Ion-channel blocker sensitivity of voltage-gated calcium-channel homologue Cch1 in Saccharomyces cerevisiae

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The Cch1 protein of the yeast Saccharomyces cerevisiae is a homologue of the pore-forming \( \alpha_1 \) subunit of mammalian voltage-gated \( \text{Ca}^{2+} \) channels (VGCCs), and it constitutes a high-affinity \( \text{Ca}^{2+} \)-influx system with the Mid1 protein in this organism. Here, we characterized the kinetic property of a putative Cch1–Mid1 \( \text{Ca}^{2+} \) channel overexpressed in S. cerevisiae cells, and showed that the L-type VGCC blockers nifedipine and verapamil partially inhibited Cch1–Mid1 activity, but typical P/Q-, N-, R- and T-type VGCC blockers did not inhibit activity. In contrast, a third L-type VGCC blocker, diltiazem, increased Cch1–Mid1 activity. Diltiazem did not increase \( \text{Ca}^{2+} \) uptake in the \( \text{cch1}^{-} \) and \( \text{mid1}^{-} \) single mutants and the \( \text{cch1}^{-} \text{mid1}^{-} \) double mutant, indicating that the diltiazem-induced increase in \( \text{Ca}^{2+} \) uptake is completely dependent on Cch1–Mid1. These results suggest that Cch1 is pharmacologically similar to L-type VGCCs, but the interactions between Cch1 and the L-type VGCC blockers are more complicated than expected.

INTRODUCTION

Mammalian voltage-gated \( \text{Ca}^{2+} \) channels (VGCCs) are classified electrophysiologically into five classes on the basis of \( \text{Ca}^{2+} \) currents: L-, P/Q-, N-, R- and T-types (Catterall, 2000; Yamakage & Namiki, 2002). All but one class (T-type) of VGCCs is inhibited by specific blockers, and these blockers are frequently used to classify VGCCs of interest. VGCCs are composed of four or five subunits: \( \alpha_1, \alpha_2/\delta, \beta \) and \( \gamma \); \( \alpha_1 \) is the most prominent subunit, and is responsible for \( \text{Ca}^{2+} \) permeation, \( \text{Ca}^{2+} \) selectivity and voltage sensing (Catterall, 2000). The structure of the \( \alpha_1 \) subunit consists of four homologous domains (termed I–IV), and each domain contains six transmembrane segments (termed S1–S6), and a membrane-associated loop (termed the pore loop or P-loop) between S5 and S6 (see Fig. 1). The P-loop has a motif, T-X-E/D-X-W, responsible for \( \text{Ca}^{2+} \) selectivity, and the S5–P–S6 region forms the pore domain. The S4 segment contains several positively charged amino acid residues, each of which is followed by two hydrophobic residues; and the segment acts as a voltage sensor.

The yeast Saccharomyces cerevisiae has one orthologue, termed Cch1, of the pore-forming \( \alpha_1 \) subunit of mammalian VGCCs (Fischer et al., 1997; Paidhungat & Garrett, 1997). Although the organization of domains and transmembrane segments of Cch1 is similar to those of \( \alpha_1 \) subunits, overall amino acid sequence identity between Cch1 and \( \alpha_1 \) subunits is low: e.g. 24 % for Cch1 versus an L-type mammalian VGCC (Paidhungat & Garrett, 1997). It is noteworthy that one of the four S4 segments of Cch1 lacks most of the positively charged residues crucial for voltage sensing. In addition, each of the remaining three S4 segments of Cch1 has four positively charged residues, while most mammalian S4 segments have five positively charged residues. Therefore, from a structural viewpoint, it is uncertain whether Cch1 functions in response to changes in membrane potential. Cch1 constitutes a high-affinity
diluting Ca2+ uptake in the cch1Δ and mid1Δ single mutants and the cch1Δ mid1Δ double mutant, indicating that the diltiazem-induced increase in Ca2+ uptake is completely dependent on Cch1–Mid1. These results suggest that Cch1 is pharmacologically similar to L-type VGCCs, but the interactions between Cch1 and the L-type VGCC blockers are more complicated than expected.

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Calcium (Ca++) influx system with another protein, Mid1 (Muller et al., 2001). Mid1 does not have any structural homologues in higher eukaryotes, but has a stretch-activated Ca2+ selectivity in animal VGCC α1 subunits. Cch1 is composed of 2039 aa residues. Fig. 1. Predicted membrane topology of Cch1, a homologue of animal VGCC α1 subunits. I–IV, putative domains; 1–6, putative transmembrane segments (S1–S6); P, putative pore loop located between the S5 and S6 segments. In the P-loop, there is a sequence homologous to the consensus T-X-E/D-X-W motif, in which E or D participates in Ca2+ selectivity in animal VGCC α1 subunits. The P-loops of domains II, III and IV of Cch1 contain E, but the P-loop of domain I has N instead of E or D. + (located in the S4 segment) represents a positively charged amino acid residue R or K that serves for voltage sensing in animal VGCC α1 subunits. Cch1 is composed of 2039 aa residues.

METHODS

Yeast strains and plasmids. Yeast strains and plasmids used in this study are listed in Table 1.

Media. Low-Ca2+ medium SD.Ca100 containing 100 μM CaCl2 as the sole Ca2+ source, and SD-Ca medium containing no CaCl2, were prepared as described previously (Iida et al., 1990).

Blockers and agonist. Stock solutions of the following compounds were prepared as follows: 50 mM nifedipine (in DMSO; Sigma-Aldrich Japan, cat. no. N7634), 100 mM verapamil hydrochloride (in H2O; Wako Jyounyaku, 222-00781), 100 mM (+)-cis-diltiazem hydrochloride (in DMSO; Sigma-Aldrich Japan, D2521), 25 mM (S)-(−)-Bay K8644 (in DMSO; Sigma-Aldrich Japan, B133), 50 mM amiloride hydrochloride hydrate (in DMSO; Sigma-Aldrich Japan; A7410), 10 mM ω-agatoxin IVA (in H2O; Sigma-Aldrich Japan, A6719), 100 mM ω-conotoxin GIVA (in H2O; Sigma-Aldrich Japan, C9915), 20 μM SNX-482 (in H2O; Sigma-Aldrich Japan, S1818), 10 mM tetrodotoxin (TTX; in 10 mM phosphate buffer, pH 7.0; Wako Jyounyaku, 207-15901), and 10 M tetraethylammonium (TEA) chloride (in H2O; Sigma-Aldrich Japan, T2265).

Ca2+-accumulation assay. Exponentially growing cultures (approx. 2 × 10^6 cells ml⁻¹) of yeast strains were incubated for 2 h at 30 °C with 45CaCl2 (PerkinElmer Japan; 185 kBq ml⁻¹; 1.81 kBq nmol⁻¹) with or without an appropriate blocker or agonist, and an aliquot (100 μl; duplicate) was taken, filtered on a Millipore filter (type HA, 0.45 μm) presoaked in 5 mM CaCl2, and washed five times with 5 ml 5 mM CaCl2. Radioactivity retained on the filter was counted with the scintillation cocktail ReadyProtein (Beckman Coulter K.K.) in a liquid scintillation counter (Beckman Coulter K.K.).

Determination of viability. The method described by Iida et al. (1990) was followed. Aliquots (100 μl) of cultures (approx. 2 × 10^6 cells ml⁻¹; at 30 °C) were taken and mixed with an equal volume of a solution containing 0.01% (w/v) methylene blue and 2% (w/v) sodium citrate. The mixture was sonicated briefly with a Sonifier (Branson model W-200P) to dissociate cell clumps. The number of methylene-blue-negative (viable) and -positive (non-viable) cells was determined by using a differential interference contrast microscope (Olympus model BX50). Viability was expressed as the percentage of the methylene-blue-negative cells in the total number of cells.

Statistics. Data are given as means ± SD. Statistical comparisons were made with Student’s t-test.

RESULTS

Kinetic characterization of Cch1–Mid1
Molecular cloning of the CCH1 gene has been unsuccessful for more than a decade since the discovery of the gene in

Table 1. Yeast strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H207</td>
<td>MATa his3-A1 leu2-3, 112 trp1-289 ura3-52 sst1-2</td>
<td>Iida et al. (1994)</td>
</tr>
<tr>
<td>H315</td>
<td>cch1:: TRP1 mid1::Δ5:: HIS3 in H207</td>
<td>Iida et al. (2004)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBCT-CCH1H</td>
<td>Ap R ARS1 CEN4 LEU2 TDH3p*::CCH1H</td>
<td>Iida et al. (2007)</td>
</tr>
<tr>
<td>pBC111</td>
<td>Ap R ARS1 CEN4 LEU2</td>
<td>Iida et al. (2007)</td>
</tr>
<tr>
<td>YCpT-MID1</td>
<td>Ap R ARS1 CEN4 URA3 TDH3p*::MID1</td>
<td>This study</td>
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*TDH3p, promoter of the glyceraldehyde-3-phosphate dehydrogenase 3 gene.
pBCT-CCH1H ensures more than a 50-fold overproduction of Cch1 and Mid1 (designated CCH1 and MID1, respectively). The resulting co-transformant which overexpresses both Cch1 and Mid1 (designated CCH1–MID1) was used to examine the properties of the putative Cch1–Mid1 Ca\(^{2+}\)-channel function, and thus can be used to compare the channel function of different strains, was approximately 12 times greater in CCH1ox MID1ox than in CCH1–MID1, and 290 times greater in CCH1ox MID1ox than in cch1Δ mid1Δ. Therefore, it was concluded that the contribution of putative Ca\(^{2+}\) channels and/or transporters other than Cch1–Mid1 was negligible in CCH1ox MID1ox cells under the conditions tested.

**Table 2.** \(K_m\) and \(V_{max}\) of various strains with altered copy numbers of CCH1 and MID1

The results presented here are mean (±SD) values based on the Hanes–Woolf plots (Segel, 1976) of the data in Fig. 2.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>(K_m) (µM)</th>
<th>(V_{max}) [pmol (10^6 cells)]^{-1} h^{-1}</th>
<th>(V_{max}/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCH1ox MID1ox</td>
<td>12 ± 6</td>
<td>49 ± 3</td>
<td>4.1</td>
</tr>
<tr>
<td>CCH1–MID1</td>
<td>31 ± 5</td>
<td>11 ± 0</td>
<td>0.35</td>
</tr>
<tr>
<td>cch1Δ mid1Δ</td>
<td>290 ± 53</td>
<td>4 ± 1</td>
<td>0.014</td>
</tr>
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</table>

Ca\(^{2+}\)-accumulation rate (\(V_{max}\)) of the putative Cch1–Mid1 channel. To do this, the cells were grown in SD.Ca100, which discloses the Ca\(^{2+}\)-uptake activity of the putative Cch1–Mid1 channel (Muller et al., 2001). Ca\(^{2+}\)-accumulation assays were conducted at 30 °C for 2 h, which was within the linear range for the cells assayed. The concentration of 45CaCl\(_2\) was varied within single experiments, and Ca\(^{2+}\)-accumulation in the cells was measured. As shown in Fig. 2, CCH1ox MID1ox, CCH1–MID1 (wild-type) and cch1Δ mid1Δ cells (lacking both Cch1 and Mid1) displayed Michaelis–Menten kinetics. Table 2 shows the apparent \(K_m\) and \(V_{max}\) values deduced from the Hanes–Woolf plots for each strain. The \(K_m\) value of CCH1ox MID1ox (12 µM) was approximately 2.6-fold lower than that of CCH1–MID1 (31 µM), and 24-fold lower than that of cch1Δ mid1Δ (290 µM). The \(V_{max}/K_m\) ratio, which measures the efficiency of Ca\(^{2+}\)-channel function, and thus can be used to compare the channel function of different strains, was approximately 10 times greater in CCH1ox MID1ox than in CCH1–MID1, and 290 times greater in CCH1ox MID1ox than in cch1Δ mid1Δ. Therefore, it was concluded that the contribution of putative Ca\(^{2+}\) channels and/or transporters other than Cch1–Mid1 was negligible in CCH1ox MID1ox cells under the conditions tested.

**Sensitivity of Cch1–Mid1 to ion-channel blockers**

To address the question of whether Cch1 is a functional homologue of mammalian VGCCs, we employed a pharmacological approach using channel blockers typical of L-, P/Q-, N-, R- or T-types of VGCCs. Since the yeast cell wall is known to provide a barrier that inhibits reagents accessing the plasma membrane (Schindler & Davies, 1975; Gorenstein et al., 1978), the channel blockers were applied in concentrations that were almost at the upper limit of their solubility in SD.Ca100 medium. CCH1ox MID1ox cells were grown overnight to exponential growth phase in SD.Ca100 medium, and further incubated for 2 h with one of the Ca\(^{2+}\)-channel blockers and 45CaCl\(_2\), and then Ca\(^{2+}\)-accumulation was measured. As shown in Fig. 3, among the Ca\(^{2+}\)-channel blockers tested, 200 µM nifedipine (L-type) and 1 mM verapamil (L-type) blocked Ca\(^{2+}\)-accumulation to levels of 55% and 71%, respectively, while α-agatoxin IVA (P/Q-type), α-conotoxin GIVA (N-type), SNX-482 (R-type) and amiloride (T-type) did not...
affect Ca$^{2+}$ accumulation, even at concentrations near their solubility limit in SD.Ca100 medium. Those blockers did not affect cell viability during the course of the experiments at any concentration used (data not shown).

Based on the data presented in Fig. 3, the $K_i$ values of nifedipine and verapamil on Cch1 were estimated from Dixon plots (Segel, 1976) to be 248 and 1750 μM, respectively (Table 3). These values were much higher than those for nifedipine and verapamil on mammalian VGCC $\alpha_1$ subunits (Table 3).

Surprisingly, the third L-type blocker diltiazem enhanced Ca$^{2+}$ accumulation, depending upon its concentration tested, although measurement errors were large (Fig. 4a). At the highest concentration tested (12 mM), diltiazem enhanced Ca$^{2+}$ accumulation 1.8-fold. At the same concentration, this compound resulted in a decrease in cell viability to 68% (Fig. 4b). Microscopic observation indicated that diltiazem-treated cells were morphologically indistinguishable from untreated cells, suggesting that the increase in Ca$^{2+}$ accumulation was not due to cell deterioration.

To examine whether the effects of diltiazem observed were mediated through Cch1–Mid1, Ca$^{2+}$ accumulation and viability were measured for this compound in cch1 $^D$mid1 $^D$, CCH1 $^ox$mid1 $^D$ and cch1 $^D$MID1 $^ox$ strains. The results showed that Ca$^{2+}$ accumulation was not increased by diltiazem in the three strains (Table 3). Viability assays indicated that this compound significantly decreased the viability of cch1 $^D$mid1 $^D$ cells to 82% at 12 mM (Fig. 4b). This observation suggests that the decrease in the viability of CCH1 $^ox$MID1 $^ox$ cells cannot be ascribed solely to the increase in Ca$^{2+}$ accumulation which potentially disturbs Ca$^{2+}$ homeostasis.

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The VGCC agonist (S)-(−)-Bay K8644 (5–100 μM) did not affect Ca$^{2+}$ accumulation (data not shown). Since the secondary structure of VGCC $\alpha_1$ subunits and Cch1 is similar to that of Na$^+$ channels and six-transmembrane K$^+$ channels (Catterall, 1995), we examined the effect of the Na$^+$-channel blocker TTX, and the K$^+$-channel blocker TEA, on Ca$^{2+}$ accumulation, and found that the blockers had no effect on Ca$^{2+}$ accumulation (10–10 000 nM TTX; 1–100 mM TEA) (data not shown).

Table 3. $K_i$ values for nifedipine and verapamil on Cch1

<table>
<thead>
<tr>
<th></th>
<th>Nifedipine (μM)</th>
<th>Verapamil (μM)</th>
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<tr>
<td>Cch1</td>
<td>248</td>
<td>1750</td>
</tr>
<tr>
<td>Mammalian $\alpha_1$ subunit</td>
<td>0.0156*</td>
<td>0.90†</td>
</tr>
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</table>

*Rat cardiac L-type VGCC (Morel et al., 1998).
†Guinea pig ileal myocyte L-type VGCC (Hashimoto et al., 2006).
DISCUSSION

The results in the present study, especially those for the effect of nifedipine and verapamil, suggest that Cch1 is pharmacologically similar to L-type VGCCs. However, this suggestion is not clear-cut because another L-type blocker, diltiazem, enhanced Ca\(^{2+}\) accumulation unexpectedly (Fig. 4a). A possible explanation for this unexpected observation is that diltiazem may specifically bind to Cch1 to activate Ca\(^{2+}\) entry, instead of blocking it. The second possibility is that diltiazem, at high concentrations, may be a stress for yeast cells, and that it indirectly stimulates the Ca\(^{2+}\)-channel activity of Cch1, because, at 12 mM, this compound kills about 32% of CCH1\(^{ox}\) MID1\(^{ox}\) cells and 18% of cch1\(^{Δ}\) mid1\(^{Δ}\) cells (Fig. 4b). Several stresses, including endoplasmic reticulum stress caused by tunicamycin (an inhibitor of N-glycosylation) or dithiothreitol (an inhibitor of disulfide bond formation), hyperosmotic stress and alkaline stress, have been reported to activate the Cch1–Mid1 channel (Bonilla et al., 2002; Matsumoto et al., 2002; Viladevall et al., 2004). The third possibility is that diltiazem at high concentrations (7.5–12 mM) causes nonspecific fluxes of ions without activating Cch1. It has been reported that diltiazem increases the cation and anion permeability of biological membranes, such as bovine photoreceptor membranes, at high concentrations (12.5–200 μM; Careta et al., 1991). However, as mentioned above, our study showed that diltiazem, even at 12 mM, did not increase Ca\(^{2+}\) accumulation in cch1\(^{Δ}\) mid1\(^{Δ}\) cells (Fig. 4a), indicating that the diltiazem-induced increase in Ca\(^{2+}\) accumulation depends on the Cch1–Mid1 channel. Therefore, the third possibility seems unlikely.

It should be noted that the half-blocking concentrations of organic Ca\(^{2+}\)-channel blockers, including nifedipine and verapamil, are in the 20 nM to 50 μM range, depending upon the type of vertebrate cells (Triggle, 1990; Hille, 1992; Catterall et al., 2005). These concentrations are much lower than those used for S. cerevisiae cells in this study. One reason for the necessity of high concentrations of the blockers could be the presence of the cell wall in S. cerevisiae cells. In the case of actinomycin D, which binds to DNA to inhibit RNA synthesis, it has been reported that spheroplasts lacking the cell wall are more sensitive to this antibiotic than intact cells of S. cerevisiae (Gorenstein et al., 1978). Since lytic enzymes that degrade the cell wall to generate spheroplasts contain proteases as well as endogluconases (Scott & Schekman, 1980), proteins protruding from the plasma membrane, including Ca\(^{2+}\) channels, would be damaged during spheroplast preparation. Therefore, careful examination is necessary to assess Ca\(^{2+}\)-uptake activity in spheroplasts.

Another reason for the need for high concentrations of nifedipine and verapamil (Fig. 3) could be a difference in the binding sites of these L-type blockers, in addition to the
presence of the cell wall. Although the three L-type blockers used in this study are classified into the same type, their binding sites are not completely identical (Mitterdorfer et al., 1998). Nifedipine, verapamil and diltiazem are members of the chemically unrelated classes of L-type blockers dihydropyridines (DHPs), phenylalkylamines (PAA) and benzothiazepines (BTZ), respectively. As shown in Fig. 5, the three classes bind to two or three of the transmembrane segments IIS5, IIS6 and IVS6 in mammalian L-type VGCCs, and the binding of each class is unique in each segment. Alignment of the corresponding transmembrane segments of Cch1 with a mammalian VGCC shows that the binding sites of Cch1 for the three classes are limited: there is no identical amino acid residue in IIS5; there is one for DHPs, and two for PAA in IIS6; and one for DHPs in IVS6 (Fig. 5). This limitation may account for the low affinities of nifedipine and verapamil to Cch1.

A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) with the amino acid sequence of the S. cerevisiae Cch1 found homologous proteins for over 30 fungal species, including pathogenic fungi. Modulation of fungal Cch1 function has been suggested to be useful in the development of cures for fungal diseases in humans caused by Candida albicans (Bonilla et al., 2002), Candida glabrata (Kaur et al., 2004), Cryptococcus neoformans (Liu et al., 2006) and Trichophyton rubrum (Yu et al., 2007), and for those in plants caused by Magnaporthe grisea (Zelter et al., 2004). This suggestion should facilitate further study on Cch1 and related Ca$^{2+}$-signalling mechanisms, and expand it to other pathogenic fungi. Our study with diltiazem indicates that when assessing the effect of a blocker of interest on any fungal species, one should always measure Ca$^{2+}$ accumulation or influx in terms of Ca$^{2+}$ signalling and curing the fungal disease.

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