ION CONDUCTIVITY OF THE OPEN KEYHOLE LIMPET HEMOCYANIN CHANNEL

R. ANTOLINI and G. MENESTRINA
Dipartimento di Fisica, Libera Università degli Studi, 38050 Povo (Trento), Italy

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1. Introduction

Hemocyanin obtained from the giant keyhole limpet *Megathura crenulata* interacts with bilayer lipid membranes (BLM) giving rise to the formation of voltage-dependent ionic channels through the membranes [1,2] whose electric properties have been detailed in [3]. In review [4] on ionic pores in BLM and their gating properties analogies were made between channels formed by hemocyanin and by excitability-inducing material (EIM) and the use of these models for understanding molecular mechanisms of gating related to the excitability of the axon and muscle membranes was shown.

Hemocyanin is an oxygen transporting blood protein of mol. wt 7×10^6, whose structure is not completely known (reviewed [5]). Therefore little is known of the channel structure formed in lipid films.

This work aims to clarify the channel structure. Experimental evidence is presented on channel conductance with different salts, obtained analyzing single channel conductance as observed from the formation bumps which follow protein incorporation into the membrane. The data presented indicate that the open channel:

(1) Discriminates between cations and anions. Cl^-, Br^-, F^-, SO_4^{2-}, HCO_3^-, CH_3COO^- cannot pass through the channel.
(2) The cation conductivities in the channel are directly related to those in the free solution. This is so at least in the case of Na^+, K^+, Rb^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, NH_4^+, tetramethylammonium$^+$ (TMA^+), tetraethylammonium$^+$ (TEA^+).
(3) Li^+ represents an exception to the last rule, its conductivity being 50% less than the expected.

2. Materials and methods

All experiments described were performed on BLM comprised of oxidized chloresterol in *n*-octane obtained as in [6]. The set-up for membrane formation and ionic conductivity measurements was as in [7]. The electrometer was a Keithley mod. 616. Keyhole limpet hemocyanin (KLH) 99.5% pure, A grade (Calbiochem) was lyophilized, stored at −20°C and dissolved in the bathing solution at the moment of the experiment. Electrolytes used were: NH_4Cl (Fluka), puriss., PA; TMACl and TEACl (Eastman Kodak); others (Carlo Erba RPE). All measurements were at 0.1 M salt and, in order to have the protein completely associated [8,9], either at pH 6.5 (phosphate buffer) or at pH 7.2 (Tris buffer). Corrections were made to account for the presence of buffer cations and for the degree of dissociation of the salt.

3. Results

After adding small amounts of KLH (1 μg/ml) to one of the two bathing solutions a step-wise current increase was observed (fig.1a), each step corresponding to the formation of one channel. Single channel conductance was deduced from the height of these steps. Membrane potential was held at −40 mV (with respect to the side containing protein) to insure that the channel was in the open state [3].

With the values of single channel conductance obtained from each different salt we built up reproducible histograms as in fig.1b. Each distribution is well fitted by a Gaussian curve. Points reported in fig.2,3 represent the mean value of those distributions and error bars correspond to twice the variance.

The result of the first set of experiments is shown in fig.2 where single channel conductance from chlorides of different cations is plotted against the equivalent ionic conductivities in free solution. Solid line represents the least-squares fit to a straight line passing through the origin.

A second set of measurements is presented in fig.3 where single channel conductance with different salts of Na^+ and K^+ is plotted against the equivalent conductivity of the respective salts. Solid lines represent the mean value of the channel conductance in the presence of Na^+ or K^+ cations, respectively.

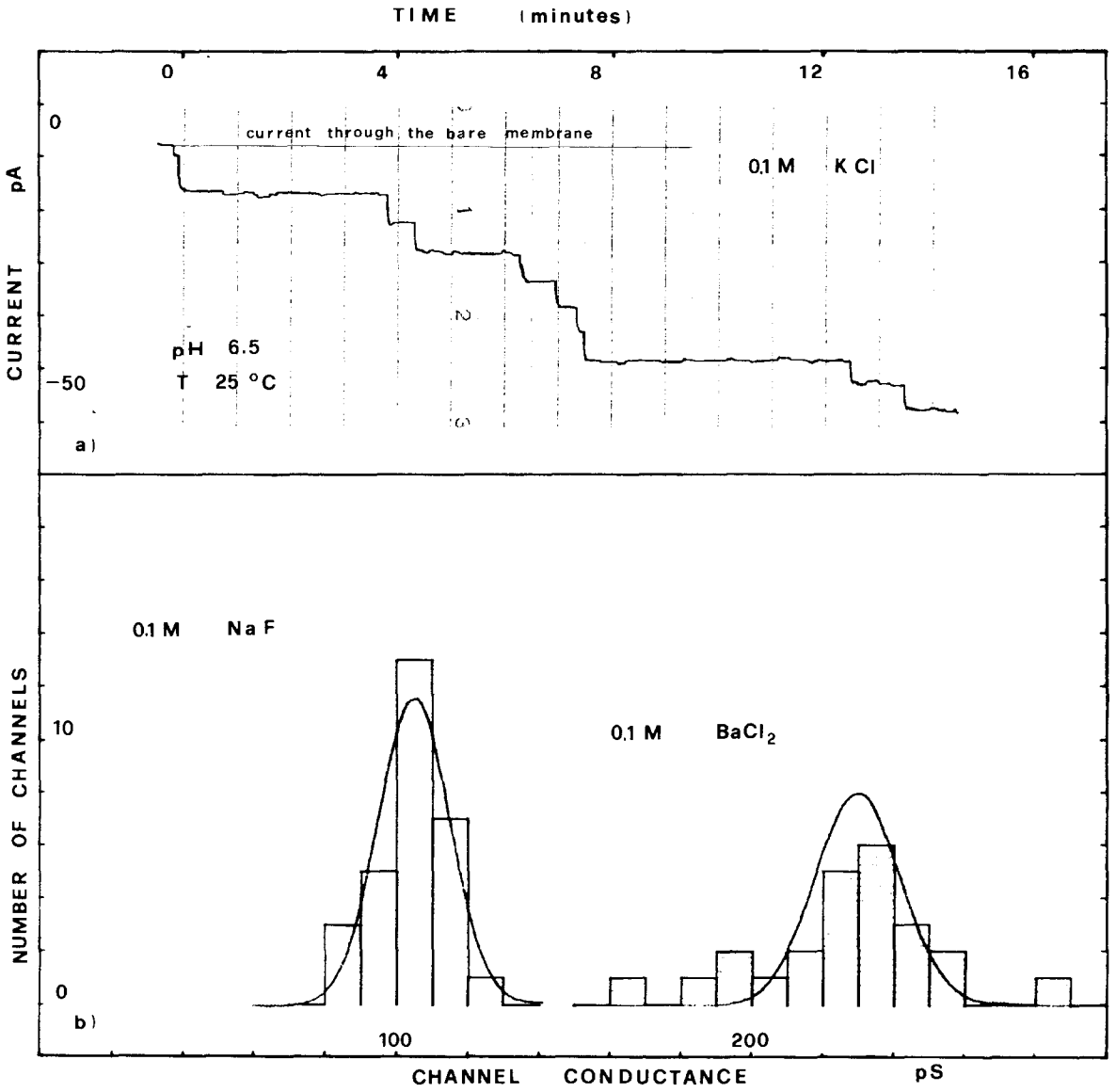

Fig.1. (a) Time course of the membrane current showing channel formation steps after the addition of 50 μg KLH to only one side. Membrane potential is held at −40 mV with respect to the KLH-containing side. The solution is 0.1 M KCl and protein 1 μg/ml. (b) Histograms of the single channel conductances for two salts: 0.1 M NaF and 0.1 M $BaCl_2$, as obtained from the heights of current steps of the kind shown in (a).

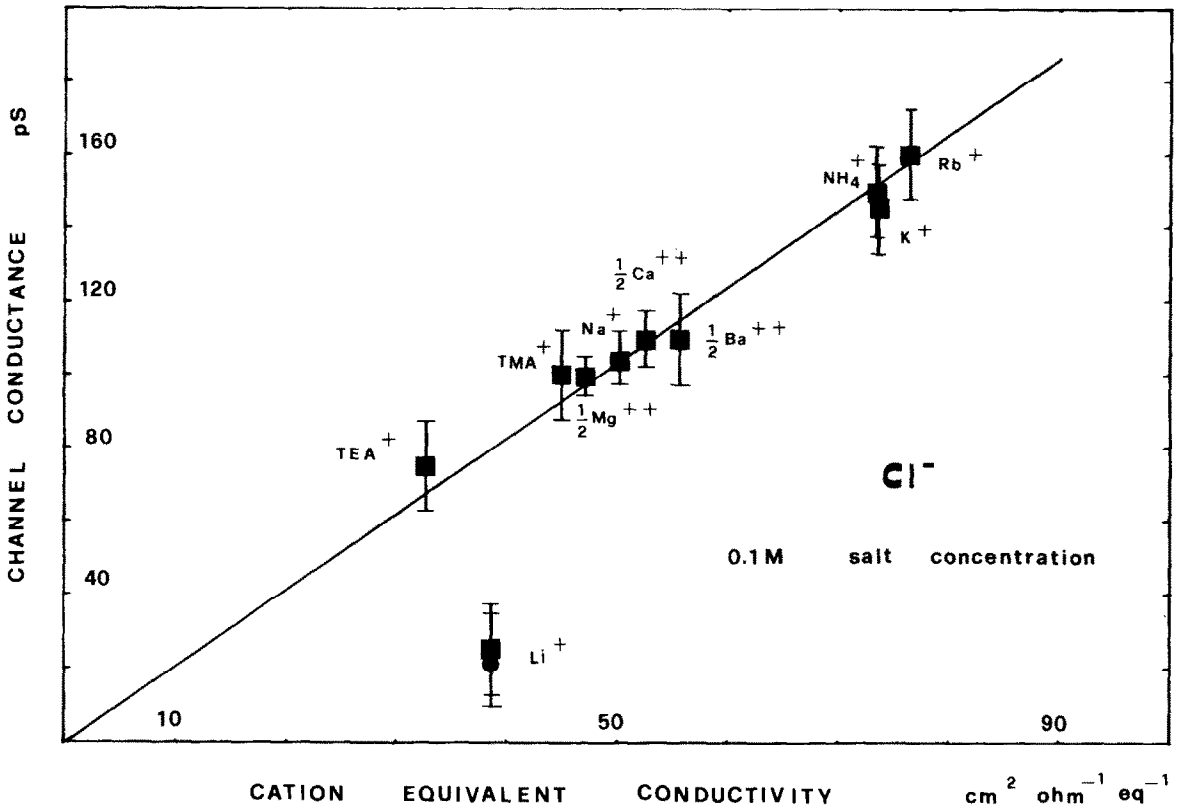

Fig.2. Single channel conductance in the presence of a 0.1 M metal–chloride, expressed in $p\Omega^{-1}$, versus the cation equivalent conductivity, expressed in $cm^2\ \Omega^{-1}\ eq.^{-1}$. Solid line represents the least-squares fit to a straight line passing through the origin.

4. Discussion

The results in fig.2 allow us to draw two conclusions about the ionic conductivity of KLH open channel:

1. The anion chloride cannot pass through the membrane. In fact, if the channel conductance were to be attributed to both cations and anions, the extrapolation of the line fitting the experimental points would intercept the horizontal axis near the value $-76.34\ cm^2\ \Omega^{-1}\ eq.^{-1}$ (which is the chloride conductivity). The least squares fit excludes this possibility and indicates, on the contrary, that channel conductance is zero when cation conductivity is zero.
2. Deducible from the linear correlation between single channel conductance and cation conductivity, is that all the cations used, except Li^+, behave in the channel in a way similar to that in free solution. The anomalous behaviour of the cation Li^+ has been observed in a study of the unit conductance channel of gramicidin A [10].

The cation selectivity is confirmed and extended by the results in fig.3. One can see in fig.3 that single channel conductance in the presence of many salts of Na^+ and K^+ does not change with the different anions but depends exclusively on the cation contribution.

The most immediate interpretation of these facts is that the channel, in the open state, may be thought of as a cylindrical pore filled with the bathing solution where cations are moving freely. Assuming that the length of the channel is that of the protein in the 100 S aggregation state (this state is predominant in solution at the experimental pH and ionic strength [8,9]), that is ~350 Å, one can estimate the channel diameter to be ~30 Å. This value is reasonable if one

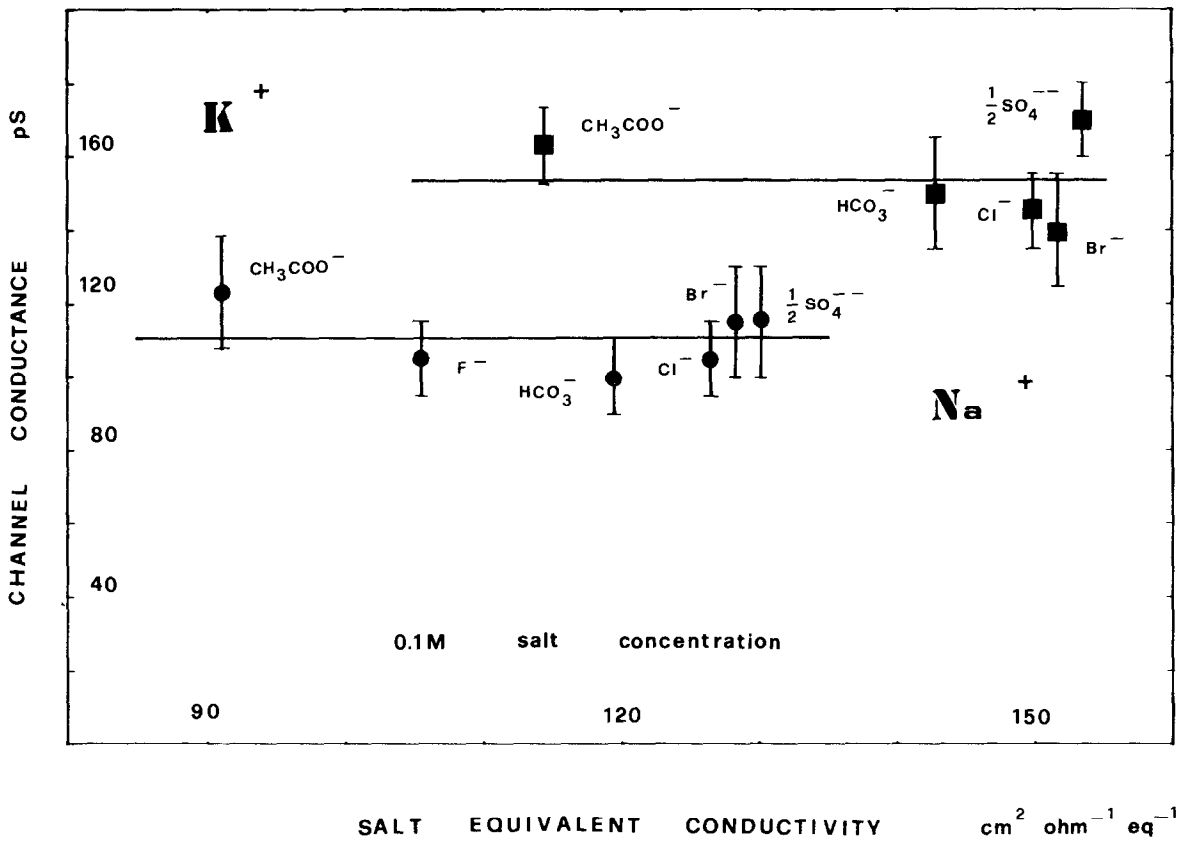

Fig.3. Single channel conductance in the presence of 0.1 M Na^+ and K^+ salts, expressed in $p\Omega^{-1}$, versus the salt equivalent conductivity, expressed in $cm^2\ \Omega^{-1}\ eq.^{-1}$. Solid lines represent the mean value of the channel conductance in the presence of Na^+ or K^+ cations, respectively.

considers that large organic cations such as TMA^+ and TEA^+ can easily pass through the channel. Each channel would contain about 15 cations at the same time at 0.1 M salt. The apparent complete impermeability of the channel to the anions may be explained with the presence of a distribution of negative charges on the inner wall of the cylinder which would prevent the income of anions while favoring the income of cations. This could explain also the saturation properties of the channel (single channel conductance versus KCl activity) observed [3]. In fact, raising cation concentration inside the channel, a limit is reached (corresponding to ~40 K^+) in which positive charges balancing the negative ones prevent the entry of further cations.

Of course, other models are possible. A channel may be occupied by a single ion [3]. However, if this were the case, the cations would have in the channel a mobility about 40-times greater than in free solution. This implies that the mechanism whereby they pass through the channel would be completely different from that in free solution in contrast with the results presented here.

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