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Manipulation of Intracellular Magnesium Content in Polymyxin B Nonapeptide-Sensitized *Escherichia coli* by Ionophore A23187

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***Escherichia coli* B cells were sensitized to ionophore A23187 by polymyxin B nonapeptide, and the induced magnesium and potassium ion fluxes were studied. Combined ionophore treatment permeabilized the cytoplasmic membrane of *E. coli* in an ion-specific manner and allowed the manipulation of intracellular Mg^{2+} content from the outside. A23187-induced Mg^{2+} efflux or influx was dependent on the free Mg^{2+} concentration gradient between the outside and inside of the cytoplasmic membrane and on the pH gradient. Most of the intracellular Mg^{2+} was bound, whereas only 1 to 2 mM was free in solution in the cellular sap.**

Magnesium plays central and multiple roles in procaryotic and eucaryotic cells as a cofactor in many enzymatic and regulatory reactions and as a structural component of biopolymers and supramolecular assemblies. One approach to characterizing these roles is to manipulate the intracellular Mg^{2+} content with ionophores specific for Mg^{2+}, such as A23187, which is proposed to induce Mg^{2+}-$2H^+$ exchange (15–17). The resistance of gram-negative bacteria to hydrophobic antibiotics has been the main hindrance in applying this strategy to wild-type *Escherichia coli*. Recently Vaara and Vaara (21) have shown that polymyxin B nonapeptide sensitizes gram-negative bacteria toward many hydrophobic antibiotics, including A23187 (1). In this study we have used polymyxin B nonapeptide-sensitized *E. coli* B cells to examine the A23187-induced Mg^{2+} transport. The data presented in this paper for *E. coli* treated with polymyxin B nonapeptide plus A23187 (defined as a "combined ionophore" treatment) show that, by supplementing the medium with $MgCl_2$, it is possible to adjust the intracellular Mg^{2+} content from undetectable amounts to that of untreated cells. Furthermore, the effects of A23187 and $MgCl_2$ supplements on the cellular growth rate and the effect of the protonophore carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) on A23187-induced Mg^{2+} transport provide an estimate of the intracellular free Mg^{2+} concentration. Our results suggest that the K^+ influx occurring concomitantly with the Mg^{2+} efflux is not directly achieved by A23187 itself, but is mediated by another transport system as a consequence of the A23187-induced Mg^{2+} transport.

MATERIALS AND METHODS

Growth of bacteria. *E. coli* strain B was grown at 37°C with continuous aeration in 1% tryptone (Difco Laboratories, Detroit, Mich.) (K^+, Ca^{2+}, and Mg^{2+} concentrations were 0.6 mM, 0.3 mM, and 60 μM, respectively, at pH 6.9 unless otherwise indicated) supplemented with 0.1 M NaCl or with 0.1 M NaCl plus 50 mM KCl. Optical density measurements at 550 nm were made with a Hitachi 101 spectrophotometer.

Chemicals. A23187, as a Ca^{2+}-Mg^{2+} salt, was purchased from Calbiochem (La Jolla, Calif.) and dissolved in dimethylformamide-ethanol (1:3) to give a stock solution of 10 mg/ml. This stock was further diluted with absolute ethanol to 1 mg/ml. In control experiments, appropriate volumes of the solvents alone were added. Polymyxin B nonapeptide was prepared as described by Vaara and Vaara (22) and was the generous gift of M. Vaara (National Public Health Institute, Helsinki, Finland). The polymyxin B content of this preparation was less than 0.2%. Aqueous stock solutions of 1 mg/ml were used for experiments. CCCP was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Determination of the intracellular contents of magnesium, potassium, sodium, and calcium. For the quantitation of intracellular cations, the bacteria were harvested by filtration (9). To check the wash procedure, samples of permeabilized (polymyxin B nonapeptide plus A23187 treatment) and untreated cells in the presence of low (2 mM) and high (20 mM) Mg^{2+} concentrations were washed with increasing volumes (1 to 10 ml per sample). In all cases, wash volumes greater than 2 ml gave constant and reproducible K^+ and Mg^{2+} contents. Accordingly, 4-ml wash volumes were used. Osmolality measurements of wash solutions and medium were made with a Knauer osmometer. Cation determinations were made on a Unicam SP 2900 atomic absorption spectrometer by H. G. Seiler.

RESULTS

Mg^{2+} and K^+ transport in polymyxin B nonapeptide-sensitized *E. coli* cells induced by A23187 treatment in the presence of various concentrations of $MgCl_2$ in the growth medium. By using the combined ionophore treatment (polymyxin B nonapeptide plus A23187), it is possible to modify the intracellular Mg^{2+} content of *E. coli* by supplementing the growth medium with different concentrations of $MgCl_2$ (Fig. 1A through D). Without the $MgCl_2$ supplement the intracellular Mg^{2+} content was lowered about 20-fold within 15 min after the treatment, and cell growth was immediately arrested (Fig. 1A). After the combined ionophore treatment in the presence of 2, 5, and 20 mM $MgCl_2$ in the tryptone medium, the intracellular Mg^{2+} content decreased to 20 to 25%, 60 to 70%, and 90 to 100%, respectively, of that before treatment (Fig. 1B through D). A sufficiently high intracellular Mg^{2+} content is correlated with growth of *E. coli*; when the intracellular level was reduced by 30 to 40%, growth was completely arrested after 10 min (Fig. 1C). The K^+ content,

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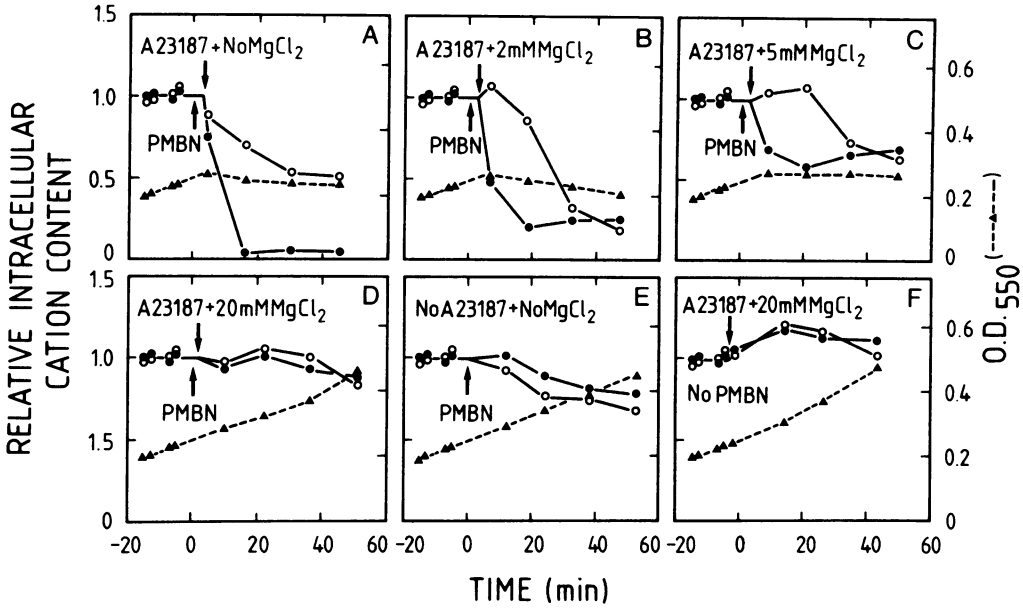

FIG. 1. Responses of the intracellular Mg^{2+} and K^+ levels in *E. coli* B after polymyxin B nonapeptide (PMBN) plus A23187 treatment at different external $MgCl_2$ concentrations. Exponentially growing cells (optical density at 550 nm [OD_{550}] of 0.25) were treated with 5 μg of polymyxin B nonapeptide (PMBN) per ml. After 1.5 to 2 min later (arrows), samples were supplemented with 1 μg of A23187 per ml and different concentrations of $MgCl_2$ (0 to 20 mM) (A to D). To determine the intracellular Mg^{2+} (●) and K^+ (○) content, 2-ml samples were withdrawn for atomic absorption spectroscopy, and the optical density at 550 nm (▲) was also measured at the same time. In control experiments, either the ionophore (E) or the polymyxin B nonapeptide (F) was omitted.

however, was not significantly changed during the first 20 min. In the control experiment in which the sensitization with polymyxin B nonapeptide was omitted (Fig. 1F), the observed 10 to 20% increase in the K^+ content is easily explained as a response to the increased osmolality of the medium due to the supplementation with 20 mM $MgCl_2$. The 15 to 20% increase in the Mg^{2+} content after the addition of the A23187–20 mM $MgCl_2$ supplement is likely to be caused by higher amounts of unfilterable A23187 colloids with bound Mg^{2+} accumulating in the absence of the outer membrane permeabilizing agent polymyxin B nonapeptide (21). The combined ionophore treatment affects Mg^{2+} and K^+ very differently. The K^+ loss was significantly delayed with respect to Mg^{2+} loss, and the length of the K^+ retention phase was inversely related to the amount of Mg^{2+} efflux (Fig. 1B and C).

The next question relates to the possibility of increasing the intracellular Mg^{2+} by a combined ionophore treatment and the addition of 100 mM $MgCl_2$ to the growth medium. Figure 2C shows that this is not possible. Only the K^+ content is increased as a consequence of the increased osmolality due to added $MgCl_2$. The situations with (Fig. 2C) and without (Fig. 2D) combined ionophore treatment are not substantially different. If we first induce a depletion of Mg^{2+} by a combined ionophore treatment (Fig. 2A and B) and 25 min later add 100 mM $MgCl_2$, then the normal intracellular Mg^{2+} content is reestablished. As in the above experiments (Fig. 2C and D), the K^+ content is increased to compensate for the osmotic pressure of the medium.

Dependency of cell growth on the dose of A23187 and outside magnesium concentration. We have assumed that the rates of Mg^{2+} efflux depend on the number of ionophores per cell (dose) and on the Mg^{2+} and H^+ concentration gradients across the cytoplasmic membrane. If there are too many ionophores per cell and a low outside Mg^{2+} concentration, e.g., in tryptone medium (50 to 60 μM Mg^{2+}), then the Mg^{2+} active transport system of *E. coli* (18) would no longer be able to compensate for the Mg^{2+} efflux, and the intracellular Mg^{2+} content would decrease with a corresponding proton influx. As a consequence, the growth rate would also decrease. Only when the outside Mg^{2+} concentration corresponds to steady-state conditions (where Mg^{2+} and H^+ gradients are approximately in equilibrium) would no net fluxes of Mg^{2+} and H^+ be induced by the combined ionophore treatment; therefore, the growth rate would be independent of the ionophore dosage.

The results of experiments designed to vary the intracellular concentration of Mg^{2+} are presented in Table 1. With a supplement of 20 mM $MgCl_2$ to the growth medium (at the moment of combined ionophore treatment; the pH of the cultures varied between 6.90 and 6.95), the growth rate of polymyxin B nonapeptide-sensitized *E. coli* cells was not affected by increasing concentration of A23187. We consider the slight reduction of the growth rate in the presence of A23187 a nonspecific consequence of the interaction of this ionophore with the cytoplasmic membrane. By the combined ionophore treatment, the growth rate was reduced with $MgCl_2$ concentrations of 15 mM or lower. We conclude that when the outside concentration of free Mg^{2+} is 20 mM and the pH is 6.90 to 6.95, then the intracellular free Mg^{2+} ion concentration is not affected by introducing ionophore A23187. Accordingly, equilibrium states of both Mg^{2+} and H^+ gradients across the cytoplasmic membrane are established.

Effect of the proton gradient (ΔpH) on magnesium transport induced by A23187 in polymyxin B nonapeptide-sensitized *E. coli*. Introducing ionophore A23187 into the cytoplasmic membrane appears to produce Mg^{2+}-$2H^+$ exchange (15). Consequently, the proton gradient (ΔpH) might affect the amount and the direction of the Mg^{2+} transport that is

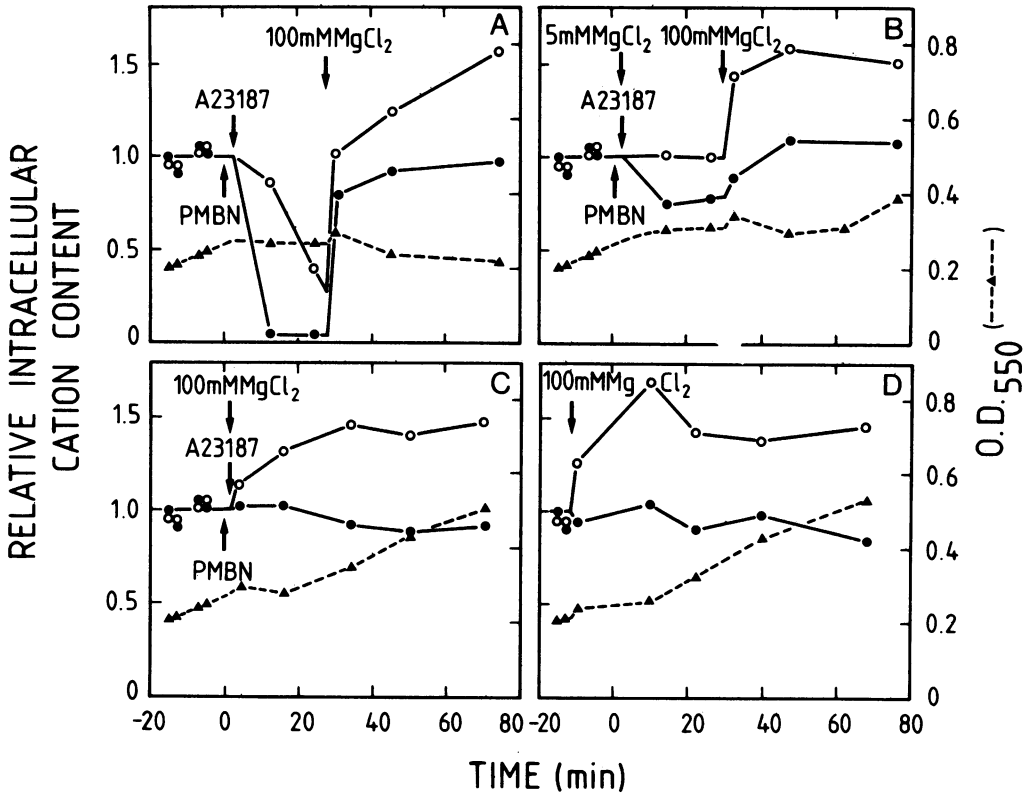

FIG. 2. Mg^{2+} influx in polymyxin B nonapeptide (PMBN)-plus A23187-permeabilized *E. coli* B mediated by $MgCl_2$ supplement in the growth medium. The cells were permeabilized with 5 μg of polymyxin B nonapeptide (PMBN) and 1 μg of A23187 per ml. The intracellular Mg^{2+} (●) and K^+ (○) content and optical density at 550 nm (OD_{550}) (▲) were determined as described in the legend of Fig. 1 and in the text.

induced by A23187. To investigate this, the proton gradient was collapsed by the protonophore CCCP (16), which was added before the combined ionophore treatment of *E. coli* in the presence of various concentrations of $MgCl_2$ and different pHs. The effect of medium pH and CCCP on the A23187-induced Mg^{2+} transport is shown in Fig. 3 and 4. In the presence of an outside Mg^{2+} concentration of 1 mM, there is always an Mg^{2+} efflux; the amount is dependent on the external pH and CCCP treatment (Fig. 3A and 4A). Cellular growth was completely inhibited at pH 6.9, but only reduced at pH 7.5 (data not shown), indicating that the amount of Mg efflux was affected by a change in external pH. The effect of CCCP on the Mg^{2+} efflux was complex. During the first 10 min at both pHs about equal Mg^{2+} efflux was observed. Surprisingly, however, at later times the responses differed; at pH 6.9 the Mg^{2+} content reached a level of about 70%, whereas at pH 7.5 the Mg^{2+} efflux continued at a lower rate to a level that was about 10% of that observed before the combined ionophore treatment. In both cases the CCCP treatment was equally effective, as concluded from the constancy of the optical density of the cultures and from the complete loss of intracellular K^+.

With an outside concentration of 5 mM, small Mg^{2+} effluxes were observed at pH 6.9 (Fig. 3D) and at pH 7.5 (Fig. 4B). However, the growth rate was reduced in the case of pH 6.9, but not at pH 7.5 (data not shown). The Mg^{2+} content in CCCP-treated control cells increased as in combined ionophore-treated cells, although at a slower rate (Fig. 4B). With an outside concentration of 20 mM Mg^{2+}, the amount and the rate of the Mg^{2+} influx in CCCP-treated cells

TABLE 1. Dependence of growth rate on A23187 concentration and external $MgCl_2$[a]

A23187 concn (μg/ml)	Relative growth rates ± SD (*n*) with the following concn of $MgCl_2$ (mM) in the growth medium:					
	1	2	5	10	15	20
0	1.00	1.00	1.00	1.00	1.00	1.00
1	0	0.27	0.65 ± 0.04 (3)	0.81 ± 0.07 (3)		0.86 ± 0.09 (3)
5		0	0.39 ± 0.04 (3)	0.61 ± 0.07 (3)	0.69	0.79 ± 0.07 (3)
10			0.14	0.37 – 0.39	0.61	0.73 ± 0.06 (3)
20				0.29 – 0.33	0.41	0.92 ± 0.19 (3)
66						0.86

[a] *E. coli* B cultures were grown in tryptone medium supplemented with 50 mM KCl at 37°C. Cells in the logarithmic growth phase were treated with 5 μg of polymyxin B nonapeptide per ml when an optical density at 550 nm of 0.25 was reached. After 1 to 3 min, subcultures were withdrawn and supplemented with A23187 and $MgCl_2$. The optical density at 550 nm of each subculture was measured every 10 min and followed for 60 min. The minimal growth rate was determined and expressed as relative growth rate compared to the appropriate control. Growth rates of controls varied from about 0.004 to 0.003 optical density units per min at 1 and 20 mM $MgCl_2$, respectively.

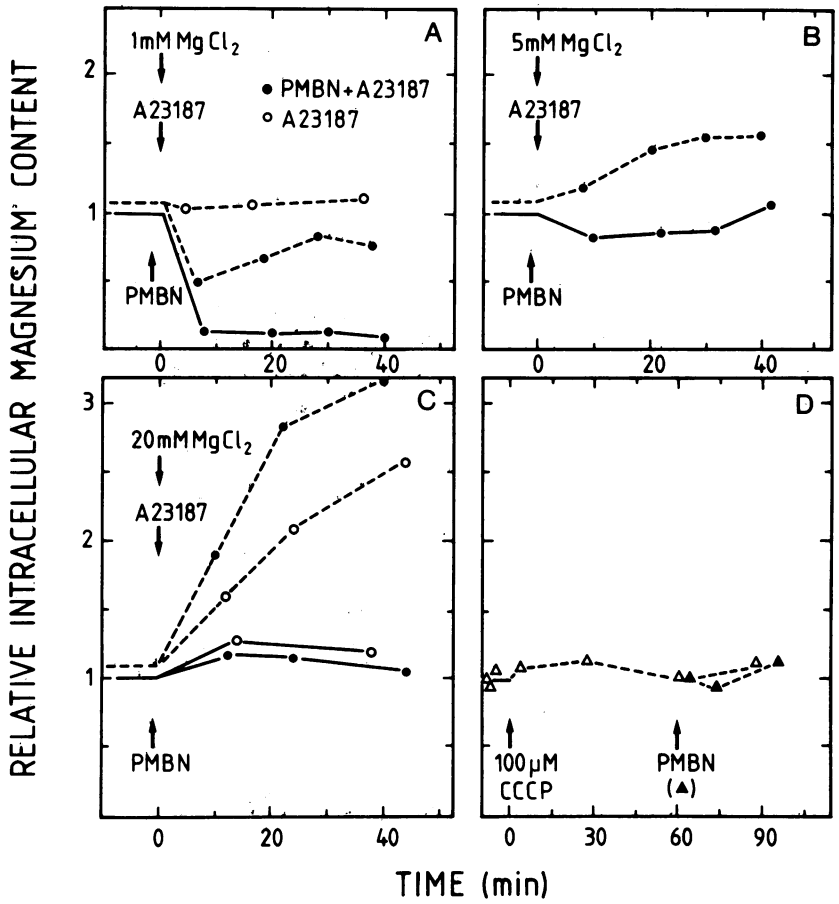

FIG. 3. Effect of external pH 6.9 and CCCP treatment on Mg^+ transport in *E. coli* B after combined ionophore treatment. Part of a culture of exponentially growing cells (optical density at 550 nm of 0.30) were pretreated for 60 min with 100 μM CCCP (– – –) and the remainder were untreated (——). At the times indicated by the arrows, the cells were then either permeabilized with 5 μg of polymyxin B nonapeptide (PMBN) per ml plus 1 μg of A23187 per ml (●) or treated with 1 μg of A23187 per ml alone (○). Panels A, B, and C represent the relative intracellular Mg^{2+} contents resulting from different amounts of $MgCl_2$ added at the times indicated by the arrows. Fig. D shows the influence of CCCP alone and together with a supplement of PMBN added 60 min later.

were enhanced by the A23187-mediated Mg^{2+} transport; the Mg content increased 2.5 to 3 times at pH 6.9 and at pH 7.5 (Fig. 3C and 4C). Surprisingly a much higher increase of the Mg^{2+} content was observed with CCCP-treated control cells at pH 6.9 than at pH 7.5. When the cellular proton gradient was not disturbed by CCCP, no increase in the Mg^{2+} content could be induced by the combined ionophore treatment in the presence of 20 mM Mg^{2+} outside the cell (Fig. 3C and 4C). CCCP treatment alone or with subsequent polymyxin B nonapeptide treatment did not affect the intracellular Mg^{2+} content (Fig. 3D and 4D). CCCP at 100 μM was sufficient to collapse the pH gradient before the combined ionophore treatment (data not shown); cell growth stopped immediately, the K^+ level dropped to zero, and the Na^+ content increased drastically (about equivalent to the amount of K^+ loss) within minutes after the CCCP pretreatment. We conclude from these results (Fig. 3 and 4) that the intracellular free Mg^{2+} ion concentration is more than 1 mM, but less than 5 mM, and that the pH affects the amount of A23187-mediated Mg^{2+} transport, as could be expected for an Mg^{2+}-H^+ exchange reaction.

K^+ transport in polymyxin B nonapeptide-sensitized *E. coli* induced by A23187 treatment in the presence of high K^+ in the growth medium. Combined ionophore treatment induced not only a Mg^{2+} efflux, but also a K^+ efflux characterized by a retention and an efflux phase (Fig. 1). To test whether it is possible to minimize this K^+ efflux, the K^+ concentration of the normal 0.1 M NaCl-containing tryptone medium (0.6 mM K^+) was increased to 50 mM. In the presence of high external K^+, the biphasic K^+ transport kinetics (mentioned above) were even more pronounced (Fig. 5A); instead of the K^+ retention phase observed in normal tryptone medium with low outside K^+ (Fig. 1A through C), we found a distinct K^+ influx phase. The K^+ content increased 20 to 25% simultaneously with a decrease in the Mg^{2+} content; the latter reached the limit of sensitivity of the atomic absorption technique employed. We did not observe a similar Na^+ influx with normal tryptone medium (data not shown). The rate of the Mg^{2+} efflux was unaffected by the presence of high K^+ and was complete within 10 to 15 min (compare Fig. 3A and 4A). After the Mg^{2+} efflux, a K^+ efflux phase was observed, the extent of which was comparable to that observed in tryptone medium not supplemented with KCl (compare Fig. 1A and 5A). In the control experiment (no A23187), a slight reduction of the intracellular Mg^{2+} and K^+ contents was observed; these were probably due to trace amounts of polymyxin B (less than 0.2%) in the polymyxin B nonapeptide preparation (Fig. 5B).

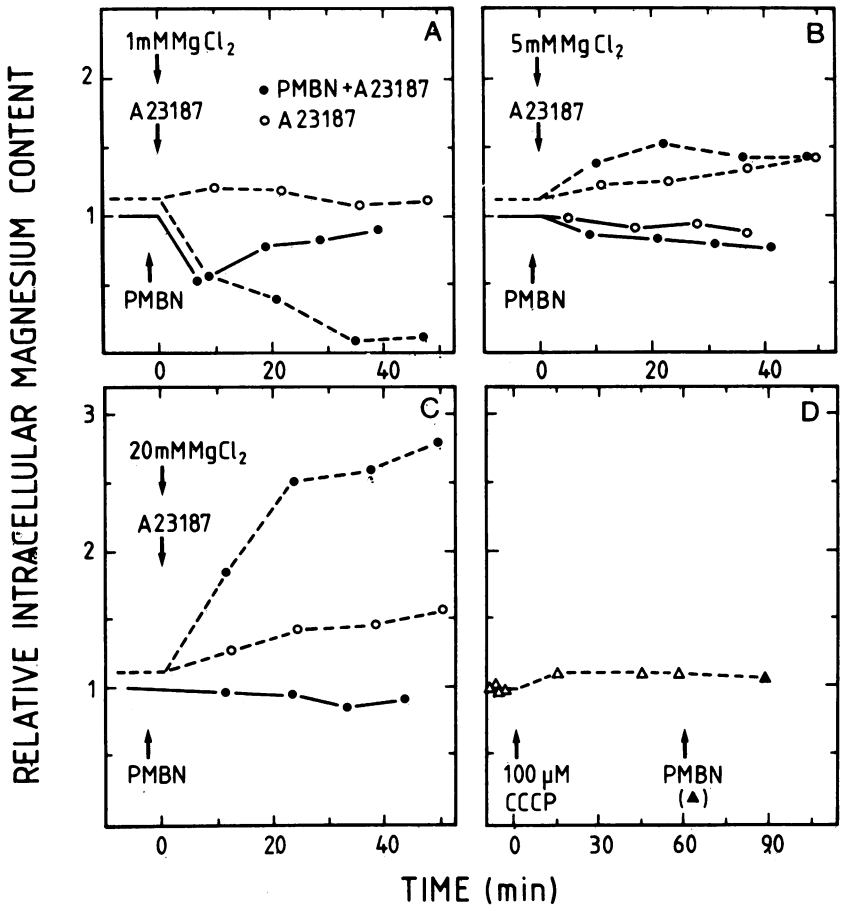

FIG. 4. Effects of external pH 7.5 and CCCP treatment on Mg^{2+} transport in *E. coli* after combined ionophore treatment. The pH of the tryptone medium was preadjusted to be 7.5 at an optical density at 550 nm of 0.30; a portion of the culture was pretreated for 60 min with 100 µM CCCP (– – –), and the remainder was untreated (——). The cells were then treated with 5 µg of polymyxin B nonapeptide (PMBN) per ml plus 1 µg of A23187 per ml (●) or 1 µg of A23187 per ml alone (○). Different concentrations of $MgCl_2$ were added as indicated by the arrows.

DISCUSSION

We have shown that after ionophore A23187 treatment of polymyxin B nonapeptide-sensitized *E. coli* cells and with various $MgCl_2$ supplements in the medium, it is possible to adjust the intracellular Mg^{2+} content from experimentally undetectable levels up to the level of normal, untreated cells (Fig. 1 and 2). However, with $MgCl_2$ concentrations as high as 100 mM in the medium, we could not push the intracellular Mg^{2+} content to higher values than normal. When we collapsed the proton gradient (ΔpH) of *E. coli* by the protonophore CCCP before the combined ionophore treatment, we could induce, with 20 mM outside Mg^{2+} concentration, higher intracellular Mg^{2+} levels than in cells treated only with CCCP (Fig. 3C and 4C). In the presence of 1 mM $MgCl_2$ in the tryptone medium, the intracellular Mg^{2+} content decreased by about 95 and 30% at pH 6.9 and 7.5, respectively. These results suggest that the A23187-induced Mg^{2+} transport is not driven only by the gradient of free Mg^{2+}, but is also driven by that of the protons. This agrees with the Mg^{2+}-$2H^+$ exchange proposed for ionophore A23187 (15). From these results (Fig. 3 and 4), we deduce that the intracellular free Mg^{2+} concentration (activity) is more than 1 mM, but less than 5 mM. This is based on the following assumptions: (i) the free intracellular Mg^{2+} concentration (used to substitute Mg^{2+} activity) is equal to the outside Mg^{2+} concentration when no pH difference exists between inside and outside; (ii) with the CCCP treatment the pH gradient is completely collapsed; and (iii) a drop in the intracellular pH caused by CCCP action does not affect the equilibrium between intracellular free and bound Mg^{2+}. However, the complexity of the effects of CCCP on Mg^{2+} content at different pHs (Fig. 3 and 4) should be considered in this context.

We observed that the growth of *E. coli* was independent of the concentration of added A23187 when the tryptone medium (pH 6.90 to 6.95) was supplemented with 20 mM $MgCl_2$ (Table 1). If we assume an intracellular pH of 7.4 to 7.8 (12, 13), we get a pH of 0.5 to 0.9 and accordingly a value of 3 to 8 for the H^+ concentration ratio between outside and inside. From this we can calculate the intracellular free Mg^{2+} concentration to be 0.3 to 2 mM, supposing that the ratio of free Mg^{2+} concentration (outside/inside) is equal to the square of the ratio of H^+ concentration (outside/inside). These results (Table 1) together with those from the CCCP experiment (Fig. 3) lead us to suggest that the intracellular free Mg^{2+} concentration in *E. coli* is most probably 1 to 2 mM. This agrees with the values estimated earlier with other methods (5, 10). However, since the intracellular Mg^{2+} concentration of *E. coli* B cells in the exponential phase of

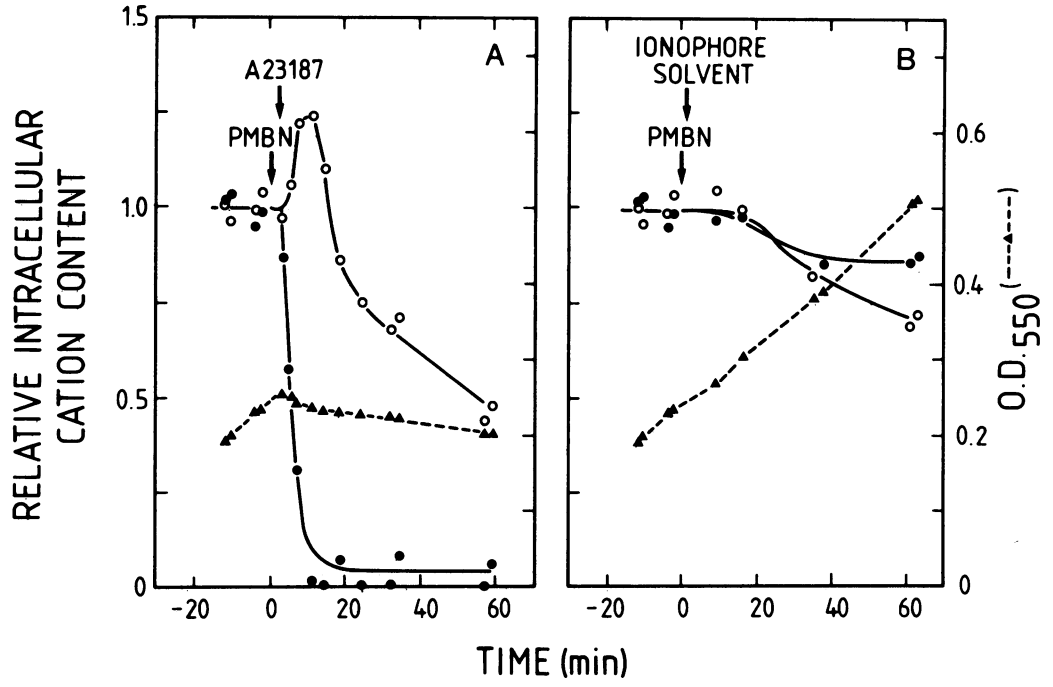

FIG. 5. Effect of KCl supplement in the medium on the K^+ transport in *E. coli* B during and after polymyxin B nonapeptide- plus A23187-induced Mg^{2+} efflux. Cells were grown exponentially in tryptone medium supplemented with 50 mM KCl at 37°C and permeabilized with 5 μg of polymyxin B nonapeptide (PMBN) per ml and 0.75 μg of A23187 per ml (A). The intracellular Mg^{2+} (●) and K^+ (○) content and optical density at 550 nm ($O.D._{550}$) (▲) were determined as described in the legend of Fig. 1 and in the text. In a control experiment (B) A23187 was replaced by the ionophore solvent.

growth has been found to be 90 to 110 mM (11), then most Mg^{2+} is in a bound form. The observed rapid and complete A23187-induced Mg^{2+} effluxes (within 10 to 15 min) show that a rapid dynamic equilibrium exists between free and bound Mg^{2+} in vivo.

The main criterion for using ionophore A23187 in biological systems has been its proposed selectivity for divalent over monovalent cations (17). However, the K^+ and Na^+ transports observed in some biological systems after A23187 treatment suggest that this is not so (4, 7, 14, 19). In other cases, the observed monovalent cation transport (at least in the case of K^+) has been interpreted as a secondary reaction that results from the Mg^{2+} transport (3, 17). Although we cannot exclude the possibility that A23187 could also mediate some K^+ and Na^+ transport under particular conditions, our results support the secondary nature of the K^+ influx observed during Mg^{2+} efflux (Fig. 5). If K^+ transport is a primary action of A23187, we would expect only K^+ efflux to occur under the conditions of our experiments. This is the opposite of the results presented in Fig. 5, where we observed a big increase (20 to 25%) in the K^+ content. Furthermore, in the presence of low outside K^+ in the tryptone medium we did not observe an influx of Na^+ (data not shown) instead of K^+ during or after Mg^{2+} efflux. The observed K^+ influx might be due to H^+-K^+ exchange that results from the increased intracellular proton concentration during Mg^{2+} efflux (2, 6, 8).

E. coli is sensitive to A23187-induced Mg^{2+} loss: a 20 to 30% reduction of the intracellular Mg^{2+} content (even with no change in the K^+ level) completely prevents growth (Fig. 2B). When looking for the determinants that control growth besides Mg^{2+} efflux, we have also to consider the A23187-induced H^+ influx. It is worth noting that a loss of 20 to 30% of the total Mg^{2+} content necessarily means a release of bound Mg^{2+} from macromolecular structures like nucleoids and ribosomes (5, 10, 20), together with a decrease of the intracellular free Mg^{2+} level. We expect that this decrease has multiple effects on bacterial metabolism rather than inhibiting only one particular Mg^{2+}-sensitive reaction. Similarly, a release of the bound Mg^{2+} might adversely affect the organization of the nucleoid and the machinery of protein biosynthesis.

We believe that the possibility for manipulating the intracellular Mg^{2+} contents, as we have shown here, opens the way for new approaches toward understanding the mechanisms that govern the functions of Mg^{2+} within the cell.

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During his stay in our laboratory, R. Cai studied the action of A23187 in comparison with ionophore W341 on gram-positive bacteria. These contributions proved invaluable to the work presented here. We are also very grateful to H. J. Seiler (Institute of Analytical Inorganic Chemistry, University of Basel) for analyzing the cations in our samples. We thank M.-A. Björnsti for critically reading the manuscript and Regula Niederhauser for typing it.

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