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BIVALENT ACTH ANTAGONISTS: INFLUENCE OF PEPTIDE AND SPACER COMPONENTS ON POTENCY ENHANCEMENT*

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Abstract—The antagonist potency of a series of bivalent adrenocorticotropic hormone (ACTH) peptides was examined using suspensions of inner zone rat adrenocortical cells. Bivalent antagonists were prepared by bis(maleimide) covalent cross-linking of carboxyl terminal cysteine sulfhydryl groups of synthetic ACTH peptides, Cys^{25}ACTH(7–25) and Cys^{39}ACTH(7–39). Antagonist potency enhancement was defined by shifts in ACTH(1–39) concentration–steroidogenic response curves relative to monovalent antagonist analogs. The EC_{50} values measured in the presence of 0.5 μM monovalent antagonist were 110 ± 28 pM for Cys^{25}ACTH(7–25)-*S*-*N*-ethylsuccinimide and 44 ± 9 pM for Cys^{39}ACTH(7–39)-*S*-*N*-ethylsuccinimide. Some bivalent ACTH antagonists displayed much greater antagonist potency than their monovalent analogs, which supports the findings of Stolz and Fauchere (*Helv Chim Acta* **71**: 1421–1428, 1988). The level of potency enhancement, however, was found to be dependent upon the spacer used to link receptor binding domains and the length of the ACTH peptide used in bivalent antagonist synthesis. The most potent inhibitor, bis(Cys^{25}ACTH(7–25)-*S*-succinimidopropionyl)2-hydroxy-1,3-propanediamine, was 28 times more potent than its monovalent analog, Cys^{25}ACTH(7–25)-*S*-*N*-ethylsuccinimide. However, a bivalent Cys^{25}ACTH(7–25) peptide containing two bis(succinimidopropionyl)2-hydroxy-1,3-propanediamine spacers that had been linked end-to-end via dithioerythritol showed no potency enhancement. Cys^{25}ACTH(7–25) based peptides containing one receptor binding domain and having the structure peptide-spacer-cysteine displayed no enhancement in antagonist potency. Bivalent Cys^{39}ACTH(7–39) linked by bis(succinimidopropionyl)2-hydroxy-1,3-propanediamine spacer exhibited only 4-fold enhancement in antagonist potency relative to Cys^{39}ACTH(7–39)-*S*-*N*-ethylsuccinimide. We therefore conclude that the potency enhancement observed with bivalent ACTH peptides: (1) is optimal with spacers less than approximately 40 Å in length, (2) is not due to direct interactions between the spacer and cell surface, and (3) is dependent on the length of the ACTH peptide component. In addition, these results indicate that electrostatic interaction between bivalent ACTH peptides and plasma membrane lipids does not adequately account for the potency enhancements observed.

Adrenocorticotropic hormone (ACTH§) stimulates glucocorticoid production by activating receptors on the surface of adrenocortical cells. Studies with truncated ACTH peptides have demonstrated the presence of functional domains within the 39 residue ACTH sequence. The amino terminal region (residues 1 through approximately 20) contains the domain responsible for stimulating steroidogenesis [1, 2]. The maximum level of steroidogenesis obtained by stimulation of rat adrenocortical cells with ACTH(5–24) is the same as that observed with the full-length peptide, ACTH(1–39), but a much higher concentration of ACTH(5–24) is required to achieve maximum stimulation [3]. ACTH(6–24) is a partial agonist that stimulates steroidogenesis to only 60% of the maximal value obtained with full agonists such as ACTH(1–39) or ACTH(5–24). ACTH peptides lacking an intact 6–9 sequence, such as ACTH(7–23) [3, 4], do not display agonist function in suspensions of rat adrenocortical cells, but instead act as competitive antagonists. These results indicate that residues 5–9 are required for full receptor activation while residues 1–4 and 10 through approximately 20 increase the affinity of the peptide for the receptor [5]. Schwyzer [6] has referred to these activation and affinity domains as message and address segments respectively.

Bivalent ligand binding to the cell surface is involved in antibody-cell surface antigen interactions and some aspects of hormone–receptor function, and has been studied using both experimental and theoretical approaches [e.g. Ref. 7]. These studies suggest that bivalent ligands may have receptor-binding properties that differ substantially from those of a monovalent ligand. Portoghese and coworkers [8–10] have synthesized bivalent opioid

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§ Abbreviations: ACTH, adrenocorticotropic hormone; Cys^{25}ACTH(7–25), a peptide containing residues 7 through 25 of the mammalian ACTH sequence with a cysteine residue substituted for the residue naturally occurring at position 25; Cys^{39}ACTH(7–39), a peptide containing residues 7 through 39 of the rat/mouse ACTH sequence with a cysteine residue substituted for the residue naturally occurring at position 39; EC_{50}, concentration resulting in 50% of the maximal response; bt_2cAMP, N^6,2′-*O*-dibutyrylcAMP; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTE, dithioerythritol; TFA, trifluoroacetic acid.

antagonists that show striking increases in receptor selectivity and affinity compared to monovalent antagonists. This approach has also been utilized by Fauchere and coworkers [11, 12] who prepared bivalent ACTH antagonists. They employed elegant solution synthesis methods for preparation of bivalent ACTH antagonists based on the 11–24 [11] and 7–24 [12] sequences. These ACTH peptides were linked via the amino groups of a lysine residue that was coupled to the carboxyl termini of two protected peptides by carbodiimide mediated peptide bond formation. The ACTH(11–24) dimer demonstrated antagonist potency that was substantially increased relative to the monovalent analog. These workers suggested that electrostatic interactions or receptor aggregation could be responsible for the enhanced potency.

Studies such as those by Portoghese and colleagues have demonstrated that the spacer used to link bivalent ligands can exert a profound influence on the potency of bivalent opioid antagonists. To determine the structural basis for enhanced potency of bivalent ACTH antagonists, we have prepared a series of bivalent ACTH antagonists by a synthetic route that allows us to examine readily the effect of specific structural components on the potency of the bivalent peptide. The scheme is based on bis(maleimide) cross-linking of synthetic ACTH peptides containing carboxyl terminal cysteine residues. These bivalent antagonists show potency enhancements up to approximately 25 times that of their monovalent analog. In this report we examine the effects of spacer structure and ACTH peptide length on the potency of these bivalent ACTH peptides and demonstrate that both the spacer and peptide component of the bivalent ligand exert substantial effects on bivalent antagonist potency.

MATERIALS AND METHODS

Materials. DTNB, bt_2cAMP, bis(3-maleimidopropionyl)2-hydroxy-1,3-propanediamine, porcine ACTH(1–39), and cysteine were purchased from the Sigma Chemical Co. ACTH(1–39) was purified by ion-exchange and reversed phase chromatography as described below. Both 2,2′-dithiopyridine and DTE were obtained from Chemical Dynamics. *N*-Ethylmaleimide was from Aldrich.

Peptide synthesis and purification. Cys^{25}ACTH(7–25) and Cys^{39}ACTH(7–39) (rat/mouse sequence) were prepared by automated solid phase methods using Fmoc-α-amino group protection. For global deblocking and cleavage of peptide from the resin, the peptide-resin was incubated for 12 hr at 22° in 90% TFA, 5% thioanisole, 3% ethanedithiol, 2% anisole. After deblocking, volatile components were removed under vacuum, and the peptide was dissolved in 0.1 M acetic acid. To generate CysACTH peptides that would not form mixed disulfides during purification, the cysteine residues of Cys^{25}ACTH(7–25) and Cys^{39}ACTH(7–39) were converted to thiopyridine disulfides by addition of an 8-fold molar excess of 2,2′-dithiopyridine. After gel filtration chromatography on Sephadex G-10 to remove low molecular weight contaminants, the deblocked peptides were purified by ion-exchange chromatography on Whatman CM-52 [13]. The peptides obtained from the CM-52 column were purified by C18 HPLC on a 25 × 1 cm Bakerbond Wide Pore column using linear gradients of 0.05% TFA (v/v) in water (solvent A) and 0.05% TFA, 50% acetonitrile in water (solvent B). Purified peptides were stored in their thiopyridine-disulfide forms at −80°.

The sulfhydryl forms of the CysACTH (i.e. CysACTH-SH) peptides used for syntheses described below were prepared by treating the thiopyridine-peptide disulfides with a 20-fold molar excess of dithioerythritol at pH 7.3. The peptide products of this reaction were isolated by C18 HPLC and taken to dryness under vacuum.

Analysis of products. The heterogeneity of purified peptides was assessed by their elution pattern on analytical C18 HPLC using a 25 × 0.46 cm (5 μm spherical packing) Bakerbond Wide Pore column. Amino acid compositions were determined on acid hydrolysates of peptides [14] in which the amino acids were converted to phenylthiocarbamyl derivatives and quantified by C18 HPLC [15]. The Tyr and Trp content of CysACTH-SH peptides isolated by C18 HPLC was determined by UV absorbance [16]. The cysteine sulfhydryl group content was then estimated by addition of DTNB. The absorbance at 412 nm due to 5-mercapto-2-nitrobenzoic acid produced from the reaction of DTNB with peptide sulfhydryl groups was measured, and the sulfhydryl group concentration calculated using $E_{412} = 13{,}600\ M^{-1}\ cm^{-1}$ [17].

The amino acid sequence of the thiopyridine form of Cys^{25}ACTH(7–25) was determined by automated Edman degradation with a MilliGen 6600 Prosequencer using protocols supplied by the manufacturer.

Preparation of monovalent and bivalent S-succinimido-ACTH peptides. The sulfhydryl forms of Cys^{25}ACTH(7–25) and Cys^{39}ACTH(7–39) were the precursors for the synthesis of the ACTH antagonists used in this study. For preparation of monovalent peptide containing a cysteine sulfur blocked by an *N*-ethylsuccinimide moiety, 200 nmol of sulfhydryl peptide was incubated with 220 nmol of *N*-ethylmaleimide in 50 mM HEPES, pH 7.3, for 20 min at room temperature (reaction volume = 0.4 mL). The reaction products were resolved by C18 HPLC. The *N*-ethylsuccinimido forms of Cys^{25}ACTH(7–25) and Cys^{39}ACTH(7–39) were obtained in yields of 76 and 87% respectively.

Antagonists with the structure: peptide-succinimide-spacer-succinimide-cysteine were prepared by reaction of bis(maleimides) with sulfhydryl forms of ACTH peptides. The structures of the bis(maleimides) spacers used in this study are shown in Fig. 1. Cys^{25}ACTH-SH peptide (100 nmol) was added to bis(maleimide) (2 μmol) in 50 mM HEPES·NaOH, pH 7.3 (total volume = 0.8 mL), and incubated for 4 min at 22°. The bis(maleimido)hexane stock solution was prepared in acetone, while the bis(3-maleimidopropionyl)2-hydroxy-1,3-propanediamine solution was prepared in water. The majority of the peptide product at this stage had its cysteine sulfur bound in a thioether linkage to a

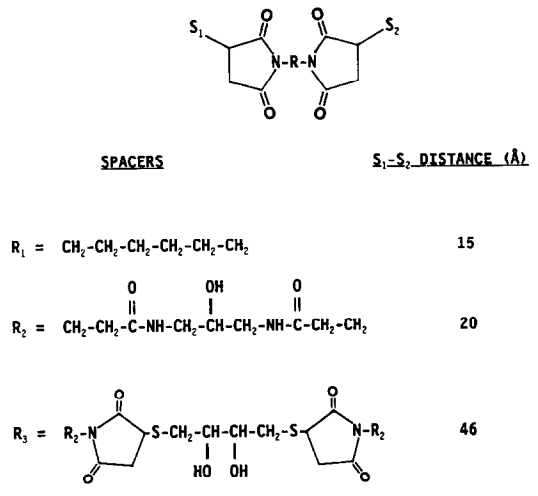

Fig. 1. ACTH peptide spacer structures. The structure at the top of the figure represents a bivalent peptide. The spacer is denoted by R. S_1 and S_2 represent sulfur atoms of carboxyl terminal cysteine residues. The numbers on the right indicate the approximate maximum distance (estimated from molecular models) in angstroms between sulfur atoms in a bivalent peptide after the cross-linking reaction has occurred.

cross-linker that contained a maleimide moiety at the distal side of the spacer (see below). Cysteine (10 μmol in 20 μL) was added to convert the peptide-linked maleimide to a non-reactive succinimido form, and the sample was incubated for an additional 5 min. Reaction products were separated by C18 HPLC. Structures of the ACTH peptide derivatives are shown in Table 1. The Cys^{25}ACTH(7–25) peptide yields were: compound **II**, 65% and compound **IV**, 81%.

Bivalent ACTH peptides with bis(succinimido)-hexane and bis(3-succinimidopropionyl)2-hydroxy-1,3-propane diamine spacers were prepared by incubation of the bis(maleimide) compounds with a slight excess of peptide sulfhydryl groups over maleimide groups. Sulfhydryl peptide (240 nmol) was incubated with bis(maleimide) (100 nmol) (i.e. 200 nmol of maleimide groups) in 50 mM HEPES, pH 7.3 (total volume = 0.3 mL), for 20 min at room temperature. The reaction products were then resolved by C18 HPLC. Bivalent Cys^{25}ACTH(7–25) peptide yields based on the starting maleimide quantity were: compound **I**, 85% and compound **III**, 82%. The bivalent Cys^{39}ACTH(7–39) peptide yield was: compound **VI**, 91%.

A bivalent Cys^{25}ACTH(7–25) derivative with a 46 Å spacer (see Fig. 1) was sy~thesized by a two-step route. In the first step CysACTH-SH peptide (100 nmol) was incubated with bis(3-maleimido-propionyl)2-hydroxy-1,3-propanediamine (2 μmol) in 0.8 mL of 50 mM HEPES, pH 7.3, for 4 min. The peptide product of this reaction was isolated by C18 HPLC. The C18 fraction was taken to dryness under vacuum. The sample was then dissolved in 0.5 mL water and a UV spectrum recorded to determine the peptide concentration. The major peptide product of this reaction contained covalently bound maleimide as judged by the presence of a component absorbing in the 300–330 nm portion of the UV spectrum. This absorbance disappeared upon addition of a sulfhydryl-containing species to the peptide sample. Also, addition of cysteine to this sample altered the C18 retention time of the peptide. In the second step, 70 nmol of maleimide-containing peptide was incubated with 33 nmol of DTE in 0.55 mL of 50 mM HEPES, pH 7.3, for 20 min. The reaction products were resolved by C18 HPLC. The yield of compound **V** based on the starting quantity of CysACTH-SH peptide was 43%.

Analysis of bivalent peptides. Bivalent peptides were analyzed by ultraviolet absorption spectroscopy, amino acid analysis, groups reactive toward DTNB and sulfhydryls, and homogeneity on analytical C18 HPLC as described above. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of peptides was carried out as described by Schagger and von Jagow [18].

Preparation of rat adrenocortical cell suspensions. Suspensions of adrenocortical inner zone cells were prepared from decapsulated adrenal glands of male Holtzman rats (150–250 g) as described by Sayers *et al.* [19]. Viable cell concentrations were determined by dye exclusion using erythrosin B [20].

Measurement of steroidogenesis. The effect of peptides on steroidogenesis was determined by incubation with rat adrenocortical cell suspensions in 12 × 75 mm capped polypropylene tubes. Cells were incubated in Krebs–Ringer bicarbonate medium that was 0.2% in glucose and 0.5% in bovine serum albumin. Prior to incubation of cell suspensions for measurement of steroidogenesis, $CaCl_2$ was added to yield a final Ca^{2+} concentration of 7.5 mM. Incubations were carried out under an atmosphere of 5% CO_2/95% air. The viable cell concentration was constant for a given experiment and ranged between 10,000 and 20,000 cells/mL in different experiments. The incubations were carried out in a shaking (30 cycles/min) water bath at 37° in a final volume of 0.5 mL after agonist/antagonist addition. Steroid production was terminated by addition of 1.0 mL dichloromethane to the cell suspension followed by mixing. Incubations were performed in triplicate. Corticosterone was determined using a fluorometric assay [21]. The data points shown represent the mean ± SD. Where not shown, the standard deviation was smaller than the symbol. The EC_{50} values were measured from concentration–response curves determined by computer spline fit of the fluorogenic steroid production values.

RESULTS

Characterization of monovalent peptide products. The synthetic route chosen for preparation of bivalent ACTH peptides utilized bis(maleimide) coupling of two deblocked peptides containing carboxyl terminal cysteine sulfhydryl groups. The parent monovalent compounds, with cysteine sulfur blocked by *N*-ethylmaleimide treatment, were used

as the reference to determine potency enhancements of bivalent peptides. Chemical characterization and biological potency assessment were therefore carried out on both monovalent and bivalent peptides.

Cys^{25}ACTH(7–25) and Cys^{39}ACTH(7–39) products (with the cysteine sulfhydryl in the thiopyridine blocked form) obtained after synthesis and purification were examined by analytical C18 HPLC and found to be greater than 98% homogeneous as estimated by peak areas from the elution profile monitored at 210 nm. The identities of the purified peptides were confirmed by amino acid analysis. Cys^{25}ACTH(7–25)-*S*-thiopyridine was also subjected to automated Edman degradation, and the expected sequence was obtained through the 13 cycles conducted.

Peptide stock solution concentrations were determined from Tyr and Trp levels using ultraviolet absorption spectroscopy as noted above [16]. This measurement gave an extinction coefficient of 6700 M^{-1} cm^{-1} at 278 nm for the sulfhydryl forms of Cys^{25}ACTH(7–25) and Cys^{39}ACTH(7–39). DTNB analysis indicated that these peptides contained 1.00 (Cys^{25}ACTH(7–25)) and 0.96 (Cys^{39}ACTH(7–39)) sulfhydryl groups per peptide. The *N*-ethylmaleimide-treated forms of Cys^{25}ACTH(7–25) and Cys^{39}ACTH(7–39) obtained from C18 separation of the reaction products were analyzed for sulfhydryl group content by reaction with DTNB and found to contain less than 0.01 mol of sulfhydryl groups per mol of peptide. Amino acid analysis of maleimide-treated peptides demonstrated the predicted number of phenylalanine and lysine residues per molecule. This indicates that these products were Cys^{25}ACTH(7–25)-*S*-*N*-ethylsuccinimide and Cys^{39}ACTH(7–39)-*S*-*N*-ethylsuccinimide, and that the parent peptides had therefore been modified only at cysteine residues, and not at the α-amino group of Phe^{7} or the ε-amino group of lysine residues. Note that all lysine residues of mammalian ACTH are located between residues 11 and 21.

Analysis of antagonist function of monovalent peptides. The steroidogenic antagonist properties of these *S*-succinimido peptides were examined in suspensions of rat adrenocortical cells. Typical ACTH(1–39) concentration–steroidogenic response curves are shown in Fig. 2. The EC_{50} (agonist concentration at which half-maximal stimulation occurs) for ACTH(1–39) in this experiment was 5.6 pM. The mean EC_{50} value obtained for ten separate cell preparations was 3.9 ± 0.4 (SE) pM, and the range of values was 2.4 to 6.3 pM. In the presence of 0.5 μM Cys^{25}ACTH(7–25)-*S*-succinimide, the ACTH(1–39) concentration–response curve was shifted 1.4 orders of magnitude (25-fold) to the right. The concentration–response curve with 0.5 μM Cys^{39}ACTH(7–39)-*S*-succinimide was shifted 0.7 orders of magnitude (5-fold) to the right. The mean EC_{50} values obtained in the presence of 0.5 μM Cys^{25}ACTH(7–25) and 0.5 μM Cys^{39}ACTH(7–39) monovalent peptides were 110 ± 28 pM (N = 6) and 44 ± 9 pM (N = 7). These results are comparable to those previously obtained by Sayers *et al.* [19] for ACTH(7–23) and Li *et al.* [22] for human ACTH(7–38), and indicate that these peptides functioned as competitive antagonists of ACTH(1–39) as anticipated. In addition, these monovalent antagonists, as well as the bivalent antagonists, at concentrations of 0.5 and 0.25 μM respectively, displayed no inhibitory effect on bt_2cAMP-stimulated steroidogenesis, consistent with their function as competitive antagonists.

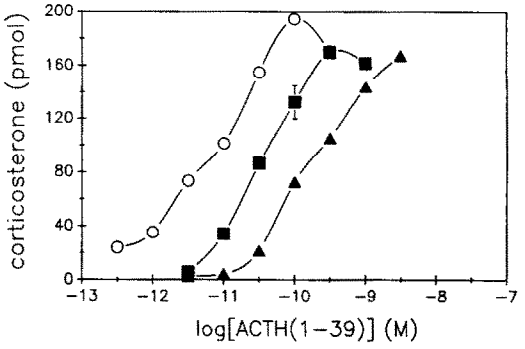

Fig. 2. Antagonist potency of monovalent ACTH peptides. Suspensions of rat adrenocortical cells (8500 viable cells/tube) were incubated with ACTH(1–39) in the absence of antagonist (○) or with 0.5 μM Cys^{25}ACTH(7–25)-*S*-*N*-ethylsuccinimide (▲) or 0.5 μM Cys^{39}ACTH(7–39)-*S*-*N*-ethylsuccinimide (■). Incubations were quenched after 60 min and analyzed for corticosterone as described in Materials and Methods. Values are means ± SD of triplicate incubations.

Characterization of bivalent ACTH peptides. Bivalent ACTH antagonists were prepared by reaction of the sulfhydryl form of monovalent ACTH antagonists with the bis(maleimide) compounds to form peptides with two ACTH receptor binding domains linked by spacers whose structures are shown in Fig. 1. These reactions were carried out with the sulfhydryl group in slight excess over the maleimide (1.2 -SH groups per maleimide, which is equivalent to 2.4 -SH groups per cross-linker) to ensure that all of the modified peptide products obtained were bivalent. The products were separated by C18 HPLC. Similar results were obtained for the four symmetrical bivalent ACTH antagonist peptides that were prepared (see Table 1) using bis(maleimide) cross-linkers. All bivalent ACTH peptides were devoid of agonist function in the concentration range between 0.1 and 10 μM, which does not support our previous preliminary report that compound **I** was a partial agonist [23].

Bivalent peptides, **I, III, V,** and **VI** were analyzed by SDS–PAGE, and each peptide exhibited a single band on silver staining whose mobility was decreased relative to that of the respective monovalent parent peptides. This shift in mobility was approximately equivalent to a doubling in apparent molecular weight when synthetic ACTH peptides were used as molecular weight standards. However, ACTH peptides as a group displayed lower mobility than standard myoglobin peptide fragments, and this may be due to the high content of basic amino acids in ACTH peptides. In addition, ACTH peptides less than 24 residues in length were not well resolved by this system.

Assessment of antagonist potencies of bivalent

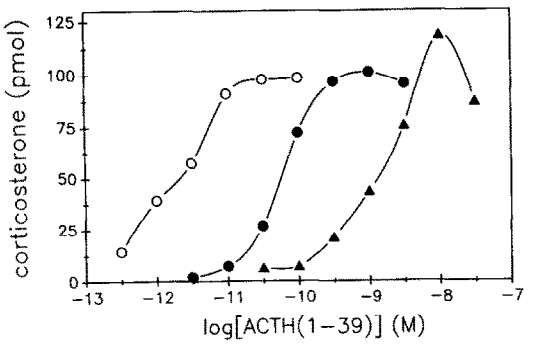

Fig. 3. Influence of spacer on potency of bivalent Cys^{25}ACTH(7–25) peptides. Suspensions of rat adrenocortical cells (10,000 viable cells/tube) were incubated as in Fig. 2 in the absence of antagonist (○) or with 0.5 μM Cys^{25}ACTH(7–25)-*N*-ethylsuccinimide (●), or 0.25 μM compound **III** (▲). Values are means ± SD of triplicate incubations.

ACTH peptides. The influence of covalent cross-linking on ACTH antagonist potency was determined by comparing bivalent ACTH antagonist peptide-induced shifts in ACTH(1–39) concentration–steroidogenic response curves to the concentration–response curves obtained with monovalent reference antagonists. Cys^{25}ACTH(7–25)-*S*-*N*-ethylsuccinimide was the reference for bivalent Cys^{25}ACTH(7–25) based peptides, and Cys^{39}ACTH(7–39)-*S*-*N*-ethylsuccinimide was the reference for bivalent Cys^{39}ACTH(7–39) based peptides. In these experiments, the bivalent peptides were present at one-half the molar concentration of the monovalent peptides. The concentration of ACTH antagonist domains (0.5 μM) was therefore the same for both monovalent and bivalent antagonist incubations.

An example of the substantial potency enhancement observed with bivalent Cys^{25}ACTH(7–25) peptides containing appropriate spacers is shown in Fig. 3. In this experiment, the EC_{50} value in the absence of antagonist was 2.5 pM. The presence of 0.5 μM Cys^{25}ACTH(7–25)-*N*-ethylsuccinimide shifted the EC_{50} value to 58 pM. In contrast, in the presence of 0.25 μM compound **III**, the EC_{50} was 1230 pM, which is more than 20-fold greater than the value observed for Cys^{25}ACTH(7–25)-*N*-ethylsuccinimide.

A summary of the results obtained with Cys^{25}ACTH(7–25) peptides is presented in Table 1. Compounds **I** and **III** showed mean potency enhancements (defined as the EC_{50} in the presence of bivalent antagonist divided by the EC_{50} in the presence of monovalent analog Cys^{25}ACTH(7–25)-*N*-ethylsuccinimide) of 19 and 28 respectively. The Cys^{25}ACTH(7–25) bivalent peptide (compound **V**) that had the longest spacer displayed no increase in antagonist potency relative to the monovalent parent compound.

To determine whether the spacer structure exerted an independent influence on the potency of bivalent antagonist, peptides were prepared that had the structure peptide-spacer-cysteine. As shown in Table 1, the presence of the spacer-cysteine structure had little, if any, effect on the potency of Cys^{25}ACTH(7–25) antagonists. These results demonstrate that the enhanced potency of the bivalent peptides requires the presence of both receptor binding domains, and is not due to the presence of the spacer *per se*.

The influence of Cys^{39}ACTH(7–39) peptides on ACTH(1–39)-stimulated steroidogenesis is shown in Fig. 4. The uninhibited EC_{50} value in this experiment was 6.3 pM. The monovalent antagonist Cys^{39}ACTH(7–39)-*N*-ethylsuccinimide (0.5 μM) shifted the EC_{50} to 20 pM. The EC_{50} value for compound **VI** was 57 pM. The results of five experiments summarized in Table 1 show that the potency enhancement observed for compound **VI** (4-fold) was considerably less than the 28-fold observed for the bivalent Cys^{25}ACTH(7–25) peptide with the same spacer (compound **III**).

DISCUSSION

Bivalent ACTH antagonists were prepared by bis(maleimide) covalent cross-linking of synthetic ACTH antagonists containing cysteine sulfhydryl groups at their carboxyl termini. This synthetic route was selected because it permits use of a single deprotected peptide precursor to prepare a series of bivalent peptides with different spacer structures. Using this approach, bivalent ACTH peptides exhibiting enhanced antagonist potency were prepared in high yield with a simple one-step chromatographic purification. Ramachandran and coworkers [25] have also demonstrated the value of carboxyl terminal sulfhydryl groups as covalent linkage sites for ACTH peptides.

Substantial enhancement of ACTH antagonist potency was observed with some bivalent antagonists, whereas others showed little or no enhancement. Fauchere and coworkers have demonstrated previously that bivalent ACTH antagonists based on the (11–24) and (7–24) sequences are potent steroidogenic antagonists. Their bivalent ACTH(7–24) peptide displayed ten times the potency of the bivalent ACTH(11–24) compound [12]. A direct measurement of potency enhancement of the bivalent ACTH(7–24) peptide relative to the monovalent compound was not presented.

We have found that both the spacer used to link the receptor binding domains and the length of ACTH peptide used to form the bivalent ligand and substantial effects on the level of potency enhancement observed. Spacer length is a particularly critical factor, as demonstrated by compounds **III** and **V**. Compound **III** contains a single R_2 spacer, while compound **V** contains two of these spacers linked by succinimide groups to a DTE-derived moiety. The spacers are thus similar in polarity, but different in length. These bivalent peptides differed substantially in antagonist potency enhancement, with compound **III** showing 28-fold enhancement and compound **V** no enhancement. This indicates that these spacer lengths must be below a certain value (20–40 Å) for potency enhancement to occur. The importance of the peptide component of the bivalent antagonist is demonstrated by the relatively small potency enhancement, 4-fold, observed with ACTH(7–39) based bivalent antagonist **VI** compared

Table 1. ACTH antagonist structures and potencies

Compound	S_1*	S_2	R	Relative potency†	Net charge at pH 7.3‡
I	Cys^{25}ACTH(7–25)	Cys^{25}ACTH(7–25)	R_1	18.6 ± 5.9§ (N = 3)	+12.6
II	Cys^{25}ACTH(7–25)	Cysteine	R_1	0.9	+6.3
III	Cys^{25}ACTH(7–25)	Cys^{25}ACTH(7–25)	R_2	28.2 ± 5.7§ (N = 5)	+12.6
IV	Cys^{25}ACTH(7–25)	Cysteine	R_2	0.8	+6.3
V	Cys^{25}ACTH(7–25)	Cys^{25}ACTH(7–25)	R_3	1.0	+12.6
VI	Cys^{39}ACTH(7–39)	Cys^{39}ACTH(7–39)	R_2	4.3 ± 1.0 (N = 5)	+4.6

* Refer to Fig. 1 for S_1, S_2, and R positions and structures of R_{1-3}.

† Potency enhancement is expressed as EC_{50} for the antagonist listed (0.25 μM for bivalent peptides and 0.5 μM for monovalent peptides) divided by the EC_{50} for Cys^{25}ACTH(7–25)-*S*-*N*-ethylsuccinimide for compounds **I–V** and Cys^{39}ACTH(7–39)-*S*-*N*-ethylsuccinimide for compound **VI**. The values presented are the means of N different cell preparations ±SEM.

‡ Net charge estimated from microconstants determined for ACTH by Noszal and Osztas [24].

§ Relative potency enhancements for compounds **I** and **III** are not significantly different (analysis of variance) at the 95% confidence level.

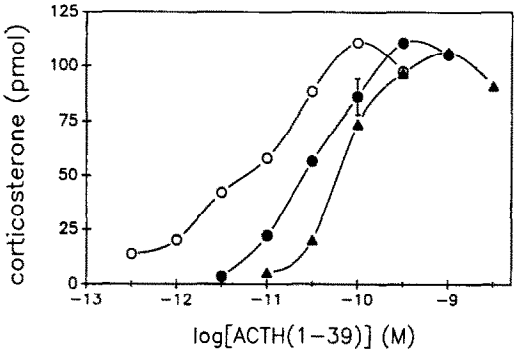

Fig. 4. Influence of spacer on potency of bivalent Cys^{39}ACTH(7–39) peptides. Suspensions of rat adrenocortical cells (8500 viable cells/tube) were incubated in the absence of antagonist (○) or with 0.5 μM Cys^{39}ACTH(7–39)-*N*-ethylsuccinimide (●), or 0.25 μM compound **VI** (▲). Values are means ± SD of triplicate incubations.

with the 28-fold with the Cys^{25}ACTH(7–25) bivalent peptide containing the same spacer (**III**). This is also consistent with a requirement for a short distance between receptor binding sites for optimum potency enhancement. Decreased potency enhancement with long spacers has been observed for bivalent opioid antagonists [9]. The C^{39}ACTH(7–39) based bivalent peptide (**VI**) is also less positively charged than the homologous Cys^{25}ACTH(7–25) based peptide (**III**). However, comparison of results with compounds **III** and **V** indicates that increased peptide charge alone does not enhance potency. This assumes that bivalent ACTH peptides, like monovalent ones [26], do not display defined secondary or tertiary structure.

Spacer structure for 15–20 Å spacers does not appear to exert large effects on potency enhancement. The bivalent Cys^{25}ACTH(7–25) derived peptide with receptor binding domains linked by a 20 Å moderately polar spacer (compound **III**) was the most potent antagonist, but the bivalent Cys^{25}ACTH(7–25) with a 15 Å hydrophobic spacer (compound **I**) also showed a large antagonist potency enhancement (see Table 1), suggesting that the spacer polarity may not be an important factor in determining bivalent potency enhancement.

The influence of direct effects of cross-linker structures on Cys^{25}ACTH(7–25) antagonist potency was examined with peptides containing one receptor binding domain per molecule (i.e. peptide-spacer-cysteine). These peptides did not display enhanced antagonist potency (Table 1). This demonstrates that cross-linker components do not increase the potency of bivalent peptides by direct interaction with cell surface components.

Two mechanisms have been suggested to account for increased potency of bivalent ACTH antagonists [11]. In the first mechanism, additional binding energy (i.e. increased receptor affinity) results from simultaneous binding of cell surface receptors by both of the receptor binding domains of the bivalent ACTH peptide. Minton [7] has provided a detailed quantitative model describing the thermodynamic basis for receptor affinity enhancement of bivalent ligands. The second proposed mechanism involves increased receptor occupancy of bivalent ACTH antagonists due to a localized concentration increase of bivalent peptide near the cell surface resulting from electrostatic attraction of the highly positively charged peptide to negatively charged membrane lipids. Schwyzer and coworkers [27, 28] have presented considerable theoretical and experimental evidence to support the suggestion that interaction of monovalent ACTH peptide–receptor binding is preceded by a preliminary non-specific, electrostatic interaction of postively charged residues of the peptide with negatively charged cell surface phospholipids. This hypothesis predicts that ACTH peptide charge acting through a Boltzmann (electrostatic) distribution is a key factor influencing the affinity of peptide–receptor interactions. The finding that ACTH(1–24) is a more potent agonist on a molar basis than ACTH(1–39) in suspensions of adrenocortical cells [e.g. Ref. 29] is consistent with this suggestion. Similarly, we observed that Cys^{25}ACTH(7–25)-*N*-ethylsuccinimide was a more

potent antagonist than Cys^{39}ACTH(7–39)-*N*-ethylsuccinimide (Fig. 2). However, our finding that Cys^{25}ACTH(7–25) based bivalent peptide **V** did not display enhanced antagonist potency suggests that the second mechanism, enhanced receptor affinity as a result of greater peptide charge, does not adequately account for potency enhancements observed with bivalent ACTH peptides. Direct evaluation of the validity of the first mechanism will require binding measurements with ^{125}I-labeled bivalent peptides.

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