Inactivation of Bradykinin in the Pulmonary Circulation

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Ferreira & Vane (1967) have shown that less than 30% of bradykinin infused into the right atrium emerges in the ascending aorta. In blood the biological half-life was found to be 17 sec. Considering the brief circulation time from right atrium to ascending aorta (2–10 sec.; Spector, 1956), they concluded that enzymes of blood could not account for the degree of inactivation that occurred in the pulmonary circulation.

A possible mechanism for the pulmonary inactivation of bradykinin is through hydrolysis by enzymes at or near the vascular endothelium. We have begun an investigation of this possibility by using a rat lung preparation *in situ* washed free of blood.

Rats, weighing 0.2–0.4 kg., were anaesthetized with pentobarbital (50 mg./kg. body wt.) given by intraperitoneal injection. Polythene cannulae were placed in the left common carotid artery and the right external jugular vein. Another cannula was placed in the trachea and the lungs were ventilated mechanically. Saline (0.9% NaCl) was infused into the venous cannula and the rat was exsanguinated through the arterial cannula. Then a cannula was inserted through a slit in the right ventricle into the pulmonary artery and another was tied into the left ventricle.

A Locke dextran solution (1 l. contained: dextran, 40 g.; NaCl, 9.0 g.; KCl, 0.42 g.; CaCl₂, 0.24 g.; D-glucose, 1.0 g.; NaHCO₃, 0.2 g.) was infused into the pulmonary artery at a rate of 4 ml./min. and the effluent was collected from the cannula in the left ventricle. The infusion solutions were heated at 37° and were aerated with O₂ + CO₂ (95:5). When the effluent became free of erythrocytes (after 30–40 min.) [2-L-proline-¹⁴C]bradykinin (28.6 µg./mg., lot no. 307–64; New England Nuclear Corp., Boston, Mass., U.S.A.) in Locke dextran solution was infused. The infusion rate was maintained at 4 ml./min. Bradykinin was infused at 2.0 or 4.0 µg./

min. In one experiment it was infused at 100 µg./min. In another experiment 2-mercaptoethanol (1.5 mg./ml.) was added to the Locke dextran solution. In each of these experiments bradykinin was infused for 20 min.

The effluent from the left ventricle was collected in an Erlenmeyer flask containing 100 ml. of 4% (v/v) trifluoroacetic acid. The vessel contents were stirred constantly with a magnetic stirring apparatus. After the infusion had been stopped the effluent was concentrated to 10–20 ml. in a rotary evaporator. This viscous solution was extracted four times with equal volumes of butan-1-ol previously equilibrated with 2% trifluoroacetic acid. Under these conditions the apparent partition coefficient of bradykinin is greater than 1.0 whereas the apparent partition coefficient of dextran is less than 0.1. The butan-1-ol extract was evaporated to dryness and the residue was resuspended in 10 ml. of 2% trifluoroacetic acid previously equilibrated with butan-1-ol. The butan-1-ol extraction was repeated and the butan-1-ol extract was evaporated to dryness. The residue was redissolved in 1.0 ml. of 6% (v/v) acetic acid.

A sample (0.1–0.5 ml.) of this solution was applied to the centre, as a line across the full width, of a strip (10 cm. × 57 cm.) of Whatman 3MM paper. Previously the paper had been wetted with a 0.1 M-acetic acid solution adjusted to pH 5 with pyridine. Electrophoresis was performed with the same buffer at 15 v/cm., 10–20 mA, for 1.5 hr. After electrophoresis, the paper was dried at 80° and then a longitudinal strip (about 2 cm. wide) was cut. This strip was developed with ninhydrin and counted in a strip counter (Atomic Accessories Inc., Bellerose, N.Y., U.S.A.). From the results thus obtained the remainder of the electrophoretogram was cut parallel to the origin in widths corresponding to radioactive peaks. Each of these strips was washed with 50–100 ml. of 6% acetic acid and this was sufficient to elute over 95% of the radioactivity. The wash solutions were evaporated to dryness and the residues were dissolved in 0.5 ml. of 6% acetic acid for further electrophoresis and chromatography. From this point, electrophoresis was performed with Whatman 1MM paper at pH 5 in the buffer described above or at pH 1.55 in formic acid-acetic acid-water (6:13:81, by vol.). With either buffer system, electrophoresis was

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carried out at 18 v/cm., 5–10 mA, for 1 hr. Ascending chromatography was done on Whatman CM-82 CM-cellulose paper with the buffer obtained by adjusting 0.2M-acetic acid to pH 5 with pyridine. Development in this system required 2 hr. Mixtures of isolated radioactive peptides and synthetic peptides were chromatographed on a Spinco model 120B amino acid analyser fitted with a Nuclear-Chicago model 6770 liquid-scintillation flow-counting system.

Bradykinin and its peptide fragments used for reference compounds were synthesized in this Laboratory by the solid-phase method (Merrifield, 1964; Stewart, 1968). [2-L-proline- ^{14}C]Bradykinin, a product of New England Nuclear Corp., was a gift of the National Institutes of Health (courtesy of Dr John Pisano).

Initially, we hoped to collect sufficient material to allow identification by quantitative amino acid analysis. This goal appeared to require that bradykinin be infused at a rate of 100 $\mu\text{g./min.}$ When bradykinin was infused at this rate, the outflow from the left ventricle fell from 4 ml./min. to less than 0.4 ml./min. A transudate formed across the surface of the lung and the trachea filled with fluid. Most of the radioactivity was found in the transudate and was identified as bradykinin by

quantitative amino acid analysis and biological activity.

However, when bradykinin was infused at 2.0 or 4.0 $\mu\text{g./min.}$, the volume of fluid emerging from the left ventricle was the same as the volume infused. Essentially all of the radioactivity infused emerged in the effluent. Electrophoresis of the effluent extract showed two bands of radioactivity. The major peak, about two-thirds of the total, migrated on electrophoresis at pH 5 like a neutral compound (see Table 1). Its migration compared with that of arginine was about 0.15. On electrophoresis at pH 1.55, this compound moved faster than proline or Pro-Pro-Gly and all larger neutral peptide fragments of bradykinin. Its migration corresponded to that of Pro-Pro. This identification was confirmed by chromatography on the long column of the Spinco model 120B amino acid analyser.

The column was packed with Spinco AA 27 resin equilibrated with pH 3.28 citrate buffer. The column temperature was 55° throughout the run. At 5 min. after the run was started the buffer was changed to pH 4.25 citrate buffer. Two peaks of radioactivity were detected. The major peak was eluted with authentic Pro-Pro at 102 min., well behind proline, which was eluted in less than

Table 1. *Electrophoretic and chromatographic behaviour of bradykinin and some of its peptide fragments*

Since [2-L-proline- ^{14}C]bradykinin was used, only peptides containing the first proline were compared.

	Relative mobility on electrophoresis at pH 5	R_F on chromatography
Basic peptides		
Arg-Pro	0.8	0.34
Arg-Pro-Pro	0.76	0.39
Arg-Pro-Pro-Gly	0.71	0.41
Arg-Pro-Pro-Gly-Phe	0.60	0.37
Arg-Pro-Pro-Gly-Phe-Ser	0.55	0.44
Arg-Pro-Pro-Gly-Phe-Ser-Pro	0.50	0.40
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	0.45	0.39
Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	0.42	0.41
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Bradykinin)	0.69	0.12
Standards		
Arginine	1.0 (~10 cm.)	
Glycine	0.18	
Neutral peptides		
	Relative mobility on electrophoresis at pH 1.55	
Pro-Pro	1.09	
Pro-Pro-Gly	0.91	
Pro-Pro-Gly-Phe	0.72	
Pro-Pro-Gly-Phe-Ser	0.71	
Pro-Pro-Gly-Phe-Ser-Pro	Not tested	
Pro-Pro-Gly-Phe-Ser-Pro-Phe	Not tested	
Standards		
Proline	0.94	
Glycine	1.0 (~17 cm.)	
Arginine	1.15	

40 min. and ahead of Pro-Pro-Gly, which was eluted at 109 min. The minor peak of radioactivity was not retained on the column and may have been the prolylproline dioxopiperazine. This dipeptide is known to be prone to dioxopiperazine formation.

The second peak of radioactivity found on preparative electrophoresis migrated at pH 5 like Arg-Pro-Pro-Gly. Its migration was slower than that of Arg-Pro or Arg-Pro-Pro and faster than that of bradykinin peptide fragments larger than Arg-Pro-Pro-Gly. It had virtually no biological activity and could not have been bradykinin.

2-Mercaptoethanol has been shown previously to decrease the apparent rate of inactivation of bradykinin in the pulmonary circulation (Stewart & Roblero, 1967). When mercaptoethanol (1.5 mg./ml.) was added to the perfusing solution, we found only one radioactively labelled compound in the effluent. At pH 5 this compound migrated towards the cathode well ahead of all neutral peptide fragments of bradykinin, but behind bradykinin itself. By this test it was not possible to distinguish the radioactive peak from Arg-Pro-Pro-Gly-Phe or Arg-Pro-Pro-Gly-Phe-Ser. However, when it was chromatographed on CM-cellulose paper, the radioactive peak corresponded to Arg-Pro-Pro-Gly-Phe-Ser. Its R_F was clearly greater than that of Arg-Pro-Pro-Gly-Phe.

From these results it appears that bradykinin is hydrolysed by enzymes at or near the endothelium of pulmonary blood vessels. So far we have identified only one primary cleavage, namely that of the Arg¹-Pro² bond. 2-Mercaptoethanol appears to inhibit that cleavage completely. The findings of Pro-Pro and Arg-Pro-Pro-Gly in control perfusions and Arg-Pro-Pro-Gly-Phe-Ser only after treatment of the preparation with 2-mercaptoethanol suggest that another primary cleavage occurs near the C-terminus of bradykinin and that the N-terminal peptide thus formed is further degraded, perhaps by a carboxypeptidase. Cleavage of the Ser⁶-Pro⁷ bond would release such a peptide. However, we have been unable to collect sufficient material to identify peptide fragments of the C-terminus of bradykinin, and unequivocal identification of the second primary cleavage site cannot yet be made.

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