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Cation-selective channels in the vacuolar membrane of *Saccharomyces*: Dependence on calcium, redox state, and voltage

(yeast tonoplast/patch clamp/sulfhydryl oxidation)

ADAM BERTL AND CLIFFORD L. SLAYMAN

Department of Cellular and Molecular Physiology, Yale School of Medicine, 333 Cedar Street, New Haven, CT 06510

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ABSTRACT **The vacuolar membrane of the yeast *Saccharomyces cerevisiae*, which is proposed as a system for functional expression of membrane proteins, was examined by patch-clamp techniques. Its most conspicuous feature, in the absence of energizing substrates, is a cation channel with a characteristic conductance of ≈120 pS for symmetric 100 mM KCl solutions and with little selectivity between K^+ and Na^+ ($P_{Na^+}/P_{K^+} \approx 1$) but strong selectivity for cations over anions ($P_{Cl^-}/P_{K^+} < 0.1$). Channel gating is voltage-dependent; open probability, P_o, reaches maximum (≈0.7) at a transmembrane voltage of −80 mV (cytoplasmic surface negative) and declines at both more negative and more positive voltages (i.e., to 0 around +80 mV). The time-averaged current–voltage curve shows strong rectification, with negative currents (positive charges flowing from vacuolar side to cytoplasmic side) much larger than positive currents. The open probability also depends strongly on cytoplasmic Ca^{2+} concentration but, for ordinary recording conditions, is high only at unphysiologically high (≥1 mM) Ca^{2+}. However, reducing agents such as dithiothreitol and 2-mercaptoethanol poise the channels so that they can be activated by micromolar cytoplasmic Ca^{2+}. The channels are blocked irreversibly by chloramine T, which is known to oxidize exposed methionine and cysteine residues specifically.**

Invention of patch-clamp techniques in 1976 (1, 2) has opened up a whole new range of biological preparations to direct electrophysiological analysis. Isolated single channel molecules can be studied in micrometer-sized patches of cell membranes, and thylakoid membranes of individual chloroplasts (3), inner and outer membranes of mitochondria (4, 5), and small microbial cells (6) have become readily accessible. This circumstance, particularly when combined with new developments in molecular biology, greatly enhances the utility of electrophysiological studies on microorganisms, which had until recently been restricted to a few fungi (7–10), slime molds (11, 12), and one species of swollen bacteria (13).

The yeast *Saccharomyces cerevisiae* seems particularly advantageous for investigation with patch electrodes, for two reasons: (*i*) the electrical properties of active transport systems in its plasma membrane can readily be compared with those already described (from measurements with penetrating electrodes) in another ascomycete fungus, *Neurospora* (14, 15), and (*ii*) *Saccharomyces* is becoming a major system for stable expression and manipulation of both animal and plant genes (e.g., see refs. 16–18).

Previous patch-clamp studies on *Saccharomyces* have reported plasma membrane K^+ channels that are voltage-dependent (19) and generally fit into an emerging pattern of outward-rectifying channels in surface membranes of plants and plant-like cells (20, 21). More recently, K^+ channels have been described as voltage-gated, opening beyond ±100 mV in wild-type strains of yeast, but at lower voltages in a mutant, pma1-105 (22). Most intriguing, this mutant is defective in the structural gene for the plasma-membrane H^+-ATPase of *Saccharomyces* (Ser-368 changed to phenylalanine), which appears to make gating of the K^+ channel sensitive to cytoplasmic ATP concentration.

Despite these successes, patch recording from yeast plasma membrane remains a treacherous and frustrating business, not because of expected difficulties in seal formation, but because plasma membrane channels in yeast are hard to activate; we have seen channel activity in only 5% of experiments with seal resistances ≥20 GΩ. Furthermore, expression of heterologous membrane transport proteins in the yeast system has proven unexpectedly difficult, thus temporarily limiting the practical utility of yeast plasma membranes for structure–function studies. To circumvent these difficulties, we have undertaken studies on tonoplast (vacuolar) membranes of *S. cerevisiae* both because plant tonoplasts have proven especially convenient for patch recording (23–25) and because genetic targeting of heterologous transport proteins to the tonoplast appears feasible (26, 27).

The following report details methods for producing yeast vacuoles for patch recording, along with some general electrophysiological properties of the tonoplast. More important at present, it describes a class of Ca^{2+}- and voltage-dependent cation channels in the tonoplast that in previous reports (28, 29) seemed to require unreasonably high Ca^{2+} concentrations for activation. We found that reducing agents could lower activating Ca^{2+} concentrations from millimolar to the (more physiological) micromolar range, whereas oxidizing agents inhibit the channels. The findings suggest a role for sulfhydryl oxidation in the often observed "rundown" behavior of diverse channels (e.g., see refs. 30–32).

MATERIAL AND METHODS

Cell and Tonoplast Preparations. Experiments were carried out on a tetraploid strain, YCC78, of *S. cerevisiae*, provided by Michael Snyder (Yale Department of Biology). The strain was chosen for its unusual size (about 1.6 times the diameter of normal haploid yeast). It requires adenine and uracil supplementation. Cells were grown overnight in small volumes of liquid YPD medium (30 ml per 125-ml Erlenmeyer flask) at 25°C with rotary shaking (90 rpm). They were harvested by centrifugation (500 × *g* for 5 min) from ≈10 ml of suspension, resuspended in 10 ml of buffer A (50 mM KH_2PO_4/40 mM 2-mercaptoethanol, brought to pH 7.2 with KOH), pelleted again, resuspended in 3 ml of buffer A, and incubated at 30°C for 30 min with occasional gentle shaking. Three milliliters of buffer B [50 mM KH_2PO_4/40 mM 2-mercaptoethanol/2.4 M sorbitol with zymolyase (2 mg/ml) and glucuronidase (2 mg/ml), titrated to pH 7.2 with KOH)] was then added, and the cells were incubated for another 45 min at 30°C. Protoplasts

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Abbreviations: DTT, dithiothreitol; ME, 2-mercaptoethanol.

were harvested by centrifugation (500 × *g*, 5 min), resuspended in buffer C (200 mM KCl/10 mM $CaCl_2$/5 mM $MgCl_2$/5 mM Tris/Mes, pH 7.2), and then transferred into the recording chamber (also containing buffer C) and allowed to settle. When the chamber was subsequently perfused for 5–10 min with buffer D (100 mM tripotassium citrate/5 mM $MgCl_2$/10 mM glucose, brought to pH 6.8 with Mes), 50–80% of the protoplasts released clean, ready-to-use vacuoles. These adhered lightly to the chamber bottom.

Recording. Patch-clamp experiments were done as generally described by Hamill *et al.* (2), using heat-polished glass capillaries (≈1-μm tip diameter) connected to a Yale model MK-V patch-clamp amplifier (design of D. Corey, now of Harvard University). Amplifier output was recorded on a Sony SL-HF 450 video tape recorder via a modified Sony PCM-501ES pulse-code modulator. Outputs from both the amplifier and the tape recorder were low-pass-filtered (8-pole Bessel filter, model 902LPF; Frequency Devices, Haverill, MA), fed through an Indec IBX data interface (Indec Systems, Sunnyvale, CA) to a PC-Limited AT110 microcomputer, and analyzed with Indec C-CLAMP software. Graphics printouts were transferred via an Appletalk network to an Apple LaserWriter.

Other Conditions. Single-channel currents were measured in excised, outside-out patches of yeast tonoplast, on which the cytoplasmic surface of the membrane was exposed to the bath solution and the vacuolar (interior) surface to the pipette solution. Such patches could be obtained by sealing the pipette to the vacuolar membrane (tonoplast), breaking the underlying membrane patch with a 10-msec voltage pulse (±0.6 V), and slowly withdrawing the pipette from the vacuole. Bath solution was continuously perfused through the recording chamber, but pipette solution was stationary. Standard pipette and bath solutions contained 100 mM KCl, 5 mM $MgCl_2$, 10 mM EGTA, and 9.6 mM $CaCl_2$ (giving 10 μM free Ca^{2+}), titrated with Tris to pH 7.0. Altered compositions (usually bath solution) are indicated in the figure legends. Sign conventions throughout this report use the vacuolar interior as reference, so that negative membrane voltages mean cytoplasmic electric potential negative to vacuolar electric potential, and positive currents mean positive charges moving from the cytoplasmic side to the vacuolar side. (The vacuolar interior thus has been treated as an enclosed extracellular space.) The data reported are representative of about 100 patches studied. The vacuole preparation used here forms gigaohm seals in more than 75% of attempts, and observable channels occur in almost all of the outside-out patches.

RESULTS

Redox and Ca^{2+} Dependence of the 120-pS Channels. The simplest property to observe in the large vacuolar channels, other than their open-state conductance under standard recording conditions, was their dependence on cytoplasmic Ca^{2+}. But in initial observations, Ca^{2+} concentrations required to elicit channel opening were observed to rise with age of the recording preparation. In other words, any given membrane patch showed decreasing channel activity as it aged ("rundown" of channel activity), but openings could still be initiated by increasing cytoplasmic free Ca^{2+} sufficiently. This is demonstrated in Fig. 1, where 10 μM cytoplasmic Ca^{2+} yielded no channel activity, but stepping to 1 mM cytoplasmic Ca^{2+} produced a rapid barrage of channel openings, revealing at least eight channels in this particular membrane patch. Numerous reagents were therefore tested for potential ability to suppress the "rundown" of channel activity (or to reactivate the channels) and to shift the required cytoplasmic Ca^{2+} concentration from the millimolar to the more physiological micromolar range.

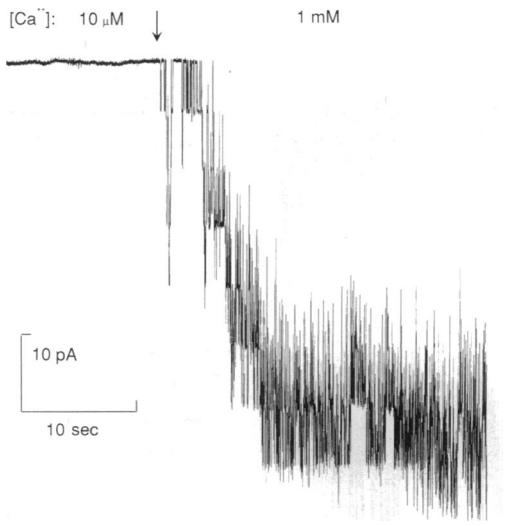

FIG. 1. Activation of yeast tonoplast channels by high cytoplasmic Ca^{2+}. All channels were closed at 10 μM Ca^{2+}, but within 10 sec of the transition to 1 mM Ca^{2+} (arrow), seven or eight channels were opening. Recording was from excised patch, with bath as cytoplasmic side of the tonoplast; voltage clamped at −40 mV, cytoplasmic side negative; standard solution (containing 100 mM KCl) in bath and pipette.

Most effective in this respect were the familiar reducing agents dithiothreitol (DTT) and 2-mercaptoethanol (ME), and the effect of the latter is demonstrated in Fig. 2 at low (10 μM) cytoplasmic Ca^{2+}. Prior to addition of ME, channel-like currents were rare. About 3 sec after addition of 10 mM ME, single open-channel incidence rose above 75% of total recording time, and coincident two-channel openings were frequent (see especially bottom record, Fig. 2 *Left*). Occa-

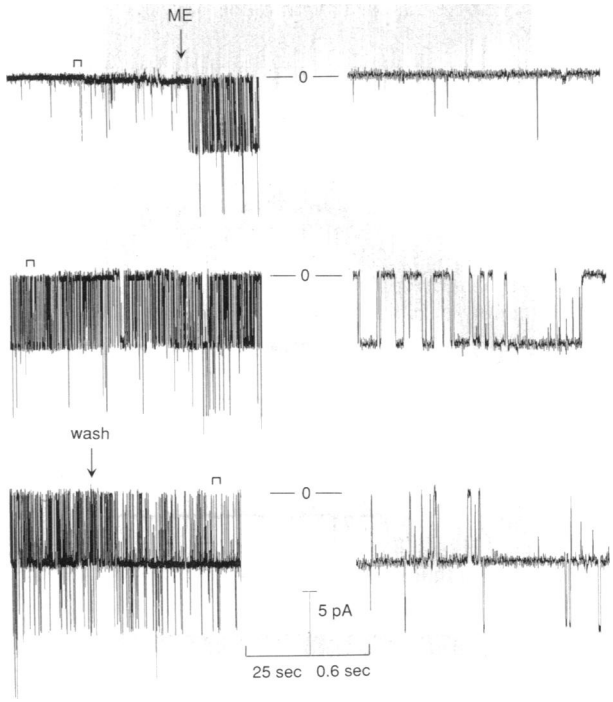

FIG. 2. Activation of channels by ME in 10 μM cytoplasmic Ca^{2+}. (*Left*) Three continuous traces at low time resolution. (*Right*) Designated segments (⊓) from *Left*, at higher time resolution. ME (10 mM) was added at first arrow, and washout began at second arrow. Standard solutions; membrane voltage, −40 mV. Baseline (all channels closed) is indicated by —0—.

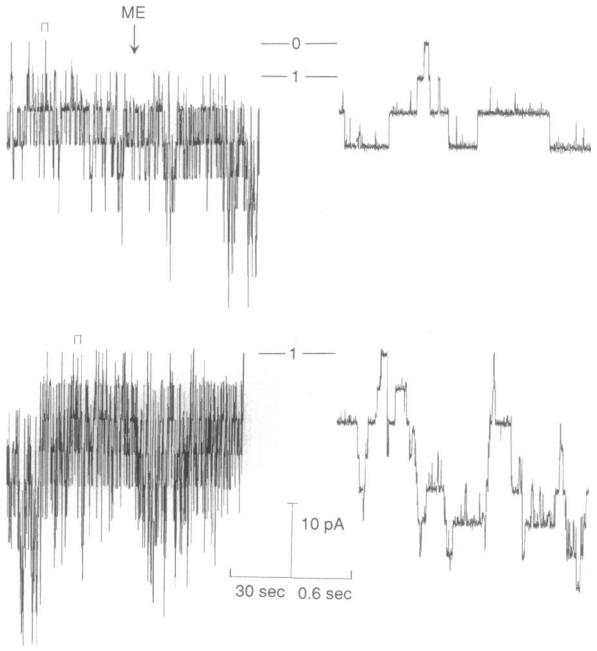

FIG. 3. Activation of channels by ME in 10 mM cytoplasmic Ca^{2+}. Description is as for Fig. 2. Current level with one open channel is indicated by —1—.

sional three-channel openings were also observed, demonstrating the presence of at least three channels in the patch. A qualitatively similar observation is demonstrated in Fig. 3 for high (10 mM) cytoplasmic Ca^{2+}. In the absence of ME, a maximum of five channels could be observed, with two or three channels being open on average. Addition of 10 mM ME gradually increased the number of active channels to at least 10 (see especially beginning of lower record, Fig. 3 *Left*).

Because both DTT and ME are known to reduce sufhydryl groups (33), other sulfhydryl reagents were subsequently tested for effects on the tonoplast channels. The sulfhydryl-binding agent *N*-ethylmaleimide proved a poor inhibitor of these channels, blocking erratically at concentrations up to 5 mM. However, chloramine T, which oxidizes exposed methionine and cysteine residues in native proteins (34, 35), was 100% effective in blocking ME-activated channels. Typically, 1 mM chloramine T blocked all channels within a period of 2–3 min after addition to the recording chamber (Fig. 4). This blockade was not reversible, either by simple washout or by prolonged (30-min) treatment with 10 mM ME or 1 mM DTT.

Voltage Dependence and Ion Specificity. Examples of channel opening are shown in Fig. 5 as functions of the applied membrane voltage, in the presence of 10 μM cytoplasmic free Ca^{2+} and 10 mM ME. At strong positive voltages (≥80 mV), the channels were nearly completely silent; but near +20 mV, single-channel openings occupied >75% of the recording time. Near −40 mV, at least one channel was open nearly 95% of the time, with occasional second-channel openings evident. By −80 mV, two channels were open for >50% of recording time, and third-channel openings occurred 5–6 times per sec. At still more negative voltages, channel openings diminished, so that at −100 mV, <60% of recording time was occupied by open channels. In both Fig. 5 and Fig. 2, at least two types of channels can be distinguished by the duration of individual openings. A "long-open" channel displays most events longer than 100 msec (e.g., in the bottom record of Fig. 2 *Right* and the −40 mV trace in Fig. 5), and a "short-open" channel displays events well under 100-msec duration. However, conductance and ion specific-

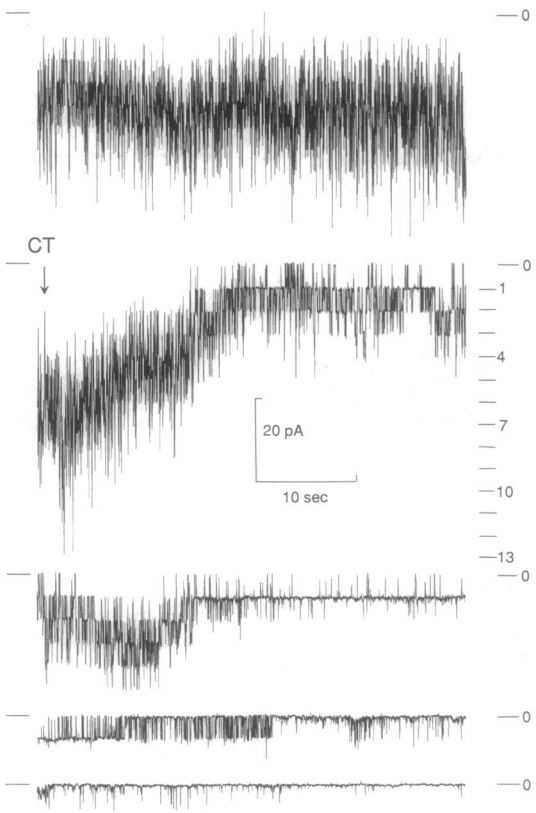

FIG. 4. Inhibition of channels by chloramine T. Channels were previously activated by 5-min treatment with 10 mM ME. Chloramine T (CT) was added at the arrow; number of open channels is indicated by lines at right margin; records are continuous. Conditions were as for Fig. 2, except 100 μM cytoplasmic Ca^{2+}.

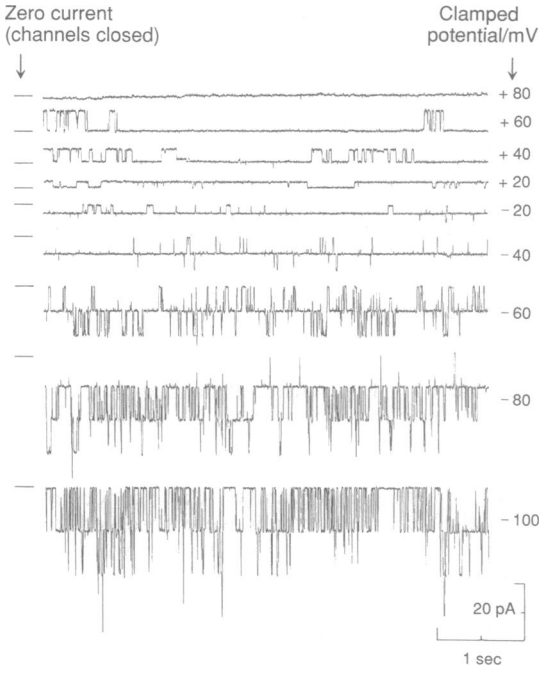

FIG. 5. Voltage dependence of channel opening. Maximal open probability is evident at −80 mV, decreasing at more positive and at more negative voltages. Standard solutions were used, with 10 μM Ca^{2+} plus 10 mM ME. Baseline (no channels open) is indicated at left, with corresponding membrane voltages at right. Upward deflections from baseline indicate positive currents; downward deflections indicate negative currents. Reversal voltage for the channel (zero current with channel open) is near 0 mV.

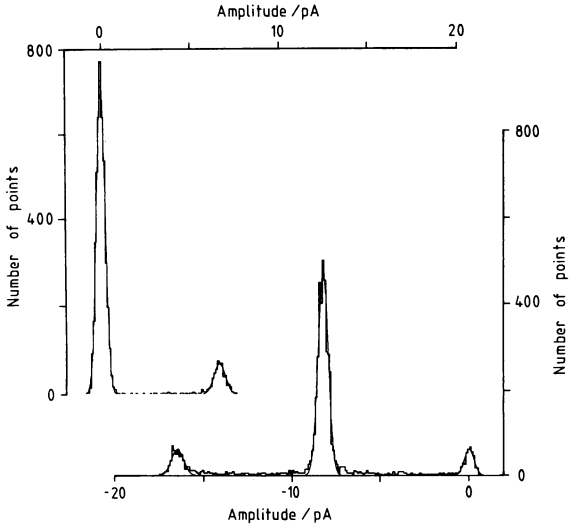

FIG. 6. Histograms for calculating channel open probabilities (P_o). (*Upper left*) Data from +60 mV trace of Fig. 5. (*Lower right*) Data from −60 mV trace of Fig. 5. Peaks around 0 pA (both plots) represent baseline noise; they define the total time with all channels closed, and are designated A_0 in the probability formula: $P_o = (A_1 + 2A_2 + \ldots + nA_n)/n(A_0 + A_1 + \ldots + A_n)$, where $A_1, A_2, \ldots A_n$ are the areas under the peaks for one open channel (at *ca.* +6.7 and −8.3 pA in the two histograms), two open channels, etc.

ity are identical for both types and only the gating pattern differs. Thus the two types probably represent different states of one channel species, rather than two different species.

"All-points" amplitude histograms (36) were constructed at each voltage, as illustrated in Fig. 6 for the +60 mV and −60 mV trace of Fig. 5, and were used to calculate mean single-channel open probabilities (P_o; see method in the legend to Fig. 6). Fig. 7 shows the resultant plot of P_o versus voltage for high (10 mM) Ca^{2+} without ME, calculated from records showing only a single, long-open channel. This shape of P_o curve, with an absolute maximum (0.7) at −80 mV and lower probabilities both at more negative voltages and at more positive voltages, implies that at least two separate steps (or independent charges) are involved in voltage-dependent gating of the channels.

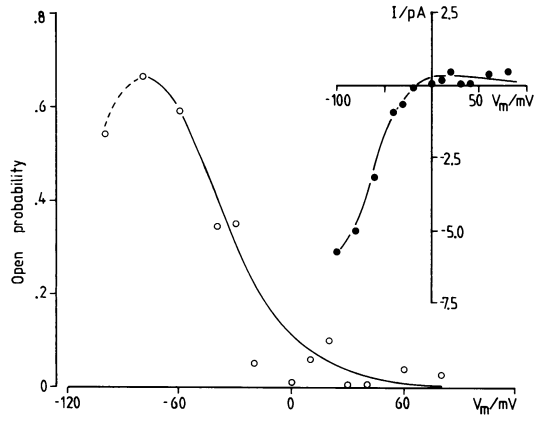

FIG. 7. Summary of the voltage dependence of open probabilities. Data are similar to those of Fig. 5, generating histograms similar to those in Fig. 6, but for a patch that displayed only a single channel. Each plotted point represents data from 80 sec of recording time in an experiment with 200 mM cytoplasmic KCl and 10 mM $CaCl_2$. (*Inset*) Time-averaged current (I) plotted against membrane voltage (V_m) for a single channel, from the same data as main curve. I was calculated as the product of P_o and open-channel current (see Fig. 8, ○).

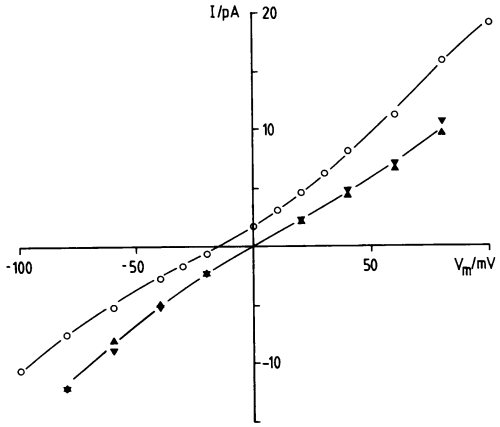

FIG. 8. Voltage dependence of single-channel currents. Upper curve: bath (cytoplasmic) KCl, 200 mM; pipette KCl, 100 mM; reversal voltage (V_m intercept), −15 mV (*cf.* Nernst potential for K^+, −16 mV). Lower curve: 100 mM bath KCl (▲) and 100 mM NaCl (▼).

The product of open probability (Fig. 7) and single-channel amplitude (Fig. 8, ○) generates the time-averaged current–voltage (I–V) curve for individual channels and yields the plot shown in Fig. 7 *Inset*. The resultant curve reveals a strong rectification, with large negative currents and small positive currents.

To explore the specificity of these 120-pS channels, experiments were carried out with various bath (cytoplasmic) conditions, and some resultant open-channel I–V relationships are summarized in Fig. 8. With pipette and bath KCl concentrations both at 100 mM, a reversal voltage of 0 mV was observed (▲), which moved to −15 mV for bath KCl at 200 mM (○). These values coincide with the respective equilibrium voltages for K^+, 0 mV and −16 mV. Since 100 mM NaCl (▼) could be substituted for 100 mM KCl without altering the I–V curve or reversal voltage, K^+ and Na^+ permeability must have been approximately equal. And since a 2-fold increase in bath KCl shifted the reversal voltage to the K^+ equilibrium voltage, K^+ permeability must have been much higher than Cl^- permeability. Thus $P_{Na^+} \approx P_{K^+} >> P_{Cl^-}$.

DISCUSSION

The primary focus of our study is a qualitative description of mechanisms by which cation channels in the tonoplast of *S. cerevisiae* can be regulated. The main characteristics of these channels, as studied by patch-clamp techniques, are (*i*) high conductance, about 120 pS in symmetrical 100 mM KCl; (*ii*) low selectivity among cations ($P_{Na^+}/P_{K^+} \approx 1$) but (*iii*) high selectivity for cations over anions ($P_{Cl^-}/P_{K^+} < 0.1$); voltage dependence, with maximal P_o at moderately negative (cytoplasmic) voltages, but declining at both more positive and more negative voltages; and (*v*) in initial observations, a need for millimolar cytoplasmic Ca^{2+} to allow channel opening. Somewhat similar characteristics, especially the high Ca^{2+} requirement, have been reported for cation channels from yeast vacuolar membrane studied in planar lipid bilayers (28, 29).

In our experiments, a key to understanding the high Ca^{2+} requirement came from investigating the rundown behavior of these channels. Other authors, examining the same phenomena in Ca^{2+} channels of excitable tissues (30–32), have noted salutory effects of cAMP, ATP, Mg^{2+}, and the catalytic subunit of cAMP-dependent protein kinase A. Our tests identified as critical, instead of protein kinase A itself, the protective antioxidant (DTT or ME) that is normally added to enzyme preparations. We have since found free Ca^{2+} con-

centrations as low as 1 μM to be highly effective in channel activation, after sustained DTT or ME treatment.

The findings suggest that oxidation of sulfhydryl groups is a major factor in the rundown of channel activity, and that the redox state of sulfhydryl groups at the cytoplasmic side of the channel (DTT or ME had no effect when added to the vacuolar side) could be a potent regulator of channel activation/inactivation. This possibility is supported by the further finding that an oxidizing agent (chloramine T) strongly inhibits yeast tonoplast channels (Fig. 4). Near neutral pH, chloramine T rapidly and specifically oxidizes exposed methionine and cysteine residues to methionine sulfoxide and cystine (34, 35) without altering other amino acids in either denatured or native proteins. The apparent irreversibility of chloramine-T blockade is probably a simple consequence of the fact that its oxidation products are not reduced by DTT or ME under ordinary conditions.

Actual physiological functions for the yeast vacuolar channels have been much more difficult to specify than we had hoped, thus repeating the experience of several other laboratories working on tonoplast channels of higher plant cells (23–25, 37, 38). The obvious proposition is agreed upon: that vacuolar channels should mediate the entry or release of metabolic storage products; but identifying the specific *substrates*, as opposed to *assay ions* like K^+ and Na^+, is not simple for these vacuolar channels. Storage contents of yeast vacuoles include lytic enzymes (e.g., proteases, peptidases, RNase, polyphosphatases; ref. 39), polyphosphates (40), Krebs-cycle anions,* protons (41–43), Ca^{2+} (44, 45), and many amino acids, particularly basic amino acids (46–49). The normal tonoplast membrane voltage is probably small ($\approx$40 mV, seen in measurements on *Neurospora* with penetrating microelectrodes: C.L.S., unpublished experiments) and oriented with the cytoplasm negative, so that only anions could be electrophoretically concentrated in the vacuole via tonoplast channels. Substitution experiments with glutamate, citrate, and gluconate have found these anions to have the same effect as Cl^- (A.B., unpublished experiments), so the channels probably do not transport physiological small anions; that in turn means the channels are probably involved in release rather than uptake by the vacuole.

Evidence for permeation of the channels by neutral molecules is not directly obtainable by patch-clamp measurements but might emerge from competition studies; i.e., do inward or outward gradients of prominently stored amino acids such as histidine and tryptophan (50) affect either channel conductivity or gating behavior in the presence of "normal" K^+ or Na^+ concentration? Evidence for permeation by the basic amino acids arginine and lysine has been sought by examining channel *I–V* curves upon direct substitution of those ions for K^+ in the bath (cytoplasmic solution). That treatment, however, destabilizes the membrane patch or seal, and we have not yet been able to study the channels themselves in the presence of high arginine or lysine concentration.

*Kulakovskaya, T. V., Matys, S. V. & Okorokov, L. A., Proceedings of Contributions to the 6th Small Meeting on Yeast: Transport and Energetics, Sept. 19–21, 1988, Dortmund, F.R.G., pp. 23–24.

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