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

Jinfeng Teng,^{1,2†} Rika Goto,^{1†} Kazuko Iida,³ Itaru Kojima² and Hidetoshi Iida^{1,4}

Correspondence

Hidetoshi Iida

iida@u-gakugei.ac.jp

¹Department of Biology, Tokyo Gakugei University, 4-1-1 Nukui kita-machi, Koganei-shi, Tokyo 184-8501, Japan

²Laboratory of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Gunma 371-8510, Japan

³Biomembrane Signaling Project 2, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan

⁴Department of Bioenvironmental Science, Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Higashiyama 5-1, Myodaiji, Okazaki, Aichi 444-8787, Japan

The Cch1 protein of the yeast *Saccharomyces cerevisiae* is a homologue of the pore-forming α_1 subunit of mammalian voltage-gated Ca^{2+} channels (VGCCs), and it constitutes a high-affinity Ca^{2+} -influx system with the Mid1 protein in this organism. Here, we characterized the kinetic property of a putative Cch1–Mid1 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 Ca^{2+} uptake in the *cch1* Δ and *mid1* Δ single mutants and the *cch1* Δ *mid1* Δ double mutant, indicating that the diltiazem-induced increase in 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.

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INTRODUCTION

Mammalian voltage-gated Ca^{2+} channels (VGCCs) are classified electrophysiologically into five classes on the basis of 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: α_1 , α_2/δ , β and γ ; α_1 is the most prominent subunit, and is responsible for Ca^{2+} permeation, Ca^{2+} selectivity and voltage sensing (Catterall, 2000). The structure of the α_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 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 α_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 α_1 subunits, overall amino acid sequence identity between Cch1 and α_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

†These authors contributed equally to this work.

Abbreviations: BTZ, benzothiazepine; DHP, dihydropyridine; PAA, phenylalkylamine; TEA, tetraethylammonium; TTX, tetrodotoxin; VGCC, voltage-gated Ca^{2+} channel.

Fig. 1. Predicted membrane topology of Cch1, a homologue of animal VGCC α_1 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 Ca^{2+} selectivity in animal VGCC α_1 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 α_1 subunits. Cch1 is composed of 2039 aa residues.

Ca^{2+} -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 Ca^{2+} -channel activity when expressed in mammalian cultured cells (Kanzaki *et al.*, 1999). Mutant cells lacking either *CCH1* or *MID1*, or both, show the same phenotypes: low Ca^{2+} -uptake activity, and mating-pheromone-induced death (Iida *et al.*, 1994; Fischer *et al.*, 1997; Paidhungat & Garrett, 1997; Muller *et al.*, 2001; Iida *et al.*, 2004). The molecular nature of a high-affinity Ca^{2+} -influx system composed of Cch1 and Mid1 is unknown. In this paper, we characterize the kinetic property of a putative Cch1–Mid1 Ca^{2+} channel, and present experimental evidence to suggest that yeast Cch1 is pharmacologically similar to L-type VGCCs.

METHODS

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

Media. Low- Ca^{2+} medium SD.Ca100 containing 100 μM CaCl_2 as the sole Ca^{2+} source, and SD-Ca medium containing no CaCl_2 , 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 H_2O ; Wako Jyunyaku, 222-00781), 100 mM (+)-*cis*-diltiazem hydrochloride (in SD.Ca100 medium; 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 μM ω -agatoxin IVA (in H_2O ; Sigma-Aldrich Japan, A6719), 100 μM ω -conotoxin GIVA (in H_2O ;

Table 1. Yeast strains and plasmids used in this study

Strain or plasmid	Genotype	Source
Strains		
H207	<i>MATa his3-Δ1 leu2-3, 112 trp1-289 ura3-52 sst1-2</i>	Iida <i>et al.</i> (1994)
H315	<i>cch1Δ::TRP1 mid1-Δ5::HIS3</i> in H207	Iida <i>et al.</i> (2004)
Plasmids		
pBCT-CCH1H	<i>Ap^R ARS1 CEN4 LEU2 TDH3p*-CCH1H</i>	Iida <i>et al.</i> (2007)
pBC111	<i>Ap^R ARS1 CEN4 LEU2</i>	Iida <i>et al.</i> (2007)
YCpT-MID1	<i>Ap^R ARS1 CEN4 URA3 TDH3p*-MID1</i>	This study
YCplac33	<i>Ap^R ARS1 CEN4 URA3</i>	Gietz & Sugino (1988)

**TDH3p*, promoter of the glyceraldehyde-3-phosphate dehydrogenase 3 gene.

Sigma-Aldrich Japan, C9915), 20 μM SNX-482 (in H_2O ; Sigma-Aldrich Japan, S1818), 10 mM tetrodotoxin (TTX; in 10 mM phosphate buffer, pH 7.0; Wako Jyunyaku, 207-15901), and 10 M tetraethylammonium (TEA) chloride (in H_2O ; Sigma-Aldrich Japan, T2265).

Ca^{2+} -accumulation assay. Exponentially growing cultures (approx. 2×10^6 cells ml^{-1}) of yeast strains were incubated for 2 h at 30 °C with $^{45}\text{CaCl}_2$ (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 CaCl_2 , and washed five times with 5 ml 5 mM CaCl_2 . 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 $\pm$ 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

Fig. 2. Michaelis–Menten plot of Ca²⁺-accumulation rate versus CaCl₂ concentration. Exponentially growing cells cultured in SD.Ca100 medium were harvested, washed twice with SD-Ca medium, and resuspended in SD-Ca medium containing varying concentrations (4.85, 9.7, 48.5, 97, 194 and 291 μM) of ⁴⁵CaCl₂ (1.81 kBq nmol⁻¹). The cell suspension was incubated at 30 °C, with shaking. The Ca²⁺-accumulation rate was determined for the initial 2 h after the start of the incubation, as described in Methods. The data were fitted by nonlinear regression of Michaelis–Menten curves. The mean of three independent experiments (±SD) is shown for each point. ■, *CCH1ox MID1ox* cells (strain H207 bearing pBCT-CCH1H and YCpT-MID1); ◆, *CCH1-MID1* cells (strain H207 bearing empty vectors pBC111 and YCplac33); ▲, *cch1Δ mid1Δ* cells (strain H315 bearing empty vectors pBC111 and YCplac33).

the *S. cerevisiae* genome (Paidhungat & Garrett, 1997), but we have recently succeeded in cloning it, using a plasmid whose copy number is kept low in *Escherichia coli* (Iida *et al.*, 2007). In the present study, we used the cloned gene to examine the *in vivo* properties of Cch1 produced from a strong promoter, *TDH3p*, on the plasmid pBC111 (Table 1). Western blotting has shown that the resulting plasmid pBCT-CCH1H ensures more than a 50-fold overproduction of Cch1 (Iida *et al.*, 2007). Since Cch1 requires Mid1 to function as a Ca²⁺-influx system (Fischer *et al.*, 1997; Paidhungat & Garrett, 1997; Iida *et al.*, 2004; Muller *et al.*, 2001), Mid1 was also designed to be produced from the same promoter on a different plasmid, YCplac33 (Table 1). Again, the plasmid thus constructed, YCpT-MID1, ensured more than 50-fold overproduction of Mid1 (data not shown). The resulting co-transformant which overexpresses both Cch1 and Mid1 (designated *CCH1ox MID1ox*) was used to assess the properties of the putative Cch1–Mid1 Ca²⁺ channel. This co-overexpression system has the advantage of reducing the perturbation effect caused by unidentified Ca²⁺-influx systems of *S. cerevisiae* cells on Ca²⁺ permeation through Cch1–Mid1.

Using cells of *CCH1ox MID1ox* and the control strains, we first estimated the affinity for Ca²⁺ (K_m), and the maximum

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–Woelf plots (Segel, 1976) of the data in Fig. 2.

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

Ca²⁺-accumulation rate (V_{max}) of the putative Cch1–Mid1 channel. To do this, the cells were grown in SD.Ca100, which discloses the Ca²⁺-uptake activity of the putative Cch1–Mid1 channel (Muller *et al.*, 2001). Ca²⁺-accumulation assays were conducted at 30 °C for 2 h, which was within the linear range for the cells assayed. The concentration of ⁴⁵CaCl₂ was varied within single experiments, and Ca²⁺ 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–Woelf 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 Ca²⁺-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 Ca²⁺ 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 Ca²⁺-channel blockers and ⁴⁵CaCl₂, and then Ca²⁺ accumulation was measured. As shown in Fig. 3, among the Ca²⁺-channel blockers tested, 200 μM nifedipine (L-type) and 1 mM verapamil (L-type) blocked Ca²⁺ accumulation to levels of 55 % and 71 %, respectively, while ω-agatoxin IVA (P/Q-type), ω-conotoxin GIVA (N-type), SNX-482 (R-type) and amiloride (T-type) did not

Fig. 3. Effect of VGCC blockers on Cch1 activity. Exponentially growing *CCH1ox MID1ox* cells grown in SD.Ca100 medium at 30 °C were incubated for 2 h with 100 µM ⁴⁵CaCl₂ (185 kBq ml⁻¹; 1.81 kBq nmol⁻¹) and an appropriate blocker, and Ca²⁺ accumulation was determined as described in Methods. The mean of three independent experiments (± SD) is shown for each point. L, P/Q, N, R and T represent the types of VGCC based upon Ca²⁺ current. *P* versus control cells: **P*=0.00369, ***P*=0.0103, ****P*=0.00008, *****P*=0.0036, ******P*=0.0010.

affect Ca²⁺ 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 α₁ subunits (Table 3).

Surprisingly, the third L-type blocker diltiazem enhanced Ca²⁺ accumulation, depending upon its concentration tested, although measurement errors were large (Fig. 4a). At the highest concentration tested (12 mM), diltiazem enhanced Ca²⁺ 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 Ca²⁺ accumulation was not due to cell deterioration.

To examine whether the effects of diltiazem observed were mediated through Cch1–Mid1, Ca²⁺ accumulation and viability were measured for this compound in *cch1Δ mid1Δ*, *CCH1ox mid1Δ* and *cch1Δ MID1ox* strains. The results showed that Ca²⁺ accumulation was not increased by diltiazem in the three strains (Fig. 4a), indicating that the diltiazem-induced increase in Ca²⁺ accumulation in *CCH1ox MID1ox* cells was mediated through Cch1–Mid1. Viability assays indicated that this compound significantly decreased the viability of *cch1Δ mid1Δ* cells to 82 % at 12 mM (Fig. 4b). This observation suggests that the decrease in the viability of *CCH1ox MID1ox* cells cannot be ascribed solely to the increase in Ca²⁺ accumulation which potentially disturbs Ca²⁺ homeostasis.

The VGCC agonist (S)-(–)-Bay K8644 (5–100 µM) did not affect Ca²⁺ accumulation (data not shown). Since the secondary structure of VGCC α₁ 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 Ca²⁺ accumulation, and found that the blockers had no effect on Ca²⁺ accumulation (10–10 000 nM TTX; 1–100 mM TEA) (data not shown).

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

The values were estimated based on the Dixon plot for non-competitive blockers (Segel, 1976). Examples of *K_i* values for the blockers on mammalian VGCC α₁ subunits are also shown.

	<i>K_i</i> (µM)	
	Nifedipine	Verapamil
Cch1	248	1750
Mammalian α ₁ subunit	0.0156*	0.90†

*Rat cardiac L-type VGCC (Morel *et al.*, 1998).

†Guinea pig ileal myocyte L-type VGCC (Hashimoto *et al.*, 2006).

Fig. 4. Effect of the L-type VGCC blocker diltiazem on Cch1 activity (a) and cell viability (b). The experimental conditions used here were the same as those described in the legend to Fig. 3. Cell viability was determined as described in Methods. The mean of three independent experiments ($\pm$ SD) is shown for each measurement. The *cch1Δ mid1Δ* strain was H315 bearing empty vectors pBC111 and YCplac33. The *CCH1ox mid1Δ* strain was H315 bearing pBCT-CCH1H and YCplac33. The *cch1Δ MID1ox* strain was H315 bearing pBC111 and YCpT-MID1. *P* versus control cells: **P*=0.005, ***P*=0.0478, ****P*=0.0005, *****P*=0.0118, ******P*=0.0068.

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 Ca²⁺ accumulation unexpectedly (Fig. 4a). A possible explanation for this unexpected observation is that diltiazem may specifically bind to Cch1 to activate Ca²⁺ 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 Ca²⁺-channel activity of Cch1, because, at 12 mM, this compound kills about 32% of *CCH1ox MID1ox* cells and 18% of *cch1Δ mid1Δ* 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 non-specific 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 μ M; Caretta *et al.*, 1991). However, as mentioned above, our study showed that diltiazem, even at 12 mM,

did not increase Ca²⁺ accumulation in *cch1Δ mid1Δ* cells (Fig. 4a), indicating that the diltiazem-induced increase in Ca²⁺ accumulation depends on the Cch1–Mid1 channel. Therefore, the third possibility seems unlikely.

It should be noted that the half-blocking concentrations of organic Ca²⁺-channel blockers, including nifedipine and verapamil, are in the 20 nM to 50 μ M 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 endo-glucanases (Scott & Schekman, 1980), proteins protruding from the plasma membrane, including Ca²⁺ channels, would be damaged during spheroplast preparation. Therefore, careful examination is necessary to assess Ca²⁺-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

Fig. 5. Amino acid sequences of the IIIS5, IIIS6 and IVS6 segments of a mammalian VGCC and Cch1. The upper sequences (written by Mitterdorfer *et al.*, 1998) are according to a rat brain Ca_v1.2 α_1 subunit (rbCII) (GenBank accession number M67515) and the lower sequences Cch1 (GenBank accession number Z73002). Italicized lower-case letters indicate DHP (*d*), PAA (*p*), and BTZ (*b*). Nifedipine is a member of the DHP group, verapamil is a member of the PAA group, and diltiazem is a member of the BTZ group. The position of these letters on the upper sequences shows the amino acid residues that contribute to blocker binding, as summarized for mammalian VGCCs (Mitterdorfer *et al.*, 1998). Asterisks indicate identical amino acids, and colons indicate conserved amino acid substitutions determined according to the CLUSTAL W program (Chenna *et al.*, 2003).

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; Striessnig, 1999). Nifedipine, verapamil and diltiazem are members of the chemically unrelated classes of L-type blockers dihydropyridines (DHPs), phenylalkylamines (PAAs) and benzothiazepines (BTZs), respectively. As shown in Fig. 5, the three classes bind to two or three of the transmembrane segments IIIS5, IIIS6 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 IIIS5; there is one for DHPs, and two for PAAs in IIIS6; 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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