J. Membrane Biol. 347–354 (1978)

Towards a Molecular Definition of Mechanisms and Pathways of Membrane Transport

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Received 30 November 1977

The title of this final note is just a convenient and, I think, appropriate envelope expression to characterize current trends in membrane science, some of which are a direct consequence of the research activity of the participants of this symposium. An attempt to summarize their contributions here appeared to me somewhat redundant, not to say dangerous. I decided instead to indulge in a few topics germane to the work presented in this meeting.

Hans Ussing and the August Krogh Principle

I used to say to my students that the syndrome of inappropriate secretion of vasopressin and Brattleboro rats look like experiments done by Nature for our own instruction, so clear-cut are the biological features of the pure syndrome as well as those of the homozygous mutant. As an extension of this type of reasoning, I came to the conclusion that for the investigation of many biological and medical problems it seems that Nature regularly provides us with a beast with unique characteristics, suitable for tackling some problem.

Any conceit of mine to original thinking in this particular was quickly dissipated two years ago by an interesting paper of Hans Krebs published in the Journal of Experimental Zoology [22]. Krebs quoted from a lecture delivered by August Krogh to the International Congress of Physiology at Boston, in August 1929. Said Krogh: "For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" [23]. Krebs named this the August Krogh principle. Among the examples he gave there were, of course, Ussing's frog skin and Leaf's toad urinary bladder.

0022-2631/78/S040-0347 $01.60

Prof. Ussing told us how he arrived at the frog skin and active Na transport by means of an "unorthodox" injection of a sample of antidiuretic hormone to an axolotl specimen [36]. Following his magic touch on frog skin, this epithelium became a privileged biological model for the study of membrane transport phenomena and the attendant aspects of cell physiology. For that purpose the skin satisfied all the cardinal requirements of a good model – simplicity, versatility, and heuristic value.

The Heuristic Value of Amphibian Epithelia

From the beginning and under the impetus of Prof. Ussing's work and ideas, amphibian epithelia have been utilized in two distinct contexts: (i) as *general* models of membrane transport in asymmetric epithelial cells; (ii) as *specific* models of certain segments of the nephron, such as the mammalian collecting duct. The first orientation led to the characterization of active and passive processes involved in the transport of chemical species such as ions, nonelectrolytes and water. The second orientation led to the identification of the cascade of cellular events implicated in the coupling of stimulus and effect of hormones such as vasopressin and aldosterone.

It would be presumptuous to try to summarize here the impressive body of knowledge generated by work done with amphibian epithelia. The reason is obvious: a good deal of it came out of the laboratories of people attending this symposium. As a convincing example, let me interject a small personal note: only in the year of 1961, while I was writing my thesis, were published the princeps papers of Dr. Crabbé on aldosterone [7], of Drs. Hays and Leaf on resistance to vasopressin *in vitro* [17] and of Drs. Orloff and Handler on the vasopressin-like effects of cAMP and theophylline [28].

Amphibian epithelia have been instrumental in the study of two major components of the biochemical cell machinery involved in membrane transport: the cyclic nucleotide system and the Na pump. Some of Sutherland's postulates were particularly well studied in toad bladder. All the key elements of the cAMP system were identified and characterized in these tissues, namely adenylyl cyclase, phosphodiesterase, cAMP-dependent kinases and phosphatases. Correlates were established between intracellular levels of cAMP and the hormonal modulation of permeability to Na and water. It is hoped that in the near future, other developments will take place, in particular, the examination of the so-called

yin-yang dualism of cAMP and cGMP and the possible role of other cyclic nucleotides; the identification of other regulatory elements of the system, e.g., the feedback regulator of Ho and Sutherland [19] or that isolated by the group of Cuatrecasas [32]; the isolation of the target molecules of cAMP which are involved in the permeability changes of the apical (or external) membrane induced by neurohypophyseal hormones and by catecholamines. A recent report [38] on photoaffinity labeling of renal membrane cAMP receptors, is encouraging with respect to this last point.

The Na pump has also been under close scrutiny in amphibian epithelia, from the abstract of Koefoed-Johnsen in 1958 on ouabain [21], to the paper of Bonting and Canady in 1964 [2], the paper of Farquhar and Palade in 1966 [11] and the quite recent report of Mills *et al.* [24] with labeled ouabain.

The use of new inhibitors of (Na+K)-ATPase, exemplified by the remarkable observations of Canessa and collaborators [3] with harmaline, may be of considerable interest for the chemical dissection of the Na extrusion mechanism. Furthermore, results obtained in the last few months in our laboratory suggest an intriguing interplay between the activities of adenylyl cyclase and (Na+K)-ATPase in amphibian skin [15]. In short, some inhibitors of the Na pump appear to stimulate the cyclase [15, 33]. A similar phenomenon was recently reported in Ehrlich cells exposed to quercetin [14]. As cAMP alters the permeability of amphibian skin to several chemical species, including Na and water, this biological model offers unique advantages for exploring the putative inter-relationship between adenylyl cyclase and ATPase.

Tracer Studies and Nonequilibrium Thermodynamics

Tracer isotope studies fully demonstrated again the usefulness and the operational simplicity of amphibian membranes when compared to most biological systems. The contributions of Prof. Ussing to this particular field are far too well known to be recalled here. Instead, I would like to digress on a note on Copenhagen in the late thirties and forties.

While doing dissection of body fluids with isotopes, inspired by the classic paper of Dr. Edelman's group [10], I come across the monograph of Francis Moore [26] on the subject and its lovely dedication to von Hevesy. This short sentence suddenly revealed to me a whole chain of subtle intellectual communication and of brilliant scientific achieve-

ments. As reported by von Hevesy in a letter to *Nature* in December, 1934 [18], the idea of tracing water in living organisms was conceived around a cup of tea that he and Moseley were taking at the Manchester Physics Laboratory. What followed this exchange of ideas is now easy to figure out if one remembers that von Hevesy went to Copenhagen where he met Niels Bohr, August Krogh, and Hans Ussing. It is difficult to imagine that biological tracer studies could have gained momentum elsewhere in the world, but the outstanding achievements of this group of people are even more remarkable if one realizes that part of the equipment they used was home-made from empty cigar boxes [35].

From tracer studies one arrives logically at Ussing's flux ratio and thermodynamics. One step further and the names of Prigogine, Katchalsky, Kedem, Caplan, and Essig appear with the application of the formalism of nonequilibrium thermodynamics to membrane transport processes. At this point, it is my real pleasure to pay tribute to my friend and teacher Alvin Essig, who introduced me to the world of Onsager's thermodynamics. Since I left his laboratory, a painstaking series of studies on the energetics of active Na transport in amphibian skin has been accomplished. Application of the same formalism to active H^+ transport has also been attempted with equally provocative results [1]. New insights may result from the use of network thermodynamics [29].

Chemical Dissection of Transport Processes

The work performed with amphibian epithelia on the cyclic nucleotide system, the Na pump, and the stimulus-effect coupling of vasopressin and aldosterone required the use of a variety of drugs capable of dissecting the different metabolic pathways involved. This type of chemical approach, largely followed in genetic molecular biology, appears to be equally promising for the identification of mechanisms and pathways of transport. The work of Drs. Cuthbert and Lindemann with an inhibitor of Na transport – amiloride – is a particularly good example of the possibilities of the method. On the other hand, the stimulation of Na transport by drugs which act directly on the apical or external membrane is also of interest, as it offers new ways of studying Na transport regulation [8]. This would be even more the case if the alleged interplay of these agents with membrane Ca^{++} and the independence of their natriferic effects from cAMP are fully demonstrated [8]. Finally, the beautiful

work of Hays and his group [16] on selective inhibition of Na, water, and urea transport across toad bladder compounds and emphasizes the validity of the chemical dissection approach in membrane transport studies.

Morpho-Functional Correlations

In this realm, amphibian epithelia behaved once more as privileged models in the attempt to correlate morphology and function. I would like to focus on just two points: the fluid-mosaic model of Singer and Nicolson and the technique of freeze-fracture.

In 1972, Finean [12] published an article in which he reviewed representative membrane models proposed since the discovery of the lipid bilayer by Gorter and Grendel in 1925 [13]. In the same year, Singer and Nicolson [34] proposed their famous fluid-mosaic model which triggered one of the most intensive research efforts ever done in the history of membrane science.

The freeze-fracture technique, previously developed in Zürich in the laboratory of Moor and Frey-Wyssling [25], gradually produced a sound morphological basis for the conceptual model of Singer and Nicolson. Despite the judicious reservations lucidly enunciated by Wade *et al.* [37] in a recent article, I think that it is fair to say that freeze-fracture still holds many promises for the advancement of our knowledge in the domain of morpho-functional correlations. At the coarse grain level of membrane structure offered by the freeze-fracture technique, the intramembranous particles may correspond indeed to the "membrane facilities" for passive and active transport to which Lars Onsager used to refer [27]. Application to these membrane components of some Markovian processes reported in solid-state diffusion [4, 30] must lead to new insights into transport processes involving carriers and narrow pores [8, 9].

Search for New Transport Models

The spectacular success obtained with the use of the favorite test-object of Prof. Ussing – the frog skin – should be an incentive for the systematic search of other biological models, carefully selected for well-defined purposes, as recommended by August Krogh [23].

Amphibia certainly are a storehouse of wonders for biologists. They startle us in many different ways. They can carry deadly poisons in their skin. They can carry a viral kidney tumor (Lucke's tumor). Some practice a unique form of parental care – gastric brooding – without digesting the offspring in HCl [6]. Amphibian membranes other than those commonly used deserve serious consideration. Outside the amphibian world, several other transporting epithelia have been studied with the Ussing technique, namely the urinary bladders of reptiles (turtles), teleosts, crabs, and, most importantly, the rabbit bladder. In my own search for new bladders I came across a most unusual one: the salivary bladder (!) of the armadillo, a strange creature already known as the best animal model for leprosy [5]. Surprisingly enough, his salivary bladder has many ultrastructural features in common with toad urinary bladder [31].

Conclusion

Collectively, all the conceptual and technological approaches briefly reviewed in this note share a common denominator: the tendency towards a molecular definition of mechanisms and pathways of transport. This goal may be achieved in not too remote a future.

Marc Kac [20] wrote once, "models are, for the most part, caricatures of reality, but if they are good, they portray some of the features of the real world." He also said, "the main role of models is not so much to explain and to predict ... as to polarize thinking and to pose sharp questions." This is, I think, exactly what was done in this symposium. Besides, the meeting had a peculiar feature to me: it never was the fair of vanities arranged in series frequently seen in specialized symposia, but a genuine tribute to somebody who did inspire most people involved in epithelial membrane transport for the last 30 years.

The excellent secretarial assistance of Mrs. A. Cergneux is gratefully acknowledged. This work was supported by the Swiss National Science Foundation, grants Nos. 3.1300.73 and 3.043-0.76.

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