Articles

Photochemical Inactivation of the Angiotensin Receptor of Rabbit Aorta by N^{α}-(2-Nitro-5-azidobenzoyl)-[1-aspartic acid,5-isoleucine]angiotensin II

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The photoaffinity label N^{α}-(2-nitro-5-azidobenzoyl)-[Asp[1],Ile[5]]angiotensin II (nitroazidobenzoyl-angiotensin II) has a pD_2 of 7.22 in the rabbit aortic strip assay compared to 8.44 for [Asp[1],Ile[5]]angiotensin II and can be completely inhibited by [Sar[1],Ala[8]]angiotensin II. Photolysis of a single dose of nitroazidobenzoyl-angiotensin II (0.1 μM) with aortic strips for 3 min with light of wavelengths greater than 320 nm, followed by washing, resulted in loss of approximately 25% of the strip's response to a subsequent dose of nitroazidobenzoyl-angiotensin II. Photolysis of three consecutive doses of label resulted in loss of 62% of the strips response to a subsequent dose. Repetitive exposure to label in the dark and repetitive photolyses without label resulted in loss of no more than 20% of the strips ability to respond to angiotensin II. Photolysis with three consecutive doses of 0.1 μM nitroazidobenzoyl-angiotensin II in the presence of 0.5 μM of the competitive inhibitor [Sar[1],Ala[8]]angiotensin II decreased the observed inhibition from 62 to 22%. The long-lasting inhibition observed after photolysis with 0.1 μM nitroazidobenzoyl-angiotensin II therefore occurred via photolysis of some part of the nitroazidobenzoyl chromophore. This inhibition was mediated via the receptor that binds [Sar[1],Ala[8]]angiotensin II and, therefore, angiotensin II.

Three criteria need to be satisfied in order to demonstrate that covalent affinity labeling of a biological receptor has occurred:[1] (1) the demonstration of an irreversible or long-lasting biological effect, (2) specific protection against this effect by the receptor's natural ligand or a competitor, and (3) stoichiometric correlation of the irreversible effect and the covalent incorporation of an affinity label.

We have previously shown that photoaffinity labeling derivatives of several peptide hormones were either long-lasting agonists (cholecystokinin carboxy-terminal pentapeptide[2] and octapeptide[3] in the exocrine pancreas) or long-lasting antagonists (oxytocin in toad bladder)[4] when photolyzed in the presence of their respective target tissues, thus satisfying criteron 1 above. In two of these cases (cholecystokinin octapeptide and oxytocin), native ligand protected against the irreversible biological effects resulting from photoaffinity labeling, satisfying criterion 2. Only one other study, with 4-N_3-Phe- and 4-NO_2-Phe-angiotensins, has demonstrated both an irreversible biological effect and protection against this effect by a competitor.[5,6] A number of recent studies have demonstrated specific radiolabeling of one or more target cell-membrane proteins, either with radiolabeled photoaffinity labeling derivatives of insulin[7-9] and epidermal growth factor[10,11] or by direct cross-linking of these membrane-bound radiolabeled hormones to their receptors with bifunctional reagents.[12] None of these studies with radiolabeled hormone have reported any long-lasting biological or biochemical effects resulting from covalent labeling.

However, Czech and co-workers have recently demonstrated both inactivation of insulin stimulated 3-*O*-methylglucose transport and labeling of a specific membrane protein complex by cross-linking insulin to fat cells with bisimidates.[13-15]

An angiotensin photoaffinity label, N^{α}-(2-nitro-5-azidobenzoylnorleucyl)-[Asp[1],Ile[5]]angiotensin II, has been prepared, but its relative affinity in the rabbit aortic strip (0.5% compared to 100% for [Asp[1],Ile[5]]angiotensin II) was judged to be too low to give a high ratio of specific to nonspecific labeling.[16] In this report, N^{α}-(2-nitro-5-azidobenzoyl)-[Asp[1],Ile[5]]angiotensin II (Figure 1, abbreviated as nitroazidobenzoyl-angiotensin II) was prepared and found to be one order of magnitude higher in relative affinity (6%) compared to N^{α}-(2-nitro-5-azidobenzoyl-norleucyl)-[Asp[1],Ile[5]]angiotensin II (0.5%). Three consecutive photolyses of N^{α}-(2-nitro-5-azidobenzoyl)-[Asp[1],Ile[5]]angiotensin II at a concentration of 0.1 μM with rabbit aortic strips reduced their ability to respond to a subsequent dose of agonist by 62%. This long-lasting inhibition was prevented by including the competitor, 0.5 μM [Sar[1],Ala[8]]angiotensin II, in the photolysis experiment. Thus, the long-lasting inactivation of rabbit aortic strips by nitroazidobenzoyl-angiotensin II occurs via the receptor

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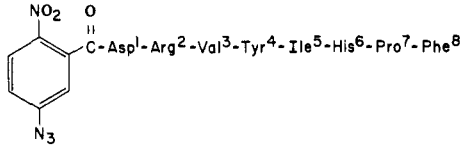

Figure 1. N^{α}-(2-Nitro-5-azidobenzoyl)-[Asp^1,Ile^5]angiotensin II.

for [Sar^1,Ala^8]angiotensin II, a competitive antagonist for [Asp^1,Ile^5]angiotensin II.[17,18]

Experimental Section

Thin-layer chromatography was performed on precoated silica gel plastic-backed plates (Merck silica gel 60, F-254) in the following systems: 1-butanol–acetic acid–water (BAW), 4:1:1; 1-butanol–acetic acid–water–pyridine (BAWP), 15:3:12:10; 1-butanol–ethyl acetate–acetic acid–water (BEAW), 1:1:1:1; ethyl acetate–pyridine–acetic acid–water (EPAW), 5:5:1:3. Peptides were visualized after exposure to ultraviolet light, by the Pauly reagent spray, or by ninhydrin.[19]

The high-pressure liquid chromatograph consisted of a 4.5 × 150 mm Partisil 10 ODS column, a Milton Roy mini pump, a Schoeffel Spectroflow monitor (SF770), and a linear recorder. The following solvent systems were used: (A) 25% acetonitrile in 0.1 M triethylammonium acetate, pH 4.4, for quantitative determination of N^{α}-(2-nitro-5-azidobenzoyl)-[Asp^1,Ile^5]angiotensin II and (B) 16% acetonitrile in 0.4 M triethylammonium acetate, pH 4.4, for determination of [Asp^1,Ile^5]angiotensin II. Column operating pressures were between 900 and 1500 psi depending on the solvent systems used and the flow rate, which was between 0.8 and 1.5 mL/min.

Amino acid analyses were performed on a Durrum 500 amino acid analyzer after hydrolysis under vacuum in 6 N hydrochloric acid containing 0.2% phenol for 24 or 48 h at 110 °C. Values are reported as molar ratios relative to phenylalanine.

Ultraviolet spectra were recorded on a Beckman 25 spectrophotometer. Extinction coefficients were calculated using the maximum absorbance of peptide in a solution of known concentration determined by amino acid analysis. Extinction coefficients were determined for solutions in distilled water and in the liquid chromatrograph systems A and B. The extinction coefficient of 2-nitro-5-azidobenzoylglycine was based on the weight of an analytically pure sample.

The fraction of peptide in solid products was calculated as percent peptide = [(grams of peptide present determined by amino acid analysis)/(grams of solid by weight)] × 100. Percent yields of peptides were calculated as [(grams isolated after purification)/(grams theoretical)] × 100.

Materials. N^{α}-(2-Nitro-5-azidobenzoyl)-[Asp^1,Ile^5]angiotensin II. This compound was prepared similarly to the analogous N^{α}-(2-nitro-5-azidobenzoylnorleucyl)-[Asp^1,Ile^5]angiotensin II.[16] The following procedures were performed in the dark. Equivalent amounts (28.3 μmol) of 1-hydroxybenzotriazole (Chemicals Procurement Laboratories, Inc., NY) and the *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoic acid[20] (0.1 M solutions in dimethylformamide) were added to 29.6 mg (28.3 μmol) of [Asp^1,Ile^5]angiotensin II prepared as previously described.[16] The volume of the reaction mixture was adjusted with an equal volume of pyridine (566 μL) and a drop of water. 1-Hydroxybenzotriazole was found to improve product yield as analyzed by high-pressure liquid chromatography. The reaction mixture was allowed to stand at room temperature for 3 days, after which the reaction product was precipitated and washed with cold acetone, collected by centrifugation, and allowed to dry in air. The resulting precipitate was combined with approximately 12 mg of reaction product made by the same procedure. The combined material was dissolved in 50% acetic acid and chromatographed on a 1.8 × 60 cm column of Bio-Rex 70 (H^+ form, Bio-Rad) equilibrated with 20% acetic acid. Elution with a gradient of 20–60% acetic acid yielded two major fractions eluting at 32 and 42% acetic acid. Lyophilization of these fractions produced 9.0 mg of free angiotensin II and 12.0 mg of nitroazidobenzoyl-angiotensin II, respectively (22% reaction yield). Amino acid analysis of nitroazidobenzoyl-angiotensin II showed it to be 73% peptide by weight: Asp, 1.04; Pro, 1.00; Val, 1.04; Ile, 1.01; Tyr, 1.01; Phe, 1.00; His, 1.02; Arg, 1.02.

High-pressure liquid chromatography in solvent A showed that 98% of all absorbance at 320 nm was due to the single nitroazidobenzoyl-angiotensin II peak and in system B that only 0.6% free angiotensin II was present. The retention time for nitroazidobenzoyl-angiotensin II was 19.4 min (system A, 1300 psi, 0.8 mL/min) and for angiotensin II was 12.2 min (system B, 1400 psi, 0.64 mL/min).

Ultraviolet absorbance spectra of nitroazidobenzoyl-angiotensin II showed ϵ_{320} 9790, ϵ_{320} (A) 11 101 (2-nitro-5-azidobenzoylglycine: ϵ_{320} 10 812; angiotensin II: ϵ_{278} 1375, ϵ_{278} (B) 1647).

Thin-layer chromatography gave the following results. Nitroazidobenzoyl-angiotensin II: R_f (BAW) 0.23, R_f (BAWP) 0.56, R_f (BEAW) 0.65, R_f (EPAW) 0.85; one spot, Pauly positive, ninhydrin negative, and visibly yellow upon exposure to ultraviolet light. Angiotensin II: R_f (BAW) 0.05, R_f (BAWP) 0.42, R_f (BEAW) 0.43 (streak), R_f (EPAW) 0.69; one spot, Pauly positive, ninhydrin positive, and not visible after exposure to ultraviolet light.

Methods. Bioassay of Nitroazidobenzoyl-angiotensin II in Rabbit Aortic Strips. Peptides were assayed on rabbit aortic strips as described by Furchgott and Bhadrakom[21] with the following modifications: aortas were removed from 4-lb male New Zealand rabbits which were sacrificed by intravenous injection of 3–4 mL USP pentobarbitol sodium (50 mg/mL). Aortas were placed in a pH 7.4 tissue culture solution containing 100 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 25 mM *N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid, 14 mM glucose, 4 mM $CaCl_2$, 0.1 mM MEM amino acids (Gibco, 50X), 0.1 mM nonessential amino acids (Gibco, 100X), 2 mM L-glutamine, 100 units/mL penicillin, 0.25 μg/mL fungizone, 0.10 mg/mL streptomycin. Aortas were stored in this solution at 4 °C if not immediately used.

Aortic strips were helically cut to be 2-cm long and 3- to 4-mm wide and suspended in 8- or 10-mL jacketed muscle baths kept at 37 °C. Air was bubbled in the baths throughout the experiments. A Grass Model 70 polygraph with FT 0.03 strain gauge was used to record isometric contraction. Strips were initially placed under 2 or 5 g of tension and allowed to equilibrate for 90 min with this tension maintained before the first dose of peptide. After response to a peptide and/or photolysis, the solution in the bath was replaced every 15 min until the tension of the strip returned to a stable base line. Dose–response curves for angiotensin II and nitroazidobenzoyl-angiotensin II were determined by the cumulative-dose method.[22] Curves were obtained by assaying each peptide simultaneously in separate, identically treated strips obtained from a single aorta.

Effect of Photolysis Light Alone on Rabbit Aortic Strips. Light is known to reversibly relax aortic smooth-muscle strips.[23] The effectof the photolysis light on the aortic strips' response to angiotensin II was tested by subjecting aortic strips to photolysis lamp exposure without the presence of peptide.

The photolysis apparatus consisted of a 450-W Hanovia high-pressure mercury lamp inserted in a 1-in. tube of Corning No. 3220 glass (which transmits only wavelengths above 320 nm) contained in a water-jacketed borosilicate immersion well. Photolysis was affected by a 3-min exposure of the aortic strip to the lamp, at a distance of approximately 2 cm from the lamp's water jacket. A 3-min photolysis was sufficient to destroy >90% of the azide functional group, as measured by infrared spectroscopy.[2] Longer photolysis times did not improve or change the observed effects of photolysis. The photolyses were performed at 40-min intervals and were always followed by replacement of bath solution (wash). Response to 0.1 μM angiotensin II was recorded before and after three photolyses as a measure of strip

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response. An experiment was performed simultaneously on a second aortic strip in the dark in which no exposure to the lamp took place, but all other conditions were identical. This protocol, 3 min of photolysis of one strip with a second strip treated identically but without photolysis, was followed in all experiments reported.

Inhibition of Nitroazidobenzoyl-angiotensin II Induced Contraction of Rabbit Aortic Strips. Aortic strips were photolyzed in the presence of nitroazidobenzoyl-angiotensin II and then restimulated with this peptide to measure the inactivation of response resulting from photolysis. Doses of 0.1 and 1 μM nitroazidobenzoyl-angiotensin II were used for these studies. Strips were exposed to nitroazidobenzoyl-angiotensin II and photolyzed at the time of maximum response to the peptide. After three photolyses the strips response to a final fourth dose or the average of a fourth and fifth dose was measured. The response of a second strip to repeated doses of nitroazidobenzoyl-angiotensin II was assayed simultaneously in the dark, without photolysis. The same photolabeling experiment and dark control was repeated at a dose of 0.1 μM nitroazidobenzoyl-angiotensin that has been previously photolyzed for 3 min at the same concentration.

Protection against the Inhibition of Nitroazidobenzoyl-angiotensin II Induced Contraction of Rabbit Aortic Strips with [Sar^1,Ala^8]Angiotensin II. After recording the response to an initial dose of 0.1 μM angiotensin II, aortic strips were subjected to simultaneous doses of 0.1 μM nitroazidobenzoyl-angiotensin II and 0.5 μM of the competitive inhibitor [Sar^1,Ala^8]angiotensin II[17,18] and photolyzed at the point in time which corresponded to the strip's maximum response to 0.1 μM nitroazidobenzoyl-angiotensin II (15 min after addition of the peptide). This latter procedure was performed three times, followed by one or two final doses of 0.1 μM angiotensin II. Identical experiments were performed at the same time in the dark without photolysis.

Statistical Treatment of Results of Biological Assays. The ED_{50}, pD_2, and the intrinsic activity of angiotensin and nitroazidobenzoyl-angiotensin II are the means of six determinations ± standard errors. (ED_{50} is the dose of agonist eliciting 50% of its maximum response and pD_2 is the negative logarithm of the ED_{50}.) In the photolysis experiments, the effect of light alone and the inhibition of the response to nitroazidobenzoyl-angiotensin II by photoaffinity labeling were determined as the means of four experiments. The protection against inhibition afforded by [Sar^1,Ala^8]angiotensin II was the mean of three experiments. Photolysis experiments were performed using two identically treated strips in which one received photolysis by exposure to the mercury lamp and one was always kept dark. Responses in each strip were normalized to the initial response obtained in that strip (either to nitroazidobenzoyl-angiotensin II or to angiotensin II). Results from pairs of aortic strips were discarded if the unphotolyzed strip kept in the dark showed a decrease to less than 75% of its original response. Using this criterion, 6 out of 10 experiments at 0.1 μM and 11 out of 18 at 1 μM nitroazidobenzoyl-angiotensin were discarded. The significance of the differences between results was calculated using the t test of significance.[24]

Results

N^{α}-(2-Nitro-5-azidobenzoyl)-[Asp^1,Ile^5]angiotensin II. This derivative was isolated in relatively low yield (22%) from the acylation of angiotensin II by the N-hydroxysuccinimide ester of 2-nitro-5-azidobenzoic acid, and a large amount of free angiotensin was recovered (~20% yield). Increasing the ratio of acylating agent to angiotensin resulted in more complete conversion to product but resulted in increasing amounts of side products containing angiotensin and the nitroazidobenzoyl chromophore which were difficult to separate from the desired derivative. This apparent reaction of the N-hydroxysuccinimide ester with nucleophiles other than the α-amino group of angiotensin is surprising considering that these esters are thought to be highly specific for amino groups.[25]

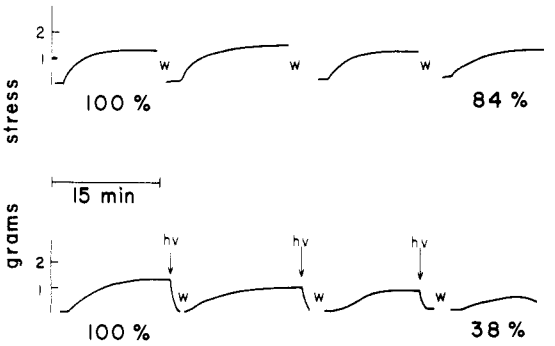

Figure 2. Inhibition of the response of a rabbit aortic strip to 0.1 μM nitroazidobenzoyl-angiotensin by repetitive photoaffinity labeling. The arrows indicate photolysis for a duration of 3 min after the maximum response to each dose of label occurred under the conditions described in the text. The upper tracing shows the control experiment performed in the dark without photolysis; the lower tracing is with photolysis. W indicates a wash of approximately 30-min duration.

The relative affinity of nitroazidobenzoyl-angiotensin II (the ratio of the ED_{50} of angiotensin II to that of nitroazidobenzoyl-angiotensin II × 100) was determined from dose–response curves for this peptide and native angiotensin II. The results of four pairs of dose–response curves gave a relative affinity of 6 ± 4% for nitroazidobenzoyl-angiotensin II compared to 100% for angiotensin II. ED_{50} values for angiotensin II and nitroazidobenzoyl-angiotensin II were $3.63 \pm 2 \times 10^{-9}$ ($pD_2 = 8.44$) and $6.04 \pm 4 \times 10^{-8}$ M ($pD_2 = 7.22$), respectively. The intrinsic activity of nitroazidobenzoyl-angiotensin II was 100 ± 30% that of angiotensin, and the strip's response to nitroazidobenzoyl-angiotensin could be completely inhibited by [Sar^1,Ala^8]angiotensin II. Nitroazidobenzoyl-angiotensin II, 0.1 μM, which had been previously photolyzed for 3 min elicited a response indistinguishable from that elicited by unphotolyzed peptide.

Inhibition of Nitroazidobenzoyl-angiotensin Induced Contraction of Aortic Strips. The inhibition of contraction of a rabbit aortic strip following photoaffinity labeling with nitroazidobenzoyl-angiotensin is shown in Figure 2. Figure 3 shows the mean results of four such experiments. The aortic strips' mean response to 0.1 μM nitroazidobenzoyl-angiotensin II dropped from 100 ± 0 to 38 ± 5% after three photolyses (Figure 3D). A simultaneous assay on a second strip in the dark without photolysis showed a mean initial response of 100 ± 0% and a final response of 84 ± 5% (Figure 3B). Figure 3 demonstrates long-lasting inhibition (inhibition persisting after a 30-min wash after each photolysis) of mean response to both 0.1 and 1 μM nitroazidobenzoyl-angiotensin II. Nitroazidobenzoyl-angiotensin II at 0.1 μM which had been previously photolyzed for 3 min showed an initial response of 100 ± 0% and a final response of 41 ± 5% after three photolyses with a strip (two experiments). In the dark, the initial response of 100 ± 0% dropped to 80 ± 3% (two experiments) for previously photolyzed nitroazidobenzoyl-angiotensin. This result suggests that the presumed nitrene initial photoproduct is not necessary for inactivation of the tissue, which was also found for nitroazidobenzoyl-cholecystokinin pentapeptide.[2]

Protection against the Nitroazidobenzoyl-angiotensin II Induced Inhibition of Contraction of Rabbit Aortic Strips with [Sar^1,Ala^8]Angiotensin II and the

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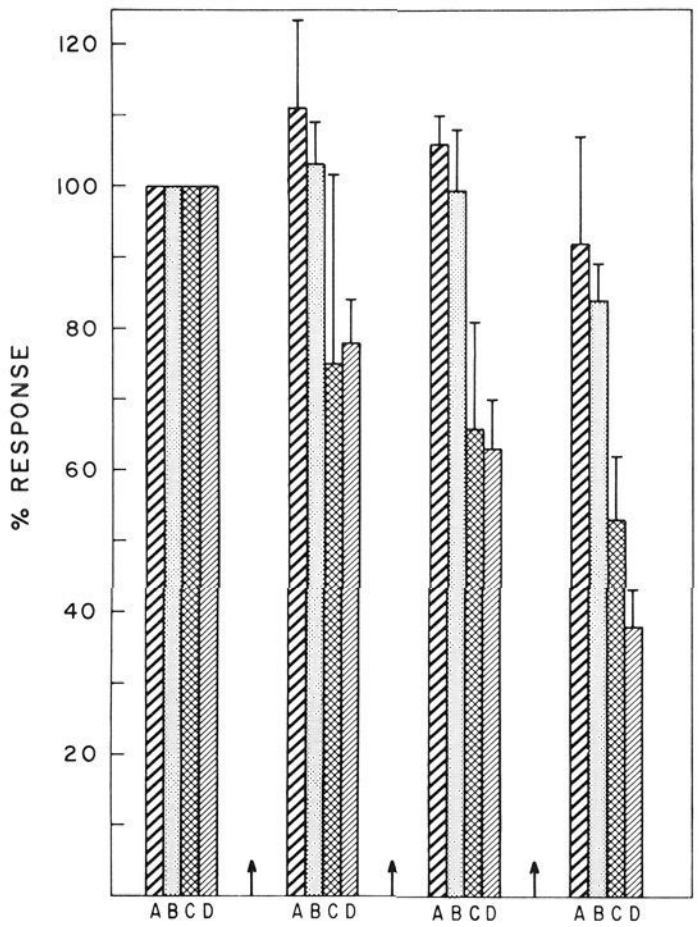

Figure 3. Inhibition of the response of rabbit aortic strips to nitroazidobenzoyl-angiotensin by repetitive photoaffinity labeling. The arrows indicate photolysis of C and D for a duration of 3 min under the conditions described in the text. The error bars show standard deviation for the means of four experiments: 1 μM nitroazidobenzoyl-angiotensin without photolysis (A) and with photolysis (C); 0.1 μM nitroazidobenzoyl-angiotensin without photolysis (B) and with photolysis (D).

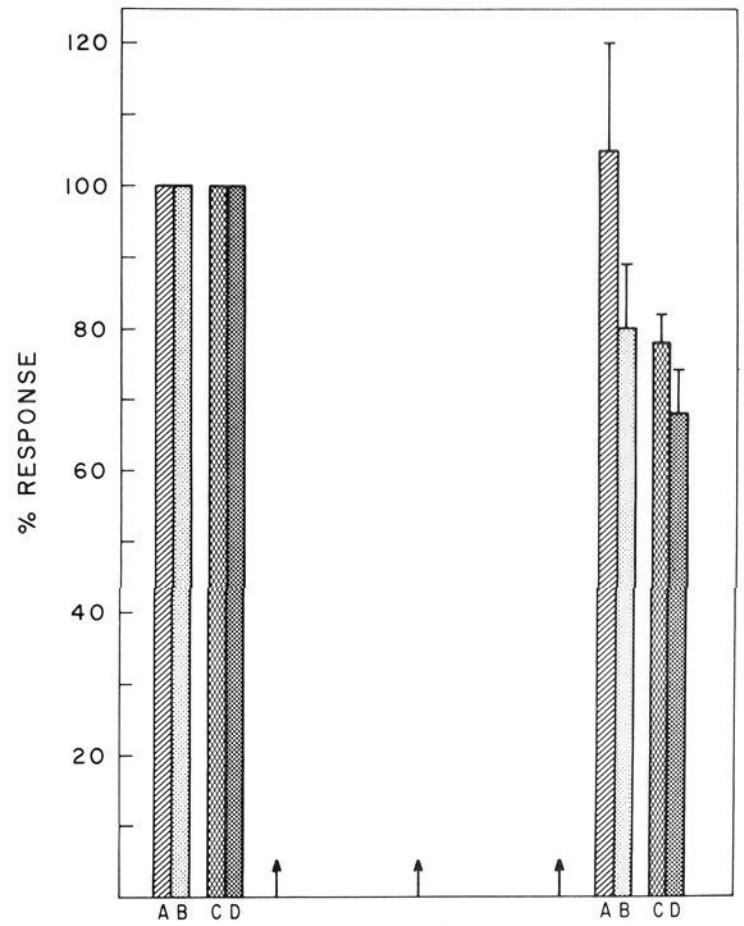

Figure 4. Protection by 0.5 μM [Sar^1,Ala^8]angiotensin II against inhibition of the response of rabbit aortic strips by repetitive photoaffinity labeling with 0.1 μM nitroazidobenzoyl-angiotensin. The arrows indicate photolysis of B and D for a duration of 3 min under the conditions described in the text. The error bars show standard deviations for the means of three experiments: 0.1 μM angiotensin II without (A) and with (B) photolysis; 0.1 μM nitroazidobenzoyl-angiotensin II plus 0.5 μM [Sar^1,Ala^8]angiotensin II without (C) and with (D) photolysis.

Effect of Photolysis in the Absence of Nitroazidobenzoyl-angiotensin II. The long-lasting inhibition of contraction by photolysis in the presence of 0.1 μM nitroazidobenzoyl-angiotensin II is shown in Figure 3D (a drop from 100 ± 0% response to 38 ± 5% response after three consecutive photolyses). Figure 4D shows that 0.5 μM [Sar^1,Ala^8]angiotensin protects against this inhibition (a drop from 100 ± 0% response to 68 ± 6%). Responses of strips exposed to identical treatment without photolysis (in the dark) were 100 ± 0% initially and 78 ± 4% at the conclusion of the experiments (Figure 4C). The effect of three consecutive photolyses in the absence of nitroazidobenzoyl-angiotensin was determined by comparing strips kept in the dark to photolyzed strips. Those strips kept in the dark showed an initial response of 100 ± 0% to 0.1 μM angiotensin II and a 105 ± 5% response at the conclusion of the assay (Figure 4A). The photolyzed strips showed a 100 ± 0% response initially and 80 ± 9% after three photolyses (Figure 4B). Responses are the means of three determinations.

Discussion

The photoaffinity label N^{α}-(2-nitro-5-azidobenzoyl)-[Asp^1,Ile^5]angiotensin II inactivates angiotensin receptor of rabbit aortic strip to the extent of 25% after a single labeling and 62% after three successive labelings. This long-lasting inhibition is both light dependent and dependent on the presence of the nitroazidobenzoyl chromophore or some photoproduct of this chromophore. It is prevented by including the competitive (reversible) antagonist [Sar^1,Ala^8]angiotensin II during photoaffinity labeling. These results are comparable to those obtained by Escher and Guillemette[5] using angiotensins substituted at positions 4 and 8 with 4′-nitrophenylalanine and 4′-azidophenylalanine. They found an average of 11 to 56% inactivation after a single labeling, and this inactivation was prevented by the inclusion of competitor during labeling. The observed inhibition of aortic strips' response by previously photolyzed nitroazidobenzoyl-angiotensin suggests that the nitro group or some photoproduct of the original chromophore could be responsible for inactivation of the receptor. Escher and co-workers[5,26] have clearly demonstrated the photolabeling ability of the aromatic nitro group. A previous study has demonstrated the long-lasting biological effect of photolysis of nitroazidobenzoyl-cholecystokinin pentapeptide after destruction of the azido group by previous photolysis.[2] Since previously photolyzed nitroazidobenzoyl-angiotensin does not inhibit the strips response without additional photolysis, a very long-lived chemically reactive photoproduct is not involved in the long-lasting inhibition. Long-lasting inhibition of the strip's response does not demonstrate covalent attachment of label to receptor but only suggests some modification of the receptor. Modification by a photooxidation involving oxygen or a free-radical reaction resulting from hydrogen abstraction by a photoproduct of the nitroazidobenzoyl chromophore could be responsible for the observed inactivation of the strip. The prevention of this inactivation by inclusion of the angiotensin competitor [Sar^1,Ala^8]angiotensin II during photolysis suggests that whatever modification is occuring does occur at the angiotensin receptor.

The results of Escher and Guillemette[5] and our results suggest either that permanent occupation of the angiotensin receptor in rabbit aortic strip inactivates the receptor's ability to respond to a subsequent dose of the hormone or that a modification of the receptor binding site for angiotensin by the photochemical intermediates de-

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stroys the receptor's ability to respond either to the affinity label or to a subsequent dose of agonist. Inactivation occurs whether the photolyzable functional group is located at the amino terminus of angiotensin (nitroazidobenzoyl-angiotensin) or at the 4 or 8 position.[5]

This long-lasting inactivation is in contrast to the results found with cholecystokinin receptor in the guinea pig exocrine pancreas. In this tissue both nitroazidobenzoylglycine-acylated carboxy-terminal tetra- and octapeptides elicited a long-lasting *agonist* effect after photoaffinity labeling.[2,3] Therefore, there must be a fundamental difference between cholecystokinin receptor in pancreas and angiotensin receptor in aorta, such as the mechanism of hormone recognition by the receptor, the activation of the receptor's initial response to the hormone, or the coupling of this response to a second messenger.

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Potential Thyroliberin Affinity Labels. 1. Chloroacetyl-Substituted Phenylalanylpyrrolidines[1]

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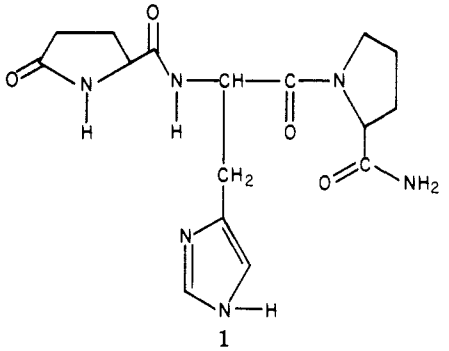
Six analogues of thyroliberin (TRH) that have a chloroacetyl substituent at the amino terminus have been prepared as potential affinity labels for the TRH receptor. These compounds are *N*-(chloroacetyl)-L-alanyl-L-phenylalanylpyrrolidine (ClAc-Ala-Phe-Pyrr; **14**), *N*-[*m*-(chloroacetyl)benzoyl]-L-phenylalanylpyrrolidine (*m*-ClAcBz-Phe-Pyrr; **11a**), *N*-[*m*-(chloroacetyl)benzoyl]-L-alanyl-L-phenylalanylpyrrolidine (*m*-ClAcBz-Ala-Phe-Pyrr; **15a**), *N*-[*p*-(chloroacetyl)benzoyl]-L-phenylalanylpyrrolidine (*p*-ClAcBz-Phe-Pyrr; **11b**), and *N*-[*p*-(chloroacetyl)benzoyl]-L-alanyl-L-phenylalanylpyrrolidine (*p*-ClAcBz-Ala-Phe-Pyrr; **15b**). Pyroglutamyl-L-phenylalanylpyrrolidine was also synthesized as a model agonist. Weak agonist activity was observed for **11a**, **11b**, and **15b**. These three analogues do not contain the amide group of the pyroglutamyl moiety that was previously thought to be essential for intrinsic activity. No significant antagonist activity was observed for these compounds at the doses tested.

Thyroliberin (TRH; 1)[2] was the first peptide hormone



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from the hypothalamus to be isolated, structurally characterized, and synthesized.[3,4] The initial knowledge that TRH influenced the secretion of thyrotropin (TSH) from the anterior pituitary[5] was followed by subsequent studies[6-10] with synthetic TRH that showed that TRH concommitantly stimulates the release of prolactin (PRL) both in vitro and in vivo. The development of a radioimmunoassay[11] for TRH led to the identification of TRH and TRH-like immunoreactive substances not only in the hypothalamus but throughout the brain[12-16] and also in the

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(2) Abbreviations used are: TRH, thyrotropin-releasing hormone; Pyrr, pyrrolidine; pGlu, pyroglutamyl; Phe, phenylalanyl; Ala, alanyl; ClAc, chloroacetyl; ClAcBz, chloroacetylbenzoyl; Z, carbobenzyloxy; IBCF, isobutyl chloroformate; NMM, *N*-methylmorpholine; TLC, thin-layer chromatography.

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