

Large Mg^{2+} -dependent currents are associated with the expression of *ALR1* in *Saccharomyces cerevisiae*

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Abstract

Two genes in *Saccharomyces cerevisiae*, *ALR1* and *ALR2*, encode proteins putatively involved in Mg^{2+} uptake. The present study identifies this role for *ALR1* and provides the first electrophysiological characterisation of this protein. The patch-clamp technique was used to measure whole-cell ion currents in protoplasts prepared from the wild-type strain, the *alr1* mutant, and a double mutant (CM66), and the *alr1* mutant whole-expressing the *ALR1* gene (CM66+*ALR1*). With 50 mM Mg^{2+} in the bath solution, the inward current in the protoplasts of CM66+*ALR1* averaged -264 ± 48 pA at -150 mV. Inward currents measured in the *alr1* mutant protoplasts were more than five-fold smaller. When Mg^{2+} was the major cation in the pipette solution, time-dependent outward currents were also detected in CM66+*ALR1* protoplasts suggesting *ALR1* can facilitate Mg^{2+} efflux as well as uptake. The large magnitude of the Mg^{2+} -dependent currents suggests that *ALR1* is a cation channel. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Magnesium; Transport; *ALR1*; Yeast; Cation channel; Aluminum

Introduction

Magnesium is the most abundant divalent cation in living cells yet, by comparison to most other macronutrients, relatively little is known about its biology. While Mg^{2+} has recognised roles in membrane stability and as a cofactor with many enzymes, it is increasingly clear that changes in free cellular Mg^{2+} concentrations can regulate different metabolic pathways in plants, animals and microorganisms. The understanding of Mg^{2+} transport mechanisms in this area, especially in plants, is limited. Mg^{2+} uptake primarily

in model plant species from the Asteraceae family was demonstrated in *Saccharomyces cerevisiae* has two genes, *ALR1* and *ALR2*, that encode proteins putatively involved in Mg^{2+} transport. These genes were first identified in a screen for Al^{3+} tolerance in *S. cerevisiae* [9] suggested that *ALR1* and *ALR2* are cation transporters because they were able to grow on a standard medium containing Al^{3+} when applied in the presence of Mg^{2+} . The expression of *ALR1* in *S. cerevisiae* [10]. They are located in the plasma membrane of *S. cerevisiae* and are conserved in other yeast mutants. *ALR1* is able to transport Mg^{2+} with a K_m of $100 \mu M$ and a V_{max} of $1.5 \mu M$ [10].

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ALR1 is a passive transporter, such as a channel, whose function depends upon the maintenance of the electrical potential difference across the plasma membrane (ΔE_{io}). Indeed the driving force generated by ΔE_{io} in yeast and bacteria is sufficient to energise Mg^{2+} uptake for normal growth [11,12]. In this study, we provide electrophysiological evidence that the *ALR1* gene encodes a transport protein involved in Mg^{2+} uptake by yeast. We conclude that ALR1 is likely to function as a cation channel.

2. Materials and methods

2.1. Yeast strains and growth conditions

Yeast strains were grown on standard synthetic medium (SC with the required auxotrophic supplements and 2% glucose) or on complete medium (YPD). Two yeast strains were derived in this study as isogenic derivatives of FY833 [13]. CM52 (MAT α *his3- Δ 200*, *ura3-52*, *leu2- Δ 1*, *lys2- Δ 202 trp1- Δ 63*) is wild-type for the two *ALR* genes while CM66 has both *ALR* genes deleted (MAT α *alr1::HIS3 alr2::TRP1 his3- Δ 200*, *ura3-52*, *leu2- Δ 1*, *lys2- Δ 202 trp1- Δ 63*). The ALR1 ALR2 deletion mutants were generated by one-step gene disruption [14] using an *alr1::HIS3* polymerase chain reaction product [9] or a *palr2::TRP1* plasmid (analogous to *palr2::URA3*) [9]. Wild-type (CM52) and CM66 were derived from segregation of the same heterozygous diploid. Growth of CM66 strains was achieved by supplementing SC or YPD with 0.25–0.4 M $MgCl_2$. Methods for yeast transformation were as described by Kaiser et al. [14] and Gietz et al. [15]. The plasmid pFLN2 was derived from the pFL61 high copy number plasmid [15] by the addition of *XhoI* and *SalI* sites to the multiple cloning region (MacDiarmid, unpublished). pFLN2 was used to express *ALR1* in CM66 using the constitutive yeast phosphoglycerate kinase promoter.

2.2. Preparation of yeast protoplasts

Yeast were grown on a rotary shaker (100 rpm at 25°C) and protoplasts were prepared using a procedure described previously [16]. Cells were harvested by centrifugation at $500\times g$ for 5 min, and resuspended in 3 ml buffer A (50 mM KH_2PO_4 pH 7.2, titrated with KOH, plus 0.2% β -mercaptoethanol). The cells were incubated in a water bath at 30°C for 30 min with slow shaking (~ 30 rpm). Buffer B (3 ml, buffer A supplemented with 2.4 M sorbitol, 2 mg ml $^{-1}$ zymolyase, 2 mg ml $^{-1}$ glucuronidase, and 50 mg ml $^{-1}$ bovine serum albumin) was added to the yeast cells and mixed gently at 30°C for 30 min. The cells were collected by centrifugation ($200\times g$, 5 min) and the protoplasts were released after resuspension in buffer C (150 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM MES, pH 7.5 adjusted with Trizma). The protoplasts were collected and resuspended in buffer C plus 1% glu-

cose and typically had diameters of 2–7 μ m. Experiments were performed on protoplasts prepared on the same day.

2.3. Whole-cell patch clamping

Patch-clamping procedures were similar to those described by Bertl et al. [16] with some differences. Uncoated, borosilicate glass pipettes (Clark Electromedical Instruments, UK, 1.5 mm O.D. and 0.86 mm I.D.) were prepared with a P-97 pipette puller (Sutter Instruments, USA). The protoplasts were transferred to a recording chamber containing a sealing solution to assist seal formation (150 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM MES, pH 7 adjusted with Tris base). The cells were allowed to settle for 10–20 min and then the debris and unattached cells were washed away by steady perfusion of sealing solution for 5–10 min. Unless stated otherwise the standard pipette solution was: 150 mM K-acetate, 5 mM Mg-acetate, 5 mM KCl, 4 Tris-ATP, 1 mM EGTA, pH 7 with Tris base. Resistance of the pipettes was 5–10 M Ω with the sealing solution in the bath. The chloride concentration in the pipette solution was kept low to avoid the possibility that inward currents generated from chloride efflux could be confused with Mg^{2+} -dependent inward currents. A G Ω seal was successfully achieved in a minority of cells after 10–20 min of constant and gentle suction. The following three criteria were used to help identify when the whole-cell patch configuration was obtained: (i) a sudden increase of membrane conductance, (ii) increase of background noise, or (iii) the appearance of a small time-dependent outward current characteristic of the DUK1 K^+ channel. Small outward currents were often observed in protoplasts from all the strains but their magnitude and the time dependence were significantly smaller than reported previously for the K^+ channel DUK1 [17]. It is likely that the low Ca^{2+} concentrations used in the pipette here inhibited the DUK1-dependent currents (A. Bertl, personal communication). Once the whole-cell configuration was obtained, the sealing solution was replaced with a bath solution that contained a high Mg^{2+} concentration (80 mM Tris acetate, 50 mM Mg-acetate, 5 mM KCl, 1 mM $CaCl_2$, 1 mM MES, titrated to pH 7 with Tris base). In some experiments the high Mg^{2+} bath solution was replaced with a Mg^{2+} -free solution (150 mM Tris-acetate, 5 mM KCl, 2 mM Ca-acetate, 1 mM MES, pH 7). The effect of aluminium on whole-cell currents was tested by using a high Mg^{2+} bath solution adjusted to pH 4.1 and then replacing it with a similar solution containing 0.5 mM $AlCl_3$. The concentration of free Al^{3+} in this solution was calculated with the GEOCHEM program [18] to be approximately 200 μ M which corresponds to an activity of 20 μ M. Current–voltage data were collected by holding the membrane potential at 0 mV and then stepping the voltage between -180 and $+80$ mV for 2 s intervals in 20 mV increments. The range of command voltages was reduced when the currents were very large or when

they appeared unstable during the pulses. Current–voltage curves were generated from currents measured near the end of 2 s voltage pulses. Currents were amplified and filtered at 2 kHz using an Axopatch 200B amplifier (Axon Instruments) and digitised online by an IBM computer at 10 kHz (pClamp8.01/Digidata 1200B, Axon Instruments). Junction potentials for the high Mg^{2+} and Mg^{2+} -free solutions were 10 mV and 12 mV respectively. The current–voltage curves are corrected for the liquid junction potentials. Leak currents were not subtracted from the data presented.

3. Results

Whole-cell currents were initially compared in three yeast strains with 50 mM Mg^{2+} in the bathing solution. Measurements were made on the CM66 (*alr1 alr2* knockout strain), CM66+ALR1 (CM66 transformed with the *ALR1* gene) and the wild-type strain (CM52). Inward and outward currents in CM66 were small and neither exceeded 200 pA in any individual cell. The average current at -150 mV was -51 ± 26 pA ($n = 12$). Currents measured in the wild-type strain (CM52) were very similar to CM66 with inward currents averaging -50 ± 27 pA ($n = 12$) at -150 mV. By contrast, large and variable inward currents were observed in 78% of the CM66+ALR1 protoplasts examined. The magnitude of these currents

ranged from -200 to -1500 pA (Fig. 1b) with a mean inward current of -264 ± 48 pA ($n = 41$) at -150 mV (Fig. 1c). The kinetics of current activation varied with voltage and between protoplasts but instantaneous and time-dependent components were observed at the more negative voltages. When Mg-acetate was removed from the bath solution and replaced with Tris-acetate, the large inward currents were reduced in 90% of protoplasts tested ($n = 9$, Fig. 2). The outward currents also became smaller when Mg^{2+} was removed from the bathing solution. In protoplasts where the inward currents were very large (> 1000 pA) the tail currents were often slower to relax to the pre-pulse levels. This has been reported previously in other yeast strains exhibiting large inward currents (see Fig. 3b in [19]).

A new set of solutions were used to determine whether ALR1 can facilitate Mg^{2+} efflux as well as influx. In these experiments the pipette solution contained 50 mM Mg^{2+} , 5 mM K^+ and 10 mM TEA-Cl to inhibit any outward K^+ currents. In these conditions, large inward and outward currents were observed in seven of 13 protoplasts examined. The remaining six protoplasts had small currents which possibly indicated that the expression of the *ALR1* was poor in these cells. The average currents measured over all 13 protoplasts were -412 ± 107 pA at -150 mV and $+212 \pm 62$ pA at 90 mV respectively (Fig. 3b). The currents averaged from the seven protoplasts showing the large currents only were -668 ± 136

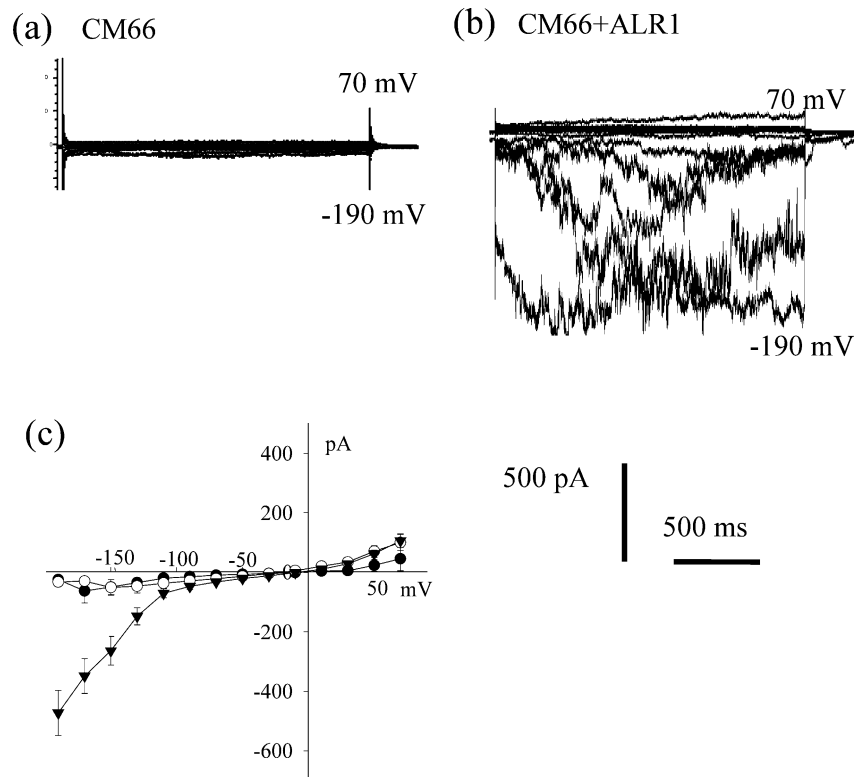


Fig. 1. Whole-cell currents measured in yeast strains with 50 mM Mg^{2+} in the bath solution. Examples of the whole-cell currents measured in (a) CM66 and (b) CM66+ALR1. c: Average current–voltage relationships collected from the wild-type strain (●), CM66 (○, $n = 12$) and CM66+ALR1 (▼, $n = 41$).

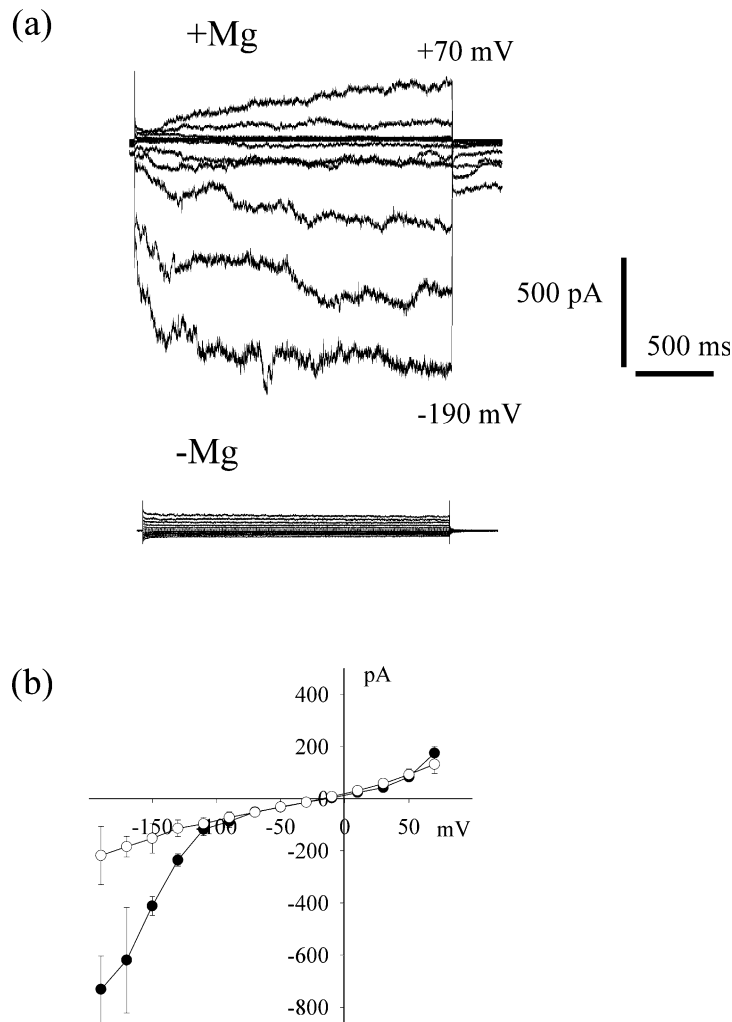


Fig. 2. The large inward currents in CM66+ALR1 protoplasts are dependent on external Mg^{2+} . a: Whole-cell currents recorded with 50 mM Mg^{2+} in the bath solution (upper) and the same cell after the 50 mM Mg-acetate is removed from the bath and replaced with 75 mM Tris-acetate (lower). b: Current–voltage relationship averaged from nine cells before (●) and after (○) 50 mM Mg^{2+} was removed from the bath solution.

pA at -150 mV and $+367 \pm 74$ pA at 90 mV. In four experiments the 50 mM Mg^{2+} -acetate in the bathing solution was replaced with 75 mM Tris-acetate. With this treatment both the inward and outward currents were significantly inhibited (Fig. 3b).

The effect of aluminium on the Mg^{2+} -dependent inward currents was measured in the CM66+ALR1 strain. High Mg^{2+} concentrations were included in the pipette and bathing solutions in these experiments and the bathing solution was adjusted to pH 4.1 to ensure that the Al^{3+} cation was the predominant species present in the bathing solution [20]. Large inward and outward currents were still observed at this pH (Fig. 4a). The addition of 500 μ M $AlCl_3$ to the bath solution (equivalent to an activity of free Al^{3+} cations of 20 μ M) significantly decreased the inward and outward currents in three separate cells. Removal of Al^{3+} led to a recovery of both currents after a 5–20-min washout period. The CM66 control was omitted from this experiment because no Mg^{2+} -dependent currents were detected in this strain.

4. Discussion

Our results provide evidence that *ALR1* encodes a protein involved in Mg^{2+} transport in yeast. We used the whole-cell patch-clamp technique to compare the Mg^{2+} -dependent currents in the *alr1 alr2* mutant (CM66) with the same strain over-expressing ALR1 (CM66+ALR1). Large Mg^{2+} -dependent inward currents were measured in a majority of CM66+ALR1 protoplasts while no comparable currents were detected in the CM66. High external Mg^{2+} concentrations were used in this study to exaggerate the effects on the whole-cell currents. While 50 mM Mg^{2+} is a higher concentration than yeasts normally experience in their environment, it is not a toxic concentration and wild-type *S. cerevisiae* can grow well in 400 mM Mg^{2+} [9].

It is unlikely that the large currents measured in the CM66+ALR1 strain are caused by other endogenous transporters, because inward currents of this magnitude are uncommon in wild-type *S. cerevisiae*. We are aware of only one report showing endogenous currents of an

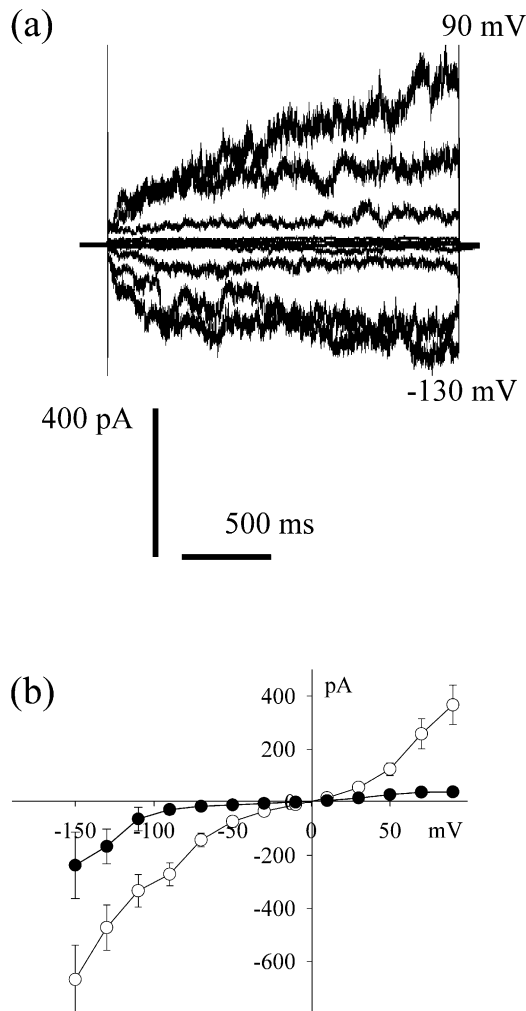


Fig. 3. ALR1 expression is associated with inward and outward currents. a: Whole-cell currents measured in a CM66+ALR1 protoplast with 50 mM Mg^{2+} in the bathing and pipette solutions. b: The current-voltage relationship averaged from all CM66+ALR1 protoplasts with 50 mM Mg^{2+} in the bathing solution (\circ , $n=13$) and after Mg^{2+} was removed from the bathing solution (\bullet , $n=4$). Pipette solution (mM): 75 Tris-acetate, 50 Mg^{2+} -acetate, 10 mM TEA-Cl, 2 Ca^{2+} -acetate, 5 KCl, 1 MES, pH 7. Bath solution with Mg (mM): 80 Tris-acetate, 50 Mg^{2+} -acetate, 2 Ca^{2+} -acetate, 5 KCl, 1 MES, pH 7. Bath solution without Mg (mM): 155 Tris-acetate, 2 Ca^{2+} -acetate, 5 KCl, 1 MES, pH 7.

equivalent magnitude. In that study, Bihler et al. [19] described large inward currents in wild-type yeast which are activated by low external divalent cation concentrations ($<10 \mu M$) and the authors attributed these currents to a non-specific cation channel designated NSC1. However, the inward currents measured in the present study cannot be attributed to NSC1 because activation of NSC1 requires a low external cation concentration while the ALR1-dependent currents were observed in the presence of 50 mM Mg^{2+} . NSC1-dependent currents are also inhibited by low external pH [19] but, in the present work, the inward currents were observed at pH 4.1. Finally, unpublished results show that the NSC1 currents are present in the CM66 strain which demonstrates conclusively that NSC1 cannot be attributed to ALR1 (H. Bihler and A.

Bertl, personal communication). It is also unlikely that the large inward currents we measured can be attributed to Trk2p, another transporter found to generate inward currents in yeast. The magnitude of Trk2p-dependent currents at pH 7.0 are very small (<-50 pA at -200 mV) and independent of external Mg^{2+} [21]. By contrast, the inward currents measured in the present study at pH 7.0 are up to 40-fold greater and dependent upon external Mg^{2+} . However, Trk2p-dependent currents do increase as pH declines because Trk2p appears to facilitate proton uptake [21]. Therefore, it remains possible that a proportion of the inward current presented in Fig. 4 is due to Trk2p activity since pH 4.1 was used in those experiments.

Sodium-coupled Mg^{2+} transport has been demonstrated in mammalian systems [22,23] and Mg^{2+}/H^+ exchange has been measured across the tonoplast of plant cells but other transporters may also exist [24]. Sodium was not included in the pipette solutions in these experiments, which precludes the involvement of ALR1 in a Mg^{2+}/Na^+ exchange reaction. In addition, the Mg^{2+} -dependent inward currents measured here were observed when the external pH was 7.1 and 4.1 (Figs. 1 and 4), making it unlikely that ALR1-dependent Mg^{2+} uptake relies on symport or antiport with H^+ . Instead, the present evidence supports the hypothesis that ALR1 functions as an ion channel, rather than another type of carrier. Furthermore, the magnitude of the inward current is too large to be attributed to a non-channel transporter which typically functions 1000-fold more slowly than channels. For instance, the currents measured in the CM66+ALR1 strain exposed to high external Mg^{2+} often exceeded 500 pA per cell and sometimes reached 1500 pA per cell. A current of 500 pA per cell is equivalent to a current density of approximately $1000 \mu A cm^{-2}$ or 3×10^7 divalent ions $\mu m^{-2} s^{-1}$ (assuming a cell diameter of 4 μm). A carrier-type transporter can facilitate ion movement at up to approximately 1000 ions s^{-1} which means that 30000 transport proteins per μm^{-2} membrane area would be required to account for the currents measured. If we assume the ALR1 protein has a diameter of 60 \AA and occupies an area of $3 \times 10^{-5} \mu m^{-2}$, then the calculated protein density exceeds the maximum protein distribution possible in the membrane. Such a density of proteins would prohibit normal growth and development. Yet the CM66+ALR1 cells have a normal phenotype with no special nutritional requirements suggesting that the other membrane proteins function normally.

The *ALR1* and *ALR2* genes were first identified from their ability to confer tolerance to aluminium toxicity when over-expressed in *S. cerevisiae* [9]. The same study showed that aluminium reduced Mg^{2+} ($^{57}Co^{2+}$) uptake by *S. cerevisiae* and it was concluded that aluminium toxicity was related to the inhibition of Mg^{2+} uptake via the ALR1 transporter. The present study supports this conclusion by demonstrating that Al^{3+} reversibly inhibits the Mg^{2+} -dependent inward currents present in CM66+ALR1 cells.

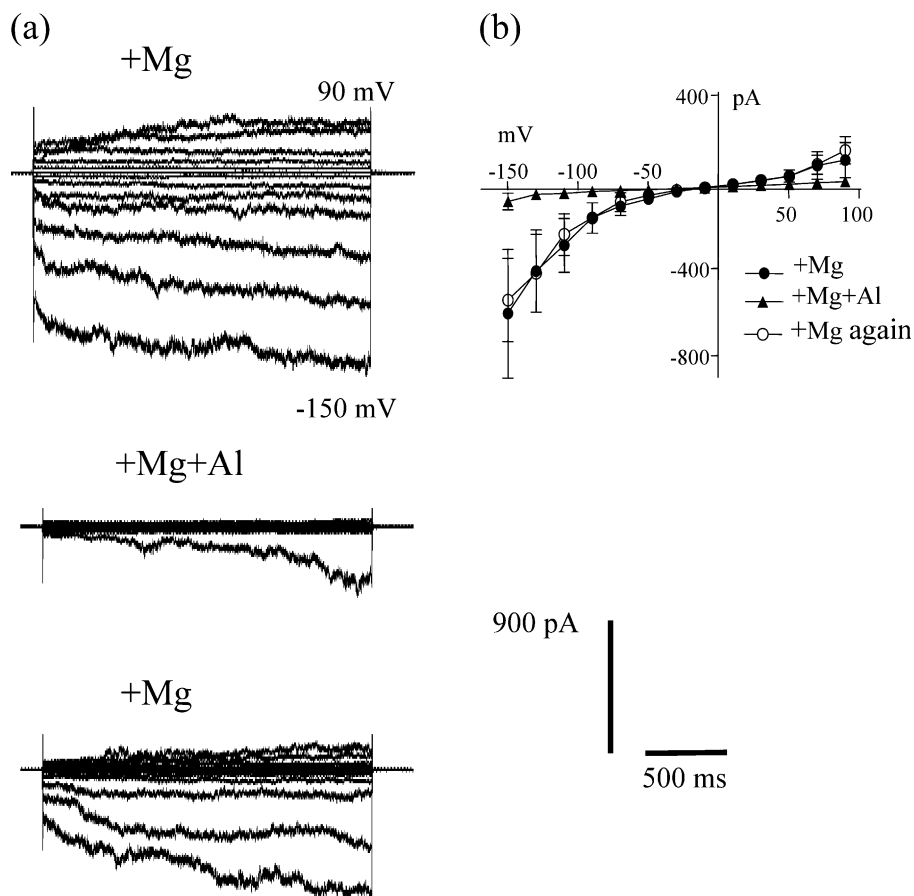


Fig. 4. Al^{3+} inhibits Mg^{2+} -dependent inward currents in CM66+ALR1 cells. a: Whole-cell currents following sequential changes in bathing solution. Addition of 0.5 mM AlCl_3 to the bathing solution inhibited the inward and outward currents. b: Current-voltage relationship of the data shown in panel a. Pipette and bath solutions contained (mM): 70 Tris-acetate, 50 Mg-acetate, 5 K-acetate, 10 TEA-Cl, pH 4.1.

The yeast ALR proteins show structural similarities to the bacterial CorA family of magnesium transporters. In particular, both the ALR and CorA proteins have a large, highly charged N-terminal domain with two hydrophobic regions at the C-terminal end. Gene fusion experiments with the *Salmonella typhimurium* CorA gene indicate that there are likely to be three membrane-spanning domains at the C-terminus of this protein [25]. The electrophysiological evidence presented here suggests that ALR1 may function as a Mg^{2+} channel and cation channels with two or three transmembrane domains have been characterised in bacteria. For instance, KcsA, a K^+ -selective channel, and MscL, a bacterial mechanosensitive non-selective cation channel, form homo-tetramers and homo-pentamers respectively (reviewed by [26]). Szegedy and Maguire [27] recently reported 'preliminary evidence' that CorA also forms pentamers.

The presence of Mg^{2+} -dependent inward and outward currents in CM66+ALR1 suggests that ALR transports Mg^{2+} into and out of yeast protoplasts. The reduction in both outward and inward currents when Mg^{2+} was removed from the bathing solution was initially surprising but both these characters have been observed for the CorA system in bacteria [7]. For instance, Mg^{2+} efflux

via the CorA transporter in *Salmonella typhimurium* is dependent on external Mg^{2+} such that when Mg^{2+} is absent from the external solution efflux from the cells ceases [28]. Snively et al. [28] concluded that the effect of external Mg^{2+} on efflux could not be explained by a simple exchange reaction. They suggested instead that external Mg^{2+} somehow regulates the CorA transporter. Indeed, Mg^{2+} has been proposed to regulate ion channel activity in a number of animal and plant cells [6,29–31]. In conclusion we suggest that the large Mg-dependent currents measured in CM66+ALR1 provide evidence that ALR1 functions as an ion channel.

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