The Characterization of High-affinity Binding Sites in Rat Brain for the Mast Cell-degranulating Peptide from Bee Venom Using the Purified Monoiodinated Peptide*

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The preparation of a pure, monoiodinated derivative of mast cell-degranulating peptide (MCD peptide), the mast cell-degranulating peptide from bee venom, has enabled us to identify binding sites in rat brain membranes that have a high affinity and specificity for this peptide. These binding sites are easily distributed throughout the brain and copurify with synaptic membranes. Saturation-binding curves, determined by rapid centrifugation or filtration assays, indicate a single population of sites with a concentration of 200 fmol/mg membrane protein in partially fractionated, lysed brain membranes. Dissociation constants of 150 and 140 pM were calculated for the iodinated and native peptides, respectively. These binding sites are probably associated with the neurotoxic action of MCD peptide in the central nervous system. No similar binding sites have been identified in peripheral tissue preparations, and other polycationic mast cell-degranulating agents including compound 48/80 show no such specificity. Specific modification of the primary amines, arginine residues, or disulfide bridges of MCD peptide results in a complete loss of binding activity. Other components of bee venom show specificity for the MCD peptide-binding site, suggesting that a class of neurotoxins in bee venom (possibly including secapin and tertiapin, but not apamin) share the specific action of MCD peptide on the central nervous system.

*Mast cell-degranulating peptide, or peptide 401, is one of several small, disulfide-rich peptides that have been purified from bee venom (1, 2). It is 22 amino acid residues long, contains two disulfide bridges, and has a strongly basic character. At neutral pH, the presence of 5 lysine, 2 arginine, and 2 histidine residues and the absence of any carboxyl groups

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The abbreviations used are: MCD, mast cell-degranulating peptide; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; 125I-MCD, monoiodinated radiolabeled mast cell-degranulating peptide.

in the MCD1 structure (Fig. 1) result in a net charge greater than +8/molecule.

In common with many other polybasic substances, MCD is known to have a potent mast cell-degranulating action (2–6). The concentrations at which MCD causes histamine release vary according to the conditions of the assay and the source of the mast cells, but half-maximal effects at concentrations as low as 2 × 10^{-11} M have been observed using rat peritoneal mast cell preparations (6, 7). In its selectivity of action on mast cells from different sources, MCD most closely resembles the histamine-releasing agent compound 48/80 (8) and is approximately equipotent on a weight basis (7). These compounds also share potent anti-inflammatory activities, which limited structure-activity studies suggest may be related to their mast cell-degranulating action, although this is not clear at present (7, 9–11).

Banks et al. (12) have reported that, in common with apamin, another bee venom peptide, MCD has a potent toxic action which is highly selective for the central nervous system. Intracerebroventricular injections of mice with 4 nmol of MCD/kg of animal cause convulsions and hyperactivity followed by death (12, 13). These authors also report that modification of the arginine residues of MCD abolishes its central activities, but has less effect on its histamine-releasing or anti-inflammatory activities. This suggests that the central and peripheral sites of MCD action may be quite distinct.

In order to investigate the mechanism of action of MCD (and because we feel that it may prove to be a useful investigative tool in view of its potency and specificity of action), we have prepared a highly radioactive, monoiodinated derivative of the peptide. We report here the preparation of this derivative and its use to identify binding sites in rat brain membrane preparations that exhibit a high affinity and specificity for MCD. We also show that the properties of this binding interaction are consistent with the action of MCD in the central nervous system, but not with its mast cell-degranulating activity, and that other components of bee venom may also bind specifically to these sites.

EXPERIMENTAL PROCEDURES

Materials

Bee venom components were kindly donated by Hugues Schweitz (this laboratory) and had been purified according to the method of Gauldie et al. (4) from the venom of the European honey bee Apis mellifera obtained from Bulgarcoop (Sofia, Bulgaria). Na91 was obtained from New England Nuclear at 2000 Ci/mmole and 100 mCi/ml. Chloramine-T, dithiothreitol, iodoacetamide, and 1,2-cyclohexanediol were all purchased from Merck; salmon sperm protamine (chloride), poly(L-lysine) (bromide, M_0 = 1000–4000), compound 48/80, and diethyl pyrocarbonate were all from Sigma. High-performance liquid chromatography was performed using Beckman 110 A pumps.
programmed through a 420 Controller and either analytical (4 × 250 mm) or semipreparative (10 × 250 mm) C18 reversed-phase columns (7-μm particle size) obtained from Merck. Samples were eluted using mixtures of water and acetonitrile, with each solvent containing 0.05% (v/v) trifluoroacetic acid, and were detected by their absorbance at 229 nm, using a Beckman 160 UV detector. γ radiation was counted using either an Intertechnique PG 4000 or a Kontron MR 480 γ counter at 80% counting efficiency.

**Purification of MCD**

MCD was further purified by elution from sulpropyl (SP)-Sephadex C-25 column (0.9 × 41 cm) which had been packed and equilibrated in 50 mM NaH₂PO₄/NaOH buffer, pH 6.0, containing 500 mM NaCl. The peptide material (58 mg) was loaded onto the column in the equilibration buffer (25 ml) and then eluted under essentially isocratic conditions using 680 mM NaCl in the same buffer and collecting 6-ml fractions at 36 ml/h. The absorbance of the eluate at 230 nm was monitored and indicated one minor component (MCD-I) eluted at 6 column volumes and one major component (MCD-II) eluted at 8–9 column volumes (Fig. 2). Fractions corresponding to these peaks were pooled separately and lyophilized, then desalted on Sephadex G-15 eluting with 200 mM AcOH, and lyophilized again. Concentration measurements of MCD-I and MCD-II were always based on absorbances, using εmax = 2.3 × 10⁵ M⁻¹ cm⁻¹ as reported for MCD elsewhere (14).

**Preparation of Iodinated MCD-II**

MCD-II was iodinated by the chloramine-T method (15) under two different sets of conditions. Iodinated peptide at low specific activity was prepared by adding a freshly prepared chloramine-T solution (50 μM) in H₂O to 250 μl of a 100 mM solution of KH₂PO₄ containing 185 nmol of MCD-II, 50 nmol of NaI, and a small quantity (2.8 × 10⁻⁹ cm³) of NaI2I⁻, Aliquots of 100 (50 nmol), 50, 25 μl and then 25 μl of the chloramine-T solution were added at 1-min intervals with rapid Vortex mixing. One minute after the last addition, the reaction was quenched with BSA as before. Reaction mixtures of either type were applied to a SP-Sephadex C-25 column (0.4 × 41 cm) that had been packed and equilibrated in a buffer consisting of 50 mM NaH₂PO₄ and 500 mM NaCl adjusted to a final pH of exactly 5.55 with a few drops of 10 M NaOH. This column was eluted at 7.8 ml/h with 10–20 ml of the equilibration buffer and then with 720 mM NaCl in the same phosphate buffer at the same final pH. In the case of preparations at high-specific activity only, the eluting buffer was also made 0.1% in BSA to carry free radioactive iodide and maximize recovery of the peptide material. The elution was continued overnight at room temperature, collecting 2-ml fractions in 5-ml hemolysis tubes. These were then monitored for absorbence and/or radioactivity and stored in a cold room at 10°C for up to 2 months.

**Preparation of Membranes**

Synaptic membranes were prepared from 2- to 3-month-old, male, Sprague-Dawley rats, using the method of Jones and Natus (16) with minor modifications. Briefly, decerebrate brains were homogenized using 10 strokes of a Teflon plunger in 10% (w/w) sucrose solution buffered with 10 mM Tris-HCl, pH 7.4, and containing 0.1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA. This whole homogenate was centrifuged at 800×g for 20 min and then the supernatant was recentlyrifuged at 100,000×g for a further 20 min. The pellet from this second centrifugation was resuspended in a large volume (approximately 2 ml/g wet weight of whole brain) of 5 mM Tris-HCl buffer, pH 8.1. This suspension was subjected to a brief homogenization and then left for 2 h in an ice bath. At the end of this period, the lysed membrane suspension was recentrifuged at 100,000×g for 40 min to form the lysed membrane pellet (P₂) used in most of the binding studies. Alternatively, the lysed membrane suspension was briefly homogenized in 2 volumes of 48% (w/w) sucrose (final sucrose concentration was 36%) and subjected to centrifugation at 60,000×g for 2 h in a sucrose gradient consisting of steps to 28.5% and 10% sucrose. Myelin (upper interface), synaptic membranes (lower interface), and mitochondria (pellet) fractions were then diluted to less than 10% sucrose and recovered as pellets by centrifugation at 100,000×g for 30 min. All pelleted membrane preparations were washed once in 10 mM Tris-HCl, pH 7.5, containing 0.1 mM phenylmethylsulfonyl fluoride and then resuspended in MCD-II as a standard, indicated concentrations of 5–15 mg/ml and stored indefinitely in liquid nitrogen before use. These preparations were thawed only once before use, and protein concentrations were determined by the method of Hartree (17) using BSA as a standard.

**Chemical Modifications of MCD II**

Acetylation of Primary Amines—To 200 μl of a solution of 1.0 × 10⁻³ M MCD-II in 50 mM pyridine (final pH 8.5) was added 15 μl of acetic anhydride with rapid Vortex mixing. The pH of this solution was then maintained at 6.0–6.5 by addition of 150 μl of pyridine as three further 15-μl aliquots of acetic anhydride were added over 15 min. The reaction mixture was left for a further 30 min at room temperature and lyophilized twice in H₂O. Fluorescamine analysis (18) of the product using the radiolabeled MCD-II as a standard, indicated that 95% of the available primary amines had been acetylated.

Irreversible Reduction of Disulfide Bonds—The double-excess method (19), using diithiothreitol and iodoacetamide, was employed to generate a peptide with the same net charge as MCD-II. To a solution of 2.7 mg of MCD-II in 200 μl of 50 mM Tris-HCl, pH 7.4, was added 50 mg of diithiothreitol. This solution was stirred and then left at room temperature. After 2.5 h, 240 mg of iodoacetamide in 1.5 ml of the same buffer were added with stirring. After a further 40 min at room temperature, 1.0 ml of this solution was eluted from a bufraterative HPLC column using a linear gradient of 0.5–50% acetonitrile in 60 min and a flow rate of 3.0 ml/min. The modified peptide eluted 24 min after the start of the gradient as a single major peak, incompletely separated from minor components that eluted slightly later. The front and center portion of this peak was collected.
and lyophilized. Using MCD-II as a standard, the yield of the reduced, alkylation peptide was estimated to be 32% by absorbence at 220 nm. 36% by fluororescine reaction, and 34% by titration of the 2 histidine residues with diethyl pyrocarbonate (performed exactly as described in Ref. 20).

Reversible Modification of Arginine Residues—The arginine residues of MCD-II were converted to \( N^\epsilon, N^\epsilon \)-[1,2-dihydroxyoxycyclohex-1,2-yleno]-arginine residues by reversible reaction with 1,2-cyclohexandione (21). A solution of 50 mM 1,2-cyclohexandione in 200 mM \( H_2BO_3/NaOH \) buffer, pH 9.0 (450 \( \mu \)l), was mixed with 50 \( \mu \)l of 1 mM MCD-II in \( H_2O \). This reaction mixture was incubated at 37 °C and, at various times during the incubation, 100 \( \mu \)l aliquots were removed and diluted 100-fold in 200 mM \( H_2BO_3/NaOH \) buffer, pH 7.4. These samples were frozen and stored at -20 °C for direct use in binding studies performed the same day. After 4-h reaction time, a 50-\( \mu \)l aliquot of the reaction mixture was added to 100 \( \mu \)l of 300 mM hydroxylamine hydrochloride/NaOH, pH 7.0, and this mixture was incubated for 16 h at 37 °C to regenerate unmodified MCD-II. Control aliquots of the reaction mixture were added to 100 \( \mu \)l of 300 mM hydroxylamine hydrochloride/NaOH, pH 7.0, and this mixture was incubated at room temperature for 16 h. The 1,2-cyclohexanedione was also performed, and these solutions were all used directly in binding studies without purification.

Binding Studies

All binding studies were performed at 4 °C in ice, using hemolysis tubes. The incubation buffer was a concentrated suspension of each fraction (final concentration 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.8 mM \( CaCl_2 \), 1.3 mM \( MgSO_4 \), and 0.1% BSA. Binding experiments were terminated either by rapid centrifugation on a Heraeus Christ Hae- nofuge for 5 min or by rapid filtration using Millipore EHPW 02500, cellulose acetate filters (0.5-\( \mu \)m pore size) that had been pre-wetted in 20 mM Tris-HCl buffer, pH 7.4. When the centrifugation technique was employed, sample volumes were always 500 \( \mu \)l and 1 ml tubes were used. After centrifugation, the supernatant was aspirated, and the pellet-containing tube was analyzed directly for radioactivity. When filtration was performed, 5-ml tubes were employed, and samples were diluted to a volume of 1.4-2.0 ml by addition, when required, of 1 \( \mu \)l of ice-cold incubation buffer immediately before filtration. The tubes and filters were then rapidly washed with 2 × 5 ml of the ice-cold buffer, and the filters were counted for radioactivity. This procedure was required to give reproducible results.

Kinetics of Binding of MCD to Membranes—The association reaction was started by rapid Vortex mixing of 50-\( \mu \)l aliquots of the concentrated membrane preparation with 2-ml aliquots of an ice-cold solution of \( ^{125}I \)-MCD in the standard incubation buffer. The dissociation reaction was started by rapid Vortex mixing of 500-\( \mu \)l aliquots of a mixture of the membranes and \( ^{125}I \)-MCD in the standard buffer that had been preincubated in ice for 20-60 min with 1-ml aliquots of 150 mM MCD-II in the same ice-cold buffer.

The association and dissociation reaction mixtures were incubated in ice, and at various times after initiation, the reactions were terminated by filtration of the whole samples. The rapid rates of reaction involved necessitated that each time point was determined individually.

Equilibrium-binding Studies—In all of these studies, membranes and ligands were incubated together for 25-35 min before analysis.

Saturation curves for \( ^{125}I \)-MCD binding to \( P_3 \) membranes were determined, using both methods of analysis, by incubating 0.1-50 \( \mu \)l of the appropriate \( ^{125}I \)-MCD-containing fractions from the SP-Sephadex C-25 elution (see text) in a final volume of 500 \( \mu \)l of buffer, both in the absence of and the presence of membranes at a final protein concentration of 0.8 mg/ml. Nonspecific binding was determined, in each case, by addition of 1 \( \mu \)M MCD-II. The final ionic composition of the incubation buffer was kept identical to standard conditions except for the necessary presence of phosphate buffer from the \( ^{125}I \)-MCD preparations. This buffer was maintained at a constant final concentration of 5 mM phosphate, and its buffering effect was compensated for by using Tris-HCl buffer at pH 7.9 instead of pH 7.4. In centrifugation assays, the concentration of free \( ^{125}I \)-MCD was determined by counting a 200-\( \mu \)l aliquot of the supernatant from each tube after centrifugation of the total incubation mixture. In filtration assays, a 100 \( \mu \)l aliquot of each incubation mixture was removed to determine the total \( ^{125}I \)-MCD concentration, before the remaining suspension was filtered as described.

The affinity of MCD-II for its binding sites in \( P_3 \) membranes was determined by incubating 2-ml samples containing \( ^{125}I \)-MCD (3.90 pm), membranes (0.19 mg protein/ml), and several dilutions of MCD-II (180 nM and lower) in the same phosphate-containing buffer that was used to determine the saturation-binding curves. The \( ^{125}I \)-MCD binding to membranes was determined by the filter assay, after subtraction of \( ^{125}I \)-MCD binding to the filters themselves when corresponding incubation mixtures containing no membranes were assayed.

The affinities of MCD-I, MCD-II, and chemically-modified MCD-II for the \( ^{125}I \)-MCD binding sites in \( P_3 \) membranes were compared by centrifugation assay, using the standard buffer with the Tris-Cl replaced by 20 mM \( NaH_2PO_4/NaOH \), pH 7.4, and the NaCl concentration reduced to 100 mM. Compound 48/80 (10 \( \mu \)g/ml) was also added to reduce the nonspecific binding of \( ^{125}I \)-MCD (measured in the presence of 100 nM MCD-II as the only competing ligand).

All other binding assays were performed using the centrifugation assay, and the standard buffer with compound 48/80 (10 \( \mu \)g/ml) added, except in the experiment described in Fig. 10, where compound 48/80 was not added to the buffer, and nonspecific binding (measured in the presence of 100 nM MCD-II) was about 25% of the total \( ^{125}I \)-MCD binding.

Amido Black Protein Quantification

The protein yields after centrifugation or filtration of \( P_3 \) membrane suspensions (0.88 mg protein/ml) under standard binding assay conditions, in triplicate, using the technique of Kuno and Kilhara (22), with BSA as a standard. Samples of the untreated membrane suspension, the pellets from centrifugation assays, and the filters from filtration assays were incubated separately overnight at room temperature in 1.2 ml of 0.1% sodium dodecyl sulfate solution in 100 mM Tris base. The protein in these solutions was then precipitated in trichloroacetic acid and collected, stained with Amido Black 10B, and determined, and quantitated as previously described (23). This method gave results for the untreated membranes that were very close to those determined by the Hartree method (17), which is sensitive to sodium dodecyl sulfate and could not be used.

HPLC Purification of Bee Venom Fractions

Small quantities of the "acapin" and "tertapiin" fractions of bee venom that had been purified according to Gauldie et al. (4) were eluted from a HPLC column, using an acetonitrile gradient of 10-60% in 50 min and a flow rate of 2.5 ml/min. Major absorbence peaks were detected in the eluate 20, 27, 30, 32, and 39 min after the start of the gradient. These fractions were collected, lyophilized, and their UV spectra were recorded, before they were tested for binding activity.

Under the conditions of the HPLC, apamin eluted 20 min after the start of the gradient, and MCD-I and MCD-II coeluted 17–19 min after the start of the gradient.

RESULTS

Preparation of Radioiodinated MCD

When MCD that had been purified from bee venom according to Gauldie et al. (4) was subjected to SP-Sephadex C-25 chromatography under isocratic conditions, a minor component was eluted before the major peptide component. Since amino acid analysis after acid hydrolysis of both of these peptides gave the composition expected for MCD, they were labeled MCD-I and MCD-II, respectively (Fig. 2). Presumably, MCD-I corresponds to the minor component previously observed during MCD purification (4). In order to separate the 2 fractions from MCD-II was essential to the preparation of a pure, radioiodinated monomeric derivative of the latter peptide. Analytical HPLC (23% acetonitrile, 1 ml/min) of MCD-I and MCD-II gave identical and anomalous elution profiles (not shown) that were very similar to those recently described for another small, highly cross-linked peptide toxin, conotoxin MI (23): each peptide eluted as two components (\( K' \) = 4.7 and 6.5) with incomplete separation, as a result of a slow conformational exchange occurring during the chromatography. Repeat HPLC of either of these components regenerated the complete elution profile. MCD-I and MCD-II were found to differ, however, in their UV spectra. At either pH 7.4 or 3.0 in 20 mM \( NaH_2PO_4/HCl \) buffer, solutions of MCD-I which ab-
the number of radioactive products. Peptides were then eluted containing 720 mM NaCl. A typical elution profile for preparations from SP-Sephadex C-25 in a phosphate buffer at pH 5.55 is shown in Fig. 3. Unreacted, radioactive iodide was eluted in the void volume together with the BSA used to quench the reaction and the reduced chloramine-T. Two major radiiodinated peptide products were then eluted after fraction numbers 36 and 46 (14 and 18 column volumes) with 720 mM NaCl elution buffer, followed by a minor radioactive component that eluted after about fraction number 59 (23 column volumes).

When MCD-II was radiiodinated at low-specific activity using 50-fold larger quantities of the reactants, the radioactivity profile and the UV absorbance profile (215 nm) could be compared (not shown). Assuming an absorption coefficient, ε_{215}, of 3.2 \times 10^4 (14) for all of the peptides eluted, the radioactivity incorporated into MCD-II corresponded to 0.95 ± 0.05 iodine atoms/peptide molecule in all fractions across the first radioactive peptide peak and the first half of the second radioactive peptide peak. MCD-II was eluted after approximately fraction number 59 (23 column volumes) and partially overlapped with the second radioactive peptide. In these radiiodinations at low-specific activity, no radioactive component coeluting with MCD-II was observed, suggesting that this component of the preparations at high-specific activity (Fig. 3, MCD-II peak) is an artifact of the purification procedure.

Under the reaction conditions we have employed, only the 2 histidine residues in the MCD-II structure should be susceptible to radiiodination. Therefore, it is reasonable to assume that the two monoiodinated peptides we have prepared are 125I-His^8 MCD-II and 125I-His^13 MCD-II. At present, however, no further analysis has been attempted, and these peptides are both referred to as 125I-MCD. Consistent with this analysis is the reported difference in pK_a values of the 2 histidine residues (pK_a = 5.7 and 6.1, see Ref. 14), which would allow the separation of the two monoiodinated products from each other as well as from the native peptide that we have obtained by ion-exchange chromatography. Presumably, 1 histidine residue is less susceptible to iodination than the other, and indeed, the yield of the first 125I-MCD peak eluted from SP-Sephadex relative to that of the second could be increased by reducing the relative proportion of MCD-II in the iodination reaction.

Predictably, in view of the small differences in pK_a involved, the separation of the two 125I-MCD peaks from each other and from MCD-II in the chromatography was very sensitive to changes in the pH of the eluting buffer. For this reason, in each preparation the fractions across the two 125I-MCD peaks were tested for their binding activities as described in the legend to Fig. 3. A profile of binding activities having two plateaux with a sharp transition between them was taken as an indication of good separation of the three peptides. Since the second 125I-MCD eluted could be prepared in higher yield and was more active in the binding assay (perhaps for related structural reasons), only uncontaminated fractions containing this radiiodinated peptide were used in the binding studies described here. Its specific activity was calculated as being equal to the specific activity of the Na125I used in the iodination reaction.

Characterization of High-affinity MCD Binding to Sites in P₅ Membranes from Rat Brain

Saturation Curves—Initial experiments indicated that specific, high-affinity binding sites for 125I-MCD exist in membrane preparations from rat brain and can be readily studied in buffers that approximate physiological conditions using a centrifugation assay. However, the determination of kinetic constants for the binding reaction necessitated the use of a filtration assay and low temperatures, despite the propensity of 125I-MCD to bind to all types of filters that we have tested. Saturation curves were, therefore, determined for the 125I-MCD binding to P₅ membranes from decerebellate rat brain using both methods of analysis and incubating the binding mixtures in ice at 4 °C. In both types of experiments, nonspecific 125I-MCD binding was determined as a function of free 125I-MCD (centrifugation assays) or total 125I-MCD (filter assays) in the presence of 1 μM MCD-II and in the presence (points B) or absence (points D) of membranes. The total 125I-MCD binding in the absence of MCD-II and the presence (points A) or absence (points C) of membranes was then adjusted for nonspecific binding using the extrapolated values B' and D', respectively. The specific 125I-MCD binding to the membranes alone was then calculated as (A - B') - (C - D')

The results of the centrifugation assay (Fig. 4) illustrate the advantages of this method over the filtration assay (Fig. 5). Displaceable binding to the centrifuge tubes was essentially negligible, whereas displaceable binding to the filters represented up to 20% of the total binding. Furthermore, although identical membrane preparations were used in these
assays at identical concentrations, the curves obtained for specific binding to the membranes by the centrifugation assay indicated a saturation point 4- to 5-fold greater than that obtained by filtration. This considerable discrepancy was mostly explained by comparing the quantities of membrane protein recovered by the two methods, using the Amido Black-staining technique (22). Membranes proteins were recovered in 68 ± 5% yield by centrifugation, but in only 21 ± 2% yield by filtration. These values were used to adjust the specific 125I-MCD binding to the membranes to 100% yield, and the results were analyzed by the Scatchard method. The centrifugation assay gave a straight line (Fig. 4, inset) corresponding to a single population of 125I-MCD-binding sites with a maximum binding number, $B_{\text{max}}$, of 230 fmol/mg of membrane protein. The filtration assay also gave a straight line (Fig. 5, inset), and the extrapolated $B_{\text{max}}$ was 160 fmol/mg of membrane protein. This is a reasonable agreement, considering the differences in the techniques and the possibility that the recovery of the binding sites upon centrifugation or filtration may not be proportional to the protein yield as has been assumed. When the equilibrium dissociation constant, $K_d^*$, for the binding reaction was calculated from the slopes of these Scatchard plots, essentially identical values of 150 and 140 fmol/mg were obtained from the centrifugation and the filtration assay, respectively.

**Kinetic Analysis**—The kinetic constants at 4 °C for the binding reaction between 125I-MCD and its binding sites in P3 membranes from rat brain were determined using the filtration technique. When the association reaction was followed as a function of the time, $t$, after addition of membranes to buffered 125I-MCD solutions, the nonspecific binding determined in the presence of 100 nM MCD-II was essentially instantaneous and constant at all time points. The specific binding of 125I-MCD to membranes, $B_s$, was therefore calculated as the total binding at time $t$ minus the total binding at $t = 0$. The association reaction was found to be very rapid and could only be followed at low peptide and membrane concentrations, causing considerable scattering of the experimental points. Fig. 6A shows the combined results from two experiments performed under identical conditions, with $B_s$ calculated as a percentage of $B_{\text{max}}$ ($t = 11$ min) and plotted as a function of time. A semilogarithmic plot of the results from the first 100 s of the reaction was fitted to a straight line through the origin, as expected for pseudo first-order kinetics (Fig. 6A, inset). From the slope of this line, the association constant, $k_a$, for the binding reaction was calculated using Equation 1,

\[
\ln \frac{[B_s]/(B_s - B_i)]}{k_d(R_{\text{ref}}/B_s)t} = 1
\]
where $K_o$ is the initial concentration of specific binding sites and $L$, is the initial $^{125I}$-MCD concentration. When $R_o$ was calculated using a value of 200-fmol binding sites/mg of membrane protein, and $B$ was calculated assuming a 21% yield of the bound radioligand in the assay (see above), the calculated value of $K_o$ was $1.1 \times 10^{-9}$ M$^{-1}$ s$^{-1}$.

The dissociation of $^{125I}$-MCD from its membrane-bound complex is a rapid, first-order reaction. $B_i$ was calculated as the difference between the total binding observed at time $t$ and that observed at $t = \infty$ (11 min) after MCD-II addition, and then expressed as a percentage of the specific binding at $t = 0$, $B_o$, and plotted against $t$ (Fig. 6B). The appropriate semilogarithmic plot for these results fitted a straight line through the origin (Fig. 6B, inset), and the slope of this line gave a dissociation rate constant, $k_d$, of $8.0 \times 10^{-3}$ s$^{-1}$. This corresponds to a half-life for the peptide-membrane complex of 86 s. The equilibrium dissociation constant, $K_d^*$, calculated from $k_d/k_o$, is 73 pm, in approximate agreement with the Scatchard analyses.

Displacement by MCD-II—The concentration dependency of MCD-II displacement of $^{125I}$-MCD from its membrane-binding sites was studied at equilibrium by the filtration technique. The results, shown in Fig. 7, fitted a smooth sigmoidal curve indicative of a noncooperative, competitive displacement of $^{125I}$-MCD by MCD-II. Low concentrations of radioligand and membranes were employed so that the equilibrium dissociation constant, $K_o$, for the MCD-II binding reaction with $P_2$ membranes could be calculated from the results with minimum error, using Equation 2 (24).

$$K_d = K_{o3}/(1 + R_o/K_d^*)$$

In this equation, $K_{o3}$ is the MCD-II concentration required to displace 50% of the specific $^{125I}$-MCD binding and was determined from Fig. 7 to be 180 pm. Using values of 200-fmol binding sites/mg of membrane protein and $K_d^* = 150$ pm, the dissociation constant for MCD-II binding was calculated to be 140 pm.

These results show that the affinity of MCD-II for its binding sites in $P_2$ membranes prepared from rat brain is essentially unmodified by the radiolabeling procedure used here, indicating that the histidine residue that has been iodinated does not play a critical role. In contrast, it is clear from the screening test for binding that we apply to each $^{125I}$-MCD preparation that MCD-II binding is more sensitive to iodination of the less reactive histidine residue. From the results in Fig. 3, it is estimated that the dissociation constant, $K_d^*$, for this radiopeptide is 300 pm, or 2-fold larger than that of the $^{125I}$-MCD used in the binding studies presented here. It may be neither histidine interacts directly with the binding site, but that one (the less reactive) is buried in the MCD structure while the other is relatively exposed on the surface. Iodination of the buried histidine would then be expected to disrupt the peptide conformation to a greater extent than would iodination of the exposed histidine, thus affecting the binding reaction to a greater extent.

Localization of the MCD-binding Site

Localization in Fractions from Rat Brain Homogenate—The various membrane fractions obtained during the purification of synaptic membranes from a crude rat brain homogenate (16) were tested for their relative abilities to bind $^{125I}$-MCD, using the centrifugation assay. The membrane fractions were
tested by equilibrating mixtures of MCD-I1 at various concentrations in standard buffer at 4 °C with °I-MCD (3.9 PM) and membranes. MCD-I1 binding as described in the text. The total °I-MCD binding measured in 100 nM MCD-I1 was approximately 25% of that measured in the absence of MCD-II.

The effects of ion concentrations on °I-MCD binding to P3 membranes from rat brain were studied using a standard buffer composed of 20 mM Tris-HC1, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.8 mM CaCl2, 1.3 mM MgSO4, and 0.1% BSA and then varying only one component at a time. The NaCl concentration dependency of specific °I-MCD binding gave a semilogarithmic plot which was used to calculate the Kd value for MCD-II binding as described in the text. The total °I-MCD binding measured in 100 nM MCD-II was approximately 25% of that measured in the absence of MCD-II.

obtained from a single preparation and were used at concentrations chosen to yield similar quantities of membrane protein in the pellet obtained in the assay. The results, summarized in Table I, show that the °I-MCD binding site was copurified with the synaptic membranes. In this preparation, synaptic membranes bound 1.6 times the amount of °I-MCD that the lysed P3 membranes bound before their fractionation on a discontinuous sucrose gradient and 2.1 times that bound by the initial whole homogenate. The myelin fraction from the sucrose gradient bound only 40% as much °I-MCD/mg of protein recovered in the assay when compared to the synaptic membranes, and the mitochondrial fraction showed a negligible binding capacity.

Localization in Regions of Rat Brain—P3 membranes were prepared from various regions dissected from rat brain and tested for °I-MCD binding at essentially identical membrane protein (0.34 mg/ml) and radioligand concentrations (22 pm). This experiment showed that the cerebellum, brainstem, hypothalamus, striatum, midbrain, and cortex all have °I-MCD-binding capacities similar to that of the whole brain, i.e. 17.3 ± 1.8 fmol of °I-MCD specifically bound per mg of protein in pellet (mean ± S.E. from three separate incubations).

Localization in Other Tissue Preparations—A preliminary search for peripheral °I-MCD-binding sites in membranes prepared from a variety of sources was made under the standard conditions used to determine °I-MCD binding to rat brain membranes. Under these conditions, little or no specific binding of °I-MCD has been observed in any of the membrane preparations listed in Table II compared to that for the lysed P3 membranes from rat brain, even when relatively high membrane protein concentrations were used.

Effect of Buffer Conditions on °I-MCD Binding

The effects of ion concentrations on °I-MCD binding to P3 membranes from rat brain were studied using a standard buffer composed of 20 mM Tris-HC1, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.8 mM CaCl2, 1.3 mM MgSO4, and 0.1% BSA and then varying only one component at a time. The NaCl concentration dependency of specific °I-MCD binding gave a

<table>
<thead>
<tr>
<th>Membrane source*</th>
<th>Membrane in assay*</th>
<th>°I-MCD specifically bound*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>0.32</td>
<td>5</td>
</tr>
<tr>
<td>Liver</td>
<td>0.79</td>
<td>2</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.34</td>
<td>11</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>0.15</td>
<td>9</td>
</tr>
<tr>
<td>Intestine (colon)</td>
<td>0.42</td>
<td>5</td>
</tr>
<tr>
<td>Lung</td>
<td>0.44</td>
<td>3</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>0.33</td>
<td>1</td>
</tr>
<tr>
<td>Erythrocytesf</td>
<td>0.78</td>
<td>0</td>
</tr>
<tr>
<td>Electric organf</td>
<td>0.40</td>
<td>0</td>
</tr>
<tr>
<td>Whole brain</td>
<td>0.31</td>
<td>100</td>
</tr>
</tbody>
</table>

*Membrane fractions obtained from various regions dissected from rat brain.

*°I-MCD binding was calculated as fmol/mg protein in pellet.

*Results are mean ± S.E. from three separate incubations.

*°I-MCD binding was 6.8 pm in the incubation mixtures.

*The yield of membrane protein from the incubation mixtures in the pellet of the binding assay varied between 60 and 80% according to the membrane source.

*Values are calculated from the °I-MCD specifically bound per mg of membrane protein in the incubates and expressed as a percentage of the value for whole rat brain (2.12 fmol/mg).

*°I-MCD binding was 6.4 pm in the incubation mixtures.

*Erythrocytes were prepared by collecting whole blood from a rat onto 20 mM EDTA, pH 7.4, and centrifugation of this suspension at 1000 x g for 10 min. The pellet obtained was used directly.

*Prepared from Electrophorus electricus as described by Wheeler et al. (26).
membranes from rat brain. A shows the specific 125I-MCD binding to membranes as a function of the NaCl concentration in buffered incubation mixtures containing 6.2 pM 125I-MCD and 0.80 mg/ml membrane protein. A, inset shows the data for 125I-MCD binding to membranes in the absence (○) and presence (●) of 100 nM MCD-II that were used to calculate the values in the main panel, illustrating the pronounced effect of the sodium ion concentration on the specific radioligand binding. B, the salts NaCl, KCl, CaCl2, MgSO4, or CaCl2 plus MgSO4 in the standard binding assay buffer were replaced with equal (monovalent cations) or double (divalent cations) concentrations of cholinium chloride in incubation mixtures containing 125I-MCD (17 pM) and membranes (0.27 mg/ml). The effect of each replacement on the specific (unshaded bars) and the nonspecific (shaded bars) 125I-MCD binding to membranes was then determined. C shows the KCl concentration dependency of specific 125I-MCD binding in incubation mixtures containing 125I-MCD (9.0 pM) and membranes (0.80 mg/ml) in otherwise buffer.

Fig. 9. Effect of structural modifications on the ability of MCD-II to displace 125I-MCD binding to P2 membranes from rat brain. Displacement curves for MCD-I (●) and MCD-II (○) were determined by assaying the specific 125I-MCD binding to membranes in incubation mixtures containing 125I-MCD (6.9 pM), membranes (0.47 mg protein/ml), and various concentrations of the nonradiolabeled peptides. Tested in the same assay were the effects on MCD-II activity of acetylation with acetic anhydride (△), reduction by dithiothreitol followed by alkylation with iodoacetamide (●), and reversible modification of the arginine residues after reaction with 2-cylohexanemethanediol at 37 °C for 240 min (▲). Also shown are the activities in this assay of solutions containing MCD-II regenerated after arginine modification by overnight incubation in 0.2 M hydroxylamine hydrochloride at 37 °C (□) compared to those of a control incubation of unmodified MCD-II (○). Inset, the modification of the arginine residues in MCD-II by reaction with 1,2-cyclohexanediol was followed by the loss in binding activity of the reaction mixture. Displacement curves for the modified peptide were determined using aliquots of the reaction mixture quenched at various times after the start of the reaction (●) and compared to that for unmodified MCD-II (○) in a single binding assay (7.3 pM 125I-MCD, 0.80 mg membrane protein/ml).

membranes from rat brain are summarized in Fig. 9. Acetylation of the amino terminus and the ε-amino groups of the 5 lysine residues in the MCD-II structure (Fig. 1) using acetic anhydride resulted in a complete (more than 10,000-fold) loss of activity. The reaction of the 2 arginine residues in MCD-II with 1,2-cyclohexanediol at pH 9.0 to form N,N'-(1,2-dihydroxycyclohex-1,2-ylene)-arginines (21), thus preserving the positive charges of the unmodified residues in the modified structure, was followed using the binding assay (Fig. 9, inset). After 4 h of reaction at 37 °C, this modification had inactivated the MCD-II to the same extent as was demonstrated for the acetylation reaction. Overnight incubation of the arginine-modified peptide at 37 °C in the presence of 200 mM hydroxylamine at pH 7.0 caused a complete recovery of the binding activity compared to MCD-II controls, indicating that possible side reactions of the arginine or lysine residues of MCD-II with the 1,2-cyclohexanediol leading to irreversible products (21, 27) had not occurred. Finally, MCD-II was reduced with dithiothreitol, and the resulting thiol groups were blocked by alkylation with iodoacetamide. This procedure, which also preserves the ionic character of the MCD-II
molecule, again resulted in a 10,000-fold loss in activity after purification of the modified peptide from unreacted or partially reacted MCD-II (about 0.5% of the total) by HPLC.

Displacement of $^{125}$I-MCD Binding by Histamine Liberators—Three very potent, polybasic mast cell-degranulating agents, proteamine, poly(l-lysine), ($M_r = 1,000-4,000$) and compound 48/80 (6, 7), were tested for their abilities to displace $^{125}$I-MCD binding. Compared to MCD-II, these compounds showed no specificity for the MCD-binding site, displacing $^{125}$I-MCD in a highly cooperative manner at concentrations 1,000-100,000 times greater than those required by MCD-II on a weight basis (Fig. 10). In particular, compound 48/80 was 30,000 times less potent than MCD-II at causing displacement of the specific $^{125}$I-MCD binding.

Displacement of $^{125}$I-MCD Binding by MCD-I and Other Fractions of Bee Venom—When the abilities of MCD-I and MCD-II to displace $^{125}$I-MCD binding to rat brain membranes were compared, MCD-I was found to be about 10-15% as potent as MCD-I1 (Fig. 9). In view of the good chromatographic separation obtained for these two components (Fig. 2), the MCD-I activity is unlikely to result from contamination of this fraction by MCD-II. Elucidation of the structural difference between these two peptides, which are clearly very closely related, will therefore provide interesting information on the specificity of this binding reaction.

Other peptide components of bee venom that share some structural features with MCD are apamin, secapin, and teriapin (2, 28). Apamin, which had been prepared in a highly purified form for previous work in this laboratory (29), was unable to displace any specific $^{125}$I-MCD binding to the P<sub>3</sub> membranes from rat brain even at 10 μM concentrations. In contrast, the secapin and teriapin fractions purified from bee venom according to Gauldie et al. (4) were found to displace $^{125}$I-MCD. These fractions which were only available in small quantities were, however, impure by analytical HPLC. They were, therefore, subjected to semipreparative HPLC to ensure their complete separation from apamin and MCD and their resolution into single components. Four major components, eluting later than MCD or apamin from the reversed-phase column in an acetonitrile gradient, were collected and each was found to displace $^{125}$I-MCD at moderately low concentrations. The most active of these fractions (eluted 30 min after the start of the gradient) was a component of the teriapin fraction and gave a half-maximal displacement of $^{125}$I-MCD at a concentration of 100-150 nM (estimating that $K_d = 5,000 \text{ M}^{-1} \text{ cm}^{-2}$).

Effects of Other Toxins, Peptides, and Drugs on $^{125}$I-MCD Binding—In an initial attempt to identify the MCD-binding site in the central nervous system and determine the mechanism of the toxic action of this peptide, a variety of agents were tested for their abilities to displace $^{125}$I-MCD binding. The substances tested are listed in Table III, together with the concentrations at which they were used, and included several basic, disulfide-rich, toxic peptides, as well as a variety of neurotransmitter agonists and antagonist. None of these substances displayed any displacing activity at low concentrations, and those $K_d$ values that were determined were 10 μM or higher, indicating essentially no specificity of action.

**DISCUSSION**

We have described herein the preparation of two highly purified, moniodinated derivatives of the mast cell-degranulating peptide isolated from bee venom. One of these derivatives, prepared in a radioactive form as $^{125}$I-MCD, has been used to identify (for the first time) specific, high-affinity binding sites for this peptide in rat brain membrane preparations. Critical factors in the characterization of this binding interaction have been the separation of the $^{125}$I-MCD from unlabeled peptide by careful ion-exchange chromatography and the use of physiological NaCl concentrations in the binding-study incubations to minimize nonspecific radioligand binding to the membranes.

The MCD-binding sites have been shown to copurify with synaptic membranes, suggesting a direct neurotoxic action for the peptide that is consistent with the striking behavioral effects that it causes upon intracerebral injection (13). The affinity of the $^{125}$I-MCD used here for these sites ($K_d = 150$...
pm), as well as that of the parent peptide, MCD-II (Kd = 140 pm), are also consistent with the toxic activity in the central nervous system. Thus, MCD was found to be toxic at doses 1 order of magnitude higher than apamin, when administered to mice (12, 13), and apamin has been found to bind to the calcium-dependent potassium channel in synaptic membranes at correspondingly lower concentrations (Kd = 10 pm, see Ref. 29). Furthermore, we have demonstrated a complete lack of any similar binding activity in a number of peripheral tissue preparations (Table II), in agreement with the nontoxicity of MCD administered peripherally, even at high doses. It is worth noting, however, that our data do not exclude the possibility that similar binding sites do exist in peripheral tissue, but at concentrations lower than the 200 fmol/mg membrane protein determined for rat brain, or in a physically or chemically modified form having a lower affinity for MCD under the conditions of our assay.

In contrast, we provide strong evidence in this study that the binding sites for MCD in rat brain membranes are not related to its mast cell-degranulating activity. Most strikingly, Fig. 10 shows that the arginine-rich peptide, protamine, as well as poly(L-lysine) and compound 48/80 have a very low affinity for these binding sites and displace [125I]-MCD binding at much higher concentrations than MCD-II and in a highly cooperative manner similar to that found for salts (Fig. 8). These compounds all release histamine from mast cells in a similar concentration range to MCD (6, 7). In particular, compound 48/80 is known to follow the selective action of MCD on different mast cell preparations quite closely (9), while it has the weakest effect in the binding assay described here. Nor is the affinity of MCD-II for the brain membrane-binding sites consistent with its histamine-releasing action, which is suggestive of a dissociation constant that is, in the best cases, about 2 orders of magnitude higher than that determined here (6). Presumably, the predominant interaction of these agents with mast cells results from their polycationic structure which must match some anionic mast cell membrane component with little other structural specificity. In this case, the highly anionic cromolyn-binding protein recently isolated by Maxurek et al. (30, 31), and identified as a calcium-gating component in histamine release from rat basophilic leukemia cells (32), seems a likely binding site for mediating the calcium-dependent-degranulating effects of these polycationic compounds. In this respect, the preparation of a radiolabeled derivative of MCD described here should prove very useful and, indeed, initial experiments show that the [125I]-MCD we have prepared does bind specifically to cells from rat peritoneal lavages.

The presence of two disulfide bridges, as well as 10 amino acid residues that carry positive charge at neutral pH and no acidic groups in the MCD structure (Fig. 1), must severely limit the conformational flexibilities that this peptide can adopt in solution as or as part of its membrane-bound complex. One likely structure for MCD in solution that is consistent with CD and NMR data has been suggested by Hider and Ragnarsson (28) on the basis of predictive parameters and consists of a spherical structure having considerable internal hydrophobic stabilization and the basic residues distributed over the external surface, as well as an exposed hydrophobic domain. The direct binding of such a simple inflexible structure to a suitable anionic site in brain membranes having a complementary hydrophilic character probably explains the very rapid association and dissociation kinetics determined for the MCD-binding reaction described here. The association constant for MCD binding (1.1 × 10^8 M⁻¹ s⁻¹) must be close to the diffusion-controlled, limiting value at this temperature (4 °C), and the dissociation reaction is also extremely rapid (kₖ = 8.0 × 10^7 s⁻¹) compared to other toxin-binding reactions. For example, the corresponding values for apamin binding to the calcium-dependent potassium channel are 2.6 × 10^5 M⁻¹ s⁻¹ (kₖ) and 3.8 × 10⁻⁴ s⁻¹ (kₖ) at the same temperature (29). These peptides possess considerable similarity, as has been pointed out elsewhere (5, 14, 25), but apamin has both less hydrophobic character and fewer charged residues than MCD and might be expected to adopt a greater variety of conformations in solution and, perhaps, undergo a larger conformational change upon binding than MCD. These peptides’ behavior may be compared to that of the larger (71 amino acid residues) peptide neurotoxin II from *Naja haje* which binds to and dissociates from the nicotinic acetylcholine receptor at much slower rates even at 20 °C (kₖ = 4.8 × 10^4 M⁻¹ s⁻¹, kₖ = 3.2 × 10⁻⁶ s⁻¹, see Ref. 33).

Despite the apparent simplicity of the MCD-binding reaction indicated by the kinetic parameters, the structure-activity relationships presented here clearly show that a very close fitting complex is formed. Thus, all of the chemical modifications of the MCD structure we have attempted result in a complete loss of binding activity. Under such circumstances, it is not possible to draw any conclusions about the relative importance of (for example) the arginine residues in the MCD structure, although their selective modification was achieved. Particularly striking, however, is the complete inability of apamin to displace specific [125I]-MCD binding to brain membrane preparations, even at high concentrations. The structural similarity of these peptides to each other and to secapin and tertiapin, two other peptides components of bee venom (28), led us to test the activities of these last two peptides in the MCD-binding assay. Our preliminary results indicate that these peptides, or copurified components from bee venom, do have significant binding activities although, at least in this system, they are less active than MCD-I or MCD-II. Further studies of these components may rapidly provide enough information to allow the design and synthesis of readily accessible peptides sharing the selective toxic action of MCD on the central nervous system, particularly as the secapin structure includes only one disulfide bridge. Clearly, the importance of such an approach will depend upon the identification of this high-affinity MCD-binding site and the elucidation of the biochemical effect that MCD binding produces.

Acknowledgments—We thank Dr. H. Schweitz for the initial purification of MCD from bee venom, Dr. M. Huges for early experiments which suggested the current method for preparation of [125I]-MCD, and C. Roulin-Bettelheim for typing the manuscript.

REFERENCES


*J. W. Taylor, unpublished results.*