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# Analysis of the binding of $Ca^{2+}$ to gramicidin A in ethanol in terms of the dimer-monomer conformational equilibrium

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This study reports the first direct observation of the binding of $Ca^{2+}$ to gramicidin A in ethanol, analysed in terms of the polypeptide dimer-monomer conformational equilibrium. High performance size-exclusion chromatography has been successfully used to elucidate the binding mechanism and to determine the rate constants as well as the stoichiometry of the processes involved. In addition, fluorescence intensity and anisotropy measurements have revealed a dependence of the number of accessible $Ca^{2+}$-binding sites on the peptide concentration.

## 1. Introduction

We have demonstrated in previous papers the advantages of high-performance size-exclusion chromatography (HPSEC) for the kinetic and thermodynamic characterization of the dimer ($M_2$)-monomer (M) conformational equilibrium of gramicidin A in a nonpolar solvent such as tetrahydrofuran (THF) [1,2]. By using this methodology it has been possible to determine the effect of polar and neutral lipids [2,3], water [4], etc. on the conformational equilibrium.

More recently, a novel HPSEC strategy has been developed which allows one to study the monomerization process of gramicidin A in a polar solvent such as ethanol [5]. Also, the usefulness of this approach to investigation of the binding of calcium to gramicidin A in this solvent has been described [5]. In this connection, although the $Ca^{2+}$-gramicidin A interaction in polar solvents (methanol, ethanol, trifluoroethanol) has been studied previously using spectroscopic techniques [6,7], the results have not been analysed in terms of the conformational equilibrium. The HPSEC approach used in the present work, besides providing a complementary point of view to previously reported spectroscopic information, allows one to study the kinetics of binding of calcium to gramicidin by directly visualizing the variation of the different conformational species as a function of time. Analysis of the kinetic results at different $Ca^{2+}$ : gramicidin A molar ratios has led to confirmation of the proposed qualitative mechanism [5] and allowed determination of both the stoichiometry and kinetic constant for the binding process.

Additional information has been obtained from fluorescence emission and anisotropy experiments. It has been found that $Ca^{2+}$ exhibits a selective

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high affinity for the monomer, the $Ca^{2+}$: gramicidinA molar ratio at which saturation occurs being dependent on the polypeptide concentration used. Since it has been reported using spectroscopic techniques that in polar organic solvents $Ca^{2+}$ binds strongly to gramicidin A whereas $Zn^{2+}$, $Na^+$ and $K^+$ exhibit little or no interaction [6,7] the effect of metal cations such as $Zn^{2+}$ or $K^+$ has also been investigated. The results are discussed in relation to experimental and theoretical data on the binding of $Ca^{2+}$ to the polypeptide incorporated into liposomes [7,8], taking into account current interest in the ion selectivity and mechanism of passage through the gramicidin A channel formed in phospholipid membranes [9–11].

## 2. Experimental

### 2.1. Materials

Gramicidin A was supplied by Koch Light and was used without further purification. *N*-Acetyl-L-tryptophanamide (NATA) was obtained from Sigma. Analytical reagent grade $CaCl_2$, potassium acetate and zinc acetate were purchased from Merck. All organic solvents were spectrograde (Merck) and were degassed and clarified through a 0.45 $\mu$m MFS regenerated cellulose filter before use.

### 2.2. HPSEC measurements

The liquid chromatograph consisted of an M-45 solvent delivery system and a U6K universal injector from Waters Associates. Samples were simultaneously monitored with a Waters 420-AC fluorescence detector (excitation and emission filter wavelength: 254 and 338 nm, respectively) and a Varian Varichrom variable-wavelength ultraviolet-visible detector set at 294 nm. The system was equipped with a 100 nm nominal pore size Ultrastyragel column (Ultrastyragel 1000 Å, 30 × 0.78 cm inner diameter) from Waters. The chromatograms were monitored using a Yokogawa Electric Works dual-channel recorder.

All chromatographic measurements were conducted at room temperature. The column was always eluted isocratically with THF at a flow rate of 1.0 ml/min (≃ 20 bar). In all experiments, a 0.5 mg/ml solution of gramicidin A in ethanol was prepared and allowed to equilibrate overnight, prior to the addition of the metal cation, according to a previously reported procedure [5]. Zero time was taken in all cases upon addition of an aliquot (from 6 to 135 $\mu$l) of a 0.1 M solution of $Ca^{2+}$ or $Zn^{2+}$ in ethanol (or $K^+$ in water) to 10 ml of the previously equilibrated polypeptide solution. The reaction mixture was immediately stirred for 1 min and transferred to a screw-cap 10 ml glass tube which was completely filled to minimize hydration and maintained at 25° C until injection. Aliquots were taken at different incubation times, diluted 1 : 25 (v/v) in THF (so that the reaction was stopped), vortex-mixed for 45 s and 50 $\mu$l of the THF-diluted sample (containing 2 $\mu$l ethanol) were always directly injected.

### 2.3. Fluorescence measurements

Fluorescence spectra were obtained on a Perkin-Elmer MPF-44B spectrofluorimeter (Uberlingen, F.R.G.). The instrument was operated in the ratio mode with correction of both excitation and emission spectra. The slit widths were 8 and 4 nm for excitation and emission beams, respectively. Excitation was at 280 nm and the fluorescence emission spectra were recorded from 290 to 430 nm at 60 cm/min scanning speed. 1-cm optical path cells were used. The temperature was maintained at 25.0 ± 0.1° C using a Lauda MT-20 compact thermostat (Koningshofen, F.R.G.).

Since eventual fluctuations in lamp intensity are expected to occur with elapsed time, in order to compare all fluorimetric measurements the values of the emission intensity, $I_f$, were corrected by measuring at different times the intensity of a solid standard (*p*-terphenyl). An inner filter correction ($10^{A}$) was employed, where $A$ is the absorbance at the excitation wavelength.

Anisotropy of the fluorescence emission of gramicidin A was measured with the corresponding accessory on the MPF-44B spectrofluorimeter. Emission was measured at 338 nm for an excita-

tion wavelength of 280 nm. The rest of the experimental conditions were similar to those indicated above. The anisotropy ($r$) values were obtained using the following relationship [12]:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where $I$ denotes the emission intensity and subscripts V and H refer to the orientation of the polarizer (vertical and horizontal, respectively). In all cases, the first subscript corresponds to the excitation polarizer whereas the second refers to the emission polarizer. The correction factor $G$ ($G = I_{HV}/I_{HH}$) was calculated from 10 independent measurements using the $p$-terphenyl standard, its value being $1.37 \pm 0.04$. Anisotropy values represent an average of 15 data acquisitions. In all cases, the relative standard deviation of intensity measurements was less than 1%, resulting in anisotropies precise to within $\pm 0.001$.

For both fluorescence emission and anisotropy measurements gramicidin A concentrations up to 0.05 mg/ml were used. In each case, a stock solution of gramicidin A in ethanol was allowed to equilibrate overnight, zero time was taken upon addition of the metal cation from a concentrated ethanolic solution (less than 1% volume change) and the reaction mixture was divided into aliquots in tightly stoppered 10-ml glass tubes which were completely filled to minimize hydration, sealed and stored in a dark room at 25 °C. For each measurement, the contents of a sealed tube were used.

Absorbance measurements were performed on a thermostatted Beckman DU 8 spectrophotometer using 1-cm optical path cells. Other details of the experimental conditions are given in the corresponding figure legends.

## 3. Results

### 3.1. HPSEC measurements

Fig. 1 depicts, as an example, some elution profiles for the reaction of calcium with gramicidin A previously equilibrated in ethanol, at 0.5 mg/ml peptide concentration, for a $Ca^{2+}$ : gramicidin A (monomer) molar ratio of $R = 0.5$. Samples were monitored simultaneously with fluorescence and absorbance detectors. However, in order not to overcrowd the figure, only the fluorescence response is shown. We have recently demonstrated that the two peaks eluting at 7.9 and 8.4 ml correspond to the free dimeric (peak I) and monomeric (peak II) species of gramicidin A [1,2], whereas the broad-tailed peak becoming apparent with elapsed time at a higher elution volume can be attributed to a $Ca^{2+}$-polypeptide complex (peak III). The chromatogram at zero time was obtained by injection of the equilibrated sample before the addition of calcium, which corresponds to a dimer mass fraction of 0.65 [5] as determined directly from the heights of the peaks recorded with ultraviolet detection at 294 nm. In order to obtain the mass fraction of dimer from the fluorescence response (which offered a higher sensitivity), the height of the monomer peak must be divided by the correction factor 1.2 (as described elsewhere [5] due to the different quantum yields of the dimeric and monomeric species in THF [4].

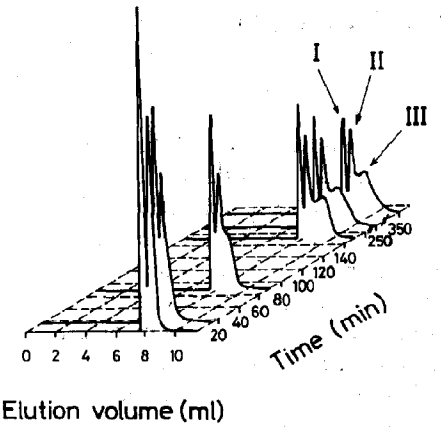


Fig. 1. Fluorescence detection-monitored elution profiles of $Ca^{2+}$-gramicidin A interaction kinetics in ethanol, at 0.5 mg/ml peptide concentration, for a $Ca^{2+}$ : gramicidin A molar ratio of $R = 0.5$. Column, Ultrastyragel 1000 Å; eluent, THF; flow rate, 1.0 ml/min. Chromatogram at zero time corresponds to injection of gramicidin A sample equilibrated before calcium addition.

Some considerations must be made concerning the mechanisms involved in the elution process,

based on chromatographic and spectroscopic data on the conformational equilibrium of gramicidin A in alcohols such as methanol and ethanol [5,13]. After injection, the 2 $\mu$l of ethanol are immediately removed from the conformational species of the polypeptide due to a size-exclusion effect. On the other hand, it is unlikely that the proportion of free dimer and monomer is altered on passage through the column, since during elution they are actually dissolved in THF (eluent) and conformational equilibrium of gramicidin A in this solvent is attained extremely slowly (> 20 days [1], whereas the time required for the analysis is only 10 min. Regarding the $Ca^{2+}$-gramicidin A complex, it is unlikely that dissociation occurs during elution because binding of the metal cation to the polypeptide will presumably be strengthened in the rather nonpolar eluent. Therefore, the chromatograms in fig. 1 can be considered as reproducing quite reliably the actual situation existing in the alcoholic solution before injection. It should be emphasized that the total amount of eluted gramicidin A after each injection did not significantly change during interaction as deduced from the ultraviolet response, since the sum of the areas of peaks I–III remained approximately constant. With respect to the residual free calcium, it is expected to precipitate after dilution in THF.

Two possible nonexclusive explanations can account for the rather large elution volume of the complex species: (i) A $Ca^{2+}$-induced conformational change in gramicidin A resulting in a lower hydrodynamic volume. Note that, in nonpolar solvents, gramicidin A exhibits a $\beta$-helical conformation [14] and particularly in THF (eluent) a $Ca^{2+}$-polypeptide complex is expected to adopt a tighter, more compact structure. (ii) Retention of the complex on the supporting hydrophobic matrix, which would result in marked tailing off of peak III. In this connection, it has been recently pointed out that, when studying the elution behavior of $Ca^{2+}$-binding proteins by hydrophobic interaction chromatography using phenyl group-derivatized supports, the elution volume depends strongly on whether calcium is bound to the protein [15], the mechanism proposed being dependent on a $Ca^{2+}$-induced conformational change in the protein, leading to preferential exposure of

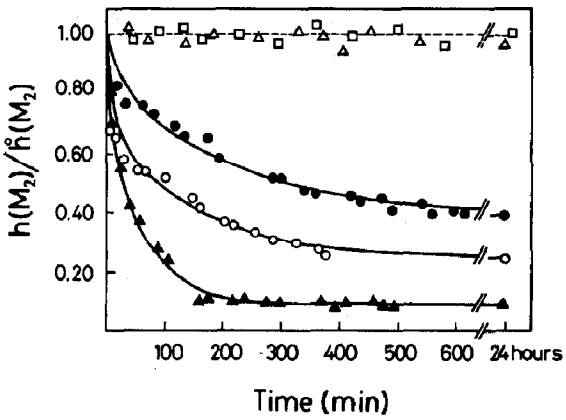


Fig. 2. (———) Variation in relative height of the free dimer peak, $h(M_2)/h^0(M_2)$, with incubation time for the $Ca^{2+}$-gramicidin A interaction in ethanol at 0.5 mg/ml peptide concentration, for molar ratios $R = 0.25$ (●), $R = 0.5$ (○) and $R = 1.5$ (▲). $h^0(M_2)$ denotes the height of the free dimer peak (in arbitrary units) at zero time. (------) Effect of $Zn^{2+}$ (△) and $K^+$ (□) under the same conditions as for $Ca^{2+}$, both at $R = 1.5$.

some hydrophobic residues. Fig. 1 also shows a very good correlation between the time courses of disappearance of free gramicidin A species and formation of the complex (measured as the increase in the semi-area of peak III as described previously [5]).

In order to obtain information on both the mechanism and stoichiometry of the $Ca^{2+}$-gramicidin A interaction, the study was extended to other molar ratios. The elution profiles obtained were qualitatively similar to those in fig. 1. Fig. 2 illustrates the results corresponding to the interaction for $R = 0.25$, 0.5 and 1.5, expressed as the variation in relative height of the free dimer peak (peak I), $h(M_2)/h^0(M_2)$, as a function of incubation time. $h^0(M_2)$ denotes the height of the free dimer peak (in arbitrary units) at zero time, i.e., that corresponding to the injection of the equilibrated gramicidin A sample before the addition of calcium. We consider it more appropriate to use the heights of the dimer peak in the kinetic plot, since the free monomer peak overlaps partially with that of the complex. As can be seen, with increasing $Ca^{2+}$ concentration, the free dimer disappears more rapidly and in greater amounts with elapsed time. Whereas for $R = 0.25$

more than 10 h are needed to reach equilibrium, at $R = 1.5$ it takes less than 3 h.

In order to analyse the results in fig. 2 in greater detail and to propose a mechanism for the interaction, let us first verify two hypotheses which seem to derive from the chromatograms, namely, that (a) equilibrium between the free dimeric and monomeric species is maintained during the reaction, and (b) $Ca^{2+}$ binds preferentially (or exclusively) to the monomeric form of the polypeptide.

In the case of hypothesis a, the apparent dissociation constant, $K_d^{app}$, for the equilibrium $M_2 \rightleftharpoons 2M$ in the presence of $Ca^{2+}$ was calculated as a function of incubation time for the three molar ratios assayed. The values obtained are summarized in table 1. The previously reported dissociation constant in ethanol (in the absence of calcium) has also been included for comparison [5,16]. It can be seen that, at relatively short times, the $K_d^{app}$ values remain practically constant and similar to that in the absence of calcium. At longer times, there is in all cases a nonsignificant, slight increase in $K_d^{app}$ probably due to overestimation of the monomer concentration as a consequence of the overlap between the peaks of the free monomer and complex. Of course, this increase is more pronounced for the molar ratio $R = 1.5$, where the area of peak III is greater. Taking all these considerations into account, it therefore seems reasonable to assume that equilibrium between free polypeptide species is maintained during the interaction, irrespective of the molar ratio assayed.

As concerns hypothesis b, we have recently obtained direct chromatographic evidence, from experiments where freshly prepared samples of either dimeric (commercial gramicidin A) or monomeric (gramicidin A lyophilized from acetic acid) gramicidin A samples containing a high $Ca^{2+}$ : polypeptide molar ratio were analysed at very short incubation times (less than 5 min), which supports the contention that $Ca^{2+}$ binds preferentially (if not exclusively) to the monomeric form of the peptide (results not shown).

Taking into account the above-described results, the following two-step kinetic mechanism for the $Ca^{2+}$-gramicidin A interaction in ethanol can be proposed in terms of the dimer-monomer conformational equilibrium. The first step would consist of a dimer $\rightleftharpoons$ two monomer equilibrium between the free species (step 1), the second involving the binding of $Ca^{2+}$ to monomer either as a quantitative reaction (step 2A) or as an equilibrium (step 2B):

$$M_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2M \text{ (step 1)}$$

with $K_d = 1.0 \times 10^{-4}$ M, $k_1 = 5.0 \times 10^{-4}$ $s^{-1}$ and $k_{-1} = 5.0$ $M^{-1}$ $s^{-1}$ (data from refs. 5 and 16) and

$$pM + qCa^{2+} \xrightarrow{k_2} \text{complex} \qquad \text{(A)}$$

Table 1

Values of the apparent dissociation constant, $K_d^{app}$, as a function of incubation time

| $K_d = (1.0 \pm 0.3) \times 10^{-4}$ M [a] | | | | | |
|---|---|---|---|---|---|
| $R = 0.25$ | | $R = 0.5$ | | $R = 1.5$ | |
| $t$ (min) | $K_d^{app}$ ($\times 10^4$) (M) | $t$ (min) | $K_d^{app}$ ($\times 10^4$) (M) | $t$ (min) | $K_d^{app}$ ($\times 10^4$) (M) |
| 5 | 1.3 | 4 | 1.1 | 3 | 1.1 |
| 18 | 1.4 | 14 | 1.4 | 11 | 0.8 |
| 32 | 1.1 | 29 | 1.0 | 24 | 0.8 |
| 64 | 1.0 | 53 | 1.1 | 45 | 1.0 |
| 85 | 0.9 | 68 | 1.2 | 58 | 1.5 |
| 119 | 1.0 | 104 | 1.0 | 91 | 1.7 |
| 136 | 0.9 | 148 | 1.0 | 105 | 2.1 |
| 178 | 1.1 | 164 | 1.3 | 167 | 2.4 |
| 194 | 1.1 | 204 | 1.3 | 179 | 3.6 |
| 284 | 1.2 | 219 | 1.4 | 222 | 3.1 |
| 299 | 1.5 | 257 | 1.5 | 235 | 3.3 |
| 341 | 1.6 | 288 | 1.5 | 280 | 3.2 |
| 360 | 1.6 | 331 | 1.5 | 295 | 3.5 |
| 420 | 1.8 | 367 | 1.9 | 370 | 3.6 |
| 436 | 1.6 | 376 | 1.8 | 395 | 3.2 |
| 474 | 1.7 | 24 h | 1.9 | 410 | 3.5 |
| 488 | 1.7 | | | 460 | 2.9 |
| 546 | 1.9 | | | 475 | 3.0 |
| 566 | 1.7 | | | 486 | 3.4 |
| 594 | 2.1 | | | 24 h | 3.3 |
| 617 | 2.0 | | | | |
| 659 | 2.1 | | | | |
| 674 | 2.1 | | | | |
| 24 h | 2.1 | | | | |

[a] Dissociation constant in ethanol (in the absence of $Ca^{2+}$) from refs. 5 and 16.

Table 2

Results from fitting of the chromatographic data to different kinetic models

Subscripts zero, e and $t$ refer to concentrations at zero time, equilibrium and time $t$, respectively.

| Mechanism | Reaction order | Integrated equation [a] | $R$ | $k_2$ ($M^{-1}$ $s^{-1}$) | Correlation coefficient |
|---|---|---|---|---|---|
| (1) $M + Ca^{2+} \xrightarrow{k_2} CaM^{2+}$ | 2 | $\frac{1}{a-b}\ln\frac{b(a-x)}{a(b-x)} = k_2 t$ | 0.25 | 0.3 | 0.99 |
| | | | 0.5 | 0.2 | 0.99 |
| | | | 1.5 | 0.2 | 0.97 |
| (2) $M + Ca^{2+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CaM^{2+}$ | 2 | $\frac{1}{m}\ln\frac{x_2(x_1-x)}{x_1(x_2-x)} = k_2 t$ | 0.25 | 0.5 | 0.95 |
| | | | 0.5 | 0.8 | 0.77 |
| | | | 1.5 | 0.3 | 0.96 |
| (3) $2M + Ca^{2+} \xrightarrow{k_2} CaM_2^{2+}$ | 2 | $\frac{1}{a-2b}\ln\frac{b(a-2x)}{(b-x)a} = k_2 t$ | 0.25 | 0.4 | 0.97 |
| | | | 0.5 | 0.6 | 0.93 |
| | | | 1.5 | 0.4 | 0.98 |
| (4) $M + 2Ca^{2+} \xrightarrow{k_2} Ca_2M^{4+}$ | 2 | $\frac{1}{b-2a}\ln\frac{a(b-2x)}{(a-x)b} = k_2 t$ | 0.25 | 0.8 | 0.91 |
| | | | 0.5 | 0.3 | 0.99 |
| | | | 1.5 | 0.2 | 0.96 |

Nomenclature code:

$a = [M]_0 = (K_d[M_2]_0)^{1/2}$ $\quad K_a = \frac{x_e}{(a-x_e)(b-x_e)}$ $\quad x_1 = \frac{1}{2}\left(a+b+\frac{1}{K_a}+m\right)$

$b = [Ca^{2+}]_0$

$x = (K_d[M_2]_0)^{1/2} - (K_d[M_2]_t)^{1/2}$ $\quad m = \left[\left(a+b+\frac{1}{K_a}\right)^2 - 4ab\right]^{1/2}$ $\quad x_2 = \frac{1}{2}\left(a+b+\frac{1}{K_a}-m\right)$

$x_e = (K_d[M_2]_0)^{1/2} - (K_d[M_2]_e)^{1/2}$ $\quad K_d = (1.0 \pm 0.3) \times 10^{-4}$ M.

[a] From refs. 17 and 18.

or

$$pM + qCa^{2+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{complex} \qquad \text{(step 2)} \qquad \text{(B)}$$

where $k_2$ and $k_{-2}$ refer to the rate constants for the forward and reverse processes, respectively. Note that the validity of the mechanism proposed implies that $k_2 \ll k_{-1}$ (since the free dimer-free monomer equilibrium is maintained during the reaction (table 1)), and that the affinity of $Ca^{2+}$ for the dimer can be considered to be negligible compared to that for the monomer.

In order to verify the validity of the above mechanism and to determine the most likely stoichiometry for the second step, the experimental kinetic results in fig. 2 were fitted to different quantitative reaction or equilibrium kinetic models, which varied in the values of the coefficients $p$ and $q$ as well as in the reaction order and molecularity. Since, as indicated above, only the dimer peak heights were reliable, the integrated equations used [17,18] were rearranged so that they

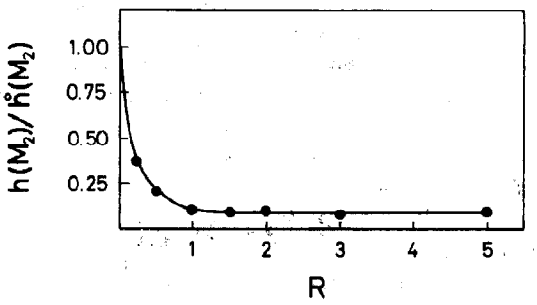


Fig. 3. Curve of titration of gramicidin A with $Ca^{2+}$. HPSEC measurements were carried out after 24 h incubation. The gramicidin A concentration was 0.5 mg/ml.

were dependent only on the free dimer concentrations. $k_2$ can be obtained in all cases directly from the slope of the straight line corresponding to each integrated velocity equation. Table 2 summarizes the general characteristics and equations for the different kinetic models as well as the results obtained from the fittings for the three $Ca^{2+}$ : gramicidin A molar ratios assayed. Based on the hypothesis that $k_2 \ll k_{-1}$, that $k_2$ must be independent of the molar ratio assayed and taking into account the values of the correlation coefficient, mechanism 1 appears to be the most likely, since it provides the lowest and most homogeneous set of values for $k_2$, the average value being $k_2 = 0.23 \pm 0.07$ $M^{-1}$ $s^{-1}$, more than 20-times lower than $k_{-1}$, which confirms the starting hypothesis.

In addition, to corroborate whether the $Ca^{2+}$ : gramicidin A monomer molar binding ratio is indeed unity, the polypeptide was titrated with calcium under the same experimental conditions as in fig. 2, by assaying all samples after 24 h incubation to ensure that equilibrium had been reached. Fig. 3 shows the titration curve at 0.5 mg/ml peptide concentration, expressed as the variation in $h(M_2)/h^0(M_2)$ vs. $R$. As can be seen, the maximum disappearance of free dimer is reached at approx. $R = 1$ whereas further addition of $Ca^{2+}$ caused no additional changes. Therefore, it appears likely that at this polypeptide concentration one $Ca^{2+}$ binds to one gramicidin A monomer. The rather puzzling observation, which we are unable to account for, that a residual free dimer peak (~ 10%) appears in all chromatograms even at high molar ratios could be due to an artifact inherent in the chromatographic strategy used. In this respect, it must be pointed out that identical results were obtained when 2 μl of the ethanolic reaction mixture at equilibrium were directly injected onto the column without prior dilution in THF. Furthermore, this residual peak may be overestimated due to a slight overlap with peak III when the complex species is largely predominant.

Since it has been reported that, unlike $Ca^{2+}$, the divalent cation $Zn^{2+}$ does not cause any changes in the infrared spectra of gramicidin A in ethanol [7] and, on the other hand, it has been pointed out from spectroscopic techniques that $Na^+$ and $K^+$ exhibit little or no interaction with gramicidin A in trifluoroethanol [6], a parallel HPSEC study was carried out to investigate the ability of $K^+$ and $Zn^{2+}$ to alter the conformational equilibrium of the polypeptide and to bind to either conformational species. Fig. 2 includes the kinetic results obtained upon addition of aliquots of these metal cations to a pre-equilibrated 0.5 mg/ml of solution gramicidin A in ethanol at a molar ratio of $R = 1.5$. No significant variation in dimer peak height or in the case of the monomer (not shown) was observed as a function of incubation time. Moreover, no distortion in peaks I and II occurred even when higher molar ratios were

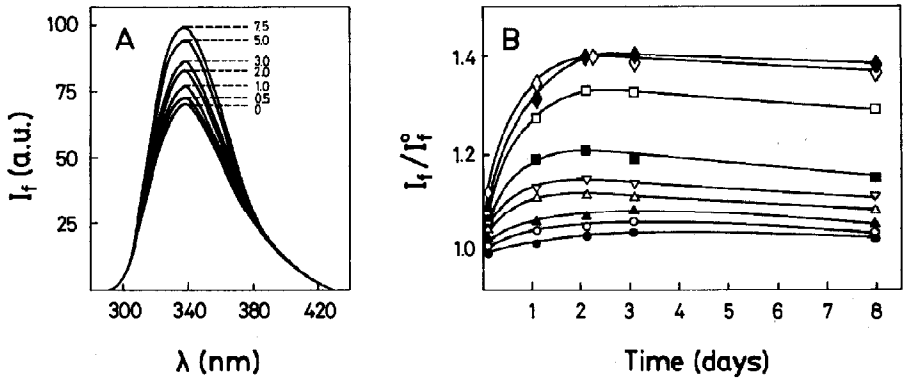


Fig. 4. (A) Corrected emission spectra of gramicidin A solutions in ethanol at different $Ca^{2+}$ : gramicidin A molar ratios (values given beside traces), recorded after 2 days incubation. (B) Time-dependent fluorescence enhancement at different $R$ values: (●) 0.5, (○) 0.75, (▲) 1.0, (△) 1.5, (▽) 2.0, (■) 3.0, (□) 5.0, (◆) 7.5, (◇) 10.0. $I_f^0$ refers to the intensity in the absence of calcium.

assayed. These results demonstrate that, in contrast with $Ca^{2+}$, $Zn^{2+}$ and $K^+$ do not alter the conformational equilibrium of gramicidin A.

### 3.2. Fluorescence measurements

In order to investigate in more depth the conformational changes induced by the binding of calcium to the gramicidin A monomer in ethanol, the kinetics of interaction at 0.01 mg/ml peptide concentration was followed by monitoring both steady-state fluorescence emission and fluorescence anisotropy, at different $Ca^{2+}$ : gramicidin A molar ratios. At this low gramicidin A concentration it can be considered that the polypeptide is quantitatively present in a monomeric form, since the molar fraction of monomers at equilibrium in this solvent is greater than 0.95. Fig. 4A depicts, as an example, some emission spectra of gramicidin A solutions in ethanol at different $R$ values, recorded after 2 days incubation. Although the addition of increasing amounts of $Ca^{2+}$ does not shift the maximum at 338 nm, it does give rise to a marked enhancement in the fluorescence intensity. Fig. 4B shows the time-dependent enhancement at $R$ values ranging from 0 to 10.0, expressed as the relative fluorescence intensity, $I_t/I_t^0$. $I_t^0$ refers to the intensity in the absence of calcium. As expected, the reaction is slower at this polypeptide concentration, the maximum intensity in all cases being reached between 2 and 3 days. On the other hand, the higher the molar ratio, the greater was the enhancement of fluorescence. The slight quenching observed at longer times is similar to that previously reported for the gramicidin A-phosphatidylcholine interaction in THF [1] and could be attributed to tighter rearrangement of bound $Ca^{2+}$ and/or to deterioration of the fluorophores due to aging of the samples. Taking into account the above results, the titration curves presented below were obtained in all cases from measurements carried out after approx. 50 h incubation.

Fig. 5 shows titration of a 0.01 mg/ml of solution gramicidin-A in ethanol (containing more than 95% monomer) with $Ca^{2+}$, followed by fluorescence emission (A) and anisotropy (B) (continuous curves). Very good agreement between

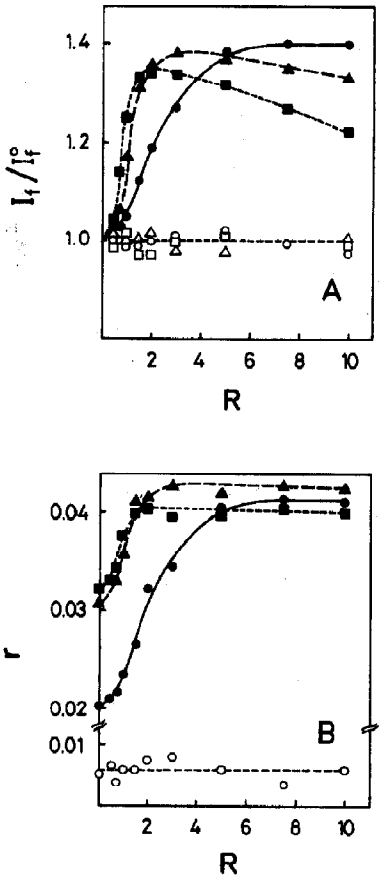


Fig. 5. Variation of $I_t/I_t^0$ (A) and $r$ (B) with the $Ca^{2+}$ : gramicidin A molar ratio, $R$, for gramicidin A samples in ethanol at concentrations of (●) 0.01, (▲) 0.025 and (■) 0.05 mg/ml. Measurements were carried out after approx. 50 h incubation. The effects of $Ca^{2+}$ on $2.15\times10^{-5}$ M NATA (○) (the same Trp concentration as for gramicidin A) and of $Zn^{2+}$ (△) and $K^+$ (□) on 0.01 mg/ml gramicidin A solutions in ethanol are also included.

both techniques is evident, the maximum $I_t/I_t^0$ and $r$ increase being obtained at $R \sim 7$ for both cases. However, the results obtained from these techniques reveal an apparent discrepancy with respect to chromatographic data, which yield a 1:1 stoichiometry for the $Ca^{2+}$-gramicidin A monomer complex. These differences suggested that the number of $Ca^{2+}$-binding sites in the monomer may be dependent on the gramicidin A concentration, so that for greater dilution of the sample, the number of accessible binding sites

increases. This prompted us to extend the fluorimetric study to peptide concentrations varying between 0.5 (used in HPSEC measurements) and 0.01 mg/ml, to verify whether the $R$ value at which saturation occurs varied between 1 and 7. The results of these experiments are also included in fig. 5 for gramicidin A concentrations of 0.05 and 0.025 mg/ml. It is interesting to note that, although for these two concentrations the absorbance of the samples is higher than 0.1, these solutions did not exhibit significant self-quenching effects. As expected, saturation for 0.05 and 0.025 mg/ml gramicidin A concentrations occurred at $R$ values of about 2 and 3, respectively, which confirms that the number of calcium-binding sites decreases as the peptide concentration increases. This point will be discussed later in greater detail.

In addition, titrations of the model fluorophore NATA were carried out over the same range of molar ratios, no significant variation being observed (see fig. 5A and B). This demonstrates that the changes in $I_f/I_f^0$ and $r$ are not due to direct interaction of the cation with the gramicidin A fluorophores but to $Ca^{2+}$-induced conformational changes in the polypeptide backbone.

Finally, the addition of $Zn^{2+}$ and $K^+$ to 0.01 mg/ml ethanolic solutions of gramicidin A caused no variation in polypeptide fluorescence (fig. 5A), which indicates a lack of interaction in agreement with the chromatographic results.

## 4. Discussion

Although a few spectroscopic studies have been previously performed on the interaction of metal cations and especially calcium with gramicidin A in organic polar solvents (such as ethanol, methanol and trifluoroethanol), we propose in the present work an HPSEC methodology which has allowed elucidation of the binding of $Ca^{2+}$ to gramicidin A in ethanol in terms of the polypeptide dimer-monomer conformational equilibrium, by taking advantage of the great resolution power of the Ultrastyragel 1000 Å column. As far as we know, this is the first time that the conformational equilibrium in the presence of calcium has been followed by visualizing separately the free and bound species. Thus, at each moment of time, the relative concentration of the existing species (free dimer, free monomer, calcium-bound monomer) can be determined with the advantages inherent to an HPLC technique (accuracy, reproducibility, rapidity, etc.).

Analysis of the kinetic results suggests the occurrence of very strong, practically irreversible (quantitative reaction) binding of one cation to one polypeptide monomer, according to the following mechanism:

$$M_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2M$$
$$k_2 \downarrow + 2Ca^{2+}$$
$$2CaM^{2+}$$

where $k_2 = (0.23 \pm 0.07)\ M^{-1}\ s^{-1}$. The high affinity of $Ca^{2+}$ for the monomer relative to the dimer may arise from the fact that the latter species has a more closed conformation in ethanol, the carbonyl groups being involved in intermolecular hydrogen bonds. In contrast, the gramicidin A monomer in ethanol has a relatively unordered structure, as pointed out by Veatch and Blout [13], which could provide greater accessibility to the $Ca^{2+}$-binding site.

As concerns the fluorimetric data, comparison between gramicidin A and NATA demonstrates that both the fluorescence enhancement and anisotropy increase induced by binding of $Ca^{2+}$ to the monomer can be mainly attributed to an increase in rigidity of the polypeptide backbone, resulting in a decrease in vibrational deactivation modes of the tryptophan residues. In this connection, it is interesting to note that the approx. 7 : 1 stoichiometry obtained for the $Ca^{2+}$-gramicidin A complex at low polypeptide concentration (fig. 5A and B) could suggest that each $Ca^{2+}$ binds to two carbonyls, which would give rise to a more ordered complex. This apparently contrasts with the 1 : 1 stoichiometry determined from HPSEC at relatively high peptide concentration (fig. 3). This can be explained taking into account that peptide-peptide interactions are quite extensive and, therefore, most $Ca^{2+}$-binding sites in the monomer become inaccessible. That the $Ca^{2+}$ : gramicidin A monomer binding ratio is dependent on the peptide concentration is not an exceptional finding, since

concentration-dependent effects have been reported previously for other proteins [19,20], and recently for the gramicidin A-phosphatidylcholine interaction [1,2]. In this regard, it has been suggested that at relatively high concentration lateral self-association of gramicidin A can occur involving intermolecular tryptophan stacking interactions [21], which would render the monomer ends more accessible to the ion; on the other hand, a $Ca^{2+}$-binding site close to the ethanolamine terminal of gramicidin A has been reported from spectroscopic data [7] and theoretical energy profile computations [8].

Although the conformation of gramicidin A in ethanol in the presence of calcium may not be equivalent to the structure in membranes, it has been pointed out that in a lipid bilayer, a gramicidin A channel formed by a head-to-head dimer (two monomers juxtaposed by their amino ends) has two calcium-binding sites which are only accessible from the adjoining aqueous solution and, thus, probably located near the COOH termini (ethanolamine end).

The cations assayed exhibit selective behavior in terms of the conformational equilibrium of gramicidin A in ethanol. The monomerization of the polypeptide, is induced in the presence of $Ca^{2+}$ (a cation which blocks the gramicidin A channel) but not with $K^+$ (which is transported through the channel) or $Zn^{2+}$ (a cation which does not block the channel). It is difficult to assess whether these results have any biological significance. HPLC experiments are currently in progress in our laboratory to verify this point using gramicidin A incorporated into liposomes in the presence of different cations.

As a conclusion, the chromatographic approach proposed in the present work allows preliminary studies on the gramicidin A dimer-monomer conformational equilibrium in nonpolar solvents [1–3] to be extended to polar ones such as ethanol or methanol. This fact is very important in the light of some future applications of this new methodology: (i) solvents such as benzene, chloroform, ethanol and methanol or mixtures are indistinctly used in the first steps of the preparation of gramicidin A-containing liposomes [11,22–25]. As the dimer/monomer ratio varies with solvent polarity, different polypeptide conformations can be expected in the vesicles, depending on the starting organic solvent used. On the other hand, gramicidin A is incorporated into organic solvent either before hydration of the sample or after the liposomes have been formed. In the latter case, the addition of gramicidin A is usually made from an ethanolic solution. Work is in progress using a novel strategy based on HPSEC for the analysis of gramicidin A incorporated into liposomes which indicates that these factors (solvent used and method of incorporation) can determine the conformation of the polypeptide in the artificial vesicle. (ii) In recent years, attention has been also directed toward the synthesis of polypeptides in order to obtain molecules with the ability to form ion channels [26–29]. However, the molecular conformations of these peptides are deduced at present basically from spectroscopic techniques both in organic solvent [26–29] and upon binding with phospholipid membranes [28,29]. In this respect, the chromatographic methodology used provides a basis for further studies of synthetic hydrophobic polypeptides in organic solvents where the occurrence of interconverting conformational species in different aggregation states is very common [26–29]. In addition, as mentioned above, characterization of the polypeptide aggregation states in the organic solvent is necessary for the subsequent HPSEC study of the peptide incorporated into liposomes.

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