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Gramicidin S inhibition of the Ca^{2+}-ATPase of human red blood cells

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The cationic amphiphilic polypeptide gramicidin S inhibits the Ca^{2+}-ATPase of human red-cell membranes by lowering the maximum velocity of the high-affinity component and the apparent affinity of the low-affinity component of the velocity-versus-ATP concentration curve of the enzyme. Gramicidin S does not alter the apparent affinity of the Ca^{2+}-ATPase for Ca^{2+}. Calmodulin is not essential for the inhibition, but increases the sensitivity of the enzyme to the inhibitor. The effects of gramicidin S on the Ca^{2+}-ATPase can be reversed with phosphatidylcholine vesicles but not with buffer solutions, suggesting that gramicidin S acts from the lipid phase of the membrane.

Introduction

The channel-forming polypeptide alamethicin increases almost 4-fold the adenylate cyclase and the $(Na^+ + K^+)$-ATPase activities of a cardiac microsomal membrane preparation, whereas it increased at most only 1.5-fold the activity of the Ca^{2+}-ATPase of the same preparation [1]. Activation by alamethicin has been attributed to the formation of channels of sufficient size to allow permeation of ATP and cofactors across the vesicles' membrane [1]. More recently, it has been reported that gramicidin S, as well as various channel-forming polypeptides, stimulate the calcium/calmodulin-dependent guanylate cyclase from the protozoa *Paramecium* [2]. After this, it was suggested that amphiphilic polypeptides may constitute a useful group of compounds which enhance the activity of membrane-bound enzymes in general [2].

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Gramicidin S is a cyclic decapeptide antibiotic and carries two cationic ornithine residues on one side and hydrophobic residues on the other side of the molecule. In contrast with alamethicin, gramicidin S has no channel-forming properties. Nevertheless, after penetrating into the lipid bilayer gramicidin S disturbs the permeability barrier of sheep erythrocytes, increasing the permeability of the membrane to K^+ [3].

This paper reports the results of experiments designed to test the effects of gramicidin S on the Ca^{2+}-ATPase from human red cell membranes, a system which like the adenylate cyclase and the guanylate cyclase, is highly dependent on calmodulin and the lipid environment [4]. Results show that gramicidin S inhibits rather than activates the Ca^{2+}-ATPase activity.

Materials and Methods

Fresh blood from haematologically normal adults collected on acid/citrate/dextrose solution was always used. Calmodulin-depleted membranes were obtained by the procedure of Gietzen et al. [5].

ATPase activities were measured estimating the release of ^{32}P from [γ-^{32}P]ATP as described previously [6]. Except when otherwise indicated in Results, the incubation media in which Ca^{2+}-ATPase activity was measured contained: 20 mM KCl, 3.75 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.4 at 37 °C), 1 mM EGTA, 1 mM ouabain, 2 mM [γ-^{32}P]ATP, 4% (v/v) dimethylsulfoxide and various concentrations of $CaCl_2$. The concentration of Ca^{2+} in the incubation media was measured with an IS-561 Ca^{2+}-selective electrode [7]. The medium in which $(Na^+ + K^+)$-ATPase activity was measured contained: 20 mM KCl, 130 mM NaCl, 3.75 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.4 at 37 °C), 1 mM EGTA, 2 mM [γ-^{32}P]ATP and 4% (v/v) dimethylsulfoxide. The reaction was started by the addition of ATP to the rest of the components of the reaction mixture at 37 °C. The amount of membranes per ml of incubation media was that equivalent to 0.05 mg of membrane protein. Ca^{2+}-ATPase activity was the difference between the activities in media with and without Ca^{2+}. $(Na^+ + K^+)$-ATPase activity was the difference between the activities in media without and with 1 mM ouabain. Measurements of enzyme activities were made in triplicate and the individual measurements did not differ from the mean by more than 10%.

Calmodulin was purified from bovine brain as described by Kakiuchi et al. [8]. Gramicidin S was dissolved in dimethylsulfoxide and added to the incubation media from a concentrated solution.

The concentration of protein was estimated by the procedure of Lündahl [9].

[γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chappell [10] except that no unlabelled orthophosphate was added to the incubation media. [^{32}P]Orthophosphate was provided by the Comisión Nacional de Energía Atómica (Argentina). Gramicidin S, ATP, enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were obtained from Sigma (U.S.A.). Phosphatidylcholine was a product of Fluka (Switzerland). Salts and reagents were of analytical reagents grade.

Results

Fig. 1 shows the results of an experiment designed to test the effect of gramicidin S at different concentrations, on Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities of red cell membranes. Ca^{2+}-ATPase activity drops to a value which at 60 μM gramicidin S represents about 10% of the control activity. The concentration of gramicidin S needed for half-maximal inhibition was 10 μM. Results in Fig. 1 also show that gramicidin S inhibits the $(Na^+ + K^+)$-ATPase activity of the red cell membranes with different kinetics than that of the Ca^{2+}-ATPase, since the $(Na^+ + K^+)$-ATPase activity remains unaltered up to 30 μM gramicidin S and then falls to a value which at 60 μM gramicidin S represents about 20% of the control.

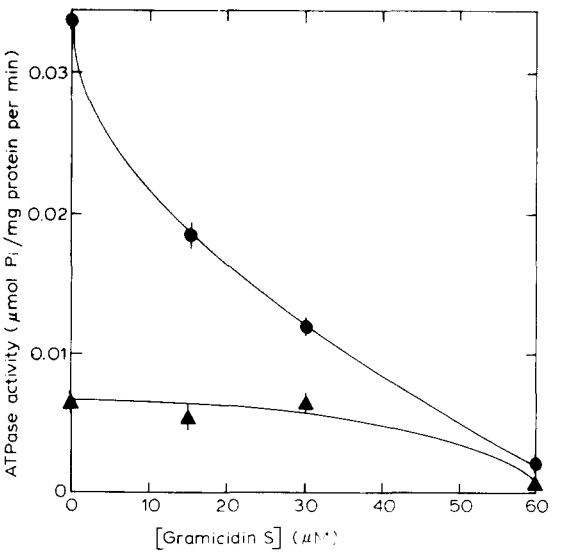

Fig. 1. Effect of varying concentrations of gramicidin S on Ca^{2+}-ATPase (●) and $(Na^+ + K^+)$-ATPase (▲) activities (± S.D.) of red cell membranes. The measurements were made simultaneously on the same preparation of membranes. The medium in which the Ca^{2+}-ATPase activity was assayed contained 15 μM Ca^{2+} and 120 nM calmodulin.

To observe the dependence of the inhibition with the membrane concentration, the effect of 30 μM gramicidin S on Ca^{2+}-ATPase activity was tested in media containing different amounts of membrane. As the concentration of membranes rose from 0.05 to 0.20 mg of membrane protein per ml, the inhibition dropped from 75% to 47% of the control in the absence of gramicidin S. After this, all assayes were performed in media containing 0.05 mg of membrane protein per ml.

To see whether washing of the membranes re-

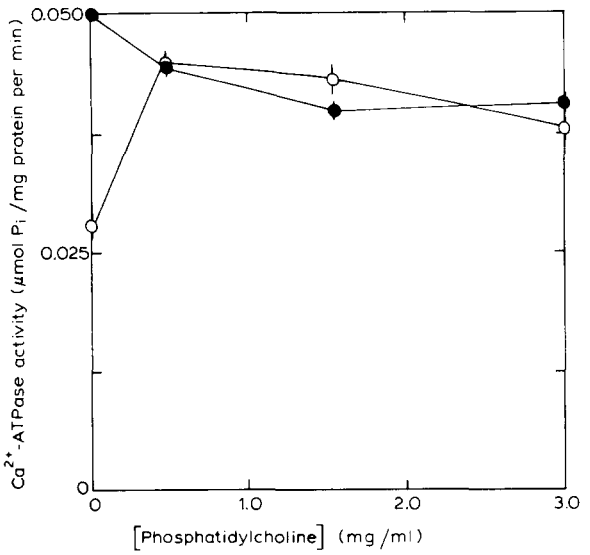

Fig. 2. Effect of the concentration of phosphatidylcholine on Ca^{2+}-ATPase activity (±S.D.) in media with (○) and without (●) 20 μM gramicidin S. The reaction media contained 15 μM Ca^{2+} and 120 nM calmodulin. Phosphatidylcholine vesicles were prepared by sonicating five times every 30 s at room temperature a mixture of 4% (w/v) phosphatidylcholine, 2% (w/v) Triton X-100 and 80 mM dithiothreitol in water. The sonicated mixture was kept at −20 °C until use.

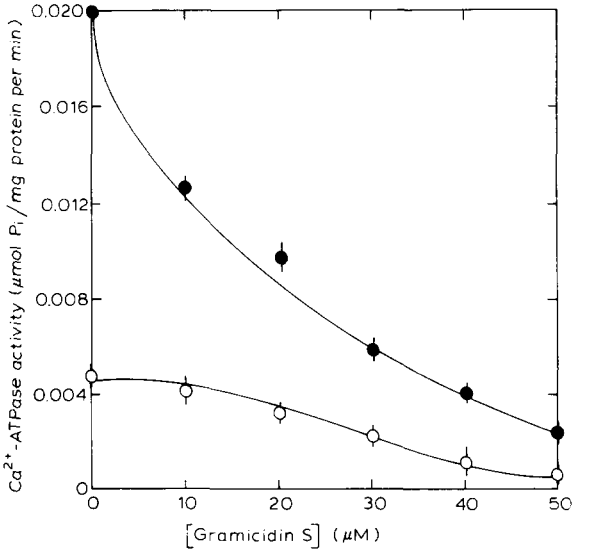

Fig. 3. Effect of varying concentrations of gramicidin S on Ca^{2+}-ATPase activity (±S.D.) in the presence (●) and absence (○) of 120 nM calmodulin. The concentration of Ca^{2+} in the reaction media was 15 μM.

stores the activity of the Ca^{2+}-ATPase after treatment with gramicidin S, red cell membranes incubated at 37 °C with 20 μM gramicidin S during 15 min were washed three times with 15 vol. of the incubation medium without gramicidin S, and their Ca^{2+}-ATPase activity was measured. The activity, which was 5% of the control at the beginning of the experiment, rose only to 17% of the control after washing. If the gramicidin S molecules that are responsible for the inhibition accumulate in the lipidic region of the cell membrane, exposing the membrane to either lipid vesicles or albumin [11] would result in removal of gramicidin S and reactivation of the enzyme. To test this point, red cell membranes treated with and without 20 μM gramicidin S were incubated with different concentrations of phosphatidylcholine vesicles and their Ca^{2+}-ATPase activity was measured. Results in Fig. 2 show that Ca^{2+}-ATPase activity, which in the presence of 20 μM gramicidin S was about 60% of the control, becomes no different to the control after treating the membranes with the phospholipid vesicles.

In all the experiments that have been described up to this point, Ca^{2+}-ATPase activity was measured in the presence of saturating concentrations of calmodulin. The effects of gramicidin S on the activity of the Ca^{2+}-ATPase in the presence and absence of calmodulin are shown in Fig. 3. The response of the activity depends on calmodulin, since in its absence inhibition was biphasic; up to 20 μM gramicidin S the drop in activity is small if compared to that of the activity with calmodulin, while at concentrations of gramicidin S 40 μM or higher, inhibition becomes almost independent of calmodulin.

Fig. 4 allows us to compare the curve of inhibition of the Ca^{2+}-ATPase from control membranes with and without calmodulin with that from membranes submitted to partial proteolysis with trypsin. Partial proteolysis drives the Ca^{2+}-ATPases into a state which, as judged by its kinetic parameters, seems to correspond to a conformation similar to that attained by combination with calmodulin [4,12]. Results show that the shape of the curve of Ca^{2+}-ATPase activity vs. [gramicidin S] of membranes treated with trypsin is similar to that of intact membranes and different from that of intact membranes in the presence of calmodulin.

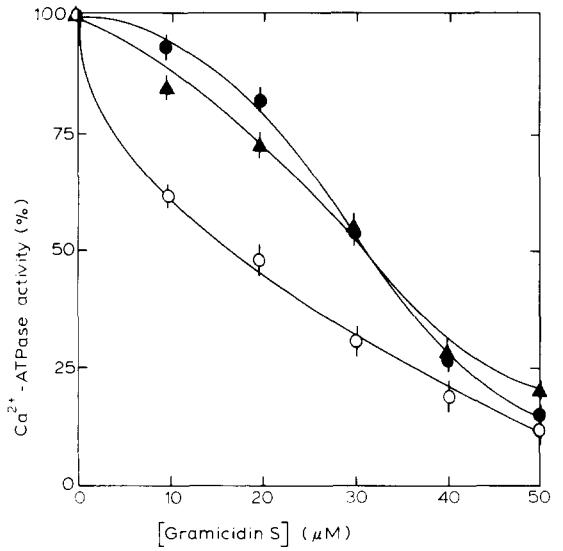

Fig. 4. Effect of varying concentrations of gramicidin S on Ca^{2+}-ATPase activity (±S.D.) of intact membranes (●), of membranes after partial proteolysis with trypsin (▲), and of intact membranes in the presence of 120 nM calmodulin (○). For partial proteolysis, the membranes (1.4 mg protein/ml) were suspended in a medium containing 30 mM Tris-HCl (pH 7.4 at 37° C), 60 μM $CaCl_2$ and 4.5 μg trypsin/ml (about 200 units/ml). The mixture was incubated at 37° C and after 2 min trypsin action was terminated by the addition of a 10-fold excess (w/w) of soybean trypsin inhibitor plus 100 μM EGTA. Activities are expressed as percent of the activities in the absence of gramicidin S which were 0.005, 0.003 and 0.02 μmol/mg protein per min for the intact membranes, the membranes after partial proteolysis, and the intact membranes in the presence of calmodulin respectively. The concentration of Ca^{2+} in the reaction media was 15 μM.

The effect of Ca^{2+} on the Ca^{2+}-ATPase activity in the presence of calmodulin was measured in media with and without 30 μM gramicidin S. Results in Table I show that, while the maximum activity was lowered to one-fourth of the control, there was no significant change in the apparent affinity of the Ca^{2+}-ATPase for Ca^{2+} upon inclusion of gramicidin S in the reaction medium. It seems worth pointing out here that the experiment in Table I was performed with saturating amounts of calmodulin and, accordingly, the value of the $K_{0.5}$ for Ca^{2+} is that characteristic of the Ca^{2+}-ATPase associated with calmodulin.

To test the effect of gramicidin S on the response of the Ca^{2+}-ATPase to ATP, the activity of the enzyme was measured over a wide range of ATP concentrations in media containing a saturating concentration of Ca^{2+} with or without 20 μM gramicidin S, with the results shown in Fig. 5. As is known [4,6], under control conditions the

TABLE I

KINETIC PARAMETERS FOR THE ACTIVATION OF THE Ca^{2+}-ATPase BY Ca^{2+}

Ca^{2+}-ATPase activity was measured at varying Ca^{2+} concentrations in the presence of 120 nM calmodulin in media with and without 30 μM gramicidin S. The values (±S.D.) are those of the Michaelis–Menten-like equation that fit best the experimental points. The reaction media contained 0.1 mM instead of 1 mM EGTA as stated in Materials and Methods.

[Gramicidin S] (μM)	$K_{0.5}$ for Ca^{2+} (μM)	V_m (μmol P_i/mg protein per min)
0	9.4±3.7	0.126±0.028
30	7.1±2.6	0.033±0.007

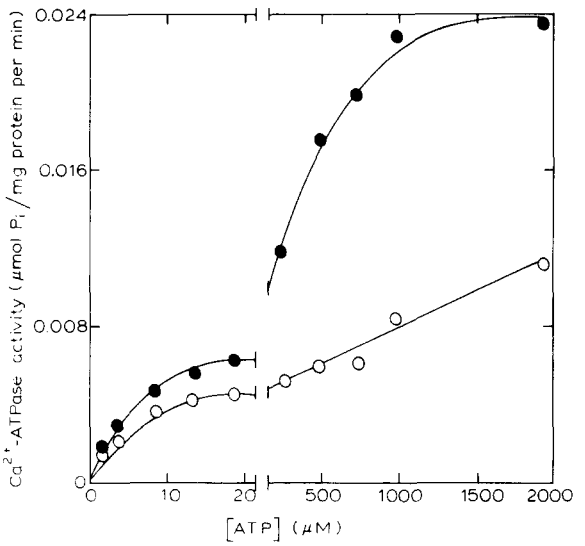

Fig. 5. The substrate curve of the Ca^{2+}-ATPase in the presence (○) and in the absence (●) of 20 μM gramicidin S. The reaction media contained 120 nM calmodulin and the concentration of Ca^{2+} was measured in each of the media and ranged from 20 to 23 μM. The curve that fits the filled circles represents Eqn. 1 in Results with (±S.D.): $K_{m1} = 5.2 \pm 3.3$ μM; $K_{m2} = 544 \pm 263$ μM, $V_1 = 0.0077 \pm 0.002$ μmol P_i/mg protein per min and $V_2 = 0.022 \pm 0.003$ μmol/mg protein per min. The curve that fits the open circles represents a modification of Eqn. 1 in which the second term on the right-hand side has been replaced by $(V_2/K_{m2})\cdot[ATP]$, with $K_{m1} = 3.0 \pm 1.0$ μM, $V_1 = 0.0050 \pm 0.0004$ μmol P_i/mg protein per min, and $(V_2/K_{m2})\cdot[ATP] = (3.63 \pm 0.47)\cdot 10^{-6}$ μmol/mg protein per min. The curve was adjusted to the experimental results by least-squares non-linear regression using the procedure of Gauss–Newton with optional damping [13].

activation curve is biphasic, with high-affinity–low-velocity and low-affinity–high-velocity components, and can be adjusted by the sum of two Michaelis–Menten equations, i.e.

$$v = \frac{V_1[\mathrm{ATP}]}{[\mathrm{ATP}] + K_{m1}} + \frac{V_2[\mathrm{ATP}]}{[\mathrm{ATP}] + K_{m2}} \quad (1)$$

where V_1 and V_2 are the maximum velocities and K_{m1} and K_{m2} are the Michaelis–Menten constants of the high- and low-affinity components, respectively. In the media with gramicidin S, Ca^{2+}-ATPase activity is lower at all the concentrations of ATP tested, and at high ATP concentrations the curve tends to a straight line of positive slope represented by $(V_2/K_{m2}) \cdot [\mathrm{ATP}]$. Comparison of the best-fitting values of K_{m1} and V_1 for the control curve and the curve in the presence of gramicidin S (see legend to Fig. 5) suggests that gramicidin S decreased V_1 without exerting any significant effect on K_{m1}. Assuming that gramicidin S does not alter the value of V_2, from the positive slope of the straight line representing the second component of the substrate curve in the presence of gramicidin S (V_2/K_{m2}) and the value of V_2 from the control curve, it can be calculated that in the presence of 20 μM gramicidin S the value of K_{m2} was about 6 mM, that is 10-fold higher than that calculated for the curve that represents the activity under control conditions.

Discussion

Results show that the cationic amphiphilic antibiotic gramicidin S inhibits the Ca^{2+}-ATPase of human red cell membranes. At a fixed concentration of gramicidin S, inhibition depends on the amount of membranes in the reaction media and at non-limiting concentration of gramicidin S the Ca^{2+}-ATPase is fully inhibited by the antibiotic. The effect is not specific for this enzyme since, although with less potency, gramicidin S also inhibits the $(Na^+ + K^+)$-ATPase from the same membrane preparation.

Washing of the membranes with incubation medium without gramicidin S does not result in recovery of the activity. Deficient recovery after washing may be indicatory of either lack of equilibrium between gramicidin S in the incubation medium and gramicidin S in the membrane or irreversible damage of the enzyme. Reactivation of the Ca^{2+}-ATPase by phospholipid vesicles demonstrates the gramicidin S causes no irreversible damage to the enzyme, and suggests rather strongly that its action is exerted from the lipid core of the membrane.

Results show that gramicidin S lowers the apparent affinity of the low-affinity component and the maximum velocity of the high-affinity component of the activity – [ATP] curve of the Ca^{2+}-ATPase. As a consequence of this in media with calmodulin and up to 2 mM ATP, which are the concentrations of ATP commonly used to assay the enzyme activity, inhibition by gramicidin S will be much more apparent on the low-affinity than on the high-affinity component of the activity – [ATP] curve. Results also show that the apparent affinity of the enzyme for Ca^{2+} remains the same regardless of whether gramicidin S is present or not. This suggests rather strongly that gramicidin S does not alter the ability of the Ca^{2+}-ATPase to combine with Ca^{2+}. Considering these results, it can be concluded that gramicidin S inhibits the Ca^{2+}-ATPase by lowering both the apparent affinity for ATP of the low-affinity component and the maximum velocity of the high-affinity component of the activity – [ATP] curve.

The response of the Ca^{2+}-ATPase to gramicidin S changes with calmodulin, since the activity – [gramicidin S] curves in the presence and absence of calmodulin show that the fraction of Ca^{2+}-ATPase activity elicited by calmodulin drops along a curve that is half-maximal at about 10 μM gramicidin S, while the activity in the absence of calmodulin from both intact and proteolyzed membranes is inhibited along a biphasic curve that reaches its half-maximal value at a concentration of gramicidin S 2- to 3-fold higher than in the presence of calmodulin. No clear-cut explanation for such behaviour can be advanced. At first hand it seems reasonable to attribute part of the decrease in Ca^{2+}-ATPase activity to inhibition of calmodulin by gramicidin S. In favour of this is the fact that the apparent affinity of the low-affinity site for ATP depends on calmodulin [14], plus the observation that the dependence of the inhibition by gramicidin S on calmodulin resembles very much the dependence on calmodulin reported by Vincenzi et al. [15] for inhibition of the Ca^{2+}-

ATPase by trifluoperazine in purified and reconstituted enzyme and by Gietzen et al. [16] for inhibition by calmidazolium in red-cell membranes. Trifluoperazine and calmidazolium have been proposed as inhibitors of calmodulin-mediated effects and share with gramicidin S the property of being amphiphilic cations of low molecular weight.

Nevertheless, experimental results in this paper are not in keeping with the idea that gramicidin S lowers the activity of the Ca^{2+}-ATPase by inhibiting calmodulin. (i) Gramicidin S does not lower the high apparent affinity for Ca^{2+} which is characteristic of the Ca^{2+}-ATPase associated to calmodulin. (ii) In the absence of calmodulin, gramicidin S inhibits the Ca^{2+}-ATPase with the same effectiveness before and after partial proteolysis. (iii) After inhibition with gramicidin S, the activity of the Ca^{2+}-ATPase associated to calmodulin can be recovered by exposing the membranes to phosphatidylcholine vesicles.

If the interaction of gramicidin S with calmodulin is discarded, it can be considered that inhibition could be brought about by direct interaction of gramicidin S from the lipid bilayer with sites in hydrophobic domains of the Ca^{2+}-ATPase. Interaction with sites in the enzyme molecule has been proposed for the inhibition of the Ca^{2+}-ATPase by trifluoperazine [15,16] which, as mentioned above, antagonizes the activity of the Ca^{2+}-ATPase in a way resembling that of gramicidin S. Nevertheless, it has to be taken into consideration that there are data in the literature showing that, among other effects, gramicidin S decreases the phase transition temperature of liposomes of neutral phospholipids like dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine [17] and increases K^+ efflux from sheep red blood cells and from liposomes prepared with phosphatidylcholine [3]. These effects suggest that penetration of gramicidin S into the lipid bilayer induces membrane disturbances to lipid and biological membranes. Such disturbances could well cause the kinetic changes that lead to the inhibition of the Ca^{2+}-ATPase, an idea that finds further justification in the strong dependence of the activity of the Ca^{2+}-ATPase on the lipids from the membranes [4]. Assuming that there are $1.5 \cdot 10^{-11}$ mol of Ca^{2+}-ATPase per mg of red-cell membrane protein [4], that under the assay conditions used in this paper 20 μM gramicidin S inhibits half of the Ca^{2+}-ATPase units, and that all the gramicidin S present in the volume (0.5 ml) of the reaction mixture has accumulated in the cell membrane, it can be calculated that $2.6 \cdot 10^4$ molecules of gramicidin S are needed to inhibit one Ca^{2+}-ATPase unit. Although this value may be a gross overestimation of the stoichiometric factor intended to be estimated, it points more to perturbation of the lipid environment than to binding of gramicidin S to a small number of specific sites in the Ca^{2+}-ATPase as the cause of the inhibition. Further studies will be necessary before proposing the site of action of gramicidin S.

This paper is the first report of a treatment with a relatively small molecule that changes the interaction of the Ca^{2+}-ATPase with ATP at the regulatory site without affecting the interaction with Ca^{2+}. A similar effect on binding of a monoclonal antibody to a Ca^{2+}-ATPase preparation from pig stomach smooth muscle has been reported recently [18]. These findings suggest that the apparent affinity for Ca^{2+} at the transport site and the apparent affinity of the low-affinity regulatory site for ATP are controlled by different processes.

The effects of gramicidin S resemble very much those of compound 48/80 on the interaction of ATP with the Ca^{2+}-ATPase [14]. Like gramicidin S, compound 48/80 has cationic and hydrophobic groups in its molecule. The main difference between the effects of gramicidin S and 48/80 is that the latter also lowers the apparent affinity of the Ca^{2+}-ATPase for Ca^{2+}.

Acknowledgements

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