Effects of magnesium and sodium ions on the outer membrane permeability of cephalosporins in *Escherichia coli*

Akihito Yamaguchi, Misa Yanai, Noribumi Tomiyama and Tetsuo Sawai

Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Chiba 260, Japan

Received 25 July 1986

Both Mg^{2+} and Na^{+} stimulated the outer membrane permeation of negatively charged cephalosporins in *Escherichia coli* without any significant alteration of the permeation of a zwitterionic cephalosporin. Such stimulation was not observed in an *E. coli* mutant lacking porins. The stimulation was caused by the direct interaction between the cations and the porin pores, which resulted in a decrease in cation selectivity of both the Omp F and Omp C porin pores.

Mg^{2+} effect *Na^{+} effect* *Membrane permeability* *Cephalosporin* (E. coli)

1. INTRODUCTION

Escherichia coli K12 strains have two major porin proteins, Omp F and Omp C [1], which form cation-selective pores in the outer membrane [2,3]. The cation selectivities of these porin pores are explained in terms of the ionic interactions between solutes and the negative charges on the inner walls of the pore [2]. This explanation is based on evidence showing that the chemical modification of amino or carboxyl groups of the porin proteins results in alteration of the selectivity, and that an amidated porin loses its cation selectivity. On the other hand, cations such as spermidine and *N*-benzyloxycarbonylglycyl-L-prolyl-L-arginine *p*-nitroanilide cause a conformational change in the Omp F porin trimer, which allows the easier diffusion of these cations even though their molecular masses are close to or a little larger than the threshold exclusion limit of the porin pores [4].

Small monovalent or divalent cations are expected to increase the outer membrane permeability of drugs or nutrients with anionic groups, because the cations would decrease the ionic repulsion between the solutes and the negative charges on the pore walls. Such an influence of small cations on drug or nutrient permeability, if it occurs, is important as to the possible environmental regulation of the pore activity. It is also of interest as to whether small cations behave like spermidine with regard to the conformational change in the porin trimer.

Here, we investigated the effects of Mg^{2+} and Na^{+} on the outer membrane permeability of cephalosporins which are known to pass through the outer membrane mainly via porin pores [5–9].

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

E. coli KY2562 is an ompB101 mutant which lacks both the Omp C and Omp F porins. *E. coli* KY2209 and KY2201 are pseudo-revertants derived from KY2562, which produce either Omp F or Omp C porin. *E. coli* KY2563 (thi, tsx, malA) is a wild strain with respect to porin. All these strains were kind gifts from T. Yura [10]. To assay the outer membrane permeation of cephalosporins by means of the method involving periplasmic β-lactamase [9], these strains were infected with an R

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

plasmid, RGN 823, which mediates type Ib penicillinase [11].

2.2. *Cephalosporins*

The cephalosporins were kindly provided by the following pharmaceutical companies: cefazolin and ceftezole, Fujisawa, Osaka; cephaloridine, Torii, Tokyo; and cephalosporin-C, Meiji Seika, Tokyo.

2.3. *Measurement of the outer membrane permeability of cephalosporins*

Exponentially growing cultures of *E. coli* in 200 ml penassay broth (Difco) were harvested by centrifugation for 15 min at 5000 × *g* at 20°C. The cells were washed once with 50 ml of 5 mM Hepes-NaOH buffer (pH 7.0) containing 200 mM sucrose and 1 mM $MgSO_4$, and then resuspended in 20 ml of the same buffer. Using these washed cells, the outer membrane permeability of cephalosporins was assayed on the basis of cephalosporin hydrolysis by periplasmic β-lactamase [9]. The assay was performed in 5 mM Hepes-NaOH buffer (pH 7.0) containing 200 mM sucrose and various concentrations of $MgSO_4$. The effect of NaCl on cephalosporin permeation was examined in the same way in medium containing 0.2 mM $MgSO_4$.

2.4. *SDS-polyacrylamide gel electrophoresis of the outer membrane proteins*

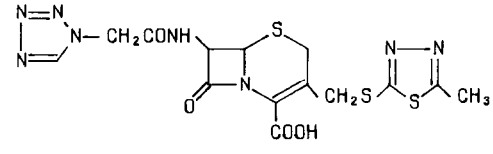
Cells grown in penassay broth were harvested and then resuspended in 50 mM phosphate buffer (pH 7.0), followed by disruption through brief sonication. The outer membrane fraction was prepared by extraction of the envelope fraction with a 1.5% sarkosyl solution in 50 mM phosphate buffer (pH 7.0), and washed twice with distilled water. Urea-SDS-polyacrylamide electrophoresis was performed by the method of Pugsley and Schnaitman [12] with the following modifications: the separation gel used for our system consisted of 7% acrylamide, 0.17% bisacrylamide, 8 M urea, 0.006% ammonium sulfate, 0.1% SDS and 0.12% *N,N,N',N'*-tetramethylethylenediamine (TEMED) in 375 mM Tris-HCl buffer (pH 8.8). The sample loading gel consisted of 4.5% acrylamide, 0.12% bisacrylamide, 0.05% ammonium sulfate, 0.1% SDS and 0.05% TEMED in 125 mM Tris-HCl (pH 6.8). The sample dissolved in a solution consisting of 0.4% SDS, 10% mercaptoethanol and 20% glycerol in 125 mM Tris-HCl (pH 6.8), and 0.04% bromphenol blue was heated in boiling water prior to electrophoresis. The electrophoresis buffer used was Tris-glycine buffer (pH 8.4), which consisted of 26 mM Tris, 190 mM glycine and 0.1% SDS. Electrophoresis was carried out at a constant current of 25 mA for about 3 h.

3. RESULTS

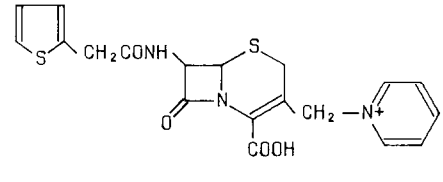
3.1. *Outer membrane permeability of cefazolin and cephaloridine*

We chose cefazolin (fig.1a) as a representative monoanionic cephalosporin because it shows average hydrophilicity and outer membrane permeability among the monoanionic cephalosporins [9]. Cephaloridine (fig.1b) is a well-known zwitterionic cephalosporin that shows slightly higher hydrophobicity than cefazolin [13].

Table 1 lists the outer membrane permeability of the two cephalosporins in *E. coli* KY strains. The porin-deficient strain, KY2562, which produces only a trace amount of Omp C, showed far lower permeability of the cephalosporins than the Omp F-producing strains, KY2563 and KY2209. The permeability of cefazolin in the Omp C-producing strain, KY2201, was about 2-fold higher than that in KY2562, but that of cephaloridine in KY2201



(a) cefazolin



(b) cephaloridine

Fig.1. Chemical structures of cefazolin and cephaloridine.

Table 1

Outer membrane permeability of cefazolin and cephaloridine in *E. coli* KY strains

Antibiotics	Permeability parameter (10^{-5} ml/min per μg dry cell)			
	KY2563 (F^+,C^+)	KY2209 (F^+,C^-)	KY2201 (F^-,C^+)	KY2562 (F^-,C^-)
Cefazolin	25.2	75.5	5.18	2.40
Cephaloridine	118	528	25.5	20.7

Permeability was measured in 5 mM Hepes, pH 7.0, containing 20 mM sucrose and 4 mM $MgSO_4$ as described in section 2. The pH was adjusted with NaOH

was slightly higher than that in KY2562. These results are consistent with the known fact that many cephalosporins use the porin channel as a permeation route, and that the Omp F porin forms more effective channels than the Omp C porin.

The high permeability of cephaloridine is also consistent with the known cation selectivity of these porin channels [6,7]. The strain lacking Omp C, KY2209, showed significantly higher permeation of cephalosporins than the wild strain, KY2563. This difference could be explained by the fact that the Omp F porin was overproduced in KY2209, probably due to overcompensation for the lack of the Omp C porin (fig.2).

3.2. *Effects of Mg^{2+} and Na^+ on the outer membrane permeability of cefazolin*

The bacterial cells were washed with 5 mM Hepes-NaOH buffer containing 200 mM sucrose and 1 mM $MgSO_4$, and then the outer membrane permeability of cefazolin in the washed cells was determined at various concentrations of $MgSO_4$ or NaCl. As shown in fig.3A,B, the cefazolin permeability was stimulated by Mg^{2+} by a factor of about 3-fold in strain KY2209 and about 5-fold in strain KY2201. The stimulation showed saturation with K_m values of about 2.5 mM (KY2209) and 10 mM (KY2201), suggesting a difference in affinity of Mg^{2+} for the porin proteins.

In the porin-deficient strain, KY2562, the permeability of cefazolin was not affected by Mg^{2+}. This fact supported the idea that the stimulation seen in the porin-producing strains was due to the direct action of Mg^{2+} on the porin pores.

Na^+ also stimulated cefazolin permeability in both KY2209 and KY2201 (fig.3C,D), although the concentration required for the same effect as

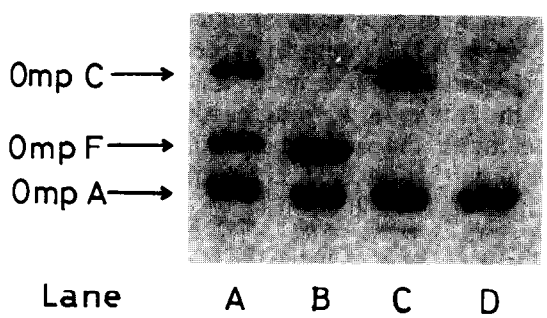


Fig.2. 8 M urea-SDS-polyacrylamide gel electrophoretic profiles of outer membrane proteins of *E. coli* KY strains. Gel electrophoresis was performed as described in section 2. Lanes: A, KY2563; B, KY2209; C, KY2201; D, KY2562.

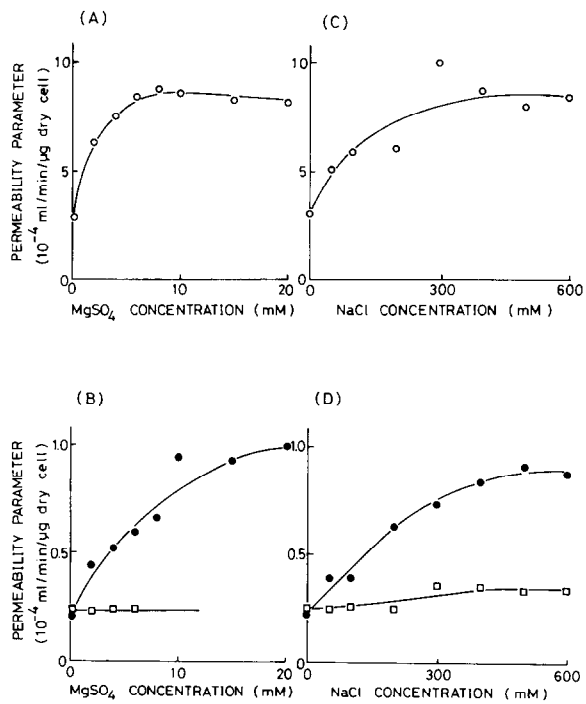


Fig.3. Effects of the concentrations of $MgSO_4$ (A,B) and NaCl (C,D) on the outer membrane permeability of cefazolin. The outer membrane permeability was measured as described in section 2 using the Omp F-producing strain, KY2209 (○), Omp C-producing strain, KY2201 (●) and porin-deficient strain, KY2562 (□).

that of Mg^{2+} was about 30-fold higher than that of the latter. The K_m values of Na^+ for the stimulation were about 110 mM (KY2209) and 320 mM (KY2201).

It should be noted that the maximum level of stimulated permeation due to Na^+ was just the same as that due to Mg^{2+}, indicating the binding of both cations to the same sites. Such a stimulatory effect was confirmed for other cephalosporins with negative charge(s) such as ceftezole and cephalosporin-C, although the degree of stimulation differed with the drug in question (not shown).

Na^+, as well as Mg^{2+}, did not show any significant stimulation of cefazolin permeability in the porin-deficient strain.

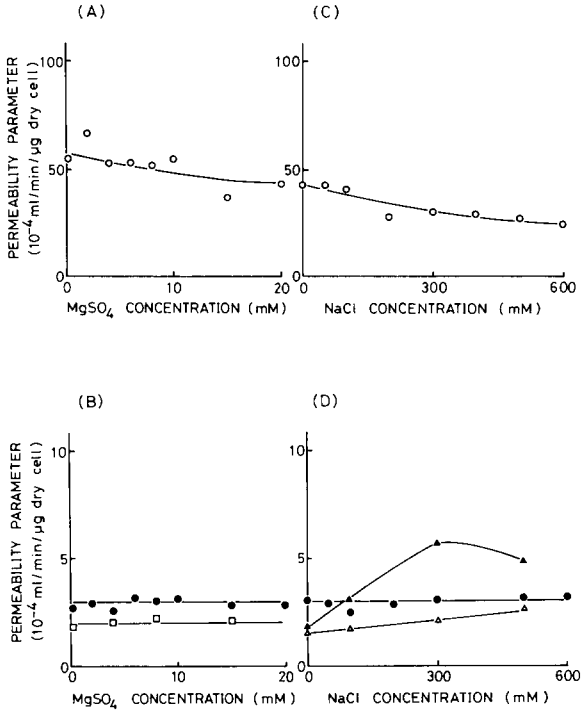


Fig.4. Effects of the concentrations of $MgSO_4$ (A,B) and NaCl (C,D) on the outer membrane permeability of cephaloridine. The permeability was measured as in fig.3 using the Omp F-producing strain, KY2209 (○), Omp C-producing strain, KY2201 (●) and porin-deficient strain, KY2562 (□), except that the sodium effect in the case of KY2562 was measured in the presence of 0.2 mM $MgSO_4$ (▲) or 5 mM $MgSO_4$ (△).

Table 2

Ratio of the outer membrane permeability of cephaloridine and cefazolin in the porin-containing strains with various ionic concentrations

Strains	Ratio (cephaloridine/cefazolin)		
	In 0.2 mM $MgSO_4$	In 20 mM $MgSO_4$	In 600 mM NaCl and 0.2 mM $MgSO_4$
E. coli KY2209 (Omp F^+, Omp C^-)	19.4	5.6	2.9
E. coli KY2201 (Omp F^-, Omp C^+)	13.4	2.8	3.7

3.3. *Effects of Mg^{2+} and Na^+ on the outer membrane permeability of cephaloridine*

As shown in fig.4B,D, the permeability of cephaloridine in the Omp C-producing strain, KY2201, was affected by neither Mg^{2+} nor Na^+. In the Omp F-producing strain, KY2209, an increase in cation concentration tended to decrease the cephaloridine permeability (fig.4A,C). As a result, the permeability was reduced about 3–5-fold in the presence of 20 mM Mg^{2+} or 600 mM Na^+ (table 2).

In the porin-deficient strain, Mg^{2+} did not affect cephaloridine permeation (fig.3C). However, Na^+ significantly accelerated cephaloridine permeation in the presence of 0.2 mM $MgSO_4$ (fig.4D). It is of interest that the stimulation by Na^+ was repressed on addition of 5 mM $MgSO_4$ (fig.4D). Therefore, the mechanism of stimulation by Na^+ in the porin-deficient strain may differ from that involving direct action of the ions on the porin pores. It might be due to the squeezing out of the divalent cations in the outer membrane by Na^+, which results in destabilization of the outer membrane [14].

4. DISCUSSION

Our observations demonstrate that both Mg^{2+} and Na^+ stimulate the porin-pore permeability of negatively charged cephalosporins without significant alteration of the permeation of a zwitterionic

cephalosporin. To the best of our knowledge, this is the first experimental demonstration of remarkable acceleration of the porin-pore permeation of anionic substances by small cations.

Two lines of evidence indicated that the stimulation was due to direct neutralization of the negative charges on the pore walls by the cations. Firstly, stimulation was not observed in the porin-deficient strain. Secondly, the permeation of cephaloridine in the porin-producing strain was not stimulated by the cations. This latter observation is analogous to the fact reported by Tokunaga et al. [15] and Benz et al. [2] that amidation of the carboxylic acids on the pore walls was found to lead to a loss or reduction of the cation selectivity of the porin pores [2,3]. Kobayashi et al. [4] reported the self-promoted permeation of large polycations, which was probably due to a conformational change of the porin pores induced by the polycations. However, the possibility of a conformational change caused by the small cations can be ruled out because cephaloridine permeability was not stimulated.

Stimulation of benzylpenicillin permeability through a lipid bilayer region by Na^+ was reported by Homma and Nakae [14] in *E. coli* cells in magnesium-free medium. This stimulation was probably due to destabilization of the outer membrane structure on exclusion of divalent cations from the membrane surface, because the stimulation was repressed by the addition of Mg^{2+} [14]. Such stimulation by Na^+ was also observed in the case of cephaloridine permeation in the porin-deficient strain (fig.4). However, the possibility of a destabilization effect of Na^+ in porin-producing strains can be ruled out because Mg^{2+} could not repress the stimulation by Na^+.

The contribution of the Donnan potential [16] to the effect of the small cations can also be excluded because, if this were the case, Na^+ should be equally effective as Mg^{2+}, however, this was clearly not the case (fig.3).

It remained possible that the binding of cations to cephalosporin molecules contributed to the stimulation. However, this possibility can be ruled out due to the difference in cation concentrations required for full stimulation between the Omp F and Omp C porins.

It is of interest to determine whether small cations play a significant role in porin-pore activity under physiological conditions.

REFERENCES

[1] Osborn, M.J. and Wu, H.C.P. (1980) Annu. Rev. Microbiol. 34, 369–422.
[2] Benz, R., Tokunaga, H. and Nakae, T. (1984) Biochim. Biophys. Acta 769, 348–356.
[3] Benz, R., Schmid, A. and Hancock, R.E.W. (1985) J. Bacteriol. 162, 722–727.
[4] Kobayashi, Y. and Nakae, T. (1985) Eur. J. Biochem. 151, 231–236.
[5] Alphen, W.V., Boxtel, R.V., Selm, N.V. and Lugtenberg, B. (1978) FEMS Microbiol. Lett. 3, 103–106.
[6] Nikaido, H., Rosenberg, E.Y. and Foulds, J. (1983) J. Bacteriol. 153, 232–240.
[7] Yoshimura, F. and Nikaido, H. (1985) Antimicrob. Agents Chemother. 27, 84–92.
[8] Kobayashi, Y., Takahashi, I. and Nakae, T. (1982) Antimicrob. Agents Chemother. 22, 775–780.
[9] Yamaguchi, A., Tomiyama, N., Hiruma, R. and Sawai, T. (1985) FEBS Lett. 181, 143–148.
[10] Sato, T. and Yura, T. (1981) J. Bacteriol. 145, 88–96.
[11] Sawai, T., Hiruma, R., Kawana, N., Kaneko, M., Taniyasu, F. and Inami, A. (1982) Antimicrob. Agents Chemother. 22, 585–592.
[12] Pugsley, A.P. and Schnaitman, C.A. (1978) J. Bacteriol. 135, 1118–1129.
[13] Yamaguchi, A., Hiruma, R. and Sawai, T. (1983) FEBS Lett. 164, 389–392.
[14] Homma, T. and Nakae, T. (1982) Tokai Exp. Clin. Med. 7, Suppl., 171–175.
[15] Tokunaga, H., Tokunaga, M. and Nakae, T. (1981) J. Biol. Chem. 256, 8024–8029.
[16] Stock, J.B., Rauch, B. and Roseman, S. (1977) J. Biol. Chem. 252, 7850–7861.