Amiloride, a Specific Inhibitor for the Na⁺-driven Flagellar Motors of Alkalophilic Bacillus*

Shigeru Sugiyama†, Edward J. Cragoe, Jr.§, and Yasuo Imae††

From the †Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464, Japan and §Merck Sharp and Dohme Research Laboratories, West Point, Pennsylvania 19486

Received for publication, December 17, 1987

Vol. 263, No. 17, Issue of June 15, pp. 8215-8219, 1988
Printed in U.S.A.

Na⁺-driven flagellar motors of alkalophilic Bacillus were found to be inhibited by amiloride, a potent inhibitor for many Na⁺-coupled systems. A concentration of 0.5 mM of amiloride completely inhibited motility but showed almost no effect on the membrane potential, the intracellular pH homeostasis, and the ATP content of the cells. Furthermore, the activity of a Na⁺-coupled amino acid transport system was reduced only by half by this concentration of amiloride. Thus, the inhibition of motility of alkalophilic Bacillus by amiloride was rather specific.

The inhibition of motility produced by amiloride was restored by increasing Na⁺ concentrations in the medium. Kinetic analysis of the data revealed that the inhibition was competitive with respect to the concentration of Na⁺ in the medium. Therefore, it is quite logical to assume that amiloride inhibits the rotation of the Na⁺-driven flagellar motors of alkalophilic Bacillus by competing with Na⁺ at the force-generating site of the motor. Some amiloride analogs known to selectively inhibit Na⁺ channels were potent inhibitors for the flagellar motors, suggesting that the Na⁺-interacting site of the motors has some similarity to that of the Na⁺ channels.

Flagellated bacteria swim by rotating their flagella, and each flagellum is powered by a motor embedded in the cytoplasmic membrane. The energy source of this tiny motor is not ATP directly but the electrochemical potential gradient of specific ions. With respect to neutrophilic bacteria such as Bacillus subtilis, Streptococcus sp., and Salmonella typhimurium, the rotation of their motors is coupled with the influx of H⁺, which is driven by the electrochemical potential gradient of H⁺, namely ΔG°H⁺ (1-3). However, the motors of alkalophilic bacteria, such as obligately alkalophilic Bacillus, are powered by the electrochemical potential gradient of Na⁺, ΔG°Na⁺ (4-6). Thus, the bacterial flagellar motors are the microengines that convert the electrochemical energy of ions to the mechanical work. Based on the coupling ions, the motors are classified into two types; the H⁺-driven motors of neutrophiles and the Na⁺-driven motors of alkalophiles. At the present time no apparent structural difference has been reported between these motors (7).

Many attempts have been made to elucidate the rotation mechanism of flagellar motors based on structural, genetical, energetical, and theoretical approaches (see Refs. 8 and 9 for reviews). According to a recent model for the motor structure proposed by Yamaguchi et al. (10), rotation of flagellar motors is suggested to occur at the interface between the membrane-embedded innermost ring (M ring) of the flagellar basal structure and the membrane proteins surrounding the M ring. The power for motor rotation is assumed to be generated by some of the surrounding membrane proteins such as mot A and mot B gene products (11).

In the case of H⁺-driven flagellar motors, at least one of the force-generating units of the motor is necessary to interact with H⁺ on the outside of the membrane and translocate it through the membrane. To analyze the interaction between H⁺ and such a force-generating unit, Conley and Berg (12) used several chemical modification reagents and suggested that some histidine residues of protein molecules might be involved in the interaction. However, this type of approach has some inherent difficulties for the identification of the force-generating units, since these chemical modification reagents interact with various proteins rather nonspecifically.

We have reported that alkalophilic Bacillus has the Na⁺-driven flagellar motors (4, 5, 13, 14). In this peculiar motor, at least one of the force-generating units is required to interact with Na⁺ on the outside of the membrane. If the Na⁺-interacting site of such a unit would have some similarities to that of other Na⁺-coupled systems, some inhibitors for the Na⁺-coupled systems would be expected to inhibit the Na⁺-driven flagellar motors. Based on this concept, we have tested several inhibitors on motility of alkalophilic Bacillus.

In this report, we describe that amiloride, a weak inhibitor of the Na⁺/H⁺ antiporter and a potent inhibitor of Na⁺ channels of various organisms (15, 16), rather specifically inhibited motility of alkalophilic Bacillus. Kinetic analysis of the data demonstrated that amiloride inhibited the Na⁺-driven flagellar motors of alkalophilic Bacillus by competing with Na⁺ in the medium.

MATERIALS AND METHODS

Bacterial Strains and Cell Growth—The obligately alkalophilic Bacillus strains used were Bacillus firmus RAB (6, 13) and alkalophilic Bacillus sp., 202-1 and YN-1 (17, 18). Cells were grown at 37 °C with shaking in AB-4 medium at pH 9.5 as described previously (7). An example of a neutrophilic Bacillus, Bacillus subtilis RM25 (19) was used, and the cells were grown as described previously (20).

Measurement of Swimming Speed—The swimming speed of alkalophilic Bacillus cells was measured by the photographic method as described previously (7). The cells at late logarithmic phase of growth were harvested by centrifugation and resuspended in TG medium consisting of 25 mM Tris-HCl buffer (pH 9.5) and 5 mM glucose. In most cases, 5 mM NaCl was added to TG medium. For the measurement of swimming speed, the cells were diluted 200-fold with TG medium supplemented with various concentrations of salts, and...
swimming speed of the cells was measured at 25 °C within a minute. For some experiments, the total salts concentration supplemented to TG medium was adjusted to 50 or 55 mM by the addition of a suitable amount of choline chloride. When amiloride or its analogs were added, swimming speed was measured 1 min after the addition of the drug.

Motility of *B. subtilis* RM125 was measured as described previously (20).

**Measurement of α-Aminoisobutyrate and Glucose Transport**—The transport of α-aminoisobutyrate (AIB) and glucose was measured by the filtration method as described previously (14). Cells were harvested, washed twice with TG medium supplemented with 5 mM NaCl, and resuspended in the same medium. For the measurement of AIB transport, cells (7 × 10⁸ cells or 0.3 mg of protein/ml) were mixed with various concentrations of amiloride and incubated for 2 min with shaking at 35 °C. Then, 50 μM [³⁵S]AIB (5 mCi/mm, DuPont-New England Nuclear) was added to the cells. After 5 min of incubation at 35 °C, samples (0.5 ml) were filtered by using a membrane filter. After washing the filter with 2 ml of the medium twice, the radioactivity trapped on the filter was measured by a liquid scintillation spectrometer (Aloka model LSC-701N). The cells treated with 10 μM gramicidin D were used as the zero incorporation control.

The transport of glucose was measured as above except that glucose was omitted from TG medium and 0.3 mm [³⁴C]glucose (0.5 mCi/mm, DuPont-New England Nuclear) was used instead of [³⁴C]AIB. Samples were withdrawn after incubation for 1 min. The cells treated with 5% toluene were used as the zero incorporation control.

**Measurement of Membrane Potential, Intracellular pH, and ATP Content**—Membrane potential and intracellular pH of the cells were measured by using [³H]triphenylmethylphosphonium ion and [³¹C]methylamine, respectively, as described previously (14). Gramicidin D-treated cells were used as controls.

ATP content of the cells was measured by the luciferin-luciferase method as described previously (6).

**Amiloride Analogs**—Amiloride and its analogs, benzamil, phenamil, 5-(N-ethyl-N-isopropyl)amiloride (EIPA), and 5-(N-methyl-N-isobutyl)amiloride (MIBA), were prepared as previously described (21).

**RESULTS**

**Motility Inhibition by Amiloride**—*B. firmus* RAB cells have been reported to require the presence of Na⁺ in the medium for their motility (6, 13). In TG medium containing 5 mM NaCl, the cells swam at the speed of about 20 μm/s, and the addition of amiloride caused an immediate reduction in the swimming speed. As shown in Fig. 1A, the swimming speed of RAB cells was gradually decreased with increasing concentrations of amiloride, and 0.5 mM amiloride was sufficient for the complete inhibition of motility.

The inhibitory effect of amiloride on the other membrane functions of RAB cells under these conditions was less significant as compared to the effect on motility. The AIB transport, which is known to be Na⁺-coupled (6, 13), was quite resistant to amiloride; the transport was reduced only by 50% at the concentration of amiloride where motility was completely inhibited (Fig. 1B). Glucose uptake, which is Na⁺-independent (13), was also reduced by half by the same concentration of amiloride (Fig. 1C).

Consistent with these results, amiloride concentrations up to 1 mM only slightly affected the bioenergetic parameters of RAB cells. The membrane potential, which is a major component of the driving force of the Na⁺-coupled systems, showed less than 10% reduction by 1 mM amiloride (Fig. 2A). The intracellular ATP level was slightly increased by the addition of amiloride (Fig. 2B). Thus, the inhibition of motility by amiloride was not due to the reduction in the cellular energy level.

High concentrations of amiloride have been reported to inhibit the Na⁺/H⁺ antiporter in some organisms (15), and the intracellular pH of RAB cells has been reported to be maintained by the function of Na⁺/H⁺ antiporter (6, 13).

**FIG. 1.** Effect of amiloride on motility and transport of RAB cells. A, swimming speed. Cells in TG medium containing 5 mM NaCl were mixed with amiloride, and motility was measured within 1 min. B, rate of AIB transport. Cells in TG medium containing 5 mM NaCl were preincubated with various concentrations of amiloride for 2 min at 35 °C. Then, the cells were incubated with [³¹C]AIB for 5 min. C, rate of glucose transport. The experimental procedure was similar to B, except that glucose was omitted from the TG medium and that [³¹C]glucose was used instead of [³¹C]AIB.

**FIG. 2.** Effect of amiloride on the membrane potential and intracellular ATP level of RAB cells. A, membrane potential and B, ATP content of the cells were measured in TG medium containing 5 mM NaCl after incubation with various concentrations of amiloride for 5 min at 35 °C.

Therefore, the disruption of the intracellular pH homeostasis in RAB cells due to the inhibition of the Na⁺/H⁺ antiporter by amiloride was assumed to be the cause of the motility inhibition. However, as shown in Table I, the addition of amiloride in concentrations up to 2 mM did not affect the intracellular pH homeostasis; the intracellular pH was main-
Effect of amiloride on the intracellular pH homeostasis of RAB cells

Cells in 50 mM 2-(cyclohexylamino)ethanesulfonate buffer (pH 9.5) containing 5 mM glucose and 5 mM NaCl were incubated with amiloride for 2 min at 35 °C. The intracellular pH was then measured by using [14C]methyamine.

Amiloride Intracellular
added pH
mM
0 8.4
1 8.6
2 8.5

Effect of various salts on the motility inhibition by amiloride

RAB cells in TG medium containing 5 mM NaCl were preincubated with 0.5 mM amiloride for 3 min, and various salts of 50 mM were then added. The swimming speed of the cells was measured 1 min later.

| Salts added          | Swimming speed (μm/s) |
|----------------------|-----------------------|
| None                 | 0                     |
| NaCl                 | 20.3                  |
| LiCl                 | 9.7                   |
| KCl                  | 11.3                  |
| Choline chloride     | 11.0                  |

Other alkalophilic Bacillus strains, 202-1 and YN-1, showed essentially the same results (data not shown). However, in the case of a neutrophilic Bacillus strain, B. subtilis RM125, amiloride at 0.5 mM showed no effect on motility, and motility was reduced only by half even at 2 mM amiloride (data not shown). These results indicate that amiloride only inhibits the Na+-driven flagellar motors.

Effect of Na+ on the Amiloride Action—Amiloride in many cases inhibits the Na+-coupled systems by competing with Na+ (15). It was found that the motility inhibition by amiloride was quickly and almost completely restored by the addition of excess NaCl (Table II). The addition of other salts, however, also caused a considerable restoration of motility, whereas these salts showed a weaker effect than NaCl. These results indicate that the motility inhibition by amiloride was reversible but significantly affected by the ionic strength of the medium.

To eliminate the effect of ionic strength on the action of amiloride, the total amount of salts added to TG medium was fixed to 50 or 55 mM by altering the amount of choline chloride in the medium. In the presence of 5 mM NaCl and 45 mM choline chloride, the swimming speed of RAB cells was gradually decreased with increasing concentrations of amiloride, and at 1 mM amiloride, the speed was reduced by half (Fig. 3). Amiloride concentrations higher than 1.5 mM caused greater inhibition of motility, but this was accompanied by a gradual decrease in the membrane potential even in the presence of 50 mM NaCl (data not shown). To avoid this complex effect of amiloride on RAB cells, the amiloride concentration was fixed at 1 mM for the following experiments.

Na+-specific restoration of the motility inhibition by amiloride—When the amount of added salts was fixed at 55 mM, the inhibition of motility of RAB cells by 1 mM amiloride was gradually restored with increasing concentrations of NaCl, and the swimming speed was almost completely restored at 55 mM NaCl (Fig. 4). Increasing concentrations of KCl was without effect, but similar studies with LiCl produced a slight increase in inhibition. Thus, employing a fixed ionic strength, only Na+ can restore the inhibited motility produced by amiloride.

Kinetic Analysis of the Action of Amiloride on Motility—To investigate the quantitative relationship between amiloride and Na+, the Na+ dependence of swimming speed of RAB cells was analyzed with and without 1 mM amiloride. The amount of salts added to TG medium was fixed at 50 mM as before by altering the amount of choline chloride. As shown in Fig. 5, swimming speed of the cells in the absence of amiloride was sharply increased by increasing the Na+ concentration up to 10 mM, and then, only a slight increase was observed with a further increase in Na+ concentration. In contrast, the swimming speed of the cells in the presence of 1 mM amiloride was moderately and rather systematically increased with increasing concentrations of Na+.

From these results, it is clear that the inhibitory effect of amiloride was more significant at lower Na+ concentrations.
was measured in the presence of amiloride, which is proportional to the Na\(^+\) influx through the flagellar motors of the cell/unit of time. Then, the work done by the cell is a function of the rate of Na\(^+\) influx through the motors, and the Na\(^+\) concentration in the medium. The swimming speed of the cell was measured as described in Fig. 4. The data were then transformed as described in Fig. 6 and shown as the Dixon plot.

**Fig. 5.** Effect of amiloride on the relationship between Na\(^+\) concentration and the swimming speed of RAB cells. Experimental procedures were as described in Fig. 4. The swimming speed was measured in the presence (●) or absence (○) of 1 mM amiloride. The data were the mean of three independent experiments.

**Fig. 6.** Double-reciprocal plot of the estimated amount of the Na\(^+\) influx through the motors and Na\(^+\) concentration. The data shown in Fig. 5 were transformed based on the equation described in the text. For the calculation of the \(\Delta \mu \text{Na}\(^+\)\), the intracellular Na\(^+\) was assumed to be 30 mM (5). For details, see the text.

**Fig. 7.** Effect of various concentrations of amiloride on the estimated Na\(^+\) influx through the motors. RAB cells in TG medium containing 2 mM ○, 5 mM △, or 20 mM ● NaCl were added by various concentrations of amiloride, and the swimming speed was measured as described in Fig. 4. The data were then transformed as described in Fig. 6 and shown as the Dixon plot.

**Fig. 8.** Effects of amiloride analogs on the swimming speed of RAB cells. Cells in TG medium containing 5 mM NaCl were mixed with various concentrations of amiloride (○), MIBA (●), EIPA (△), phenamil (△), or benzamil (▲). The swimming speed of the cells was measured within 1 min after the addition of analogs.

**DISCUSSION**

Amiloride, a well known inhibitor for many Na\(^+\)-coupled systems in various organisms, was found to be a potent
Inhibitor for the flagellar motors of alkalophilic Bacillus. We showed that the inhibition of motility by amiloride was not a secondary effect on the reduction in the cellular energy pool such as the membrane potential and ATP content or the disruption of the cellular pH homeostasis. We also showed that the cellular transport systems including a Na+-coupled amino acid transport were only slightly affected by amiloride. From these results, we can conclude that amiloride is a rather specific inhibitor for the flagellar motors of alkalophilic Bacillus.

At a fixed ionic strength, the inhibition of motility by amiloride was specifically and completely restored by increasing Na⁺ concentrations in the medium. Kinetic analysis of the data shown in Fig. 6 revealed that amiloride inhibited motility by competing with Na⁺ in the medium. For the analysis, we assumed that the efficiency of the motor, ϵ, is virtually constant. Recently, Lowe et al. (26) reported that the efficiency varied with the rotation speed of the motor. So, we re-evaluated our kinetic data with the assumption that ϵ is 0.9 at zero speed, decreases linearly with the increase of swimming speed, and reaches 0.05 at the maximum speed as shown by Meister et al. (23). Fortunately, however, we again obtained the results indicating that amiloride inhibits motility by competing with Na⁺ in the medium (in this case, Kᵢ for amiloride was 0.2 mM and showed no change, and the Kᵢ values for Na⁺ were also only slightly decreased as 1.3 and 16.7 mM in the absence and presence of 1 mM amiloride, respectively). Thus, all these kinetic results confirm that the Na⁺ influx drives the flagellar motors of alkalophilic Bacillus and indicate that amiloride inhibits this Na⁺ influx by competing with Na⁺ in the medium. Therefore, it is reasonable to speculate that the competition between amiloride and Na⁺ occurs at the Na⁺-interacting site located in the medium side of the energy-coupling unit of the Na⁺-driven flagellar motors.

The kinetic data also indicate that the relationship between Na⁺ concentration in the medium and the Na⁺ influx through the motor is apparently of the Michaelis-Menten type. This means that the interaction between Na⁺ and the Na⁺-interacting site on the motor is sufficiently fast and not a rate-limiting step. In the case of the H⁺-driven flagellar motors of Streptococcus sp., Khan and Berg (22) reported the absence of the isotope effect on the torque generation of the motors of tethered cells. This suggests that the interaction between H⁺ and the H⁺-accepting site of the motor was not rate-limiting. Recently, Meister et al. (23) measured the rotation frequency of flagellar bundles in free swimming cells of Streptococcus sp. and showed some isotope effect. However, the authors suggested that the rate-limiting process might be in the H⁺ transfer step from the H⁺-accepting site to the mechanical coupling site. There are two theoretical models of the rotation mechanism of bacterial flagellar motors; one is that the motor rotation is tightly coupled with the ion translocation (22, 24), and the other is that the coupling is assumed to be loose (25). Although our experiments were not aimed to evaluate these models, our results suggest that in any models, the first interaction of any ions with the energy coupling unit is reversible and not rate-limiting. In the case of the Na⁺-driven motors, amiloride seems to compete with Na⁺ at this first interaction site.

We showed that besides amiloride, benzamil and phenamil were potent inhibitors for the flagellar motors of alkalophilic Bacillus. These analogs are known to be very potent Na⁺ channel inhibitors with little or no effect on the Na⁺/H⁺ exchanger (27–29). The relative potencies of amiloride, benzamil, and phenamil on the Na⁺ channels (27, 28) are parallel to their relative potencies on the flagellar motors of RAB cells. Thus, the structure of the Na⁺-interacting site of the Na⁺-driven flagellar motors may have some similarity with that of Na⁺ channels but not with that of Na⁺/H⁺ exchangers.

For the identification of the Na⁺-interacting unit in the motors, the use of the radiolabeled probe which can specifically interact with and covalently bind to the target protein is quite helpful. In the case of the Na⁺ channels or Na⁺/H⁺ exchangers in some organisms, it has been reported that some amiloride analogs, such as iodoamiloride or bromobenzamil, covalently bound to target proteins after UV irradiation and irreversibly inhibited their functions (30, 31). Furthermore, a 65-kilodalton protein related to the rat renal Na⁺/H⁺ exchanger was identified by using [14C]bromo-EIPA (32). All the inhibitors reported in this paper are the reversible type and may not be useful for such a purpose. However, it is quite likely that some other amiloride derivatives might have the ability to bind covalently with the Na⁺-interacting subunit of the motor and cause irreversible inhibition on the motor. This type of research is now in progress.

Acknowledgments—We thank Professors T. A. Krulwich, K. Hotikoshi, and Y. Nosoh for bacterial strains. We also thank Professor F. Oosawa for helpful discussions.

REFERENCES

1. Matsushita, S., Shiio, J., and Imae, Y. (1977) FEBS Lett. 82, 187–190
2. Mansson, M. D., Tedesco, P., Berg, H. C., Harold, P. M., and Van der Drift, C. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3690–3694
3. Eisenbach, M., and Adler, J. (1981) J. Biol. Chem. 256, 8007–8014
4. Hirota, N., Kitada, M., and Imae, Y. (1981) FEBS Lett. 132, 278–280
5. Hirota, N., and Imae, Y. (1985) J. Biol. Chem. 260, 10571–10581
6. Kitada, M., Guffanni, A. A., and Krulwich, T. A. (1985) J. Bacteriol. 152, 1096–1104
7. Imae, Y., Matsukura, H., and Kobayashi, S. (1986) Methods Enzymol. 125, 582–592
8. Berg, H. C., Mansson, M. D., and Conley, M. P. (1982) Symp. Soc. Exp. Biol. 35, 1–31
9. Macnab, R. M., and Aizawa, S. (1984) Annu. Rev. Biophys. Biophys. Chem. 13, 51–93
10. Yanagisako, S., Aizawa, S., Kihara, M., Isomura, M., Jones, C. J., and Macnab, R. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 70–70
11. Block, S. M., and Berg, H. C. (1984) Nature 309, 470–472
12. Conley, M. P., and Berg, H. C. (1984) J. Bacteriol. 158, 382–345
13. Sugiyama, S., Matsukura, H., and Imae, Y. (1985) FEBS Lett. 178, 265–269
14. Sugiyama, S., Matsukura, H., Koyama, N., Nosoh, Y., and Imae, Y. (1986) Biochim. Biophys. Acta 827, 187–190
15. Beno, D. J. (1982) Am. J. Physiol. 242, C131–C145
16. LaBèze, E. F., Woodward, P. L., and Cragoe, E. J., Jr. (1983) J. Biochem. Biophys. Acta 778, 133–144
17. Nakamura, N., Watatane, K., and Horikoshi, K. (1973) Biochim. Biophys. Acta 279, 186–193
18. Ohita, K., Ejoyama, N., and Nosoh, Y. (1976) J. Gen. Microbiol. 86, 259–266
19. Uozumi, T., Hoshino, T., Miwa, K., Hiroinoue, S., Beppu, T., and Arima, K. (1977) Mol. Gen. Genet. 153, 65–69
20. Shiio, J., Matsushita, S., and Imae, Y. (1980) J. Bacteriol. 144, 891–897
21. Crapo, E. J., Jr., Woltersdorf, O. W., Sicking, J. B., Kwong, C. F., and Jones, J. H. (1977) J. Med. Chem. 20, 66–75
22. Khan, S., and Berg, H. C. (1987) Cell 52, 913–919
23. Meister, M., Lowe, C. L., and Berg, H. C. (1987) Cell 52, 641–650
24. Lauger, P. (1977) Nature 268, 360–362
25. Ormos, F., and Massal, J. (1982) J. Phys. Soc. Jpn. 51, 631–641
26. Lowe, G., Meister, M., and Berg, H. C. (1987) Nature 325, 637–640
27. Guthbert, A. W., and Fanetti, G. M. (1978) Br. J. Pharmacol. 63, 139–149
28. Asher, C., Crapo, E. J., Jr., and Garty, H. (1987) J. Biol. Chem. 262, 8566–8573
29. Sinchovits, L., and Cragoe, E. J., Jr. (1986) Mol. Pharmacol. 30, 112–120
30. Cobh, M. H., and Scott, W. N. (1981) Exp. Opin. Biol. 15, 65–69
31. Kleyman, T. R., Yulo, T., Ashbaugh, C., Lardry, D., Crapo, E. J., Jr., Kalin, A., and Aj Aweke, G. (1984) J. Biol. Chem. 261, 2335–2343
32. Friedrich, T., Sallotini, J., and Berckarde, G. (1986) J. Membr. Biol. 94, 253–266