OPINION

Water channel proteins: from their discovery in Cluj-Napoca, Romania in 1985, to the 2003 Nobel Prize in chemistry and their implications in molecular medicine

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Introduction: Definition of Water Channels

Water transport in cells and tissues is essential for life. When water molecules move into or out of the cell or from one subcellular compartment to another they have to cross the cell membranes, which form the barrier between the interior and exterior of cell or between cell organelles and cytosol. I have edited a two volume book providing in depth presentations of water transport in a variety of biological membranes. Since the membrane proteins confer to biological membranes a much higher water permeability compared to the lipid counterpart, by water channels we understand in fact the water channel proteins, those transmembrane proteins that have as their main (specific) function the transport of water. In 1993 the water channel proteins were called aquaporins.

Evolution of Concepts on Water Transport across Biological Membranes

The evolution of concepts on water transport across biological membranes matches the advances of membraneology, that can be divided into four periods: the “Early Period” (prior to 1940), the “Classical Membraneology” (the period between 1940 and 1970), the “Modern Membraneology” (1970–1984) and the “Molecular Membraneology” (since 1985).

The red blood cell (RBC) appeared to be ideally suited for investigating water permeability because of its availability and simple structure, lacking internal membranes. In the “Early Period of Membraneology” (prior to 1940) the first major concept, that of a membrane enclosing the cell contents was born from permeability studies dating back to the second half of 19th century, when it was observed that marine eggs and RBCs behaved as osmometers: the cells would swell in dilute media and shrink in concentrated media. The conclusion was that the cells are surrounded by a membrane allowing water exchanges between cell and medium.

The second major concept was the quantitative definition of permeability as a permeability coefficient (resistance) using diffusion equation (derivation of the Fick’s equation). Overton studying the movement of water and nonelectrolytes across cell membranes demonstrated that the most important determinant of permeability was the lipid solubility of the solute; however, since small hydrophilic solutes could also penetrate the membrane in an inverse relationship to molecular size, he proposed a mosaic membrane largely lipid but with aqueous patches.

The third major concept of the “Early Period” was the proposition by Gorter and Grendel, from studies of RBCs, that the plasma membrane consists of lipid structure in a bilayer arrangement. Ten years later studies on fish eggs led Danielli and Davson to propose that the permeability barrier of the cell surface was a lipid bilayer sandwiched between protein monolayers.

In the “Classical Membraneology Period” (1940–1970) studies of transport held center stage owing to the

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availability of radioactive isotopes which allowed easy and accurate measurements of fluxes of ions and molecules and also the use of inhibitors or “chemical probes” that could perturb specific transport systems. Stein and Danielli proposed that hydrophilic pores in the membrane could account for the movement of water and ions.

Solomon and coworkers (after 1950) introduced the techniques to measure the RBC osmotic water permeability (named also the filtration or hydraulic conductivity, measured by the net flux of water in response to a hydrostatic or osmotic pressure gradient), and the RBC diffusional water permeability. Since in the case of RBC the osmotic permeability coefficient ($P_f$) is 2.5 times greater than the diffusional permeability coefficient ($P_d$), in lipid bilayers the ratio is unity (in accord with expectations for a nonporous structure) the difference between the two rates of water transport in RBC has been interpreted as indicating the presence of water-filled channels or pores in the membrane. An estimate of the “equivalent radius” of these channels (on the assumption of uniform right cylindrical pores) was performed employing hydrodynamic principles from the ratio of the $P_f$ and $P_d$. The “equivalent radius” of the water channels was first calculated as equal to 3.5 Å, and the effective area of pores in the human RBC was estimated to lie between 0.01–1% of the total surface.

Unfortunately, Solomon changed later several times his estimation giving at some time values of 4.5 Å for the equivalent pore radius in the human RBC and 6.1 Å in dog RBC and finally concluded that water crosses the human RBC membrane through an equivalent pore whose radius of 6.5 Å is large enough to permit the passage of ions and nonelectrolytes.

Macey and Farmer found that incubation of RBCs with the sulfhydryl (SH) reagents p-chloromercuribenzoate (PCMB) and p-chloromercuribenzenesulfonate (PCMB) can produce a dramatic decrease (up to 90%) in RBC osmotic water permeability and this could be reversed by cysteine. As the maximal inhibitory effect of mercurials was to reduce the osmotic water permeability by a factor of 10, i.e. to a value similar to the diffusional permeabilities obtained with lecithin-cholesterol bilayers, Macey and Farmer suggested that “the action of PCMB and PCMB can be to inhibit water flow through aqueous channels (pores) leaving the lipid portions of the membrane as the only alternative for water transport”. There was no idea where the water channels were localized in the RBC membrane and which of the membrane proteins could accommodate these channels.

In the “Modern Membraneology Period” (1970–1984) a series of technological advances have been followed by rapid exploitation (see various chapters in Benga): the formulation of the fluid mosaic model for the structure of biological membranes; the concept of transmembrane proteins and the beginning of understanding of their functional architecture; the use of “probes” to “mark” functional proteins that lead to the identification of some transport proteins (such as band 3 protein, the anion transporter in the RBC membrane); sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) for separating and identifying membrane proteins; purification of transporters and their functional reconstitution in model membranes and others.

The RBC continued to be one of the most favored cells for studies of water permeability to this preferred use contributing the fact that the rather detailed knowledge of molecular structure of RBC membrane became available, regarding both the lipid and the protein counterpart.

On the other hand new methods for measuring water permeability became available. In 1976 together with Vasile Morariu we began systematic studies of the human RBC water permeability, by using a nuclear magnetic resonance (NMR) technique described by Conlon and Outhred. This method was evaluated by us and then I used it extensively to characterize various aspects of water permeability of human RBC and resealed ghosts, not only during this period, but also in the “Molecular Membraneology Period” (since 1985).

We have shown for the first time by NMR that the parameters characterizing water permeability such as $P_d$, and the activation energy ($E_{a,d}$), are the same in RBCs and resealed ghosts and have reported the largest series of determinations of water diffusional permeability of RBCs available in the literature. We have studied in detail the effects of various inhibitors of water permeability, uncovering new findings, such as the irreversible inhibition induced by fluoresceinmercuriacetate or the powerful inhibition induced by mersaly. We have also shown that water permeability of RBCs is not affected by proteolytic enzymes, i.e. that the RBC water channels proteins are impervious to proteolytic digestion. In addition, the enzymic treatment of membranes, or the preincubation with SH reagents that are not inhibitors of water permeability, such as N-ethyl-maleimide (NEM) or iodoacetamide (IAM), did not prevent the inhibition induced by mercurials. No ultrastructural changes could be seen by electron microscopy after incubation of RBCs with SH reagents.

In this period it was accepted that the water transport through the RBC membrane involves two parallel paths: a) through the water channels formed by integral membrane proteins, and b) via diffusion through the background lipid bilayer. The inhibition of water transport by SH group blocking reagents was interpreted as an indication of water channel blockage. Consequently,
experiments aimed at associating water channels with specific membrane proteins using radioactive-sulfhydryl labeling methods became feasible. Brown et al. were the first to report the results of labeling experiments using \(^{14}\text{C-DTNB}\) (dithiobisnitrobenzoate) after pre-incubation of the cells with \(N\)-ethyl-maleimide (NEM) and iodoacetamide (IAM). A binding of \(^{14}\text{C-DTNB}\) to the band 3 protein was found and they suggested that band 3 is involved in water transport, on the assumption that DTNB is an inhibitor of this process. However, no marked inhibition of water diffusion could be induced by DTNB, so this experiment is not relevant. Later work by Sha’afi and Feinstein presented evidence for selective labeling of band 3 with \(^{14}\text{C-PCMBS}\) after pre-incubation of RBCs with IAM, NEM and mersalyl, compounds that were considered not to inhibit transport. However, we demonstrated that mersalyl is a strong inhibitor of diffusional water permeability; consequently this labeling experiment is also not relevant.

In 1982 I organized together with Fred Kummerow (Burnsides Research Laboratory, University of Illinois at Urbana-Champaign) a Romanian-American Workshop in New York City and the proceedings were published next year by The New York Academy of Sciences. At the Workshop Solomon reported the localization of \[^{203}\text{Hg}\text{-PCMBS}\] on band 3 following incubation with human RBC ghosts at 0°C for 2 min, at 0.1 mM final concentration. However, the inhibition of water permeability was not estimated. Solomon, Verkman and coworkers continued to claim that no specific water channel exists and that a protein migrating as band 3 on the electrophoretogram of RBC membranes is a common pore for water, cations, anions and non-electrolytes.

After my return to Cluj-Napoca we found that longer incubation times or higher temperatures are needed in order to induce the inhibition of water transport.

In conclusion none of these labeling experiments provided clear indication which of the RBC membrane proteins could accommodate the water channel.

**The First Discovery of a Water Channel Protein**

I believe that everybody agrees with the following definition of discovery: “to discover” means “to learn or see for first time”.

In 1985 my group performed the labeling experiment by which the presence and location of a water channel protein in the human RBC membrane was clearly demonstrated for the first time; the results were published in 1986. The water channel protein was identified among polypeptides migrating in the region of 35–60 kDa on the electrophoretogram of RBC membranes. This channel was labeled with \[^{203}\text{Hg}\text{-p-chloromercuribenzenesulfonate (PCMBS)}\] under conditions of specific inhibition of water diffusion. It is worth discussing in detail the experimental conditions that allowed us to obtain a very selective labeling of the water channel protein as illustrated in Figure 2 (a) of our landmark publication.

Resealed ghosts, suspended at a cytocrit of 25% in an isotonic medium (150 mM NaCl, 5 mM sodium phosphate buffer, pH 7.5), were preincubated with 2 mM \(N\)-ethyl maleimide (NEM) for 60 min at 2°C. In this way we blocked almost all SH groups that are not involved in water transport. After the preincubation the ghosts were diluted with the same isotonic medium containing 2 mM NEM at 10% and incubated with 0.1 mM \[^{203}\text{Hg}\text{-PCMBS}\] (the inhibitor of water transport), for 15 min at 0°C or for 5 min at 37°C.

NMR measurements of water permeability showed that at 0°C there was no inhibition of water permeability; 5 min at 37°C is the minimum time required to see a significant inhibition. After the incubation, resealed ghosts were washed three times in 20 volumes of a hypotonic medium (50 mM NaCl, 4 mM sodium phosphate, pH 7.5, 2 mM NEM), by centrifugation at 8000 g for 10 min. at 4°C. Purified membranes were prepared from resealed ghosts to remove \[^{203}\text{Hg}\text{-PCMBS}\] that may have bound to haemoglobin and other cytoplasmic components. Membrane polypeptides were separated by electrophoresis, the gel cut into 2 mm slices and the radioactivity measured. The protein binding PCMBS under these conditions were identified by superposition of the radioactivity on the densitometric tracing obtained by scanning gels stained with Commassie Blue. The nomenclature devised by Fairbanks et al. was used to identify the membrane proteins.

Under conditions where marked inhibition of water transport was first obtained (after the incubation at 37°C) the inhibitor was bound to the membrane proteins migrating as band 3 and band 4.5 (actually the polypeptides migrating in the region of 35–60 kDa on the electrophoretogram, as can be seen in Fig. 2 (a) in our landmark publication. This binding pattern suggested that either or both band 3 and 4.5 proteins could be associated with water channels. However, we pointed out that polypeptides migrating in this region have already been identified in other transport functions, notably anion exchange (band 3 protein) and the transport of glucose and nucleosides (band 4.5 protein). There was no evidence that a specific inhibitor of one of these processes will inhibit water transport.

Consequently, I concluded: “It remains possible that a minor membrane protein that binds PCMBS is involved in water transport”, since the only way to explain our results was to postulate that PCMBS is bound to a protein in the RBC membrane that has not been previously seen. I also indicated the way in which the specific protein could be further characterized: by purification and reconstitution in liposomes.
In the same year the labeling experiments were confirmed and extended. In the following 2–3 years I described the novelty of our work in several reviews. It should be emphasized that our first landmark paper was published in a well known American journal, the invited reviews were published in well known international series and, moreover, I reviewed again our work in the second volume of the book that I edited for CRC Press, Boca Raton.

In 1988, Agre and coworkers purified a new protein from the RBC membrane, nicknamed CHIP28 (channel-forming integral membrane protein of 28 kDa). However, in addition to the 28 kDa component, the protein had a 35–60 kDa glycosylated component, i.e., the one we detected as the binding site of PCMB under conditions for the inhibition of water transport across the RBC membrane. They suggested that CHIP28 may play a role in the linkage of the membrane skeleton to the lipid bilayer.

In 1990, Parker first suggested in personal discussion to Agre that the novel protein might be the water channel, and in 1992 Agre and coworkers based on Windager’s suggestion to use oocyte expression as a mechanism to study water transporters, found that oocytes from Xenopus laevis microinjected with in vitro-transcribed CHIP28 RNA exhibited increased osmotic water permeability. The water permeability was inhibited by mercuric chloride, therefore, it was suggested that CHIP28 is a functional unit of membrane water channels. By reconstitution in liposomes it was shown that CHIP28 is a water channel itself rather than a water channel regulator. In 1993 CHIP28 was renamed aquaporin 1.

Since 1993 water channel proteins became a very hot area of research; more than 200 members of the aquaporin family have been found in bacteria, plants, animals and humans; a great diversity of physiological and pathological implications are being uncovered. In 2003 Peter Agre was awarded the Nobel Prize in Chemistry for the discovery of water channels.

It is obvious and overwhelmingly documented from the facts presented above that the first water channel protein, aquaporin 1, discovered? If a comparison with the discovery of The New World of America is made, the first man who has “seen” a part, very small indeed, of The New Land was Columbus; later, others, including Amerigo Vespucci (from whom the name derived), have better “seen” a larger part of the new Continent and in the subsequent years many explorers discovered the complexity of the Americas!

“No one had seen this protein before” said Agre. However, this protein was first seen by my group in Cluj-Napoca, Romania, in 1985, reported in publications in 1986 and reviewed in subsequent years. Consequently, a fair and clear cut answer to the above mentioned question would be: the first water channel protein, now called aquaporin 1, was identified or “seen” in situ in the human RBC membrane (hence discovered) by Benga and coworkers in 1985. It was again “seen” when it was by chance purified by Agre and coworkers in 1988 and it was again identified (hence re-discovered) when its main feature, the water transport property, was found by Agre and coworkers in 1992. It appears that our seminal contributions published in 1986 were grossly overlooked by Peter Agre and by the Nobel Prize Committee.
I presented the above mentioned claim in a PETITION on October 18th, 2003 at The 8th World Congress on Advances in Oncology, and 6th International Symposium on Molecular Medicine (Hersonissos, Crete, Greece) and immediately scientists from a lot of countries have signed in support of my PETITION. After the PETITION and additional information was placed on the web site of the Ad Astra Association (www.ad-astra.ro/benga) thousands of science-related professionals from hundreds of academic and research units, as well as participants in several international scientific events, have signed as supporters of my priority, that is also mentioned in comments on the 2003 Nobel Prize in Chemistry.5,1 I have also received many messages from scientists who considered that I was the first discoverer of the first water channel protein and that my omission from the Nobel Prize is a mistake. In addition the above mentioned facts were presented and acknowledged at over 25 international scientific events in 2004 and 2005, including two world conferences, as well as in seminars at many European, American and Japanese universities in 2003–2005, steering favorable reactions; as can be seen on the above mentioned site the recognition of Gheorghe Benga as a discoverer of the first water channel protein from the human RBC membrane is growing.

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