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COMPLEX MODULATION OF CATION CHANNELS IN THE TONOPLAST AND PLASMA MEMBRANE OF *SACCHAROMYCES CEREVISIAE*: SINGLE-CHANNEL STUDIES

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Summary

Detailed patch-clamp studies have been made of ion channels in the plasma membrane and tonoplast of the yeast *Saccharomyces cerevisiae*. The predominant tonoplast channel is a high-conductance cation-selective inward rectifier (passing ions easily *into the cytoplasm* from the vacuole), with its open probability (P_o) peaking at about −80 mV (cytoplasm negative) and falling to near zero at +80 mV. It has a maximal slope conductance of approximately 150 pS in 100 mmol l^{-1} KCl, and conducts Na^+, K^+ and Ca^{2+}. Elevated cytoplasmic Ca^{2+} concentration, alkaline pH and reducing agents can activate the channel, its likely physiological function being to adjust cytoplasmic Ca^{2+} concentration from the vacuolar reservoir. The predominant plasma-membrane channel is a strongly outward rectifying K^+ channel (passing K^+ easily *out of the cytoplasm* to the extracellular medium), which is activated by positive-going membrane voltages as well as by elevated cytoplasmic Ca^{2+} concentration and alkaline pH. Interaction between membrane voltage and $[Ca^{2+}]_{cyt}$ is complex and defines three parallel closed states for the channel: a Ca^{2+}-independent brief closure (I), a calcium-inhibited long closure (G) and, at large positive voltages, a calcium-induced brief blockade (B). This channel is likely to function in steady-state turgor regulation and in charge balancing during proton-coupled substrate uptake.

Introduction

Direct study of charge-translocating processes in the yeast vacuole by means of patch electrodes began against a background of considerable knowledge about the chemistry and structure of yeast vacuolar H^+-ATPase (V-ATPase; Uchida *et al.* 1988; Hirata *et al.* 1990). The initial aim was to characterize charge movements through that ATPase *and* through associated channels which perforce must operate in parallel with the ATPase. For technical reasons, however, the channels have proved much simpler then the V-ATPase to study *via* patch electrodes, and the experimental work has focused on vacuolar cation channels: their fundamental properties, their regulation and their comparison with plasma membrane cation channels in the same organism. Implications for understanding the interactions between vacuolar and plasma-membrane channels, as well as between the channels and their corresponding ATPases, will be considered in the last section.

Key words: yeast channels, vacuolar transport, outward rectifier, calcium blockade, calcium activation.

The care and feeding of yeast membranes

Earlier electrophysiological studies on *Saccharomyces* (Conway, 1960; H. Kuroda and C. L. Slayman, unpublished experiments, 1978) showed the organism to be nearly intractable for work with conventional penetrating microelectrodes. The situation improved considerably, at least for study of protoplasts, with the adoption of patch electrodes by Gustin *et al.* (1986, 1988), whose experiments piqued our current interest. Because the plasma membranes of simple wild-type yeast protoplasts proved to be treacherous material even for patch recording, we adopted a tetraploid strain (YCC78) and developed a recipe for growing larger protoplasts in order to increase the membrane surface area and improve adhesiveness for attaching patch electrodes.

Protoplasts can be liberated from most strains of *Saccharomyces* by partial digestion of the cell walls from late log-phase cells with a zymolyase/glucuronidase mixture (2 mg each per milliliter of buffer) in osmotically protective medium (Bertl and Slayman, 1990). We routinely used 1.2 mol l^{-1} sorbitol, buffered with 50 mmol l^{-1} KH_2PO_4 at pH 7.2 (titrated with KOH) and containing 40 mmol l^{-1} β-mercaptoethanol. After incubation for 45 min at 30 °C, which releases protoplasts through 'cracked' walls, the cells were harvested by centrifugation, washed and resuspended in saline glucose (200 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} glucose and 5 mmol l^{-1} Mes brought to pH 7.2 with Tris base; 25 °C). Protoplasts were incubated in this medium for up to several days, during which time they could increase three- to fivefold in diameter.

Yeast protoplasts in different stages of enlargement are shown in Fig. 1. Fig. 1A,B compares fresh protoplasts from a diploid strain (R757/R1838) and a tetraploid strain (YCC78); Fig. 1C,D illustrates the expansion – especially the relative vacuolar expansion – with incubation of tetraploid protoplasts for 2 days and longer than 4 days, respectively. Most older preparations contained modest numbers of giant cells (Fig. 1D). For experiments on *vacuolar* membranes, 2- to 3-day-old protoplasts were used, and vacuoles suitable for patch recording were obtained by gentle acid lysis: several drops of the protoplast suspension were placed in the recording chamber and the chamber was pefused with 100 mmol l^{-1} potassium citrate at pH 6.8 (other components as in saline glucose). Experiments on yeast *plasma* membranes yielded the highest success rates of giga-seal formation with protoplasts incubated for only 1–2 h.

Standard patch-clamp techniques (Hamill *et al.* 1981) were used, and primary data were recorded on video cassettes *via* a pulse-coded modulator, with an overall system time constant of approximately 20 μs. Data for display and analysis were obtained by low-pass filtering of the tape records, usually at 200 Hz, with sampling at 0.5–2 kHz. All voltages, for both plasma membrane and tonoplast, are reported as cytoplasmic electrical potential minus extracytoplasmic electrical potential; i.e. the vacuole interior and the extracellular space are treated equivalently, and signs for current and voltage are independent of the physical orientation of the membrane. By this convention, a negative voltage across the tonoplast means cytoplasm negative with respect to the vacuolar interior, and a negative voltage across the plasma membrane means cytoplasm negative with respect to the cell exterior. Negative or 'inward' *currents* are constituted by positive charge flowing into the cytoplasm, and have been drawn downward in the plots. Positive

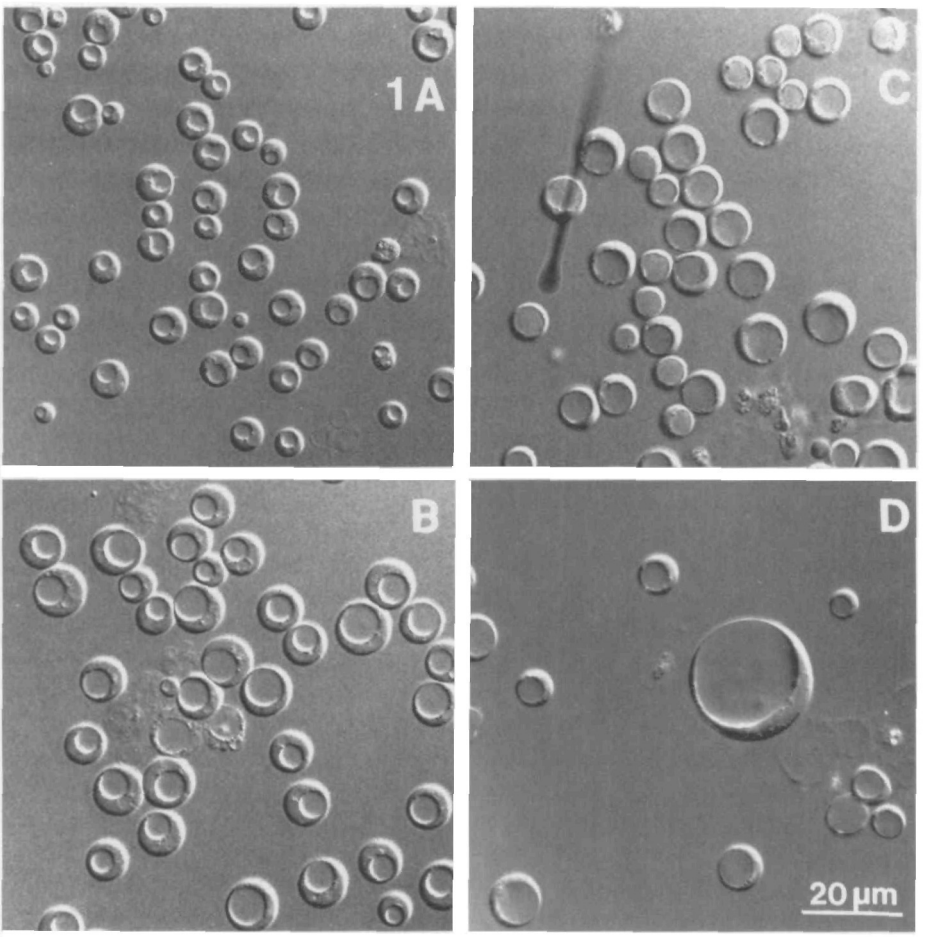

Fig. 1. Growth of yeast protoplasts. (A,B) Comparison of fresh protoplasts from a diploid strain (R757/R1838) and a tetraploid strain (YCC78). (B,C,D) Comparison of protoplasts incubated 0, 2 and more than 4 days, respectively, in saline glucose. Note especially the increased fractional volume occupied by vacuoles, and the appearance of giant cells, as *Saccharomyces* protoplasts age. Nomarski optics.

or 'outward' currents occur by flow of positive charge out of the cytoplasm and have been drawn upward in the plots. Isolated tonoplast patches were routinely used in the outside-out orientation, and plasma-membrane patches in the inside-out orientation, in both cases exposing the cytoplasmic surface to the bathing solution.

For recording from the plasma membrane, typical bath (cytoplasmic) solutions contained 200 mmol l^{-1} KCl, 5 mmol l^{-1} Mes titrated to pH 7.0 with Tris base, plus Ca^{2+} (unbuffered at 1 mmol l^{-1} or above; buffered with EGTA at 100 μmol l^{-1} or below) as indicated in the respective figure legends; pipette (extracellular) solutions were 50 mmol l^{-1} KCl, 100 μmol l^{-1} $CaCl_2$, 250 mmol l^{-1} sorbitol, pH 5.5–5.7 (unbuffered). For recording from the tonoplast, cytoplasmic (bath) solutions contained 100 mmol l^{-1} KCl, 5 mmol l^{-1} Mes titrated to pH 7.0 with Tris base, plus Ca^{2+} as indicated; pipette

solutions were 100 mmol l^{-1} KCl, 10 μmol l^{-1} free Ca^{2+} (buffered with EGTA), adjusted to pH 7.0 with Tris base.

A Ca^{2+}-activated, Ca^{2+}-conducting maxi-cation channel in yeast tonoplast

The most easily observed channel in the yeast vacuolar membrane, which we have designated YVC1, was first identified by Wada *et al.* (1987) in bilayer-fusion experiments, where its activation depended on millimolar Ca^{2+} levels; our first patch electrode experiments confirmed its basic properties, including the requirement for high calcium levels. A search for possible physiological modulators (such as protein kinases), however, revealed the channels to be persistently active – and in the micromolar range of calcium concentrations – when conventional reducing agents were present. In Fig. 2, for

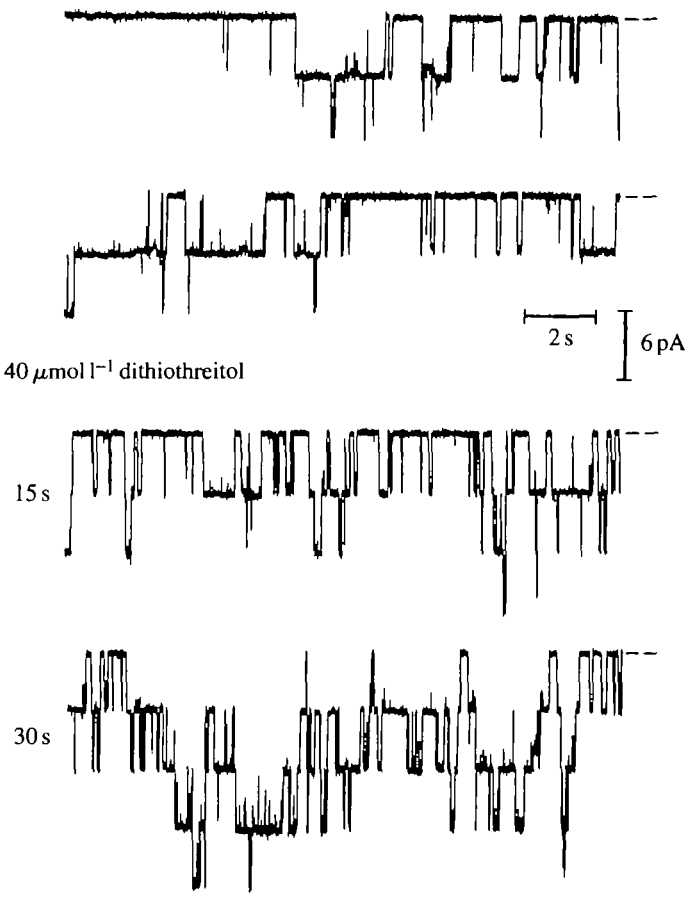

Fig. 2. Enhancement of activity of tonoplast channels (YVC1) by the reducing agent dithiothreitol (DTT). Outside-out patch from YCC78 vacuolar membrane, containing at least four channels. The dashed line designates baseline (current with zero channels open). Clamped membrane voltage, −40 mV. Bath solution: 100 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} Mes/Tris at pH 7.0. Filter frequency, 200 Hz; sample frequency, 500 Hz.

example, a patch containing at least four channels displayed one open channel approximately 40 % of the time, occasionally two open channels, and very rarely three open channels, for an overall open probability (P_o) of approximately 0.1. Within 30 s of the addition of 40 μmol l^{-1} dithiothreitol (DTT), four open channels were easily visible, and overall open probability had risen above 0.3.

Dependence of channel activation upon bath (cytoplasmic) [Ca^{2+}] *per se* was indicated initially by the need for stepwise elevation of the concentration of that ion to counter a conspicuous rundown of channel activity over recording periods of 5–30 min. And even in the presence of DTT or β-mercaptoethanol (ME), changes in $[Ca^{2+}]_{cyt}$ strongly modulated channel activity, as shown for the three-channel patch of Fig. 3. In this case the tonoplast voltage was clamped at –40 mV, and 40 μmol l^{-1} DTT was present

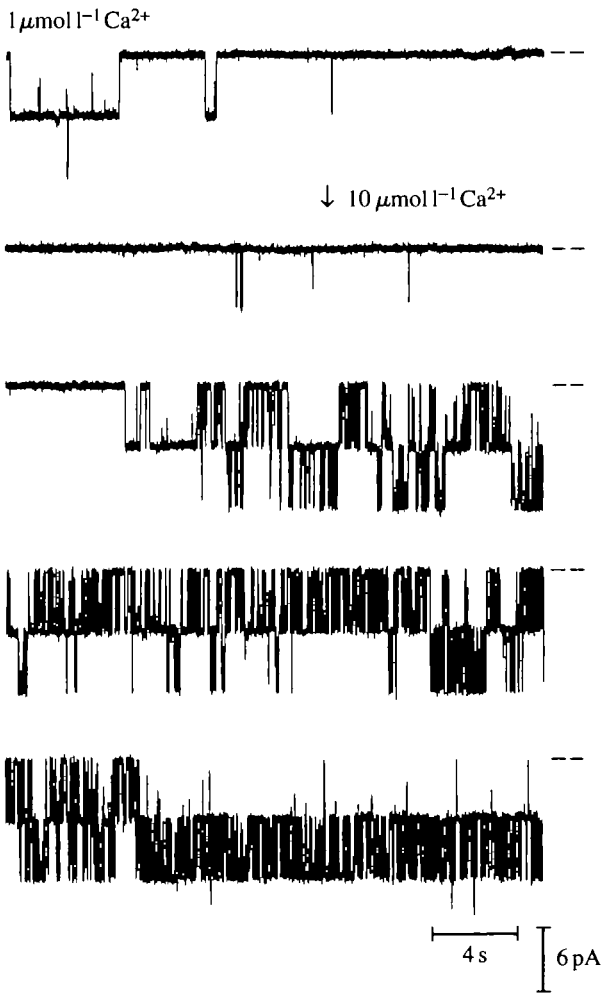

Fig. 3. Calcium modulation of tonoplast channel activity. Conditions as in Fig. 2, except 40 μmol l^{-1} DTT present throughout and cytoplasmic [Ca^{2+}] changed as indicated. Patch containing at least three channels.

throughout the recording period. In 1 μmol l^{-1} Ca^{2+}, P_o was less than 0.04; but by 10–15 s after addition of 10 μmol l^{-1} Ca^{2+}, P_o had risen approximately 10-fold, to about 0.35, with two channels open for a major fraction of the recording time.

Other physiological factors involved in YVC1 regulation include calmodulin and cytoplasmic pH. The central observation of the initial calmodulin experiments is that channels in isolated patches which have been silenced by calcium extraction cannot be reactivated by restoring even millimolar Ca^{2+} alone, but only by incubation in the presence of both Ca^{2+} (approximately 100 μmol l^{-1}) and calmodulin (approximately 0.5 μmol l^{-1}), either of homologous or heterologous origin. After such treatment, normal responses to changing $[Ca^{2+}]_{cyt}$ are observed. Consequences of altering cytoplasmic pH are shown in Fig. 4, for a patch containing at least nine channels. At pH 5.5, P_o was very low (<<0.01) with the records dominated by brief and infrequent single-channel openings. But 10–15 s after bath pH had been elevated to the more physiological level of 7.5, multiple-channel openings predominated, and overall P_o had risen above 0.3. The pH effect was quickly and fully reversible. Finally, channel gating was also found to be *voltage*-dependent (Bertl and Slayman, 1990), but in a biphasic fashion. From a maximum near 0.7 at −80 mV, P_o fell (approximately exponentially) to less than 0.01 at +80 mV and to about 0.5 at −100 mV.

Since for technical reasons ion-channel surveys are routinely carried out in salt-rich Ringer-like solutions, YVC1 was initially defined as being permeable to the alkali cations K^+ and Na^+ but not to Cl^- (Bertl and Slayman, 1990). Physiological considerations, however, argued that the evolved function of the channel was not likely to be transport of K^+ or Na^+, but rather demand-activated release of cations or neutral molecules specifically stored in the yeast vacuole. The principal candidate ions, then, seemed to be basic amino acids (lysine, arginine), which can be accumulated by fungal vacuoles to nearly molar concentrations (Wiemken and Dürr, 1974; Martinoia *et al.* 1979; Cramer *et al.* 1980), and calcium, whose normal cytoplasmic concentration is approximately 300 nmol l^{-1} (Halachmi and Eilam, 1989). Checks for arginine and lysine permeability of the channel failed, because replacement of bath K^+ by either amino acid destabilized seals.

But finite calcium permeability was revealed by a less severe kind of experiment, illustrated in Fig. 5. Here mean open-channel currents (I_o) were determined as functions of membrane voltage for three conditions: the control, with identical solutions containing 100 mmol l^{-1} K^+ and 10 μmol l^{-1} Ca^{2+} in the pipette and bath; low-K^+, low-Ca^{2+} solution, with 10 mmol l^{-1} K^+ and 10 μmol l^{-1} Ca^{2+} in the bath; and low-K^+, high-Ca^{2+} solution, with 10 mmol l^{-1} of each in the bath. At the fixed low $[Ca^{2+}]$, the reversal voltage (E_r) for the channel varied with $[K^+]$ as expected for a potassium electrode; i.e. for $[K^+]_{cyt}$=100 mmol l^{-1}→$[K^+]_{cyt}$=10 mmol l^{-1}, E_r=+2 mV→E_r=+51 mV, compared with similar equilibrium voltages for K^+: E_K=0 mV→E_K=+53 mV (considering the changing *activity*, rather than concentration). Elevating $[Ca^{2+}]_{cyt}$ to 10 mmol l^{-1} at the *low* $[K^+]_{cyt}$ revealed an appreciable calcium effect on the reversal voltage: E_r=+53 mV→E_r=+21 mV. These numbers yield a rough estimate of the channel's permeability ratio, P_{Ca}/P_K, of approximately 5 (calculated, for example, from the Henderson equation).

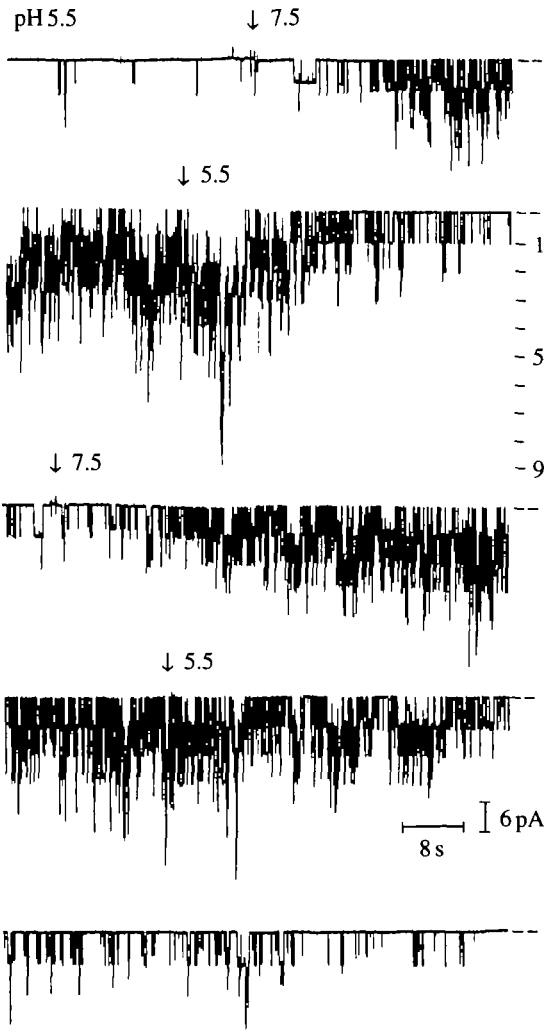

Fig. 4. Effect of varied cytoplasmic pH upon tonoplast channel activity. Conditions as in Fig. 2, except 10 mmol l^{-1} β-mercaptoethanol and 100 μmol l^{-1} Ca^{2+} present throughout, and pH shifted as indicated. Patch containing at least nine channels. Note progressive increase of channel activity following each alkaline shift and progressive decrease following each acid shift.

Overall, when YVC1 is open, its behavior is not far from expectation for a cationic Nernst–Planck diffusion regime, with little or no rectification. However, because of the voltage-dependence of channel gating, discussed above, its *time-averaged* behavior (which – under steady-state conditions – is the physiologically relevant property) is strongly inward-rectifying; i.e. current passes easily into the cytoplasm from the vacuole, but not in the reverse direction. This property is demonstrated in Fig. 6, which compares the time-averaged single-channel current from an isolated patch with the steady-state current through a whole vacuolar membrane when its voltage was stepped from the

reversal value (−17 mV) to the plotted value. The clear physiological implication of this result, plus the channel's cation selectivity, is that – if the principal substrate is ionic rather than electroneutral – then the channel must release cations into the cytoplasm from vacuolar storage. If the principal substrate is also an activator of the channel, as in the likely case of calcium ions, then initiation of channel openings creates a positive feedback process.

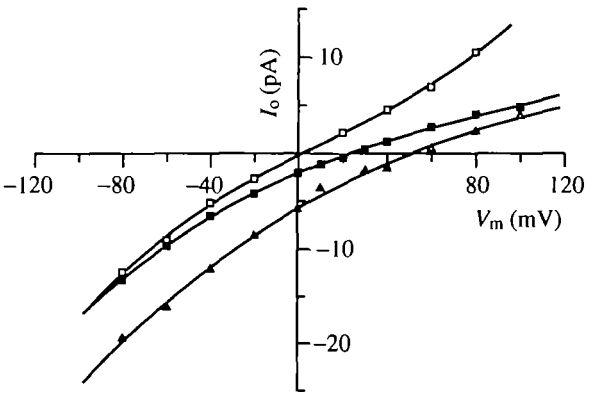

Fig. 5. Open-channel current–voltage curves for YVC1, demonstrating finite permeability to Ca^{2+}. General conditions as in Fig. 3, but with the following cytoplasmic-side K^+ and Ca^{2+} concentrations. (□): 100 mmol l^{-1} K^+, 10 μmol l^{-1} Ca^{2+}; (△): 10 mmol l^{-1}, 10 μmol l^{-1}; (■): 10 mmol l^{-1}, 10 mmol l^{-1}. Current measurements (I_o) made at different clamped membrane voltages between −80 and +100 mV, as plotted.

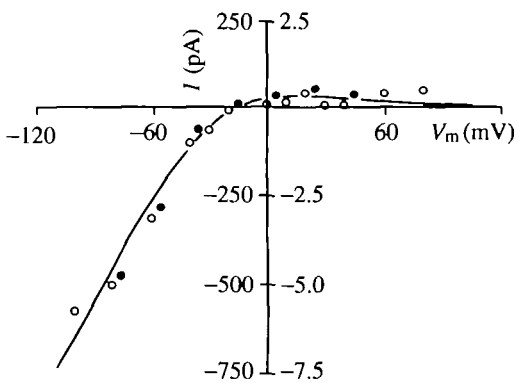

Fig. 6. Time-averaged current–voltage relationship for YVC1 channels. Comparison of steady-state whole-vacuole currents (●) with averaged single-channel currents (○) obtained as the product P_oI_o for an experiment similar to those in Fig. 5, but with bathing medium containing 200 mmol l^{-1} KCl and 1 mmol l^{-1} Ca^{2+}. Whole-cell currents on left of ordinate scale, single-channel currents on right. This is a quantitative demonstration that YVC1 channels account for the resting membrane conductance of the yeast tonoplast and that approximately 100 such channels are present.

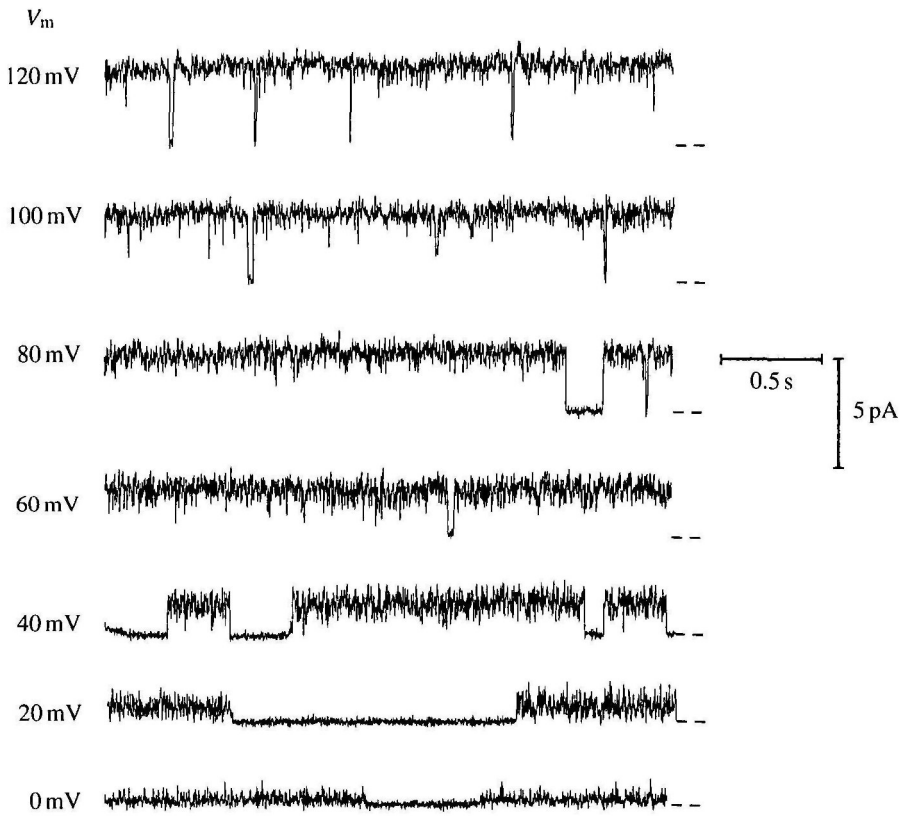

Fig. 7. Basic characteristics of the *Saccharomyces* plasma-membrane K^+ channel (YPK1). Inside-out patch from YCC78 protoplast plasma membrane, containing probably only one active channel. Membrane voltages clamped as designated at the left of each record. Bath solution: 200 mmol l^{-1} KCl, 1 μmol l^{-1} $CaCl_2$, 5 mmol l^{-1} Mes/Tris at pH 7.0. Filter frequency, 200 Hz; sample frequency, 1000 Hz. Note especially: positive sign for all clamped voltages; increased current fluctuations (flickering) during channel opening; long open periods, and long closed periods at low voltages.

A K^+-release channel in the yeast plasma membrane

Thus far two types of ion channels have been described in the *Saccharomyces* plasma membrane from patch electrode measurements: an outward-rectifying K^+ channel and a 'stretch'-sensitive channel (Gustin *et al.* 1986, 1988). Additionally, an assortment of channel-like currents have been reported from studies of bilayers containing fused yeast membrane fragments (Gómez-Lagunas *et al.* 1989).

By far the simplest of these channels to observe is the outward-rectifying K^+ channel, which we have designated YPK1, whose properties are summarized in the records of Fig. 7, from an inside-out patch. The obvious properties which differentiate this from the tonoplast channel just described are (i) its small open-channel conductance: e.g. approximately 35 pS for the 100-mV record in Fig. 7; (ii) its bias to open at positive membrane voltages, rather than negative ones; (iii) its apparent tendency to 'hang' either

open or closed for long periods (0.1 s and upward); (iv) its large current fluctuations during channel opening (channel flicker); and (v) its selectivity for K^+ over other alkali metal cations, such as Na^+, illustrated in the 2-min continuous record of Fig. 8.

In this experiment 200 mmol l^{-1} bath (cytoplasmic) KCl was replaced with 200 mmol l^{-1} NaCl, beginning at the arrow in the top record. Open-channel current amplitude declined continuously, reaching the baseline in about 20 s. Upon readmission of KCl (arrow in the third record), open-channel amplitude increased, again continuously, over a 45-s period. The strong asymmetry of time course in apparent open-channel current amplitude with sodium entry *versus* sodium replacement suggests that Na^+ plays a more specific role than simply as a non-conducted ion. In fact, addition of Na^+ to the cytoplasmic side of the membrane (while keeping cytoplasmic $[K^+]$ constant) decreased K^+ currents, indicating fast, voltage-dependent Na^+ blockade.

Stronger evidence for ionic blockade of the channel was provided by a systematic survey of calcium effects, illustrated in Fig. 9. Channel activity was recorded at constant membrane voltage (100 mV) with different $[Ca^{2+}]_{cyt}$, ranging from 1 μmol l^{-1} to 10 mmol l^{-1}. At the lowest $[Ca^{2+}]$, flickery open channel records were obtained with occasional long gaps and few if any discrete brief closures. As the calcium concentration was raised, discrete but brief closures (several milliseconds) became conspicuous (cf. record at 100 μmol l^{-1}), then dominant (1 mmol l^{-1}), then obliterated the open-channel characteristic (10 mmol l^{-1}), creating a kind of fuzzy baseline from which few full openings could be observed. Other divalent cations, such as Mg^{2+} had the same effect, but

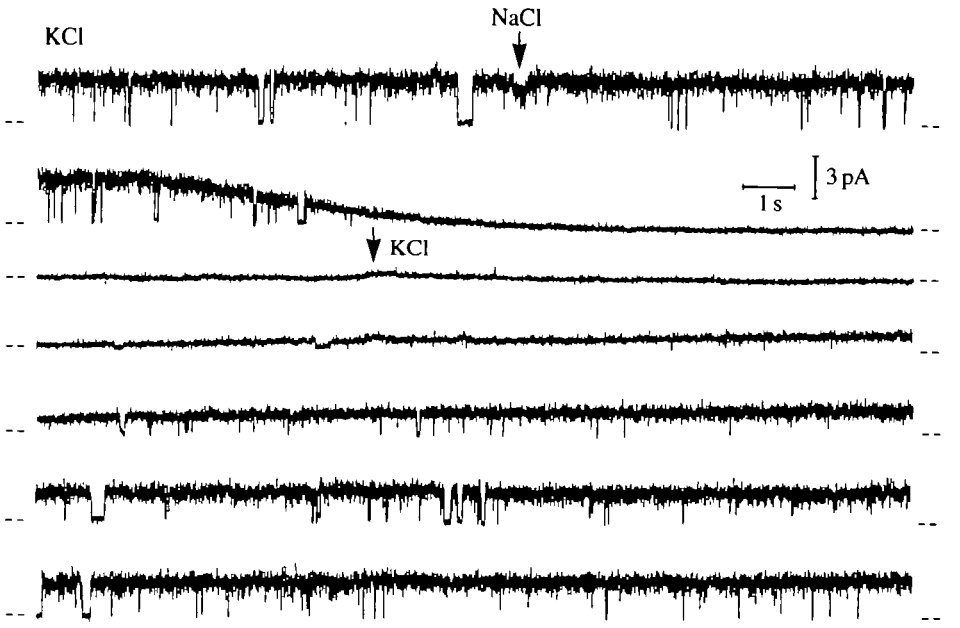

Fig. 8. Demonstration of ion specificity in YPK1. Conditions as for Fig. 7, but continuous record with membrane voltage clamped at 100 mV. Bath KCl (200 mmol l^{-1}) replaced by NaCl, beginning at arrow in top record; KCl restored, beginning at arrow in third record. Note gradual decline in open-channel current with KCl removal, and very slow recovery with KCl re-entry.

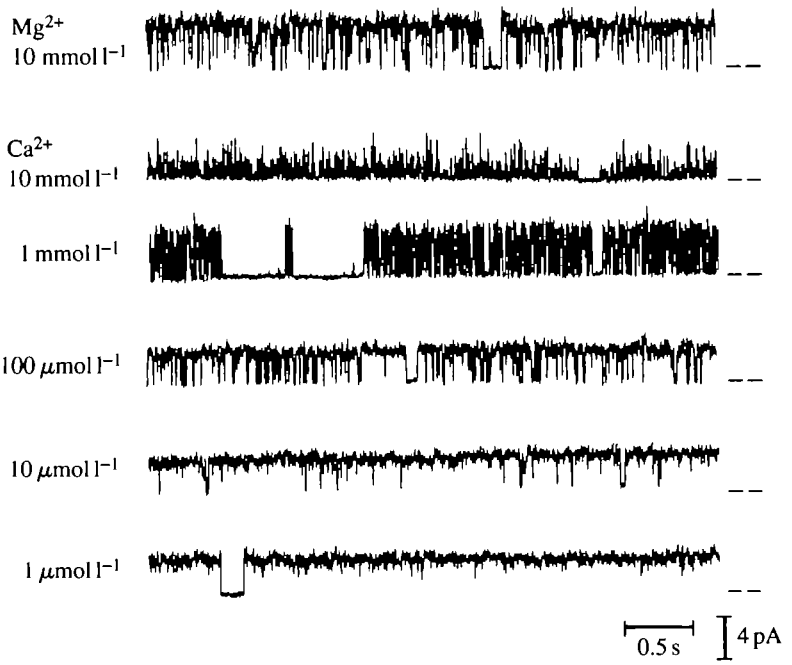

Fig. 9. Calcium (and magnesium) blockade of yeast plasma-membrane channels. General conditions as in Fig. 7, but membrane voltage set at 100 mV for all six records, and cytoplasmic $[Ca^{2+}]$ or $[Mg^{2+}]$ set as indicated. Note increased frequency of brief closures with steps of increasing $[Ca^{2+}]$, until open-channel intervals are essentially obliterated at 10 mmol l^{-1} Ca^{2+}; magnesium effect at 10 mmol l^{-1} is similar to calcium effect at 100 μmol l^{-1}. Records from two different patches (top three, bottom three).

required higher concentrations (compare 10 mmol l^{-1} Mg^{2+} with 100 μmol l^{-1} Ca^{2+}). Finally, we can add that statistical analysis of the flickery *open* channel records, by means of a beta distribution function, yielded an interpretation in terms of very brief closures (<1 ms). Long-lasting, flickery channel openings are therefore *bursts*, consisting of brief channel openings and closings, which were not resolved by the recording and sampling circuits, but did contribute to the mean level of apparent open-channel current. Quantitative analysis of the data revealed that elevating membrane voltage (+) tends to reduce the closed-channel duration, and to increase the open-channel duration during bursts.

In summary, the records of Figs 7 and 9 present evidence for three different kinds of closed states of the YPK1 channel, which we have labelled as follows: *Interrupts*, very brief closures, usually <1 ms, creating flickery open channel currents; *Blocks*, calcium-induced brief closures, 2–3 ms long; and *Gaps*, the long quiet closures, usually >100 ms, conspicuous at low positive voltages.

Each of these modes of closure appears to be independent of the others, and each has its own peculiar voltage-dependence, which can be most simply described in terms of the effect of membrane voltage upon the thermodynamic stability constant (K_I, K_B, K_G) for each closed state. Two of the closed states are also calcium-dependent: obviously, the

blocks are induced by calcium and, not so obviously, the gaps are suppressed by calcium (shortened duration). From detailed quantitative analysis of these and related experiments (A. Bertl, C. L. Slayman and D. Gradmann, in preparation), the overall calcium- and voltage-dependence of these stability constants can be written explicitly as follows:

$$K_I = 3.3\{\exp(zu)\}^{-1},$$

$$K_B = 2.7\times10^3\{[Ca^{2+}]\exp(zu)\}^2$$

and

$$K_G = 1.9\times10^{-4}\{[Ca^{2+}]\exp(zu)\}^{-1}, \quad (1)$$

in which the numerical coefficients are the *standard* values for the stability constants (with V_m=0 mV, and $[Ca^{2+}]_{cyt}$=1 mol l^{-1}), z (=1) is the charge number, and u is the 'reduced' membrane voltage (Läuger and Stark, 1970) calculated as FV_m/RT, in which F, R and T have their usual meaning.

Because increasing cytoplasmic calcium or positive membrane voltage tends to enhance blocks, but to suppress the interrupts and gaps, the net open probability (P_o) of the channel ($P_o = 1/\{1+K_I+K_B+K_G\}$) is necessarily peaked as a function of either $[Ca^{2+}]_{cyt}$ or V_m. Representative curves for the open-probability function are shown in Fig. 10.

The physiological significance of observed channel open probabilities

Observations on the stochastic behavior of single molecules, for example in patch-clamp surveys of ion channels in biological membranes, can introduce a very severe bias: *selection for species and conditions* in which the required behavior (i.e. channel opening) is maximized. For many types of channels, such bias is a necessary condition of observation, because most biological channels would – if kept open – catalyze rapid ionic equilibration, which would make them lethal. So, to carry out their selected physiological functions, most channels must be *preponderantly closed*, that is, nearly invisible to patch-clamping.

This point is well illustrated by the two *Saccharomyces* channels. Rough estimates of channel densities indicate approximately 1 YPK1 channel per 2 μm^2 of surface in the plasmalemma and approximately 1 YVC1 channel per 1 μm^2 in the tonoplast, which for yeast cells and vacuoles of *normal* size implies about 45 YPK1 per cell and 30 YVC1 per vacuole. (Experiments in Fig. 6 were carried out on enlarged vacuoles, having approximately 100 channels per vacuole.) For a *single* plasma-membrane K^+ channel of 40 pS, open at 0 mV with E_K=−100 mV (=the disequilibrium voltage, $E_K - V_m$), cytoplasmic K^+ depletion would occur at 0.5 mmol l^{-1} s^{-1}. For a single vacuolar cation channel of 150 pS, open with a disequilibrium voltage of −20 mV, vacuolar $[K^+]$ would rise at approximately 1 mmol l^{-1} s^{-1} and cytoplasmic $[K^+]$ would fall at approximately 0.25 mmol l^{-1} s^{-1}. Even for a less abundant ion like calcium ($[Ca^{2+}]_{vac} \approx 1$ mmol l^{-1}, $[Ca^{2+}]_{cyt} \approx 300$ nmol l^{-1}) shift rates of approximately 60 μmol l^{-1} s^{-1} (vac) or approximately 15 μmol l^{-1} s^{-1} (cyt) could be expected, when the higher permeability ($P_{Ca}/P_K \approx 5$) and larger disequilibrium voltage ($E_{Ca} - V_m \approx 140$ mV) are considered.

If the selected function of the vacuolar channel were, say, to 'restore' cytoplasmic $[Ca^{2+}]$ from 200 to 300 nmol l^{-1} (Miller *et al.* 1990; Halachmi and Eilam, 1989; Iida *et al.*

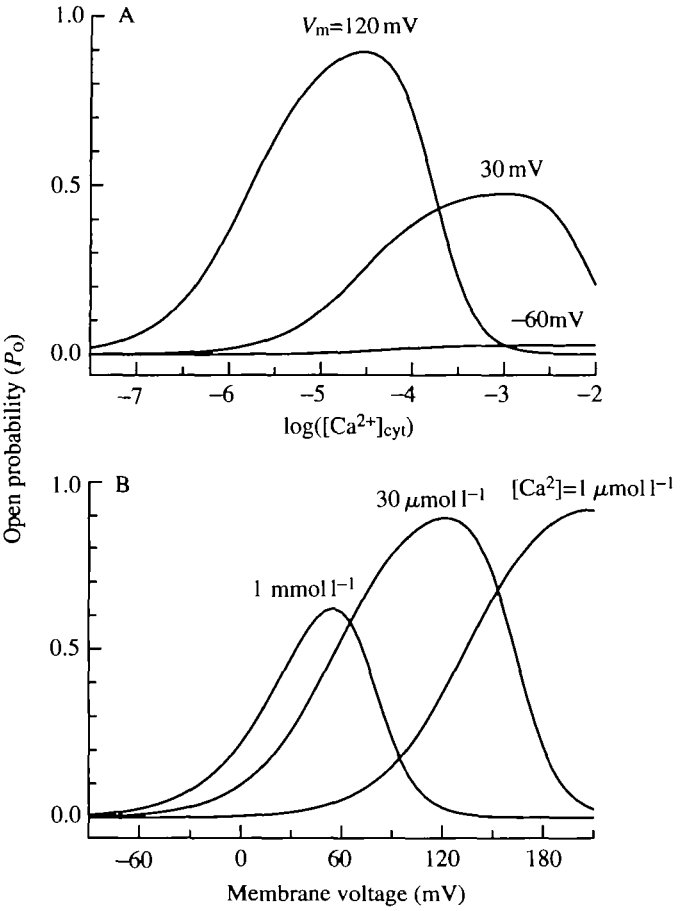

Fig. 10. Quantitative summary of calcium and voltage interactions in gating YPK1 channels. (A) Effect of calcium at three representative membrane voltages; (B) effect of voltage at three representative Ca^{2+} concentrations. Theoretical curves calculated from the reaction parameters given in equation 1.

1990), the job would be done by a single channel open for 7 ms. This implies a functional open probability of $1/(150\times30)\approx0.0002$, which belies the observed P_o values in the range 0.1–0.5 (Bertl and Slayman, 1990) near the expected tonoplast voltage. Thus, physiological regulation of YVC1, whether *via* calmodulin, pH or redox potential, must operate at *much lower values of* P_o *than those required for observation.*

The situation is similar, but not so drastic, for the plasma-membrane K^+ channel. Opening of a single 40-pS K^+ channel in the yeast plasmalemma would yield a cell-membrane resistance of $2.5\times10^{10}\,\Omega$, compared with measured fungal plasma-membrane resistivities which can exceed $100\,k\Omega\,cm^{-2}$ (Blatt and Slayman, 1983), implying approximately $10^{11}\,\Omega$ for a normal yeast cell. Then with 45 YPK1 channels per cell, P_o under resting conditions should be approximately 0.006 or smaller. Thus, for $V_m > 0$ mV, $[Ca^{2+}]_{cyt}$ levels of $1\,\mu mol\,l^{-1}$ or even lower would yield functionally plausible values of

P_o, as indicated in Fig. 10. An outward rectifying K^+ channel, as observed, could well serve for controlled release of potassium during periods of depolarization.

At normal resting membrane voltages, negative to −100 mV, however, the numbers are rather different. In that range, calculated values of P_o fall far below 10^{-5} at $[Ca^{2+}]_{cyt}$ levels below 1 μmol l^{-1}. Whether there could be a physiological 'need' for such channels is questionable, and the curves of Fig. 10 (extrapolated) raise a new question. Is the important voltage-gating parameter absolute V_m, as plotted in Fig. 10, or the disequilibrium voltage for potassium, $[E_K - V_m]$? In practical experiments, this possibility is easily obscured by operational bias: use of 200 mmol l^{-1} bath (cytoplasmic) KCl with 50 mmol l^{-1} pipette KCl (outside; $E_K \approx -39$ mV), instead of the more usual $[K^+]_{out}$ of approximately 2 mmol l^{-1} or less for ordinary growth conditions ($E_K \approx -120$ mV).

The same kind of bias is imposed in almost all patch-clamp studies, on plant or animal tissues. As a result, most of the single-channel literature focuses on absolute voltage as the important gating parameter. It has often been noted, however, that whole-cell ionic conductances, particularly for K^+, behave as if $(E_K - V_m)$ were the important gating parameter. Ciani *et al.* (1978) coined the descriptive 'electrochemical-potential-dependent gating' in modelling anomalous rectification (K^+) in *Nordora* eggs (starfish; Hagiwara and Takahashi, 1974), taking off from earlier observations on striated muscle (Hodgkin and Horowicz, 1959; Nakamura *et al.* 1965; Adrian, 1969). Recently, too, related effects of the electrochemical gradient for Cl^- have been observed upon the gating modes of Cl^- channels in *Torpedo* electroplax (Richard and Miller, 1990).

We were not suprised to find (A. Bertl, preliminary experiments), therefore, that for the yeast plasma-membrane K^+ channel, varying cytoplasmic $[K^+]$ changes P_o as if $(E_K - V_m)$ were indeed the critical gating parameter. This would mean that the curves in Fig. 10B should be shifted substantially to the left for physiological concentrations of extracellular potassium.

Integrated functioning of plasma-membrane and tonoplast channels

Yeast vacuoles are storage compartments for a wide range of metabolites and, as such, serve – or are assumed to serve – many distinct roles in the cellular economy. They are lysosomes (Wiemken *et al.* 1979), buffering reservoirs for critical ions such as H^+ and Ca^{2+}, and warehouses for amino acids, nucleosides, organic and inorganic phosphates, and carbohydrates. The accumulative transport implied for all of these roles is fueled largely by the V-ATPase, pumping protons out of the cytoplasm into the vacuole, and is mediated by a variety of H^+- or voltage-linked secondary and tertiary transport systems (Okorokov *et al.* 1985).

It has been suggested, from cation measurements made on differential lysates of *Saccharomyces*, that yeast vacuoles play a significant role in modulating cytoplasmic K^+ levels (Lichko *et al.* 1982). Although vacuolar cation channels could act to adjust $[K^+]_{cyt}$, modulating $[Ca^{2+}]_{cyt}$ seems more likely as a *selected* function for YVC1, particularly since cytoplasmic redox state and calmodulin have emerged as probable channel regulators. From a strictly energetic point of view, the direct cost of Ca^{2+} modulation *via* these channels is small, since that ion is accumulated only to millimolar levels and is

effective as a messenger at $<1\,\mu mol\,l^{-1}$, compared – for example – with tens to hundreds of millimolar for alkali metal cations, amino acids, carbohydrates and phosphates. But use of YVC1 for calcium modulation would have significant associated *indirect* costs, because of the channel's lack of selectivity among cations. Potassium leak through open channels should exceed Ca^{2+} leak by about 30-fold (see numbers above); and in normal circumstances, when the $[K^+]_{cyt}/[K^+]_{vac}$ is kept fixed, the energy for restoration would be supplied, directly or indirectly, *via* the vacuolar ATPase.

Such indirect energy loss during Ca^{2+} homeostasis probably serves an important function: to stabilize the driving force for calcium movement through the channels. Channels permeable only to Ca^{2+} would, upon opening, move the tonoplast voltage quickly toward the equilibrium voltage (E_{Ca}) for calcium diffusion, thus abolishing the driving force for Ca^{2+} exit through the channel. But channels dominated by K^+ and Na^+ permeability should move the tonoplast voltage toward the Goldman voltage ($E_{K,Na}$) for those two ions. This should be a small value, positive or negative, compared with approximately 100 mV for E_{Ca}, thus sustaining a large fraction of the initial driving force for calcium transfer. Presumably, similar principles apply to the release of other vacuolar reserves in response to metabolic changes, but demonstration and characterization of the implied array of release pathways remain to be carried out.

Thus, the general characteristics of YVC1 accord well with the current understanding of vacuolar function and suggest an obvious physiological role for such channels. Outward-rectifying K^+ channels (YPK1) in the yeast plasma membrane, however, are more bizarre, and their suggested functions are necessarily more speculative. From measurements on both isolated patches and cell-attached patches (*in vivo*), YPK1 should be mostly closed under normal conditions. Depolarization or a (perhaps transient) rise in $[Ca^{2+}]_{cyt}$ might activate these channels, but the existence of either of these precedent activities must be identified.

One such activity may be osmotic regulation. The large operating voltage (several hundred millivolts) of the plasma-membrane ATPase (a P-type ATPase) is useful for driving uptake of rarefied nutrients, but can also lead to hyperaccumulation of plentiful cations, such as K^+. As has also been suggested for plants (Slayman, 1992), an effective antidote to K^+ engorgement would be periodic salt loss *via* coordinated opening of anion channels and outward-rectifying cation channels. Anion currents seem to dominate the slow action potentials seen in fungi (Fuller and Picard, 1972; Slayman *et al.* 1976; Müller *et al.* 1986; Caldwell *et al.* 1986), and the accompanying depolarization would be a clear way to activate YPK1 channels.

Another physiological activity may be charge balancing during rapid influxes of amino acids or sugars, or during subsequent metabolic events. H^+-coupled symport of maltose, for example, has been reported to activate passive K^+ efflux from *Saccharomyces* (Serrano, 1977). Under certain circumstances, the associated charge influx is fast enough to short-circuit the yeast membrane, which – like anion action potentials – could activate YPK1. With sugars such as glucose, however, which is absorbed without ion coupling (Bisson and Fraenkel, 1982; Cirillo, 1989), a different trigger or pathway is required for the observed K^+ efflux. But glucose influx, followed by fast oxidation, produces other metabolic changes, including a rise of cytoplasmic $[Ca^{2+}]$ (Eilam *et al.* 1990; Eilam and

Othman, 1990), a transient rise of cytoplasmic reducing equivalents (Ghosh and Chance, 1964; Polakis and Bartley, 1966), and rapid cytoplasmic alkalization (Gillies *et al.* 1982). Since each of these changes would tend to activate YVC1 channels, the vacuole is a prime candidate-source for the *sustained* elevation of $[Ca^{2+}]_{cyt}$, which in turn would sustain activation of the plasma-membrane K^+ channels.

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