Design, Synthesis, and Characterization of a Model Peptide Having Potent Calcitonin-like Biological Activity: Implications for Calcitonin Structure/Activity†

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ABSTRACT: A calcitonin analogue, MCT-II, having the potential to form an amphiphilic α-helix from residue 8 to residue 22 with a continuous surface of aliphatic leucine side chains on the hydrophobic face of the helix has been synthesized, and its physical and biological properties have been characterized. Properties exhibited by this peptide, including self-association in the micromolar concentration range with a concomitant increase in the percentage of α-helical structure, formation of stable monolayers at the air–water interface, and adsorption to the surface of egg lecithin single-bilayer vesicles, demonstrate that MCT-II can readily form an amphiphilic α-helical structure. Though MCT-II has minimal sequence homology to any particular natural analogue from residue 8 to residue 22, it has biological activity similar to that of salmon calcitonin I for receptor binding in brain and kidney membranes, for activation of adenylate cyclase, and in hypocalcemic potency in vivo. The amphiphilic α-helical structure of MCT-II, therefore, is important for binding to calcitonin receptors. It is also apparent that a hydrophilic residue commonly occurring on the hydrophobic face (position 15) in the natural calcitonins is not required for high biological activity. Comparison of the physical and biological properties of MCT-II with a previously described model peptide [Moe, G. R., Miller, R. J., & Kaiser, E. T. (1983) *J. Am. Chem. Soc. 105*, 4100], MCT-I, which differs structurally from MCT-II in having a tryptophan in the middle of the hydrophobic face of the α-helix, suggests that, in addition to differences in amphiphilicity and α-helix-forming potential, the lower activity of the mammalian hormones compared to those of ultimobranchial origin may be due to the presence of one or more aromatic residues in positions 12, 16, and 19 on the hydrophobic face of the former.

Amphiphilic secondary structures have been shown to be important elements in the functional properties of many peptides and segments of larger proteins (Kaiser & Kézdy, 1984, 1983; Osterman et al., 1984). Recently, we proposed a structural model for the 32 amino acid calcium-regulating hormone calcitonin as a basis for studying the structure/function relationships of this group of peptides (Moe et al., 1983). The model describes three structural regions likely to be important in calcitonin's biological activity. These are a seven-residue cyclic segment at the N-terminus formed by a disulfide bond between cysteines at positions 1 and 7, an amphiphilic α-helix from residue 8 up to the helix-breaking proline at position 23, and a hydrophilic random-coil segment from residue 23 to the carboxyl-terminal proline amide.

We designed a model peptide, MCT-I, to optimize amphiphilic α-helical structure in the region from residue 8 to residue 22 while having as little sequence homology to any natural calcitonin as possible, and characterized its chemical and biological properties (Moe et al., 1983). The model peptide exhibited higher α-helicity than salmon calcitonin I (SCT-I) in aqueous solution and behaved as an amphiphilic molecule, forming stable monolayers at the air–water interface. However, MCT-I was approximately 10-fold less potent than SCT-I, the most active natural hormone, in both in vitro and in vivo biological assays. The biological activity of MCT-I was comparable to that of porcine calcitonin. As a group, mammalian hormones are between 10 and 50 times less potent than those from fish or of ultimobranchial origin (Guttmann, 1981). From our studies of MCT-I it was apparent that other features, in addition to the relative helix-forming potential of the amino acids in the 8–22 region and the amphiphilicity of the helix, must be important in receptor binding.

If binding of the proposed amphiphilic α-helical segment to the receptor is mediated by hydrophobic interactions, then this binding should be most sensitive to structural changes on the hydrophobic face of the helix. Therefore, the important differences between the structures of SCT-I and MCT-I are likely to involve the glutamate (position 15) of SCT-I and the tryptophan (position 12) of MCT-I on the hydrophobic face of the α-helical segment (Figure 1). All of the natural calcitonins have hydrophilic amino acids, usually aspartate or glutamate, in position 15. In addition, the mammalian hormones all have aromatic residues in the central regions of the hydrophobic face, i.e., in positions 12, 16, and 19, while the more active ultimobranchial peptides do not. In order to understand the possible role of hydrophilic residues in position 15 and the effect of aromatic residues in positions in the middle of the hydrophobic face, we have designed and synthesized a second model peptide, MCT-II. Structurally, this analogue is nearly identical with MCT-I except for having a continuous hydrophobic surface consisting of leucine side chains (Figure 1). We report here the physical and biological properties of this peptide compared with the corresponding properties of MCT-I and SCT-I and relate these to the biological activities

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A

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                                  5                  10                  15
SCT-I   H2N-CYS-SER-ASN-LEU-SER-THR-CYS-VAL-LEU-GLY-LYS-LEU-SER-GLN-GLU-LEU-

MCT-I   H2N-CYS-GLY-ASN-LEU-SER-THR-CYS-LEU-LEU-GLN-GLN-TRP-GLN-LYS-LEU-LEU-

MCT-II  H2N-CYS-SER-ASN-LEU-SER-THR-CYS-LEU-LEU-GLN-GLN-LEU-GLN-LYS-LEU-LEU-

                      20                  25                  30
HIS-LYS-LEU-GLN-THR-TYR-PRO-ARG-THR-ASN-THR-GLY-SER-GLY-THR-PRO-NH2

GLN-LYS-LEU-LYS-GLN-LEU-PRO-ARG-THR-ASN-THR-GLY-SER-GLY-THR-PRO-NH2

GLN-LYS-LEU-LYS-GLN-TYR-PRO-ARG-THR-ASN-THR-GLY-SER-GLY-THR-PRO-NH2
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B

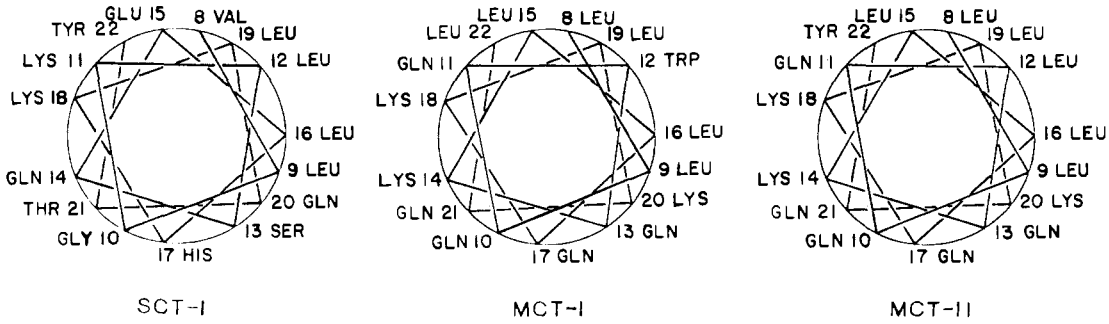

FIGURE 1: (A) Amino acid sequences of SCT-I, MCT-I, and MCT-II. (B) Axial projection of the α-helical region (residues 8–22) of SCT-I, MCT-I, and MCT-II.

of the naturally occurring calcitonins.

Materials and Methods

$Na^{125}I$ was purchased from New England Nuclear Corp., Boston, MA. [α-^{32}P]ATP (400 Ci/mmol) and [^{3}H]cAMP (30–50 Ci/mmol) were obtained from Amersham, Arlington Heights, IL. Synthetic SCT-I (lot K887-110, 4770 IU/mg) was provided by Armour Pharmaceutical Co., Kankakee, IL, and was used without further purification. Egg yolk lecithin was purchased from Avanti Polar-Lipids Inc., Birmingham, AL. Calcium standards and lithium buffer were obtained from Chem-Chek Consulting Inc., South Bound Brook, NJ. Amino acid derivatives were purchased from either Peninsula Laboratories, San Carlos, CA, or Bachem, Torrance, CA. Male Sprague-Dawley (SD) rats were purchased from Charles River Breeding Laboratories, Wilmington, MA. All other reagents were the highest quality available from commercial sources.

MCT-II was synthesized by the solid-phase method with a benzhydrylamine-substituted polystyrene resin cross-linked with 1% divinylbenzene (Hruby et al., 1977) in a Beckman 990 peptide synthesizer. The C-terminal amino acid Boc-Pro was coupled to the resin by using *N*-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC); thereafter, symmetric anhydrides of Boc-Arg(N^g-Tos), Boc-Cys(*S*-4-MeOBzl), Boc-Gln, Boc-Gly, Boc-Leu, Boc-Lys(N^ϵ-2-ClZ), Boc-Pro, Boc-Ser(OBzl), Boc-Thr(OBzl), and Boc-Tyr(*O*-2,6-Cl_2Bzl) were used. An exception was Boc-Asn, which was coupled by the HOBt/DCC method. The deprotection and coupling program used was similar to that employed by Yamashiro & Li (1978). Cleavage from the resin and removal of all the remaining protecting groups were accomplished by treatment with anhydrous liquid HF in the presence of anisole (7:1 v/v) at 0 °C for 45 min. Crude peptide was removed from the resin by washing with 10% aqueous acetic acid. A 200-mg portion of the residue remaining after lyophilization was treated with excess dithiothreitol in 5 mL of 50 mM sodium phosphate buffer at pH 7.5. The intramolecular disulfide bond between cysteine residues 1 and 7 was formed by diluting the peptide solution to a volume of 4 L in the same buffer and adding a solution of 20 mM $K_3Fe(CN)_6$ slowly with stirring until a persistent yellow color was obtained. The resultant dilute peptide solution was concentrated by passing it through a CM-Sephadex C-25 column and then eluted with a linear gradient of NaCl from 0.0 to 0.3 M, employing the same phosphate buffer. Fractions from this column were desalted on a Sephadex G-15 column and purified further by loading them onto a Du Pont Zorbax C_{18} semipreparative HPLC column equilibrated with 0.2 M sodium phosphate buffer, pH 2.5, containing 20% CH_3CN. Peptide was eluted from the column with a linear gradient from 20% to 50% CH_3CN in the same buffer. Fractions from HPLC were desalted on the G-15 column and lyophilized. The purified peptide eluted as a single symmetrical peak from an Altex Ultrasphere ODS analytical column in the same gradient and buffer as above. The results of its amino acid analysis after hydrolysis with HCl/TFA (2:1) at 150 °C for 45 min were as follows: Arg, 1.04 (1); Asx, 1.67 (2); Glx, 5.20 (5); Gly, 1.75 (2); Leu, 7.00 (7); Lys, 3.15 (3); Ser, 2.18 (3); Thr, 2.94 (4); Tyr, 0.98 (1).

The circular dichroic spectra of MCT-II solutions buffered with 20 mM sodium phosphate at pH 7.4 containing 0.16 M KCl were measured from 250 to 200 nm on a Cary 60 spectropolarimeter. The spectrometer cells used were washed with concentrated nitric acid, rinsed throughly with deionized water, then treated with a 1% aqueous poly(ethylene glycol) (M_r 15K–20K) solution for 1 h, and finally rinsed with water before using.

The molecular weights of MCT-I, MCT-II, and SCT-I (1 mM peptide in 20 mM sodium phosphate buffer, pH 7.4, containing 0.16 M KCl and 7 mg/mL Dextran T-40) were determined by sedimentation equilibrium on a Beckman Spinco Airfuge (Pollet et al., 1979). The relative peptide concentration in each successive 10-μL aliquot removed from the

centrifuge tube was determined by derivatizing with fluorescamine and measuring the fluorescence intensity (λ_{ex} 390 nm, λ_{em} 480 nm) on a Perkin-Elmer 650-40 fluorescence spectrometer.

For surface monolayer studies, a subphase of 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, with 0.1 M NaCl was prepared with glass-distilled water. Surfactant contaminants were removed by bubbling air through the solution for 10 min and then aspirating the surface of the buffer. Insoluble monolayers were spread from a 0.28 mM solution of MCT-II in 0.1 mM HCl. The surface pressure, π (dyn/cm), of the MCT-II monomolecular layer was measured as a function of area, A (cm^2), by using a Lauda film balance.

Egg lecithin single-bilayer vesicles were prepared by rapid injection of an ethanolic lecithin solution into aqueous 0.16 M KCl (Batzri & Korn, 1973) and purified by filtration through a 2.5 cm × 87 cm column of Fractogel TSK HW-75(F). After purification, vesicles were characterized by gel filtration through a 1.5 cm × 40 cm column of Sepharose CL-4B (Batzri & Korn, 1973). The concentrations of peptides and vesicles in stock solutions were determined by amino acid analysis and by phosphate assay (Ames & Dubin, 1960), respectively. In a typical binding experiment, various concentrations of peptide were incubated for 45 min at 21 °C with vesicles (0.7 mM lecithin) in 20 mM sodium phosphate buffer, pH 7.4, containing 0.16 M KCl in a total volume of 300 μL. Free peptide was separated from vesicle-bound peptide by rapid filtration through a 1.4 cm × 6.3 cm column of Sepharose CL-6B in the same buffer as above as eluent. The concentration of peptide in each fraction was determined by comparing the fluorescence intensity to that of a standard after first disrupting the vesicles with 2-propanol (20% by volume) and derivatizing the peptide with fluorescamine.

^{125}I-SCT-I (sp act. 150 μCi/μg) was prepared by the method of Hunter & Greenwood (1962). Iodinated hormone was purified by ion-exchange chromatography on SP-Sephadex C-25 and stored until needed in 50-μL aliquots of ~3000 cpm/μL at −20 °C.

Crude rat brain membranes were prepared as described by Nakamuta et al. (1981) using 250–300-g male SD rats. Membranes were either used immediately or stored at −80 °C for no more than 3 days. Crude rat kidney membranes used for binding studies and adenylate cyclase assays were prepared as described by Schwartz et al. (1981) with a minor modification. The dissected kidney cortices were first homogenized briefly with a Polytron at low speed ("5") before complete homogenization with a loose-fitting Dounce homogenizer (Wheaton, pestle B). Protein concentrations were determined with Coomassie Brilliant Blue G-250 (Sedmak & Grossberg, 1977) and crystallized bovine serum albumin (BSA, Sigma) as the standard. All preparations of kidney membranes were carried out at 4 °C, and the membranes were used immediately for binding studies or adenylate cyclase assays.

In both brain and kidney binding studies, a set of 1.5-mL polyethylene tubes were first pretreated with a specific concentration of MCT-II or SCT-I, ^{125}I-SCT-I tracer, and 50 mM Tris-HCl buffer, pH 7.4, containing 35 μM bacitracin. The mixture was aspirated after a 1-h incubation, and these tubes were used for all experiments, one set for brain membrane binding experiments and one set for kidney membrane binding experiments. In the binding assays, each pretreated tube contained membrane suspension (0.5–1 mg of protein), 15 000–20 000 cpm ^{125}I-SCT-I, and from 0 to 1 μM cold peptide in the same Tris buffer described above in a final volume of 1 mL. Incubations were continued for 30 min at 4 °C (brain membranes) or 40 min at 21 °C (kidney membranes) and then stopped by centrifugation of the tubes at 13 000 rpm for 5 min (Beckman Microfuge II). The supernatant was aspirated and the pellet collected by aspiration into a 200-μL disposable pipet tip packed with glass wool. Radioactivity in each tip was quantitated on a Beckman Gamma 5500 counter.

Peptide stimulation of adenylate cyclase activity in crude kidney cortical membranes was measured by methods similar to those described by Marcus et al. (1971). The reaction mixture contained 1 mM [α-^{32}P]ATP (sp act. 70–100 cpm/pmol), 1 mM [^{3}H]cAMP (approximately 40 000 cpm), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.5), 10–20 μg of membrane protein, hormone (except for control tubes), and an ATP-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/mL creatine kinase, and 20 μg/mL myokinase in a total volume of 100 μL. The reaction was initiated by the addition of membranes. Incubations were continued for 10 min at 37 °C and terminated with the addition of 100 μL of a solution containing 10 mM cAMP, 40 mM ATP, and 1% sodium dodecyl sulfate, followed by boiling the mixture for 3 min. cAMP was isolated by the method given by Birnbaumer et al. (1976).

The relative hypocalcemic potency of MCT-II and SCT-I was assessed by giving 3–4-week-old male SD rats subcutaneous injections of hormone in 0.9% saline containing 0.1% BSA. In each experiments, 25 rats were divided into five groups of five rats, and each group was given either saline alone or saline with hormone. Blood samples were obtained by cardiac puncture from the ether-anesthetized rats 1 h after injection. A total of 200 μL of the blood plasma was diluted in 5 mL of 5% aqueous trichloroacetic acid/1000 ppm lithium and centrifuged before determining the calcium concentration by plasma emission spectrometry (Beckman Spectra Span IV).

RESULTS

Design of the Model Calcitonin Peptides. The sequence of amino acids from 8 to 22 for the model peptide was chosen to maximize α-helix-forming potential (Chou & Fasman, 1978) and amphiphilicity of the helix with as little sequence identity to any particular natural analogue as possible. However, the number and position of basic amino acids as well as the balance of hydrophilicity to hydrophobicity were designed to be consistent with the general structure of the native hormones. Except for a tyrosine retained in position 22 as a potential site for introducing a radioactive label, leucine was used exclusively for the hydrophobic residues of MCT-II. Residues 1–7 and 23–32 are identical with the sequence of SCT-I.

Solution Properties of MCT-II. The molar ellipticities obtained from the circular dichroic (CD) spectra of MCT-II from 240 to 204 nm were fitted to a linear combination of three different basis functions derived from the spectra described by Greenfield & Fasman (1969) for α-helix, β-sheet, and random-coil structure. This analysis suggests that MCT-II exists in solution as a mixture of α-helical and random-coil structure. Since there was no significant contribution to the spectrum from β-sheet structure, we used the mean residue molar ellipticity at 222 nm, $[\theta]_{222}$, to estimate changes in the percent α-helical structure (Morrisett et al., 1973). $[\theta]_{222}$ exhibited a concentration-dependent decrease above 0.42 μM MCT-II (Figure 2) that is consistent with a monomer/trimer equilibrium. $[\theta]_{222}$ for the monomer was −5200 deg·cm^2/dmol

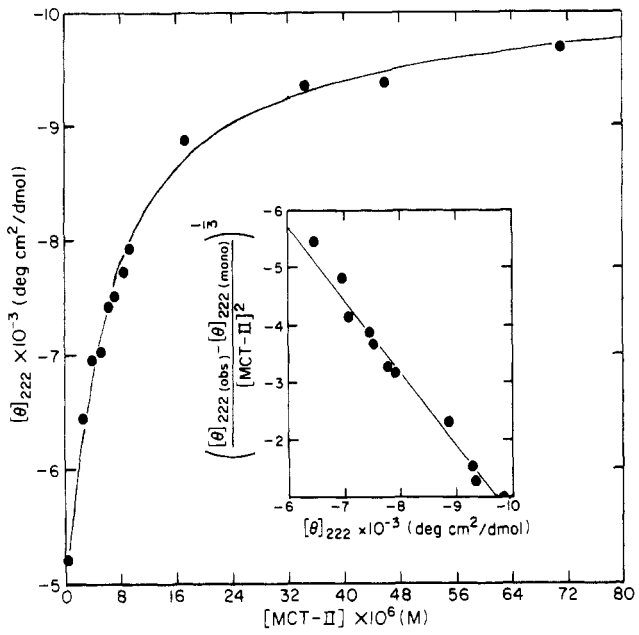

FIGURE 2: Concentration dependency of $[\theta]_{222}$ for MCT-II. Data points and a theoretical curve derived for trimerization with $[\theta]_{222(monomer)} = -5200$ deg·cm^2/dmol, $[\theta]_{222(trimer)} = -10475$ deg·cm^2/dmol, and $K_d = 5.06 \times 10^{-11}$ M^2 are shown. Each data point represents the mean from two experiments. (Inset) Linear regression analysis of the data points fitted to the equation $[([\theta]_{222(obsd)} - [\theta]_{222(mono)})/[MCT\text{-}II]^2]^{1/3} = [3/K_d([\theta]_{222(tri)} - [\theta]_{222(mono)})^2]^{1/3}([\theta]_{222(tri)} - [\theta]_{222(obsd)})$.

(20% α-helix) and −10475 deg·cm^2/dmol (35% α-helix) for the trimer. A plot of $[\theta]_{222}$ vs. $[([\theta]_{222} - [\theta]_{222}$ monomer)/$[MCT\text{-}II]^2]^{1/3}$ (Taylor, 1983; inset in Figure 2) yielded a dissociation constant for the oligomer of 5.06×10^{-11} M^2. This dissociation constant corresponds to 4.5 kcal/mol of stabilization energy per monomer for trimerization. Under similar conditions and over the same concentration range, $[\theta]_{222}$ for MCT-I and SCT-I remained constant at −7800 deg·cm^2/dmol and −4600 deg·cm^2/dmol, respectively (Moe et al., 1983).

The M_r of MCT-II determined by sedimentation equilibrium was 10045 ± 645 or 2.8 times the monomer M_r of 3563 and is consistent with CD evidence for a monomer/trimer equilibrium. MCT-I also appeared to be trimeric at 1 mM concentration with M_r 10658 ± 213 (3.0 times that of the monomer) despite our not detecting self-association in CD experiments up to 0.1 mM (Moe et al., 1983). This suggests MCT-I may be aggregating only at relatively very high concentrations. SCT-I appears to be monomeric at 1 mM concentration with M_r calculated to be 3456 ± 60 compared with the theoretical monomer M_r of 3435.

Surface Properties of MCT-II. MCT-II forms stable monolayers when spread from concentrated solutions. The force–area curve between 0 and 21 dyn/cm can be described by the equation $\pi[A - A_\infty(1 - \kappa\pi)] = nRT$, where A_∞ is the limiting molecular area extrapolated to zero surface pressure and κ is a constant reflecting the compressibility of the monolayer. The parameters calculated from the entire π–A curve (Taylor, 1983) indicate that MCT-II forms a compact, relatively rigid structure at the air–water interface. A_∞ was 362 ± 10 Å^2 compared with 434 ± 7 Å^2 for MCT-I and 559 ± 18 Å^2 for SCT-I. Monolayers of SCT-I, MCT-I, and MCT-II were successively less compressible with κ having values of 0.03 cm/dyn for SCT-I, 0.02 cm/dyn for MCT-I, and 0.01 cm/dyn for MCT-II. Collapse of the MCT-II monolayer occurred at 22 dyn/cm, which was slightly lower than the 24 dyn/cm observed for MCT-I and higher than that for the SCT-I monolayer, which collapses at 12 dyn/cm (Moe et al., 1983).

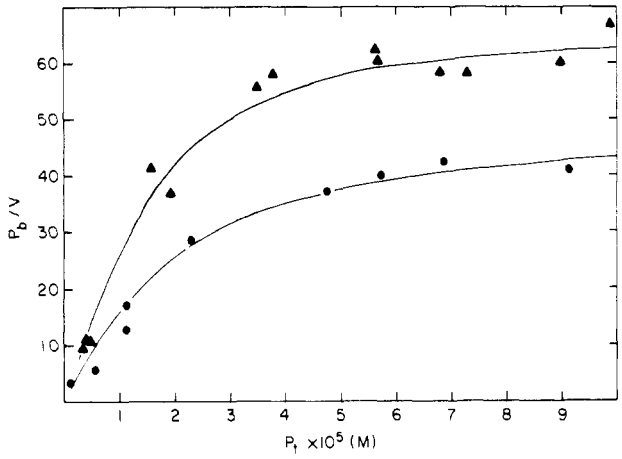

FIGURE 3: Binding of MCT-I (●) and MCT-II (▲) to vesicles as a function of peptide concentration. The symbols and conditions are described in the text.

As shown in Figure 3, both MCT-I and MCT-II adsorb to the surface of lecithin vesicles at concentrations of peptide between 1 and 100 μM. There was no detectable adsorption of SCT-I to vesicles under similar concentration ratios of peptide to vesicles at either pH 7.4 or pH 3.0. The data for MCT-I and MCT-II were analyzed according to the equation $P_f = (NVP_f/P_b) - K$ (Adamson, 1976), where P_f and P_b are the molar concentrations of free and bound peptide, respectively, V is the initial molarity of vesicles (3200 phospholipids per vesicle; Kupferberg et al., 1981), N is the upper limit of P_f/V, and K is the dissociation constant. Plots of VP_f/P_b vs. P_f were linear and gave $K = 12.9 \pm 4.8$ μM with $N = 50 \pm 5$ for MCT-I and $K = 6.7 \pm 1.7$ μM with $N = 68 \pm 2$ for MCT-II. The products, $A_\infty N$, where A_∞ was obtained from the monolayer studies discussed earlier, are nearly the same [$A_\infty N_{(MCT\text{-}I)} = (2.2 \pm 0.2) \times 10^4$ Å^2, $A_\infty N_{(MCT\text{-}II)} = (2.5 \pm 0.1) \times 10^4$ Å^2], suggesting that the relatively larger size of MCT-I at the vesicle surface could account for the difference in the observed values of N. $A_\infty N$ is also in reasonable agreement with estimates of the available surface area between the phospholipid head groups of 230-Å diameter vesicles [$(1.8 \pm 0.6) \times 10^4$ Å^2; Kupferberg et al., 1981]. Analytical Sepharose CL-4B gel filtration of vesicles after incubation with MCT-I or MCT-II at concentrations above 0.1 mM showed that their structure was disrupted, resulting in the formation of a smaller molecular weight species, possibly peptide/lipid micelles.

In Vitro Biological Assays. Preliminary binding experiments with both brain and kidney membranes were hampered by nonspecific binding of MCT-II to the plastic incubation tubes. This nonspecific binding was not inhibited by 35 μM bacitracin or BSA at concentrations as high as 2%. However, use of tubes pretreated with peptide as described under Materials and Methods allowed us to obtain curves for displacement of ^{125}I-SCT-I by MCT-II comparable to those for SCT-I (Figures 4 and 5). As shown in Figure 4, the curve for MCT-II in the brain binding experiment had an unusual shape. A theoretical curve for competitive binding of single ligands to independent binding sites[1] (Figure 4) could be fitted to the data points for concentrations of MCT-II above 0.3 nM. Thus, the anomaly may be an artifact of interactions between

[1] Percent specific binding = $100C/(1 + P_0/K_p)$, where C is the ratio of the total concentration of receptors in the presence of competitive ligand to the concentration in the absence of competitive ligand and P_0 and K_p are respectively the initial concentration and dissociation constant of the competitive ligand.

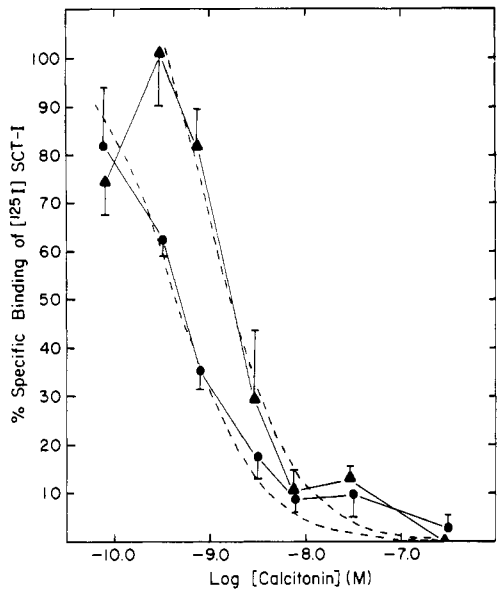

FIGURE 4: Competitive inhibition of ^{125}I-SCT-I binding to rat brain particulate fraction by SCT-I (●) and MCT-II (▲). Each point represents the mean of three triplicate determinations. Dashed lines were calculated from the equation described in the text. Error bars indicate the SE.

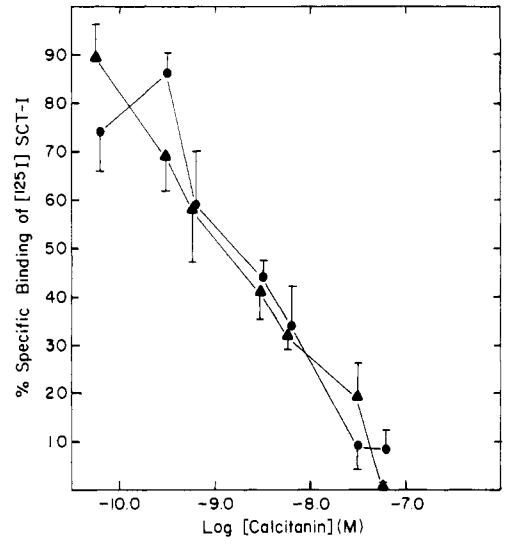

FIGURE 5: Competitive inhibition of ^{125}I-SCT-I binding to crude rat kidney cortical membranes by SCT-I (●) and MCT-II (▲). Each point represents the mean of three triplicate determinations. Error bars indicate the SE.

MCT-II and the membrane vesicles resulting in an apparent increase in the concentration of tracer accessible receptors. For brain receptors, IC_{50} values calculated from theoretical curves fitted to the data (Figure 4) were 0.4 nM for SCT-I and 0.6 nM for MCT-II. With the crude kidney cortical membranes, SCT-I and MCT-II appeared to have identical potency and IC_{50} values of approximately 1.5 nM (Figure 5). The IC_{50} for SCT-I in the brain membrane binding experiments was slightly lower than that determined in control experiments carried out in tubes that had not been pretreated (IC_{50} = 1 nM), but was unchanged in experiments employing kidney cortical membranes under both conditions.

Both SCT-I and MCT-II stimulated adenylate cyclase over basal activity (Figure 6). SCT-I produced a slightly higher maximal stimulation and was slightly more active over the same range of concentration. However, this difference is probably not large enough to be significant when the high nonspecific binding of MCT-II encountered in membrane

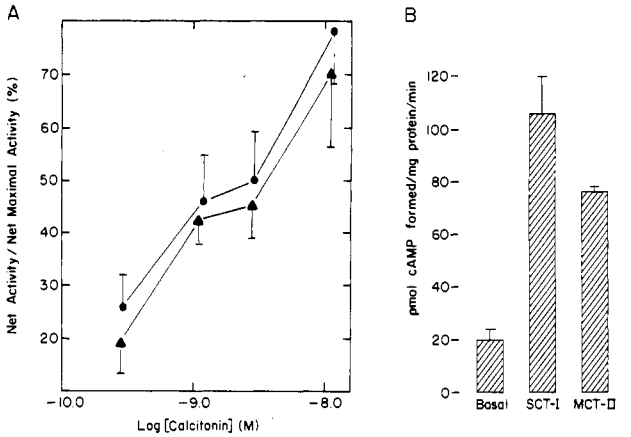

FIGURE 6: (A) Effect of increasing concentrations of SCT-I (●) and MCT-II (▲) on rat kidney adenylate cyclase activities. Adenylate cyclase activity is expressed as the enzyme activity above basal stimulated by SCT-I or MCT-II (net activity) divided by the maximal stimulated enzyme activity above basal (net maximal activity) × 100. Each point is the mean of duplicate determinations from two separate assays. Error bars indicate the SE. (B) The mean maximal adenylate cyclase activity expressed as pmol of cAMP formed (mg of protein)$^{-1}$ min^{-1}. Error bars indicate the SE.

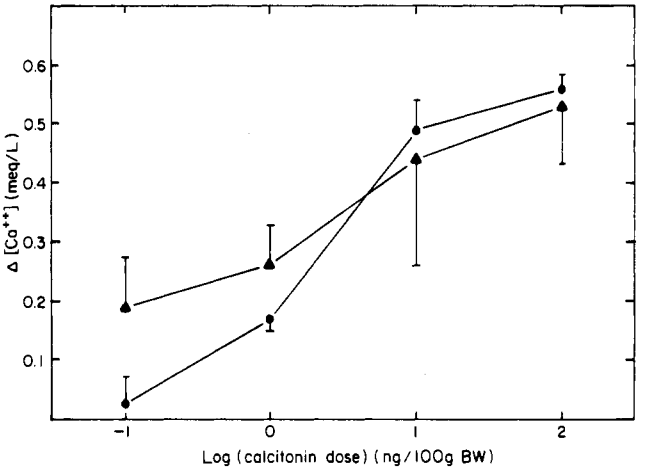

FIGURE 7: The log dose–response curves for SCT-I (●) and MCT-II (▲). Each point represents the difference between the mean serum [Ca^{2+}] for rats given only saline and the mean for those given a particular dose of either SCT-I or MCT-II in two separate experiments as described in the text. Error bars indicate the SD.

receptor assays and the similar potency observed for SCT-I and MCT-II in these assays and in the in vivo assay are considered.

In Vivo Biological Assay. In two assays of each peptide, using different groups of rats, MCT-II produced a decrease in serum calcium similar to that elicited by our preparation of SCT-I over the same dosage range (Figure 7). While this experiment does not permit us to estimate quantitatively the potency of MCT-II, it shows that the relative in vivo biological activity of the model peptide corresponds closely to that observed in the in vitro experiments.

Discussion

The solution and surface properties show that MCT-II can readily form an amphiphilic α-helical structure in environments favoring structural amphiphilicity. Moe et al. (1983) have demonstrated that MCT-I and SCT-I are also capable of forming similar amphiphilic structures. Yet, there are striking differences in the comparative physical properties of these three peptides. Relative to MCT-II, MCT-I appears to aggregate at higher concentrations (>0.1 mM) and bind less strongly to vesicles than MCT-II, while SCT-I does not self-associate

or adsorb to vesicles at all. At the air–water interface, the model peptides form more compact and less compressible structures than SCT-I, while MCT-II has a substantially smaller limiting molecular area and compressibility than MCT-I.

The observed differences in amphiphilic properties of MCT-I and SCT-I compared with those of MCT-II are clearly related to structural variations on the hydrophobic face of the helical segments. The glutamate in position 15 of SCT-I places a negative charge on the hydrophobic face, where it is likely that unfavorable electrostatic interactions prevent self-association and adsorption of the peptide to lecithin vesicles. In addition, decreasing the amphiphilicity of the helical structure formed by SCT-I decreases its stability in the air–water interface. In contrast, the nearly identical collapse pressures of MCT-I and MCT-II suggest that they form structures of similar amphiphilicity. However, having tryptophan in position 12 rather than leucine, as in MCT-II, results in an increase in the concentration at which self-association is detected, in the dissociation constant for vesicle binding, and in the limiting molecular area for MCT-I compared to MCT-II. This can be attributed to steric interactions involving the bulkly indole ring of tryptophan.

The amino acid sequence of MCT-II differs considerably from that of SCT-I from residue 8 to residue 22 (only 33% homology); yet, in the variety of in vitro and in vivo assays used to compare them, MCT-II was indistinguishable from SCT-I. This strongly suggests that the amphiphilic α-helical structure shared by both peptides is important for binding to calcitonin receptors. In addition, the hydrophilic residue commonly occurring in position 15 of the natural analogues does not appear to be required for high biological activity, though it may be important for decreasing nonspecific binding. Comparison of the biological activities of MCT-I and MCT-II suggests that receptor binding is sensitive to steric interactions with the hydrophobic face of the α-helix; this point is of particular importance with respect to the biological activities of the mammalian calcitonins. It is apparent that, in addition to differences in α-helix-forming potential and amphiphilicity of the helix formed, aromatic residues in positions 12, 16, and 19 of the mammalian hormones may be responsible for their lower activity compared to the ultimobranchial peptides.

The relationship between aromatic residues in positions 12, 16, and 19 and lower activity was suggested earlier by Maier et al. (1976). They were able to show that sequentially substituting leucines for aromatic residues in human calcitonin significantly increased its hypocalcemic potency. However, they did not characterize the secondary structure of their analogues or obtain analogues with biological activity equal to that of SCT-I. Our approach has been to idealize the sequence of model peptides such that they are structurally related to each other in a simple way. This has enabled us to show that differences in physical and biological properties are directly related to the presence or absence of tryptophan in position 12 on the hydrophobic face.

With MCT-II, we have demonstrated that a peptide hormone designed to possess amplified amphiphilic α-helical structure in an appropriate structural region can retain very high activity in a hormone receptor system presumably involving many complex interactions between ligand and receptor. Further, we have shown that simplifying the structural relationships between analogues can be quite useful in elucidating the nature of these interactions. At the present time, we are attempting to identify the role of the C-terminal segment of the calcitonin molecule by the synthesis and characterization of new analogues.

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