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EFFECTS OF APLYSIA HEMOCYANIN ON THE CONDUCTANCE OF OXIDIZED CHOLESTEROL BLACK LIPID MEMBRANES

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Treating oxidized cholesterol black lipid membranes with *Aplysia* hemocyanin induces the formation of channels with two conductivity states; at the fundamental level of conductance, the lifetime is several hours. Transitions from this state to a different conductivity state occur. Membranes with many of these channels have a voltage-dependent conductance and transitions between different conductivity values occurring in a few ms. Thus molluscan hemocyanins can be considered as a general class of pore-forming proteins.

Hemocyanins are respiratory copper proteins of large molecular weight present in the blood of a number of invertebrates. In 1975 one of these, purified from the hemolymph of the giant keyhole limpet *Megathura crenulata*, was shown to induce the formation of ionic channels in black lipid membranes [1,2]. Voltage gating [3] and cation selectivity [4] are among the properties that make these channels interesting models of channels of excitable tissues [5,6]. The structure of these channels has been examined by means of electron microscopy [7]. Interacting keyhole limpet hemocyanin with different lipid complexes McIntosh et al. could observe a ring-shaped proteic structure which they called annulus. Other hemocyanins (from *Busycon, Helix, Octopus* and *Pila*) neither increased the bilayer conductance nor induced the formation of the annulus. They suggested that only the keyhole limpet hemocyanin induces the formation of channels in black lipid membranes and that the annulus is the pore-forming structure. Testing whether other hemocyanins can increase the conductance of bilayers we found and we report here evidence that the hemocyanin from *Aplysia* interacts with oxidized cholesterol bilayers, forming ionic channels.

All experiments were performed at room temperature on black lipid membrane made with oxidized cholesterol obtained as in Ref. 8. The apparatus for membrane formation and ionic conductivity measurements was as described in Ref. 10. The hole in the Teflon septum had a diameter of about 2 mm. The electrolytic solution was 0.1 M KCl (Carlo Erba ACS) buffered at pH 7.0 by 0.01 M BisTris (Sigma). *Aplysia* hemocyanin at a concentration of about 15 mg/ml in sucrose solution was stored at −20°C and dissolved in small amounts in the bathing solution at the moment of the experiment. Current was detected through Ag-AgCl electrodes and amplified by means of an Analog Devices 515-K operational amplifier, with a 10^8 Ω resistor and a 50 pF capacitor in the feedback-loop (time constant 5 ms). Current and voltage signals were monitored on a Tektronix 7613/7A22 and 7A18 storage oscilloscope, and recorded on a frequency modulation magnetic tape and a chart recorder. Potentials are referred to the potential of the compartment with the protein.

When *Aplysia* hemocyanin is added to the reference compartment (final concentration about 20 μg/ml) the membrane conductance increases in discrete steps (Fig. 1a). These high conductance steps, which

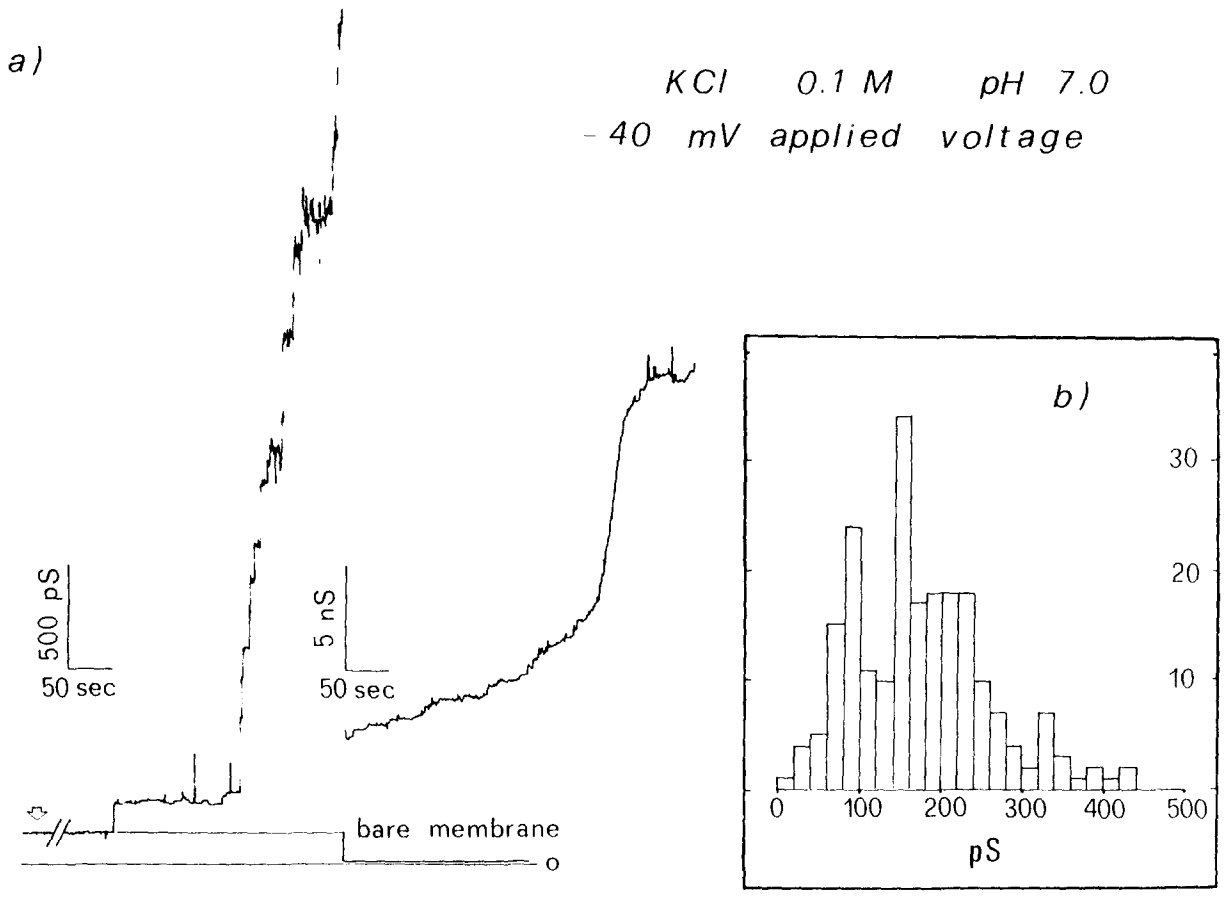
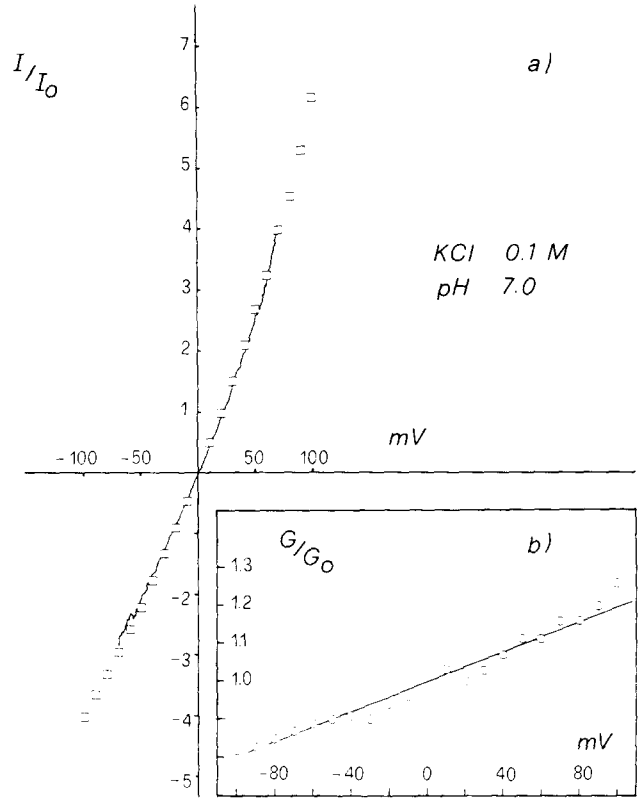


Fig. 1. Black lipid membrane conductance increase induced by *Aplysia* hemocyanin. (a) After about 100 s from the addition, indicated by the arrow, of 200 μg/ml protein to the reference compartment membrane conductance increased in discrete steps. Note the change in conductance scale. Untreated membrane and zero conductance are indicated. (b) Frequency distribution of the conductance change. 214 steps, under voltage clamp conditions, were analysed from records like that of Fig. 3a.

are not always of the same size but are rather broadely distributed between 20 and 450 pS (Fig. 1b), can be attributed to the formation of ionic channels in the black lipid membrane. We frequently observed a decrease in the current, indicating transition to another state of low conductance of the channels. After 1 h, a steady conductance is reached which is three orders of magnitude greater than that of untreated black lipid membrane. In these membranes we measured the *I-V* curves. A typical result is presented in Fig. 2a. Voltage pulses of different amplitudes (lasting a few seconds) were applied. This procedure generated the open circles in the graph. The solid line instead is obtained applying a voltage ramp lasting 50 s. It may be seen that the result is essentially the same with the two pulse procedures. The *I-V* curve is

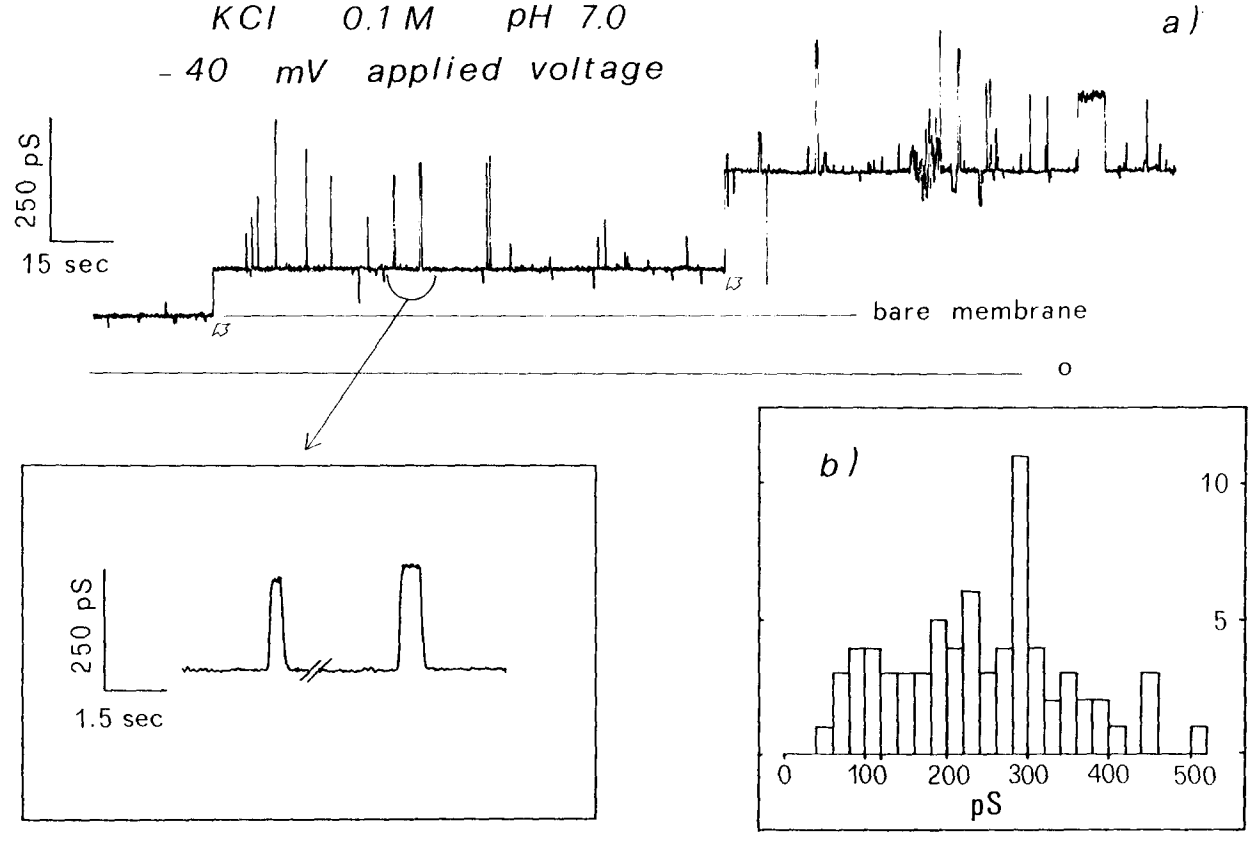
Fig. 3. Single *Aplysia* hemocyanin channels in oxidized cholesterol membranes. (a) In presence of 10 μg/ml protein few channels can be observed for long periods. Channel formation is defined by a long lasting current step (indicated by the arrow) followed by short jumps between that level and an upper one. Two jumps are shown in a 10-fold enlarged time scale. Conductance of the untreated membrane and zero conductance level are indicated. (b) Occurrence of a given conductance jump from the fundamental level in a single channel recording lasting 68 min. 72 conductance jumps are analyzed.



slightly non-linear, with the conductance increasing almost linearly with the applied field (Fig. 2b). The rearrangement between different conductance values occurs in less than 5 ms.

Addition of protein to a final concentration of 10 μg/ml induced the formation of a few channels (Fig. 3a). From these records we concluded that the elementary event is a current step, virtually irreversible, lasting for hours and accompanied by a number of further transitions between that level of conductance and different, generally higher levels (see lower left side inset in Fig. 3). These jumps usually have a life-

Fig. 2. Electrical properties of membranes containing a large number of *Aplysia* hemocyanin channels. (a) Normalized current vs. voltage characteristic, I_0 is the current at +20 mV. Points are obtained applying short voltage pulses (I_0 = 240 pA). Solid line is recorded using a voltage ramp lasting 50 s. (I_0 = 580 pA.) (b) Normalized conductance vs. voltage characteristic. G_0 is the conductance at +20 mV. Points are replotted from part (a) (G_0 = 12 nS). Solid line is a fit to the points using a straight line.



time of a few seconds but occasionally might last some minutes. The amplitudes of the observed jumps are distributed quite continuously between 50 and 450 pS. Fig. 3b shows the frequency distribution computed analysing the records from a black lipid membrane with an elementary event which lasted more than 1 h. After each jump the channel always returned to the lower level (generally about 100 pS in 0.1 M KCl). Single channel properties can be used to explain the wide distribution of current jumps shown in Fig. 1a.

The main result is that *Aplysia* hemocyanin (like *Paludiana* [9] and *Megathura crenulata* [1] hemocyanins) can induce the formation of pores in oxidized cholesterol bilayer membranes. These channels have two levels of conductance, the frequency distribution of the second transition to a usually higher level of conductance is a continuous function. This behavior is different from that of all other channels studied, including the two other hemocyanin channels [3,9]. There are some similarities with the alamethicin channel, which can also fluctuate between several quite different conductance levels [11,12]. In the case of alamethicin, however, current jumps are reversible and strongly voltage dependent, while for *Aplysia* hemocyanin, the first jump is voltage independent and virtually irreversible. Multi-channel membranes showed some voltage dependent conductance. Due to the great variability in size of both the first conductance state and the following fluctuations of each single channel, attempts to correlate the properties of a multi-channels to a single channel membrane are difficult. In our opinion many hemocyanins can form pores in black lipid membrane. Furthermore, since the three hemocyanin channels studied have different electrical properties, we propose that the hemocyanin-lipid complex has different structures according to the protein used.

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