Stabilization of Vasoactive Intestinal Peptide by Lipids

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ABSTRACT

An anionic phospholipid, phosphatidylglycerol (PG), induced vasoactive intestinal peptide (VIP) to adopt a helical conformation, determined by circular dichroism studies. PG inhibited the trypsin-catalyzed, antibody-catalyzed and uncatalyzed cleavage of VIP, measured by radiometric and HPLC methods. Phosphatidylcholine, a neutral lipid, did not alter the circular dichroism spectra of VIP, and it was without detectable effect on the rates of VIP cleavage. Tryptsin-catalyzed cleavage of Boc-Ile-Glu-Arg-methylcoumarinamide, a substrate unrelated to VIP, was decreased by about 5-fold compared with aqueous VIP. These observations indicate that certain phospholipids and liposomes can be applied to circumvent the rapid loss of VIP in vitro and in vivo due to degradative processes.

The biological actions of neurotransmitters and neuropeptides are terminated rapidly after their release from neurons. In the case of the 28-amino acid neuropeptide VIP, three types of degradative pathways have been identified: 1) cleavage by enzymes such as serine proteases (Caughey et al., 1988; Tam et al., 1990) and neutral endopeptidase (Hachisu et al., 1991), 2) antibody-catalyzed cleavage, which is described to occur in certain disease states (Paul et al., 1991; Gao et al., 1994), and 3) uncatalyzed spontaneous hydrolysis, a process that occurs at comparatively high rates at low VIP concentrations (Mody et al., 1994). These processes are generally assumed to be responsible for the rapid disappearance of VIP from blood (T1/2 = 0.4–1.0 min) after administration of the peptide to experimental animals and humans (Domschke et al., 1978; Mitchell et al., 1982; Hassan et al., 1994).

VIP is a remarkably versatile peptide. It is broadly distributed in the CNS and peripheral nervous system, and it exerts diverse biological effects, including smooth muscle relaxation, anti-inflammatory and immunomodulatory effects, regulation of the production of certain hormones, regulation of transport phenomena across excocrine epithelia and modulation of cell proliferation (for reviews see Yanaihara, 1992; Paul and Ebadi, 1993). Deficiencies of VIP have been described in the airways in asthma (Ollerenshaw et al., 1989), in the skin in cystic fibrosis (Heinz et al., 1985), in the penis in impotent males (Crowe et al., 1983) and in the GI tract in ulcerative colitis (Surrenti et al., 1993). Administration of exogenous VIP in patients with such deficiencies could potentially be of therapeutic benefit. Even in instances where a deficit of VIP is not a pathophysiological factor, it may be possible to treat the disease with VIP. For instance, the potent anti-inflammatory effects of VIP (for reviews see Yanaihara, 1992; Paul and Ebadi, 1993) suggest its possible broad utility in controlling tissue inflammation.

It is generally held that the rapid degradation of VIP is a major factor limiting the potential clinical applications of this peptide. VIP can be modeled as a cationic amphipathic helix (Musso et al., 1988), which is a common structural motif found in lipid-binding proteins and peptides (Sansom, 1991). In an effect consistent with its predicted lipophilicity, VIP displays reversible and saturable binding to protein-free lipid bilayers (Noda et al., 1994). In the present report, we describe the ability of a negatively charged phospholipid, and of liposomes, to confer helical character to VIP, reduce the uncatalyzed and catalyzed degradation of VIP in vitro and increase the longevity of circulating VIP in mice. These studies suggest that the lipophilicity of VIP can be applied to developing therapeutically useful formulations of the peptide.

Study of the kinetics of antibody-catalyzed VIP cleavage indicated that the inhibition by PG was due to decreased affinity for VIP, suggested by observations of increased Kmax values and unaltered Vmax values. Incorporation of VIP in the liposomes and the liposomal surface permitted maintenance of the peptide in essentially undegraded form at 37°C for 8 days. The longevity of liposomal VIP administered i.v. to mice was increased by about 5-fold compared with aqueous VIP. These observations indicate that certain phospholipids and liposomes can be applied to circumvent the rapid loss of VIP in vitro and in vivo due to degradative processes.

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ABBREVIATIONS: BSA, bovine serum albumin; CD, circular dichroism; PG, phosphatidylglycerol; PC, phosphatidylcholine; TCA, trichloroacetic acid; VIP, vasoactive intestinal peptide.
Materials and Methods

VIP. Synthetic human VIP (HSDAVFDTNYTRLLKGAVKLY
LNSILN-NH2; peptide content 81%; Bachem, Torrance, CA) was labeled with 125I using chloramine-T. (Tyr10-125I]VIP was separated by reverse-phase HPLC on a NovaPak C18 column (Waters, Milford, MA) using an ISCO HPLC apparatus (Mody et al., 1994). The radiolabeled VIP eluted from the HPLC column at 40% acetonitrile in 0.1% trifluoroacetic acid was diluted with an equal volume of 0.2 M acetic acid containing 0.5% BSA (Sigma, St. Louis, MO, RIA grade) and stored at -80°C in aliquots. Maintenance of the VIP at acidic pH and in the presence of a stabilizing protein is necessary to minimize the uncatalyzed degradation of the peptide (Mody et al., 1994). The specific activity of the peptide was ~2000 Ci/nmol. Unlabeled VIP synthesized at the University of Florida, Gainesville, and purified by preparative reverse-phase HPLC on a C-18 column was used in certain experiments. The peptide content of this preparation was 83%, and its purity was confirmed by amino acid analysis and N-terminal sequencing at the University of Nebraska Protein Structure Core Facility.

Peptide hydrolysis. Antibodies to VIP were purified from the plasma of a human subject (code #80) by ammonium sulfate precipitation, protein G-Sepharose chromatography and affinity chromatography on VIP-Sepharose (Paul et al., 1991). The final antibody preparation was electrophoretically homogeneous as assessed by SDS-gel electrophoresis under reducing and nonreducing conditions (Paul et al., 1991). Antibody-catalyzed cleavage of [Tyr10-125I]VIP (30-100 pm) was determined by incubation at 37°C in 200 μl of 0.05 M HEPES buffer, pH 7.4, 0.025% Tween-20, 0.1% BSA, 0.02% sodium azide (Paul et al., 1991). Uncatalyzed and trypsin-catalyzed cleavage of VIP were assayed similarly, except that BSA was not included in the assay diluent. For these assays, the [Tyr10-125I]VIP was thawed, diluted 50-fold with in 0.1% trifluoroacetic acid and extracted on a SepPak C18 cartridge as described previously (Mody et al., 1994). This step was necessary to remove the stabilizer BSA present in the radiolabeled peptide. Hydrolysis of the peptide was measured by addition of TCA (final concentration 10% w/v) and carrier BSA (25 μL of 10 mg/ml solution) followed by centrifugation and counting of the acid-precipitated pellet in a γ-spectrometer (approximate efficiency 80%). The amount of radioactivity rendered acid-soluble represents the extent of VIP degradation. Estimates of peptide hydrolysis by this method are essentially identical to those observed by separating degraded and intact peptide via reverse-phase HPLC (Mody et al., 1994). Cleavage of Boc-Ile-Glu-Arg-methylcoumarinamide (Peptides International, Louisville, KY) was estimated as the fluorescence of the coumarin leaving group (λex=460nm, λem=370nm) using a Perkin-Elmer LS50 fluorometer (Gao et al., 1994). Bovine pancreatic trypsin (3X crystallized) was from USB Corp.

Lipids and liposomes. Chloroform solutions of egg yolk PC (10 mg), PG (5 mg, dioleoyl, ammonium salt) and cholesterol (6.4 mg) (Sigma) were mixed in a pear-bottomed flask and dried under reduced pressure in a rotary evaporator. The lipids were rehydrated with 100 μl of 0.15 M sodium chloride containing BSA-free [Tyr10-125I]VIP and, in certain experiments, 0.7 mg of unlabeled synthetic VIP. The suspension was subjected to five cycles of freeze-thawing using a dry ice-acetone bath and a water bath maintained at 37°C. The volume was made up to 500 μl with 0.15 M NaCl, and the liposomes were separated from the unincorporated VIP by passage through a Sepharose 4B column (Pharmacia, Uppsala, Sweden; 10 ml gel packed in a 1.5 x 12 cm disposable column). The liposomes were recovered at the column void volume. The average incorporation of VIP in the liposomes in six preparations was 39% (±10%, S.D.), determined as (cpm radioactivity recovered in the liposome fraction/original cpm radioactivity) x 100. Incorporation of phospholipids into the liposomes was measured on the basis of their inorganic phosphorous content (Noda et al., 1994): it was routinely greater than 90% of the initial phospholipid concentration. When unlabeled VIP was used, 0.004 ± 0.0008 mol of VIP was incorporated per mole of phospholipid. The size of the liposomes, analyzed by quasi-elastic light scattering (Nicomp model 270 submicron particle sizer, Pacific Scientific, Menlo Park, CA), was 675 ± 71 nm.

Peptide release and degradation in liposome formulation. VIP-containing liposomes were incubated in the absence and presence of human serum (25% v/v in 0.15 M NaCl) at 37°C. Released VIP was separated from liposomal VIP by passing the incubation mixture through a Sepharose 4B column (2 ml gel; 0.8 x 4 cm column) equilibrated with 0.15 M NaCl. The liposomes were recovered quantitatively from the column at V, 0.8 ml, on the basis of measurement of the turbidity at λmax, and counted for residual radioactivity. Percent release of the VIP was calculated as: [cpm 125I-VIP applied to the column - cpm 125I-VIP contained in the liposome fraction]/cpm 125I-VIP applied to the column x 100. The amount of degraded peptide in the liposome fraction was measured by two cycles of freeze-thawing (immersion in dry ice/acetone bath; rapid thawing at 37°C) followed by separation of intact and degraded VIP by precipitation with 10% TCA or HPLC.

VIP half-life in circulation. Aqueous [Tyr10-125I]VIP or liposomal VIP in 10 mM sodium phosphate, pH 7.4, 0.15 M sodium chloride was injected into the tail vein of 6-week-old Swiss-Webster mice (106 cpmmouse). The mice were euthanized 2, 5, 15, 30, 60 and 120 min later by carbon dioxide asphyxiation. Blood from the heart was collected in protease inhibitors (final concentration 100 U Trasylol/ml, 5 μM Pepstatin A, 50 μM phenylmethylsulfonyl fluoride, 0.003% w/v EDTA; Sigma) and centrifuged for 15 min at 400 xg. The plasma samples were subjected to two cycles of freeze-thawing, as described in the preceding paragraph, to disrupt the liposomes. Peptide degradation was estimated as above. Values of half-lives were estimated using the computer program Pharmkit (version 3 Beta, by A. Johnston and R. Wollard).

CD. Synthetic VIP (20 μM) in 0.05 M HEPES, 0.025% Tween-20, pH 7.4, was mixed with phospholipid stock solutions in methanol (final methanol concentration 0.5%). CD spectra were recorded at 25°C using a Jasco J-710 spectropolarimeter equipped with a DP-500 data processor. All measurements were made using quartz cuvettes with 1-mm path length. Calibration was with d-10-camphorsulfonic acid. Five spectra were accumulated for each sample, and average spectra are shown. Structure assignments were computed as in (Sreerama and Woody, 1993).

Results

Conformation and stability of aqueous VIP. The CD spectra of VIP were markedly different in the absence and presence of the anionic phospholipid PG (fig. 1). The intensity of the negative ellipticity at 222 nm was reproducibly in-
creased \((n = 3)\) in the presence of PG, a result that suggests increased helicity. In comparison, the CD spectra of VIP in the absence and presence of the neutral phospholipid PC were nearly indistinguishable. Nominal values of the proportion of peptide present in the form of an \(\alpha\)-helix, \(\beta\)-sheet, \(\beta\)-turn, PII helix and unordered structure were 14, 25, 6, 29 and 26\%, respectively, in buffer without phospholipid, compared with values of 54, 12, 11, 12 and 12\%, respectively, in a 30 \(\mu\)M PG solution, computed from representative spectra. Dose-response studies (not shown) suggested that the concentrations of PG required to induce the increase in negative ellipticity at 222 nm were between 10 \(\mu\)M and 50 \(\mu\)M.

Inclusion of PG in the assay diluent resulted in decreased trypsin-catalyzed (fig. 2 A) and autoantibody-catalyzed (fig. 2 B) cleavage of radiolabeled VIP. An equivalent concentration (30 \(\mu\)M) of PC was without effect on the rate of VIP cleavage by either catalyst. PG (50 \(\mu\)M) did not inhibit the trypsin-catalyzed hydrolysis of Boc-Ile-Glu-Gly-Arg-MCA (\(\Delta F\) in arbitrary units, 145 ± 9/15 min and 135 ± 7/15 min in the absence and presence of PG, respectively; trypsin, 1 nM; substrate, 3.3 \(\mu\)M), which suggests that the interaction with VIP, rather than the catalyst, underlies the inhibitory effect of the phospholipid. The inhibition by PG was concentration-dependent, with sharply reduced trypsin-catalyzed and autoantibody-catalyzed cleavage observed between 10 and 100 \(\mu\)M PG (not shown). This is also the concentration range at which PG was observed to inhibit the uncatalyzed cleavage of VIP (fig. 2 C). Because no catalysts are present in the latter reaction, the inhibition can be interpreted to derive from a direct interaction of the PG with VIP.

The initial rates of the antibody-catalyzed reaction measured at increasing concentrations of VIP were consistent with the Michaelis-Menten-Henri equation. The \(K_m\) value for the antibody was in the low nanomolar range, which is consistent with observations that antibody catalysis is characterized by high-affinity recognition of the substrate (Paul et al., 1991; Gao et al., 1994). Inclusion of PG in the solvent resulted in a 5-fold increase in \(K_m\) without a significant change in the \(V_{max}\) value for the reaction (table 1). Thus decreased hydrolysis in the presence of PG may be attributed to impaired recognition of the substrate by the antibody, with no evident effect on the catalytic rate constant.

**Stability and release of liposomal VIP.** The stabilization of VIP was even more pronounced by incorporation of VIP in liposomes. Measurement of the rates of cleavage at increasing trypsin concentrations suggested that the liposomal VIP was 3 to 4 orders of magnitude less susceptible to the trypsin-catalyzed reaction than was aqueous VIP (fig. 3). To validate the method employed for estimation of the cleavage of liposomal VIP (i.e., freeze-thawing of the liposome suspension followed by separation of the intact peptide and its cleavage products by TCA precipitation), we analyzed by RP-HPLC selected reaction mixtures of the peptide incubated with trypsin (fig. 4). The two methods yielded essentially identical values of liposomal VIP cleavage (RP-HPLC, 60.2\%; TCA precipitation, 69.4\%). The retention times of the major radioactivity peaks corresponding to the product fragments of aqueous and liposomal VIP were similar. However, the degradation products of liposomal VIP eluted more sharply, with most of the radioactivity focused at retention times of 18 to 19 min. Because VIP contains multiple peptide bonds susceptible to cleavage by trypsin (arg\(_{12}\), leu\(_{13}\), arg\(_{14}\), lys\(_{15}\), lys\(_{20}\), lys\(_{21}\), lys\(_{21}\)-tyr\(_{22}\)), it is possible that interactions with lipids can direct the cleavage to occur at certain peptide bond(s).

**Table 1**

<table>
<thead>
<tr>
<th>PG ((\mu)M)</th>
<th>(K_m) (nM)</th>
<th>(V_{max}) (nM VIP/40 nM Ab/8 h)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>21.8 ± 6.2</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>30</td>
<td>108.2 ± 29.8</td>
<td>13.2 ± 1.5</td>
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Values are means from three experiments, each of which was performed using two replicates for each substrate concentration ± S.D.

**Fig. 2.** Effect of PG on uncatalyzed and catalyzed hydrolysis of VIP. In panels A and B, [Tyr\(_{10}\)-\(^{125}\)I]VIP (40 nM) was incubated with trypsin (1 nM) and affinity-purified catalytic autoantibodies (21 nM), respectively, at 37°C. The reaction mixtures contained 30 \(\mu\)M PC or PG as indicated. VIP hydrolysis was determined as the acid-soluble radioactivity expressed as percent of total available radioactivity after subtraction of the background acid-soluble peptide (<10% of total radioactivity) observed by incubation in assay diluent without catalysts. Background values of acid-soluble radioactivity were essentially identical in the absence and presence of the phospholipids. In the uncatalyzed cleavage reaction (panel C) [Tyr\(_{10}\)-\(^{125}\)I]VIP (40 nM) was incubated at 37°C in diluent without stabilizing proteins at increasing PG concentrations, and the acid-soluble radioactivity was measured at 6, 24 and 48 h. Data represent percent of total available radioactivity and are means of three replicates ± S.D.
Less than 25% of the radiolabeled VIP incorporated into liposomes was released into the aqueous phase monitored over 8 days at 37°C (fig. 5). Inclusion of plasma in the incubation mixture increased the release of the peptide only marginally. Of the residual radioactivity associated with the liposomes over the 8-day period, more than 90% was observed to represent the undigested peptide. It may be concluded that the liposomal VIP is not susceptible to the uncatalyzed cleavage reaction observed with aqueous VIP preparations.

Stability of liposomal VIP in vivo. As described previously by other investigators (Domschke et al., 1978; Mitchell et al., 1982; Hassan et al., 1994), aqueous VIP administered i.v. to mice was rapidly removed from the blood (fig. 6). At 2 min and 5 min after administration, 5.6-fold and 4.5-fold greater amounts of the liposomal VIP were recovered in the undegraded state compared to the aqueous VIP formulation. The elimination curve for liposomal VIP was biexponential, corresponding to $T_{1/2}^\alpha$ and $T_{1/2}^\beta$ values of 5.9 min and 242.1 min, respectively. Precise $T_{1/2}$ values for the aqueous VIP cannot be reliably ascertained from the present data, because only 5.6% of the administered VIP was recovered in the undegraded state at the earliest time-point studied (2 min; calculated from the data in fig. 1 assuming a total plasma volume of 0.9 ml). Previous estimates of the $T_{1/2}$ of VIP in the human, pig and rat are 1.0 min (Domschke et al., 1978), 1.0 min (Mitchell et al., 1982) and 0.4 min (Hassan et al., 1994), respectively.

Discussion

VIP is a basic peptide (3 lys, 2 arg; 2 acidic residues) offering multiple sites at which ion pairing with negatively charged phospholipids could occur. The CD observations described here suggest that interaction with the anionic phospholipid PG induces a helical conformation of VIP. This conclusion is consistent with the studies of Robinson et al. (1982)
showing increased helicity of VIP solutions in water in the presence of sodium dodecylsulfate and phosphatidic acid. The decreased proteolysis of VIP in PG solutions suggests that the helical conformation of VIP is comparatively resistant to antibody-catalyzed and trypsin-catalyzed breakdown. The basic residues in VIP are found in the central segment, spanning residues 12 to 22, which is also the region with the highest propensity for amphipathic helix formation (Robin-son et al., 1982; Musso et al., 1988). The peptide bonds in VIP cleaved by antibodies (Paul et al., 1991) and trypsin (McMaster et al., 1987; Tam et al., 1990) are also located in this segment of VIP. It may be hypothesized that conformational flexibility in the central region of VIP is required for its efficient binding by the catalyst. Assumption of a stable helical conformation in the presence of the anionic phospholipid may therefore limit recognition of the peptide by the catalyst.

In the case of the antibody recognition phenomenon, PG reduced the affinity of binding (increased $K_m$) without altering the rate of the catalytic step (unchanged $V_{max}$ value), indicating that once bound by the antibody, cleavage of the VIP proceeds at similar rates in the absence and presence of PG. Analogous kinetic analyses of the PG effect on the trypsin-catalyzed reaction were not performed, because the large $K_m$ value for trypsin (380 μM) requires the use of millimolar concentrations of VIP and PG in the assays, which introduces difficulties related to solubility and the possibility of nonspecific effects of the lipid on polypeptide conformation.

Consideration of the elimination pathways operational at picomolar to femtomolar concentrations of VIP is important, because the peptide has been observed to elicit biological responses in neurons and several other cell types in this concentration range (Brenneman et al., 1990; Miguel et al., 1992). The biological significance of the uncatalyzed VIP cleavage reaction (Mody et al., 1994) is yet to be determined, but this phenomenon may be an important degradative mechanism at picomolar concentrations of VIP, at which binding (and thus elimination) of the peptide by enzymes and catalytic antibodies is limited by the comparatively low affinities of these proteins (nanomolar to millimolar range). PG was observed to inhibit the uncatalyzed cleavage of VIP. Thus the phospholipid interferes with all the known path-
tween the liposomes and other lipidic structures in the vasculature and peripheral tissues. Furthermore, nonspecific removal of the liposomes by the reticuloendothelial system may result in the comparatively rapid removal of liposomal VIP from blood. The use of sterically stabilized liposomes, which are taken up at reduced levels by the reticuloendothelial system, offers a promising means of improving the longevity of VIP in blood (Sejourne et al., 1997). In view of the improved stability and biological activity of the liposomal VIP, further optimization of the lipid constitution of the formulation intended to prolong the life of VIP in vivo is warranted.

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References


Diabetes 32:875–881.


