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Rapid Report

A trans-membrane pore can account for protein movement across zymogen granule membranes

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Abstract

We have reported that the membrane of zymogen granules, secretion vesicles from the exocrine pancreas, is permeable to its contained proteins by measuring both the loss and accumulation of protein in response to mass action forces [1–3] However, the mechanism of transport has remained unknown. Here we consider evidence that this transport occurs through trans-membrane pores. Using freeze-fracture electron microscopic methods, Cabana et al. [4] have reported the presence of a 15 nm intramembrane particle in zymogen granule membrane which contains a 5 nm (±0.1 nm, S.D.) diameter lucent center. In this article, we propose that this structure is a pore through which proteins can be transported, and test this hypothesis by comparing the predicted phenomenological permeability coefficient for transport by diffusion via this structure, to that calculated from protein flux measurements on granules using an X-ray microscope. The predicted and experimental values were essentially identical and hence support the hypothesis that this structure could be a protein transporting channel.

Keywords: Membrane pore; Membrane protein transport; Permeability coefficient; Secretion; X-ray microscopy

The theoretical permeability coefficient of a membrane porous to protein was derived according to Nikaido and Rosenberg [5] as $P_t = (D/l)(a_t/A)(R)$, where D is the diffusion coefficient of protein, l is the length of the pore, a_t is the total cross sectional area of pores and, A is the surface area of the membrane. R is the Renkin coefficient, a correction factor that takes into account the protein's size on its rate of penetration through the pore. It is expressed as $R = (1 - (d/\Delta)^2(1 - 2.104(d/\Delta) + 2.09(d/\Delta)^3 - 0.95(d/\Delta)^5)$, where the variables (d) and (Δ) are the respective diameters of the protein and pore [6]. The length of the pore was assumed to be the same as the membrane bilayer thickness, approx. 5 nm. The number of pores per micron square area is known (26) from data of Cabana et al. for zymogen granule membrane, and the area of one pore (a_o) was calculated from the radius. Hence a_t, total pore cross sectional area, was calculated as $a_t = (26a_o)$. A value of P_t, was determined by first calculating P for each granule protein known to be transported, some 20 different digestive enzymes, comprising over 90% of granule protein contents. Each value of P was then weighted according to its relative proportion in the pancreas [7] before being averaged to obtain P_t (see Table 1). The values for both variables D and R depend on the size of the protein passing through the pore. The diameters of the digestive enzymes were determined from their molecular weights (Table 1), assuming sphericity and a protein density of 1.3 g/cm^3. They were found to range in size from 3.2 nm (ribonuclease) to 4.9 nm (amylase). This demonstrates that all of the proteins known to be transported can be accommodated by the pore. The value of D for each protein was calculated from the Stokes-Einstein equation (298 K), $D = kT/3\pi d\eta$, where k is the Boltzman constant, T is temperature in units of Kelvin and η is the viscosity coefficient.

The experimental permeability coefficient, P_{ex} (expressed per unit area), was determined from Fick's first law of diffusion: $J/A = P_e A(C_{in} - C_{out})$, where J is protein efflux, A is the membrane surface area and C_{in} and C_{out} are the concentrations of soluble protein inside and outside of the membrane, respectively. The values for these variables were calculated from measurements of the protein content and size of isolated granules made on

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Table 1
The molecular weight (M_r) and mass proportion (%) of the different types of digestive enzymes produced by the acinar cell of rat pancreas and stored in zymogen granules

Digestive enzyme	M_r	%	Digestive enzyme	M_r	%
Amylase 1	55.0	11.5	Proelastase 1	26.0	2.7
Amylase 2	53.0	17.3	Proelastase 2	28.5	3.9
Lipase	50.0	4.9	Chymotrypsinogen 1	25.0	12.9
Procarboxypeptidase A_1	49.0	6.3	Chymotrypsinogen 2	25.0	4.8
Procarboxypeptidase B_1	47.0	5.7	Trypsinogen 1	21.0	14.3
Procarboxypeptidase B_2	47.0	4.0	Trypsinogen 2	21.0	1.7
Procarboxypeptidase B_3	47.0	1.8	Trypsinogen 3	22.5	7.5
			Ribonuclease	14.0	0.7

The following information is from protein analysis of rat pancreatic juice after stimulation of the pancreas [7]. The relative amount of each of the different types of enzymes that comprise the complete set of digestive enzymes are shown as the mass proportion (%). These values should be considered as averages as the relative proportions of each enzyme will vary.

images (Fig. 1) obtained on an X-ray microscope [8]; average values are shown in Table 2. Protein flux (J) was calculated as the difference in protein content of a granule between the first and second image divided by the amount of elapsed time and is considered the initial rate of release. Granule membrane surface area (A) was calculated using the diameter as measured in the initial image. The concentration of soluble protein outside the granules (C_{out}) was zero or close to zero, as the solution in which the granules were suspended was continuously perfused. The concentration of soluble protein (C_{in}) was set at 75 μg/ml, as determined in other studies on isolated granules [9].

A value for P_t was calculated and compared to P_{ex}, for

Table 2
Average values for protein mass and granule diameter for two populations of isolated granules suspended in different solutions and imaged at two different times

Suspending solution	Average values for isolated zymogen granules		
	mass 1 (fg)	mass 2 (fg)	diameter 1 (μm)
Distilled water (pH 6.0)	182 ± 9	126 ± 7	0.92 ± 0.02
0.6 M sucrose (pH 6.0)	214 ± 29	158 ± 22	1.08 ± 0.07

Mass '1' refers to the first time point and mass '2' refers to the second time point, approx. 1 h later. These values are averages (± S.E.) for the populations and as such, were not used to calculate the experimental permeability coefficients shown in Table 3. Instead, P was calculated for each individual granule and P_{ex} averaged from these data. It should be noted that the general morphology of these granules is such that unlike red blood cells, they do not appear to be affected by hypo- or hyperosmotic solutions, i.e., granules do not shrink, swell or lyse in these conditions [19,20]. Indeed, in the current experiments as well as in other experiments [1,8], granules are observed to be stable over time (up to 5 h) in a range of non-ionic solutions of different osmolarity, including water. As such, most of the protein inside granules is predominantly held in an osmotically inactive aggregate with some associated water. The aqueous phase within granules, which contains a small amount of soluble protein, equilibrates readily with non-ionic solutions of different osmolarity without noticeable changes in object size. It should be noted however, that granules are quite sensitive to ionic solutions which can cause them to either shrink or swell as well change the rate at which protein is released [19–21].

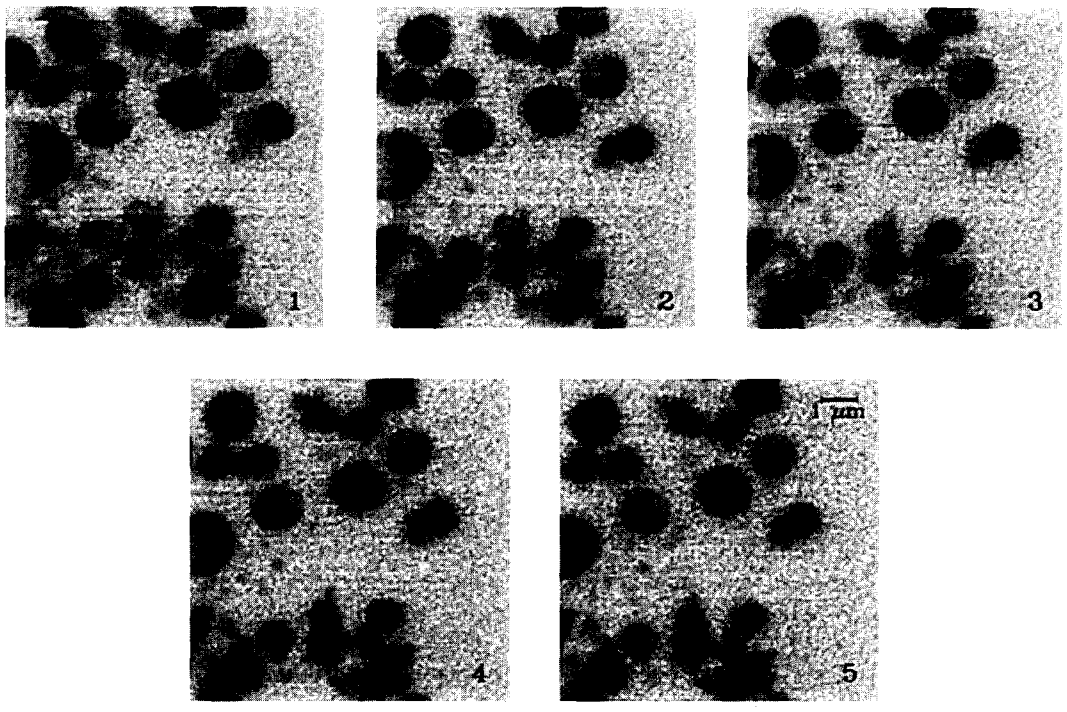

Fig. 1. Zymogen granule images obtained with an X-ray microscope. This series of images shows the same group of granules, suspended in an aqueous environment, over a period of approx. 4 h. Each image was generated by detecting the number of X-ray photons that passed through the specimen at discrete locations, pixels, and stored digitally. As X-rays in the energy range that was used (2.3–4.4 nm) are readily absorbed by protein but not water, these images can be viewed as protein density maps of the objects, enabling one to see the structure of protein within the granules at relatively high resolution (~ 50 nm). From knowledge of the number of transmitted photons and the atomic composition of protein the protein content, or 'mass', can be quantitatively calculated for each granule. A more detailed description of the experimental method is presented elsewhere [1,18].

Table 3
A comparison of predicted and measured permeability coefficients for the transport of proteins across the membrane of pancreatic zymogen granules suspended in solutions of different viscosity

Suspending solution	Permeability coefficient (cm/s)	
	predicted	measured
Distilled water (pH 6.0)	$(2.7 \pm 0.5) \cdot 10^{-6}$	$(2.8 \pm 0.3) \cdot 10^{-6}$ (51)
0.6 M sucrose (pH 6.0)	$(1.4 \pm 0.3) \cdot 10^{-6}$	$(1.5 \pm 0.3) \cdot 10^{-6}$ (11)

The errors shown for the predicted values were determined from the variability in the pore diameter measured by Cabana et al. [4]. Measured values are given as average ± S.E. (number of granules).

granules suspended in water. The two values were essentially the same (Table 3) and as such provide confirmation for the pore hypothesis. As a further test of the hypothesis, the values of both variables were compared for granules suspended in a solution of higher viscosity (0.6 M sucrose). If movement of protein is indeed a result of diffusion through aqueous channels, then increasing the viscosity of the suspending medium would decrease the diffusion coefficient, and hence the permeability coefficient, in accordance with the Stokes-Einstein equation. From the table, it can be seen that P_{ex} is indeed lower. In addition, the experimental and theoretical values were again essentially the same.

Thus, the results are consistent with the hypothesis that protein transport across this membrane is mediated through a channel with characteristics similar to those of the 15 nm particle described by Cabana et al. The correspondence between the predicted and measured permeability also suggests that the following assumptions made in the calculations are appropriate. First, folded protein can pass through the channel. This does not exclude the possibility that partial unfolding takes place, as suggested by the recent discovery of two chaperones in the granule [10]. Second, the estimate for soluble protein concentration in the granule is suitable.

Evidence of such a protein transporting channel in zymogen granule membrane lends further credence to an idea that is gaining increasing experimental support; viz., that protein-accepting channels are important mechanisms for the sorting and transport of diverse proteins across biomembranes. For example, protein transporting pores have been reported in the nucleus [11,12], mitochondrion [13] and in *Escherichia coli* inner and outer membranes [14–16]. Indeed, a subset of ATP binding cassette proteins, involved in the transport of macromolecules, are thought to function as protein-accepting channels [17]. In recent work, one of these proteins (the cystic fibrosis transmembrane conductance regulator) has been implicated in the transport of protein across zymogen granule membrane [18] although the relationship between this protein and the intramembrane particle of Cabana et al. has yet to be established.

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