Vol. 84, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 29, 1978 Pages 306-312

DISPOSITION OF MEMBRANE PROTEINS AS AFFECTED BY CHANGES IN THE ELECTROCHEMICAL GRADIENT ACROSS MYCOPLASMA MEMBRANES

A. Amar, S. Rottem, and S. Razin

Biomembrane Research Laboratory, Department of Clinical Microbiology,
The Hebrew University-Hadassah Medical School,
Jerusalem, ISRAEL

Received July 28,1978

SUMMARY: The effect of ionophores on the exposure of Acholeplasma laidlawii membrane proteins to the aqueous surroundings was studied by the lactoperoxidase-mediated radio-iodination procedure. The iodination values of intact cells pre-treated with valinomycin and/or carbamylcyanide m-chlorophenylhydrazone (CCCP) were much lower than those of untreated cells. The iodination values of isolated membranes from treated or untreated cells were, however, the same. Our results suggest that membrane proteins are less exposed to the aqueous external surroundings when electrical gradients of ions across the cell membrane are collapsed.

Mycoplasma membranes are useful tools for studying the molecular organization of biomembrane components (1). In previous studies the disposition of polypeptides in mycoplasma membranes was studied using the lactoperoxidase-dependent radio-iodination technique. The data obtained suggested an asymmetrical distribution of the polypeptides that carry the iodine binding sites in the membranes, with more polypeptides facing the cytoplasm than the aqueous external surroundings of the cell (2,3). Very little is known, however, about the factors controlling polypeptide disposition and exposure in biomembranes (4). The central feature of energy conservation and exposure in bacteria involves the maintaining of an electrochemical gradient of protons across the cell membrane (5). Although mycoplasmas generate ATP primarily by substrate level reactions rather than by phosphorylation linked to electron transport (6,7), a proton gradient is maintained (Zilberstein and Amar, unpublished data) and K^+ is accumulated in the cells by an energy-dependent process

0006-291X/78/0842-0306$01.00/0

(8). In this communication we examine the effect of proton and potassium specific ionophores upon the exposure of iodine-binding sites on the membrane. Our results suggest that the availability of the iodine-binding sites on membrane proteins located on the external side of A. laidlawii membranes is influenced by the electrochemical gradients of ions across the membrane.

MATERIALS AND METHODS

Acholeplasma laidlawii (oral strain) was grown in a modified Edward medium containing 3% horse serum and Na_2HPO_4 instead of K_2HPO_4 and adjusted to a pH of 8.5 (9). Growth inhibition by valinomycin (Sigma, St. Louis, Mo.) was assessed in test tubes containing two-fold dilutions of the inhibitor (0.4-10 μM). The test tubes were inoculated with an overnight culture at an inoculum level of 5% and incubated at 37°C for 24 hr. Growth was followed by measuring the absorbancy of the cultures at 640 nm. For determination of the effect of inhibitors on macromolecular synthesis and on iodination of surface proteins, the organisms were grown in 250 ml volumes to the mid-exponential phase of growth (A_{640} = 0.2). Valinomycin or CCCP (5-10 μM) was then added to the medium, followed by the addition of either [1-^{14}C]acetate (60 mCi/mmole) or [^{14}C]protein hydrolysate (56 mCi/mAtom), products of the Radiochemical Centre, Amersham, England, to a final concentration of 0.25 μCi/ml. At various time intervals of up to 2 hr, 10 ml volumes were withdrawn and filtered through a millipore HA 0.45 μm pore size filter (Millipore Corp., Bedford, Mass.). The filters were washed with 20 ml of cold 10% trichloroacetic acid, air dried, transferred to scintillation vials, and counted using a toluene-triton scintillation mixture (10). For the iodination of membrane proteins, A. laidlawii cultures (A_{640} = 0.2) were harvested by centrifugation at 12,000 x g for 15 min, washed once, and resuspended in cold 0.15 M NaCl containing 0.05 M phosphate buffer, pH 7.5 Cell membranes were isolated by osmotic lysis of the washed organisms (9). The membranes were washed twice with deionized water and were resuspended in 0.25 M NaCl. Protein was determined in the suspensions of cells or membranes according to Lowry et al. (11).

The lactoperoxidase-mediated radio-iodination was carried out as previously described (3) in a reaction mixture (2 ml) containing 50 mM phosphate buffer pH 7.5, 150 mM NaCl, 50 μg of lactoperoxidase (EC 1.11.1.7), 10 μCi of K^{125}I in 10 μM unlabeled KI, 10 units of glucose oxidase (Miles Laboratories, Kankakee, Ill.), and washed cells or isolated membrane preparations (1 mg membrane protein). Glucose (100 μg) was added to start the reaction and another portion of 100 μg glucose was added 2 min later. After 10 min incubation at room temperature, the reaction was terminated by the addition of 0.1 ml of a 100 mM solution of NaN_3 followed by 8 ml of 50 mM phosphate buffer pH 7.5 in 150 mM NaCl. The cells or membranes were then centrifuged, washed twice and resuspended in 10 μM KI in 50 mM phosphate buffer, pH 7.5, in 150 mM NaCl. Aliquots were taken for radioactivity measurements in a Packard Auto Gamma spectrometer.

Membrane proteins were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (3). When radioactive iodine-labeled membranes were analyzed, the stained gels were sliced into 2-mm sections and counted in a Packard Auto Gamma Spectrometer. Electron paramagnetic resonance spectroscopy was carried out on membranes spin-labeled with 12-doxylstearate or 5-doxylstearate (Syva, Palo Alto, Calif.) by exchange from bovine serum albumin as previously described (12). Spectra were measured at various temperatures with a Varian E-4 spectrometer equipped with

a temperature control unit. The large hyperfine splitting ($2T_{\parallel}$) and motion parameter (τ_0) were measured and calculated as previously described (13).

RESULTS

Valinomycin inhibited the growth of Acholeplasma laidlawii (Fig. 1), the lowest inhibitory concentrations being affected by the serum content of the growth medium. When grown in Edward medium containing 3% horse serum, complete inhibition was reached at a concentration of 0.5 μM valinomycin. The growth inhibition induced by low valinomycin concentrations (up to 2 μM) was partially reversed by KCl added to the growth medium (Fig. 1). Fig. 2 shows the effect of valinomycin on the incorporation of radioactive precursors into A. laidlawii cells. The incorporation of [^{14}C]acetate into the chloroform-methanol extractable material, and that of the [^{14}C]protein hydrolysate into the trichloroacetic acid-insoluble material, were similarly inhibited (∿50% of that of untreated cells). Thus, after treating A. laidlawii cells with 10 μM valinomycin for 1-2 hr, the lipid-to-protein ratio of the cell membrane preparations did not change, resulting in an almost identical density of membrane preparations from treated (1.170 g/ml) and control cells (1.171 g/ml). Furthermore, no major differences in membrane protein composition were discernible by electrophoretic analysis, and the fluidity of the two membrane preparations was very similar, as revealed by the freedom of motion of 5-doxylstearate or 12-doxylstearate incorporated into the membranes. Freedom of motion was determined from the correlation time (τ_0) of 12-doxylstearate and from the large hyperfine splitting ($2T_{\parallel}$) of 5-doxylstearate. The inhibitory effect of valinomycin on the incorporation of [^{14}C]acetate into complex lipids was almost completely reversed by adding KCl (10 mM) to the growth medium. However, the effect of valinomycin on the incorporation of [^{14}C]protein hydrolysate into the protein fraction was not reversed by KCl.

The lactoperoxidase radio-iodination procedure was applied to test for changes in the disposition of membrane proteins after treating A. laidlawii cells with valinomycin and CCCP. Table 1 compares the iodination values of

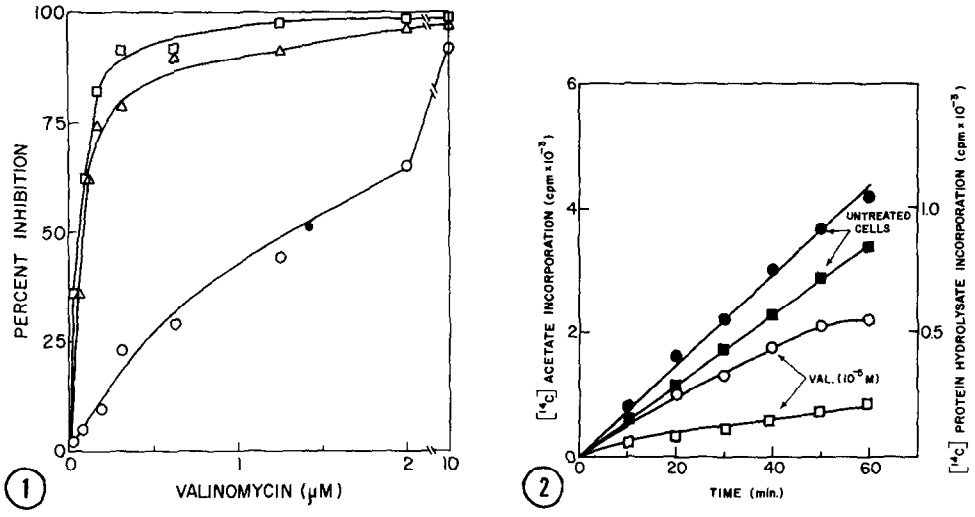

Fig. 1 Effect of valinomycin on growth of Acholeplasma laidlawii in a medium with 100 mM KCl (o), 10 mM KCl (Δ) and without KCl (□).

Fig. 2 Effect of valinomycin on the incorporation of radioactive precursors into Acholeplasma laidlawii macromolecules. [1-^{14}C]acetate, (●,o); [^{14}C]protein hydrolyzate, (■,□).

intact cells and isolated membrane preparations of treated and control cells. The iodination values of isolated membranes always exceeded those of membranes of intact cells, indicating that in A. laidlawii as in other mycoplasmas (2,3), most membrane proteins are located on the inner half of the membrane facing the cytoplasm. The iodination values of isolated membranes were almost the same for treated and control cells. However, the iodination values of intact cells, representing the iodine binding sites exposed on the cell surface, were markedly decreased in the order of control > valinomycin treated > CCCP treated > valinomycin + CCCP treated cells. This resulted in an increase in the labeling ratio of isolated membranes to intact cells from 4 to about 11. The addition of KCl did not reverse the decreased iodination values obtained with CCCP- or valinomycin-treated cells.

Determination of the distribution of the iodine label in sodium dodecyl sulfate-containing polyacrylamide gels indicated that the lower iodination values obtained with valinomycin and/or CCCP-treated cells were due to lower

Table 1. The effect of valinomycin and CCCP on the lactoperoxidase-mediated ^{125}I-labeling of membrane polypeptides of intact cells and isolated membranes of A. laidlawii

Treatment before iodination	Radioactivity (cpm x 10^{-5}/mg membrane protein)		Labeling ratio (membranes/cells)
	Membranes	Cells	
None	30.9	7.5	4.2
Valinomycin (10 µM)	30.5	5.8	5.3
Valinomycin (10 µM) + KCl (100 mM)	30.4	3.8	7.9
CCCP (10 µM)	30.4	5.3	5.7
CCCP + Valinomycin (10 µM each)	30.8	2.7	11.2
CCCP + Valinomycin (10 µM each) + KCl (100 mM)	30.6	2.2	13.6

* A suspension of washed A. laidlawii cells (10 mg cell protein/ml) in 0.15 M NaCl containing 0.05 M sodium phosphate buffer, pH 7.5, was treated with the ionophores for 10 min at 37°C. Membranes were prepared from part of the cell suspension and iodination of intact cells and membrane preparations was performed as described in Materials and Methods.

iodination values of every polypeptide band rather than to a low iodination value of specific polypeptide bands.

DISCUSSION

When valinomycin was used to dissipate the K^+ gradient in Acholeplasma laidlawii cells, growth was inhibited, but as in the case of Streptococcus

faecalis (14), A. laidlawii cells grow almost normally in the presence of low valinomycin concentrations under conditions (100 mM KCl, pH 8.5) that compensated for the decrease in the intracellular K^+. These results suggest that a K^+gradient across the membrane is not necessary for biosynthetic activities. However, at a high valinomycin concentration (5-10 μM), growth inhibition could not be reversed by KCl. Our results indicate that valinomycin and/or CCCP affect the degree of availability of A. laidlawii membrane polypeptides to hydrophilic substances such as iodine. The effect of changing the microviscosity of erythrocyte membranes on the vertical displacement of membrane proteins was recently reported (15). The possibility that valinomycin and/or CCCP, by being inserted into the cell membrane, affected membrane microviscosity in a way similar to colicin E_1 (16) was challenged by demonstrating that the freedom of motion of spin labeled probes incorporated into ionophore-treated A. laidlawii membranes did not significantly differ from that in membranes from control cells. Furthermore, if valinomycin or CCCP affect the exposure of iodine binding sites through an effect on membrane microviscosity, one would expect to find changes in the iodination values of isolated membranes as well as in membranes of intact cells. It is therefore suggested that the effect of the ionophores on the iodination values of intact cells is due to the dissipation of the electrochemical gradient of ions rather than to changes in membrane viscosity. The effect of valinomycin and CCCP was most pronounced when the two ionphores were combined.
This might be due to the enhancement of proton permeability by pre-treatment of the cells with valinomycin similar to that described by Pavlasova and Harold (17). The dissipation of the electrochemical gradient decreases the availability of iodine binding sites on the externally located polypeptide chains rather than affecting the disposition of the polypeptide chains. Thus, the decrease in iodination values was noticeable immediately after the addition of the ionophore to the intact cells, due to a decrease in the labeling intensity of all externally located polypeotide bands. Although the basis

for the effect of membrane potential on the vertical displacement of membrane proteins is yet unclear, there is accumulating evidence for membrane proteins that undergo pronounced changes in response to fluctuating membrane potentials (18). Examples of such proteins are the lac carrier of Escherichia coli (19), the adenosine triphosphatase of chloroplast membranes (20) and the mitochondrial cytochrome oxidase (21), all of which underwent conformational changes induced by the electrical potential. Our results suggest that similar changes may be a general characteristic of the externally located polypeptide chains exposed to the aqueous medium.

REFERENCES

1. Razin, S. (1975) Progress in Surface and Membrane Science 9, 257-312.
2. Amar, A., Rottem, S. and Razin, S. (1974) Biochim. Biophys. Acta 352, 228-244.
3. Amar, A., Rottem, S., Kahane, I. and Razin, S. (1976) Biochim. Biophys. Acta 426, 258-270.
4. Rothman, J.E., and Lenard, J. (1977) Science 195, 743-753.
5. Harold, F.M. (1972) Bacteriol. Rev. 36, 172-230.
6. Van Denmark, P.J. (1967) Ann. N.Y. Acad. Sci. 143, 77-84.
7. Tarshis, M.A., Bekkouzjin, A.G., Ladygina, V.G., and Panchenko, L.F. (1976) J. Bacteriol. 125, 1-7.
8. Rottem, S., and Razin, S. (1966) J. Bacteriol. 92, 714-722.
9. Razin, S., and Rottem, S. (1976) In Biochemical Analysis of Membranes (Maddy, A.H., ed.), pp 3-26, Chapman and Hall, London.
10. Rottem, S., Markowitz, O., and Razin, S. (1978) Eur. J. Biochem. 85, 451-456.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Rottem, S., Hubbell, W.L., Hayflick, L., and McConnell, H.M. (1970) Biochim. Biophys. Acta 219, 104-113.
13. Rottem, S., and Leive, L. (1977) J. Biol. Chem. 252, 2077-2081.
14. Harold, F.M., and Van Brunt, J. (1977) Science 197, 372-372.
15. Borochov, H., and Shinitzky, M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4526-4530.
16. Halgerson, S.L., Cramer, W.A., Harris, J.M., and Lytle, F.E. (1974) Biochemistry 13, 3057-3061.
17. Pavlasova, E., and Harold, F.M. (1969) J. Bacteriol. 98, 198-204.
18. Schuldiner, S., and Kaback, H.R. (1977) Biochim. Biophys. Acta 472, 399-418.
19. Schuldiner, S., Kerwar, G., Weil, R., and Kaback, H.R. (1975) J. Biol. Chem. 250, 1361-1370.
20. Ryrie, I., and Jagendorf, A.T. (1972) J. Biol. Chem. 247, 4453-4459.
21. Lindsay, J.G., and Wilson, O.F. (1972) Biochemistry 11, 4613-4621.