Biochimica et Biophysica Acta, 389 (1975) 444–448

BBA 76956

VOLTAGE-DEPENDENT CONDUCTANCE INDUCED BY HEMOCYANIN IN BLACK LIPID FILMS

OSVALDO ALVAREZ[a], ELIECER DIAZ[a] and RAMON LATORRE[b]

[a] *Universidad de Chile, Facultad de Ciencias, Departamento de Biologia, Casilla 653, Santiago (Chile) and* [b] *Laboratory of Biophysics, IR, National Institutes of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)*

(Received November 18th, 1974)

SUMMARY

When hemocyanin is added to a black lipid film, the conductance increases in discrete steps. For negative potentials the single step conductance is constant, but for positive potentials the step conductance appears to decrease as the potential increases. At high positive potentials the conductance fluctuates between several levels. These data suggest that, in lipid membranes, hemocyanin conducts ions through discrete channels. The voltage-dependent conductance observed at high levels of conductance seems to be a consequence of the properties of the conductance of the single channel.

Hemocyanin is the characteristic blood pigment of gastropods, cephalopods, molluscs and crustaceans. Its molecular weight is greater than 1 000 000 and it carries copper atoms that combine with oxygen [1, 2]. It has been shown that this substance causes dramatic changes in the electrical properties of black lipid films. Electrical conductance increases as much as six orders of magnitude and is voltage dependent [3].

Excitability-inducing material, a protein of still unknown structure [4], and the polypeptide antibiotic alamethicin [5] also induce voltage-dependent conductances when interacting with lipid membranes. In both cases, this voltage-dependent conductance arises via independent statistical units called channels or pores [6–9].

The voltage dependence of the excitability-inducing material and the alamethicin conductances are similar to the voltage dependences of comparable parameters for natural electrical excitation. These characteristic shapes seem to be a feature of all electrical excitation processes.

In this paper, we examine the nature of the voltage-dependent conductance induced in thin lipid films by hemocyanin.

All experiments were done in oxidized cholesterol membranes formed using the brush technique [10]. KCl solution (0.1 M) buffered at pH 7 was used in all the experiments. Lyophilized keyhole limpet hemocyanin, 99.9 % pure, was purchased

from Calbiochem, dissolved in 0.1 M KCl, pH 7, at a final concentration of 1 mg/ml and stored at 4 °C. Membrane potential was measured with a pair of Ag/AgCl electrodes connected to a high input impedance differential amplifier. Another pair of electrodes was used to provide and to measure current. The sensitivity of the current-measuring circuit was up to 10 pA/V. The system is capable of responding to a step current change of 10 pA in 1 ms. For higher currents the response time was proportionally shorter. Positive potentials are defined to correspond to cation flow into the compartment containing hemocyanin.

When small amounts of hemocyanin are added to an oxidized cholesterol membrane the current increases in discrete steps. When one side of the membrane is held at 100 mV negative with respect to the hemocyanin-containing side, lower trace of Fig. 1, the value of the unit conductance step is $2 \cdot 10^{-10}\ \Omega^{-1}$. The upper trace in Fig. 1 is the current recorded when the membrane potential is held at +100 mV. There are two significant differences between this record and the one shown in the lower part of Fig. 1. The first is that the unit conductance step has a smaller value, approx. $0.9 \cdot 10^{-10}\ \Omega^{-1}$. The second difference is that the conductance relaxes back through intermediate levels of conductance. At low positive polarizations, intermediate levels of conductance are seldom seen and behavior is similar to the lower trace of Fig. 1. Conductance of the formation step (the conductance change indicated by the arrows in Fig. 1) was measured at different potentials. In 0.1 M KCl and for negative potentials, current increases linearly with potential and an average conductance of $(2.0 \pm 0.2) \cdot 10^{-10}\ \Omega^{-1}$ for the hemocyanin unit was calculated. For positive potentials, the apparent conductance of the formation step decreases as potential increases (see Fig. 3A).

When large amounts of hemocyanin have been incorporated into the black lipid film, the current response to step changes in potential can be examined at high

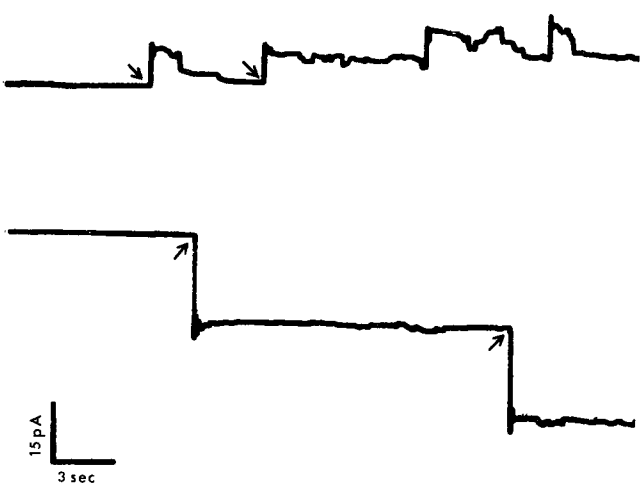

Fig. 1. Current steps arising when small amounts of hemocyanin are added to a bilayer. Hemocyanin concentration, $2 \cdot 10^{-8}$ g/ml. Upper trace was obtained at +100 mV, lower trace was obtained at −100 mV. In the upper trace, formation steps of approx. 9 pA are seen, followed by small on-off steps. The lower trace shows two larger formation steps of approx. 20 pA. Membranes were formed in 100 mM KCl, pH 7. Temperature, 26 °C.

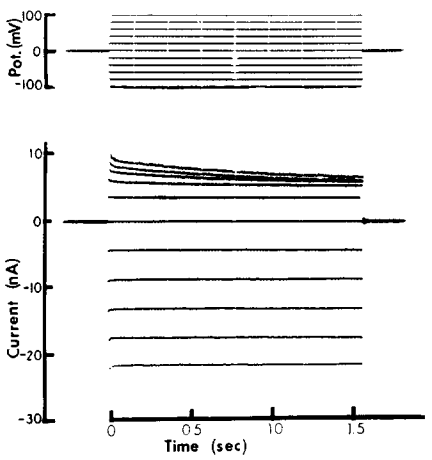

Fig. 2. The response of the hemocyanin-induced current in a membrane containing a large amount of hemocyanin when the potential is suddenly changed from zero to ± 20, ± 40, ± 60, ± 80 and ± 100 mV. Hemocyanin concentration, $2 \cdot 10^{-6}$ g/ml.

levels of conductance. This is shown in Fig. 2 for both positive and negative potentials. Conductance remains constant in the negative potential range. After a sudden change of potential, the current at the very beginning of the pulse is smaller for positive than for negative potentials. For potential higher than +30 mV, steady-state conductance in a membrane with many hemocyanin units is generally obtained between 3 and 4 min after the potential is applied. If the current is measured at these times, the current vs voltage curves show the characteristic negative resistance which was first described by Pant and Conran [3]. This region always appears in the positive quadrant between +30 and +70 mV.

Fig. 3A shows the voltage dependence of the relative conductance of the hemocyanin formation step. Relative conductance remains constant at negative potentials and decreases as the potential increases. Fig. 3B shows the voltage dependence of the relative conductance in a membrane with many hemocyanin units. Conductances are measured at different times after the potential steps are applied. When negative potentials are applied the relative conductance remains constant, but for positive potentials a time-variant, voltage-dependent conductance is seen.

The experimental value of $2 \cdot 10^{-10}\ \Omega^{-1}$ for the maximum conductance of the hemocyanin unit in 0.1 M KCl corresponds to a flow of about $1.2 \cdot 10^{8}$ ions/s across the membrane for a driving force of 100 mV. By comparison, the transport rate of the complex formed by K^+ and a valimomycin carrier is $2 \cdot 10^{4}$ ion/s [11]. Thus the ion fluxes are too large to be accounted for by a carrier mechanism. This indicates that the increase in membrane conductance when hemocyanin is added to the bathing solution results from the formation of channels through the membrane.

Further conclusions about the properties of the hemocyanin channel can be drawn for Figs 3A and 3B. Similarity of the single-channel and many-channel conductance vs voltage curves at 2 ms indicate the independence of separate channels. The instantaneous conductance in many-channel membranes is voltage independent, indicating that the maximum conductance state of the channel is ohmic. The channel

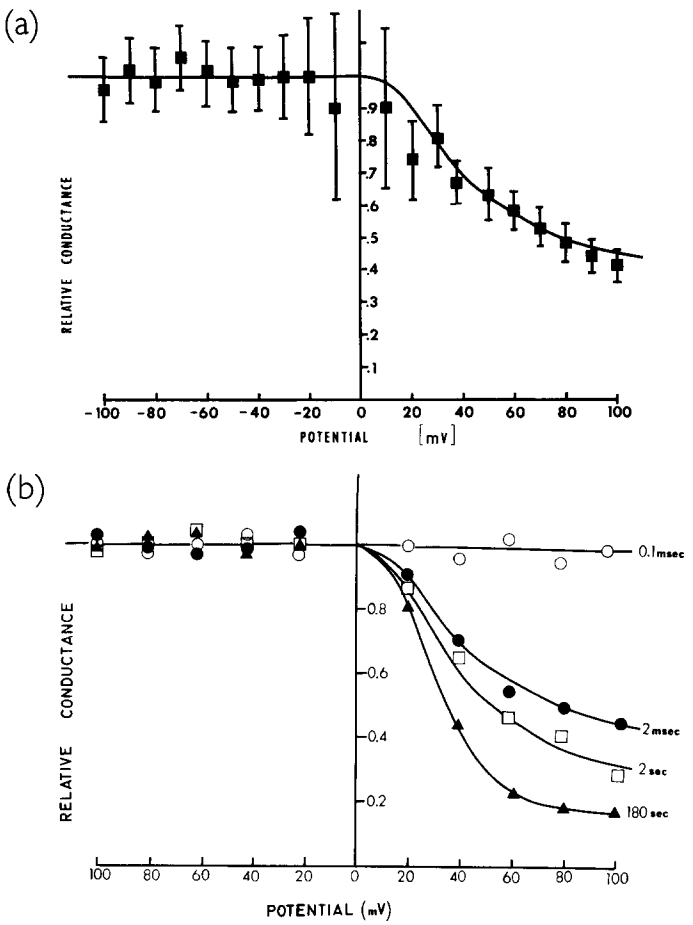

Fig. 3. (A) Voltage dependence of the relative conductance of the hemocyanin formations steps. For negative potentials, the step height is a linear function of membrane potential. A regression line was calculated using the least squares method. Relative conductance is the ratio of the observed step height and the conductance calculated from the regression line. At least 10 steps were measured at each membrane potential. The solid line connecting the points is the 2-ms curve shown in B. Time response of the current measuring system 1 ms. (B) Voltage dependence of the relative conductance in a membrane with many hemocyanin channels. Conductances were measured at the times indicated in the figure. Relative conductance is defined as the ratio of the observed conductance and the average conductance obtained at negative potentials. The charging time of the circuit was 10 μs.

must be undergoing fast conductance transitions from maximum to intermediate levels of conductance when positive potentials are applied. This can explain the shape of the conductance vs voltage curve shown in Fig. 3A. These fast transitions will be not seen in the single channel measurements due to bandwidth limitations of the current-measuring system. Although the number of levels and their voltage dependences have not yet been adequately resolved, at later times, for positive potentials, the channels assume different lower conductance levels (cf. Fig. 1). The transitions to these lower levels is responsible, at least in part, for the decrease in conductance with increasing potential shown in Fig. 3B for many-channel membranes.

Although apparent similarities exist between the excitability-inducing material and the hemocyanin characteristics in black lipid films, there can be no question that their modes of action are quite different in several respects. (a) For hemocyanin (Fig. 3A) the conductance vs voltage curve at 2 ms is strongly voltage dependent, whereas, under identical conditions, the current vs voltage relationship for the single excitability-inducing material channel is ohmic [6]. (b) The early current rectification shown in Fig. 2 is not present in excitability-inducing material-doped membranes. (c) For steady-state measurements, excitability-inducing material shows two well defined negative resistance regions, for both positive and negative potentials [12]. Hemocyanin, on the other hand, has only one negative resistance region (for positive potentials). For negative potentials, the conductance is constant over the complete voltage range tested (Fig. 3B). (d) The hemocyanin channel conductance saturates at high electrolyte concentrations (0.8–1 M KCl), in contrast to the excitability-inducing material channel where the conductance increases linearly with concentration up to 1 M KCl [13] (Latorre, R., Alvarez, O. and Diaz, E., unpublished results).

A question may be asked regarding the purity of the hemocyanin preparation, and if the hemocyanin itself is the active component or if a contaminant at the level of 0.1 % is causing the conductance changes. Currently we have obtained identical conductance characteristics for the hemocyanin channel using three different commercial batches. Furthermore, hemocyanin obtained by direct crystallization from snail blood has given the same results. This is noteworthy, since the blood of invertebrates does not contain appreciable amounts of other proteins.

ACKNOWLEDGEMENTS

We are greateful to Drs Gerald Ehrenstein and Sidney Simon for useful suggestions and help with the manuscript. This work was supported in part by Oficina Tecnica de Desarrollo Cientifico y Creacion Artistica, Universidad de Chile, Grant 623.

REFERENCES

1 Florey, E. (1967) An introduction to General and Comparative Animal Physiology, W. B. Saunders Co., Philadelphia, London
2 Biology Data Book, (1972) (Altman, P. L. and Dittmer, D. S., eds), Fed. Am. Soc. Exp. Biol., Bethesda, Md.
3 Pant, H. and Conran, P. (1972) J. Membrane Biol. 8, 357–362
4 Mueller, P. and Rudin, D. O. (1963) J. Theor. Biol. 4, 268–280
5 Mueller, P. and Rudin, D. O. (1968) Nature 217, 713
6 Ehrenstein, G., Lecar, H. and Nossal, R. (1970) J. Gen. Physiol. 55, 119–133
7 Latorre, R., Ehrenstein, G. and Lecar, H. (1972) J. Gen. Physiol. 60, 72–85
8 Gordon, L. G. M. and Haydon, D. A. (1972) Biochim. Biophys. Acta 255, 1014–1018
9 Eisenberg, M., Hall, J. and Mead, C. A. (1973) J. Membrane Biol. 14, 143–176
10 Mueller, P., Rudin, D. O., Tien, H. T. and Wescot, W. C. (1962) Nature 194, 979–980
11 Stark, G., Ketterer, B., Benz, R. and Lauger, P. (1971) Biophys. Biochim. Acta 11, 981–994
12 Bean, R. C. (1973) Membranes, Vol. 2, Lipid Bilayers and Antibiotics (Eisenman, G., ed.), Marcel Decker, Inc., New York
13 Bean, R. C. (1972) J. Membrane Biol. 7, 15–28