Biochemical Pharmacology, Vol. 28, pp. 553-556.

0006-2952/79/0215-0553/$02.00/0

SUBSTANCE P DEGRADATION BY RAT BRAIN PEPTIDASES: INHIBITION BY SQ 20881.

C.M. Lee, A. Arregui and L.L. Iversen

MRC Neurochemical Pharmacology Unit, Medical School, Cambridge CB2 2QD, U.K.

(Received 9 November 1978; accepted 23 November 1978)

The nonapeptide SQ 20881 (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) and the compound SQ 14225 (2-D-methyl-3-mercapto-propanoyl-L-proline) have been described as specific inhibitors of the angiotensin-converting enzyme (ACE) (EC 3.4.15.1) (1-4). Both compounds possess antihypertensive actions when administered in vivo, but it has been suggested recently that this may be due in part to a mechanism unrelated to the renin-angiotensin system (5). It is possible, for example, that the hypotensive effects of these compounds may be due to an inhibition of the degradation of endogenous vasodilator substances such as substance P (SP). We report here that the nonapeptide SQ 20881 has a potent inhibitory effect on the activity of a substance P degrading enzyme (SP-DE) in rat brain, and that the SP-DE differs from ACE in terms of its chloride ion requirement, subcellular distribution and sensitivity to inhibition by various agents. Our results suggest caution in interpreting the actions of SQ 20881.

Brains from adult, male Sprague-Dawley rats were homogenized in 20 vol of 0.32 M sucrose. Subcellular fractions were prepared by differential centrifugation to yield P1, P2, P3 pellets and a postmicrosomal supernatant fraction (1,000 x g for 10 min, 27,000 x g for 20 min and 100,000 x g for 60 min, respectively). The pellets were resuspended twice in distilled water to remove endogenous SP and the final washed pellet resuspended in 20 vol of 0.32 M sucrose before assay for SP-DE activity.

The assay involved measuring the disappearance of exogenous SP immunoreactivity by a radioimmunoassay procedure (6), with a lower limit of sensitivity of 0.01 pmol of SP. Preliminary experiments revealed a predominant localization (> 75%) of SP-DE activity in the supernatant fraction (S3) of rat brain homogenates. In addition, an appreciable amount of SP-DE activity was found in the washed P2 fraction, while the P1 and P3 fractions contained only negligible amounts of SP-DE activity. The SP-DE activity in the supernatant fraction had a pH optimum of 7.4, which was different from that of the P2 SP-DE activity (pH 8.6).

Preliminary experiment also indicated that the SP-DE activity was independent of chloride ions since its activity in the presence of 150mM Cl^- was not different from that in the absence of Cl^-. Subsequent studies compared the actions of various potential inhibitors of SP-DE in the supernatant and P2 fractions. All enzyme assays were performed in duplicate at 37°C. Enzyme preparations (1-5 mg tissue equivalent) were preincubated in 50 mM potassium phosphate buffer, pH 7.4 (supernatant fraction), or in 50 mM barbital acetate buffer, pH 8.6 (P2 fraction) in the presence of several peptidase inhibitors for 15 min at 37°C. The reaction (final vol 0.4 ml) was started by adding synthetic SP (Peninsula Laboratories, Inc., San Carlos, Calif.) at a final concentration of 0.2 µM and stopped at appropriate time intervals by adding 100 µl 1N acetic acid and placing the tubes in a boiling water bath for 10 min. Boiled enzyme preparations exhibited no significant SP-DE activity and were used as controls. The samples were cooled and their pH adjusted to 8.6 with 1N NaOH and barbital buffer. The denatured proteins in the reaction mixture were sedimented by centrifugation at 3,000 x g for 10 min. Aliquots of the clear supernatant were then assayed for SP immunoreactivity in duplicate. None of the inhibitors tested interfered with the SP radioimmunoassay at the concentrations used. In control experiments, gel chromatographic studies using Sephadex G-15 were performed on the remaining SP. This revealed the presence of only 1 major peak of SP immunoreactivity with elution characteristics comparable to those of synthetic SP, indicating that there was no detectable accumulation of SP-degradation products that could cross-react with the SP-antisera during the in vitro assay of SP-DE activity.

Results and Discussion Among the several peptidase inhibitors that were tested for their ability to prevent the breakdown of SP in the supernatant and P2 fractions, it was found that benzamidine (1 mM) 1,10-phenathroline (0.1 mM), EDTA (2 mM) and puromycin (1 mM) were completely ineffective (Table 1). This suggests that serine-endopeptidases, metallo-endopeptidases and amino-exopeptidases are unlikely to be involved in SP degradation. However, a possible role of thio-endopeptidases in SP degradation cannot be ruled out, since p-chloromercuribenzoate (PCMBA) exhibited a dose-dependent inhibitory action on both the P2 and supernatant SP-DE activities (Figure 1), although iodoacetate (0.1 mM) was ineffective.

In addition to the differences in pH optima and subcellular localization, the P2 and supernatant SP-DE activities could be differentiated by the following pharmacological characteristics: 1) bacitracin, which had a dose-dependent inhibitory action on SP breakdown, was a more effective inhibitor of the P2 SP-DE activity than that in the supernatant (Table 1).

Table 1. Effect of peptidase inhibitors on particulate (P2) and supernatant (S3) substance P-degrading enzyme activities in rat brain homogenates.

INHIBITOR	CONC. (mM)	% CONTROL ACTIVITY P2	S3
BENZAMIDINE	1.0	98 ± 2	99 ± 2
1,10-PHENANTHROLINE	0.1	96 ± 3	98 ± 4
EDTA	2.0	101 ± 3	98 ± 5
IODOACETATE	0.1	99 ± 2	97 ± 2
PUROMYCIN	1.0	94 ± 7	94 ± 7
BACITRACIN	0.035	69 ± 3	85 ± 4
	0.070	48 ± 3	73 ± 7
	0.180	11 ± 5	55 ± 7

Values represent the mean ± s.e.m. for 3 experiments. Controls enzyme activities for P2 and S3 fractions are 68.3 ± 1.7 and 452.2 ± 13.7 pmol SP degraded/hr/mg tissue respectively.

2) PCMBA inhibited the supernatant activity more potently (IC_{50}= 5 μM) than the P2 SP-DE (IC_{50}= 50 μM) (Figure 1). 3) SQ 14225 (1 mM) had no effect on the soluble enzyme but exerted a weak inhibitory action (25%; $p<0.02$) on the particulate enzyme (Figure 1). SQ 20881 (1 mM) had only a weak inhibitory action (30%; $p<0.001$) on the particulate enzyme, but was a potent inhibitor of the soluble enzyme (IC_{50}=5 μM) (Figure 1).

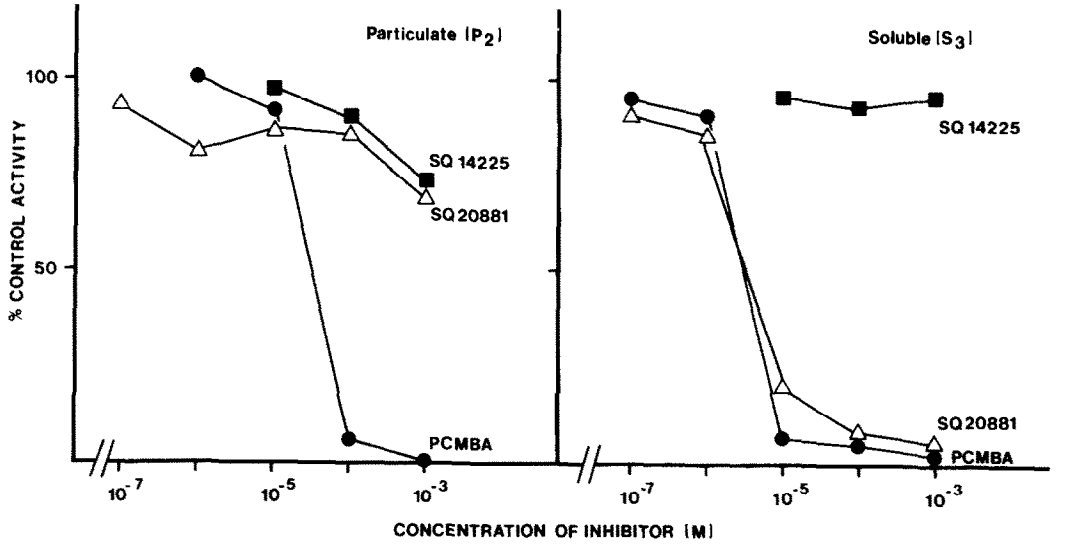

Figure 1. Inhibition of particulate and soluble substance P degrading enzyme activities of rat brain homogenates. Control activities for P2 and S3 fractions are the same as in Table 1. Each point represents the mean of 4-12 determinations.

These observations suggest that the membrane bound (P2) and the cytoplasmic (S3) SP-DE may represent different enzymes.

In the rat brain, ACE has been found to be dependent on chloride ions and to have a preferential localization in the particulate (microsomal) fractions, with little or no activity in the post-microsomal supernatant (1,7,8). This enzyme is inhibited by EDTA, 1,10-phenanthroline, SQ 20881 and SQ 14225, but unaffected by the presence of PCMBA (1,2,7). On the contrary, there is a predominantly cytoplasmic localization of SP-DE activity, and its activity does not depend on the presence of chloride ions. In addition, EDTA and 1,10-phenanthrolineare ineffective in preventing SP breakdown while PCMBA exerts a profound inhibitory effect on SP degradation. Although SQ 20881 inhibits both SP-DE and ACE in rat brain homogenates, it inhibits ACE activity more potently (IC_{50}= 0.2 μM) than the supernatant SP-DE (IC_{50}= 5 μM). Thus, the biochemical and pharmacological evidence suggests that SP-DE is quite distinct from ACE. Since SQ 20881 and SQ 14225 inhibit SP-DE to a greater or less extent, as well as enkephalin degradation (3,9), they cannot be considered specific ACE inhibitors. The degradation of substance P in plasma has also been shown to be inhibited by SQ 20881 (10). It is conceivable that the hypotensive actions of these compounds may be due to their capacity to inhibit the degradation of vasodilator peptides (e.g. substance P) in addition to their action on ACE.

ACKNOWLEDGEMENTS : C.M.L. is a Li Po Chun Scholar and A.A. a Wellcome Trust Fellow. We thank Dr Z.P. Horovitz (Squibb Institute, N.J.) for supplying SQ 20881 and SQ 14225.

REFERENCES :

1. Benuck, M. & N. Marks, J. Neurochem. 30, 729 (1978).
2. Erdös, E.G, Fed. Proc. 36, 1760 (1977).
3. Erdös, E.G., A.R. Johnson & N.T. Boyden, Biochem. Pharmacol. 27, 843 (1978).
4. Ondetti, M.A., B. Rubin & D.W. Cushman, Science 196, 441 (1977).
5. Vollmer, R.R., J.A. Boccagno, D.N. Harris & V.S. Murthy, Eur. J. Pharmacol. 51, 39 (1978).
6. Powell, D., S.E. Leeman, G.W. Tregear, H.D. Niall & J.T. Potts Jr., Nature, New Biol. 241, 252 (1973).
7. Yang, H.-Y.T. & N.H. Neff, J. Neurochem. 19, 2443 (1972).
8. Arregui, A. & L.L. Iversen, Eur. J. Pharmacol. (in press).
9. Erdös, E.G., A.R. Johnson & N.T. Boyden, in Adv. in Biochem. Psychopharmacol. 18, (Eds. E. Costa & M. Trabucchi) pp. 45-49. Raven Press, N.Y. (1978).
10. Cannon, D., P. Skrabanek & D. Powell, Irish J. Med. Sci. 146, 301 (1977).