

A Molecular Target for Viral Killer Toxin: TOK1 Potassium Channels

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Summary

Killer strains of *S. cerevisiae* harbor double-stranded RNA viruses and secrete protein toxins that kill virus-free cells. The K1 killer toxin acts on sensitive yeast cells to perturb potassium homeostasis and cause cell death. Here, the toxin is shown to activate the plasma membrane potassium channel of *S. cerevisiae*, TOK1. Genetic deletion of *TOK1* confers toxin resistance; overexpression increases susceptibility. Cells expressing *TOK1* exhibit toxin-induced potassium flux; those without the gene do not. K1 toxin acts in the absence of other viral or yeast products: toxin synthesized from a cDNA increases open probability of single TOK1 channels (via reversible destabilization of closed states) whether channels are studied in yeast cells or *X. laevis* oocytes.

Introduction

Most strains of *Saccharomyces cerevisiae* carry one or more double-stranded RNA (dsRNA) viruses (Wickner, 1992). Killer strains secrete virally encoded protein toxins that are lethal to virus-free yeast strains; killer cells are immune to the toxin they release (Bussey, 1981; Bostian et al., 1984; Boone et al., 1986). The virus thereby confers a growth advantage to its host, increasing its survival in ecosystems of clinical, environmental, and industrial significance (Starmer et al., 1992; Wickner, 1996; Magliani et al., 1997). Killer strains are exploited in commercial fermentation (Van Vuuren and Jacobs, 1992), may influence the pathophysiology of opportunistic infections (Murphy et al., 1986; Pettoello-Mantovani et al., 1995), and offer candidates for novel antimycotic medications (Polonelli et al., 1986). Yeast viruses show striking parallels to pathogenic dsRNA viruses of higher eukaryotes (Cheng et al., 1994).

The type 1 killer system has at least two cytoplasmic dsRNA components, L-A and M1. L-A is a 4.6 kb helper virus encoding a Gag-Pol fusion protein that mediates

viral transcription, replication, and encapsulation (Icho and Wickner, 1989). M1 is a 1.8 kb satellite that carries the determinants for toxicity and immunity in a single open reading frame that encodes a K1 preprotoxin peptide (Thiele and Leibowitz, 1982; Bostian et al., 1984; Skipper et al., 1984). The precursor peptide has a δ - α - γ - β domain organization and is processed by *S. cerevisiae* proteases. The δ domain is a leader sequence that mediates folding and secretion; the γ domain is N-glycosylated and required for maturation. Mature toxin is secreted as an unglycosylated α - β heterodimer stabilized by three intersubunit disulfide bonds. Mutational analysis confirms the α - β product is necessary and sufficient to mediate cytotoxicity (Sturley et al., 1986; Zhu and Bussey, 1991). The β subunit (83 amino acids) is essential for binding to whole cells, while the α subunit (103 residues) is required to kill cells separated from the cell wall (spheroplasts) (Skipper et al., 1984; Boone et al., 1986; Sturley et al., 1986; Zhu and Bussey, 1991).

K1-mediated cytotoxicity proceeds in two steps. First, mature toxin binds to a 1,6- β -D-glucan receptor on the yeast cell wall; the receptor is present on both sensitive and immune cells, and its expression requires activity of *KRE* genes (Boone et al., 1990), most of which encode cell wall biosynthetic enzymes. Subsequently, toxin disrupts regulated ion flux across the plasma membrane, leading to cell death (Bussey et al., 1973; de la Pena et al., 1981). While most *kre* mutants do not bind toxin (and are therefore resistant to its action), spheroplasts produced from these strains are toxin sensitive (Bussey et al., 1973, 1990). In contrast, *kre3* and *kre12* mutants display normal toxin binding to the cell wall but yield spheroplasts that are toxin resistant (Al-Aidroos and Bussey, 1978; Schmitt and Compain, 1995). This argues that the final determinant of killing resides in the plasma membrane rather than the cell wall.

K1 toxin is known to produce uncontrolled leakage of potassium, protons, ATP, and other small molecules from target cells (Bussey and Skipper, 1976; de la Pena et al., 1981). The idea that cell death is principally due to potassium efflux (Skipper and Bussey, 1977; de la Pena et al., 1980) gained support when high levels of external potassium were demonstrated to be protective (Kurzweilova and Sigler, 1993). Subsequently, it was widely accepted that the mechanism underlying killing was de novo formation of ion channels by K1 toxin. This conclusion was based on observation of ion channel activity in toxin-treated spheroplasts or liposomes into which concentrated yeast cell culture supernatants were incorporated (Martinac et al., 1990). Here, we have tested and confirmed an alternative hypothesis: that K1 toxin activates ion channels endogenous to yeast cell plasma membranes.

In this report, a plasma membrane target for K1 toxin is identified. TOK1 is an outwardly rectifying, potassium-selective ion channel with two pore-forming P domains within each subunit (Ketchum et al., 1995). The channel is expressed in the plasma membrane of yeast cells (Loukin et al., 1997) and remains functional in yeast spheroplast preparations (Fairman et al., 1999). Among

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proteins encoded in the *S. cerevisiae* genome, only TOK1 channels contain P domains, a structural feature common to all known potassium-selective ion channels. Genetic, biochemical, and electrophysiologic evidence is presented to show that K1 killer toxin targets TOK1 potassium channels.

Results

TOK1 Deletion Confers Resistance to K1 Killer Toxin
If K1 toxin acts via TOK1 channels, deletion of the *TOK1* gene from target cells should confer toxin resistance. Conversely, overexpression of *TOK1* might lead to increased toxin sensitivity if naturally occurring levels of the channel were insufficient for maximal toxin efficacy. Cocultures of killer and toxin sensitive, nonkiller cells were used to validate both predictions as follows. Killer and nonkiller cells were mixed and incubated in fluid cocultures for 16 hr. Subsequently, cultured cells were spread on plates at low cell density for enumeration. As the killer strain, K12 (Bussey et al., 1973), carries an *ade2* mutation, it yields red colonies. The nonkiller strain, NY (TerBush et al., 1996), is wild type at this locus and produces white colonies. Counting white colonies indicated the number of nonkiller cells that survived coculture with K12 cells secreting virally encoded K1 toxin.

Figure 1 shows that deletion of *TOK1* from NY cells is protective against the cytotoxic effects of K12 cells, while overexpression of *TOK1* decreases survival. K12 cells were incubated with wild-type NY cells, NY+*TOK1* cells (in which *TOK1* was constitutively overexpressed behind the ADH promoter of a nonintegrating plasmid), or NYΔ*TOK1* cells (in which the 2.2 kb *TOK1* gene was deleted). Growth of few NY+*TOK1* colonies and many NYΔ*TOK1* indicated their differential susceptibility to K1 toxin released by K12 cells. In the experiment shown in Figure 1A, survival was 5-fold greater for cells without *TOK1* than for those overexpressing the gene. As the number of K12 cells in cocultures was increased, survival of nonkiller cells overexpressing *TOK1* decreased sharply (Figure 1B, hatched bars). Conversely, cells deleted of *TOK1* showed only a small change in survival even at high killer cell inocula (Figure 1B, open bars). Survival in seven coculture experiments, with a 1:1 ratio of K12 to nonkiller cells, was $30\% \pm 5\%$ for NY cells, $15\% \pm 2\%$ for NY+*TOK1* cells, and $70\% \pm 8\%$ for NYΔ*TOK1* cells compared to nonkiller cells cultured alone (Figure 1B).

Construction of a complete cDNA copy of the coding sequence for K1 preprotoxin behind a *PHO5* promoter allows tightly regulated production of mature K1 toxin in nonkiller cells by adjusting inorganic phosphate levels (Sturley et al., 1986). This strategy was used to produce K1 toxin in NY cells in the absence of M1 virus; toxin concentration was estimated by the killing zone method (Experimental Procedures). When NY+*TOK1* cells were incubated in ~ 50 nM K1 toxin, survival was $30\% \pm 6\%$ when compared to cells treated with control supernatant, while it was $72\% \pm 5\%$ for NYΔ*TOK1* cells deleted of the gene (Figure 1C). This supported the conclusion drawn from cocultures that deletion of *TOK1* protected NY cells from the cytopathic effects of K1 toxin. Moreover, as these experiments employed plasmid-encoded, virus-free toxin produced in NY target

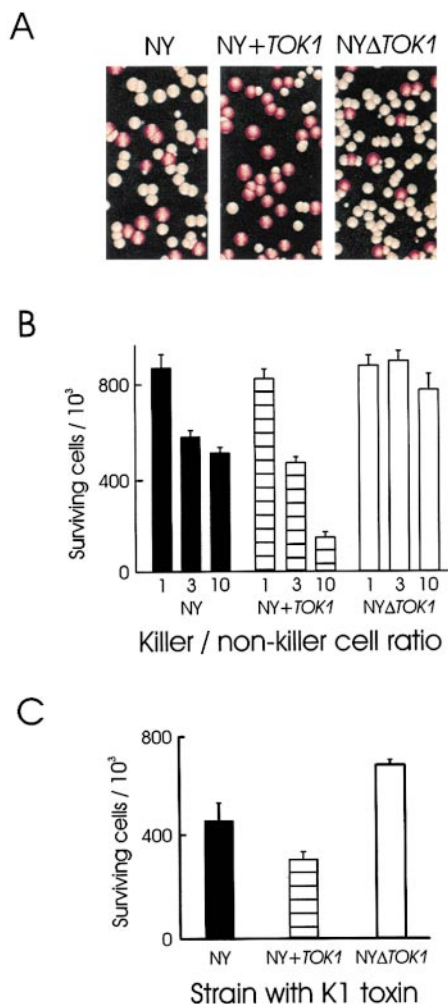


Figure 1. *TOK1* Deletion Confers Resistance to K12 Killer Cells and Cell-Free K1 Toxin

(A) Disruption of *TOK1* increases target cell survival upon coculture with K12 killer cells. Representative grids from plates used to assess survival of target cells (white colonies) after 16 hr coculture with K12 killer cells (red colonies) as in Experimental Procedures. More NYΔ*TOK1* cells were recovered from cocultures than NY or NY+*TOK1* cells.

(B) NYΔ*TOK1* cells are insensitive to increasing numbers of K12 killer cells in cocultures. In target cells, *TOK1* was wild type (NY, solid), overexpressed (NY+*TOK1*, cross-hatch), or deleted (NYΔ*TOK1*, open). Assay as in (A); recovery after culture with K12 cells or alone; a ratio of 1 indicates 1.8×10^7 cells of each type in a 3 ml culture. Mean \pm SEM for seven experiments.

(C) Disruption of *TOK1* increases target cell survival upon incubation with K1 toxin. In target cells, the *TOK1* gene was wild type (NY, solid), overexpressed (NY+*TOK1*, cross-hatch), or deleted (NYΔ*TOK1*, open). Assay as in (A); recovery after incubation with cell-free toxin or control solution (Experimental Procedures). Mean \pm SEM for three experiments.

cells, non-toxin viral effects and yeast cell mating pheromones could be eliminated as potential confounding variables.

Potassium Flux Induced by K1 Toxin Requires the *TOK1* Gene Product

Enhanced survival of NYΔ*TOK1* cells after coculture with killer cells or incubation with cell-free toxin indicated

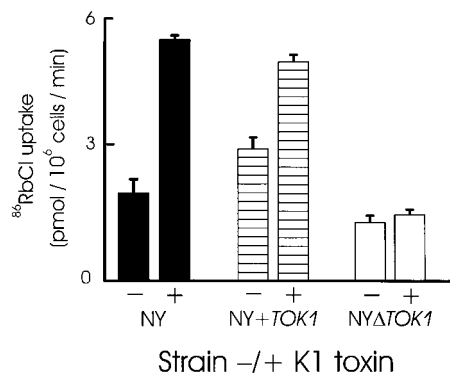


Figure 2. K1 Toxin Induces Potassium Flux in Yeast Cells Expressing TOK1

Uptake of ⁸⁶rubidium (200 μM) in the presence of control (–) or K1 toxin (+) solution. In target cells, the TOK1 gene was wild type (NY, solid), overexpressed (NY+TOK1, cross-hatch), or deleted (NYΔTOK1, open). Transport assays were performed in triplicate (Experimental Procedures). Mean ± SEM for six experiments.

only that TOK1 played a central role in the cytopathic mechanism of K1 toxin. It did not provide evidence that TOK1 channels mediated permeability changes in target cells or that killing involved altered potassium transport. To assess these issues, ~20 nM K1 toxin, ⁸⁶rubidium (an analog of potassium), and a rapid filtration technique were used (Experimental Procedures). If TOK1 channels and potassium flux were responsible for killing, flux of ⁸⁶rubidium was expected to accelerate on exposure to toxin if target cells expressed TOK1 channels.

As predicted, K1 toxin acted on whole yeast cells in a TOK1-dependent fashion to increase transport of the potassium analog. Thus, incubation with toxin increased ⁸⁶rubidium flux in wild-type NY cells expressing TOK1 and in NY+TOK1 cells that overexpressed the gene but had no effect on NYΔTOK1 cells in which the gene was deleted (Figure 2). Moreover, basal flux of ⁸⁶rubidium was least in NYΔTOK1 cells, slightly greater in NY cells, and pronounced in NY+TOK1 cells (Figure 2), consistent with the idea that the level of TOK1 channel expression and ⁸⁶rubidium flux were directly related to TOK1 gene dosage. These findings supported two ideas: first, that increased permeability of yeast cell plasma membranes to potassium was a step in the cytotoxic process mediated by TOK1 channels; second, that K1 toxin increased potassium flux by increasing the activity of TOK1 channels, perhaps by direct toxin:channel interaction.

K1 Toxin Increases the Activity of Native TOK1 Channels in Yeast Spheroplasts

As suspected based on flux studies, K1 toxin was found to open single native TOK1 channels in yeast cell plasma membranes. To show this, membrane patches were excised from yeast spheroplasts in outside-out configuration, thereby exposing the extracellular face of the channels to a solution that could be varied during a recording session; channel function was then evaluated by voltage clamp technique in the absence and presence of K1 toxin (Experimental Procedures). Three yeast strains were studied: wild-type NY, NYΔTOK1, and CY162, a strain deleted of the potassium-selective transport proteins TRK1 and TRK2 (Anderson et al., 1992); CY162

cells proved most amenable to patch recording and were used extensively after the strain was cured of M1 virus (Experimental Procedures).

TOK1 channels were readily recognized in patches excised from spheroplasts based on their characteristic gating kinetics, ion selectivity, current rectification, and unitary conductance (Figure 3; Table 1). In all cases, patches with one or more native TOK1 channels showed increased channel activity when treated with ~20 nM K1 toxin (Figures 3A and 3B and Table 1; wild-type NY, n = 2 patches; CY162, n = 12). Conversely, all patches that displayed no TOK1 channel activity under control conditions showed no new currents when exposed to K1 toxin (Figure 3C; wild-type NY, NYΔTOK1, and CY162 cells, n = 3–5 patches).

TOK1 Channels Expressed in *Xenopus laevis* Oocytes Are Activated by K1 Toxin

To explore the notion that K1 toxin enhances K⁺ transport via direct interaction with the channel, TOK1 was studied outside its natural yeast cell environment. TOK1 channels can be expressed in *X. laevis* oocytes by microinjection of TOK1 cRNA and studied after excision of membrane patches under voltage clamp (Ketchum et al., 1995). K1 toxin was found to specifically and reversibly increase the activity of TOK1 channels expressed in oocytes in the absence of other viral or yeast cell proteins (Figures 4 and 5; Table 1). As in yeast spheroplasts, all patches with one or more TOK1 channels (n = 36) showed increased channel activity when treated with ~20 nM K1 toxin (Figure 4A); this effect was reversible, as washing patches with control solution returned TOK1 channel activity to baseline levels (data not shown, n = 5). Conversely, all patches that displayed no TOK1 channel activity under control conditions also showed no new currents when exposed to K1 toxin (data not shown, n = 11 patches).

K1 Toxin Increases TOK1 Channel Open Burst Duration and Frequency

The mechanism by which K1 toxin increased TOK1 channel activity was next considered in oocytes. In both the absence and presence of ~20 nM K1 toxin, TOK1 channels opened in bursts, periods when the channel moved rapidly and repeatedly between the open state and a short-lived closed state (Figures 3A and 4A). Bursts of openings were interrupted by longer closures, some lasting seconds. Visual inspection suggested that K1 toxin increased open probability by increasing the duration and frequency of open bursts at the expense of dwell time in closed channel states. This impression was confirmed when single-channel transitions were idealized and dwell times estimated (Table 1) by the method of Colquhoun and Sigworth (1995).

Closed time distributions for single TOK1 channels in control conditions were well-fit by three exponential components (Figure 4B). Consistent with this analysis, three closed states were apparent in single-channel current recordings (Figure 4A). Very long closures (*C_{verylong}*) were seen in condensed traces, long closures (*C_{long}*) in 10-fold expansions of the records (Figure 4A, inset a), and short intraburst closures (*C_{short}*) in 100-fold expansions (Figure 4A, inset b). Alternatively, open time histograms were well fit by a single exponential (Figure 4B),

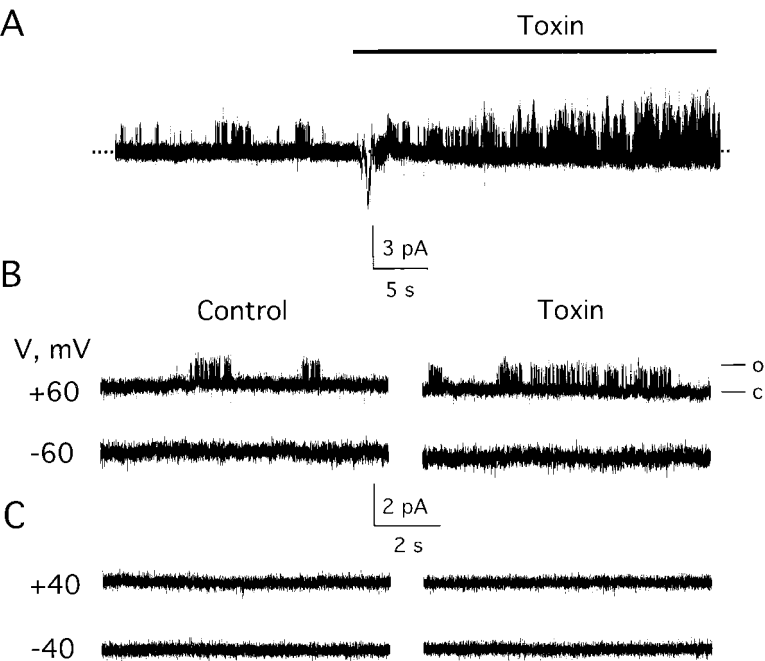


Figure 3. K1 Toxin Increases the Activity of Native TOK1 Channels in Yeast Cell Spheroplasts

Outside-out patches containing native TOK1 channels were excised from spheroplasts and studied with control or toxin solution (Experimental Procedures). Filtered at 1 kHz, sampled at 5 kHz.

(A) A multichannel patch from an M1 virus-cured CY162 spheroplast in control solution and then ~20 nM K1 toxin solution shows activation of TOK1 channels.

(B) Representative single native TOK1 channel in a patch excised from a CY162 spheroplast studied at 60 and -60 mV in control or ~20 nM K1 toxin solution. Closed (C) and open (O) states are indicated. The toxin increases channel activity recorded at 60 mV but does not produce new currents at -60 mV; this indicates the channels remain outwardly rectifying.

(C) Representative membrane patch excised from a CY162 spheroplast studied at 40 and -40 mV in control or ~20 nM K1 toxin solution, showing that no new currents are induced if no TOK1 channels are observed under control conditions.

and data from multichannel patches gave no evidence for additional open components (data not shown). When single TOK1 channels were exposed to ~20 nM K1 toxin, current recordings and dwell time histograms continued to reveal three closed and one open state (Figure 4B; Table 1).

Application of K1 toxin increased the single-channel open probability of TOK1 channels (Figure 5A). This was associated with decreased dwell time in all three closed states (Figure 5C), no change in the time constant for the open state (Figure 5D), and increased mean duration and frequency of open channel bursts (Figure 5E). Changes with ~20 nM K1 toxin appeared to be maximal, as application of a 3-fold higher concentration of toxin had no additional effect (data not shown). While K1 toxin increased open probability, it did not modify other attributes of TOK1 channels.

Toxin activation did not alter the open probability/voltage relationship for single TOK1 channels (Figure

5A). Nor did K1 toxin change the unitary slope conductance of TOK1 channels (Figure 5B; Table 1). Toxin application did not modify the effect of membrane potential on closed state dwell times (Figure 5C) or open channel burst duration and frequency (Figure 5E). Nor did toxin modify the characteristic outward rectification of TOK1 currents; TOK1 was the first non-voltage-gated, outwardly rectifying potassium channel to be cloned—it passes large outward potassium currents at potentials positive to the equilibrium reversal potential for potassium and only small inward currents negative to this potential (Ketchum et al., 1995; Loukin et al., 1997; Vergani et al., 1997). K1 toxin did not induce measurable inward currents through single TOK1 channels at voltages negative to 0 mV, despite the presence of near equal levels of potassium on both aspects of patches (Figures 3B, 4A, and 5A). Nor did K1 toxin modify selectivity of TOK1 channels for potassium over sodium and chloride. While inward TOK1 currents are too small to

Table 1. K1 Toxin Alters Function of Single TOK1 Channels in Oocytes and Spheroplast Membranes

	C_{verylong} (ms)	C_{long} (ms)	C_{short} (ms)	O (ms)	O_{burst} (ms)	Burst Frequency (s^{-1})	P_o	γ_s (pS)
Oocytes								
Control	250 ± 140	15 ± 7	1.2 ± 0.4	0.8 ± 0.1	37 ± 16	4.2 ± 0.6	0.15 ± 0.06	32 ± 2
Toxin	180 ± 70	10 ± 5	0.9 ± 0.1	0.8 ± 0.1	54 ± 18	6.4 ± 0.7	0.30 ± 0.04	33 ± 3
Spheroplasts								
Control	320 ± 60	13 ± 3	1.5 ± 0.2	0.8 ± 0.1	39 ± 13	3.9 ± 0.6	0.12 ± 0.08	35 ± 4
Toxin	149 ± 5	9 ± 1	1.0 ± 0.1	0.8 ± 0.0	63 ± 24	5.5 ± 0.8	0.26 ± 0.1	33 ± 3

Time constants for three closed states and one open state of single TOK1 channels studied at 60 mV in outside-out patches excised from M1 virus-free CY162 spheroplasts or *X. laevis* oocytes microinjected with TOK1 cRNA in the absence or presence of ~20 nM k1 toxin (Experimental Procedures). Closed and open state dwell time histograms were constructed from 5,000 to 50,000 events and time constants calculated by maximum-likelihood fits to the data. All values are mean ± SEM for 3–6 patches. At 40 mV, K1 toxin increased open probability of single native TOK1 channels and channels in oocytes 2.8 ± 0.1 fold and 2.6 ± 0.2 fold, respectively. Our most important potential source of error in evaluating steady-state open probability would be missed brief openings. That error is negligible in these measurements, as O_{burst} is ~40–60 ms, considerably longer than the minimum detectable event duration of 180 μs at the analysis bandwidth (1 kHz). Mean burst duration was calculated according to $[W_{cs}/(1 - W_{cs})][C_{\text{short}} + O] + O$, where W_{cs} is the weight of C_{short} . Mean burst frequency was calculated according to $(P_o/\text{mean burst duration})$.

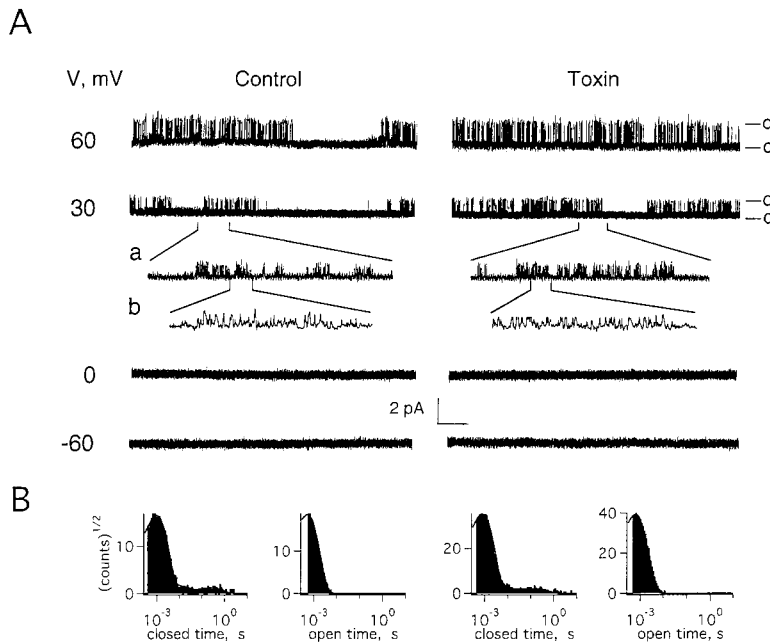


Figure 4. K1 Toxin Activates TOK1 Channels Expressed in *X. laevis* Oocytes

Outside-out patches containing single TOK1 channels were excised from oocytes and studied in 140 mM symmetrical potassium with cell-free K1 toxin (Experimental Procedures).

(A) Representative single channel recorded in an outside-out patch with symmetrical 140 mM potassium solution at the indicated voltages in the presence of control or ~ 20 nM K1 toxin solution (Experimental Procedures). The closed (C) and open (O) states are indicated. (Inset a) 10-fold expansion of the indicated portion of the record at 30 mV to highlight open channel bursts. (Inset b) 100-fold expansion shows rapid transitions between the open and short closed state within an open burst. Scale bar represents 2.0 s, 0.2 s (inset a), and 0.02 s (inset b). Filtered at 1 kHz and sampled at 5 kHz.

(B) Representative closed and open state dwell time histograms for a single TOK1 channel in control or ~ 20 nM K1 toxin solution at 60 mV; each histogram represents 1–3 min of recording analyzed by a 50% threshold crossing method (Experimental Procedures) and maximum-likelihood fits to the data.

study at the single-channel level, they are sufficiently large in patches containing many channels to assess macroscopic current reversal potential (Ketchum et al., 1995); reversal potentials in asymmetric potassium solutions were the same in the absence and presence of ~ 20 nM K1 toxin ($E_{rev} = -75 \pm 3$ mV, $n = 3$). The effect of toxin was specific in that ~ 20 nM K1 toxin had no effect on two unrelated potassium channels, I_{Ks} and I_{Kr} (Abbott et al., 1999) ($n = 3$, data not shown).

Native TOK1 Channels Function like Those Heterologously Expressed in Oocytes

Single TOK1 channels in patches excised from spheroplasts were similar to channels expressed in *X. laevis* oocytes when evaluated under control conditions and in their response to K1 toxin (Table 1). Thus, TOK1 channels from the two cell types were similar in appearance (Figure 6A). Quantitative analysis explained the resemblance: native and expressed channels had similar closed state time constants (Figure 6B), open state time constants (Figure 6C), unitary slope conductances (Figure 6D), and single channel open probability/voltage relationships (Figure 6E). TOK1 channels from the two cell types were also similar in mean open burst duration and frequency (data not shown).

So, too, K1 toxin altered the function of native TOK1 channels, as it had channels expressed in oocytes (Table 1). Single TOK1 channels in patches excised from spheroplasts in outside-out mode and exposed to ~ 20 nM toxin showed increased open probability, decreased closed state dwell times, invariance of open state dwell time, and increased mean duration and frequency of open channel bursts. As in oocytes, K1 toxin did not alter unitary current magnitude or outward rectification of single native TOK1 channels (Table 1; Figure 3B).

Discussion

The killer phenomenon has been recognized for over three decades (Bevan and Makower, 1963). Mediated by dsRNA viruses, killer cells secrete virally encoded toxins that are lethal to yeast cells that do not harbor virus. Study of these mycoviruses has been driven by their conspicuous similarity to RNA viruses that infect humans and their importance to commercial and medical mycology. Nonetheless, the target for K1 killer toxin and its mechanism of action have remained elusive. In this report, a potassium channel native to yeast cell plasma membranes is shown to be one such target. K1 toxin is seen to induce aberrant activity of TOK1 channels leading to dysregulated potassium homeostasis. Thus, deletion of *TOK1* from target cells confers resistance to K1 toxin whether virally encoded (Figures 1A and 1B) or synthesized from a cDNA template (Figure 1C). Likewise, K1 toxin accelerates flux of the potassium analog ^{86}Rb only if yeast cells express *TOK1* but not if the channel gene is deleted (Figure 2). The mechanism of action of K1 toxin on TOK1 channels is described (Figures 3–6; Table 1): toxin increases the open probability of single TOK1 channels by destabilizing closed channel states. The toxin does not alter other attributes of TOK1 channels, including current rectification, ion selectivity, or unitary current amplitude.

Previously, K1 toxin was thought to damage sensitive cells by directly inserting into the plasma membrane to form holes that allowed unregulated ion leakage (Martinac et al., 1990). We see no evidence for direct insertion in our studies. In all cases, membrane patches that showed no evidence for TOK1 channel activity under control conditions demonstrated no new currents upon application of either virally encoded or recombinant K1 toxin; this was true whether patches were excised from spheroplasts or *X. laevis* oocytes. Conversely, native

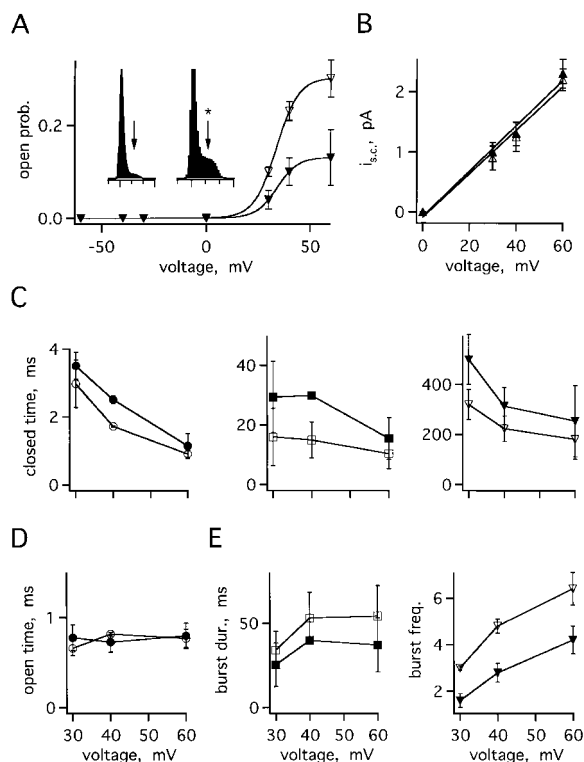


Figure 5. K1 Toxin Increases the Open Probability of TOK1 Channels

Single TOK1 channels were studied in outside-out patches excised from *X. laevis* oocytes in the absence or presence of ~ 20 nM K1 toxin (Experimental Procedures).

(A) K1 toxin increases the open probability of single TOK1 channels. Plot of the single channel open probability/voltage relationships in control (filled) or K1 toxin (open) solution; the mean \pm SEM for 2–6 patches at each voltage was fit to the Boltzmann function $A/(1 + \exp[(V_{1/2} - V)/V_s])^{-1}$, where A is a constant, $V_{1/2}$ is the half-maximal voltage, and V_s the slope factor; the values for control and toxin were $V_{1/2} = 34 \pm 5$ and 34 ± 4 mV and $V_s = 5.0 \pm 0.7$ and 5.4 ± 0.3 , respectively. K1 toxin increased open probability 2.6 ± 0.2 fold at 40 mV and 2.3 ± 0.1 fold at 60 mV (Table 1). (Inset) All points amplitude histograms show the presence of K1 toxin (+) increases occupancy of the open state (arrow).

(B) K1 toxin does not alter the unitary conductance of TOK1 channels. Plot of the single channel current/voltage relationships in control (filled) or K1 toxin (open) solution for the patches in (A); slope conductances were calculated by linear regression (Table 1).

(C) K1 toxin decreases the dwell times of single TOK1 channels in the short, long, and very long closed states. Time constants in control (filled) or K1 toxin (open) solution were determined as in Figure 4B.

(D) K1 toxin does not alter the dwell time of single TOK1 channels in the open state. Time constants in control (filled) or K1 toxin (open) solution were determined as in Figure 4B.

(E) K1 toxin increases the mean burst duration and frequency of single TOK1 channels. Values for control (filled) or K1 toxin (open) conditions calculated from time constants as in Table 1.

TOK1 channels recorded in spheroplasts and those expressed heterologously in *X. laevis* oocytes were, in all cases, activated by K1 toxin (Figures 3, 4, 5; Table 1). One discrepancy between our findings and those of Martinac and coworkers (1990) can now be understood. TOK1 channels are strongly selective for potassium over sodium and are blocked by cesium (Ketchum et al.,

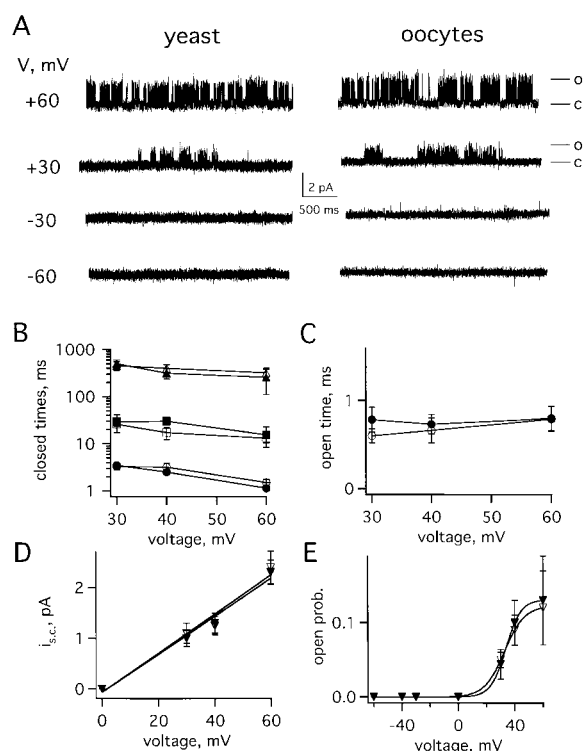


Figure 6. Native TOK1 Channels Function like Channels Expressed in *X. laevis* Oocytes

Outside-out patches containing single TOK1 channels were excised from spheroplasts or oocytes and studied as in Figures 3 and 4.

(A) A single native TOK1 channel in a patch excised from an M1 virus-cured CY162 spheroplast is similar in appearance to a single TOK1 channel excised from an oocyte when studied at the indicated voltages. Closed (C) and open (O) states are indicated. Filtered at 1 kHz and sampled at 5 kHz.

(B) Dwell times of single native TOK1 channels in the short, long, and very long closed states are similar to those in oocytes. Time constants for native (open) or heterologously expressed channels (filled).

(C) Dwell time of single native TOK1 channels in the open state is similar to that in oocytes. Time constants for native (open) or heterologously expressed channels (filled).

(D) Unitary conductance of single native TOK1 channels is indistinguishable from that in oocytes. Plot of the single channel current/voltage relationships for native (open) or heterologously expressed channels (filled); slope conductances calculated by linear regression (Table 1).

(E) The open probability of single native TOK1 channels is similar to that in oocytes. Plot of the single channel open probability/voltage relationships for native (open) or heterologously expressed channels (filled); the mean \pm SEM for 2–5 patches at each voltage fit as in Figure 5; $V_{1/2}$ and V_s were 33 ± 6 mV and 6 ± 1 , respectively, for native channels, and 34 ± 5 mV and 5.0 ± 0.7 for channels in oocytes.

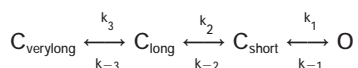
1995). Martinac and colleagues did not observe TOK1 channels in spheroplasts nor altered TOK1 channel activity because their solutions were formulated with cesium and/or explicitly excluded potassium.

Consistent with the idea that K1 toxin interacts with a low abundance target on yeast cell plasma membranes (like TOK1 channels), killing is associated with occupancy of as few as 10^4 sites per cell (Palfrey and Bussey, 1979) and toxin concentrations of ~ 50 nM (Bussey et

al., 1979). That recombinant K1 toxin activates TOK1 channels in *X. laevis* oocytes (Figures 4 and 5; Table 1) indicates that additional viral or yeast products are not required and suggests that K1 toxin interacts directly with TOK1 channels. While toxin may act via an intermediary plasma membrane receptor present in both yeast cells and oocytes, this seems unlikely. Conversely, it appears that K1 toxin can also damage yeast cells by a second cytopathic pathway; while *TOK1*-deleted NY cells resist K1 toxin, cells do die after prolonged exposure to killer cells (Figure 1B) or concentrated toxin (Figure 1C). We speculate that K1 toxin activates another transporter endogenous to yeast cells, perhaps the large conductance ion channel observed by Martinac and co-workers (1990). Our results explicitly exclude a role for the potassium-selective transporters TRK1 and TRK2 (Ko and Gaber, 1991; Schachtman and Schroeder, 1994), as they are present in NYΔ*TOK1* cells and this strain showed no acceleration of ⁸⁶Rb flux when treated with toxin (Figure 2).

Altered potassium flux has been a recognized feature of K1 toxin-mediated killing, but its basis was uncertain (Skipper and Bussey, 1977; de la Pena et al., 1980; Kurzweilova and Sigler, 1993). Here, we show that activation of TOK1 channels is necessary and sufficient to account for changes in potassium flux (Figure 2). Support for the theory that increased TOK1 channel activity is sufficient to kill yeast cells is found in the work of Loukin and colleagues (1997), who identified three lethal *tok1* mutants that activated more readily and closed more slowly than wild-type channels. The changes in kinetics they observed were attributed to accumulation of mutant channels in a pre-open state (analogous to our C_{short}) and were similar in magnitude to the effects we observe when wild-type TOK1 channels are exposed to K1 toxin (Table 1).

Gating transitions of single TOK1 channels in membrane patches excised from yeast cells and oocytes (Table 1) support a model for function like that derived from macroscopic current measurements (Ketchum et al., 1995; Loukin et al., 1997; Vergani et al., 1997):



Movement between C_{short} and O represents the rapid state changes observed within single-channel open bursts and the nearly instantaneous rise and fall of macroscopic currents associated with voltage steps (Ketchum et al., 1995; Loukin et al., 1997; Vergani et al., 1997). Transit between longer closed states and C_{short} represents movement into and out of the open burst state and contributes to time-dependent phases in macroscopic current recordings. That K1 toxin decreases dwell time in closed states but not O suggests it increases the forward rate constants k_3 , k_2 , and k_1 without altering the rate for leaving the open state, k_{-1} . Structural and functional commonalities support the idea that similar mechanisms may underlie gating of classical voltage-gated potassium channels (Perozo et al., 1998; Yellen, 1998) and two P domain channels like TOK1 and dORK1 (Ketchum et al., 1995; Vergani et al., 1997; Goldstein et al., 1998; Ilan et al., 1999).

Yeast strains with impaired potassium transporter function (*trk1 trk2*) are now widely used to clone and study potassium-selective ion channels and carriers (Anderson et al., 1992; Sentenac et al., 1992; Schachtman and Schroeder, 1994; Goldstein et al., 1996; Bertl et al., 1998; Fairman et al., 1999; Minor et al., 1999). Laboratory strains of *S. cerevisiae* often harbor one of three recognized killer systems (Wickner, 1992). Thus, CY162 is a K1 killer strain that was cured of M1 virus for use in these studies (Experimental Procedures). Our results suggest it is prudent to assess the killer phenotype of a strain prior to its use for studies of potassium transport and that reports on the biophysical attributes of TOK1 channels in CY162 cells (or strains that remain uncharacterized with regard to killer status) should be considered with caution.

Peptide toxins that act directly on potassium channels to inhibit their function have been isolated from scorpions, spiders, snakes, and snails (Miller, 1995; MacKinnon et al., 1998; McIntosh et al., 1999). These toxins have proven useful for channel purification, subtype identification, and interrogation of channel structure and function. Activators of potassium channels identified to date are small nonpeptide molecules that act through accessory proteins; some are used clinically to decrease excitability. K1 toxin is novel both because it is virally encoded and a polypeptide that activates a potassium channel. Elucidation of the structural basis for K1 toxin action and the isolation of agonists and antagonists could prove relevant to commercial fermenting, protection of plants of environmental and agronomic importance, and medical antimycotic therapy.

Experimental Procedures

Strains, Plasmids, and Toxin Production

Strains

Wild-type NY is NY1489 (MATa *ura3-52 trp1 his 3-200*) and was provided by P. Novick (TerBush et al., 1996). In NYΔ*TOK1*, the 2.2 kb open reading frame for *TOK1* is deleted and replaced by *Trp1*; this was achieved with a PCR-mediated gene disruption strategy (Brachmann et al., 1998) using *Trp1* amplified from a pRS304 (Sikorski and Hieter, 1989) with primers containing 40 nucleotides of the *TOK1* ORF. Primer AA1 was 5'TATTTTACCGATTCTATATATATGACAAGG-TTCATGAAGTGTGCGGTATTTACACCG3'; primer AA2 was 5'AGAGAATACATCCGTAGTAGTCTACAGTAAGATATAAATCAGATGTACTGAGAGTGCAC3'. To confirm *TOK1* deletion, genomic DNA was amplified with AA1/AA2 to reveal a 1 kb *Trp1* insertion rather than the 2.2 kb *TOK1* gene. NY+*TOK1* is wild-type NY transformed with p*TOK1*, a nonintegrating plasmid carrying *TOK1*. K12 is a K1 killer strain (Bussey et al., 1973) that is (MATα *ade2 arg*). Isolates of CY162 (*ura3-52 trk1 trk2*) were provided by R. F. Gaber (Anderson et al., 1992) and C. Slayman (Bertl et al., 1998) and cured of virus by repetitive passage at 40°C, as described (Wickner, 1974). S6 yeast cells (a/α) are ultrasensitive to K1 toxin (Bussey, 1981).

Plasmids and K1 Toxin Production from cDNA

pSH6 contains K1 preprotoxin gene (Sturley et al., 1986) behind a PHO5 promoter, while p1A1R1-15 is the plasmid without the gene. pRS316 is a CEN/ARS, URA3, ampR shuttle vector modified to include an ADH promoter and terminator (Goldstein et al., 1996). p*TOK1* contains the 2.2 kb *TOK1* ORF in pRS316. K1 toxin was made as before (Sturley et al., 1986). Briefly, wild-type NY cells were transformed with pSH6 or p1A1R1-15, grown in high phosphate, and induced in low-phosphate solution at room temperature; cells were removed by filtration and supernatants concentrated 100-fold in an Amicon cell with a YM10 membrane to produce toxin or control solutions. Activity was judged on 0.03% methylene blue yeast peptone dextrose (YPD) plates (Sturley et al., 1986) seeded with sensitive

cells. Toxin activity was assessed by a standard killing zone assay (Bussey, 1981) and diluted to similar potency; judged by published relationships for protein concentration to killing activity (Bussey et al., 1979; Palfree and Bussey, 1979), the level of K1 toxin in our stock solutions was roughly estimated to be ~100 to 300 nM and was diluted for use as indicated.

Killing and Flux Assays

Cocultures

Wild-type NY and NY Δ TOK1 cells were transformed with pRS316. Separate cultures of wild-type NY, NY Δ TOK1, NY+ TOK1, and K12 were grown overnight in uracil-deficient liquid YNB (pH 5.9) at 30°C. A 0.1 ml aliquot was grown in 4 ml to OD₆₀₀ = 1.0 (~3 × 10⁷ cells per milliliter) and 0.6 ml diluted into a 6 ml culture with various innocula of K12 cells for incubation with agitation at 20°C. Control cultures were grown without K12-1. Coculture samples were spread on YNB plates without uracil at a cell density of 200–600 colonies/plate. K12 cells are red, and the NY target cells are white; survival was assessed by counting white colonies from control and cocultures. Killing was slightly underestimated, as ~0.25% to 1% of K12 cells produced white colonies after overnight incubation. Killer target cell mating was not a confounding variable, as white cells surviving culture remained unable to kill S6 yeast cells.

Killing with K1 Toxin

Target cells (9 × 10³) in 0.25 ml were incubated for 12 hr with an equal volume of concentrated K1 toxin produced from pSH6 in wild-type NY cells or control supernatant that was concentrated extracellular medium from the same strain expressing a vector-only plasmid lacking the toxin gene (p1A1R1-15). Thirty microliter aliquots were plated for enumeration. Killing was determined by counting colonies from control and toxin incubations.

Flux of ⁸⁶Rubidium

Cells cultures grown to saturation were washed twice in sterile water, resuspended in 50 mM Tris-succinate (pH 5.9), and incubated in the absence of potassium for 16 hr at 30°C on a rotary shaker. Uptake assays were performed using a transport buffer containing 50 mM Tris-succinate, 2.5 mM glucose, and 0.25 mM ⁸⁶rubidium solution (pH 5.9) and rapid filtration (Goldstein et al., 1996). Briefly, uptake was initiated by mixing 125 μl of cells (3 × 10⁷ cells per milliliter) with 125 μl transport buffer and 25 μl control or toxin solution; after 5 min, cells were separated from buffer by vacuum filtration using 0.45 μm Durapore membranes (Millipore Corp., Bedford, MA). Filters were washed four times with 5 ml 50 mM Tris-succinate (pH 5.9) and immersed in Ecoscint scintillant fluid (National Diagnostics, Atlanta, GA) for evaluation. Statistical analysis was performed by one-way ANOVA. Flux of ⁸⁶rubidium was linear for the initial 10 min of the assay.

Electrophysiology

Oocytes

X. laevis oocytes were injected with 46 nl of TOK1 cRNA (~5 ng) in sterile water as before (Ketchum et al., 1995). TOK1 channels were recorded in excised outside-out patches with 140 mM KCl solution in the pipette (in mM): 140 KCl, 1 MgCl₂, 5 EGTA, 10 HEPES (pH 7.0 with KOH) and in the bath, 140 KCl, 1 MgCl₂, 0.3 CaCl₂, 10 HEPES (pH 7.0 with KOH).

Yeast Cell Spheroplasts

Preparation was similar to reported methods (Martinac et al., 1990) with the addition of a sucrose gradient purification of spheroplasts from intact cells and debris as in a previously reported method (Roberts et al., 1999). Channels were recorded in excised outside-out patches with the following solution in the pipette (in mM): 175 KCl, 5 MgCl₂, 1 EGTA, 5 HEPES, 0.0001 CaCl₂, 4 ATP (pH 7.0 with KOH) and in the bath, 150 KCl, 5 MgCl₂, 10 CaCl₂, 5 HEPES (pH 7.0 with KOH). Channels in spheroplasts were studied at pH 7.0 for comparison to oocytes; they were also evaluated in pH 4.7 solution, analogous to liquid media used for killing assays; channels were similarly activated by toxin at the lower pH (data not shown).

Experiments were performed at room temperature using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), an ITC-16 A/D converter (Instrutech, Port Washington, NY), a Macintosh Quadra 800 computer. Data were recorded to videotape, sampled at 5 kHz, and filtered at 1 kHz. Single channel data were stored to

tape for analysis off-line using a 50% crossing method (Colquhoun and Sigworth, 1995) and TAC software (Bruxton, Seattle, WA).

Acknowledgments

This work was supported by National Institutes of Health grants to S. A. N. G.; S. L. S. is an Established Investigator of the American Heart Association. We are grateful to F. Sigworth and Rosana Gonzalez-Colaso for extensive consultations during the course of this work and to D. Goldstein and G. W. Abbott for help with the figures.

Received July 27, 1999; revised September 16, 1999.

References

- Abbott, G.W., Sesti, F., Splaswki, I., Buck, M., Lehmann, M.H., Timothy, K.W., Keating, M.T., and Goldstein, S.A.N. (1999). MiRP1 forms IKr potassium channels with HERG and is associated with cardiac arrhythmia. *Cell* 97, 175–187.
- Al-Aidroos, K., and Bussey, H. (1978). Chromosomal mutants of *Saccharomyces cerevisiae* affecting the cell wall binding site for killer factor. *Can. J. Microbiol.* 24, 228–237.
- Anderson, J.A., Huprikar, S.S., Kochian, L.V., Lucas, W.J., and Gaber, R.F. (1992). Functional expression of a probable Arabidopsis thaliana potassium channel in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 89, 3736–3740.
- Bertl, A., Bihler, H., Reid, J.D., Kettner, C., and Slayman, C.L. (1998). Physiological characterization of the yeast plasma membrane outward rectifying K⁺ channel, DUK1 (TOK1), in situ. *J. Membr. Biol.* 162, 67–80.
- Bevan, E.A., and Makower, M. (1963). The physiological basis of the killer character in yeast. *Proc. Xth Intl. Congr. Genet.* 1, 202–203.
- Boone, C., Bussey, H., Greene, D., Thomas, D.Y., and Vernet, T. (1986). Yeast killer toxin: site-directed mutations implicate the precursor protein as the immunity component. *Cell* 46, 105–113.
- Boone, C., Sommer, S.S., Hensel, A., and Bussey, H. (1990). Yeast KRE genes provide evidence for a pathway of cell wall beta-glucan assembly. *J. Cell. Biol.* 110, 1833–1843.
- Bostian, K.A., Elliott, Q., Bussey, H., Burn, V., Smith, A., and Tipper, D.J. (1984). Sequence of the preprotoxin dsRNA gene of type I killer yeast: multiple processing events produce a two-component toxin. *Cell* 36, 741–751.
- Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J.C., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14, 115–132.
- Bussey, H. (1981). Physiology of killer factor in yeast. *Adv. Microb. Physiol.* 22, 93–122.
- Bussey, H., and Skipper, N. (1976). Killing of *Torulopsis glabrata* by *Saccharomyces cerevisiae* killer factor. *Antimicrob. Agents Chemother.* 9, 352–354.
- Bussey, H., Sherman, D., and Somers, J.M. (1973). Action of yeast killer factor: a resistant mutant with sensitive spheroplasts. *J. Bacteriol.* 113, 1193–1197.
- Bussey, H., Saville, D., Hutchins, K., and Palfree, R.G. (1979). Binding of yeast killer toxin to a cell wall receptor on sensitive *Saccharomyces cerevisiae*. *J. Bacteriol.* 140, 888–892.
- Bussey, H., Boone, C., Zhu, H., Vernet, T., Whiteway, M., and Thomas, D.Y. (1990). Genetic and molecular approaches to synthesis and action of the yeast killer toxin. *Experientia* 46, 193–200.
- Cheng, R.H., Caston, J.R., Wang, G.J., Gu, F., Smith, T.J., Baker, T.S., Bozarth, R.F., Trus, B.L., Cheng, N., and Wickner, R.B. (1994). Fungal virus capsids, cytoplasmic compartments for the replication of double-stranded RNA, formed as icosahedral shells of asymmetric Gag dimers. *J. Mol. Biol.* 244, 255–258.
- Colquhoun, D., and Sigworth, F.J. (1995). Fitting and statistical analysis of single-channel records. In *Single-Channel Recording*, B. Sakmann and E. Neher, eds. (New York: Plenum Press), pp. 483–588.
- de la Pena, P., Barros, F., Gascon, S., Ramos, S., and Lazo, P.S.

- (1980). Primary effects of yeast killer toxin. *Biochem. Biophys. Res. Commun.* **96**, 544–550.
- de la Pena, P., Barros, F., Gascon, S., Lazo, P.S., and Ramos, S. (1981). Effect of yeast killer toxin on sensitive cells of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **256**, 10420–10425.
- Fairman, C., Zhou, X.L., and Kung, C. (1999). Potassium uptake through the TOK1 K⁺ channel in the budding yeast. *J. Membr. Biol.* **168**, 149–157.
- Goldstein, S.A.N., Price, L.A., Rosenthal, D.N., and Pausch, M.H. (1996). ORK1, a potassium-selective leak channel with two pore domains cloned from *Drosophila melanogaster* by expression in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **93**, 13256–13261.
- Goldstein, S.A.N., Wang, K.W., Ilan, N., and Pausch, M. (1998). Sequence and function of the two P domain potassium channels: implications of an emerging superfamily. *J. Mol. Med.* **76**, 13–20.
- Icho, T., and Wickner, R.B. (1989). The double-stranded RNA genome of yeast virus L-A encodes its own putative RNA polymerase by fusing two open reading frames. *J. Biol. Chem.* **264**, 6716–6723.
- Ilan, N., Zilberberg, N., Gonzalez-Colaso, R., and Goldstein, S.A.N. (1999). Molecular dissection and single-channel analysis reveals gating of an open-rectifier (leak) potassium channel by its cytoplasmic tail. *Biophys. J.* **76**, A411.
- Ketchum, K.A., Joiner, W.J., Sellers, A.J., Kaczmarek, L.K., and Goldstein, S.A.N. (1995). A new family of outwardly-rectifying potassium channel proteins with two pore domains in tandem. *Nature* **376**, 690–695.
- Ko, C.H., and Gaber, R.F. (1991). TRK1 and TRK2 encode structurally related K⁺ transporters in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**, 4266–4273.
- Kurzweilova, H., and Sigler, K. (1993). Factors affecting the susceptibility of sensitive yeast cells to killer toxin K1. *Folia Microbiol.* **38**, 524–526.
- Loukin, S.H., Vaillant, B., Zhou, X.L., Spalding, E.P., Kung, C., and Saimi, Y. (1997). Random mutagenesis reveals a region important for gating of the yeast K⁺ channel Ykc1. *EMBO J.* **16**, 4817–4825.
- MacKinnon, R., Cohen, S.L., Kuo, A., Lee, A., and Chait, B.T. (1998). Structural conservation in prokaryotic and eukaryotic potassium channels. *Science* **280**, 106–109.
- Magliani, W., Conti, S., Gerloni, M., Bertolotti, D., and Polonelli, L. (1997). Yeast killer systems. *Clin. Microbiol. Rev.* **10**, 369–400.
- Martinac, B., Zhu, H., Kubalski, A., Zhou, X.L., Culbertson, M., Bussey, H., and Kung, C. (1990). Yeast K1 killer toxin forms ion channels in sensitive yeast spheroplasts and in artificial liposomes. *Proc. Natl. Acad. Sci. USA* **87**, 6228–6232.
- McIntosh, J.M., Olivera, B.M., and Cruz, L.J. (1999). Conus peptides as probes for ion channels. *Methods Enzymol.* **294**, 605–624.
- Miller, C. (1995). The charybdotoxin family of K⁺ channel-blocking peptides. *Neuron* **15**, 5–10.
- Minor, D.L., Masseling, S.J., Jan, Y.N., and Jan, L.Y. (1999). Transmembrane structure of an inwardly rectifying potassium channel. *Cell* **96**, 879–891.
- Murphy, N., Buchanan, C.R., Damjanovic, V., Whitaker, R., Hart, C.A., and Cooke, R.W. (1986). Infection and colonisation of neonates by *Hansenula anomala*. *Lancet* **1**, 291–293.
- Palfree, R.G., and Bussey, H. (1979). Yeast killer toxin: purification and characterisation of the protein toxin from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **93**, 487–493.
- Perozo, E., Cortes, D.M., and Cuello, L.G. (1998). Three-dimensional architecture and gating mechanism of a K⁺ channel studied by EPR spectroscopy. *Nat. Struct. Biol.* **5**, 459–469.
- Pettoello-Mantovani, M., Nocerino, A., Polonelli, L., Morace, G., Conti, S., Di Martino, L., De Ritis, G., Iafusco, M., and Guandalini, S. (1995). *Hansenula anomala* killer toxin induces secretion and severe acute injury in the rat intestine. *Gastroenterology* **109**, 1900–1906.
- Polonelli, L., Lorenzini, R., De Bernardis, F., and Morace, G. (1986). Potential effect of yeast killer toxin. *Mycopathologia* **96**, 103–107.
- Roberts, S.K., Fischer, M., Dixon, G.K., and Sanders, D. (1999). Divalent cation block of inward currents and low-affinity K⁺ uptake in *Saccharomyces cerevisiae*. *J. Bacteriol.* **181**, 291–297.
- Schachtman, D.P., and Schroeder, J.I. (1994). Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. *Nature* **370**, 655–658.
- Schmitt, M.J., and Compain, P. (1995). Killer-toxin-resistant kre12 mutants of *Saccharomyces cerevisiae*: genetic and biochemical evidence for a secondary K1 membrane receptor. *Arch. Microbiol.* **164**, 435–443.
- Sentenac, H., Bonneaud, N., Minet, M., Lacroute, F., Salmon, J.M., Gaymard, F., and Grignon, C. (1992). Cloning and expression in yeast of a plant potassium ion transport system. *Science* **256**, 663–665.
- Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27.
- Skipper, N., and Bussey, H. (1977). Mode of action of yeast toxins: energy requirement for *Saccharomyces cerevisiae* killer toxin. *J. Bacteriol.* **129**, 668–677.
- Skipper, N., Thomas, D.Y., and Lau, P.C. (1984). Cloning and sequencing of the preprotoxin-coding region of the yeast M1 double-stranded RNA. *EMBO J.* **3**, 107–111.
- Starmer, W.T., Ganter, P.F., Aberdeen, V., Lachance, M.-A., and Phaff, H.J. (1992). The ecological role of killer yeasts in natural communities of yeasts. *Can. J. Microbiol.* **33**, 783–796.
- Sturley, S.L., Elliot, Q., LeVitre, J., Tipper, D.J., and Bostian, K.A. (1986). Mapping of functional domains within the *Saccharomyces cerevisiae* type 1 killer preprotoxin. *EMBO J.* **5**, 3381–3389.
- TerBush, D.R., Maurice, T., Roth, D., and Novick, P. (1996). The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *EMBO J.* **15**, 6483–6494.
- Thiele, D.J., and Leibowitz, M.J. (1982). Structural and functional analysis of separated strands of killer double-stranded RNA of yeast. *Nucleic Acids Res.* **10**, 6903–6918.
- Van Vuuren, H.J.J., and Jacobs, C.J. (1992). Killer yeasts in the wine industry: a review. *Am. J. Enol. Vitic.* **43**, 119–128.
- Vergani, P., Miosga, T., Jarvis, S.M., and Blatt, M.R. (1997). Extracellular K⁺ and Ba²⁺ mediate voltage-dependent inactivation of the outward-rectifying K⁺ channel encoded by the yeast gene TOK1. *FEBS Lett.* **405**, 337–344.
- Wickner, R.B. (1974). "Killer character" of *Saccharomyces cerevisiae*: curing by growth at elevated temperature. *J. Bacteriol.* **117**, 1356–1357.
- Wickner, R.B. (1992). Double-stranded and single-stranded RNA viruses of *Saccharomyces cerevisiae*. *Annu. Rev. Microbiol.* **46**, 347–375.
- Wickner, R.B. (1996). Prions and RNA viruses of *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **30**, 109–139.
- Yellen, G. (1998). The moving parts of potassium channels. *Quart. Rev. Biophys.* **31**, 239–295.
- Zhu, H., and Bussey, H. (1991). Mutational analysis of the functional domains of yeast K1 killer toxin. *Mol. Cell. Biol.* **11**, 175–181.