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Inhibition by melittin of phospholipid-sensitive and calmodulin-sensitive Ca^{2+}-dependent protein kinases

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Effects of melittin, an amphipathic polypeptide, on various species of protein kinases were investigated. It was found that melittin inhibited the newly identified phospholipid-sensitive Ca^{2+}-dependent protein kinase (from heart, brain, spleen and neutrophils) and the cardiac myosin light-chain kinase, a calmodulin-sensitive Ca^{2+}-dependent enzyme. In contrast, melittin had little or no effect on either the holoenzymes of the cardiac cyclic AMP-dependent and cyclic GMP-dependent protein kinases or the catalytic subunit of the former. Kinetic analysis indicated that melittin inhibited phospholipid-sensitive Ca^{2+}-dependent protein kinase non-competitively with respect to ATP ($K_i = 1.3\,\mu M$); although exhibiting complex kinetics, its inhibition of the enzyme was overcome by phosphatidylserine (a phospholipid cofactor), but not by protein substrate (histone H1) or Ca^{2+}. On the other hand, melittin inhibited myosin light-chain kinase non-competitively with respect to ATP ($K_i = 1.4\,\mu M$) or Ca^{2+} ($K_i = 1.9\,\mu M$), and competitively with respect to calmodulin ($K_i = 0.08\,\mu M$); although exhibiting complex kinetics, its inhibition of the enzyme was reversed by myosin light chains (substrate protein). The present findings indicate the presence of functionally important hydrophobic or hydrophilic loci on the Ca^{2+}-dependent protein kinases, but not on the cyclic nucleotide-dependent class of protein kinase, with which melittin can interact. Moreover, the kinetic data suggest that melittin inhibited myosin light-chain kinase by interacting with a site on the enzyme the same as, or proximal to, the calmodulin-binding site, thus interfering with the formation of active enzyme–calmodulin–Ca^{2+} complex.

Melittin, a lytic polypeptide of 26 amino acids, is the primary organic component of bee venom (for a review, see Habermann, 1972). Melittin exerts its effects, via the hydrophobic part of the molecule, by interacting with phospholipids in membranes or activating phospholipase A_2 (another major component of dry bee venom), or both, leading to altered properties of membranes and activities of membrane-associated enzymes (Repke & Portius, 1963; Sessa *et al.*, 1969; Vogt *et al.*, 1970; Habermann, 1972; Mollay & Kreil, 1973, 1974; Mollay *et al.*, 1976; Ladd & Shier, 1979; Gelehrter & Rozengurt, 1980; Georghiou *et al.*, 1981). The toxic manifestations of bee venom on many tissues, however, are most likely due to the combined actions of melittin and phospholipase A_2.

Myosin light-chain kinase is a calmodulin-sensitive Ca^{2+}-dependent protein kinase. The mechanism of activation of this enzyme, and of other enzymes (such as phosphodiesterase) also stimulated by calmodulin and Ca^{2+}, has been suggested to involve interactions of hydrophobic regions of the enzymes with a hydrophobic region of calmodulin, a Ca^{2+}-binding protein (Klee *et al.*, 1980; Tanaka & Hidaka, 1980; Weiss & Wallace, 1980). A new species of Ca^{2+}-dependent protein kinase that requires phospholipid (such as phosphatidylserine) instead of calmodulin as a cofactor has been reported (Takai *et al.*, 1979; Kuo *et al.*, 1980; Mori *et al.*, 1980; Wrenn *et al.*, 1980). Although the mechanism by which phospholipid activates this enzyme is unclear, it is likely that a functionally important hydro-

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phobic region may be present on the enzyme, with which the phospholipid cofactor can interact. It is possible that melittin may potentially regulate activities of myosin light-chain kinase and phospholipid-sensitive Ca^{2+}-dependent protein kinase. In the present paper we report the findings regarding inhibition by melittin of these two species of Ca^{2+}-dependent protein kinases, and possible inhibitory mechanism of the surface-active toxin.

Experimental

Materials

Melittin (from bee venom, containing up to 20 units of phospholipase A/mg of solid), phospholipase A_2 (from bee venom, 1500 units/mg of solid), lysine-rich histone (type III-S, histone H1), mixed histone (type II) and phosphatidylserine (from bovine brain) were from Sigma Chemical Co., St. Louis, MO, U.S.A. The pure melittin (free of phospholipase A_2 activity) was a gift from Dr. Günther Kreil, Institute of Molecular Biology, Austrian Academy of Sciences, Vienna, Austria.

Methods

Phospholipid-sensitive Ca^{2+}-dependent protein kinase was purified over 10000-fold (about 90% homogeneous) from the bovine heart extract by the steps of $(NH_4)_2SO_4$ fractionation and DEAE-cellulose, controlled-pore glass, Sephacryl S-200 and phosphatidylserine–Affigel 102 chromatographies (B. C. Wise, R. L. Raynor & J. F. Kuo, unpublished work). The enzyme was also purified about 300–400-fold (20–30% homogeneous) from extracts of the pig spleen (R. C. Schatzman & J. F. Kuo, unpublished work) or the rat brain (R. S. Turner & J. F. Kuo, unpublished work) by the steps of DEAE-cellulose and Sephacryl S-200 chromatographies. Myosin light-chain kinase was purified to homogeneity from the bovine heart extract by the procedure essentially the same as that described by Walsh *et al.* (1979). Homogeneous myosin light chains from the bovine heart were prepared by the method of Perrie & Perry (1970), with modifications by Katoh *et al.* (1973). Pure calmodulin was prepared from the pig brain extract by the fluphenazine affinity method of Charbonneau & Cormier (1979). Cyclic AMP-dependent protein kinase (Kuo *et al.*, 1970) and cyclic GMP-dependent protein kinase (Shoji *et al.*, 1977), both from the bovine heart extract, were partially purified as described elsewhere. The homogeneous catalytic subunit of bovine heart cyclic AMP-dependent protein kinase, prepared by the method of Bechtel *et al.* (1977), was a gift from Dr. David B. Glass, Department of Pharmacology, Emory University School of Medicine. [γ-^{32}P]ATP was prepared by the method of Post & Sen (1967), and protein was determined by the method of Lowry *et al.* (1951). Ca^{2+}-EGTA buffers were prepared by the method of Pires & Perry (1977).

Phospholipid-sensitive Ca^{2+}-dependent protein kinase was assayed essentially as described by Kuo *et al.* (1980). Briefly, the incubation mixture (0.2ml) contained 5μmol of Tris/HCl (pH7.5), 2μmol of $MgCl_2$, 40μg of histone H1, 5μg of phosphatidylserine, 0.05μmol of EGTA, with or without 0.1μmol of $CaCl_2$, and 0.001μmol of [γ-^{32}P]ATP, containing (0.9–1.2)$\times 10^6$c.p.m. Myosin light-chain kinase was assayed in the incubation mixture (0.2ml) that contained 5μmol of Tris/HCl (pH7.5), 2μmol of $MgCl_2$, 40μg of myosin light chains, 2μg (0.11nmol) of calmodulin, 0.01μmol of EGTA, with or without 0.02μmol of $CaCl_2$, and 0.1μmol of [γ-^{32}P]ATP containing about 1×10^6c.p.m. Cyclic AMP-dependent or cyclic GMP-dependent protein kinase, and the catalytic subunit of the former, was assayed as described previously (Kuo *et al.*, 1970; Shoji *et al.*, 1977), in the incubation mixtures (0.2ml) containing 40μg of mixed histone and 0.5μM cyclic nucleotides. Phosphorylated substrate proteins (histones or myosin light chains) were recovered by precipitating them from 5% trichloroacetic acid containing 0.25% tungstate.

Results

Melittin inhibited the cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase in a concentration-related manner, without appreciably affecting its basal activity seen in the absence of $CaCl_2$ (Fig. 1*a*). The inhibition curves obtained for the commercial and pure preparation of melittin were superimposable, with a 50% inhibition seen at about 4μM (containing 0.05 units of phospholipase A_2/0.2ml of incubation mixture using the commercial melittin) for both, indicating that the slight contamination by phospholipase A_2 in the commercial melittin preparation (up to 20 units/mg of solid, or 60 units/μmol) had no effect on the enzyme activity under the present assay conditions. We found that 1.3 units of phospholipase A_2 were required to lower the protein kinase activity by 60%, presumably by hydrolysing phosphatidylserine to a less effective phospholipid cofactor, lysophosphatidylserine (B. C. Wise, N. Katoh & J. F. Kuo, unpublished work). Melittin also inhibited cardiac myosin light-chain kinase without affecting its basal activity, with a 50% inhibition similarly seen at about 4μM (Fig. 1*b*); phospholipase A_2 (up to 10 units/0.2ml) was without effect on the enzyme (results not shown).

Phospholipid-sensitive Ca^{2+}-dependent protein kinase from different sources (bovine heart, rat brain, pig spleen and human neutrophils) and different states of purity, and the partially purified bovine heart myosin light-chain kinase, were all

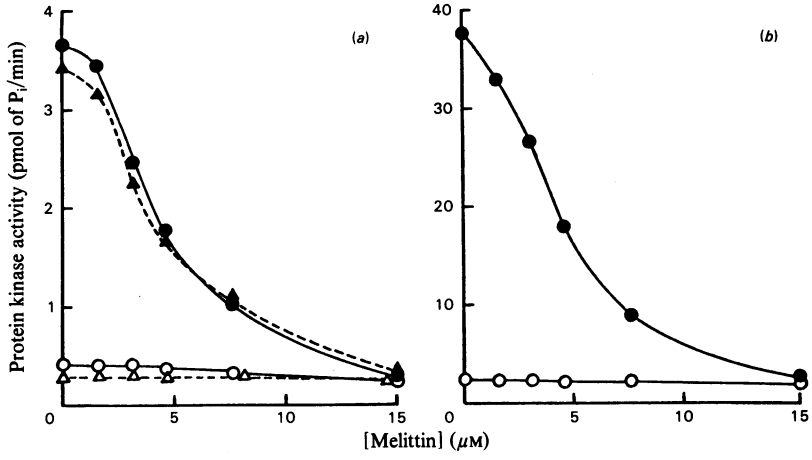

Fig. 1. *Inhibition of two species of Ca^{2+}-dependent protein kinases, both from the bovine heart, by two preparations of melittin*

The assay conditions for the enzymes were as described in the Experimental section, in the presence of various concentrations of melittin, as indicated. (*a*) Phospholipid-sensitive Ca^{2+}-dependent protein kinase (0.02 μg) was incubated in the presence of phosphatidylserine (5 μg/0.2 ml), with (●, ▲) or without (○, △) $CaCl_2$ (0.1 mM), using a commercial (——) or pure (– – – –) preparation of melittin. (*b*) Myosin light-chain kinase (1.4 μg) was incubated in the presence of calmodulin (2 μg/0.2 ml, or 0.56 μM), with (●) or without (○) $CaCl_2$ (0.1 mM), using a commercial melittin. The commercial preparation of melittin (Sigma Chemical Co.) was employed in all subsequent experiments reported in the present paper.

Table 1. *Comparative effects of mellitin on various Ca^{2+}-dependent and cyclic nucleotide-dependent protein kinases*

Various kinds and amounts of protein kinases from different sources were incubated in the presence or absence of melittin (15 μM), $CaCl_2$ (500 μM for phospholipid-sensitive Ca^{2+}-dependent enzyme and 100 μM for myosin light-chain kinase), cyclic AMP (0.5 μM) or cyclic GMP (0.5 μM), as indicated. Other assay conditions were as indicated in the legend to Fig. 1 and the Experimental section.

Kind, source and amount of enzymes	Addition	Enzyme activity (pmol of P_i/min) Control	+Melittin
Phospholipid-sensitive Ca^{2+}-dependent protein kinase			
Bovine heart (0.5 μg)	None	0.4	0.3
	Ca^{2+}	8.4	0.3
Rat brain (1.4 μg)	None	0.9	0.6
	Ca^{2+}	8.8	0.6
Pig spleen (1.1 μg)	None	0.6	0.4
	Ca^{2+}	2.7	0.5
Human neutrophils (15.6 μg)*	None	0.4	0.4
	Ca^{2+}	9.3	0.4
Myosin light-chain kinase from bovine heart (12 μg)	None	2.1	1.9
	Ca^{2+}	38.0	2.0
Cyclic AMP-dependent protein kinase from bovine heart (30 μg)	None	0.5	0.5
	Cyclic AMP	8.9	7.8
Cyclic GMP-dependent protein kinase from bovine heart (82 μg)	None	0.7	0.6
	Cyclic GMP	3.6	3.3
Catalytic subunit of cyclic AMP-dependent protein kinase from bovine heart (0.01 μg)	None	2.0	1.9

* Leukaphoresed blood was obtained from healthy human volunteers. The neutrophils were further purified by hypo-osmotic lysis of the erythrocytes as previously described (Himmelhoch *et al.*, 1969) followed by centrifugation over lymphocyte-separation medium (Bionetics Laboratory Products, Kensington, MD, U.S.A.). The resulting preparation of cells, containing >90% neutrophils, was treated with di-isopropyl fluorophosphate (5 mM) as previously described (Amrein & Stossel, 1980), and washed twice with Hanks balanced salt solution. The cells were then sonicated in 5 vol. of buffer containing 50 mM-Tris/HCl (pH 7.5), 50 mM-2-mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride, 2 mM-EGTA, and 0.3% Triton X-100. The sonicated material was gently stirred for 1 h at 4°C and then centrifuged at 100000 *g* for 1 h. The resulting supernatant was used as the source of enzyme.

similarly and totally inhibited by melittin at 15 μM (Table 1), the same as that seen for inhibition of the essentially homogeneous cardiac enzymes shown in Fig. 1. These findings clearly suggested the lack of specificity of the melittin effect on the phospholipid-sensitive enzyme from different tissues and species. It should be noted here that the activity of the enzyme in the human neutrophils was at least as high as that in the rat brain and spleen, tissues previously shown to be the richest sources of the

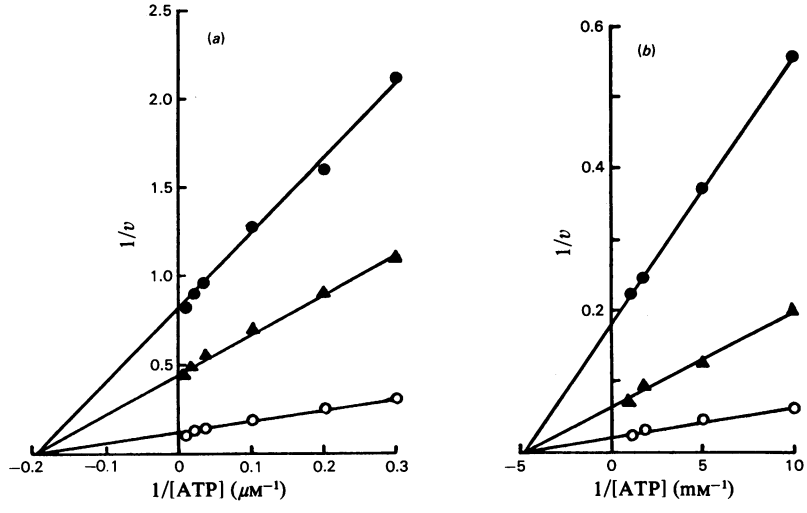

Fig. 2. *Double-reciprocal plots showing inhibition of two species of Ca^{2+}-dependent protein kinases by melittin as a function of ATP concentration*

The assay conditions for the enzymes were as described in the Experimental section and the legend to Fig. 1, in the presence of various ATP concentrations, as indicated. (*a*) Phospholipid-sensitive Ca^{2+}-dependent protein kinase (0.02 μg) was incubated in the presence of phosphatidylserine (5 μg/0.2 ml) and $CaCl_2$ (0.1 mM), without (○) or with 4 μM- (▲) and 8 μM-melittin (●). (*b*) Myosin light-chain kinase (1.4 μg) was incubated in the presence of calmodulin (0.56 μM) and $CaCl_2$ (0.1 mM), without (○) or with 4 μM- (▲) and 8 μM-melittin (●). Units of v were pmol of P_i/min.

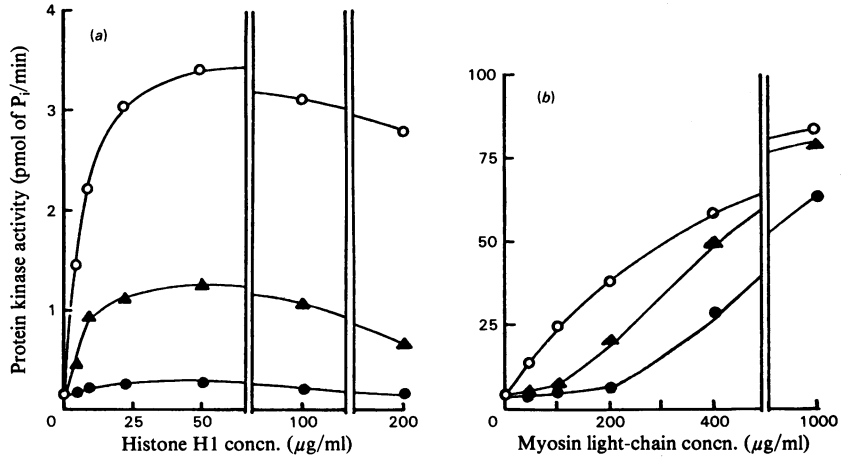

Fig. 3. *Inhibition by melittin of two species of Ca^{2+}-dependent protein kinases as a function of their substrate protein concentration*

The assay conditions for the enzymes were as described in the Experimental section and the legend to Fig. 2, except for the varying concentrations of (*a*) histone H1 for phospholipid-sensitive Ca^{2+}-dependent protein kinase and (*b*) myosin light chains for myosin light-chain kinase, as indicated.

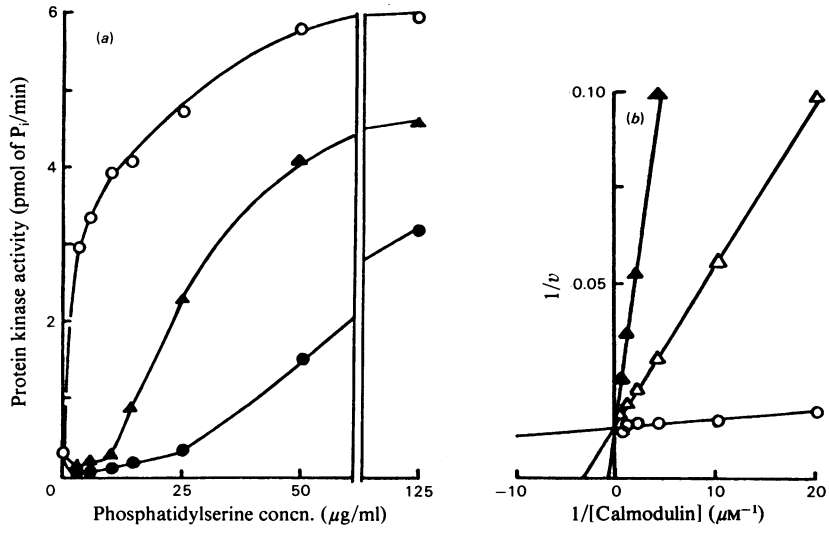

Fig. 4. *Inhibition by melittin of two species of Ca^{2+}-dependent protein kinases as a function of their cofactor concentration*
The assay conditions were as described in the Experimental section, in the presence of varying concentrations of their respective cofactors, as indicated. (*a*) Phospholipid-sensitive Ca^{2+}-dependent protein kinase (0.02 μg) was incubated in the presence of $CaCl_2$ (0.1 mM) and various concentrations of phosphatidylserine, without (○) or with 4 μM- (▲) and (●) 8 μM-melittin. (*b*) Myosin light-chain kinase (1.4 μg) was incubated in the presence of $CaCl_2$ (0.1 mM) and various concentrations of calmodulin, without (○) or with 2 μM- (▲) and (●) 4 μM-melittin. Units of *v* were pmol of P_i/min.

enzyme (Kuo *et al.*, 1980). In comparison, melittin had little or no effect on the partially purified bovine heart cyclic AMP-dependent protein kinase, the homogeneous preparation of its catalytic subunit, or the partially purified cyclic GMP-dependent protein kinase (Table 1). The possibility that the lack of melittin inhibition of the holoenzymes of cyclic nucleotide-dependent protein kinases may be due to the presence of melittin-binding substances in these impure enzyme preparations seemed to be excluded, because the ability of melittin to inhibit Ca^{2+}-dependent enzymes was not affected by the presence of the impure cyclic nucleotide-dependent enzymes (results not shown).

Kinetic analysis revealed that melittin inhibited both the phospholipid-sensitive Ca^{2+}-dependent enzyme (Fig. 2*a*) and myosin light-chain kinase (Fig. 2*b*) non-competitively with respect to ATP, with average K_i values of 1.3 and 1.4 μM respectively. Its inhibition of both enzymes with respect to their substrate proteins did not appear to obey classical Michaelis–Menten kinetics; the inhibition of the phospholipid-sensitive enzyme was not reversed by histone H1 (Fig. 3*a*), whereas that of myosin light-chain kinase appeared to be overcome by myosin light chains (Fig. 3*b*).

Additional kinetic studies were carried out to elucidate how the melittin inhibition is related to the cofactors (phosphatidylserine and calmodulin) and the activator (Ca^{2+}) of the enzymes. Melittin was found to totally inhibit the phospholipid-sensitive enzyme in the presence of low phosphatidylserine concentrations, and this inhibition was reversed by high concentrations of the phospholipid (Fig. 4*a*). These observations suggest hydrophobic interactions exist between melittin and phosphatidylserine, which may account for the failure of melittin inhibition to obey the classical kinetics noted under the experimental conditions. In contrast, melittin inhibited myosin light-chain kinase competitively with calmodulin, with an average K_i of 0.08 μM (Fig. 4*b*). Inhibition of the phospholipid-sensitive enzyme by melittin, in contrast with its reversal by phosphatidylserine seen earlier in Fig. 4(*a*), was not reversed by increasing Ca^{2+} concentrations (Fig. 5*a*). This inhibition again failed to obey classical kinetics. Melittin, however, was found to inhibit myosin light-chain kinase non-competitively with respect to Ca^{2+}, with an average K_i of 1.9 μM (Fig. 5*b*).

Discussion

The present studies demonstrated that melittin inhibited the Ca^{2+}-dependent class of protein kinases without affecting appreciably the cyclic nucleotide-dependent class of protein kinases. This specific action of melittin appears to indicate the presence in Ca^{2+}-dependent protein kinases of certain loci that can interact with the surface-active polypeptide, and

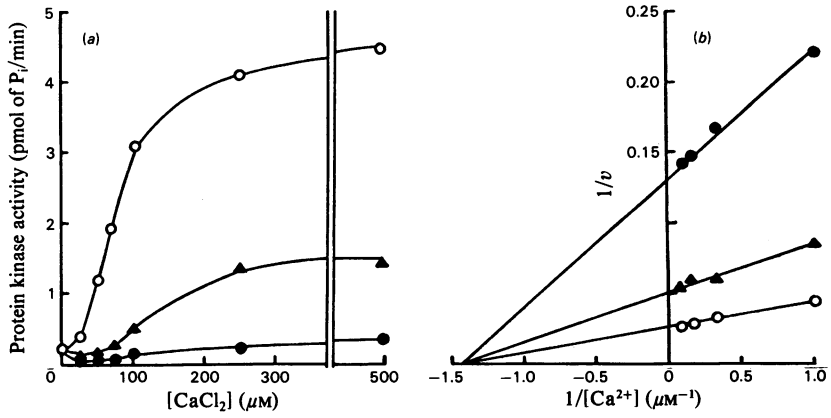

Fig. 5. *Inhibition by melittin of two species of Ca^{2+}-dependent protein kinase as a function of $CaCl_2$ concentration*
The assay conditions for the enzymes were as described in the Experimental section and the legend to Fig. 2, except for varying concentrations of $CaCl_2$ for (*a*) phospholipid-sensitive Ca^{2+}-dependent protein kinase and (*b*) myosin light-chain kinase, as indicated.

that these loci appear to be lacking in cyclic nucleotide-dependent enzymes. Functional importance of hydrophobic regions in enzymes stimulated by calmodulin/Ca^{2+} (such as myosin light-chain kinase in the present studies) has been suggested (Klee *et al.*, 1980; Tanaka & Hidaka, 1980; Weiss & Wallace, 1980). Although the molecular structure of phospholipid-sensitive species of Ca^{2+}-dependent protein kinase has yet to be determined, it is likely that a hydrophobic region is also functionally crucial for the enzyme. This notion is based on the following observations. (1) Phosphatidylserine is essential for supporting the Ca^{2+}-dependent activity of the enzyme (Takai *et al.*, 1979; Kuo *et al.*, 1980). (2) Phosphatidylserine–Affigel 102 (phosphatidylserine is coupled to Affigel 102 via the carboxy group) not only binds but also activates the enzyme (B. C. Wise, R. L. Raynor & J. F. Kuo, unpublished work), presumably involving an interaction between the enzyme and the free hydrophobic aliphatic chain of the fatty acid. (3) Other lipophilic compounds, such as adriamycin (Katoh *et al.*, 1981*a*) and palmitoylcarnitine (Katoh *et al.*, 1981*b*) also inhibit the phospholipid-sensitive Ca^{2+}-dependent phosphorylation of histone or endogenous proteins from the heart. It is noteworthy that these two compounds, like melittin, are essentially without effect on cyclic nucleotide-dependent protein kinases (Katoh *et al.*, 1981*a*,*b*).

The effects of melittin on intact cells have often been attributed to the accumulation of lysophospholipids. However, Fain *et al.* (1981) and Gelehrter & Rozengurt (1980) have shown that the effects of melittin on adipocytes and 3T3 fibroblasts respectively are unrelated to lysophospholipid accumulation. These results support the hypothesis that melittin effects are secondary to binding to proteins with hydrophobic sites or to phospholipid components of cell membranes. A unique feature of protein kinases employed in the present studies is that they are soluble enzymes and do not behave like integral membrane proteins.

Kinetic analysis suggests that melittin exerted its action on myosin light-chain kinase by interfering with the formation of the active enzyme–calmodulin–Ca^{2+} ternary complex, the site of melittin binding on the enzyme being the same as, or proximal to, the calmodulin-binding site. This tentative conclusion was supported by the observations that (1) melittin had little or no effect on the basal activity of the enzyme seen in the absence of added calmodulin and Ca^{2+} (Fig. 1*b*) and (2) it non-competitively inhibited the enzyme with respect to ATP (Fig. 2*b*) or Ca^{2+} (Fig. 5*b*), but competitively inhibited it with respect to calmodulin (Fig. 4*b*). The site of interaction of melittin on phospholipid-sensitive Ca^{2+}-dependent enzyme, on the other hand, could not be determined kinetically, because this lytic polypeptide could potentially interact with enzyme, phospholipid cofactor, substrate protein and/or complexes. It seems clear, however, that melittin was not interacting with the free enzyme, since it was without effect on the basal activity of the enzyme (Fig. 1*a*). Melittin and phospholipase A_2 are the two major toxins of bee venom (Habermann, 1972). Phospholipase A_2 hydrolyses and inactivates the phospholipid cofactor for the phospholipid-sensitive enzyme (B. C. Wise & J. F. Kuo, unpublished work), whereas melittin could either interact with and inactivate the

phospholipid cofactor or directly interact with and inactivate the enzyme itself. It is conceivable, therefore, that the present phospholipid-sensitive Ca^{2+}-dependent protein kinase may represent an enzyme system that can be markedly inhibited by bee venom. We have shown previously that the enzyme exists in both the soluble and particulate form (Kuo *et al.*, 1980; Wise *et al.*, 1981; Katoh *et al.*, 1981*b*). It is possible that the hydrophilic (positively charged) part, instead of, or in addition to, the hydrophobic part, of melittin might be involved in its action; this possibility, although unlikely, cannot be eliminated at present.

Several chemically unrelated substances, in addition to melittin, have been shown to inhibit, with comparable potencies, both the calmodulin-sensitive Ca^{2+}-dependent enzymes (e.g. myosin light-chain kinases and certain cyclic nucleotide phosphodiesterases) and phospholipid-sensitive Ca^{2+}-dependent protein kinase. Phenothiazine antipsychotic drugs, such as trifluoperazine and chlorpromazine, reported previously to inhibit calmodulin-sensitive enzymes by interacting with calmodulin–Ca^{2+} complex (for a review, see Weiss & Wallace, 1980), also inhibit the phospholipid-sensitive Ca^{2+}-dependent phosphorylation of histone (Mori *et al.*, 1980; Schatzman *et al.*, 1981) and endogenous proteins from the brain (Wrenn *et al.*, 1980). Compound W-7 [*N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide], shown previously to be an inhibitor of calmodulin-sensitive enzymes (Hidaka *et al.*, 1980), also inhibits the phospholipid-sensitive enzyme (B. C. Wise & J. F. Kuo, unpublished work). In addition, we recently reported that palmitoylcarnitine (Katoh *et al.*, 1981*b*) and adriamycin (Katoh *et al.*, 1981*a*) inhibit Ca^{2+}-dependent phosphorylation of histone or cardiac proteins specifically stimulated by calmodulin or phospholipid. These observations, taken collectively, seem to hint at some common features for the calmodulin-sensitive and phospholipid-sensitive Ca^{2+}-dependent enzymes regarding their activation by respective cofactors and inhibition by a diversity of substances. It is worth noting here that, in the light of the above-mentioned findings, it is erroneous to assume that phenothazines or compound W-7 block exclusively, primarily or preferentially the calmodulin/Ca^{2+}-stimulated enzyme systems or biological processes. This is particularly true in certain tissues (such as brain and spleen) where the newly recognized phospholipid-sensitive Ca^{2+}-dependent protein kinase is exceptionally abundant (Kuo *et al.*, 1980).

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