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Expression of pI_{Cln} in *Escherichia coli* gives a strong tolerance to hypotonic stress

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Abstract We amplified the coding region DNA sequence from a rat renal pI_{Cln} cDNA by PCR and expressed the protein in *Escherichia coli* cells. The cells were exposed to hypotonic conditions followed by spreading them onto LB plates for subsequent colony survival assay. The present study demonstrated that the cells expressing pI_{Cln} exhibit a strong resistance to hypotonic stress. Moreover, the resistance was specifically inhibited by extracellular ATP and some anion channel inhibitors. These findings indicate that the expression of pI_{Cln} directly confers tolerance to hypotonic stress, and pI_{Cln} is concluded to be an important molecule for cell-volume regulation.

Key words: Putative volume (or osmo)-sensitive Cl^- channel; Channel regulator; pI_{Cln} protein; Expression; Hypotonic tolerance; *Escherichia coli*

1. Introduction

It is essential for every cell to maintain a constant cell volume [1,2]. Although the phenomenon of regulatory volume decrease (RVD), by which a swollen cell loses solute and water to restore its constant volume, has been observed for decades, the molecular mechanism responsible for the volume control is not well-known [3].

As one of the possible candidates for a volume-regulated anion channel, in 1992, Paulmichl et al. [4] reported the cloning of a cDNA that codes a 235-amino acids protein termed pI_{Cln} (I = current, Cl = chloride, n = nucleotide-sensitive) from MDCK cells. When overexpressed in *Xenopus* oocytes, pI_{Cln} gives rise to an outwardly rectifying chloride conductance displaying a feature consistent with the swelling-induced chloride current ($I_{Cl.swell}$) seen in several cells [5–8]. pI_{Cln} was initially proposed as a membrane-spanning Cl^- channel with an antiparallel β-barrel after several analyses. Shortly after the publication of these studies, in 1994, Krapivinsky et al. [9] demonstrated that abundantly cytoplasmic localization and soluble characteristics of this protein are unexpected for an ion channel; pI_{Cln} was therefore concluded to be a Cl^- channel regulator but a channel-forming protein itself. As another possibility, in 1996, Strange et al.[10] proposed an 'anchor ⇄ insertion channel' model for pI_{Cln}. Based on their speculations, cell osmotic swelling might release pI_{Cln} from its cytosolic binding site, allowing the protein to insert spontaneously into the cell membrane to form a channel. However, pI_{Cln} was recently described to be neither a channel itself by Buyse et al. [11] nor a regulator of a volume-regulated anion channel (VRAC) by Voets et al. [12]. At present, the dispute over channel vs. regulator surrounding pI_{Cln} is seemingly shifting to over whether it has anything at all to do with $I_{Cl.swell}$ which is critical to cell-volume regulation.

Much controversy over pI_{Cln} still remains unresolved, and the function of pI_{Cln} has yet to be discovered [13]. A close link between pI_{Cln} and $I_{Cl.swell}$ had been reported by experiments in which the $I_{Cl.swell}$ was inhibited after treatment of cells with antisense oligonucleotides or anti-pI_{Cln} monoclonal antibodies [14,9]. More recently, this link was also confirmed in the epithelium by Chen et al. [15]. Importantly, we previously observed that pI_{Cln} is predominantly present on the lumenal surface membranes of the distal tubules, where it faces the lowest osmolality in the nephrons; thus the function of pI_{Cln} was suggested to be the protection of the cells from osmo-swelling against intra-tubular hypotonic solution [16]. It is of interest to ask whether pI_{Cln} can confer hypotonic resistance. In the present study, we subcloned and expressed pI_{Cln} in *Escherichia coli* cells. Using the cells, hypotonic tolerances were investigated.

2. Materials and methods

2.1. Subcloning of pI_{Cln} gene into an expression vector

The gene for pI_{Cln} was amplified from rat renal pI_{Cln} cDNA (DDBJ/GenBank/EMBL databases, accession numberD13985) which was kindly given by Dr. Takaaki Abe [17]. The selected primers were 5′-CGCC**GGTACC**AATGAGCTTCCTCAAAAG-3′ with a *Kpn*I site (including start codon) and 5′-CGCC**TCTAGA**TCAGTGGTCAACGTCTGC-3′ with a *Xba*I site (including stop codon). The reaction was carried out with KOD DNA Polymerase Kit (Toyobo, Osaka, Japan) in a Gene Amp 2400-R PCR System (Perkin-Elmer, CA, USA) and performed according to the following program: an initial cycle of 94°C (30 s), 50°C (30 s), and 74°C (1 min) was followed by 25 cycles of 98°C (15 s), 65°C (2 s), and 74°C (30 s).

The PCR product was treated with phenol extraction, followed by digestion with restriction endonucleases *Kpn*I and *Xba*I (New England Biolabs). The corresponding digestion was applied to expression plasmid pThioHis (Invitrogen, CA, USA). Both fragments were run on an 1% agarose gel and isolated with the GeneClean II Kit (Bio 101, CA, USA). The pI_{Cln} gene fragment was then subcloned into pThioHis by T4 DNA ligase reaction (Boehringer-Mannheim) to obtain pI_{Cln}-expressing plasmid. After transformation in *E. coli* cells and plasmid preparation (Qiagen), the correct construction was confirmed by redigestion with *Kpn*I and *Xba*I.

2.2. Expression of tag-pI_{Cln} in E. coli

After transformation of the expression vector in *E. coli* TOP10 (Invitrogen, CA, USA), a single colony was inoculated into 10 ml LB medium (1% tryptone and 0.5% yeast extract, both from Gibco BRL; 1% NaCl, pH 7.0) containing ampicillin (100 mg/l). After incubation for 8 h at 37°C, the culture was transferred to 1 l of the same medium in a 3-l flask. Incubation was continued in a shaking incubator at 37°C until an optical density of 0.5 at 550 nm was reached. At this point, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After shaking for different times,

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cells were used for protein-expression checking with SDS-PAGE, for hypotonic tolerance study (2 h) or for pI_{Cln} purification (8 h).

As the control, the plasmid pThioHis (Invitrogen, CA, USA) was used to express an only tag peptide (thioredoxin) by the same way. The expression of the tag peptide was confirmed with immunoblot assay using a specific anti-thioredoxin antibody (Invitrogen, CA, USA).

2.3. Purification of tag-pI_{Cln} and N-terminal amino acid sequencing

All operations of the purification were carried out at 0–4°C. pI_{Cln}-expressing cells were harvested by centrifugation (Tomy SRX-201, 10 000×*g*, 15 min) after 8 h of IPTG induction, and were resuspended in 3 ml of 10 mM Tris-HCl buffer (0.1% Triton X-100, pH 8.0) per gram of cells (wet weight). Cell suspension was sonicated on ice with two or three 10 s bursts at 150 W, the lysate frozen by liquid nitrogen, and then quickly thawed at 37°C. The treatments were repeated three times. The soluble fraction was obtained by centrifugation (22 000×*g*, 20 min) of cell lysate. The soluble lysate was applied to an anion exchanger DE52 (Whatman, UK) column which had previously been equilibrated with 10 mM Tris-HCl buffer containing 0.5 mM EDTA, 0.1% Triton X-100, pH 8.0. After washing the column with the equilibrating buffer, proteins were eluted with a linear gradient of 0–0.5 M NaCl in the buffer. All fractions were analyzed by SDS-PAGE. The 50 kDa protein-enriched fractions were collected and dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.0. The sample was applied to a hydroxyapatite (Wako, Tokyo, Japan) column pre-equilibrated with the same buffer, and eluted with a linear gradient of 10–500 mM potassium phosphate buffer, pH 7.0. The 50 kDa protein fractions were collected, and digested with lysylendopeptidase (EC 3.4.21.50, Wako, Osaka, Japan). The resulting peptides were separate on a reverse-phase column of Wako-pak (Wakosil 5C18) connected with an HPLC apparatus (Pharmacia LKB, Sweden).

N-terminal sequence analysis of peptides was performed on an Applied Biosystem 491 Procise Sequencer (Perkin-Elmer, CA, USA) with standard methods.

2.4. Electrophoresis and immunoblot

Aliquots (20 μg proteins) of samples were subjected to SDS-PAGE by the method of Laemmli [18]. Coomassie brilliant blue R-250 (CBB) was used for the staining of the proteins. Western blot assay was performed by the method of Towbin et al. [19] with slight modifications [20]. Protein concentrations were determined by Lowry's method [21]. Anti-tag peptide (thioredoxin) antibody (Invitrogen, CA, USA; dilution: ×1000) and goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad, Richmond, CA, USA) (dilution: ×1000) were used. The substrate was 3,3-diaminobenzidine tetrahydrochloride.

2.5. Hypotonic tolerance assay

Two types of *E. coli* cells harboring the plasmid with or without pI_{Cln} genes were grown in LB medium as described above. After IPTG induction, incubations were continued at 37°C for 2 h. 100 μl of each culture was transferred to 100 ml distilled water (dH_2O) to reach a harsh hypotonic stress (0.17 mM of final NaCl concentration). Three samples from each hypotonic suspension were removed into 5 ml of 0.7% TOP agarose (10 g tryptone, 5 g NaCl, 7 g agarose per liter) at 15, 30, and 60 min, followed by spreading them onto LB plus ampicillin plates for colony survival assay.

Colony survival assay was performed according to previously described methods [22,23]. The spreading plates were incubated overnight at 37°C before counting the number of single colonies (cell survival number) on each plate. The colony-forming units (CFU) were calculated, and the hypotonic tolerance was reflected by each 'survival rate' (% of total cells) as shown: $R_{survival} = CFU_{hypo}/CFU_{non-hypo} \times 100\%$. The dH_2O containing 171 mM NaCl as the same as in normal LB medium was used for the control ($CFU_{non-hypo}$).

2.6. Effects of ATP and anion channel inhibitors on E. coli with the hypotonic tolerance

Aliquots (10 μl) of pI_{Cln}-expressing *E. coli* culture after 2 h of IPTG induction were added to 10 ml of distilled water pre-mixed with ATP or each inhibitor at different final concentrations. After 30 min, three aliquots of each cells suspension were mixed with 5 ml of 0.7% TOP agarose and spread onto LB plates followed by a colony survival assay as described above.

3. Results and discussion

3.1. Construction of pI_{Cln}-expressing vector

A double-strand pI_{Cln} gene encoding a 236 amino acid sequence was amplified from rat renal cDNA (see Fig. 1A) by

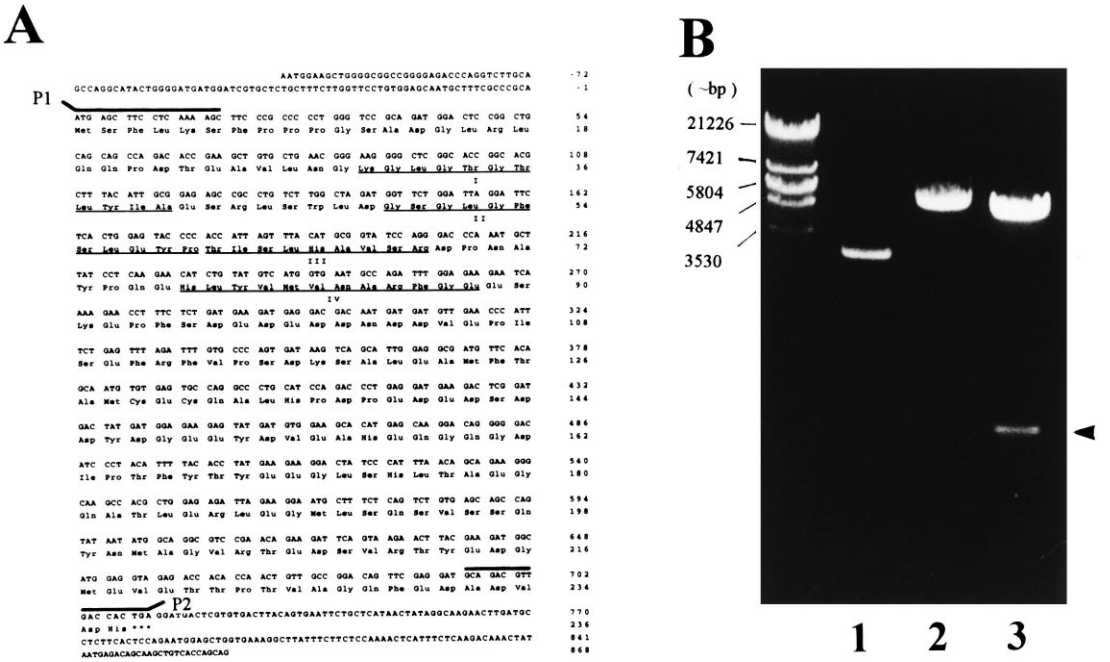

Fig. 1. Construction of pI_{Cln}-expressing plasmid. A: Alignment of rat renal pI_{Cln} cDNA (see [17]). The pI_{Cln} coding region was amplified by PCR using the two primers (p1 and p2). The underlines (I–IV) are putative trans-membrane domains. B: The correct construction was confirmed on agarose electrophoresis. Amplified pI_{Cln} gene was inserted into an expression plasmid. The constructed circular plasmid (lane 1) was linearized with *Kpn*I (lane 2), or with *Kpn*I and *Xba*I (lane 3). The arrow indicates inserted pI_{Cln} genes.

PCR. The PCR product was a single band of 732 bp. After digestion with two restriction enzymes, the pI_{Cln} gene fragment was constructed with sticky ends (5′-end: *Kpn*I; 3′-end: *Xba*I).

Plasmid pThioHis (4.4 kb) carries a 357 nucleotide fragment encoding a tag-peptide (thioredoxin) followed by a polylinker. The pI_{Cln} was inserted between the *Kpn*I and *Xba*I sites. The correct insertion of the pI_{Cln} gene was confirmed by a re-cut of the constructed plasmid with the restriction enzymes. As shown in Fig. 1B, the constructed circular pI_{Cln}-expressing plasmid (lane 1) was linearized by *Kpn*I (lane 2) or was digested into an insert and a plasmid fragment bands (lane 3) by *Kpn*I plus *Xba*I.

3.2. Expression of pI_{Cln} in E. coli cells

After transformation of the above-mentioned plasmid with or without the pI_{Cln} gene in the *E. coli* cells, as shown in Fig. 2A CBB, in comparison with wild-type cells (lane 1), a 15.6 kDa protein was expressed in the control cells which harbor only the plasmid (lane 2), and a 50 kDa protein was expressed in the cells harboring a plasmid with the pI_{Cln} gene (lane 3). On an immunoblot (Fig. 2A Blot), the protein band (lane 2) migrating at 15.6 kDa was specifically immunostained with an antibody against the tag-peptide (thioredoxin). This confirmed

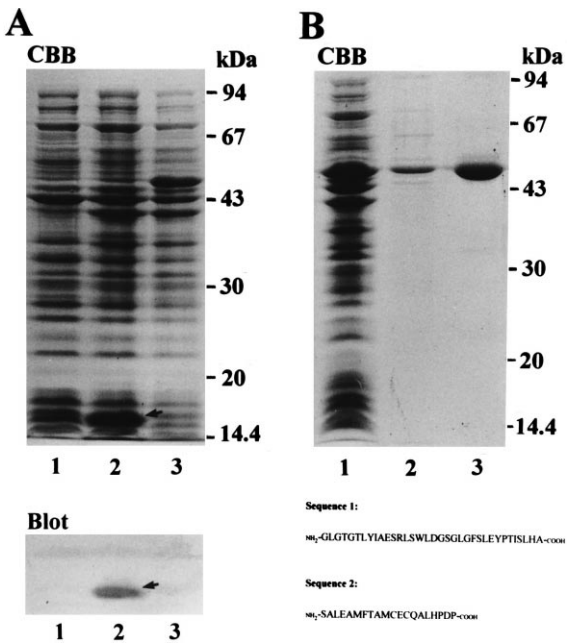

Fig. 2. Expression of pI_{Cln} and confirmation with amino acid sequencing. A: 50 kDa fusion protein and tag peptide were expressed in *E. coli* cells. The cells transformed with pI_{Cln}-expressing plasmid or with the plasmid only were induced to express the proteins by adding IPTG to the cultures. Samples were taken after 2 h of IPTG induction, and subjected to 12.5% SDS-PAGE followed by staining with CBB (CBB), or immunoblotting using a specific anti-tag peptide antibody (Blot). Samples were lysates from *E. coli* cells of wild-type (lane 1), only plasmid (lane 2), and plasmid with pI_{Cln} gene (lane 3). The arrows are indicating tag-peptide (15.6 kDa). B: Purification and amino acid sequencing. The 50 kDa protein was purified from the pI_{Cln}-expressing cell lysate by two column chromatograpies. SDS-PAGE of the purification was stained with CBB. Samples were from cell lysate (lane 1), DE52 column (lane 2), hydroxyapatite column (lane 3). The purified tag-pI_{Cln} (50 kDa) was digested into peptide fragments followed by amino acid sequencing. The analyzed two sequences are 100% consistence with amino acids 31–65 and 119–138 of the pI_{Cln} sequence (see Fig. 1).

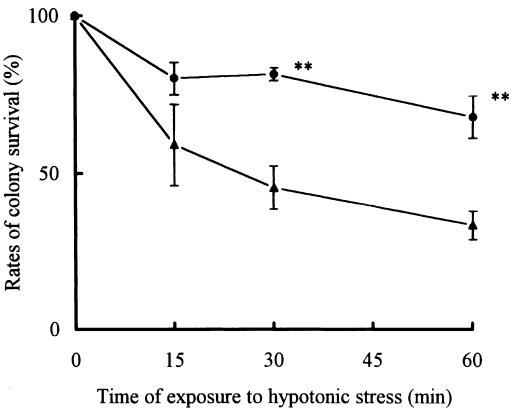

Fig. 3. The pI_{Cln}-expressing *E. coli* cells exhibit a strong hypotonic tolerance. pI_{Cln}-expressing cells and the control cells were removed into hypotonic solution after 2 h IPTG induction. At 15, 30, and 60 min, three aliquots of each cell suspension were spread onto LB plates, and incubated overnight at 37°C followed by colony survival assay. The colony survival rates reflecting their hypo-tolerance were compared between the tag-pI_{Cln} expressing cells (●) and tag only expressing cells (▲) ($n=3$; $^{**}P<0.01$).

the expression of tag-peptide (15.6 kDa band) in control *E. coli* cells.

To further verify whether pI_{Cln} is contained in the 50 kDa fusion protein, we substantially purified the 50 kDa fusion protein from the cell lysate by two column chromatographies (Fig. 2B). The purified 50 kDa protein was digested into peptide fragments with lysylendopeptidase, followed by N-terminal amino acid sequence analysis. As shown in Fig. 2B (lower pattern), the two analyzed partial sequences are 100% consistent with amino acids 31–65 and 119–138 of the pI_{Cln} sequence (see Fig. 1A).

3.3. Hypotonic tolerance observed in the pI_{Cln}-expressing E. coli cells

Expression of exogenous proteins in *E. coli* cells to explore its function is a widely used method [22–24], and no pI_{Cln} homologue is found in *E. coli* genomic DNA by a homology search. On exposure of *E. coli* cells to hypotonic conditions, some cells will be osmotically killed by cell lysis, and the number of remaining cells can be checked by a colony survival assay.

The survival rates (% of total cells) under hypotonic stress were compared at different times between pI_{Cln}-expressing *E. coli* and its control cells in the present study. Surprisingly, as shown in Fig. 3, the survival rates of pI_{Cln}-expressing cells were significantly higher than that of control cells at the time points 30 min and 60 min. In addition, after hypotonic stress, obvious cell lysis was observed on control cell glass slides in comparison with that of pI_{Cln}-expressing cells by optical microscopy (data not shown). The expression patterns of cellular proteins are not apparently different between pI_{Cln}-expressing cells and control cells (see Fig. 2A), with the exception of the 15.6 kDa tag and 50 kDa tag-pI_{Cln} fusion proteins. These results indicate that the expression of pI_{Cln} in the cells directly contributed to the strong tolerance to hypotonic stress.

3.4. Specific inhibition of the hypotonic tolerance by extracellular ATP

pI_{Cln} was initially cloned as a nucleotide-sensitive Cl^- chan-

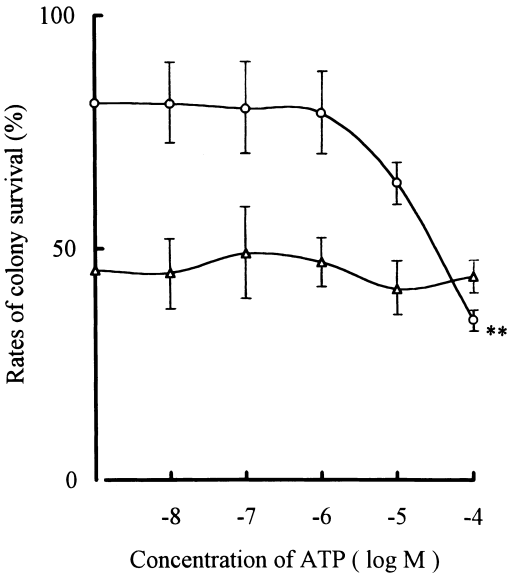

Fig. 4. Specific inhibition of the hypotonic tolerance by extracellular ATP. *E. coli* cells after 2 h IPTG induction were transferred to hypotonic solution pre-mixed with absence or presence of ATP at different concentrations. After 30 min of exposure to the hypotonic conditions, three aliquots of each cell suspension were taken and spread onto LB plates for a subsequent colony survival assay. The survival rates of *E. coli* in each concentration of ATP were compared with that of non-ATP treatment ($n=3$; $^{**}P<0.01$). The tag-pI_{Cln}-expressing cells (○); tag-expressing cells (△).

nel, and its outwardly rectifying Cl^- current induced by expression of this protein in oocytes was well-known to be inhibited by treatment of the cell with nucleotides including ATP [4]. To confirm the link between hypo-tolerance and expression of pI_{Cln}, inhibition experiments were performed with a series of pI_{Cln}-sensitive extracellular ATPs. As shown in Fig. 4, a specific inhibition of ATP at 10^{-5}–10^{-4} M on the survival rate of pI_{Cln}-expressing cells was observed, while at the same range of ATP concentrations, the survival rates of control cells were not significantly affected. The results indirectly indicate that the hypotonic tolerance was due to the expression of pI_{Cln} in the *E. coli* cells. A higher concentration of ATP ($>10^{-3}$ M) could also decrease the survival rate of control cells (data not shown). The nucleotide-binding site has been proposed to be the GXGXG motif localized at the outside of the pI_{Cln} channel pore [4]. Recently, however, Voets et al. [12] reported that this GXGXG motif is not important for the sensitivity of the I_{Cln} to nucleotides by electrophysiological experiments after injection of a mutant cRNA (GXGXG→AXAXA) into *Xenopus* oocytes. The mechanism of how extracellular ATP inhibits the hypo-tolerance or the current (I_{Cln}) is still unknown.

3.5. Effects of anion channel inhibitor drugs on the hypo-tolerance

The Cl^- current (I_{Cl}) induced by expression of pI_{Cln} in *Xenopus* oocytes was blocked with some channel inhibitors including NPPB or DIDS [4,11,12]. As shown in Fig. 5, the survival rates of pI_{Cln}-expressing cells were also blocked by three inhibitor drugs, while the control cells were not significantly affected. The inhibitory sequence for the hypotonic tolerance is DIDS > NBBP≥DPC. These results indicate that the hypotonic tolerance is closely linked to the activation of an anion channel. Although the pI_{Cln} was also found in membrane fractions (data not shown), it is still uncertain whether the expressed pI_{Cln} is inserted into the membrane to form the anion channel as proposed by Paulmichl et al. [4] or Strange et al. [10]. However, the present results explain that pI_{Cln} can activate the endogenous anion channel originally existing in the *E. coli* membrane to restore the hypo-induced swollen cells.

3.6. Conclusions

We have unambiguously shown that *E. coli* cells expressing rat pI_{Cln} exhibit a strong tolerance to hypotonic stress. The tolerance was sensitive to extracellular ATP or some anion channel blockers. Taken together, the present results and our previous data showing that pI_{Cln} is predominantly localized at the membranes of the distal tubules where it is exposed to the most diluted intra-tubular solution, pI_{Cln} is concluded to play a critical role in cell-volume regulation through an anion channel.

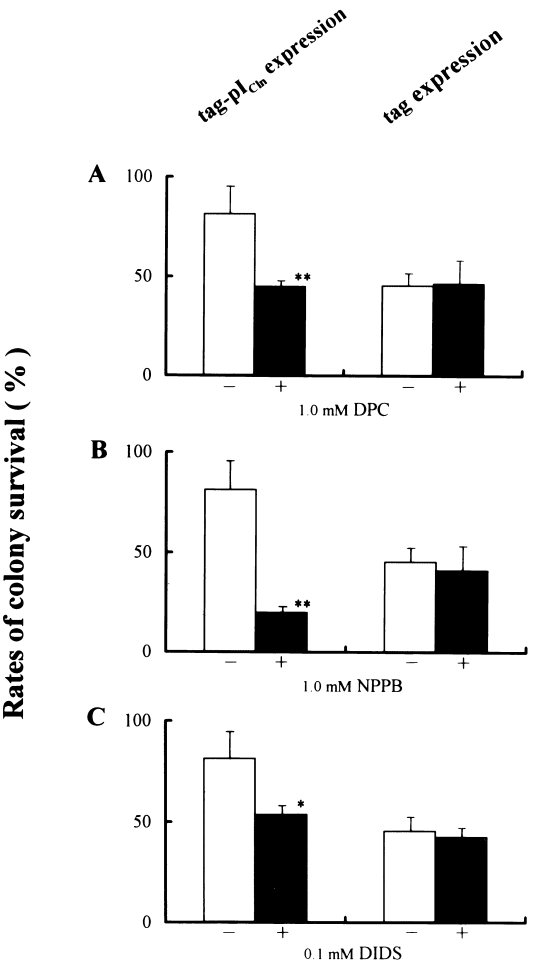

Fig. 5. Effects of channel blocker drugs on the hypotonic tolerance. pI_{Cln}-expressing cells and the control cells were transferred to the hypotonic solution pre-mixed with absence or presence of channel blocker drugs at different concentrations. After 30 min of exposure to hypotonic stress, three aliquots of each cell suspension were spread onto LB plates for subsequent colony survival assay. The *E. coli* cells survival rates were compared between the two hypotonic solutions with and without the drugs ($n=3$; $^{*}P<0.05$; $^{**}P<0.01$).

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