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Biochemistry

Biological function of gramicidin: Selective inhibition of RNA polymerase*

(peptide antibiotics/sporulation/RNA polymerase–DNA complex/actinomycin D/*Bacillus brevis*)

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ABSTRACT **This paper describes a novel biochemical effect of gramicidin, a class of peptide antibiotics produced by *Bacillus brevis* during the transition from vegetative growth to sporulation. Gramicidin inhibits RNA synthesis by purified RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2. 7.7.6) by interfering with the binding of RNA polymerase to DNA. This effect seems to involve the destabilization of the "open" RNA polymerase–DNA complex, a mode of action consistent with the control of promoter selection. Selectivity in the inhibition of RNA synthesis by gramicidin is observed when transcription is partially blocked by low levels of actinomycin D. Since the inhibition of RNA synthesis by gramicidin is obtained in a highly purified system devoid of membranes, it must be distinct from the ionophoretic activity of the antibiotic. It is possible that this new mode of action reflects the function of gramicidin during bacterial sporulation.**

The gramicidins are a group of linear pentadecapeptide antibiotics (2) that are produced by certain strains of *Bacillus brevis* during the transition from vegetative growth to sporulation (3, 4). They can increase the permeability of various membranes to monovalent cations (5–8), an effect that is probably due to their helical dimer structure in organic solvents (9, 10) and that seems to be responsible for their antibacterial activity (7). Recently, it has been proposed that the biological function of peptide antibiotics is not their antibacterial action but is related to sporulation of the producing organism (4, 11). This hypothesis has now been confirmed by the isolation of gramicidin-negative mutants of *B. brevis* that are unable to form normal spores unless provided with the antibiotic (12).

The question of whether the mechanism by which gramicidin acts in the producing organism is based on its antibacterial activity or whether it involves targets other than biological membranes is therefore of considerable interest. In preliminary experiments, we had found that gramicidin can act as an inhibitor of RNA synthesis (4). This paper presents a more detailed analysis of this mode of action. Our results reveal that gramicidin has relatively specific effects on transcription by purified RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). This represents a hitherto unrecognized mode of action of gramicidin and raises the possibility that the antibiotic may exert its biological function by a mechanism altogether different from its antibacterial action.

EXPERIMENTAL PROCEDURES

Materials. RNA polymerase was purified from exponentially growing cells of *B. brevis* ATCC 8185 as described previously (1). The purified enzyme was about 75% pure, with a specific activity of 990 units/mg of protein, and contained a stoichiometric amount of σ factor. RNA polymerase from *Escherichia coli* MRE 600 was purchased from Boehringer, [5-^{3}H]UTP and [5-^{3}H]thymidine from New England Nuclear, gramicidin† from Nutritional Biochemicals, heparin from Organon, actinomycin D, rifampicin, and salmon sperm DNA from Calbiochem, poly(dA-dT) from Boehringer, and poly(dG-dC) from PL Laboratories. DNA from *B. brevis* ATCC 8185 was isolated by the method of Marmur (13) and further purified by CsCl density gradient centrifugation.

Preparation of ^{3}H-Labeled DNA. Bacteriophage T7 DNA labeled with ^{3}H was prepared as described by Hinkle and Chamberlin (14), with a specific radioactivity of 5×10^4 cpm/μg of DNA. DNA from *B. brevis* ATCC 8185 was labeled by the addition of [5-^{3}H]thymidine to exponentially growing cultures; it was isolated by the procedure of Marmur (13) and further purified by methylated albumin-kieselguhr chromatography (15). Its specific radioactivity was 2×10^4 cpm/μg of DNA.

Assay of RNA Polymerase. RNA polymerase was assayed by incubation at 37° for 15 min in the presence of 20 μM [5-^{3}H]UTP/1 mM each of ATP, GTP, and CTP/50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/10 mM 2-mercaptoethanol/0.4 mM potassium phosphate/gelatin, 50 μg/ml, and appropriate amounts of enzyme and DNA in a final volume of 0.1 ml. For the assay of RNA polymerase from *E. coli*, 0.1 M KCl was added to the incubation mixture. Gramicidin was added in 5 μl of either ethylene glycol or ethanol, and equivalent amounts of the corresponding solvent were added to all incubation mixtures. RNA synthesis was always initiated by the addition of RNA polymerase. The reaction was terminated by the addition of trichloroacetic acid and the precipitate was collected as described previously (16). One unit of RNA polymerase catalyzes the incorporation of 1 nmol of UMP under these conditions, with bacteriophage ϕe DNA as template (1).

Assay of Binding of DNA to RNA Polymerase. The binding reaction was studied at 37° in the presence of 40 mM Tris·HCl, pH 8.0/4 mM $MgCl_2$/10 mM 2-mercaptoethanol/gelatin, 25 μg/ml, and appropriate amounts of ^{3}H-labeled DNA and enzyme (added last) in a final volume of 0.1 ml. The complex was collected on a nitrocellulose filter by the method of Jones and Berg (17) except that dilution and washing were done at 37°. In all experiments, RNA polymerase was not saturating and bound 50–80% of the added DNA.

RESULTS

Inhibition of RNA Synthesis by Gramicidin. Gramicidin inhibited RNA synthesis by purified RNA polymerase from *B.*

* Some of the results described here were presented at the ICN–UCLA Winter Conference in Molecular and Cellular Biology, March 23, 1976, Keystone, CO (ref. 1).

† Gramicidin refers to the mixture of linear pentadecapeptides produced by *B. brevis* ATCC 8185. It consists of approximately 85% gramicidin A, 10% gramicidin B, and 5% gramicidin C (2).

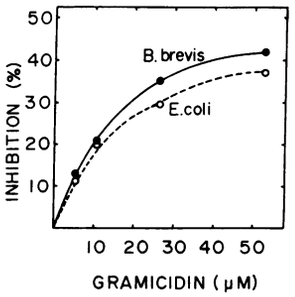

FIG. 1. Inhibition of RNA synthesis by gramicidin. RNA synthesis was measured as described under *Experimental Procedures* with 2 μg of bacteriophage T7 DNA and 0.9 μg of RNA polymerase from *B. brevis* (●) or 0.16 μg of RNA polymerase from *E. coli* (○), with gramicidin added as indicated.

brevis, with half-maximal inhibition at 10 μM (Fig. 1). Maximal inhibition was only about 50%. This may be due in part to the limited solubility of gramicidin, but it also suggests that the synthesis of some classes of RNA is not inhibited by the antibiotic. Inhibition of RNA synthesis by gramicidin was also observed with RNA polymerase from *E. coli* (Fig. 1) and with various DNA templates such as DNA from bacteriophages T7 and φe, *B. brevis*, salmon sperm, poly(dA-dT), and poly(dG-dC) (not shown). On the other hand, DNA synthesis by partially purified extracts of *B. brevis*, studied with a homologous DNA template, was not significantly affected by 54 μM gramicidin.

Inhibition by Gramicidin of RNA Polymerase Binding to DNA. The binding of RNA polymerase to DNA was inhibited by gramicidin to nearly the same extent as RNA synthesis (Fig. 2). As with RNA synthesis, the inhibition of RNA polymerase–DNA complex formation by gramicidin also occurred with RNA polymerase from *E. coli* and with DNA from bacteriophage T7 and *B. brevis* (not shown). Prior washing of nitrocellulose filters with solutions containing gramicidin had no effect on the RNA polymerase-dependent retention of DNA, showing that inhibition was not an artefact resulting from the interaction of gramicidin with the filter material.

In order to determine whether the inhibition of RNA polymerase binding to DNA was responsible for the inhibition of RNA synthesis by gramicidin, we tested the competence of the gramicidin-sensitive binary complex to initiate RNA synthesis. RNA polymerase was incubated with DNA in the presence or absence of an inhibitory concentration of gramicidin, and the mixture was then diluted by the simultaneous addition of the four ribonucleoside triphosphates and rifampicin (to inhibit further complex formation) to initiate RNA synthesis while decreasing the concentration of gramicidin to a noninhibitory level. As shown in Table 1, the presence of gramicidin during

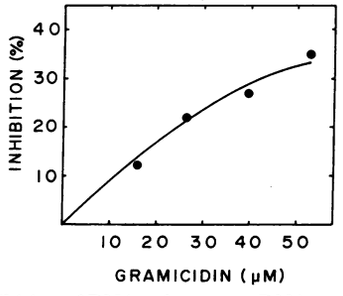

FIG. 2. Inhibition of RNA polymerase–DNA complex formation by gramicidin. Binary complex formation was measured as described under *Experimental Procedures* with 0.18 μg of *B. brevis* RNA polymerase and 0.14 μg of ^{3}H-labeled bacteriophage T7 DNA, at the indicated concentrations of gramicidin.

Table 1. Effect of gramicidin on competence of RNA polymerase–DNA complex to engage in RNA synthesis

Gramicidin present, μM		[^{3}H]UMP	
1st incubation	2nd incubation	incorporated, pmol	Inhibition, %
0	0	5.0	—
27	5.4	2.8	44
0	5.4	5.0	0

RNA polymerase (0.36 μg) and DNA (0.8 μg) from *B. brevis* were incubated under conditions of complex formation, described under *Experimental Procedures*, with or without gramicidin as indicated, for 15 min at 37° in a volume of 0.04 ml (1st incubation). A mixture (0.16 ml) containing [^{3}H]UTP (20 μM), ATP, GTP, and CTP (1 mM each), rifampicin (0.4 μg/ml), and gramicidin to give the final concentration indicated, was then added and incubation was continued for 10 min at 37° to permit RNA synthesis (2nd incubation). The mixtures were then analyzed for the incorporation of [^{3}H]UMP into RNA as described under *Experimental Procedures*.

complex formation led to almost 50% inhibition of subsequent RNA synthesis, whereas the addition of an equivalent amount of gramicidin during synthesis alone had no significant effect.

Characterization of the Gramicidin-Sensitive Binary Complex. RNA polymerase can form several types of complex with DNA which differ in stability and response to agents such as heparin and salt (18). Accordingly, we investigated the properties of the binary complex formed under the conditions of our experiments. As shown in Table 2, the formation of a complex between RNA polymerase and DNA from *B. brevis* was inhibited by 0.2 M KCl, suggesting that it represents an "open" complex (18). This was supported by the observation that the complex, once formed, was relatively resistant to heparin at concentrations at which this polyanion completely

Table 2. Effect of inhibitors on RNA polymerase–DNA complex

Exp.	Additions	[^{3}H]DNA bound, cpm	Inhibition, %
1	None	1515	0
	0.2 M KCl, before complex formation	111	93
2	None	1031	0
	Heparin, before complex formation	10	99
	Heparin, after complex formation	681	34
3	None	1198	0
	Gramicidin, before complex formation	500	58
	Gramicidin, after complex formation	484	60

B. brevis RNA polymerase (0.11 μg) and ^{3}H-labeled *B. brevis* DNA (0.1 μg) were incubated for 10 min at 37° for binary complex formation, as described under *Experimental Procedures*, with 0.2 M KCl, heparin (0.1 μg/ml), or gramicidin (27 μM) as indicated (before complex formation). The binding reaction was terminated by the addition of denatured salmon sperm DNA (10 μg) followed by incubation for 10 min at 37°, whereupon more heparin or gramicidin was added as indicated (after complex formation). After 10 min at 37°, the complex was collected on nitrocellulose filters as described under *Experimental Procedures*.

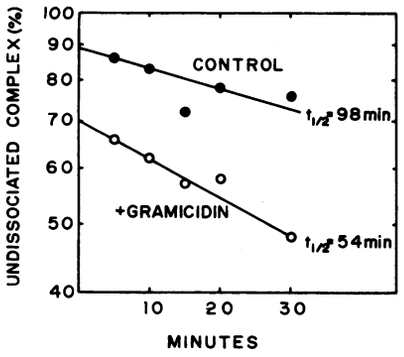

FIG. 3. Effect of gramicidin on the dissociation of the RNA polymerase–DNA complex. Binary complex was formed with 0.23 μg of *B. brevis* RNA polymerase and 0.16 μg of ^{3}H-labeled bacteriophage T7 DNA as described under *Experimental Procedures*. After 10 min of incubation at 37°, unlabeled bacteriophage T7 DNA (0.7 μg) was added without (●) or with (○) gramicidin (27 μM). Samples were removed at the indicated times for measurement of ^{3}H-labeled DNA bound to RNA polymerase. The percentage of radioactive complex dissociated was calculated by using an appropriate correction factor for the small amount of reassociation of RNA polymerase with radioactive RNA.

prevents complex formation when present initially. It was of interest, therefore, that, unlike heparin, gramicidin had the same effect when added before or after completion of complex formation, suggesting that the peptide antibiotic actually promotes the dissociation of the "open" complex.

This effect could be demonstrated directly by studying the effect of gramicidin on the rate of dissociation of the RNA polymerase–DNA complex. This rate can be measured by diluting the complex, formed with radioactive DNA, with an excess of unlabeled DNA to prevent the reassociation of dissociated RNA polymerase molecules with labeled DNA (14). As Fig. 3 shows, the addition of unlabeled bacteriophage T7 DNA led to a rapid loss of about 10% of the bound radioactive T7 DNA, followed by a slower first-order decay with a half-time of about 98 min. If gramicidin was added at the same time as unlabeled DNA, the fraction of the labeled complex dissociating rapidly was increased to 30%, and the remainder decayed at about twice the rate as in the absence of the antibiotic, with a half-time of 54 min.

The detailed mechanism of the destabilization of the binary complex has not yet been studied. However, it should be noted that gramicidin does not bind to DNA, as shown by the fact that it does not promote the binding of radioactive DNA to nitrocellulose filters under conditions where tyrocidine, another peptide antibiotic that binds to DNA, leads to complete retention (19).

Selectivity of Inhibition of Transcription by Gramicidin. The possibility that gramicidin inhibits the transcription of only certain classes of genes was examined by comparing its effect with that of actinomycin D, a drug that inhibits RNA synthesis by binding to GC base pairs of the DNA template (20). We found that, if RNA synthesis is inhibited 75% by the addition of low levels of actinomycin D, the residual transcription becomes totally refractory to inhibition by gramicidin (Fig. 4).

As shown in Table 3, this effect is not seen with templates that do not bind actinomycin D, such as poly(dA-dT) and denatured DNA, and must therefore involve the interaction of the drug with DNA. On the other hand, the effect is highly specific for actinomycin D and is not observed with other agents that inhibit transcription by binding to DNA, such as chromomycin, daunomycin, netropsin, acridine orange, ethidium bromide, and

Table 3. Effect of actinomycin D and gramicidin on transcription of different DNA templates

Template	Additions	[^{3}H]UMP incorporated, pmol	Inhibition by gramicidin, %
B. brevis DNA (1.0 μg)	None	18	—
	Gramicidin	9	50
	Actinomycin	4.5	—
	Actinomycin + gramicidin	4.6	0
Denatured salmon sperm DNA (6.7 μg)	None	35	—
	Gramicidin	23	34
	Actinomycin	34	—
	Actinomycin + gramicidin	19	44
Poly(dA-dT) (0.8 μg)	None	26	—
	Gramicidin	17	35
	Actinomycin	24	—
	Actinomycin + gramicidin	15	38

RNA synthesis was measured as described under *Experimental Procedures*, with the DNA preparations shown and *B. brevis* RNA polymerase (0.5 μg), in the presence or absence of gramicidin (54 μM) and actinomycin D (0.15 μg/ml with *B. brevis* DNA; 0.1 μg/ml with other templates) as indicated.

tyrocidine (not shown). The results shown in Table 3 also reveal a difference in the specificity of inhibition by gramicidin and actinomycin D: the transcription of poly(dA-dT) and denatured DNA is resistant to actinomycin D but sensitive to inhibition by gramicidin. This means that the resistance of transcription of *B. brevis* DNA to inhibition by gramicidin, observed in the presence of actinomycin D, is not simply due to restriction of transcription to AT-rich or denatured nucleotide sequences of the template. In fact, at a slightly higher concentration (0.5 μg/ml), actinomycin D completely inhibited transcription of *B. brevis* DNA. It should also be noted that inhibition by actinomycin D and its effect on gramicidin inhibition were the same with either UTP or CTP as the labeled precursor for RNA synthesis (not shown).

DISCUSSION

The ability of gramicidin to modify the cation permeability of lipid membranes has been studied in considerable detail (e.g.,

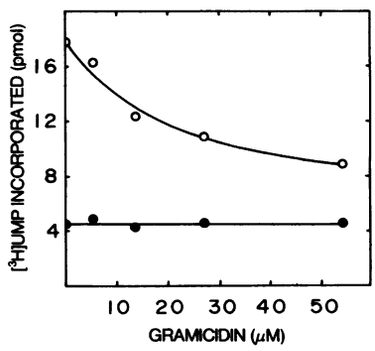

FIG. 4. Effect of actinomycin D on the inhibition of RNA synthesis by gramicidin. RNA synthesis was measured as described under *Experimental Procedures* with *B. brevis* RNA polymerase (0.5 μg) and *B. brevis* DNA (1.0 μg), without (○) or with (●) actinomycin D (0.15 μg/ml), at the gramicidin concentrations indicated.

refs. 5–8), but little attention has been given to the question of whether gramicidin can also exert other types of biochemical effects. In view of the fact that recent evidence implicates gramicidin as an essential element in bacterial sporulation and questions the biological significance of its antibacterial activity (4, 12), a search for other possible modes of action of the antibiotic was indicated. Indeed, we found that gramicidin, at concentrations corresponding to those found in the producing organism, is a potent inhibitor of purified RNA polymerase (4), and this result was confirmed in another laboratory (19). Because this effect of gramicidin was observed in a completely soluble system devoid of membrane structures, it must be mediated by a mechanism distinct from that underlying its ionophoretic activity.

Accordingly, we studied in some detail the mechanism by which gramicidin inhibits RNA synthesis. We found that the antibiotic inhibits the binding of RNA polymerase to DNA to nearly the same extent as it inhibits overall RNA synthesis and that the presence of gramicidin during the binding reaction decreases subsequent RNA synthesis. These observations, together with the earlier finding (1) that gramicidin has no significant effect on the initiation and elongation of RNA chains, suggest that the formation of the RNA polymerase–DNA complex is the primary target of gramicidin inhibition of RNA synthesis.

The interaction of RNA polymerase with DNA proceeds in several stages. The initial recognition of a promoter involves the formation of a weak "closed" complex which, at temperatures above 20°, can be transformed to the more stable "open" complex in which RNA polymerase is bound to the partially unwound promoter region, ready to initiate the synthesis of RNA chains (18). The two types of complex can be distinguished by their sensitivity to salt and to agents such as heparin. Our finding that the complex formed under our experimental conditions is sensitive to 0.2 M KCl but is relatively resistant to low concentrations of heparin permits its identification with the "open" complex (18, 21). The inhibition of "open" complex formation appears to involve destabilization of the complex by gramicidin, because the antibiotic increases the first-order rate constant for its dissociation. It is of interest that destabilization of RNA polymerase–DNA complexes is often encountered as a mechanism for the control of transcription. For example, polypeptides synthesized after infection of *B. subtilis* with bacteriophage SP01 associate with RNA polymerase and specifically destabilize its complex with heterologous DNA such as that of bacteriophage ϕ29 (22). Similarly, σ factor suppresses nonspecific binding of RNA polymerase to DNA by increasing the rate of dissociation of weak binary complexes (18).

Evidence that gramicidin selectively inhibits transcription of some genes but not others came from experiments in which certain sites on *B. brevis* DNA were blocked by the addition of low levels of actinomycin D. At a molar ratio of actinomycin D to nucleotide residues of 1:250, transcription was inhibited by 75%, but the residual RNA synthesis was totally resistant to inhibition by gramicidin. Actinomycin D had no effect on the inhibition by gramicidin of RNA synthesis with poly(dA-dT) and single-stranded DNA as templates, showing that this effect requires the interaction of actinomycin D with DNA. Moreover, the fact that transcription of poly(dA-dT) and single-stranded DNA is inhibited by gramicidin but resistant to actinomycin D, while the gramicidin-resistant transcription of *B. brevis* DNA can be totally inhibited by slightly higher concentrations of actinomycin D, speaks against the idea that the observed effect is due to transcription of DNA sequences that cannot bind actinomycin D. The possibility that the loss of sensitivity to gramicidin observed in the presence of actinomycin D is some kind of artefact resulting from decreased RNA synthesis is ruled out by the observation that the inhibition of RNA synthesis by other drugs that bind to DNA (chromomycin, daunomycin, netropsin, acridine orange, ethidium bromide, and tyrocidine) does not produce this effect. Rather, it appears that actinomycin D, at the low concentrations used in these experiments, exerts its effect as a result of relatively subtle differences in DNA base composition, perhaps near the promoter region. Further experiments are necessary to clarify the mechanism by which actinomycin D can exert selective effects on the transcription of different groups of genes (20). In spite of this uncertainty, our results clearly demonstrate that the transcription of a class of bacterial genes that are relatively insensitive to actinomycin D is completely resistant to gramicidin. This means that gramicidin can exert a selective effect on RNA synthesis and thus has the potential of regulating transcription.

At this stage, it is of interest to ask whether the mode of action of gramicidin presented in this paper would be consistent with a function in sporulation. We had pointed out earlier (4) that many peptide antibiotics that are produced during the early stages of sporulation are strong inhibitors of vegetative growth of the producing organism. The fact that peptide antibiotics inhibit a process that is essential for growth but not for sporulation would be consistent with a regulatory role during the transition from vegetative to sporulation metabolism. The properties of gramicidin-negative mutants of *B. brevis*, which we have recently isolated, support this view (12). These mutant strains produce defective spores unless they are supplied with gramicidin within a critical period during the transition from growth to sporulation. Since this corresponds to the time when changes in the specificity of transcription are first observed (23), one is tempted to speculate that peptide antibiotics might function to inhibit the transcription of genes that are required for vegetative growth but not for sporulation. The observations described in this paper—that gramicidin can indeed inhibit transcription, that inhibition occurs at the level of promoter recognition, and that it affects the transcription of only some classes of genes—are consistent with such a regulatory function. On the other hand, the possibility that gramicidin may regulate ion movement during sporulation (11, 24) also deserves consideration. The detailed study of the biochemical defects of gramicidin-negative mutants (12) should permit us to decide between these alternatives.

Undoubtedly, the control of sporulation in *B. brevis* involves factors in addition to gramicidin. Of special interest in this regard are the tyrocidines, a group of cyclic decapeptide antibiotics that are produced by *B. brevis* ATCC 8185 at the same time as the linear gramicidins (3, 4). The tyrocidines are also inhibitors of RNA synthesis (4, 19, 25) and seem to act by binding to DNA (25, 26). It appears that, under certain conditions, gramicidin can counteract the effect of tyrocidine (19, 26), and the possibility must be considered that the regulation of sporulation may involve complex interactions between these groups of antibiotics.

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