Mutations in a Bacterial Mechanosensitive Channel Change the Cellular Response to Osmotic Stress*

(Received for publication, August 12, 1997, and in revised form, October 7, 1997)

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MscL is a channel found in bacterial plasma membranes that opens a large pore in response to mechanical stress. Here we demonstrate that some mutations within this channel protein (K31D and K31E) evoke a cellular phenotype in which the growth rate is severely depressed. Increasing the osmolarity of the growth medium partially rescues this “slowed growth” phenotype and decreases an abnormal cytosolic potassium loss observed in cells expressing the mutants. In addition, upon sudden decrease in osmolarity (osmotic downshock) more cytoplasmic potassium is released from cells expressing the mutants than cells expressing wild-type MscL. After osmotic downshock, all cells remained viable; hence, the differences in potassium efflux observed are not due to cell lysis but instead appear to be an exaggeration of the normal response to this sudden change in environmental osmolarity. Patch clamp studies in native bacterial membranes substantiate the hypothesis that these mutant channels are more sensitive to mechanical stresses, especially at voltages approaching those estimated for bacterial membrane potentials. These data are consistent with a crucial role for MscL in the adaptation to large osmotic downshock and suggest that if the normally tight regulation of MscL gating is disrupted, cell growth can be severely inhibited.

The ability to detect mechanical force, mechanosensation, is an inherent property of essentially all living organisms. Many animals, including humans, need mechanosensation for the senses of touch, hearing, and balance, as well as for regulating cardiovascular function; plants have the ability to detect wind and gravity; even many microorganisms have a primitive sense of touch, hearing, and balance, as well as for regulating amino acid metabolism and stress responses (6–8). Mechanosensitive ion channels are the ion channels that are evoked by mechanical stress and gating by mechanical stress, bacterial MS channels have been studied extensively in E. coli (9–12) and other bacteria (13–15), but their function of importance in general has not been entirely clear. The current view is that MS channels mediate the response to sudden changes in environmental osmolarity (16–18). These changes are thought to cause an opening of the channel, allowing water to enter and increasing the cell volume. This leads to a decrease in the cytoplasmic potassium concentration and a decrease in the cytoplasmic pH. The decrease in potassium concentration is thought to be caused by the entry of potassium ions into the cell. This decrease in potassium concentration is thought to be caused by the entry of potassium ions into the cell. This decrease in potassium concentration is thought to be caused by the entry of potassium ions into the cell.

Recently, we generated a number of site-directed mutants with altered channel properties as assayed by patch clamp of native membranes (26). Here we demonstrate that one of these mutants, K31E, inhibits the growth of the bacterial cell. Analysis of this phenomenon by whole cell and electrophysiology has provided evidence that is consistent with MscL being one of the pathways for the normal release of cytoplasmic components upon osmotic downshock, and that Lys-31, a charge in or near the first transmembrane domain (18), is an important residue for normal gating of MscL in the bacterial cell.
EXPERIMENTAL PROCEDURES

Constructs, Strains, and Cell Growth—The K31D MscL mutation was generated from an M13 construct containing the mscL open reading frame (18) using the Sculpture IVM kit (Amersham) employing the following mutating oligonucleotide: GCC ATT CGG GTA TAT TGT CTC T. The mutation was confirmed by sequencing, then subcloned using the pBluescript construct (5). Generation of other mutants has been described (26). All mutants were expressed in the pBluescript construct (5). The mscL-null PB104 bacterial strain (18, 26) was used to host these expression constructs. In all experiments shown, the wild-type control was a PB104 strain containing the p5-2-2 plasmid (pB10a containing the wild-type mscL open reading frame) (5). For plate phenotypic studies (Fig. 1) and growth for electrophysiological studies (Table I and Fig. 7), cells were grown in Luria-Bertani medium (LB). For growth and whole cell physiological studies (Figs. 2–4), cells were grown in minimal medium, K10 (27): 46 mM Na2HPO4, 23 mM NaH2PO4, 8 mM (NH4)2SO4, 0.4 mM MgSO4, 6 μM FeSO4, 1 μg/ml thiamine, 10 mg/ml histidine, leucine, threonine, valine, and isoleucine, 0.2% glucose, 10 mM KCl, and 100 μM ampicillin. K10 was assumed to be 208 mouse. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM for the induction of wild-type and mutant mscL transcription; normal induction time was 1 h, 2 h for electrophysiological studies. By estimating the number of channels seen by patch clamp in an area of membrane equivalent to that of a single cell, we estimate that there are normally 10 to 100 functional MscL channels in a normal bacterial cell, absolutely zero in the mscL-null, zero to six in a mscL-null that contains the p5-2-2 expression plasmid but has not been induced by IPTG, and 50 to 200 in a mscL-null containing the p5-2-2 plasmid and has been induced. The PB104 strain contains the p5-2-2 plasmid, with or without IPTG, all had similar growth rates, demonstrating that the modest increase in MscL expression in this expression system (about 5 times endogenous levels) had little physiological consequences in normal laboratory conditions. No obvious differences in the number of functional channels per patch were observed between cells expressing the wild-type and mutant MscL. Western analysis has shown that the amount of MscL protein in intact cells expressing the wild-type was indistinguishable from the K31E mutant (26). All growth and warming of buffers for the potassium experiments was at 37 °C.

Potassium Content and Efflux Experiments—To measure the potassium loss over 15 min, cells were grown to midlog phase in K10 with or without 325 mM NaCl. After induction of MscL expression for 1 h, 10 ml of culture was harvested by filtration onto a 47-mm diameter 0.45-μm nitrocellulose filter (Millipore). The filter was placed on the wall of a 25-mm diameter 0.45-μm nitrocellulose filter (Millipore). The filter was then washed with 3 ml of wash. A normal 3-ml wash took approximately 3 to 5 s. The first 3 ml of wash was contained in a 50-ml plastic beaker and dried at 80 °C to 95 °C overnight, and then the solutes were resuspended in 3 to 4 ml of double-distilled water. The solution was then assayed for potassium by flame photometry (Buck Scientific, PFP7).

Cell Viability Studies—Cells grown in K10 to midlogarithmic growth were incubated for an additional 1 h induced or uninduced. The OD650 of each culture was determined at harvest, and the cells were serially diluted into LB medium and plated in triplicate onto LB plates containing 100 μg ampicillin. Colony forming units (CFU) were determined per OD650 unit. To determine the viability after downshock, the same procedure was used except that the cells were grown in K10 with 325 mM NaCl. The second time point was taken at 15 min and was again 1 ml of culture. The potassium efflux assays were designed to measure the decrease of potassium due to osmotic downshock and were performed as negative voltages (positive pipette voltages), but channel currents are presented as upward. The pipette solution was: 200 mM KCl, 90 mM MgCl2, 10 mM CaCl2, and 5 mM HEPES adjusted to pH 6.0; the bath solution was the same plus 0.3 M sucrose. Pressure was assessed using a pressure transducer (Micro Switch; Omega, Stamford, CT) calibrated by a pneumatic transducer tester (Bio-Tec, Winooski, VT) and is presented normalized to MscS (applied pressure/threshold pressure required to open MscS in the same patch) as previously established (26). Data were acquired with 10 kHz filtration and a sampling rate of 30 kHz and analyzed with pCLAMP6 software.

RESULTS

Expression of Mutant MscL with a Charge Change at the Lys-31 Position Leads to a Slowed-growth Plate Phenotype—Cells expressing one of two site-directed MscL mutants, K31D or K31E, were assayed for a plate phenotype. Because the wild-type and mutated mscL genes were under the transcriptional control of an inducible promoter, expression could be chemically induced by IPTG. As expected, on plates that did not contain IPTG, all cells grew equally well (Fig. 1, left). This growth was comparable to that of the wild-type strains or mscL-null mutants (not shown). However, on plates containing IPTG, the growth of cells expressing the K31D or K31E mutant was severely depressed (Fig. 1, right). No growth retardation was observed on IPTG plates for cells expressing K31R, K31C, or K31I (not shown), suggesting that the plate phenotype observed was due to the charge reversal at the Lys-31 position.

Cells Expressing the K31D or K31E Mutant MscL Show a Slowed-growth Phenotype in Liquid Medium That Is Partially Reversed by Increasing the Osmolarity of the Medium—Growth rates of cells expressing one of these mutants or wild-type MscL were measured in a liquid medium (Fig. 2A). Induction of expression led to a severe slowed-growth phenotype for cells expressing the mutants but not the wild-type MscL. Interestingly, the slowed-growth phenotype of the mutants in these liquid cultures did not appear as pronounced as on plates, presumably because of differences in the assays. Viability studies demonstrated that the slowed increase in OD650 of liquid cultures expressing the mutants cannot be explained by death of a fraction of the population. In a typical experiment, the number of viable K31E-containing cells prior to induction (1.5 × 108 CFU/OD650 unit) remained the same after 1 h of...
induction (1.5 $\times 10^8$ CFU/OD$_{650}$ unit) and was not different from the cultures expressing the wild-type MscL (1.5 $\times 10^8$ and 1.4 $\times 10^8$ CFU/OD$_{650}$ unit before and after a 1-h induction, respectively).

Addition of 325 mM NaCl increased the growth rate of the cells expressing the mutant MscL (Fig. 2B). Although the growth rate of cells expressing the MscL wild-type was decreased with increasing NaCl concentrations (Fig. 3, top), as little as 100 mM NaCl increased the growth rate of the cells expressing the K31D and K31E mutants, and maximum growth rates were observed at 200–300 mM NaCl (Fig. 3, bottom). As the concentration of NaCl increased to 500 mM, the growth rate of the cells expressing these mutants approached that of cells expressing the wild-type MscL.

To determine whether the remediation of the slow-growth phenotype was specific for NaCl, or a function of increased medium osmolarity, we tried two other osmolites: KCl and sorbitol. As seen in Fig. 4, all three osmolites inhibited growth of all uninduced cells. As expected, the growth rates of induced wild-type MscL cultures were similar to that of uninduced cells in all media tested. However, the poor growth of the cells expressing the K31D or K31E mutant was remediated by each of the osmolites. These data are consistent with the hypothesis that medium osmolarity is the stimulus for remediation of the slow-growth phenotype.

IPTG-induced cultures that were placed in medium containing 325 mM NaCl grew as fast in the first 0.5 h as in the next 1.5 h (wild-type: 0.41 versus 0.38 h$^{-1}$; K31D: 0.23 versus 0.23 h$^{-1}$; K31E: 0.25 versus 0.21 h$^{-1}$ for the two growth rates); there was no significant lag to the remediation of the slowed-growth phenotype. Therefore, it does not seem likely that time-dependent transcriptional and translational events are responsible for the osmotic remediation.

Cells Expressing K31D or K31E Mutant MscL Show an Abnormally Large Potassium Loss That Is Partially Reversed by Increasing the Osmolarity—To determine if the cells expressing the mutants lost cytoplasmic solutes at an increased rate, we measured the intracellular potassium of cells after being placed in K0, a potassium-free buffer (Fig. 5). As expected, if uninduced cells were grown in K10, then placed in K0, they lost only a small proportion of their potassium over the course of 15 min (about 30%). In contrast, if the cells were induced with IPTG for 1 h prior to being placed in buffer, the cells expressing mutant MscL not only started out with less internal potassium (60% that of cells expressing wild-type MscL), but lost substantially more potassium in 15 min (cells expressing mutants lost 70 to 80%, cells expressing wild-type lost 30%). Uninduced cells grown at high osmolarity accumulated about 1.5 times as much internal potassium as those grown in lower osmolarity medium and lost only a small amount of potassium upon being placed in K0 for 15 min (10–15%). Of induced cells grown at high osmolarity, those expressing the mutants started at internal potassium levels 85% of that of cells expressing wild-type MscL. Note that although the cells expressing mutants still lost more potassium than wild-type expressing cells (30% versus 12%), this loss was significantly less than the loss of potassium in low osmotic medium (30% versus 70–80%). These data demonstrate that cells expressing the mutants lose more potassium than cells expressing wild-type MscL and that this loss is exacerbated by low osmolarity.

Upon Osmotic Downshock, Cells Expressing K31D or K31E...
Mutants Retain Less Cytoplasmic Potassium than Cells Expressing Wild-type MscL—When bacteria are challenged with a rapid osmotic downshock, many of the smaller cytoplasmic components are jettisoned into the medium, yet the bacteria remain viable (21–25). One of the characteristics of this phenomenon is that the solute efflux is very rapid and transient in nature (24). We therefore tested whether the cells expressing mutants lost more potassium than cells expressing wild-type MscL when subjected to such an osmotic downshock. In contrast to the experiments above where changes in potassium levels were measured over a long time course (15 min) after essentially no changes in osmolarity, in this experiment the cells were osmotically downshocked using the wash solution; hence, rapid changes in potassium concentration occurring in less than 5 s were being observed (see "Experimental Procedures"). As seen above in Fig. 5, when induced for 1 h after growth in high osmolarity medium, no significant differences were observed between the total amount of internal potassium in the cells expressing wild-type versus the K31D or K31E mutants (all contained about 300 μM/OD650 unit). If, however, the cells were exposed to an osmotic downshock, from the starting 858 mosM to less than 550 mosM, less internal potassium was measured. As shown in Fig. 6, less potassium remained in the cells expressing K31D and K31E mutants relative to the wild-type when the shocking buffer was between 600 and 200 mosM. These data cannot be attributed to differences in MscL expression because it has been previously demonstrated by Western blot of membrane proteins that the K31E mutant is expressed at levels indistinguishable from that of the expressed wild-type MscL (18). Nor can the results of these experiments be attributed to lysis of the mutant expressing cells because even at the largest downshock, the cells retained 100% viability.
makes it difficult to acquire data at these voltages. However, once a pressure at which Mscl activity is observed at $\pm 20$ mV is clamped, the voltage potential can sometimes be changed to $\pm 100$ mV for short periods of time, then returned to $\pm 20$ mV without breaking the seal of the patch. When successfully performed with the wild-type channel, an increase in the probability of opening ($P_o$) is observed (Fig. 7A, left). Similar results were obtained with the K31E mutant (Fig. 7A, right); however, the increase in $P_o$ consistently seemed greater for the mutant relative to wild-type Mscl.

Quantitation of the fold increase in $P_o$ between $\pm 20$ mV and either $\pm 80$ or $\pm 100$ mV demonstrated that as the voltage became more negative, approaching that of the normal resting potential of E. coli, the increase in $P_o$ was greater for the K31E mutant than wild-type Mscl (Fig. 7B).

These data are consistent with the hypothesis that a charge change at the Lys-31 position leads to more severe changes in single channel properties as voltages approach the bacterial resting potential; therefore, the data presented in Table I on the pressure sensitivity of the Lys-31 mutants may be a gross underestimate of the increase in pressure sensitivity of these mutants in vivo.

**DISCUSSION**

Previously, we expressed wild-type and mutant Mscl channels in a mscl-null E. coli strain and identified several site-directed mutations that led to changes in Mscl channel activities as assayed by patch clamp (26). We found that mutations at two sites, Lys-31 and Gln-56, could lead to changes in channel kinetics and/or shifts in the pressure sensitivity curve. We have assayed several of these mutants, including all that led to dramatic changes in channel dwell times or pressure sensitivity, for their ability to evoke a plate phenotype when expressed (not all are shown in this study). We previously found two mutations that led to channels that opened with less pressure, K31E and Q56P. These two mutants were the only ones that evoked an obvious plate phenotype. The Q56P mutation led to a slow growth plate phenotype that is less severe than the K31E mutation and is only seen at a lower temperature (22 °C) (not shown). Patch clamp studies, however, initially suggested that Q56P was a more severe mutation than K31E because it had larger shift in the pressure sensitivity curve at moderate membrane potentials (26).

Furthermore, under the patch clamp conditions used, the Lys-31 Mscl mutant channels still opened at higher pressures than Mscl. Therefore, the observed
FIG. 7. The increase in channel open probability (Po) observed at more negative membrane potentials is greater for the K31E mutant than the wild-type MscL. A, single-channel recordings from an excised patch from cells expressing wild-type (left) or K31E (right) MscL. The top traces show the channel recordings at −20 mV with the pressure clamped at approximately 150 mm Hg. The middle traces show the same patch after the membrane voltage was changed to −100 mV; note there is an increase in Po even though the pressure is the same as in the upper traces and that this increase appears larger for the mutant than the wild-type. The baseline (no MscL channels open) is marked in these traces with a dashed line; note that for simplicity, openings in all cases are shown as upward. The bottom traces are from the same patch at the same pressure several seconds after the membrane potential was returned to −20 mV. B, the fold increase in Po at different voltages as compared with that observed at −20 mV. The points and error bars are the mean ± S.E. for 3 or more independent measurements; a total of 99 measurements are represented.

increase in sensitivity to pressure does not seem sufficient to explain the phenotype if poor growth is due to excessive loss of cytoplasmic solutes; the critical pressure for solute release from MS channels ought to be set by the lower threshold of MscS and not MscL. Presumably, differences in environment between the intact cell and the membrane patch account for this discrepancy. One of these differences is the membrane potential. Here we present evidence that suggests that the pressure sensitivity of wild-type MscL is voltage-sensitive; the channel becomes more sensitive to membrane tension at more negative membrane potentials. This voltage dependence of the pressure sensitivity is apparently exaggerated for the K31E mutant; the baseline (no MscL channels open) is marked in these traces with a dashed line; note that for simplicity, openings in all cases are shown as upward. The bottom traces are from the same patch at the same pressure several seconds after the membrane potential was returned to −20 mV. B, the fold increase in Po at different voltages as compared with that observed at −20 mV. The points and error bars are the mean ± S.E. for 3 or more independent measurements; a total of 99 measurements are represented.

Because of the rapid kinetics of growth recovery when cells expressing the mutants are placed in high osmotic medium, it is possible that the phenotype observed here is not directly due to the inhibition of the MS channels but to something else. For example, it is possible that the expression of the mutants changes the cytoplasmic environment in such a way that it affects the growth of the cell. This possibility is currently being investigated.

One crucial piece of evidence to support the assertion that bacterial MS channels are the pathway through which bacteria jettison cytoplasmic components upon osmotic downshock was the demonstration of a direct correlation between the concentration of Gd³⁺ required to block bacterial MS channels and the rate of potassium efflux (9). This correlation suggests that the MS channels are responsible for the efflux of potassium (and presumably other solutes) when the cell is exposed to osmotic downshock.

Several studies have demonstrated the rapid release of some of the cytoplasmic contents into the medium when a bacterial cell is subjected to an osmotic downshock (21–25). The components released include potassium, proline, glutamate, ATP, lactose, and trehalose. Many of these molecules are osmoprotectants that are either synthesized in, or transported into, the cytoplasm to a high concentration when growth is in high osmolarity medium. This rapid efflux is thought to be a means by which bacteria rapidly adjust to hypopsomotic stress. The initial discovery of channels in the membrane of E. coli with very large conductances and gated by tension (9) suggested pathways for this release of components. A few studies have provided evidence consistent with this hypothesis. MS channel activities in E. coli, with conductances consistent with those of MacM and MacS, have been observed in response to osmotic changes when assayed in the excised (34) as well as the whole-cell (protoplast) (35) patch clamp configuration (MacL apparently was not observed because the greater stimulation required to open this channel is difficult to achieve in the whole-cell configuration). Also, the MS channel activities (35, 36) and the MacL protein (18, 20) have both been localized to the inner membrane, the principal cellular barrier of the E. coli cell. Finally, it is known that gadolinium (Gd³⁺) blocks MS channels in a wide range of systems including bacteria (4), and a direct correlation has been demonstrated between the concentration of Gd³⁺ required to block bacterial MS channels and the rate of potassium efflux (9). This correlation suggests that the major pathway for these molecules may normally be independent of the MS channels blocked in this study. None of these studies, however, is without its caveats, nor do the studies directly demonstrate that the MS channel activities observed in patch clamp play a role in the efflux response upon osmotic downshock.

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placed in hyperosmotic medium, and maximal changes are not seen until 30 to 35 min. One might expect that these changes may even occur more slowly in the cells expressing the K31D or K31E mutants, which have a doubling time of about one-fourth of their normal rate. On the other hand, potassium pump activity has been reported to occur very rapidly when the osmolarity of the medium is increased (40). Perhaps this accounts for the observation that when KCl is used as an osmolyte, the cells have a significantly faster growth rate than when NaCl or sorbitol is used (Fig. 4; p < 0.05 but > 0.01). However, it seems unlikely that this increased potassium pumping activity can account for all of the data because the potassium loss observed over time in potassium-free buffer is partially inhibited by increasing the buffer osmolarity (Fig. 5); in this experiment there should be no potassium to pump into the cell. Hence, the simplest interpretation is that the partial growth rescue is at least partially due to a direct effect, perhaps a slight decrease in turgor force across the cell membrane in the higher osmotic medium, that allows for better growth of the mutants by eliminating improper gating of the mutant channel. In this scenario, the increase in turgor at lower osmolarity would cause the channel to open in vivo with some low frequency, transiently decreasing the turgor by causing the release of solutes and the breakdown of the proton gradient that serves as a major energy source (see Refs. 41–43 for reviews). Thus, the cells expressing these mutant MscLs would be in a state of catch-up in their metabolic energy, as well as having to resynthesize or recover any lost osmolites, metabolites, or amino acids. Preliminary results suggest that the average internal pH of a population of cells expressing the K31D and K31E mutants is similar to that of cells expressing wild-type MscL (not shown). This implies that the mutant MscL channels open infrequently in vivo and that the proton gradient, and presumably also the turgor and metabolic state, quickly recovers. This is perhaps not surprising, given the observation that cells expressing the K31D and K31E MscL still grow, albeit at a slower rate.

The increased efflux of potassium upon osmotic downshift strongly supports the model that, in vivo, the mutant MscL channels open more easily upon membrane tension caused by low or rapidly decreasing osmolarity. These studies are complemented by the in vitro patch clamp studies that demonstrate that the mutant channels gate more easily by suction in the pipette, especially at voltages approaching those predicted for bacterial membrane potentials. Hence, the data suggest not only that MscL is one of the pathways for cytoplasmic components to efflux upon osmotic downshift, but that if the normally tight regulation of MscL gating is disrupted, the rate of cell growth can be severely inhibited. Genes predicted to encode MscL homologues have been found in several bacterial species, including some pathogens (7). Therefore, if misgating of MscL channels could be accomplished pharmacologically rather than genetically, it could be the genesis of a new class of antimicrobial agents.

A plate phenotype, such as that observed for the K31D and K31E mutants (Fig. 1), demonstrates that screens may be developed to select for functional mutations within MscL and other MS channels. The use of an expression plasmid that allows one to turn on or off expression within the cell and the ability to selectively mutagenize only the target gene are some of the advantages this system has for such studies. A simple screen for colonies that show a “slowed or no growth” phenotype when a randomly mutagenized MscL is expressed has already been employed, leading to the isolation of 19 single-site mutations. Interestingly, one of the randomly generated mutations is at Lys-55, only one amino acid away from the Gln-56 mutants presented previously (26). More impressive, however, is the observation that 14 of the mutants isolated are clustered between amino acid residues 13 and 30, very close to the Lys-51 mutants described here. Seven of these fourteen not only are in this region but, like K31D and K31E, cause the MscL protein to become more electrically negative. Given the proximity of this critical region to the membrane (18), an obvious question is whether this protein domain contributes to the pore or gate of the channel. In the future, the use of genetic screens to identify new functional regions within MscL as well as to identify new proteins involved with osmotic adaptation, in combination with site-directed mutagenesis, whole cell physiology, and patch clamp techniques, promises to give insight into the physiological and functional significance of domains of the MscL protein and other MS channels in bacteria.

Acknowledgments—We thank Drs. S. I. Sukharev, X. Ou, and P. C. Moe for their helpful discussions and critical reading of the manuscript. We also thank Dr. I. R. Booth and all of the members of his laboratory for their helpful discussions and infinite patience while teaching P. B. several techniques during his visit to the University of Aberdeen, Marischal College.

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