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EFFECTS OF CALMODULIN AND RELATED PROTEINS ON THE HEMOLYTIC ACTIVITY OF MELITTIN

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SUMMARY: The calcium-dependent binding of melittin by calmodulin effectively inhibits the hemolytic activity of melittin in suspensions of washed rabbit erythrocytes. Protection is also obtained with troponin C ($\pm Ca^{++}$), denatured phosphorylase kinase, and denatured calcineurin but not with whole troponin or the native enzymes. These effects can be used both in assays for melittin in venom samples and in determinations of calmodulin or related proteins.

A number of small peptides which range upwards in size from 9 or 10 amino acid residues and contain certain common structural features--clusters of two or more strongly basic amino acid residues, associated hydrophobic sequences, a low incidence of glutamyl residues, and a propensity for either an α-helical or "random coil" conformation--undergo efficient calcium-dependent binding by calmodulin (cf review by Anderson and Malencik (1)). Several cytoactive peptides from insect venoms--melittin (3,4), the mastoparans (2,4,5), and crabrolin (Malencik and Anderson, in preparation)--display affinities for calmodulin which rival those of calmodulin-dependent enzymes. The dissociation constants for their complexes with calmodulin are generally in the nanomolar range or less when calcium is present. Although none of these particular peptides probably associates with calmodulin in vivo, they are interesting and important both as models for binding and as potentially useful calmodulin antagonists (1).

The tenacity of binding and strong calcium dependence obtained with the venom peptides have several additional implications. For example, they suggest that calmodulin could be useful in assays for the peptides and that affinity

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chromatography procedures based on calmodulin binding could expedite peptide purification. To speculate further, calmodulin may even become a valuable therapeutic agent for the treatment of venomous insect bites. This paper describes the effects of calmodulin and related proteins--troponin C, the regulatory myosin light chains, and parvalbumin--on the melittin-induced hemolysis of rabbit erythrocytes. Melittin, a 26-residue polypeptide, and phospholipase A_2 are the principal toxic components of honey bee venom--accounting for ~50% and ~12%, respectively, of its dry weight (6).

MATERIALS AND METHODS

Proteins were prepared using published procedures: porcine brain calmodulin (7); rabbit muscle troponin & troponin C (8); dogfish parvalbumin (9); smooth muscle myosin light chain, modification of (10); turkey gizzard myosin light chain kinase (11); rabbit muscle phosphorylase kinase (12); and bovine brain calcineurin (13). The concentrations of calmodulin and myosin light chain kinase were matched in stoichiometric fluorescence titrations (14). Synthetic melittin was obtained from Peninsula Laboratories. Lyophilized honey bee venom and a fraction containing 70% by weight melittin were purchased from Sigma Chemical Co. Titrations of the latter sample with calmodulin are consistent with the stated melittin content (1, also this work). $NaDodSO_4$ electrophoresis showed that the lyophilized venom sample contained 14% by weight of phospholipase A_2 and the 70% melittin fraction, 4.6% of phospholipase (the remaining 25% is mostly non-protein). Treatment of the crude venom with phenylmethanesulfonyl fluoride had no effect on the properties studied here. Honey bee venom apparently contains no proteases (15).

Rabbit erythrocytes were separated from citrated whole blood and washed according to standard procedures. They were stored at 0° in a buffer containing 155 mM NaCl, 2.2% D(+) glucose, and 10 mM sodium phosphate, pH 7.2 (16). Most experiments were completed within 48 hours after the blood was drawn. Hemolysis experiments were performed in microfuge tubes containing 1.3 ml of 155 mM NaCl, 5 mM Mops, specified concentrations of melittin, and either 1 mM EDTA or 1 mM $CaCl_2$ (pH 7.2, 37°). The erythrocytes were always added last, usually to a final concentration of 7.7×10^6 cells/ml. Following mixing and 30 min of incubation, the lysates were centrifuged at 4500 rpm for 3 min in a Beckman microfuge 11. The amount of hemoglobin in the supernatant solutions was measured spectrophotometrically at 413 nm. Parallel experiments in which cells were lysed in distilled H_2O provided the standards corresponding to 100% hemolysis (17). In cases where proteins such as calmodulin were also present, several different orders of mixing were tested. The addition of melittin to cell suspensions already containing calmodulin--or the final addition of cells to solutions containing calmodulin and melittin--gives the same result. The latter procedure, was followed uniformly. Unless one of the protective proteins is present, appreciable hemolysis occurs as soon as the erythrocytes are mixed with melittin.

RESULTS AND DISCUSSION

Figure 1 presents the results of experiments to determine the threshold concentrations of melittin needed for hemolysis under our conditions which include the addition of either 1 mM $CaCl_2$ or 1 mM EDTA to the standard solutions. Two samples of melittin were examined, one being a homogeneous synthetic preparation and the other a partially purified fraction reported by the supplier to contain 70% melittin and up to 20 units/mg of phospholipase A_2. When 1 mM $CaCl_2$ is pre-

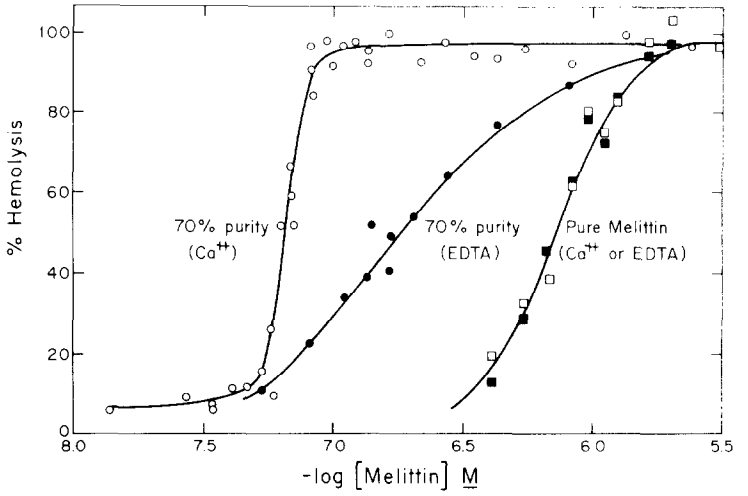

Fig. 1. Effect of melittin concentration on the hemolysis of rabbit erythrocytes. Results are shown for synthetic melittin in the presence of 1 mM $CaCl_2$ (□) and of 1 mM EDTA (■) and for a phospholipase A_2-containing fraction of melittin in the presence of calcium (○) and of EDTA (●). The cell suspensions (7.7 x 10^6 cells/ml) were incubated at 37° for 30 min. In addition to the above components, the medium contained 155 mM NaCl and 5 mM Mops, pH 7.2.

sent, the concentration of melittin required to produce 50% hemolysis ranges from 0.066 μM with the phospholipase-containing specimen to 0.72 μM with the synthetic peptide. The eleven fold greater sensitivity found in the first case is consistent with the well known synergism between melittin and honey bee phospholipase A_2 (18). Stimulation of phospholipase by calcium (6) also accounts for the different effects obtained with the two samples when hemolysis is performed in solutions containing 1 mM EDTA and no added calcium.

Since the dissociation constant for the calcium-dependent melittin-calmodulin complex is 3 nM (3) or less (1,2), the addition of calmodulin to the erythrocyte suspensions should completely inhibit hemolysis provided that its concentration equals or exceeds that of melittin and calcium is present. This prediction applies equally to the two melittin preparations. Figure 2 shows the hemolysis occurring in solutions which contain 3 μM pure melittin and varying proportions of calmodulin. Hemolysis obtained in the presence of calcium declines from ~90% to ~10% as the calmodulin concentration increases from 1.7 μM to 2.7 μM. This range is consistent with the threshold levels of pure melittin previously demonstrated. The slight hemolysis detected when [CaM] ≥ 3 μM is the

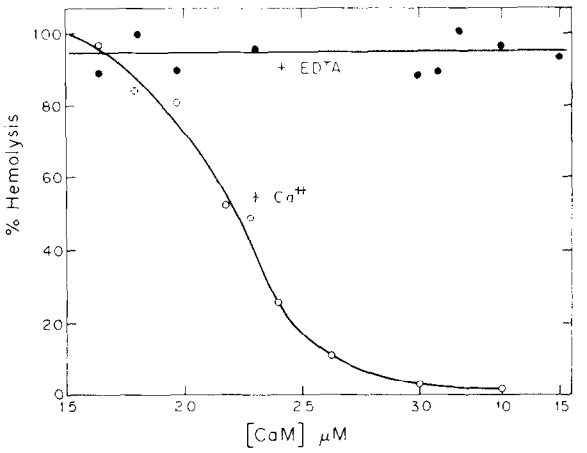

Fig. 2. Effect of calmodulin on the melittin-induced hemolysis of rabbit erythrocytes. Hemolysis was determined for samples containing 3.0 μM synthetic melittin and varying concentrations of calmodulin in the presence of 1 mM $CaCl_2$ (O) or 1 mM EDTA (●). Other conditions are given under Fig. 1.

same as that found in the absence of melittin. In the absence of calcium, even 15 μM solutions of calmodulin fail to reduce the free melittin concentration below threshold levels. This substantiates the strong calcium dependence of melittin binding since the hemolysis alone is calcium independent.

Hemolysis in the presence of a fixed concentration of calmodulin and 1 mM $CaCl_2$ yields precise upper limits for the quantity of melittin present in both raw bee venom and the phospholipase A_2-containing sample (Fig. 3). The range of melittin concentrations over which hemolysis is initiated in these cases is so narrow that intermediate results are seldom observed. Interpolation of the amount of lyophilized sample needed for 50% hemolysis in the presence of 1.8 μM calmodulin gives values of 7.3 μg/ml for the partially purified sample (consistent with the composition of 70% by weight melittin) and of 8.8 μg/ml for whole venom (corresponding to 58% melittin). The latter figure is within the reported range of melittin, 40%-60%, in honey bee venom samples (19). Whole venom has a fractional amount of hemolytic activity, 10%-20%, which is not effectively inhibited by calmodulin. The range of venom concentrations used produces essentially complete hemolysis in the absence of calmodulin. Calmodulin confers no protection in solutions containing 1 mM EDTA.

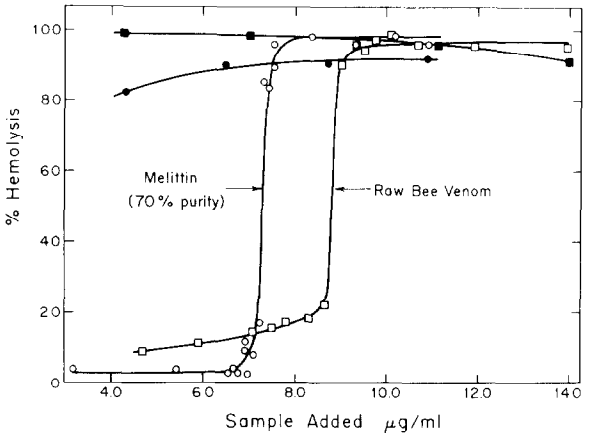

Fig. 3. Hemolysis obtained in the presence of a fixed concentration of calmodulin (1.8 μM) and varying amounts of raw bee venom or of the phospholipase A_2-containing melittin fraction. Measurements on the latter were performed in solutions containing either 1 mM $CaCl_2$ (○) or 1 mM EDTA (●). The venom sample was characterized in both the presence (□) and absence of calmodulin (■), with 1 mM $CaCl_2$ included in both cases. See Fig. 1 for other details.

The interactions of melittin with the erythrocyte and calmodulin are specific: the inclusion of 10 mg/ml human serum albumin in the assays has no effect on the hemolysis obtained in the presence or absence of calmodulin. Using fixed concentrations of both calmodulin (1.8 μM) and the phospholipase-containing melittin sample (1.6 μM melittin), we find complete protection occurring over a wide range of erythrocyte concentrations (7.7×10^6 to 5.8×10^8 cells/ml). This suggests that calmodulin could be effective in the treatment of honey bee and other insect stings. The availability, stability, and low antigenicity of calmodulin support this speculation.

Several proteins that are ancestrally related to calmodulin have distinctive effects on hemolysis. Experiments using both pure melittin and the phospholipase A_2-containing preparation show that isolated rabbit skeletal muscle troponin C reduces the free melittin concentration below the threshold levels for hemolysis--both in the presence and absence of calcium (Table I). The results obtained with varying concentrations of the partially purified melittin and a constant level of troponin C (2 μM) reveal sharp endpoints similar to those observed with calmodulin in the presence of calcium (Fig. 3). The melittin binding site of troponin C may be inaccessible in native troponin, which exerted

Table I. Effects of Calmodulin and Related Proteins on the Melittin-Induced Hemolysis of Rabbit Erythrocytes

Protein Added	Conc. μM	Melittin Source	[Melittin] μM	[Ca^{++}] mM	[EDTA] mM	% Hemolysis
CaM	3.0	A	3.0	1.0	0	3.0
CaM	3.0	A	3.0	0	1.0	94±5
CaM	1.8	B	1.73	1.0	0	6.7±3
CaM	1.8	B	1.73	0	1.0	90±3
TnC	3.0	A	3.0	1.0	0	6.3±1.4
TnC	3.0	A	3.0	0	1.0	6.9±1.4
TnC	2.0	B	1.34-1.8	1.0	0	7.5±2
TnC	2.0	B	1.34-1.8	0	1.0	10-15
Parvalbumin	50	A	3.0	1.0	0	88±3
Myosin LC	16	A	3.0	1.0	0	39±4
Troponin	2.6	B	0.8-2.2	1.0	0	91.5±4
Phos. Kinase	1.0	B	0.27	1.0	0	96±3
Boiled Kinase	1.0	B	0.27	1.0	0	5.3
Boiled Kinase	1.0	B	0.27	0	1.0	89
Calcineurin	1.5	B	0.54	1.0	0	96
Boiled Calcineurin	1.5	B	0.54	1.0	0	8
MLCK	1.82	B	0.90	1.0	0	100
MLCK-CaM	1.82	B	0.90	1.0	0	6.1-7.5

A=synthetic melittin. B=phospholipase A_2-containing melittin sample. See text for details.

no protective action in these trials. The 20,000 dalton smooth muscle myosin light chain forms a complex with melittin in the micromolar concentration range --reflected in the partial protection found with the synthetic peptide. Althougl this association is weak, it needs to be considered whenever melittin is applied as a calmodulin antagonist in activity measurements of myosin light chain kinase. Parvalbumin, believed to contain no specific protein recognition sites (20), has minimal effect on hemolysis.

The definite stoichiometry applying to the amount of calmodulin needed to inhibit the lytic activity of melittin indicates that this assay could be used to detect and quantitate the calmodulin present in protein complexes and possibly tissue samples. Phosphorylase kinase is an unusual calcium-dependent enzyme containing calmodulin as an integral part of its quaternary structure (21). Like troponin, phosphorylase kinase shows no high affinity interactions with melittin--even after 20 min. pre-incubation (Table I). However, boiling phosphorylase kinase for 10 min. in solutions containing 1 mM EDTA releases calmodulin with accompanying denaturation of the other subunits (22). The boiled fraction exhibits the protective effects characteristic of calmodulin. Calcineurin, the calcium-dependent phosphatase which contains an integral subu-

nit similar to calmodulin (23), also interacts well with melittin only after denaturation.

Most calmodulin-dependent enzymes associate reversibly with calmodulin in the presence of calcium, giving complexes with dissociation constants in the nanomolar range. The dissociation constant for the turkey gizzard myosin light chain kinase-calmodulin complex is ~2.0 nM in 0.20 N KCl, pH 7.3, 25° (14,24). In contrast to the results obtained with phosphorylase kinase, melittin loses its lytic activity in the presence of myosin kinase and calmodulin, even when [Melittin] < [CaM] < [Melittin] + [MLCK]. (The enzyme alone has no hemolytic activity.) The hemolysis obtained with the phospholipase A_2-containing melittin sample is independent of the order of mixing of the proteins and indicative of a free melittin concentration less than 0.066 μM. Calculation indicates that ([Melittin]/[CaM-Melittin])/([MLCK]/[CaM-MLCK]) < $\big((0.066)/(0.83)\big)/\big((0.83)/(0.99)\big)$ = 0.095. This value agrees with experiments showing that melittin dissociates the myosin kinase-calmodulin complex with a high degree of efficiency, suggesting a dissociation constant for the melittin-calmodulin complex which is an order of magnitude smaller than that for the enzyme. The calcium-dependent complex of melittin with calmodulin possesses both extraordinary stability and responsiveness (1,2). Most of the experiments described in this paper have also been performed with human erythrocytes. The results were essentially the same in both cases.

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