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CHARACTERIZATION OF A CONTINUOUS FLUOROGENIC ASSAY FOR CALPAIN I. KINETIC EVALUATION OF PEPTIDE ALDEHYDES, HALOMETHYL KETONES AND (ACYLOXY)METHYL KETONES AS INHIBITORS OF THE ENZYME

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Abstract: Z-Leu-Arg-(7-methoxynaphthyl)amide (**1**) is a substrate for calpain I. The specificity constant for **1** (k_{cat}/K_m = 1405 ± 40 $M^{-1}s^{-1}$) is 10x greater than for any previously reported fluorogenic substrate. Using this substrate, a sensitive, continuous fluorogenic assay was developed permitting the identification of Z-(D)Ala-Leu-Phe-(OCO-2,6-Fl_2-Ph) (**69**) as the first selective (>100-fold versus cathepsins B and L) time-dependent inhibitor of the enzyme.

Calpain (EC 3.4.22.17) is a Ca^{2+}-dependent cysteine protease which is found in the cytosolic and microsomal compartments of most mammalian neurons and other cells.[1] Two distinct subclasses of calpains have been identified - calpain I or μ-calpain (low Ca^{2+} (5-20 μM) requiring) and calpain II (high Ca^{2+} (0.5-1 mM) requiring).[2] The calpains hydrolyze a large number of intracellular proteins[3] and are thought to be essential for turnover of cytoskeletal elements *in vivo*.[1,2] Although extensively investigated over the past 25 years, there are few reports of kinetic studies using synthetic substrates[3] or of calpain selective inhibitors.[4] In this Letter, we describe the kinetic characterization of some commercially available fluorogenic peptide substrates and determine second order rate constants of inactivation for over sixty peptide α-chloromethyl- and α-(acyl)aryloxymethyl ketones.[5]

Sasaki and co-workers published specificity constants for a large number of peptidyl 4-methylcoumarylamides.[3] Our attempts to use several of the substrates having the highest specificity constants to develop a continuous assay were unsuccessful. As a result, we elected to screen some 10-15 commercially available fluorogenic peptides to assess their potential as calpain I substrates. The screening protocol was straightforward: substrate (1 mM) and buffer were equilibrated for 5 minutes, enzyme was added[6] and the change in fluorescent units was recorded over time. Figure 1 shows the results of a substrate screen. Initial data obtained for each of the peptides revealed that peptides **1-7** possessed superior activity and they were selected for further kinetic characterization (Table 1).

The dipeptide Z-Leu-Arg-MNA (**1**) emerged as one of the most promising substrates. The rate of hydrolysis of Z-Leu-Arg-MNA is linear with increasing enzyme concentration (data not shown). Figure 2 shows the hydrolysis of 130 μM Z-Leu-Arg-MNA by calpain as a function of time.[7] The hydrolysis is linear for the first 5 to 7 minutes, but eventually plateaus. Addition of more substrate (A) at the plateau region of the reaction produces no further increase in fluorescence, while the addition of more enzyme (B) restimulates the fluorogenic effect. Calpain is well known for its autocatalytic properties[2,8] and these results suggest that under the assay conditions, calpain deactivates itself over time. A study was done to compare the autocatalytic effect at 8 °C, 22 °C and 37 °C. The decrease in rate (autocatalysis) over time is virtually absent at 8 °C (data not shown) and all subsequent studies were therefore conducted at this temperature.

Amide **1** exhibits Michaelis-Menton kinetics with a calculated K_m of 370 ± 7 mM and a k_{cat} of 0.52± 0.07 sec^{-1} (Figure 3; k_{cat}/K_m = 1405 ± 40 $M^{-1}s^{-1}$, n=8).[7] The specificity constants of Z-Leu-Arg-

coumarinylamides **2** and **3** are 10x poorer than naphthylamide **1**. The difference in magnitude of the specificity constants is a reflection of 10-fold decrease in k_{cat} as the K_m values are within *ca.* 1.5x of one another. The other fluorogenic peptides **4-7** are inferior to **1**.

In order to demonstrate that this assay is measuring the protease activity of calpain, the inhibitory effect of calpastatin,[9a] a specific, endogenous inhibitor of calpain was examined (Figure 4). In this study, calpastatin was evaluated in both the new fluorogenic assay and in a calpain assay where ^{3}H-acetyl casein is used as the substrate.[10] The inhibitory activity of calpastatin is identical in both assays. Since calpastatin is known to inhibit only the calpains, these results demonstrate that the activity measured in the fluorogenic assay can be attributed to the action of calpain hydrolyzing amide **1**. The protease inhibitors leupeptin,[4b] antipain,[9b] and chymostatin[9b] were also examined in both assays. The inhibitors exhibit identical potencies in the two assays (data not shown). For the time-dependent irreversible inhibitor E-64 (L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane),[11] the apparent second order rate constant for inactivation ($k_{obs}/[I]$) of 2,500 $M^{-1}s^{-1}$ (ten inhibitor concentrations, n = 4) was measured in the fluorogenic assay. This value compares favorably with the inactivation rate constant obtained in an 'endpoint' (non-continuous) calpain assay at a higher temperature ($k_{obs}/[I]$ = 7,500 $M^{-1}s^{-1}$; 37 °C).[11b,c]

A series of peptide aldehydes **8-23** and affinity labels **24-70** were examined for their ability to inhibit calpain I (Tables 2 and 3).[12] These compounds were prepared in connection with our discovery program to identify a potent, selective inhibitor of calpain I. Although a number of reversible and irreversible inhibitors have been described for calpain,[4] they collectively suffer from inadequate selectivity with respect to two lysosomal cysteine proteases, cathepsins B and L. Calpain selectivity versus cathepsin B has been achieved;[4,8] however, agents selective against cathespin L are unknown. For example, Z-Leu-Leu-Tyr-CHN_2, as reported by Shaw,[13] possesses inactivation rate constants of 230,000 $M^{-1}s^{-1}$, 1,300 $M^{-1}s^{-1}$ and 1,500,000 $M^{-1}s^{-1}$ against calpain II, cathepsin B and cathepsin L, respectively.

Calpain I prefers hydrophobic amino acid residues at P_1-P_3 as based on extensive compilation of peptide substrate data. Peptide aldehydes containing such residues (e.g., MDL 28170: Z-Val-Phe-H, IC_{50} = 17 nM)[12a] are inhibitors of the calpains.[4] Aldehydes **8-23** prepared in this study lend support that Leu and Phe are the P_2/P_1 specificity preferences for calpain I (Table 2). A P_1 aromatic residue (Phe or Tyr) in combination with Leu or Val at P_2 furnishes potent (IC_{50}s <100 nM) calpain inhibitors (**8, 9, 16-19**). Introducing a smaller Ala (**10**) residue or sterically demanding Phe (**13**) or t-Butylgly (**14**) residues at P_2 results in a loss of potency. Similar replacement of Phe/Tyr with larger P_1 residues (1-Naphthyl)ala (**21**), Homophe (**22**), Pro (**23**)) also gives rise to inhibitors with attenuated potency as compared to **8** or **9**. Aldehyde **16** is the most potent inhibitor of its class reported against calpain I (IC_{50} = 20 nM for **16** versus MDL 28170, IC_{50} = 40 nM, in our assay).

Using Z-Leu-Phe and Z-Leu-Tyr as optimized backbone scaffolds, a series of α-chloromethyl- and α-(acyl)aryloxymethyl ketones **24-71** were synthesized and evaluated as time-dependent (irreversible) inhibitors of calpain (Table 3). To some extent, the potency trends exhibited by dipeptides **24-60**, parallel that observed for the peptide aldehydes **8-23**. The P_2 Leu/Val and P_1 Phe/Tyr residues generally display greater activity than other amino acids at these positions (compare: chloromethyl ketone **41** versus **25**, **29** and **33**; 2,6-dichlorobenzoyloxymethyl ketones **49**, **50** and **57** versus **36**, **37** and **42**; 2,6-dichloro-3-morpholinoethoxybenzoyloxymethyl ketones **55**, **58** and **60** versus **30**, **43** and **44**). *Most striking, is the profound influence of leaving group structure on potency which can override calpain's putative P_1-P_2 specificity preferences.* Dipeptides **56** and **59** contain a P_1 glycine residue and are not expected to have appreciable enzyme affinity.[2,4,8] Yet, by virtue of the leaving group (2,6-dichloro-3-morpholinylsulfonamidobenzoate and chloride), **56** and **59** possess rapid inactivation rates between 20,000 - 30,000 $M^{-1}s^{-1}$. This is in direct contrast to glycine analogs **27**, **34**, **37**, **42** and **43** with rates of <6000 $M^{-1}s^{-1}$. Similarly, the activity of **51** versus **37** is leaving group dependent. This effect of leaving group structure on potency is not restricted to calpain, but rather is broad-based in nature (for cathepsin B: compare **41** versus **50** and **58**; for cathepsin L: compare **50** to **58**).[14]

Selectivity data obtained for the more potent inactivators **50-60** reveal that agents **50**, **57**, and **58** are calpain:cathepsin B selective (>10x). None of the dipeptides exhibit calpain:cathepsin L selectivity. Neutral P_3 amino acids were then introduced into the Leu-Phe (P_2-P_1) backbone (tripeptides **61-71**) in an attempt to achieve exclusive calpain specificity. Gly, Leu and Pro (**64-67**) did not provide selectivity enhancement. Insertion of Ala at P_3 yields inhibitor **67** which is *ca.* 10-fold selective for calpain over cathepsins B and L. Of greater significance

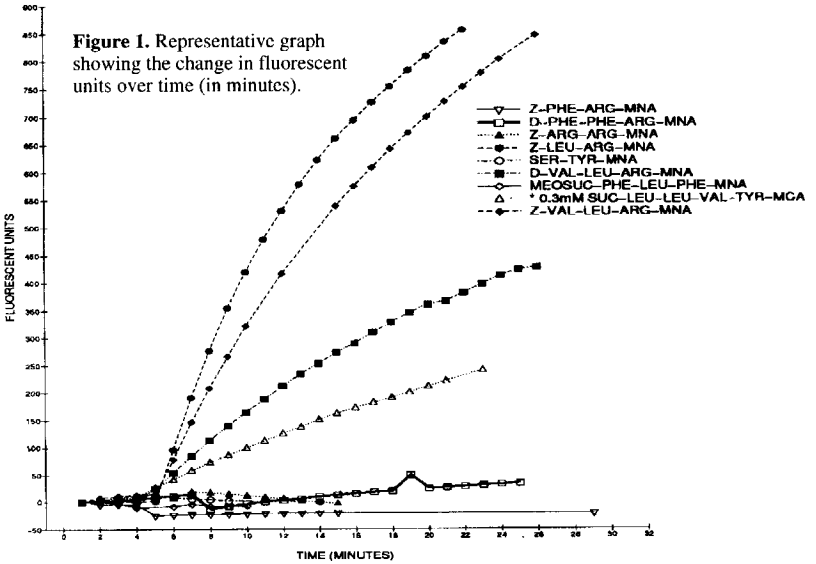

Figure 1. Representative graph showing the change in fluorescent units over time (in minutes).

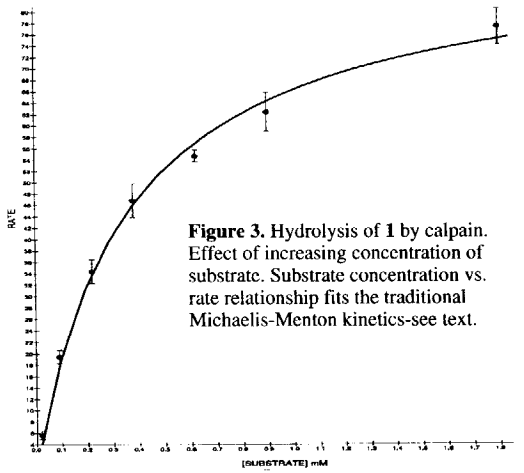

Figure 3. Hydrolysis of **1** by calpain. Effect of increasing concentration of substrate. Substrate concentration vs. rate relationship fits the traditional Michaelis-Menton kinetics-see text.

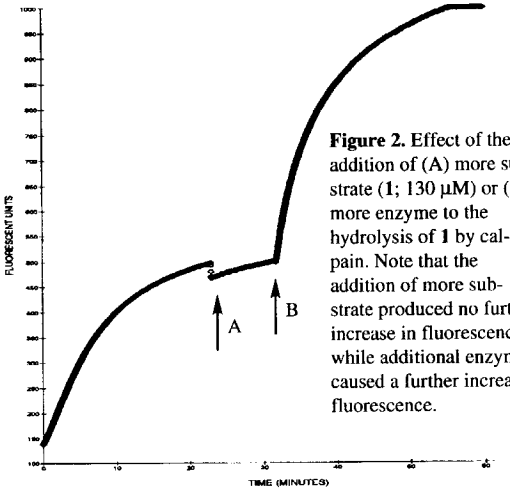

Figure 2. Effect of the addition of (A) more substrate (**1**; 130 μM) or (B) more enzyme to the hydrolysis of **1** by calpain. Note that the addition of more substrate produced no further increase in fluorescence, while additional enzyme caused a further increase in fluorescence.

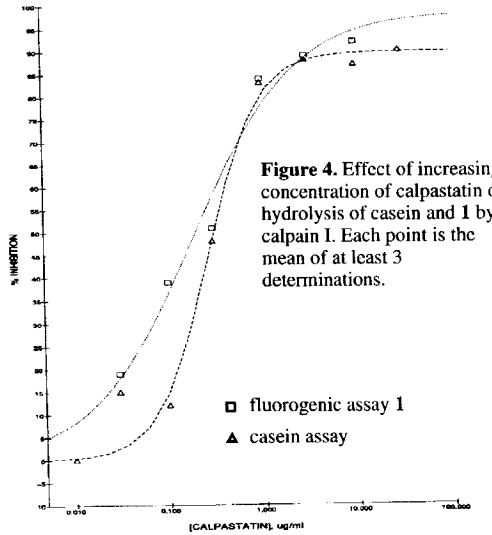

Figure 4. Effect of increasing concentration of calpastatin on hydrolysis of casein and **1** by calpain I. Each point is the mean of at least 3 determinations.

TABLE 1. Kinetic constants for fluorogenic peptide substrates.[a,c]

Substrate[b]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Z-Leu-Arg-MNA (**1**)	0.37	0.52	1405
Z-Leu-Arg-AMC (**2**)	0.19	0.02	105
Z-Leu-Arg-AFC (**3**)	0.46	0.07	153
Suc-Leu-Leu-Val-Try-AMC (**4**)	0.16	0.42	262
Suc-Leu-Leu-Val-AMC (**5**)	0.20	0.29	141
BOC-Val-Leu-Lys-AMC (**6**)	5.90	0.49	83
Z-Val-Leu-Arg-MNA (**7**)	0.10[c]	d	d

(a) Standard error for each value <5% (n>4). (b) MNA = 7-methoxynaphthylamine; AMC = 7-amino-4-methylcoumarin; AFC = 7-amino-4-trifluoromethylcoumarin; (c) K_s = 0.2 μM, see ref. 16. (d) Not calculated. (e) Other peptides found as inferior substrates include: Boc-Val-Leu-Lys-AMC; Z-Phe-Pro- Arg-MNA; (D)-Val-Leu-Lys-MNA; (D)-Phe-Phe- Arg-MNA; (D)-Val-Leu-Arg-MNA; Z-Arg-Arg- MNA; Z-Arg-Gly-Phe-Phe-Leu-MNA; Z-Val-Lys- Lys-Arg-MNA; Z-Phe-Arg-MNA; Z-Ser-Tyr- MNA, see Figure 1.

TABLE 2. Inhibition of calpain I by peptidyl aldehydes.[a]

Inhibitor	IC_{50} (nM)	Inhibitor	IC_{50} (nM)
Z-Leu-Phe-H (**8**)	35	Z-Leu-Tyr-H (**16**)	20
Z-Val-Phe-H (**9**)	40	Z-Val-Tyr-H (**17**)	25
Z-Ala-Phe-H (**10**)	140	Z-Val-Tyr(OMe)-H (**18**)	30
Z-t-Butylala-Phe-H (**11**)	100	Z-Ile-Tyr-H (**19**)	55
Z-Phenylgly-Phe-H (**12**)	100	Z-Val-Homophe-H (**20**)	100
Z-Phe-Phe-H (**13**)	400	Z-Val-(1-Naphthyl)ala-H (**21**)	100
Z-t-Butylgly-Phe-H (**14**)	500	Z-Homophe-Phe-H (**22**)	270
Z-(1-Naphthyl)ala-Phe-H (**15**)	>1000	Z-Val-Pro-H (**23**)	>1000

(a) Obtained from ^{3}H-acetyl-casein assay at 8 °C, ref 10.

is the P_3 (D)-Ala containing inhibitor **69**. This inhibitor not only possesses the desired >100-fold specificity for calpain, but also a 4-fold rate enhancement as compared to the dipeptide congener **48**. Once again a dramatic leaving group effect is observed for the series. Cathepsin L selectivity is eliminated upon exchange of the 2,6-difluorobenzoyloxy- (DFB) for the 2,6-dichloro-3-morpholinylsulfonamidobenzoyloxy- (DCMorB) departing group (**69** versus **70**). Enzyme affinity is also modulated by the choice of the P_3 N-capping functionality. In the case of the DFB series, loss of both calpain potency and lysosomal selectivity is seen upon N-benzyloxycarbamoyl to N-methoxycarbamoyl exchange (**67** versus **63**). In the structurally related DCMorB series, calpain potency is retained but cathepsin L selectivity is lost (**70** versus **71**).

In summary, a continuous assay for calpain I was developed using Z-Leu-Arg-MNA (**1**) as a new substrate. The k_{cat}/K_m for **1** is superior to any calpain substrate reported to date. Using the assay, the apparent second order rate constant of inactivation ($k_{obs}/[I]$) for a series of time-dependent inhibitors was determined. One of the most intriguing observations concluded from the data is the dramatic impact which the leaving group imparts to potency and enzyme selectivity. Lastly, Z-(D)-Ala-Leu-Phe-DFB (**69**) as discovered here, is presently the only known rapid inactivator of calpain (31,000 $M^{-1}s^{-1}$, 8 °C) displaying >100x selectivity against both cathepsin B and cathepsin L.[15] Inhibitor **69** will undoubtedly be an important agent to assist in unraveling the biological role of calpain I, exclusive of lysosomal enzymes.

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6. Calpain I preparation: One unit of in-dated packed red blood cells 4 to 10 days old (*ca.* 350 mL) was diluted with an equal volume of

TABLE 3. Inhibition of calpain I by peptidyl α-substituted methyl ketones and selectivity data.

Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	
	calpain I[a]	cathepsin B[b]
Dipeptides[d]		
Z-Leu-Tyr-CH_2-Br (**24**)	<500	50
Z-Phe-Phe-CH_2-Cl (**25**)	<500	---[e]
Z-Ala-Gly-CH_2-DCB (**26**)	<500	---
Z-Phe-Gly-CH_2-DCB (**27**)	<500	---
Z-Phe-Ala-CH_2-DCB (**28**)	<500	360000[f]
Z-Phe-Ala-CH_2-Cl (**29**)	<500	220000
Z-Phe-Ala-CH_2-DCMorB (**30**)	<500	150000[f]
Z-N(Me)-Leu-Phe-CH_2-DFB (**31**)	<500	---
Z-Leu-Ala-CH_2-ACB (**32**)	500	---
Z-Asn-Phe-CH_2-Cl (**33**)	600	3000
Z-Leu-Gly-CH_2-ACB (**34**)	600	---
Z-Val-Phe-CH_2-DMB (**35**)	700	---
Z-Gly-Phe-CH_2-DCB (**36**)	700	---
Z-Val-Gly-CH_2-DCB (**37**)	800	---
Z-Gly-Phe-CH_2-DCSGB (**38**)	900	---
Z-Leu-Tyr-CH_2-Cl (**39**)	1500	2000
Ts-Leu-Phe-CH_2-DFB (**40**)	1500	---
Z-Leu-Phe-CH_2-Cl (**41**)	5500	2500
Z-Leu-Gly-CH_2-DCB (**42**)	6000	---
Z-Leu-Gly-CH_2-DCMorB (**43**)	6000	---
Z-Leu-Ala-CH_2-DCMorB (**44**)	6000	58000
Z-Leu-Ala-CH_2-Cl (**45**)	6000	43000
Z-Leu-Tyr-CH_2-DFB (**46**)	7000	---
Boc-Leu-Phe-CH_2-DFB (**47**)	8000	---
Z-Leu-Phe-CH_2-DFB (**48**)	9000	---
Z-Val-Phe-CH_2-DCB (**49**)	11000	---

Inhibitor	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)		
	calpain I	cathepsin B	cathepsin L[c]
Z-Leu-Phe-CH_2-DCB (**50**)	11000	250	2700
Z-Val-Gly-CH_2-DCSGB (**51**)	12000	600000	120000
Z-Leu-Ala-CH_2-DCSMorB (**52**)	14000	---	---
Z-Leu-Tyr-CH_2-DCSMorB (**53**)	14000	---	---
Z-Leu-Phe-CH_2-DCSMorB (**54**)	17000	---	---
Z-Val-Phe-CH_2-DCMorB (**55**)	20000	5500	63000
Z-Leu-Gly-CH_2-DCSMorB (**56**)	23000	260000	160000
Z-Leu-Tyr-CH_2-DCB (**57**)	28000	1600	33000
Z-Leu-Phe-CH_2-DCMorB (**58**)	30000	80	38000
Z-Leu-Gly-CH_2-Cl (**59**)	31000	170000	500000
Z-Leu-Tyr-CH_2-DCMorB (**60**)	54000	2600	50000
Tripeptides			
Z-(D)-Ala-Leu-Gly-CH_2-DCMorB (**61**)	<500	---	---
Z-(D)-Phe-Leu-Phe-CH_2-DFB (**62**)	1000	100	100
Cme-(D)-Ala-Leu-Phe-CH_2-DFB (**63**)	1400	700	400
Z-Gly-Leu-Phe-CH_2-Cl (**64**)	3300	2700	---
Z-Leu-Leu-Phe-CH_2-Cl (**65**)	9500	30000	---
Z-Gly-Leu-Phe-CH_2-DFB (**66**)	15000	800	14000
Z-Ala-Leu-Phe-CH_2-DFB (**67**)	19000	2200	1600
Z-Pro-Leu-Phe-CH_2-DFB (**68**)	26500	11000	---
Z-(D)-Ala-Leu-Phe-CH_2-DFB (**69**)	31000	100	300
Z-(D)-Ala-Leu-Phe-CH_2-DCMorB (**70**)	32000	400	28000
Cme-(D)-Ala-Leu-Phe-CH_2-DCMorB (**71**)	43000	4700	90000

(a) Data generated using Z-Leu-Arg-MNA as substrate at 8 °C; std. error <10%; n = 3. (b) Data obtained at 8 °C, ref 17a . (c) Data obtained at 8 °C, ref. 17b. (d) DCB = OCO-2,6-Cl_2-Ph; DCMorB = OCO-2,6-Cl_2-3-OCH_2CH_2morpholine-Ph; DFB = OCO-2,6-F_2-Ph; ACB = OCO-2-NHCOMe- 6-Cl-Ph; DMB = OCO-2,6-Me_2-Ph; DCSGB = OCO-2,6-Cl_2-3-SO_2NHCH_2COOBn-Ph; DCSMorB = OCO-2,6-Cl_2-3-SO_2morpholine-Ph; Boc = t-BuOCO; Cme = MeOCO; TS = 4-Me-$PhSO_2$; Z = BnOCO. (e) Not determined. (f) $k_{obs}/[I]$ = 104,000 (**28**) and 180,000 (**30**) against cathepsin L at 8 °C.

diluting/wash buffer (150 mM NaCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol, 140 μg/mL bacitracin) and centrifuged at 1000 x g for 20 min. The supernate was discarded and the procedure was repeated. The washed cell pellet (*ca.* 300 mL) was lysed with 700 mL of lysing solution (5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol, 140 mg/mL bacitracin) and centrifuged at 40,000 x g for 30 min to remove cell debris. The supernate (membrane-free hemolysate) was added to 500 mL DEAE-sephacel and the slurry was stirred gently at 4 °C for 1 h. Batch elution was done using DEAE-sephacel wash solution to remove a large amount of unwanted protein, most of which was hemoglobin. The DEAE-sephacel was equilibrated/washed with 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 5 mM 2-mercaptoethanol and 140 mg/mL bacitracin and then eluted with 150 mM NaCl (pH 7.5) containing 1 mM EDTA and 5 mM 2-mercaptoethanol. The DEAE-sephacel slurry was poured into a 750 mL column connected in tandem to phenyl-sepharose CL-4B column. Under these conditions calpastatin (an endogenous inhibitor of calpain) does not adhere to the phenyl-sepharose CL-4B column and is separated from calpain. The phenyl-sepharose CL-4B column was equilibrated with the same buffer used to elute the DEAE-sephacel, eluted first with 75 mM of NaCl and then with 0 mM NaCl wash solution. The fractions were collected and activity monitored using the ^{3}H-acetyl casein method (see reference 10). The active fractions were pooled and concentrated (*ca.* 50-fold) using an Amicon stirred cell equipped with a YM-10 membrane. Calpain was stored at 4 °C with 5 mM EDTA and 5 mM 2-mercaptoethanol. Protein values for the enzyme were determined by Peterson's method of Lowry (Peterson, G. L. *Anal. Biochem.* **1977**, *83*, 346). We thank Ms. V. Thompson of Dr. D. Goll's Muscle Biology Group, Univ. of Arizona, Tuscon, AZ, for providing pure enzyme.

7. Hydrolysis of Fluorogenic Substrates: Progress curves were initially determined at 25 °C but the temperature was lowered to 8 °C after assay optimization to minimize autolysis. In a total volume of 2 mL, 1 μg of calpain I was incubated with 0.01-2.0 mM substrate, 60 mM imidazole (pH 7.3), 0.1 mg/mL BSA, 5 mM cysteine, 2.5 mM 2-mercaptoethanol, 1.0 μM aprotinin. The reaction was initiated by adding the activated enzyme (calpain that has been incubated in the presence of calcium for 2 min) and continued at 8 °C for 5-7 min. The increase in fluorescence (excitation at 340 nm, emmission at 425 nm for MNA) was continuously monitored using a Perkin Elmer LS50 and the rate of hydrolysis was calculated from the linear portion of the graphs. Control runs were done in the presence of 5 mM EDTA. For studying the inhibitors, the compounds were dissolved in 100% DMSO and diluted 100 to 1 into the assay mixture containing 0.2 mM 1 (1% DMSO, final concentration). Calculations: The K_m and k_{cat} values were calculated by fitting the substrate concentration versus the rate to the Michaelis Menton equation: $V=V_{max}*K_m/(K_m + [S])$. The mode of inhibition was determined by fitting the data to equations for non-competitive, uncompetitive, competitive and mixed-noncompetitive variables and compared using routines for 2 independent variables and determined the best fit by using analysis of variance and F-test paradigms. For studing the inhibitory effects of the irreversible inhibitor E-64 and (acyl)aryloxymethyl ketones, apparent second order rate constants were estimated by $k_{obs}/[I]$. The kinetic constant, k_{obs} (observed pseudo-first order rate constant for inactivation in the presence of substrate), was derived by fitting the progress curves (ten inhibitor concentrations) to the integrated first order equation $Y=v_s t+((v_o-v_s)(1-e^{(-kobs(t))})/k_{obs})+A_o$.

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(b) For a discussion of these endogenous proteinase inhibitors see: *Proteinase Inhibitors*; Barrett, A. J.; Salvensen, G., Eds., Elsevier Science Publishers B. V.: Amsterdam, 1985.

10. Casein Assay: the tritiated assay is a modification of that described by Golpalakrishna, R.; Barsky, S. H. *J. Biol. Chem.* **1986**, *261*, 13936. The reaction mixture contains 0.1 mg casein (0.004 mg, *ca.* 150,000 dpm, ^{3}H-acetyl casein) in 50 mM HEPES (pH 7.5) containing 2 mM DTT. A basic assay was set up in the the following manner: HEPES buffer (250 mM; 50 μL, final conc = 50 mM), $CaCl_2$ (5 mM; 50 μL, final conc = 1 mM), enzyme (50 μL, 1.7 μg/tube), ^{3}H-acetyl-casein (1 mg/mL; 100 μL, 0.1 mg/tube (0.004 mg hot/tube)). All reagents, with the exception of the substrate (casein) were combined in the order shown above in 1 mL polystyrene titer plates. The plates were preincubated at 20 °C for 5 min with gentle shaking prior to the addition of substrate. The incubation was continued for an additional 2 h and was terminated by the addition of 0.5 mL ice cold 5% TCA. Unlabeled casein was added (2 mg/0.25 mL), the samples were centrifuged at 3,6000 x g for 5 min and 0.5 mL of the supernatant was counted in 5 mL of Ready Protein liquid scintillation cocktail for 2 min.

11. Barrett, A. J.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K.; Kembhavi, A. *Biochem. J.* **1982**, *201*, 189. (b) Parks, C.; Kembhavi, A. A. Barrett, A. J. *Biochem. J.* **1985**, *230*, 509. (c) A comparable value for the inactivation of calpain II by E-64 is reported in ref. 14a.

12. For general synthetic routes to these agents see: (a) Mehdi, S.; Angelastro, M. R.; Wiseman, J. S.; Bey, P. *Biochem. Biophy. Res. Commun.* **1988**, *157*, 1117. (b) ref. 5.

13. Crawford, C.; Mason, R. W.; Wikstrom, P.; Shaw, E. *Biochem. J.* **1988**, *253*, 751.

14. The influence of leaving group structure on potency has been previously established for the (acyloxy)methyl ketones and cathepsin B (ref. 5). This has been attributed to the different leaving group abilities (pKa) of the leaving groups. Some of the effects on potency and selectivity observed here appear to be unrelated to the pka of the leaving group. For additional examples of leaving group-potency effects see: (a) Pliura, D. H.; Bonaventura, B. J.; Smith, R. A.; Coles, P. J.; Krantz, A. *Biochem. J.* **1992**, *288*, 759. (b) Dolle, R. E.; Singh, J.; Whipple, D.; Osifo, I. K.; Speier, G.; Graybill, T. L.; Gregory, J. S.; Harris, A. L.; Helaszek, C. T.; Miller, R. E.; Ator, M. A. *J. Med. Chem.* **1994**, *37*, 3865.

15. Inhibitors **63** and **69** have $t_{1/2}$ = 40 and 85 minutes, respectively, in human plasma at 37 °C.

16. The data for Z-Val-Leu-Arg-MNA, characterized by a decrease in rate at higher substrate concentrations (data not shown) was found to better fit an equation for substrate inhibition than the Michaelis-Menton equation where K_m = 0.1 mM and K_s = 0.2 μM.

17. (a) Cathepsin B assay: Knight, C. G. *Biochem. J.* **1980**, *189*, 447. Cathepsin L assay: Mason, R. W.; Green, G. D. J.; Barrett, A. J. *Biochem. J.* **1985**, *226*, 233.

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