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CYCLOPHILIN-FACILITATED BRADYKININ INACTIVATION IN THE PERFUSED RAT LUNG

MARILYN P. MERKER*†‡§ and CHRISTOPHER A. DAWSON‡‖

Department of *Anesthesiology, ‖Physiology and †Pharmacology/Toxicology, Medical College of Wisconsin, Milwaukee, WI 53226; and ‡V. A. Medical Center, Milwaukee, WI 53295, U.S.A.

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Abstract—*Cis* and *trans* isomers of X-proline (X-Pro) bonds can influence some aspects of the kinetics of peptide metabolism. We previously used the peptidyl-prolyl *cis-trans* isomerase, cyclophilin, to show that angiotensin converting enzyme (ACE) preferentially hydrolyzes the *trans* isomer of a synthetic tripeptide that contains a C-terminal proline (Dawson *et al., Am J Physiol* **257**: H853–H865, 1989; Merker *et al., J Appl Physiol* **75**: 1519–1524, 1993). Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) exists as both *cis* and *trans* isomers at all three X-Pro bonds, and although its inactivation in the lung by pulmonary endothelial peptidases is extensive, commonly a small fraction of the peptide survives passage through the lung. To determine whether the presence of *cis* X-Pro bonds might limit the extent of bradykinin metabolism in the lung, we studied inactivation of bradykinin by the isolated perfused rat lung using the rabbit jugular vein superfused with the pulmonary venous effluent as a bioassay for bradykinin. A large fraction (>90%) of the bradykinin in a bolus injection was inactivated in a single transit through the pulmonary circulation, but a detectable fraction emerged in the venous effluent. The addition of cyclophilin to the bradykinin in the bolus reduced the bradykinin emerging from the lungs to virtually undetectable levels. When the isomerase inhibitor cyclosporin A was included with bradykinin and cyclophilin in the injectate, this effect of cyclophilin was reversed. These observations suggest that the fraction of bradykinin that normally survives passage through the lungs contains isomers that have at least one X-Pro bond that is refractory to enzymatic inactivation and whose isomerization time constant is significantly longer than the pulmonary capillary transit time.

Key words: kinins; lung metabolism; cyclophilin; cyclosporin A

Proline-containing peptides can exist as relatively slowly equilibrating mixtures of *cis* and *trans* rotational isomers at X-Pro imide bonds [1–3]. The potential physiological significance of this phenomenon has received attention recently due to the discovery of a family of immunosuppressant binding proteins, the cyclophilins and FK binding proteins, that are also peptidyl-prolyl *cis-trans* isomerases [4, 5]. Since these ubiquitously distributed enzymes [6–8] catalyze *cis-trans* isomerization, the search for physiological processes that they might influence has intensified. For example, *cis-trans* isomerization has been identified as a rate-limiting process in the maturation of some proteins [9–11]. These enzymes are also useful for examining the role that *cis-trans* isomerization plays in kinetic processes involving proline-containing peptides [12], as in the present study.

Although the role of *cis-trans* isomerization in the function of proline-containing vasoactive peptides is not well understood, some peptidases preferentially hydrolyze *trans* isomers of proline-containing peptides in solution [1, 13–17], and preferentially bind *trans* isomers of their inhibitors [18–20]. Angiotensin converting enzyme ACE¶, a peptidase found on the luminal surface of the pulmonary endothelium that hydrolyzes a variety of physiologically important peptides in the pulmonary vasculature [21], preferentially hydrolyzes *trans* conformers of tripeptide substrates containing C-terminal proline residues *in vitro* and in the intact perfused lung [12, 22, 23]. Since *cis-trans* isomerization time constants of the prolyl-peptide bonds in these substrates are longer than the pulmonary capillary transit time (80–170 sec vs 2–3 sec, respectively), a fraction of peptide nearly equal to the equilibrium *cis* fraction is spared from hydrolysis and emerges in the lung venous effluent. The ACE preference for *trans* isomers of its proline-containing vasoactive substrates could have significant consequences with respect to the fate of vasoactive peptides on passage through the lungs.

Although bradykinin undergoes extensive inactivation by endothelial peptidases, including ACE, on passage through the lungs, commonly a small fraction of the peptide survives [24–32]. Bradykinin contains three proline residues, and the *cis-trans* ratio of each of the imide bonds has been estimated to be up to 10% at equilibrium [33, 34]. For the Ser^6-Pro^7 bond specifically, *cis-trans* ratios of 0.15 and 0.13 have been reported for the bradykinin fragment Ser-Pro-Phe-Arg and for the same bond in [*p*-flouro-Phe^8]bradykinin, respectively [34, 35]. The $T_{1/2}$ for the *cis-trans* isomerization about the Ser^6-Pro^7 bond in the [*p*-fluoro-Phe^8] bradykinin is about 14 sec [35], again longer than for transit through the pulmonary capillary bed. If the pulmonary peptidases involved in bradykinin hydrolysis preferentially cleave peptides at or near *trans* X-Pro bonds, this might account for the observation that some fraction of bradykinin can survive passage through the lung.

In this study, we examined the hypothesis that *cis* isomers of the imide bonds in bradykinin limit the extent of its inactivation in the pulmonary vasculature. We took advantage of the finding that *cis-trans* isomerization of the Ser^6-Pro^7 peptide bond of [*p*-fluoro-Phe^8] bradykinin has been shown to be catalyzed by the peptidyl-prolyl

§ Corresponding author: Dr. Marilyn P. Merker, Department of Anesthesiology, Research Service - 151, VA Medical Center, 5000 West National Ave., Milwaukee, WI 53295. Tel. (414) 384-2000, Ext. 1394; FAX (414) 382-5374.

¶ Abbreviation: ACE, angiotensin converting enzyme.

cis-trans isomerase, cyclophilin [35]. We measured bradykinin surviving passage through perfused rat lungs in the presence or absence of cyclophilin or cyclophilin with its specific inhibitor, cyclosporin A. We used a bioassay technique because it was a rapid, sensitive assay for the small fraction of injected bradykinin surviving passage through the lung, and because bradykinin cleavage products are not vasoactive in this assay preparation [24, 36].

MATERIALS AND METHODS

Bradykinin was purchased from the Sigma Chemical Co. (St. Louis, MO) or Bachem Bioscience, Inc. (King of Prussia, PA). Purified human recombinant cyclophilin was prepared with the assistance of Dr. Wei Li in the laboratory of Professor Robert E. Handschumacher, Yale University School of Medicine (New Haven, CT). The cyclophilin was >95% pure, and was active in an assay for peptidyl-prolyl *cis-trans* isomerase activity, which measures the extent to which cyclophilin accelerates chymotrypsin-dependent hydrolysis of the substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide [2, 4]. The cyclophilin concentration was determined in our laboratory by titration with tritiated cyclosporin A [37]. Cyclosporin A was the gift of Dr. B. Ryffel of Sandoz Pharma Ltd., Basel, Switzerland. [Gly^6]bradykinin was purchased from Genosys Biotechnologies, Inc. (The Woodlands, TX).

For the bioassay tissue, two pieces of 22 μm tungsten wire were threaded through a segment of rabbit jugular vein that was ~1.5 mm long. The wires were stretched over the jaws of two stainless steel rings, one of which was anchored and the other attached to a force transducer, as previously described [38]. A 750-mg load was applied to the jugular ring, and the vessel segment was allowed to equilibrate in the perfusion system in the Krebs–Ringer bicarbonate buffer (pH 7.4; P_{O_2} = 100 Torr, P_{CO_2} = 40 Torr) containing 5 mM glucose and 2.5% bovine serum albumin at 37° for 60 min. The tissue response to bradykinin was then tested, and afterward the isolated rat lung was connected into the perfusion circuit so that the jugular vein was superfused with the venous effluent from the lung.

The isolated rat lungs from male Sprague–Dawley rats weighing between 330 and 450 g were perfused at a flow rate of 11 to 12.6 mL/min with the perfusate described above using previously described techniques [22]. This produced a pulmonary artery pressure of 5.7 ± 2.1 (SD) Torr with the venous pressure set at zero. The pulmonary artery pressure did not change significantly throughout the course of the experiments. The lungs were ventilated at 30 breaths/min with 6% CO_2 and 15% O_2 with end inspiratory and end expiratory pressures of 9.2 ± 2.4 (SD) and 2.5 ± 0.5 (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 distal to the lung into the venous outflow (tissue injection site) (Fig. 1).

Bolus injectates containing bradykinin (or [Gly^6]-bradykinin) with or without cyclophilin or cyclophilin plus cyclosporin A in a final volume of 0.1 mL were prepared immediately before injection. Sequences of injectates containing bradykinin with cyclophilin (+Cyp) or bradykinin with cyclophilin and cyclosporin A (+Cyp+CsA) always included a bolus of bradykinin

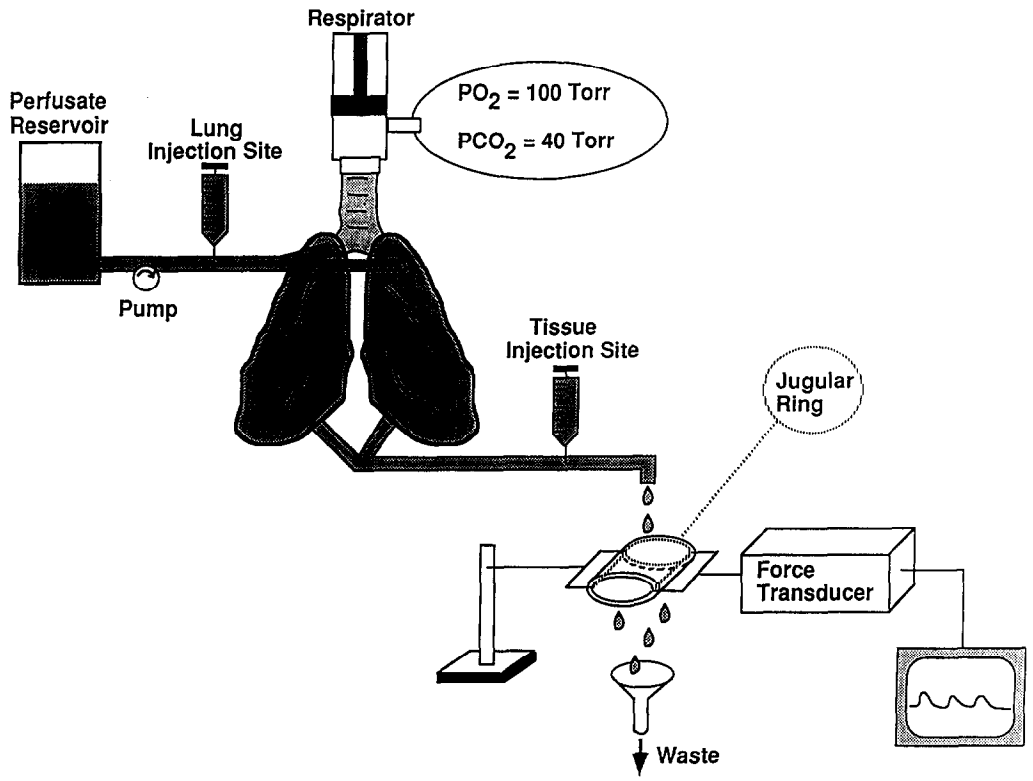

Fig. 1. Diagram of the rabbit jugular ring superfused with the venous effluent of the isolated perfused rat lung. Note in particular the location of the lung and tissue injection sites.

alone before the sequences (BK1). A second bolus of bradykinin alone (BK2) was also included at the end of each sequence.

The dose of bradykinin was adjusted for each jugular ring-lung pair to give a submaximal contraction. For all of the experiments that included cyclophilin and cyclophilin with cyclosporin A, we used doses varying from 2.5 to 40 pmol/0.1 mL of bradykinin for boluses injected at the tissue injection site and 200–600 pmol/0.1 mL for boluses injected into the lung injection site. When cyclophilin was included in the boluses, it was present at 3.5 to 19 nmol/0.1 mL. In the experiments that included cyclophilin plus cyclosporin A boluses, cyclophilin and cyclosporin A were present at 7 and 25 nmol, respectively, per 0.1 mL bolus for one lung-jugular ring pair or 3.5 and 8.5 nmol, respectively, per 0.1 mL bolus for three lung-jugular ring pairs.

All injectates had the same concentrations of the phosphate buffer that was used to prepare bradykinin or [Gly^1]bradykinin solutions and cyclophilin and cyclosporin A vehicles. Thus, injectates containing bradykinin only (BK1 and BK2) also contained cyclophilin and cyclosporin A vehicle, and injectates containing bradykinin and cyclophilin (+Cyp) contained cyclosporin A vehicle. The cyclophilin vehicle was potassium phosphate buffer (pH 7.2; 20 mM) containing NaCl (100 mM) and 2-mercaptoethanol (5 mM), and the cyclosporin A vehicle was ethanol. The final concentration of ethanol in all injectates was 2.5%. To ensure that the cyclosporin A would be available to bind to cyclophilin, it was always added to the injectate mixtures after the addition of the cyclophilin and bradykinin.

To summarize the data, the magnitude of the contractions elicited by each injectate in a sequence was normalized to the magnitude of the contraction elicited by the initial bradykinin bolus (BK1) in each sequence. The data were analyzed for statistical significance by one-way ANOVA with repeated measures followed by the Newman–Keuls test.

RESULTS

The characteristics and dose dependence of jugular ring contractions to bradykinin boluses injected into the tissue injection site are shown in Fig. 2. To demonstrate that bradykinin inactivation by the rat lung was detectable in our preparation, the jugular ring response to bradykinin doses injected at the lung and tissue injection sites for a lung-jugular ring pair are shown in Fig. 3.

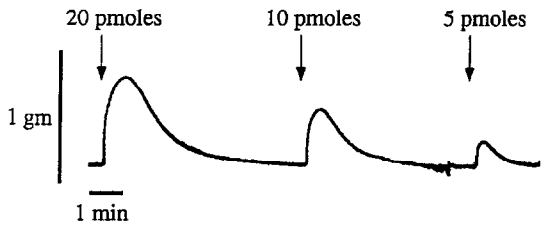

Fig. 2. Dose-dependent contractions of the jugular ring to bradykinin boluses injected into the tissue injection site such that the boluses did not pass through the lung. The arrows denote the time of injection of the bradykinin boluses. The amount of bradykinin in each bolus is also indicated.

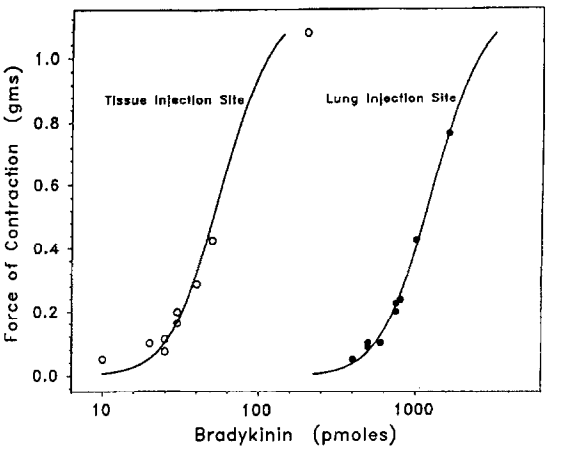

Fig. 3. Contractions of a rabbit jugular ring induced by bolus injections of bradykinin introduced into the lung (●) or tissue (○) injection sites, such that the bradykinin either did or did not pass through the lungs, respectively, before reaching the jugular ring. To control for any changes in responsiveness of the tissue or metabolic status of the lung throughout the course of the experiment, injections into the lung and tissue injection sites were alternated. Data are from one lung-jugular ring pair.

Passage through the lung increased the dose required to obtain a contraction equivalent to one in which the bradykinin dose did not pass through the lung by about 20-fold. The doses of bradykinin used in subsequent studies were always below the inflection point of the dose–response curve so that the responses were submaximal.

Neither cyclophilin nor cyclophilin with cyclosporin A significantly affected the magnitude of contractions induced by bradykinin when injections were made into the tissue injection site such that the boluses did not pass through the lung (Fig. 4). However, when cyclophilin

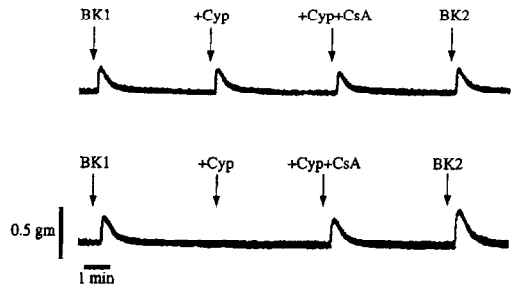

Fig. 4. Effects of cyclophilin or cyclophilin with cyclosporin A on bradykinin-induced contraction of the jugular ring. *Upper tracing:* Injections were made into the tissue injection site such that the boluses did not pass through the lungs as follows: bradykinin alone (BK1); bradykinin and cyclophilin (+Cyp); bradykinin and cyclophilin plus cyclosporin A (+Cyp+CsA), and a final dose of bradykinin (BK2). Boluses contained 2.5 pmol of bradykinin, 3.5 nmol of cyclophilin, and 8.5 nmol of cyclosporin A. *Lower tracing.* Injections were made into the lung injection site such that the boluses passed through the lungs as follows: bradykinin alone (BK1); bradykinin and cyclophilin (+Cyp); bradykinin and cyclophilin plus cyclosporin A (+Cyp+CsA), and a final dose of bradykinin (BK2). Boluses contained 50 pmol of bradykinin and cyclophilin and cyclosporin A as above.

was included with the bradykinin in a bolus injection into the lung injection site, the jugular ring contraction was essentially abolished, an effect that was reversed when cyclosporin A was also included in the bolus (Fig. 4).

A summary of the data from all of the experiments of the kind shown in Fig. 4 is shown in Figs. 5 and 6. The force of bradykinin-induced contractions was unaffected by cyclophilin (+CyP) or cyclophilin and cyclosporin A (+Cyp+CsA) as compared with bradykinin alone (BK1 and BK2) when boluses were introduced into the tissue injection site (Fig. 5), but when the boluses were introduced into the lung injection site, the magnitude of the bradykinin-induced contraction was decreased to a mean of less than 6% of that elicited by bradykinin alone (BK1 and BK2) when cyclophilin (+Cyp) was present (Fig. 6). In six of these eleven experiments, in which cyclophilin was included in the bradykinin boluses injected into the lung injection site, no detectable contraction of the jugular ring occurred. When cyclosporin A was included in the bolus (+Cyp+CsA), the mean magnitude of the contractions was not significantly different from that elicited by bradykinin alone (BK1 and BK2; Fig. 6).

To determine whether cyclophilin might act to diminish the force of the bradykinin-induced contractions by causing the lung to release a physiological antagonist of bradykinin, we first determined that it took 5 sec for a bolus to traverse the tubing and lung between the lung

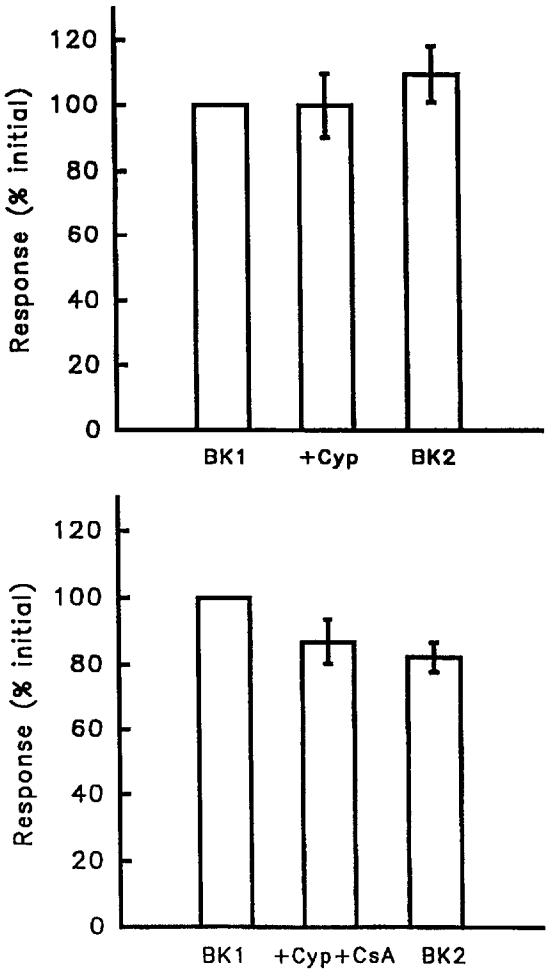

Fig. 5. Summary of data demonstrating that neither cyclophilin nor cyclophilin with cyclosporin A affected the magnitude of bradykinin-induced constractions of the jugular ring when injections were made into the tissue injection site such that the boluses did not pass through the lungs. The injection sequences were as follows: *Upper panel:* (BK1), bradykinin alone; (+Cyp), bradykinin and cyclophilin; and (BK2), a final dose of bradykinin. Data are from seven jugular rings. The mean force of contraction elicited by the initial bradykinin boluses (BK1) was 0.25 ± 0.18 (SD) g. *Lower panel:* (BK1), bradykinin alone; (+Cyp+CsA), bradykinin and cyclophilin plus cyclosporin A, and (BK2), final dose of bradykinin. Data are from three jugular rings. The mean force of contraction elicited by the initial bradykinin boluses (BK1) was 0.29 ± 0.17 (SD) g. Values are given as means and ± SD. The data were analyzed for statistical significance by ANOVA followed by a Newman–Keuls test. No significant differences were detected.

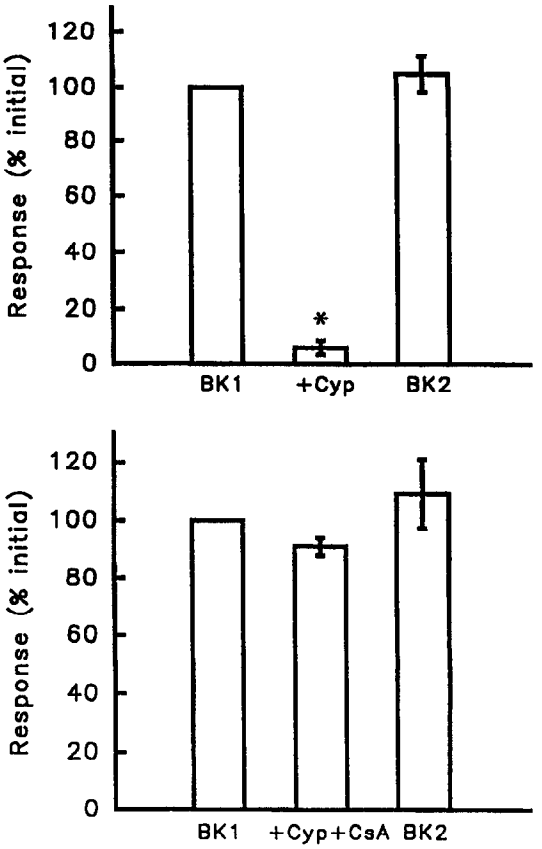

Fig. 6. Summary of data demonstrating that cyclophilin decreased the magnitude of bradykinin-induced contractions of the jugular ring when the cyclophilin and bradykinin were injected into the lung injection site such that the boluses passed through the lungs, and that cyclosporin A reversed this effect. The injection sequences were as follows: *Upper panel:* (BK1), bradykinin alone; (+Cyp), bradykinin and cyclophilin; and (BK2) a final dose of bradykinin. Data are from eleven lung-jugular ring pairs. The mean force of contraction elicited by the initial bradykinin boluses (BK1) was 0.19 ± 0.11 (SD) g. *Lower panel:* (BK1), bradykinin alone; (+Cyp+CsA), bradykinin and cyclophilin plus cyclosporin A; and (BK2), a final dose of bradykinin. Data are from four lung-jugular ring pairs. The mean force of contraction elicited by the initial bradykinin boluses (BK1) was 0.29 ± 0.16 (SD) g. Values are given as means ± SD. The data were analyzed for statistical significance by ANOVA followed by a Newman–Keuls test. Key: (*) significantly different from the other responses, $P < 0.01$.

and tissue injection sites. A bolus containing cyclophilin alone was injected into the lung injection site, and 5 sec later, a bolus of bradykinin alone was injected into the tissue injection site. Under these conditions, the magnitude of the contraction induced by the bradykinin that did not pass through the lungs was the same whether the bolus was injected into the lung injection site contained cyclophilin or vehicle only.

The results obtained with the bradykinin analog, [Gly^6]bradykinin, were similar to those obtained with bradykinin (Fig. 7).

DISCUSSION

When bradykinin boluses were injected into the lung injection site, passing through the lung en route to the jugular ring bioassay tissue, we observed significant intrapulmonary inactivation of bradykinin, to the extent that as little as 5% of the injected dose survived passage through the lungs (e.g. Fig. 3). This observation is consistent with the many published studies of bradykinin metabolism in the lung [24–32]. When cyclophilin was included in a bolus of bradykinin injected into the lung injection site, the fraction of bradykinin that ordinarily survived passage through the lungs fell to much lower, often undetectable, levels. Thus, we conclude that cyclophilin facilitated the inactivation of the bradykinin that normally survives a single transit through the pulmonary capillary bed.

The observation that the cyclophilin effect required that the bradykinin pass through the lung is consistent with the hypothesis that bradykinin inactivation in the lung is isomer specific, involving one or more of its X-Pro bonds. This is consistent with observations that many peptidases preferentially hydrolyze peptide conformers containing *trans* X-Pro bonds [1, 12–17]. Since a fraction of the bradykinin is not inactivated in the lung under normal conditions, our result also implies that the *cis-trans* isomerization rate constant of one or more of the three X-pro bonds is longer than the capillary transit time.

London *et al.* [35] showed that cyclophilin catalyzed *cis-trans* isomerization of the Ser^6-Pro^7 and Gly^6-Pro^7 bonds, respectively, of [*p*-fluoro-Phe^8]bradykinin and its Gly^6 analog. They found that at a bradykinin concentration of 2.2 mM, the spontaneous isomerization rate constant (0.048 sec^{-1}) was increased by 1.2 sec^{-1} per μM cyclophilin. In a typical experiment in our study, the cyclophilin concentration in the injectates was 32 μM. This represents an upper boundary on the concentration of cyclophilin in the bolus passing through the lung, which is reduced by mixing and dispersion in the perfusate flowing through the lungs. Based on the results of London *et al.* [35], this concentration of cyclophilin would result in a catalyzed isomerization rate constant for bradykinin (2.2 mM) in their experiments of 40 sec^{-1}. Although we do not know the K_m for this reaction, K_m values for other peptides are generally in the millimolar range [4, 39]. In our boluses, bradykinin was present in micromolar concentrations. Therefore, the fact that cyclophilin had a significant effect on the isomerization within the time course of a pulmonary capillary transit time of about 2–3 sec appears to be consistent with the influence of cyclophilin on the rate of [*p*-fluoro-Phe^8]bradykinin isomerization measured by London *et al.* [35].

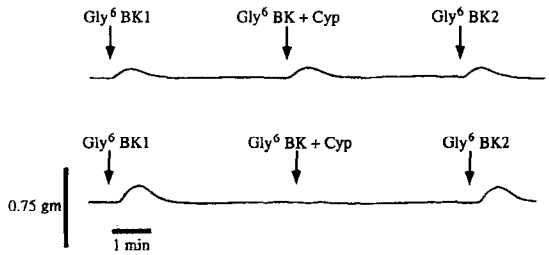

Fig. 7. Effect of cyclophilin on [Gly^6]bradykinin-induced jugular ring contractions. *Upper tracing:* Effects of [Gly^6]bradykinin with and without cyclophilin when boluses were injected into the tissue injection site. The injection sequence was as follows: [Gly^6]bradykinin alone (Gly^{6}BK1); [Gly^6]bradykinin and cyclophilin (Gly^{6}BK+Cyp); and a final dose of [Gly^6]bradykinin (Gly^{6}BK2). Boluses contained 37.5 pmol of [Gly^6]bradykinin and 3.5 nmol of cyclophilin. *Lower tracing:* Effects of [Gly^6]bradykinin with and without cyclophilin when boluses were injected into the lung injection site. The sequence of injections was as above. Boluses contained 600 pmol of [Gly^6]bradykinin and cyclophilin as above.

London *et al.* [35] studied only the Ser^6-Pro^7 bond, but the study did not exclude the possibility that *cis-trans* isomerization of the two remaining X-Pro bonds could also be catalyzed by cyclophilin. Since the length of the substrate N-terminal to the imide bond is thought to be important for efficient catalysis by cyclophilin [2], the Arg^1-Pro^2 bond may not be an important site of cyclophilin-catalyzed *cis-trans* isomerization in bradykinin. However, since the Arg^1-Pro^2 and Pro^2-Pro^3 bonds of bradykinin have not been specifically studied in this regard, we cannot predict the extent to which their isomerization rates contributed to the results of our study.

Although the shift between the lung and tissue injection site dose–response curves is probably not a highly accurate measure of the extent of intra-pulmonary bradykinin inactivation since there is a difference in the dispersion that occurs after injection at the different sites, fractional inactivation of bradykinin in the perfused lung appears to exceed 90% (Fig. 3). If each X-Pro bond exists as up to 10% *cis* conformer [33, 34], the data may suggest that not every X-Pro bond is functionally involved in the inactivation, that there are bradykinin peptidases that do not prefer *trans* isomers, or under the conditions of the study, that the uncatalyzed isomerization time constant of one or more of these bonds approaches the capillary transit time. Alternatively, if the relevant *cis* fractions are somewhat smaller than under the conditions of the NMR studies, more than one of the X-Pro bonds may contribute to the peptidase resistant conformation(s).

Given the degree to which ACE is thought to contribute to bradykinin inactivation in the lung [21, 25, 31, 40], it seems likely that the *cis* isomer of the Ser^6-Pro^7 bond may be relevant. It is also probable that lung peptidases other than ACE have a preference for *trans* isomers of proline-containing substrates. For example, aminopeptidase P, which is also thought to contribute to the intrapulmonary inactivation of bradykinin [25, 31], cleaves bradykinin at the Arg^1-Pro^2 bond, and there is direct and indirect evidence suggesting that it also has a *trans* preference [14, 41].

We used cyclophilin as a tool to evaluate the impact of

cis and *trans* prolyl-peptide isomers on bradykinin hydrolysis in the lung. This is a predominately intracellular enzyme that is probably not present in plasma in sufficient quantity to have a substantial effect on the rate of *cis-trans* isomerization of imide bonds that are involved in regulating bradykinin hydrolysis in the lung [42]. Therefore, it is not expected that cyclophilin plays an important role in facilitating inactivation of proline-containing peptides in the lung vasculature under normal physiological conditions. On the other hand, the abundant *cis-trans* isomerases in intracellular compartments [7, 8, 42] and, by extension, their inhibitors, the immunosuppressive drugs cyclosporin A and FK506, may be important modulators of peptide activation and inactivation by converting enzymes and peptidases inside cells.

One possible implication of our results is that at the beginning of the arterial system, at least one of the imide bonds in any bradykinin that survives passage through the lungs is predominately in the *cis* conformation. Although we do not know which of the *cis* conformations is most relevant, we might take as an example a form that has a *cis* isomer at the Ser^6-Pro^7 bond. Based on the spontaneous isomerization rate constant that has been measured for this bond in [*p*-fluoro-Phe^8]bradykinin (0.048 sec^{-1}) [35], half the bradykinin could still be in this peptidase resistant conformer 14 sec after it has entered the systemic circulation from the venous outflow of the lung. This finding may be of further physiological relevance if *cis* and *trans* isomers can affect the conformation of an entire peptide [1], and, as has been suggested, bradykinin receptor subtypes are sensitive to different bradykinin conformations [33, 35]. If the proportions of circulating bradykinin occurring in alternative isomeric conformations vary throughout the circulation, the fraction of bioactive bradykinin available to receptor subtypes at different levels of the systemic circulation would also vary. Hence, for the wide variety of circulating proline-containing physiological peptides, the lung may regulate not only the extent of their conversion to active or inactive forms, as has been known for a long time, but also the nature of their activity.

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