Receptor binding of guinea pig and pig vasoactive intestinal peptides by rat lung

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Guinea pig vasoactive intestinal peptide (gpVIP) differs from other mammalian VIPs in four of its 28 amino acid residues. In the present study, the gpVIP displaced ^{125}I -labelled pig VIP (pVIP) binding by rat lung membranes with 7.7-fold lower potency than pVIP. Degradation of gpVIP by rat lung membranes, assessed by radioimmunoassay and h.p.l.c., was 1.9-fold greater than that of pVIP. This difference in degradation of the two peptides was not large enough to account for the lower receptor-binding potency of gpVIP. The amino acid residues that distinguish pVIP from gpVIP are likely to contribute to the interaction of VIP with receptors and peptide hydrolases in lung membranes.

INTRODUCTION

The primary structures of human, dog, cow, pig, goat and rat vasoactive intestinal peptides (VIP) are identical (Mutt & Said, 1974; Carlquist *et al.*, 1979; Itoh *et al.*, 1983; Dimaline *et al.*, 1984; Eng *et al.*, 1986). Guinea pig VIP (gpVIP) is unique in that four of its 28 amino acid residues are different from those of other mammalian VIPs (Du *et al.*, 1985). To evaluate the contribution of the non-conserved amino acids to the biological activity of VIP, we have compared the binding and degradation of gpVIP and pig VIP (pVIP) by rat lung membranes.

MATERIALS AND METHODS

Chemicals

Synthetic gpVIP was purchased from Peninsula Laboratories, Belmont, CA, U.S.A. pVIP purified from the intestine (Said & Mutt, 1972) was kindly provided by Dr. S. I. Said, University of Illinois College of Medicine, Chicago, IL, U.S.A. The peptide content of the gpVIP and pVIP preparations was 73% and 80%, respectively. Peptide stock solutions (1 mg/ml of 0.1 M-acetic acid) were stored in aliquots at -70°C . The purity of the peptides was ascertained by reverse-phase h.p.l.c. on a Novapak-C18 column (Waters Associates, Milford, MA, U.S.A.) using an ISCO h.p.l.c. apparatus (Lincoln, NE, U.S.A.). Elution was with a gradient of 8–72% acetonitrile in 0.1% trifluoroacetic acid and absorbance of the effluent was monitored at 214 nm. The gpVIP eluted as a single peak with a retention time of 23.2 min. The VIP preparation exhibited a major peak (retention time 22.9 min) and a minor contaminant peak (retention time 24.7 min) with an area 1.9% that of the major peak. The contaminant did not react with antiserum against pVIP in a radioimmunoassay test performed according to Paul *et al.* (1984). The gpVIP and pVIP peaks observed by h.p.l.c. of 2 μg of each of these peptides were nearly equivalent in height and area. H.p.l.c.-grade solvents were from Fisher Scientific, Fairlawn, NJ,

U.S.A. Other chemicals were from Sigma, St. Louis, MO, U.S.A.

^{125}I -labelled VIP

pVIP was labelled with ^{125}I by the chloramine-T method and the ^{125}I -pVIP was purified by reverse-phase h.p.l.c. on a Novapak-C₁₈ column to a specific activity of 2.0 Ci/ μmol (Paul *et al.*, 1987).

Membrane preparation

Lungs were excised from anaesthetized rats (Sprague–Dawley) and perfused with Krebs buffer (Hamasaki *et al.*, 1984). Crude membrane preparations from the lungs were obtained according to Paul *et al.* (1988) by homogenization of the tissues in 10 mM-Trizma/HCl, pH 7.2 (4 $^\circ\text{C}$) containing 0.25 M-sucrose, 1 mM-EDTA (sodium salt), 100 units of aprotinin/ml, 5 μM -pepstatin A and 50 μM -phenylmethanesulphonyl fluoride (homogenization buffer), filtration through medical gauze and centrifugation at 30000 g (4 $^\circ\text{C}$, 30 min). The protein content of the membranes, assayed according to Lowry *et al.* (1951), was adjusted to 6 mg/ml of homogenization buffer.

Receptor binding

^{125}I -VIP binding assays were performed according to Paul *et al.* (1987). Briefly, 50 μl of ^{125}I -pVIP (55 pM), 50 μl of membrane suspension (50 μg of protein) and increasing unlabelled VIP concentrations (30 pM–1 μM) or assay diluent (100 mM-Trizma/HCl, pH 7.2, containing 5 mM-MgCl₂ and 1% bovine serum albumin) were incubated for 30 min at 23 $^\circ\text{C}$. Assay diluent (0.5 ml) was added to each tube and bound VIP was separated by centrifugation (12000 g, 3 min, 4 $^\circ\text{C}$) in a Beckman microcentrifuge and aspiration of the supernatants. Radioactivity in the pellets was measured in a gamma spectrometer at an efficiency of 70%. Non-specific binding was determined by incubation in the presence of pVIP (1 μM). The binding assays were done using three replicates of each type of incubation. Time-

Abbreviations used: VIP, vasoactive intestinal peptide; gpVIP, guinea pig VIP; pVIP, pig VIP; IR-pVIP and IR-gpVIP, immunoreactive pVIP and gpVIP.

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course experiments (results not shown) indicated that steady-state saturable binding was achieved under these conditions. Analysis of the displacement of ^{125}I -pVIP binding by unlabelled pVIP and gpVIP was with the computer programs Equilibrium Binding Data Analysis and Ligand (Biosoft-Elsevier, Cambridge, U.K.) (McPherson, 1985; Munson & Rodbard, 1980) using a value of 0.38 nM as the K_d of ^{125}I -labelled pVIP binding. This value was determined by measuring saturable binding at increasing ^{125}I -pVIP concentrations (5–500 pM) and analysis of the data with the Ligand program. Statistical comparison was by the t test for independent observations.

Degradation of VIP

gpVIP or pVIP (6 nM) was incubated with lung membranes under the conditions utilized for the receptor assay. Trifluoroacetic acid was added to 0.1%, the tubes placed in a boiling water bath for 8 min, the assay mixtures were cooled and then extracted on Seppak-C18 cartridges (Waters Associates, Milford, PA, U.S.A.) (Paul *et al.*, 1987). VIP was measured by radioimmunoassay (Paul *et al.*, 1984) using pVIP or gpVIP as standards (0.62–160 pg/assay tube) and rabbit anti-pVIP antiserum (no. 89N; final dilution 1:360000). Data reduction was with a Beckman DP 5500 system (logit/log calculation mode). Cross-reactivity of the anti-pVIP antiserum with gpVIP, determined as the ratio of the concentrations of pVIP and gpVIP that inhibited binding of ^{125}I -VIP by 50% (IC_{50}), was 39.7%. The minimal detectable dose for gpVIP, corresponding to 5–7% inhibition of ^{125}I -VIP binding, was lower than that for pVIP (0.8 ± 0.2 and 1.7 ± 0.2 pg/assay tube, respectively; means $\pm$ S.E.M.; $n = 8$). The presence of multiple antibody species in the rabbit anti-VIP antiserum with different relative affinities for gpVIP and pVIP could explain the apparent discrepancy between IC_{50} and minimal detectable dose values for the two peptides. The immunoreactivity present in Seppak-C18 extracts of peptides incubated with lung membranes was analysed by reverse-phase h.p.l.c. (see above). The column fractions were dried (Speedvac Model SVC100H; Savant, Hicksville, NY, U.S.A.), reconstituted in 0.5 ml of radioimmunoassay diluent and assayed for immunoreactive VIP.

RESULTS AND DISCUSSION

Progressively increasing concentrations of pVIP, as well as gpVIP, competitively inhibited the saturable binding of ^{125}I -pVIP by membranes prepared from rat lung (Fig. 1). The IC_{50} values for pVIP and gpVIP were, respectively, 1.14 ± 0.12 nM and 8.80 ± 0.77 nM (means $\pm$ S.E.M.; $n = 3$; $P < 0.001$). Thus, gpVIP was 7.7-fold less potent than pVIP in inhibiting the binding by the membranes. Computer-assisted analysis of these data according to McPherson (1985) suggested the presence of high affinity pVIP and gpVIP binding sites with apparent K_d values of 0.44 ± 0.08 nM and 6.45 ± 0.42 nM (means $\pm$ S.E.M.; $P < 0.001$), respectively. Maximum-binding-capacity values (in pmol of peptide/mg of membrane protein) for these pVIP and gpVIP binding sites were 1.3 and 0.7, respectively. Consistent with previous studies of VIP receptors in the lung and other tissues (Rosselin, 1986; Paul & Said, 1987), a second low affinity (K_d 341 ± 202 nM), high capacity (192 pmol of

Fig. 1. ^{125}I -pVIP binding by rat lung membranes: competitive inhibition by unlabelled pVIP (O) or gpVIP (●)

Values are means $\pm$ S.D. of three replicates from a representative experiment, expressed as % of specific binding obtained in the absence of unlabelled peptide.

Fig. 2. Degradation of pVIP (O) or gpVIP (●) by rat lung membranes

The peptides (6 nM) were incubated with membranes for increasing lengths of time, extracted on Seppak-C18 cartridges and the extracts assayed for immunoreactive pVIP and gpVIP (see the Methods section). Values (means $\pm$ S.D.) represent percentage reduction in immunoreactive peptide concentrations relative to the starting concentrations.

pVIP/mg of protein) binding site was suggested by Scatchard plots (not shown) of the pVIP binding data. In contrast, analyses of gpVIP binding did not suggest a second binding site. The binding of gpVIP by the low affinity pVIP binding site was presumably too small to be detected.

The receptor binding was measured in the presence of the peptide hydrolase inhibitors aprotinin, pepstatin A and phenylmethanesulphonyl fluoride. Nevertheless, a difference in the enzymic inactivation of pVIP and gpVIP

was a potential factor in the receptor-binding potency of the peptides. We measured, therefore, the residual immunoreactive pVIP (IR-pVIP) and gpVIP (IR-gpVIP) after incubation with rat lung membranes under conditions used for the receptor-binding assay. The rate of disappearance of IR-gpVIP was greater than that of IR-pVIP (Fig. 2). After incubation with membranes for 30 min, $62\% \pm 12\%$ of the IR-gpVIP had disappeared, compared with only $32\% \pm 13\%$ of IR-pVIP ($P < 0.025$; $n = 3$). gpVIP and pVIP recovered after incubation for 30 min with the membranes were subjected to reverse-phase h.p.l.c. (see the Methods section). More than 90% of the IR-gpVIP and IR-pVIP were observed to elute as single peaks with retention times of 22.9 and 23.3 min, respectively. The retention times for gpVIP and pVIP that had not been incubated with membranes were 23.2 and 22.9 min, respectively, values close to those for IR-gpVIP and IR-pVIP. These observations suggest that the bulk of the observed immunoreactivities represented undegraded gpVIP and pVIP.

The concentrations of gpVIP and pVIP used in the receptor-binding assay were corrected for degradation observed at 30 min of incubation with the membranes. The IC_{50} values for gpVIP and pVIP obtained from these data were 3.34 nM and 0.77 nM, respectively ($P < 0.001$). The apparent K_d values for the gpVIP binding site and the high affinity pVIP-binding site, computed after correction for peptide degradation, were 2.10 ± 0.08 nM and 0.30 ± 0.03 nM, respectively.

Guinea pig VIP contains leucine, threonine, methionine and valine, respectively, at positions 5, 9, 19 and 26 (Du *et al.*, 1985) instead of valine, asparagine, valine and isoleucine present at the corresponding positions in other mammalian VIPs (Mutt & Said, 1974; Carlquist *et al.*, 1979; Itoh *et al.*, 1983; Dimaline *et al.*, 1984). In the present study, receptors in rat lung membranes were observed to bind gpVIP with a lower affinity than pVIP. Thus, one or more of the four amino acid residues that distinguish pVIP from gpVIP are likely to play a role in the interaction of VIP with its receptors in the rat lung. A clearly demarcated sequence within the VIP molecule that functions as a receptor-binding domain has not been identified. Previous studies observed the fragments VIP(10–28) (Turner *et al.*, 1986) and VIP(14–28) (Couvineau *et al.*, 1984) to possess a low receptor-binding affinity, suggesting that the receptor-binding sequence may be a part of these fragments. On the other hand, chemical modification or removal of amino acid residues lying outside these fragments has been shown to result in large decreases in receptor binding (Couvineau *et al.*, 1984; Pandol *et al.*, 1986). Thus, further studies are required to define the relative contribution of the individual sequence differences between gpVIP and pVIP in VIP–receptor interactions.

Peptide hydrolases are likely to be of physiological importance in terminating the neurotransmitter or

neuromodulator actions of VIP. The existence of enzymes that degrade VIP preferentially has been previously documented (Keltz *et al.*, 1980), but these enzymes remain to be fully characterized: The observation that gpVIP is degraded more efficiently than pVIP by rat lung membranes suggest that peptide bonds involving a non-conserved amino acid(s) are likely targets for enzymic hydrolysis.

In summary, this study observed significant differences in the receptor-binding affinity and degradation of gpVIP and pVIP by rat lung membranes. The lower receptor-binding affinity of gpVIP and its more efficient degradation suggest that the intrinsic biological activity of this peptide is likely to be lower than that of pVIP.

This work was supported by NIH grants 35506 and 38382. The authors thank Dr. Said for providing pure pig VIP and anti-VIP antiserum.

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