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Differential Inhibition of Calpain and Proteasome Activities by Peptidyl Aldehydes of Di-Leucine and Tri-Leucine

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To explore membrane-permeable synthetic inhibitors that discriminate between endogenous calpain and proteasome in cells, we examined the inhibition profiles against calpain and proteasome *in vitro* and *in vivo* of peptidyl aldehydes possessing di-leucine and tri-leucine. The tripeptide aldehyde benzyloxycarbonyl-leucyl-leucyl-leucinal (ZLLLal) strongly inhibited calpain and proteasome activities *in vitro*. The concentration required for 50% inhibition (IC_{50}) of the casein-degrading activity of calpain was 1.25 μM, and the IC_{50}s for the succinyl-leucyl-leucyl-valyl-tyrosine-4-methylcoumaryl-7-amide (Suc-LLVY-MCA)- and benzyloxycarbonyl-leucyl-leucyl-leucine-4-methylcoumaryl-7-amide (ZLLL-MCA)-degrading activities of proteasome were 850 and 100 nM, respectively. On the other hand, the synthetic dipeptide aldehyde benzyloxycarbonyl-leucyl-leucinal (ZLLal) strongly inhibited the casein degrading activity of calpain (IC_{50} 1.20 μM), but the inhibition of proteasome was weak (IC_{50}s for SucLLVY-MCA- and ZLLL-MCA-degrading activities were 120 and 110 μM, respectively). Thus, while calpain was inhibited by similar concentrations of ZLLal and ZLLLal, the inhibitory potencies of ZLLLal against the ZLLL-MCA- and SucLLVY-MCA-degrading activities in proteasome were 1,100 and 140 times stronger than those of ZLLal, respectively. To evaluate the effectiveness of these inhibitors on intracellular proteasome, the induction of neurite outgrowth in PC12 cells caused by proteasome inhibition was examined. ZLLLal and ZLLal initiated neurite outgrowth with optimal concentrations of 20 nM and 10 μM, respectively, again showing a big difference in the effective concentrations for the proteasome inhibition as *in vitro*. As for the effect on intracellular calpain, the concentrations of ZLLLal and ZLLal required for the inhibition of the autolytic activation of calpain in rabbit erythrocytes were 100 and 100 μM or more, respectively. The almost equal inhibitory potencies of ZLLLal and ZLLal were in agreement with the inhibition of calpain *in vitro*. These differential effects of inhibitors against calpain and proteasome are potentially useful for identifying the functions of calpain and proteasome in cell physiology and pathology.

Key words: calpain, neurite outgrowth, peptide aldehyde, protease inhibitor, proteasome.

Intracellular proteolysis is important in cell function for both protein catabolism and protein processing. Calpain and proteasome are the main nonlysosomal intracellular proteases and have recently drawn much attention. Calcium-activated neutral protease (calpain; EC 3.4.22.17) is an intracellular cysteine protease that exists ubiquitously in various animal tissues and is activated by calcium ions, important modulators of cell function. Calpain has two isoforms, μ-calpain and m-calpain, which are activated at μM and mM concentrations of calcium ions, respectively (*1, 2*). Proteasome, a multicatalytic protease, has also been identified in many species. It is composed of 14 α- and 14 β-subunits and possesses three or four different peptidase activities, including trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide hydrolyzing activities. Two types of proteasome are distinguishable based on differences in sedimentation coefficients: 26S proteasome (ATP-dependent, molecular mass about 2,000 kDa) and 20S proteasome (ATP-independent, molecular mass about 750 kDa) (*3-5*).

To elucidate the roles of intracellular proteases, protease

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Abbreviations: SucLLVY-MCA, succinyl-leucyl-leucyl-valyl-tyrosine-4-methylcoumaryl-7-amide; ZLLL-MCA, benzyloxycarbonyl-leucyl-leucyl-leucine-4-methylcoumaryl-7-amide; Boc-VLK-MCA, tertiary-butyloxycarbonyl-valyl-leucyl-lysine-4-methylcoumaryl-7-amide; ZLLE-NA, benzyloxycarbonyl-leucyl-leucyl-glutamic acid-β-naphthylamide; ZLLLal, benzyloxycarbonyl-leucyl-leucyl-leucinal; ZLLal, benzyloxycarbonyl-leucyl-leucinal; DMEM, Dulbecco's modified Eagle's medium; N2 medium, the F12/DMEM base medium supplemented with transferrin (100 μg/ml), progesterone (5 μg/ml), putrescine (5 μg/ml), selenium (52 μg/ml), and insulin (5 μg/ml); PBS, phosphate-buffered saline; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PVDF, polyvinylidene fluoride; TBS, Tris-buffered saline; IC_{50}, concentration required for 50% inhibition.

species-specific and cell-permeable inhibitors offer powerful tools. Many data on calpain and proteasome inhibitors have been reported, because calpain and proteasome are intracellular proteases expected to play very important roles *in vivo*. Among these inhibitors, peptide aldehydes inhibit calpain and proteasome activities. Leupeptin (*N*-acetyl-leucyl-leucyl-argininal), a typical peptide aldehyde calpain inhibitor, also inhibits the trypsin-like activity of proteasome (*6*). Calpain inhibitor 1 (*N*-acetyl-leucyl-leucyl-norleucinal) and calpain inhibitor 2 (*N*-acetyl-leucyl-leucyl-methioninal) are synthetic tripeptide aldehydes developed as calpain inhibitors (*7*), but they inhibit various substrate-degrading activies of proteasome *in vitro* (*8*). In experiments using peptide aldehydes *in vivo*, it is difficult to identify which protease, calpain or proteasome, is inhibited and involved in cellular functions, because peptide aldehydes may inhibit both calpain and proteasome activities. In our experiments on the neurite outgrowth in PC12 cells, it became clear only recently that proteasome is involved, a finding made using a synthetic tripeptide aldehyde, benzyloxycarbonyl-leucyl-leucyl-leucinal (ZLLLal), developed in our laboratory (*9, 10*). ZLLLal initiates neurite outgrowth in PC12 cells at a low concentration (30 nM) and is a very strong inhibitor of 20S proteasome. On the other hand, benzyloxycarbonyl-leucyl-leucinal (ZLLal), a synthetic dipeptide aldehyde, initiates neurite outgrowth in PC12 cells at a higher concentration of 100 μM (*9*). Recently, it became evident that the induction of neurite outgrowth in Neuro 2A mouse neuroblastoma cells by lactacystin (*11, 12*) is also due to the inhibition of proteasome (*13*). Together these facts suggest that ZLLLal and ZLLal have different inhibitory effects against 20S proteasome. Therefore we measured the inhibition of purified 20S proteasome by ZLLLal or ZLLal *in vitro* and the induction of neuritogenesis in PC12 cells to evaluate the effectiveness of these inhibitors. At the same time, we determined the inhibitory activities of these inhibitors against purified *m*-calpain *in vitro* and the autolytic activation of intracellular calpain in rabbit erythrocytes.

MATERIALS AND METHODS

Materials—*m*-Calpain and 20S proteasome were purified from bovine lung and bovine brain, respectively, as described (*10, 14*). Peptide aldehydes and ZLLL-MCA were synthesized according to the previous methods (*10*). A monoclonal antibody specific for the large subunit of μ-calpain ($1A_8A_2$) was obtained as described previously (*15*). Reagents and chemicals were purchased from the following manufacturers: SucLLVY-MCA and tertiary-butyloxycarbonyl-valyl-leucyl-lysine-4-methylcoumaryl-7-amide (BocVLK-MCA), Peptide Institute (Osaka); alkaline phosphatase-conjugated anti-mouse IgG, Biosource International (USA); collagen type I, Koken (Tokyo); DMEM and F12, Life Technologies (USA); Alkaline Phosphatase Substrate Kit I, Vector Laboratories (USA); calcium chloride dihydrate, Wako Pure Chemical Industries (Osaka); benzyloxycarbonyl-leucyl-leucyl-glutamic acid-β-naphthylamide (ZLLE-NA) and calcium ionophore A23187, Sigma Chemical (USA).

Measurement of Inhibitory Activities of ZLLal and ZLLLal against m-Calpain and 20S Proteasome—Inhibitory activities of ZLLal and ZLLLal against *m*-calpain and 20S proteasome were measured by previously described methods (*16, 17*). For the *m*-calpain inhibitory assay, the 0.5 ml reaction mixture contained 0.24% alkali-denatured casein, 28 mM 2-mercaptoethanol, 0.94 unit of *m*-calpain, ZLLal or ZLLLal, 6 mM $CaCl_2$, and 0.1 M Tris-HCl (pH 7.5). The reaction was started by the addition of *m*-calpain solution and stopped by the addition of 0.5 ml of 10% trichloroacetic acid after incubation at 30°C for 15 min. After centrifugation at $1,300\times g$ for 10 min, the absorbance of the supernatant at 280 nm was measured. The reaction mixture for the 20S proteasome inhibitory assay contained 0.1 M Tris-acetate, pH 7.0, 20S proteasome, ZLLal or ZLLLal, and 25 μM substrate dissolved in dimethyl sulfoxide in a final volume of 1 ml. After incubation at 37°C for 15 min, the reaction was stopped by the addition of 0.1 ml of 10% SDS and 0.9 ml of 0.1 M Tris-acetate, pH 9.0. The fluorescence of the reaction products was measured. To determine the IC_{50}s against *m*-calpain and 20S proteasome, various concentrations of the synthetic peptide aldehydes were included in the assay mixture.

Neurite Outgrowth of PC12 Cells in the Presence of ZLLal and ZLLLal—PC12 cells were cultured as previously described (*18*). In brief, PC12 cells were plated at about 1×10^4 cells/cm^2 onto collagen-coated culture dishes in N2 medium (N2 medium consists of the F12/DMEM base medium supplemented with 100 μg/ml transferrin, 5 μg/ml progesterone, 5 μg/ml putrescine, 52 μg/ml selenium, and 5 μg/ml insulin) containing various concentrations of ZLLal or ZLLLal. The cells were cultured at 37°C in a 5% CO_2 incubator for 48 h and the number of cells with neurites longer than the diameter of the cell was measured in 200-400 cells.

Inhibition of Calpain Autolysis in Rabbit Erythrocytes by ZLLal and ZLLLal—Experiments were performed according to the previous method (*15*). Rabbit erythrocytes were prepared from rabbit blood by 4 cycles of centrifugation at $2,000\times g$ for 10 min and washing the packed erythrocytes with 5 volumes of 5 mM Tris-HCl containing 150 mM NaCl, pH 8.0. The washed erythrocytes were suspended at a concentration of 5×10^8/ml, and the suspension (200 μl) was preincubated at 25°C for 5 min in the presence or absence of 20 μM A23187 and/or inhibitors at various concentrations. The reaction was started by the addition of $CaCl_2$ solution to 400 μM and stopped after 15 min by the addition of an equal volume of sodium dodecyl sulfate (SDS) sample buffer. The samples were heated to 100°C for 5 min and electrophoresed in the presence of SDS in 12.5% polyacrylamide gels (*19*) at 5-10 mA for 6 h at room temperature. After the proteins had been transferred onto a polyvinylidene fluoride (PVDF) membrane, the membrane was blocked for 2 h with 5% skim milk in TBS (50 mM Tris-HCl, 200 mM NaCl, pH 7.4), washed with 0.1% Tween 20/TBS and incubated for 1 h at 37°C with anti-μ-calpain antibody ($1A_8A_2$) diluted 1:1,000 in 0.1% Tween 20/TBS. The membrane was then washed with 0.1% Tween 20/TBS and incubated with the second antibody (goat anti-mouse IgG coupled to alkaline phosphatase diluted 1:1,000) for 45 min at room temperature. The membrane was washed again with 0.1% Tween 20/TBS, and the proteins were developed with a Vector Alkaline Phosphatase Substrate Kit I.

RESULTS

Inhibition of m-Calpain and 20S Proteasome by ZLLal and ZLLLal—Profiles for the inhibition of *m*-calpain and 20S proteasome activities by ZLLal and ZLLLal are shown in Fig. 1. The IC_{50}s of ZLLal and ZLLLal for the alkali-denatured casein-degrading activity of *m*-calpain were both around 1.2 μM. These peptide aldehydes inhibited the casein-degrading activity of *m*-calpain at similar concentrations. On the other hand, ZLLLal strongly inhibited the ZLLL-MCA-degrading activity of 20S proteasome, while ZLLal inhibited it only weakly. The IC_{50}s for the ZLLL-MCA-degrading activity of 20S proteasome by ZLLLal and ZLLal were 100 nM and 110 μM, respectively. Thus, ZLLLal is 1,100 times more effective than ZLLal in inhibiting the ZLLL-MCA-degrading activity of 20S proteasome. Similar inhibition profiles were obtained for SucLLVY-MCA (a well-known substrate for measuring the chymotrypsin-like activity of proteasome) degrading activity in proteasome with IC_{50}s of 850 nM and 120 μM for ZLLLal and ZLLal, respectively. ZLLLal inhibited the degradation of BocVLK-MCA (a substrate for the trypsin-like activity of proteasome) only partially (about 25%) at 20 μM and did not inhibit at all that of ZLLE-NA (a substrate for the peptidylglutamyl-peptide hydrolyzing activity of proteasome), while ZLLal inhibited neither activity at 80 and 100 μM, respectively (data not shown).

Induction of Neurite Outgrowth in PC12 Cells by ZLLal and ZLLLal—To test the abilities of ZLLal and ZLLLal to inhibit intracellular proteasome, their effects on neurite outgrowth in PC12 cells were examined, since cells differentiate in response to proteasome inhibition as described in

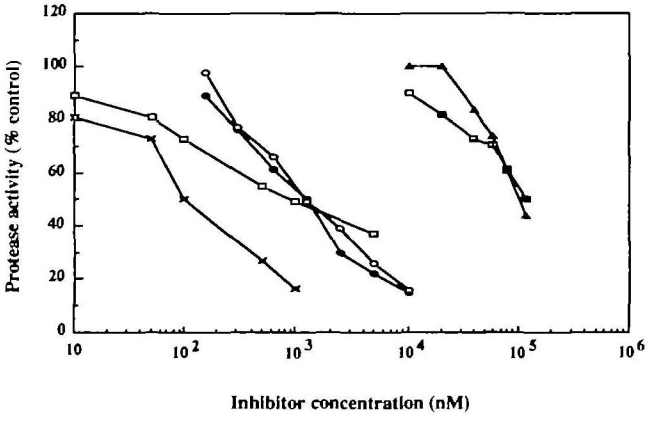


Fig. 1. **Inhibition of *m*-calpain and 20S proteasome by ZLLal and ZLLLal.** Inhibition of the alkali-denatured casein-degrading activity of *m*-calpain: ●, ZLLal; ○, ZLLLal. Inhibition of the ZLLL-MCA-degrading activity of proteasome: △, ZLLal; ×, ZLLLal. Inhibition of the SucLLVY-MCA-degrading activity of proteasome: ■, ZLLal; □, ZLLLal. Activities of *m*-calpain and 20S proteasome in the absence of inhibitors were designated as 100%.

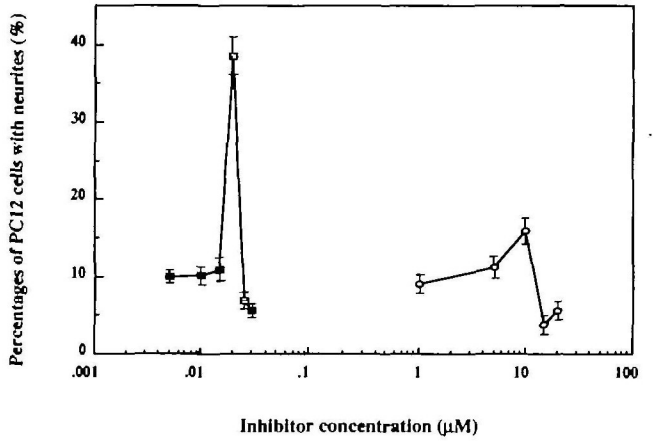


Fig. 2. **Neurite outgrowth of PC12 cells treated with ZLLal and ZLLLal.** Cells were incubated at 37°C for 48 h. The percentages of PC12 cells with neurites longer than the diameter of the cell in the presence of ZLLLal (■) and ZLLal (○) were determined using 200-400 cells; measurements were carried out in triplicate. The percentage of control cells with neurites (no inhibitor) was 1.88±0.15%. Data are expressed as mean±SD.

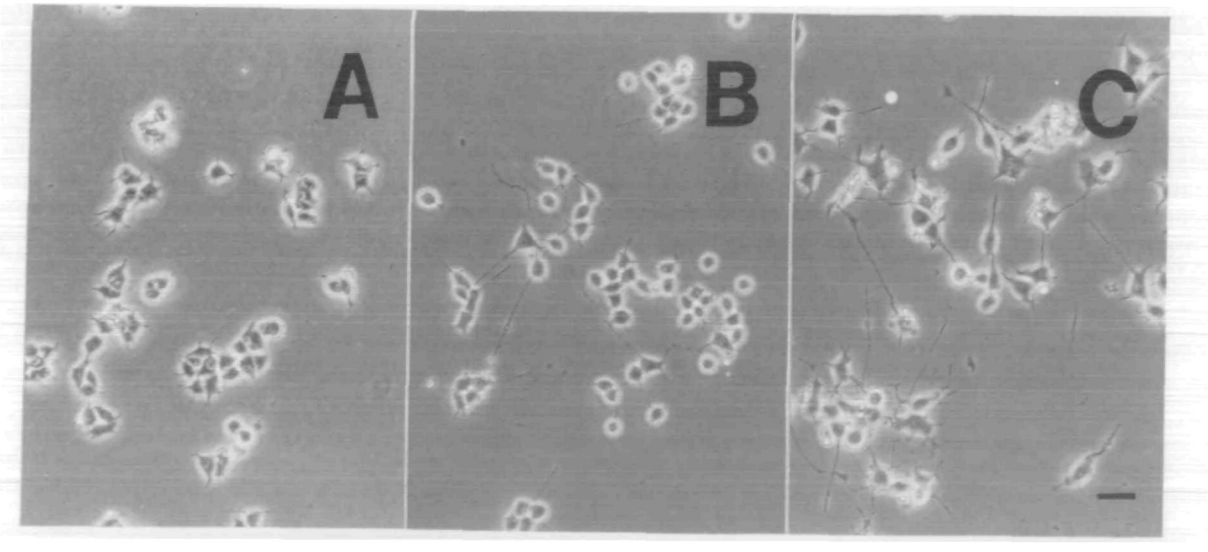


Fig. 3. **Phase contrast photomicrographs of PC12 cells treated with ZLLal and ZLLLal.** Cells were incubated at 37°C for 48 h. A: control cells, B: ZLLal-treated cells (10 μM), C: ZLLLal-treated cells (20 nM). Bar=35 μm.

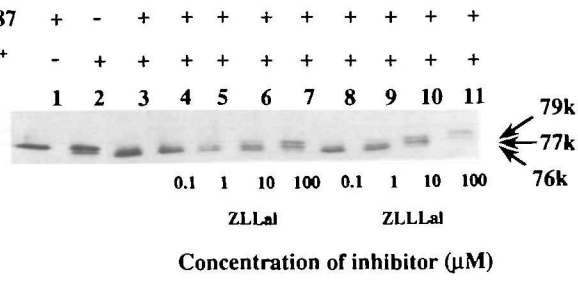


Fig. 4. **Inhibition of calpain autolysis in rabbit erythrocytes by ZLLal and ZLLLal.** A23187 (20 μM) (lanes 1, 3-11); Ca^{2+} (400 μM) (lanes 2-11); no inhibitor (lanes 1-3); ZLLal (lanes 4-7); ZLLLal (lanes 8-11).

the introduction. ZLLLal initiated neurite outgrowth in PC12 cells at an optimal concentration of 20 nM, while the optimal concentration for ZLLal was 10 μM, 500 times higher (Figs. 2 and 3). Moreover, the percentages of PC12 cells with neurites longer than the diameter of the cell in the presence of 20 nM ZLLLal or 10 μM ZLLal were 38.56 ± 1.78 and 15.97 ± 0.87%, respectively (data expressed as means $\pm$ SD). Thus, ZLLLal is much more effective than ZLLal with respect to the concentration required for neurite outgrowth and the extent of neurite outgrowth. This means that the former inhibits intracellular proteasome activity more effectively than the latter, as observed *in vitro*.

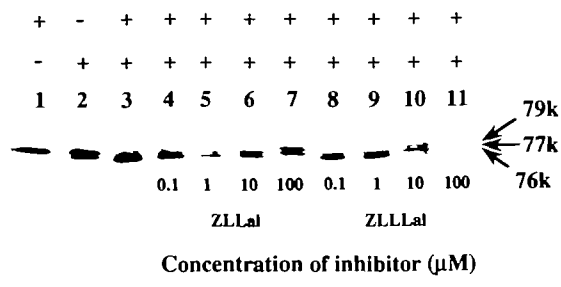
Inhibition of Calpain Autolysis in Rabbit Erythrocytes by ZLLal and ZLLLal—Next, the inhibition of intracellular calpain by ZLLLal and ZLLal was evaluated using the erythrocyte system (*15*). As shown in Fig. 4, a concentration-dependent inhibition of calpain autolysis, an indicator of calpain activation, by ZLLLal and ZLLal in rabbit erythrocytes was observed at elevated intracellular Ca^{2+} concentration. When A23187 (20 μM) and Ca^{2+} (400 μM) were added to rabbit erythrocytes, most of the original intracellular calpain 79-kDa large subunit was degraded to a 76-kDa form. ZLLal inhibited the autolysis of calpain at concentrations higher than 100 μM, and ZLLLal also inhibited autolysis at a concentration of about 100 μM. That ZLLLal and ZLLal inhibit intracellular calpain at similar concentrations shows that both inhibitors penetrate cell membranes at a similar rate and inhibit calpain with almost equal efficiencies as *in vitro*.

DISCUSSION

As described above, ZLLLal inhibits efficiently 20S proteasome and calpain activities both *in vitro* and *in vivo*. An inhibitor with one more leucine residue, ZLLLLal (benzyloxycarbonyl-leucyl-leucyl-leucyl-leucinal, a tetrapeptide aldehyde), also strongly inhibited calpain and 20S proteasome activities at similar concentrations (data not shown). On the other hand, ZLLal strongly inhibited only calpain activity, while its inhibition of 20S proteasome activity was weak. These facts show that the inhibition of calpain by these peptide aldehydes is not influenced by peptide length, but the inhibition of proteasome is. Recently, a peptide-degrading mechanism for archaebacterial *Thermoplasma acidophilum* 20S proteasomes was suggested based on an analysis of degradation products from insulin B-chain and human hemoglobin (*20*). According to that hypothesis, peptide degradation by the 20S proteasome occurs inside the cylinder structure of the 20S proteasome molecule generating peptides with certain lengths. This suggests that proteasome possesses an active site which accommodates several amino acid residues of a substrate and cleaves it to peptides of definite sizes like a molecular ruler. This characteristic is reflected in the inhibition profile by peptide aldehydes. That is, to bind the catalytic site of proteasome tightly and strongly, it is necessary for peptide aldehydes to consist of three or more amino acid residues. Shorter peptide aldehydes cannot interact properly with the catalytic site of 20S proteasome. This may be why the tri- and tetrapeptide aldehydes inhibit proteasome more strongly than the dipeptide aldehyde. On the other hand, the substrate specificity of calpain is rather fuzzy and low. Thus, it is likely that even a dipeptide aldehyde can bind tightly to the active site and inhibit calpain activity as strongly as longer aldehydes. This speculation is supported by the finding that calpeptin (benzyloxycarbonyl-leucyl-norleucinal), a dipeptide aldehyde, inhibits calpain effectively (*21*) but inhibits proteasome weakly (*8*), showing that the length of the peptide aldehyde is very important for the inhibition of proteasome activity.

These differing effects of ZLLal and ZLLLal on calpain and proteasome activities may be useful for identifying the functions of calpain and proteasome in cell physiology and pathology. Hitherto, it has been difficult to distinguish calpain and proteasome using the peptide aldehyde inhibitors, especially *in vivo*. For example, we had concluded from indirect evidence that proteasome is the target protease for the induction of neurite outgrowth of PC12 cells by ZLLLal. But not until the differing effects of ZLLLal and ZLLal were found could our conclusion be confirmed. ZLLLal initiates neurite outgrowth in PC12 cells at an optimal concentration of 20 nM, while the optimal concentration of ZLLal is 10 μM. The optimal concentration is that at which the percentage of cells with neurites longer than the diameter of the cell is maximum. At the optimal concentration, ZLLLal induces neurite outgrowth in PC12 cells acceleratively as a neurite outgrowth initiator. At concentrations higher than the optimal concentration, the cytotoxicity of the peptide aldehydes damages the cells, resulting in shortening of neurites. Futher, cell death occurs and the percentage of cells with neurites longer than the diameter of the cell decreases. The possibility that calpain is the target protease was excluded, since both inhibitors penetrate into cells equally and inhibit calpain at almost the same concentrations, as shown in the erythrocyte experiment.

In our *in vivo* experiments, however, the absolute concentrations of inhibitors required for inhibition were different from those required *in vitro*. The concentrations required for neurite outgrowth were lower than those required for proteasome inhibition *in vitro*. One possibility is that since the cells were exposed to inhibitors for a long time (48 h), a partial inhibition was amplified in cells. On the other hand, the concentrations required for the inhibition of calpain autolysis in erythrocytes were higher than those required to inhibit calpain *in vitro*. In this case, the cells were exposed to inhibitors for a short period (20 min) and the penetration of the inhibitors was not complete, although the rates were the same for both inhibitors.



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Another possibility is that since we evaluated inhibitor effectiveness by calpain autolysis rather than by catalytic activity toward substrates, the inhibition was less prominent. It was recently reported that calpain is active without autolysis (*22, 23*). If that is so, autolysis is a side-reaction that occurs after substrate digestion, and calpain activity can be inhibited at lower inhibitor concentrations.

The development of specific inhibitors against proteasome and calpain has long been awaited because protease inhibitors are one of the most powerful tools for clarifying the function of proteases in cells and tissues. The only calpain-specific inhibitor known so far is endogenous calpastatin. But calpastatin cannot be used in cell studies because it is a large protein and not membrane-permeable. Thus, some tripeptide aldehydes were designed and designated as "calpain inhibitors." However, they were later found to inhibit proteasome as well. As we found, dipeptide aldehydes are more effective in inhibiting calpain than proteasome, and it is now possible to distinguish calpain and proteasome in inhibition profiles by using ZLLal and ZLLLal. It was recently found that lactacystin is a specific inhibitor of proteasome which conjugates to the N-terminal threonine residue of the β-subunit. Lactacystin inhibits three distinct peptidase activities of proteasome (trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide hydrolyzing activities) (*13*). The inhibitory potency of lactacystin against chymotrypsin-like activity in proteasome is 20 times stronger than that against trypsin-like activity, and that against peptidylglutamyl-peptide hydrolyzing activity is weakest. On the other hand, ZLLLal inhibits the ZLLL-MCA-degrading and chymotrypsin-like activities rather specifically (the inhibitory potencies against the ZLLL-MCA-degrading and chymotrypsin-like activities are at least 200 times and 20 times stronger than those against trypsin-like activity, respectively). Calpain inhibitors 1 and 2 and calpeptin also inhibit the chymotrypsin-like activity of proteasome comparatively well (*8*), but the IC_{50}s are higher. Thus, ZLLLal is a useful tool for determining the specific functions of the hydrophobic amino acid residue-degrading activity of proteasome.

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