Effect of Gramicidin on Methanogenesis by Various Methanogenic Bacteria

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Received 19 February 1985/Accepted 18 April 1985

Methanogenesis by *Methanobacterium thermoautotrophicum* strains was extremely sensitive to gramicidin, total inhibition being observed at 0.2 µg/ml. In contrast, methane synthesis by *Methanococcus voltae*, *Methanogenium marisnigri*, *Methanosarcina mazei*, and *Methanospirillum hungatei* were resistant to the highest concentrations of gramicidin tested (40 µg/ml), although spheroplasts of *Methanospirillum hungatei* were extremely sensitive. Other species tested showed intermediate sensitivity to gramicidin, methanogenesis inhibition occurring at 4 to 20 µg/ml.

Gramicidin is a peptide ionophore which dissipates Na⁺, K⁺, and H⁺ gradients (4). As a consequence, it dissipates both the electrical and chemical components of protonmotive force and it can also cause declines in intracellular ATP concentrations of various bacteria, including methanogenic bacteria (8, 9, 13). It can also be a powerful inhibitor of methanogenesis in methanogens, but thus far its use has been limited (8, 13).

Against *Methanobacterium bryantii*, gramicidin D (10 µg/ml) inhibits nickel transport (7) and lowers the electrical potential and ΔpH (8, 9). It also causes a large decline in intracellular ATP levels (8) and is a powerful inhibitor of methanogenesis. In contrast, gramicidin (at the same concentration) had no significant effect on methane synthesis, ATP concentration, protonmotive force, or Na⁺-dependent isoleucine transport in the fragile *Methanococcus voltae* (K. Jarrell and G. D. Sprott, unpublished data). Differential sensitivity to gramicidin by a host of methanogens was reported by Hilpert et al. (5), who used an agar diffusion test. They found that two *Methanobacterium thermoautotrophicum* strains were sensitive to the ionophore but all other strains tested, including *M. bryantii*, were insensitive.

Several workers have noted the lack of data on the susceptibility of methanogens to ionophores and inhibitors, the mode or site of action of these agents, and the need for such data for useful bioenergetic experiments (3, 10). It has been shown that some agents that are effective against eubacteria are not effective against methanogens or do not act in the classical fashion (8, 13). The reducing conditions used in methanogen studies and the unusual walls and membranes of methanogens, which may act as barriers, have been suggested as possible reasons for the observed differences (8, 13). Since gramicidin can be of importance in elucidating problems involving energy coupling in methanogens, we have further examined the effect of this ionophore on methane synthesis by many methanogens.

All organisms were obtained from Dennis Sprott, National Research Council of Canada, except for *Methanobacterium thermoautotrophicum* Marburg, which was obtained from David Nagle, University of Oklahoma, Norman. Methanogens were cultured in the following media: *Methanococcus voltae* was grown in the defined medium of Whitman et al. (14); *Methanobacterium thermoautotrophicum* strains,

Methanobrevibacter smithii, *Methanobrevibacter arboriphilus* AZ, *Methanobacterium formicicum*, *Methanobacterium G2R*, *Methanobacterium bryantii* strains, and *Methanosarcina barkeri* MS were all grown in JM medium (6); *Methanospirillum hungatei* strains were grown in JMA medium (6); *Methanogenium marisnigri* was grown in Balch medium III (2); and *Methanosarcina mazei* was grown in JM medium supplemented with 2% (wt/vol) yeast extract and 2% (wt/vol) tryptone. All strains were grown in 1-liter bottles under CO₂-H₂ (1:4, vol/vol) headspace. *Methanosarcina mazei* was grown on methanol. All strains were grown at 35°C except for *Methanogenium marisnigri* (room temperature) and *Methanobacterium thermoautotrophicum* (60°C).

Spheroplasts of both *Methanospirillum hungatei* strains,

TABLE 1. Sensitivity to gramicidin of methane synthesis by various methanogens

Organism	Methanogenesis rate ^a (µmol hr ⁻¹ mg ⁻¹ [dry wt])	Inhibitory concn of gramicidin ^b (µg/ml)
<i>Methanobacterium thermoautotrophicum</i>		
ΔH	49.7	0.2
Marburg	45.7	0.2
GC1	126.0	0.2
<i>Methanobacterium formicicum</i>	20.1	4
<i>Methanobacterium G2R</i>	28.1	4
<i>Methanobacterium bryantii</i>		
MOH	10.3	4
MOHG	29.0	4
<i>Methanobrevibacter smithii</i>	31.0	20
<i>Methanobrevibacter arboriphilus</i>	28.4	20
<i>Methanosarcina barkeri</i>	12.8	20
<i>Methanosarcina mazei</i>	3.0	>40 ^c
<i>Methanococcus voltae</i>	14.6	>40
<i>Methanogenium marisnigri</i>	0.6	>40
<i>Methanospirillum hungatei</i> GP1		
Cells	29.4	>40
Spheroplasts	13.2	0.1
<i>M. hungatei</i> JF1		
Cells	23.8	>40
Spheroplasts	8.1	0.1

^a Rate of methanogenesis in the ethanol control.

^b Concentration giving at least 95% inhibition of methanogenesis.

^c Highest concentration tested.

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FIG. 1. Effect of gramicidin on methane synthesis in gramicidin-resistant (*Methanococcus voltae*), moderately sensitive (*Methanobrevibacter smithii*), and extremely sensitive (*Methanobacterium thermoautotrophicum*) methanogens. Gramicidin was added to the following final concentrations (micrograms per milliliter): *Methanococcus voltae* (A) and *Methanobrevibacter smithii* (B), 0 (○), 10 (□), 20 (▲), and 40 (●); *Methanobacterium thermoautotrophicum* (C), 0 (○), 0.04 (□), 0.1 (▲), and 0.2 (●).

active in methanogenesis, were prepared by the alkaline pH-dithiothreitol method (12) as modified previously (13).

For the study of methanogenesis inhibition by gramicidin, 5-ml aliquots of actively growing cells were anaerobically distributed into 60-ml serum bottles under CO₂-H₂ (1:4, vol/vol). The cells were treated with various concentrations of gramicidin D (Sigma Chemical Co., St. Louis, Mo.) dissolved in ethanol or with ethanol alone (control). Methane synthesis was followed over the course of several hours, with a Perkin-Elmer model Sigma 3 gas chromatograph fitted with a flame ionization detector, under the following conditions: oven temperature, 100°C; injector and detector temperature, 150°C; Porapak T column (10 ft [ca. 3 m] by 3 in. [7.62 cm] in outer diameter; Chromatographic Specialties, Brockville, Ontario, Canada).

The different species of methanogens examined exhibited a wide range of sensitivities to the action of gramicidin (Table 1). Four species were found to be resistant to gramicidin in that the rate of CH₄ synthesis was not significantly inhibited at the highest concentration of gramicidin tested, 40 μg/ml. These resistant strains were *Methanococcus voltae*, *Methanogenium marisnigri*, *Methanosarcina mazei*, and the two *Methanospirillum hungatei* isolates. At the other end of the spectrum were the three thermophiles, *Methanobacterium thermoautotrophicum* strains ΔH, GC1, and Marburg. Methane synthesis by these strains was totally inhibited by 0.2 μg of gramicidin per ml. The other, mesophilic strains examined, representing many of the major groups of methanogens, showed intermediate sensitivity, total inhibition of methanogenesis occurring at gramicidin concentrations of 4 to 20 μg/ml.

The responses of representatives of the resistant, moder-

ately sensitive, and extremely sensitive methanogens to various gramicidin concentrations are shown in Fig. 1. Note the different gramicidin concentrations used in each case.

The gramicidin sensitivity of the thermophiles was further examined and shown not to be due simply to the higher incubation temperature. *Methanobacterium thermoautotrophicum* Marburg was examined at 60 and 45°C. There was no noticeable increase in the concentration of gramicidin needed to cause total inhibition of methanogenesis at the two temperatures (data not shown). Methanogenesis at 45°C was 50% of the rate observed at 60°C. This finding indicates that the extreme sensitivity of the thermophiles to gramicidin is not due to the higher incubation temperature but more likely to the membrane adaptations of *Methanobacterium thermoautotrophicum* for life at 60°C (11).

The two *Methanospirillum hungatei* isolates were further studied, since it had been shown that the sheath acted to protect the intact cells from the action of several ionophores (13). When the spheroplasting technique (12) was used to remove the sheath, the resulting spheroplasts, active in methanogenesis, were extremely sensitive to the action of gramicidin, in stark contrast to results obtained with the intact cells (Fig. 2). Total inhibition of methanogenesis by spheroplasts was obtained at a gramicidin concentration of 0.1 μg/ml, an increase in sensitivity of more than 400 times compared with that seen with the intact cells.

The results of this study indicate that gramicidin can be a very effective agent against some methanogens, although other methanogens are resistant to high concentrations of this ionophore. Previous work has shown that if methanogenesis is affected by gramicidin, then one can expect decreases in intracellular ATP, protonmotive force, and

FIG. 2. Effect of gramicidin on methane synthesis by *Methanospirillum hungatei* GP1 whole cells (A) and spheroplasts (B). Gramicidin was added to the following final concentrations (micrograms per milliliter): (A) 0 ($\circ$), 10 ($\square$), 20 ($\triangle$), and 40 ($\bullet$); (B) 0 ($\circ$), 0.02 ($\square$), 0.04 ($\triangle$), and 0.1 ($\bullet$).

transport (8). On the other hand, if gramicidin does not inhibit methane synthesis, it is not likely to affect these other parameters (K. F. Jarrell and G. D. Sprott, unpublished data).

In general terms, it seems that gramicidin is effective against all members of the family *Methanobacteriaceae*, which includes the genera *Methanobacterium* and *Methanobrevibacter*. These isolates all have a pseudomurein wall and contain C-20 and C-40 isoprenoid glycerol ethers in their membranes (1). *Methanospirillum hungatei* is resistant to the action of the ionophore unless the sheath is removed, whereupon the spheroplasts become extremely sensitive. *Methanospirillum hungatei* has C-20 and C-40 lipids (1).

Gramicidin was not effective against *Methanococcus voltae*, *Methanogenium marisnigri*, or *Methanosarcina mazei*, all of which possess walls and membranes quite distinct from those of the sensitive strains. The resistant isolates all lack pseudomurein and, instead, possess either a wall composed of protein or an acid heteropolysaccharide (1). Furthermore, *Methanococcus* and *Methanosarcina* isolates contain only C-20 lipids in their membranes. The lipid composition of *Methanogenium* strains has not been reported (1).

As seems the case with whole cells of *Methanospirillum hungatei*, the resistance of *Methanococcus voltae*, *Methanogenium marisnigri*, and *Methanosarcina mazei* is likely due to the inability of gramicidin to reach its target site. Whether this is due to unusual walls preventing gramicidin from reaching the cytoplasmic membrane or to the unusual C-20 lipid composition of the cytoplasmic membrane itself is yet to be shown. Interestingly, Hilpert et al. (5) reported that all the extreme halophiles tested, which contain only C-20 glycerol ether lipids and lack a pseudomurein

type wall, were resistant to gramicidin in their diffusion assay.

Regardless of the mechanism of resistance of gramicidin, this study has shown that this useful ionophore should be employed with caution, especially on methanogens that are not members of the family *Methanobacteriaceae*.

This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to K.F.J.

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