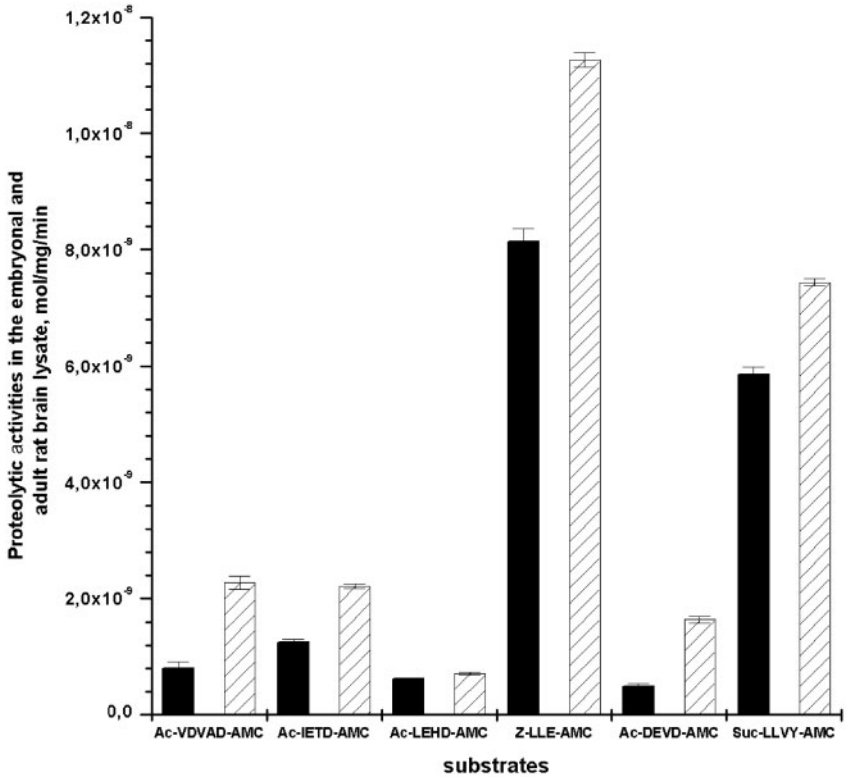

Fig. 1 AdaAhx(3)L(3)VS-sensitive proteolytic activity in adult and embryonal (stage E19) rat brain lysates. The initial velocity of the reaction was calculated with different substrates: the black columns indicate the activities in the adult brain lysates; the striped columns indicate the activities in the embryonal lysates. Total activity for each substrate was calculated as the difference between total activity and activity without a protein probe (blank). In each column, activity was calculated as the total level minus the activity in the presence of 1 μM of AdaAhx(3)L(3)VS for the first 10 min of reaction. For example, Ac-VEID-AMC and Ac-YVAC AdaAhx(3)L(3)VS-sensitive activities (nmol/mg protein/min) were equal to 3.5 ± 0.2 and 2.9 ± 0.2 in the adult brain lysates and 6.8 ± 0.3 and 6.0 ± 0.5 in embryos, respectively. The total level activity without inhibitor was calculated at 100%.

respect to rat age. The proteolytic cleavage of all of these tested substrates was inhibited by AdaAhx(3)L(3)VS with equal efficiency (Fig. 2A). The analysis of the caspase substrates cleaved by the proteases in the tissue lysates indicates that the caspase aldehyde inhibitors did not possess selectivity against their amino acid–analogous substrate peptides (Supplementary Table SI). All the studied aldehyde inhibitors also decreased the proteolysis of Z-LLE-AMC (Fig. 2B) and Ac-GPLD-AMC (Fig. 2C) in a manner that was very similar to the inhibition of the Ac-VEID-AMC cleavage (Fig. 2D).

The inhibitory potential of the aldehyde inhibitors can be divided into several groups, in each of which the efficiency of inhibition is similar to the range of IC_{50} values. Ac-VEID-CHO was the most effective inhibitor ($IC_{50} = 0.13 \pm 0.04$ μM) for all of the substrates especially to Ac-GPLD-AMC. The inhibition of Ac-GPLD-AMC cleavage was in the lower range of concentrations compared with other studied substrates. Ac-YVAD-CHO inhibited the activity of all of the proteases with a comparatively uniform efficiency ($IC_{50} = 1.08 \pm 0.05$ μM). The IC_{50} values of Ac-DQMD-CHO, Ac-LEHD-CHO and Ac-IETD-CHO were 0.43 ± 0.19, 0.72 ± 0.21 and 0.6 ± 0.28 μM, respectively. Ac-VDVAD-CHO did not significantly inhibit protease activity at concentrations ranging from 10 nM to 10 μM, but it demonstrated an inhibitory potential towards Z-LLE-and Ac-GPLD-ase activities (Fig. 2A–D and Supplementary Table SI).

The existence of oligopeptidase proteolytic activity in brain lysates can be indicated by the presence of active tripeptide–peptidase 2 (*40*, *41*). AAF-CMK (Ala–Ala–Phe–CMK • TFA) was tested as an additional irreversible inhibitor of this enzyme. This inhibitor had no effect on the caspase-like enzyme activity in brain lysates for any of the investigated caspase substrates. AAF-CMK in the presence of a chymotrypsin-like substrate inhibited activity at a concentration range of 10–100 μM (maximally at 30%) but had no effect on Boc-LRR-ase or Z-LLE-ase activity (data not shown).

Trypsin-like activity was usually inhibited in the tissue extracts by a protease inhibitor cocktail (mostly by leupeptin and AEBSF) ('Materials and Methods' section). To investigate trypsin-like activity, the protease cocktail was omitted from the reactions. Among all of the caspase inhibitors, only Ac-DQMD-CHO influenced the cleavage of Boc-LRR-AMC, increasing the proteolytic activity in a dose-dependent manner by up to ~20%. The caspase aldehyde inhibitors had no influence on Boc-LRR-ase cleavage in the presence of the protease inhibitor cocktail.

In the presence of the protease inhibitor cocktail, none of the caspase inhibitors affected the

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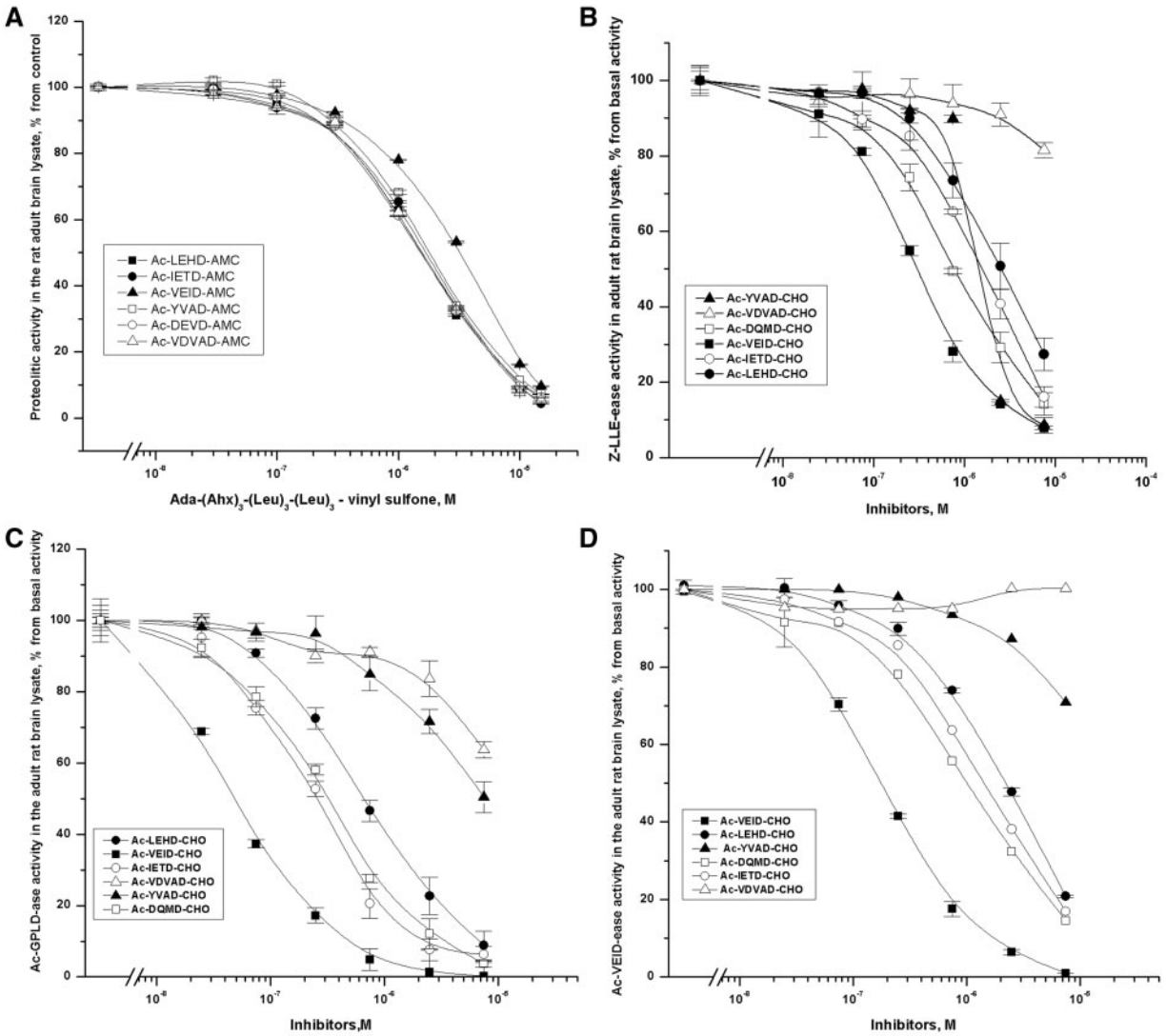

Fig. 2 Influence of different inhibitors on caspase-like protease activity in adult rat brain lysates. AdaAhx(3) L(3)VS-sensitive inhibition of the cleavage of different caspase substrates (A). Influence of different caspase inhibitors on Z-LLE-ase- (B), Ac-GPLD-ase- (C) and Ac-VEID-ase- (D) specific activity, calculated as the difference between the total activity and the activity in the presence of 1 μM of AdaAhx(3)L(3)VS.

Suc-LLVY-ase activity in rat brain lysates. In the absence of the cocktail, caspase inhibitors at concentrations >1 μM decreased the chymotrypsin-like activity to 2.8–4% (Ac-YVAD-CHO, Ac-DQMD-CHO, AC-LEHD-CHO) and 7–8% (Ac-VEID-CHO and Ac-IETD-CHO). In the adult rat brain tissue lysates, epoxomicin inhibited both chymotrypsin-like activity (up to ~90%) and cleavage of caspase substrates in a saturating manner with an IC_{50} of 0.25 ± 0.04 μM for Suc-LLVY-AMC and all of the studied caspase substrates, except for Z-LLE-AMC (IC_{50} was equal to 11 ± 0.1 nM). The most effective peptidylglutamyl-peptide hydrolyzing activity was obtained for Ac-VEID-AMC/AFC cleavage. The Ac-VEID-ase activity demonstrated in saturation concentrations (>0.5 μM) was 92% and 97% inhibition by epoxomicin and AdaAhx(3)L(3)VS, respectively.

AdaAhx(3)L(3)VS was used as an irreversible inhibitor that selectively binds to each of the three types of proteasome active centres. AdaAhx(3)L(3)VS maximally inhibited the enzymatic activity to background levels, with approximately equal efficiency against all caspase substrates ($IC_{50} = 1.05 \pm 0.03$ μM) (Fig. 2A).

Protease activities in the extracts from embryonal rat brains

To induce the activation of caspases, cytochrome C and dATP were added for 30 min at 34°C to a solution of lysates purified on Sephadex G-10 columns, after cold incubation with irreversible inhibitors [epoxomicin, Q-VD-OPh, Z-VAD-FMK, or AdaAhx(3)L(3)VS] or with the same concentration of DMSO (as a control). The specific AdaAhx(3)L(3)VS)-sensitive proteolysis of the various substrates was higher in the brain neural tissue of stage E18–E22 rat embryos than in newborn rats and adult animals (Figs 1, 3A and B). The apoptosis-induced Q-VD-OPh- or Z-VAD-FMK-sensitive caspase activities were obtained in the tissue lysates of the brain (maximally versus other tissues), heart and liver in embryos only. We could not obtain similar induced caspase activities in the adult tissues. Non-inducible

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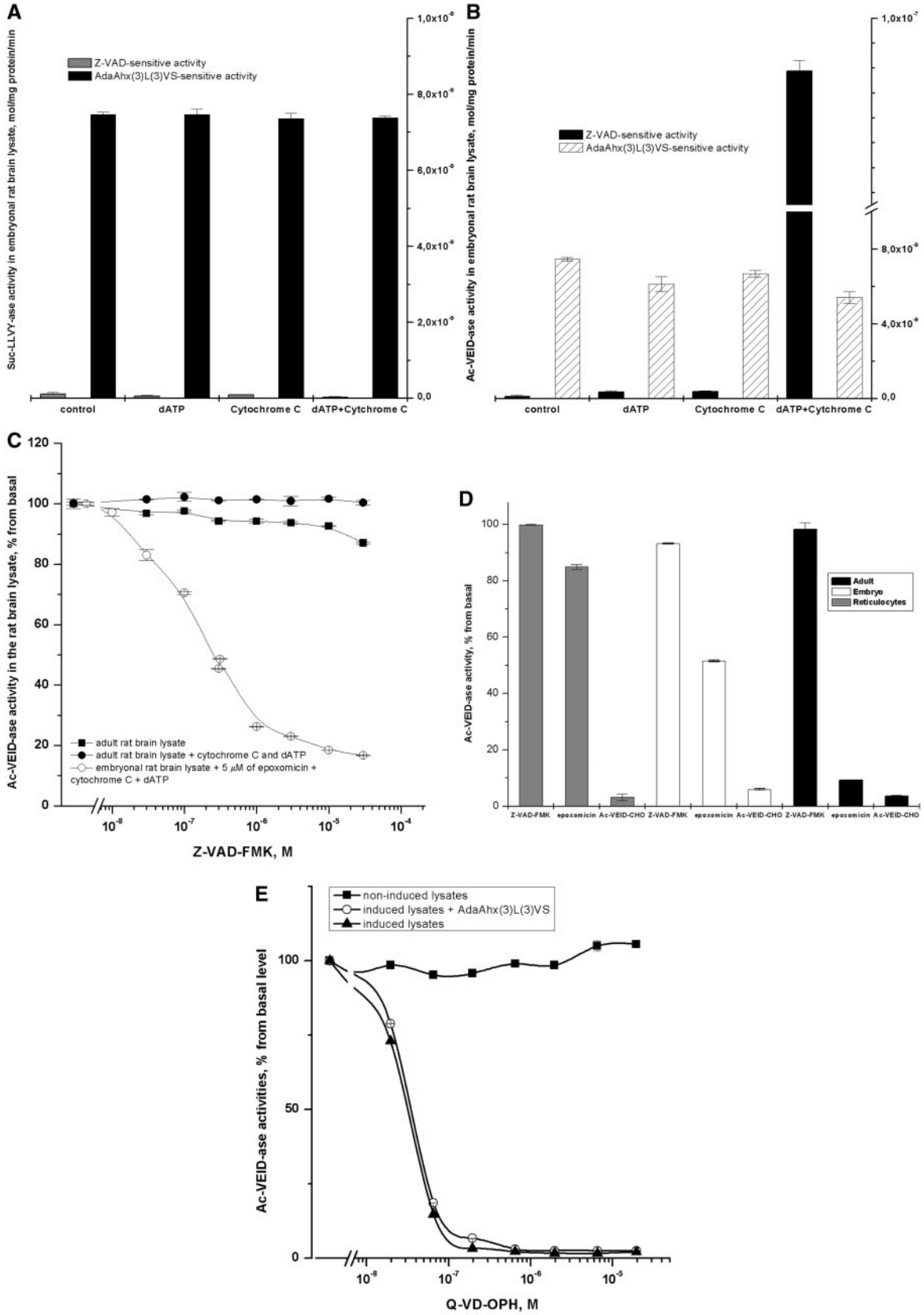

Fig. 3 Comparative effects of AdaAhx(3)L(3)VS, epoxomicin and Z-VAD-FMK on embryonal (stage E22) rat brain lysates. Treatment with the inhibitors was performed before apoptosis induction *in vitro*. Suc-LLVY-ase (A) and Ac-VEID-ase (B) activities. The black or striped columns in (A) represent Z-VAD-FMK-(5 mM) or AdaAhx(3)L(3)VS-sensitive (10 mM) activity, respectively. The control indicates the result without the induction of *in vitro* apoptosis. (C) The influence of Z-VAD-FMK on Ac-VEID-AMC cleavage. Epoxomicin-sensitive Ac-VEID-ase activity in the control adult (filled circle) or control adult with cytochrome C and dATP (filled square) rat brain lysates. In the cases with filled symbols, the basal activity is determined as the difference between the total activity and the activity after treatment with 5 μM epoxomicin without apoptosis

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Q-VD-OPh- or Z-VAD-FMK-sensitive cleavage of the caspase substrates was identified in white blood cells (after the elimination of the erythrocytes with ammonium sulphate) in adults.

The treatment of brain lysates with cytochrome C or dATP alone did not lead to Z-VAD-FMK-sensitive caspase activation. Pre-incubation with Z-VAD-FMK completely inhibited apoptosis induction but did not affect the chymotrypsin-like activity (Fig. 3A–C). The inhibitory effect of epoxomicin on the activity of proteases against caspase substrates in nervous tissue changed with age. In lysates from the rat embryonal brain, epoxomicin inhibited Ac-VEID-ase activity by nearly 50%. The same activity in adult rat brain lysates demonstrated a >90% sensitivity to epoxomicin. In contrast to the influence of epoxomicin, this activity was equally sensitive to Ac-VEID-CHO in both adult and embryonal brain lysates (Fig. 3D).

The Q-VD-OPh- and Z-VAD-FMK-sensitive caspase activities could be induced only in the lysates of embryonal or newborn rats and not in adult rat brain tissue lysates. Pre-incubation with cytochrome C and dATP triggered caspase-like activity, which was not inhibited by epoxomicin or AdaAhx(3)L(3)VS but was effectively inhibited by all studied peptide aldehyde inhibitors, Z-VAD-FMK (Fig. 3C) and Q-VD-OPh. The last compound was the most potent inhibitor among all tested (Fig. 3E).

There was a several fold increase in protease activity that could be monitored for all of the investigated substrates with different efficacies after the apoptosis induction in the samples of embryonal lysates. The initial velocity of the caspase proteolysis reaction was investigated with substrates whose cleavage was monitored by adding AdaAhx(3)L(3)VS or Q-VD-OPh into the incubation mixture. Examples of such experiments can be illustrated by the most and least effective peptides. The initial velocity calculated for Ac-VEID-AFC/-AMC ($n=4$, mean $\pm$ SD, nmol mg^{-1} protein min^{-1}) was as follows: the total inhibitor-free activity was equal to 91.1 ± 2.4, which included 83.1 ± 6.1 of Q-VD-OPh-sensitive activity and 6.8 ± 0.3 of AdaAhx(3)L(3)VS-sensitive activity. In comparison with VEID peptide, the total activity for Ac-DEVD-AMC was calculated as 9.63 ± 0.32 nmol mg^{-1} protein min^{-1}, which was 9.5-fold less effective for the monitoring of caspases. The initial velocity for Ac-DEVD-AMC was equal to 8.45 ± 0.8 nmol mg^{-1} protein min^{-1} in the presence of AdaAhx(3)L(3)VS, indicating that caspase activities are predominant, with a minor component of proteasome participation as shown by the rate of 1.64 ± 0.06 nmol mg^{-1} protein min^{-1} in the presence of Q-VD-OPh.

Protease activity can also be monitored using the peptide Z-VAD-AFC that is often used as a non-selective pan-caspase substrate. In our experiments, Z-VAD-ase activity was at least ~25–30 times lower than the cleavage of Ac-VEID-AMC in caspase-induced lysates from all of the investigated tissues (not only the brain). The cleavage of Z-VAD-AFC was effectively inhibited by AdaAhx(3) L(3)VSand the profiles of the inhibitory action of this molecule in the presence and absence of 5 μM Z-VAD-FMK were similar. The addition or withdrawal of the protease inhibitors cocktail has no influence on the profile of AdaAhx(3)L(3)VS-sensitive activity (Supplementary Fig. S3). Thus, the 'pan-caspase substrate' did not show specificity in relation to caspases as opposed to proteasomes.

Protease activities in the preparations of purified proteasomes. To compare the features of the caspase-like activity with the proteasomal features in nervous tissue, we studied the enzymatic activity of commercially available 26S and crude proteasome preparations. The ratios between the chymotrypsin- and caspase-like activities in these preparations were quite different from those of the brain lysates. However, the preparations did demonstrate activity in the presence of all of the investigated caspase substrates (Supplementary Table SII). The ratio of Ac-VEID-ase or Ac-IETD-ase activity to Z-LLE-ase activity was ~5.2 and 1.9%, respectively, in the crude proteasomes from reticulocytes; 24.8 and 11.2%, respectively, in the 26S proteasome preparation from human erythrocytes. The kinetic data for the inhibition of Z-LLE-ase activity by Ac-VEID-CHO in the preparations of purified proteasomes demonstrated that Ac-VEID-CHO acted as a competitive inhibitor (*42*, *43*) (Fig. 4A). All of the caspase substrates were cleaved in the crude and 26S proteasome preparations. An example of the effectiveness of this cleavage is given in Supplementary Table SII. In proteasomes from different manufacturers, cleavage of the caspase substrates seemed to occur at the peptidyl-glutamyl-peptidase site. The structural caspase aldehyde inhibitors increased the proteosomal trypsin-like activity in the absence of the protease inhibitor cocktail, inhibited

Fig. 3 Continued
induction *in vitro*. (open circle) epoxomicin-independent Ac-VEID-ase activity after apoptosis induction in the embryonal (stage E23) rat brain lysates. In cases with unfilled symbols, basal activity was calculated as the difference between the total activity in the presence of 5 μM epoxomicin and the background value (without a protein probe). The treatment with the inhibitor was performed before apoptosis was induced *in vitro* by cytochrome C and dATP. (D) The comparative effects on Ac-VEID-ase activity inhibition of Z-VAD-FMK, epoxomicin and Ac-VEID-CHO with respect to the enzyme activity source. Lanes 1, 4 and 7: the influence of Z-VAD-FMK (5 μM) on the proteolysis of Ac-VEID-AMC in proteasomal fraction 2 from rabbit reticulocytes, embryonal brain lysates and adult brain lysates, respectively. Lanes 2, 5 and 8: the influence of epoxomicin (3 μM) on the proteolysis of Ac-VEID-AMC in proteasomal fraction 2 from rabbit reticulocytes, embryonal brain lysatesand adult brain lysates, respectively. Lanes 3, 6 and 9: the influence of Ac-VEID-CHO (5 μM) on the proteolysis of Ac-VEID-AMC in proteasomal fraction 2 from rabbit reticulocytes, embryonal brain lysatesand adult brain lysates, respectively. (E) The influence of Q-VD-OPH on Ac-VEID-AMC cleavage in embryonal (stage E22) rat brain lysates. (filled square) Ac-VEID-ase activity control lysates. (open circle) Ac-VEID-ase activity in the Cytochrome C + dATP - induced lysates in the presence of 10 μM AdaAhx(3)L(3)VS. For the control probe and the AdaAhx(3)L(3)VS-sensitive probes, 100% activity was calculated as the difference between the total activity and the background value (without a protein probe). (filled triangle) Ac-VEID-ase activity in induced lysates without AdaAhx(3)L(3)VS. One hundred percent activity The difference between the total activity and the activity in the presence of 10 μM AdaAhx(3)L(3)VS was calculated as 100%.

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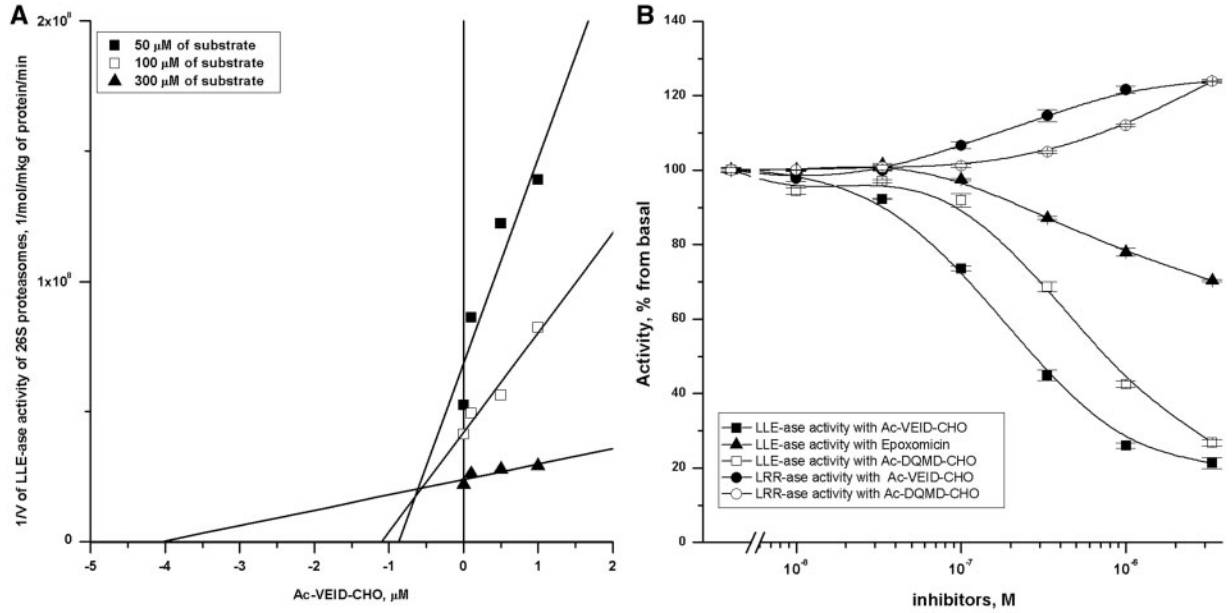

Fig. 4 Influence of caspase inhibitors on the protease activities of commercially available purified preparations of proteasomes. (A) The determination of the inhibition type and the inhibition constant from the dependence on $1/v$ of the Z-LLE-ase activity of 26S proteasomes against inhibitor Ac-VEID-CHO (according to *43*). The specific activity was calculated as the difference between the total Z-LLE-ase activity and the activity in the presence of 1 μM AdaAhx(3)L(3)VS. (B) The dependence of the proteolysis of Z-LLE-AMC and boc-LRR-AMC by a proteasome fraction 2 preparation from rabbit reticulocyte cell extracts on Ac-VEID-CHO, Ac-DQMD-CHO and epoxomicin. The specific activity was calculated in the absence of the protease inhibitor cocktail as the difference between the total activity and the activity in the presence of 1 μM AdaAhx(3)L(3)VS.

Z-LLE-ase ($IC_{50} = 0.15 \pm 0.06$ μM for Ac-VEID-CHO, 0.38 ± 0.1 μM for Ac-DQMD-CHO) and either did not affect Suc-LLVY-ase activity or increased it by 10% (Ac-VEID-CHO) (Fig. 4B). The profiles of the inhibition of Suc-LLVY-ase activity by AdaAhx(3)L(3)VS were similar in the purified proteasomes and the brain lysate preparations, as were the profiles of caspase substrate cleavage inhibition by AdaAhx(3)L(3)VS ($IC_{50} = 1.15 \pm 0.02$ μM).

In the purified proteasome preparations, AAF-CMK inhibited Ac-VEID-ase activity in the absence of the protease inhibitor cocktail, with a maximal inhibition of ~30%. This chemical had no effect on Z-LLE-ase activity at concentrations of ~100 μM and had a stimulating influence (~5%) on Boc-Leu–Arg–Arg-ase and Suc-LLVY-ase activities (data not shown).

The non-specific caspase inhibitor Z-VAD-FMK had a slight inhibitory effect on caspase-like activity in both the 26S proteasome preparations and the adult rat brain lysates. Z-VAD-FMK (but not Q-VD-OPh) increased the chymotrypsin-like activity in both the preparations of commercially available proteasomes and the adult, non-induced rat brain lysates in a dose-dependent manner, but its modulating influence (the stimulation of chymotrypsin-like activity by ~10%) was clear only at higher (>30 μM) concentrations of the drug, as in the case of the aforementioned inhibitory action. Additionally, we observed that Z-VAD-FMK can bind to the caspase-like sites of proteasomes in the adult brain lysates with very low affinity and can inhibit their activities weakly (8–15% for different substrates).

In commercial proteasome preparations from rabbit reticulocyte extracts under the same conditions, epoxomicin inhibited Ac-VEID-ase activity slightly (Fig. 3D).

All of the data indicate that the pharmacological characteristics of the caspase-like proteolysis enzymes in rat brain lysates are similar to the properties of proteasomes and that Q-VD-OPh- and Z-VAD-FMK-sensitive activity can only be stimulated in embryonal or newborn brain lysates.

Comparison of caspase immunoreactivities in preparations with caspase-like activities

Polyclonal and monoclonal rabbit antibodies to caspases-3, -8 and -9 were used to identify changes in the MW of proteins corresponding to activated caspases (*16*). The position and amount of immunopositive bands corresponding to caspase-8 (*44*) (i.e. proteins with MWs of 18 and 50 kDa) did not change in the lysates of adult or embryonal brain tissue and preparations of purified or crude proteasomes (data not shown). Band positions corresponding to non-activated caspase-9 (*22*) in the embryonal and adult animals also did not change during the control reactions. The band corresponding to activated caspase-9 (typical MWs of 17–25 kDa) (reference *18*, *22*, Supplementary Fig. S1) appeared in the presence of apoptosis inducers in the preparations of embryonal and newborn neural tissues only. The pre-incubation of the lysates with Z-VAD-FMK or Ac-VEID-CHO after the induction of apoptosis did not prevent the appearance of these protein fragments in the embryonal nervous tissue (Supplementary Fig. S1, line 2).

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Bands with an MW characteristic of activated caspase-3 (*27*) were only found in the presence of apoptosis inducers in preparations of embryonal (but not adult) nervous tissue. The appearance of these bands was blocked by incubation with Z-VAD-FMK or Ac-VEID-CHO (Supplementary Fig. S2). The activated caspase-3-related bands appeared after 10 min and reached saturation after 30 min of incubation of the embryonal neuronal lysates with the apoptosis inducers. The incubation of adult nervous tissue lysates for ~4 h with or without substrates or in the presence of apoptosis inducers did not lead to specific procaspase degradation products. Commercially available specific peptides and activated and control cell lysates were used to confirm the specificities of all observed changes.

Proteasomes purified from human erythrocytes (Enzo Co.) and crude proteasome fraction 2 from rabbit reticulocyte cell extracts (Enzo Co., Sigma) were examined using antibodies to caspases-3, -8 and -9. A protein specifically recognizable by anti-caspase-9 antibodies, but not by antibodies to caspases-3 or -8, was present in both preparations. The MW of this protein (35/37 kDa) identified it as a fragment of activated caspase-9. A fragment with an MW of 13/15 kDa was not detected (Supplementary Fig. S3).

The immunolocalization and quantification of the 35/37 kDa protein did not change during a 4-h incubation at 37°C. The addition of adult rat brain tissue lysates to the preparations did not lead to the appearance of immunoreactive bands specific to low MW fragments of activated caspase-9. The amount of caspase-9 in the reticulocyte cell extract was significantly greater than that in the purified proteasome preparations, as it was identified with antibodies to caspase-9.

Comparative investigation of caspase-like and chymotrypsin-like activities in lysates from adult and embryonal rat brains by native PAGE

Monitoring chymotrypsin proteasomal activity with Suc-LLVY-AMC demonstrated the presence of two high MW bands in native gels after the electrophoresis of the lysates (Fig. 5A). After two or three cycles of freezing in liquid nitrogen and thawing on ice, the highest MW band decreased and eventually disappeared (Fig. 5A). The cleavage of Suc-LLVY-AMC was inhibited by epoxomicin and AdaAhx(3)L(3)VS (but not by the aldehyde inhibitors or Z-VAD-FMK) in lysates from adult and embryonal nervous tissue.

The proteasomal caspase-like substrate Z-LLE-AMC and one of the caspase substrates were added simultaneously in order to visualize the activities of these proteases separately with the different inhibitors (Fig. 5B–D). In these experiments, the presence of both (or one) of the previously mentioned proteasomal bands was confirmed by the pre-incubation of sample proteasomes with irreversible antagonists that reduced the fluorescence of the bands to the background level (Fig. 5C and D).

The induction of apoptosis in the embryonal brain lysates resulted in the appearance of another appreciably bright fluorescent signal in the gel, which manifested as a spot that disappeared in the presence of Z-VAD-FMK but did not change in the presence of AdaAhx(3)L(3)VS or epoxomicin. This spot was visible near the lowest position of the proteasome band. Z-VAD-FMK and the caspase aldehyde inhibitors eliminated only this activity in lysates from embryonal neural tissue. The appearance of a Z-VAD-FMK-sensitive fluorescent spot with MW slightly lower than the AdaAhx(3)L(3)VS-sensitive bands indicated the presence of caspase activity in the higher MW protein complexes (apoptosomes) only. The localization of caspase activity in native gels near the higher MW proteasomal complexes and the absence of low MW spots also confirmed this observation (Fig. 5B–D).

A western blot of the native PAGE strips followed by SDS–PAGE demonstrated the presence of activated fragments of caspases-3 (Fig. 5E) and -9 (data not shown), the appearance of which was sensitive to Z-VAD-FMK. It should be noted that immunoblotting with commercially available antibodies to caspases can result in the visualization of intact proteins or of proteins that have been cleaved partially or completely. This fact and the presence of non-specific binding of the secondary antibodies (or Streptavidin–HRP conjugate) to proteins in the lysates allowed us to identify as caspase bands only those whose occurrence depended on the influence of caspase inhibitors and/or the presence of control antigen peptides. The position of the only low MW band (between 10 and 15 kDa) visualized with the anti-caspase-3 antibodies was dependent on the presence of caspase inhibitors, but the positions of all other bands did not change with the addition of caspase inhibitors or incubation without primary antibodies.

Binding of biotinylated AdaAhx(3)L(3)VS in lysates from adult and embryonal rat brains and preparations of 26S proteasomes

Biotinylated AdaAhx(3)L(3)VS was used to identify the individual proteasome β-subunits in the presence of the protease inhibitor cocktail. The incubation of adult brain lysates with biotinylated AdaAhx(3)L(3)VS allowed us to identify three bands with molecular masses corresponding to the individual proteasomal β-subunits. The incubation of the lysates with epoxomicin before the incubation with biotin-AdaAhx(3)L(3)VS led to the disappearance of two of the bands, whereas pre-incubation with Ac-VEID-CHO resulted in the disappearance of only the third band (Fig. 6A, B). The same effect of the caspase and proteasome inhibitors was observed with biotinyl-AdaAhx(3)L(3)VS binding to the β-subunits of the purified 26S proteasomes (Fig. 6C). In the embryonal brain lysates, it was possible to identify only two bands corresponding to the β-subunits. Binding of the lower MW band was inhibited by epoxomicin, whereas binding of the other band was inhibited by Ac-VEID-CHO. Pre-incubation with Z-VAD-FMK, cytochrome C and/or dATP did not cause any noticeable change in the bands of the biotinylated ligand-binding proteins.

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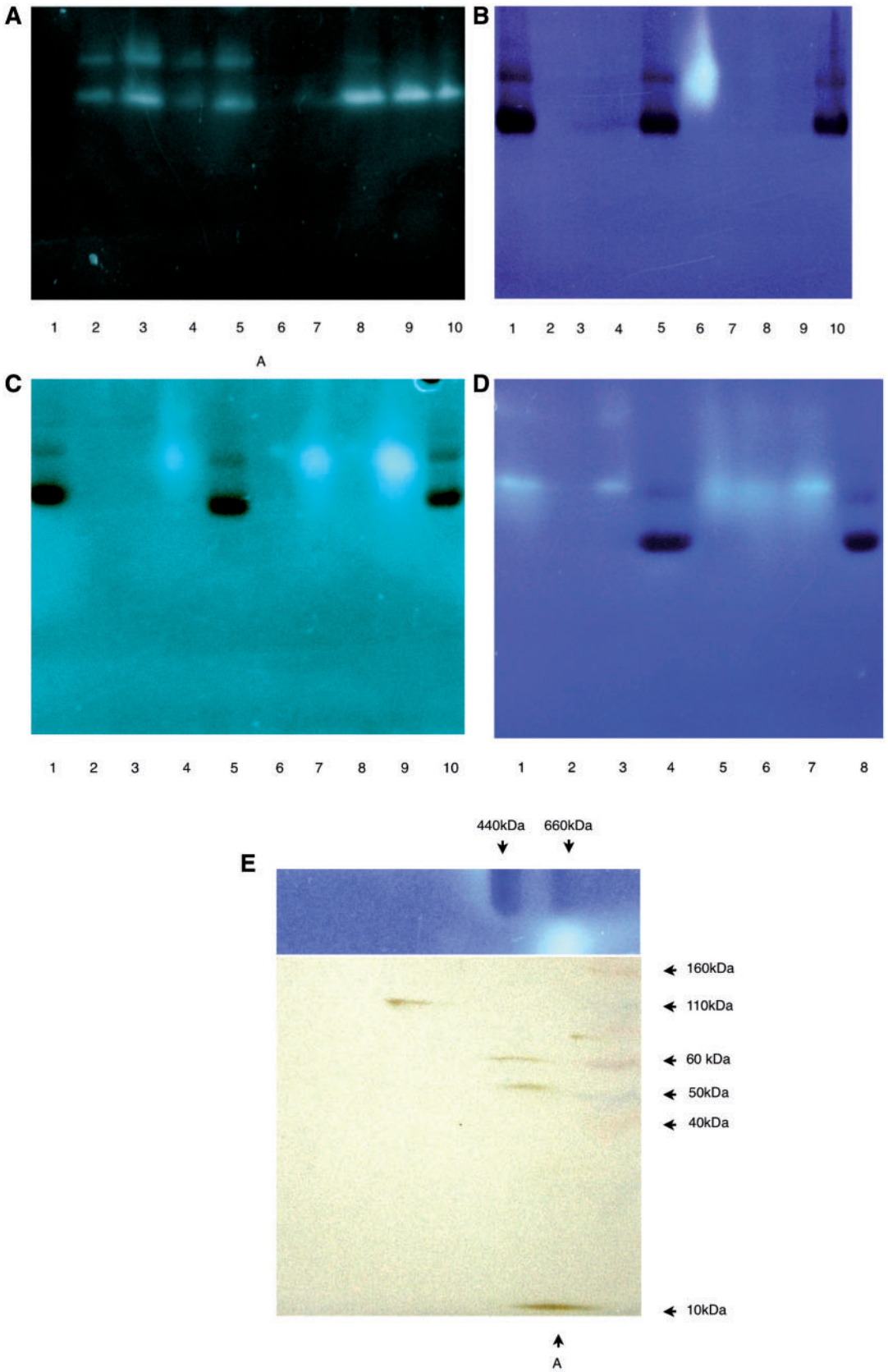

Fig. 5 Protease activities in embryonal and adult rat brain lysates in native gels. (A) Suc-LLVYase activity before and after freezing. Lane 1: standards; 2: adult cortex; 3: stage E19 brain; 4: adult hippocampus; 5: newborn brain; 6: adult cortex + 1 μM AdaAhx3L3VS; 7: stage E19 brain + 1 μM AdaAhx3L3VS; 8: stage E19 brain + 1 freeze–thaw cycle; 9: stage E19 brain + 2 freeze–thaw cycles; 10: stage E19 brain + 3 freeze–thaw cycles. (B) Ac-VDVAD-ase activity after treatment with 1 μM AdaAhx3L3VS**.** Lanes 1, 5 and 10: standard; 2: adult brain lysate + cytochrome C + dATP; 3: adult brain lysate + cytochrome C + dATP + 10 μM Z-VAD-FMK; 4: adult brain lysate; 6: embryonal brain lysate + cytochrome C + dATP; 7: embryonal brain lysate + cytochrome C + dATP + 10 μM Z-VAD-FMK; 8: embryonal brain

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Unlabelled biotin inhibitor completely blocked binding to the biotin analogue (Fig. 6B, C).

ATP and geldanamycin dependence of protease activities in the extracts

We found that the inhibitory and stimulatory actions of ATP or ATPγS on proteasomal activity in the crude lysates were not obvious. For example, the action of ATPγS may be confused with the direct or non-direct activity of HSP90. Pre-incubation of the lysates with geldanamycin (10 μM), a selective HSP90 inhibitor, increased the basal Suc-LLVY-ase activity by up to ~30%, accompanied by a slightly stimulating effect from the ATPγS. At the same time, the geldanamycin significantly increased the inhibitory effect of ATPγS on Z-LLE-ase proteolysis and inhibited the basal levels of this activity (Fig. 7). Additionally, the simple fractionation of the adult rat brain lysates on a Sepharose 4CL column (1 cm × 30 cm) altered the inhibitory action of 0.2 mM ATP to a stimulatory effect on Suc-LLVY-ase cleavage (data not shown) and produced a significant increase in the stimulatory effect of 0.2 mM ATPγS on the Ac-VEID-ase proteasome activity.

Discussion

Proteasomes have high MWs, can be activated by ATP and can hydrolyze specific substrates. Combining these features can be sufficient to identify proteasomal activity (*45*). It is generally accepted that ATP (ATPγS) stimulates the activity of the 26S proteasome, whereas crude proteasomes are less sensitive to ATP (*46*, *47*). In contrast, the proteolytic activity of the crude proteasome can be stimulated with SDS or other detergents or with fatty acids, such as cardiolipin (*48*, *49*).

Nevertheless, there are data that contradict this universal conception of the proteasomal machinery. Diversity has been observed within gate proteasomal regulatory proteins from different tissues of genetically identical mice. Additionally, 26S preparations from the heart and liver differ in their protein kinase, phosphatase and arginine methyltransferase compositions. Proteasome preparations from different tissues have varying sensitivities to inhibitors and substrates. The proteasomal activities of different tissues change during the life of the animal in different ways (*46*, *49*). Finally, different parts of the mouse brain demonstrate selective proteasomal dysfunction in ischaemia (*50*).

The influence of ATP on proteasome activity has been discussed in a range of publications. There are publications in which authors suggested that ATP exerted bi-directional regulation on the 26S proteasome *in vitro*, with the optimal ATP concentration (between 50 and 100 μM) stimulating proteasome chymotrypsin-like activities. The manipulation of intracellular ATP levels led to bidirectional changes in the levels of proteasome-specific protein substrates in cultured cells. Finally, the ability of proteasomes to inhibit the induction of cell death increased when the levels of intracellular ATP were raised, while decreasing intracellular ATP attenuated this opportunity. Thus, authors suggested that endogenous ATP within the physiological concentration range could exert a negative impact on proteasome activities, allowing the cell to rapidly up-regulate proteasome activity via ATP reduction under stress conditions (*51*).

The optimal conditions for proteasomal activities are typically obtained by choosing the concentration of ATP needed to stimulate the proteasome activity in the brain tissue lysates. However, it has been observed that concentrations of ATP in the range of 10^{-3} M inhibit proteasome proteolytic activity in brain tissue extracts (*52*).

In the present study, nucleotide di-and triphosphates had various effects on proteolytic activity in tissue lysates, including a dose-dependent inhibitory effect (Fig. 7). The ATP-dependent chaperone HSP90 mediates the assembly and activity of 26S proteasomes (*53*). The mechanisms underlying the stimulating action of ATP may include the stimulating effect of phosphorylation by protein kinase A on the proteolytic activity of proteasomal β-1, β-2 and β-5 subunits (*49*). Thus, the multiplicity of modes of nucleotide di- and triphosphate action on proteasome activity does not clarify the interactions of different regulatory mechanisms in screening experiments but complicates matters instead.

At low concentrations (0.01–0.02%), Triton X-100 behaves as an allosteric activator for peptidyl-glutamyl-peptide hydrolyzing activities, suppressing the co-operativity and decreasing the half-saturation substrate concentration. Triton X-100 fully suppressed the high K_m and high V^m components of the saturation curve for the LLE peptide in the absence of detergent (*54*). Considering the ambiguity of the modes of action of the nucleotide di- and triphosphates on the studied process, we used Triton X-100 as a stimulator of proteasome activity. Use of this detergent allowed us to obtain more accurate data on the caspase-like activity of proteasomes by enhancing the specificity of substrate proteolysis.

Fig. 5 Continued
lysate; 9: embryonal brain lysate + 10 μM Z-VAD-FMK. (C) Ac-VEID-ase + Z-LLE-ase activity. Lanes 1, 5 and 10: standards; 2: adult brain lysate; 3: embryonal brain lysate + 1 μM AdaAhx3L3VS; 4: embryonal brain lysate; 6: embryonal brain lysate + cytochrome C + dATP + 10 μM Z-VAD-FMK; 7: embryonal brain lysate + cytochrome C + dATP + epoxomicin; 8: embryonal brain lysate + cytochrome C + dATP + 1 μM AdaAhx3L3VS + 10 μM Z-VAD-FMK; 9: embryonal brain lysate + cytochrome C + dATP. (D) Ac-IETD-ase + Z-LLE-ase activity in apoptosis-induced newborn and stage E19 brain lysates. Lane 1: embryonal brain lysate; 2: embryonal brain lysate + 1 μM AdaAhx3L3VS + 10 μM Z-VAD-FMK; 3: embryonal brain lysate + 10 μM Z-VAD-FMK. Lanes 4 and 8: standards; 5: embryonal brain lysate + epoxomicin; 6: newborn brain lysate + epoxomicin; 7: newborn brain lysate. (E) Visualization of Z-VAD-FMK-sensitive caspase activity after *in vitro* apoptosis induction. Ac-VEID-ase activity of embryonal brain lysate after native gel electrophoresis followed by SDS–PAGE in the second (vertical) dimension and identification of caspase-3 by western blot. (A), The low MW fragment of activated caspase-3 is indicated by the arrow.

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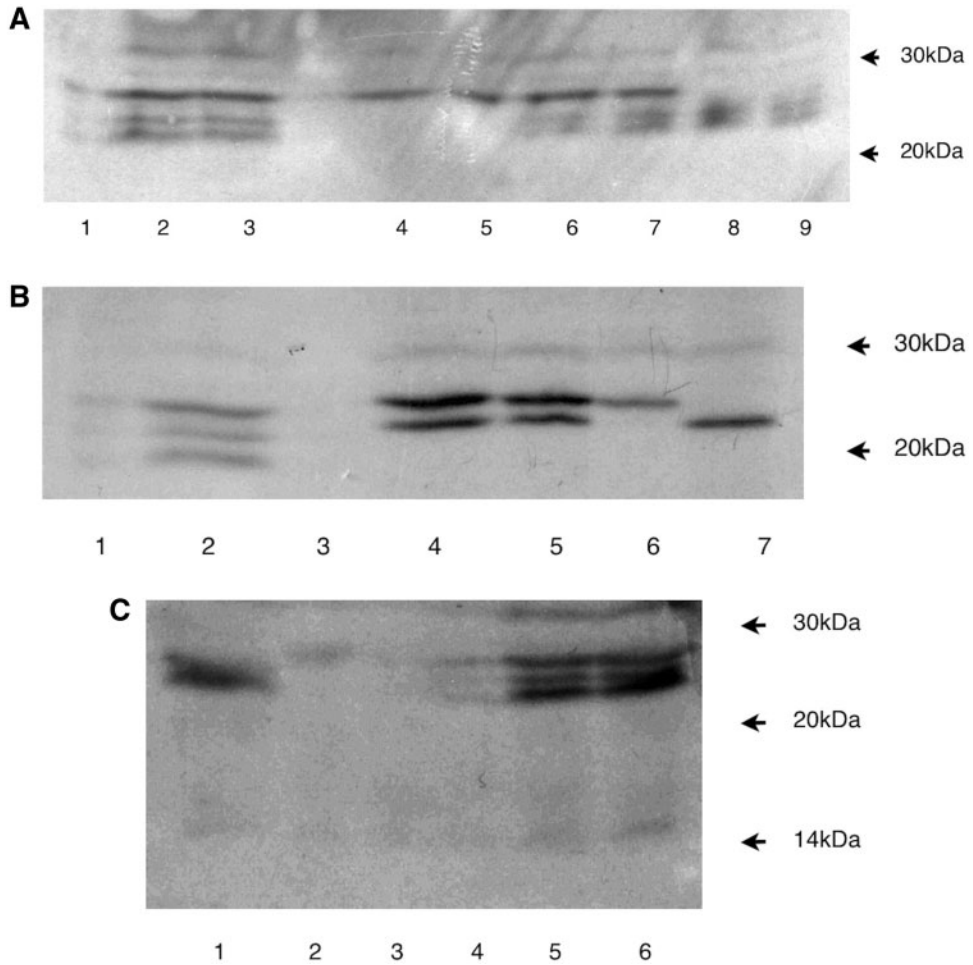

Fig. 6 Identification of the proteasome catalytic subunits using biotinyl-AdaAhx3L3VS. (A) Lysates (50 μg/probe) from the brains of adult rats. Lane 1: 1 μM AdaAhx3L3VS; 2: control; 3: cytochrome C + dATP; 4: 1 μM Epoxomicin; 5: 1 μM epoxomicin + cytochrome C + dATP; 6: 10 μM Z-VAD-FMK; 7: cytochrome C + dATP + 10 μM Z-VAD-FMK; 8: cytochrome C + dATP + 10 μM Ac-VEID-CHO; 9: 10 μM Ac-VEID-CHO. (B) Lysates from the brains of rat embryos and adult rats (50 μg/probe). Lane 1: adult brain lysate + 1 μM AdaAhx3L3VS; 2: adult brain lysate; 3: embryonal brain lysate + 1 μM AdaAhx3L3VS; 4: embryonal brain lysate; 5: embryonal brain lysate + 10 μM Z-VAD-FMK; 6: embryonal brain lysate + 1 μM epoxomicin; 7: embryonal brain lysate + 10 μM Ac-VEID-CHO. (C) Preparations of purified 26S proteasomes (2.5 μg/well) from human erythrocytes. Lane 1: 10 μM Ac-VEID-CHO; 2: 1 μM epoxomicin; 3: 1 μM epoxomicin + 1 μM AdaAhx3L3VS; 4: 1 μM AdaAhx3L3VS; 5: 10 μM Z-VAD-FMK; 6: control.

The main aim of this research was to show that commonly used caspase substrates and inhibitors are not specific to certain groups of caspases (*35*) but also target proteasomes, specifically the β1-subunit of proteasomes that possesses caspase-like activity. To prove this, we used purified proteasomes and lysates of nervous tissue in which apoptosis had been induced *in vitro* by cytochrome C and dATP.

Proteasome substrate specificity has been well studied and several researchers have shown the presence of Ac-VDVAD-ase, Ac-YVAD-ase, Ac-IETD-ase and Ac-DEVD-ase activities in proteasome preparations (*33*). We obtained an immunopositive reaction with antibodies to caspase-9 in both purified and partly purified proteasome preparations, perhaps indicating the presence of this caspase protein in the proteasome preparations. Caspase-9 activity is transient and the presence of a protein with a weight of 32/35 kDa may suggest that the caspase was once activated and became inactivated (*18*, *19*).

Neither the addition of adult rat neuronal tissue lysates to the commercial proteasome preparations nor long-term incubation at 37°C (without or with cytochrome C and dATP) resulted in a catalytically active low MW fragment (human or rabbit). Neither of these conditions increased the caspase-like activity, even in the presence of a comparatively large amount of 35/37-kDa caspase-9 in the rabbit reticulocyte extracts. At the same time, selective proteasome inhibitors in lysates and in the commercial proteasome preparations inhibited the studied caspase-like activity. In contrast, all of the tested caspase aldehyde inhibitors inhibit 26S proteasomes that activity was detected with Z-LLE-AMC and Ac-GPLD-AMC. This observation confirms that the activity of 32/35-kDa caspase-9 does not explain the caspase-like activity of the purified proteasomes.

The ratios of the Ac-VEID-ase and Ac-IETD-ase activities relative to Z-LLE-ase revealed that the 26S proteasome preparations had five times more

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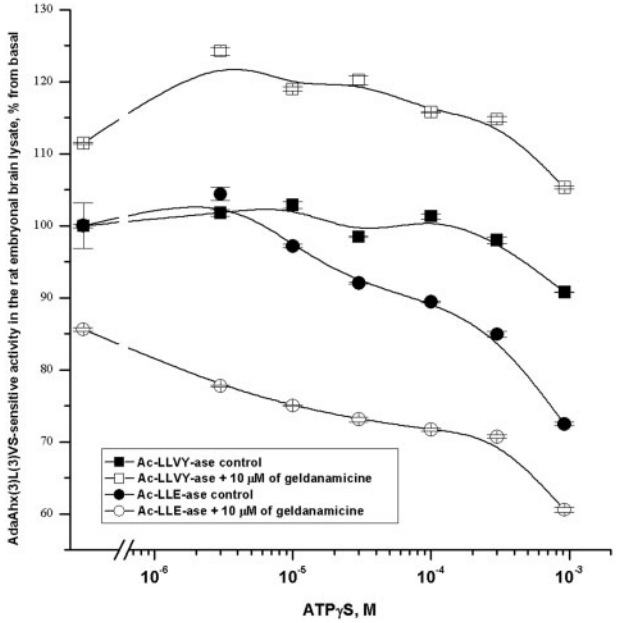

Fig. 7 The comparative influence of ATPγS in embryonal rat brain lysates on the cleavage of various proteasome substrates and in the presence of HSP90 inhibitor. The specific activity was calculated as the difference between the total activity and the activity in the presence of 1 μM AdaAhx(3)L(3)VS. Geldanamicin was added at 10 μM for 30 min (4°C) before the start of the reactions.

Ac-VEID-ase- and Ac-IETD-ase-specific activities than preparations from the reticulocytes. At the same time, immunoreactivity to caspase-9 in the 26S proteasome preparations was ~90-fold less than that in preparations from reticulocytes, but the caspase-like activities in both preparations were comparable (24 and 17 pmol $min^{-1} mg^{-1}$ protein, respectively). These results also indicate that the caspase substrates were only cleaved by proteasomes.

Published data about the presence of various caspases in the brain of adult rats are contradictory. The presence of activated caspases-6, -3, -9, -8, -10 and -14 in embryonic nervous tissue and in injured areas of the brain of various mammals has been shown in several studies, indicating that caspase gene expression is only significantly increased after injury (*21*, *22*, *44*). There is some evidence that the adult rat brain normally expresses procaspases-6, -3, -9, -8 and -10, but their gene expression levels are low and significantly enhanced by injury (*44*, *55*). The induction of apoptosis in the rat brain *in vitro* may only be possible during the embryonic period (*21*, *23*). We found that the new caspase-like proteolytic activity after apoptosis induction is sensitive to Z-VAD-FMK, Q-VD-OPh and aldehyde inhibitors, but not to the selective proteasome inhibitors epoxomicin and AdaAhx(3)L(3)VS.

Based on the signature pattern of the crude core subunits, 5 of the 11 protein complexes visualized by Blue-Native PAGE were unequivocally identified as proteasomes (*3*). To visualize proteasomal activities in-gel, we used a variation of Blue-Native PAGE (previously termed Clear-Native PAGE), using similar gel conditions but omitting the coomassie G-250 dye in the cathode buffer and reducing the aminocaproic acid (as an alternative active component of the cathode buffer instead of coomassie dye) concentration to 50 mM. Coomassie G-250 is a potent inhibitor of chymotrypsin-like activity (90% inhibition), whereas high concentrations of aminocaproic acid (500 mM) mildly reduce (50%) cleavage of the substrate Suc-LLVY-AMC (*38*) and addition of 50 mM aminocaproic acid to the reaction inhibited cleavage of Z-LLE-AMC by 10% (in our experiment).

The application of Clear-Native PAGE results in lower resolution and a more complex staining pattern with anti-proteasome subunit antibodies than does Blue-Native PAGE. Nevertheless, mass spectrometry and antibodies to different proteasome subunits identified five distinct 20S proteasome complexes that co-migrated with PA28-capped and 19S-capped proteasomes. The in-gel assays demonstrated that four of these complexes retained peptidase activity. The size of these complexes and the relative intensity of the PA28-contained protease activity staining indicate that they represent singly and doubly 19S-capped (and PA28-capped also) proteasomes and have MWs of ~900 and ~1100 kDa, respectively. At the same time, free 20S particles had a MW of ~750 kDa. As expected, isolated 20S particles lacked proteolysis activity and it is assumed that the heterogeneous bands containing 19S- and PA28-capped proteasome subunits from the mammalian tissues were visualized with the fluorogenic substrate Suc-LLVY-AMC by Clear-Native PAGE only (*3*).

Similar results were previously observed in an investigation of rat brain extracts. In this study, the rat cortex contained 14% doubly capped 26S proteasomes, 43% singly capped and nearly 43% free 20S proteasomes. These samples were purified by the affinity method, which is based upon the high affinity of 26S proteasomes for the ubiquitin-like domain of Rad23B. This approach allows the gentle, rapid isolation of the 26S proteasomes with their associated proteins. At the conditions of in-gel protease activities, these investigators demonstrated that two major bands are related to doubly and singly capped (by 19S) complexes of 20S subunits; this finding was confirmed by both mass spectroscopy and western blotting with antibodies specific to the proteasome subunits (*26*).

We propose that two major singly and doubly capped (by 19S) 20S subunit complexes were visualized in-gel without freezing of the samples and that a singly capped complex was obtained after freeze–thaw cycles.

Similar to Blue-Native PAGE, mass estimation by Clear-Native PAGE of soluble basic or uncharged proteins is precluded or difficult, unless the proteins bind to the mixed anionic/neutral micelles that were added to the cathode buffer for Clear-Native PAGE. Since, it is not straightforward to test for binding to the mixed micelles, the mass estimation of soluble proteins by Clear-Native PAGE remains rather ambiguous: a coefficient of at least 0.8 was proposed to determine the mass of standard proteins (*56*). Taking into account such data, ferritin was used in our experiments as a black-coloured ultraviolet-light marker mainly for

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the preliminary detection of border migration for high MW proteins.

A comparison of the published data on proteasome separation and visualization in native gels with our data shows that visual localization of proteasomes in our work does not differ from the results of various studies in which Suc-LLVY-AMC was used as a substrate for proteasomes. We used the peptide substrates and inhibitors for the monitoring of caspases, as well as for the visualization of caspase-like activities in native gels. In our investigations, a clear pharmacological difference was achieved in the identification of caspase and proteasome activities. Additionally, these observations indicate that proteasome and caspase activities cannot be differentiated by their MWs because the protein complexes in which they occur are similar in size. The caspase activity of the apoptosomes, which is visualized with caspase substrates in native gels, shows that these proteins have an MW that is only slightly smaller than that of proteasomes, which can also be visualized with tools specific for the investigation of proteasome activities (Fig. 5).

Binding of the irreversible proteasome inhibitor biotinyl-AdaAhx(3)L(3)VS to one of the catalytic subunits of the proteasome was competitively and selectively blocked by caspase aldehyde inhibitors, but not by Z-VAD-FMK. This finding confirms our assumption that the point of action of these inhibitors is the catalytic site of the β1-subunit of the proteasome.

All three proteasome activities are functionally united due to the existence of allosteric functional interactions between the different β-subunits (*28*, *32*, *57*). A complex set of allosteric interactions in proteasomes makes it difficult to predict the exact functional consequences of altering a particular active centre. However, the internal mechanisms of these allosteric interactions have been revealed. Using atomic force microscopy, Osmulski *et al.* (*7*) observed a strict correlation between the specific states of the β-subunit active centres and gate opening or closing in the α-ring. Each active centre of a β-subunit in the catalytic chamber of the 20S proteasome was suggested to be allosterically coupled to the α-ring gate, with a tetrahedral transition state at an active centre serving as the likely trigger for gate opening (or for inhibiting its closing) (*7*). However, it should be remembered that the proteasomal activities and the exact content of proteasomal enzymes and related proteins are not constant over time (*4*, *6*, *58–60*). Apart from such observations, the well-known allosteric modulation of caspase-like activity by chymotrypsin-like or trypsin-like sites does not simplify the identification of 'true' caspases. In our experiments, the allosteric modulation of chymotrypsin-like sites by caspase-like sites with weak proteasome inhibitors such as Z-VAD-FMK (at higher concentrations of the last) could lead to stimulation of the chymotrypsin-like site and inhibition of the caspase-like activity simultaneously. Taking into account that the working concentrations of this caspase inhibitor and others are often very high (100 μM, for example, ref. *60*), the real target for this drug cannot be caspase protein only.

Another inhibitor, epoxomicin, known to be a specific, irreversible inhibitor that block chymotrypsin-like, trypsin-like and PGPH activities of the proteasome, was tested in our experiments. We found that epoxomicin inhibited caspase-like activity in lysates from the brains of embryos and adults with different effectiveness. Additionally, in contrast to fermentation reactions, the discovery that the epoxomicin-inhibited sites of the biotinyl-AdaAhx(3)L(3)VS binding were not the same sites that are sensitive to aldehyde inhibitors was unexpected. This result was reflected in experiments involving the binding of biotinyl-AdaAhx(3)L(3)VS to proteasome proteins in the presence of epoxomicin, which revealed the existence of two bands that are sensitive to this inhibitor in adults and one such band in embryos, none of which was sensitive to aldehyde inhibitors (Fig. 6). We propose that the epoxomicin-sensitive inhibition of biotinyl AdaAhx(3)L(3)VS-derivative binding more accurately reflects the presence of chymotrypsin-like sites of the proteasome. It is possible that the binding of epoxomicin results in a decrease in caspase-like activity allosterically. The appearance of a second chymotrypsin-like, epoxomicin-sensitive binding site in the adult brain amplifies allosteric influence on the caspase-like sites and strengthens the inhibitory action of the drug. The existence of age-dependent, incomplete inhibition of caspase-like activities by epoxomicin, which was shown in the comparison of embryonal to adult brain, confirms this suggestion. The weaker, reversible and non-covalent binding of epoxomicin to caspase-like sites can also show the same picture of biotinyl-AdaAhx(3)L(3)VS binding, but the partial inhibition (~20%) of caspase-like activity and the saturated form of the curve of the inhibitory influence of epoxomicin on caspase-like proteasomal activity in 26S preparations is contrary to such a suggestion. Thus, we hypothesize that the inhibition of the chymotrypsin-like sites by epoxomicin leads to the allosteric inhibition of caspase-like sites. Conversely, inhibition of the caspase-like sites by the tested caspase inhibitors resulted in stimulation of the chymotrypsin-like activity of proteasomes.

The use of caspase inhibitors that can bind to proteasomes and both inhibit their caspase-like sites and allosterically modulate their chymotrypsin-like activities results in an underestimation of the role of proteasomes in the physiology of cellular process and an exaggeration of the importance of caspases.

In our preliminary experiments, we could not distinguish between specific caspase activities (those that can be inhibited by Q-VD-OPh or Z-VAD-FMK or induced with cytochrome C and dATP) and alterations in proteasomal caspase-like activity in the hippocampus of adult rats after a 3-week period of immobilization stress. Additionally, we obtained considerable differences in the total activity of proteasomes in the samples from adult rat brains as in the case of different rat lines and families or individually. We conclude that functional experiments involving the induction of apoptosis *in situ* and/or specific caspase inhibitors would help in the discrimination of true caspases and proteasomal caspase-like activities.

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We propose that in the adult brain, in the case of identification of Q-VD-OPh or Z-VAD-FMK-sensitive activity, the sources of injury-activated caspases could be blood leucocytes, endothelial cells or resident macrophages that are non-neuronal cells.

Leukocytes can express permanently proteins that are close or equal to interleukin-1 converting enzyme (caspase-1) or granzyme B. The basal activity of these proteins can be measured with the substrates of caspases and their cleavage refers to the process of apoptosis, but at the same time, apoptosis of leukocytes is absent. It has been reported that granzyme B-dependent caspase-3 activation occurs in stimulated T-lymphocytes, without associated apoptosis. The presence of cathepsin C in the cytolytic granules is a necessary condition for the maturation of granzyme B, as is the deactivation of nucleocytoplasmic protease inhibitor 9. It was proposed that, upon stimulation of CD8 (+) T lymphocytes, some granzyme B is released from the cytolytic granules into the cytosol, where it escapes nucleocytoplasmic protease inhibitor 9 and thereby mediates apoptotic T-cell death at sites of acute or chronic tissue inflammation injury (*62*).

Cell death during development is a major factor shaping the body. This death is mainly caspase-mediated apoptosis. After the embryonal waves of developmental apoptosis have passed, this death machinery is turned off and its components are only partially present. Different types of cellular death are utilized instead of embryonal apoptosis and they are named apoptotic, necrotic or autophagic, or some combination of these processes. Among the types of cellular death in the adult tissues, the loss of enterocytes at the tips of the intestinal villi and the maturation of oocytes are described most carefully. Anoikis, i.e. apoptosis induced by detachment from the extracellular matrix, is thought to be involved in controlling enterocyte survival. The EGFR-dependent phosphorylation of E-cadherin, a key protein in cell–cell adhesion that is involved in the control of anoikis in normal intestinal epithelial cells, is the triggering mechanism of enterocyte apoptosis. It was shown that, upon the loss of their anchorage, normal enterocytes execute a programme of apoptosis within minutes, via caspase-3 activation in a Bcl-2-regulated, caspase-9-dependent pathway (*63*). Apoptosis in the reproductive tissues of both males and females is thought to occur after the withdrawal of crucial hormonal support. Apoptosis in the ovary and the uterus is linked to oestrus. Atresia, a process that involves apoptosis of granulosa cells, oocytes and eventually theca cells, can occur during any stage of follicular development. During each oestrus cycle, other ovarian cell types in addition to the follicular cells may also undergo apoptosis. The ovarian surface epithelial cells covering the protruding pre-ovulatory follicles degenerate from the ovarian surface by apoptosis just prior to ovulation to facilitate the release of the oocyte. All these events in the female gonads occur with the participation of Fas, Fas ligand, Bcl-2, Bax and caspase-3. The levels of these mRNAs and the corresponding quantity of the related proteins vary greatly in dependence on the presence of sex hormones in the tissue (*64*).

Consistent with multiple configurations of proteasomes in cells, proteasome-mediated degradation also occurs in various manners. In the canonical ubiquitin-dependent pathway, proteins are degraded by the 26S proteasome in an ATP-dependent and ubiquitin-dependent manner. However, the 20S proteasome also exists in free form and some proteins, including oxidized proteins, are degraded by the 20S proteasome in an ATP-independent and ubiquitin-independent manner. In rare instances, proteins are degraded by the 26S proteasome in an ATP-dependent but ubiquitination-independent process. Intracellular proteolysis can show a biphasic response to oxidative stress. Mild to moderate oxidative stress increases the susceptibility of proteins to degradation and enhances the proteolytic capacity of the cell, therefore promoting intracellular protein degradation. In contrast, extensive but non-lethal oxidative stress impairs the functioning of the proteolytic system, reducing intracellular protein degradation and inducing intracellular accumulation of damaged or abnormal proteins. Both substrate ubiquitination and the degradation of ubiquitinated substrates by the 26S proteasome require ATP. The assembly of the 26S proteasome is also an ATP-dependent process. In contrast, the 20S proteasome degrades proteins in an ATP-independent manner. A number of studies have demonstrated that ATP has no stimulating effect on the degradation of oxidized proteins in cell lysates. In some cases the addition of ATP to the cell lysate reduced the degradation of oxidized proteins in cell-free lysates (*65*).

There are numerous investigations of tissue injury evoked by ischaemic reperfusion or similar pathological conditions and natural cellular death in adult tissues or in culture conditions that have used caspase substrates and inhibitors, such as Z-VAD-FMK, as apoptosis markers. The cleavage of caspase substrates by proteasomes clearly exists, as we found. Z-VAD-FMK added at high concentrations (100 μM) can interact with proteasomes, where it modulates their chymotrypsin-like activity and weakly blocks their caspase-like sites. Both types of these apoptosis-related chemicals are usually used without needed controls and/or in concentrations that are not specific for caspases. Together, these observations lead us to emphasize the necessity of using proteasomal inhibitors that suppress all of the activities of proteasomes to discriminate between caspase and caspase-like proteasomal protease activities. There are no preferential substrates for the investigation of the activated caspases. Without specific or particular considerations, there is no need to add ATP; the proteasomal regulatory mechanism involved in activity modulation includes different forms of HSP90 action. The separation of preparations according to MWs or dividing lysates into lower and higher MW components is not helpful because the weights of both active protease complexes are similar.

Thus, most of our current knowledge about caspase activity has been obtained from technically flawed experiments that did not allow investigators to distinguish between the activity of caspases and the caspase-like activities of proteasomes and this trend

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has led to an overestimation of the role of caspases in our current vision of cellular life.

Supplementary Data

Supplementary Data are available at *JB* online.

Funding
This work was partially supported by National Academy of Sciences of Ukraine by grant # 0107U005336.

Conflict of interest
None declared.

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