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Yeast K1 killer toxin forms ion channels in sensitive yeast spheroplasts and in artificial liposomes

(protein toxin/plasma membrane/patch clamp/cation conductances)

BORIS MARTINAC*, HONG ZHU†, ANDRZEJ KUBALSKI*, XINLIANG ZHOU*, MICHAEL CULBERTSON*, HOWARD BUSSEY†‡, AND CHING KUNG*

*Laboratory of Molecular Biology, University of Wisconsin–Madison, Madison, WI 53706; and †Department of Biology, McGill University, Montreal, PQ H3A 1B1, Canada

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ABSTRACT **The patch-clamp technique was used to examine the plasma membranes of sensitive yeast spheroplasts exposed to partially purified killer toxin preparations. Asolectin liposomes in which the toxin was incorporated were also examined. Excised inside-out patches from these preparations often revealed at 118 pS conductance appearing in pairs. The current through this conductance flickered rapidly among three states: dwelling mostly at the unit-open state, less frequently at the two-unit-open state, and more rarely at the closed state. Membrane voltages from −80 to 80 mV had little influence on the opening probability. The current reversed near the equilibrium potential of K^+ in asymmetric KCl solutions and also reversed near 0 mV at symmetric NaCl vs. KCl solutions. The two levels of the conductance were likely due to the toxin protein, as treatment of spheroplasts or liposomes with extracellular protein preparations from isogenic yeasts deleted for the toxin gene gave no such conductance levels. These results show that *in vivo* the killer-toxin fraction can form a cation channel that seldom closes regardless of membrane voltage. We suggest that this channel causes the death of sensitive yeast cells.**

K1 killer toxin is a secreted heterodimeric protein produced by strains of *Saccharomyces cerevisiae* that contain an M1 virus with a double-stranded RNA genome (for a review, see ref. 1). The K1 killer toxin kills sensitive yeast cells lacking the M1 virus, whereas M1 virus-containing cells are immune to the toxin they produce. Toxin production and specific immunity to the toxin are encoded on the M1 double-stranded RNA component of the viral genome (2–5).

The secreted toxin is processed from a larger precursor molecule that consists of an N-terminal leader, followed by the two toxin subunits, α and β which are separated by a central glycosylated γ peptide (ref. 4 and for a review, see ref. 6). Analysis of the predicted amino acid sequence of preprotoxin shows the α subunit possessing two highly hydrophobic regions (residues 72–91 and 112–127) separated by a short highly hydrophilic stretch of amino acids. It has been suggested that the hydrophobic α subunit is involved in ion-channel formation (4). The β subunit, however, lacks potential membrane-spanning regions. By analogy to the abrin and ricin class of toxins (7), this β subunit has been proposed to function by binding to a cell wall receptor required for toxin action (4). Previous genetic and biochemical studies of K1-killer-toxin action on sensitive yeast cells indicate a set of specific cell surface interactions. These include binding to a $(1 \rightarrow 6)$-β-D-glucan-containing cell wall receptor (8, 9). After wall binding, energy-dependent processes result in lethal physiological changes. Ion leakage at the plasma membrane appears to be a primary cause for the lethality. In metabolically active cells, a rapid inhibition of net proton pumping from the cells, along with an inhibition of K^+ and amino acid uptake, upon exposure to the killer toxin has been reported (10). Furthermore, intoxicated cells showed a reduced proton gradient across the plasma membrane, accompanied by acidification of the cytoplasm and K^+ efflux (11). At later stages of killer-toxin treatment, K^+, ATP, and small metabolites were lost from cells (12). Based on such data, it was suggested that the killer toxin perturbed an energized membrane state. Whether the toxin inhibited some component of the proton pump or acted more directly by forming a transmembrane protein channel remained unclear. The amphipathic character of killer-toxin protein (4) is consistent with the proposal that this toxin directly perturbs the cell membrane. Kagan (13) showed that a killer-toxin preparation from the yeast *Pichia kluyveri*, with physiological properties similar to the K1 killer toxin of *S. cerevisiae*, could form ion channels *in vitro* in a planar lipid bilayer.

Here, we report results of patch-clamp experiments that show that a fraction enriched in K1 toxin forms ion channels *in vivo* in sensitive yeast spheroplasts and *in vitro* when the K1 toxin is incorporated into artificial liposomes.

METHODS

Cells. The following strains of *S. cerevisiae* were used: (*i*) T158C/S14a, *MATa/MATα his4C-864/HIS4 ade2-5/ADE2* (ATCC 26427), a K1 killer strain containing the M1 virus. (*ii*) T158C/S14a HC, a sensitive nonkiller strain derived from T158C/S14a by heat curing of the M1 virus (14, 15). These strains were used as sources for killer-toxin preparations and toxin-free controls. (*iii*) AH22, *MATa leu2-3, 2-112 his4-519 can1*, a sensitive yeast strain, was used as a toxin-sensitive strain in studies designed to detect toxin-induced channel activity by patch-clamp examination of its spheroplasts. AH22 was transformed with a killer expression plasmid, pL308 (5, 16); such transformants secreted K1 killer toxin. In addition AH22 was transformed with a control plasmid pL361 (16), which lacked the killer toxin gene. (*iv*) H4A, *MATα his3 ura3*, was transformed with a killer-toxin mutation plasmid, ZH20, in which Ile-129 in the C terminus of the α subunit was replaced by Arg-129. Concentrated extracellular protein preparations from these transformants were used as sources of killer toxin or as toxin-free controls.

Yeast Concentrated Culture Filtrates and Partially Purified Toxin Preparations. Yeast cells were grown at 18°C in liquid minimal medium (pH 4.7) containing 1× Halvorson salt (17), 2% (wt/vol) glucose, and 10% (vol/vol) glycerol. After the cultures entered the stationary phase of growth, the cells were pelleted by centrifugation. The medium was removed and concentrated between 200 and 1000 times by ultrafiltra-

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‡To whom reprint requests should be addressed.

tion using a PM10 membrane (Amicon). Active wild-type killer toxin (from strain T158C/S14a) was partially purified by affinity chromatography using pustulan, a wall receptor (1→6)-β-D-glucan analog coupled to Sepharose as described (8). Some 70% of the protein in such preparations was K1 killer toxin, based on Coomassie brillant blue staining after SDS/polyacrylamide gel electrophoresis.

Toxin Activity Assay. Toxin activity was tested by a standard killing-zone assay. Sensitive yeast cells (AH22) were seeded into a YEPD agar plate. An aliquot of concentrated toxin culture filtrate was then spotted on top of the plate. The inhibition of cell growth was shown by formation of a clear zone around the spotted culture filtrate (18).

Yeast Spheroplast Preparation. Killer-sensitive strain, AH22, was grown at 30°C YEPD (pH 4.7) to the early stage of logarithmic growth, and the affinity-purified toxin was added at ≈5 μg/ml of cell culture, which was allowed to grow for another 15–20 min. The yeast cells were then subjected to yeast lytic enzyme (73,400 units/g from ICN) digestion at 80 μg/ml in a solution of 1 M sorbitol, 0.1 mM Na_2EDTA, and 5 mM Hepes (pH 7.2) for 20 min at 30°C (19). The yeast spheroplasts were patch-clamped immediately in the whole-cell mode. The pipet and bath solutions contained CsCl or NaCl instead of KCl to suppress the activity of the K^+ channel reported in yeast spheroplasts (20). To maintain killer-toxin activity, the bath solution was always adjusted to pH 4.7 by Mes and the pipet solution was adjusted to pH 7.2 by Hepes.

Preparation of Liposome Vesicles Incorporating Toxin. Liposome vesicles were prepared by using asolectin (Sigma catalog no. P 5638) as described by Delcour *et al.* (21), with the following modifications. The pH difference between pipet and bath solution was as described for spheroplasts. The K1 killer toxin was incorporated into the lipids at a toxin/lipid molar ratio of 1:1000 in the solution of 100 mM KCl plus 1× Halvorson salts (18) containing 50 mM succinic acid, 30 mM $(NH_4)_2SO_4$, 50 mM K_2HPO_4, 2.7 mM $CaCl_2$, and 4.1 mM $MgSO_4$, by using either partially purified toxin or toxin-containing concentrated culture filtrates from killer strain T158C/S14a or AH22 transformed with killer expression plasmid pL308. Toxin incorporation was tested by spotting the redissolved lipid/protein mixture onto an agar plate seeded with sensitive yeast cells and scoring for the formation of a killing zone. Concentrated culture filtrates without killer toxin came from either strain T158C/S14aHC or from AH22 transformed with pL361 (the expression plasmid lacking the toxin gene) and were used as controls for incorporation with lipids under the conditions described above.

Patch-Clamp Techniques. Conventional patch-clamp techniques were used (22) at room temperature. All recordings from liposomes were obtained from inside-out membrane patches excised from toxin-incorporated azolectin liposomes. The recordings from spheroplasts exposed to the partially purified toxin were obtained from inside-out excised patches or from whole spheroplasts. Data were stored in an FM tape recorder (Gould 6500), digitized off-line at a rate of 0.2 msec, and analyzed on computer (Indec) with a program developed by Yoshiro Saimi (University of Wisconsin, Madison). Amplitude histograms and current–voltage relations were plotted using this program.

RESULTS

Spheroplasts from sensitive cells previously treated with partially purified killer toxin yielded patches that often contained a characteristic channel activity (Fig. 1*B*) (9 out of 14 patches). In contrast, out of 14 patches excised from spheroplasts from cells incubated with concentrates of secreted

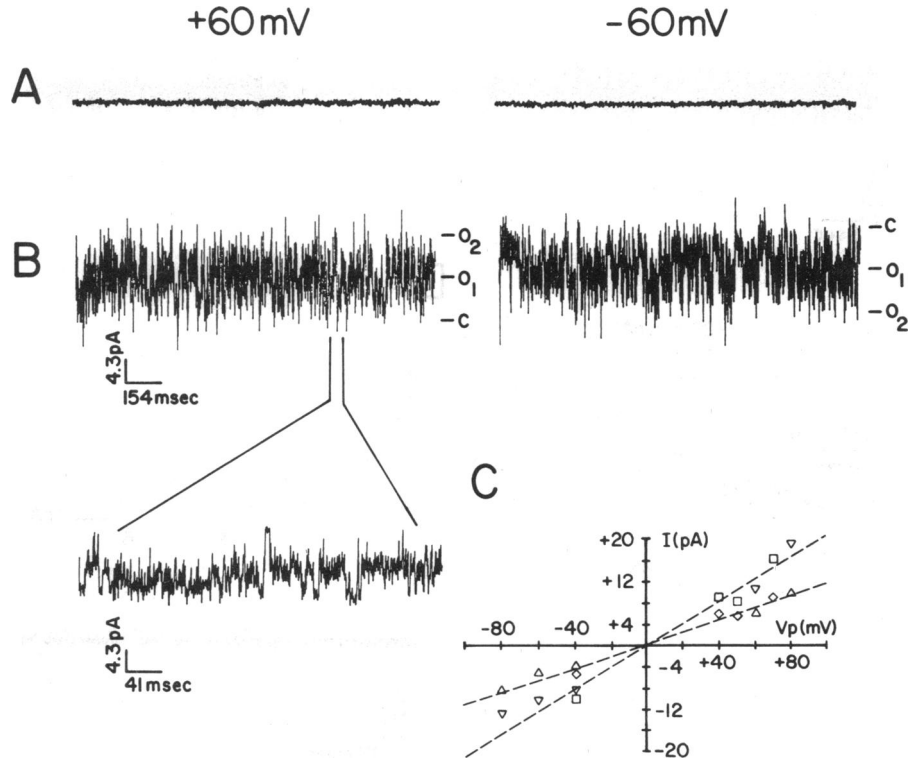

FIG. 1. (*A*) Absence of killer-toxin channel activity in excised patches and whole spheroplasts containing toxin-free extracellular proteins from T158C/S14a HC. (*B*) Channel activity of excised patch from yeast spheroplasts exposed to partially purified killer toxin from T158C/S14a. Channel activity was measured at ±60 mV in symmetric Cs. The trace at +60 mV is expanded below at a higher time resolution. C and O_1 and O_2 denote closed and one-unit- and two-unit-open levels of the channel, respectively. (*C*) Average single-conductance levels from two experiments were 114.4 and 220.7 pS. NP_o [opening probability of the total unknown number of channels (*N*) in the patch] values for these two traces were 2.1 and 2.0. This indicates that the channel is not voltage sensitive. I, current; V_p, voltage.

yeast proteins from an isogenic strain lacking the toxin gene, none was found to have the activity of such a channel (Fig. 1*A*). This activity was also never encountered in >500 patches from untreated yeast spheroplasts examined for this study or for other investigations of ion channels native to the yeast plasma membrane (refs. 19 and 20 and X.Z., unpublished observations). The current through this conductance flickered rapidly among three states with kinetics largely beyond the resolution of the present recording system (5 kHz), hence the spiky appearance of the records. This conductance often occurs in a pairwise manner. Currents through this conductance appear to dwell mostly at a one-unit level but frequently visit a two-unit level, or a zero-current (closed) level (marked O_1, O_2, C, respectively, in Fig. 1*B*). The levels were determined by the rarer events when longer dwell times provide the flat-top or flat-bottom records as shown in the lower trace of Fig. 1*B*. The kinetics of the conductance are not markedly affected by voltage (compare the left and right sides of Fig. 1*B*). The current–voltage relation from two experiments shows the unit conductance to be 114 pS in magnitude (Fig. 1*C*). In these two cases, no change in the open probability of these channels was observed in the range of voltages plotted. We found both spheroplast patches and whole spheroplasts from toxin-treated cells to be unstable and easily broken, especially at high voltages (≥60 mV). We therefore concentrated on using the toxin-incorporated liposomes to characterize the toxin-related channels.

The channels found in toxin-incorporated liposomes had the same fingerprint as those in spheroplasts from toxin-treated cells (Fig. 2*B*). With the toxin/lipid ratio of our incorporation experiments, we encountered this channel about 25 times out of 80 patches. No such channel activities were observed in liposomes incorporated with the non-toxin-containing control filtrates (Fig. 2*A*). As in spheroplasts, the conductance flickered rapidly among the three states, which could be resolved at higher time resolution (Fig. 2*D*, lower trace). An amplitude histogram of such activities over 30 sec shows that the conductance most often appeared at its unit-open (O_1) state (Fig. 2*D*). A separate distribution peaking at the two-unit-open (O_2) state is also evident. The closed state (C) was apparently rarely visited, as shown by the lack of points at the zero-current level. Fig. 2*C* shows the unit currents and the two-unit currents at different voltages (between −80 and +80 mV) from two separate experiments. Linear regression analysis shows the unit conductance to be 118 pS and the two-unit conductance to be 222 pS.

We altered the pipet solution and the bath solutions to investigate the ion specificity of this conductance. With asymmetric KCl solutions, the reversal potential of the unit current is near the calculated equilibrium potential for K^+, indicating little or no Cl^- permeation (Fig. 3*A*). In Na^+/K^+

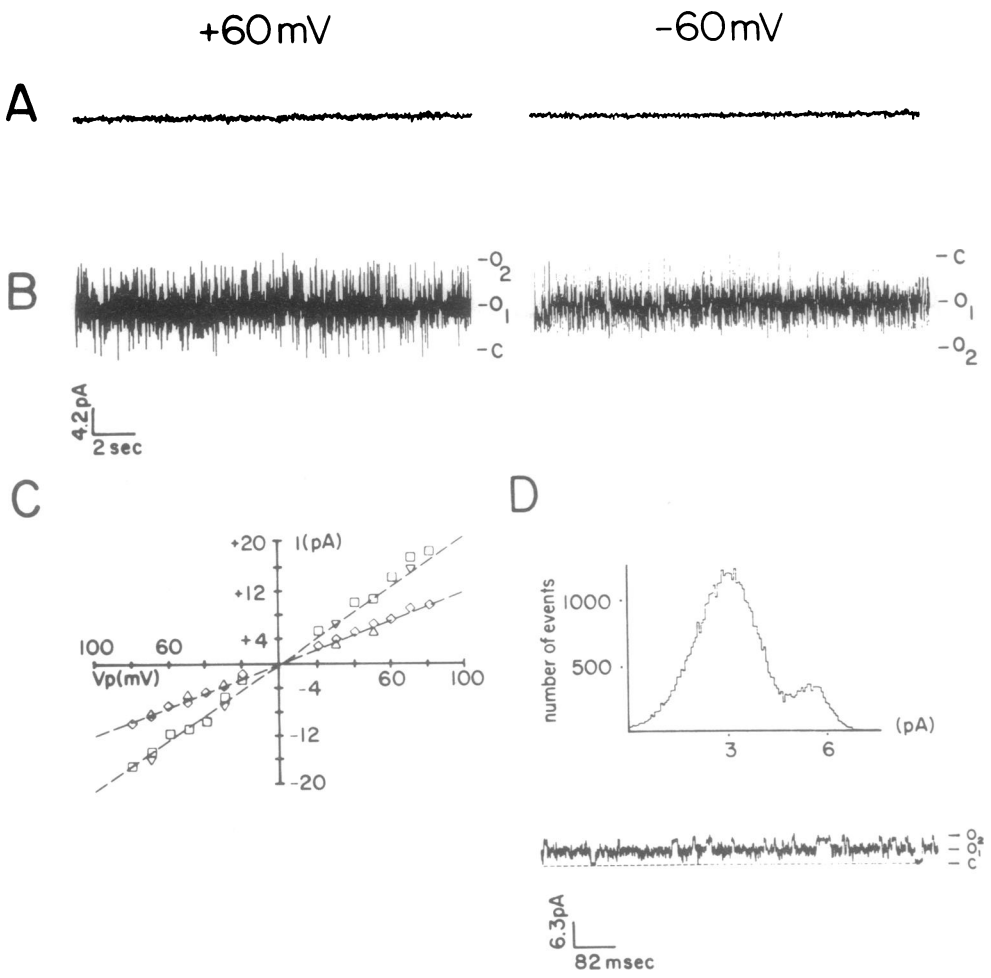

FIG. 2. (*A*) Activity of patches from liposomes containing extracellular protein extracts of yeast strain T158C/S14a HC lacking killer toxin. (*B*) Traces of channel activity from reconstituted killer toxin recorded in symmetric 150 mM K. This channel activity could be seen in one out of three or four patches (+60 and −60 mV). (*C*) Current–voltage (I–V_p) plots from two experiments. As can be seen in *B*, there were two levels of conductance. In these two experiments average single conductance was 118.4 pS (diamonds and triangles) and average double conductance was 221.7 pS (squares and inverted triangles). (*D*) Amplitude histogram for +30 mV, trace shown below (reconstituted system, symmetric 150 mM K).

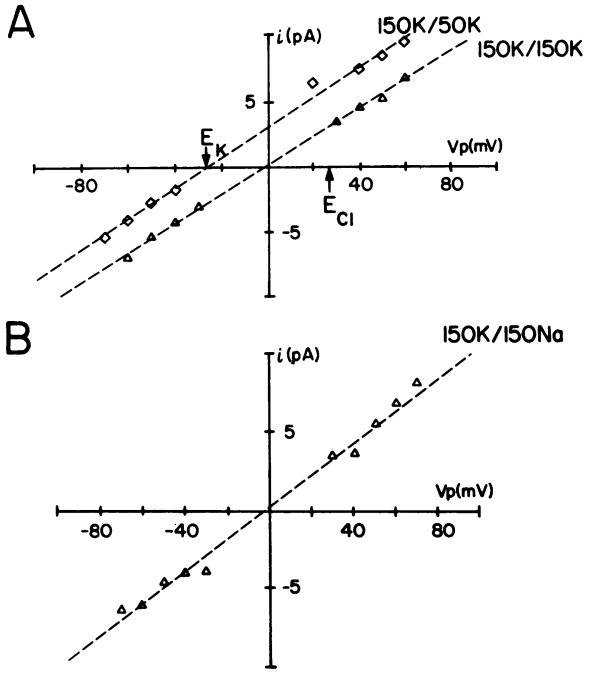

FIG. 3. (*A*) Current–voltage (i–V_p) plot for asymmetric K solution (150/50 mM K pipet/bath reconstituted system). The obtained reversal potential E_{rev} = −25.4 mV and the expected E_K, equilibrium potential of K^+, is −27.7 mV (−25.9 mV, after activity-coefficient correction). The expected E_{Cl} is +27.7 mV. Similar results were obtained in three other experiments. (*B*) Current–voltage (i–V_p) plot for K/Na (150 mM KCl in the pipet/150 mM NaCl in the bath reconstituted system; the same experiment as in *A*). E_{rev} in K/Na is −2.4 mV near 0 mV expected in K/K. There is no significant difference in conductance between K/K (110.1 pS) and K/Na (104.5 pS).

bi-ionic conditions, the current reversed near the origin, indicating no discrimination between these two ions (Fig. 3*B*).

The possible voltage sensitivity of this conductance was examined. No obvious changes in channel opening or closing with membrane voltages were encountered in all the patches examined (Figs. 1*B* and 2*B*). In three patches for which quantitative analyses were made, the opening probabilities of the conductance showed no systematic variations with membrane voltage from −80 to 80 mV (Fig. 4).

We also examined a mutant toxin from a strain harboring a mutation in the α subunit coding region of the toxin gene, ZH20. This mutant toxin, which has a specific activity some 10% of the wild type on yeast cells and spheroplasts, was

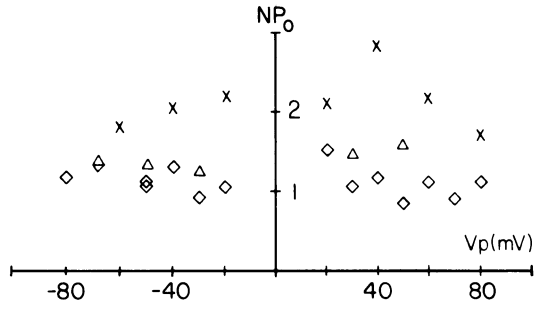

FIG. 4. Data points from three experiments indicate that the channel is not voltage sensitive. Average NP_o [opening probability of the total unknown number of channels (N) in the patch] value (X) was between 2 and 3, which indicates that more than one pair of channels was present in the patch. In spite of the large scatter however, there was no evident voltage dependency of the channel. V_p, voltage.

likely altered in some channel function. Out of 36 patches excised from liposomes incorporated with this ZH20 mutant toxin, we found no channel activities of the character described in this paper. In 8 cases we saw activities with different characteristics (data not shown), consistent with the detection of a mutant channel.

DISCUSSION

We have discovered the activities of a type of ion channel associated with a fraction highly enriched for the K1 toxin from killer strains of *S. cerevisiae*. The cation channel is capable of passing K^+, consistent with the observed K^+ bleeding during the process of cell intoxication (12). Such properties befit a destructive device that has evolved to kill sensitive cells by draining them electrically and energetically. We found that the channel does not discriminate between K^+ and Na^+. Testing H^+ permeation electrically is difficult with single channels because of its low concentration. That this toxin channel also drains the H^+ gradient, as suggested by de la Pena *et al.* (11), remains quite possible, although the present study shows that K^+ loss through this channel is apparently independent of cytoplasmic acidification. These conductance activities were not seen in untreated spheroplasts or liposomes, but how confident can we be that they are killer toxin specific? To demonstrate biochemically that the toxin causes the conductance is inherently difficult, for no matter how pure the protein toxin, one can argue that some very minor contaminant may be responsible for the currents detected by the extremely sensitive biophysical device—the patch clamp. Here although the toxin is the principal component of the partially purified extracellular protein preparations, other proteins are clearly present in minor amounts. The best evidence for specificity of toxin action is genetic. We are able to effectively delete the toxin gene in two independent ways, by deletion of the killer virion or by deletion of the toxin gene from an expression plasmid. When such deletions are made in otherwise isogenic yeast backgrounds, we find that preparations of extracellular proteins lacking only the toxin protein do not form the observed conductances in spheroplasts or liposomes. In addition to these negative controls, we have examined the conductance activities of a mutant toxin that, when incorporated into liposomes, show an altered activity. These controls are consistent with the conductance activities being killer toxin dependent and strongly suggest that these channels are the basis of killing by K1 toxin.

By using planar lipid bilayers, Kagan (13) showed that extracellular extracts containing a toxin from the yeast, *P. kluyveri*, form an *in vitro* channel with broadly similar features to that seen with K1 toxin, but the channels of the two toxins appear to differ considerably in detailed characteristics. The *P. kluyveri* toxin channel exists in two conductance states, of 140 and 220 pS, as compared to the 118- and 222-pS conductances seen with K1 toxin. These twin conductances hint at complexity, but as Kagan (13) has pointed out, whether these represent two distinct molecular species or two conductance states of the same channel is unclear. By using the present techniques, we cannot determine the structure of the channel, although presumably the hydrophobic α subunit, identified by site-directed mutagenesis as being necessary to kill spheroplasts, is involved. One may conjecture on structural grounds that a single monomer of the α subunit of the toxin, with its two transmembrane domains, is insufficient to line a water-filled pore. In this context, the known oligomerization of the toxin up to at least an octamer (23) may assume functional significance. The *P. kluyveri* toxin channel showed a marked voltage dependence at greater than 40 mV and less than −40 mV and only a weak selectivity for common physiological ions in the sequence

$K^+ > Na^+ > Ca^{2+} > Cl^-$. With K1 toxin, the conductance appeared to be voltage independent from −80 to 80 mV and to show a preference for monovalent cations. The kinetics of opening and closing of the *P. kluyveri* conductance were considerably slower than that seen with the K1 toxin, though this may be merely because the two toxins were examined by different techniques. An additional common feature of these toxins is that both function at acidic pH *in vivo*, although the basis for this pH dependence is unclear. Both here and in the Kagan study (13), toxin conductances were measured at pH 4.6, though detailed study of the pH dependence of these channels has not been made. With the K1 toxin, liposome incorporation of toxin was done at pH 4.6 but, after incorporation, toxin conductance appeared to be independent of a pH gradient from pH 4.6 to pH 7.2.

Our work demonstrates that killer toxin induced conductances *in vivo* in sensitive yeast spheroplasts and implicates such channels in the killing process. The fact that such conductances can be demonstrated *in vitro* in artificial azolectin liposomes does not necessarily imply that there are no additional components involved *in vivo* in toxin action at the plasma membrane. It was reported (24) that K1 toxin can kill spheroplasts made from insensitive cells of *Candida albicans*, *Candida utilis*, *Kluyveromyces lactis*, and *Schwanniomyces alluvius* but fails to kill spheroplasts made from the yeasts *Candida buinensis*, *Hansenula mrakii*, and *P. kluyveri*. Biologically there is additional complexity in the process of toxin immunity in toxin-producing cells. Immunity occurs at the plasma membrane and is conferred by the product of the toxin precursor gene, where mutations map to the region encoding the α subunit of the toxin (5, 25). To effect immunity, the precursor protein must in some way interfere with toxin-channel formation, but how this is achieved remains to be determined.

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