INTRACELLULAR POTENTIALS IN FROG SKIN AND TOAD BLADDER

FRED M. SNELL AND TUSHAR K. CHOWDHURY[1]

Department of Biophysics, State University of New York at Buffalo, Buffalo, New York

Since the early work of Huf [12] and Krogh [14], electrical potentials across frog skin have been known to be associated with the transport of sodium chloride. Using the isolated skin of *Rana temporaria,* Ussing [24] first showed that this electrical potential was unambiguously associated with the transport of Na^+. Ussing and Zerahn [26] made an elegant demonstration that this tissue, when the transtissue electrical potential was reduced to zero by an external source (the so-called short-circuited state), gave rise to a transmembrane current that was equivalent to the net flux of Na^+, measured isotopically. Since then, a number of laboratories have directed their attention to this sodium transport system, utilizing not only a variety of frog species but also toad skin and the urinary bladder of the toad. The amphibian skin is a rather complex epithelial structure, squamous in character, multilayered, and composed, therefore, of cells at all levels of maturity. This has given rise to uncertainties as to which cells are actually engaged in the transcellular transport of sodium ion. The toad bladder preparation (Leaf [15]), on the other hand, is a much simpler epithelial structure and, when moderately stretched, consists of virtually a single layer of epithelial cells on a basement membrane. The associated loose connective tissue, a small amount of smooth muscle, and a serosal epithelium appear to be nonfunctional with regard to the transtissue sodium transport system. Otherwise, the characteristics of the amphibian skin and the toad bladder preparation have much in common.

Our concern in this paper is primarily with the electrical potential manifested by the sodium transport system. We are interested ultimately in the relation of this electrical potential to possible mechanisms involved in sodium transport, but our discussion will concern itself primarily with the possible intracellular distribution. The electrical potential is manifest across the entire epithelium, but one may

[1] Present Address: Department of Physiology, George Washington University, Washington, D.C.

ask what are its relations to the bounding cell membranes. Is it generated at these bounding cell membranes or does it arise in the cytoplasm or intracellular spaces? First, we shall review some of the more important behavioral characteristics of the sodium transport systems in the amphibian skin and toad bladder.

Transport of sodium occurs from the outside (mucosal) bathing solution to the inside (serosal) bathing solution. This transport can be carried out against large concentration differences of Na^+, and it is almost uniquely specific for Na^+ (Li^+ may be substituted for Na^+ to a degree). The Na^+ flux characteristically bears a nonlinear relation to the Na^+ concentration in the solution bathing the outside surface $(Na)_o$, exhibiting typically a rectangular hyperbolic relation, the form associated with "saturation" kinetics. The system derives its energy from metabolic sources, presumably adenosine triphosphate (ATP), and is, thus, inhibited by any poisons interfering with ATP production. The electrical potential, manifest in the open-circuit state, is positive on the inside with respect to that on the outside surface of these tissues, i.e., $\psi_i - \psi_o > 0$. The epithelial layer of cells is permeable to chloride ion and presumably other univalent anions. However, it is only slightly permeable, negligibly so in the "best" of preparations, to divalent anions, such as sulfate and presumably other multivalent anions. Similarly, it is negligibly permeable to K^+, divalent cations such as Mg^{++}, and organic cations such as the tetraalkyl ammonium ions or choline. If these preparations are bathed in the usual Ringer's solutions, chloride comprises the principal co-ion of the sodium transport system, moving presumably under the influence of the electrical potential difference. If, however, the Ringer's solution is prepared with sulfate salts, the conductance path of the chloride is removed, and the electrical potential difference, $\psi_i - \psi_o$, is increased. Under these circumstances, this electrical potential difference was shown by Koefoed-Johnsen and Ussing [13] to be a linear function of the logarithm of both the Na^+ concentration bathing the external surface $(Na)_o$ with the linear coefficient being equal to the $2.3\ RT/F$,[2] and K^+ bathing the internal surface $(K)_i$ with the coefficient equal to $-2.3\ RT/F$. Other investigators have confirmed these observations to varying degrees. In general, the magnitude of the linear coefficients is reported to be somewhat less, and the functional relation is not always linear throughout the entire range (Curran,

[2] R is the gas constant, T is the absolute temperature, and F is the Faraday.

[8], Leaf, [17]; Snell and Chowdhury [22]). Lindley and Hoshiko [18] have rationalized with some success these smaller coefficients and the slight nonlinearities in the logarithmic relations on the basis of leakage or shunt paths of other ions.

On the basis of the functional dependency of the transmembrane potential on $(Na)_o$ and $(K)_i$ together with other characteristics of the active sodium transport system, Koefoed-Johnsen and Ussing [13] conceived of a hypothesis for the system. In its essence, this model postulates that the outward facing cell membrane of a single active layer of cells is selectively permeable to Na^+ and Cl^-, whereas the inward facing membrane of these cells is selectively permeable to K^+ and Cl^-. The active transport process is assumed to exist at the inner boundary. Presumably via a carrier mechanism, sodium is transported from the cytoplasmic fluid to the solution bathing the inside surface and potassium in the opposite direction. With this system, the cytoplasmic sodium concentration, $(Na)_c$, is thus maintained relatively low and the cytoplasmic K^+ concentration, $(K)_c$, relatively high. The total transmembrane electrical potential is conceived to be composed, therefore, of two parts. At the outer membrane, it is due to the selective diffusion of Na^+; and at the inner membrane, it is due to the selective diffusion of K^+. One may write for the total transmembrane potential

$$\psi_{io} \equiv \psi_i - \psi_o = \frac{RT}{F}\left[\ln \frac{(Na)_o}{(Na)_c} + \ln \frac{(K)_c}{(K)_i}\right]$$

which serves also to define the double subscript notation for the potential difference.

In the Koefoed-Johnsen and Ussing hypothesis, it must be assumed that the electrical field vanishes in the cytoplasm and is discontinuous across the bounding membranes. Furthermore, it must be presumed that the cytoplasmic ion concentrations are maintained at least partly uniform throughout by processes of diffusion and possibly other forms of mixing. The role of the cytoplasm is thus assumed to be secondary except that it provides a source of energy through the usual biochemical transformation to enable the carrier system to perform its thermodynamic work.

There are a large number of experimental observations which support the concept of the Koefoed-Johnsen and Ussing model (Curran [7]) and yet, there has been a sufficiency of observations that have been so difficult to reconcile with the model as to cause a modicum

of skepticism (Snell and Chowdhury [22]; Cereijido and Curran [1]; Farquhar and Palade [10]). Recently, both Ussing and Windhager [25] and Leaf [16] have introduced modifications of the model based upon additional experimental evidence. These modifications have been confined, however, to considerations of the organization of the functional cells into layers with conceivable intercellular channels, and to considerations of additional functional barriers in series at the outer facing membrane. Since our concern here is primarily with that of the electrical potential, these additional considerations need not be detailed. We proceed, therefore, to review critically some of the experimental work from other laboratories as well as our own laboratory concerning the electrical potential manifestations. At the outset, it may be stated that the results of our own microelectrode studies (Chowdhury and Snell, [3, 4, 5, 6]) are at variance with those of others. Reviewed critically one can only conclude that the prior concepts of the sodium transport system are much too elementary and oversimplified to stand even as a working hypotheses.

In an effort to ascertain the potential profile across these tissue membranes engaged in the active transport of sodium, a number of investigators have made microelectrode studies. Such studies should provide crucial evidence in relation to the Koefoed-Johnsen and Ussing model. All have used the salt-filled micropipette electrode introduced by Ling and Gerard [19]. Engbaek and Hoshiko [9], using frog skin, showed that, in general, there were two positive going potential steps as the microelectrode was advanced into and through the preparation from the outside surface. These two steps were of about equal magnitude and were presumed to be located in the epithelium itself which has a total thickness of about 30–70 μ. Scheer and Mumbach [21] similarly reported two steps in the potential profile, but in that the distance between the two steps, as measured during the advance of the electrode, was of the order of 2000 μ; they suggested that the corium and subcutaneous layers were implicated in the sodium transport system. It should be pointed out, however, that the total thickness of the usual abdominal skin of the frog is not more than several hundred microns, and thus the results of Scheer and Mumbach are open to criticism in that with their experimental procedure there was undoubtedly severe tissue distortion and indentation, followed by sudden penetration of the electrode. Whittembury [27] attempted to resolve the question of the spatial localization of the potential steps by coupling the microelectrode potential measurements with an

injection of dye. The location was then determined by histological examination. He again found that there were two positive going potential steps in his toad skin preparation and localized the plateau region to the epithelium. However, spatial resolution was insufficient to define more precisely which layers of the epithelial structure were involved. Frazier [11] carried out microelectrode studies with the toad bladder preparation and also found that there were two positive going potential steps on penetration from the mucosal surface, even though this tissue represents a much simpler epithelial structure.

In reviewing these studies it should be pointed out that many of these investigators noted exceptions to the observations of two potential steps. Occasionally, more steps were found. Initial attempts with microelectrodes in our laboratory (Chowdhury [2]), in which the experiments were designed for the primary purpose of localizing more crucially the sodium selective boundary and the potassium selective boundary, showed that during the controlled advance of the microelectrode there was considerable surface and cellular distortion under direct microscopic observations. This was manifest in the form of indentation prior to actual puncture, the indentation appearing to be released in sudden steps. Feeling that this may have contributed to the step changes noted by others, we devised an apparatus to fabricate electrodes with considerably finer tips than heretofore used. The conventional microelectrode has tip diameters in the range of 0.3 to 0.5 μ. Our ultrafine microelectrodes on the basis of the taper angle at the tip and the measured electrical resistance had tip diameters as small as 100 Å. Simultaneously we introduced the technique of applying an axial vibration to the microelectrode during penetration, driving the electrode with axial excursions up to 3000 Å at audio frequencies (Snell, Chowdhury, and Burke [23]). Either the use of ultrafine micropipette electrodes or the use of vibration with conventional microelectrodes appeared to eliminate the microscopically observable tissue indentation. More importantly, the electrical potential was now observed to be more or less a smooth and monotonically increasing function of the depth of penetration, once the region in which the potential is manifest was approached. This was true in both frog skin preparations and toad bladder preparations. Figure 1 illustrates a typical result from an experiment with toad bladder in which the total transmembrane potential was manifest over a distance of only some 6 μ. Could such smooth potential profiles have arisen as artifacts? It is conceivable that they could have as a result

of cell membrane rupture with concomitant electrical leakage paths around the electrode, or perhaps as a result of defects in the micropipette electrodes themselves, such as cracks along the electrode wall near and about the tip. Both of these conditions might suggest that the microelectrode would measure some average potential along its axis and lack definition as to the precise point of its measurement. However, the fact that the conventional microelectrode when advanced with axial vibration, or the ultrafine tipped electrode when advanced without vibration gave more or less identical results speaks against these possibilities. Furthermore, we were successful in demonstrating with the coarser tipped electrodes that multiple steps in the potential profile could be obtained at will. In these experiments, the electrode,

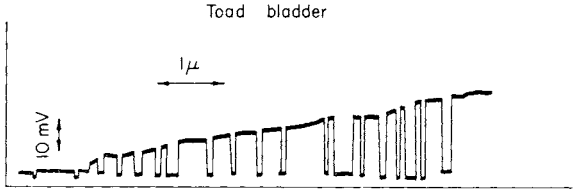

FIG. 1. Recorded electrical potential from a fine tipped microelectrode during advance into toad bladder from the mucosal surface. Upper line: open circuit state; lower line: short-circuit state.

after it had touched the tissue surface, was advanced without vibration for a distance approaching that of a total thickness of the region manifesting the potential, being careful to avoid any vibration during this maneuver. At this point, the advancement mechanism was stopped, and the apparatus was gently vibrated by tapping the microscope stage. Associated more or less with each tap the electrical potential changed abruptly in small steps. Such an experiment is illustrated in Fig. 2. It is tempting to conclude that rather than the more or less smooth potential profile, as we observed, being artifactual, the generally observed two-step potential profile observed by others is entirely a result of mechanical distortion of the cells. Apparently the limiting membranes as well as possible cytoplasmic structures become distorted during advance of electrode followed by a sudden real penetration. The two steps suggest that they are two regions which offer greater mechanical resistance than most other regions.

These observations on the electrical potential profile speak for

themselves on the untenableness of the simple Koefoed-Johnsen and Ussing model. However, we have carried out other experiments, more crucial with respect to the model, and hopefully free from objections arising from ambiguities of absolute potential measurements with such liquid junction electrodes.

With the microelectrode placed in the region of the frog skin or toad bladder manifesting the electrical potential, the Na^+ concentration in the solution bathing the external surface, $(Na)_o$, and the K^+ concentration bathing the internal surface, $(K)_i$, were changed in several steps, taking extreme care to avoid altering the position of the microelectrode. Na^+ was replaced by choline and K^+ by Na^+ in

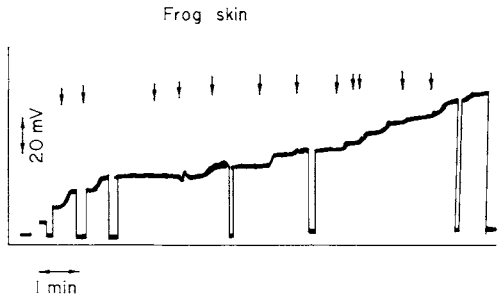

FIG. 2. Recorded electrical potential from a coarse tipped microelectrode advanced to indent but not penetrate the toad bladder. Penetration occurred as the apparatus was gently tapped at points indicated by the arrows.

altering the concentrations. The Ringer's solution was prepared with sulfate salts to avoid changes in cytoplasmic composition (MacRobbie and Ussing [20]). With a macroelectrode in the solution bathing the outside surface serving as a reference electrode, the potential of the microelectrode, $\psi_m - \psi_o = \psi_{mo}$, was plotted as a function of the total transmembrane potential, ψ_{io}. The results gave straight lines within experimental error for both Na^+ changes and K^+ changes. However, the slopes of these lines, $d\psi_{mo}/d\psi_{io}$, varied with the depth of penetration. This depth was conveniently ascertained in terms of the fraction of the total electrical resistance of the tissue encountered by the microelectrode and measured as $[\psi_{mo}(oc) - \psi_{mo}(sc)]/\psi_{io}(oc)$, where the (oc) and the (sc) refer to open-circuit and short-circuit states of preparation, respectively. The results of all experiments performed are collected in Fig. 3, in which the measured slopes for the

$(Na)_o$ changes and the slopes for the $(K)_i$ changes are plotted against the fractional resistance, r. It is quite apparent that at all positions within the tissue, the microelectrode "sees" as much of the potential change effected by Na^+ in the outside solution as that effected by K^+ in the inside bathing solution. This is a situation clearly impossible to realize in terms of the Koefoed-Johnsen and Ussing model, especially in the single-layered epithelium of toad bladder.

In considering the reliability of the results presented in Fig. 3, one is still faced with the possibility that the microelectrode may be

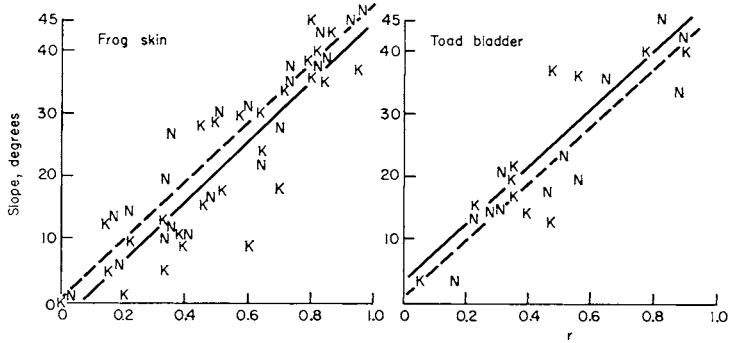

FIG 3. Plots of the slope, $d\psi_{mo}/d\psi_{io}$, as described in text, against the relative position of the microelectrode. The changes in the potentials were achieved by altering either $(Na^+)_o$, designated as N, or $(K^+)_i$, designated as K. Least squares regression lines: broken—$(Na^+)_o$; solid—$(K^+)_i$.

measuring some average potential distributed along its axis. Also, it is not inconceivable that what is measured by the electrode is simply the potential along some shunt pathway, the origin of the potential still being located at the outer and inner bounding surface as envisaged in the Koefoed-Johnsen and Ussing model. Both such objections can be eliminated by assessing the time of the first detectable change in the microelectrode potential with reference to that in the transmembrane potential, following a step change in the ionic concentrations bathing the bounding surfaces. In actual fact, the bounding solutions cannot be changed in a true step function because of the experimental necessity of rigidly holding the tissue in a fixed position. However, the first detectable response of the microelectrode can be easily measured and referred to the time of the first de-

tectable potential change measured by the macroelectrodes emersed in the solutions bathing each surface. This indeed is more what is desired in eliminating the objections. If there is no delay in a $\Delta\psi_{mo}$ over the first detectable $\Delta\psi_{io}$ when K^+ is changed in the solution bathing the internal surface, or in $\Delta\psi_{mi}$ over $\Delta\psi_{oi}$ when Na^+ is changed in the solution bathing the outer surface, then either a shunt pathway is being monitored by the microelectrode or it is monitoring some macroregion distributed along its axis. However, any time delay

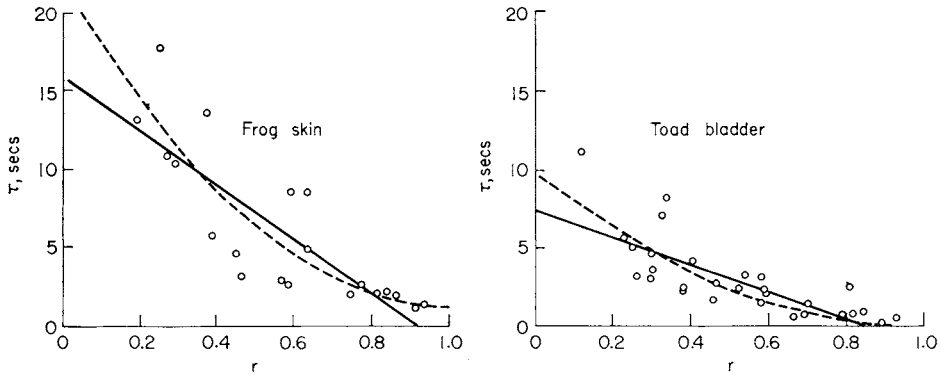

FIG. 4. The time delay, τ, of the first detectable change in potential of the microelectrode with reference to the time of the first detectable change in total transmembrane potential following change in $(K)_i$, plotted as a function of the fractional resistance, r, measured from the outside (mucosal) surface. The solid and interrupted lines are least square linear and curvilinear regressions, the latter based upon the supposition of diffusional delay from an infinite plane following a step change.

greater than that expected from the resistance-capacitance time constant of the system would definitively eliminate these objections. The results, clearly showing time delays, are presented in Fig. 4 for the $(K)_i$ changes and in Fig. 5 for the $(Na)_o$ changes. These delays measured in seconds are far greater than possible on the basis of any RC network, since the RC time constant is in the order of milliseconds, as evident from the electrical response of the microelectrode in stepping from the open-circuit state to that of the short-circuit state (see Figs. 1 and 2). It is clear that the delay times are larger the further the microelectrode is positioned (in terms of fractional resistance) from the surface where the ionic changes are instituted.

Although there is considerable scatter of the data, the relations are suggestively curvilinear such as predicted were the delay due to diffusion from an infinite plane. It is also to be noted that the delays in the case of the $(Na)_o$ changes are almost an order of magnitude smaller than those from $(K)_i$ changes.

It is indeed difficult to imagine an interpretation of all of these results, which in brief show a more or less uniform electric field, a sensitivity to alterations of $(Na)_o$ and $(K)_i$ distributed throughout that region of the tissue manifesting an electric field, and time delays in the potential changes of the positioned microelectrode in response

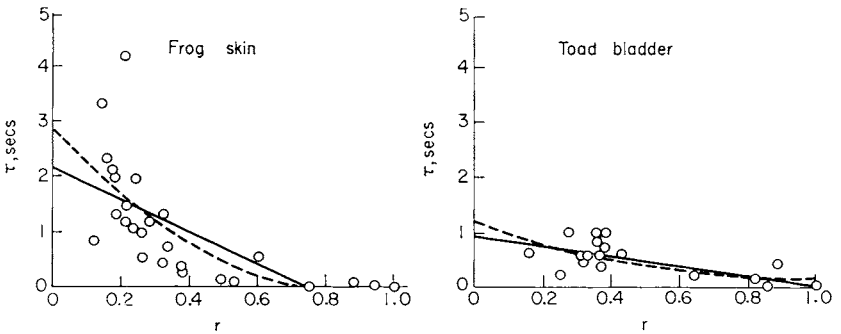

FIG. 5. The time delay, τ, of the first detectable change in potential of the microelectrode with reference to the time of the first detectable change in total transmembrane potential following change in $(Na)_o$, plotted as a function of the fractional resistance, r, measured from the inside (serosal) surface. Solid and interrupted lines as in Fig. 4.

to changes in $(Na)_o$ and $(K)_i$, in terms of the simple Koefoed-Johnsen and Ussing model. It is also difficult to imagine how such results could arise in their entirety by experimental artifact, although it is quite plausible to see how discontinuities of the electric field could arise from mechanical factors associated with penetration by electrodes whose tips are in the order of 2000 to 5000 Å. These evidences, coupled with the fact that changes in $(K)_i$ affect the $(Na)_o$-potential relations and changes in $(Na)_o$ affect the $(K)_i$-potential relations in a reciprocal manner (Snell and Chowdhury [22]), strongly suggest that the processes and mechanisms associated with the active transport of sodium across these tissues are not to be entirely relegated to the bounding and limiting cell membranes. One

must implicate some direct cytoplasmic function as well. Any model that suggests itself is rather complex and currently defies rational analysis. Our thoughts, however, are being directed toward cytoplasmic flow processes, a kind of microcirculation, perhaps arising on the basis of osmotic differences across cytoplasmic membranes, the flow being guided and directed in the numerous channels formed by cytoplasmic membrane structures. Such flow can readily give rise to potential gradients in terms of streaming potentials. Also one can imagine how these may be functionally dependent on the bounding ionic solutions. Suffice it to say, however, that many such mathematical models are under study at the present time and that it is premature to elaborate further detail.

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