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Journal of Microbiological Methods 49 (2002) 325–328

Journal of Microbiological Methods

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Note

Potassium release, a useful tool for studying antimicrobial peptides

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Received 28 August 2001; received in revised form 19 November 2001; accepted 19 November 2001

Abstract

We used an ion-selective electrode to measure potassium release from bacteria treated with antimicrobial peptides. This broadly applicable and simple technique for assessing membrane integrity deserves greater use.

Keywords: Electrode; Bacteria; Potassium

There is growing interest in the role of antimicrobial peptides in innate immunity. Although these peptides vary in structure, many act by inducing permeability changes in the membranes of susceptible bacteria. Several methods have been used to measure this effect. However, the technique described below is seldom applied.

Ion-selective electrodes can measure extracellular concentrations of H^+, Ca^+, NH^{4+} and K^+, and—as microelectrodes—have been used to measure net ion fluxes in bacteria (Shabala et al., 2001). Because the internal ionic environment of bacteria and eukaryotic cells is generally potassium-rich, leakage of this ion has been used to monitor membranolytic events in erythrocytes (Ozawa et al., 1999), yeast cells (Enriquez-Freire et al., 1999), liposomes (Sokolov et al., 1999) and bacteria (Katsu et al., 1986; Matsuzaki et al., 1997).

We used two antimicrobial peptides in these experiments. PG-1 is a porcine protegrin with two intramolecular disulfide bonds and a β-hairpin structure (Kokryakov et al., 1993). Its primary sequence is RGGRLCYCRRRFCVCVGR-amide. SMAP-29 is an α-helical cathelicidin that is expressed by ovine leukocytes (Skerlavaj et al., 1999). Its primary sequence is VRGLRRLGRK IAHGVKKYGP TVLRIIRIAG. These peptides have potent antimicrobial properties and were shown to permeabilize the cytoplasmic membrane of *E. coli* ML-35p by a method based on detecting loss of crypticity of their cytoplasmic β-galactosidase (Lehrer et al., 1988; Skerlavaj et al., 1999).

The organisms used in this report were *E. coli* ML 35p (Lehrer et al., 1988) and *S. aureus* ATCC 33591. To obtain organisms for testing, 50 ml of trypticase soy broth was inoculated with bacteria from a single colony. After overnight incubation at 37 °C, the culture was washed three times with 10 mM Tris–acetate buffer containing 100 mM NaCl, pH 7.4. The washed organisms were resuspended in this buffer at $\sim 2.5 \times 10^8$ CFU/ml ($OD_{620} = 1.0$), kept on ice, and used within 30 min.

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0167-7012/02/$ - see front matter
PII: S0167-7012(01)00383-9

Our experiments were performed with an Orion SensorLink PCM-700 pH/ISE meter with a MI-442 potassium electrode (Microelectrodes, Bedford, NH) and an SDR-2 reference electrode (World Precision Instr., Sarasota, FL). The bridge contained 2 M NaCl in a 1% agarose gel. The K^+ electrode was thoroughly washed with distilled water and with 0.7% octylglucoside, a readily removed detergent (Silberstein et al., 1999). Relative voltage data were collected every second and stored on a PC, using a Thermo-Orion SensorLink® PCMCIA card and software. Before each experiment, the electrode was calibrated with standard solutions that contained 0.01, 0.1 or 1.0 mM KCl in 100 mM NaCl. The measured electrode voltage (V_{meas}) showed linear dependence on $\log_{10}[K^+]$ between 10 μM and 1 mM KCl (Fig. 1, inset A). Linear regression analysis of the calibration data provided Eq. (1), which related V_{meas} to $\log_{10}K^+$ concentration.

$$V_{meas} = m\log_{10}[K^+] + z \quad (1)$$

We simulated complete potassium release from the bacteria by disrupting them with prolonged sonication

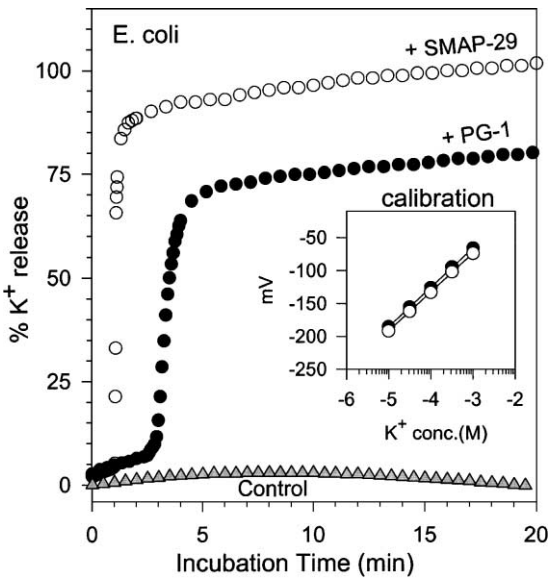

Fig. 1. Effect of antimicrobial peptides on K^+ release by *E. coli*. One to two minutes after these recordings began, *E. coli* ML-35p was exposed to 25 μg/ml of either PG-1 or SMAP-29. Almost immediately, the treated bacteria sustained a massive loss of potassium. In contrast, the untreated bacterial controls showed little loss of intracellular K^+. The inset shows three typical electrode calibration curves.

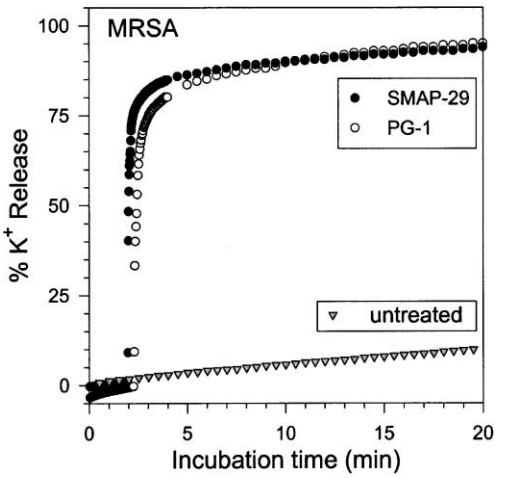

Fig. 2. Effect of antimicrobial peptides on K^+ release by *S. aureus*. One to two minutes after these recordings began, *S. aureus* ATCC 33591, a methicillin-resistant strain, was exposed to 25 μg/ml of either PG-1 or SMAP-29. Almost immediately, massive potassium efflux occurred in the treated bacteria, whereas the untreated controls retained their potassium.

(3 min at 85% power) by a Bronwill Biosonik IV sonicator (VWR Scientific, San Francisco, CA). The concentrations of K^+ in the medium initially (K_{init}) and after sonication (K_{total}) were calculated from the measured voltages by applying Eq. (1). The potassium concentration of the medium at each time point was calculated from the measured electrode voltage (V_{meas}) with Eq. (2).

$$[K^+] = 10^{(V_{meas}-z)/m} \quad (2)$$

where z and m were the linear regression equation coefficients from Eq. (1). The calculations were performed with the Sigma Plot graphics program (SPSS Science, Chicago, IL), using its transform functions. Finally, data were converted to percent potassium release with Eq. (3).

$$\%\ \text{release} = ([K^+]_{meas} - [K^+]_{init})/([K^+]_{tot} - [K^+]_{init}) \times 100. \quad (3)$$

Incubations were performed in 12 × 40-mm round-bottom polystyrene tubes containing a tiny, Teflon-coated bar magnet. These tubes—made by cutting standard 12 × 75-mm tubes (Falcon 2058, Becton Dickinson, Franklin Lakes, NJ) with a scalpel—were

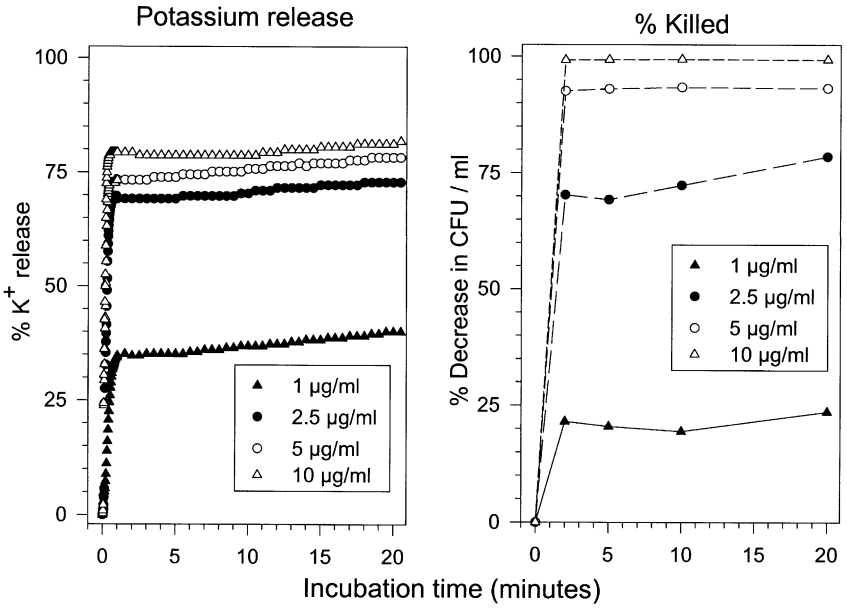

Fig. 3. Comparison of K^+ release and microbicidal activity. *S. aureus* ATCC 33591 was exposed to different concentrations of SMAP-29 from 1 to 10 μg/ml. Potassium release is shown in the left panel. The right panel shows the percentage reduction in colony-forming units (CFU)/ml at 2, 5, 10 and 20 min after addition of SMAP-29.

inserted into a 50-ml, water-jacketed reaction vessel (Kimble/Kontes, Vineland, NJ). The vessel was maintained at 37 ◦C by a circulating water bath and mounted on a magnetic stirring unit. The continuously stirred incubation tube held, in a final volume of 250 μl, 6×10^7 CFU of washed, stationary phase bacteria in 100 mM NaCl with 10 mM Tris–acetate, pH 7.4. Peptide or an equivalent volume of vehicle was added as required.

Figs. 1 and 2 illustrate the effect of the antimicrobial peptides on potassium release. Note that the addition of 25 μg/ml of either protegrin PG-1 or SMAP-29 caused rapid and profound efflux of potassium from *E. coli* (Fig. 1) and from *S. aureus* ATCC 33591, a methicillin-resistant (MRSA) strain (Fig. 2). Fig. 3 shows an experiment performed with lower concentrations of SMAP-29 and the MRSA strain. During this experiment, two chambers were set up in parallel. One chamber was used to monitor potassium efflux. From the other chamber, aliquots were withdrawn at intervals, diluted, plated and incubated overnight to obtain colony counts. Note that the rapid efflux of potassium was matched by an equally rapid fall in viability, and that these events were similar in extent. Very similar data were obtained when we tested PG-1 against the MRSA strain in the same manner (data not shown).

Acknowledgements

This study was supported by grants from the NIH AI 22839 and 43934. We thank Dr. Huiyuan Wu for her expert technical assistance.

References

Enriquez-Freire, E., Lopez, R., Pena, A., 1999. Potassium ion efflux by cationic compounds in yeast. Biochim. Biophys. Acta 1418, 147–157.

Katsu, T., Kobayashi, H., Fujita, Y., 1986. Mode of action of gramicidin S on *Escherichia coli* membrane. Biochim. Biophys. Acta 860, 608–619.

Kokryakov, V.N., Harwig, S.S., Panyutich, E.A., Shevchenko, A.A., Aleshina, G.M., Shamova, O.V., Korneva, H.A., Lehrer, R.I., 1993. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Lett. 327, 231–236.

Lehrer, R., Barton, A., Ganz, T., 1988. Concurrent assessment of inner and outer membrane permeabilization and bacteriolysis in

E. coli by multiple-wavelength spectrophotometry. J. Immunol. Methods 108, 153–158.

Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N., Miyajima, K., 1997. Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. Biochim. Biophys. Acta 1327, 119–130.

Ozawa, Y., Sato, S., Matsumoto, K., Nishi, S., Shishido, S., Imafuku, Y., Miura, Y., Yoshida, H., 1999. Determination of potassium flux activity of viable human erythrocytes by measuring the release-influx ratio. Clin. Chim. Acta 279, 125–132.

Shabala, L., Ross, T., Newman, I., McMeekin, T., Shabala, S., 2001. Measurements of net fluxes and extracellular changes of H^+, Ca^{2+}, K^+, and NH^{4+} in *Escherichia coli* using ion-selective microelectrodes. J. Microbiol. Methods 46, 119–129.

Silberstein, A., Mirzabekov, T., Anderson, W.F., Rozenberg, Y., 1999. Membrane destabilization assay based on potassium release from liposomes. Biochim. Biophys. Acta 1461, 103–112.

Skerlavaj, B., Benincasa, M., Risso, A., Zanetti, M., Gennaro, R., 1999. SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes. FEBS Lett. 463, 58–62.

Sokolov, Y., Mirazbekov, T., Martin, D.W., Lehrer, R.I., Kagan, B.L., 1999. Membrane channel formation by antimicrobial protegrins. Biochim. Biophys. Acta 1420, 23–29.