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Inhibition of β-Bungarotoxin Binding to Brain Membranes by Mast Cell Degranulating Peptide, Toxin I, and Ethylene Glycol Bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic Acid†

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ABSTRACT: The presynaptically active snake venom neurotoxin β-bungarotoxin (β-Butx) is known to affect neurotransmitter release by binding to a subtype of voltage-activated K^+ channels. Here we show that mast cell degranulating (MCD) peptide from bee venom inhibits the binding of ^{125}I-labeled β-Butx to chick and rat brain membranes with apparent K_i values of 180 nM and 1100 nM, respectively. The mechanism of inhibition by MCD peptide is noncompetitive, as is inhibition of ^{125}I-β-Butx binding by the protease inhibitor homologue from mamba venom, toxin I. β-Butx and its binding antagonists thus bind to different sites of the same membrane protein. Removal of Ca^{2+} by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid inhibits the binding of ^{125}I-β-Butx by lowering its affinity to brain membranes.

β-Bungarotoxin (β-Butx)[1] is a snake venom neurotoxin with a Ca^{2+}-dependent phospholipase A_2 activity (Wernicke et al., 1975; Strong et al., 1976; Howard & Gundersen, 1980). The toxin has a M_r of 20 500 and consists of two subunits (Kelly & Brown, 1974). The A subunit (M_r 13 500) carries the phospholipase A_2 activity; the B subunit (M_r 7000) has sequence homology to protease inhibitors (Kondo et al., 1978a,b; Lee, 1979). β-Butx inhibits neurotransmission by interacting with the presynaptic terminal (Abe et al., 1976, 1977). In chick brain, the toxin binds to a membrane protein of M_r 430 000, which contains smaller polypeptide subunits (Rehm & Betz, 1982–1984). Electrophysiological and binding data

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[1] Abbreviations: β-Butx, β-bungarotoxin; MCD peptide, mast cell degranulating peptide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography.

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indicate that the β-Butx binding protein belongs to a subclass of voltage-gated K^+ channels (Rehm & Betz, 1984; Peterson et al., 1986). A detailed analysis of its function and structure has been hampered by the virtual absence of a pharmacology for this binding site. With the exception of Ca^{2+}-chelating agents like EDTA (Rehm & Betz, 1982), only toxin I and two closely related polypeptides, dendrotoxin and toxin K, have been shown to inhibit β-Butx binding (Othman et al., 1982; Rehm & Betz, 1984). These binding antagonists are protease inhibitor homologues of M_r 7000 from mamba snake venom which facilitate neurotransmitter release (Harvey & Karlsson, 1982; Halliwell et al., 1986). They also antagonize the electrophysiological effects of β-Butx at the neuromuscular junction (Harvey & Karlsson, 1982).

MCD peptide is a basic 22 amino acid peptide from bee venom which has a potent mast cell degranulating activity (Breithaupt & Habermann, 1968; Gauldie et al., 1978). In brain, high-affinity binding sites for radioiodinated MCD peptide have been demonstrated which are not related to its mast cell degranulating activity (Taylor et al., 1984). In this paper we show that MCD peptide is a noncompetitive inhibitor of β-Butx binding to brain membranes.

Experimental Procedures

Materials. MCD peptide was kindly provided by M. Lazdunski, Nice, France. Toxin I was a gift of M. Hollecker, Heidelberg, FRG. Newly hatched chickens were obtained from Geflügelzucht Hockenberger, Elsenz-Eppingen, FRG. Other materials were as described in Rehm and Betz (1982).

Preparation of Brain Membranes. Membranes were prepared from the brains of adult rats or newly hatched chicken as described by Rehm and Betz (1982), except that the sucrose gradient step was omitted. After DNase I treatment, the membranes were frozen in liquid nitrogen and stored at −70 °C. Before use in binding assays, the thawed membranes were washed once in buffer A [20 mM K-HEPES, pH 7.4, 115 mM KCl, 1 mM $CaCl_2$, 0.01% (w/v) NaN_3] and collected by centrifugation for 20 min at 25000*g*.

Binding of ^{125}-I-β-Butx to Brain Membranes. β-Butx was labeled with ^{125}I (Rehm & Betz, 1982) to an initial specific activity of 1100–1800 Ci/mmol. Binding of ^{125}I-β-Butx was determined according to Rehm and Betz (1982) with some modifications. Membranes (about 250 μg of protein per assay) were incubated in buffer A with 0.02–2.0 nM ^{125}I-β-Butx in a total volume of 200 μL for 45–90 min at room temperature. The samples were then diluted with 3.5 mL of buffer A and immediately filtered on cellulose–acetate filters (Sartorius, 0.20 μm). The filters were washed once with 3.5 mL of buffer A and counted at 75% efficiency in a γ-counter. All data shown represent the mean ± SD (indicated if larger than size of symbol) of triplicate determinations. All dilutions of ^{125}I-β-Butx, β-Butx, or polypeptide inhibitors were in buffer A containing 0.1% (w/v) bovine serum albumin.

Miscellaneous Methods. For HPLC analysis, MCD peptide dissolved in 70% (v/v) formic acid was loaded on a C18 reverse-phase column and eluted with a linear gradient of 10–70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Protein was determined according to Lowry et al. (1951) as modified by Larson et al. (1986) with bovine serum albumin as a standard.

Results

Micromolar concentrations of MCD peptide inhibited the binding of ^{125}I-β-Butx to chick and rat brain membranes (Figure 1). This inhibition was specific: basic peptides like apamin or cytochrome *c* had no significant effect on ^{125}I-β-Butx binding (Table I). Another component of bee venom, phospholipase A_2, also inhibited β-Butx binding [Table I and Rehm and Betz (1982)]. A contamination of the MCD peptide preparation with phospholipase A_2 could, however, be excluded:

(i) Upon HPLC analysis, MCD peptide (10 μg) behaved as a pure preparation; in particular, no material was detected at the position of bee venom phospholipase A_2 (data not shown).

(ii) In contrast to the inhibition of ^{125}I-β-Butx binding by MCD peptide, inhibition by bee venom phospholipase A_2 exhibited a marked temperature sensitivity and therefore probably resulted from enzymatic activity (Table I).

The apparent inhibitory constant (K_i) of MCD peptide was calculated according to Cheng and Prusoff (1973) and found to be 180 nM for chick and 1100 nM for rat brain membranes. For comparison, toxin I, an established antagonist of β-Butx binding (Othman et al., 1982), exhibited a K_i value of 0.4 nM for chick brain membranes (Figure 1). Its affinity thus is similar to that of native β-Butx (K_i of 0.13 nM; Figure 2C).

Scatchard plots of ^{125}I-β-Butx binding with different concentrations of MCD peptide (Figure 2A), or toxin I (Figure 2B), revealed an apparent reduction of the total number of ^{125}I-β-Butx binding sites. In order to exclude unrelated irreversible processes like inactivation, proteolysis, etc., membranes preincubated with toxin I, or MCD peptide, were examined for a time-dependent decrease in the number of ^{125}I-β-Butx binding sites. Preincubation with MCD peptide or toxin I, for up to 5 h at room temperature or 37 °C, did not alter the

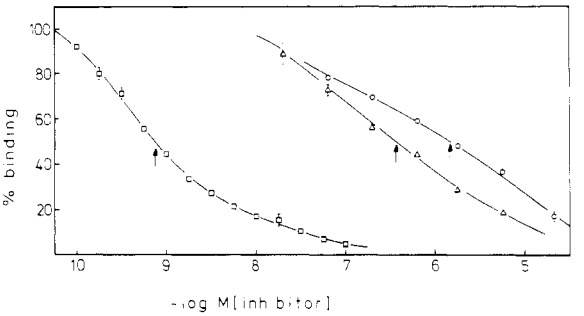

FIGURE 1: Inhibition of ^{125}I-β-Butx binding to brain membranes by MCD peptide and toxin I. The preparation of membranes and the binding of ^{125}I-β-Butx (final concentration 0.18 nM) were performed as detailed under Experimental Procedures. The inhibition of ^{125}I-β-Butx binding to chick brain membranes by toxin I (□) and MCD peptide (△) and to rat brain membranes by MCD peptide (○) is shown. Control binding was 13 ± 0.2 fmol/mg of protein for chick and 22 ± 0.4 fmol/mg of protein for rat brain membranes, respectively. The IC_{50} values are marked by arrows.

Table I: Effect of Bee Venom Components and Cytochrome *c* on ^{125}I-β-Butx Binding to Brain Membranes[a]

peptide added	μg/assay (μM)	^{125}I-β-Butx bound (% of control) 23 °C	4 °C
MCD peptide	0.1 (0.19)	74 ± 2	76 ± 9
	1.0 (1.9)	39 ± 2	41 ± 8
phospholipase A_2	0.1 (0.032)	94 ± 3	85 ± 8
	1.0 (0.32)	19 ± 3	80 ± 8
apamin	3.3 (8.1)	106 ± 7	nd
cytochrome *c*	20.0 (8.3)	97 ± 9	nd

[a] Chick brain membranes were prepared, and the specific binding of ^{125}I-β-Butx (final concentration 0.18–0.25 nM) was determined as detailed under Experimental Procedures. The peptides tested were added 30 min prior to the addition of ^{125}I-β-Butx. The incubations were performed at 23 °C, or 4 °C, as indicated. The data are from different experiments with control binding ranging from 9 to 12 fmol/mg of protein. nd, not determined.

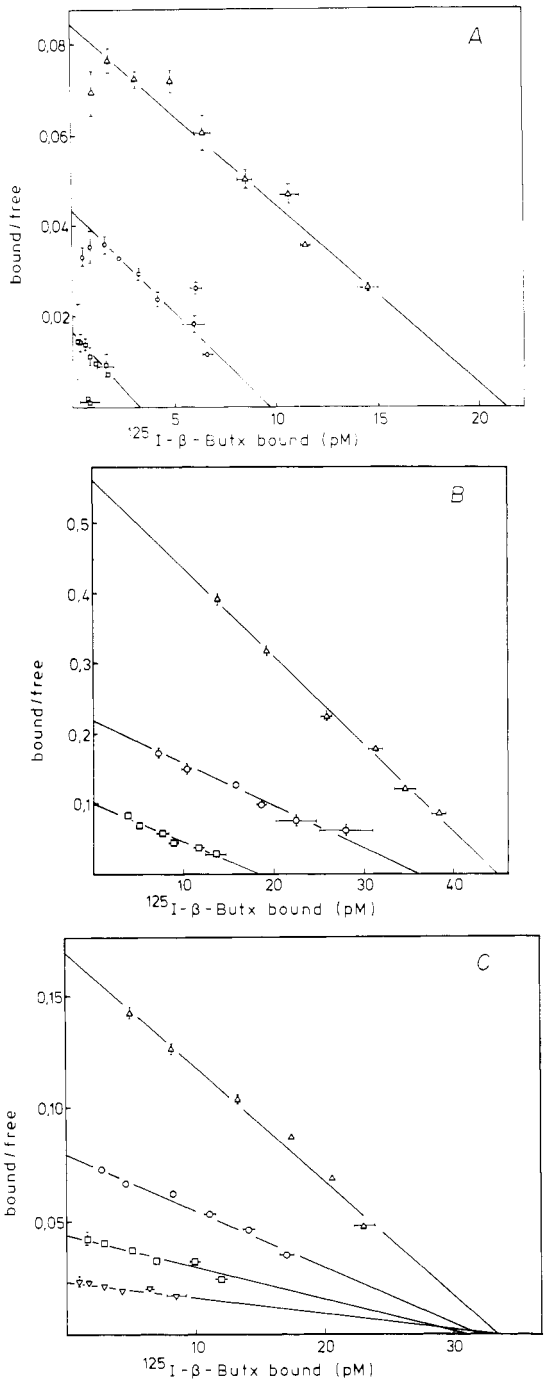

FIGURE 2: Scatchard analysis of ^{125}I-β-Butx binding to chick brain membranes in the absence and presence of MCD peptide, toxin I, and β-Butx. The preparation of membranes and the binding of ^{125}I-β-Butx were performed as detailed under Experimental Procedures. Binding data obtained at various concentrations of ^{125}I-β-Butx (final concentration 0.02–0.6 nM) were plotted according to Scatchard (1949). (A) Binding in the presence of MCD peptide [(○) 0.1 μM; (□) 1.0 μM]. (B) Binding in the presence of toxin I [(○) 0.45 nM; (□) 2.5 nM]. (C) Binding in the presence of unlabeled β-Butx [(○) 0.12 nM; (□) 0.24 nM; (▽) 0.49 nM]. Control binding values are indicated by (△).

degree of inhibition obtained (not shown). We therefore conclude that the inhibition of β-Butx binding by MCD peptide and toxin I is noncompetitive. As expected, native β-Butx in contrast gave a competitive inhibition pattern (Figure 2C).

Removal of Ca^{2+} also inhibits ^{125}I-β-BTX binding to chick brain membranes (Rehm & Betz, 1982). Here, the Ca^{2+} dependence of ^{125}I-β-Butx binding was determined for both chick and rat brain membranes (Figure 3). In both species, ^{125}I-β-Butx binding was Ca^{2+}-dependent, though to a different

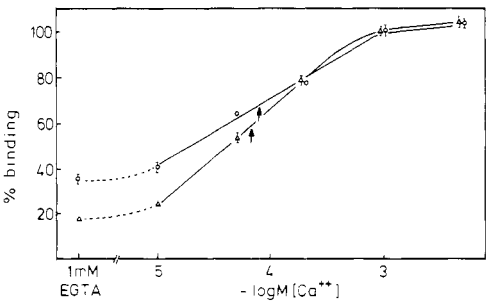

FIGURE 3: Effect of Ca^{2+} on ^{125}I-β-Butx binding to chick and rat brain membranes. The preparation of membranes and the determination of ^{125}I-β-Butx binding (final concentration 0.14 nM) was performed as detailed under Experimental Procedures. In this experiment, the membranes were washed once with buffer A containing 6 mM EDTA and twice with buffer A containing no Ca^{2+} but 10 μM EGTA. Control binding at 1 mM Ca^{2+} was 13 ± 0.2 fmol/mg of protein for chick (△) and 24 ± 0.5 fmol/mg of protein for rat (○) brain membranes. Arrows indicate the concentration of Ca^{2+} required for half-maximal stimulation of ^{125}I-β-Butx binding.

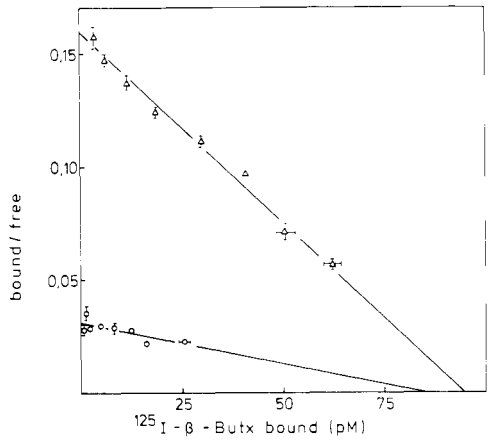

FIGURE 4: Scatchard analysis of ^{125}I-β-Butx binding to rat brain membranes in the presence of Ca^{2+} and EGTA. The binding of ^{125}I-β-Butx (final concentration 0.04–2.0 nM) in the presence of 1 mM Ca^{2+} (△) or 1 mM EGTA (○) was determined as described under Experimental Procedures. The membranes were washed as detailed in the legend to Figure 3.

extent. Maximal inhibition obtained with EGTA-containing buffer was 82% in chick and 65% in rat. However, the amount of Ca^{2+} needed to relieve half of this inhibition was nearly identical for both species: 70 μM and 80 μM Ca^{2+} for membranes from chick and rat, respectively.

Scatchard analysis of ^{125}I-β-Butx binding to rat brain membranes in the presence of Ca^{2+}, or EGTA, showed that the inhibition of ^{125}I-β-Butx binding by EGTA was due to a 5-fold reduction in apparent toxin affinity (Figure 4).

The classical inhibitors of K^+ channels [for review, see Hille (1984)], tetraethylammonium (25 mM) and 4-aminopyridine (5 mM), had no effect on ^{125}I-β-Butx binding (data not shown).

DISCUSSION

This paper establishes MCD peptide as an additional ligand of the β-Butx binding protein, a putative neuronal K^+ channel. The inhibition of ^{125}I-β-Butx binding by MCD peptide is noncompetitive. Therefore, the binding sites for MCD peptide and β-Butx on the β-Butx binding protein are probably not identical. Similar results were obtained for the inhibition of ^{125}I-β-Butx binding by toxin I. This toxin exhibits a high degree of sequence homology to the B subunit of β-Butx (Harvey & Karlsson, 1982). This was previously taken as evidence that β-Butx binds via its B subunit (Othman et al.,

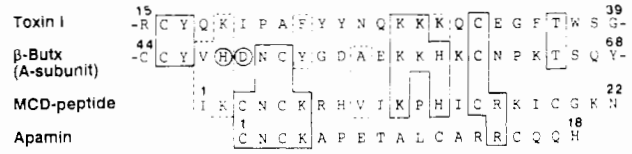

FIGURE 5: Amino acid sequence comparison of β-Butx A subunit, toxin I, MCD peptide, and apamin. Partial amino acid sequences of β-Butx (A-1 chain; Kondo et al., 1982b) and toxin I (Strydom, 1973) are aligned with the sequences of MCD peptide (Gauldie et al., 1978) and apamin (von Haux et al., 1967). The amino acid residues His-48 and Asp-49 located in the phospholipase A_2 active site of β-Butx (Kondo et al., 1978b; Ikeda & Hayashi, 1983) are indicated by circles. Blocks drawn around common residues indicate invariant amino acids. Isofunctional residues (Dayhoff et al., 1978) are marked by dotted lines. The IUPAC one-letter notation for amino acids is used.

1982). However, arguments were also raised against this interpretation (Rehm & Betz, 1982; Rehm, 1984). Interestingly, MCD peptide has some sequence homology with the A subunit of β-Butx in a region which is considered to be important for the neurotoxicity of this snake toxin [Figure 5 and Kondo et al. (1982a,b)]. Apamin, a blocker of Ca^{2+}-activated K^+ channels, does not inhibit ^{125}I-β-Butx binding and has little homology to the A subunit despite being identical with MCD peptide at other amino acid positions. Toxin I also has some sequence homology to the A subunit of β-Butx and to MCD peptide (Figure 5). We therefore speculate that the K^+ channel sensitive to β-Butx has at least two interacting binding sites of related structure.

The binding of β-Butx to brain membranes is stimulated by Ca^{2+}. The Ca^{2+} concentrations required for half-maximal stimulation (70–80 μM) are similar for chick and rat. Species differences were, however, observed for both residual binding in the presence of EGTA and the apparent K_i of MCD peptide. Ca^{2+} is known to bind to β-Butx with a K_D of 100–200 μM (Abe et al., 1977; Ikeda & Hayashi, 1983). This binding induces a conformational change of the toxin (Abe et al., 1977). The stimulation of ^{125}I-β-Butx binding by Ca^{2+} thus may reflect changes of the toxin rather than of the binding protein. We suggest that Ca^{2+} induces a state of higher binding affinity of β-Butx. Previously published data support this hypothesis. In a study using tritiated β-Butx, Othman et al. (1982) could not detect a Ca^{2+} dependence of toxin binding to rat brain membranes. This toxin preparation had no detectable phospholipase A_2 activity. As Ca^{2+} is essential for the phospholipase activity of β-Butx (Abe et al., 1977), the tritiated toxin probably lost its capacity to bind Ca^{2+}. Binding of [^{3}H]-β-Butx would then reflect only Ca^{2+}-independent binding. Interestingly, the neurotoxicity of the tritiated β-Butx preparations used by Othman et al. (1982) was found to be 5 times lower than that of native toxin. In our hands, removal of Ca^{2+} reduced the apparent affinity of ^{125}I-β-Butx for rat brain membranes by a factor of 5. These data are consistent with the idea that β-Butx binds with high affinity only when complexed with Ca^{2+} ions.

The data presented in this paper provide a basis for the future biochemical analysis of the β-Butx binding protein. β-Butx, MCD peptide, and toxin I all qualify as potential ligands for the affinity purification of this neuronal K^+ channel protein. By use of a β-Butx matrix, selective elution from the affinity column may be achieved by Ca^{2+} removal. Initial experiments (R. R. Schmidt, unpublished results) indicate that this approach may be indeed successful.

ADDED IN PROOF

Bidard et al. (1987) recently reported an interaction of MCD peptide and dendrotoxin binding sites in rat brain. Their data complement the findings presented in this paper.

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Clotting of Bovine Fibrinogen. Calcium Binding to Fibrin during Clotting and Its Dependence on Release of Fibrinopeptide B†

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ABSTRACT: Polymerization of bovine fibrinogen acted upon by thrombin is accompanied by binding of Ca^{2+} and a concomitant decrease of the free Ca^{2+} concentration. The latter can be recorded by a Ca^{2+}-selective electrode as a shift in the electrode potential. The shift shows marked dependence on the initial free Ca^{2+} concentration, being maximal at about $10^{-4.1}$ M and decreasing sharply on either side of this. Thus, the effect is limited to the 10^{-3}–10^{-5} M free Ca^{2+} concentration range. From the initial and the final value of the electrode potential during a clotting experiment, the amount of Ca^{2+} bound to fibrinogen and fibrin, respectively, can be calculated. The difference between the two, plotted against free Ca^{2+} concentration, gives a bell-shaped curve. This indicates that the reason for the Ca^{2+} binding is a shift of the pK of some groups from a lower to a higher value. The recordings can be used for evaluation of the kinetics of the Ca^{2+} uptake. However, they have to be corrected for the effect of the continuous shift in the free Ca^{2+} concentration during the experiment. The reaction does not follow simple kinetics, showing a lag period. Therefore, rates were estimated from inverse half-reaction times. Half-times of the corrected curves show that the reaction is first order with respect to thrombin. Moreover, the rate of Ca^{2+} uptake is identical with that of the conformational change seen in differential scanning calorimetry [Donovan, J. W., & Mihalyi, E. (1985) *Biochemistry 24*, 3434]. The inverse rate and the final corrected Ca^{2+} uptake increase linearly with the initial fibrinogen concentration. Concomitant estimates of fibrinopeptide A and B release showed that the Ca^{2+} uptake runs parallel to the release of fibrinopeptide B. Fibrinopeptide A was released largely during the lag period of the Ca^{2+} uptake. In agreement with this, clotting with Ancrod, an enzyme that liberates only fibrinopeptide A, was not accompanied by binding of Ca^{2+}. Thus, polymerization is not sufficient for the Ca^{2+} uptake to occur; liberation of fibrinopeptide B seems to be obligatory. Further support for this was obtained with experiments with the polymerization inhibitor Gly-Pro-Arg-Pro. The tetrapeptide inhibits polymerization and also, proportional to this, release of fibrinopeptide B [Hurlet-Jensen, A., Cummins, H. Z., Nossel, H. L., & Liu, C. Y. (1982) *Thromb. Res. 27*, 419; Lewis, S. D., Shields, P. P., & Shafer, J. A. (1985) *J. Biol. Chem. 260*, 10192]. Calcium uptake was also depressed by the tetrapeptide in a way similar to its effect upon firbinopeptide B release.

The fibrinogen molecule undergoes a conformation change during polymerization manifested by an increase of the enthalpy of denaturation of the D domain and a shift of the transition curve of this domain to higher temperature (Donovan & Mihalyi, 1985). These two changes are affected in different ways by the presence of calcium ions: a large increase of the enthalpy of unfolding occurs in the absence of calcium ions, and this increases only moderately further by increasing concentrations of calcium ions. On the other hand, the transition temperature is not affected by polymerization without calcium, while increasing concentrations of the latter elicit an increasing shift that eventually levels off.

Estimates of the shift as a function of free calcium concentration were used to calculate the pK value of the calcium binding sites governing this reaction. This can be compared with the values determined by equilibrium dialysis for the binding constants of calcium to bovine (Marguerie et al., 1977) and to human fibrinogen (Nieuwenhuizen et al., 1981). For human fibrinogen, the pK from the titration curve coincided with that of the high-affinity binding sites, whereas with bovine fibrinogen it was more than 1 unit below this. The data suggest that the sites governing the shift of the transition temperature may be different from the high-affinity calcium binding sites. They may be new sites created by the polymerization, or sites shifted from lower to higher affinity. In either case, it is expected that polymerization will be associated with additional binding of calcium and a corresponding decrease in free calcium concentration. This paper describes studies with the calcium electrode to test this hypothesis.

Experimental Procedures

Materials. Bovine fibrinogen, Pentex (lot 28, 95% clottable), was obtained from Miles Laboratories, Inc., Research Products Division, Naperville, IL. Calcium-free fibrinogen was prepared as described (Donovan & Mihalyi, 1985). An

† This is paper 3 in the series "Clotting of Fibrinogen". For paper 2, see Mihalyi and Donovan (1985).