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Calcium- and voltage-dependent ion channels in *Saccharomyces cerevisiae*

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SUMMARY

Ion channels in both the tonoplast and the plasma membrane of *Saccharomyces cerevisiae* have been characterized at the single channel level by patch-clamp techniques. The predominant tonoplast channel is cation selective, has an open-channel conductance of 120 pS in 100 mM KCl, and conducts Na^+ or K^+ equally well, and Ca^{2+} to a lesser extent. Its open probability (P_o) is voltage-dependent, peaking at about −80 mV (cytoplasm negative), and falling to near zero at +80 mV. Elevated cytoplasmic Ca^{2+}, alkaline cytoplasmic pH, and reducing agents activate the channel. The predominant plasma membrane channel is highly selective for K^+ over anions and other cations, and shows strong outward rectification of the time-averaged current-voltage curves in cell-attached experiments. In isolated inside-out patches with micromolar cytoplasmic Ca^{2+}, this channel is activated by positive going membrane voltages: mean P_o is zero at negative membrane voltages and near unity at 100 mV. At moderate positive membrane voltages (20–40 mV), elevating cytoplasmic Ca^{2+} activates the channel to open in bursts of several hundred milliseconds duration. At higher positive membrane voltages, however, elevating cytoplasmic Ca^{2+} blocks the channel in a voltage-dependent fashion for periods of 2–3 ms. The frequency of these blocking events depends on cytoplasmic Ca^{2+} and membrane voltage according to second-order kinetics. Alternative cations, such as Mg^{2+} or Na^+, block the yeast plasma-membrane K^+ channel in a similar but less pronounced manner.

1. INTRODUCTION

Cytoplasmic calcium is now accepted as a ubiquitous second messenger which plays a vital role in all living cells. In the common yeast, *Saccharomyces cerevisiae* (see, for example, Davies & Thorner 1986), Ca^{2+} involvement has been demonstrated in several processes, including mating (Iida *et al.* 1990) and growth and cell division (Ohya *et al.* 1986), which have also been reported as calmodulin-dependent (Davies *et al.* 1986; Geiser *et al.* 1991). Elevation of cytoplasmic Ca^{2+}, as a signal, can also be triggered by a variety of stimuli, such as pheromones (Iida *et al.* 1990) or glucose uptake (Eilam & Othman 1990; Eilam *et al.* 1990).

Elevation of cytoplasmic Ca^{2+} can, in principle, occur either by entry of extracellular Ca^{2+} through the plasma membrane or by release of Ca^{2+} from intracellular stores. Some evidence has been presented showing that a rapid, transient rise which occurs 3–5 min after glucose application can be explained as a voltage-dependent influx across the plasma membrane (Eilam & Chernikovsky 1987). A slower rise in cytoplasmic Ca^{2+} can be observed 60–80 min after application of glucose (Eilam & Othman 1990), and 30–40 min after application of the mating pheromone, α-factor (Iida *et al.* 1990). In the latter case, elevation of cytoplasmic Ca^{2+} probably depends upon a metabolic product(s) to trigger release of Ca^{2+} from internal stores.

In *Saccharomyces cerevisiae*, a prominent intracellular source for Ca^{2+} is the central vacuole, which acts as a storage compartment not only for Ca^{2+}, but also for phosphates (ortho-, pyro-, and poly-phosphates), lytic enzymes, amino acids, and protons (Okorokov *et al.* 1980). Uptake of highly accumulated substrates, such as Ca^{2+} or basic amino acids into the yeast vacuole is mediated by an H^+/Ca^{2+} antiporter (Ohsumi & Anraku 1983; Eilam *et al.* 1985), and H^+/amino-acid antiporters (Ohsumi & Anraku 1981), respectively, and is coupled to an electrochemical gradient for protons generated by the vacuolar H^+-ATPase (Ohsumi & Anraku 1981; Kakinuma *et al.* 1981).

Although those primary and secondary active transport systems had been characterized in considerable detail, very little was known about the mechanisms by which accumulated substrates are released from the yeast vacuole, until ion channels were identified in the yeast tonoplast by electrophysiological experiments with vacuolar vesicles fused into lipid bilayers (Wada *et al.* 1987; Tanifuji *et al.* 1988). After initial patch-

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clamp experiments had confirmed the existence of these channels in intact vacuolar membranes (Bertl & Slayman 1990), we argued that their poor selectivity and particular voltage-dependence would favour a primary role in releasing stored cations or neutral substrates into the cytoplasm, upon metabolic demand.

Signal transduction in *Saccharomyces* may operate not only via chemical messengers such as Ca^{2+} (Eilam & Othman 1990), pH (Gillies 1982), or NADH (Polakis & Bartley 1966), but also via electrical signals. This possibility is raised by observations of plasma-membrane depolarization and concomitant K^+-efflux during co-import of protons with amino acids or disaccharides (Cirillo 1961; Eddy 1978, 1982; Eddy & Hopkins 1989; Serrano 1977). Potassium efflux can also be observed during uptake of glucose (Borst-Pauwels *et al.* 1988), which is not coupled to the electrochemical H^+-gradient, but occurs via facilitated diffusion (Heredia *et al.* 1968; Bisson & Fraenkel 1982).

Potassium transport across the yeast plasma membrane appears to be mediated by a dual-affinity transport system (Rodriguez-Navarro & Ramos 1984). A gene (*TRK1*) that is required for high-affinity K^+ transport in *S. cerevisiae* has been cloned and sequenced (Gaber *et al.* 1988). It has been shown, furthermore, that high-affinity K^+ uptake occurs via a transporter which is functionally distinct from both the plasma-membrane H^+ pump and a low-affinity K^+ uptake activity (Gaber *et al.* 1988). A likely candidate for the high-affinity K^+ transport system has been described in *Neurospora* (Rodriguez-Navarro *et al.* 1986; Blatt *et al.* 1987), where K^+ uptake is driven by the electrochemical gradient for protons, built up by the plasma membrane H^+-ATPase. Thus, the H^+–K^+ symporter is indirectly dependent on H^+-pump activity. On the other hand, low-affinity K^+ transport across the yeast plasma membrane may be mediated by a K^+ channel. This could be clarified by patch-clamp experiments on yeast mutants lacking the *TRK2* gene that encodes a transporter required for low affinity K^+ transport (Ko & Gaber 1991; Ko *et al.* 1990). So far, at least one type of K^+ channel has been described in the yeast plasma membrane (Gustin *et al.* 1986). In mutants (*pma1*-105) of *S. cerevisiae* this K^+ channel has been reported to be activated by intracellular application of ATP, and blocked by the ATPase inhibitor *N,N′*-dicyclohexylcarbodiimide (DCCD; Ramirez *et al.* 1989). Interestingly, the mutation is in the structural gene (*PMA1*) encoding the plasma membrane H^+-ATPase. Because the wild-type channels are not sensitive to ATP or DCCD, Ramirez *et al.* (1991) suggested a tight coupling between the K^+ channel and the H^+-ATPase.

In this paper we present data on the identification and characterization of ion channels in the plasma membrane and tonoplast of *Saccharomyces cerevisiae*, and on their modulation by cytoplasmic factors such as free Ca^{2+}, pH, and redox state. Possible physiological roles for these channels, in mediating potassium efflux and elevation of cytoplasmic calcium during the uptake of metabolic substrates (such as glucose and maltose), are outlined.

2. RESULTS AND DISCUSSION

(*a*) *Experimental conditions*

(*i*) *Preparation of protoplasts*

Although patch-clamp experiments are usually not limited by the size of the cells, large cells remain more convenient and easier to work with than small ones. We therefore carried out the bulk of these experiments on a tetraploid strain, YCC78, of *Saccharomyces cerevisiae* whose surface area per cell is about 2.5-fold that of normal haploid yeast. Cells were grown, and protoplasts and vacuoles isolated, as described by Bertl & Slayman (1990). The size of the protoplasts varied in the range of 4–6 μm in diameter, with central vacuoles occupying 15–25% of the cell volume. Larger protoplasts and vacuoles were obtained by incubating freshly liberated protoplasts for 24–48 h in glucose-supplemented saline (250 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM glucose, and 5 mM MES titrated to pH 7.2 with Tris base) at 25°C. This allowed the protoplasts to enlarge without either dividing or regenerating cell walls, and yielded protoplasts up to 15 μm in diameter after 24 h, at which time the vacuoles occupied more than 90% of the cell volume.

(*ii*) *Recording conditions*

The standard pipette solution for tonoplast studies contained 100 mM KCl, 5 mM $MgCl_2$, 10 μM free Ca^{2+} buffered with EGTA, set at pH 7.0 with Tris base; that for plasma-membrane experiments contained 50 mM KCl and 100 μM $CaCl_2$, equilibrated with air (pH 5.5–5.8). Standard bath (cytoplasmic) recording solutions contained 100 mM KCl for tonoplast experiments, and 200 mM KCl for plasma-membrane experiments; MES titrated to pH 7 with Tris (except in figure 4); Ca^{2+} unbuffered at 1 mM and above, and buffered with EGTA at 100 μM and below. Deviations from these compositions are given in the appropriate figure legends.

Output from the patch-clamp amplifier was recorded on video tape via a pulse-code modulator (Bertl & Slayman 1990) with a system time constant near 20 μs. The tape records were then low-pass filtered, usually at 200 Hz, and digitized at 500 Hz–2 kHz.

(*iii*) *Definitions and conventions*

Throughout this report, membrane voltage will be given as the electrical potential of the cytoplasm minus that of the extracytoplasmic solution. This traditional convention is used here for measurements on both the plasma membrane and the vacuolar membrane, regardless of the actual physical orientation of the membrane being studied (i.e. outside-out, inside-out, or cell-attached). Correspondingly, currents resulting from the flow of positive charges from the cytoplasmic side to the extracytoplasmic side are defined as positive, are plotted upward, and are termed outward currents. For simplicity, we use the

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term 'cytoplasmic Ca^{2+}' to mean the concentration of free calcium at the cytoplasmic side of the membrane.

(*b*) *The vacuolar cation channel*

The main ion channel detected so far in the tonoplast of *Saccharomyces cerevisiae* is cation selective and has an open-channel conductance of about 120 pS in 100 mM KCl (Wada *et al.* 1987; Tanifuji *et al.* 1988; Bertl & Slayman 1990). Its open probability depends strongly upon membrane voltage, favoring movement of positively charged substrates from the vacuolar side to the cytoplasmic side of the membrane.

(*i*) *Rundown and Ca^{2+}-dependence*

Initial experiments on the vacuolar cation channel from *Saccharomyces* reconstituted into lipid bilayer membranes (Tanifuji *et al.* 1988) showed channel opening to require cytoplasmic Ca^{2+}, but at unphysiologically high concentrations (1 mM and above). Bertl & Slayman (1990) confirmed this property in the course of patch-clamp experiments from isolated yeast vacuoles, but found the high Ca^{2+} requirement to arise gradually during prolonged recording. Figure 1 demonstrates this in a continuous record from an isolated tonoplast patch, with the cytoplasmic side facing the bath solution. In 1 μM cytoplasmic Ca^{2+}, initial channel open probability was very high (one channel open almost all the time and occasional opening of a second channel), but declined gradually to less than 2% of the initial value after about 2 min. Such rundown of channel activity usually continued over periods of 20–40 min, but the silenced channels could be reactivated with progressively higher concentrations of cytoplasmic Ca^{2+} (mM).

Figure 2 shows the mean open probability (P_o) of a rundown channel as a function of cytoplasmic Ca^{2+}. P_o was near zero for cytoplasmic Ca^{2+} less than 1 mM, rose sigmoidally with increasing cytoplasmic Ca^{2+}, and was half maximal at about 2.5 mM. This corresponds well with the Ca^{2+}-dependence of macroscopic currents (Wada *et al.* 1987) and also with the Ca^{2+}-dependence of time-averaged single channel currents in lipid bilayers (Tanifuji *et al.* 1988).

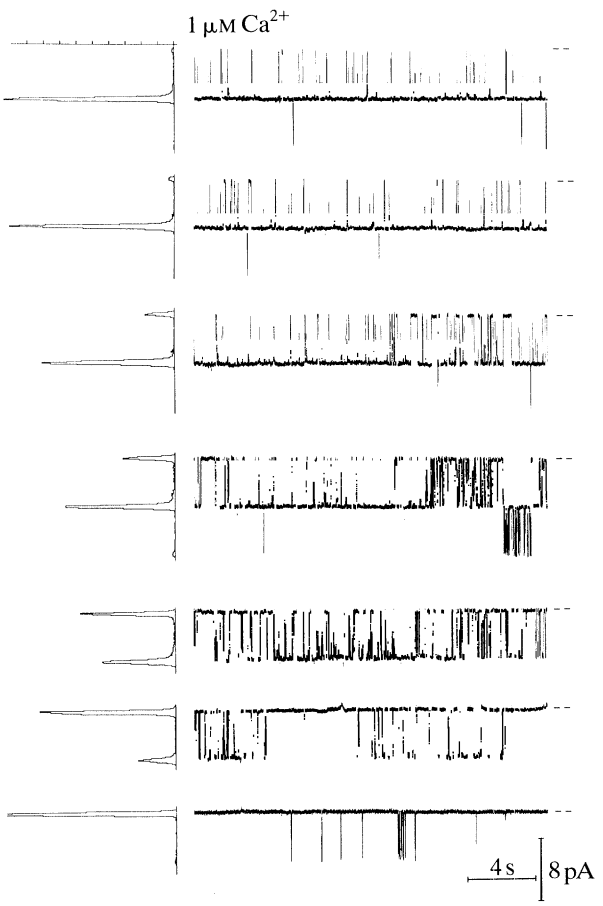

Figure 1. Spontaneous rundown of tonoplast cation channels. Continuous record from an excised patch showing the time-dependent decrease in channel activity. Bathing solution (cytoplasmic): 100 mM KCl with 1 μM Ca^{2+}. Membrane voltage clamped at −40 mV. All-points amplitude histograms are shown for each trace, at the left. –– Denotes the baseline with all channels closed.

(*ii*) *Modulation by reducing agents*

Reducing agents, such as β-mercaptoethanol (ME), dithiothreitol (DTT) or reduced glutathione (GSH) prevent decline of channel activity and reactivate rundown yeast tonoplast channels (Bertl & Slayman 1990).

A corollary of the enhancement of channel activity by reducing agents is a large reduction of cytoplasmic Ca^{2+} required for channel activation, from a very unphysiological range (millimolar) to the micromolar range. This is illustrated in figure 3, which displays the Ca^{2+}-dependence of multiple channels in a patch which had previously gone silent in 1 mM cytoplasmic Ca^{2+} and was then reactivated with 10 mM ME. In all experiments on yeast tonoplast channels, the presence of reducing agents yielded considerable channel activ-

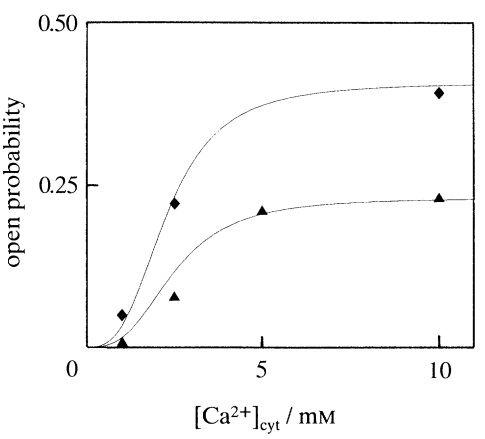

Figure 2. Effect of cytoplasmic calcium concentration upon the open probability of yeast tonoplast channels in freshly excised patches. 100 mM KCl in both the pipette and the bath solution ('symmetric KCl'). Measurements were made approximately 30 min after vacuole isolation, and each data point represents approximately 3 min recording time. Membrane voltage clamped at +20 mV for the lower curve and −20 mV for the upper curve.

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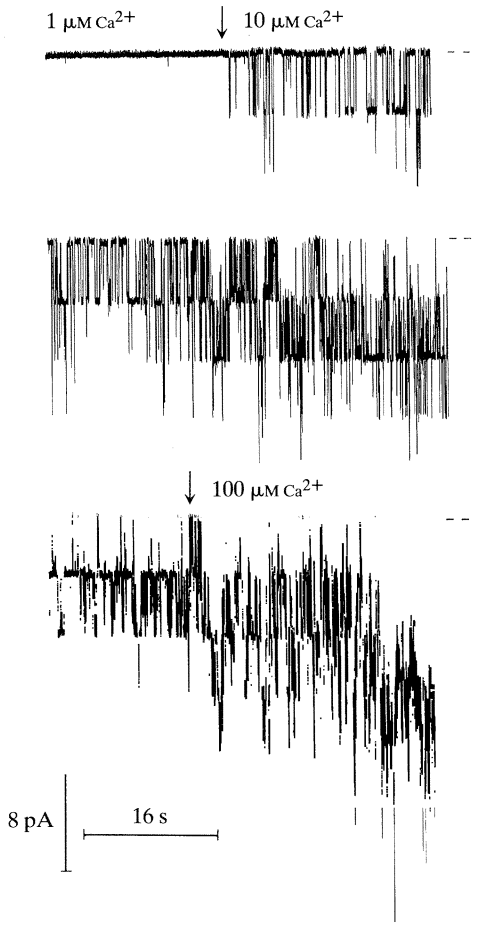

Figure 3. Calcium-dependence of channel opening in the presence of β-mercaptoethanol (ME). Tonoplast patch containing at least eight channels; symmetric 100 mM KCl; clamped membrane voltage = −40 mV; 10 mM ME. Almost no channel activity was observed in 1 μM cytoplasmic Ca^{2+} (only rare, partially resolved openings are visible), but 10 μM Ca^{2+} activated several channels (one or two open on average), and 100 μM activated as many as eight (lower right of bottom record; about three open on average). –– Denotes baseline with all channels closed.

ity at micromolar cytoplasmic Ca^{2+}, thus suggesting that physiological reducing agents may play an essential role in ion permeation through this channel.

An explicit molecular role for redox modulation of channel behaviour has been noted more recently in studies of animal K^+ channels, expressed in oocytes (Ruppersberg *et al.* 1991). These K^+ channels are transiently activated upon voltage steps and show a characteristic inactivation, with time constants in the neighbourhood of 10 ms. Fast inactivation was lost in excised patches when the channels were removed from the cytoplasmic environment. But pushing the patch pipette back into the cytoplasm of the oocyte, or adding reducing agents (e.g. DTT or reduced glutathione) to the bath solution, restored the physiological behaviour (fast inactivation) of these channels. Thiel (1991) reported that the sulphydryl group reagent p-chloromercuribenzenesulfonate (pCMBS) inhibits K^+ currents in *Chara* plasma membrane, and DTT partly reverses the pCMBS effect. In patch clamp experiments on plant vacuolar channels Johannes *et al.* (1992) and Weiser & Bentrup (1991) did not explicitly demonstrate channel activation by reducing agents, although they used 0.5–1 mM DTT (on the cytoplasmic side) in their experiments, which probably indicates that reducing agents were necessary for channel activity. Thus, reducing agents have been demonstrated to be important for the physiological response of ion channels. It is not yet known, however, whether such effects represent bona fide regulatory mechanisms, or whether a constantly reduced cytoplasmic environment is essential for functioning of these channels, as it is for many cytoplasmic enzymes. Nevertheless, cytoplasmic concentrations of physiological reducing agents, such as glutathione or NADH, can change up to 10-fold upon environmental stimuli and can reach concentrations as high as 0.8 mM (Polakis & Bartley 1966). This suggests that the redox-dependence of ion channels (Bertl & Slayman 1990; Ruppersberg *et al.* 1991) is physiologically important, and not simply a laboratory artifact.

The redox and calcium sensitivities of the yeast vacuolar cation channel were abolished by the strong oxidizing agent chloramine T, which is also known as a strong inhibitor of calmodulin (Thiry *et al.* 1980). This result prompted us to some preliminary experiments on depletion and replacement of calmodulin, and the results are indeed consistent with its involvement in regulating this channel.

(iii) pH-dependence

Even in the presence of reducing agents, concentrations of 1–10 μM cytoplasmic Ca^{2+} were necessary to activate yeast cation channels. This concentration range is close to, but still somewhat higher than, the cytoplasmic Ca^{2+} expected in yeast cells under physiological conditions (Halachmi & Eilam 1989; Iida *et al.* 1990). However, it is well known that other ionic constituents greatly affect calcium binding to Ca^{2+}-sensitive proteins. Haiech *et al.* (1981) showed that the affinity of calmodulin for Ca^{2+} decreased as K^+, Mg^{2+} or H^+ concentrations increased. No data are available yet on effects of K^+ or Mg^{2+} on the Ca^{2+}-dependence of the yeast tonoplast cation channel, but modulation of these channels by protons is clearly demonstrated in figure 4. At constant cytoplasmic Ca^{2+} (0.1 mM), channel openings were rare at pH 5.5, but cytoplasmic alkalinization to pH 7.5 greatly increased open probability. A similar pH-dependence in open probability has been reported recently for K^+ channels from rat muscles (Laurido *et al.* 1991).

Although the normal cytoplasmic pH of aerobic *Saccharomyces* is about pH 7.2, the measured pH can vary with changing physiological conditions, and may range at least between pH 5.5 and pH 7.8 (Borst–Pauwels 1981; Salhany *et al.* 1975; Gillies 1982; A. Ballarin–Denti and C. L. Slayman, unpublished experiments). Thus, pH in yeast is apparently not as stable as in plant cells (Bertl *et al.* 1984; Felle & Bertl 1986; Kurkdjian & Guern 1989). Most of our tonoplast experiments were carried out at pH 7.0, and the results shown in figure 4 indicate that in a slightly alkaline environment, the Ca^{2+} requirement for

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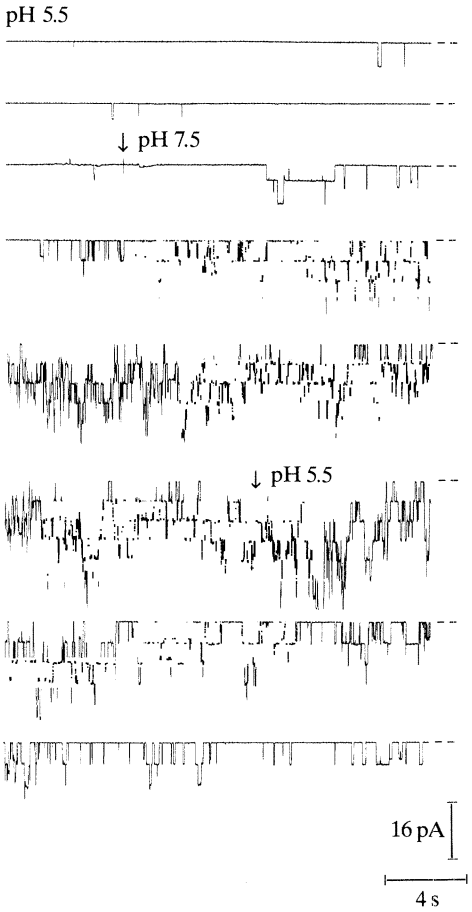

Figure 4. Modulation of tonoplast channel activity by cytoplasmic pH. Continuous record (top to bottom) from an excised patch; symmetric 100 mM KCl, with 100 μM cytoplasmic Ca^{2+} plus 1 mM DTT; membrane voltage = −40 mV. Channel openings were rare at pH 5.5, but up to eight distinct channels were seen after the transition to pH 7.5. Channel activity decreased again after switching back to pH 5.5. Baseline with all channels closed is marked by −−.

channel activity would be further reduced, from 1–10 μM to less than 1 μM.

(*iv*) *Selectivity*

A prominent ion channel in the vacuolar membrane of many plant cells (often referred to as the 'SV-channel') has been reported to be rather unspecific, with relative permeabilities for K^+:Na^+:Cl^- of about 1:1:0.2 (for reviews, see Hedrich *et al.* 1988; Hedrich & Schroeder 1990; Tester 1990). The main ion channel in the yeast tonoplast discriminates poorly between cations, but is impermeable to anions (Wada *et al.* 1987; Tanifuji *et al.* 1988; Bertl & Slayman 1990). However, results from an experiment with 100 mM vacuolar and 10 mM cytoplasmic KCl (in the presence of 10 mM cytoplasmic $CaCl_2$) seemed to render this statement invalid, as shown in figure 5. Under these conditions, the equilibrium voltage for K^+ as calculated by the Nernst equation using ion activities (rather than concentrations) would be about 50 mV. In contrast, the records show the open channel currents to reverse at about 20 mV. That the

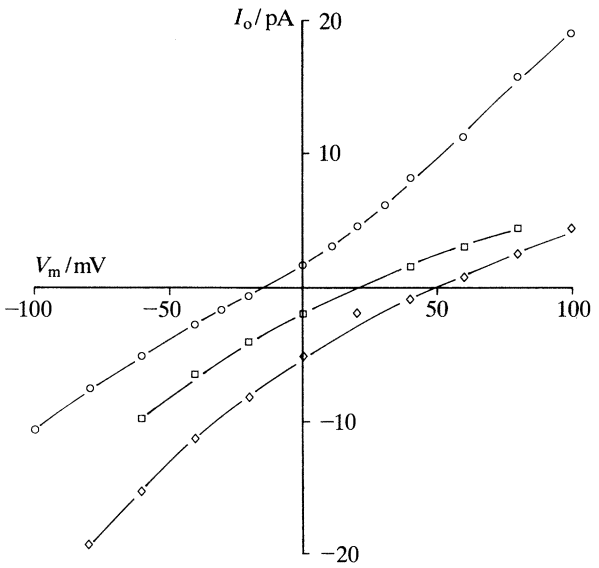

Figure 5. Influence of both (cytoplasmic) ion composition upon the voltage dependence of single-channel currents. Excised tonoplast patch. Pipette contained 100 mM KCl and 10 μM buffered free Ca^{2+} for all three curves. Bath: 200 mM KCl, 10 mM $CaCl_2$ (upper curve); 10 mM KCl, 10 mM $CaCl_2$ (middle curve); 10 mM KCl, 10 μM $CaCl_2$ (lower curve).

inferred Cl^- effect was spurious, however, was shown by reducing cytoplasmic Ca^{2+} from 10 mM to 10 μM, which shifted the reversal voltage for the same K^+ gradient (100 mM/10 mM) from 20 mV to the expected 50 mV. This result indicates, instead, that the yeast tonoplast channel is significantly permeable to Ca^{2+}. Thus, the cation channel in the vacuolar membrane of *Saccharomyces* may well be a device for elevating cytoplasmic Ca^{2+} in response to environmental stimuli.

It is important to realize that this result is qualitative, not quantitative. The data themselves make clear that calculations of relative permeability for two ionic species, from altered reversal potentials, must incorporate information about the channel's partial conductance for all significant ionic species present. We will therefore postpone the quantitative calculation of relative Ca^{2+} permeability until more complete data on the open-channel current and conductance are available.

(*c*) *Plasma-Membrane Channels*

(*i*) *Overview*

Prior reports have noted two distinct types of ion channels in the plasma membrane of *Saccharomyces cerevisiae*: (i) potassium channels with an open-channel conductance of ~20 pS (in symmetrical 100 mM KCl; Gustin *et al.* 1986), and with high selectivity for K^+ over both Na^+ and Cl^-; and (ii) rather unspecific channels of 30–40 pS and apparent mechanosensitivity (Gustin *et al.* 1988). Putative mechanosensitive (or stretch-activated) channels have now been identified in a wide variety of cells and tissues, including plants (Cosgrove & Hedrich 1992), fungi (Gustin *et al.* 1988; Zhou *et al.* 1991), and bacteria (Martinac *et al.*

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1987). Such channels are surely interesting for their physical interaction with membrane (surface) forces, but whether or not they have a bona fide physiological role as sensors or transducers is thus far unresolved (Morris & Horn 1991).

More recently, K^+ channels in yeast plasma membrane have been described as voltage-gated, opening at trans-membrane voltages beyond ± 100 mV (Ramirez *et al.* 1989). The most interesting aspect of this property is that in a particular mutant, *pma1*-105, cytoplasmic ATP appears to narrow the gating threshold to ± 60 mV; but ATP has no such effect on wild-type cells. Because the mutant carries an altered structural gene for the plasma membrane H^+-ATPase, Ramirez *et al.* (1989) suggested a tight physical coupling between the ATPase and the K^+ channel.

The species of K^+ channel first described by Gustin *et al.* (1986) has proven the easiest to identify under controlled conditions, and has dominated our studies of the yeast plasma membrane. Its behaviour, seen in a cell-attached experiment in figure 6, contrasts in many respects with that of the tonoplast cation channels (see above). It has a much smaller apparent conductance, about 30 pS in symmetric 200 mM K^+, has essentially zero open probability (P_o) for negative membrane voltages, but shows increasing P_o with increasingly positive membrane voltages (figure 7);

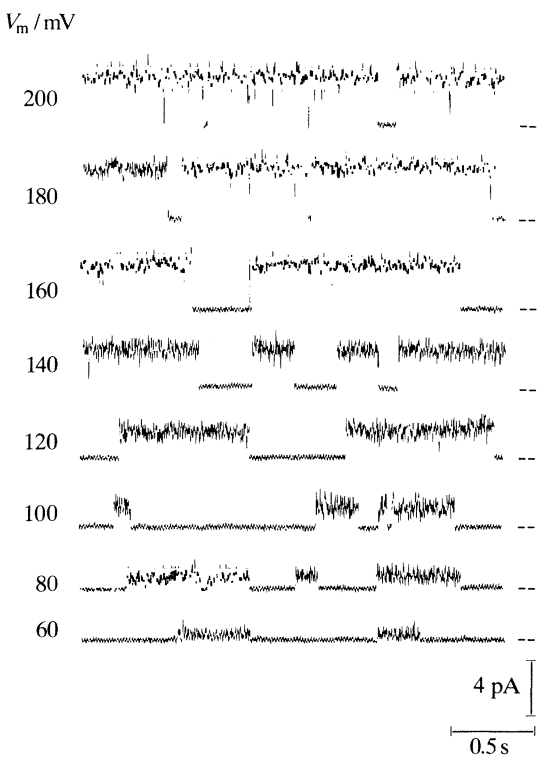

Figure 6. Examples of single-channel currents from the yeast plasma membrane. Cell-attached recording configuration, with 200 mM K^+ in pipette and bath. Normal cytoplasmic K^+ concentration is in the range of 100–200 mM, so the resting voltage should have been near zero; then the clamp voltage (V_m) approximately equals the actual membrane voltage. Note the noisy open channel currents. Data were low-pass filtered at 200 Hz and sampled at 2 kHz. Baseline is marked by ––.

also, its open-channel current appears very noisy. At moderate positive membrane voltages, elevating cytoplasmic Ca^{2+} activates the plasma-membrane K^+ channels to open for periods of several hundred milliseconds; but at large positive membrane voltages, high cytoplasmic Ca^{2+} leads to channel blockade.

(ii) Open channel noise

Compared with the symmetric (Gaussian) noise of the baseline, all-points amplitude histograms of open-channel currents showed asymmetrically enlarged noise (Bertl *et al.* 1992*b*). This phenomenon can be explained by very brief openings and closings, which are incompletely resolved by the recording apparatus; i.e. the apparent long-lasting openings shown in figure 6 would in fact be bursts. Then the mean open- and closed-times within these bursts, as well as the true open-channel currents, can be estimated by fitting a beta-distribution (convoluted by the Gaussian noise of the baseline) to the asymmetric histograms at each membrane voltage tested (Yellen 1984). Quantitative analysis of the data showed mean open times to increase by about fivefold per (+) 58 mV increase of membrane voltage, whereas the mean closed times decrease about two fold per 58 mV. These numbers yield an open probability, within the bursts, which bends from a saturating value near unity at positive voltages toward a 10-fold decrease per -58 mV at more negative voltages ($P_o = 0.5$ at about 40 mV). The simplest interpretation of this result is that a barrier to a single-charge gating movement lies approximately two-thirds of the way through the membrane. These switching kinetics do not depend on cytoplasmic Ca^{2+}.

Thus the apparent open-channel currents, which are filtered averages, must be less than the true open-channel currents; and because of the particular switching kinetics, the apparent currents fell well

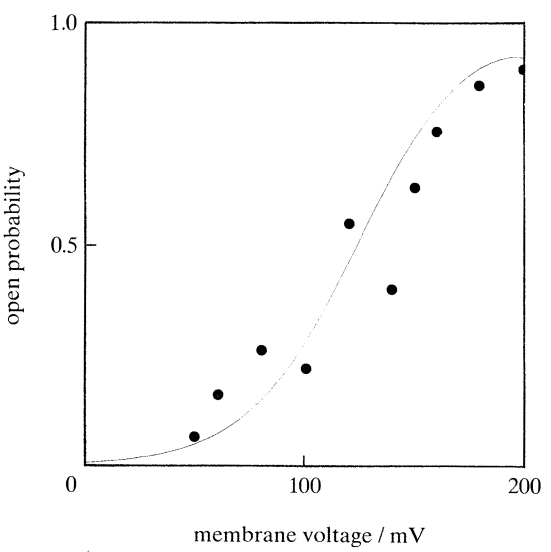

Figure 7. Open probability of the *Saccharomyces* plasma membrane K^+ channel, as a function of voltage. Data taken from the experiment in figure 6; each plotted point represents data from about 2 min recording time.

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below the true values at low positive (or negative) membrane voltages, but approached the true values at high positive voltages.

(iii) Calcium- and voltage-dependent blockade

In solutions with 1 μM cytoplasmic free Ca^{2+}, the plasma-membrane K^+ channel had the same general properties in isolated inside-out patches (figure 8) as it did in cell-attached patches: strong outward rectification, noisy open-channel current, mean burst durations in the range of several hundred milliseconds, and low conductance. (Open-channel conductance was somewhat larger in isolated patches, probably because of differing compositions of cytoplasm and the saline solution (200 mM KCl) used with inside-out patches.) Elevation of cytoplasmic Ca^{2+}, however, drastically altered the gating properties of this channel. With 10 mM cytoplasmic Ca^{2+}, and at +100 mV membrane voltage, brief closures or blockades occurred at a frequency greater than 50 per second, as shown in the first four traces of figure 9. Reducing cytoplasmic calcium to 1 μM (middle of trace 4) gradually reduced the frequency of the blocking events. A detailed analysis of this behaviour (Bertl *et al.* 1992*b*) showed that the frequency of the blocking events depends upon both cytoplasmic Ca^{2+} and membrane voltage, with second order kinetics. The mean lifetime of the blocking events (2–3 ms), however, was independent of both membrane voltage and cytoplasmic Ca^{2+}.

Additional studies have shown that other cations also interact with the yeast plasma membrane K^+ channel. Mg^{2+} mimicked calcium in blocking the channel with similar lifetimes of the blocking events,

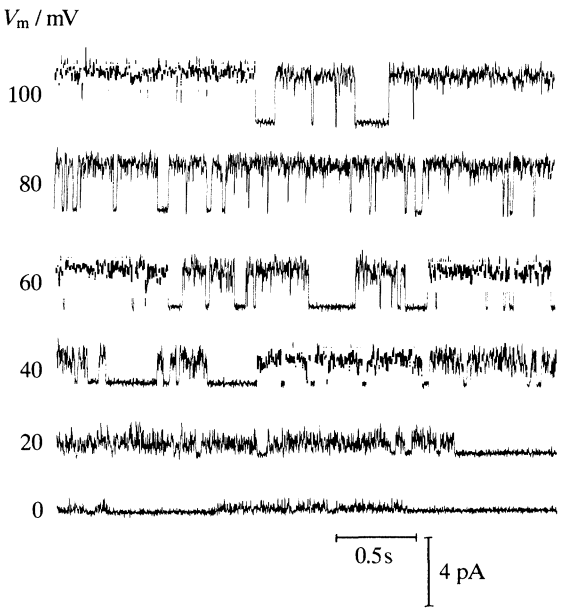

Figure 8. Voltage-dependence of single-channel K^+ currents in an excised, inside-out patch. 50 mM KCl in the pipette, and 200 mM KCl plus 1 μM Ca^{2+} in the bath (cytoplasm). In these inside-out patches, open-channel conductance was somewhat higher than in the cell-attached experiment of figure 6, probably due to different cytoplasmic salt contents (detailed cytoplasmic composition is unknown). Note again the high noise level, compared with the baseline, which is most prominent at moderate voltages (see 40 mV trace). Asymmetry in the noise is visible in a preponderance of brief (incomplete) upspikes in the 20 mV record, and of brief downspikes in the 60 mV record. Data were low-pass filtered at 200 Hz and sampled at 2 kHz.

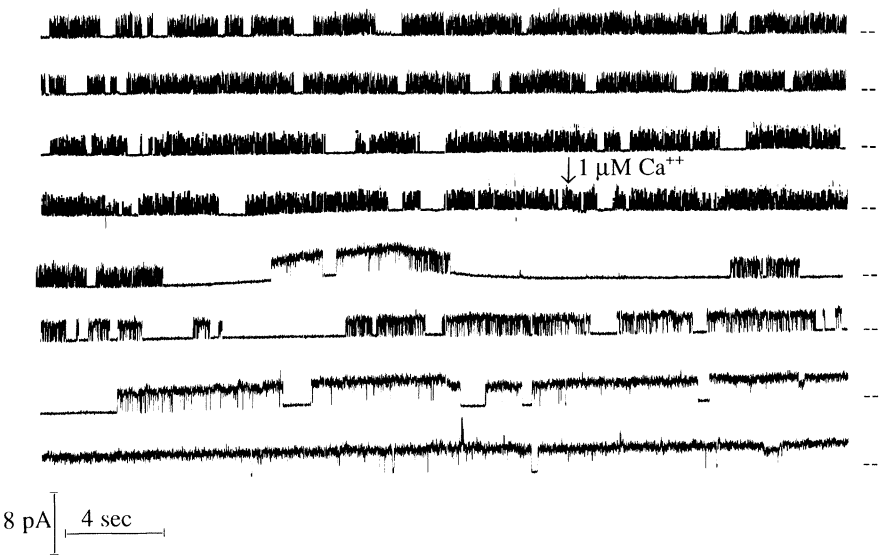

Figure 9. Calcium blockade of the yeast plasma-membrane K^+ channel. Continuous record from an inside-out patch; 50 mM KCl in the pipette, 200 mM KCl in the bath (cytoplasmic) solution; membrane voltage clamped at 100 mV. In 10 mM cytoplasmic Ca^{2+} (top four traces), long channel-open intervals were interrupted by many brief blocking events at a frequency of about 50 per second. Transition to 1 μM cytoplasmic Ca^{2+} (trace 4) gradually reduced the frequency of blockades, with little change either in the duration of channel-open intervals or in the duration of individual blocking events (not evident at the time resolution shown). Also note the high noise level of open-channel currents, visible in the lower four traces when calcium blockade was infrequent. The baseline is marked by --. (The hump in the fifth trace is an artifact due to a discontinuity in solution exchange.)

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but with a weaker voltage-dependence. Cytoplasmic Na^+, added to K^+, reduced open-channel currents without visible signs of blocking events, suggesting that blockade by Na^+ is very fast. Voltage-dependent blockades of potassium channels by other cations (Ca^{2+}, Mg^{2+}, Cs^+, Na^+) have frequently been reported (Armstrong *et al.* 1982; Lansman *et al.* 1986; Bertl 1989; Thiel & Blatt 1991; Liu & Strauss 1991), but it is still unclear which of these actions of cations are significant for the physiological function of K^+ channels, and which are simply essential physical consequences of channel structure.

(*d*) *Physiological implications*

Uptake of sugars, such as maltose (Serrano 1977) or glucose (Van de Mortel *et al.* 1988) has been reported to activate plasma membrane K^+ channels and to result in K^+ efflux from *S. cerevisiae*. Maltose uptake via an H^+/maltose symport system (Serrano 1977) seems to be accompanied by depolarization of the plasma membrane. Sufficient depolarization per se could activate voltage-dependent K^+ channels, as shown in figures 6 and 7; and K^+ efflux would result if the membrane voltage became more positive than E_{K^+}. Such a mechanism for K^+-channel activation cannot account for the K^+ efflux observed during glucose uptake (Van de Mortel *et al.* 1988), however, because *S. cerevisiae* takes up that sugar by facilitated diffusion (Bisson & Fraenkel 1982; Heredia *et al.* 1968; Cirillo 1961, 1989) without ion coupling, so that changes in membrane voltage are not to be expected. In fact, in low external K^+ (5 μM) glucose uptake is accompanied by potassium efflux and reported hyperpolarization of the plasma membrane (Van de Mortel *et al.* 1988). It is therefore more likely that glucose induced K^+-efflux is linked to a transient increase in cytoplasmic Ca^{2+} which is stimulated by that particular sugar (Eilam *et al.* 1990). By activating plasma-membrane K^+ channels (Bertl *et al.* 1992*a*) when the resting voltage is positive to E_{K^+}, elevated cytoplasmic Ca^{2+} could explain both the observed K^+ efflux and the apparent hyperpolarization.

A somewhat subtle point which must be emphasized in this connection is that the voltage dependence of the yeast plasma-membrane K^+ channel is a bias, not a wall, against channel opening; the gating function describes continuous behaviour of the channel, rather than threshold behaviour. Therefore, in a whole cell having many such channels, increasing cytoplasmic [Ca^{2+}] could have a physiologically significant effect in increasing channel openings, despite the counterbias of a negative membrane voltage. Demonstration that cytoplasmic Ca^{2+} does in fact play such a role would require more extensive and quantitative experiments.

A fast and transient rise in cytoplasmic Ca^{2+} (2–5 min) induced by glucose has been reported to depend on membrane voltage and to occur via calcium influx from the extracellular solution (Eilam & Chernichovsky 1987; Eilam & Othman 1990). But a slower and longer lasting rise in cytoplasmic Ca^{2+} (60–80 min after glucose addition; Eilam & Othman 1990) depends on cell metabolism, and probably occurs via release from internal stores. The tonoplast cation channel has been shown to conduct Ca^{2+} (see figure 5) and would be a good effector for glucose-induced release of vacuolar Ca^{2+}, since sudden feeding of glucose causes cytoplasmic alkalinization (Gillies 1982) and a surge of reducing equivalents (NADH; Polakis & Bartley 1966). And both of these changes activate the tonoplast cation channel.

This general arrangement would make a signalling ion (Ca^{2+}) the important physiological substrate for the tonoplast cation channel, rather than the more abundant alkali metal cations, a circumstance which has a number of interesting aspects. One of these is a positive feedback effect: increased cytoplasmic pH or redox potential (NADH itself, or perhaps reduced glutathione) could activate the channel slightly, leading to initial release of Ca^{2+} from the vacuole, with local accumulation of cytoplasmic Ca^{2+} near the vacuolar membrane. This in turn should further activate the cation channel, for secure as well as fast release of stored Ca^{2+}. A second interesting aspect is functional voltage-clamping by the alkali metal ions. If the channels were highly specific for Ca^{2+}, opening them would polarize the vacuolar membrane towards the calcium equilibrium (near +120 mV), thus diminishing the driving force for Ca^{2+} release. However, since the channel has a high permeability for K^+ (normally distributed near its own equilibrium), the membrane should instead be clamped near E_{K+}, thus maintained a large driving force on Ca^{2+} and, again, enhancing its release.

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