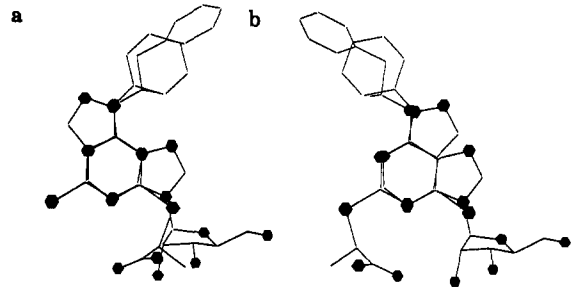

Figure 5. (a-b) The superimpositions of α-((4-thioxo-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio)propionamide (6) against the two orientations of (*R*)-(phenylisopropyl)adenosine (2).

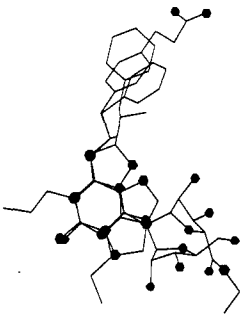

Figure 6. Superimpositions of (*R*)-(phenylisopropyl)adenosine (2), 5′-*N*-ethyl-2-[[4-(2-carboxyethyl)phenethyl]amino]-adenosine-5′-uronamide (3), and 1,3-dipropyl-8(*R*)-(phenylisopropyl)xanthine (5), illustrating the common hydrophobic binding domain occupied by the N^6, C2, and C8 hydrophobic groups and the remaining important binding domains of the central aromatic ring and the ribose domain which varies between the A1 and A2 receptors.

phenylisopropyl recognition unit of each molecule occupy the same space,[11] consistent with our proposal for one hydrophobic binding site. This fit is different to that proposed for theophylline and adenosine[20] where the 6:5 rings of the heterocycle were superimposed, and the fit provided good steric and electrostatic correlations. Since no aryl group was present, however, lipophilic factors were not taken into account.

α-((4-Thioxo-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)-thio)propionamide (6) was aligned with the two possible conformations of (*R*)-(phenylisopropyl)adenosine (2) to maximize lipophilic factors. These fits are shown in parts a and b of Figure 5. Figure 5a gives the highest electrostatic (Table I), steric, and lipophilic correlations between the molecules. A similar orientation against *N*-ethyladenosine-5′-uronamide (7) places the amide functionalities of *N*-ethyladenosine-5′-uronamide and α-((4-thioxo-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-yl)thio)propionamide in close proximity. This provides further evidence for the hydrophobic group of (*R*)-(phenylisopropyl)adenosine being over the five-membered ring. We postulate that all active compounds in the classes represented will align in a similar manner to the sample compounds.

In conclusion, we present a model that rationalizes the existing N^6, C2, and C8 binding domains by demonstrating that they are the one region of the receptor (Figure 6). The model takes into account steric, hydrophobic, and electrostatic properties that may contribute to receptor binding potency. The model may be of value in development of novel structures with selective agonistic and antagonistic activity. Recent independent confirmation that the N^6 and C8 binding domains are common[11] provides support for our model. Our model is more expansive including the role of the ribose binding domain and accommodates the C2-substituted analogues.

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Registry No. 1, 58-61-7; 2, 38594-96-6; 3, 120225-54-9; 4, 85872-51-1; 5, 130324-52-6; 6, 134896-38-1; 7, 35920-39-9; 8, 102146-07-6.

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Inactivation of Calpain by Peptidyl Fluoromethyl Ketones

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The syntheses of Z-Leu-Leu-Tyr-CH_2F (1) and Z-Tyr-Ala-CH_2F (3) are described. The ability of Z-Leu-Leu-Tyr-CH_2F (1) and Z-Leu-Tyr-CH_2F (2) to inactivate in vitro calcium-activated proteinase from chicken gizzard are compared. Like the analogous diazomethyl ketones 4 and 5, these inhibitors were also found to inactivate cathepsin L in common with other inhibitors under current investigation. However, other specific inactivators for cathepsin L are available, for example, the fluoromethyl ketone 3 and diazomethyl ketone 6 of Z-Tyr-Ala-OH, which have no effect on the calcium-activated proteinase and therefore provide control inhibitors for observations made with Z-Leu-Leu-Tyr-CH_2F (1).

Introduction

Calpain, the calcium-activated neutral proteinase(s) of the cytoplasm, is a cysteinyl proteinase of considerable interest in a number of physiologically important roles such as signal transduction across membranes[1,2] possibly reflected in cytoskeletal alterations. A major area for this selective proteolysis role may be in the central nervous

*Abbreviations used: Z, benzyloxycarbonyl; AMC, 4-methyl-7-coumarinylamine; SDS, sodium dodecyl sulfate; Suc, succinyl.

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system.[3] In the hope of achieving an effective inactivator for calpain that would permit us to examine some of the postulated intracellular functions of the enzyme, we have been seeking irreversible inhibitors of the proteinase that act by affinity labeling.[4,5] The advantages of irreversible inhibition include maximal effectiveness and an additional important property, the ability to label altered cellular constituents so that the system may be analyzed after exposure to the inhibitor to see whether or not the postulated site of action was, in fact, covalently labeled, a prerequisite for understanding its mechanism of action. In addition, the investigator may see what other components of the system become labeled, requiring some caution in interpretation of the results.

A number of reports of small molecular inhibitors of calpain have appeared. Early among these were the action of leupeptin and antipain[6,7] and later other peptidyl aldehydes and carbonyl derivatives[8-11] all of which inhibit serine proteinases as well as cysteinyl proteinases. Furthermore, even if they achieve tight binding possibly due to hemimercaptal (or hemiketal) formation with an active center SH (OH) group, they are reversible inhibitors, displaceable by protein substrates. Of great difficulty also in this work is the failure to consider specificity among the cysteinyl proteinases, let alone proteinase mechanistic class.

A naturally occurring epoxide, E-64,[12] irreversibly inactivates cysteinyl proteinases but not serine proteinases[13-16] and thus is more specific than the foregoing reagents. Some members of this class, i.e., the ethyl ester E-64d, penetrate platelets and apparently inactivate intracellular calpain.[17]

We have been examining peptidyl diazomethyl ketones and monofluoromethyl ketones for the inactivation of calpain. These are irreversible inhibitor classes which are largely effective against cysteinyl proteinases.[5,6] Di- and trifluoromethyl ketones derived from peptides are, instead, effective inhibitors of serine but not cysteinyl proteinases.[18-20] Peptidyl diazomethyl ketones permit a ready evaluation of inhibitor structure when hydrophobic amino acid side chains are dealt with. The monofluoromethyl ketones are more tedious to arrive at, requiring extensive purification. On the other hand, they have been found to be more effective in some cases, for example, about 50-fold in the case of cathepsin B.[21] In the initial phase of our work, we departed from the observation of Sasaki et al.[22] that calpain cleaves at peptide bonds of amino acids preceded by a leucine residue and explored the effect of structure–activity relationships in a group of peptidyl diazomethyl ketones. Z-Leu-Leu-Tyr-CHN_2, in which the tyrosine had been included as an eventual radioiodination site, emerged as an effective inhibitor.[4] However, concurrent examination of its specificity with respect to other cellular cysteinyl proteinases, namely cathepsin B and L, showed that cathepsin L is also inactivated by this inhibitor. In this work and subsequent extensions, it appeared that this dual action would be difficult to resolve. Cathepsin L functions intracellularly within lysosomes where it has a role in protein turnover. Its inactivation could cause some unforeseen metabolic difficulty. However, peptidyl diazomethyl ketones with a phenylalanine or tyrosine residue in the P_2 position, while effective in inactivating cathepsin L, have no action of calpain. Labeled derivatives of ths type are proving useful in cellular studies due to ready penetrability and selectivity and offer an additional tool for the analysis of the site of action of a potential calpain inactivator.[23,24]

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Therefore, Z-Leu-Leu-Tyr-CH_2F (1) has been synthesized, examined as a calpain inactivator in vitro, and compared with some structurally related inhibitors. Studies with platelets,[25] demonstrate the superiority of Z-Leu-Leu-Tyr-CH_2F (1) in vivo to covalently modify calpain and to block certain platelet functions when a calcium ionophore is added to activate the intracellular proteinase.

Experimental Section

Z-Leu-Tyr-CH_2F (2)[26] and Z-Leu-Leu-Tyr-CHN_2 (4)[4] were prepared as described. Z-Leu-Nle-H was purchased from Novabiochem AG, CH-4448 Läufelfingen, Switzerland. Peptide intermediates were purchased from Bachem Feinchemikalien AG, CH-4416 Bubendorf, Switzerland.

Z-Leu-Leu-Tyr($COCH_2F$)-CH_2F [[(Benzyloxycarbonyl)-leucylleucyl-D,L-*O*-(fluoroacetyl)tyrosyl]fluoromethane] (7). (Benzyloxycarbonyl)leucylleucyltyrosine (4.03 g, 7.7 mmol), fluoroacetic anhydride (4.25 g, 30.8 mmol), and triethylamine (2.15 mL, 15.4 mmol) were combined and cooled to 0 °C. 4-(Dimethylamino)pyridine (47 mg, 0.385 mmol) and methylene chloride (3 mL) were added. After 5 min the cooling bath was removed, and the mixture was stirred for 1.5 h. The orange viscous solution was diluted with methylene chloride (4 mL) and stirred for a further 1.5 h. The reaction mixture was diluted with ethyl acetate (200 mL), washed with 1 N HCl (50 mL), saturated $NaHCO_3$ (2 × 10 mL), and brine (50 mL), dried over $MgSO_4$, filtered, and evaporated. The resulting yellowish solid foam was chromatographed over silica gel with methylene chloride/ethyl acetate (9:1, v/v). The obtained yellowish solid foam was dissolved in ethyl acetate and precipitated with hexane to yield a colorless solid (289 mg, 6.3% yield): mp 197–199 °C; ^1H NMR (DMSO) δ 0.72, 0.77, 0.84, 0.86 (4 d, 12 H, J = 7 Hz, 4 CH_3), 1.10–1.30 (m, 3 H, $CHCH_2CH(CH_3)_2$), 1.30–1.48 (m, 2 H, $CHCH_2CH(CH_3)_2$), 1.48–1.60 (m, 1 H, $CHCH_2CH(CH_3)_2$), 2.70–3.16 (m, AB part of the ABX system, 2 H, $OC_6H_4CH_2CH$), 3.97–4.08, 4.08–4.18 (2 H, 2 m, $2CHCH_2CH(CH_3)_2$), 4.52–4.63 (m, X part of the ABX system, 1 H, $OC_6H_4CH_2CH$), 5.00 (s, 2 H, $C_6H_5CH_2O$), 5.04, 5.16, 5.20, 5.31 (4d (d of the AB system), J_{HF} = 47 Hz, J_{HH} = 12 Hz, 2 H, CH–CO–CH_2F), 5.30 (d, 2 H, J = 47 Hz, C_6H_5O–CO–CH_2F), 7.08, 7.29 (2 d, 4 H, J = 9 Hz, C_6H_4), 7.35 (s, 5 H, C_6H_5), 7.43, 7.98, 8.55 (3 d, 3 H, J = 7 Hz, 3 NH); MS (field desorption) m/z (rel intensity) 617 (M^+) ($C_{32}H_{41}F_2N_3O_7$) formula M_r 617.690. Anal. ($C_{32}H_{41}F_2N_3O_7$) C, H, F, N.

Z-Tyr($COCH_2F$)-Ala-CH_2F [[*N*-(Benzyloxycarbonyl)-*O*-(fluoroacetyl)tyrosyl-D,L-alanyl]fluoromethane] (8). The procedure as above was used. Chromatography on silica gel with chloroform/hexane (9:1, v/v) followed by recrystallization from the same solvent gave fine needles (7% yield): mp 141–149 °C; ^1H NMR (DMSO) δ 1.13, 1.21 (3 H, 2 d, J = 7 Hz, $CHCH_3$, racemate), 2.68–3.08 (2 H, m, AB part of the ABX system, $C_6H_5CH_2CH$), 4.18–4.42 (2 H, m, $C_6H_5CH_2CH$ (X part of the ABX system), $CHCH_3$), 4.94 (2 H, d, J = 5 Hz, $C_6H_5CH_2O$), 5.00–5.38 (2 H, m, d of the AB system, CH_2F), 5.30 (2 H, d, J = 45 Hz, C_6H_4O–CO–CH_2F), 7.08–7.18, 7.18–7.44 (4 H, 2 m, C_6H_4), 7.30 (5 H, s, C_6H_5), 7.62, 7.64 (1 H, 2 d, J = 7 Hz, NH, racemate), 8.50, 8.56 (1 H, 2 d, J = 7 Hz, NH, racemate); MS (field desorption) $(M)^+$ 462 ($C_{23}H_{24}F_2N_2O_6$ formula M_r 462.449). Anal. ($C_{23}H_{24}F_2N_2O_6$) C, H, F, N.

Z-Leu-Leu-Tyr-CH_2F [[(Benzyloxycarbonyl)leucyl-leucyl-D,L-tyrosyl]fluoromethane] (1). [(Benzyloxycarbonyl)leucylleucyl-D,L-*O*-(fluoroacetyl)tyrosyl]fluoromethane (7) (210 mg, 0.35 mmol) was hydrolyzed in acidic 80% methanol [1 N HCl (1.75 mL), water (1.75 mL), and methanol (14 mL)] for 48 h at room temperature. The reaction mixture was concentrated and extracted with ethyl acetate (50 mL). The organic phase was washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL), dried over $MgSO_4$, filtered, and evaporated. The colorless viscous oil was chromatographed over silica gel with methylene chloride/3% methanol. The obtained oily solid was taken up in ethyl acetate and precipitated with hexane to yield a colorless soild (88 mg, 46%): mp softening 85–90 °C; ^1H NMR (DMSO) δ 0.66–0.94 (m, 12 H, 4 CH_3), 1.12–1.58 (m, 6 H, 2 $CHCH_2CH(CH_3)_2$), 2.80–3.00 (m, AB part of the ABX system, 2 H, $OC_6H_4CH_2CH$), 3.98–4.10, 4.10–4.30 (2 m, 2 H, 2 $CHCH_2CH(CH_3)_2$), 4.30–4.52 (m, X part of the ABX system, 1 H, $OC_6H_4CH_2CH$), 4.76–5.34 (m, d of the AB system, J_{HF} = 47 Hz, 2 H, CH_2F), 5.02 (s, 2 H, $C_6H_5CH_2O$), 6.60–6.70, 6.92–7.04 (2 m, 4 H, C_6H_4), 7.32 (s, 5 H, C_6H_5), 7.43, 7.93 (2 d, J = 7 Hz, 2 H, 2 NH), 8.35, 8.42 (2 d, J = 7 Hz, 1 H, NH, racemate), 9.24 (s, 1 H, OH, exchangeable with D_2O); MS (field desorption) m/z (rel intensity) 557 (M^+) ($C_{30}H_{40}FN_3O_6$) formula M_r 557.663. Anal. ($C_{30}H_{40}FN_3O_6$) C, H, F, N.

Z-Tyr-Ala-CH_2F [[*N*-(Benzyloxycarbonyl)tyrosyl-D,L-alanyl]fluoromethane] (3). The same procedure as above was used. The resulting viscous oil was chromatographed on silica gel with chloroform/1% methanol to yield colorless crystals (18% yield) on precipitation from chloroform and hexane: mp 145–150 °C; ^1H NMR (DMSO) δ 1.14, 1.23 (3 H, 2 d, J = 7 Hz, CH_3, racemate), 2.58–2.76, 2.76–2.94 (2 H, 2 m, AB part of the ABX system, $HOC_6H_4CH_2CH$), 4.10–4.24 (1 H, m, $CHCH_3$), 4.24–4.40 (1 H, m, X part of the ABX system, $HOC_6H_4CH_2CH$), 4.98 (2 H, s, $C_6H_5CH_2O$), 4.98–5.37 (2 H, m, d of the AB system, CH_2F), 6.62–6.71, 7.02–7.14 (4 H, 2 m, C_6H_4), 7.22–7.40 (5 H, s, C_6H_5), 7.53, 7.56 (1 H, 2 d, J = 7 Hz, NH, racemate), 8.44, 8.49 (1 H, 2 d, J = 7 Hz, NH, racemate), 9.21 (1 H, s, OH, exchangeable with D_2O); MS (field desorption) $(M)^+$ 402 ($C_{21}H_{23}FN_2O_5$ formula M_r 402.422). Anal. ($C_{21}H_{23}FN_2O_5$) C, H, F, N.

Enzymic Studies. Calpain II was prepared from chicken gizzard by a modification of the method of Crawford et al.[27] Essentially, a 30–45% $(NH_4)_2SO_4$ pellet was obtained from the calpain II pool after the DEAE-Sepharose step. The pellet was dissolved in a minimum volume of 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA, 0.5 M NaCl, 0.01% thioglycerol, and 0.01% NaN_3 (buffer A) and chromatographed on an Ultrogel AcA 34 column. Active fractions were pooled and applied to a column of Cibacron 3GA-Agarose. After unbound protein was washed with buffer A, calpain was eluted with buffer A without NaCl. A final purification was carried out on a Mono Q column as described by Crawford et al.[27]

Calpain II was assayed fluorometrically in 50 mM Tris/acetate buffer, pH 7.5, containing 5 mM cysteine, 5 mM $CaCl_2$, and 0.02% (v/v) mercaptoethanol at 37 °C, using Suc-Leu-Tyr-AMC (2.5×10^{-4} M) as substrate. Mixtures of enzyme and inhibitor were prepared, and timed aliquots were removed for assay with the fluorogenic substrate.

Cathepsin L from rat liver,[28] stabilized as a mercury salt, was kindly provided by Dr. H. Kirschke. The enzyme was preactivated with DTT in 0.05 M Na acetate buffer, pH 5.4, 1 mM in EDTA and assayed in the same buffer with 0.01% Brij [poly(oxyethylene) glycol dodecyl ether] and containing mercaptoethanol (10 μL per 50 mL). Z-Phe-Arg-AMC at 2×10^{-5} M was used as substrate.[29]

Z-Leu-Nle-H was examined as a reversible inhibitor and initial velocities were measured. For the irreversible inhibitors, mixtures of enzyme and inhibitor were prepared and timed aliquots were removed for assay with the fluorogenic substrate.

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Table I. In Vitro Rates of Inactivation by Affinity Labeling Reagents[a]

inhibitor		concn (μM)	$t_{1/2}$ (s)	k_2 (M^{-1} s^{-1})
Calcium-Activated Protease (Calpain)				
Z-Leu-Tyr-CHN_2	(5)	1–6		1470
Z-Leu-Tyr-CH_2F	(2)	0.08	510	17000
Z-Leu-Leu-Tyr-CHN_2	(4)	0.1–0.2		230000
Z-Leu-Leu-Tyr-CH_2F	(1)	0.4	60	28900
Z-Tyr-Ala-CHN_2	(6)	20–80		<10
Z-Tyr-Ala-CH_2F	(3)	0.1		0[b]
Cathepsin L				
Z-Leu-Tyr-CHN_2	(5)	1–3		14960
Z-Leu-Tyr-CH_2F	(2)	0.5	120	12000
Z-Leu-Leu-Tyr-CHN_2	(4)	0.005–0.05		1500000
Z-Leu-Leu-Tyr-CH_2F	(1)	0.01	102	680000
Z-Tyr-Ala-CHN_2	(6)	0.1–0.5		176600
Z-Tyr-Ala-CH_2F	(3)	0.001	94	7400000

[a] Data for peptidyl diazomethyl ketones from ref 4. [b] Not distinguishable from spontaneous inactivation.

Results and Discussion

The tripeptide Z-Leu-Leu-Tyr-OH was obtained by hydrolysis of Z-Leu-Leu-Tyr-OEt which was synthesized from Z-Leu-Leu-OH by coupling with H-Tyr-OEt by the standard mixed-anhydride procedure. The fluoromethyl group in this tripeptide and in the dipeptide Z-Tyr-Ala-OH was introduced by the method of Dakin–West[30] with 4 equiv of fluoroacetic anhydride[5] and 2 equiv of triethylamine. The formed peptidyl fluoromethane is a mixture of diastereomers in which the C-terminal amino acid is fully racemized. Racemization of the α-amino ketone is inherent in the Dakin–West reaction[5] and was detectable by NMR: the α-amino proton of the peptide bond, which is normally a doublet, shows two doublets; the protons of the methyl group of alanine, which normally show a doublet, show two doublets (see NMR data in the Experimental Section). During the Dakin–West reaction tyrosine is fluoroacetylated. Free tyrosine derivative was obtained by acidic hydrolysis.

In the inactivation of calpain by the peptidyl fluoromethyl ketones, the time-dependent loss of activity was expected to be the result of covalent bond formation following the initial formation of a reversible complex as with typical affinity labeling reagents:[6]

$$I + E \rightleftharpoons E\cdots I \rightarrow E\text{-}I$$

Conceivably, the time dependence could have been due to a slow binding step. However, the formation of a covalent bond is indicated by the stability of the enzyme product formed with radioiodinated inhibitor to denaturation in SDS as demonstrated with the derivative from Z-Leu-Leu-Tyr-CH_2F (1).[25] We have shown this to be observable also with Z-Leu-Tyr-CH_2F (2). Apart from the demonstration of the formation of E–I, in cellular studies the ability to identify the target protein permits a more secure interpretation of the mechanism of action of an inhibitor.

The rates of inactivation given in Table I are apparent second-order rate constants observed under conditions such that the constants are independent of inhibitor concentration (saturation effects are avoided) and thus permit comparison of different inhibitors (Figure 1). Similar apparent second-order rate constants have been reported for the interaction of calpain with peptidyl oxides[16] and diazomethyl ketones,[4] but K_i values are generally lacking. The instability of calpain is a deterrent, in part. The chicken gizzard preparation is more stable than the protease from other sources; although an apparent steady amidase activity is observable with small substrates for 15 min or more when calcium is added,[31] the active protease is undergoing autodigestion and ascribing the K_i value to a particular molecular species of protease would not be possible.

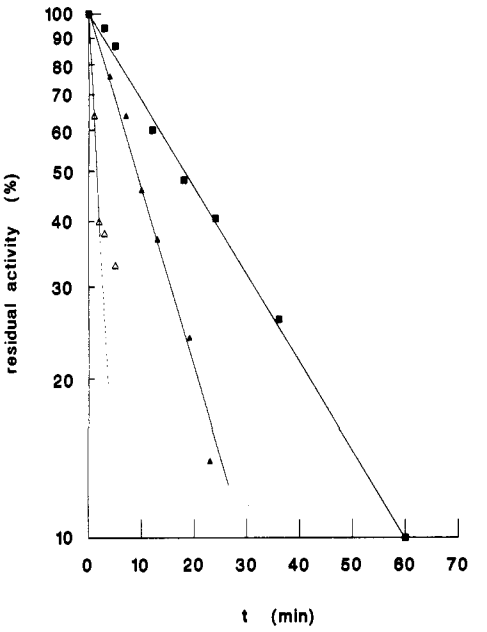

Figure 1. Inactivation of calpain by Z-Leu-Tyr-CH_2F and Z-Leu-Leu-Tyr-CH_2F. △ Z-Leu-Leu-Tyr-CH_2F (1) = 2×10^{-7} M; ▲ Z-Leu-Tyr-CH_2F (2) = 0.8×10^{-7} M; ■ Z-Leu-Tyr-CH_2F (2) 0.4×10^{-7} M; calpain = 0.75 μg/mL.

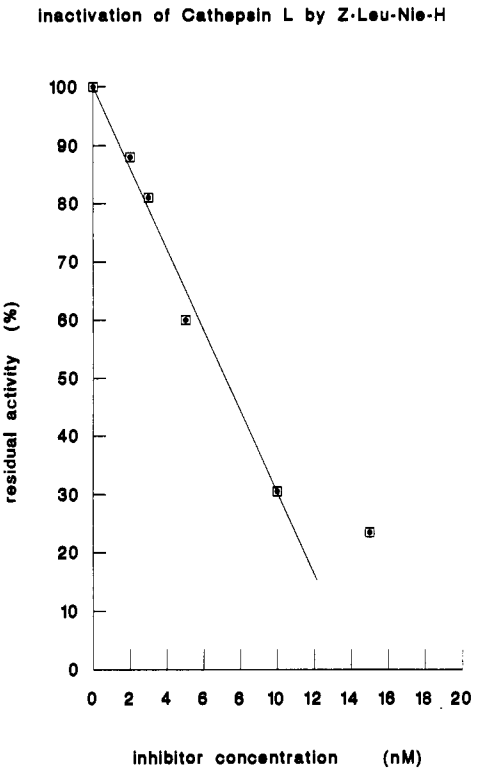

Figure 2. Inhibition of cathepsin L by Z-Leu-NLe-H. Initial velocities of cathepsin L on 2×10^{-6} M Z-Phe-Arg-AMC were measured in various concentrations of Z-Leu-NLe-H.

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The peptidyl fluoromethyl ketones were synthesized in view of earlier observations that they were sometimes significantly more effective than the corresponding diazomethyl ketones. The in vitro results summarized in Table I show that this was true in some comparisons in the present series. For example, the second-order rate constant for inactivation of calpain by Z-Leu-Tyr-CH_2F (2) is at least 1 order of magnitude greater than that determined for Z-Leu-Tyr-CHN_2 (5). On the other hand, the tripeptide derivatives 1 and 4 have the converse relationship. However, these pairs of inhibitors were comparable in their inhibition of cathepsin L. The difference between the corresponding diazomethyl ketones and fluoromethyl ketone is much less. However, the change in length from dipeptide to tripeptide has a pronounced effect, increasing the rate of inactivation 50–100-fold.

Although Z-Leu-Leu-Tyr-CHN_2 (4) is more effective in inactivating calpain in vitro than the fluoromethyl ketone 1, in intact platelets the latter was more effective[25] under the conditions studied. A preincubation with 10 μM of fluoromethyl ketone 1 was able to protect components of the cytoskeleton from calcium-triggered degradation, whereas diazomethyl ketone 4 at this concentration was not effective. The fluoromethyl ketone 1 may penetrate platelets more readily.

Z-Leu-NLe-H has been shown to inhibit the calcium-activated proteinase in vitro and in intact platelets.[9] We expected this inhibitor also to be a powerful cathepsin L inhibitor in view of the results described here and in ref 5. In fact, during the present studies we have shown that this material will produce a graded inhibition in the presence of 2×10^{-5} M Z-Phe-Arg-AMC. From a plot (Figure 2) it was established that at pH 5.4, the concentration required for 50% inhibition is 7.2 nM. The choice of leucine in the penultimate position has been based on the observations of Sasaki.[22] A calpain inhibitor with greater selectivity would be desirable. Meanwhile, the use of two irreversible inhibitors permits the exploration of the cellular role of the calcium-activated proteinase.

Registry No. 1 (diasteromer 1), 137490-22-3; 1 (diasteromer 2), 133410-84-1; 2, 123392-26-7; 3 (diastereomer 1), 137362-97-1; 3 (diastereomer 2), 123392-25-6; 4, 116614-45-0; 7 (diastereomer 1), 137362-98-2; 7 (diastereomer 2), 137490-23-4; 9 (diastereomer 1), 137362-99-3; 8 (diastereomer 2), 137393-18-1; Cbz-Leu-Leu-Tyr-OH, 137363-00-9; Cbz-Tyr-Ala-OH, 23018-09-9; $(FCH_2CO)_2O$, 407-33-0; calpain, 78990-62-2; cathepsin L, 60616-82-2.

Structure–Function Studies in a Series of Carboxyl-Terminal Octapeptide Analogues of Anaphylatoxin C5a

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The synthesis and structure–activity relationships of C-terminal octapeptide analogues of anaphylatoxin C5a have been studied. The introduction of hydrophobic amino acids into the N-acetylated native octapeptide (N-Ac-His-Lys-Asp-Met-Gln-Leu-Gly-Arg-OH) (1) has led to an analogue with 100 times more activity than the native octapeptide in inhibiting the binding of ^{125}I-labeled anaphylatoxin C5a to human neutrophil membrane receptors. The observed apparent binding K_i's for the compounds (8–10) are in the range of 1–3 μM, and they possess nearly full agonist activity, despite the fact that these analogues are one-eighth or -ninth the size of the natural ligand anaphylatoxin C5a.

Introduction

Numerous investigations have been performed studying the inflammatory role of anaphylatoxin C5a (hereafter abbreviated C5a), a glycopeptide generated by proteolytic cleavage of the fifth complement component C5 during complement activation.[1,2] C5a, a relatively large molecule containing 74 amino acids, is a potent inflammatory mediator and potentially plays an important role in the pathogenesis of a number of inflammatory diseases.[3] In addition, recent studies have suggested that C5a may also be important in mediating inflammatory effects of phagocytic mononuclear cells that accumulate at sites of chronic inflammation[4] and may have a further proinflammatory role by enhancing the local production of antibodies at inflammatory sites.[5] Thus, a C5a antagonist would have significant inhibitory effects on these events, and therefore the discovery of a C5a antagonist is of particular

(1) Abbreviations follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature for the amino acids and peptides: *Eur. J. Biochem.* **1984**, *158*, 9. Additional abbreviations used herein are Cha, cyclohexylalanine and Ac, acetyl.

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