Synthetic Tools for Adrenocorticotropin Receptor Identification†

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ABSTRACT: Biotinylated photoaffinity derivatives of adrenocorticotropin (ACTH) are potentially useful tools for the identification of ACTH receptors. The hormone can be attached covalently to its receptor by photoactivation, and the presence of biotin in the molecule facilitates isolation of the solubilized hormone–receptor complex on columns of immobilized succinoylavidin (Suc-avidin). Six photoprobes of $ACTH_{1-24}$ have been prepared by reacting $ACTH_{1-24}$, [25-biocytin]$ACTH_{1-25}$ amide, and [25-dethiobiocytin]$ACTH_{1-25}$ amide with either 4- or 5-azido-2-nitrophenylsulfenyl (4-NAPS and 5-NAPS, respectively) chlorides in acetic acid. The homogeneity of the photoprobes was carefully monitored by thin-layer chromatography and amino acid analyses of acid hydrolysates. The presence of underivatized starting material in the photoprobes was critically scrutinized by high-pressure liquid chromatography and was estimated to be <0.5%. Both the 4- and 5-NAPS derivatives stimulated maximal steroidogenesis (as compared with $ACTH_{1-24}$) in calf adrenal cortical cells. However, the potencies of the two isomers differed significantly. The ED_{50} for steroidogenesis with 5-NAPS-$ACTH_{1-24}$ was 100-fold greater than the standard ($ACTH_{1-24}$) while that for 4-NAPS-$ACTH_{1-24}$ was only approximately 7 times greater. Although 4-NAPS-$ACTH_{1-24}$ was capable of stimulating maximal adenosine cyclic 3′,5′-phosphate (cAMP) production, the 5-NAPS derivative was usually not. The level of stimulation with the 5-NAPS derivative varied considerably from cell preparation to cell preparation. $ACTH_{1-24}$-induced cAMP production was inhibited by 5-NAPS-$ACTH_{1-24}$ or 5-NAPS-[25-dethiobiocytin]$ACTH_{1-25}$ amide. In order for these derivatives to be useful for isolation of covalent photoprobe–receptor complexes, it was important to ascertain that they bind to adrenal cells in the presence of Suc-avidin. [25-Biocytin]$ACTH_{1-25}$ amide retained 10%, on a molar basis, of its steroidogenic activity and cAMP stimulating activity when assayed in the presence of an equimolar amount of Suc-avidin. The activity did not decrease further when the molar ratio of Suc-avidin to [25-biocytin]-$ACTH_{1-25}$ amide was increased to 10:1. The findings that the 5-NAPS derivatives stimulate cAMP and steroid production and inhibit cAMP formation due to $ACTH_{1-24}$ indicate that they interact with ACTH receptors. It could be expected that if irradiation of a photoaffinity-labeled hormone caused covalent attachment of the hormone to its cell surface receptor, then activation of hormone-dependent events should persist even after the cells had been washed free of unattached hormone. Such was the case with stimulation of cAMP production by 5-NAPS-[25-dethiobiocytin]$ACTH_{1-25}$ amide. In the absence of irradiation, the photoprobe was apparently removed from the cells by washing. Photoactivation of the hormone analogue resulted in persistent activation when the cells were subsequently incubated without hormone. In contrast, the 4-NAPS derivatives failed to elicit elevated cAMP production after the cells had been washed. These results suggest, but do not prove conclusively, that irradiation of calf adrenal cortical cells exposed to 5-NAPS derivatives of ACTH brings about covalent attachment of the hormone to or near the ACTH receptors.

In our efforts to gain information relating to the adrenocorticotropin (ACTH)[1] receptor, we have synthesized six derivatives of ACTH which embody within their structures a photoaffinity label in addition to biotin or dethiobiotin. The structures of these compounds are shown in Figure 1.

Biotinylated photoaffinity derivatives of ACTH are molecules that are potentially useful for isolating ACTH receptors since the hormone can be attached covalently to its receptor by photoactivation and the hormone–receptor complex may be retrievable on immobilized Suc-avidin affinity columns (Hofmann & Finn, 1985). Romovacek et al. (1983) have described the synthesis of a number of biotinylated derivatives of ACTH and have shown that these compounds are just as active as the parent hormone with regard to the stimulation of cAMP and corticoid production in bovine adrenal cortical cells. 2-Nitrophenylsulfenyl chlorides can be attached covalently and highly selectively to the 2-position of the indole ring of the tryptophan residue of ACTH (Scoffone et al., 1968); the 4- and 5-azido derivatives of 2-nitrophenylsulfenyl chloride (Demoliou & Epand, 1980; Muramoto & Ramachandran, 1980) react similarly. Attachment of the 2-nitrophenylsulfenyl group to the single tryptophan residue of ACTH substantially lowers its ability to stimulate steroidogenesis and cAMP formation (Seelig & Sayers, 1973). However, Muramoto & Ramachandran (1980) have reported that photoactivation of 5-NAPS-$ACTH_{1-38}$ in the presence of isolated rat adrenal cells resulted in persistent stimulation of both steroidogenesis and cAMP formation, suggesting that covalent attachment of the

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[1] Abbreviations: ACTH (adrenocorticotropin) signifies $ACTH_{1-25}$ (Synacthen); Bct, biocytin (N^{ϵ}-biotinyl-L-lysine); DTBct, dethiobiocytin (N^{ϵ}-dethiobiotinyl-L-lysine); cAMP, adenosine cyclic 3′,5′-phosphate; 4-NAPS, 2-nitro-4-azidophenylsulfenyl; 5-NAPS, 2-nitro-5-azidophenylsulfenyl; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; Suc-avidin, succinoylavidin; CF_3COOH, trifluoroacetic acid.

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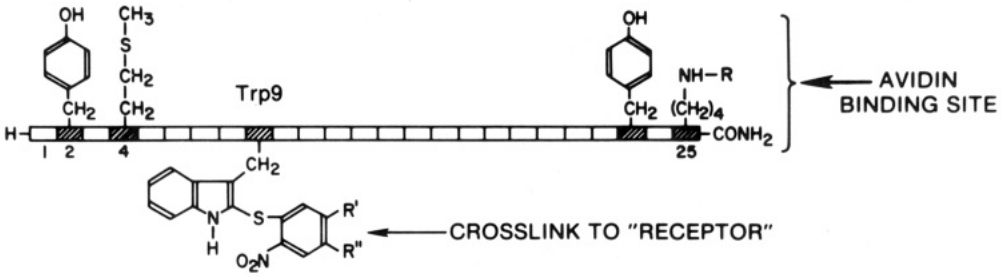

FIGURE 1: Chemical structures of photoprobes.

no.	compd	R	R′	R″	no.	compd	R	R′	R″
I	4-NAPS-ACTH	H	H	N_3	IV	5-NAPS-[Bct^{25}]ACTH$_{1-25}$ amide	biotin	N_3	H
II	5-NAPS-ACTH	H	N_3	H	V	4-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide	dethiobiotin	H	N_3
III	4-NAPS-[Bct^{25}]ACTH$_{1-25}$ amide	biotin	H	N_3	VI	5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide	dethiobiotin	N_3	N

photoaffinity-labeled hormone analogue to the ACTH receptor had taken place.

The homogeneity of the photoprobes (Figure 1) was carefully assessed, and their ability to stimulate cAMP and corticoid production in calf adrenal cortical cells was determined. In addition, the effect of Suc-avidin on the cAMP- and steroid-stimulating activity of one of these compounds has been explored. Preliminary results on photolabeling of ACTH receptors on calf adrenal cortical cells with some of the photoprobes are presented also.

Experimental Procedures

Materials

Collagenase (CLSPA) and trypsin were purchased from Worthington; trypsin inhibitor (soybean), bovine serum albumin (fraction V), penicillin G, streptomycin, avidin, and 3-isobutyl-1-methylxanthine were from Sigma. ACTH (Synacthen) and 4- and 5-NAPS chlorides were gifts from Dr. Rudolf Andreatta of Ciba-Geigy, Basel. The cAMP ^{125}I radioimmunoassay kit was from New England Nuclear; minimum essential medium (Earle's), L-glutamine solution (200 mM), and calf serum were from M. A. Bioproducts. Silica gel G for TLC was from Brinkmann Instruments, Sephadex resins were from Pharmacia, and IRA-400 was from Bio-Rad. Photolysis was conducted with a Blak-Ray longwave ultraviolet lamp (principle wavelength of radiation 366 nm) obtained from Ultra-violet Products, Inc., and the filter for the working lamp was a Kodak Safelight 1A filter. Suc-avidin was prepared as described (Finn et al., 1980), and the syntheses of the Bct and DTBct derivatives of ACTH have been described elsewhere (Hofmann & Kiso, 1976; Romovacek et al., 1983).

Methods

Preparation of NAPS Derivatives. All operations were performed essentially in the dark using only the working lamp as a light source. The appropriate sulfenyl chloride (3.7 mg, 16 μmol) was added to a solution of the desired peptide (26 mg, 8 μmol) in glacial acetic acid (2 mL), and the solution was stirred at room temperature for 2 h. The solution was diluted with water (1 mL) and was applied to a column of Sephadex G-25 (2.5 × 60 cm) equilibrated with 10% acetic acid to remove excess reagent (Veronese et al., 1970). The column was eluted with 10% acetic acid, and fractions containing peptide were pooled and lyophilized. The residue was purified by partition chromatography (Yamashiro, 1964) on a Sephadex G-50 column (3 × 55 cm) using the solvent system 1-butanol/pyridine/0.1% aqueous trifluoroacetic acid, 5:3:11. The flow rate was 0.7 mL/min, and 4-mL fractions were collected. To each fraction containing peptide was added

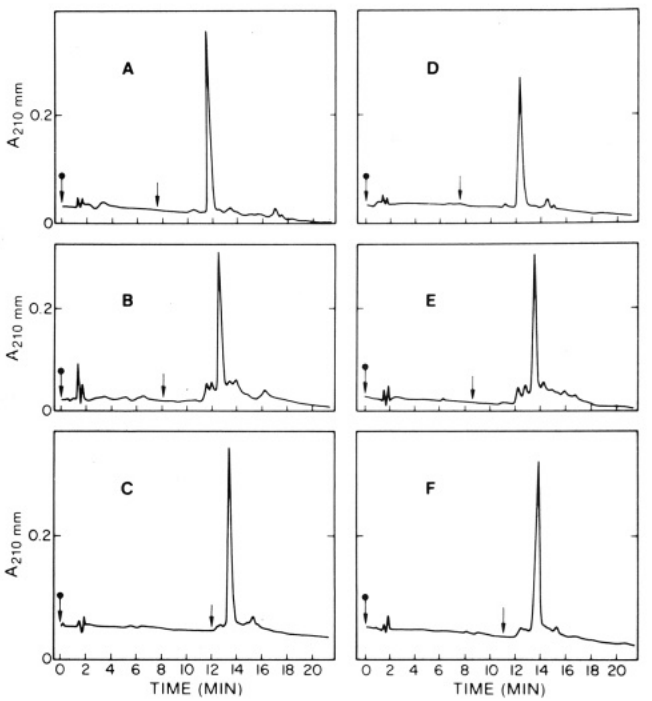

FIGURE 2: HPLC of NAPS derivatives of ACTH and biotinylated congeners: (A) 5-NAPS-ACTH; (B) 5-NAPS-[Bct^{25}]ACTH$_{1-25}$ amide; (C) 5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide; (D) 4-NAPS-ACTH; (E) 4-NAPS-[Bct^{25}]ACTH$_{1-25}$ amide; (F) 4-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide. Downward arrows indicate positions of underivatized controls: (A and D) ACTH; (B and E) [Bct^{25}]ACTH$_{1-25}$ amide; (C and F) [DTBct^{25}]ACTH$_{1-25}$ amide. A Bondapak C_{18} column using the following solvent system was employed: (pump A) 0.05% trifluoroacetic acid (CF_3COOH); (pump B) 50% acetonitrile in 0.05% CF_3COOH. The linear gradient was 40–80% pump B over 20 min with a pumping speed of 2 mL/min. Samples of approximately 10 μL were applied.

ethanol (0.5 mL), and an aliquot of the clear solution (0.05–0.1 mL) was diluted to 1 mL with glacial acetic acid for spectral examination. Fractions having the absorbance ratios A_{280}:A_{390}(4-NAPS) = 6.5 and A_{282}:A_{325}(5-NAPS) = 1.4 (Muramoto & Ramachandran, 1980) were pooled, evaporated to a small volume, and lyophilized. The material was converted to the acetate salt in 10% acetic acid on the ion-exchanger IRA-400 (5 mL), and fractions containing peptide were lyophilized, average yield 19 mg. All the compounds exhibited a single, chlorine-positive (Rydon & Smith, 1952) spot (R_f 0.5) on TLC in the system 1-butanol/pyridine/glacial acetic acid/water (30:20:6:24), and the UV spectra were identical with those reported for 4- and 5-NAPS-ACTH$_{1-38}$, respectively (Muramoto & Ramachandran, 1980; curves not shown). The HPLC patterns of the NAPS derivatives are illustrated in

Table I: Amino Acid Ratios in Acid Hydrolysates of Synthetic Photoprobes[a]

amino acid	peptide					
	I	II	III	IV	V	VI
Trp						
Lys	4.01 (4)	4.19 (4)	4.62 (5)	4.82 (5)	5.13 (5)	4.93 (5)
His	0.89 (1)	1.10 (1)	0.73 (1)	0.92 (1)	0.74 (1)	0.91 (1)
Arg	3.03 (3)	3.05 (3)	2.69 (3)	3.27 (3)	2.82 (3)	3.02 (3)
Ser	1.96 (2)	2.11 (2)	2.28 (2)	2.24 (2)	1.85 (2)	2.01 (2)
Glu	1.01 (1)	0.93 (1)	1.02 (1)	1.09 (1)	1.01 (1)	1.04 (1)
Pro	3.03 (3)	3.37 (3)	3.15 (3)	3.04 (3)	2.89 (3)	2.89 (3)
Gly	2.02 (2)	1.83 (2)	2.14 (2)	1.98 (2)	1.85 (2)	1.88 (2)
Val	3.08 (3)	2.72 (3)	3.23 (3)	2.84 (3)	3.28 (3)	3.12 (3)
Met	0.81 (1)	0.98 (1)	0.83 (1)	0.96 (1)	0.84 (1)	0.97 (1)
Tyr	1.93 (2)	1.91 (2)	2.09 (2)	1.92 (2)	2.13 (2)	2.08 (2)
Phe	1.04 (1)	0.85 (1)	1.21 (1)	1.02 (1)	1.15 (1)	1.11 (1)

[a] Samples were hydrolyzed at 110 °C for 24 h with constant-boiling HCl containing 0.1% phenol. Roman numerals designate the various compounds (see Figure 1). Values in parentheses are the theoretical values.

Figure 2, and the amino acid ratios in acid hydrolysates are summarized in Table I.

Preparation, Storage, and Assay of Cells. Calf adrenal cells were prepared by the method used for steer adrenals (Finn et al., 1976). L-Glutamine solution (5 mL), calf serum (50 mL), penicillin G (31.4 mg), and streptomycin (50 mg) were added to minimum essential medium (445 mL), previously gassed with O_2/CO_2 (95%/5%) for 10 min, and cells obtained from approximately 10 g of tissue were suspended in this medium. The suspension was blanketed with O_2/CO_2, sealed, and stored at 4 °C for approximately 18 h. Cells could be stored overnight a second time provided that the medium was replaced. Only cell preparations in which steroidogenesis could be stimulated 5-fold or more above basal were used for further experimentation.

The cell suspension was centrifuged at 174*g* for 10 min, and the pellets were resuspended in 100 mL of Krebs–Ringer bicarbonate buffer containing 0.2% glucose and 0.5% bovine serum albumin (KRBGA) and centrifuged as before. The pellets were resuspended in 100 mL of KRBGA containing 8 mM calcium and incubated at 37 °C for 15 min in an atmosphere of O_2/CO_2. The incubates were filtered through one layer of organdy and centrifuged as before. The pellets were resuspended in 100 mL of KRBGA containing 8 mM calcium and 1 mM 3-isobutyl-1-methylxanthine. The cells were counted, and the suspension volume was adjusted to a final concentration of (2–5) × 10^6 cells/mL. Aliquots (0.9 mL each) of the suspension were added to Teflon beakers containing hormone dissolved in 0.1 mM HCl/0.9% NaCl (vehicle) or vehicle alone (0.1 mL). In the experiments where Suc-avidin was used, mixtures of the biotinyl hormone analogue and Suc-avidin in vehicle were added instead.

The suspensions were incubated at 37 °C for 1 h under an atmosphere of O_2/CO_2. Portions of the incubates (0.1 mL) were mixed with 50 mM sodium acetate buffer pH 6.2 (0.3 mL), and heated at 90 °C for 10 min. These samples were assayed for cAMP by using a cAMP ^{125}I radioimmunoassay kit. The remainder of the incubates was extracted with 5 mL of methylene chloride, and steroids were measured fluorometrically as described (Finn et al., 1976).

Photolysis. Cells [(2–5) × 10^6 cells/mL] were incubated for 30 min at 30 °C under O_2/CO_2 in KRBGA containing 8 mM calcium (medium) (9–18 mL) to which hormone analogue dissolved in vehicle (1–2 mL) or the same volume of vehicle alone was added. The incubation vessels (250-mL siliconized beakers) were protected from light (dark controls) or covered with a dish containing a 3-mm layer of an aqueous

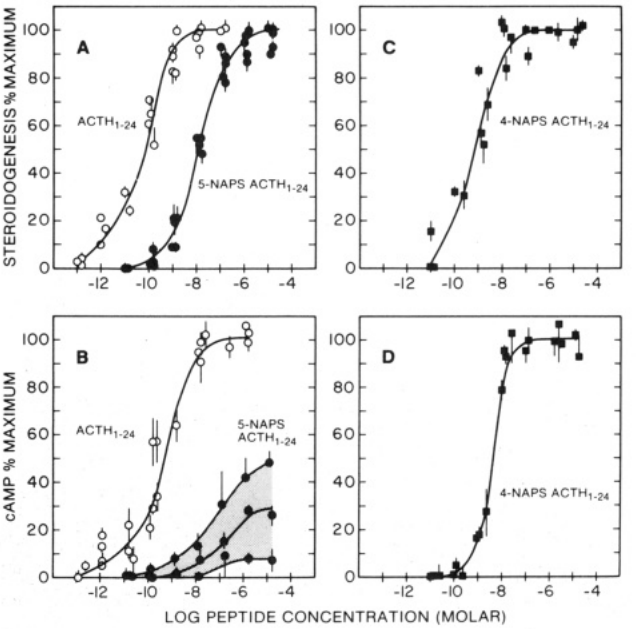

FIGURE 3: Log dose–response curves for stimulation of steroidogenesis and cAMP production in bovine adrenal cortical cells by (○) ACTH, (■) 4-NAPS-ACTH, and (●) 5-NAPS-ACTH. Assays were performed in triplicate, and the curves are the composite of several assays except for those in the stippled area which denote values obtained with three different batches of cells. Since the number of cells varies in different assays, peptide concentrations have been normalized to the concentration per 10^6 cells. Vertical bars indicate standard deviation. For assay conditions, see Experimental Procedures.

0.01% solution of potassium acid phthalate (Demoliou & Epand, 1980) and irradiated for 4 min with a Blak-Ray UV lamp positioned 15 cm above the cell suspension. Photolysis was repeated for an additional 4 min with a 2-min pause between to prevent overheating. Cell suspensions (diluted with 1.5 volumes of medium) were centrifuged at 250*g* for 5 min, the supernatant was aspirated, and the cells were resuspended in the same volume of fresh medium, recentrifuged, and resuspended in the original volume. This entire process [incubation, photolysis, and washing (preincubation)] was performed 4 times. Finally, the cells were resuspended in medium to which 3-isobutyl-1-methylxanthine (final concentration 1 mM) had been added and were incubated (0.9-mL aliquots) with hormone analogue in vehicle (0.1 mL) or vehicle alone (0.1 mL) for 60 min at 37 °C under an atmosphere of O_2/CO_2. A portion of each sample was used for the cell count. Results are normalized to 10^6 cells.

Results

The amino acid compositions of acid hydrolysates of the various peptide derivatives (Table I) were in agreement with theory indicating that Trp was the only amino acid that was derivatized. Evaluation of the photoprobes by TLC did not reveal the presence of underivatized material which differed in R_f value from the derivatized peptides in the system employed. In addition, we have critically examined the NAPS derivatives by HPLC (Figure 2). When 100 μg of 5-NAPS-ACTH or 5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide was subjected to HPLC under conditions where 10 μg produced full-scale deflection, no peak was visible at the position corresponding to the underivatized compound. As little as 0.5 μg of ACTH is readily detected under these conditions. From this observation, we conclude that the NAPS derivatives contain <0.5% of underivatized starting material.

Both the 4- and 5-NAPS derivatives have the ability to stimulate maximum steroidogenesis (as compared with

Table II: Inhibition of ACTH-Stimulated Production of cAMP by 5-NAPS Derivatives of ACTH[a]

inhibitor:ACTH ratio	cAMP[b]
Inhibitor: 5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ Amide	
ACTH alone	46.8 ± 5.6
0.85:1	43.7 ± 6.5
8.5:1	42.8 ± 3.4
85.0:1	28.2 ± 6.2
inhibitor alone	23.9 ± 1.2
Inhibitor: 5-NAPS-ACTH	
ACTH alone	38.5 ± 2.1
0.94:1	33.6 ± 4.4
9.4:1	31.4 ± 4.6
94.0:1	23.7 ± 3.1
inhibitor alone	18.5 ± 3.1

[a] For assay of 5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide, 1.3 × 10^6 cells/mL were used; for 5-NAPS-ACTH, 2.1 × 10^6 cells/mL were used. The concentration of ACTH was 0.34 μM in both experiments. [b] Cyclic AMP values are picomoles per 10^6 cells ± standard deviation; n = 6.

ACTH) in cells isolated from calf adrenals (Figure 3A,C). However, the potencies of the two isomers are significantly different. The ED_{50} for steroidogenesis with 5-NAPS-ACTH is 100-fold greater than the standard while that for 4-NAPS-ACTH is only approximately 7 times greater than the standard.

Although 4-NAPS-ACTH (Figure 3D) is capable of stimulating maximal cAMP production (ED_{50} approximately 10 times that of the ACTH standard), 5-NAPS-ACTH (Figure 3B) is usually not. Furthermore, the level of stimulation with the 5-NAPS derivative varies considerably from one cell preparation to another. The results of assays with three different batches of cells are depicted in Figure 3B. The degree of stimulation has varied from 100% (data not shown) to 6%, but only in the latter case was less than full steroidogenesis observed. The 4- and 5-NAPS derivatives of [Bct^{25}]- and [DTBct^{25}]ACTH$_{1-25}$ amides behaved similarly (data not shown).

ACTH-induced cAMP production is inhibited by 5-NAPS-ACTH or 5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide (Table II). Since both analogues are capable of stimulating cAMP production, the activity of ACTH is diminished but not abolished in their presence.

In order for these derivatives to be useful for isolation of covalent photoprobe–receptor complexes, it is important to ascertain that they bind to adrenal cells in the presence of Suc-avidin. The results of such a study (Figure 4) show that [Bct^{25}]ACTH$_{1-25}$ amide retains 10%, on a molar basis, of its steroidogenic and cAMP-stimulating activity when assayed in the presence of an equimolar amount of Suc-avidin. The activity does not decrease further when the molar[2] ratio of Suc-avidin to [Bct^{25}]ACTH$_{1-25}$ amide is increased to 10:1.

Photolysis. Adrenal cells subjected to four cycles of preincubation (see Experimental Procedures) were incubated in the dark for 60 min at 37 °C with or without hormone analogue, and steroids and cAMP were measured. The results of such experiments with 4- and 5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amides are presented in Table III.

The experimental design was the same for each derivative. Samples 1 and 2, preincubated without hormone and protected from irradiation, served as controls for basal and hormone-stimulated levels of cAMP and steroid production, respectively.

[2] Since avidin contains four subunits, each with a biotin binding site, a molar ratio of Suc-avidin to [Bct^{25}]ACTH$_{1-25}$ amide of 10:1 is a ratio of 40:1 in terms of binding sites.

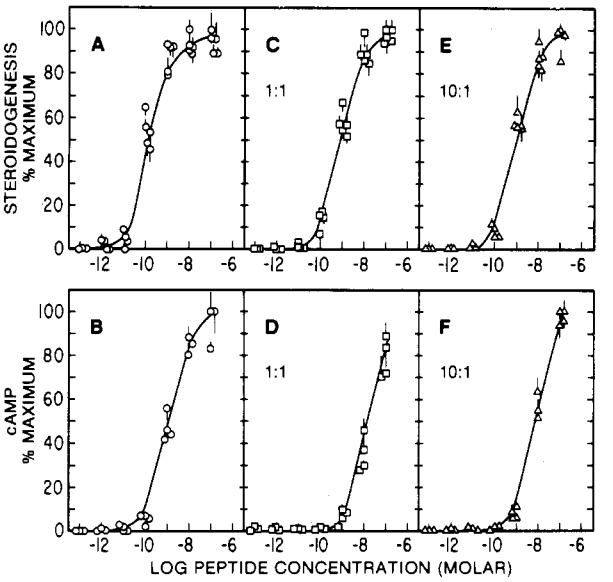

FIGURE 4: Effect of Suc-avidin on the ability of [Bct^{25}]ACTH$_{1-25}$ amide to stimulate steroidogenesis and cAMP production in calf adrenal cortical cells. (A and B) [Bct^{25}]ACTH$_{1-25}$ amide; (C and D) 1:1 ratio of Suc-avidin:[Bct^{25}]ACTH$_{1-25}$ amide; (E and F) 10:1 ratio of Suc-avidin:[Bct^{25}]ACTH$_{1-25}$ amide. Assays were performed in triplicate, and the curves are the composite of several assays. Since the number of cells varies in different assays, peptide concentrations have been normalized to the concentration per 10^6 cells. Vertical bars indicate standard deviation. For assay conditions, see Experimental Procedures.

Table III: Photolysis of NAPS Derivatives of [DTBct^{25}]ACTH$_{1-25}$ Amide[a]

sample	preincubation hormone[b]	preincubation light	incubation with hormone	assay cAMP[c]	assay steroids[d]
4-NAPS-[DTBct^{25}]ACTH$_{1-25}$ Amide					
1	–	–	–	8.6 ± 1.2	1.8 ± 0.1
2	–	–	+	30.4 ± 2.1	9.2 ± 0.6
3	–	+	+	20.3 ± 1.1	5.8 ± 0.4
4	+	–	–	6.6 ± 1.4	2.6 ± 0.1
5	+	+	–	7.5 ± 1.1	2.6 ± 0.1
5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ Amide					
1	–	–	–	4.1 ± 1.5	1.2 ± 0.02
2	–	–	+	23.6 ± 3.2	6.9 ± 0.40
3	–	+	+	20.2 ± 1.8	4.7 ± 0.20
4	+	–	–	5.5 ± 0.6	2.4 ± 0.10
5	+	+	–	16.6 ± 2.5	2.2 ± 0.02

[a] Values are averages ± standard deviation; n = 6. For preincubation and incubation conditions, see Experimental Procedures. [b] 4-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide and 5-NAPS-[DTBct^{25}]ACTH$_{1-25}$ amide. Concentrations were 29 and 290 nM, respectively. [c] Picomoles per 10^6 cells. [d] Relative fluorescence per 10^6 cells after 1-h incubation.

Sample 3 is the same as sample 2 except that it was exposed to irradiation during the preincubation cycles. Samples 4 and 5 were preincubated with the hormone and washed after each cycle. Hormone was omitted from the final incubation. Only sample 5 was irradiated, and thus, the difference between cAMP and steroid production by this pair should indicate whether the photoprobe has become attached covalently to receptor (persistant activation).

Production of cAMP is considered to be a more reliable indicator of persistant activation than is steroidogenesis for reasons discussed later. In the case of the 4-NAPS derivative, cAMP production was (within the limits of error) not increased by preincubation and irradiation of the photoaffinity probe (compare samples 4 and 5). Irradiation significantly decreased the ability of the cells to produce steroids (compare samples 2 and 3) when they were subsequently stimulated with hor-

mone. A decrease was also seen in cAMP production in this experiment, but this was not generally true (five examples).

The 5-NAPS derivative showed a marked difference in cAMP production when the photoprobe was irradiated. A 3-fold increase in cAMP was obtained (compare samples 4 and 5) when the 5-NAPS derivative was photoactivated. Steroidogenesis was not increased by irradiation, and, as above, irradiation of the cells before addition of the hormone analogue diminished their ability to respond to subsequent stimulation by the hormone analogue.

Discussion

Biological investigations involving the use of derivatized hormones, i.e., materials obtained by modifications of the parent compound, suffer from the disadvantage that it is difficult to remove unmodified starting material quantitatively. In the present study, we have made a serious effort to detect unmodified starting peptide in the derivatives by using TLC and HPLC. Evaluation of the products by TLC did not reveal the presence of underivatized material which exhibits a different R_f value in the system employed. In addition, we have critically examined the NAPS derivatives by HPLC (Figure 2). We conclude that our NAPS derivatives contain <0.5% of the underivatized starting material. Small proportions of unidentified impurities are present in some of the NAPS derivatives, but it appears highly unlikely that they would affect the biological findings.

In a recent study (Finn et al., 1984), we have investigated the effect of Suc-avidin on the ability of biotinyl-X-insulins (X = spacer) to stimulate glucose oxidation in rat epididymal adipocytes. Some of the compounds investigated retained 20–30% of their stimulating potency in the presence of a 100-fold excess of the avidin derivative. This observation led us to the conclusion that these insulin derivatives were indeed capable of binding simultaneously to insulin receptors and to Suc-avidin. Analogous experiments have now been conducted using the calf adrenal cell assay as a measure for receptor binding. Approximately 10 times as much of the [Bct^{25}]-$ACTH_{1-25}$ amide is required in the presence of Suc-Avidin to achieve levels of steroidogenesis and cAMP production comparable to those produced in its absence (Figure 4). Furthermore, the inhibition produced by a 10:1 ratio of Suc-avidin to peptide is identical with that caused by a 1:1 ratio. Previous studies (Romovacek et al., 1983) have shown that [Bct^{25}]-$ACTH_{1-25}$ amide forms a strong complex with Suc-avidin with a $t_{1/2}$ for dissociation of 20 days. Thus, the activity remaining in the presence of Suc-avidin is assumed to represent that of the [Bct^{25}]$ACTH_{1-25}$ amide–Suc-avidin complex and indicates that the complex is capable of interacting with the receptor on the adrenal cells.

In general, the principal determinant of the biological behavior of the NAPS derivatives is the position of the azido group on the 2-nitrophenylsulfenyl ring. That is to say, 5-NAPS-ACTH behaves in the same manner as 5-NAPS-[$DTBct^{25}$]$ACTH_{1-25}$ amide and 5-NAPS-[Bct^{25}]$ACTH_{1-25}$ amide, but their behavior differs markedly from that of the corresponding 4-NAPS derivatives. Shifting the azido group from the 4- to the 5-position exerts a marked effect on biological activity. The 4-NAPS derivatives, as exemplified by 4-NAPS-ACTH, are approximately 10% as active as ACTH, with concern to both stimulation of steroidogenesis and formation of cAMP (Figure 3). In contrast, the 5-NAPS derivatives are only approximately 1% as active. Furthermore, although steroidogenesis can be stimulated maximally by both derivatives, the degree of stimulation of cAMP production by the 5-NAPS hormone analogues is usually less than maximal and varies from one cell preparation to another (Figure 3B). Thus, the 4-NAPS derivatives are full agonists as concerns steroidogenesis and cAMP formation; the 5-NAPS derivatives are full agonists of steroidogenesis but only partial agonists of cAMP production. These findings are at variance with those described by Ramachandran et al. (1981). These authors reported 5-NAPS-$ACTH_{1-38}$ to be a partial agonist both for steroidogenesis and for cAMP production in rat adrenal cells. The possiblity cannot be overlooked that the observed discrepancies may reflect differences between the analogues employed.

Steroidogenesis is a poor measure of the interaction of ACTH with its receptor and consequently is of little value in assessing the degree of covalent attachment of the photoaffinity-labeled ACTH analogues to their receptors. The amplification of the signal (cAMP) produced in response to receptor occupation by hormone is such that the percent of maximum response for cAMP is always less for a given peptide concentration than is the percent of maximum response for steroidogenesis. Furthermore, the reactions involved in steroid production are removed by many steps from the locus of hormone–receptor binding.

The variability in the biological activity of the 5-NAPS compounds (Figure 3) may be attributable both to their low potency and to the phenomenon of spare receptors (Stephenson, 1956; Seelig & Sayers, 1973). Preparation of isolated cells for biological assays involves proteolytic digestion of tissue slices. It is reasonable to assume that ACTH receptors are damaged during this process and that the number of intact receptors remaining may vary from one cell preparation to another. As concerns such weakly active ACTH derivatives as 5-NAPS-ACTH, the number of remaining viable receptors may not be sufficient to fully activate adenylate cyclase, but the low level of cAMP elaborated suffices to elicit a maximal steroidogenic response (spare receptors).

When increasing amounts of 5-NAPS-ACTH or 5-NAPS-[$DTBct^{25}$]$ACTH_{1-25}$ amide are mixed with ACTH (at a concentration less than that required for full stimulation), a decrease in cAMP production, as compared with that elicited by ACTH alone, is observed (Table II). Since both analogues are capable of stimulating cAMP production, the activity of ACTH is diminished but not abolished in their presence. In the example where the effect of 5-NAPS-[$DTBct^{25}$]$ACTH_{1-25}$ amide on ACTH was evaluated, full activity (that stimulated by ACTH alone) corresponds to the production of 46.8 pmol of cAMP, and the basal level, i.e., that due to 5-NAPS-[$DTBct^{25}$]$ACTH_{1-25}$ amide alone, is 23.9 pmol. Consequently, a decrease in the level of ACTH-stimulated cAMP production from 46.8 to 35.4 pmol would correspond to 50% inhibition, and this requires approximately 20 times as much of the 5-NAPS-[$DTBct^{25}$] derivative as ACTH. In the case of inhibition by 5-NAPS-ACTH, 50% inhibition corresponds to a decrease in cAMP production to 28.5 pmol. This requires a ratio of approximately 26:1 (5-NAPS-ACTH:ACTH).

These results indicate that the 5-NAPS derivatives have less affinity for the receptor than ACTH and this may be partially responsible for their decreased activity. Our findings differ significantly from those reported by Ramachandran et al. (1980). On the basis of work with rat adrenal cells, these authors state that 5-NAPS-$ACTH_{1-38}$ is a potent inhibitor of $ACTH_{1-39}$-stimulated cAMP production (molar inhibition ratio 0.8). They suggest that 5-NAPS-$ACTH_{1-38}$ binds to ACTH receptors with slightly higher affinity than $ACTH_{1-39}$. In addition, they find that the 5-NAPS derivative inhibits $ACTH_{1-39}$-stimulated steroidogenic activity. We are unable

to confirm this latter finding with our 5-NAPS derivatives which are full agonists as concerns steroidogenesis. Inhibition of either steroidogenesis or cAMP formation by the 4-NAPS derivatives could not be investigated since these analogues are full agonists with respect to both activities.

The findings that the 5-NAPS derivatives stimulate cAMP and steroid production and inhibit cAMP stimulation due to ACTH indicate that they interact with ACTH receptors.

One would predict that if irradiation of a photoaffinity-labeled hormone caused covalent attachment of the hormone to its cell surface receptor then activation of hormone-dependent events should persist even after the cells have been washed free from unattached hormone. such is the case with stimultion of cAMP production by 5-NAPS-[DTBct^{25}]-$ACTH_{1-25}$ amide (Table III). In the absence of irradiation, the photoprobe is apparently removed from the cells by washing since the cAMP production by the dark control (sample 4) is essentially at the basal level. Photoactivation of the hormone analogue results in significant persistant activation when the cells are subsequently incubated without hormone. In contrast, the 4-NAPS derivative does not cause elevated cAMP synthesis after the cells have been washed. This suggests, but does not prove, that covalent attachment does not occur with this derivative.

The steroidogenesis response is complicated by several factors, the first of which is that the ability of cells to produce steroids following irradiation is significantly reduced. Furthermore, because photolysis generates a low yield of covalently attached hormone, it is necessary to incubate and photolyze repeatedly, and Johns (1976) observed that repeated exposure of adrenal cells to ACTH results in refractoriness insofar as steroid production is concerned. This can be seen by comparing the cAMP production by cells irradiated in the presence of the 5-NAPS derivative (3-fold greater than the dark control) with the level of steroidogenesis (essentially the same as the dark control). For all of these reasons and for the fact that activation of adenylate cyclase is an event more closely coupled to receptor occupation than is steroid production, we consider that the cAMP data provide evidence that photoactivation of the 5-NAPS derivative results in its covalent attachment at or near the ACTH receptor. Covalent attachment does not appear to occur with the 4-NAPS analogue. The 4-NAPS derivative is better able to interact with receptor than the 5-NAPS derivative, presumably because in the 4-position the azido group is oriented away from the binding site and interferes less with formation of an active complex. For this same reason, the 4-NAPS azido group is not in the correct orientation to form a covalent bond with the receptor.

In summary, we have observed (1) that both 4- and 5-NAPS-ACTH and derivatives stimulate cAMP and corticoid production in calf adrenal cortical cells, (2) that 5-NAPS-ACTH and derivatives are partial agonists for cAMP stimulation and inhibit the action of ACTH in this regard, (3) that Suc-avidin inhibits partially but does not destroy the ability of 5-NAPS-[Bct^{25}]$ACTH_{1-25}$ amide to stimulate cAMP and corticoid production and, (4) that photolysis of calf adrenal cells exposed to 5-NAPS-ACTH and derivatives results in persistant activation of cAMP stimulation. These results suggest, but do not prove conclusively, that irradiation of calf adrenal cells exposed to 5-NAPS derivatives of ACTH brings about covalent attachment of the hormone to or near the ACTH receptors.

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