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Photoaffinity Labeling of a Peptide Secretagogue Receptor in the Exocrine Pancreas

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SUMMARY

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The photoaffinity label 2-nitro-5-azidobenzoyl-Gly-Trp-Met-Asp-Phe-NH_2 (NAB-CCK-5) mimics cholecystokinin and the more potent secretagogue cerulein in stimulating discharge of exportable proteins from acinar cells of the guinea pig pancreas *in vitro*. Photolysis of this affinity label in the presence of pancreatic lobules (small clusters of acini) causes irreversible stimulation of protein discharge which is indistinguishable in rate, magnitude, and morphological aspects from that observed with the most potent doses of cerulein. The irreversible agonist activity cannot be removed by extensive washing of the lobules, and is blocked only by metabolic inhibition. Irreversible agonist activity is not observed without photolysis, nor is it observed after photolysis of lobules in the presence of 2-nitro-5-azidobenzoic acid, 2-nitro-5-azidobenzoyl-angiotensin II, 2-nitro-5-azidobenzoyl-Gly-Gly-Met-Asp-Phe-NH_2, or cerulein, or with previously photolyzed NAB-CCK-5. Protection by native peptide secretagogues against irreversible labeling by NAB-CCK-5, however, could not be demonstrated. Although the biologically active sites which we have labeled appear to be those responsible for peptide secretagogue-mediated release of exportable proteins, the absence of competition by native secretagogues for labeling shows that the mechanism of labeling NAB-CCK-5 is not understood at this time.

INTRODUCTION

The photoaffinity label 2-nitro-5-azidobenzoyl-Gly-Trp-Met-Asp-Phe-NH_2 is a derivative of the COOH-terminal pentapeptide of cholecystokinin. NAB-CCK-5[1] has been shown to bind irreversibly to bovine serum albumin (1) after photolysis. This result and the known biological activity of peptides of this length in mimicking CCK stimulation of protein secretion from the exocrine pancreas (2) suggest that NAB-CCK-5 might be used to label CCK receptors on pancreatic acinar cells. The use of photoactivated derivatives of peptides to label their binding sites has been suggested by Schwyzer and Caviezel (3) and Levey (4). The advantages of photoactivated affinity labels over chemically reactive affinity labels, with particular reference to the labeling of peptide binding

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[1] The abbreviations used are: CCK, cholecystokinin; NAB-, the radical 2-nitro-5-azidobenzoyl; NAB-CCK-5, 2-nitro-5-azidobenzoyl-Gly-Trp-Met-Asp-Phe-NH_2; NAB-angiotensin II, 2-nitro-5-azidobenzoyl-Asn-Arg-Val-Tyr-Val-His-Pro-Phe; the structures for CCK-8, CCK-8-des-SO_3, and CCK-4 are given in Table 1.

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sites, have been discussed previously (1, 3, 4). Derivatives of several peptide hormones have been employed as chemical affinity labels for their respective receptors with varying degrees of success (4–8). For convenience the primary structures and secretory potencies in the exocrine pancreas of CCK, NAB-CCK-5, and other secretagogues are shown in Table 1.

The objective of this study was to test the feasibility of photoaffinity labeling receptors for NAB-CCK-5 in lobules (small clusters of acini) of guinea pig pancreas. The theoretical pathway of the photoaffinity labeling process can be described by the following two reactions:

$$\text{NAB-CCK-5} + \text{receptor} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\text{NAB-CCK-5: receptor complex}] \quad (A)$$

$$[\text{NAB-CCK-5:receptor complex}] \xrightarrow[h\nu]{k_3} \text{NAB-CCK-5:receptor covalent complex} \quad (B)$$

In reaction A, NAB-CCK-5 and receptor form a reversible complex with association rate constant k_1, dissociation rate constant k_2, and equilibrium association constant $K_a = k_1/k_2$. In reaction B, photolysis of the reversible NAB-CCK-5:receptor complex produces an irreversible, covalent NAB-CCK-5:receptor complex with the forward rate constant k_3, where $k_3 >> k_2$.[2] The short lifetime of the nitrene intermediate resulting from photolysis of aryl azides ensures that the equilibrium in reaction A will not change during the course of reaction B, and thus the amount of covalent complex formed in B should be directly proportional to the amount of reversible complex present in A at the time of photolysis. In addition, reaction B should enable one to "freeze" permanently the ligand-receptor interaction, which is fast with respect to its biological effect (discharge of secretory proteins), and which can then be measured with a conveniently slow assay method.

We show here that photoaffinity labeling of a secretagogue receptor on pancreatic exocrine cells irreversibly stimulates discharge of secretory proteins and that irreversible stimulation of discharge shows great specificity for the affinity label. However, native peptide secretagogues do not compete for or protect against this irreversible stimulation of discharge. Irreversible stimulation of discharge has been used to measure the time course of the affinity labeling. These results have been reported in preliminary form (11).

[2] The lifetime of dinitrenobiphenyl in ethanol is about 200 μsec (9). This lifetime reflects hydrogen abstraction from a carbon-hydrogen bond and should be similar to the lifetime of the nitrene when reacting with a carbon-hydrogen bond in a protein. Although no data for the quantitative binding of peptides to receptors in exocrine pancreas are known, the lifetime of the hormone-receptor complex for gastrin I with gastric mucosal membranes has been reported to be approximately 3 min (10). Thus, although k_3 and k_2 are not known for NAB-CCK-5 in guinea pig pancreatic lobules, it seems likely that $k_3 >> k_2$.

MATERIALS AND METHODS

Materials. 2-Nitro-5-azidobenzoic acid and 2-nitro-5-azidobenzoyl-Gly-Trp-Met-Asp-Phe-NH_2 were prepared as described previously (1). 2-Nitro-5-azidobenzoyl-[2-^{3}H]Gly-Trp-Met-Asp-Phe-NH_2 (specific activity, 50 μCi/μmole) was prepared as previously described (1), and was repurified before use by chromatography on Bio-Rad AG1-X2 (acetate form), eluting with a gradient of 10–50% acetic acid. Trp-Met-Asp-Phe-NH_2 and tritiated leucine were obtained from Schwarz/Mann. 2-Nitro-5-azidobenzoyl-Gly-Gly-Met-Asp-Phe-NH_2 was prepared in the same manner as the above peptides from Gly-Met-Asp-Phe-NH_2 (an inactive analogue of tetragastrin), which was the generous gift of Dr. J. S. Morley of Imperial Chemical Industries, Ltd. 2-Nitro-5-azidobenzoyl-[Asn^1,Val^5]-angiotensin II was prepared by acylating synthetic [Asn^1,Val^5]-angiotensin II (12) with the *N*-hydroxysuccinimide ester of 2-nitro-5-azidobenzoic acid in dimethylformamide–pyridine in the presence of 1 Eq of 1-hydroxybenzotriazole. The product was purified by chromatography on Bio-Rex 70 (H^+ form), eluting with a gradient of 10–50% acetic acid.

Other natural and synthetic peptide se-

cretagogues were the kind gifts of the following scientists, whose generosity and interest in our studies we gratefully acknowledge: cerulein, Drs. Bruno Camerino and Robert Costiglione, Farmitalia, Milan; cholecystokinin, Dr. Victor Mutt, Karolinska Institute, Stockholm; cholecystokinin octapeptides, Dr. Miguel Ondetti, Squibb Institute for Medical Research, Princeton; gastrins, Dr. Morton Grossman, Wadsworth Veterans Administration Center, Los Angeles; and secretin, Dr. H. C. Beyermann, Technische Hogeschool, Delft, and Dr. Rolf Geiger, Hoechst Aktiengesellschaft, Germany. Tritiated angiotensin II was obtained from New England Nuclear. All solvents and buffers were prepared from reagent-grade chemicals.

Assay of released protein. The pancreas was removed from 350–450-g male guinea pigs (fasted for 24 hr, with water given ad libitum), placed in cold Krebs-Ringer-bicarbonate solution (13, 14), expanded with this solution, and dissected into lobules (small clusters of acini, about 2 mm in diameter) (15, 16). The dissected lobules were then placed in Erlenmeyer flasks (4–10/flask) at either 4° or 37°, containing either 2 or 5 ml of Krebs-Ringer solution and one disc (1 cm in diameter) of Nytex nylon cloth, 150-μm mesh. Since the lobules adhered to the edge of the disc, the affinity labeling experiments, including all washings and transfers to new medium, were conveniently performed with lobules attached to the discs.

The labeling and release of secretory proteins were carried out as described for pancreatic slices (17), with each disc in a separate flask. The discs were placed in medium containing 50 μCi/ml of tritiated leucine, plus 0.4 mM nonradioactive leucine. The temperature was kept at 4° for 10 min to allow the labeled leucine to diffuse into the lobules, and was then increased to 37° for 10 min to initiate incorporation of label into exportable proteins (pulse labeling). Each disc was then rinsed in assay medium containing 4.0 mM nonradioactive leucine and reincubated in 5 ml of assay medium for 3 hr at 37°. Then the tissue and medium were collected separately and the amount of acid-precipitable radioactive secretory protein was determined in each as previously described (13, 14, 17). Except for experiments involving photoaffinity labeling, secretagogue was present for the whole 3-hr assay period. The flasks were gassed every 30 min with 95% O_2–5% CO_2, and the pH remained constant at 7.4.

The amount of labeled protein released is expressed as percentage discharge or percentage response. Percentage discharge measures the amount of labeled protein discharged by the tissue in 3 hr relative to the total radioactivity present in protein after the labeling procedure:

$$\%\ \text{discharge} = \frac{\text{radioactivity released}}{\text{radioactivity released} + \text{radioactivity remaining in tissue protein}} \times 100$$

Percentage response expresses the percentage discharge relative to the discharge obtained with maximal stimulation in the same experiment:

$$\%\ \text{response} = \frac{\%\ \text{discharge}}{\%\ \text{discharge with maximal stimulation}} \times 100$$

Tissue was stimulated maximally during the assay period either by photoaffinity labeling with 5 μM NAB-CCK-5 or by 0.1 nM cerulein. Maximally stimulated discharge was normally between 40% and 70% in 3 hr.

For all affinity labeling experiments reported here, pulse labeling of secretory proteins with tritiated leucine followed photolysis. This protocol, rather than the reverse, was chosen so that the wave of pulse-labeled proteins would move through all cells in all flasks in synchrony to their site of release from zymogen granules. In this protocol, cells irreversibly stimulated with affinity label will discharge secretory proteins during the pulse; these proteins, however, will not be radioactive, since 20–40 min elapse before pulse-labeled proteins accumulate in zymogen granules (14). The kinetics of release of labeled proteins during the assay period will be unaffected. In the alternative protocol, discharge of labeled proteins would be asynchronous because of the variable times of previous incubation, especially in experiments measuring the time course of

affinity labeling and washout of affinity label.

The morphological changes associated with the secretory response were observed by light and electron microscopy of fixed, Epon-embedded tissue (17).

All experiments reported here were performed in duplicate on the same day (using a single experimental animal), and most results are averages of two to four duplicate experiments.

Photolysis of 2-nitro-5-azidobenzoic acid derivatives. The photolysis apparatus consisted of a 450-W Hanovia high-pressure mercury lamp inserted in a 1-inch tube of Corning No. 3220 glass (which transmits only wavelengths above 320 nm) contained in a water-jacketed borosilicate immersion well, and has been described (1). The immersion well was inserted horizontally in a rectangular Lucite water bath filled with ice water or held at a higher temperature by means of a Lauda K2/R circulating water bath. Photolysis was always carried out at the same temperature as the prephotolysis incubation of the tissue. For photolysis times less than 1 min, the Erlenmeyer flask was held by hand above the lamp, resting on the immersion well, and gently agitated. For photolysis times greater than 1 min, flasks were clamped to a horizontal rod parallel to the long axis of the lamp and attached to an Eberbach reciprocating shaker operating at 160 excursions/min.

The kinetics of photolysis of 2-nitro-5-azidobenzoic acid [ϵ_{317} (max) = 14,000] at concentrations of 5 mM, 0.5 mM, and 5 μM in water and in 1% ammonium bicarbonate was monitored under the above conditions by ultraviolet spectroscopy of the solutions at selected times. The disappearance of the azide band in the infrared region (2100 cm^{-1}) was followed at 5 mM under the same conditions.

Irreversible stimulation of discharge: dose-response curves. To obtain dose-response curves resulting from photoaffinity labeling, lobules were prepared and placed under subdued lighting in flasks containing a range of concentrations of the affinity label. The tissue was incubated in the dark at 4° or 37° for the specified times, and then photolyzed for 10 sec at the same temperature. The discs with attached lobules were rinsed with four changes of Krebs-Ringer solution (50 ml each) at 4° to remove excess affinity label, placed in holding flasks containing 10 ml of Krebs-Ringer solution at 4° in the dark until all samples had been photolyzed, and then labeled with tritiated leucine and assayed for discharge as described above. The total elasped time between the end of the photolysis and placement in the holding flasks was approximately 30 sec. Total wash time following photolysis, including pulse labeling and the postpulse wash, was more than 40 min.

Time course of photoaffinity labeling by NAB-CCK-5 in pancreatic lobules. The time course of photoaffinity labeling by NAB-CCK-5 was determined by measuring irreversible stimulation of the secretory response after photoaffinity labeling as a function of the time during which NAB-CCK-5 was present before photolysis. Lobules were prepared as usual, and at zero time were placed in flasks containing NAB-CCK-5 at either 5 or 0.5 μM at 4° or 37° in Krebs-Ringer solution in the dark. At time t, the flask was photolyzed for 10 sec, and the disc with attached lobules was removed, washed, and placed in a holding flask containing 10 ml of Krebs-Ringer solution in the dark until photolysis of the last flask was completed. All flasks were then held for a further 10 min at 4°, and the discharge assay was carried out in the usual way.

The time course of the washout of NAB-CCK-5 from lobules was also determined by measuring the extent of irreversible stimulation of discharge which occurred when photolysis was carried out at various times after initiation of washout. Lobules were prepared and placed in flasks containing 2 ml of 5 μM NAB-CCK-5 at either 4° or 37°. After a 30-min incubation in the dark at 4° to ensure attachment of lobules to discs and equilibration of NAB-CCK-5 with tissue, all discs but one (zero-time flask) were rinsed once in Krebs-Ringer solution at 4° under subdued lighting and transferred to new flasks containing 5 ml of fresh solution at either 4° or 37°. The

flasks were then photolyzed at various times, and the discs were rinsed once and placed in 10 ml of solution in a holding flask at 4°. For dissociation at 4°, each disc was transferred to fresh solution every 7 min until the time of photolysis in order to inhibit reassociation. For dissociation at 37°, each disc was transferred to fresh solution every 2 min until the time of photolysis. The tissues were then assayed as usual for discharge.

For both types of kinetic experiments the total washout time for unbound peptide after photolysis was always more than 40 min, including the time in the holding flasks and washes incurred during pulse labeling with tritiated leucine prior to assay.

Direct measurement of washout of tritiated NAB-CCK-5 from pancreatic lobules. The washout of tritiated NAB-CCK-5 from pancreatic lobules was measured at 4° and 37° directly for comparison with washout as determined by photoaffinity labeling.

Lobules were prepared and equilibrated with 5 μM tritiated NAB-CCK-5 in Krebs-Ringer solution for 30 min at either 4° or 37° in a single flask in the dark. At zero time the disc was rinsed rapidly once and transferred within 10 sec to a flask containing fresh solution at either 4° or 37°. At fixed time intervals each disc was then transferred to a flask containing fresh solution, and the amount of radioactivity released into each flask was measured and used to calculate the rate of loss from the tissue. The entire procedure was performed under subdued lighting. Tritium remaining in whole tissue was determined by dissolving lobules in hot 88% formic acid, followed by scintillation counting. The washout of tritiated angiotensin II from pancreatic lobules was performed in an identical manner. In order to confirm independently whether diffusion into the lobules had reached equilibrium, lobules were prepared, divided between two flasks, and equilibrated with 5 μM tritiated NAB-CCK-5 at 4° or 37° for 30 min. The tissue was then photolyzed, rinsed, fixed, and examined by light microscope radioautography after 9 weeks of exposure, as described for pancreatic slices (14, 18). The amount of tritiated NAB-CCK-5 bound to the tissue by photolysis was calculated by normalizing to the number of cells present (which were largely exocrine cells) as determined by assaying for DNA (17) and employing the factor 4 pg of DNA per cell.

Protection against affinity labeling by native peptide secretagogues. The affinity labeling with NAB-CCK-5 was performed as described, except that a competing peptide secretagogue was added simultaneously to the prephotolysis incubation along with NAB-CCK-5. The competing secretagogue was always present either at the concentration at which it alone elicited a maximal response from the tissue, or at a concentration 10-fold higher. The tissue with NAB-CCK-5 and competitor was incubated at 4° for 30 min before photolysis, and washed for 30 min at 4° after photolysis (which is sufficient to wash out all competing secretagogues as determined in control experiments), except when the competitor was cerulein, for which a greater variety of conditions was tested, including variations in temperature, concentration, order of addition of peptides, and duration of prephotolysis incubation.

RESULTS

Photolysis of 2-nitro-5-azidobenzoic acid. The half-time for the disappearance of 2-nitro-5-azidobenzoic acid at 5 mM, determined by ultraviolet spectroscopy under our photolysis conditions, was 5–10 min. A precise end point for light-induced change in the ultraviolet spectrum of 2-nitro-5-azidobenzoic acid at this concentration did not occur, and the observed change as a function of time was complex and without an isosbestic point. This change appeared, however, to be resolvable into a fast process with a half-time of about 5–10 min, followed by a further change in the ultraviolet spectrum at a much slower rate. The faster photolytic process observed in the ultraviolet spectrum is therefore probably associated with loss of the azide functional group.

At 0.5 mM, photolysis of 2-nitro-5-azidobenzoic acid monitored in the ultraviolet region showed a process occurring with a half-life of about 5 sec and an isosbestic

point at 278 nm. At 5 μM, photolysis of 2-nitro-5-azidobenzoic acid for 5 sec yielded an ultraviolet spectrum which already corresponded to the end point of the reaction monitored at 0.5 mM. Therefore the half-time for the disappearance of the azide at 5 μM is probably much less than 5 sec. All these results were identical in water and in 1% ammonium bicarbonate, pH 8. The concentration dependence of the disappearance rate of 2-nitro-5-azidobenzoic acid was probably due to the lamp output intensity becoming limiting at high concentrations of azide. Below the concentration at which the light intensity was no longer limiting (undetermined, but less than 0.5 mM), the disappearance half-time may approach the lifetime measured for dinitrenobiphenyl, about 200 μsec (9).

Irreversible stimulation of secretion: dose-response curves. The irreversible stimulation of the secretory response observed with 5 μM NAB-CCK-5 was maximal for all photolysis times from 10 sec to 20 min. A photolysis duration of 10 sec was chosen as the shortest time conveniently reproducible without employing shutters or other mechanical devices for regulating photolysis time.

Figure 1 shows the dose-response curves obtained with cerulein, NAB-CCK-5, CCK-8, gastrin II, and CCK-4. NAB-CCK-5 had nearly 100 times the potency of the peptide from which it was prepared, CCK-4. 2-Nitro-5-azidobenzoyl-Gly-Gly-Met-Asp-Phe-NH_2, prepared from an inactive analogue of tetragastrin, elicited only minimal discharge at the highest concentration tested, 10 μM. Table 1 gives the doses eliciting 50% response for these and other peptide secretagogues in guinea pig pancreatic lobules.

Figure 2 shows the dose-response curves obtained after affinity labeling with NAB-CCK-5. Equilibration with NAB-CCK-5 for 10 min (Fig. 2b) or 60 min (Fig. 2e) at 4° before photolysis, or for 10 min at 37° (Fig. 2d) before photolysis, gave similar dose-

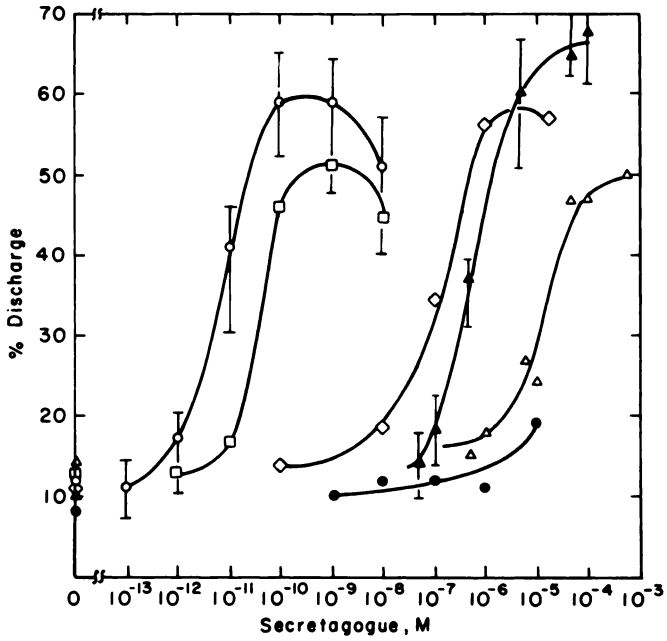

FIG. 1. *Dose-response curves for several peptide hormone secretagogues without photoaffinity labeling*
The secretagogues were present throughout the 3-hr assay period. The dose-response curves for NAB-CCK-5 which had been photolyzed for 10 sec or 5 min were identical with that of unphotolyzed NAB-CCK-5. The dose-response curves for 2-nitro-5-azidobenzoyl peptides were obtained in the dark. ○, cerulein; □, CCK-8; ◇, gastrin II; △, CCK-4; ▲, NAB-CCK-5; ●, NAB-Gly-Gly-Met-Asp-Phe-NH_2. The vertical bars include the range of six individual values averaged to give the final curves for cerulein and NAB-CCK-5.

TABLE 1

Peptide secretagogues for exocrine pancreas

Secretagogue	Primary structure	Concentration producing 50% response
		M
	SO_3H	
CCK	Lys-. . . Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2	10^{-10}
Cerulein[a]	Glu[b]-Gln ——— Thr ———	10^{-11}
CCK-8	——— Met ———	10^{-10}
CCK-8-des-SO_3	——— Tyr ———	10^{-7}
Gastrin I	Glu-. . . Glu-Glu-Glu-Ala-Tyr ———	10^{-7}
	SO_3H	
Gastrin II	——— Tyr ———	10^{-7}
CCK-4	Trp-Met-Asp-Phe-NH_2	10^{-5}
NAB-CCK-5	2-Nitro-5-azidobenzoyl-Gly-Trp-Met-Asp-Phe-NH_2	5×10^{-7}
Secretin[c]	27 amino acids, unrelated sequence	10^{-6}

[a] The horizontal bars indicate identity with the preceding sequence.

[b] 2-Pyrrolidone-5-carboxylic acid.

[c] A complete dose response for secretin probably was not obtained; 10 μM (the highest concentration tested) elicited only 33% discharge.

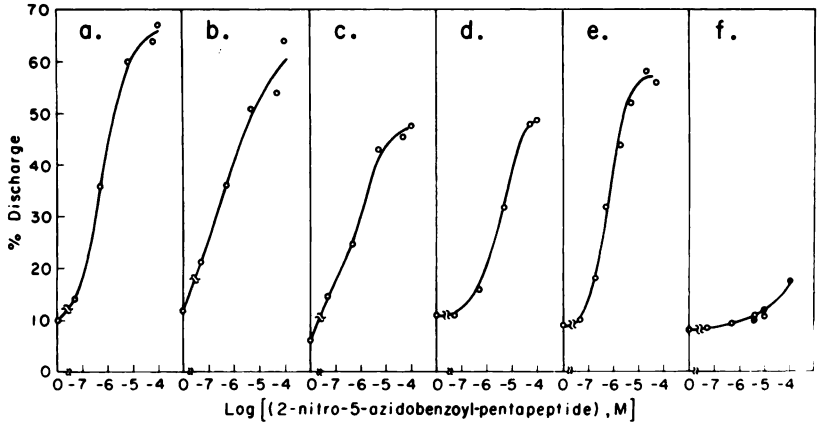

FIG. 2. *Dose-response curves obtained after photoaffinity labeling followed by washing out of all free peptide, compared with curves obtained with secretagogue present throughout 3-hr assay period*

For photoaffinity labeling, NAB-CCK-5 was equilibrated with lobules in the dark and then photolyzed for 10 sec. The lobules were then rinsed and discharge measured as described in MATERIALS AND METHODS. a. Equilibrated for 10 min at 4° without NAB-CCK-5; assay carried out in the presence of NAB-CCK-5. b. Equilibrated for 10 min at 4° with NAB-CCK-5, photolyzed, washed, and assayed. c. Equilibrated for 10 min at 37° without NAB-CCK-5; assay carried out in the presence of NAB-CCK-5. d. Equilibrated for 10 min at 37° with NAB-CCK-5, photolyzed, washed, and assayed. e. Equilibrated for 60 min at 4° with NAB-CCK-5, photolyzed, washed, and assayed. f. ●, equilibrated for 10 min at 4° with NAB-CCK-5, washed, and assayed; ○, equilibrated for 10 min at 4° with NAB-CCK-5 that had been photolyzed for 10 sec, then washed and assayed. Total wash times in experiments b, d, e, and f, were more than 40 min.

response curves. They were indistinguishable from the curves obtained in the continuous presence of NAB-CCK-5 in the dark at 4° (Fig. 2a) and 37° (Fig. 2c). Figure 2f shows that insignificant release occurred if NAB-CCK-5 (unphotolyzed or previously photolyzed) was added and washed out of the tissue without photolysis before the assay was performed.

The amount of protein released after photoaffinity labeling with 5 μM NAB-CCK-5 increased linearly with time during the initial hour of the 3-hr release period, and was indistinguishable from that obtained in the presence of 0.1 nM cerulein. The time-dependent morphological changes accompanying the secretory response produced by photoaffinity labeling with 5 μM NAB-CCK-5 observed on stained thick Epon sections were also identical with those produced by the continuous presence of cerulein or carbamylcholine (17). Photolysis itself had no effect on the ability of the tissue to respond to secretagogues, and the presence of secretagogues in the medium after photoaffinity labeling with 5 μM NAB-CCK-5 did not enhance the irreversible stimulation of discharge. Irreversible discharge could be blocked only by metabolic inhibition (by performing the 3-hr assay at 0° or under 95% nitrogen–5% carbon dioxide atmosphere, or in the presence of an uncoupler of oxidative phosphorylation), as is characteristic of normal discharge (19).

Finally, experiments not shown here indicated that photolysis in the presence or absence of NAB-CCK-5 did not compromise the ability of the tissue to incorporate labeled amino acids into proteins, a further index of functional integrity.

Time course of affinity labeling of guinea pig pancreatic lobules by NAB-CCK-5. Figure 3 shows the time course of association of NAB-CCK-5 with guinea pig pancreatic lobules determined by irreversible stimulation of discharge after photoaffinity labeling. The percentage discharge from the unstimulated but photolyzed control tissue (an average of about 10% background discharge) was subtracted and the values normalized with respect to the maximal discharge obtained with 5 μM NAB-CCK-5 (an average of about 40% above background) to obtain percentage response. The rate of association was concentration- and temperature-dependent.

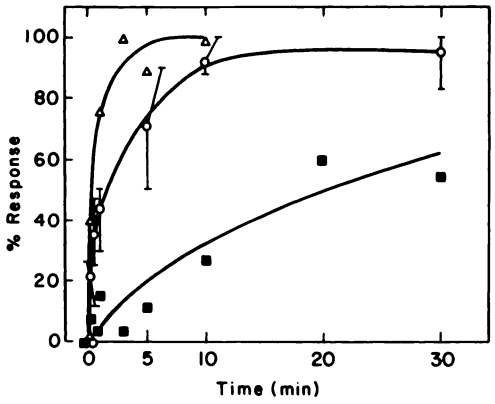

FIG. 3. *Time course of association of NAB-CCK-5 with guinea pig pancreatic lobules, measured by irreversible stimulation of discharge resulting from photoaffinity labeling*

○, 5 μM NAB-CCK-5 at 4° (vertical bars show the range of individual determinations); △, 5 μM NAB-CCK-5 at 37°; ■, 0.5 μM NAB-CCK-5 at 4°. The experimental procedure is described in MATERIALS AND METHODS.

Direct labeling studies showed that after exposure to 5 μM tritiated NAB-CCK-5 for 10 min at 4°, the tissue bound 5 × 10^6 molecules of NAB-CCK-5 per cell. After photolysis and thorough washing, 4 × 10^6 molecules/cell remained bound. Thorough washing without photolysis still left 2 × 10^6 molecules bound per cell.

Time course of washout of NAB-CCK-5 from lobules determined by photoaffinity labeling and with tritiated NAB-CCK-5. Figure 4 shows the washout of NAB-CCK-5 from guinea pig pancreatic lobules determined by photoaffinity labeling, and the dissociation of tritiated NAB-CCK-5 and tritiated angiotensin II from the tissue at 4° in the dark. The observed half-time for all three of these washout curves was about 20 min at 4°. The washout of NAB-CCK-5 from lobules was also followed at 37° after equilibration at 4° for 30 min. The observed half-time for the washout of 5 μM NAB-CCK-5 by both procedures, and also of tritiated angiotensin II, was about 2 min at 37°. The observed half-time for washout

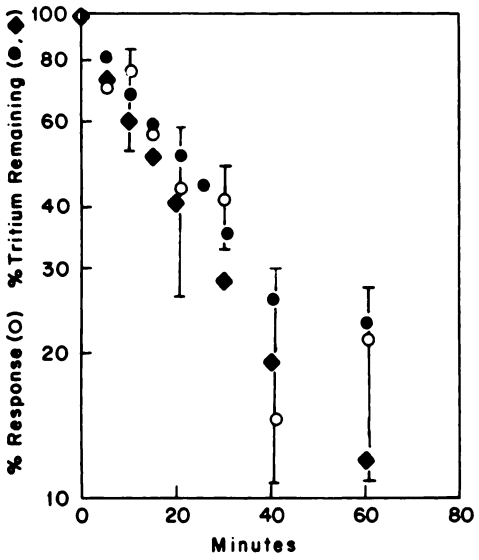

FIG. 4. *Time course of washout at 4° of 5 μM NAB-CCK-5 from guinea pig pancreatic lobules, measured by irreversible stimulation of discharge resulting from photoaffinity labeling* (○), *and time course of release of 5 μM tritiated NAB-CCK-5* (●) *and 5 μM tritiated angiotensin II* (◆) *from lobules*

The vertical bars include the range of four values averaged for irreversible stimulation of discharge (○). The experimental procedures are described in MATERIALS AND METHODS. Linear regression lines separately fitted to each set of points were not statistically significantly different.

at 37° was similar after equilibration at 4° or 37°, suggesting that diffusion into the tissue was complete at both temperatures.

Light microscopic radioautography of lobules equilibrated for 30 min at 4° or 37° with 5 μM NAB-CCK-5, photolyzed, rinsed, and immediately fixed showed a uniform distribution of silver grains throughout the tissue, confirming that diffusion of peptide into the lobules was complete. Localization to specific cell sites could not be ascertained because of the limits of resolution of the technique.

Mechanism of photoaffinity labeling. The mechanism of the irreversible photoinduced stimulation of discharge by NAB-CCK-5 was tested by the experiment of Ruoho *et al.* (20). According to these authors, a large reduction in the observed amount of affinity labeling (measured by irreversible stimulation of discharge) in the presence of 10 mM 4-aminobenzoic acid would indicate that labeling does not occur from NAB-CCK-5 bound to its receptor at the time of photolysis, but rather from free photoactivated NAB-CCK-5, which subsequently binds irreversibly to its receptor. The results (Table 2) indicate that irreversible stimulation of discharge appears to occur by photolysis and reaction of NAB-CCK-5 within its receptor binding site, according to the criteria set forth by Ruoho *et al.* (20).

Specificity of labeling with respect to affinity label. The weak secretagogue effect of NAB-Gly-Gly-Met-Asp-Phe-NH_2, prepared from an inactive analogue of tetragastrin, is shown in Fig. 1. Table 3 shows the results of photolyzing guinea pig pancreatic lobules with NAB-Gly-Gly-Trp-Met-Asp-Phe-NH_2 and several other potential affinity labels. Irreversible stimulation of discharge was observed only after photolysis with NAB-CCK-5. Photolysis with NAB-Gly-Gly-Met-Asp-Phe-NH_2 did not block the subsequent response to either cerulein or NAB-CCK-5.

NAB-CCK-5 that has previously been photolyzed for 10 sec still showed some irreversible stimulation upon further photolysis in the presence of tissue. This unexpected effect is commented upon below.

Protection against affinity labeling by native peptide secretagogues. If NAB-CCK-5 labels a single peptide hormone receptor site on pancreatic acinar cells, cerulein (or another peptide secretagogue with structural homology) and NAB-CCK-5

TABLE 2

Testing mechanism of photoaffinity labeling

Conditions	Discharge
	%
Assay directly without secretagogue	8
Assay with 10 mM 4-aminobenzoic acid	10
Assay with 0.1 nM cerulein	47
Assay with 10 mM 4-aminobenzoic acid and 0.1 nM cerulein	42
Photoaffinity labeling[a]	38
Photoaffinity labeling[a] in the presence of 10 mM 4-aminobenzoic acid	33

[a] NAB-CCK-5 (5 μM) was equilibrated for 10 min at 4°, then photolyzed for 10 sec, washed, and assayed as described in MATERIALS AND METHODS.

TABLE 3

Specificity of affinity labeling

Photoaffinity label[a]	Secretagogue present during assay	Discharge
		%
None	None	12
2-Nitro-5-azidobenzoic acid, 10 μM	None	11
NAB-Gly-Gly-Met-Asp-Phe-NH_2, 5 μM[b]	None	13
NAB-Gly-Gly-Met-Asp-Phe-NH_2, 5 μM	Cerulein, 0.1 nM	59
NAB-Gly-Gly-Met-Asp-Phe-NH_2, 5 μM	NAB-CCK-5, 5 μM	58
NAB-angiotensin II, 5 μM[b]	None	10
Cerulein, 10 or 0.1 nM	None	9
NAB-CCK-5, 5 μM, previously photolyzed 10 sec	None	26
NAB-CCK-5, 5 μM, previously photolyzed 5 min	None	14

[a] The affinity label was equilibrated for 10 min at 4° in the dark (except for cerulein, which was equilibrated for 30 min), followed by 10 sec of photolysis for all compounds in this column. After photolysis, each disc was rinsed in Krebs-Ringer solution at 4° for 10 min and assayed for discharge as usual.

[b] This concentration present during the assay elicited only background secretion.

TABLE 4

Competition by peptide secretagogues for labeling by 5 μM NAB-CCK-5

Competitor	Discharge			
	Photolysis[a]		Washout[b]	
	+Competitor	−Competitor	+Competitor	−Competitor
	%	%	%	%
CCK-4, 100 μM	43	39	16	14
CCK-8-des-SO_3, 50 μM	51	50	24	14
CCK-8, 10 nM	40	44	20	16
Cerulein, 1 nM	50	44	14	11
Gastrin I, 10 μM	42	38	15	17
Gastrin II, 10 μM	38	38	19	17
Cholecystokinin, 1 nM	42	38	17	9
Secretin, 10 μM	51	52	15	13

[a] Equilibration of tissue with NAB-CCK-5, with or without the competitor present, followed by photolysis, washout, and assay.

[b] Equilibration of tissue with NAB-CCK-5, with or without the competitor present, followed by washout and assay only (no photolysis). The experimental details are given in MATERIALS AND METHODS.

should compete for the same site. The results of attempts to protect against the irreversible effect of photolyzing NAB-CCK-5 by employing a large number of native peptide secretagogues as competitors are shown in Table 4. The first column shows percentage discharge found for the competition experiment. The second column shows percentage discharge for photolysis with NAB-CCK-5 alone. The third and fourth columns show percentage discharge for washout in the dark of the competitor plus NAB-CCK-5 and of NAB-CCK-5 alone, respectively. For cerulein, variation of neither the prephotolysis equilibration time, temperature, duration, nor order of addition of peptides produced protection against the irreversible affinity labeling effect. Competition by combinations of two or more native peptide secretagogues present simultaneously with NAB-CCK-5 could not be demonstrated because of difficulty in washing out the peptides.

DISCUSSION

Irreversible stimulation of discharge by affinity labeling the guinea pig exocrine pancreas with the secretagogue NAB-CCK-5 suggests that this peptide becomes irreversibly attached to its receptor by a photochemical reaction.

Photolysis of 2-nitro-5-azidobenzoic acid. The observed half-time for the photolytic loss of the azide functional group of 2-nitro-5-azidobenzoic acid at 5 μM monitored by ultraviolet spectroscopy was less than 5 sec. The affinity labeling of secretagogue receptors in guinea pig pancreatic lobules by NAB-CCK-5 at 5 μM was complete in less than 10 sec of photolysis. Therefore the photochemical affinity labeling reaction occurring in tissue is probably

the same one observed indirectly by ultraviolet (and directly by infrared) spectroscopy, i.e., loss of the azide functional group. However, direct participation of the nitro functional group in the photochemical affinity labeling reaction, as demonstrated by Escher and Schwyzer (21), cannot be ruled out. We observed that NAB-CCK-5 previously photolyzed for 10 sec still exhibited some ability to affinity label lobules, while NAB-CCK-5 that had been photolyzed for 5 min did not (Table 3). These results suggest that the photochemical affinity labeling reaction observed in our experiments may involve more than the azide functional group, since complete photolysis of the azide group (in 10 sec) did not completely destroy the activity of the peptide as an affinity label. The labeling that we consistently observed after a previous 10-sec photolysis probably occurs by a different mechanism than the labeling observed without prior photolysis. Neither the former nor the latter mechanism is known at this time.

Irreversible stimulation: dose-response curves. The similarity of the curves in Fig. 2a and b suggests that affinity label reversibly bound to receptor at a given concentration is efficiently attached by photolysis. Inefficient attachment would have moved the curve in Fig. 2b to the right; delayed binding to receptors occurring from NAB-CCK-5 initially not bound to the receptor could have moved the same curve to the left. Variation of the temperature at which association of NAB-CCK-5 and tissue occurs (compare Fig. 2c and d) or variation of association time at 4° (compare Fig. 2a and e) did not produce significantly different dose-response curves.

Time course of affinity labeling and washout of NAB-CCK-5 from pancreatic lobules. Figure 3 shows that 10 min of equilibration at either 4° or 37° are sufficient to reach saturation of sites affinity-labeled by NAB-CCK-5 at 5 μM. As discussed below, the equilibration rates shown in Fig. 3 probably measure the rate of diffusion of peptide into the tissue. Figure 4 shows that the rates of washout of tritiated NAB-CCK-5, tritiated angiotensin II, and NAB-CCK-5 measured by the irreversible affinity labeling response are the same, with a half-time of about 20 min at 4°. Therefore diffusion of the peptide in intact tissue is probably the rate-limiting step in both the association and dissociation of NAB-CCK-5 in pancreatic lobules and is a distinct limitation on studies using intact tissues compared with those using single cells, where diffusion barriers are absent [see discussion by Colquhoun *et al.* (22) for other tissues]. The use of either dispersed pancreatic acinar cells (23) or a simpler acinar preparation recently devised in our laboratory, which is highly sensitive to secretagogues and apparently devoid of diffusion barriers, should allow the determination of the hormone-receptor association and dissociation rates. In addition, the enormous number of NAB-CCK-5 molecules bound to the tissue by photolysis (2×10^6/cell) suggests a lack of binding specificity for this peptide in whole tissue, since studies in many other systems have demonstrated only 10^3–10^5 peptide hormone receptors per cell (24).

Mechanism and specificity of photoaffinity labeling. The results in Table 2 suggest that photoaffinity labeling occurs directly from receptor-bound NAB-CCK-5 to the receptor, according to the criteria of Ruoho *et al.* (20). In addition, labeling is very specific with respect to the affinity label (Table 3). However, the inability of any native peptide secretagogues to protect against labeling by NAB-CCK-5 (Table 4) suggests either that labeling and the resulting response are not specific, that the peptide secretagogues tested and NAB-CCK-5 do not bind to the same receptors, that there exists a large excess of functional receptors for either or both secretagogues, or that NAB-CCK-5 stimulates secretion by occupying most or all of the receptors for the various secretagogues tested. An example of two hormones which elicit nearly identical responses in a single tissue but do not bind to the same receptor is glucagon and secretin in the fat cell. Both hormones, which possess similar sequences, stimulate lipolysis with similar potencies, but do not compete for the same receptor (25, 26). Pancreatic exocrine cells may therefore possess separate receptors

for cholecystokinin and perhaps gastrin, both of which are labeled by NAB-CCK-5. Alternatively, for example, a large excess of "spare" functional receptors has been observed for both insulin (27) and gonadotrophin (28), and may exist for cholecystokinin in the exocrine pancreas.

In conclusion, we believe that we have irreversibly photoaffinity-labeled a receptor site in the guinea pig exocrine pancreas for the secretagogue NAB-Gly-Trp-Met-Asp-Phe-NH_2. Photolysis of this peptide in the presence of pancreatic lobules causes irreversible stimulation of the discharge of exportable proteins from the tissue, which is specific for this peptide. However, the absence of protection against irreversible labeling by any other peptide secretagogue suggests that the physiologically significant receptor for cholecystokinin may not have been the one labeled with NAB-CCK-5. Nevertheless, the technique of affinity labeling may be of use in characterizing a biologically significant secretagogue receptor on the exocrine pancreatic cell.

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