Contributions of Arginines-43 and -94 of Human Choriogonadotropin β to Receptor Binding and Activation As Determined by Oligonucleotide-Based Mutagenesis†

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Received March 22, 1991; Revised Manuscript Received July 16, 1991

ABSTRACT: Members of the glycoprotein hormone family contain a common α subunit and a hormone-specific β subunit. Human choriogonadotropin (hCG) β is a 145 amino acid residue protein glycosylated at 6 positions (2 N-linked and 4 O-linked oligosaccharides). In an effort to elucidate receptor determinants on hCGβ, we have used site-directed mutagenesis to prepare and express several mutant cDNAs with replacements at arginines-43 and -94. Arg-43 is invariant in all known mammalian CG/lutropin β amino acid sequences, and Arg-94 is conserved in 10 of the 12 sequences. Moreover, various studies involving synthetic peptides and enzymatic digestions of intact β chains suggest that these residues may be important in hCG receptor binding. Point mutants were made in which these two arginines were replaced with the corresponding residues in human follitropin β, Leu-43 and Asp-94. The wild-type and mutant β chains were expressed in CHO cells containing a stably integrated gene for bovine α, and heterodimer formation occurred. These heterologous gonadotropins were active in assays using transformed Leydig cells, competitive binding with standard ^{125}I-hCG, and cAMP and progesterone production, but the potency was considerably less than that associated with the hCGβ wild-type-containing gonadotropin. The double-mutant protein Arg-43 to Leu/Arg-94 to Asp also associated with bovine α, but the resultant heterodimer exhibited only low activity. Replacement of each arginine with lysine yielded heterodimers that were at least as potent as bovine α-hCGβ wild type, but the Lys-43-containing β chain appeared to exhibit a low degree of subunit association or reduced stability relative to the expressed hCGβ wild type. These results demonstrate that arginines-43 and -94 contribute to receptor binding through a positive charge.

The four human glycoprotein hormones are heterodimers, all of which share a common α subunit (Pierce & Parsons, 1981). The hormone-specific β subunits are homologous, with the greatest similarity occurring between human choriogonadotropin β (hCGβ)[1] and hLHβ (Ward et al., 1990). hCGβ contains a C-terminal O-glycosylated extension, not present in the other human β subunits, but this extension has no effect on in vitro potency (El-Deiry et al., 1989, 1991); it does, however, appear to increase the circulatory half-life of the hormone (Matzuk et al., 1990).

Various experimental approaches, including susceptibility to trypsin (Birken et al., 1987), chemical modifications (Gordon & Ward, 1985), synthetic peptides (Keutmann et al., 1987, 1988, 1989), and construction of chimeric hormones via protein engineering (Campbell et al., 1991), have identified several amino acid residues and regions of hCGβ that may be important receptor contact sites. One such putative region is the "determinant loop" comprising residues 93–100 of hCGβ, CRRSTTDC, where a disulfide has been proposed between cysteines-93 and -100 (Mise & Bahl, 1981). The net charge of this sequence was suggested by Ward and co-workers (Moore et al., 1980) to be important in determining CG/LH vs FSH or TSH receptor binding; namely, a net positive or neutral charge was proposed to favor the CG/LH receptor and a net negative charge either the FSH or the TSH receptor.

A cyclic decapeptide, based on the amino acid sequence of hCGβ 93–101 and containing an additional Tyr residue at the amidated C-terminus, was synthesized by Keutmann et al. (1989) and found to inhibit binding of hCG to rat luteal cells with an ED_{50} of 0.2 mM, although the peptide did not promote steroidogenesis. By systematically replacing various residues, they showed that net charge was important; moreover, the linear peptide was ineffective. A determinant loop peptide based on the amino acid sequence of hFSHβ was found by Santa Coloma and Reichert (1990) to inhibit binding of hFSH to its receptor, but in contrast to the findings of Keutmann et al. (1989) on the hCGβ determinant loop peptide, that based on hFSHβ-stimulated steroidogenesis in granulosa cells.

Of considerable interest is the recent report by Campbell et al. (1991) that a chimeric hCG/hFSHβ chain, corresponding to hCGβ amino acid residues 1–93 with a C-terminal region (residues 94–114) based on the amino acid sequence of hFSHβ (the hFSHβ numbering would be 88–108), yielded a heterodimer that exhibited full FSH activity in vitro and minimal CG/LH activity. Using site-directed mutagenesis to produce truncated forms of hCGβ, we found that des-(101–145)hCGβ, coexpressed with bovine α, yielded an active gonadotropin, albeit with a lower potency than that associated with hCGβ wild type (Chen & Puett, 1991). In contrast, the deletion mutant des(93–145)hCGβ failed to associate with α,

† This research was supported by NIH Grant DK33973. A preliminary portion of these results was presented (Chen & Puett, 1990).

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[1] Abbreviations: CG, choriogonadotropin (chorionic gonadotropin); ED_{50}, effective dose for 50% change (inhibition or stimulation); FSH, follitropin (follicle-stimulating hormone); h, human; LH, lutropin (luteinizing hormone); TSH, thyrotropin (thyroid-stimulating hormone).

0006-2960/91/0430-10171$02.50/0

and, not surprisingly, there was no discernible activity. These results suggest an important role of the 93–100 region in subunit assembly, as well as receptor binding.

In other studies, Keutmann et al. (1987, 1988) showed that a synthetic (cyclic) peptide corresponding to residues 38–57 of hCGβ, CPTMTRVLQGVLPALPQVVC, not only inhibited hCG binding to rat ovarian membranes with an ED_{50} of about 0.15 mM but also enhanced testosterone production in rat Leydig cells with an ED_{50} of 36 μM. The amino acid sequence of hLHβ in this region is similar to that of hCGβ, and Keutmann et al. (1988) found that replacement of Arg-43 by Asp or Ala yielded inactive peptides. Peptides from this region, on the basis of the amino acid sequence of hFSHβ, are also effective at high concentrations in inhibiting hFSH binding to receptor and stimulating steroidogenesis in vitro (Sluss et al., 1986; Santa Coloma et al., 1991). Bousfield and Ward (1988) reported that incubation of ovine LH and ovine LHβ with endoproteinase Arg-C cleaved one of the peptide bonds on either side of Arg-43. This "nicked" form of ovine LHβ was capable of reassociating with the α subunit, but the heterodimer was only about 2% as effective as native ovine LH in inhibiting the binding of ^{125}I-labeled equine LH to rat Leydig cells. In contrast to these results, Campbell et al. (1991) found that replacement of hCGβ amino acid residues 39–58 with those present in hFSHβ led to a heterodimer with full CG/LH activity in vitro.

Lastly, Birken et al. (1987) showed that incubation of hCGβ with trypsin led to cleavages at several arginines (residues 43, 74, 95, and 114) and at one lysine (residue 122), with a partial cleavage at Lys-2. The cleavages at Arg-114 and Lys-122 removed the C-terminal region of the protein, but the remaining tryptic core, i.e., des(115–145) with cleavages at residues 43, 74, and 95, remained disulfide-linked. This hCGβ tryptic core associated with hCGα, but the heterodimer was essentially inactive. Since other studies have shown that the C-terminal extension is not required for activity (El-Deiry et al., 1989, 1991; Matzuk et al., 1990), receptor contact sites might involve residues 43, 74, and 95 or their neighboring residues.

These independent investigations suggest that the determinant loop region corresponding to amino acid residues 93–100 in CG/LHβ is a possible receptor contact site and that one or two arginines may be particularly important. The studies discussed above have also suggested that Arg-43 and, more generally, the region corresponding to amino acid residues 38–57 in hCGβ may also be important in receptor binding and activation but these results are not consistent with the findings of Campbell et al. (1991) using a chimeric hCG/hFSHβ chain. We have used site-directed mutagenesis to determine the contributions of Arg-43 and Arg-94 to receptor binding and activation in intact hCGβ. Arg-94 was chosen instead of Arg-95 since in 12 amino acid sequences of mammalian CGβ/LHβ, Arg occurs at position 94 in all except equine CGβ/LHβ (Ward et al., 1990). On the other hand, Arg occurs at position 95 only in the β subunits of primate CG/LHs. Significant reductions in activity were found when each residue in hCGβ was replaced with the corresponding residue in hFSHβ (Arg-43 to Leu and Arg-94 to Asp), and the double mutant was nearly inactive. In contrast, replacement of the arginines with lysine did not reduce activity. Interestingly, subunit assembly occurred with all the mutant hCGβs.

Experimental Procedures

Materials. ATP, bovine serum albumin, lysozyme, and isobutylmethylxanthine were obtained from Sigma Chemical Co. (St. Louis, MO); methorexate was from Bristol Laboratories (Syracuse, NY) and DMSO from Fisher (Fair Lawn, NJ). The site-directed mutagenesis kit Muta-gene M13 was purchased from Bio-Rad (Richmond, CA), and the SEQUANSE version 2 kit was from USB (Cleveland, OH). Competent DH5α cells were prepared from stock cells obtained from Bethesda Research Laboratories (Gaithersburg, MD). Progesterone was from Steraloids (Wilton, NH), and [1,2,6,7-(N)-^{3}H]progesterone (94 Ci/mmol) and [α-^{35}S]dATP (500 Ci/mmol) were purchased from New England Nuclear (Boston, MA). The cAMP RIA kit, a polyclonal antiserum against the hCGβ subunit (free and dimeric forms), and ^{125}I-hCGβ (100–150 μCi/μg) were from ICN (Horsham, PA). The TANDEM-RHCG kit (Hybritech, San Diego, CA) was used to determine heterodimer concentrations of hCG, and the progesterone antiserum was from Radioassay Systems Laboratories (Carson, CA). P^{z523} columns were obtained from 5 Prime-3 Prime, Inc. (Paoli, PA), and Centricon 10 columns were from Amicon (Danver, MA).

Mutant hCGβ cDNA Preparation. The cDNA for hCGβ was kindly provided by Dr. John Fiddes (Talmadge et al., 1983) and inserted into the unique *Hind*III site of M13mp18 (El-Deiry et al., 1989; Chen & Puett, 1991). Dr. Rudolf Werner synthesized the various deoxyoligonucleotides (21 bases each) on an Applied Biosystems Model 380B DNA synthesizer, with the DNA sequences corresponding to residues 43 and 94 changed from CGC (Arg) to CTC (Leu), AAA (Lys), and GAC (Asp). Following in vitro mutagenesis, the mutant phage clones were identified by sequencing (Sanger et al., 1977), and each mutant gene was subcloned into a P^{rsv} expression vector, obtained by self-ligation of the one containing the hCGβ wild-type gene.

Gonadotropin Expression. Using the polybrene method (Chaney et al., 1986), the mutant and wild-type expression plasmids were transfected into CHO cells containing a stably integrated gene for bovine α [kindly provided by Dr. John Nilson (Kaetzel et al., 1985)]. The transfected cells were maintained as described elsewhere (El-Deiry et al., 1989; Chen & Puett, 1991), and medium was collected between 4 and 10 days after transfection. The medium was concentrated, if desired, with Centricon 10 columns, and the concentrations of total β and heterologous hormone were determined by radioimmunoassays with hCG as standard. As shown earlier, the medium from the transfected cells can be assayed directly for gonadotropic activity without interference from other components (El-Deiry et al., 1989).

In Vitro Assays: Competitive Binding and Progesterone-/cAMP Responses. The gonadotropin-responsive, transformed murine Leydig cells, MA-10, were kindly provided by Dr. Mario Ascoli and grown as recommended (Ascoli, 1981). The various in vitro assays were performed as described elsewhere (Chen & Puett, 1991). Briefly, competitive binding assays were performed at room temperature for 16 h with 1×10^6 cells in Waymouth's medium, containing 1 mg of bovine serum albumin/mL, different concentrations of expressed hormone, and 10^5 cpm of ^{125}I-hCG, in a final volume of 0.7 mL, and nonspecific binding was determined in the presence of 10 μg of hCG. After being washed, the trypsinized cells were counted in a γ counter. Progesterone concentrations were determined in cell media after a 4-h incubation, and intracellular cAMP levels were measured after a 1-h incubation in the presence of 0.8 mM isobutylmethylxanthine. In both assays, the cells were maintained at 37 °C in Waymouth's medium supplemented with 1 mg of bovine serum albumin/mL. All assays were performed 2 or 3 times, and excellent

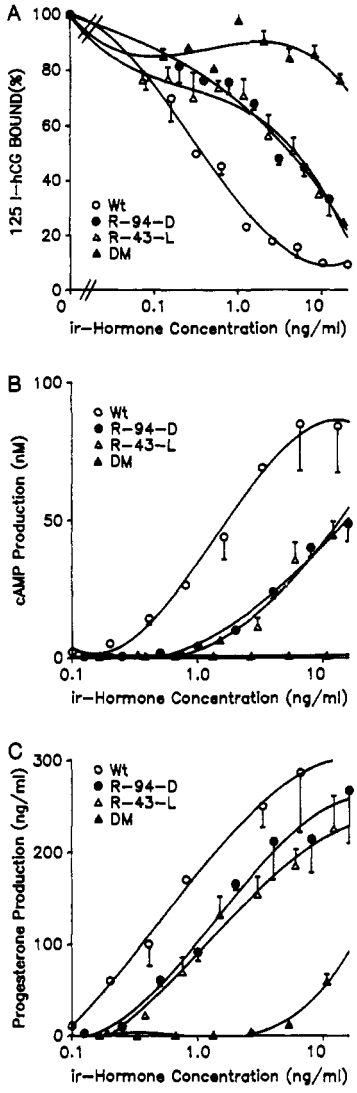

FIGURE 1: Characterization of expressed heterologous gonadotropins in three in vitro assays: (A) a competitive binding assay with ^{125}I-hCG (16 h, room temperature); (B) intracellular cAMP production in the presence of isobutylmethylxanthine (1 h, 37 °C); (C) extracellular progesterone production (4 h, 37 °C) in cultured Leydig tumor cells (MA-10). Wt, bovine α-hCGβ (wild type); R-94-D, bovine α-hCGβ (Arg-94 to Asp); R-43-L, bovine α-hCGβ (Arg-43 to Leu); DM, bovine α-hCGβ (Arg-43 to Leu and Arg-94 to Asp).

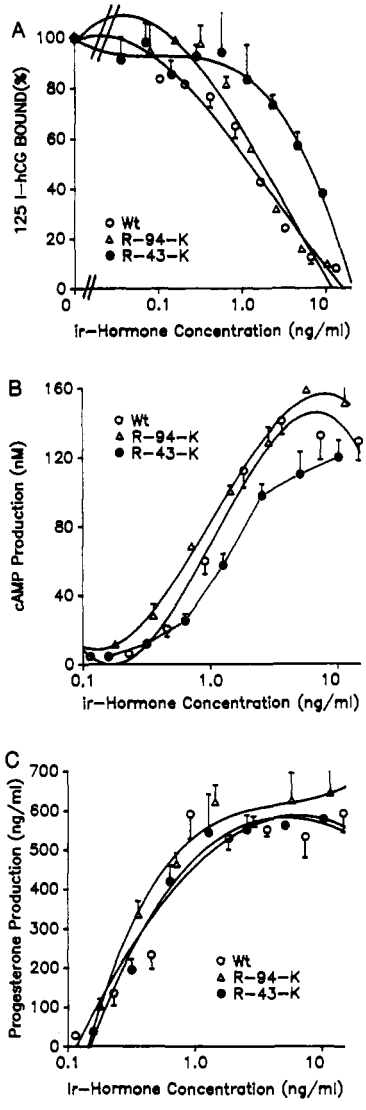

FIGURE 2: Characterization of expressed heterologous gonadotropins in the in vitro assays described in Figure 1. Wt, bovine α-hCGβ (wild type); R-94-K, bovine α-hCGβ (Arg-94 to Lys); R-43-K, bovine α-hCGβ (Arg-43 to Lys).

reproducibility was obtained. Results are presented for a typical assay, and the data are expressed as mean ± SEM. Concentrations of expressed heterologous hormones are referred to the heterodimer.

RESULTS

The mutant and wild-type clones were sequenced in the regions of the mutations, and only the desired changes were found. For example, the codon for Arg-43 in the wild-type clone was CGC, while it was CTC and AAA, respectively, for the Leu and Lys replacements. Likewise, that for position 94 was CGC (Arg, wild type), GAC (Asp replacement), and AAA (Lys replacement). Following subcloning into the P^{rsv} expression vector, restriction mapping was used to select plasmids with the correct orientation.

Under the conditions used, we found that 60–80% of the total immunoreactive hCGβ wild type expressed in the medium is associated with the bovine α subunit. This same range was obtained with the various heterologous mutant hormones, with the exception of the Arg-43 to Lys replacement, which yielded only about 30% heterodimeric β.

The results of competitive binding studies, cAMP production, and progesterone production with the two point mutants Arg-43 to Leu and Arg-94 to Asp and the double-mutant Arg-43 to Leu/Arg-94 to Asp are shown in Figure 1. The point mutants inhibit ^{125}I-hCG binding and increase cAMP and progesterone production, but the apparent potencies, as estimated from the respective ED_{50} values, are reduced (Table I). Interestingly, the double mutant exhibits only very weak activity.

Figure 2 shows comparable data obtained with the two point mutants, Arg-43 and Arg-94 each to Lys. The potencies of these mutant gonadotropins in cAMP and progesterone production are comparable to expressed bα-hCGβ wild type (Table I), and the Lys-94-containing subunit seems to have increased potency. The inhibition of ^{125}I-hCG binding appears reduced in the Lys-43-containing mutant, perhaps arising from subunit dissociation.

DISCUSSION

These results offer strong evidence in an intact hCGβ chain that arginines-43 and -94 contribute to receptor binding, although either can be replaced with that occurring in hFSHβ with some retention of CG/LH activity. Whether these arginines contribute directly, e.g., contact sites, or indirectly, e.g.,

Table I: Summary of the Relative Potencies of Expressed Heterologous Gonadotropins (Bovine α-hCGβ, Wild-Type or Mutant)

hCGβ	binding competition[a]	cAMP production[a]	progesterone production[a]
Wt	1.0	1.0	1.0
R43→L	0.1	0.1	0.3
R43→K	0.2[b]	0.7	1.1
R94→D	0.1	0.1	0.4
R94→K	0.7	1.3	1.4
R43→L/R94→D	<0.04[c]	<0.1[c]	<0.07[c]

[a] In each assay, the ED_{50} for bα-hCGβ wild type (Wt) was normalized to 1.0, and the ED_{50} for the mutant β-containing heterodimer is expressed relative to that value. The ED_{50}'s for bα-hCGβ wild type in the competitive binding assay, cAMP production, and progesterone production were in the ranges of 0.4–1.4, 1.3–1.4, and 0.5–0.75 ng/mL, respectively. [b] This value is low compared to that expected from the cAMP and progesterone ED_{50}'s. As discussed in the text, this may reflect heterodimer subunit dissociation, i.e., reduced hormone stability, during the relatively long period of the competition binding assay relative to the assays involving cellular responses. [c] These values are based on the highest heterodimer concentration used. Extrapolating the data on progesterone production to estimate the ED_{50} yields a relative potency of about 0.02.

via conformational determinants, to receptor recognition cannot be assessed from these studies. In 12 mammalian amino acid sequences of CG/LHβ, Arg-43 is invariant, while hydrophobic residues occur at this position in the 11 known FSHβ/TSHβ sequences (Ward et al., 1990). Residue 94 is Arg in 10 of the 12 known CGβ/LHβ amino acid sequences; the horse contains Gln at this position in its CGβ/LHβ chains (identical amino acid sequences), and Asp and Asn residues occur in the 11 known FSHβ/TSHβ structures (Ward et al., 1990). Thus, the highly conserved nature of arginines-43 and -94 argues for an important functional role in CG/LH activity. Replacement of either Arg-43 or Arg-94 with Lys has only a modest effect on in vitro bioactivity; indeed, the Lys-94 replacement in hCGβ seems to yield a more potent gonadotropin than the wild type. Thus, it appears that a positive charge at Arg-43 and -94 is important in receptor recognition. Replacing these residues with the corresponding ones in hFSHβ leads to a significant reduction in CG/LH activity, e.g., 60–70%, as estimated by progesterone production. The double mutant containing Leu-43 and Asp-94 exhibits only low in vitro bioactivity. From our data, it is not possible to conclude whether the two changes are affecting binding and activity in an additive or cooperative fashion.

These results greatly extend and complement those obtained using synthetic peptides based on CGβ/LHβ sequences (Keutmann et al., 1987, 1988, 1989), as well as endoprotease cleavages of hCGβ (Birken et al., 1987) and ovine LHβ (Bousfield & Ward, 1988). They are not, however, easily reconciled with the observations of Campbell et al. (1991) on the chimeric hCGβ/hFSHβ subunit in which replacement of hCGβ amino acid residues 39–58 with those occurring in hFSHβ gave a heterodimer with full CG/LH activity. Also, our findings that replacement of Arg-94 with a negatively charged residue (Asp) greatly diminished CG/LH activity, while replacement with a positively charged group (Lys) had only a small effect on binding, if anything, enhancing bioactivity, are consistent with the importance of charge in the determinant loop region, i.e., residues 93–100, as originally proposed by Moore et al. (1980) and elegantly demonstrated by Campbell et al. (1991).

It should be emphasized that the three in vitro assays used in this study to characterize the mutant hCGβ chains are quite different and exact correspondence cannot be expected in terms of ED_{50} values. For example, the competition assay with ^{125}I-hCG is a nonequilibrium assay and is not capable of distinguishing mutant gonadotropins that bind to or dissociate from the cell-surface receptor with kinetics quite different from those of standard hCG, or are internalized differently. The assays for intracellular cAMP and extracellular progesterone are based on different times of incubation, and the former is in the presence of a phosphodiesterase inhibitor. Also, it has been well established, first with rat Leydig cells (Catt & Dufau, 1973), that maximal testosterone production occurs when only a limited number of the receptors are occupied and intracellular cAMP levels are increased to only a fraction of the maximal value at saturating gonadotropin concentrations. In the MA-10 cells, Ascoli (1981) has shown there is a better correlation between receptor occupancy and progesterone production than in rat Leydig cells, but in both cases, the ED_{50} for binding is higher than that for steroid production.

Our estimates of the expressed heterologous gonadotropin concentrations are based on standard radioimmunoassays developed for hCG and thus are subject to error, particularly if the mutant hormones interact differently than wild type with the antibodies. If the antigen–antibody affinity is diminished by an amino acid residue replacement, then the concentration of the heterodimer is underestimated and the apparent potency is overestimated. However, none of the epitopes mapped by Moyle et al. (1990) include Arg-43 and Arg-94. In all cases, the estimated ED_{50} values are referred to bα-hCGβ wild type, which is present as an internal standard. The interassay variations were quite low for cAMP and progesterone dose–response determinations, and thus comparisons of relative potency within a given assay should be quite reliable. The variation was somewhat greater in the radioreceptor assay, perhaps reflecting differences in the labeling of standard hCG or in cell passage number. Despite these caveats, it is noteworthy that the rank order of potency for the various mutant hormones is the same for the three various assays.

Interestingly, none of the amino acid residue replacements affected subunit assembly, with the possible exception of Arg-43 to Lys. The percent heterodimer to total expressed β was much lower in this mutant chain, and the apparent binding inhibition of ^{125}I-hCG is much less than one would expect from the ED_{50}'s associated with cAMP and progesterone production. This finding could be explained if the hormone stability is low, such that in a long-term incubation more of the mutant gonadotropin would dissociate, a factor that would not be as pronounced in short-term incubations. Aside from this possible exception, our finding that Arg-43 and Arg-94 replacements did not affect subunit assembly is consistent with our earlier results on tryptic digestion of hCGβ, where a tryptic core consisting of des(115–145)hCGβ, with cleavages at Arg-43, -74, and -95, was capable of associating with hCGα (Birken et al., 1987). Also, Bousfield and Ward (1988) found that ovine LH, nicked on either side of Arg-43, could associate with ovine LHα.

In summary, our results show that Arg-43 and Arg-94 contribute, in large part through their positive charge, to receptor binding and subsequent intracellular signaling.

Registry No. CG, 9002-61-3; cAMP, 60-92-4; progesterone, 57-83-0.

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Conformation–Activity Relationship of Tachykinin Neurokinin A (4–10) and of Some [Xaa^8] Analogues

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Received March 18, 1991; Revised Manuscript Received June 27, 1991

ABSTRACT: NKA(4–10), the C-terminal heptapeptide fragment (Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2) of tachykinin NKA, is more active than the parent native compound in the interaction with the NK-2 receptor. Substitution of Gly^8 with the more flexible residue β-Ala^8 increases its selectivity with respect to other two known receptors (NK-1 and NK-3), whereas substitution with either D-Ala^8 or GABA^8 deprives the peptide of its biological activity. These findings can be interpreted by a conformational analysis based on NMR studies in DMSO-d_6 and in a DMSO-d_6/H_2O cryoprotective mixture combined with internal energy calculations. NKA(4–10) is characterized by a structure containing a type I β-turn extending from Ser^5 to Gly^8, followed by a γ-turn centered on Gly^8, whereas for [β-Ala^8]NKA(4–10) is possible to suggest a type I β-turn extending from Ser^5 to β-Ala^8, followed by a C_8 turn comprising β-Ala^8 and Leu^9 and by another β-turn extending from β-Ala^8 to the terminal NH_2. The preferred conformation of [β-Ala^8]NKA(4–10) is not compatible with models for NK-1 and NK-3 agonists proposed on the basis of rigid peptide agonists [Levian-Teitelbaum et al. (1989) *Biopolymers 28*, 51–64; Sumner & Ferretti (1989) *FEBS Lett. 253*, 117–120]. The preferred solution conformation of [β-Ala^8]NKA(4–10) may thus be considered as a likely bioactive conformation for NK-2 selective peptides.

Neurokinin A (NKA)[1] is a neuropeptide of sequence His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2, expressed by mammalian neurons (Kimura et al., 1983; Kanagawa et al., 1983) in the same precursor, γ-preprotachykinin A (Nawa et al., 1983), containing the sequences of substance P (SP) and neurokinin B (NKB). The wide spectrum of action of NKA and its potential usefulness in the field of antiasthmatic drugs (Nawa et al., 1984) has stimulated many structure–activity relationship (SAR) investigations (Dion et al., 1987a;

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[1] Abbreviations: Aib, three-letter code for aminoisobutyric acid; β-Ala, β-alanine; BFGS, Broyden–Fletcher–Goldfarb–Shanno algorithm; DMSO-d_6, perdeuteriodimethyl sulfoxide; DQF-COSY, double-quantum-filtered correlation spectroscopy; EM, energy minimization calculations; GABA, γ-aminobutyric acid; GPI, guinea pig ileum; NK-1, substance P receptor; NK-2, NKA receptor; NK-3, NKB receptor; NKA, neurokinin A; NKB, neurokinin B; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RPA, rabbit pulmonary artery; RPV, rat portal vein; SAR, structure–activity relationship; SP, substance P; TMS, tetramethylsilane.

0006-2960/91/0430-10175$02.50/0