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Ca^{2+}-dependent high-affinity complex formation between calmodulin and melittin

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The amphiphatic polypeptide melittin migrates as an equimolar complex with bovine brain calmodulin when monitored by gel disc electrophoresis or gel filtration in the presence of Ca^{2+}, even in 4 M-urea. The complex disppsociates in the presence of EDTA and urea. The affinity is of the same order as that of calmodulin for its target enzymes, and more than 1000-fold higher than that of calmodulin for basic peptide hormones or hydrophobic drugs. The activation of brain phosphodiesterase by calmodulin is inhibited by melittin. The kinetics of inhibition suggest competition between the enzyme and melittin for calmodulin. The calmodulin–melittin interaction may constitute a model for that existing between calmodulin and its target enzymes.

Complex-formation between calmodulin and different target enzymes requires the binding of 3 Ca^{2+} by calmodulin (Cox *et al.*, 1981*b*, 1982; Malnoë *et al.*, 1982), displays an affinity of approx. $10^9\ M^{-1}$ and involves both hydrophobic and ionic interactions (Blumenthal & Stull, 1982). Since nothing is known about the recognition sequence in the target enzymes that bind calmodulin, it is of interest to study the interaction of calmodulin with drugs and polypeptides of known chemical structure. Calmodulin binds in a Ca^{2+}-dependent manner to small basic peptides of known primary structure, such as the hormones corticotropin, glucagon, β-endorphin and Substance P (Malencik & Anderson, 1982; Sellinger-Barnette & Weiss, 1982). However, the affinity is not very high ($K_d > 1\ \mu M$), indicating that the interaction between calmodulin and these polypeptides does not constitute the best model for the interaction existing between calmodulin and its target enzymes. Calmodulin also binds in a Ca^{2+}-dependent way to myelin basic protein and histone H2B (Grand & Perry, 1980). However, the affinity is at least 60-fold lower than that of calmodulin for the target enzymes (Malencik *et al.*, 1982). Furthermore, the latter two polypeptides are not good model compounds because of their high molecular weights (above 15000).

Here we report that calmodulin forms a high-affinity complex with melittin, the major peptide of the venom of the common honey bee. Melittin is a small basic peptide (26 residues; mol.wt. 2840). It assumes an amphiphilic α-helical conformation upon interaction with hydrophobic surfaces, such as membranes and detergents, or upon aggregation into a tetramer (Terwilliger & Eisenberg, 1982). Although it was first recognized as a potent haemolytic agent, more-recent reports have emphasized its action on various membrane-bound enzymic systems (Lad & Shier, 1980; Boone & Skalka, 1980; Bishop & Kenrick, 1980; Vesely, 1981). The formation of a complex between calmodulin and melittin was first suggested to us by the work of Katoh *et al.* (1982), who claimed that melittin inhibits myosin light-chain kinase by interacting with a site on the enzyme close to the calmodulin-binding site. Their data, however, did not exclude an interaction of melittin with the biologically active site on calmodulin itself.

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Materials and methods

Bovine brain calmodulin and calmodulin-deficient phosphodiesterase were prepared by the method of Watterson *et al.* (1976), and melittin and [^{3}H]-monoacetylmelittin as described by Maulet *et al.* (1980, 1982). Rabbit skeletal-muscle troponin C was prepared as previously described (Cox *et al.*, 1981*a*); the sarcoplasmic-reticulum Ca^{2+} binding proteins of vertebrates and invertebrates were prepared as described recently by Wnuk *et al.* (1982). Soya-bean trypsin inhibitor and bovine β-lactoglobulin were purchased from Sigma, St. Louis, MO, U.S.A.

Gel disc electrophoresis was performed on 10 or 15% polyacrylamide-gel columns in the presence or absence of 4 M-urea as described by Head & Perry (1974), or in the presence of 0.1% sodium dodecyl sulphate, as described by Laemmli (1970). Phospho-

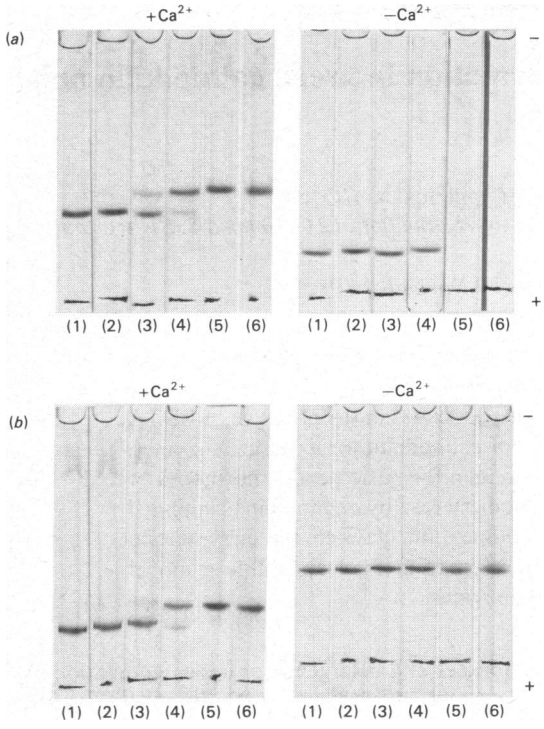

Fig. 1. *Complex-formation between calmodulin and melittin monitored by 10%-polyacrylamide-gel disc electrophoresis in the absence (a) and presence (b) of 4 M-urea and in the presence of 1 mM-$CaCl_2$ ($+Ca^{2+}$) or 1 mM-EDTA ($-Ca^{2+}$)*

Free melittin does not migrate in this electrophoretic system. All samples contain 0.18 nmol of caldmodulin. (1) Calmodulin alone. The other gels contain melittin in the following amounts: (2), 0.03 nmol; (3), 0.08 nmol; (4), 0.1622 nmol; (5), 1.622 nmol; (6), 8 nmol.

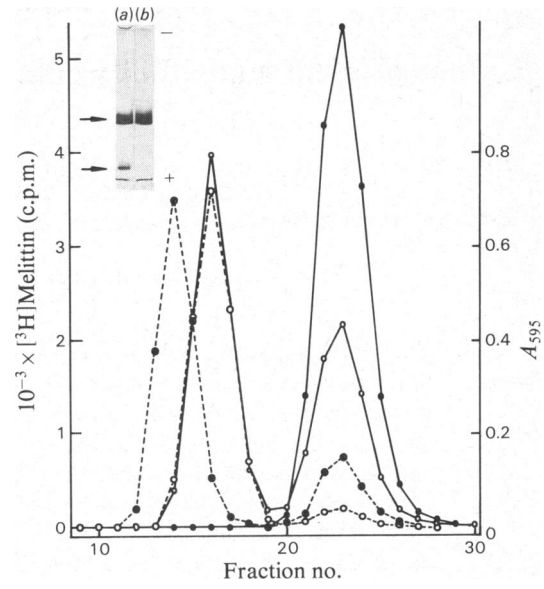

Fig. 2. *Complex formation between calmodulin and melittin monitored by gel filtration at room temperature on a column (60 cm × 0.9 cm) of Sephadex G-75 equilibrated in 20 mM-imidazole/HCl, pH 7.0, 0.1 M-NaCl, 4 M-urea in the presence of 1 mM-$CaCl_2$ (○) or 1 mM-EDTA (●)*

The column was loaded with a 1 ml solution containing 60 μM-calmodulin, 90 μM-mono-acetylmelittin and 0.7 μM-[^{3}H]mono-acetylmellitin. Fractions were of 1.25 ml. Total protein content (A_{585}, ----) was measured by the method of Bradford (1976). [^{3}H]Acetylmelittin (——) was monitored by liquid-scintillation counting. The inset shows analysis of the calmodulin-containing fractions by 15% polyacrylamide-gel disc electrophoresis in the presence of 0.1% sodium dodecyl sulphate. (*a*) Fraction 16 of the elution profile in the presence of Ca^{2+}; (*b*) fraction 14 of the profile in the absence of Ca^{2+}. The upper and lower arrows represent the positions of pure calmodulin and melittin respectively.

diesterase activity was assayed at 30°C by the method of Boudreau & Drummond (1975).

Results and discussion

When the ratio of melittin to calmodulin was increased, electrophoresis in the presence of Ca^{2+} revealed the appearance of a new protein band with a mobility slightly slower than that of calmodulin (Fig. 1*a*). This band reaches maximal intensity at equimolar concentrations of both proteins, suggesting the formation of a melittin$_1$–calmodulin$_1$ complex. No other complexes of different stoichiometry could be observed. In the presence of EDTA, no complex is formed except at 10-fold or higher excess amounts of melittin over calmodulin: the calmodulin band disappers completely without appearance of a distinct new protein band on the gels (Fig. 1*a*), suggesting that the complex or protein aggregate in the presence of EDTA does not migrate in our gel system, similarly to melittin. It should be noted that the latter type of complex or aggregate is not formed in the presence of Ca^{2+}, even at high melittin/calmodulin ratios. Fig. 1(*b*) shows that in the presence of Ca^{2+} the calmodulin$_1$–melittin$_1$ complex is stable even in 4 M-urea, whereas the interaction between the two proteins in the absence of Ca^{2+} is abolished by urea.

The Ca^{2+}-dependent complex formation was also monitored by gel filtration in the presence of 4 M-urea (Fig. 2). Radiolabelled melittin alone or in the presence of calmodulin and EDTA elutes as a single peak with maximum at fraction 23, whereas it co-elutes with calmodulin, provided Ca^{2+} is present. Sodium dodecyl sulphate/polyacylamide-gel disc electrophoresis on fraction 16 (elution profile in the

presence of Ca^{2+}) confirmed the existence of a calmodulin–melittin complex (Fig. 2, inset, *a*). The electrophoresis pattern of the calmodulin-containing fraction in the elution profile in the absence of Ca^{2+} (fraction no. 14) revealed only the presence of the latter protein without traces of melittin (Fig. 2; inset, *b*).

The stoichiometry of the calmodulin–melittin complex in the presence of Ca^{2+} was determined after gel-filtration experiments similar to those of Fig. 2. For this purpose, 5 mg of calmodulin and a 2.6-fold molar excess of melittin over calmodulin were loaded on the Sephadex G-75 column. In the complex-containing fractions, the concentrations of calmodulin (two tyrosine residues, no tryptophan residues) and melittin (no tyrosine residues, one tryptophan residue) were measured by the spectrophotometric method for the determination of tyrosine and tryptophan of Edelhoch (1967). In the absence of urea, calmodulin binds 0.94 mol of melittin per mol of protein, whereas a value of 0.86 is obtained in the presence of 4 M-urea. These data confirm that the complex has the composition calmodulin$_1$–melittin$_1$.

The affinity of the calmodulin–melittin complex was determined as follows: 2.36 nM-calmodulin was pre-incubated at room temperature for 3 h with various amounts of [^{3}H]mono-acetylmelittin in 20 mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid) buffer, pH 7.0, containing 0.1 M-NaCl and 1 mM-$CaCl_2$, and loaded on a column containing 1 ml of carboxymethyl-Sephadex equilibrated in the same buffer. The resin retains free melittin but not the complex with calmodulin, which was collected in a Lubrol PX solution to a final concentration of 0.01% and quantified by liquid-scintillation counting. A control experiment indicated that no appreciable dissociation occurred during the time of separation on the resin (10 min). The Scatchard plot of the experimental data (not shown) yields a straight line intersecting the abscissa at a value of 0.93 mol of melittin bound/mol of calmodulin, and a dissociation constant of 3.0 mM.

Nanomolar concentrations of melittin completely neutralize the activation of bovine brain phosphodiesterase by calmodulin; this inhibition can be overcome by adding excess amounts of calmodulin (Fig. 3). A control experiment indicated that melittin does not affect the enzymic activity of phosphodiesterase in the absence of calmodulin. These results suggest that melittin competes with the enzyme for the biologically active site of calmodulin. From this experiment the affinity of calmodulin for melittin can be estimated, based on the two following principles (Cox *et al.*, 1981*b*): (1) the concentration of the enzyme in the assay is negligible compared with that of calmodulin; (2) at a given activation of phosphodiesterase, the concentration of free calmodulin–$Ca^{2+}{}_3$ is constant, irrespective of the presence of melittin. Hence, from a standard curve in the absence of melittin, it is possible to calculate the concentration of free calmodulin in samples where melittin is present. Subsequently, the concentrations of calmodulin–melittin complex are calculated as the difference between total and free calmodulin. The Scatchard plot (Fig. 3, inset) of the data thus obtained shows a straight line intersecting the abscissa at a value of 1.0 mol of calmodulin bound/mol of melittin, and a dissociation constant of 5.9 mM.Taken together these values are similar to those obtained in the direct binding assay.

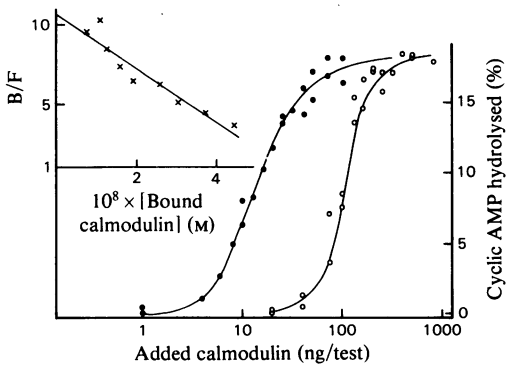

Fig. 3. *Inhibition by melittin of the activation of bovine brain phosphodiesterase by calmodulin*

The phosphodiesterase activity (expressed as percentage cyclic AMP hydrolysed) was measured as a function of the concentration of calmodulin in the presence (○) or absence (●) of melittin (60 nM). Calmodulin, melittin and phosphodiesterase were pre-incubated in the presence of $CaCl_2$ at room temperature for 20 min; the reaction was started by the addition of final (in 300 μl) 20 mM-Tris/HCl, pH 7.5, 3 mM-$MgCl_2$, 50 μM-cyclic AMP, 2.5 μCi of [^{3}H]cyclic AMP/ml, 0.3 mM-dithiothreitol, 0.1 mg of bovine serum albumin/ml and 200 μM-$CaCl_2$. The inset represents the Scatchard plot of the binding of calmodulin to melittin as determined with the enzyme assay. B/F is the ratio of bound calmodulin to free calmodulin.

The specificity of Ca^{2+}-dependent complex-formation between melittin and different intracellular Ca^{2+}-binding proteins belonging to the calmodulin family was studied by gel disc electrophoresis (Fig. 4). No interaction was observed between melittin and the sarcoplasmic-reticulum Ca^{2+}-binding proteins of fish, annelids and crustacea, nor with the acidic proteins soya-bean trypsin inhibitor and β-lactoglobulin. A discrete complex band was observed in the case of tropinin C. This interaction was fully Ca^{2+}-dependent and yielded, as in the case of calmodulin, a troponin C$_1$–melittin$_1$ complex. In

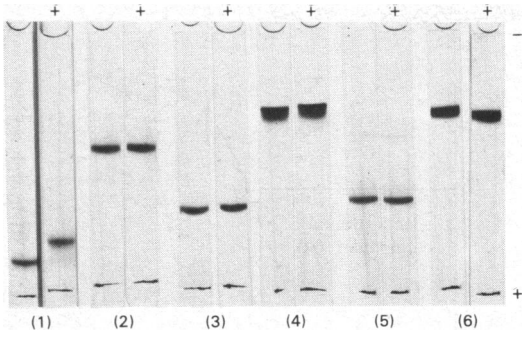

Fig. 4. *Screening of different acidic proteins for their ability to form complexes with mellitin monitored by polyacrylamide (10%)-gel disc electrophoresis in the presence of 4 M-urea and 1 mM-Ca^{2+}*

+ above a gel indicates the presence of 7.5 μg of melittin per gel. All gels contain 3–10 μg of the following acidic proteins: (1), skeletal-muscle troponin C (pI = 4.0); (2), perch parvalbumin (pI = 4.1); (3), sandworm sarcoplasmic-reticulum Ca^{2+}-binding protein (pI = 5.0); (5), soya-bean trypsin inhibitor (pI = 4.5); (6), bovine milk β-lactoglobulin (pI = 5.1).

this respect, it should be noted that in certain cases troponin C functionally replaces calmodulin (Marcum *et al.*, 1978; Cohen, 1980), and both proteins, in contrast with parvalbumin and other sarcoplasmic-reticulum Ca^{2+}-binding proteins, interact with phenothiazines (Marshak *et al.*, 1981).

Head *et al.* (1977) first described the formation of a urea-stable complex between calmodulin and skeletal troponin I. A similar finding was later reported by Grand & Perry (1980) for myelin basic protein and histone H2B. Their affinity for calmodulin, however, appears to be one to two orders of magnitude lower (LaPorte *et al.*, 1981; Malencik *et al.*, 1982) than that of melittin for calmodulin. Furthermore, these proteins are large and their tridimensional structure is not known. This is the first report of a direct high-affinity interaction of calmodulin with a small polypeptide, the three-dimensional structure of which is known (Terwilliger & Eisenberg, 1982) and the conformational states in solution extensively studied (Brown *et al.*, 1980). Since melittin and the target enzymes bind to the same biologically active site of calmodulin, as suggested by Fig. 3, then the different calmodulin-target enzymes probably have a calmodulin-binding site that is structurally very similar to melittin and the interaction calmodulin–mellitin may constitute a model for that existing between calmodulin and its target enzymes. Furthermore this interaction may provide information on the amphiphatic character of melittin.

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