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Channel Formation in Phospholipid Bilayer Membranes by the Toxin of *Heminthosporium maydis*, Race T

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Summary. Southern Corn Leaf Blight is caused by a toxin produced by a virulent form of *Helminthosporium maydis* (Race T). The toxin has been shown to uncouple oxidative phosphorylation and dissipate Ca^{2+} gradients in mitochondria isolated from susceptible, but not resistant, corn. The possibility that the toxin acted by increasing the permeability of membranes to ions was tested using a planar bilayer membrane system. Addition of the toxin to the bilayer system, under voltage-clamp conditions, resulted in stepwise increases in current across the phospholipid bilayer, a response characteristic for channel formers. Single-channel conductance in 1 M KCl is 27 pS which corresponds to 1.7×10^7 ions sec^{-1} $channel^{-1}$ at 100 mV applied potential. The toxin channels are: (i) fairly uniform in conductance, (ii) ideally selective for K^+ over Cl^-, and (iii) most conductive to H^+. The channel showed the following selectivity for alkali metal cations: $Rb^+ > K^+ > Cs^+ > Na^+ > Li^+$ (16 : 9 : 7 : 3 : 1) based on the most frequently observed conductance in 1 M chloride salts. The toxin showed no voltage dependence over the range of −100 to +100 mV. Channel formation was also a property of a synthetic analog (Cmpd IV) of the toxin. The ability of the native toxin to form channels may be a mode of toxin action on mitochondrial membranes from susceptible corn.

Key Words host-specific pathotoxin · ionophore · cations · permeability · corn

Introduction

Race T of the fungus *Helminthosporium* (*Cochliobolus*) *maydis* is the causative agent of Southern Corn Leaf Blight. A host-specific pathotoxin (HmT toxin), isolated from the fungal culture medium, produces the symptoms of the disease on susceptible corn (Smedegard-Peterson & Nelson, 1969; Hooker et al., 1970). Corn with Texas male-sterile cytoplasm is susceptible to the toxin, while normal male-fertile corn is resistant. Both the characteristics of male-sterility and susceptibility to the toxin are maternally inherited, indicating that these characteristics may be coded for by extranuclear genomes, such as the mitochondrial DNA (Levings & Pring, 1979). HmT toxin is composed of at least seven closely related polyketol components with the hydrocarbon chain length varying from 35 to 45 carbons (Kono & Daly, 1979; Kono et al., 1980, 1981). The structure of the most common component of the toxin mixture (Band 1) is shown in the inset of Fig. 1. Four major components have been separated and shown to have the same activity as the native toxin mixture (Payne et al., 1980*a*).

The mode of toxin action is not understood (Daly, 1981 and references therein). Miller and Koeppe (1971) showed that mitochondrial respiration in susceptible corn was affected by the toxin. A stimulation of respiration and uncoupling of oxidative phosphorylation were seen in response to the toxin when respiration was driven by exogenous NADH or succinate (Miller & Koeppe, 1971; Peterson et al., 1975; Payne et al., 1980*b*). The behavior of the toxin in susceptible mitochondria was not completely consistent with that of a classic uncoupler, as respiration was inhibited when malate was the substrate in these studies. This result suggested the toxin inhibited Complex I, or NADH-Ubiquinone oxidoreductase, of the electron transport chain. Recently, Kimber and Sze (1984) showed that the toxin decreased Ca^{2+} accumulation into susceptible mitochondria. The decrease was caused by an increase in mitochondrial membrane permeability to Ca^{2+} and, perhaps, H^+, induced by the toxin (Berville et al., 1984; Holden & Sze, 1984). These effects could be seen with 1 to 10 ng/ml (1.3 to 13 nM) of purified HmT toxin, while mitochondria from resistant corn showed little or no effect when treated with toxin up to 1 μg/ml. HmT toxin may dissipate Ca^{2+} and H^+ gradients in susceptible mitochondria by either indirectly facilitating the movement of ions across the membrane or directly in the manner of an ionophore, as has been suggested (Payne et al., 1980*b*; Daly, 1981). We investigated this question by studying the behavior of HmT toxin in a model membrane system, planar

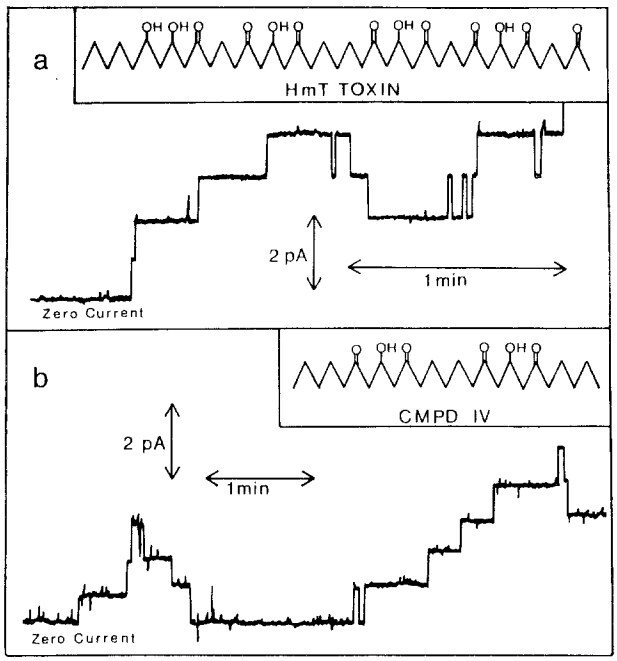

Fig. 1. (*a*) Recorder tracing of step changes in current across an asolectin bilayer subsequent to the addition of HmT toxin (1 ng/ml, final concentration) to the bilayer chamber containing 1 M KCl. The voltage was clamped at 40 mV. *Inset:* Assigned structure of Band 1 (mol wt = 768), the most common component of the native HmT toxin mixture, as determined by Kono and Daly (1979). (*b*) Recorder tracing of an experiment conducted in the same manner with the synthetic analog Cmpd IV (mol wt = 440). Final concentration was 40 ng/ml

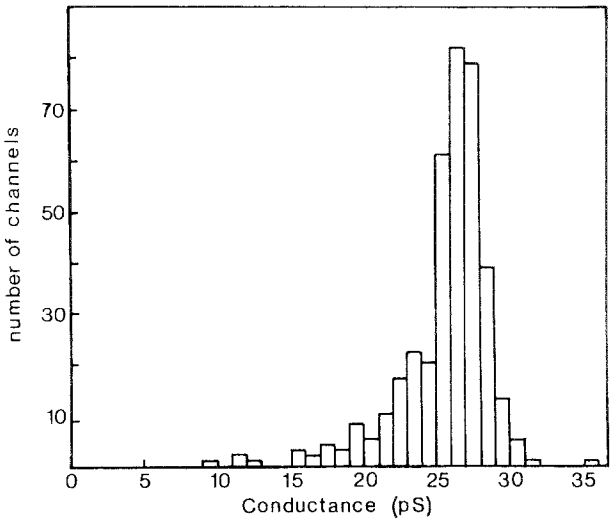

Fig. 2. Histogram of the conductances of single channels formed by HmT toxin in an asolectin bilayer (1 M KCl + 1 mM $CaCl_2$). Measurements were made using recorder tracings of current across bilayers with only a few channels present at any point in time, as in Fig. 1

phospholipid bilayers. Here, we show that HmT toxin forms ion channels in phospholipid bilayers.

Materials and Methods

Planar phospholipid bilayers were made by apposition of monolayers across a hole (0.1 to 0.2 mm in diameter) in a Saran® (Dow Chemical Co.) partition separating the two compartments of a Teflon® chamber, according to the method of Montal and Mueller (1972) with the modifications of Schein, Colombini and Finkelstein (1976). The partition was pretreated with a thin layer of petrolatum. All experiments were performed under voltage-clamp conditions. Either calomel electrodes, with saturated KCl bridges, or Ag-AgCl electrodes, without bridges (with symmetrical salt solutions and Cl^- as the anion), were used to interface with 5 ml of an aqueous solution bathing each side of the bilayer.

Phospholipids used for these studies were either asolectin (soybean phospholipids), purified by the method of Kagawa and Racker (1971), or a single phospholipid species, diphytanoyl phosphatidylcholine. HmT toxin (5 to 50 μl dissolved in dimethyl sulfoxide) was added to aqueous solutions (5 ml volume) on both sides of the membrane in most experiments (the observed behavior was the same if the toxin was added to only one side). Dimethyl sulfoxide (DMSO) alone, at concentrations up to 7% (vol/vol), did not cause any increased membrane permeability to ions in control experiments.

Asolectin was purchased from Sigma Chemical Co., St. Louis, Mo., and diphytanoyl phosphatidylcholine (DPPC) from Avanti Biochemicals Inc., Birmingham, Alabama. All other chemicals were reagent grade. Purified HmT toxin and the toxin analog, Cmpd IV, were generous gifts of Drs. J. M. Daly, University of Nebraska, Lincoln, Neb. and Y. Suzuki, Institute of Physical and Chemical Research, Wako-Shi, Saitama 351, Japan.

Results

Ion Channels

The addition of an aliquot of HmT toxin to 1 M KCl solution bathing the membrane (final concentration of 0.5 to 1.0 ng/ml HmT toxin; 0.1% DMSO) resulted in discrete step increases in current in the presence of an applied electrical potential (Fig. 1*a*). These steps were detected within one to a few minutes after the addition of the toxin to the chamber and correspond to increases in the ion permeability or conductance of the membrane. The stepwise increase in transmembrane current is characteristic of channel-forming molecules. The individual current increments induced by the native toxin had discrete lifetimes ranging from a few seconds to a few minutes.

The possibility that contaminants in the toxin sample were responsible for the observed channels, was tested by determining whether a synthetic analog of the toxin would also form channels. Several synthetic analogs to the toxin have been prepared (Suzuki et al., 1982*a*, 1983). One of these, Cmpd IV,

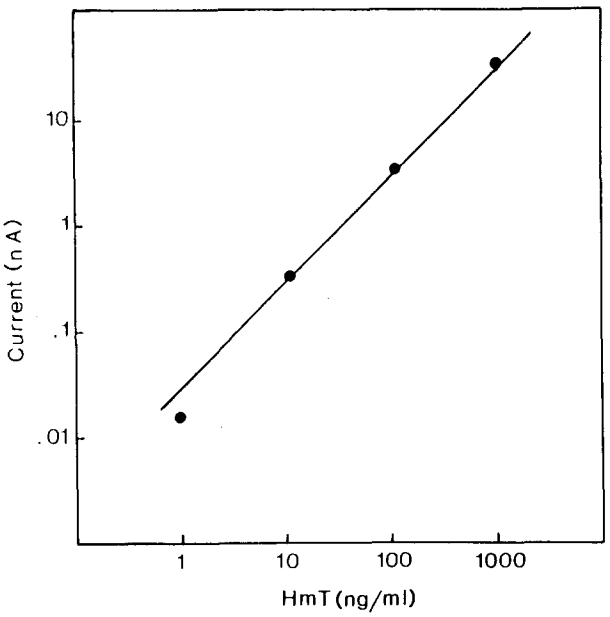

Fig. 3. The steady-state current across an asolectin bilayer as a function of the toxin concentration. Bathing solution was 1 M KCl + 1 mM $CaCl_2$ and voltage was clamped at −40 mV. With each addition of HmT toxin, the current was allowed to reach a steady state prior to the next toxin addition. The line is drawn with a slope of one. DMSO concentration reached 1.3% with the final toxin addition

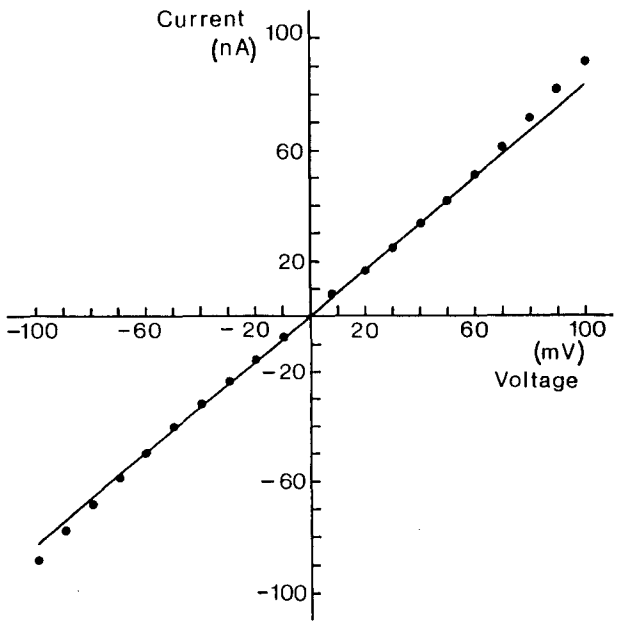

Fig. 4. Effect of applied voltage on current flow through toxin channels in an asolectin bilayer. The aqueous phase was 1 M KCl and the HmT toxin concentration was 1 μg/ml. The straight line shows the linear relationship. At high voltages there are slight deviations from linearity

has a chain length of 25 carbons and a structure that is analogous to the midportion of the toxin hydrocarbon chain. Cmpd IV has similar qualitative effects on susceptible mitochondria as the native toxin (Suzuki et al., 1982*b*; Holden & Sze, 1983). A 10-fold increase in concentration was required to show the same quantitative effects as the native toxin. Figure 1*b* shows that single channels were observed upon the addition of Cmpd IV to bilayer membranes in the presence of 1 M KCl. Thus channel formation is a property of the native toxin. Studies were undertaken to characterize the channel formed by the native toxin.

It was possible to record many individual step increases and decreases in conductance if the final concentration of HmT toxin in the bulk salt phase was kept low (1.0 ng/ml or less). A histogram of these conductance fluctuations (Fig. 2) indicates a fairly uniform channel population with the most commonly observed conductance being 27 pS. A small population of slightly lower conductances may also be present.

The observed membrane current was proportional to the amount of toxin added to the aqueous phase. Increased amounts of toxin resulted in an increase in the number of conductance events. With each toxin addition, an increase in current was observed which reached a steady state (15 to 30 min) indicating that an equilibrium had occurred between toxin in the membrane and toxin in the aqueous phase.[1] Figure 3 shows the steady-state conductance as a function of final toxin concentration. For each 10-fold increase in toxin concentration, a 10-fold increase in conductance was noted. Since the channels are ohmic (Fig. 4), the results show that the conductance is proportional to the toxin concentration to the first power. The linear dependence of conductance on concentration suggests that a single toxin molecule is capable of forming a channel.

The possibility that the channel formed by HmT toxin was voltage dependent was investigated by measuring current flow through the channels as a function of voltage. Figure 4 shows a nearly linear dependence of current on applied voltage, in the range of −100 to +100 mV. This generally ohmic behavior indicates that the conductance of the toxin channel does not depend on the transmembrane voltage. The slight deviation from ohmic behavior at high voltages may be due to the energetics of ion translocation. This result would be expected if the rate-limiting step to ion flow through the channel

[1] Perfusion of the chamber with toxin-free solution, after a steady-state current was achieved, resulted in a drop in conductance at about half the rate of channel insertion.

Table. Selectivity sequence for monovalent cations

Cation	Conductance (pS)[a]	Relative selectivity
Rb^+	47	16
K^+	27	9
Cs^+	20	7
Na^+	9	3
Li^+	3	1

[a] Conductance values correspond to the conductance most frequently observed in the presence of 1 M chloride solutions of the alkali cations + 1 mM $CaCl_2$ (asolectin membranes were used). A histogram of the conductances of 100 to 300 individual channels was constructed (as in Fig. 2) for each cation.

were not at the channel's mouth but in the middle of the channel (Krasne, 1980).

Channel Selectivity

The selectivity of the toxin channel for cations (K^+) versus anions (Cl^-) was determined. A twofold gradient of KCl was imposed across the membrane (0.2 *vs.* 0.1 M). Sufficient sucrose was added to the low-salt solution to equalize the osmotic pressures. Addition of HmT toxin to the aqueous phase resulted in the generation of a current whose reversal potential was determined to be −15.0 mV (±0.29 mV, 1 SD), negative on the high-salt side. This value represents the average of four experiments. As an internal control, sufficient valinomycin was added to increase the membrane conductance 10- to 30-fold, thus masking the current due to the toxin. The reversal potential of this valinomycin-induced current was −15.0 mV (±0.32 mV). The mean difference between the HmT toxin and the valinomycin reversal potentials (HmT toxin − valinomycin, for each experiment) was determined to be 0.0 mV (±0.14 mV). Valinomycin has been shown to be ideally selective for cations over anions (Mueller & Rudin, 1967). Since there is no significant difference between the toxin reversal potential and the valinomycin reversal potential, the toxin is also considered ideally selective to cations.

The selectivity of HmT toxin for alkali cations was explored by measuring the single-channel conductance in the presence of 1 M chloride salt solutions of the alkali metal cations. Additionally each solution contained 1 mM $CaCl_2$ for membrane stability. A histogram of single-channel conductances was prepared for each cation and the most frequently observed conductance was used to determine the selectivity sequence. Results show that Rb^+ was the most permeable ion and Li^+ the least permeable (Table).

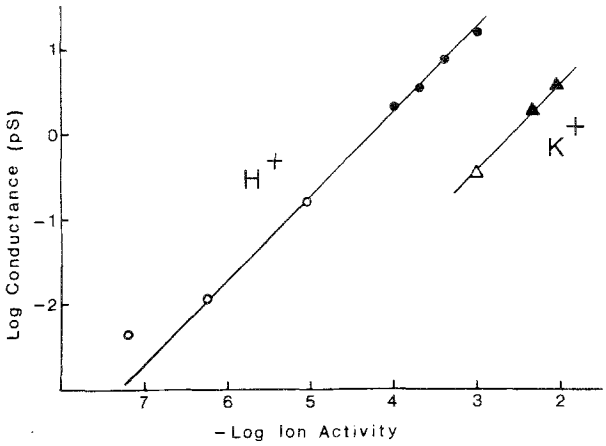

Fig. 5. Channel conductance as a function of proton and potassium concentration in diphytanoyl phosphatidylcholine bilayers. Proton concentration was varied over the range of pH 7 to pH 3 using a buffer system of Tris-succinate. Additionally, 5 mM Tris-Cl (pH 7) was included as Ag-AgCl electrodes were used. KCl solutions were buffered to pH 7.0 with Tris-succinate. Conductance was measured either directly or indirectly. Between pH 3 and pH 4 as well as 5 mM and higher concentrations of KCl it was possible to observe single channels. Many individual channels were measured and the plotted value represents the averaged value (closed symbols). At lower concentrations of H^+ and K^+, an indirect method was used. Measurement was made of a total stable current in the presence of toxin (30 to 200 ng/ml). Sufficient concentrated KCl was added to bring the [KCl] to 0.1 M. The conductance in 0.1 M KCl was divided by the single-channel conductance in 0.1 M KCl (value obtained from single-channel measurements) in order to obtain the total number of channels in the membrane. The conductance, prior to the addition of the concentrated KCl, was then divided by the number of channels in order to obtain a measure of the conductance per channel (open symbols)

The conductance of the toxin channel to protons over the range of pH 7 to pH 3 was investigated. Due to the low ion concentration, uncharged membranes were used (diphytanoyl phosphatidylcholine). The experimental points of single-channel conductance as a function of pH, fit nicely to a line of slope one (Fig. 5) indicating that, in this concentration range, the toxin's proton conductance is directly proportional to the proton activity.[2] Similar results were obtained for low K^+ concentrations. The single-channel conductance is 17.8 pS for 1 mM H^+ and 0.39 pS for 1 mM K^+. Thus the channels are 46 times more permeable to H^+ than K^+.

[2] The lines were drawn with a slope of one (rather than statistical best fit) because we expect the conductance to vary linearly with the ion activity. The only significant deviation is at pH 7. Here it is likely that the extra current is being carried by either the buffer or contaminating ions in the solution since there were five orders of magnitude more cations in the medium than protons.

Discussion

Planar phospholipid bilayers, normally quite impermeable to ions, can be rendered permeable to ions by the addition of purified HmT toxin. Increasing concentrations of toxin added to the bulk phase solution bathing the membrane resulted in increasing current across the membrane in response to an applied potential. This current was due to the formation of channels in the bilayer. That HmT toxin is a channel-former, rather than a carrier, is indicated by the magnitude and uniformity of the stepwise conductance increments. The calculated ion flux through the channel at 100 mV applied potential and in the presence of 1 M KCl is 1.7×10^7 ions per second, a value considered in excess of the capability of a carrier.

The channel observed with HmT toxin is a property of the native toxin molecule and not a contaminant. This conclusion is supported by the finding that channel formation is also a characteristic of the synthetic analog, Cmpd IV. However, as compared to the native HmT toxin, a higher concentration of Cmpd IV was required to generate an equivalent current. This may be related to the requirement for 10-fold higher concentrations of Cmpd IV, than of HmT toxin, in bioassays using mitochondria isolated from susceptible corn (Suzuki et al., 1982*b*; Holden & Sze, 1983). These results suggest that the toxin's chain length is important for optimal activity (Suzuki et al., 1982*b*).

We suggest that a single toxin molecule may be capable of forming a channel. The transmembrane current was linearly dependent on the amount of added toxin (Fig. 3). There are two obvious explanations for this behavior: (1) a single toxin molecule is capable of forming a channel, or (2) a regular aggregate exists in aqueous solution which inserts as a unit into the bilayer. While we cannot rule out the latter possibility, it is less likely for the following reason. Aggregation normally results in a polydispersed form rather than in a single form of defined composition. Since a linear function was observed over a concentration range spanning three orders of magnitude, the toxin is, most likely, monodispersed in the salt solution in this concentration range (significant aggregation would result in a sublinear behavior at the higher toxin concentrations). The experimental approach used here has proven to be valid when applied to other channel formers such as gramicidin (Tosteson et al., 1968; Goodall, 1970). We conclude that a single toxin molecule forms a channel.

While we do not yet have enough data to seriously propose a model for channel formation by the toxin, the single power dependence of the conductance on the toxin concentration seems to leave us with very few options. Space-filling models demonstrate that the toxin can form a helix with a nonpolar exterior and a hydrophilic interior. Helixes which were large enough to allow the passage of Cs^+ were only 12 to 14 Å long. For this to span a 40 to 45 Å bilayer would require a great deal of deformation. Such a deformation has been proposed by Marty and Finkelstein (1975) for the half-pore formed by nystatin added to only one side of a membrane. An alternate model proposes that the molecule forms a "hairpin" (28 Å in length). Figure 1 shows that the molecule contains four polar clusters, each consisting of three polar groups. In this hairpin model two binding sites are formed by pairing of these clusters. Although this model is more likely to span the membrane, it proposes that a two-stave barrel can form a channel.

Diffusion potential experiments showed that, within the limit of resolution, the toxin is ideally selective for K^+ over Cl^-. From this we infer that the toxin channel is cation selective. In an osmotically balanced salt gradient, the magnitude of the reversal potential for the toxin was essentially equal to that of valinomycin in the same membrane. Valinomycin is ideally selective for cations over anions and so serves as a control for the toxin (Mueller & Rudin, 1967; Rosenberg & Finkelstein, 1978).

A marked preference of the channel for protons was demonstrated by the 46-fold higher conductance of H^+ as compared to the conductance of K^+ at equivalent activity. Channel selectivity among alkali metal cations is not as great, being only 16-fold for Rb^+ over Li^+, the ions at opposite ends of the alkali metal spectrum. Thus, under the measurement conditions, no one alkali metal cation is highly favored. The selectivity sequence observed is Eisenman's Sequence III, signifying that the channel is characterized by low field-strength ligands binding the ions (Eisenman, 1961; Diamond & Wright, 1969; Eisenman & Horn, 1983). This is consistent with the known chemical structure of the toxin, which has carbonyl and hydroxyl groups as the only polar residues. The channel may also conduct divalent cations. Preliminary experiments indicate channel permeability to Ca^{2+} at 0.1 mM.

It is not clear how the ability of the toxin to form channels in soybean phospholipids or diphytanoyl phosphatidylcholine bilayers is related to the specific action of HmT toxin on mitochondria from susceptible corn. One possibility is that unique properties of the lipid components of susceptible mitochondria facilitate the partition of the hydrophobic toxin into the membrane allowing formation of ion channels. Thus, susceptible mitochondria might be more permissive to channel formation be-

cause the properties of the membrane lipids mimic in some fashion the lipids used to form the bilayer membrane. Another working model postulates a protein component of the susceptible mitochondrial membrane binding to the toxin. The arrangement of toxin molecules bound to the protein(s) may be similar to that required for channel formation in phospholipid bilayers. At high toxin concentrations (e.g. a few μg/ml), mitochondria from normal corn show slight sensitivity to HmT toxin (Payne et al., 1980*b*; Kimber & Sze, 1984). Susceptible mitochrondria may be quantitatively much more susceptible to channel formation due to either protein or lipid component differences. We do not yet know whether the dose of toxin needed to induce a given permeability in a bilayer membrane is comparable to that needed to induce the same permeability in susceptible mitochondria. These possibilities need to be tested.

In summary, both HmT toxin and a synthetic analog form cation-selective channels in phospholipid membranes. Formation of ion channels by the toxin in bilayers is consistent with the ability of the toxin to dissipate ion gradients in mitochondria isolated from susceptible corn (Payne et al., 1980*b*; Berville et al., 1984; Holden & Sze, 1984). These results support the idea that channel formation may be one mechanism of toxin action in susceptible mitochondria.

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