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# ACTIVATION OF MICROSOMAL GUANYLATE CYCLASE BY A CYTOTOXIC POLYPEPTIDE: MELITTIN

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SUMMARY: Melittin, a 26 amino acid polypeptide, activated membrane-associated guanylate cyclase in a manner suggesting that membrane phospholipids play an important role in regulating the enzyme activity. Melittin was unique in that it activated only the particulate activity in a dose-dependent manner in the concentration range 15 to 200 µg/ml, and, unlike other known activators, solubilization of enzyme did not occur. The effects of melittin on guanylate cyclase showed a lag of two minutes and were not blocked by inhibitors of prostaglandin synthetase or phospholipase suggesting that the effect was not mediated by prostaglandin endoperoxides or phospholipase products but may be due to the ability of melittin to alter membrane lipid properties.

The enzyme guanylate cyclase is found in both soluble and membrane-associated forms. The membrane-associated enzyme is latent in its nature; as a result, maximal activity is observed only in the presence of synthetic detergents like Triton X-100 and Lubrol PX (1-2) or in the presence of lysolecithin, a natural detergent found in most mammalian tissues, (3-4). The activation by these agents involves partial disruption of the membrane; therefore the effects of another membrane perturbing agent, melittin, on rat heart microsomal guanylate cyclase were studied. The polypeptide, melittin, is a major cytolytic component of bee venom and has surface-active properties (5). Melittin, an amphipathic molecule perturbs membrane by interacting with phospholipids (6-8) and activating endogenous phospholipases A (9-10). The ability of melittin to interact with phospholipids seems to be involved in the action of melittin on microsomal guanylate cyclase.

## MATERIALS AND METHODS

Melittin and other biochemicals were obtained from Sigma Chemical Co. Fisher strain F/322 male rats weighing 180-260g (Simenson Laboratories) were used to prepare rat heart microsomes (2,4). The purity of melittin (Lot #47C-0175 and 55C - 0029) was examined by polyacrylamide gel electrophoresis and gel filtration chromatography.

Guanylate cyclase assay. Guanylate cyclase was assayed by the method of White and associates (11,12). Assays were carried out in a final reaction volume of 150 µl containing 40 mM HEPES-Na, pH 7.6, 2 mM $MnCl_2$, 0.2 mM GTP,

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0.5 mM $Na_4$ EDTA, 0.5 mM dithiothreitol, 0.25 M sucrose, 10 mM theophylline, 20 mM caffeine and 31.25 µg of crystalline bovine serum albumin. All assays were done at 37° for 6 or 7 minutes. Protein content was determined by measuring tryptophan fluoresence in 0.05% aqueous sodium dodecylsulfate (excitation, 288 nm, emission, 345 nm) with bovine serum albumin as standard. Assays were performed in triplicate and the results presented as the mean ± standard error. The experiments reported here are representative of two or more experiments performed.

## RESULTS

Melittin activated the membrane-associated guanylate cyclase from rat heart (Fig. 1) in a dose-dependent manner. Maximal activation of guanylate cyclase in most experiments was observed at melittin concentrations of 100-120 µg/ml and it was comparable to that obtained with the nonionic detergent Lubrol PX. In contrast, another phospholipase activator, the direct lytic factor from African Ringhals cobra, exhibited no detectable effect on the activity guanylate cyclase in all conditions tested. Soluble guanylate cyclase was not stimulated by melittin at concentrations up to 100 µg/ml even though it was stimulated almost 2-fold in the presence of lysolecithin (0.4 mg/ml) or Lubrol PX (0.2%). As observed by Kimura and Murad (13), rat heart microsomal guanylate cyclase exhibited non-ideal kinetics ($S_{0.5}$ =0.4-0.6mM) in the absence of detergents or melittin. However, melittin-treated microsomal guanylate

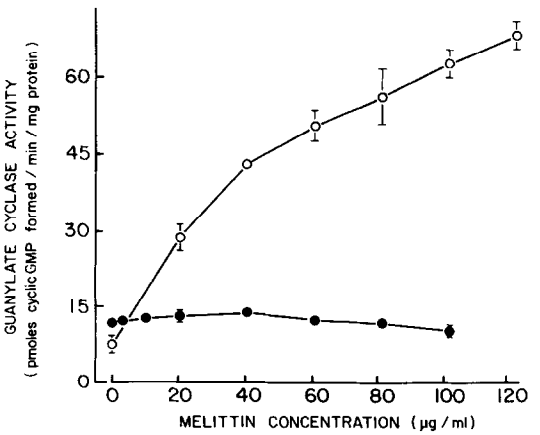


Fig. 1. Effect of melittin on the activities of guanylate cyclases. Guanylate cyclase activity was assayed in the microsomal (-O-) and soluble (-●-) fractions from rat heart for 7 min at 37° following 30 min preincubation at 4° with the indicated concentrations of melittin.

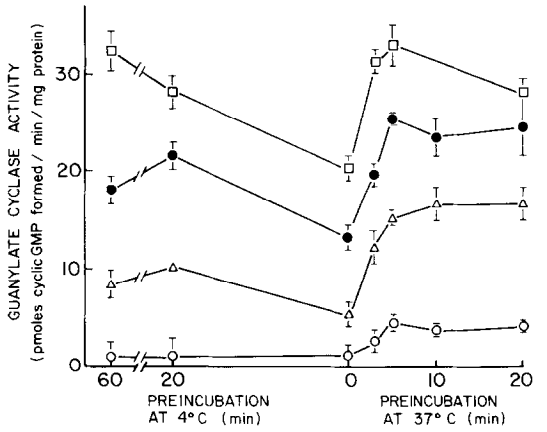


Fig. 2. Effect of preincubation with melittin on the activation of rat heart microsomal guanylate cyclase. Microsomal preparations were preincubated at 4°C or 37°C for the indicated times with no melittin (-O-), or with melittin at 30 μg/ml (-Δ-), 60 μg/ml (-●-), and 100 μg/ml (-□-).

cyclase exhibited a $K_m$ for GTP equivalent to Lubrol PX-solubilized enzyme ($K_m$ 0.17-0.2 mM). Mammalian guanylate cyclase shows specificity for cofactor $Mn^{2+}$ (13,14); rat heart microsomal guanylate cyclase with or without melittin (100 μg/ml) showed similar specificity for $Mn^{2+}$.

As illustrated in Fig. 2, rat heart microsomal guanylate cyclase activation is optimal after a 20 min preincubation at 4° or a 2 to 5 min preincubation at 37° at all melittin concentrations tested. In experiments comparing the reaction rates the enzyme treated with 100 μg/ml melittin exhibited an initial lag (1-2 min) followed by a linear rate for at least 15 min, whereas Lubrol PX-treated and untreated enzyme preparations exhibited linear rate up to 6-8 min with no lag. The enzyme preincubated with melittin for 2 or 5 min exhibited no lag.

At least partial solubilization of membrane-associated guanylate cyclase is evident upon the optimal activation of the enzyme by surface active agents such as lysolecithin, Lubrol PX and Triton X-100 (2,4). Hence, it was of interest to determine if melittin treatment also resulted in solubilization of the enzyme. As indicated in Table I, compared to the control, a range of melittin concentrations did not solubilize a significant amount of guanylate

Table I. Solubilization of microsomal guanylate cyclase by melittin.

| Melittin (μg/ml) | Guanylate Cyclase Activity (picomoles cyclic GMP formed/min by the total fraction) | | | | | |
|---|---|---|---|---|---|---|
| | Original Microsomal preparation | | Pellet | | Supernatant | |
| | -LPX | +LPX | -LPX | +LPX | -LPX | +LPX |
| 0 | 7.7 | 131.8 | 3.6 | 111.8 | 8.1 | 23.5 |
| 30 | 14.9 | 132.2 | 11.4 | 97.0 | 13.8 | 22.3 |
| 50 | 25.3 | 126.2 | 17.6 | 93.2 | 16.1 | 20.7 |
| 70 | 21.3 | 108.9 | 23.0 | 64.5 | 18.3 | 18.6 |
| 90 | 31.1 | 90.3 | 36.1 | 64.9 | 15.5 | 8.2 |
| 110 | 46.1 | 95.8 | 35.6 | 70.3 | 18.4 | 5.6 |
| 150 | 48.7 | 108.0 | 40.4 | 73.3 | 13.0 | 7.4 |
| 200 | 65.6 | 100.5 | 43.1 | 67.8 | 8.0 | 4.6 |

Aliquots (2 ml) of the rat heart microsomal preparation were added to centrifuge tubes and mixed with 1 ml of melittin solutions that gave the indicated final concentrations. An aliquot (0.4 ml) was removed from each suspension and stored on ice while the remainder was centrifuged at 100,000 xg for 1 hr. The resulting pellets (resuspended in 2.6 ml of the buffer) the supernatants and the uncentrifuged preparations were assayed for guanylate cyclase activity in the presence (+LPX) and absence (-LPX) of 0.5% Lubrol PX.

cyclase activity associated with a microsomal fraction. Approximately 15% or less of the activity was found in the supernatant of samples treated with melittin or in the control (0 μg/ml melittin). Less than 15% of the total microsomal protein was solubilized by melittin even at high concentrations.

Melittin has been shown to activate endogenous phospholipases in cultured cells leading to the release of lysolecithin, free fatty acids and prostaglandins (9), these compounds are also reported to activate guanylate cyclase (3-4,15-16). Therefore agents that have been reported to inhibit phosphilipase A activity or prostaglandin synthesis were examined for their effect on melittin activation of guanylate cyclase. Indomethacin, which at concentrations of 10 μM or greater inhibits the synthesis of prostaglandin endoperoxides

Table II. Effect of various inhibitors on rat heart microsomal guanylate cyclase.

| Inhibitor | Concentration (μM) | Guanylate cyclase activity (picomoles cyclic GMP formed/min/mg protein) in the presence of | | |
|---|---|---|---|---|
| | | Buffer | Lubrol PX (0.25%) | Melittin (100 μg/ml) |
| NONE | - | 1.0 ± 0.6 | 34.6 ± 2.0 | 25.7 ± 0.4 |
| dexamethasone | 1.0 | 1.1 ± 0.7 | 34.2 ± 1.3 | 27.9 ± 1.2 |
| prednisone | 1.0 | 1.0 ± 0.4 | 34.2 ± 1.2 | 28.1 ± 1.9 |
| indomethacin | 0.1 | 1.8 ± 0.5 | 32.0 ± 1.1 | 25.0 ± 1.1 |
| indomethacin | 10.0 | 2.7 ± 0.3 | 27.5 ± 0.6 | 22.6 ± 2.2 |
| indomethacin | 100.0 | 4.6 ± 1.0 | 13.22± 0.9 | 6.12± 0.9 |

Rat heart microsomes were treated with various inhibitors for 10 min at 4° and then mixed with buffer, melittin or Lubrol PX and kept on ice for 25 minutes. Guanylate cyclase activity was determined at 37° for 7 minutes.

by fatty acid cyclooxygenase (17), did not block the activation of guanylate cyclase by melittin except at 100 μM (Table II). However, this concentration of indomethacin also inhibited 0.25% Lubrol PX-activated guanylate cyclase, suggesting that the effect of indomethacin at that concentration is due to inhibition of guanylate cyclase rather than inhibition of fatty acid cyclooxygenase. Prednisone or dexamethasone did not affect basal, Lubrol PX- or melittin-activated activities. The effect of melittin on rat heart endogenous phospholipase A activity was also examined. Microsomes were prepared in the same manner from rats administered [methyl-$^{14}$C] choline as described previously (9), and assayed for phospholipase activity under the conditions used to assay guanylate cyclase. Melittin even at a high concentration (100 μg/ml) did not activate sufficient hydrolysis of microsomal lecithin to account for the activation of guanylate cyclase by generation of endogenous lysolecithin.

## DISCUSSION

These studies demonstrate that melittin activates guanylate cyclase of rat heart microsomes. The activation due to melittin is unique. Although it

involves an elevated $V_{max}$ and lowered $K_m$ for GTP, as observed with detergent treated enzyme, activation is achieved without solublizing the enzyme. If the elevated $V_{max}$ in the presence of detergents represents increased accessibility of substrate to the enzyme, then the use of melittin demonstrates that solubilization of the enzyme is not necessary to achieve this.

The results presented above also establish that the effects of melittin on guanylate cyclase are not mediated through modification of the lipid composition of the membrane by activation of endogenous phospholipase A. This conclusion is supported by the following observations: (1) guanylate cyclase is not solubilized as would be expected if activation was mediated by endogenously generated lysolecithin; (2) melittin-activated phospholipase A in mouse fibroblasts exhibits a marked dependence on free calcium ions (9), whereas no calcium dependence of melittin activation of guanylate cyclase was observed; (3) the direct lytic factor from H. hemachatus venom activates endogenous phospholipase A (18) but it does not modify guanylate cyclase activity; (4) direct examination of the rate of hydrolysis of rat heart microsomal lecithin in the presence of melittin indicated that insufficient lysolecithin was generated under the conditions of the assay to activate guanylate cyclase.

Melittin-treated microsomal guanylate cyclase remained activated after centrifuging it away from soluble components (Table I), suggesting that melittin induced permenant changes by binding to membranes. The lag observed may be the time required for such binding. The binding of melittin to phospholipids in the liquid crystalline state is well decumented (6-8). A blue shift in the intrinsic fluorescence of the tryptophan residue of melittin is evident in the presence of phospholipid vesicles or membranes, suggesting that tryptophan residue is going from a polar to non-polar environment (10,19). Hegner et al. (20), from the studies with spin labelled membranes suggested that melittin exerts its effects mainly through retraction of the apolar region of the membrane. The effect of melittin at low concentrations

involved increased fluidity of the apolar fatty acid chains, whereas high melittin concentrations (i.e., a melittin-to-lipid molar ratio > 1:130) caused decreased fluidity of the apolar fatty acid chains (20). Verma et al. (6,21) using infrared absorption spectroscopy confirmed that melittin decreases hydrocarbon chain mobility in phospholipid bilayers, and induces a reorganization of polar head groups, these organizational changes in the phospholipid matrix leading to an increase in the membrane fluidity may be regulating membrane-associated guanylate cyclase activity. Such a role for membrane fluidity in regulation of adenylate cyclase (22-23) and ($Na^+$, $K^+$) ATPase (24) has been proposed.

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REFERENCES

1) Ishikawa, E., Ishikawa, S., Davis, J.W., and Sutherland, E.W. (1969) J. Biol. Chem. 244, 6371-6376.
2) White, A.A. (1975) in Advances in Cyclic Nucleotide Research, (Drummond, G.I., Greengard, P., & Robinson, G.A., Eds.), Vol. 5, pp. 353-373, Raven Press, New York.
3) White, A.A. and Lad, P.J. (1975) Fed. Proc. 34, 232.
4) Shier, W.T., Baldwin, J.H., Nilsen-Hamilton, M., Hamilton, R., and Thanassi, N.M. (1976) Proc. Natl. Acad. Sci. USA 73, 1586-1590.
5) Habermann, E. (1972) Science 177, 314-322.
6) Verma, S.P., Wallach, D.F.H. and Smith, I.C.P. (1974) Biochim. Biophys. Acta 345, 129-140.
7) Williams, J., and Bell, R.M. (1972) Biochim. Biophys. Acta 288, 255-262.
8) Mollay, C.W., and Kreil, G. (1973) Biochim. Biophys. Acta 316, 196-203.
9) Shier, W.T. (1979) Proc. Nat. Acad. Sci. USA 76, 195-199.
10) Mollay, C., Kreil, G., and Berger, H. (1976) Biochim. Biophys. Acta 426, 317-324.
11) White, A.A. and Zenzer, T.V. (1971) Anal. Biochem. 41, 372-396.
12) White, A.A., Crawford, K.M., Patt, C.S. and Lad, P.J. (1976) J. Biol. Chem. 251, 7304-7312.
13) Kimura, H., and Murad, F. (1974) J. Biol. Chem. 249, 6910-6916.
14) Garbers, D.L., Dyer, E.L., Hardman, J.G. (1975) J. Biol. Chem. 250, 382-387.
15) Asakawa, T., Scheinbaum, I., and Ho, R.J. (1976) Biochem. Biophys. Res. Commun, 73, 141-148.
16) Glass, D.B., Garrard, J.N. Townsend, D., Carr, D.W., White, J.G., and Goldberg, N.D. (1977) J. Cyclic Nucl. Res. 3, 37-44.
17) Vane, J.R. (1971) Nature New Biol. 231, 232-235.
18) Shier, W.T. (1977) Biochem. Biophys. Res. Commun, 78, 1168-1174.
19) Dufourcq, J., and Faucon, J. (1977) Biochim. Biophys. Acta 467, 1-11.
20) Hegner, D., Schummer, U., and Schnepel, G.H. (1973) Biochim. Biophys. Acta. 291, 15-22.
21) Verma, S.P. and Wallach, D.F.H. (1976) Biochim. Biophys. Acta 426, 616-623.
22) Engelhard, V.H., Esko, J.D., Storm, D.R., and Glaser, M. (1976) Proc. Nat. Acad. Sci. USA 73, 4482-4486.
23) Dipple, I., and Houslay, M.D. (1978) Biochim. J. 174, 179-190.
24) Kimelberg, H.K., and Papahadjopoulos, D. (1974) J. Biol. Chem. 249, 1071-1080