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ALAMETHICIN AND SYNTHETIC PEPTIDE FRAGMENTS AS UNCOUPLERS OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION. EFFECT OF CHAIN LENGTH AND CHARGE

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SUMMARY Alamethicin, its derivatives and some synthetic fragments have been shown to be uncouplers of oxidative phosphorylation in rat liver mitochondria. A minimum peptide chain length of 13 residues is necessary for this activity. Peptide esters are more efficient uncouplers than the corresponding peptide acids. Esterification of the Glu(18) γ-COOH group in alamethicin does not diminish uncoupling activity. The structural requirements for uncoupling activity parallel those determined for ionophoretic action in small, unilamellar liposomes.

INTRODUCTION

Alamethicin (I), a 20 residue polypeptide channel forming ionophore, induces excitability phenomena in model membrane systems (1). A number of related

1 5 10
Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-
15 20
Val-Aib-Aib-Glu-Gln-Phol.

(I)

α-aminoisobutyric acid (Aib)[1] containing polypeptides have also been reported to be ionophores (2,3), stimulating considerable interest in the conformational analysis of Aib peptides, with a view towards establishing the structural requirements for their biological activity (4,5). Alamethicin has been demonstrated to activate the membrane bound enzymes, Ca^{2+} and (Na^+, K^+)-ATPases (6,7) and recently,

Abbreviations : Aib, α-aminoisobutyric acid; Z, benzyloxycarbonyl; OMe, methyl ester; OBz, benzyl ester: Ac, acetyl; CTC, chlortetracycline.

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along with the hypelcins, shown to stimulate the state 4 respiration of mitochondria (8). We have earlier reported on the effects of chain length and charge of synthetic alamethicin fragments on the divalent cation permeability of unilamellar phospholipid vesicles (9). In this report we examine the effect of synthetic alamethicin fragments and derivatives on the stimulation of the state 4 respiration of mitochondria and correlate the results so obtained with the divalent cation translocating activity of the same peptides. It is shown that fragments 10 residues, or less, in length, neither translocate ions nor uncouple mitochondria; the 1-13 fragments are marginally effective, while the 1-17 fragments and alamethicin and its derivatives are very effective both in ion translocation and in uncoupling mitochondria.

MATERIALS AND METHODS

Alamethicin, its synthetic fragments and derivatives were synthesized by solution phase procedures described elsewhere (10). All peptides were checked for homogeneity by TLC and characterized by 270 MHz ^{1}H NMR and amino acid analysis. Egg phosphatidyl choline, Sephadex G-50, CTC and ADP were from Sigma Chemical Co. All other chemicals were of analytical grade.

Cation translocation across phospholipid membranes was followed as previously described (9), by a fluorescence technique using CTC in small, unilamellar liposomes. Mitochondria were isolated from adult, male rats by the method of Johnson and Lardy (11). The respiratory rates of mitochondria were measured on a Gilson Model K-ICT-C Oxygraph fitted with a Clark oxygen electrode at 30°C, in a medium containing 53 mM sucrose, 2.1 mM EDTA, 7.1 mM $MgCl_2$, 110 mM Tris-HCl and 21 mM potassium phosphate, pH 7.4 in a total volume of 1.4 mL. Succinate (18 mM) was used as substrate. Peptide (in ethanol) was added to state 4 mitochondria such that no more than 5 μL of solution was added. 5 μL of ethanol did not affect the respiration of mitochondria. Due to the hydrophobicity of the peptides the cell had to be rinsed with mitochondria between experiments to remove the last traces of uncoupler. Protein was estimated by the biuret method (12).

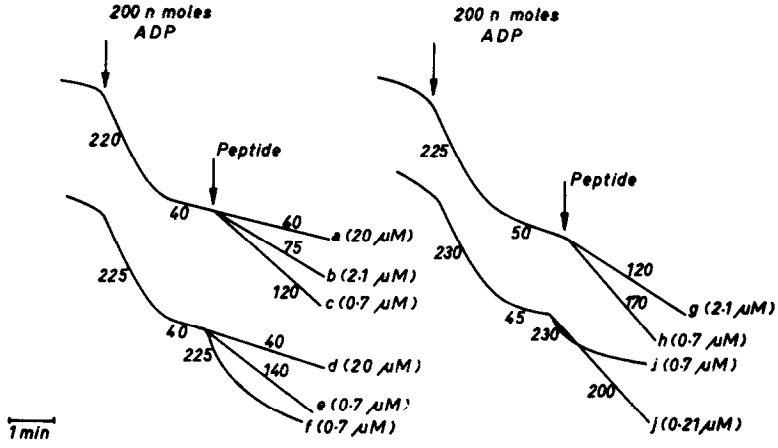

Fig.1 Effect of peptides on the state 4 respiration of rat liver mitochondria. Additions and concentrations as shown in the figure. Numbers adjacent to the traces are respiratory rates in n atoms O/mg. protein/min. a) Z-1-13-OH; b) Z-1-13-OMe; c) Z-1-20-OBz; d)Z-1-10-OMe; e) Ac-1-17-OMe; f) alamethicin; g) Z-1-17-OH; h) Z-1-17-OMe; i,j) Ac-1-20-OBz.

RESULTS AND DISCUSSION

Fig.1 shows the effect of synthetic peptides on mitochondrial respiration. The sharp increase in the rate of oxygen consumption, on adding peptide after the consumption of added ADP, indicates uncoupling. The efficiency of uncoupling varies with peptide chain length. While fragments less than 13 residues in length do not show any uncoupling activity, the 13 residue protected ester (Z-1-13-OMe) shows weak activity. The longer peptides, Z-1-17-OMe and synthetic alamethicin are extremely efficient uncouplers. The peptide acids, Z-1-13-OH and Z-1-17-OH were considerably less effective than the corresponding esters. In fact, no uncoupling activity could be detected for Z-1-13-OH upto a concentration of 20 μM. Alamethicin possesses a negatively charged carboxylate group (Glu-18-γ-COOH). It was of

interest to examine the effect of blocking this negative charge on the state 4 respiration of mitochondria. The effect of addition of alamethicin benzyl ester (Ac-1-20-OBz) on the state 4 respiration of mitochondria is shown in Fig.1. It can be seen that this derivative is a very efficient uncoupler. The amino terminal benzyloxycarbonyl derivative of alamethicin benzyl ester (Z-1-20-OBz) also exhibits uncoupling activity. Peptide concentrations were varied to determine the concentration at which half-maximal activity ($\Phi_{1/2}$) was obtained, the maximum possible stimulation being to the state 3 rate. The sequence of effectiveness as uncouplers for these peptides ($\Phi_{1/2}$ values in μM in parentheses following the peptide) is Ac-1-20-OBz (0.1) $\sim$ alamethicin (0.2) $>$ Z-1-20-OBz (0.7) $\sim$ Z-1-17-OMe (0.5) $>$ Ac-1-17-OMe (1.8) $\sim$ Z-1-17-OH (2.3) $>$ Z-1-13-OMe (4). It should be noted that at sufficiently high concentrations of the peptides (from 0.7 μM for Ac-1-20-OBz to about 15 μM for Z-1-13-OMe) all the uncoupling peptides stimulated respiration to the state 3 rate. Further, subsequent inhibition of respiration is seen at these concentrations (Fig.1f and i).

Fig.2 shows the ionophoretic activity of alamethicin, Z-1-20-OBz and Ac-1-20-OBz monitored on a unilamellar liposome system (9). Both alamethicin and its benzyl ester function as efficient translocators of Ca^{2+}. Z-1-20-OBz is significantly less active. In an earlier report we have shown that the effectiveness of the alamethicin fragments as ionophores varied in the sequence alamethicin $>$ Z-1-17-OMe $>$ Z-1-17-OH $\sim$ Ac-1-17-OMe $>$ Z-1-13-OMe, while

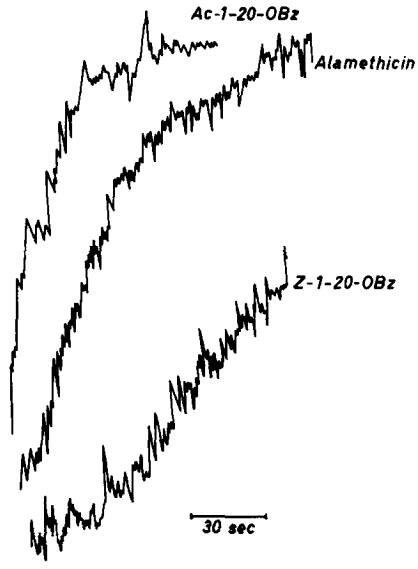

Fig.2 Time dependent changes in CTC-Ca^{2+} fluorescence in response to ionophore addition. An increase in fluorescence corresponds to an influx of Ca^{2+} into the liposomes. Lipid 200 μg/mL, CTC 25 μM, Ca^{2+} 1 mM. Ionophore (6 μM) was added just before the start of the recording.

Z-1-13-OH and smaller fragments were ineffective (9). The above results, together with the results in Fig.2 show that the order of effectiveness of the peptides as cation translocators parallels their efficiency in uncoupling mitochondrial oxidative phosphorylation.

Alamethicin, its active derivatives and fragments presumably form channels in membranes. Z-1-13-OMe started uncoupling appreciably only above 2 μM, indicating that a critical peptide concentration is necessary before uncoupling occurs. This, in turn, may reflect a requirement for the aggregation of peptide molecules into a functional channel - either in the membrane or in the aqueous phase. Evidence for the aqueous aggregation of hydrophobic Aib peptides has been reported (13).

Boheim and Kolb (14) have proposed that alamethicin pre-aggregates enter the membrane, while others suggest reversible lipid-phase aggregation (1, 15, 16) to account for the Black Lipid Membrane conductivity of alamethicin.

Experiments with valinomycin and nigericin have demonstrated that mitochondria can be uncoupled by breaking down the transmembrane proton gradient (17). These experiments required net K^+/H^+ exchange and a K_m of 5-10 mM was determined for K^+. Neither valinomycin nor nigericin alone is a particularly effective uncoupler. Both antibiotics, however, act in concert. We have used $\sim$ 40 mM K^+ in the medium for our experimentation. It is likely that transmembrane channels formed by alamethicin and related peptides are non-specific and may affect both H^+ and K^+ fluxes.

Most of the known uncouplers like 2,4-dinitrophenol, pentachlorophenol, p-trifluoromethoxycarbonylcyanine phenylhydrazone etc., have been shown to be proton ionophores (18), and probably act by alteration of the mitochondrial pH gradient (19). The alamethicin channel is likely to consist of an aggregate of 3_{10} helical monomers (9) with a hydrophobic channel interior, which must contain extensively structured water. Such an ordered water array may be extremely efficient for H^+ translocation. (D.T. Edmonds, Oxford University, personal communication).

The sharp difference in the uncoupling abilities of Z-1-13-OMe and Z-1-13-OH indicate that in the former charge repulsion between the terminal COOH groups prevents

effective aggregation to form the channel. This charge effect diminishes with increasing length - being small, but distinct, for the 1-17 fragments and negligible for alamethicin. Similarly, the hydrophobicity of the benzyloxycarbonyl group is evident in the 17 residue fragments, where Z-1-17-OMe is more active than Ac-1-17-OMe, but not so in alamethicin, where the peptide chain itself is probably sufficiently hydrophobic to ensure efficient partitioning into the membrane.

The results presented establish that the structural requirements for ionophoretic activity and for uncoupling mitochondria in the case of alamethicin fragments are the same and that these two parameters can be used to study the relationship between structure and activity for alamethicin and similar channel forming polypeptide ionophores.

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