Biochemical Pharmacology, Vol. 32, No. 19, pp. 2929–2933, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

INHIBITION OF CALMODULIN ACTIVITY BY INSECT VENOM PEPTIDES

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(*Received* 24 *January* 1983; *accepted* 25 *March* 1983)

Abstract—Several peptides found in insect venom, including melittin, apamin and mastoparan, inhibited the activity of calmodulin-stimulated phosphodiesterase at concentrations that had no appreciable effect on basal phosphodiesterase activity; the K_i value of melittin for inhibiting calmodulin activity was 30 nM. Acetylation of the peptides reduced their inhibitory effect on calmodulin, suggesting that a net positive charge was an important determinant of anti-calmodulin activity. An examination of other structural features of these peptides suggested that the most potent inhibitors of calmodulin had an α-helical conformation. Equilibrium dialysis experiments showed that melittin inhibited the calcium-dependent binding of ^{3}H-chlorpromazine to calmodulin (IC_{50} 0.9 μM); kinetic analyses of these data indicated that this inhibition was non-competitive, suggesting that melittin and chlorpromazine act at different sites on calmodulin. Since calmodulin regulates a number of processes that these peptides inhibit, our results raise the possibility that the inhibition of calmodulin activity by these insect venom peptides may explain some of their biochemical or toxicological effects.

Calmodulin, a ubiquitous, acidic, calcium binding protein, functions as an intracellular receptor for calcium [1] and mediates the effects of calcium on a wide variety of enzymes and physiological processes [2]. Accordingly, agents that inhibit the action of calmodulin could exert profound pharmacological actions. Among the calmodulin inhibitory compounds described so far are calcium chelating agents [3], antipsychotic drugs [4, 5], smooth muscle relaxants [6], several local anesthetics [7] and certain endogenous neuropeptides and proteins [8–12]. The mechanism by which antipsychotics, smooth muscle relaxants and neuropeptides block calmodulin-dependent processes is through a direct interaction of these substances with calmodulin [6, 9, 13].

Studies of the relationship between the structure and anti-calmodulin activity of a number of compounds show that antipsychotic drugs and the neuropeptide inhibitors of calmodulin share similar physicochemical characteristics [14, 15]; both groups of compounds have a hydrophobic region and a net positive charge at physiological pH [14, 15]. Since several peptides found in insect venom have these same structural features, we determined if these insect peptides could also inhibit calmodulin activity.

METHODS

A calmodulin-sensitive form of phosphodiesterase was purified from rat brain by preparative polyacrylamide gel electrophoresis [16]. Calmodulin was prepared from bovine brain according to the procedure of Watterson *et al.* [17]. Phosphodiesterase activity was determined by a modification of the luciferin luciferase technique [18].

To determine the effects of peptides on calmodulin activity, various concentrations of each peptide were added to the preparation of phosphodiesterase, and enzyme activity was determined in the absence (basal) and presence (activated) of 1 unit of calmodulin. One unit of calmodulin is defined as that amount required to increase phosphodiesterase activity to 50% of the maximum activation of the enzyme; in these studies, calmodulin stimulated phosphodiesterase activity approximately 10-fold, and one unit of calmodulin was approximately 1.2 pmole.

Peptides were acetylated with acetic anhydride according to a modification of the procedure of Montelaro and Rueckert [19]. Under these conditions, only lysine residues are acetylated. Briefly, 2 mg of melittin and 1 mg of mastoparan were dissolved in 1 ml of deionized distilled water and reacted with 100 μl of acetic anhydride for 20 min at room temperature. The samples were neutralized with 10 M NaOH, and protein content was assayed by the method of Lowry *et al.* [20]. The extent of acetylation was estimated from the decrease in free amino groups as determined with a ninhydrin reagent [21].

To determine if the peptides interact with calmodulin, we measured the ability of melittin to displace [^{3}H]-chlorpromazine from calmodulin. The binding of [^{3}H]-chlorpromazine to calmodulin was determined by equilibrium dialysis [13].

Melittin and phospholipase A_2 were obtained from the Sigma Chemical Co. (St. Louis, MO), and mastoparan, polistes mastoparan and granuliberin R from Peninsula Laboratories Inc. (Belmont, Ca). [^{3}H]-Chlorpromazine was purchased from the New England Nuclear Corp. (Boston, MA), and unla-

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beled chlorpromazine was donated by Smith Kline & French Laboratories (Philadelphia, PA).

RESULTS

Melittin, a basic peptide found in bee venom, produced a dose-dependent inhibition of calmodulin-stimulated phosphodiesterase activity without altering the basal activity of this enzyme (Fig. 1). The concentration of melittin required to inhibit by 50% the activation of phosphodiesterase by calmodulin was 0.1 μM. A kinetic analysis of the calmodulin-stimulated phosphodiesterase by melittin indicated an apparent inhibitory constant (K_i) for melittin of 30 nM (Fig. 2).

The commercial preparation of melittin used in these experiments is contaminated with phospholipase A_2. Therefore, we examined the effect of purified phospholipase A_2 on both basal and calmodulin-stimulated phosphodiesterase by mellipase A_2, even at twenty times the concentration present in our sample of melittin, had no effect on either basal or calmodulin-stimulated phosphodiesterase activity, nor did it alter the inhibition of calmodulin activity produced by melittin (data not shown).

Wasp and bee venom contain several basic peptides structurally similar to melittin. Like melittin, these peptides have a high proportion of hydrophobic amino acid residues and a number of basic residues located at one end of the peptide chain. An examination of their effects on basal and calmodulin-stimulated phosphodiesterase activity showed that all of these peptides selectively inhibited the calmodulin-activated form of the enzyme (Table 1). Mastoparan was the most potent inhibitor of calmodulin, with an IC_{50} value of 20 nM; melittin and polistes mastoparan were equipotent, and apamin and granuliberin R were the least active of this series.

Earlier studies showed that a positively-charged amino group is important for the ability of phenothiazine antipsychotics to bind to and inhibit calmodulin [8, 23]. To determine if positively-charged amino groups in melittin and mastoparan are important for anti-calmodulin activity, we acetylated the lysine residues on these peptides and measured their effects on calmodulin-stimulated phosphodiesterase activity. This procedure acetylated between two and three lysines, as evidenced from the percent change in the slope in a ninhydrin reaction curve. The results showed that acetylation markedly reduced, but did not eliminate, the inhibitory action of these peptides on calmodulin-stimulated phosphodiesterase (Table 1). This suggested that, like the phenothiazine antipsychotic drugs, the presence of positively-charged amino groups in these peptides is important for anti-calmodulin activity. Further, since blockade of the ε-amino groups on melittin does not reduce the surface activity of this peptide [24], the results also suggest that its interaction with calmodulin is not due solely to its hydrophobicity.

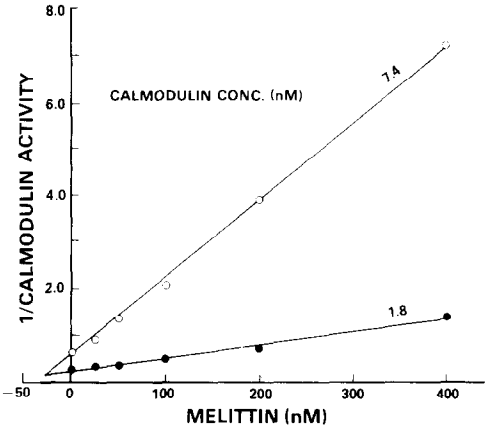

Fig. 2. Kinetic analysis of the inhibition by melittin of calmodulin-stimulated phosphodiesterase activity. The calmodulin-induced increase in phosphodiesterase activity was determined in the presence of various concentrations of melittin. Each point represents the mean value of three experiments. The intersect of the two lines indicates the K_i for melittin (30 nM) [22].

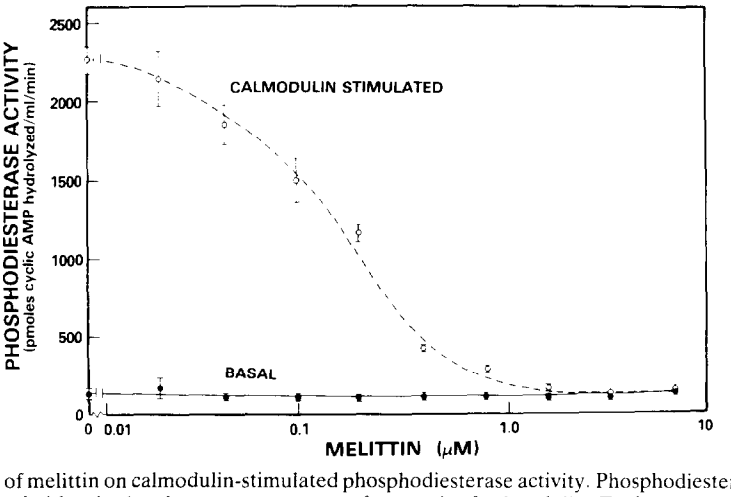

Fig. 1. Effect of melittin on calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was determined either in the absence or presence of one unit of calmodulin. Each point represents the mean of five determinations. Vertical brackets indicate the standard error.

Table 1. Inhibition of calmodulin-sensitive phosphodiesterase activity by insect venom peptides*

Peptide	IC_{50} (μM) Calmodulin-activated phosphodiesterase	Basal phosphodiesterase activity
Mastoparan	0.02	> 100†
Melittin	0.1	> 200†
Polistes mastoparan	0.09	> 100
Acetylmelittin	0.4	> 10
Acetylmastoparan	0.8	> 5 ‡
Apamin	10	> 200
Granuliberin R	30	> 200

*The IC_{50} value is the concentration of peptide required to inhibit by 50% the activation of phosphodieaterase induced by 1 unit of calmodulin. Each value in the table represents the average of two experiments; in each experiment, the effects of at least six concentrations of each peptide were determined and each concentration was assayed in quadruplicate.

†At concentrations above 10 μM, melittin and mastoparan increased the basal phosphodiesterase activity.

‡Acetylmastoparan inhibited the basal phosphodiesterase activity by about 50% at 5 μM. However, no further inhibition activity was seen at 10 μM.

Antipsychotic drugs and the neuropeptide β-endorphin inhibit the actions of calmodulin by binding directly to it. This was shown in experiments utilizing radiolabeled compounds [9, 13]. Since we were unable to obtain radiolabeled melittin, we measured the binding of melittin to calmodulin indirectly by determining the ability of melittin to displace [^{3}H]-chlorpromazine from calmodulin. Figure 3 shows that melittin reduced the Ca^{2+}-dependent binding of [^{3}H]-chlorpromazine to calmodulin; the IC_{50} value was 0.9 μM. An Eadie–Hofstee [25] plot of these data (Fig. 4) suggests that this inhibition is non-competitive since the maximum binding was reduced by one-half (y-intercept) but the slope, an indication of the dissociation constant, remained the same. Thus, melittin apparently reduces the total number of [^{3}H]-chlorpromazine binding sites on calmodulin without altering the K_d, suggesting that melittin and phenothiazine drugs interact at different sites on calmodulin.

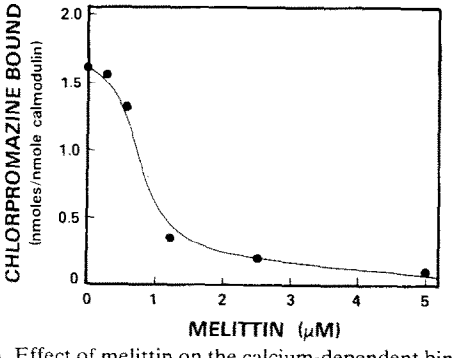

Fig. 3. Effect of melittin on the calcium-dependent binding of [^{3}H]-chlorpromazine to calmodulin. Samples of calmodulin were dialyzed against 5 μM [^{3}H]-chlorpromazine in the presence of various concentrations of melittin. Each point represents the mean value of three experimental determinations.

DISCUSSION

The results presented in this paper show that several peptides found in wasp and bee venom are potent and selective inhibitors of the calmodulin-induced activation of phosphodiesterase. For example, the K_i value for the inhibition of calmodulin by melittin was 30 nM. Katoh *et al.* [26] recently reported that melittin inhibited calmodulin-sensitive protein kinase, but at concentrations about ten times higher than those that we found to inhibit the calmodulin-sensitive phosphodiesterase. Structurally, these peptides are similar in that they all contain a high proportion of hydrophobic amino acids and they all are positively-charged at physiological pH.

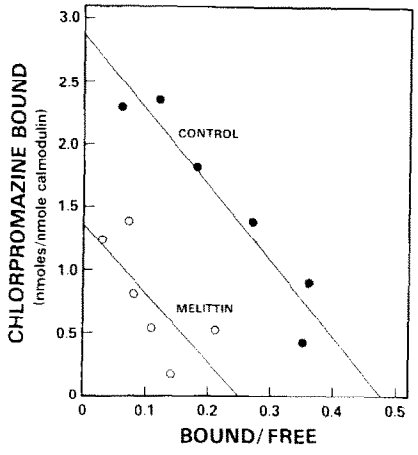

Fig. 4. Eadie–Hofstee plot of the inhibition by melittin of calcium-dependent binding of [^{3}H]-chlorpromazine to calmodulin. Samples of calmodulin were dialyzed against several concentrations of [^{3}H]-chlorpromazine in the absence or presence of 1 μM melittin. Each point represents the mean value of three experiments. The lines were drawn by linear regression analysis. The correlation coefficients were −0.96 for controls and −0.72 for melittin.

To determine the relative importance of hydrophobicity and charge on anti-calmodulin activity, we measured the effects of acetylating the lysine residues of melittin and mastoparan on calmodulin inhibitory activity. Eliminating the positive charge on the ε-amino nitrogen of the lysine residues decreased the potency of both peptides: about 4-fold for melittin and 40-fold for mastoparan, suggesting that positively-charged groups on the peptides are important for interacting with the negatively-charged aspartic or glutamic residues on calmodulin. However, although acetylation markedly reduced the potency of the peptides, the compounds still retained some anti-calmodulin activity. Since acetylation reduces the net positive charge on these peptides but does not alter the surface activity [24], these results suggest that the positive charge on the molecule as well as hydrophobicity are important determinants of anti-calmodulin activity. A similar conclusion was reached in studies of the calmodulin-inhibitory activity of a large number of drugs from several different pharmacological classes [14, 23]. The binding of calmodulin to calmodulin-sensitive enzymes may also involve hydrophobic and ionic interactions [27].

Although all peptides examined had a net positive charge and a region of hydrophobic amino acids, we found that these peptides were not equipotent in inhibiting calmodulin activity. The reason for this difference may be the presence of a secondary structure in the form of an α-helix. Whereas most peptides form random coils in solution, melittin possesses a high degree of α-helical content [28]. There are no reports regarding the nature of the secondary structure for mastoparan, but based on a set of empirical rules that expresses the probability with which peptides form a particular secondary structure, one could predict that mastoparan forms an α-helix extending from residue 1 through 11 [29]. The importance of the α-helix in determining the anti-calmodulin activity of these peptides can be further demonstrated by comparing the potencies of polistes mastoparan and granuliberin R. Both have the same number of basic amino acids, yet polistes mastoparan is 300 times more potent than granuliberin R in inhibiting calmodulin. Polistes mastoparan can be predicted to contain an α-helix extending from residues 8 through 14; no such sequence can be found in granuliberin R. Of the endogenous neuropeptides, β-endorphin, the most potent, also possesses an α-helical segment [30]. Thus, in addition to a hydrophobic region and a net positive charge, the ability to form an α-helix may be an important structural feature for conferring calmodulin inhibitory activity on a peptide.

Since both antipsychotic drugs and β-endorphin inhibit calmodulin activity by binding directly to it, we were interested in determining if the venom peptide melittin also binds to calmodulin. Our findings showed that melittin inhibited the calcium-dependent binding of chlorpromazine; however, the concentration was approximately 10-fold higher than that required to inhibit the calmodulin-induced activation of phosphodiesterase. These results suggest that chlorpromazine and melittin might be binding to different sites on calmodulin. Indeed, an analysis of the data by the method of Eadie–Hofstee[25] showed that melittin reduced the total number of binding sites for chlorpromazine without decreasing the affinity of these sites for the phenothiazine. This type of non-competitive interaction may be contrasted with the results showing that several different chemical classes of antipsychotic drugs apparently compete with the phenothiazines for the same site on calmodulin[13].

Other recent findings also support the idea that calmodulin inhibitors may act at different sites on calmodulin. For example, verapamil and proafidin (SKF-525A), which interact with calmodulin, increase the fluorescence of a hydrophobic probe; by contrast, the phenothiazine antipsychotic drugs decrease the fluorescence of this probe [31, 32]. There also is evidence that drugs may produce differential inhibition of different calmodulin-dependent processes. Katoh [33] has shown that palmitoylcarnitine inhibits the activation of cardiac myosin light chain kinase by calmodulin but does not inhibit the activation of phosphodiesterase, and Van Belle [34] reported that the compound R24-571produced a differential inhibition of calmodulin-sensitive ATPase, protein kinase and phosphodiesterase. Thus, it is possible that different calmodulin inhibitors might produce selective pharmacological effects by preventing the interaction of calmodulin with one enzyme without altering its interaction with another.

Currently, it is not known whether the anti-calmodulin activity of the venom peptides reported here is important in producing their toxic effects. It might be noted, however, that a number of processes affected by these peptides are also regulated by calmodulin. For example, melittin produces hypotension [35]and inhibits the activity of adenylate cyclase [36]and ATPase [37]. Both melittin and mastoparan release histamine from mast cells [38, 39] and both are hemolytic [37, 39]. Since calcium and calmodulin play an important role in all of these processes [2], and since calmodulin is embedded within the membrane matrix [40], it is possible that the mechanism for the membrane effects of these peptides may be explained by their interaction with calmodulin.

In conclusion, we have shown that a number of peptides found in insect venom selectively inhibit calmodulin-activated phosphodiesterase. The most potent of these have a net positive charge at physiological pH, have a hydrophobic region, and an α-helical secondary conformation. Since these structural features are shared by the endogenous neuropeptide inhibitor of calmodulin β-endorphin, we believe that these properties may also be found in other endogenous calmodulin inhibitory peptides and may provide an insight into the structural characteristics present in calmodulin-sensitive enzymes.

Note—Comte *et al.* demonstrated the formation of a complex between melittin and calmodulin in the presence of calcium. (M. Comte, Y. Maulet and J. A. Cox *Biochem. J.* **209**, 269, 1983).

Acknowledgements—We thank Ms. Becky Simon for her excellent technical assistance. This work was supported by Grant MH30096 awarded by the National Institute of Men-

tal Health, and by funds from the Department of Public Welfare of the Commonwealth of Pennsylvania and the Scottish Rite Schizophrenia Research Program, N.M.J., U.S.A.

REFERENCES

1. A. R. Means and J. R. Dedman, *Nature*, **285**,73 (1980).
2. B. Weiss and T. L. Wallace, in *Calcium and Cell Function* (Ed. W. Y. Cheung), p. 329. Academic Press, New York (1980).
3. Y. M. Lin, Y. P. Liu and W. Y. Cheung, *Fedn. Eur. Biochem. Soc. Lett.* **49**, 356 (1975).
4. B. Weiss, R.Fertel, R. Figlin and P. Uzunov, *Molec. Pharmac.* **10**, 615 (1974).
5. R. M. Levin and B. Weiss, *Molec. Pharmac.* **12**, 581 (1976).
6. H. Hidaka, T. Yamaki, T. Totsuka and M. Asano, *Molec. Pharmac.* **15**, 49 (1979).
7. M. Volpi, R. I. Sha'afi, P. M. Epstein, D. M. Andrenyak and M. B. Feinstein, *Proc. natn. Acad. Sci. U.S.A.* **78**, 795 (1981).
8. B. Weiss, W. Prozialeck, M. Cimino, M. Sellinger-Barnette and T. L. Wallace, *Ann. N.Y. Acad. Sci.* **356**, 319 (1980).
9. M. Sellinger-Barnette and B. Weiss, *Molec. Pharmac.* **21**, 86 (1982).
10. D. A. Malencik and S. R. Anderson, *Biochemistry* **21**, 3480 (1982).
11. C. B. Klee and M. H. Krinks, *Biochemistry* **17**, 120 (1978).
12. R. J. A. Grand and S. V. Perry, *Biochem. J.* **189**, 227 (1980).
13. R. M. Levin and B. Weiss, *J. Pharmac. exp. Ther.* **208**,454 (1979).
14. B. Weiss, W. C. Prozialeck and T. L. Wallace, *Biochem. Pharmac.* **31**, 2217 (1982).
15. M. Barnette and B, Weiss, Adv. Cyclic. Nucleotide Res., in press.
16. P. Uzunov and B. Weiss, *Biochim. biophys. Acta* **284**, 220 (1972).
17. D. M. Watterson, W. G. Harrelson, P. M. Keller, F. Sharief and T. C. Vanaman, *J. biol. Chem.* **251**, 4501 (1976).
18. B. Weiss, R. Lehne and S. J. Strada, *Analyt. Biochem.* **45**, 222 (1972).
19. R. C. Montelaro and R. R. Rueckert, *J. biol. Chem.* **250**, 1413 (1975).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. E. W. Yemm and E. C. Cockung, *Analyst, Lond.* **80**, 209 (1955).
22. M. Dixon and E. C. Webb, *Enzymes*, p. 171. Academic Press, New York (1958).
23. W. C. Prozialeck and B. Weiss, *J. Pharmac. exp. Ther.***222**, 509 (1982).
24. E. Haberman and H. Kowallek, *Hoppe-Seyler's Z. physiol. Chem.* **351**, 884 (1970).
25. B. H. J. Hofstee, *Science* **116**, 329 (1952).
26. N. Katoh, R. L. Raynor, B. C. Wise, R. C. Schatzman, R. S. Turner, D. M. Helfman, J. N. Fain and J. F. Kuo, *Biochem. J.* **202**, 217 (1982).
27. D. K. Blumenthal and J. T. Stull, *Biochemistry* **21**, 2386 (1982).
28. J. Bello, H. Bello and E. Granados, *Biochemistry* **21**, 461 (1982).
29. P. Y. Chou and G. D. Fasman, *A. Rev. Biochem.* **47**, 251 (1978).
30. C-S. Wu, N. Lee, H. H. Loh, J. T. Yang and C. H. Li, *Proc. natn. Acad. Sci. U.S.A.* **76**, 3656 (1976).
31. P. M. Epstein, K. Fiss, R. Hachisu and D. M. Andrenyak, *Biochem. biophys. Res. Commun.* **105**, 1142 (1982).
32. D. C. LaPorte, R. M. Wierman and D. R. Storm, *Biochemistry* **19**, 3814 (1980).
33. N. Katoh, D. M. Helfman, R. W. Wrenn and J. F. Kuo, *Biochim. biophys. Acta* **714**, 129 (1983).
34. H. Van Belle, *Cell Calcium* **2**, 483 (1981).
35. E. Habermann and G. Zeuner, *Naunyn-Schmiedeberg's Arch. Pharmac.* **270**, 1 (1971).
36. G. H. Cook and J. Wolff, *Biochim. biophys. Acta* **498**, 255 (1977).
37. E. Haberman, *Science* **177**, 314 (1972).
38. A. M. Rothschild, *Br. J. Pharmac. chemother.* **25**, 59 (1965).
39. Y. Hirai, M. Kuwada, T. Yasuhara, H. Yoshida and T. Nakajima, *Chem. pharm. Bull., Tokyo* **27**, 1945 (1979).
40. K. Sobue, R. Yamazaki, S. Yasuda and S. Kakiuchi, *Fedn. Eur. Biochem. Soc. Lett.* **129**, 215 (1981).