Volume 321, number 2,3, 145–148 FEBS 12343 April 1993

Microcin E492 forms ion channels in phospholipid bilayer membranes†

Rosalba Lagos, Marcela Wilkens*, Cecilia Vergara, Ximena Cecchi and Octavio Monasterio

Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

Received 12 February 1993; revised version received 9 March 1993

Microcin E492, a polypeptide antibiotic, has been shown to have an M_r of 6,000 by urea-SDS-polyacrylamide gel electrophoresis of the fluorescently labelled compound. It is known that the bactericidal action of microcin involves a loss of the transmembrane potential. In this study we show that microcin forms cation-selective channels in planar phospholipid bilayers. The channels have two main conductance states the current–voltage curves of which rectify. The reversal potentials measured under biionic conditions indicate a permeability sequence of $NH_4^+ > K^+ = Rb^+ = Cs^+ > Na^+ = Li^+ > Tris^+$. The results suggest that membrane potential dissipation induced by microcin is a consequence of the formation of pores in the bacterial membrane.

Ion channel; Lipid bilayer; Microcin E492; Antibiotic; *Klebsiella pneumoniae*

1. INTRODUCTION

Microcins are a family of low molecular weight antibiotics produced and excreted by enterobacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. These antibiotics share several characteristics such as resistance to certain proteases and to extremes of pH and temperature, and solubility in methanol [1]. Microcin E492, which has been reported to be a peptide of M_r between 5,000 and 7,000 [2], is produced by the *K. pneumoniae* RYC492 strain. *E. coli* preloaded with the lipophilic ion tetraphenylphosphonium (TTP^+) is unable to retain this ion in the presence of exogenous microcin E492, probably due to a rapid loss of the transmembrane potential [2]. It is therefore reasonable to think that this microcin, as some colicins, acts by making ion channels in the plasma membrane. In this work we demonstrate the ability of microcin E492 to form ion channels in a lipid bilayer. This approach has been used to study the action of colicins A, K and other channel-forming colicins [3–5].

2. MATERIALS AND METHODS

2.1. *Isolation, purification, and fluorescent labelling of microcin E492*

Microcin E492 was extracted from the supernatant of cultures of the producer strain *Klebsiella pneuminiae* RYC492 grown in citrate medium, by passage of this supernatant through a cartridge of Sep Pak C18 (Waters) on which the microcin was retained [6]. The peptide was eluted with 95% methanol and its activity was estimated by the critical dilution method [7]. Microcin was concentrated by evaporation in a boiling water bath without a significant loss of activity, and used immediately for incorporation in planar bilayers.

Electrophoresis of microcin samples was performed on urea-SDS-polyacrylamide gels as described by Hashimoto et al. [8], except that urea was added in a 3.5–7.0 M linear gradient instead of 7 M (C. Retamal and J. Babul, personal communication). Electrophoresis was performed at a constant current of 20 mA/gel. Fluorescent labelling of the microcin was performed by adjusting samples to 0.04 M sodium borate, pH 8.0 and then adding an equal volume of 0.5 mg/ml of fluorescein isothiocyanate (FITC) in dimethyl sulfoxide. After electrophoresis the bands were visualized under short-wave UV light [9].

Microcin treatment with trypsin and chymotrypsin was performed according to de Lorenzo [6]. After incubating microcin with these proteases, the samples were labelled with FITC as described above.

2.2. *Single channel recordings*

Bilayers were formed by the technique of Mueller et al. [10] using a 4:1 mixture of palmitoyl- oleoyl phosphatidylethanolamine (POPE) and phosphatidylcholine (PC), to give a final lipid concentration of 20 mg/ml in decane. The membrane separated two aqueous compartments containing 0.1 M KCl, 0.01 M 2-*N*(morpholino)ethanesulfonic acid (MES)-KOH pH 7.0 unless otherwise stated. One of the bilayer compartments (*cis* side) was connected to an adjustable DC potencial source that allowed the application of potentials from 0 to ± 200 mV. The current flowing through the membrane as a result of the applied potential was measured by a current to voltage transducer connected to the other compartment (*trans* side), that therefore was virtual earth. Current was simultaneously monitored on a chart recorder, on an oscilloscope, and recorded on a video tape for subsequent analysis. Microcin was added to the solution in the *cis* compartment. In order to measure anion versus cation selectivity, concentrated KCl was added to the *cis* compartment and the current–voltage curve was determined in this asymmetric condition. To measure relative permeabilities among cations, the current–voltage curve was determined under biionic conditions after perfusing the *cis* compartment with the different cations as their chloride salts at 0.1 M. The data presented in Fig. 4 were fitted by a non-linear regression subroutine of SigmaPlot 5.0.

Correspondence address: R. Lagos, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile. Fax: (56) (2) 271 3891.

**Permanent address:* Departamento de Química, Facultad de Ciencia, Universidad de Santiago, Av. Ecuador 3425, Santiago, Chile.

†This paper is dedicated to the memory of Dr. Hermann Niemeyer F., deceased June 7, 1991.

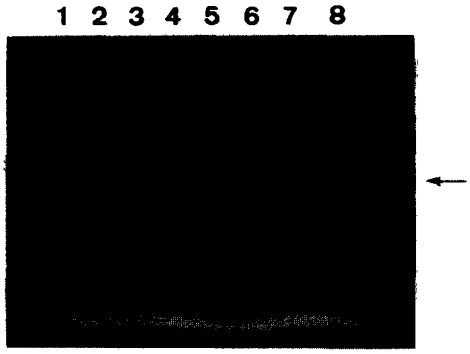

Fig. 1. Urea-SDS-polyacrylamide gel electrophoresis of microcin E492 covalently linked to FITC. Details are given in section 2. Lane 6 shows the migration of fluorescently labelled microcin, indicated by the arrow; microcin previously treated with trypsin (lane 3) or chymotrypsin (lane 4). Controls included fluorescent labelling mixture without microcin (lane 1), and lanes 2 and 7, trypsin and chymotrypsin without microcin, respectively. Molecular weight markers (lanes 5 and 8) were myoglobin fragments of M_r 14,400, 8,160, and 6,210.

3. RESULTS

Previous reports by de Lorenzo [6], and de Lorenzo and Pugsley [2], concluded that an accurate determination of molecular weight of microcin by polyacrylamide gel electrophoresis systems was not possible because microcin E492 could not be detected directly in traditionally stained gels after electrophoresis. In this report we introduce the use of fluorescently labelled microcin in order to visualize the band corresponding to microcin on gels.

Fig. 1 shows the results of electrophoretic gel analysis in a urea-SDS-polyacrylamide system of microcin obtained after the purification in a C18 cartridge and fluorescent labelling as described in section 2. A fluorescent band with a migration close to M_r 6,000 is observed (lane 6). We identified this M_r 6,000 band as microcin since growth inhibition zones were observed when slices from the gel were cut and incubated on plates seeded with 5×10^7 CFU of *E. coli* RYC1000 in LB agar. No other regions of the gel caused such growth inhibition. The assignment of this band to microcin was further confirmed by experiments using trypsin and chymotrypsin. De Lorenzo [6] reported that the biological activity of microcin E492 was trypsin-resistant but very sensitive to chymotrypsin. The band assigned to microcin remained unaltered when treated with trypsin (lane 3) and disappeared after the treatment with chymotrypsin (lane 4).

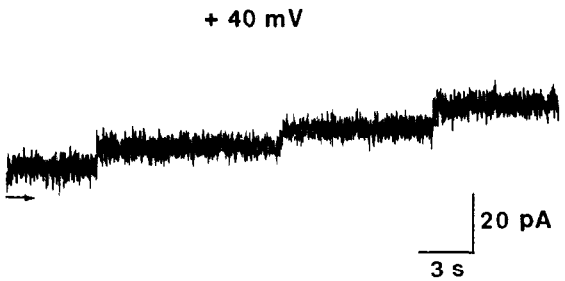

Fig. 2. Current steps arising when several microcin E492 channels are incorporated in a phospholipid bilayer. The arrow at the left indicates zero current.

We tested the ability of microcin E492 to induce an ion conductance in artificial lipid bilayers. When microcin E492 was added in the range of 0.3–0.5 μg/ml, a continuous increase in the membrane current was induced. Using lower protein concentrations, individual channels were resolved. Fig. 2 shows the increase of the membrane conductance that resulted from the formation of individual pores. At the applied voltage of +40 mV an average stepwise increase of 130 pS was observed in 0.1 M KCl. Fig. 3 shows the current flowing through a single microcin E492 channel applying ± 70 mV. The channel had several conductance states, states labelled 1 and 2 being the preferred ones. Microcin E492 also presented other less frequent conductance states. From state 2, the channel can go to two different states of smaller conductance and from state 1 the channel undergoes very fast transitions to a higher conductance state. At least 15 independent experiments with single channels were performed. All the recorded channels presented a similar kinetic pattern with variation in the conductance values for each state of no more than 15%.

Fig. 4 shows three current–voltage curves: state 1 (triangles); transition from state 1 to state 2 (circles); and a scaled-down macroscopic current (squares). It can be observed that the single channel and the macroscopic current behave as rectifiers. The conductances for state 1 were 143 and 95 pS at ± 60 mV, respectively; and for the transition from state 1 to state 2 at the same potentials were 92 and 40 pS, respectively. The single channel curves were drawn using data of three independent experiments. The scaled-down macroscopic current was obtained after dividing the macroscopic current by a factor resulting from the ratio of the macroscopic and single channel current at −50 mV. The macroscopic curve is very similar to the single channel *I/V* curve, indicating that the 52 microcin channels incorporated in this membrane behaved similarly. The reversal potential found in asymmetric ion conditions (0.3/0.1 M KCl *cis/trans*), indicates that the microcin channels are selective for potassium over chloride (not shown).

Measurements under biionic conditions for state 1 and the transitions between states 1 and 2 were performed. The permeability ratios with respect to K^+ calculated from the reversal potentials gave the following permeability sequence: NH_4^+ (1.50) > K^+ (1.00) = Rb^+ (1.00) = Cs^+ (1.00) > Na^+ (0.71) = Li^+ (0.71) > $Tris^+$ (0.25).

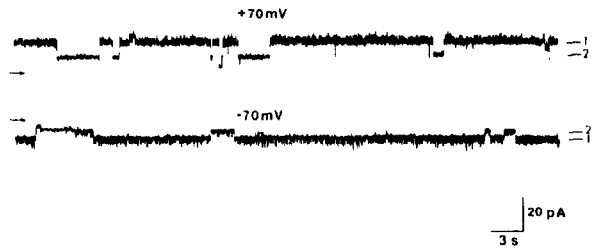

Fig. 3. Conductance fluctuations induced by microcin E492 in a phospholipid bilayer. The arrows at the left indicate zero current. States labelled 1 and 2 are the more frequently observed.

4. DISCUSSION

It has been described that colicins A, E1, K, Ia, and Ib can form ion channels as demonstrated by the stepwise increase in conductance observed after the addition of colicins to lipid bilayer membranes [3–5]. The conductance in 1.0 M NaCl for colicin E1 channels is 21 pS [11], for colicin K channels in 0.1 M KCl is 1.5–10 pS [4] and for colicins A and B in 1.0 M KCl are 14 and 16 pS, respectively. In spite of the fact that these determinations were done under different conditions (salt concentration and pH), it seems that the microcin E492 channel presents conductances in 0.1 KCl which are substantially higher (40–143 pS) than that of colicins. Colicins E1- and K-induced channels are partially cation-selective (Na^+ to Cl^- of 4:1) [4,11], whereas microcin E492 is completely selective to cations. Colicins K, Ia, Ib, E1, A and B form voltage-dependent channels [3,12], and some of them such as E1 and B present rectification of the *I/V* curve, as does the microcin E492 channel. However, a strict comparison of the sequence of permeability between colicins and the microcin E492 is difficult, because even though the data for some of them exist [12], the determinations were performed under different experimental conditions. It is necessary to know the primary structure of microcin E492 in order to classify it as belonging to a particular group of ion channel-forming proteins as has been done with colicins [3]. This study is currently under progress.

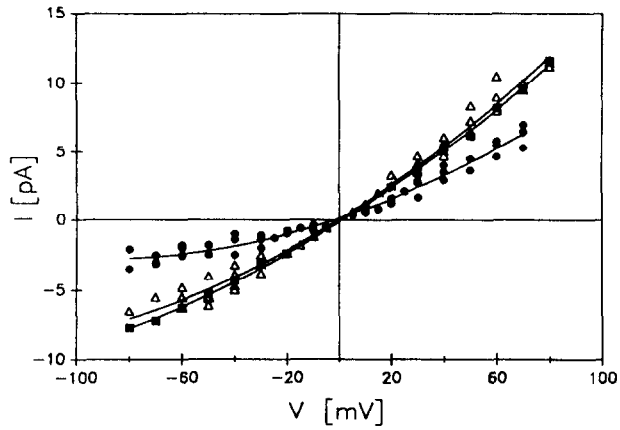

Fig. 4. Scaled-down macroscopic and single channel current-voltage curves of microcin E492. (△) state 1; (●) transition from state 1 to state 2; (□) macroscopic current.

The ability of microcin E492 to form ion channels in planar phospholipid bilayers suggests that its bactericidal action resides in the capacity to form pores in the cytoplasmic membrane, as has been described for the colicins mentioned above. Microcin being a protein of M_r 6,000, the channels recorded are most probably formed by the aggregation of several molecules. The rectification of the *I/V* curve could represent an asymmetric structure of the channel. Further studies to characterize this channel are in progress.

The small molecular weight of microcin as compared to colicins represents an advantage for the analysis of the relationship between its structure and function. The three-dimensional structure of microcin can be studied in solution through bidimensional NMR, a method that can replace X-ray crystallography when rather small proteins are analyzed. The three-dimensional structure determined for the pore-forming domain of colicin A has led to a hypothetical model for membrane insertion and channel formation [13], which is indeed useful but limited, because of the possible influence that the other domains of the protein may have on the three-dimensional structure. Microcin E492 therefore, could become a model system for studying mechanisms of polypeptide insertion into membranes [13], or for understanding mechanisms of conduction, selectivity, and gating at the molecular level. The knowledge of the three-dimensional structure of the microcin monomer would be the first step in the study of whole structure of the channel, which is probably an oligomer.

Acknowledgements: We thank Dr. José Luis San Millán for providing us with the *K. pneumoniae* RYC492 and *E. coli* RYC1000 strains. We are grateful for critical appraisals of Drs. Ramón Latorre, Osvaldo Alvarez, and Daniel Wolff. We also acknowledge Dr. Juan Bacigalupo for his suggestions and Dr. Catherine Connelly for helping us to improve the English of this manuscript. This work was supported by Grants 1203-90, 0005-92 and from the 1078-91 Fondo Nacional de Desarrollo Científico y Tecnológico.

REFERENCES

[1] Baquero, F. and Moreno, F. (1984) FEMS Microbiol. Lett. 23, 117–124.
[2] de Lorenzo, V. and Pugsley, A. (1985) Antimicrob. Agents Chemother. 27, 666–669.
[3] Lazdunski, C.J., Baty, D., Geli, V., Cavard, D., Morlon, J., Lloubes, R., Howard, S.P., Knibiehler, M., Chartier, M., Varenne, S., Frenette, M., Dasseux, J.-L. and Pattus, F. (1988) Biochim. Biophys. Acta 947, 445–464.
[4] Schein, S.J., Kagan, B.L. and Finkelstein, A. (1978) Nature 276, 159–163.
[5] Latorre, R. and Alvarez, O. (1981) Physiol. Rev. 61, 77–150.
[6] de Lorenzo, V. (1984) Arch. Microbiol. 139, 72–75.

[7] Mayr-Harting, A., Hedges, A.J. and Berkeley, C.W. (1972) Methods Microbiol. 7A, 315–422.
[8] Hashimoto, F., Horigome, T., Kanbayashi, M., Yoshida, K. and Sugano, H. (1983) Anal. Biochem. 129, 192–199.
[9] Ragland, W.L., Pace, J.L. and Kemper, D.L. (1974) Anal. Biochem. 59, 24–33.
[10] Mueller, P., Rudin, D., Tien, H.T. and Wescott, W. (1963) J. Phys. Chem. 67, 534–535.
[11] Bullock, J.O., Cohen, F.S., Dankert, J.R. and Cramer, W.A. (1983) J. Biol. Chem. 258, 9908–9912.
[12] Pressler, U., Braun, V., Wittmann-Liebold, B. and Benz, R. (1986) J. Biol. Chem. 261, 2654–2659.
[13] Parker, M.W., Tucker, A.D., Tsernoglou, D. and Pattus, F. (1990) Trends Biochem. Sci. 15, 126–129.