Kallikrein inhibitors[1,2]

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ABSTRACT

So far the $C\bar{1}$ inactivator, α_2-macroglobulin, antithrombin III (in the presence of heparin), and α_1-antitrypsin have been identified as inhibitors of plasma kallikrein; α_1-antitrypsin reacts slowly also with tissue kallikreins. Of the various naturally occurring kallikrein inhibitors the basic trypsin–kallikrein inhibitor of bovine organs, aprotinin (the active substance of Trasylol®), has attained by far the most interest. This inhibitor, which is produced by mast cells, has unusual properties due to its compact tertiary structure. Additional topics of aprotinin and structurally related inhibitors discussed are the mechanism of enzyme–inhibitor complex formation, the production of chemical mutants of aprotinin, the structural basis of kallikrein inhibition, and selected aspects regarding aprotinin medication.—**Fritz, H., E. Fink and E. Truscheit.** Kallikrein inhibitors. *Federation Proc.* 38: 2753–2759, 1979.

SPECIFIC AND NONSPECIFIC KININOGENASES

A common feature of trypsin and trypsin-like enzymes with broader substrate specificity, e.g., plasmin and sperm acrosin, is the cleavage of arginyl– and lysyl–peptide bonds; that is, as *nonspecific* kininogenases they normally also liberate bradykinin from the kininogens, the natural substrates of the *specific* kininogenases or kallikreins. The kallikreins are characterized, on the other hand, by their limited substrate specificity: Tissue kallikreins (M_r ~ 25,000–35,000) from submandibular glands, pancreas, urine (kidney), colon, etc. react preferably with low molecular weight kininogen yielding kallidin (13), whereas plasma kallikrein has *two* preferential biological substrates, high molecular kininogen and the Hageman factor (36).

PLASMATIC INHIBITORS OF KALLIKREIN

Both plasma kallikrein and high molecular weight kininogen are involved in the solid phase activation of the intrinsic blood clotting cascade (36). The significance of simultaneous kinin liberation is not yet fully understood. The relationship of plasma kallikrein to the clotting factors is outlined also by its inhibitory specificity (see Table 1): Plasma kallikrein exhibits highest affinity to the inactivator of the C1 esterases, but inhibition by α_2-macroglobulin also occurs under in vivo conditions (1, 22, 36, 41). Antithrombin III reacts relatively slowly with plasma kallikrein; complex formation is observed only when the concentration of the C1 inactivator is low and heparin is administered simultaneously in therapeutical dosage (5, 31). Inhibition of plasma kallikrein by α_1-antitrypsin proceeds too slowly to be observed under in vivo conditions (14).

So far, of all plasmatic inhibitors only α_1-antitrypsin has been unequivocally identified as an inhibitor of tissue kallikreins; it is a progressive, slowly reacting inhibitor (14). In addition, the presence of a fast reacting inhibitor of tissue kallikreins in human plasma has also been reported (13). Tissue kallikreins, to our present knowledge, act primarily as kinin-generating enzymes.

Inhibition by the plasmatic inhibitors is under in vivo conditions *irreversible*. In the case of α_2-macroglobulin the proteinases are entrapped after cleavage of a suitable peptide bond of the inhibitor (41); in this cage the enzyme is still accessible to lower molecular weight substrates and inhibitors but normally not to the natural higher molecular weight substrates (41). The plasma kallikrein–α_2-macroglobulin complex is one of the few exceptions; it still reacts with kininogens (36). In the form of the α_2-macroglobulin complex the proteinase is rapidly eliminated from the circulation by cells of the reticuloendothelial system (2, 37). Irreversible inhibition of proteinases by the other plasmatic inhibitors occurs by formation of a covalent bond between the serine residue of the active site of the proteinase and an amino acid residue near the *C*-terminal region of the inhibitor after cleavage of a proteinase-sensitive bond of the inhibitor (6, 21, 38).

THE KALLIKREIN INHIBITOR FROM BOVINE ORGANS

Inhibitors of plasma and/or tissue kallikreins are produced by various plants like potatoes and peanuts (15), by animal tissues like kidneys (20), and by bacteria such as the microbial peptides antipain and leupeptin (15). The inhibitor, however, that has attained by far the most interest of biochemists, pharmacologists, and

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[2] Dedicated to Prof. Dr. E. Auhagen on the occasion of his 75th birthday.

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TABLE 1. Inhibitors of plasma kallikrein of physiological or medical relevance (preferentially inhibited enzymes in italics)

In human plasma	Inhibits plasma kallikrein and: (36)
$C\bar{1}$ inactivator	*$C\bar{1}s$, $C\bar{1}r$*, plasmin
α_2-Macroglobulin	*Neutral and acidic proteases from leukocytes and lysosomes,*[a] *pancreatic proteases,*[b] bacterial and mold proteases; plasmin
Antithrombin III + heparin	*Thrombin, factor Xa*; IXa, XIa, XIIa, $XIIa_{frag}$, VIIa; $C\bar{1}s$; plasmin
α_1-Antitrypsin[c]	*Neutral proteases from leukocytes and lysosomes, pancreatic proteases,*[b] tissue kallikreins, plasmin
Aprotinin from bovine lung	*Tissue kallikreins, plasmin, trypsin*, chymotrypsin

[a] Elastase, cathepsin G, collagenase, cathespin B and D, e.g. [b] Chymotrypsin, trypsin. [c] Slowly reacting with plasma and tissue kallikreins or plasmin, therefore probably without physiological relevance regarding the in vivo inhibition of these proteases.

physicians is the kallikrein inhibitor from bovine organs, aprotinin or Trasylol®, see Table 1. It was discovered 50 years ago by Frey, Kraut and Werle as "kallikrein inactivator" in bovine lymph nodes and, 6 years later, independently by Kunitz and Northrop as trypsin inhibitor in bovine pancreas (13, 48, 49).

Properties and structure

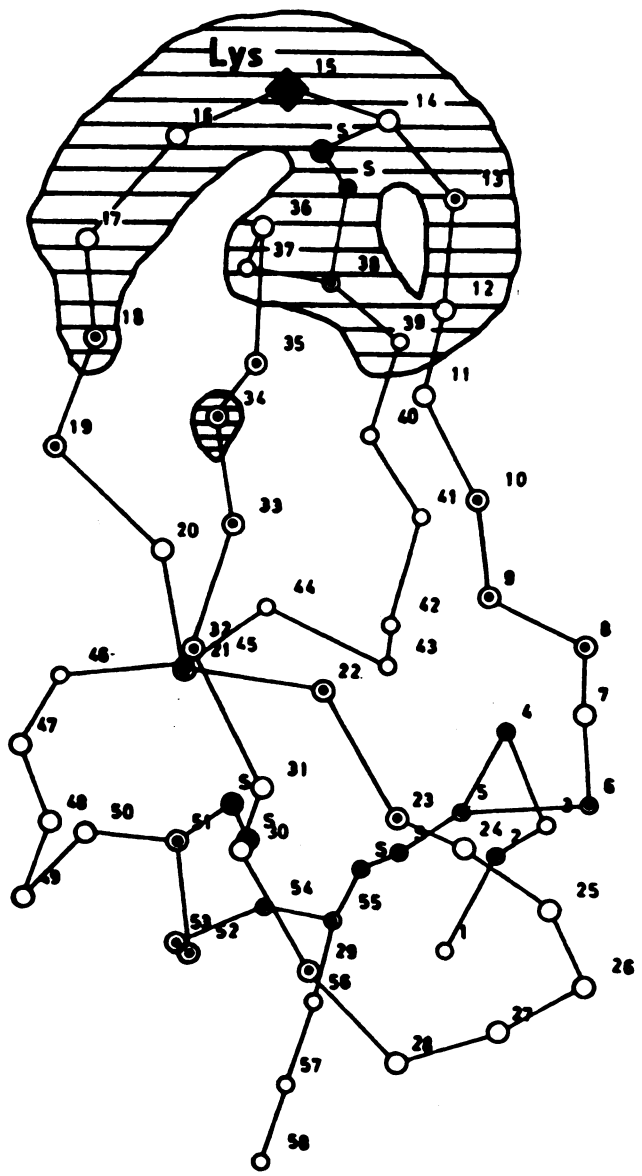
The following properties of aprotinin are especially striking. *1*) It forms equimolar and reversible complexes with trypsins, chymotrypsins, plasmins, and plasma as well as glandular kallikreins of various species (13, 48, 49). *2*) Its high stability is due to the low molecular weight of 6,500 daltons and an unusually rigid tertiary structure (7). *3*) Its high basicity (the isoelectric point is close to 10.5) is responsible for the marked affinity of this protein to negatively charged groups, causing adsorption of the inhibitor to mucoprotein layers (23) and fixation to the brush border membrane of the kidney after therapeutical administration (29). *4*) It exhibits low toxicity and antigenicity (13, 48, 49).

The structure of the aprotinin molecule is shown in Fig. 1 (7, 49): 58 amino acid residues are aligned to a single polypeptide chain, which is cross-linked by three disulfide bridges. The polypeptide chain is folded in such a way that a pear-shaped molecule results with the hydrophobic residues concentrated in the interior of the pear and the hydrophilic residues exposed to the surrounding medium. This arrangement leads to a very compact tertiary structure, which is the main reason for the unusual stability of aprotinin against denaturation by high temperature, acid, alkali, organic solvents, or proteolytic degradation (30). The high dipole character of the aprotinin molecule is due to concentration of negatively charged residues near the bottom of the pear.

Mechanism of complex formation

The region of the inhibitor molecule that is in close contact with the enzyme in the trypsin–inhibitor complex is indicated by the hatched area at the

Figure 1. Tertiary structure of the aprotinin molecule as revealed by X-ray crystallographic studies (26). The polypeptide chain, consisting of 58 amino acid residues cross-linked by three disulfide bridges, is folded in such a way that a pear-shaped molecule results. The reactive peptide bond Lys^{15}-Ala^{16} is localized at the top of the pear. The region of the inhibitor molecule that is in close contact with the enzyme in the trypsin–inhibitor complex is indicated by the hatched area.



top of the pear (26): 12 residues of aprotinin, including its reactive site peptide bond Lys^{15}-Ala^{16}, are involved. All these contacts (11–13 hydrogen bonds, a salt bridge, more than 200 Van der Waals interactions) contribute to the free energy of 19 kcal/mol, the driving force of the complex formation, resulting in an extremely low dissociation constant, K_i of 6×10^{-14} mol/liter (32).

As schematically outlined in Fig. 2, formation of such a tight enzyme–inhibitor complex is normally associated with *i*) a perfect fit of the reactive-site residue of the inhibitor into the specificity pocket of the enzyme; *ii*) the formation of a tetrahedral intermediate by an attack of the nucleophilic hydroxyl group of the serine residue of the active site of the enzyme on the carbonyl group of the lysine residue of the reactive peptide bond of the inhibitor, and *iii*) stabilization of the adduct by additional weaker interactions in the vicinity of the specificity pocket and reactive peptide bond, respectively (26, 27, 32). Taking into account the surplus of energy gained by the numerous contacts between enzyme and inhibitor, it is not surprising that not both, formation of the tetrahedral intermediate and the perfect fit of the reactive site residue of the inhibitor (Lys in Fig. 1 and 2) into the specificity pocket of the enzyme are absolutely necessary for complex formation. In fact, the enzymatically inactive anhydro-trypsin forms also a tight complex with aprotinin (see Table 2) (25, 32).

The energy gained by the contact of the complementary regions is even high enough to enable the aprotinin to push the conformation of trypsinogen into the conformation of the active enzyme without the activation peptide being cleaved off (Table 2) (4, 46). On the other hand, the relatively low affinity of aprotinin to chymotrypsin in comparison to trypsin reflects primarily the disturbed fit of the Lys^{15}-residue into the specificity pocket of this enzyme (3).

In the case of the plasmin–aprotinin complex the contact regions of the partners should be distinctly smaller and/or their fit not nearly as perfect as in the trypsin–aprotinin complex (see Table 2). The same should hold true for the complexes formed between various kallikreins and aprotinin, as may be deduced from the K_i values compiled in Table 3 (10, 17). In this respect it is interesting that, based on the primary structure of porcine pancreatic kallikrein as elucidated recently (45), 57% of the amino acid residues of trypsin mediating the contact in the complex with aprotinin are preserved in kallikrein; especially the residues participating in more than just one interaction with the inhibitor remain unchanged. Of the numerous interactions in the trypsin–aprotinin complex about 70% also seem to be possible in the complex with porcine pancreatic kallikrein, especially the contacts around the reactive site peptide bond Lys^{15}-Ala^{16} of the inhibitor (45).

Figure 2. Schematic representation of the formation of tight but reversible proteinase–inhibitor complexes. Details are described in the text.

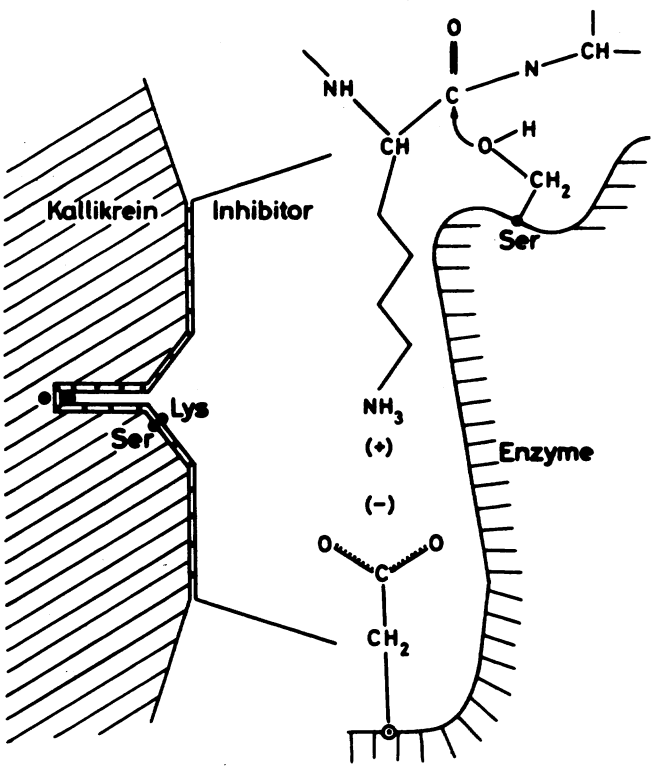


TABLE 2. Dissociation constants (K_i) of complexes between aprotinin and various partners

Enzyme/ partner	Species	K_i (mol/liter)	pH
Trypsin	Bovine	6.0×10^{-14}	8
Anhydro-trypsin	Bovine	$<3.0 \times 10^{-13}$	8
Tryp-sinogen	Bovine	1.8×10^{-6}	8
Chymo-trypsin	Bovine	9.0×10^{-9}	8
		6.0×10^{-9}	7
Plasmin	Porcine	4.0×10^{-9}	8
	Human	2.3×10^{-10}	8

Structurally homologous inhibitors—structural basis of kallikrein inhibition

Another possible approach for obtaining an insight into the structural basis of kallikrein inhibition by aprotinin is a comparison of the inhibition specificities and reactive site sequences of structurally homologous inhibitors (Table 4) (10, 24). From the results of the crystallographic work it is known that the amino acid residues of aprotinin with the most intimate contact to trypsin in the complex are, besides Cys^{15} and Cys^{38},

TABLE 3. Dissociation constants of complexes between aprotinin and various kallikreins

Kallikrein	Species	K_i (mol/liter)	pH
Pancreatic	Porcine	1×10^{-9}	8.0
Subman-dibular	Porcine	1.6×10^{-9}	9.0
Urinary	Porcine	1.7×10^{-9}	9.0
	Human	1×10^{-10}	8.0
Plasma	Human	3×10^{-8}	8.0
	Porcine	1×10^{-7}	7.8

TABLE 4. Identified and mutual reactive site residues of structurally related trypsin–chymotrypsin inhibitors of the aprotinin type

Inhibitor (from)	Positions of the residues									Inhibition of		
	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_{23}'	P_{24}'	Plasmin	Tissue kallikreins	Plasma kallikreins
(Position[a])	14	15	16	17	18	19	20	38	39			
Aprotinin	Cys	Lys-	Ala	Arg	Ile	Ile	Arg	Cys	Arg	++	++	+
SAI, sea anemone	Cys	Arg-	Ala	Arg	Phe	Pro	Arg	Cys	Arg	++	++	+
HPI_K, snail	Cys	Lys-	Ala	Ser	Phe	Arg	Gln	Cys	Arg	++	+	+
RVV II, snake venom	Cys	Arg-	Gly	His	Leu	Arg	Arg	Cys	Gly	+	+	+
NNV II, snake venom	Cys	Lys-	Ala	Arg	Ile	Arg	Ser	Cys	Gly	ϕ	++	+
HHV II, snake venom	Cys	Lys-	Ala	Tyr	Ile	Arg	Ser	Cys	Gly	++	++	+
CTI, cow colostrum	Cys	Lys-	Ala	Ala	Leu	Leu	Arg	Cys	Gln	++	–	–
Kunitz, soybean[b]	Tyr	Arg-	Ile	Arg	Phe	Ile	Ala			++	–	++

[a] In aprotinin. [b] Structurally not homologous. ++ strong, + weaker, – no inhibition; ϕ not tested.

the "specificity pocket" residue Lys^{15}, Ala^{16} and the basic residues Arg^{17} and Arg^{39} (26). Regarding the inhibition specificities of the structurally homologous inhibitors, it is evident that the basic nature of the residues in positions 17 and 39 is in favor of the relatively strong interaction of aprotinin with the kallikreins. Exchange of both amino acid residues in these positions by neutral residues (see the cow colostrum inhibitor) completely abolishes the affinity of aprotinin-type inhibitors to both tissue and plasma kallikreins.

If, on the other hand, a basic residue is present in position 19 (see snake venom inhibitor HHV II), kallikrein inhibitory activity is exhibited again. Obviously, in an aprotinin-type inhibitor the basic nature of amino acid residues in one or more of the positions 17, 19, and 39 is necessary to gain enough energy for complex formation with a kallikrein.

In contrast, the affinity of an aprotinin-type inhibitor to plasmin and trypsin is not significantly affected by such amino acid exchanges in the reactive site region. It may be concluded, therefore, that inhibition of plasma and particularly of glandular kallikreins presupposes additional structural requirements in specific subsite positions of a trypsin or plasmin inhibitor, thus reflecting the more restricted substrate specificity of the kallikreins compared to trypsin or plasmin. On the other hand, the conclusion is then justified that the active site regions of all kallikreins that form a complex with aprotinin are very similar, especially if the K_i values of the complexes are so closely related, as in the case of porcine tissue kallikreins (Table 5) (10).

The reactive site bond—chemical mutants of aprotinin

There is general agreement that aprotinin is a single-headed inhibitor with the Lys^{15}-residue in its active center. Location of the reactive site peptide bond within the sequence of an inhibitor is normally proved by limited proteolysis in slightly acidic solution, resulting in the formation of an equilibrium between virgin and modified (with the reactive site bond hydrolyzed) inhibitor and resynthesis of the virgin inhibitor from the modified one under suitable conditions (12), see Fig. 3 (upper part). Due to the high stability of the virgin inhibitor molecule these reactions proved to be especially difficult to achieve in the case of aprotinin (43, 44).

The modified inhibitor was used to produce chemical mutants of aprotinin (28, 43). As expected, replacement of Lys^{15} by Arg via several steps (cf. left and lower part of Fig. 3) did not influence the inhibitory properties of aprotinin, whereas replacement of the basic active site residue by Phe or Trp caused a significant increase in the affinity of the inhibitory protein for chymotrypsin and decrease in the affinity for trypsin. The total synthesis of aprotinin achieved recently opens a way to study the influence of amino acid exchanges on the inhibitory properties of this molecule in detail (42).

TABLE 5. Dissociation constants K_i of complexes between aprotinin-type inhibitors (cf. Table 4) and porcine tissue kallikreins

Structurally homologous inhibitor from	Kallikrein from porcine		
	Pancreas	Submand. gland	Urine
Bovine lung TKI	0.8	0.9	1.0
Sea anemone SAI 5-II	0.8	0.6	0.8
Snake venom NNV-II	1.1	1.3	1.3
Snake venom HHV-II	8.3	210	109
Snail HPI_K	27.7	210	204
Cow colostrum CTI	–	–	–

Values in nanomoles per liter. –, no inhibition.

Selected aspects of aprotinin medication

In view of the application of aprotinin (e.g., as Trasylol, the registered trade name in favor of Bayer AG, Germany) in medical therapy and experimental animal studies, some of its properties are of special interest.

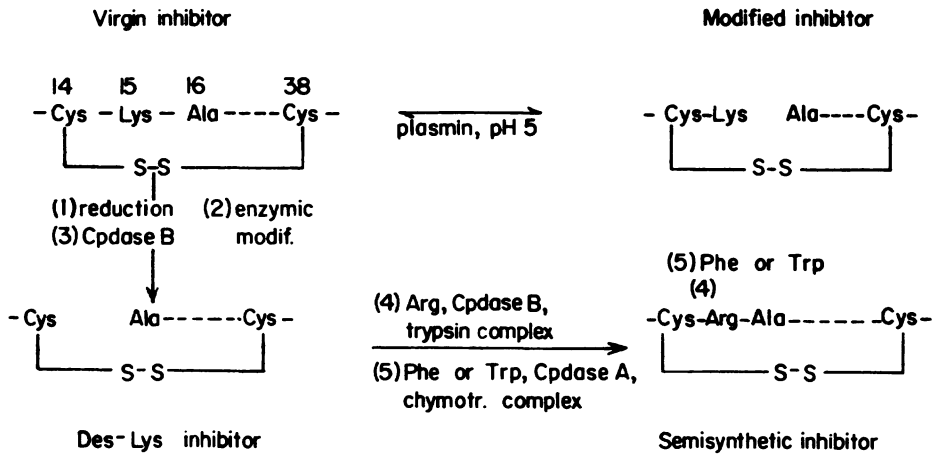


Figure 3. Modification of aprotinin by limited proteolysis. By the action of catalytic amounts of plasmin at pH 5 a thermodynamic equilibrium is accomplished between virgin inhibitor (reactive site peptide bond intact) and modified inhibitor (reactive site bond hydrolyzed), cf. upper part. Semisynthetic reactive site modified inhibitors are obtained by a series of chemical and enzymic reactions as indicated in the left and lower part of the figure: *1*) selective reduction of the Cys^{14}-Cys^{38} disulfide bridge, *2*) selective proteolytic cleavage of the Lys^{15}-Ala^{16} peptide bond, *3*) removal of Lys^{15} by treatment with carboxypeptidase B, *4*) insertion of Arg and resynthesis of the reactive site peptide bond via the trypsin complex, *5*) insertion of Phe or Trp and resynthesis of the reactive site peptide bond via chymotrypsin complex.

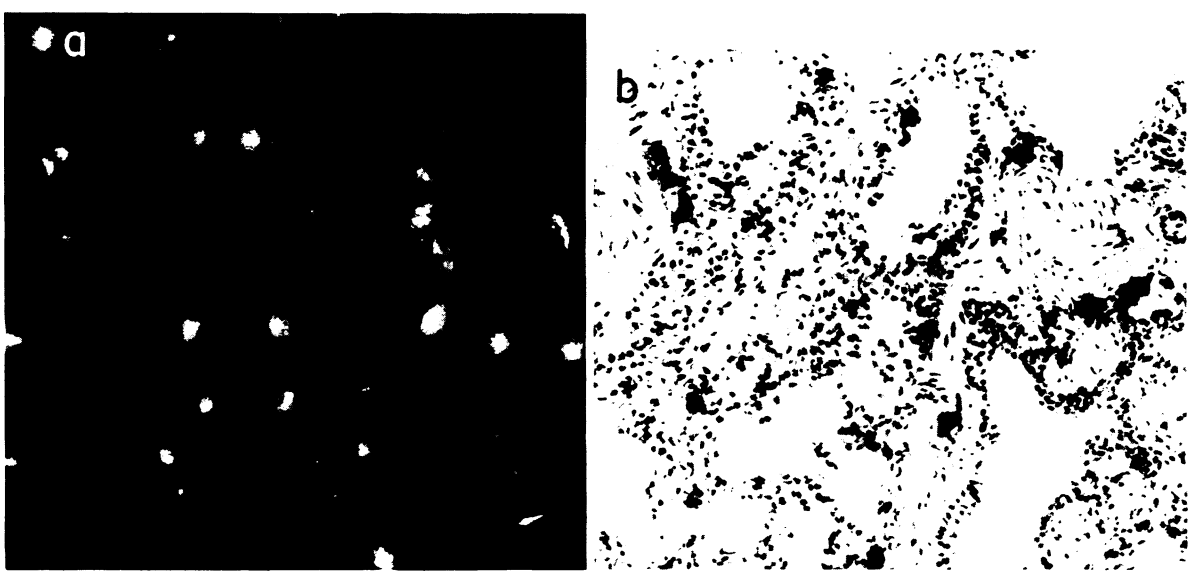
Aprotinin strongly inhibits both human cationic and anionic trypsin but rather weakly inhibits human chymotrypsin I and human pancreatic protease E or elastase; human chymotrypsin II is not inhibited (11, 34, 35). Tissue kallikreins and plasma kallikrein of man, pig, and cattle are relatively strongly inhibited by aprotinin, whereas the corresponding kallikreins of guinea pig and dog are not inhibited (47, 48). We emphasize especially the rather strong inhibition of human plasmin and human tissue (urinary, submandibular, pancreatic) kallikrein—K_i values close to 0.1 nmol/liter were reported for the corresponding complexes, cf. Tables 2 and 3—compared to the clearly weaker inhibition of human plasma kallikrein—the K_i value of this complex is higher than 10 nmol/liter, cf. Table 3. Due to this significant difference in the affinity of aprotinin to plasmin and plasma kallikrein it seems possible to regulate the desired effect, namely inhibition of plasmin and thus fibrinolysis or of plasma kallikrein and hence blood clotting, by the amount of inhibitor administered (16, 39). Regarding the release of appreciable amounts of neutral leukocytic proteinases during the inflammatory response, inhibition of granulocytic elastase by aprotinin is also of interest (33).

The trypsin-like acrosomal proteinase acrosin, which assists the spermatozoa to penetrate the zona pellucida of the ovum, is also, though rather weakly, inhibited by aprotinin (18). In vivo application of aprotinin for contraceptive purposes is hindered further by the high concentration (about 10 mmol/liter) necessary to achieve passage of the inhibitor across sperm head membranes (W.-B. Schill, G. Feifel and H. Fritz, ms in preparation). Implantation of blastocysts into rabbit uteri could be prevented, however, in the presence of much lower concentrations (about 1 μmol/liter) of aprotinin probably via inhibition of a trypsin- or kallikrein-like trophoblast proteinase (8, 9).

Occurrence in bovine tissue and possible biological function

Although detailed knowledge of the biochemistry, pharmacology, and mechanism of action of aprotinin has accumulated, its physiological function in the bovine organism has so far remained obscure (13, 48, 49). This was due, at least partly, to its widespread distribution in functionally totally different organs such as lung,

Figure 4. Localization of aprotinin in mast cells as revealed by immunofluorescence (19). *a*) Distribution pattern of the fluorescent spots in tissue sections of bovine lung after treatment with aprotinin-specific antibodies (control sections treated with unspecific antibodies showed no fluorescence). *b*) Distribution pattern of the mast cells as revealed by metachromatic staining with toluidine blue. Magnification: ×128.



parotid gland, lymph nodes, liver, pancreas, seminal vesicles, ovary, heart, etc. The availability of aprotinin antibodies stimulated us to look for the origin of this inhibitor at the cellular level, hoping to find in this way an indication of its biological function. Figure 4*b* shows the distribution of mast cells in tissue sections of bovine lung as revealed by selective staining with toluidine blue. The similar pattern of fluorescent spots obtained after treatment of acetone-fixed tissue sections with aprotinin-directed antibodies using the indirect immunofluorescence technique (Fig. 4*a*) indicates strongly that aprotinin is localized in mast cells (19). In order to corroborate the mast cell character of the cells showing specific fluorescence, additional combined light and electron microscopic studies were performed (19). The granulated cells thus identified were identical with the cells showing metachromatic staining with toluidine blue or specific fluorescence. An additional indication of the presence of aprotinin in mast cells is the similar distribution of mast cells and aprotinin in the tissues of the bovine organism; tissues known to contain high numbers of mast cells are also rich in aprotinin and vice versa. The ubiquitous occurrence of mast cells in all connective tissues of the organism offers a simple explanation for the occurrence of aprotinin in nearly every organ or tissue of cattle.

The presence of aprotinin in such a unique and highly specialized cell population as the mast cells (50) implies an important biological function of this molecule. Regarding our knowledge of the biochemistry of aprotinin, this inhibitory protein is most likely involved in the regulation of mast cell proteinase activities either intra- or extracellularly. The chymotrypsin- or trypsin- or kallikrein-like substrate specificity of mast cell proteinases (40) is in favor of such an assumption. Inhibition of the pancreatic proteinases and of plasmin and plasma kallikrein should be due to the relationship of all these proteinases as members of the serine proteinase family of enzymes. Considering the species specificity of such proteinases, aprotinin-like inhibitors which do not inhibit or only weakly interact with the pancreatic enzymes normally used for the search of inhibitors could be present also in other animals and men.

The discovery that aprotinin originates from tissue mast cells does, on the one hand, terminate a long debate concerning its unusual overall distribution throughout the bovine organism. On the other hand, we expect a stimulating effect regarding the search for the biological function of this fascinating molecule as well as the biochemistry and pharmacology of mast cell proteinases. FP

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