findings of the present experiments. However, it is evident from the electronprobe X-ray analytical studies that early focal events are taking place in the dorsal fascia which represents a calcification process and that these reactions are not detectable by standard histochemical techniques.

Therefore the earliest stages in the calcification reaction of local calcergy in the mouse are represented by the close association of Pb, Ca and P ions. As the reaction progresses, Pb is not inevitably associated with Ca and P and after 1 week, it is undetectable. Therefore Pb acts as a trigger for the calcification reaction and when this reaction becomes detectable by histochemical techniques, Pb is apparently unimportant in the progression of the reaction.

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Inhibitors of the acrosomal proteinase acrosin: Human urinary trypsin inhibitor (UTI) and 4-(2-carboxyethyl) phenyl trans 4-aminomethylcyclohexanecarboxylate hydrochloride (DV-1006)

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Summary. Human urinary trypsin inhibitor (UTI) and (4-(2-carboxyethyl) phenyl trans 4-aminoethylcyclohexanecarboxylate hydrochloride (DV-1006) competitively inhibited the human acrosomal proteinase acrosin; Ki values were 1.2×10^{-8} M and 9.4×10^{-7} M, respectively.

The acrosomal proteinase acrosin (EC 3.4.21.10) is essential for sperm passage through the zona pellucida of the ovum, and is thus the key enzyme in the fertilization process[1,2]. Recently, we purified urinary trypsin inhibitor (UTI) as an apparently homogeneous protein from normal human urine, estimated its mol. wt to be 67,000 (UTI-I) by gel filtration[3-5], and succeeded in transforming it into lower molecular weight forms (mol.wt 45,000, 22,000, and $<10{,}000$) with several proteinases such as urinary enzyme[3,4], papain[6,7], and pronase[6,7]. The highly purified form of this inhibitor reacted with trypsin and to a lesser extent, with chymotrypsin and the fibrinolytic enzyme plasmin[4,8]. In this paper, we demonstrate the strong inhibitory effect of UTI (UTI-I) and of a synthetic trypsin-plasmin inhibitor 4-(2-carboxyethyl) phenyl trans 4-aminomethylcyclohexanecarboxylate hydrochloride (DV-1006)[9] on human acrosin.

Materials and methods. The following substances were used: soybean trypsin inhibitor (SBTI) and leupeptin from Sigma Chemical Co., USA; DV-1006 from Daiichi Kagaku, Tokyo; UTI, purified by affinity chromatography as reported previously[5], 1210 U/mg protein, mol. wt 43,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (mol.wt 67,000 by gel filtration); human acrosin, purified by the method of Zaneveld et al.[1] except that Sephadex G-75 (Pharmacia, fine) was used for the gel filtration in place of Sephadex G-50 gel, 1100 mU/mg protein. Acrosin activity was measured using N^{α}-Benzoyl-L-arginine methylester (Bz-Arg-OEt; Sigma Chemical Co., USA) as substrate. Details are given in the table. 1 milliunit (mU) was defined as the amount of enzyme which caused a change in absorbance of 0.001/min at 253 nm[1].

I_{50} and Ki values for acrosin hydrolysis of Bz-Arg-OEt

Inhibitor	Inhibitor concentration for 50% inhibition (M)	Ki(M)
UTI	5.9×10^{-8}	1.2×10^{-8}
DV-1006	3.3×10^{-6}	9.4×10^{-7}
SBTI	1.1×10^{-6}	-
Leupeptin	5.0×10^{-7}	2.1×10^{-7}

Constant amounts of acrosin (13.5 mU) were incubated with increasing amounts of inhibitor in 2.0 ml of 0.1 M borate buffer containing 0.05 M $CaCl_2$, pH 8.0, for 15 min at room temperature (22-23 °C). The enzymatic reaction was initiated by the addition of Bz-Arg-OEt in deionized water; end volume: 3.0 ml. In the experiments where I_{50} values were determined, the substrate concentrations were 0.2 mg/ml.

Results and discussion. Comparison of the effects of UTI and DV-1006 with known acrosin inhibitors, SBTI[1] and leupeptin[10] were made. The concentrations of the inhibitor required for 50% inhibition (I_{50}) are shown in the table. UTI and DV-1006 were found to be very powerful inhibitors of acrosin. By the method of Dixon[11], the inhibitors were shown to be competitive and the dissociation constant (Ki) values evaluated were approximately 1.2×10^{-8} M for UTI (assuming mol. wt 43,000) and 9.4×10^{-7} M for DV-1006. The Ki value of UTI is lower than that of leupeptin ($Ki=2.1\times10^{-7}$ M), which is the most powerful inhibitor of acrosin known at present (with boar acrosin; $Ki=8.6\times10^{-8}$ M (Fritz et al.[10]).

In 1971, DV-1006 was first reported by Muramatu[9] as an excellent synthetic inhibitor of trypsin, plasmin, kallikrein, and thrombin. It is now used in Japan for the clinical treatment of gastric peptic ulcers. UTI is also a trypsin-plasmin inhibitor and is non-antigenic for humans. In the presence of serine proteinase inhibitors such as tosyllysine chloromethyl keton and diisopropylfluorophosphate, fertilization is prevented both in vitro and in vivo[1,2]. Therefore, the use of these inhibitors as antifertility agents may be possible.

The relation of the UTI molecule to other naturally occurring acrosin inhibitors is interesting; the antigenicity of UTI is identical to serum inter-α-trypsin inhibitor, which also inhibits acrosin[1] and its concentration decreases during the ovulatory cycle[12]. Schumacher and Zaneveld[12], and Wallner et al.[13] have purified other new acrosin inhibitors from human cervical mucus. Hochstrasser et al.[14] showed that the anti-inter-α-trypsin inhibitor cross-reacts with the human mucus trypsin inhibitor.

Studies on the molecular structure and physiological significance of these inhibitors are now in progress.

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Synthesis and contractile activity of the C-terminal heptapeptide of substance P with N^5-dimethyl glutamine in the 6-position. Active site studies

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Summary. The synthesis and testing of [N^5-dimethyl-Gln^6]-SP_{5-11} showed 37 ± 12% contractile activity relative to SP, and intrinsic efficacy 98 ± 4%. This finding indicates that the carboxamide groups of the dual Gln^5-Gln^6 moiety are not equally related with the contractile response of the C-terminal heptapeptide of SP.

It is well known that the C-terminal tripeptide and tetrapeptide of substance P (SP) are inactive, while the pentapeptide has a weak spasmogenic effect on the guinea-pig ileum[2-5]. However, the hexapeptide and heptapeptide do not differ in their activity (on the guinea-pig ileum) from synthetic SP (SSP)[2-5]. In other words, the N-terminal increase of the C-terminal pentapeptide by glutaminyl residue(s) leads to the enchancement of biological activity. A feature common to the biologically active hexapeptide and heptapeptide is the presence of the hydrophilic side chain of the glutaminyl carboxamide group, at positions 5 and 6. Provided that the carboxamide groups at these positions are exposed and possess maximal structural freedom, they should be more available to the receptor. Recent evidence[6] indicates that a C-terminal heptapeptide analogue of SP, namely [N^5-dimethyl-Gln^5]-SP_{5-11} was found to exhibit only 25% activity relative to SP and about 30% antagonist action, when tested for contractile activity on the guinea-pig ileum; these effects were achieved by the mere change of the carboxamide hydrogens by methyl groups of the glutaminyl residue at position 5 of the hormone.

In the light of this finding and its significance for active-site studies on this hormone, it was decided to investigate whether the same modification of the glutaminyl residue at position 6 would lead to identical or different biological response. Thus, the synthesis of [N^5-dimethyl-Gln^6]-SP_{5-11} (figure) was performed by stepwise solution techniques from H-Phe-Phe-Gly-OBzl[7]. Boc-Glu [$N(CH_3)_2$]-OH[6,8] was preactivated with DCC and 1-hydroxy-benzotriazole[9] and coupled to the tetrapeptide ester to give Boc-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-OBzl, m.p. 128–130 °C; $[\alpha]_D^{20}$ −18.61° (c 0.5, DMF). Thereafter, elongation of the peptide chain, using the same coupling process, afforded Boc-Gln-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-OBzl, m.p. 212–214 °C; $[\alpha]_D^{20}$ −23.53° (c 0.5, DMF). Catalytic hydrogenation of the latter produced the acid, Boc-Gln-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-OH, m.p. 193–195 °C; $[\alpha]_D^{20}$ −23.2° (c 0.5, DMF), which was coupled with H-Leu-Met-NH_2[10] in the same manner as above. The resulting heptapeptide derivative, Boc-Gln-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-Leu-Met-NH_2, m.p. 216–219 °C; $[\alpha]_D^{20}$ −40.89° (c 0.5, DMF), was deprotected with CF_3COOH in the presence of anisole and was permitted to remain in ethanol solution containing thioglycolic acid for 24 h at room temperature. Following the desalting of the product (50 mg) by ion-exchange chromatography (Dowex-1X8) with ethanol as the eluent, purification was attained by gel filtration on Sephadex G-15 (80 × 2.5 cm) with 2 M acetic acid (elution volume 242 ml). At a flow rate of 21 ml/h fractions of 7 ml were collected (32–39) containing the major peak of peptide material and lyophilized; yield 38 mg. A portion (20 mg) was finally purified by partition chromatography on a column (76 × 1.5 cm) of Sephadex G-25 equilibrated with n-BuOH-AcOH-H_2O (4:1:5). The peptide was eluted with the upper phase at a flow rate of 6 ml/h. Fractions of 2 ml were collected (30–38) and lyophilized to give homogenous product (15 mg): m.p. 232–237 °C; $[\alpha]_D^{20}$ −58.5° (c 0.18, DMF); TLC showed a single spot with R_f 0.39 in n-BuOH-AcOH-H_2O (4:1:1), R_f 0.72 in n-BuOH-AcOH-H_2O-pyridine (30:6:24:20) and R_f 0.34 in n-BuOH-AcOH-H_2O (4:1:5, upper phase). Amino acid analysis gave the following molar ratios: Glu, 2.05; Phe, 2.01; Gly, 1.00; Leu, 1.03; Met, 0.89; NH_3, 1.99. Elemental analysis gave the following values: $C_{43}H_{64}N_{10}O_9S$ calculated. C 57.57; H, 7.19; N, 15.61; found C, 57.62; H, 7.24; N, 15.39.

Biological test[1]. Terminal ileum (2 cm) of guinea-pigs (250–300 g) was incubated in a 5-ml bath for 20 min before testing. The bath solution was composed of 9 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.1 g $MgCl_2$, 1 g glucose, 0.12 g tris-(hydroxy-methyl)-aminomethane per 1000 ml distilled water while the temperature was kept at 37 °C and the pH at 7.4. Substances were dissolved in distilled water, diluted

a H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
1 2 3 4 5 6 7 8 9 10 11

b H-Gln-Glu[$N(CH_3)_2$]-Phe-Phe-Gly-Leu-Met-NH_2
5 6 7 8 9 10 11

a Amino acid sequence of substance P (SP). *b* [N^5-dimethyl-Gln^6] C-terminal heptapeptide of SP; numbers indicate sequence positions of individual residues. Amino acids, with the exception of glycine, are of the L-configuration.