Catalytic Hydrolysis of Vasoactive Intestinal Peptide by Human Autoantibody

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Vasoactive intestinal peptide (VIP) labeled with ^{125}I, [Tyr^{10}-^{125}I]VIP, can be hydrolyzed by immunoglobulin G (IgG) purified from a human subject, as judged by trichloroacetic acid precipitation and reversed-phase high-performance liquid chromatography (HPLC). The hydrolytic activity was precipitated by antibody to human IgG, it was bound by immobilized protein G and showed a molecular mass close to 150 kilodaltons by gel filtration chromatography, properties similar to those of authentic IgG. The Fab fragment, prepared from IgG by papain treatment, retained the VIP hydrolytic activity of the IgG. Peptide fragments produced by treatment of VIP with the antibody fraction were purified by reversed-phase HPLC and identified by fast atom bombardment–mass spectrometry and peptide sequencing. The scissile bond in VIP deduced from these experiments was Gln^{16}-Met^{17}. The antibody concentration (73.4 fmol per milligram of IgG) and the K_d (0.4 nM) were computed from analysis of VIP binding under conditions that did not result in peptide hydrolysis. Analysis of the antibody-mediated VIP hydrolysis at varying concentrations of substrate suggested conformity with Michaelis-Menton kinetics (K_m). The values for K_m ($37.9 \times 10^{-9} M$) and the turnover number k_{cat} (15.6 min^{-1}) suggested relatively tight VIP binding and a moderate catalytic efficiency of the antibody.

ANTIBODIES, LIKE ENZYMES, ARE CAPABLE OF CATALYSIS (*1–3*). The diversity and specificity of the immune response hold the promise that antibodies can be designed to catalyze specific chemical reactions outside the range of naturally occurring enzymes. It is generally believed that many of these antibodies obtain their catalytic property, like enzymes, from their ability to bind to the transition state of the ligand better than to its ground state. A number of catalytic antibodies have been produced by immunization with analogues of the presumed transition states of certain chemical reactions and application of hydridoma techniques. The antibodies obtained in this way have been shown to catalyze various chemical reactions, including acyl group transfers (*1*) and pericyclic rearrangements (*2*).

The natural immune response to pathogens and autoantigens most often involves antibodies to peptide epitopes. A question that emerges from the above considerations is whether antibodies found in infection and autoimmune disease are capable of catalytic hydrolysis of specific peptide bonds. This is a significant issue because antibody-catalyzed hydrolysis of peptide bonds is likely to inactivate the target protein substrate, the peptide bond hydrolysis is likely to be sequence specific, and, by definition, a single catalytic antibody molecule is likely to hydrolyze multiple substrate molecules. Compared to the types of chemical reactions known to be catalyzed by antibodies to transition state analogues, the hydrolysis of peptide bonds is more energy-demanding. We now describe the catalysis of peptide bond hydrolysis by an antibody.

We have previously described a humoral autoimmune response to the neuropeptide vasoactive intestinal peptide (VIP) in man (*4, 5*). The chemical and biological properties of the autoantibodies to VIP are not fully understood. Experiments to study the biological actions of these autoantibodies have now led to the finding of catalytic antibody-mediated hydrolysis of a peptide bond in VIP.

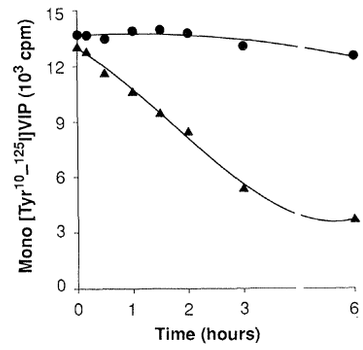

Fig. 1. Hydrolysis of [Tyr^{10}-^{125}I]VIP treated with 42.5 μg of human IgG (▲) containing antibodies to VIP, compared to an equivalent concentration of a nonimmune IgG (●). The IgG was prepared from plasma collected in protease inhibitors by chromatography on DEAE-cellulose and protein G–Sepharose, and then ultrafiltered on a YM 10 membrane filter (10-kD cutoff, Amicon) to approximately 25 mg/ml. The [Tyr^{10}-^{125}I]VIP (approximately 30 pM) was incubated with immune or nonimmune IgG at 38°C in (200 μl volume) 50 mM tris-HCl, 100 mM glycine, 0.025 percent Tween-20 (Sigma), and 0.1 percent bovine serum albumin (BSA), *p*H 8.0. Trichloroacetic acid was added to 10 percent (v/v), the precipitate was separated by centrifugation (5800*g*, 20 minutes), the supernatant was removed by aspiration and the radioactivity was measured at 70 percent efficiency (Beckman model 5500 gamma spectrometer). The starting radioactivity in each tube was 15,040 cpm. Values are means of duplicates. More than 90 percent intact [Tyr^{10}-^{125}I]VIP was precipitated at the trichloroacetic acid concentration used to measure VIP hydrolysis.

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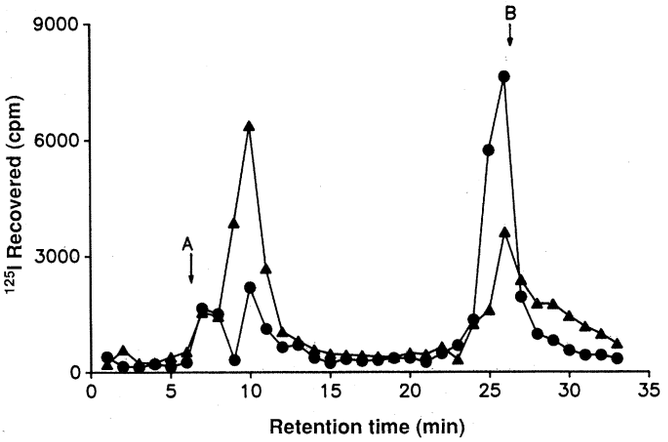

Fig. 2. Reversed-phase HPLC of [Tyr^{10}-^{125}I]VIP treated with IgG (▲) from a human subject. The IgG (75 μg of protein as determined on the assumption that 0.8 mg of IgG per milliliter corresponds to one A_{280} unit) prepared by chromatography on protein G–conjugated Sepharose (*4*) was incubated with [Tyr^{10}-^{125}I]VIP (72 p*M*) for 20 hours at 30°C. The reaction mixture was extracted on an Extractclean C-18 cartridge (Altech) previously equilibrated with 5 ml each of 0.1 percent trifluoroacetic acid (TFA) in water (solvent A) and 0.1 percent TFA in 80 percent acetonitrile (solvent B); the cartridges were washed with solvent A, and the retained peptide was eluted (3 ml of solvent B). Acetonitrile was removed with dry nitrogen and the sample was chromatographed on a Novapak-C18 column (Waters) with a gradient of acetonitrile in TFA [30 to 60 percent solvent B in solvent A over a 45-minute period (0.5 ml/min)]. Effluent radioactivity in 0.5-ml fractions was measured. (A) and (B) show the elution positions of Na ^{125}I (ICN) and [Tyr^{10}-^{125}I]VIP, respectively. The black code shows the profile obtained with IgG heated at 100°C for 10 minutes prior to incubation with [Tyr^{10}-^{125}I]VIP.

Human autoantibodies to and hydrolysis of VIP. VIP is a 28–amino acid peptide originally isolated from the intestine, but now known as a neuropeptide widely distributed in the central and peripheral nervous systems (*6, 7*). There is evidence that VIP modulates neural transmission by classical transmitters and appears to be a neurotransmitter in its own right (*7*). In the human airway, VIP is believed to be a mediator of nonadrenergic, noncholinergic relaxation of smooth muscle (*8*). Asthma, a disorder of airway hyperresponsiveness, may arise from imbalance in autonomic contraction and relaxation of the smooth muscle (*9*). Since a deficiency of VIP may be a causative factor in asthma (*8*), we studied autoantibodies to this peptide in asthma subjects (*4*). The VIP-specific autoantibodies were present in a subpopulation of asthma patients (*4*) and also in some healthy individuals with a history of habitual muscular exercise (*5*). The distinctive property of the autoantibodies from asthma patients, compared to those from healthy subjects, appears to be their higher VIP binding affinity [mean K_d, 0.13 n*M* and 7.7 n*M*, respectively (*4*)].

VIP labeled with ^{125}I, designated [Tyr^{10}-^{125}I]VIP (*10*), was used to study hydrolysis of the peptide by antibody. To compare antibody-mediated and spontaneous hydrolysis of VIP, the labeled VIP was treated with immunoglobulin G (IgG) purified from an individual carrying the antibody to VIP, or with IgG from a nonimmune individual. The precipitation of [Tyr^{10}-^{125}I]VIP with trichloroacetic acid was used as the criterion of VIP hydrolysis. This method had been used previously to measure hydrolysis of mid-sized peptides, including that of VIP (*11*). Increasing duration of treatment with the immune IgG progressively reduced the amount of labeled VIP precipitated by trichloroacetic acid to 37 percent of the starting radioactivity at 6 hours (Fig. 1). In comparison, 92 percent of [Tyr^{10}-^{125}I]VIP incubated with nonimmune IgG was still precipitable by trichloroacetic acid at this time point. Analysis of the antibody-treated peptide by reversed-phase HPLC (Fig. 2) showed a reduced amount of intact [Tyr^{10}-^{125}I]VIP (retention time 25 minutes) and the presence of an early eluting peak of radioactivity with a retention time of 10 minutes (69.9 percent of the initial radioactivity). Heat treatment of the IgG before incubation with [Tyr^{10}-^{125}I]VIP caused a reduction in the amount of radioactivity in the peak with a retention time of 10 minutes. When [Tyr^{10}-^{125}I]VIP was incubated in buffer instead of the IgG, most of the radioactivity was recovered in the form of intact peptide (retention time, 25 minutes), and only 13.9 percent in the peak which had a retention time of 10 minutes. These data suggest that the IgG hydrolyzed a peptide bond in VIP. The detection of only one radioactive peak distinct from intact [Tyr^{10}-^{125}I]VIP in the reversed-phase HPLC indicates that the IgG cleaved a single peptide bond in VIP. To

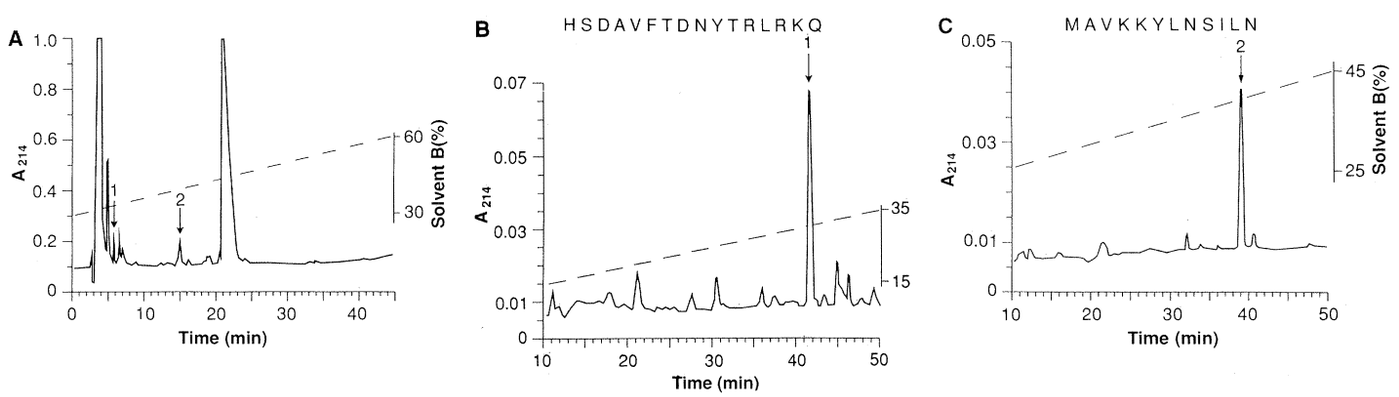

Fig. 3. Identification of VIP fragment produced by anti-VIP. VIP (50 μg) was treated with 525 μg of immune or nonimmune IgG as before, except that bovine serum albumin was absent in the reaction mixture and incubation was for 3.5 hours at 38°C. The reaction mixtures were extracted on Extractclean C-18 cartridges as in Fig. 2, the extracts were dried by centrifugation at reduced pressure (Speedvac, Savant), redissolved in solvent A, and subjected to reversed-phase HPLC on a Novapak C-18 column (Waters Associates, Millipore, Milford, Massachusetts) and an ISCO (Lincoln, Nebraska) HPLC apparatus. Elution was with a gradient of solvent B (flow rate: 0.5 ml/min). The absorbance of the eluate was monitored at 214 nm and 0.5-ml fractions were collected. Most of the A_{214} absorbing material seen after treatment of VIP with the antibody (**A**) was in a peak with retention time similar to that of intact VIP (21.3 minutes). The peaks labeled 1 and 2 were missing in chromatograms of VIP treated with buffer or a nonimmune antibody fraction. Peptides 1 and 2 were purified by rechromatography (**B** and **C**, respectively) at lower starting concentrations of solvent B, and shallower gradients for elution. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

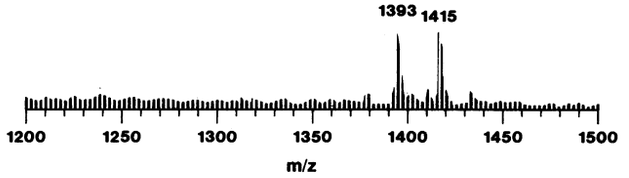

Fig. 4. Partial positive ion fast atom bombardment–mass spectrum (*m/z* 1200 to 1500) of VIP(17–28) produced by antibody-mediated hydrolysis of VIP. Peptide 2 from Fig. 3 was subjected to fast atom bombardment–mass spectrometry in the positive ion mode with a VG Analytical ZAB-2SE spectrometer (acceleration potential, 8 kV) with peptides dissolved in 5 percent acetic acid and thioglycerol or glycerol or *m*-nitrobenzyl alcohol matrices. Mass calibration was with cesium iodide and a mixture of cesium iodide and glycerol. The signal at 1393 daltons corresponds to the molecular ion of VIP(17–28).

identify the VIP fragments produced in this reaction, we used unlabeled VIP as substrate. Reversed-phase HPLC of unlabeled VIP treated with the immune IgG revealed two peptide peaks (labeled 1 and 2 in Fig. 3A) that were absent in the VIP preparation treated with nonimmune IgG. These peptides were purified by a second round of HPLC (Fig. 3, B and C) and sequenced with a pulsed liquid phase sequenator (Applied Biosystem, model 477A) with on-line phenylthiohydantoin amino acid detection. Peptide 1 was a 16–amino acid peptide that started with an NH_2-terminal His, ended with a COOH-terminal Gln, and was identical in sequence with VIP(1–16). Peptide 2 had an NH_2-terminal Met, a COOH-terminal Asn, and was identical in sequence with VIP(17–28). Confirmatory evidence that fragment 2 is VIP(17–28) was obtained by fast atom bombardment–mass spectrometry (Fig. 4). The mass spectrum indicated a molecular mass of 1393 daltons for this peptide, a value corresponding closely to the theoretical mass of VIP(17–28). The additional signal at 1415 daltons probably represents the sodium adduct of VIP(17–28). Intact VIP(1–28) gave a signal at 3325 daltons that corresponds well to the molecular mass of the peptide. The radioactive peak produced in Fig. 2 (retention time, 10 minutes) by treatment of [Tyr^{10}-^{125}I]VIP with IgG is probably [Tyr^{10}-^{125}I]VIP(1–16). The difference in retention times (2.6 minutes) between the iodinated peptide and unlabeled VIP(1–16) purified in Fig. 3A is attributable to the size of the ^{125}I atom (*12*).

The possibility that the IgG was contaminated with a conventional peptidase was considered. While cell surface receptors (*13*) and intracellular regulators of IgG secretion (*14*) are well known, tight binding of conventional peptidases with IgG is unlikely. Evidence that the VIP hydrolytic agent was an antibody, not a contaminating peptidase, is summarized as follows: (i) The IgG did not contain detectable nonimmunoglobulin material, as indicated by polyacrylamide gel electrophoresis and subsequent silver staining and immunoblotting (*15*); (ii) the Fab fragment of IgG, prepared by papain treatment and chromatography on immobilized protein A, exhibited a molecular mass close to 50 kD and it hydrolyzed VIP (Fig. 5); (iii) the hydrolytic activity of intact IgG was retained on immobilized protein G, an agent that binds IgG via its Fc region, and then released by low *p*H treatment (Fig. 6); (iv) gel filtration chromatography of the IgG revealed a single peak of hydrolytic activity with a molecular mass close to 150 kD, as determined by comparison with marker proteins (*16*); (v) of the original VIP hydrolytic activity present in IgG purified by DEAE-cellulose chromatography, 78 and 80 percent was preserved in the retentate after ultrafiltration on a 100-kD cutoff filter and in the ammonium sulfate precipitable fraction, respectively (*17*); (vi) treatment of the IgG with antibody to human IgG and removal of the immunoprecipitate decreased the hydrolytic activity by 75 percent (*18*); (vii) only two of six immune IgG preparations showed hydrolytic activity, and nonimmune IgG was without activity; (viii) gel filtration of the IgG under conditions that favor disruption of noncovalent protein interactions did not remove the hydrolytic activity of the IgG (*19*); (ix) the observed K_m value (see below) suggests hydrolysis of VIP by a relatively tight binding agent, such as an antibody [K_m values for VIP hydrolysis by peptidases are higher by three to four orders of magnitude (*20, 21*)]; and (x) the scissile bond observed in our study (Gln^{16}-Met^{17}) is different from the known peptidase-sensitive bonds in VIP (*21*).

Catalytic nature of autoantibody-mediated VIP hydrolysis. The amount of [Tyr^{10}-^{125}I]VIP rendered soluble in trichloroacetic acid (TCA) by the antibody was a faithful indicator of hydrolysis of the peptide. The amount of radioactivity rendered TCA-soluble after treatment with the antibody correlated well with the early eluting peak of radioactivity noted in the HPLC analysis (74.2 and 69.9 percent of the total radioactivity analyzed, respectively). Hydrolysis of [Tyr^{10}-^{125}I]VIP by the antibody fraction, measured as the amount of TCA-soluble radioactivity, was dependent on the *p*H of the assay diluent. Maximal hydrolytic activity was evident at *p*H 8 to 8.5 (*22*). The kinetics of antibody-mediated hydrolysis were examined by incubating IgG with increasing concentrations of unlabeled VIP mixed with a fixed concentration of [Tyr^{10}-^{125}I]VIP as tracer. The hydrolysis was saturable with increasing VIP concentrations and the curves of the reciprocal of the velocity plotted as a function of the reciprocal of the substrate were essentially linear (Fig. 7A), suggesting that the reaction conformed to Michaelis-Menton kinetics. The K_m (±SE) for the reaction was 37.9 (±5.5) n*M*, a value smaller than that for peptidases, suggesting relatively tight binding of VIP by antibody. The Scatchard plot of VIP binding by the antibody, under conditions that did not lead to VIP hydrolysis (*23*), was linear (Fig. 7B). The slope for the Hill plot was close to unity (1.02). These data suggested a single antibody class with K_d of 0.4

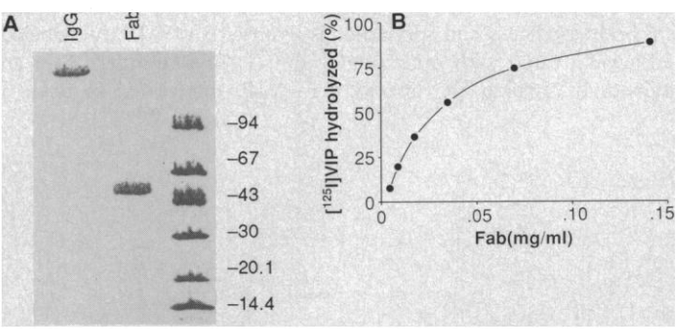

Fig. 5. VIP hydrolysis by Fab prepared from IgG. The IgG (10 mg in 0.5 ml) was treated with papain conjugated to agarose (Pierce) (1.5 ml in 20 m*M* sodium hydrogen phosphate, *p*H 7.0, 10 m*M* EDTA and 20 m*M* cysteine) for 5 hours at 38°C with vigorous shaking. The mixture was centrifuged, and Fab in the supernatant was purified by chromatography on protein A conjugated to agarose (2.2-ml gel; Pierce). The column was washed with 10 m*M* tris-HCl, *p*H 7.5, to recover unretained Fab. This fraction was concentrated by ultrafiltration on an Amicon YM-10 filter. The retentate, the parent IgG fraction, and marker proteins (right lane) were subjected to electrophoresis on an 8 to 25 percent gradient polyacrylamide gel (Phastgel) (Pharmacia) and stained with Coomassie blue (**A**). In (**B**), increasing concentrations of the Fab preparation were incubated with [Tyr^{10}-^{125}I]VIP in 0.05*M* tris-HCl, 0.1*M* glycine, *p*H 8.0, containing 0.025 percent Tween 20 for 3 hours at 38°C. Trichloroacetic acid was added to 10 percent (v/v), the tubes were centrifuged (5800*g*, 20 minutes), the supernatants were aspirated and the radioactivity (^{125}I) in the pellets was determined. Values are means of three replicates (the SD values were 0.6 to 3.1 percent) expressed as the percentage of radioactivity rendered soluble in trichloroacetic acid by the Fab.

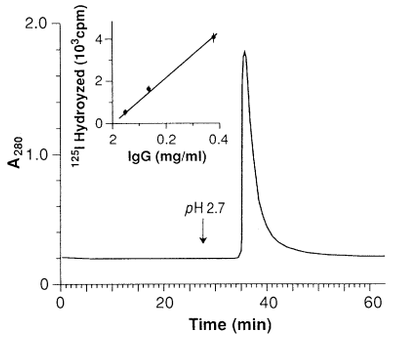

Fig. 6. Chromatography of the VIP hydrolytic IgG on immobilized protein G. Immunoglobulin G purified on a DEAE-cellulose column was chromatographed on protein G conjugated to Sepharose (Pharmacia) in 50 m*M* tris-HCl, *p*H 7.3. All of the A_{280} was retained by the protein G, and then released upon application of a low *p*H buffer (0.1*M* glycine-HCl, *p*H 2.7). The eluate fractions were adjusted to *p*H 8 with 1*M* tris-HCl, *p*H 9, pooled, and assayed for VIP hydrolytic activity (inset) as in Fig. 1.

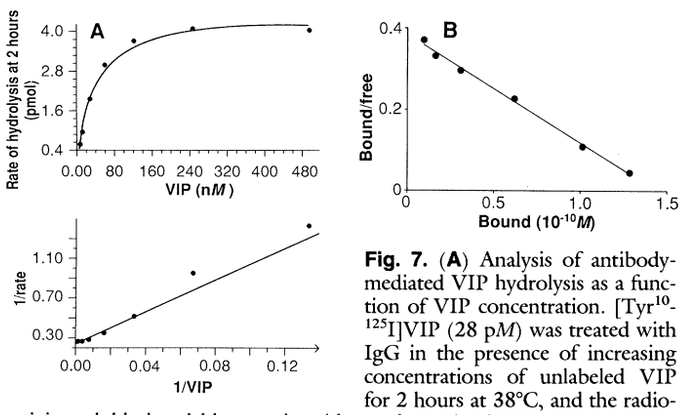

Fig. 7. (**A**) Analysis of antibody-mediated VIP hydrolysis as a function of VIP concentration. [Tyr^{10}-^{125}I]VIP (28 p*M*) was treated with IgG in the presence of increasing concentrations of unlabeled VIP for 2 hours at 38°C, and the radioactivity soluble in trichloroacetic acid was determined. (Upper panel) The values plotted represent the amount of hydrolysis (picomoles per 2 hours) determined at each concentration of VIP (nanomolar). The lower panel represents the reciprocals of the values in the upper panel. Data were fitted to the Michaelis-Menton equation by means of the ENZFITTER program (Elsevier). (**B**) Scatchard analysis of VIP binding by the IgG. Binding of [Tyr^{10}-^{125}I]VIP (40 p*M*) with IgG (1 mg/ml, prepared without performing the ultrafiltration step) was in the presence of increasing concentrations of unlabeled VIP in 200 μl of radioimmunoassay diluent for 20 hours at 4°C; and bound peptide was separated with polyethylene glycol (*4*). Data were analyzed by means of EBDA and LIGAND (*27*).

n*M* and IgG concentration 73.4 fmol/mg (if we assume antibody bivalency). The turnover number and k_{cat}/K_m values for the antibody, computed on the basis of kinetics of hydrolysis and antibody concentration obtained from binding data, were 15.6 ± 0.6 min^{-1} and 4.1 × $10^7 M^{-1}$ min^{-1}. These values suggest that the antibody to VIP (anti-VIP) acts catalytically to hydrolyze VIP. The catalytic efficiency of the anti-VIP is not large compared to classical peptidases, but is comparable to previously described catalytic antibodies to transition state analogues of acyl group transfer reactions (*1*).

The mechanism of antibody-catalyzed hydrolysis of the Gln^{16}-Met^{17} bond in VIP is not known. Amino acid residues within the antibody combining site may participate actively in the hydrolysis of this bond, in the same way that enzymes cleave peptide bonds. Alternatively, antibody binding to VIP could introduce electronic strain in the Gln^{16}-Met^{17} bond, or favor a peptide conformation that accelerates intramolecular nucleophilic attack of the peptide bond carbonyl by the nitrogen in the side chain of glutamine. Enhanced cleavage rates of peptides at the carboxyl side of Asn has been suggested to occur via a similar intramolecular reaction pathway (*24*). The HPLC profiles of dilute solutions of [Tyr^{10}-^{125}I]VIP kept in buffer showed a small peak of radioactivity with a retention time similar to that of [Tyr^{10}-^{125}I]VIP(1–16). It is possible that the Gln^{16}-Met^{17} linkage in VIP may be relatively unstable, facilitating antibody-mediated cleavage of this bond.

Catalytic antibodies to peptides, their disease-causing potential, and possible therapeutic application. We observed catalytic cleavage of a peptide bond in VIP by a human autoantibody. The demonstration of peptide bond hydrolysis by an antibody fulfills a major goal in catalytic antibody research. While this article was in editorial review, Iverson and Lerner (*25*) described an antibody that hydrolyzed a Gly-Phe bond, provided that a metal cofactor was present. The turnover number described for this antibody is smaller than the catalytic VIP autoantibody we have identified by more than two orders of magnitude (6 × 10^{-4} sec^{-1} compared to 0.26 sec^{-1}, respectively).

The identification of antibodies that have a high catalytic efficiency and act as site-specific proteases may provide new ways of treating disease. These antibodies could also serve as reagents analogous to nucleic acid restriction enzymes. One approach in generating such antibodies is to utilize transition state analogues of specific peptide bonds with appropriate flanking sequences as the immunogen. The second approach consists of further exploration of the autoimmune repertoire to identify catalytic antibodies that may be useful as laboratory reagents or for therapeutic purposes. Further studies on the mechanism of peptide bond cleavage by the VIP autoantibody are likely to provide clues for designing high-efficiency catalytic antibodies. The catalytic property of the autoantibody may derive from its ability to bind the transition state conformation of VIP better than the ground state conformation. The antigenic structure responsible for provoking the formation of the catalytic autoantibody to VIP is not known. The possibility that a fleeting transition state encountered during hydrolysis of susceptible bonds in VIP by peptidases serves as the immunogen cannot be ruled out.

Antibodies capable of catalytic peptide bond hydrolysis are likely to inactivate target antigens at higher rates than antibodies that bind antigens but do not mediate their hydrolysis. Catalytic autoantibodies to peptide epitopes thus have a high potential for contributing to the pathophysiology of autoimmune diseases. Autoantibodies to VIP are present in some asthma patients. Although the pathophysiology of catalytic VIP autoantibody was not the subject of our study, such an autoantibody could be a major factor in disorders that may arise from a deficiency of VIP, such as asthma.

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10. Synthetic porcine VIP (3 μg; Bachem) was labeled for 30 seconds with ^{125}I in the presence of 10 μg of chloramine T [S. Paul, K. Wood, S. I. Said, *Peptides* **5**, 1085 (1984)], except that bacitracin was not added as stabilizer. Reversed-phase HPLC (Fig. 3) resolved a major peak of radioactivity that had a retention time of 25.3 minutes and reacted with rabbit antiserum to VIP in radioimmunoassay. In order to obtain sufficient peptide for chemical identification, 50 μg of VIP was labeled with ^{127}I containing a small amount of ^{125}I. Amino acid sequencing of the iodinated VIP yielded radioactivity mainly in cycle 10. The HPLC characteristics of the amino acid obtained in cycle 10 were identical to those of standard monoiodotyrosine (Calbiochem), indicating that the radioactive peptide was [Tyr^{10}-^{125}I]VIP.

Although oxidation of methionine is possible during iodination of peptides with chloramine T, amino acid composition analysis showed no evidence for the presence of methionine sulfoxide or sulfone in [Tyr^{10}-^{125}I]VIP.
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12. The retention time of unlabeled VIP is smaller than that of [Tyr^{10}-^{125}I]VIP for the same reason (21.6 and 25.3 minutes, respectively).
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15. IgG (50 μg) was subjected to electrophoresis in 12 to 20 percent polyacrylamide gels. Silver staining revealed one major IgG band and a minor light chain band of 150 kD and 25 kD, respectively. A nitrocellulose blot of the gel was first treated with rabbit antibody to human IgG that was conjugated to peroxidase (Accurate) and then stained with diaminobenzidine and hydrogen peroxide. Both bands were reactive with the antibody to human IgG.
16. Gel filtration of the protein-G purified IgG was on a Superose-12 column (Pharmacia) in 50 m*M* tris-HCl, *p*H 8 (0.5 ml/min). Marker proteins used for size determination were ferritin, catalase, bovine serum albumin, and chymotrypsinogen.
17. The IgG was diluted to 0.1 mg/ml and subjected to ultrafiltration (Amicon YM-100) to 2.2 mg/ml. The IgG was precipitated with 50 percent saturated ammonium sulfate and the precipitate was redissolved in assay diluent (legend to Fig. 1), dialyzed, and assayed for hydrolytic activity.
18. The immune IgG (450 μg, 100 μl) was incubated with 700 μl of assay diluent or goat antibody to human IgG (purified by chromatography on immobilized protein G as in Fig. 4, diluted 13.5-fold; Antibodies, Inc.) for 45 minutes at 4°C; the precipitate was removed by centrifugation and the supernatants were tested for VIP hydrolytic activity. The retention of a proportion (25 percent) of the starting VIP hydrolytic activity in the supernatant (see text) was probably due to incomplete IgG precipitation.
19. IgG purified by chromatography on immobilized protein G was brought to *p*H 2.7 with 0.1*M* HCl, and chromatographed on a Proteinpak 300 sw gel filtration column (Waters) at *p*H 2.7; the effluent fractions were neutralized with 1*M* tris-HCl, *p*H 9. The chromatography at *p*H 2.7 resulted in delayed elution of the IgG (compared to *p*H 8.0), probably because of interaction of IgG with the column matrix. Fractions corresponding to elution volumes of 7.5 to 10 ml had [Tyr^{10}-^{125}I]VIP hydrolytic activity (523 to 1950 cpm). Each of these fractions exhibited a single protein band in SDS–polyacrylamide gel electrophoresis that was identified as IgG on the basis of its molecular size (150 kD) and reaction with antibody to human IgG in immunoblots.
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22. The *p*H of the assay diluent was varied from 4.0 to 9.0 by addition of HCl or NaOH.
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