Br. J. Pharmac. (1969), **35**, 51–61.

Sensitization of smooth muscle to plasma kinins: effects of enzymes and peptides on various preparations

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1. The influence of various materials, principally hydrolases and peptides, on the sensitivity to stimulating substances has been studied in a wide range of isolated and *in vivo* smooth muscle preparations.

2. Chymotrypsin raised the susceptibility of the guinea-pig isolated ileum to peptides related to bradykinin and also sensitized isolated ileum and fundus of rat and albino gerbil to bradykinin. No sensitization to the kinin occurred in the following preparations: rat, gerbil and rabbit duodenum; rat colon and urinary bladder; dog tracheal chain; and rabbit jejunum. Chymotrypsin and peptides structurally related to the active centre of the enzyme did not affect the permeability increasing property of bradykinin in guinea-pig skin microcirculation vessels. In contrast, intravenously administered chymotrypsin markedly augmented the bronchoconstrictor action of the kinin.

3. Ficin and pronase increased the sensitivity of guinea-pig ileum to bradykinin. Pronase also sensitized the gerbil ileum and this effect was abolished by previous treatment with di-isopropylfluorophosphonate (dyflos). Pronase destroyed bradykinin after incubation.

4. Reduced glutathione potentiated the response to bradykinin of guinea-pig ileum but did not affect its sensitivity. The potentiation also occurred in a chymotrypsin-treated preparation.

5. It is assumed that specific sensitization elicited by proteinases might derive from an effect on the protein envelope of smooth muscle membrane.

Previous work (Edery, 1965a) has shown that the guinea-pig isolated ileum and rat isolated uterus became sensitized to plasma kinins after brief exposure to chymotrypsin or trypsin. Similarly, Graham & Al-Katib (1966b) reported that these enzymes augmented the response of the guinea-pig isolated vas deferens to bradykinin, but diminished or abolished the response to other contracting compounds. Recently Rocha e Silva, Reis & Ferreira (1967) have studied different classes of chymotrypsin and found that the δ-type is the most potent as far as the sensitizing action on guinea-pig ileum is concerned.

In the present investigation the influence of hydrolases and other materials on the sensitivity of a wide range of isolated and *in vivo* smooth muscle preparations has been examined. Some preliminary results have already been reported (Edery, 1966a; 1967a).

Methods

Isolated preparations were suspended in a 5 ml. organ-bath of Tyrode solution at 35° C for guinea-pig ileum and at 37° C for rabbit duodenum and jejunum, rat fundus, albino gerbil (Naftali & Wolf, 1955) ileum and fundus as well as for dog tracheal chain. Rat uterus, duodenum and colon as well as albino gerbil duodenum were bathed in de Jalon solution at 29° C and rat urinary bladder in McEwen solution at 35° C. Rat ileum was suspended in Krebs solution at 35° C. In all preparations drugs were allowed to act for 60 sec, except for the rat and gerbil fundus in which they acted for 90 sec. Other experimental details were described previously (Edery, 1965a).

Bronchiolar tone was recorded in guinea-pigs anaesthetized with urethane according to the method of Konzett & Rössler (1940). Substances were injected at 5 min intervals into the cannulated left jugular vein. In order to regain the baseline after a drug had caused bronchoconstriction, the lungs were forcibly inflated as described by Collier, Holgate, Schachter & Shorley (1960).

Effects on the permeability of microcirculation vessels were examined in guinea-pigs injected intracardially with pontamine sky blue 60 mg/kg. Substances were injected into the clipped skin of the abdomen at a volume of 0.1 ml. The animals were killed 30 min later and the diameter of blue spots appearing on the inner side of the skin was measured.

The drugs used were: acetylcholine chloride (Light), histamine dihydrochloride (Fischer), 5-hydroxytryptamine creatinine sulphate (Sigma); dosages refer to the base; dyflos (di-isopropylfluorophosphonate), dimethylsulfoxide (Fluka), bradykinin (Sandoz), angiotensin (Ciba), vasopressin (Sandoz), reduced glutathione (Boehringer), oxidized glutathione (Boehringer), methyl-glutathione (Zion Chemicals), SRS-A (Brocklehurst, 1960). The following peptides were synthetized (Schröder, 1965) and kindly supplied by Dr. E. Schröder (Germany): (Phe-Lys-Arg)^1-Bradykinin (I), (Ser-Lys-Met-Lys-Arg)^1-Bradykinin (II), (Lys-Lys-Arg)^1-Bradykinin (III), (Met-Lys-Arg)^1-[Gly^6-Bradykinin] (IV), Gly^1-Kallidin (V). Two samples of peptide Gly-Asp-Ser-Gly (VI) (van de Linde, Kienhuis, Verwej & van der Holst, 1961) were used, one supplied by Dr. H. Kienhuis (Holland) and the other synthetized by solid-phase method by Dr. B. Merrifield (New York). Both materials gave identical results. The peptide HCl.H-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-OCH_3 (VII) (Laufer & Blout, 1967) was obtained through the courtesy of Professor E. Blout (Boston). These peptides will be referred to in the text by Roman numerals. The enzymes α-chymotrypsin (Nutritional Biochem.), trypsin (Nutritional Biochem.), collagenase (Calbiochem), bacterial collagenase (Calbiochem), pronase (Calbiochem), ficin (Nutritional Biochem.) and ribonuclease (Boehringer) were used.

All substances were diluted in 0.9% NaCl solution, while phosphate buffer, 0.2M at pH 7.3 was used in experiments requiring incubation.

Results

Effects on isolated smooth muscle preparations

The effects on isolated smooth muscle preparations are summarized in Table 1. Chymotrypsin sensitized the guinea-pig ileum not only to bradykinin, but also to peptides I, II and III, while there was either very slight or no sensitization to peptide IV. Figure 1 illustrates some of these findings.

Pronase and ficin sensitized the guinea-pig ileum to bradykinin. After pronase, following the initial enhancement of bradykinin-contractions, which coincided with a diminished responsiveness to acetylcholine and angiotensin (Fig. 2), there was a gradual reduction of the height of kinin-responses until they reached a constant level. Supplemental introduction of 200–300 μg of pronase brought about a concomitant increase of sensitivity. Maximal sensitization was usually attained after the fourth or fifth dose with no further increase on subsequent additions.

TABLE 1. *Effects of enzymes and peptides on the response of smooth muscle isolated preparations to various contracting substances*

Isolated preparation (number of experiments)	Enzyme or Peptide (μg)	BK 1–30		I 60		II, III IV 30–150		V 30		Ag 10–20		Va 1·5U		5-HT 5–40		A 10–500		SRS-A 6U		E 5	
		P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S
Guinea-pig ileum (20)	Chymotrypsin (200–500)	+	+	+	+	+	+	−	−	−	−	−	−			−	−	−	−	−	−
	Pronase (300–500)	−	+							−	−					−	−				
	Ficin (500)	+	+							−	−			−	−	−	−			−	−
	Collagenase (500)	+	−							−	−					−	−				
	Bacterial collagenase (500)	+	−							−	−					−	−				
	Ribonuclease (500)	−	−							−	−					−	−				
	Reduced glutathione (200)	+	−			+	−	+	−												
	Oxidized glutathione (300)	−	−																		
	Methyl glutathione (300)	−	−							−	−					−	−				
	Peptide VI (500)	−	−							−	−										
	Peptide VII (500)	+	−														−				
Rat uterus (10)	Chymotrypsin (300)	+	+	+	+							−	−	−	−	−	−				
	Pronase (300)	−	+																		
	Bacterial collagenase (500)	+	+											−	−						
	Peptide VI (500)	−	−																		
	Peptide VII	+	−														−				
Rat fundus (5)	Chymotrypsin (400–600)	+	+							−	−			−	−	−	−				
Rat ileum (4)	Chymotrypsin (500)	+	+							−	−					−	−				
	Trypsin (500)	+	+							−	−										
Gerbil ileum (4)	Chymotrypsin (400)	+	+							−	−					−	−				
	Pronase (200)	−	+																		
	Dimethyl sulfoxide (20 mg)	−	−																		
Gerbil fundus (4)	Chymotrypsin (500)	+	+							−											

When in the presence of the test substance, the standard agonist elicited a response which was two or more times greater than the control, the effect was recorded as potentiation (P) and as sensitization (S) when it was so after washing. The stimulants (doses in ng) were: bradykinin (BK), peptides I, II, III, IV and V (for structures see **Methods**), vasopressin (Va), angiotensin (Ag), eledoisin (E), 5-hydroxytryptamine (5-HT), acetylcholine (A) and slow-reacting substance (SRS-A). For comparative purposes, some results from a previous paper (Edery, 1965a) are included.

When 500 μg of pronase was added to the bath 15 sec before 20 μg of bradykinin, there was no response of the guinea-pig ileum, although thereafter it became sensitized to the kinin as described. The former effect seemed due to the fact that pronase destroyed the peptide. This hypothesis was tested as follows: 400 μg of bradykinin was incubated at 35° C with 1 mg of pronase. Each substance was dissolved in 1 ml. of buffer solution. In a separate tube bradykinin was incubated with buffer only as control. After 5 and 10 min incubation, aliquots of 0.4 ml. were taken from the incubated mixtures, boiled for 5 min and tested on the isolated guinea-pig ileum. No experimental sample caused contraction in contrast to the control. These experiments were repeated three times with identical results.

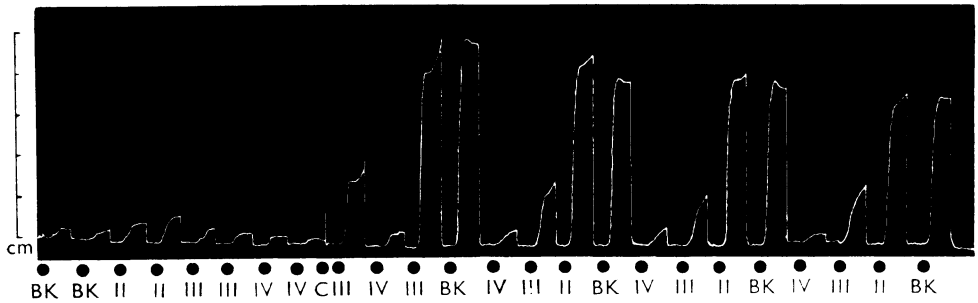

FIG. 1. Guinea-pig isolated ileum suspended in 5 ml. of Tyrode solution; 1 min contacts at 5 min intervals. The preparation was washed twice after each application of the drug. Responses to bradykinin (BK, 20 ng) and to peptides II (150 ng), III (150 ng) and IV (30 ng) (see structures in **Methods**). Chymotrypsin (C, 500 μg) was introduced for 1 min (kymograph stopped) and the preparation washed. Chymotrypsin caused a marked sensitization to all peptides except peptide IV.

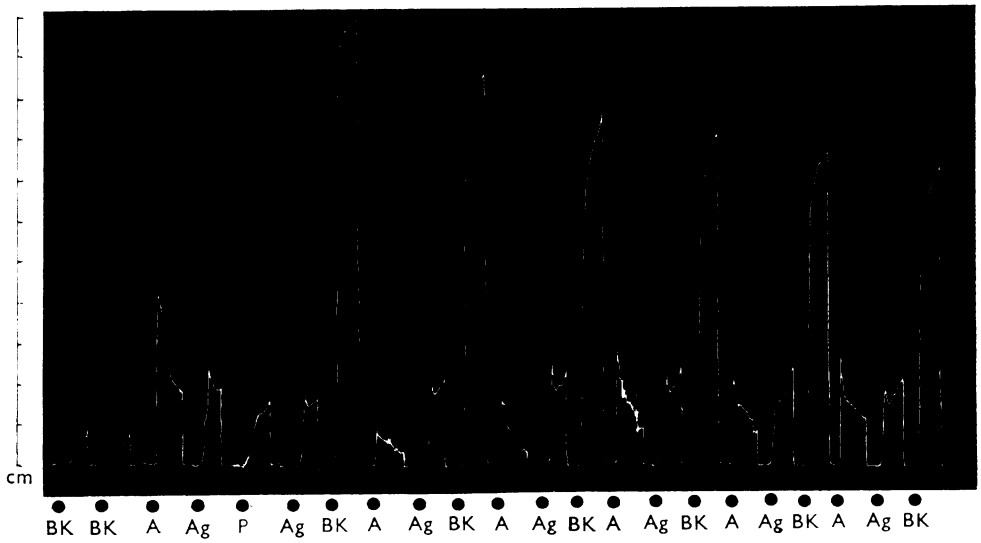

FIG. 2. Guinea-pig isolated ileum preparation with conditions similar to those in Fig. 1. Contractions were in response to bradykinin (BK, 20 ng), acetylcholine, (A, 10 ng) and angiotensin (Ag, 10 ng). After pronase (P, 300 μg), the preparation became sensitized to bradykinin, while there was a transitory reduction of the responsiveness to acetylcholine and angiotensin.

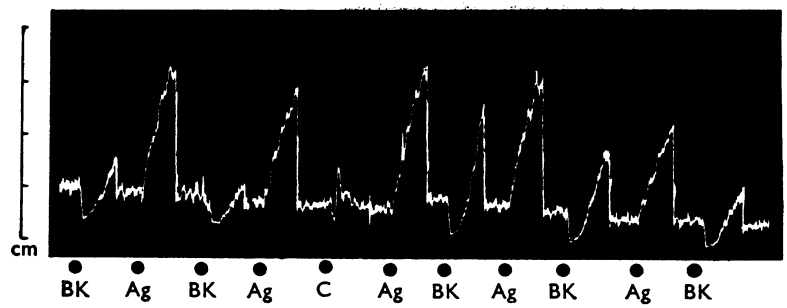

FIG. 3. Rat ileum suspended in 5 ml. of Krebs solution. Other conditions as described in Fig. 1. Responses to bradykinin (BK, 20 ng) and angiotensin (Ag, 5 ng). Note the biphasic response to bradykinin. Only the contracting phase was augmented after chymotrypsin (C, 500 μg).

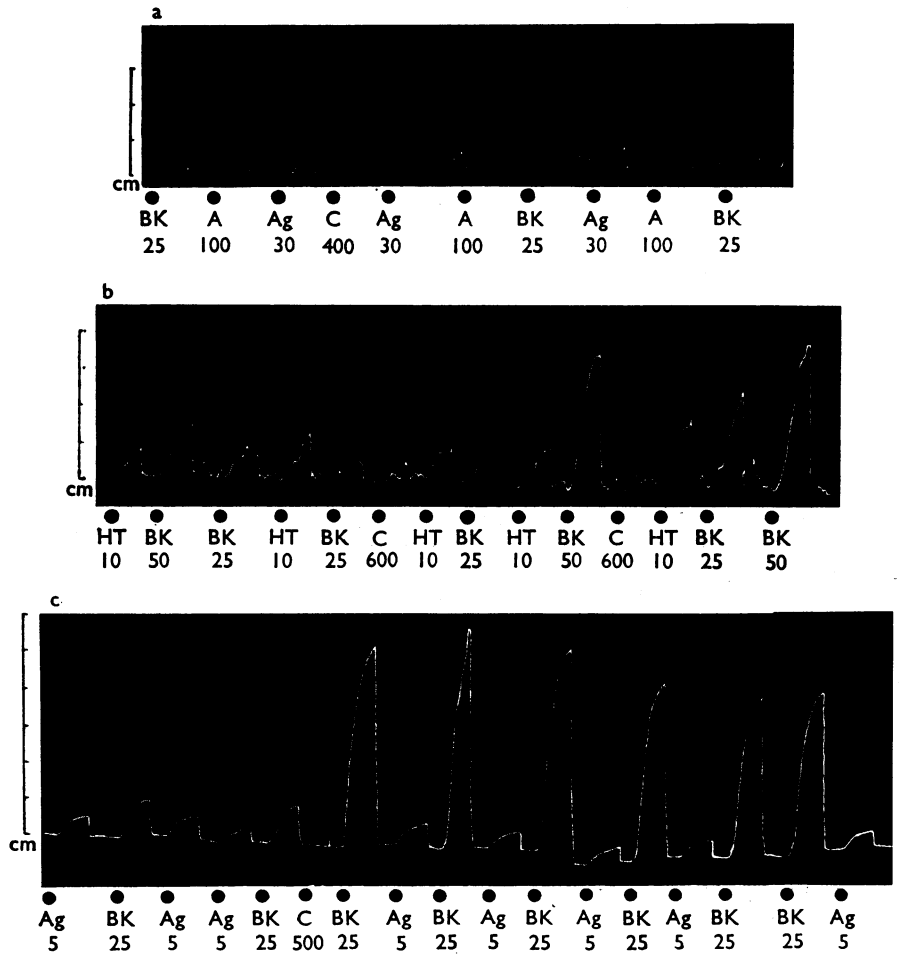

FIG. 4. Isolated fundus preparations of rat (a and b), and of gerbil (c) suspended in 5 ml. of Tyrode solution at 37° C. Contacts, 90 sec; other conditions as in Fig. 1. Responses to bradykinin (BK), acetylcholine (A), angiotensin (Ag) and 5-hydroxytryptamine (HT). Doses in ng. After chymotrypsin (C, doses in μg) the preparations became specifically sensitized to bradykinin.

In the rat ileum bradykinin caused relaxation followed by contraction. This latter phase of the response was enhanced following the presence of chymotrypsin (Fig. 3). This hydrolase also sensitized ileum and fundus of both rat and gerbil to the kinin, as is shown in Fig. 4. On the other hand, chymotrypsin did not affect the sensitivity of rat, gerbil and rabbit duodenum, rat colon and rat urinary bladder ; dog tracheal chain or rabbit jejunum.

Pronase sensitized the gerbil ileum to bradykinin. The enzyme (300 μg) did not cause such an effect after incubation with dyflos (10^{-4}M) at 35° C for 45 min.

Both collagenase and bacterial collagenase potentiated the action of bradykinin in the guinea-pig ileum and rat uterus but only the latter became thereafter sensitized to the peptide.

Chymotrypsin did not affect responses of the rat uterus to vasopressin, whereas the contraction elicited by peptide V was potentiated and after washing the preparation became highly sensitive to this peptide.

It seemed of interest to examine the action of peptides VI and VII, because their structure coincides with the alleged active centre of chymotrypsin (Oosterbaan & Cohen, 1964). Peptide VI left in the bath for up to 6 min neither potentiated the response of guinea-pig ileum and rat uterus to bradykinin nor changed the sensitivity of these preparations to it. When peptide VI (500 μg) remained in contact with rat uterus for 1 hr there was no change of sensitivity towards bradykinin or peptide V. In one experiment out of four, however, after washing out peptide VI, the preparation showed greater sensitivity to kinins, but this effect rapidly faded away. After incubating peptide VI (500 μg) with bradykinin (40 μg) for 35 min at 35° C, the latter retained its full contracting action on guinea-pig ileum. Peptide

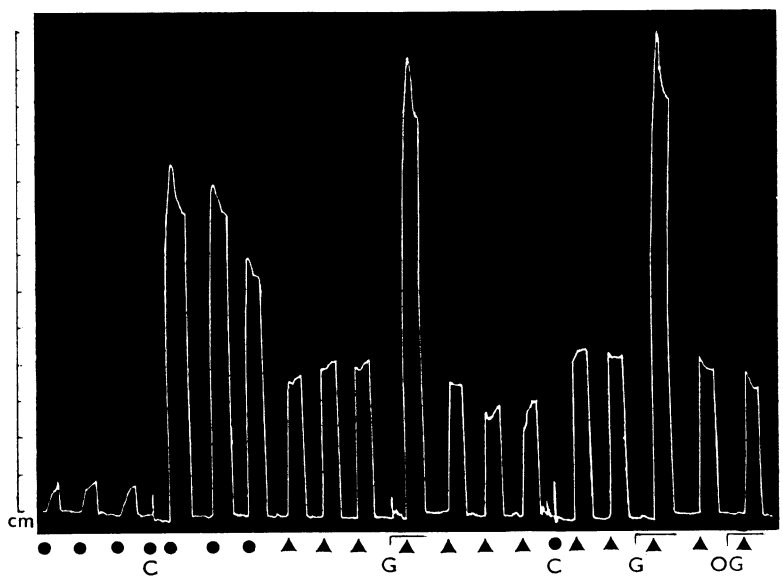

FIG. 5. Guinea-pig isolated ileum containing atropine and mepyramine. Contacts and intervals as in Fig. 1. Contractions were elicited by bradykinin (●, 30 ng ; ▲, 15 ng). After the sensitization caused by chymotrypsin (C, 500 μg ; kymograph stopped), the response to bradykinin was potentiated by reduced glutathione (G, 500 μg) but not by oxidized glutathione (OG, 500 μg). Bars indicate that the preparation was not washed after addition of drug.

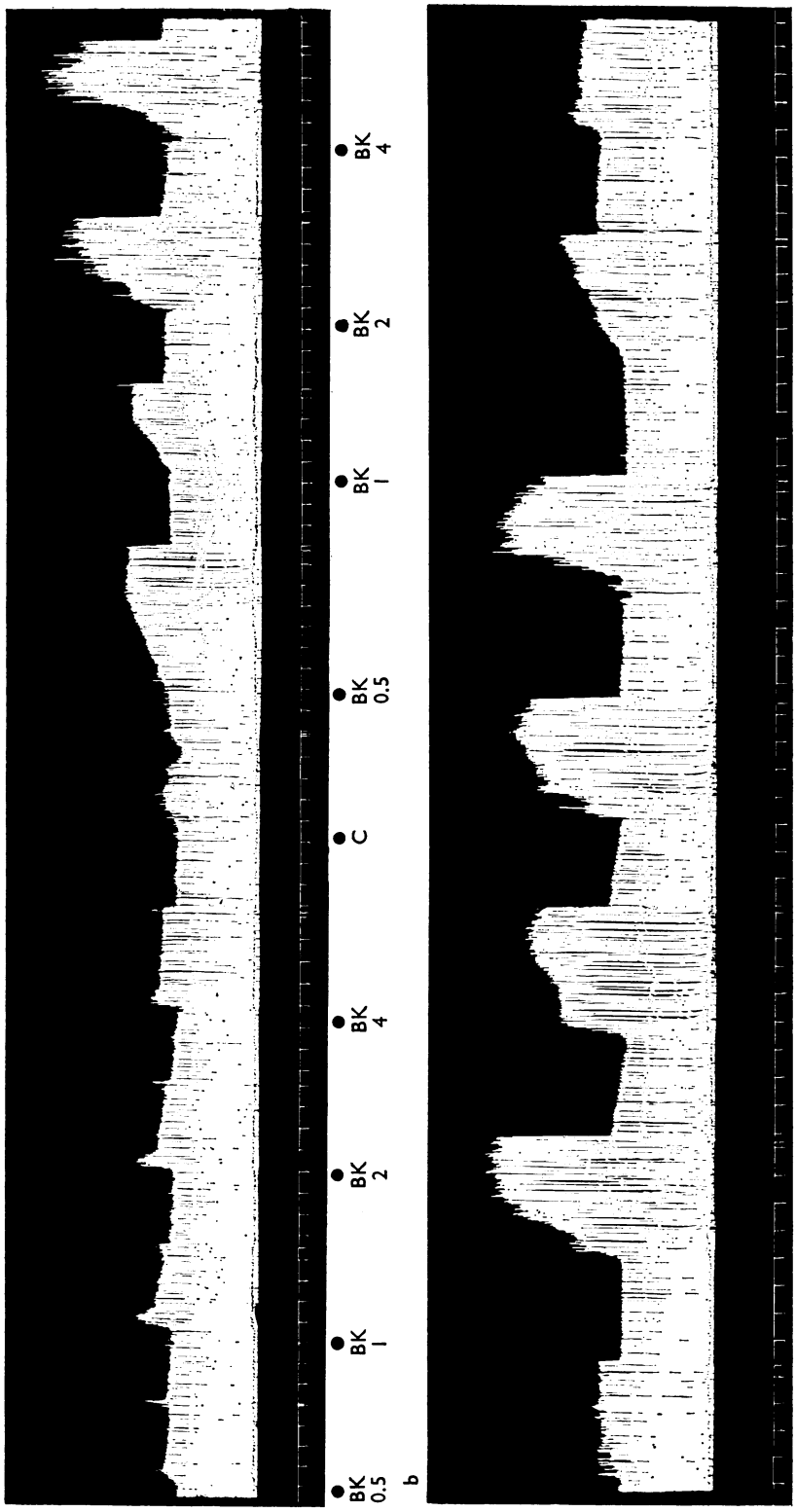

FIG. 6. Guinea-pig, 580 g, anaesthetized with urethane. Record of bronchiolar tone. (a) and (b) are successive tracings of the same experiment. Intrajugular injection of bradykinin (BK, doses in μg), chymotrypsin (C, 4 mg) and calcium acetylsalicylate (As, 5 mg). Time marker, 30 sec. Intervals between injections 5 min (kymograph stopped) during which the lungs were forcibly inflated to regain the base-line. After chymotrypsin there was an enhancement of the bronchoconstrictor effect of bradykinin, which was antagonized by calcium acetylsalicylate.

VII (500 μg) caused a rapid contraction followed by a quick relaxation of the guinea-pig ileum. The presence in the bath of peptide VII for 2 or 30 min did not modify the subsequent response of the guinea-pig ileum to bradykinin or to acetylcholine. Likewise, when peptide VII remained in contact with rat uterus for 1 hr, its response to bradykinin was not altered. For control, at the end of all these experiments, chymotrypsin (300–400 μg) was introduced into the bath for 1 min and the subsequent responses to bradykinin were increased 3–5 times.

Reduced glutathione (300 μg) potentiated the contraction of the guinea-pig ileum elicited by bradykinin but did not sensitize the preparation to it. Previous experiments (Edery & Grunfeld, 1966) have shown that glutathione inhibited the kininase activity of intact guinea-pig ileum but not that of the homogenate, while chymotrypsin inhibited both. It therefore seemed of interest to examine the action of the tripeptide on a chymotrypsin-sensitized ileum. A typical experiment is illustrated in Fig. 5; it shows that here also reduced glutathione potentiated the response to bradykinin, but the sensitivity remained unaltered.

Effects on bronchiolar tone of guinea-pig

After injection of bradykinin (0.25–2 μg) there was a gradual increase of bronchiolar resistance to inflation. The preparations became tachyphylactic on repeated injections and the dose therefore had to be increased in order to obtain a bronchoconstrictive response of about the same magnitude as that produced by the preceding one. These findings were in agreement with those previously described by Collier *et al.* (1960) and Bisset & Lewis (1962).

TABLE 2. *Effects of peptides VI and VII (for structure see* **Methods**), *chymotrypsin and bradykinin on permeability of microcirculation vessels*

Substance	Dose (μg)	Diameter of spot (mm)	Remarks
Peptide VI	5	Prick only	
Peptide VI	50	Prick only	
Peptide VI and BK	2·5 1	11 (10–12)	Inc.
BK	1	13 (12–15)	
ChT	2·5	4 (0–7)	
ChT	5	9 (7–10)	
ChT	5	3 (2–4)	Boil.
ChT and BK	2·5 1	4·7 (3–6)	Inc.
ChT and BK	2·5 1	12 (10–15)	No inc.
Peptide VII	25	Prick only	
Peptide VII	50	3	
Peptide VII and BK	25 1	12 (11–14)	No inc.
Peptide VII and BK	25 1	13 (10–17)	Inc.
Saline (0·1 ml.)		2	
Phosphate buffer (0·1 ml.)		4 (3–6)	

Substances were injected into the skin of abdomen of guinea-pigs administered with pontamine-sky blue. Diameter of the spots appearing in the inner side of skin represent the average obtained in at least four animals (number in parentheses indicates range).
Inc., mixture incubated for 20 min at 35° C.
No inc., mixture injected immediately after preparation.
Boil., substance boiled for 5 min before injection.
ChT, chymotrypsin; BK, bradykinin.

Chymotrypsin (2–4 mg/kg) caused bronchoconstriction which could be overcome by forceful inflation. Subsequently, the bronchoconstrictor effect of bradykinin was greatly enhanced, as illustrated in Fig. 6. This occurred in five out of ten experiments. No obvious reason for the variability was found. In some experiments, after repeated injections of chymotrypsin, the response to bradykinin became biphasic; that is, the peptide first caused bronchodilation followed by bronchoconstriction. In all cases calcium acetylsalicylate (5 mg/kg) antagonized bradykinin, confirming a previous report of Collier & Shorley (1960).

Effects on permeability of microcirculation vessels

The findings are presented in Table 2. Peptides VI and VII neither enhanced permeability by themselves nor affected the increment caused by bradykinin. Moreover, these peptides, in contrast to chymotrypsin, neither destroyed the kinin upon incubation nor suppressed its permeability effects. Chymotrypsin (5 μg) produced a well circumscribed blue spot extending around the site of injection, whereas the boiled enzyme did not. On the other hand chymotrypsin did not potentiate the permeability increasing action of bradykinin.

Discussion

A salient feature emanating from the present work is the remarkable specificity of the sensitization caused by proteases, which is two-fold. On the one hand, the hydrolases affected exclusively a certain type of smooth muscle preparations; and on the other, these became sensitized only to bradykinin and some structurally related peptides. A notable exception was peptide IV.

No satisfactory reason for the mechanism of sensitization has yet been found. A simple explanation could be through the inhibition of kininase in the sense that organophosphates increase the sensitivity to acetylcholine by inhibiting cholinesterase. This postulate could be applicable to chymotrypsin which has been found to inhibit kininase of the guinea-pig isolated ileum (Edery, 1966b); though glutathione, which exerted a similar effect, nevertheless caused no sensitization. Glutathione potentiated the response of bradykinin not only in a normal but also in the chymotrypsin-treated guinea-pig ileum—that is, with kininase inhibited. Thus potentiation of bradykinin responses could not have been caused through blockade of the enzymic destruction of kinin. A similar conclusion was reached by other workers (Graham & Al-Katib, 1966a; Auerswald & Doleschel, 1967) analysing the potentiation by 2-halogenoalkylamines and sulphydryl compounds of bradykinin contractions on plain muscle.

Seeking a more likely mechanism, it seems reasonable to speculate in terms of membrane effects. The early concept of plasma membrane being formed by a bimolecular layer of lipids sandwiched between two layers of protein (Davson & Danielli, 1943) has subsequently received considerable support (Robertson, 1959; Gray, 1964; Finean, 1966). The presence of pores and tortuous channels traversing the cell membrane has also been repeatedly postulated (Burgen, 1957; Kavanau, 1966; Reid, 1967). Moreover, it appears that the process of facilitated diffusion (Finean, 1966; Kavanau, 1966) is mainly dependent on the chemical structure of proteins lining these pores and channels. It could then be assumed that in the present work proteases could have interacted with peptide bonds of muscle cell membrane, thus unmasking additional kinin receptors, and also with those proteins

bordering the fenestrations. Consequently, only molecules of a particular size and shape, such as bradykinin and very closely related peptides, could accommodate themselves to the new stereo-chemical configuration and their attachment to the receptors be greatly facilitated. Conversely, passage of molecules unrelated to kinins could have been hampered. This latter assumption is supported by the fact that *pari passu* with the increase of sensitivity of preparations to bradykinin there was a decrease towards other stimulating substances. The fact that some preparations were sensitized to plasma kinins while others were not could be interpreted that no chemical interaction with proteases took place at membrane level. In this connection it should be recalled that evidence exists which points to the heterochemical composition not only of lipids (Cuthbert, 1967) but also of proteins of the membrane envelope, depending on the type of cell considered (Maddy, 1966 ; Ashworth & Green, 1966 ; Reid, 1967).

The lack of activity of peptides VI and VII was somewhat contrary to expectation because these compounds derive from chymotrypsin (van de Linde *et al.*, 1961). It could be argued that mere resemblance to the primary structure of the active centre of the enzyme could hardly confer biological activity on these peptides. Nevertheless, other related peptides have been shown to possess enzymic activity (Sheehan, Bennett & Schneider, 1966) which is essential for the sensitizing action of chymotrypsin (Edery, 1965a) and pronase as shown here. Moreover peptides VI and VII are structurally similar to the β-fibrinopeptide which has been reported to sensitize the rat isolated uterus to bradykinin (Osbahr, Gladner & Laki, 1964).

The chymotrypsin-bradykinin interrelationship seems to differ greatly whether the visceral or vascular smooth muscle is considered. In the latter, in contrast to the former, there was no interaction. The enzyme potentiated neither the permeability increasing activity of the kinin nor its vasodilatatory effect (Edery, 1965b). These findings would suggest structural differences of the bradykinin receptor for both types of smooth muscle.

The reason whereby chymotrypsin increased the susceptibility of guinea-pig bronchioles to bradykinin seems obscure at present. It could be due to the uncovering of spare receptors located in the Reisseissen or perialveolar muscles ; but there is as yet no evidence in support of this hypothesis.

Finally, it may be noted that the chymotrypsin sensitization phenomenon has already found practical interest. It has been applied to demonstrate the formation in various conditions of minute amounts of plasma kinins (Greenbaum & Kim, 1967 ; Rocha e Silva *et al.*, 1967 ; Zacest & Marshford, 1967) as well as to identify a newly synthesized bradykinin (Edery, 1967b).

Our thanks are due to Drs. E. Schröder, B. Merrifield, E. Blout, H. Kienhuis, J. Zeitlin and V. O. Marquis for generous supplies of some of the materials used in this work. The efficient technical help of Miss Dvorah Vischner and Mrs. Simona Rosenthal is gratefully acknowledged.

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(*Received May* 14, 1968)