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Limitations of SLLVY-AMC in calpain and proteasome measurements

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Abstract

Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (SLLVY-AMC) is a fluorogenic substrate used to measure calpain activity and the “chymotrypsin-like” activity of the 20s proteasome. The goal of this study was to determine the relative role of calpains and the proteasome on SLLVY-AMC cleavage in attached and suspended renal epithelial cells (NRK-52E). The proteasome inhibitor epoxomicin did not inhibit purified calpain 1 or calpain 10 cleavage of SLLVY-AMC. Epoxomicin inhibited 11% of total SLLVY-AMC cleavage in attached cells and increasing concentrations of the calpain inhibitor calpeptin were additive. In contrast, cell suspensions had a 3.5-fold higher rate of SLLVY-AMC cleavage, epoxomicin inhibited cleavage 65% and calpeptin inhibited cleavage an additional 26%. Calpeptin alone also inhibited proteasomal activity. In conclusion, 1) SLLVY-AMC is cleaved in cells by calpain and the proteasome, 2) proteasome activity can be measured with epoxomicin, and 3) calpeptin can inhibit proteasome activity in some cases; thus limiting the use of SLLVY-AMC and calpeptin.

Keywords

calpain; proteasome; SLLVY-AMC; calpeptin; epoxomicin

Introduction

Calpains are a ubiquitously expressed, 15-member family of non-lysosomal, Ca^{2+} -activated, cysteine proteases that have been implicated in numerous pathologies related to loss of Ca^{2+} homeostasis and/or their genetic mutations [1;2;3;4]. Calpains 1 and 2, the first described members of the calpain family, are better understood than some of the more recently described calpain family members; however, the molecular mechanisms of activation and regulation, physiological functions, and disease pathologies are still highly debated topics regarding the entire family. Physiological functions of calpains include cell proliferation, signal transduction, apoptosis, cell motility, and regulation of gene expression [2]. Genetic mutations in the genes encoding calpain 3 and calpain 10 are thought to be associated with limb girdle muscular dystrophy and type 2 diabetes, respectively [5;6]. Abnormal activation of calpains has been

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implicated in pathologies associated with loss of Ca^{2+} homeostasis, including Alzheimer's disease and ischemic injury [1;2;7]. Despite these physiological and pathological associations, clearly defined molecular roles for specific calpains remain to be identified, emphasizing the need for additional research tools.

Currently, many tools for calpain research not only lack isoform specificity but also lack calpain specificity, in that they inhibit proteases other than calpain [8]. For example, in addition to inhibiting calpains, both calpain inhibitor 1 (ALLN) and calpain inhibitor 2 (ALLM) also inhibit the proteasome and cathepsins B and L at concentrations near their respective IC_{50} for calpain [9;10;11]. Calpeptin, one of the most widely used calpain inhibitors, also inhibits cathepsins L and K [11;12;13] and has been reported to inhibit the "chymotrypsin-like" activity of the isolated bovine proteasome at concentrations of 50–100 μM [10], concentrations commonly used in calpain research. The ubiquitin proteasome system (UPS) is a multicatalytic cytosolic protease that is vital to many important cellular functions, including removal of short-lived, mis-folded, and damaged proteins; control of the cell cycle; apoptosis; stroke; and cancer [14;15]. Another issue that plagues calpain and proteasome research is the lack of specific substrates for measuring their activities. SLLVY-AMC is one of the most widely used substrates to measure calpain, but is also commonly used to measure "chymotrypsin-like" 20s proteasome activities [16;17;18;19;20;21].

Many inhibitors used to study the proteasome are also non-specific. For example, MG132, a commonly used proteasomal inhibitor, also inhibits both calpains and cathepsins [14]. Epoxomicin, the most selective and potent inhibitor of the proteasome, has no effect on the activities of calpains, cathepsins, or any other proteases that have been tested to date [14;22]. Therefore, epoxomicin may be suitable for specific evaluation of proteasomal activity.

Whereas non-specific inhibitors and substrates are acceptable for use in purified enzyme assays, they are not adequate for studying these proteases in complex cellular environments. Consequently, the purpose of this study was to clearly address the issue of non-specific and overlapping characteristics of these commonly used tools.

Materials and Methods

Reagents

Calpeptin and purified porcine calpain 1 were obtained from Calbiochem (La Jolla, CA). SLLVY-AMC (Suc-Leu-Leu-Val-Tyr-AMC) and epoxomicin were purchased from Bachem (King of Prussia, PA) and Biomol (Plymouth Meeting, PA), respectively. Dulbecco's Modified Eagle Medium and calf serum were obtained from Invitrogen (Carlsbad, CA). Normal rat kidney epithelial (NRK-52E) cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cell culture

NRK-52E cells were cultured as previously described [23] on either 35-mm or 48-well culture plates in DMEM (high glucose) supplemented with 10% fetal calf serum. Confluent monolayers were either assayed for calpain activity directly on the plate or removed from the dish by gentle scraping for analysis of cell suspensions. After preparation of cell suspensions, cell viability was determined via trypan blue exclusion and only preparations with greater than 90% viability were used for analysis.

Isolation of rabbit renal mitochondrial matrix

Mitochondria were isolated from the kidney cortex of female New Zealand White rabbits (2 kg) as previously described [24;25]. After isolation of kidney mitochondria, further

fractionation was performed to yield a mitochondrial matrix fraction, a concentrated source of calpain 10 [24], as previously described [26].

Measurement of calpain activity

Calpain activity was measured spectrofluorometrically using the calpain substrate, SLLVY-AMC, as previously described [24;27]. Briefly, cellular suspensions in an isotonic calpain activity buffer (150 mM KCL, 20 mM HEPES, pH 7.4) and 0.45 mM CaCl₂ were assayed for SLLVY-AMC (100 μ M) cleavage in a 96-well plate (5 $\times$ 10⁴ cells/well) for 20 min. Attached cells were assayed for SLLVY-AMC cleavage directly on the 48-well plate in culture media. Mitochondrial matrix fractions (50 μ g protein/well) were diluted in the isotonic calpain activity buffer containing SLLVY-AMC (50 μ M). Calpain 1 activity was measured in a similar manner using 5 μ g purified porcine calpain 1 diluted in a calpain 1 buffer (150 mM KCL, 20 mM HEPES, 5 mM DTT, 1.5 mM CaCl₂) containing SLLVY-AMC (50 μ M). In all cases, activity was measured under linear conditions as a function of SLLVY-AMC hydrolysis using excitation and emission wavelengths of 355 nm and 444 nm, respectively.

Statistical analysis

Separate passages of NRK-52E represent one experiment (n=1). Mitochondrial matrix isolated from one animal represents one experiment (n=1). Data are presented as means $\pm$ SE and were subjected to the student's t-test or one-way ANOVA. Multiple means were compared using Student-Newman-Keuls test. Means with a value of p<0.05 were considered statistically different from one another.

Results and Discussion

To determine the effects of epoxomicin on calpain activity, we utilized two sources of calpain activity, purified calpain 1 and enriched calpain 10 (mitochondrial matrix [24]). Epoxomicin (10 μ M) had no effect on the activity of purified calpain 1 or calpain 10, indicating that the 20s subunit proteasome inhibitor does not inhibit calpain activity (figure 1). As expected, calpeptin (1 μ M) completely inhibited cleavage of SLLVY-AMC by both calpain 1 and calpain 10. These results provide evidence that epoxomicin is a specific proteasomal inhibitor.

As stated above, the calpain substrate SLLVY-AMC is known to be cleaved by the proteasome, specifically the "chymotrypsin-like" protease of the 20s subunit [16;17]. It also has been reported that 50–100 μ M concentrations of calpeptin can inhibit proteasome activity [10]. To determine the relative contribution of calpain and the proteasome to SLLVY-AMC cleavage in attached cells, NRK-52E cells were assayed for SLLVY-AMC cleavage with varying concentrations of calpeptin and/or epoxomicin. Calpeptin inhibited SLLVY-AMC cleavage in a concentration-dependent manner (50% inhibition at 100 μ M) (figure 2). Epoxomicin (10 μ M) inhibited SLLVY-AMC cleavage 11% with concentrations up to 50 μ M having no additional inhibition (figure 2, data not shown). Increasing concentrations of calpeptin, in the presence of epoxomicin, further decreased SLLVY-AMC cleavage in an additive manner to 69%. In summary, we suggest that 11% of SLLVY-AMC cleavage in attached NRK-52E cells is due to proteasome activity and that calpains are responsible for an additional 58%. Furthermore, it appears that calpeptin and epoxomicin are selective calpain and proteasome inhibitors with these concentrations and conditions. It is not known what protease(s) are responsible for the remaining cleavage of SLLVY-AMC.

We conducted similar experiments using the same cells in suspension to determine whether differences in proteasome and calpain activities existed between attached cells and cell suspensions. We first confirmed that the process of removing the cells from the plate and preparing a cellular suspension did not injure the plasma membrane using trypan blue exclusion

(> 90% trypan blue negative after scraping, data not shown). Initial experiments revealed that cells in suspension cleaved SLLVY-AMC at a rate that was 3.5 times greater than in attached cells (1.3 ± 0.3 versus 0.4 ± 0.1 fluorescence units/min/ μ g cellular protein; $p < 0.05$, $n = 3$). Calpeptin inhibited SLLVY-AMC cleavage in a concentration-dependent manner (75% inhibition at 100 μ M) (figure 3). Epoxomicin (10 μ M) inhibited SLLVY-AMC cleavage 65% (figure 3) with concentrations up to 50 μ M providing no additional inhibition (data not shown). These results provide evidence that the increase in SLLVY-AMC cleavage is due to increased proteasome activity. Increasing concentrations of calpeptin, in the presence of epoxomicin, decreased SLLVY-AMC cleavage to 91% (figure 3). Therefore, we suggest that 65% of SLLVY-AMC cleavage in NRK-52E cell suspensions is due to proteasome activity and that calpains are responsible for an additional 26%. Furthermore, the increased SLLVY-AMC cleavage in cell suspensions, compared to attached cells, is the result of increased proteasomal activity. It is not known what protease(s) are responsible for the remaining cleavage of SLLVY-AMC.

These data also provide evidence that calpeptin is not specific for calpain and inhibits the proteasome under these conditions. Because the proteasome and calpains are responsible for approximately 65% and 26% of SLLVY-AMC cleavage, respectively, then calpeptin alone should not inhibit more than 26% of SLLVY-AMC cleavage. However, we observed calpeptin-mediated inhibition of SLLVY-AMC in excess of 35% at concentrations of 50 μ M, indicating that calpeptin is capable of inhibiting the proteasome at these and possibly lower concentrations under these conditions.

Interestingly, SLLVY-AMC cleavage was 3.5-fold higher in the cell suspensions than in attached cells. Although the mechanism of this increase in SLLVY-AMC cleavage has not been elucidated, we suggest that the increase in total SLLVY-AMC cleavage in the suspension model is primarily the result of increased proteasome activity. However, we cannot rule out the possibility that increased calpain activity may also contribute to this observation.

In conclusion, we have demonstrated that SLLVY-AMC is cleaved by both calpain and the proteasome in cells, and that the relative amount of SLLVY-AMC cleavage by the proteasome and calpains can be determined in both attached cells and cell suspensions through the use of epoxomicin and calpeptin. The type of cellular model can influence the relative amounts of calpain and proteasomal activities. In addition, under certain conditions, calpeptin can inhibit proteasomal activity at concentrations of 50 μ M and possibly lower. Therefore limitations exist in the use of calpeptin and SLLVY-AMC alone to measure calpain and proteasome activities in cells.

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Figure 1. Effect of protease inhibitors on calpain 1 and calpain 10 activity

Calpain 1 (a) or mitochondrial matrix (calpain 10) (b) were incubated with SLLVY-AMC (50 μ M) and either vehicle control, epoxomicin (10 μ M), or calpeptin (1 μ M) for 1 hour. Activity was measured under linear conditions and presented as percent of vehicle. Bars represent the means $\pm$ SEM, $n=3$. Asterisks (*) indicate statistically different means ($p < 0.05$).

Figure 2. Calpain and proteasome activity as measured by cleavage of SLLVY-AMC in attached NRK-52E cells

NRK-52E cells were incubated with SLLVY-AMC (100 μ M) and either vehicle control or inhibitors at indicated concentrations for 20 min. Activity was measured under linear conditions and presented as percent of vehicle. Bars represent the means $\pm$ SEM, n=3. Bars with different letters indicate statistically different means ($p < 0.05$).

Figure 3. Calpain and proteasome activity as measured by cleavage of SLLVY-AMC in NRK-52E cells in suspension

NRK-52E cells were incubated with SLLVY-AMC (100 μ M) and either vehicle control or inhibitors at indicated concentrations for 20 min. Activity was measured under linear conditions and presented as percent of vehicle. Bars represent the means $\pm$ SEM, n=3. Bars with different letters indicate statistically different means ($p < 0.05$).