Proline in vasoactive peptides: consequences for peptide hydrolysis in the lung

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Many proline-containing peptides exist as relatively slowly equilibrating mixtures of cis and trans rotational isomers at X-Pro imide bonds (11, 33). Some peptidases preferentially hydrolyze trans isomers of proline-containing peptides in solution (11, 12, 14–16, 23, 25) and preferentially bind trans isomers of peptidase inhibitors (2, 30, 32). Peptidases on the luminal endothelial surface of the lung also preferentially hydrolyze trans conformers of certain proline-containing peptides (5, 23–25). Underlying this phenomenon is that the cis-trans isomerization time constants for the relevant imide bonds in these peptides are considerably longer than the pulmonary capillary transit time, thus sparing a fraction of peptide equivalent to the equilibrium cis fraction from hydrolysis during passage through the lung.

To further explore the hypothesis that cis bonds protect physiologically relevant proline-containing peptides from hydrolysis in the pulmonary circulation, we have taken advantage of the fact that two closely related vasoactive peptides that are substrates for lung peptidases, bradykinin and [Gly6]bradykinin, have different equilibrium cis-to-trans ratios at the X6-Pro7 bond (17, 20). In the present study, the fractional hydrolysis of bradykinin and [Gly6]bradykinin in the intact perfused rat lung was compared with both a bioassay technique and high-performance liquid chromatography (HPLC). A mathematical model was developed for kinetic interpretation of the hydrolysis data to estimate cis-trans isomerization rate constants and equilibrium cis-to-trans ratios of the peptides within the lung. We also examined the impact of changing the cis-trans isomerization rates on the hydrolysis kinetics of the peptides in the lung using the peptidyl prolyl cis-trans isomerase cyclophilin, for which both bradykinin and [Gly6]bradykinin are substrates (17, 24).

METHODS

Materials

Bradykinin was purchased from Peninsula Laboratories (Belmont, CA), and [Gly6]bradykinin was from Genosys (The Woodlands, TX). HPLC columns were purchased from MetaChem (Torrance, CA). Fluorescamine, acetonitrile, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Purified human recombinant cyclophilin was prepared under the guidance of Drs. Wei Li and Robert Handschumacher (Yale University School of Medicine, New Haven, CT). The cyclophilin was >95% pure as determined by polyacrylamide gel electrophoresis. The cyclophilin concentration was determined by titration with tritylated cyclosporin A (22), which was obtained from Amersham Life Sciences (Arlington Heights, IL). Unlabeled cyclosporin A was the kind gift of Sandoz Pharmaceutical (Basel, Switzerland). Stock solutions of bradykinin and [Gly6]bradykinin (1 mg peptide/ml H2O) were prepared, divided into 100-μl aliquots, and lyophilized, and the lyophilized aliquots were stored at −20°C. The peptide was redissolved in H2O (100 μl) immediately before use. For HPLC, all H2O was filtered in a Quad Style Nanopure water system containing the type I ORGANICfree cartridge kit (Barnstead) to obtain reagent grade H2O (18.0 MΩ·cm resistivity).

Bioassay Studies

For the bioassay tissue, two 22-μm tungsten wires were threaded through an ~1.5-mm segment of rabbit jugular vein. The wires were stretched over the jaws of two stainless steel
steel rings, one of which was anchored and the other attached to a force transducer as previously described (21, 24). A 750-mg load was applied to the jugular ring, and the vessel segment was allowed to equilibrate in the perfusion system in Krebs-Ringer bicarbonate buffer (KRB; pH 7.4; Po2 100 Torr, PCO2 40 Torr) containing 5 mM glucose and 2.5% bovine serum albumin at 37°C for 60 min. The artificial perfusate was used to minimize any possible contribution of plasma angiotensin-converting enzyme to peptide metabolism. The tissue response to bradykinin was then tested, and afterward, the isolated rat lung was connected to the perfusion circuit so that the jugular vein was superfused with the venous effluent from the lung.

The isolated rat lungs from eight male Sprague-Dawley rats weighing between 285 and 355 g were perfused with KRB as previously described (23, 24). This produced a pulmonary arterial pressure of 6.2 ± 1.6 (SD) Torr, with the venous pressure set at zero. The lungs were ventilated at 30 breaths/min with 6% CO2 and 15% O2, with end-inspiratory and end-expiratory pressures of 5.9 ± 1.3 (SD) and 2.0 ± 0.7 (SD) Torr, respectively. Two injection ports were included in the perfusion circuit such that a 0.1-ml bolus could be introduced either proximal to the lung into the arterial inflow (lung injection site) or into the venous outflow (tissue injection site). The arterial injection resulted in the bolus passing through the lung before it reached the jugular ring. Injection into the venous outflow resulted in the bolus passing directly onto the jugular ring without passing through the lung (Fig. 1). To control for any changes in responsiveness of the tissue or metabolic status of the lung throughout the course of the studies, injections into the lung and tissue sites were alternated.

The dose of peptide required to give contractions of equal force was determined from the log dose-response curves at the lung and tissue injection sites as previously described (24), and the data were compared for significance with the Mann-Whitney rank sum test.

HPLC Studies

Lung perfusion. The lungs from 10 male Wistar rats (235–363 g) were perfused with Krebs bicarbonate buffer containing 5 mM glucose and 2.5% Ficoll (mol wt ~70,000) (23, 24). The lungs were perfused at a flow rate of 0.50 ml/s, which produced a pulmonary arterial pressure of 12 ± 3 (SD) Torr, with a venous pressure of zero. The total volume of the recirculation system including the reservoir, tubing, and lung was ~8.9 ml. Studies of the fraction of peptide surviving passage through the lung were initiated by the addition of bradykinin (2.4, 9.4, or 37.8 nmol) or [Gly6]bradykinin (0.6, 2.4, or 9.6 nmol) to the reservoir with or without cyclophilin (300 nmol). Perfusate samples (~300 µl) were collected from the venous outflow tubing at timed intervals. Figure 2 is a diagram of this recirculating lung perfusion system.

Immediately after collection of each sample, 200 µl of the sample were added to 2 ml of ethanol on ice, and the mixture was centrifuged (5 min at 1,600 g) to remove precipitated Ficoll. The pellet was discarded, and the supernatant was dried under N2 at 37°C and stored at −20°C for HPLC analysis for intact peptide. Standards of bradykinin and [Gly6]bradykinin were prepared in perfusate, the Ficoll was precipitated with ethanol, and the standards were centrifuged and dried under N2 in the same manner as described above for the samples collected from lung perfusate.

HPLC. To detect the small quantities of unhydrolyzed bradykinin and [Gly6]bradykinin remaining in the recirculating reservoir, it was necessary to develop a sensitive HPLC assay for these peptides. The HPLC system consisted of a Hewlett-Packard series 1050 autosampler (model 79852A) fitted with a 100-µl sample loop, a Hewlett-Packard series 1050 dual-piston pump (model 79852A), a Hewlett-Packard helium degasser (model 79865A), a MetaChem Inertsil 5-µm octadecylsilane column (30 × 250 mm, 5 µm), and a Perkin-Elmer model 650-10S fluorescence spectrophotometer containing a 20-µl flow cell as a detector.

The optimal reaction conditions for fluorescamine derivatization of bradykinin were determined by carrying out the derivatization reaction at pH 6.05, 7.01, 8.00, and 9.00 and varying the time of reaction from 1 to 5 min. The maximum fluorescence intensity of the peptide derivative was obtained at pH 8.00 and 9.00 during the first 1–2 min of reaction time, and after 1–2 min, the fluorescence intensity steadily decreased over 5 min. The fluorescence intensity of the reaction was ~20–25% less in solutions having a pH of 6.05 or 7.01 than in the solutions with the higher pH. On the basis of these results, for all studies, the derivatization was carried out at pH 8.00 for 1 min before injection onto the HPLC column.
Samples to be analyzed for bradykinin or [Gly6]bradykinin were dissolved in 200 µl of 0.1 M borate buffer (pH 8.00). The solution was filtered by centrifugation (1,600 g) through a 0.45-µm syringe filter (Titan) to remove any particulate material. A 60-µl portion of the filtrate was transferred to a 250-µl capacity glass conical insert that was placed into a 2.0-ml capacity amber autosampler vial. The derivatization, which was carried out automatically, consisted of drawing 40 µl of the sample and 10 µl of fluorescamine (0.3 mg/ml acetonitrile) into the autosampler loop in which the solutions were mixed at a rate of 0.5 ml/min for 1 min. After 1 additional min to allow for derivatization, the derivatized sample was automatically injected onto the Inertsil column that had been equilibrated in 15% acetonitrile in H2O.

The components of the injected sample were separated at a flow rate of 0.5 ml/min, with a mobil phase consisting of a gradient of 15–45% acetonitrile in H2O over a period of 6 min, followed by an isocratic elution with 45% acetonitrile in H2O for 4 min. The acetonitrile concentration was then increased to 100% over a 3-min period, and the HPLC column was washed in 100% acetonitrile for 3 min. The system was then reequilibrated in 15% acetonitrile in H2O for 10 min. The H2O and acetonitrile were sparged with helium continuously during the chromatography, and the separation was run at room temperature (23°C). The chromatograms were recorded on a Hewlett-Packard model 3396 integrator using the peak area for quantitation against a standard curve.

Typical standard curves representing the fluorescence intensity of the fluorescamine derivative as a function of the amount of bradykinin or [Gly6]bradykinin were linear over the range of 0.3–48 pmol of peptide injected. The retention time was 9.61 ± 0.17 (SD) min for 22 standard injections of bradykinin and 9.62 ± 0.18 (SD) min for 15 standard injections of [Gly6]bradykinin, with amounts of peptide-fluorescamine derivative that spanned the linear range of fluorescence intensity. The amount of unaltered peptide remaining in each reservoir sample injected was calculated from standard curves prepared the day the experiment was carried out. Chromatograms showing the bradykinin present in the venous effluent of the perfused lung at various times after the peptide was introduced into the recirculating reservoir are shown in Fig. 3.

RESULTS

To examine the hypothesis that the equilibrium cis-to-trans ratio of the X6-Pro7 bond of bradykinin and [Gly6]bradykinin is a factor determining the extent of peptide hydrolysis in a single pass through the pulmonary capillary bed, the effects of passage through the lungs on contraction of the rabbit jugular vein were examined for both peptides. Figure 4 shows representative jugular vein contraction dose-response curves for bradykinin and [Gly6]bradykinin injected into the tissue or lung injection sites. The rightward shift of the dose-response curves obtained after lung site injections

![Diagram of lung recirculation studies in which amount of peptide emerging in venous effluent is measured by HPLC.](http://ajplung.physiology.org/)

![HPLC chromatograms of fluorescamine-derivatized bradykinin prepared from lung venous effluent at indicated times after addition of bradykinin to reservoir. Standard, representative chromatogram of a sample collected from perfusion system without the lung (reservoir and tubing only and equivalent to 0 time). Arrows, elution time of bradykinin peak.](http://ajplung.physiology.org/)
compared with tissue site injections reveals peptide hydrolysis in the lungs. The shift was greater for bradykinin than for [Gly6]bradykinin, consistent with a larger fractional hydrolysis of bradykinin. On average, for all such studies carried out with bradykinin, passage through the lung increased the dose required by 25.6 ± 6.4 (SE)-fold to produce a contraction equivalent to one in which bradykinin did not pass through the lung (P < 0.001). With [Gly6]bradykinin, passage through the lung increased the dose required by 7.0 ± 1.4 (SE)-fold to produce a contraction equivalent to one in which [Gly6]bradykinin did not pass through the lung (P < 0.001), which was significantly less than that for bradykinin (P < 0.003).

To obtain more quantitative information regarding the contribution of cis-trans isomerization to the hydrolysis kinetics of the two peptides in the pulmonary circulation, the amount of bradykinin or [Gly6]bradykinin emerging in the venous effluent of the lung perfused from a recirculating reservoir containing the peptides was measured by HPLC (Figs. 5 and 6). For both peptides, there were two phases to the hydrolysis reaction, an initial rapid phase and a subsequent slower phase. The rapid phase is reflected by the fact that by the first sampling time (10 s), there was a substantially lower concentration of peptide in the venous effluent than was originally in the reservoir. The peptide surviving the initial rapid phase was subsequently much more slowly hydrolyzed over a period of ~40 s as shown for bradykinin in Fig. 5. The fraction of [Gly6]bradykinin surviving passage through the lung at the earliest time point was higher than that of bradykinin, but like bradykinin, the peptide surviving the rapid phase subsequently decreased much more slowly until there was no detectable [Gly6]bradykinin remaining in the venous effluent after 80 s (Fig. 6). In three control experiments for each peptide, with no lung in the perfusion circuit, the rate of peptide degradation over the time course of the experiments was not significant, indicating that the disappearance of intact peptide from the lung venous effluent samples required that the peptide pass through the lung. Over the range of peptide doses studied, no systematic effect of dose on the fraction of peptide remaining with respect to time was detected, indicating that lung hydrolysis was first order under the conditions of the study. The addition of cyclophilin to the reservoir containing either peptide

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Fig. 4. Contraction of rabbit jugular vein ring induced by bolus injections of bradykinin (A) or [Gly6]bradykinin (B) introduced into tissue and lung injection sites. In this example, passage through lung increased amount of peptide required to produce a contraction equivalent to one in which peptide did not pass through lung by ~12.5-fold for bradykinin and 3.6-fold for [Gly6]bradykinin.

Fig. 5. Hydrolysis of bradykinin in lung perfused in series with recirculating reservoir containing bradykinin or bradykinin plus cyclophilin. Solid lines, Eqs. 5, 6, 10, 11, and 16 fit to the data, with resulting estimate for initial fraction of peptide in cis (unhydrolyzed) form (\(u\)) of 0.11 and reaction rate constant (\(k_1\)) of 0.14 s\(^{-1}\) for bradykinin and 2.43 s\(^{-1}\) for bradykinin plus cyclophilin.

Fig. 6. Hydrolysis of [Gly6]bradykinin in lung perfused in series with recirculating reservoir containing [Gly6]bradykinin or [Gly6]bradykinin plus cyclophilin. Solid lines, Eqs. 5, 6, 10, 11, and 16 fit to the data, with resulting estimate for \(u\) of 0.31 and \(k_1\) of 0.051 s\(^{-1}\) for [Gly6]bradykinin and 1.81 s\(^{-1}\) for [Gly6]bradykinin plus cyclophilin.
increased both the fraction of peptide hydrolyzed in the lung in the rapid phase of the reaction and the rate of hydrolysis of the slower phase (Figs. 5 and 6).

To further evaluate the hypothesis that the trans isomers of bradykinin and [Gly⁶]bradykinin were preferentially hydrolyzed in the lung and to estimate the fraction of cis peptide at equilibrium and cis-trans isomerization rate constant for each peptide, we carried out the following kinetic analysis, the underlying hypotheses of which are presented in Fig. 7. The total concentration (b) of unaltered peptide at any time (t) was defined as the sum of the concentrations of the cis (c) and the trans (t) forms

\[ b(t) = c(t) + t(t) \]  

To interpret the hydrolysis-process curves [b(t)], we hypothesized the following kinetic processes, which included the kinetics of cis-trans isomerization and the enzymatic hydrolysis of the trans isomer(s) of the peptides

\[ k_1 \]
\[ \frac{c}{X} \]
\[ \frac{t}{X} \]
\[ k_2 \]  

where \( c \) is the concentration of bradykinin and [Gly⁶]bradykinin peptides in the lung, \( k_1 \) and \( k_1 \) are the cis-trans and trans-cis isomerization rate constants, respectively, and \( k_2 \) is the rate constant of the formation of the trans peptide isomer-enzyme complex \( (te) \), which either dissociates to form \( e \) and \( t \) with the rate constant \( k_2 \) or proceeds to form the hydrolysis product \( (p) \) with the rate constant \( k_4 \).

Initially, before exposure to the peptides in the lung, the cis and trans forms are in equilibrium, and we defined the initial fraction of peptide in the cis (c) form \( (\theta) \) as

\[ \theta = \frac{c(0)}{b(0)} = \frac{k_{-1}}{k_{-1} + k_1} = \frac{K_d}{1 + K_d} \]  

where the dissociation constant \( (K_d) \) is \( k_{-1}/k_1 \).

Under the assumption that the reservoir is well mixed, the concentrations of the cis and trans forms of peptide in the reservoir are described by the following species balance equations

\[ V_R\frac{dc_R}{dt} = F_{in}(c_L - c_R) + V_R(t)(k_1c_R - k_{-1}c_T) \]

and

\[ V_R\frac{dc_T}{dt} = F_{in}(c_L - c_T) + V_R(t)(k_{-1}c_T - k_1c_R) \]

with the following initial conditions

\[ c_R(0) = \theta b(0) \]

\[ t_R(0) = (1 - \theta)b(0) \]

where \( c_R \) and \( t_R \) are the concentrations (nmol/ml) of the cis and trans isomers, respectively, in the reservoir; \( V_R(t) \) is the volume (ml) of the reservoir at time \( t \); and \( F_{in} \) is the flow (ml/s) into the reservoir. During sampling

\[ F_{in} = 0 \]

and

\[ V_R(t + \Delta t) = V_R(t) - F\Delta t \]

otherwise

\[ F_{in} = F \]

and

\[ V_R(t + \Delta t) = V_R(t) \]

where \( F \) is the perfusate flow (ml/s) into the lung.

The concentrations of the cis and trans forms of the peptide in the tubing connecting the lung and the reservoir at distance \( x \) from the inlet to the tubing at time \( t \) are described by the following species balance equations

\[ \frac{\partial c_T}{\partial t} + W_T \frac{\partial c_T}{\partial x} = k_{-1}t_T - k_1c_T \]

and

\[ \frac{\partial t_T}{\partial t} + W_T \frac{\partial t_T}{\partial x} = k_1c_T - k_{-1}t_T \]

with the following initial conditions

\[ c_T(x, 0) = 0 \]

and

\[ t_T(x, 0) = 0 \]

and boundary conditions

\[ c_T(0, t) = c_R(t) \]

and

\[ t_T(0, t) = t_R(t) \]

where \( c_T(x, t) \) and \( t_T(x, t) \) are the concentrations of the cis and trans forms, respectively, of the peptide within the tubing at distance \( x \) from the inlet to the tubing at time \( t \) and \( W_T \) is the average flow velocity (cm/s) in the tubing.

The spatial and temporal variations in the concentrations of the cis and trans forms of the peptide in the lungs at distance \( x \) from the inlet and time \( t \) are
described by the following species balance equations

\[
\frac{\partial c_L}{\partial t} + W_L \frac{\partial c_L}{\partial x} = -\frac{V_{\text{max}}}{K_m} t_L + k_1 c_L - k_{-1} t_L \tag{14}
\]

and

\[
\frac{\partial c_L}{\partial t} + W_L \frac{\partial c_L}{\partial x} = -k_1 c_L \tag{15}
\]

where \(V_{\text{max}} = ek_w\), the maximum rate of peptide hydrolysis (nmol/s); \(K_m = (k_{-1} + k_2)/k_2\), the concentration of peptide resulting in \(V_{\text{max}}/2\); \(W_L\) is the average flow velocity in the lungs; and \(c_L(x,t)\) and \(t_L(x,t)\) are the vascular concentrations of the cis and trans forms, respectively, of the peptide within the lungs at distance \(x\) at time \(t\). Under the assumption that \(V_{\text{max}}/K_m \gg F\), Eqs. 14 and 15 reduce to

\[
\frac{\partial c_L}{\partial t} + W_L \frac{\partial c_L}{\partial x} = -k_1 c_L \tag{16}
\]

with the following initial condition

\[c_L(x, 0) = 0\tag{17}\]

and boundary condition

\[c_L(0, t) = c_L(z, t)\tag{18}\]

where \(x = z\) is the tubing outlet.

The only model parameters are \(k_1\) and \(k_{-1}\) because \(k_{-1} = k_1/(1 - \theta)\). Furthermore, because the only impact of cyclophilin is to increase \(k_1\), only \(k_{-1}\) was allowed to change when the model equations were fit to the data obtained when cyclophilin was present, and \(\theta\) was kept constant. Thus in the four studies with bradykinin and the three with \([\text{Gly}\text{6}]\text{bradykinin}\) that included experiments both with and without cyclophilin, optimization for the three parameters, \(\theta\) and the two \(k_1\) values (without and with cyclophilin), was carried out with a modified Levenberg-Marquardt algorithm to find the model parameter values for which the numerical solution of Eqs. 5, 6, 10, 11, and 16 produce the best fit to the data sets from experiments with and without cyclophilin simultaneously. In the three studies with bradykinin and the three with \([\text{Gly}\text{6}]\text{bradykinin}\) in which only the peptides in the absence of cyclophilin were studied, optimization was carried out for only two parameters, \(\theta\) and \(k_1\) without cyclophilin.

Examples of the model solutions calculated with the parameters estimated from Eqs. 5, 6, 10, 11, and 16 are shown in Figs. 5 and 6. Also in Figs. 5 and 6 are the data representing the amount of bradykinin or \([\text{Gly}\text{6}]\text{bradykinin}\), respectively, remaining in the venous effluent expressed as a percentage of the amount expected if none had been metabolized. The mean estimates for the model parameters obtained from all of the studies are shown in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(n)</th>
<th>Mean (\pm) SE</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\theta)</td>
<td>7</td>
<td>0.13 (\pm) 0.013</td>
<td>0.024 (\pm) 0.017</td>
</tr>
<tr>
<td>(k_1, \text{s}^{-1})</td>
<td>7</td>
<td>0.074 (\pm) 0.011</td>
<td>0.028 (\pm) 0.018</td>
</tr>
<tr>
<td>(k_1, \text{CyP}, \text{s}^{-1})</td>
<td>4</td>
<td>1.9 (\pm) 0.19</td>
<td>0.67 (\pm) 0.290</td>
</tr>
</tbody>
</table>

Parameter estimates were obtained by fitting model Eqs. 5, 6, 10, 11, and 16 to data obtained from HPLC-recirculation studies; \(n\), no. of lung experiments from which data were obtained. c, Unhydrolyzed (cis) fraction; \(k_1\), rate of cis-to-trans conversion; \(k_1, \text{CyP}\), rate of cis-to-trans conversion with cyclophilin present. The 95% confidence interval is a measure of precision of parameter estimates from data from each lung (\(\pm SD\) among the \(n\) lungs studied). (13).

DISCUSSION

The results are consistent with the hypothesis that cis conformers of at least one of the X-Pro bonds in bradykinin and \([\text{Gly}\text{6}]\text{bradykinin}\) are refractory to enzymatic hydrolysis in the lung. In addition, the cis-trans isomerization time constants for both peptides are longer than the pulmonary capillary transit time. Three types of evidence support this hypothesis. The first is that for both peptides, in both the single-pass bioassay and recirculation-HPLC studies, there was a fraction of peptide that was not hydrolyzed during transit through the lungs. In both kinds of experiments, a greater fraction of bradykinin than \([\text{Gly}\text{6}]\text{bradykinin}\) was in the more rapidly hydrolyzable form, an observation consistent with the fact that the equilibrium cis-to-trans ratio of the Gly\(6\)-Pro\(7\) bond of \([\text{Gly}\text{6}]\text{bradykinin}\) (~0.38–0.4) has been found to be greater than that of the Ser\(6\)-Pro\(7\) bond of bradykinin (~0.1–0.13) as measured by NMR (17–20). Finally, in the recirculation-HPLC study, the hydrolysis rates of the more slowly hydrolyzed fractions of both peptides were increased by adding the peptidyl prolyl cis-trans isomerase cyclophilin.

In the bioassay studies, when the peptide passed through the lungs before reaching the jugular vein rings, it took ~25.6 times as much bradykinin and 7 times as much \([\text{Gly}\text{6}]\text{bradykinin}\) to produce the same jugular vein contraction as was produced when the peptide did not pass through the lungs, consistent with a substantially larger fraction of \([\text{Gly}\text{6}]\text{bradykinin}\) than of bradykinin surviving passage through the lung. Additionally, the bioassay results reveal that \([\text{Gly}\text{6}]\text{bradykinin}\) had ~40% of the activity of bradykinin as an agonist for contraction of the jugular vein, which is consistent with the findings of London et al.
(20) and Stewart (34) that [Gly\(^6\)]bradykinin had from
\(\sim 20\) to \(70\%\) of the activity of bradykinin in bioassays for
contraction of rat uterus muscle or guinea pig ileum.

In the lung recirculation-HPLC studies of bradykinin
and [Gly\(^6\)]bradykinin hydrolysis kinetics, the ability of
the model to fit the data is also consistent with the
hypothesis that the trans isomers are the preferred
peptidase substrates and that the effect of cyclophilin is
to increase the rate of cis-trans isomerization, reflected
by the increase in \(k_1\). There were two phases to the
hydrolysis reactions, rapid and slow. The fraction of
peptide hydrolyzed in the rapid phase was higher for
bradykinin than for [Gly\(^6\)]bradykinin, which is reflec-
ted in the estimated values of the equilibrium cis
fractions (\(n\)) for bradykinin and [Gly\(^6\)]bradykinin (0.13
and 0.43, respectively) obtained from the model fits to
the data. These values are close to NMR measurements
of the cis fractions of these peptides at the Ser\(^6\)-Pro\(^7\)
(\(\sim 0.1–0.13\)) and Gly\(^6\)-Pro\(^7\) (0.38–0.40) bonds, respec-
tively (17–20). The increase in the rate of cis-trans
isomerization caused by cyclophilin allowed for a suffi-
cient amount of cis isomer to be converted to trans
within the capillary transit time such that a fraction of
peptide in excess of the equilibrium trans fraction could
be hydrolyzed within the lung.

The results of the bioassay and HPLC studies can be
quantitatively compared as follows. Assuming a rat
lung capillary mean transit time of \(\sim 3\) s for a flow rate
of 0.17 ml/s [assuming that the pulmonary capillary
blood volume-to-lung weight ratio is about the same in
a rat as in a rabbit (1)], taking into account the cis-trans
isomerization rate constants obtained from the recircu-
lation-HPLC studies, and all else being equal, an
estimated equilibrium cis fraction for bradykinin and
[Gly\(^6\)]bradykinin from the bioassay studies would be
7.5 \(\pm 1.8\) (SE) and 24.0 \(\pm 5.6\%\) (SE), respectively, based
on the shift in the dose-response curves between the
lung and tissue injection sites. The reason for the
quantitative differences between such estimates for
the equilibrium cis peptide fraction obtained from the
bioassay and HPLC-recirculation studies is not clear,
but factors that might contribute to an underestima-
tion of the cis peptide fraction from the bioassay results
include differences in dispersion of the bolus reaching
the bioassay tissue between the two injection sites; the
possibility that bradykinin might release other vasoac-
tive agents from the lung that may affect the bioassay
tissue response (35); and, finally, the possibility that
bradykinin receptors in the jugular vein are preferen-
tially activated by one isomer or the other, a con-
that has been previously suggested (18, 20). Although
such estimates of the equilibrium cis contents of the
peptides from the bioassay study are lower than those
calculated from the HPLC-recirculation data, the rela-
tive differences are similar; i.e., in the bioassay and
HPLC experiments, the unhidrolyzable fraction was
3.1- and 3.2-fold higher in the two kinds of experiments,
respectively, for [Gly\(^6\)]bradykinin than for bradykinin.

These relative differences in the equilibrium cis
fraction estimated by the bioassay and HPLC studies
are consistent with NMR measurements. Bradykinin
contains three proline residues, and in the studies of
London and colleagues (18, 19), the cis content of each
of the imide bonds was estimated to be up to 10% at
equilibrium with NMR spectroscopy, and it also has
been suggested that all of the observed cis proline
resonances observed in bradykinin were probably asso-
ciated with Pro\(^7\) (20). Other studies of the Ser\(^6\)-Pro\(^7\)
and [p-fluoro-Phe\(^8\)]bradykinin, respectively (17, 19). Car-
bon-13 NMR spectroscopic studies of [90%-1,2-\(^13\)C\(^2\)-
Gly\(^6\)]bradykinin and of the [p-fluoro-Phe\(^8\)] analog of
[Gly\(^6\)]bradykinin indicate cis-to-trans ratios of 0.38 and
0.4, respectively (17, 20). Thus, with the data obtained
from the [p-fluoro-Phe\(^8\)] analogs, in which the two
peptides were directly compared, [Gly\(^6\)]bradykinin has
a 3.1 higher equilibrium cis content than the bradyki-
nin, very close to the relative difference between the
estimates obtained by both the bioassay and HPLC
studies. Also consistent with our observations, the
cis-trans isomerization rate constants for the Ser\(^6\)-Pro\(^7\)
and Gly\(^6\)-Pro\(^7\) bonds in [p-fluoro-Phe\(^8\)]bradykinin and
the [p-fluoro-Phe\(^8\)] analog of [Gly\(^6\)]bradykinin were
found to be 0.048 and 0.021 s\(^{-1}\), respectively, under the
NMR conditions of the study (17), which compares
reasonably well with 0.074 and 0.049 s\(^{-1}\), respectively,
under the conditions of the present study.

Angiotensin-converting enzyme has been shown to
account for the majority of pulmonary hydrolysis of
bradykinin (9, 10, 28). The results of the present study
are consistent with this finding because the only differ-
ce between bradykinin and [Gly\(^6\)]bradykinin is the
substitution of Gly for Ser at the sixth position in the
peptide, and the differences in the kinetics of peptide
hydrolysis can be accounted for by the magnitude of the
difference in the cis-to-trans ratio at the X\(^6\)-Pro\(^7\) bond.
Therefore, it seems likely that a preference of angioten-
sin-converting enzyme for a trans isomer at the X\(^6\)-Pro\(^7\)
and [p-fluoro-Phe\(^8\)] analog at the Pro\(^7\) bond is respon-
sible for the fact that a fraction of peptide equivalent
to the cis fraction at that bond escapes inactivation in the
lung. However, aminopeptidase P also hydrolyzes bradykinin in the pulmonary cir-
culation (27), and there is evidence that it also has a
preference for trans isomers (15, 31). Thus the impact
of any potential cis-trans isomerization at the Arg\(^1\)-Pro\(^2\)
and Pro\(^2\)-Pro\(^3\) bond may also be involved in determin-
ing the extent of peptide inactivation in the lung.

To our knowledge, there is no evidence that the
concentration of cyclophilins in plasma is sufficient to
affect the rate of cis-trans isomerization of imide bonds
involved in the hydrolysis of peptides in the lung (7, 8,
29). Thus, under physiological conditions, our results
predict that cis peptides in the venous blood would
escape hydrolysis in the lung. Although the relevance of
this finding to the bioactivity of peptides is as yet
unknown, it has been suggested that different subtypes
of bradykinin receptors may preferentially bind brady-
kinin conformers containing either cis or trans isomers
(18, 20). In fact, the 50–70% lower activity of
[Gly6]bradykinin compared with that of bradykinin in some bioassay preparations was interpreted as a possible indication that the cis peptide either did not bind to the receptor or had a lower affinity than the trans peptide (20). Because one-half of the bradykinin entering the systemic circulation could still be in the cis conformation 9 s after leaving the lung [about two times the resting arterial mean transit time (26)], there could be a concentration gradient of differing bradykinin conformers throughout the circulation. This phenomenon might play a role in regulating the proportion of bradykinin conformers with varying biological activity available to different receptor subtypes in the vasculature.

Comments on HPLC Method

Analytic information regarding bradykinin inactivation in the lung has been obtained with several different techniques including radio- and chemiluminoimmunoassay (4, 6) and HPLC of radioactive bradykinin peptides (27). However, many of these assays have the drawback that they make use of radioactive bradykinin analogs and/or antibodies to bradykinin for which there are no available [Gly6]bradykinin counterparts. Therefore, this study required that we develop a sensitive analytic methodology that could be used to directly compare the kinetics of hydrolysis of these peptides under first-order conditions. We chose 4-phenylspiro[ furan-2(3H),1′-phtalan]-3,3′-dione (fluorescamine) derivatization of peptides in the lung perfusate followed by HPLC detection of intact peptides. Advantages of this technique include that the derivatization reaction takes place rapidly and requires a single step that can be carried out at room temperature and that the product is highly fluorescent (3, 36) and thus sensitive. Most importantly, bradykinin and [Gly6]bradykinin were equally amenable to derivatization and could be separated from other fluorescent components with the same HPLC methodology. In addition, neither the fluorescamine nor its rapidly produced hydrolysis products are fluorescent (36). The problem of instability known to occur with fluorescamine derivatives was overcome by using automated precolumn derivatization of individual samples immediately before injection onto the HPLC column. Although Boppana et al. (3) previously explored the utility of automated precolumn fluorescamine derivatization with a variety of peptides, their studies of bradykinin did not include studies in biological fluids or physiological solutions. Thus, to our knowledge, detection of bradykinin and [Gly6]bradykinin by precolumn derivatization with fluorescamine followed by HPLC represents a new method for the quantitation of these peptides in physiological solutions such as lung perfusate.

APPENDIX

The motivation for the model hypothesis that $V_{\text{max}}/K_m$ is very large relative to $F$, that is, that all of the trans isomer is hydrolyzed within the transit time of the pulmonary capillary bed, came from the results of a previous study by Merker and Dawson (24) that revealed that when cyclophilin was included in a bolus of bradykinin injected into the arterial inflow of the lung, bradykinin was not detectable in the venous effluent. Under the assumption that cyclophilin did not affect bradykinin peptidase activity, the conclusion was that the cis content rather than the hydrolysis rate constant ($V_{\text{max}}/K_m$) determined the extent of hydrolysis. The results of the present study are consistent with this conclusion to the extent that the model fits the data. However, the model also provides an additional means of evaluating the hypothesis as illustrated in Fig. 8. The value of $V_{\text{max}}/K_m$ was successively decreased until the model fit to the data deteriorated. This deterioration is revealed by the sharp rise in the coefficient of variation between the model fit and the data (Fig. 8, ○). This occurred at a $V_{\text{max}}/K_m$ of $\sim 2$ ml/s for both bradykinin and [Gly6]bradykinin, thus indicating a lower bound on $V_{\text{max}}/K_m$ consistent with the data of $\sim 2$ ml/s. Setting $V_{\text{max}}/K_m$ at this lower bound and fitting the model to the data had little effect on the estimated values of $\theta$ or $k_1$ without cyclophilin. However, when cyclophilin was present, the higher value of $k_1$ resulted in a high correlation between $V_{\text{max}}/K_m$ and $k_1$ with cyclophilin. In this case, the lower bound on $V_{\text{max}}/K_m$ resulted in a correspondingly higher estimate of $k_1$ with cyclophilin. Thus the values of $k_1$ with cyclophilin in Table 1, obtained under the assumption that $V_{\text{max}}/K_m \gg F$, actually represent lower bounds on $k_1$. When this assumption is relaxed, and the

Fig. 8. Impact of decreasing values of hydrolysis rate constant [ratio of maximum rate of peptide hydrolysis to concentration of peptide resulting in $V_{\text{max}}/2(V_{\text{max}}/K_m)$] on estimates of model parameters: $V_{\text{max}}/K_m$ (A) and $k_1$ without ($k_1$; B) and with cyclophilin ($k_1$CyP; C). ○, Coefficient of variation (CV) between model fit and data; ■, parameter values obtained for bradykinin; ●, parameter values obtained for [Gly6]bradykinin.
V_{\text{max}}/K_{\text{m}} values are set at their lower bounds (~2 ml/s), the estimated values for k_{i} with cyclophilin represent upper bounds. The estimated upper bounds on k_{i} in the absence of cyclophilin were, on average, ~90 and 37 s^{-1} for bradykinin and [Gly^6]bradykinin, respectively. To put the lower bounds on V_{\text{max}}/K_{\text{m}} in perspective, if V_{\text{max}}/K_{\text{m}} were actually close to the lower bound of ~2 ml/s, the fraction of the trans form of either bradykinin or [Gly^6]bradykinin surviving hydrolysis on passage through the lungs would still be ~2%. Thus the overall conclusion from this evaluation is that the estimated values of \theta and k_{i} without cyclophilin are virtually independent of the values of V_{\text{max}}/K_{\text{m}} that are consistent with the data, whereas the values of k_{i} with cyclophilin estimated under the assumption that V_{\text{max}}/K_{\text{m}} \gg F and with no additional experimental data could be considered the most conservative estimate for the cyclophilin-stimulated cis-to-trans conversion rate constant.

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REFERENCES


