J. Membrane Biol. 184, 203–210 (2001)
DOI: 10.1007/s00232-001-0103-4

The Journal of
Membrane
Biology

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Hans Ussing, Experiments and Models

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Received: 15 August 2001

Abstract. The article describes work and impact of Hans H. Ussing, a founder of epithelial physiology. Emphasis is on Ussing's model of epithelial transport, which showed early how a complex function can arise from a few basic principles. The KJU-model was developed 1958 for the amphibian epidermis and later applied and adapted to many epithelia, but especially to those that express amiloride-sensitive sodium channels in their apical membrane. Some of the subsequent research dealing with such channels and their cellular environment is briefly reviewed. The ideas of Hans Ussing were and are an inspiration to many of us, who continue to work in the way Ussing has taught us.

Fig. 1. Hans H. Ussing in his lab in 1964. Drawing by Hans Bendix. Courtesy of Else K. Hoffmann, Copenhagen.

Introduction

The useful and intended output of scientific work is scientific insight, yet a scientist is assessed not only by his insights, but equally by his personality, his personal "look and feel". This is especially true for a speaker addressing an audience.

I remember some of Hans Ussing's lectures for just this reason. They were not only lectures dealing with topics of interest to me, but also had a strong "human side". They conveyed imagination paired with attention to experimental detail. They conveyed resort to physical principles paired with refreshing fantasy in the invention of cellular and supra-cellular models; concern about experimental difficulties paired with great fun in the lab, fun doing all this. They also conveyed what probably caused the most admiration in the large audiences, authority paired with modesty.

The Beginning

Working in the intellectual tradition of great Danish biologists, first in the lab of August Krogh, Ussing's early research, right after the war, used a new tool that had just become available: radioactive isotopes of sodium and potassium as tracers to assess the movement of these ions through biological membranes and tissues [60–62]. Today this method seems nearly forgotten. Like other once widespread devices—who still remembers Warburg's respirometer?—it was superseded by the more direct and convenient techniques of later years. In the 1940's, however, the tracer method offered a unique chance to demonstrate something very fundamental: active transport.

Scientists increasingly need to explain their work to the public. When explaining what active transport is and why it is interesting, we necessarily make use of those concepts that have been coined in the period from Nernst to Ussing: chemical and electrical gradients drive the diffusion of ions. Thus, movement in the absence of macroscopic gradients or against them would be unexpected, interesting. Tracer fluxes can

be measured simultaneously in two opposing directions to reveal the flux ratio as an indicator of the interactions underpinning such strange behaviour. A flux-ratio exponent of unity over a wide range of gradients: no interaction, just diffusion; an exponent above unity: single filing. But an asymmetric flux ratio over a wide range of gradients: possibly active transport.

Frog skin soon became the principal object of Ussing's transport studies. Mounted, of course, in the famous "Lucite chamber", which was fitted with "Krogh pumps". The Ussing chamber allowed the manipulation of chemical and electrical gradients. In a typical experiment the two half-chambers contained identical solutions and were held at the same electrical potential [64]. Thus, diffusional driving forces were absent and any net movement of ions, if it nevertheless occurred, was due to active forces, generated in the epithelial cells by chemical reactions.

How were the electrical potentials kept equal? In the early days, I was told, Ussing's technician did this. With a potentiometer she changed a current flowing through the chamber such that the voltage across the skin remained zero. The current flowing, the short-circuit current, was the net sodium flow (multiplied with Faraday's constant), as proven with bi-directional flux measurements. More advanced designs included a motor that reacted to the potential difference, moving the potentiometer. It took some time, a scrutiny of methods sections in papers on nerve fibers and the advent of operational amplifiers before we all realized how a voltage clamp can be built without moving parts.

The Two-Membrane Model

Frog skin held a fascination of its own. From pond water containing only 1 mM NaCl it would absorb sodium ions into a more than 100-fold concentration on the interstitial side. This was due to active transport, yes, but how did it work? Ussing and colleagues found out that the voltage generated by the skin was dependent on the presence of sodium ions on the pond side. It varied with a near-Nernstian slope when Na^+ ions were substituted by choline$^+$. Also, the voltage was dependent on the presence of potassium ions on the interstitial side, sodium ions on this side having little effect. Thus the two surfaces differed: under the conditions specified (absence of permeant anions being perhaps the most important), the outer surface was selectively permeable for sodium and the inner surface was selectively permeable for potassium.

These observations led the lab in Copenhagen to conceive a model for epithelial sodium uptake, the two-membrane model for the (isolated) amphibian epidermis. It is best known as the Koefoed-Johnsen-Ussing (KJU) model [30]. The epidermis is complex,

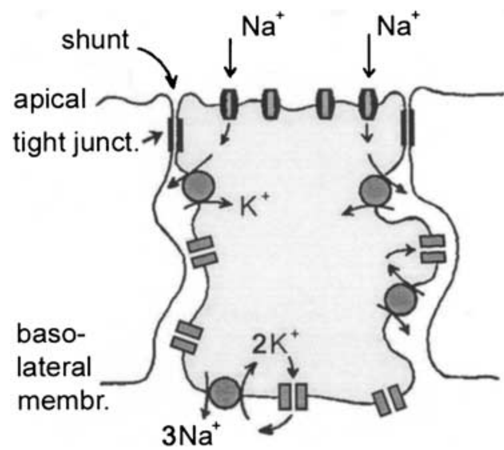

Fig. 2. A modern view of the Koefoed-Johnsen and Ussing model of sodium uptake by polarized principal cells of the short-circuited amphibian epidermis. Sodium channels blockable by amiloride are found on the apical membrane, facing the environment. Potassium channels (rectangles) and Na^+/K^+-pumps (circles) are found on the basolateral membrane, facing the interstitial compartment. While modifications were introduced later and more transporters were subsequently specified for the principal cells [2, 44], the main features of transepithelial sodium uptake are already captured in the KJU-model.

made of several layers of epithelial cells. Yet, the principle of its function can be understood from the properties of only two plasma membranes. The one facing the outer world is permeable to sodium ions (as well as water), while the one facing the inner world of the animal, the interstitial space, has a high permeability for potassium ions. And this inner membrane is the seat of a pump exchanging cellular sodium for interstitial potassium, at the expense of metabolic energy (Fig. 2).

The use of inhibitors amply confirmed the KJU-model. The short-circuit current was sensitive to ouabain added to the interstitial side, blocking the pump. It was also sensitive to barium ions added to the same side, blocking passive potassium pathways. And the short-circuit current was sensitive to amiloride added to the outer side, blocking passive sodium transport. Thus, for the first time, sodium uptake by an epithelium could be understood from the asymmetric transport properties of two plasma membranes acting in series. The outer membrane takes up sodium ions passively, the flow being driven by the local electrochemical potential difference for sodium. The current thus generated continues through the inner membrane. Without active transport, or in a fast transient mode, it continues as a potassium current. This, however, causes the cells to gain sodium and loose potassium, stopping sodium uptake rather soon. But with the pump running, sodium is exchanged for potassium, thus limiting the rise in intracellular sodium concentration. When more cellular sodium becomes available, the pump works more

rapidly while apical sodium uptake slows down. Thus a steady state is established, in which sodium uptake continues as long as the epithelial cells are able to supply ATP as fuel for the pump. In the steady state the sodium concentration inside the cells remains relatively small, although the throughput of sodium is large.

The KJU-model of sodium uptake [30, 63] showed how a complex biological function may arise from a few basic principles. Its power and beauty inspired the formulation of similar models to explain the function of other sodium-retrieving "distal" epithelia. These included the distal nephron, the urinary bladder, the distal colon, ducts of sweat glands and salivary glands, the lining of trachea and bronchi, the olfactory epithelium (excluding the sensory cells) and the oral epithelium, including sensory cells of taste buds dealing with salt taste. The diversity of tissues brought along expansions of the original model. Even the amphibian epidermis required modifications for different experimental conditions [2, 28]. Yet, the Ussing model remained starting point and reference system and continued to provide the principal idea of two series membranes having different transport properties, and a transport compartment between them.

The Na^+ Permeability Is Due to Channels

Below I shall trace some of the research which evolved in subsequent years and which was inspired by the description of a sodium-selective membrane in the 1958 model by Koefoed-Johnsen and Ussing.

The sodium permeability in the outward-facing membrane of frog skin, toad bladder and related epithelia was investigated in detail by several laboratories. Its selectivity of sodium over potassium was found to be very high, in the range 100–1000 [49] and its Na^+ flux ratio exponent was unity [8, 46], confirming the absence of active sodium transport at this membrane. The apical sodium permeability was reversibly and fully blockable by the diuretic amiloride in low-micromolar concentrations. This inhibition allowed an estimate of the speed of sodium translocation at single transport sites by analysis of the blocker noise, as initiated by Willi Van Driessche and colleagues [41, 65]. The high rate of more than one Na^+ ion per microsecond suggested diffusion through channels. The channel conductance was found to be moderate, below 10 pS in amphibian epithelia. In the absence of amiloride the spontaneous open-close rate of the channel was remarkably low.

Subsequent patch-clamp studies confirmed the channel nature of the apical sodium permeability [23, 51] and expression cloning of ENaC subunits from rat colon finally revealed its molecular nature [10–12, 43]. This highly significant advance opened the way to understand the apical sodium channel of epithelia as a member of a large and widespread family of ENaC-related channels, as described in other contributions of this volume.

The large functional diversity of members of the ENaC/Deg family, which we know today, tends to explain the differing single-channel conductances, ion selectivities and amiloride sensitivities previously noted in different tissues and animal species [20, 50] and would seem to resolve many a controversy of the past.

Inhibition by Amiloride

The high affinity of the sodium channel for amiloride allowed a convenient preliminary identification of ENaC in tissues and later in expression systems. The charged form of amiloride enters the outer channel structure at the onset of a blocking event and both the formation and the dissociation of the blocking complex with the channel are influenced by membrane voltage [47, 66, 67]. On the amiloride molecule, the chemical moieties responsible for the binding were identified and those responsible for the on-rate and others responsible for the lifetime of the blocking complex could be distinguished [29, 34, 35]. Based on these results, it was suggested that amiloride forms a transient complex with the channel entrance prior to forming a blocking complex of longer lifetime. Accordingly, in ENaC expressed in oocytes more than one motif for binding of amiloride could be identified in the channel entrance [26, 45, 57]. Thus, the binding moieties of the amiloride molecule are known and the amino acid sequences that bind amiloride to the outer loop of ENaC subunits are also, at least partially, known. But which moiety binds to which amino acid remains to be determined.

In addition to the binding within the channel entrance, which causes inhibition, amiloride and some of its analogues also stimulate transport through the channel [36, 72]. This effect was confirmed for BNC1, a member of the ENaC/Deg family expressed in neurons [1]. Interestingly, binding of amiloride to the stimulatory site was not influenced by membrane voltage, suggesting a location at the outer surface of the channel protein.

Regulation and Self-Inhibition

The function of epithelial sodium channels is regulated by a number of agents and processes, including peptide and steroid hormones [20], extracellular proteases [13], voltage [53], intracellular Ca^{2+} [19, 25], intracellular Na^+ [7, 25, 27] and intracellular H^+ ions [48, 52, 64]. In addition, channel function is, at least in some tissues, inhibited by extracellular Na^+ ions.

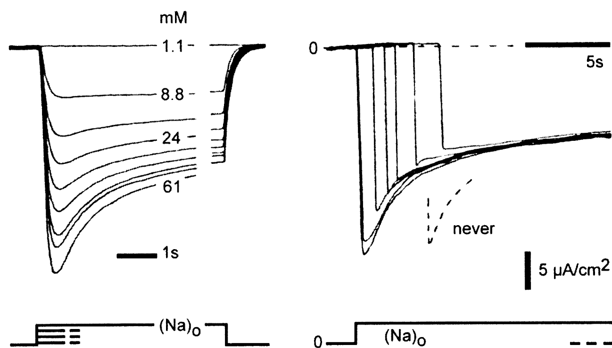

Fig. 3. *Left:* Time course of sodium inward current in response to steps of sodium concentration (parameter) at the outer side of 1 cm^2 of frog skin, recorded in the absence of permeant anions. The secondary (delayed) change from peak to plateau is due to self-inhibition. *Right:* When the flow of sodium current was suppressed during and after the sodium step (current clamp), the inhibition nevertheless developed, as evidenced on switching back to voltage clamp. Thus traces like the dashed one were never observed.

In frog skin the latter effect, an inhibition of sodium uptake by sodium in the outside (pond) compartment, seemed especially interesting. It suggested that the skin of these animals has a rapid, sodium-triggered turn-off device, which prevents flooding of the tissue with Na^+ ions when the animal immerses in water of high salinity. This seemed to make sense because at the German coast frogs live in an environment where both fresh and brackish water is available to them.

With the consent of Professor Ussing, his young collaborator Erik Hviid Larsen came to our lab to investigate the so-called self-inhibition, the lowering of the sodium permeability, or open probability of the channel, by extracellular sodium ions. Together, we managed to achieve a sudden increase in Na^+ concentration with a fast solution switch, which allowed flow rates sufficiently high to decrease the unstirred layer of 1 cm^2 skin to below 20 μm. The machine developed for this purpose consumed 40 ml of mucosal solution per second. On a good day we used 50 liter, and there were many good days. This work with Erik H. Larsen and Wolfgang Fuchs was great fun.

In response to a step increase in Na^+ concentration, the inhibition became noticeable in a peak-recline-plateau time course of the sodium current, i.e., as a fast secondary decrease in inward current [18]. The delayed time course (Fig. 3) excluded a saturation of the diffusion pathway leading through the channel pore with Na^+ ions as the mechanism causing the inhibition. The reason is that the rate of Na^+ turnover in the diffusion pathway is high, exceeding 10^6 binding events per second per site. Therefore, saturation of the channel pore would establish itself without a measurable delay. This is why channel saturation, which has been shown to occur to varying degrees [25, 31, 54], depending on the tissue and other experimental circumstances, cannot explain the self-inhibition, even though it also shows up as a decrease of permeability with increasing sodium concentration.

From early on there was a need to distinguish the self-inhibition from other forms of secondary inhibition, especially those based on increases in cellular Na^+ and Ca^{2+} concentrations (feedback inhibition). One way to deal with this difficulty is depicted in Fig. 3. The sodium concentration steps were performed at zero current (current clamp) and sodium transport allowed to begin at a later time, by a delayed switch-over to voltage clamp. Such experiments showed that suppression of Na^+ current in time intervals just following the concentration step did not prevent the inhibition to develop.

It may be speculated that the self-inhibition is initiated by occupation of a sodium binding site somehow associated with the channel. This site may be in the pore region, in the channel vestibule, outside the vestibule on the outer aspect of the channel or on a separate, as yet unidentified protein. The delay may be caused by a low frequency of Na^+ binding events or by a slow conformational change closing the channel once the site is occupied. If the site is one engaged in Na^+ transfer, its occupation per se cannot cause the block (*see above*). Yet, a large mean time of occupation of such a site might lead to a delayed allosteric change that, in turn, gives rise to the block. Such a model of a "use-dependent" block would be excluded if the Na^+ affinity of transfer and block were found to be different.

As noted above, the self-inhibition was first observed with amphibian skin, a tissue sufficiently robust to allow fast sodium steps in macroscopic experiments. Further experiments with more fragile tissues (amphibian and mammalian) used slower steps or steady-state approaches to test for the inhibition [16, 22, 31, 37, 42, 54, 65]. In noise and patch-clamp data, channel saturation shows up as a change in single-channel current, while self-inhibition shows up as a change in open probability and/or number of transporting channels. Especially in noise analysis these quantities are calculated from the data with equations derived from kinetic models, and there will always be concern whether the model used is applicable. Therefore, additional data, generated with fast sodium-steps, remain interesting to test for the inhibition.

Can the self-inhibition be demonstrated with molecularly identified channels? A first indication of self-inhibition in an heterologous expression system was obtained when size-fractionated poly(A)$^+$-rich RNA from bovine trachea was injected into oocytes [33]. The inhibition became evident as a decrease in sodium permeability with increasing sodium concen-

tration, exceeding what may be expected from channel saturation. The crude nature of the injected material did not exclude the possibility that the inhibition required an additional protein, which needed to be coexpressed with the channel protein and which contained the regulatory sodium binding site. However, this argument seemed less applicable when RNA specific for ENaC subunits was expressed in oocytes and tested for inhibition by extracellular sodium, as described below.

When expressing cloned ENaC subunits from *Xenopus*-derived A6 cells in oocytes [55], it was found that the subunit combination αβγ2 of XENaC gave rise to a bimodal I_{Na} versus $[Na]_o$ plot. The plot indicated a high-affinity Na^+ transfer mechanism and a lower-affinity inhibitory mechanism. It may be noted that the difference in Na^+ affinities found excluded a use-dependent block of the kind described above. Furthermore, the subunit combination αβγ2 but not αβγ caused an especially clear increase of the apparent binding constant for amiloride with the external Na^+ concentration. This suggested competition of Na^+ and amiloride for the inhibitory site. The effect was taken to be indicative of self-inhibition. Based on additional experiments with expressed γ/γ2 chimeric constructs, a Na^+ binding site in the extracellular loop of the γ2 subunit was postulated to mediate the "sodium sensing" [56].

The results obtained by Puoti et al. (1997) suggest that the inhibitory effect of extracellular Na^+ ions is mediated by subunits of ENaC and is especially strong with, or perhaps exclusively tied to the γ2 subunit of the amphibian sodium channel. Future studies may expand on these findings by using concentration-step experiments with the expressed XENaC. Should such experiments not show the expected delay to be associated with the γ2 inhibition, then it will be worthwhile to look for yet another inhibitory Na^+ binding site, located, perhaps, on a separate protein.

In conclusion, then, the sodium self-inhibition—operationally defined as a delayed decrease of sodium permeability or as a decrease in open probability and/or number of active channels—is a property of some of the ENaC-expressing tissues. There are first indications that the inhibition may also be functional in ENaC complexes expressed in heterologous systems, even though the speed of the inhibition has not yet been tested for in such systems. The molecular mechanism remains to be investigated.

Sodium taste

The epithelium lining the oral cavity of vertebrates is continuous with the skin epithelium and thus appears to be an extension of the epidermis. It may not be surprising, therefore, that the oral epithelium expresses sodium channels blockable by amiloride. Furthermore, since the receptor cells of taste buds are derived from epithelial cells, they may also express the sodium channels and perhaps use them as sensors for salt taste. Thus it seemed a good idea to check whether salt taste is blockable by amiloride.

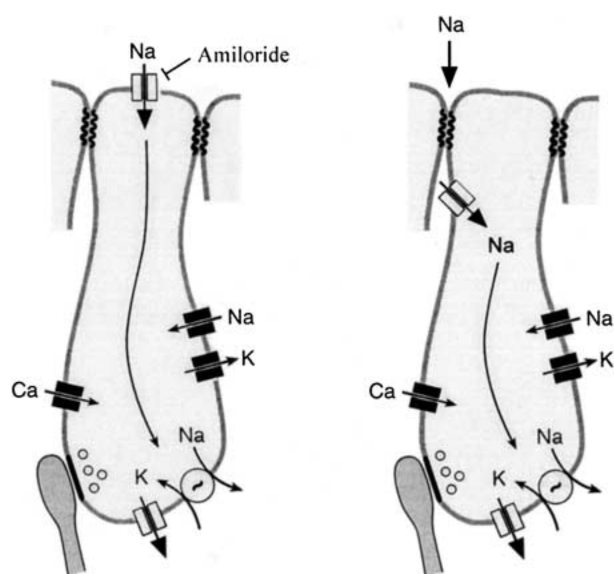

Fig. 4. Model of salt-receptor cell with ENaC-type Na^+ channels blockable by amiloride, basolateral K^+-channels and a Na^+/K^+-pump. In addition to these transporters, known from the KJU-model (Fig. 2), the receptor cell expresses voltage-gated Na^+ channels, K^+ channels and Ca^{2+} channels (black) and develops synapses with sensory nerve fibres [39, 40]. *Left:* The sodium channels are present on the apical membrane, where they can be blocked by oral amiloride. *Right:* The sodium channels are located in a protected space below the tight junction, where oral amiloride cannot reach them.

The seminal experiments in the lab of John DeSimone showed that high concentrations of mucosal NaCl caused current to flow into the lingual epithelium of the dog or rat tongue. The current could be blocked by amiloride, even though a high concentration was required. Furthermore, recordings from the taste nerve showed mucosal amiloride to block the taste response to mucosal NaCl [15, 24], The findings were confirmed in subsequent experiments with rat and hamster, animals which do have a Na^+-(and Li^+)-specific salt taste arising from the anterior tongue. This taste was blocked by mucosal amiloride, even in low micromolar concentrations, as evidenced by recordings from the taste nerve, from the taste pore and by psychophysical experiments [6, 9, 14, 21, 59, 71].

The concept that salt taste—or the sodium-specific component thereof—is mediated by amiloride-blockable sodium channels located in the apical membranes of taste cells, is an attractive one. When located apically (Fig. 4), the channels would permit current to flow into the receptor cell and the strength

of this current would mirror the Na^+ concentration in the oral compartment. Furthermore, the current would necessarily pass outward through the basolateral membrane which, in consequence, would depolarize and release transmitter. A sodium-specific receptor cell mediating salt taste would thus be possible, based on principles already familiar from Ussing's polarization model of Na^+-retrieving epithelia (Fig. 2).

At the cellular level, the first demonstration of amiloride-blockable inward currents was achieved with taste receptor cells (TRCs) isolated from taste buds of the frog. The cells, which are of slender, bipolar shape, were patch-clamped and the whole-cell current and the cell potential recorded [3]. The data indicated that Na^+ ions can enter a subset of TRCs through channels that are blockable by amiloride in sub-micromolar concentrations. (In addition, there were other pathways, which also supported Na^+ inward current, but which were not blocked by amiloride.) Membrane patches containing 20–50 of such channels could be excised from the somal membrane of frog TRCs [4, 5]. These observations provided a first indication that the epithelial Na^+ channel, believed to be always apically located, may also be present in basolateral membranes of some types of epithelial cells.

Surprisingly, recordings from the taste nerve of frogs did not show a convincing sensitivity of the salt response to amiloride. This is a main obstacle in understanding the salt taste of these animals. To offer a speculation, part of the inward Na^+ current may flow not through channels in the apical membrane but through the tight junctions. It would then enter the taste cells via amiloride-blockable channels contained in the basolateral membrane close to the tight junction (Fig. 4). Supposing that Na^+ ions but not amiloride can diffuse through the tight junction, the lack of sensitivity to mucosal amiloride would be explained. (A related model was first put up by others to explain the so-called anion paradox of salt taste in mammals [69, 70]). The advantage of such a design is obvious: The Na^+ channels and other sensors are located in a *protected space* where they are difficult to reach by xenobiotics like amiloride, chemicals which might be present in the nutriments and able to interfere with the taste process or other cellular functions.

Later research confirmed, especially for the anterior tongue of rodents, that the model of Na^+ taste transduction outlined above applies. It predicts an apical and sub-apical location of the epithelial Na^+ channel, which is now known as ENaC [10–12, 43]. The channel acts as a salt taste receptor by providing a specific pathway for sodium current into taste cells, given that Na^+ ions are present in the oral space in sufficient concentration [6, 32, 38]. The current triggers action potentials at the basolateral membrane of the taste cell, presumably followed by synaptic events (Fig. 4).

Of the three essential subunits of ENaC, at least one is under inductive control by a steroid hormone, aldosterone [38]. Thus the sensitivity of sodium taste is increased in animals in sodium-need via induction of more ENaC channels. A systemic Na^+-deficiency, which leads to salt craving, occurs regularly in herbivores but can also be observed in rodents and humans [68]. The induction of ENaC subunits in taste receptor cells by circulating aldosterone provides an instructive example for adaptive tuning of taste acuity in a state of nutritional deficiency.

In humans, however, the amiloride-sensitivity of salt taste, so helpful for the investigation of rodent responses, is less pronounced [58]. This may suggest the involvement of another channel, as yet unspecified. Alternatively, part of the human salt taste might be mediated by ENaC located below the tight junction, where the channels cannot be reached by amiloride present in the oral cavity. It is strange to see how little is known especially about the molecular foundation of human salt taste.

Outlook

While epithelial sodium uptake and salt taste have been studied by many and with many techniques, the models invoked are mostly elaborations on the two-membrane scheme which arose in Copenhagen. This shows once again the power of the ideas put forward rather modestly by Koefoed-Johnsen and Ussing in 1958. We must indeed be grateful for their integrative transport model, which, as is obvious, has passed the test of time.

As described in other contributions of this volume, Hans Ussing concerned himself in later years increasingly with the coupling of ion and water transport in epithelia. This work, which required a long succession of experiments and models, was of a kind that physiologists will do increasingly in the future [17]. Because once molecular biology and proteomics have defined the proteins expressed in differentiated cells, there will still be a need to understand how these players play together, taking account not only of biochemical but also of morphological and biophysical detail. As Hans Ussing has taught us.

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