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Physiological Characterization of the Yeast Plasma Membrane Outward Rectifying K^+ Channel, DUK1 (TOK1), *In Situ*

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Abstract. The major voltage-dependent ion channel in the plasma membrane of *Saccharomyces cerevisiae*, a conspicuous outwardly rectifying K^+ channel, was first dubbed YPK1 and later renamed according to its registered gene names (*DUK1, TOK1*). It has proven novel in both structure and function. Whole-cell patch-clamp studies of the channel directly on yeast protoplasts now extend our earlier description obtained from isolated patches of yeast membrane (Bertl & Slayman, 1992; Bertl et al., 1993), and provide new data both on the contributions of channel properties to yeast physiology and on possible contributions of molecular structure to channel properties. Three recording tactics produce completely equivalent results and thereby allow great flexibility in the design of experiments: whole-cell voltage clamp with sustained voltage steps (~2.5 sec), whole-cell voltage clamp with slow voltage ramps (5 sec, −40 to +100 mV), and time-averaging of single-channel currents.

Activation of Duk1 channels under steady-state conditions is dependent upon ATP in the cytoplasmic solution, and the absence of ATP results in channel ''run-down''—decreasing numbers of activable channels—over periods of 10 min to 1 hr from the start of patch recording. Several putative serine- and threonine-phosphorylation sites, as well as a variant ATP-binding fold, exist in the molecule as potential mediators of the ATP effects. The channel runs down similarly following cytoplasmic acidification, but is almost completely insensitive to extracellular pH changes (8.0 to 5.5 tested). This remarkable asymmetry may depend on the protein's strongly asymmetric distribution of histidine residues, with 10 out of 12 predicted to lie close to the membrane-cytoplasm interface.

Further data confirm the well-recognized observation that changes of K^+ concentration, intracellular or extracellular, can shift the gating voltage of Duk1p in the direction of E_K. Among the other alkali-metal cations tested, extracellular Rb^+ and Cs^+—but not Na^+—substitute almost completely for K^+. Extracellular TEA^+ inhibits whole-cell K^+ currents through Duk1p with a K_I of 2.8 mM, and does so probably by reducing the single-channel current.

Key words: Potassium channel — Yeast — Outward rectifier — K^+-dependent gating — ATP-dependent channel — pH-dependent channel

Introduction

The feasibility of studying transport systems in the plasma membrane of the yeast, *Saccharomyces cerevisiae*, by the patch-clamp technique was first demonstrated by Gustin et al. (1986). That investigation discovered an outward rectifying channel in the yeast plasma membrane that was subsequently described in considerable detail by Bertl et al. (1992*b*, 1993) and dubbed YPK1 (**Y**east **P**lasma-membrane $\mathbf{K^+}$ Channel **#1**). Somewhat later, a sequence in the yeast genome database, YJL093c (J0911; Miosga, Witzel & Zimmermann, 1992), was discovered to encode a putative membrane protein containing motifs reminiscent of the H5 region of voltage gated K^+-channels (named *TOK1;* Ketchum et al, 1995). Expression of *TOK1* in *Xenopus* oocytes induced K^+-selective outward currents with characteristics very similar to those already reported for the

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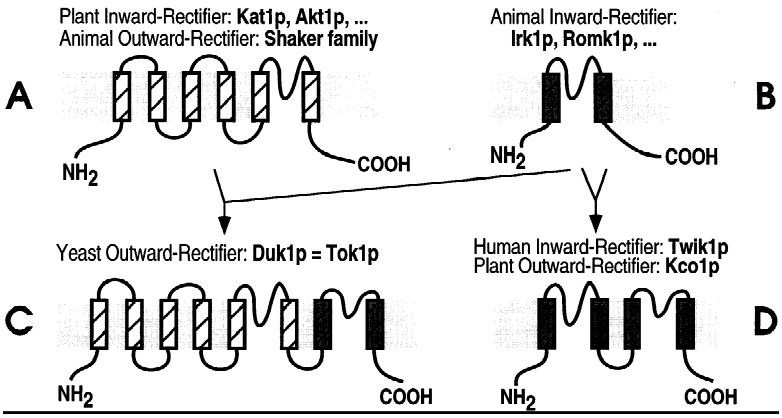

Fig. 1. Structural cartoons of four classes of potassium channels, inferred from known gene sequences and hydropathy diagrams. (*A*) The P1 motif, containing 6 transmembrane helices, with a ''pore'' segment between the fifth and sixth, characteristic of animal outward rectifying channels and plant inward rectifiers. (*B*) The P2 motif, with just two transmembrane helices bracketing the ''pore'' segment, characteristic of animal inward rectifiers. (*C*) The yeast outward rectifier, comprised by a P1 + P2 motif; and (*D*) The P2 + P2 motif found in plant outward rectifiers and in certain weak inward rectifiers of animal cells (incl. many in *Caenorabditis elegans,* inferred from genome data). In all four diagrams the cytoplasmic face of the membrane and channels is drawn downward.

yeast plasma membrane outward rectifier, YPK1. Despite the obvious similarity in key characteristics and the yeast origin of *TOK1,* Ketchum et al. (1995) did not identify the gene product with YPK1. The identity was demonstrated later in two independent reports on K^+-channel activity measured directly in the plasma membrane of yeast cells and assigning the names *DUK1* (Reid et al., 1996) and *YKC1* (Zhou et al., 1995) to the gene. Independent rediscoveries of the same sequence were reported by André (1995) and by Lesage et al (1996*a;* renaming it YORK). [To avoid any confusion, from here on we shall use *DUK1* (Reid et al., 1996) to refer to this gene, since ''*YPK1*'' had already been registered for the gene encoding the yeast protein kinase p40.]

The first detailed functional description of this channel (Bertl & Slayman, 1992; Bertl et al., 1992*b*, 1993), in addition to confirming its role as a K^+-selective outward rectifier, identified a minimum of three closed states of the channel (designated **I** = ''interrupt,'' **G** = ''gap,'' and **B** = ''block'') which could be reached in parallel from a single open state (**O**), and which were poised so that the channel should be preponderantly closed under physiological conditions of membrane voltage and cytoplasmic free calcium concentration (Bertl et al., 1993).

Other novel properties of the channel were also observed in the yeast membrane: a sigmoidal open-channel current-voltage relationship (over the voltage range +/−120 mV), dual action of calcium on the channel (activation and blockade), and an apparent dependence of channel gating on the equilibrium voltage for the substrate ion, K^+ (Bertl & Slayman, 1992), which was later elaborated upon expression of the channel in *Xenopus* oocytes (Ketchum et al., 1995; Vergani et al., 1997).

The discovered nucleotide sequence encoding this channel, YJL093c, identified it as the prototype for a new superfamily of potassium channels, which contain two distinct clusters of transmembrane helices and associated ''pore'' domains: one (**P1**) consisting of 6 putative transmembrane helices (S1–S6), with a ''pore'' domain (containing the diagnostic sequence GLGD), between S5 & S6, and a second (**P2**) consisting of two putative transmembrane helices (S7–S8), with another ''pore'' domain (containing GYGD) between, as diagrammed in Fig. 1. Since the first reports of this novel K^+-channel structure in yeast, more than 20 allied sequences have been inferred from the genome of *Caenorhabditis elegans,* and several more have been identified in higher animal and plant systems (*TWIK-1:* Lesage et al., 1996*b; ORK1:* Goldstein et al., 1996; *KCO1:* Czempinski et al., 1996). Except for that in yeast, however, all are P2-P2 structures (4 predicted transmembrane helices), rather than P1-P2 structures. Whether several related structures, with putative 4S-P-4S-P-2S motifs, found in prokaryots, actually function as channels remains to be determined (*see* review in Jan & Jan, 1997).

With reference to the vast channel literature on animal cells, P1 sequences are characteristic of outward-rectifying channels, and P2 sequences are characteristic of inward rectifying channels. Functionally, the ''2-P'' channels from yeast (P1-P2) and plants (P2-P2) are clear outward rectifiers, whereas the animal ''2-P'' channels (P2-P2) which have been tested are either weak inward rectifiers or open channels (e.g., the *Drosophila* 2-P channel, Ork1p, shows no significant voltage dependence; Goldstein et al., 1996). At present, such dicotomies add further complexity to attempts to identify the structural features of ion channels that determine gating and rectification (*see,* e.g., Stühmer, et al., 1989; Strong, 1990; Jan & Jan, 1992), an enterprise already saddled with the finding that simple P1-channels in plants are actually *inward* rectifiers (Anderson et al., 1992; Sentenac et al., 1992; Ketchum & Slayman, 1995). Yet another complication in such comparisons is the fact that the Duk1p sequence contains no plausible transmembrane charge string—such as the $(RXX)_n$-repeat characterizing the S4 helix in animal outward rectifiers and postulated to be the critical gating element (Jan & Jan, 1992; Yang, George & Horn 1996).

The purpose of the present manuscript is to complete our general description of Duk1p, as it behaves in its native yeast plasma membrane, adding—to the properties already outlined above—definitive information about the channel's sensitivity to cytoplasmic pH, to extracellular pH, to cytoplasmic ATP; and about its interactions with a wider range of monovalent and divalent cations. This provides the necessary background for more detailed analyses of the interactions of K^+, Na^+, and Ca^{++} with both the ion transit process and the channel gating process.

Materials and Methods

Detailed methods for growing and handling cells of *Saccharomyces,* and for carrying out the patch-clamp experiments, have already been described (Bertl et al., 1992*b,* 1993, 1995, 1997). The present experiments employed two recording geometries: the whole-cell configuration and the isolated (inside-out) patch configuration. Data were obtained in response to both voltage-pulse (long) and voltage-ramp stimuli, and direct comparisons were made between whole-cell behavior and time-averaged single-channel behavior.

Except as specifically noted in the figure legends, most data were obtained on yeast strain Y588 (referred to as wild type; Mirzayan, Copeland & Snyder, 1992, donated by Dr. Michael Snyder, Yale Department of Biology). Y588 is an adenine-requiring tetraploid strain adopted mainly because of its large cells and consequent mechanical convenience in patch-clamp experiments.

Standard whole-cell recording solutions were as follows: *Pipette solution* (cytosolic): 175 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, 100 nM free Ca^{++}, 1 mM EGTA brought to pH 7.0 with KOH. *Bath solution* (extracellular): 150 (135) mM KCl, 5 mM $MgCl_2$, 10 mM $CaCl_2$ buffered to pH 7.5 with 0.1 mM Tris/MES. Standard isolated-patch solutions were: *Pipette solution* (extracellular): 50 mM KCl, 0.1 mM Ca^{++}, 250 mM sorbitol. *Bath solution* (cytosolic): 200 mM KCl, 1 μM free Ca^{++}, 1 mM EGTA, brought to pH 7.0 with KOH. Isolated-patch data were filtered at 400 Hz and sampled at 1000 Hz, and whole-cell data were filtered at 100 Hz and sampled at 400 or 1000 Hz. For both recording geometries, membrane voltages were always calculated and displayed as physiological *inside minus* physiological *outside* (Bertl et al., 1992*a*). Whole-cell currents in these experiments have been corrected for linear leaks.

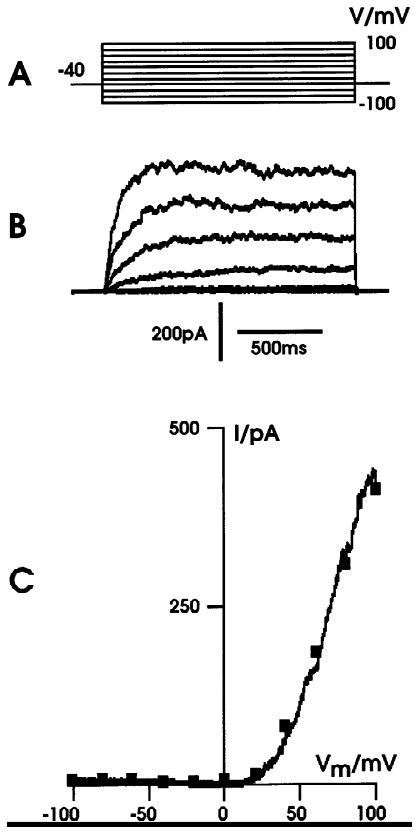

Fig. 2. Equivalence of different recording modes for assessing the behavior of Duk1p. (*A*) Superimposed traces for the voltage protocol applied during whole-cell recording; a staircase of 2.5-sec pulses, at 20-mV intervals between −100 and +100 mV, was used routinely, with membrane voltage held at −40 mV for 0.5 sec between pulses. (*B*) Superimposed display of typical membrane currents measured by the voltage clamp during protocol shown in *A.* (*C*) Filled squares (■), plot of steady-state membrane currents in *B vs.* clamp voltage; currents were measured at the end of each voltage pulse. *Jagged trace,* continuous record of membrane current during a 5-sec ramp of voltage from −100 to +100 mV; same cell as in *B.*

Results

Coincidence of Whole-cell and Single-channel Currents

Activation and deactivation of Duk1p could readily be demonstrated in yeast plasma membranes by the appearance of time-dependent outward currents, when the whole-cell membrane voltage was stepped from −40 mV to values ⩾ +20 mV; or by currents whose mean amplitude increased progressively as membrane voltage was *slowly* ramped positive (~20 to 40 mV per sec) from −100 mV (or −200 mV). Data from both kinds of current-voltage (''*I-V*'') experiments are shown in Fig. 2*B* and *C* for a typical spheroplast of the tetraploid yeast strain Y588. Equivalence of results obtained by the two methods is clearly demonstrated by ready superposition of the ramp current upon the steady-state currents from the pulse experiment: end-of-trace values in *B* plotted as filled squares, ■, in *C.*

The origin of whole-cell outward currents (Fig. 2*B* and *C*) as ensemble responses of the single channels previously described in isolated inside-out patches (Bertl & Slayman, 1992; Bertl et al., 1993) was demonstrated by comparing time-averaged single-channel currents with whole-cell ramp currents. Single-channel currents for such calculations are shown in Fig. 3*A*, which is a series of records obtained from an isolated inside-out patch of yeast plasma membrane, clamped at 20-mV intervals between −80 and +100 mV. Time-averaged currents were calculated for the full experiment, 30–60 sec at each voltage, and have been plotted as open circles

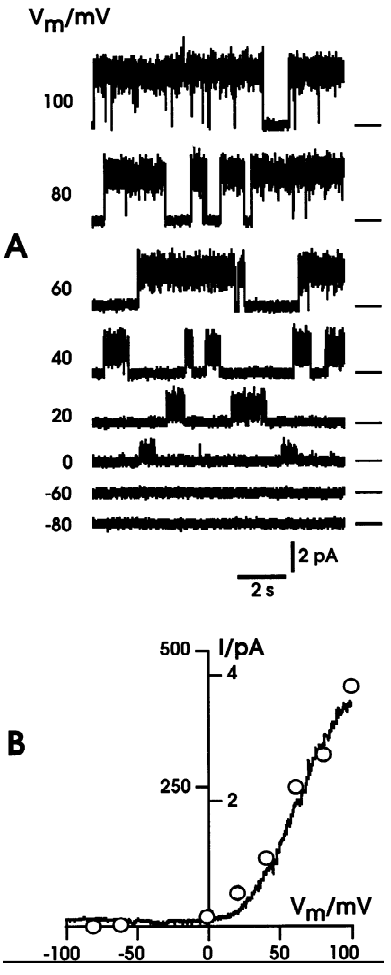

Fig. 3. Qualitative equivalence of time averaged single channel currents and whole cell currents. (*A*) Typical single-channel currents recorded from an inside out patch with 50 mM K^+ on the external side and 200 mM K^+ on the cytosolic side of the membrane. Membrane voltages indicated to the left of each trace; baseline currents (zero open channels) marked by dashes at the right of each trace. (*B*) *Open circles* (○), time-averaged single-channel currents for Duk1p, representing data extended (30–60 sec) from the records in *A.* Values to be read on the right ordinate scale. *Jagged trace,* whole cell current in response to a voltage ramp, with 50 mM external K^+. Pipette (cytosolic) solution as in Material and Methods.

(○) in Fig. 3*B.* Evidently, the resultant *I-V* relationship can readily be superimposed on that from the whole-cell currents recorded under similar experimental conditions (jagged trace). Since no other channel type has been seen in single channel recordings from yeast plasma membranes under the conditions of these experiments, the ordinate-scale ratio in Fig. 3*B* provides an estimate of the number of Duk1 channels present in the plasma membrane of a single yeast cell (strain Y588): in these experiments: 450 pA/4 pA ≅ 110, which compares with ~50, estimated previously from isolated-patch measurements (Bertl et al., 1993). [Note that cytosolic Ca^{++} was

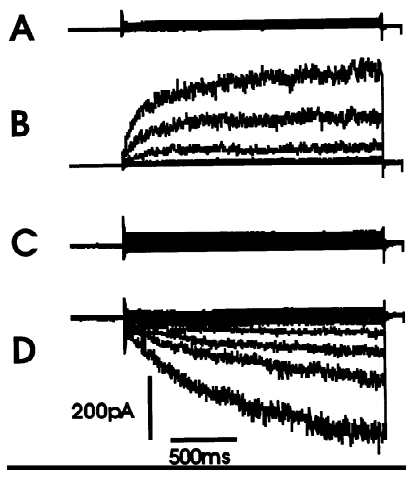

Fig. 4. Demonstration that Duk1p is encoded by the DNA sequence YJL093c. (*A*) Whole-cell currents in a haploid strain having the YJL093c (*DUK1*) sequence disrupted by replacement of a 1.8-kb central region (NsiI–EcoRV) with the yeast *HIS3* gene (strain JRY378; Reid et al., 1996). (*B*) and (*C*) Whole-cell currents in a *DUK1*-deletion strain augmented by a plasmid-borne copy of the gene, behind a *GAL1/10* promoter (strain JRY384). (*B*) Currents measured in cells grown and maintained on 55 mM galactose, which activates the *GAL1/10* promoter. (*C*) Currents measured in cells grown on 55 mM glucose, which represses the promoter. (*D*) Absence of a *plasmid* effect: Currents in a strain (JRY391) with YJL093c disrupted and carrying a plasmid-borne gene for a different channel, the plant inward-rectifier *AKT1,* from *Arabidopsis thaliana.* Note the complete absence of time-dependent *outward* (upward) currents in panels *A, C,* and *D.* Voltage protocol as in Fig. 2*A,* above, but ranging between −240 and +100 mV in *D.*

1 μM in the single channel recording and 0.1 μM in the whole-cell experiment. Therefore, the number of channels/cell, as given above, is very likely *under*estimated.]

Identification of Duk1p with the Product of Coding Sequence YJL093c

Despite the appearance of two previous papers on this point (Zhou et al., 1995; Reid et al., 1996), doubts have been raised about whether the outward rectifier described in yeast plasma membranes does represent the genetic entity *DUK1.* The data in Fig. 4 certify a positive answer to the question. The strategy for proof was (i) to disrupt the coding sequence and demonstrate the absence of slowly activating outward currents in cells carrying the disruption; then (ii) to reintroduce the intact sequence into the disruption strain, on a yeast plasmid, and demonstrate restoration of the currents; and finally as a control against expression artifacts, (iii) to introduce a different (and heterologous) membrane transporter into the disruption strain. The voltage-dependent whole-cell currents for this sequence of experiments are shown in Fig. 4.

Disruption of the YJL093c (*DUK1*) sequence abolished the large, time-dependent component of outward current, as is shown in Fig. 4*A.* When the same yeast strain was subsequently transformed with an integrating plasmid (YI-plac128) bearing *DUK1* behind the *GAL1/10* promoter, growth of the cells on *galactose* yielded whole-cell current records (Fig. 4*B*) like those in Fig. 2*B:* that is, with time-dependent outward currents reaching several hundred pA (stead-state amplitude) at +100 mV. However, growth of the cells on *glucose* yielded traces (Fig. 4*C*) essentially identical to those in the simple disruption strain, viz., totally lacking the time-dependent outward current. Thus, switching the GAL1/10 promoter and its controlled *DUK1* sequence on or off, with galactose or glucose respectively, completely determined the presence or absence of characteristic time-dependent outward rectifying currents.

In all cases the *inward* currents (downward) were small and time-independent, regardless of the status of the *DUK1* gene. And the residual outward currents (upward) were likewise small and time-independent, with the coding sequence either disrupted or switched off. Despite the precision and clarity of these results, one caveat cannot be absolutely excluded: that YJL093c might encode a separate protein which is either a regulatory factor or an essential functional subunit of the active channel. But these possibilities appear remote, since expression of YJL093c alone has been shown to produce Duk1p-like currents in the heterologous system of *Xenopus* oocytes (Ketchum et al., 1995; Reid et al., 1996; Lesage et al., 1996*a*), where such complementary effects should be qualitatively distinct.

Two other caveats *can* definitely be ruled out, however. The first is that the absence of Duk1p-like currents in the disruption strain might result from derangement of the whole-cell recording geometry. In cells as small as yeast, transitions between on-cell recording and whole-cell recording are not always accompanied by the clear changes of input capacitance and conductance which accompany such transitions in larger cells (Hamill et al., 1981). Furthermore, since (we have found that) spontaneous resealing of previously disrupted patches can occur with yeast membranes–resulting in reversion from whole-cell to on-cell recording geometry, the absence of (or disappearance of) time-dependent currents can be difficult to interpret. The obvious control against such artifacts is transformation of YJL093c-disrupted yeast with the same plasmid but carrying a gene for a functionally different ion channel. In the experiment illustrated in Fig. 4*D*, the YJL093c-disrupted yeast strain was transformed with a plasmid bearing the *Arabidopsis AKT1* gene, coding for an *inwardly* rectifying K^+-channel. The resultant currents, for a voltage-clamp protocol extending to −240 mV, were large, slowly developing, and inward (Fig. 4*D*), typical for Akt1p in *Saccharomyces* plasma membranes (Bertl et al., 1997). Most important for present purposes, however, is the fact that these conspicuous *inward* currents were clearly visible in the complete absence of time-dependent *outward* currents, thus ruling out cryptic derangement of whole-cell recording geometry as a possible cause for disappearance of the characteristic yeast channel currents. A fringe benefit of this experiment is the ruling out, also, of spurious plasmid effects as a potential source of the restored Duk1p-like currents in Fig. 4*B.*

Influence of Metabolic Conditions upon the Activation of Duk1p

Initial experiments with whole-cell patch-recording in yeast yielded small outward currents but no convincing, stable, channel activation/recruitment (A. Bertl, *unpublished experiments*). Because the elementary properties of Duk1p were already known (Bertl & Slayman, 1992; Bertl et al., 1993), and an estimate of channel density in the yeast membrane (~50 per cell) could be made from isolated-patch experiments, it was clear that negative results in whole-cell experiments depended upon the exact conditions of recording. Also, since Duk1p is involved in K^+-efflux, which has been shown to be triggered by glucose feeding of K^+-starved yeast (Serrano, 1977; Van de Mortel et al., 1988), it seemed likely that parameters related to cellular energy balance would be involved in K^+-channel regulation and could be causing low conductance (rarely open ion channels) in the early whole-cell studies. Two of the most conspicuous factors to change in yeast upon glucose feeding were known to be cytoplasmic ATP concentration and cytoplasmic pH (Navon et al., 1979; Den Hollander et al., 1981), so these were examined first in the search for conditions to activate Duk1p in whole-cell experiments.

ATP, added to the cytoplasmic solution, in fact proved to be the critical ingredient for Duk1p currents, as is demonstrated in Fig. 5. ATP at 4 mM produced the maximal effect in stabilizing channel activation, with currents—elicited either by the voltage-step protocol or by the voltage-ramp protocol (Fig. 5*A*)—sustained at maximum for the duration of the patch recording, in some cases for several hours. Observed variations around the maximum (*cf.* for example the 0- min and 30-min traces in Fig. 5*A*) were within the normal scatter. By contrast, whole-cell recording achieved without ATP in the pipette (Fig. 5*B*) resulted in a rundown of the outward currents, typically over a period of about 1 hr. This is illustrated in Fig. 5*B*, where the time constant for quasi-exponential decay was 33 min. The decay reflected a progressive decline in the *number* of activable Duk1 channels, as will be described below, after considering the effects of intracellular pH.

The observed effects of lowered intracellular pH

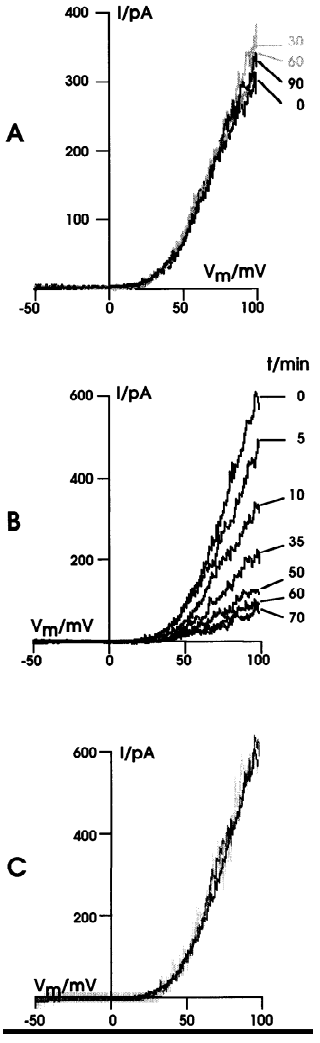

Fig. 5. Dependence of whole-cell outward currents upon cytosolic ATP. Current traces in response to 5-sec voltage ramps, imposed at the indicated times after formation of the tight seal. (*A*) 4 mM ATP/4 mM $MgCl_2$ (pH 7.0) present in the pipette (intracellular) solution. (*B*) Corresponding current traces from a different cell, with a patch pipette lacking ATP. (*C*) Rescaled current traces from *B*, demonstrating that the rundown of whole-cell current in the absence of cytosolic ATP influences only the number of channels active, not the shape of the *I-V* characteristic; black trace: 0 min in *B;* dark grey: 10 min in *B*, scaled up 1.9×; light grey: 50 min in *B*, scaled up 4.8×.

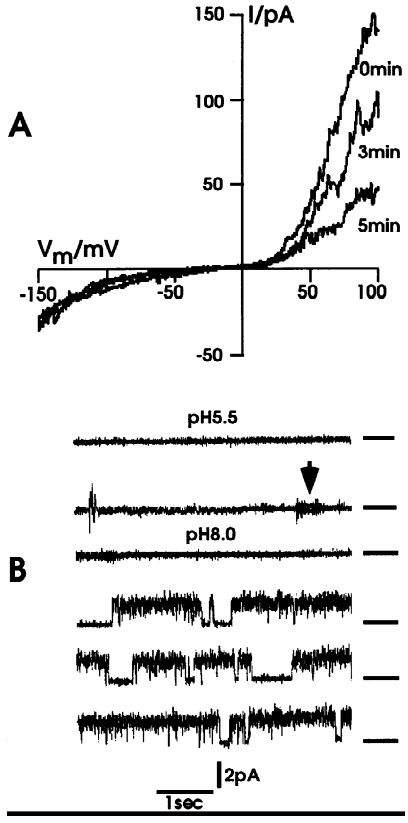

Fig. 6. Inhibition of the outward rectifier by cytosolic acidification. (*A*) Whole-cell currents, in response to the standard voltage ramp, recorded at intervals after the onset of internal acidification. Whole-cell recording achieved under standard conditions with 135 mM KCl and 4 mM Mg-ATP in the bath. Acidification was produced by adding 5 mM acetic acid to the bath solution (extracellular pH, 5.0), immediately after the 0-min record. (*B*) Single-channel record from an isolated inside-out patch (standard conditions for isolated patches; 0 ATP in both solutions), with membrane voltage clamped at +80 mV. Bath solution shifted to pH 5.5 ~5 sec before the start of this record, then to 8.0 at the *down arrow* (↓). Dashes to the right of each trace indicate baseline currents with zero open channels.

(pH_c) were even more dramatic, as shown in Fig. 6*A*. Lowering of pH_c by addition of 5 mM acetic acid to the extracellular solution diminished whole-cell ramp currents to ~30% of control values within 5 min, the process having a quasi-exponential time constant of ~4 min. In isolated-patch measurements, where ATP was routinely absent, the same effect could be demonstrated on the single channel level: shifting bath (cytosolic) pH from 5.5 to 8 activated Duk1 channels within 5–10 sec, as shown in Fig. 6*B*.

An important additional point about both cytosolic acidification and ATP is that the effects were on channel open probability (P_o), over the entire voltage range, not on the voltage-dependence function nor on the single-channel conductance. These points are especially clear from the two sequences of records in Figs. 5*B* and 6*A*, where the only significant difference from curve-to-curve within each set is a scaling factor, as demonstrated explicitly in Fig. 5*C*. That is, no change in *shape* occurred with time at zero ATP or at acid pH_c; no shift occurred along either the voltage axis or the current axis; and there was no significant change in the noise level.

Reinforcing the notion that the ATP and pH_c effects are metabolic in nature was the finding that lowering of *extracellular* pH had no effect on either the amplitude or the kinetics of whole-cell outward currents. The ramp current for a cell maintained 10 min in extracellular (bath) solution at pH 5.1 is illustrated in Fig. 7*A*, in

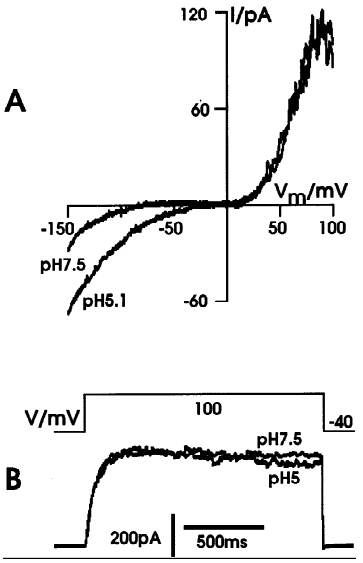

Fig. 7. (*A*) Demonstration that extracellular acidification does not affect outward currents through Duk1p, although it does *enhance* native yeast inward currents (*TRK*-related). Whole-cell currents in response to the standard 5-sec voltage-ramp protocol, for a cell maintained in standard buffer (pH 7.5), then 10 min after shifting to pH 5.1. (*B*) pH_o-independence of activation kinetics, for voltage step to +100 mV.

comparison with the corresponding curve obtained under standard whole-cell conditions, $pH_o = 7.5$. The outward (upward) segments of the two curves—produced by Duk1p—were indistinguishable, even though the inward (downward) segments clearly differed. Evidently, the *amplitude* of steady-state current through Duk1p, as estimated by the ramp measurement, was independent of extracellular pH. That the *kinetics* of Duk1p activation were also pH_o-independent is demonstrated by Fig. 7*B*, wherein currents in response to single voltage steps from −40 to +100 mV, at pH 5.0 and 7.5, could be superimposed, too.

Data in the left half of Fig. 7*A*, showing pH_o-sensitive inward currents, have been presented as general controls, to demonstrate that extracellular acidification does alter *something* visible to the whole-cell recording. These inward currents, which display a clear rightward (positive) shift of apparent activation voltage and a less conspicuous change in shape (kinetics) of the voltage-dependence function, are related to expression of the TRK transporters, mediating potassium uptake in yeast (Ko & Gaber, 1991).

Summary of Interactions of Duk1p with Monovalent and Divalent Cations

As was noted in the Introduction, potassium plays at least two roles in the yeast outward rectifying channel: it is the primary (permeant) substrate for the channel, and it is one of several factors controlling the gating process. A qualitative appreciation of the latter fact is readily obtained from whole-cell *I-V* curves generated in response to voltage ramps at different extracellular K^+ concentrations, as shown in Fig. 8*A* for two separate yeast cells. Shifting extracellular [KCl] from 150 to 50 mM again shifted the *I-V* curves ~25–30 mV negative, with little or no change in the shape. Similarly, lowering extracellular [KCl] from 50 to 5 mM moved the *I-V* curves further (~50 mV) negative, although at this extreme a progressive (time-dependent) flattening of the *I-V* relationship also occurred. The latter effect is of unknown origin. The leftward shift of gating voltages closely approximates the imposed changes of equilibrium voltage for potassium (E_K), which has led other investigators (Ketchum et al., 1995; Lesage et al., 1996*a*) to postulate actual coupling of the gating voltage to E_K, rather than simply to the membrane voltage. It must be added that the simple relationship between $[K^+]_o$ and channel activation held only when KCl removal was osmotically balanced (e.g., by addition of a nonionic solute such as sorbitol).

Predictions of single-channel behavior, based on such whole-cell results for the outward-rectifying channels were easily verified and are displayed in Fig. 8*B*. Under standard conditions (incl. $[K^+]_o = 50$ mM, $[K^+]_i = 200$ mM) this channel behaved essentially the same as that in Fig. 3*A*, and further elevation of cytosolic $[K^+]$, to 500 mM, pushed the channel open probability to >0.9 at +80 mV, as determined from the total record, lasting >60 sec. Subsequent lowering of cytosolic $[K^+]$ to 50 mM, which set E_K at 0 mV, had two obvious effects: (i) a reduction of the single channel current amplitude, just as expected for lowering substrate concentration, and (ii) an increase of the long-lasting closed times (G state) which is evident from a simple inspection of corresponding current traces from the two panels. Therefore, lowering cytosolic $[K^+]$ would result in a shift of the time-averaged *I-V* curve towards positive voltages.

Quite a different result was seen upon replacing extracellular potassium with Na^+ or with the conventional potassium analogues, Rb^+ and Cs^+. Whole-cell *I-V* curves for this comparison are shown in Fig. 9. In the control experiment, again 150 mM KCl was present in the bath solution; and in the three test cases, the KCl was successively replaced by 150 mM NaCl, then by 150 mM CsCl, and finally by 150 mM RbCl. Replacing external K^+ completely by Na^+ shifted the *I-V* curve about 80 mV negative along the voltage axis, just as lowering $[K^+]_o$ from 150 to 5 mM had done (*see also* Fig. 8*A*, above).

The observed virtual absence of shifts in gating voltage, upon replacement of K^+ by Rb^+ or—especially—Cs^+, suggests that the channel cannot discriminate between K^+ and Rb^+ or Cs^+, at least in the gating process. Although the slopes (conductances) of the *I-V* curves at higher positive voltages varied somewhat from K^+ to Rb^+ or Cs^+, such variations were not systematic among

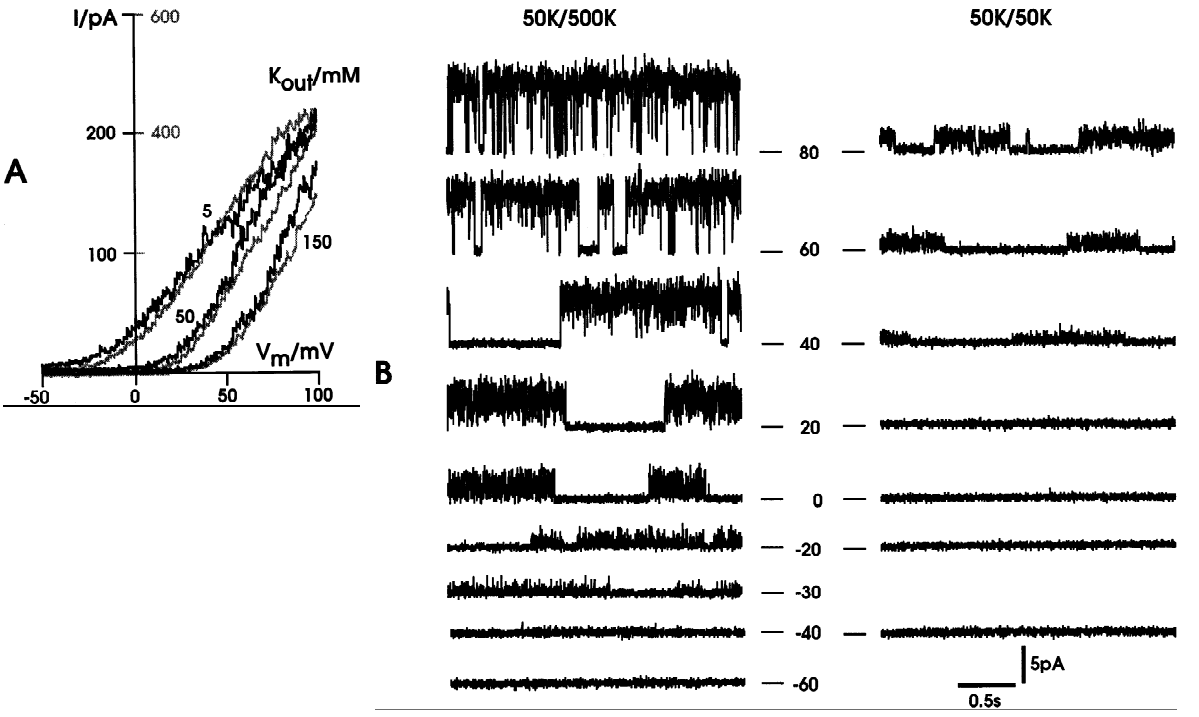

Fig. 8. Potassium effect on gating of the yeast outward rectifier. (*A*) Whole-cell currents in response to the standard 5-sec voltage ramp, for two different cells (grey traces and right ordinate scale; black traces and left ordinate scale) and three different extracellular K^+ concentrations. Each trace was obtained within 5 min after either initiation of whole-cell recording or partial replacement of bath KCl with osmotically equivalent sorbitol. (*B*) Complementary demonstration on an isolated inside-out patch containing a single activable K^+ channel. Pipette solution (extracellular) containing 50 mM K^+ and bath solution (cytosolic) containing first (left panel) 500 mM K^+ and then (right panel) 50 mM K^+. Header notation: 50K/500K = 50 mM extracellular K^+/500 mM cytosolic K^+. Membrane voltages (in mV) and baseline currents are indicated by the numbers and horizontal lines, respectively, between corresponding traces in the two panels.

independent experiments, and probably resulted from spurious intracellular actions of Rb^+ and Cs^+. [No attempt was made, in these short-term experiments, to control ion leakage via the yeast TRK systems (Ko & Gaber, 1991).] The decrement of slope for Na^+ at higher positive voltages, however, *was* systematic and arose from blockade of outward K^+ current by intracellular sodium (Bertl & Slayman, 1992). The latter effect was enhanced by prolonged exposure to extracellular Na^+ and also by deletion of the gene for the principal sodium extrusion pump in yeast, *PMR2 = ENA1* (A. Bertl, *unpublished experiment*).

Among traditional K^+-channel inhibitors, the tetraethylammonium ion (TEA^+), in extracellular solutions, had a surprisingly strong and clear-cut effect on the yeast outward rectifier, at least by comparison with its reported actions on most plant K^+ channels (Schachtman et al., 1992; Bertl et al., 1997; Wegner, DeBoer & Raschke, 1994). Figure 10 demonstrates that—over the range 0–25 mM—TEA^+ scaled down the current at all voltages, but had no effect on the kinetics of Duk1p gating. Panels *A* and *B* in Fig. 10 show corresponding whole-cell data obtained from both voltage-ramp and voltage-pulse (to +100 mV) protocols, which were in excellent agreement.

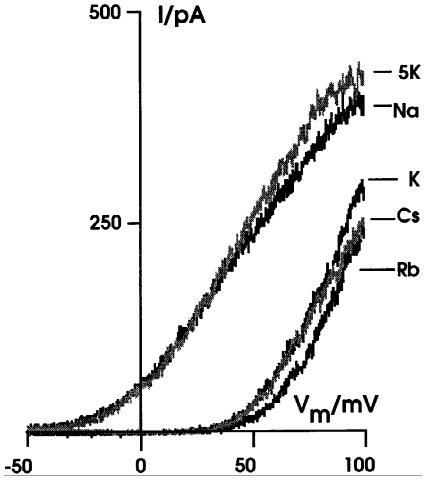

Fig. 9. Effects of K^+-like and nonK^+-like monovalent cations on Duk1p currents. Whole cell currents from cells bathed in buffers containing chloride salts of 150 mM K^+ (the control), 5 mM K^+ (+ osmotically equivalent sorbitol; *cf.* Fig. 8, above), or 150 mM Na^+, Rb^+, or Cs^+. Measurements made with standard voltage-ramp protocols, ~5 min after each solution replacement.

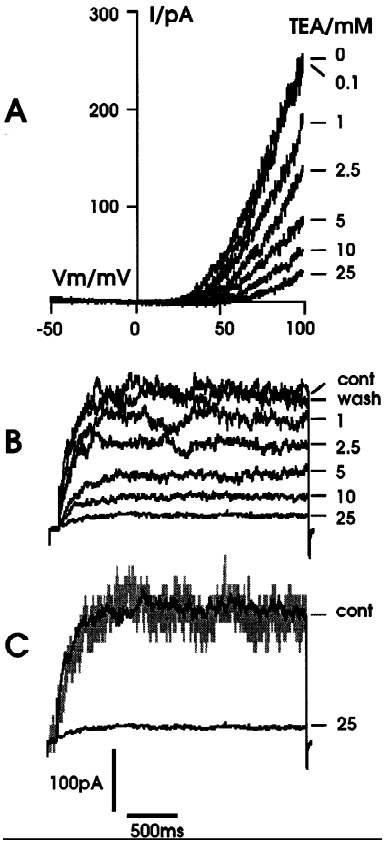

Fig. 10. Inhibition of outward currents through Duk1p by extracellular tetraethylammonium (TEA^+) ions. (*A*) Whole-cell currents in response to the standard voltage-ramp protocol, for different concentrations of TEA^+ *added* to the standard bath solution. Measurements made ~2 min after each addition. Inhibitor washed out for at least 5 min between traces. (*B*) Whole-cell currents in response to a standard voltage-step: $-40 \rightarrow +100$ mV for all traces. Same cell and general conditions as in panel *A*. Wash curve shown is that following 25 mM TEA^+. (*C*) Demonstration that TEA^+ reduces channel current without altering gating kinetics. Comparison of 0-TEA current (black tracing: control trace in panel *B*) with superimposed trace for 25 mM TEA^+ (grey tracing), rescaled by 9×. Current scale bar for all traces in *B* and *C*, except for the rescaled, grey tracing. Overall, TEA^+ inhibits K^+ current through Duk1p with a $K_i \approx 2.8$ mM; the computed maximal current for this experiment was ~255 pA at +100 mV.

Examination of the rising portion of the traces in *B* reveals no systematic change in shape with increasing TEA^+, but only a progressive decline in the steady-state current. This impression is reinforced by panel *C*, in which the 25-mM curve from *B* has been rescaled (grey tracing) and superimposed on the control 0-TEA^+ tracing. The steady-state data in Fig. 10*A* yielded a K_I for TEA^+ of ~2.8 mM, about half the value inferred from data on Duk1p expressed in *Xenopus* oocytes (Lesage et al., 1996*a*). Preliminary analysis of the conspicuous reduction in noise with increasing $[TEA^+]_o$ (*cf.* traces at 10 and 25 mM TEA with those at 0 or 1 mM in Fig. 10*B*) suggests that the main effect of TEA^+ was to reduce single-channel conductance.

Discussion

Homologous Recording

Several properties of the *Saccharomyces* membrane system make the yeast outward-rectifying channel, Duk1p (Tok1p) particularly convenient for study. Most significantly, of course, yeast genetics can readily be applied to structure-function studies on this channel, and numerous laboratories around the world are now commencing just such experiments (*see* e.g., Loukin et al., 1997).

It is likely that most efforts will settle on the heterologous expression system of *Xenopus* oocytes for the functional analyses (*see* e.g., Ketchum et al., 1995; Lesage et al., 1996*a;* Vergani et al., 1997), and such experiments will be especially useful for comparative purposes, when particular properties of the channel are well-established and quantified in the yeast membrane itself. Two interesting examples of the latter point are the pH-dependence and apparent E_K-dependence of channel activation (Bertl et al., 1992, 1993; Ketchum et al., 1995; Lesage et al., 1996*a*). Although the relevant *I-V* plots of Lesage et al. (Fig. 5*b;* 1996*a*) are displaced about 40 mV negative to those in Fig. 6*A* above, the observed suppression of channel openings (isolated patch records) is very similar between the two groups of experiments. Likewise, the displacement of whole-cell *I-V* curves leftward (negative) along the voltage axis by lowered extracellular $[K^+]$, as reported by Ketchum et al. (1995), is essentially superimposable upon that described in Fig. 7*A* above. It is fair to say, then, that these two properties of Duk1p are intrinsic to the channel protein and have not been introduced by site factors peculiar either to yeast or to the vertebrate expression system. A third property of Duk1p reported in oocytes, the continuous open state or instantaneous activation with positive voltage steps (Ketchum et al., 1995; Lesage et al., 1996*a*), has not been found in the experiments on yeast, and is likely to have resulted from the particular experimental conditions in the oocyte system.

In a more general sense, however, heavy emphasis on *Xenopus* oocytes, or other heterologous systems, is likely to prove to be fundamentally a digression, since the essential maneuvers of random mutagenesis and selecting or screening for explicit phenotypic changes must be carried out on yeast to begin with. An obvious additional handicap is the transient nature of expression in the oocyte system. Finally, the background physiology of the oocyte membrane is very different from that of the yeast membrane: e.g., in membrane lipid composition, free running membrane voltage, glycosylation signals,

targeting signals, etc. This fact introduces the strong likelihood that important properties of the channel will be distorted by expression in the heterologous system, particularly since several cases of such distortion have already been recognized for other heterologous transporters expressed in the *Xenopus* system (Attali et al., 1993; Spruce & Moody, 1995; Wang & Goldstein, 1995; Tzounopoulos, Maylie & Adelman, 1995).

Metabolic Effects and Possible Structural Correlates

Metabolic dependence—particularly ATP-dependence—of ion channels is well-known in both animal (Rorsman & Trube, 1990; Inagaki et al., 1996; Quast, 1996) and plant systems (Hedrich, Busch & Raschke, 1990; Katsuara, Mimura & Tazawa, 1990; Spalding & Goldsmith, 1993; Colombo, Cerena & Giromini, 1994). The yeast outward rectifying channel, as explored thus far, appears solely to be activated by ATP, that is, ATP (mM) must be present in the cytoplasmic solution for channel gating to be observable during whole-cell recording. In this property Duk1p resembles a small class of plant channels (Hedrich et al., 1990; Colombo et al., 1994; Spalding & Goldsmith, 1994) said to be ATP *dependent,* and contrasts with the much larger group of channels, mostly from animal epithelial and excitable tissues (Rorsman & Trube, 1990; Quast, 1996) identified as ATP *sensitive.* The latter are most easily spotted by deactivation in the presence of mM ATP; but their recovery following sustained deactivation may require either displacement of competing nucleotides (e.g., ADP) or a cycle of ATP removal and readdition (Ribalet, Ciani & Eddlestone, 1989; Hussain & Wareham, 1994). Although a few of the ATP-sensitive channels may react directly with ATP (Weik & Neumcke, 1989; McNicholas et al., 1996*a*), in many cases modulation of channel activity is complex and can involve phosphorylation/dephosphorylation, protein kinases, cyclic nucleotides (Ribalet et al., 1989), changes in cytoplasmic pH (Davies, 1990; Fan et al., 1993), and even the participation of cytoskeletal elements (Terzic & Kurachi, 1996) or sulfonylurea receptors (Lee et al., 1996; McNicholas et al., 1996*b*). Thus, the fact that Duk1p is responsive to the presence of cytoplasmic ATP opens the channel to legion experiments on the actual control mechanism.

Particularly interesting would be studies involving three distinct features of the protein structure. First, the relatively short cytoplasmic segment S4→S5 contains a possible variant P-loop, 229-GYKLGKYPPTFNLL-242, which might serve directly as an ATP-binding site. [Compare the consensus sequence GXXXXGKXXX-XXXXI—in which M, V, and L are sometimes substituted for the terminal I (Walker et al., 1982; Walker, Saraste & Gay, 1984; Ho et al., 1993).] Second, there are nine putative phosphorylation sites on the protein, all but one of which should lie on the cytoplasmic face of the channel (*see* Fig. 11), and four of which are clustered together—between Ser346 and Lys376—in the cytoplasmic loop S6→S7. [A preliminary effort to investigate potential PKC regulation has been reported on Duk1p in oocyte membranes, but with ambiguous results (Lesage et al., 1996*a*).] The same region, cytoplasmic loop S6→S7, is also rich in basic amino acids. Cytosolic domains containing a high density of basic amino acids have been shown to contribute to the inactivation gating of certain voltage-sensitive Na^+ and K^+ channels in excitable cells (Aldrich, Hoshi & Zagotta, 1990; Patton et al., 1993); and highly charged segments displaying multiple phosphorylation sites have been postulated to regulate CFTR-chloride channels (Rich et al., 1991) and an inwardly rectifying K^+ channel from mammalian kidney (ROMK1; Ho et al., 1993). Finally, the long C-terminus (235 residues) of Duk1p is festooned with clumps of basic amino acids and separate acidic amino acids. This arrangement is reminiscent of N-type inactivating structures in excitable cells (Aldrich et al., 1990; Patton et al., 1993) and is distinctly different from the C-type inactivation sequences (C-termini) in voltage-dependent K^+ channels (ShA, ShB; Schwarz et al., 1988; Hoshi, Zagotta & Aldrich, 1991). On these purely structural grounds it is worth searching for experimental conditions which might display inactivation of the channel, even though such a process has not yet been reported for Duk1p.

For the pH effects, histidine residues are the most likely sites involved, since they are the only amino acid with a pK in the physiologically relevant range, viz., around the pH of the cytosol. The prominent effect of intracellular pH and the lack of any measurable sensitivity to external pH correlates with a sharply unequal distribution of histidine residues along the channel protein (Fig. 11): of the total of 12 histidine residues, 10 are predicted to be located in hydrophilic segments facing the cytoplasm, and none are predicted to lie clearly at the extracellular face. To assess the involvement of ''cytosolic'' histidines in pH-dependent channel regulation, we are currently constructing yeast mutants with Duk1 channels mutated in these residues.

Summary of Effects of Various Extracellular Cations

(1) **H^+:** no influence on whole-cell currents, over the 1000-fold concentration range from 10^{-8} to 10^{-5} M. This is remarkable in that it presumably means that neither sites involved in gating nor sites involved in ion transit through the open channel react with extracellular protons.

(2) **K^+:** changing $[K^+]_o$ or $[K^+]_i$ slides the *I-V* relationship along the voltage axis in the same direction as

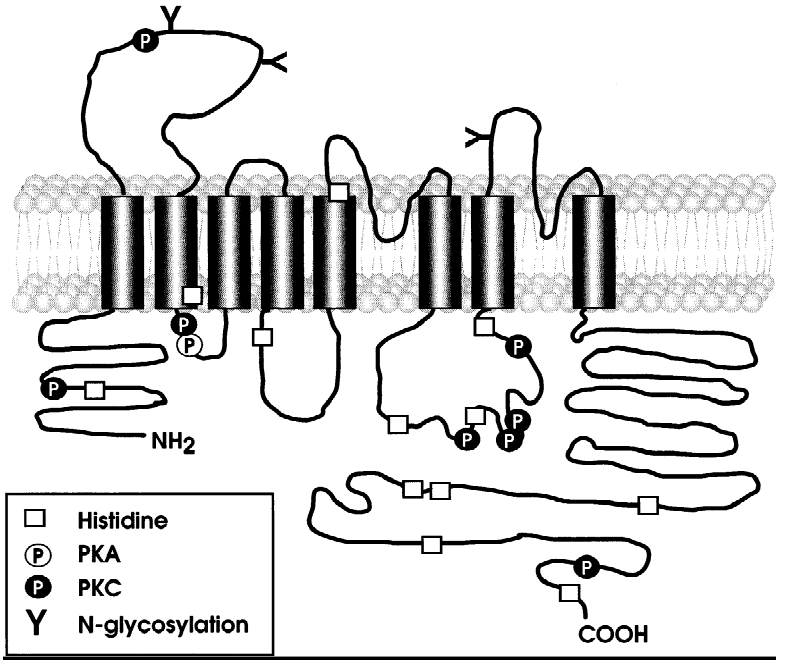

Fig. 11. Locations of potential critical reactive sites in the Duk1 protein, inferred from the gene sequence. *Open squares* (□): Histidine residues, His33, 159, 226, 271, 337, 349, 378, 596, 616, 619, 651, 687. *Open circle* (○): Potential protein kinase A site, Thr165. *Filled circles* (●): Potential protein kinase C sites, Thr37; Ser107, 161, 346, 353, 358, 374, 679. Y: Potential N-glycosylation sites (extracellular), Asn110, 120; 405. In addition, there is a potential P-loop (nucleotide binding region) in the S4→S5 segment, spanning from Gly229 to Leu242: GYKLGKYPPTFNLL, which compares with the consensus sequence GXXXXGKXXXXXXXI/V (Walker et al., 1984).

E_K and by approximately the same amount, so that in certain circumstances shifts of membrane voltage and E_K have interchangeable effects. The actual mechanism of this behavior remains to be worked out, but it probably should not be viewed as a literal linkage between E_K (or $V_m - E_K$) and gating. For present purposes, we note that only one reaction, the **G—O** transition, in our original 4-state model (*see* Introduction above; Bertl et al., 1993) is sensitive to $[K^+]_i$ and $[K^+]_o$; one reaction, **I→O,** seems to be sensitive to $[K^+]_i$ only; while the third reaction is not sensitive to K^+ at all. This would require two distinct binding sites for potassium, being accessible from the extracellular side and from the cytosolic side of the channel, respectively. Thus, it is very likely that the yeast outward rectifier senses K^+ concentrations, rather than transmembrane K^+ gradients, which correlates with the notion that Duk1p plays a central role in osmoregulation and K^+-homeostasis in yeast.

(3) **Na^+:** no influence—other than that of potassium removal—upon whole cell currents at short times and/or low positive voltages. No binding to the postulated extracellular binding site. Decrement of both whole-cell and single-channel currents at long times and high positive voltages. The latter process represents a very fast voltage-dependent block contingent upon leakage of sodium ions into the cells (Bertl & Slayman, 1992) and will be described in detail elsewhere.

(4) **Rb^+** and **Cs^+:** bind to the proposed extracellular binding site, leading to almost complete replacement of K^+.

(5) **TEA^+:** the evident down-scaling of whole-cell currents and current noise (Fig. 10), without effect on the time-course or voltage-dependence of channel opening, implicates two alternative modes of action of extracellular TEA^+: reduction in the total number of activable channels, or reduction in open-channel conductance. Preliminary quantitative analysis of the noise data favors the latter. The findings are generally consistent with TEA^+ blockade of animal outwardly rectifying K^+ channels.

(6) **Ba^{++}:** reduces whole-cells currents with an apparent K_I near 3 mM at short times (several minutes) and *tends* to reduce currents further at long times (>10 min), but is quantitatively unstable and unpredictable in these effects (A. Bertl, unpublished experiments; Zhou et al., 1995; Lesage et al., 1996*a*). Evidently, therefore, at least one variable critical to barium's actions on Duk1p is not yet under control.

Physiological Significance of Some Cation Effects

From the point of view of yeast physiology, this spectrum of ionic effects may be the most significant properties of the Duk1 channel. As was demonstrated previously (Bertl et al., 1993), the normal conditions for patch-clamp experiments—with 50–200 mM K^+ in the extracellular solution and 1μM–10 mM Ca^{++} in the cytosolic solution—poise the channel to be activated only at very *un*physiological membrane voltages: 0 to 200 mV *positive,* depending on the exact conditions. The peak value of P_o (~0.2, near +10 mV for $[Ca^{++}]_c$ = 10 mM) shifts approximately 45 mV positive for each decanal decrease of $[Ca^{++}]_c$ (~0.9, near +190 mV for $[Ca^{++}]_c$ = 1 μM).

While these numbers *in abstract* have meaning for the physical properties of Duk1p itself, they have mean-

ing for the growth and survival of yeast mainly in relation to the normal physiological parameters. The potassium concentration in the usual growth media (~20 mM in YPD, ~10 mM in synthetic complete medium), for example, is well below the range in patch-clamp solutions. That difference has at least two major consequences, which can be inferred from extensive electrophysiological measurements on the related ascomycete, *Neurospora* (Slayman, 1965, 1974; Blatt & Slayman, 1983; Blatt et al., 1987), and which are qualitatively supported by pilot experiments on yeast itself (with penetrating microelectrodes; A. Bertl, unpublished experiments). First, with sustained low (1–10 mM) extracellular salt, the resting membrane voltage in yeast cells lies in the range −100 to −200 mV, far negative to the range for peak activation of Duk1p. Indeed, the shift of membrane voltage between low and high (50–200 mM) extracellular salt can be superNernstian, due in part to the shift in E_K but due *largely* to a change in K^+-shunting of H^+ current through the proton pump. With regard to the functionality of Duk1p, this shift of membrane voltage is offset in *direction* by the nearly Nernstian shift of voltage-dependent gating, demonstrated in Figs. 8 and 9.

The second consequence is that, in sustained low salt, the expected time-average membrane resistance of a single yeast cell should lie near 10^{10} ohms, such that only a single Duk1 channel would be open at any one time in haploid yeast, and only ~3 in tetraploid yeast. With a normal channel density in the *Saccharomyces* membrane of ~100/cell, the implied physiological upper limit of P_o is in the range 0.01–0.03. Furthermore, the fact that many other transport systems in the yeast membrane also carry current (and contribute to conductance), means that the actual upper limit of P_o for Duk1p in vivo must be still smaller, perhaps as much as 10-fold smaller.

A general implication of these arrangements, plus the fact that potassium efflux from yeast is reported to be activated by depolarizing sugar uptake (Serrano, 1977), is that the physiological function of Duk1 can change, depending upon the conditions under which it is activated. When few channels are open and the resting membrane voltage is far negative to E_K, this outwardly rectifying channel can mediate net potassium *uptake*/influx, which is ultimately driven by the voltage effect of the primary proton pump. Such behavior of fungal outward rectifiers, under steady-state conditions, is likely to occur as well with plant K^+ channels, where membrane voltages often also rest considerably negative to E_K, but to occur rarely with animal cells, where membrane voltages are almost always positive to E_K. Indeed in animal cells, *inwardly* rectifying K^+ channels can *release* potassium when the resting membrane voltage is positive to E_K; that is a logical mirror image for K^+ uptake through the yeast outward rectifier.

The other side of this argument is that activation of the outward rectifier by sufficient depolarization can serve to stabilize the yeast membrane voltage, holding it near E_K. That action, in effect, substitutes stored potassium ions for pumped protons as the energy source (voltage) driving H^+-coupled nutrient import systems in yeast. Outward rectifiers in plant *and* animal cells should play this same role, albeit substituting stored K^+ for the pumped Na^+ (not H^+) in animal cells.

pH effects on the yeast outward rectifier also reflect significantly on yeast physiology. Since *Saccharomyces* can survive and grow at very low extracellular pH values (as low as 2), it must have defense mechanisms against membrane damage by low pH_o. One is that the gating of any major current-carrying channels must not be disrupted by extremes of pH_o, especially not toward uncontrolled opening. Although we have not investigated the full range of pH_o tolerated by yeast (pH 2–pH 9), the results in Fig. 7 are an impressive start: no effect on Duk1p behavior over 2.5 pH units, a 300-fold change in $[H^+]_o$. This statement encompasses several aspects of the channel behavior: total current, gating kinetics, number of channels active, and current through single open channels.

The situation is much different, of course, for *intracellular* (cytosolic) pH, and its influence on membrane transport processes may be complex. Again using established electrophysiological results on *Neurospora* as a guide (Sanders, Hansen & Slayman, 1981; Blatt & Slayman, 1987), cytosolic acidification should accelerate the yeast proton pump in proportion (first-order kinetics) to the increase of $[H^+]_c$. When the acid shift is carried out without adding permeant anions (e.g., as in Fig. 6, with acetic acid), increased proton pumping should be accompanied by hyperpolarization and an increased return current, which can do useful work: for example driving net K^+ influx and thence countermanding the cytosolic acidification because of net H^+ efflux. In K^+-starved *Neurospora*, this process operates through a derepressed K^+–H^+ symport (Blatt & Slayman, 1987). We don't yet know how it would work in *Saccharomyces*, normal or K^+-starved, but there is no reason in *principle* why it could not work via simple K^+ channels.

The results in Figs. 6 and 7, above, demonstrate that strong cytosolic acidification deactivates Duk1 channels, though not completely—at least until very long times. Although we do not have detailed data for smaller acidifying steps, the results on yeast are quantitatively consistent with those sketched out on *Xenopus* oocytes (Lesage et al., 1996); viz., about 50% deactivation of Duk1p with a ''physiological'' acidification from pH 7.2 to pH 6.6.

Since total pump current through the membrane of a single yeast cell rarely exceeds 20 pA, and a single molecule of Duk1p admits 4 pA at 100 mV, a yeast cell with ~100 of these channels in its membrane, even half deac-

tivated, still has plenty of capacity to regulate cytosolic pH via K^+ channels. In this context it will be important to determine how Duk1p is modulated by prolonged K^+ starvation of yeast, and what the actual effect of these channels is, upon cytosolic pH under acid stress. We are currently designing yeast strains which are suitable for such experiments.

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