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EFFECTS OF MEMBRANE CHANNEL-FORMING POLYPEPTIDES ON MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION. A COMPARISON OF ALAMETHICIN, GRAMICIDIN A, MELITTIN AND TETRAACETYL MELITTIN

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Summary Transmembrane channel-forming polypeptides can function as uncouplers of mitochondrial oxidative phosphorylation. The observed effects are dependent on the phosphate ion (P_i) concentration in the medium. At low P_i (2.5 mM) the order of uncoupling efficiencies is gramicidin A >> alamethicin > tetraacetyl melittin > melittin. The remarkably high activity of gramicidin A suggests insertion of preformed channel dimers into the membrane. It is also suggested that lipid phase association of peptides is necessary in the other cases. At P_i = 100 mM inhibitory effects are observed for alamethicin and tetraacetyl melittin. Less pronounced inhibition is seen for melittin, while no such effect is noted for gramicidin A. The site of inhibition is shown to be complex IV, and the differences in the behavior of the peptides are rationalized in terms of channel structures.

Introduction

Peptide ionophores function as uncouplers of mitochondrial oxidative phosphorylation, presumably by dissipating electrochemical gradients across the inner mitochondrial membrane (1). Transmembrane channel-forming polypeptides like alamethicin and gramicidin A have been shown to act as uncouplers of rat liver mitochondria (2-4). While gramicidin channels are selective for small cations (5), alamethicin ones exhibit much less discrimination (6-9) and are permeable even to larger ions like Tris or Hepes (10) and can also serve to transport anions (9, 10). Alamethicin-induced uncoupling has also been shown to be dependent on the concentration of phosphate (P_i) ions in the medium (3, 11). The role of P_i in this process is unclear. We compare in this report the effects of the peptides alamethicin, gramicidin A, melittin and its tetraacetyl derivative on oxidative phosphorylation in rat liver mitochondria, as a function of P_i concentration in the medium. Evidence for a P_i-dependent transition from uncoupling to

Abbreviations used: BSA, bovine serum albumin; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; P_i, inorganic phosphate; RCI, respiratory control index; TMPD, N,N'-tetramethyl-p-phenylenediamine; Tris, Tris (hydroxymethyl)aminomethane.

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inhibitory behavior is presented for alamethicin and tetraacetyl melittin. Differences in the effects of the various peptides are rationalized in terms of known structural features of these membrane channels.

Materials and Methods

Alamethicin was a gift sample from Upjohn Co., Kalamazoo, Michigan, and was used without further purification. Gramicidin A, melittin and antimycin were from Sigma Chemical Co. All other chemicals were of the highest available grade. Melittin was purified by chromatography over Sephadex G-50 (0.1 M acetic acid), followed by desalting over Sephadex G-10. Tetraacetyl melittin was prepared as described (12). Both melittin and tetraacetyl melittin were found to be > 90% pure by HPLC on a Lichrosorb RP-18 column, using an 80-90% methanol-water gradient, with 0.5% trifluoroacetic acid. Reduced cytochrome c was prepared from horse heart cytochrome c (Sigma, Type III) as described (13).

Mitochondria were prepared from rat liver (Wistar) by standard procedures (14). O_2 consumption was monitored using a Hansatech O_2 electrode. The assay solution (2 ml) contained D(-) mannitol (220 mM), sucrose (70 mM), HEPES (2 mM), EGTA (0.5 mM) and $MgCl_2$ (2.5 mM), pH 7.4. Succinate was added to a level of 7.5 mM, and P_i concentrations were varied by addition of KH_2PO_4. Alamethicin and gramicidin A stock solutions were prepared in ethanol and aliquots added to the mitochondrial suspension such that the alcohol volume did not exceed 10 µl. Melittin and tertraacetyl melittin were dissolved in water. All experiments were carried out at 33°C.

Results and Discussion

Figure 1 lists the suquences of the peptides examined in this study. The effects of peptide addition on the O_2 consumption rate of state 4 mitochondria are shown in Figure 2. The influence of P_i is compared at concentrations of 2.5 mM and 100 mM. At P_i = 2.5 mM all four peptides act as classical uncouplers. The efficiencies of uncoupling were determined by measuring the changes in respiratory control index (RCI) as a function of peptide concentration. The $\emptyset_{1/2}$ values (concentration of peptide necessary for half-maximal uncoupling activity) determined for the four peptides at 2.5 mM P_i are as follows: gramicidin A, 0.0005; alamethicin, 0.16; tetraacetyl melittin, 1.08; and melittin, 2.17 nanomoles per mg mitochondrial protein.

At 100 mM P_i, addition of 3 µM alamethicin clearly results in an inhibition of respiration (Figure 2). An initial short burst of O_2 consumption immediately follows peptide addition, prior to the onset of inhibition. A similar effect is noted in Figure 2 for tetraacetyl melittin. The transition to inhibitory behavior is much less pronounced for melittin, while no inhibitory tendencies are observed for gramicidin A at the concentrations studied. It should be noted that while the RCI values at 100

Alamethicin I (II): Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val
(Aib)
-Aib-Aib-Glu-Gln-Phol

Gramicidin A : For-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp
-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$

Melittin : Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu
-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2

Tetraacetyl Melittin : Melittin acetylated at α-amino group and ϵ-amino group of Lysines 7, 21, 23

Fig. 1. Sequences of peptides used.

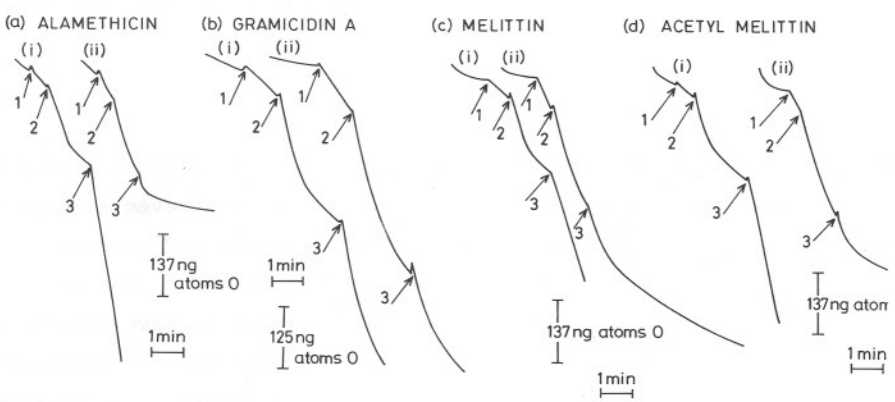

Fig. 2. A comparison of the effects of a) alamethicin b) gramicidin A c) melittin and d) tetraacetyl melittin on state 4 respiration of rat liver mitochondria suspended in media containing i) 2.5 mM P_i and ii) 100 mM P_i. Peptide concentrations, ∿ 3 μM; mitochondrial protein concentration, ∿ 0.5 mg/ml. Arrows 1, 2, 3 indicate points of addition of succinate (7.5 mM), ADP (72 μM [a, c, d] or 131 μM [b]) and peptide, respectively. Oxygen concentrations are specified assuming a saturating concentration of 480 nanogram atoms/ml at room temperature. All measurements were made at 33°C. However, no correction has been made for the saturating oxygen concentration.

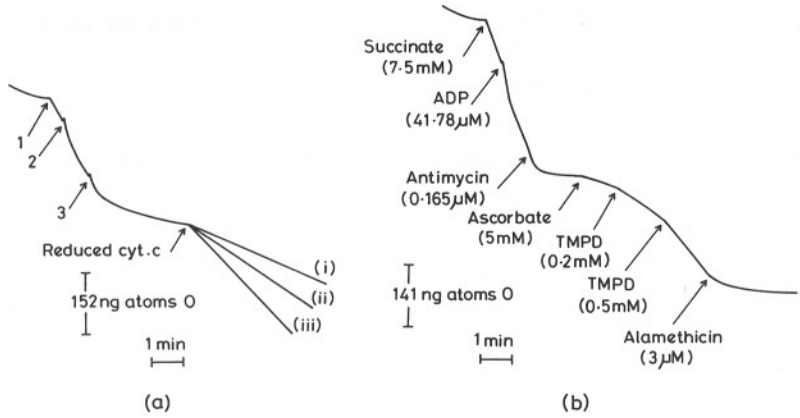

Fig. 3. a) Effect of addition of cytochrome c (reduced) on alamethicin-inhibited mitochondria at 100 mM P_i. Mitochondrial protein concentration, ∿ 0.4 mg/ml. Arrows 1, 2, 3 mark the points of addition of succinate (7.5 mM), ADP (41 μM) and alamethicin (3 μM), respectively. Reduced cytochrome c was added at concentrations of i) 0.42 μM, ii) 0.83 μM, or iii) 1.24 μM. No further change in O_2 consumption rate was observed at higher concentrations of cytochrome c. b) Effect of alamethicin on antimycin-inhibited mitochondria using ascorbate-TMPD as electron source. All conditions as described in Figure 3a. Additions to the mitochondrial suspension are indicated in the Figure.

mM P_i (RCI∿2-4) are significantly lower as compared to those at 2.5 mM P_i (RCI∿5-6), a significant degree of coupling does exist even at high P_i levels. Respiration studies were carried out in the presence and absence of BSA (7.2 μM). At high P_i concentrations (> 50 mM) the presence of BSA results in an enhancement of the RCI, but no significant differences have been noted for peptide-induced effects. Under the conditions used in the present study the inhibitory effects noted for alamethicin were only partial at peptide concentration less than 3 μM.

The inhibitory effects of alamethicin and tetraacetyl melittin are reversed by addition of reduced cytochrome c. Figure 3a shows the effect of varying concentraions of cytochrome c on the O_2 consumption rate of mitochondria inhibited by alamethicin at 100 mM P_i. Mitochondria inhibited by blocking electron transport at complex III with antimycin can be induced to consume O_2 by permitting electron flow to complex IV, by addition of ascorbate and N,N'-tetramethyl-p-phenylenediamine (TMPD) (15). Addition of alamethicin in such a situation also results in inhibition (Figure 3b). These observations suggest that the inhibitory effects of the peptide are mediated

by interactions involving complex IV of the electron transport chain (cytochrome c - cytochrome oxidase system).

The results presented above demonstrate that gramicidin A is the most efficient uncoupler of the peptides studied. $\emptyset_{1/2}$ values determined for gramicidin A are at least three orders of magnitude lower than those for the other three peptides. This observation is pertinent in view of the suggestion that gramicidin A adopts a dimeric structure in homogeneous solutions even at low concentrations (16). A head-to-head π_{LD} structure has been strongly favoured for the functional transmembrane channel (17, 18). It is likely that preformed peptide dimers insert into the membrane from the aqueous interface, thus allowing channel formation and uncoupling activity, even at very low peptide concentration. In contrast, alamethicin channels involve aggregation of several peptide molecules in the lipid phase (6-8,19,20). It is then likely that much higher peptide concentrations are necessary before formation of functional channels can occur. Similar membrane phase association of melittin may be implicated in its activity as a membrane lytic agent (21) and in formation of ion channels across artificial lipid bilayers (22). The enhanced activity of the peracetylated derivative may reflect its more facile association in the lipid phase to form funcitonal channels. Electrostatic repulsions between the basic C-treminal segments may destabilize melittin aggregates in the membrane.

The observed inhibition at complex IV by alamethicin at high P_i concentrations may be a consequence of direct peptide interactions with the cytochrome oxidase system (10) or a result of P_i equilibration across the inner mitochondrial membrane. This may be a consequence of the transport of the anion through alamethicin channels. At high P_i levels, mitochondria swell to considerable extents. Under these conditions interactions among membrane components, including peptide aggregates, may be modified. It is noteworthy that cytochrome oxidase activity in Keilin-Hartree particles has been shown to be significantly affected at $P_i > 25$ mM. The high-affinity active site on beef heart cytochrome oxidase is inhibited under these conditions (23). While complete abolition of cytochrome oxidase activity has not been noted in earlier studies at high P_i, it is likely that the behavior of the enzyme in intact mitochondria may differ from that observed in purified or partially purified enzyme preparations. The reversal of alamethicin inhibition by addition of cytochrome c supports the view that P_i effects on cytochrome c - cytochrome oxidase interactions (23) may be responsible for the observed phenomenon. A direct effect of the peptide on this interaction cannot be definitely excluded at this time.

Tetraacetyl melittin presumably functions as an inhibitor by a similar mechanism. The reduced effect of melittin may reflect its lower efficiency in equilibrating P_i across the membrane. The observed lack of inhibition in the case of gramicidin A may then be indicative of the inability of the peptide channel to permit passage of the P_i anion. This is consistent with the known properties of the gramicidin channel, where specificity of cation transport is ensured by interactions with the negative end of carbonyl group dipoles lining the channel interior (5,17,18). On the contrary, alamethicin channels have been postulated to have a nonpolar inner surface with a transmembrane aqueous core, permitting passage of both anions and cations (6,20). The above results suggest that peptide effects on mitochondrial respiration may prove a convenient means for examining peptide channel characteristics in natural membranes. These observations may also be of value in further studies of protein-protein interactions at complex IV of the respiratory chain.

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