CURRENT-VOLTAGE CURVE OF
SODIUM CHANNELS AND CONCENTRATION DEPENDENCE
OF SODIUM PERMEABILITY IN FROG SKIN

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(Received 20 July 1976)

SUMMARY

1. The inward facing membranes of in vitro frog skin epithelium were
depolarized with solutions of high K concentration. The electrical prop-
erties of the epithelium are then expected to be governed by the outward
facing, Na-selective membrane.

2. In this state, the transepithelial voltage \( V \) was clamped to zero and
step-changes of Na activity in the outer solution \( (\text{Na}_o) \) were performed
with a fast-flow chamber at constant ionic strength, while the short-
circuit current was recorded.

3. At pre-selected times after a step-change of \( (\text{Na}_o) \) the current re-
response \( (I) \) to a fast voltage staircase was recorded. This procedure was
repeated after blocking the Na channels with amiloride to obtain the current–voltage curve of transmembrane and paracellular shunt pathways. The current–voltage curve of the Na channels was computed by subtracting the shunt current from the total current.

4. The instantaneous \( I_{\text{Na}}-V \) curve thus obtained at a given \( (\text{Na}_o) \)
could easily be fitted with the constant field equation in the range between
\(-50\) and zero mV. This fit yielded approximate estimates of \( P_{\text{Na}} \), the Na–
permeability of the Na-selective membrane (at this \( (\text{Na}_o) \)) and the cellular
Na activity, \( (\text{Na})_c \). As residual properties of the serosal membrane were
ignored the computed values are expected to underestimate the true ones.

5. At constant \( (\text{Na})_c \), the steady-state value of \( 1/P_{\text{Na}} \) increases linearly
with \( (\text{Na}_o) \). Error analysis and the effect of drugs show that the dependence is not due to the residual properties of the inward facing membranes but reflects the true behaviour of \( P_{\text{Na}} \).

6. The steady-state \( P_{\text{Na}} \) at a given \( (\text{Na}_o) \) is smaller than the transient
\( P_{\text{Na}} \) observed right after a stepwise increase of \( (\text{Na}_o) \) to this value. The
time constant of \( P_{\text{Na}} \)-relaxation is in the order of seconds.
In conclusion, Na transport through open Na-selective channels of the outward facing membrane of the stratum granulosum cells can be described as an electrodifussion process which as such does not saturate with increasing (Na)₀. However, when added to the outer border of the membrane Na causes a decrease of P_Na within several seconds. It is considered that binding of Na results in closure of Na channels.

INTRODUCTION

It has long been known that the outer surface of frog skin has the properties of a 'Na electrode' (Koefoed-Johnsen & Ussing, 1958). In the absence of permeating anions the transepithelial electrical potential difference was reported to respond with a change of about 58 mV to a ten-fold variation of the Na concentration of the outer bathing solution. Similarly, the inner surface of the skin was shown to behave like a 'K electrode'. These observations formed the experimental basis of the two-membrane hypothesis. It proposes that transport of Na across the skin consists of a passive entry from the outer medium into the transporting cells of the epithelium, followed by active extrusion from the cells towards the inner solution.

Good evidence has since accumulated that the membrane responding to changes of the sodium activity of the outer solution, (Na)₀, is the apical cell membrane of the stratum granulosum cells (Fig. 1A). Recently, tracer kinetic studies of Na transport between the outer solution and the epithelium revealed that the inward movement of Na occurs through a selective, saturating transport system (Rotunno, Villalonga, Fernandez & Cereijido, 1970; Biber, Cruz & Curran, 1972; Erlij & Smith, 1973). This transport was shown to be voltage sensitive (Biber & Sanders, 1973). However, the driving force for the Na translocation has not yet been evaluated because the electrochemical potential difference for the Na ions between the granulosum cells and the outer solution is difficult to estimate.

Membrane potentials of the outward facing membrane have been recorded with micro-electrodes (e.g. Ussing & Windhager, 1964; Rawlins, Mateu, Fragachan & Whittembury, 1970; Hviid Larsen, 1973; Nagel, 1976; see also Ehrenfeld, Nelson & Lindemann, 1976), but the Na activity (Na)ₑ of the transporting cells was unknown under all experimental conditions. Direct measurements with Na-selective micro-electrodes (Janáček, Morel & Bourguet, 1968) are technically difficult and include a risk of diffusional Na leakage through the puncture channel (Lindemann, 1975). Calculations based on isotope measurements of intracellular Na pools or on intracellular Na concentrations obtained by flame photometry may be misleading (see Leblanc, 1972), because they represent mean values from
an unknown fraction of cells of the epithelial population. Determinations by X-ray spectroscopy have a better spatial resolution (Dörge, Gehring, Nagel & Thurau, 1974) but like those by flame photometry include an unknown fraction of bound or sequestered Na which does not contribute to the Na activity at the inner surface of the Na-selective membrane.

In this situation one might think of using the Na-selective membrane itself as an electrode for the determination of the intracellular Na activity. This should be possible in the absence of membrane-permeating small anions, were it not for the presence of a significant paracellular shunt pathway (Ussing & Windhager, 1964). This shunt partially short-circuits the voltage across the Na-selective membrane and makes it an unreliable indicator of \((\text{Na})_c\).

A convenient determination of \((\text{Na})_c\) may become possible, however, by making use of the hitherto unknown current–voltage relationship of the Na channels in the Na-selective membrane. This approach has the additional advantage of determining the Na permeability, \(P_{\text{Na}}\), at the same time. Furthermore, the shape of the current–voltage curve may lead to conclusions regarding the nature of the transport mechanism.

To use this rather simple approach, three experimental requirements must be fulfilled. (1) Voltage clamping of the Na-selective membrane. Potential and resistance of other membranes in series to it must be negligible. (2) Na currents at different voltages are to be computed from total currents. Therefore, the current–voltage curve of cellular and extra-cellular shunt pathways (Fig. 1B) must be determined while the Na-specific channels are completely blocked. (3) Exposure to solutions with different \((\text{Na})_o\) must be brief enough to leave \((\text{Na})_c\) almost unchanged. We attempted to fulfil these requirements by: (1) depolarizing the inner membranes with inner bathing solutions of high K concentration (Rawlins et al. 1970); (2) blocking the Na channels with 30 \(\mu\text{M}\) amiloride (Ehrlich & Crabbé, 1968; Dörge & Nagel, 1970; Cuthbert & Shum, 1974); and (3) using a fast-flow chamber which allows a change of the outer solution within 20 msec and reduces the unstirred layer at the outer surface of the Na-selective membrane to about 15 \(\mu\text{m}\) (Lindemann, Gebhardt & Fuchs, 1972).

A brief description of our results was presented at the 1975 spring meeting of the Deutsche Physiologische Gesellschaft (Fuchs, Hviid Larsen & Lindemann, 1975).

**METHODS**

Abdominal skins of the frog *Rana esculenta* or its close relative *Rana ridibunda* were used. Skins of *Rana temporaria* were used occasionally. The isolated skin was aerated in \(\text{SO}_4\)-Ringer solution of high K concentration containing 2.5 or 0 \(\text{mm}\)-Na (see below) for at least 30 min before mounting in the fast-flow chamber.
Fast-flow chamber. This instrument has been described in detail before (Lindemann et al. 1972; Gebhardt & Lindemann, 1974). Its function will be discussed here only briefly. The outer surface of 1 cm² of the skin forms the bottom of a flow channel which has a height of only 1 mm. Its ceiling is made up of a Ringer–agar plug which serves as a current electrode. In the centre of the plug is a lucite tubing filled with 3 m-KCl agar to which the outer voltage electrode is connected. Similar electrodes make contact with a Ringer–agar plug on which the inner surface of the skin is resting. The flow channel is constantly perfused at a low rate of 3 ml./sec with pre-equilibration solution (P-solution). Its exchange with a test-solution (T-solution) of different composition is done as follows. On pressing a start switch, the flow of P-solution is accelerated to 40 ml./sec and remains constant for a preset time, for instance 5 sec. By means of a fluid switch moved by electromagnets, access of P-solution to the flow channel is cut off while access of T-solution is now permitted. Photometric and resistance measurements have shown that the half-time of change of solution in the flow channel right above the membrane is 10 msec. Pressure transients are no larger than 0.05 atm at this location. When the preset time of exposure with T-solution is over, the switch moves back and T-solution is replaced by P-solution flowing at the same rate. After another preset interval, the flow of P-solution is reduced to the initial low rate. The interval between subsequent flow cycles was 2 min or larger.

The anatomical position of the Na-selective membrane is shown in Fig. 1A. With the high rates of flow used, the unstirred layer on top of this membrane is no thicker than 12–15 µm, including the stratum corneum of about 5 µm in thickness (Fuchs, Gebhardt & Lindemann, 1972). The main purpose of the fast-flow technique is to introduce step changes of ionic concentrations or drug concentrations at the outer surface of the apical membrane of the Na transporting cells. The exposure to T-solution is made brief such that the ionic concentrations of the intraepithelial compartments remain almost constant. In such an ideal situation, voltage and resistance changes recorded during one P-T-P cycle develop only across the apical membranes and tight junctions of the outermost living cell layer.

Voltage clamp. Two methods have been developed which make it possible to operate a voltage clamp while the resistivity of the flowing solution is purposely and rapidly changed (Brennecke & Lindemann, 1974a, b; Gebhardt, 1974). However, in the experiments reported here changes of solution resistivity were avoided by keeping the total ionic concentration of the outer solution constant (Na–K substitution). Thus, a conventional voltage clamp with continuous feed-back current and an open loop gain of 1000 could be used. During the pre-equilibrium period the clamp voltage $V$ was held at zero mV and maintained at zero mV for a period of 6 or 10 sec after each change of P- to T-solution. This was followed by a 250 msec interval during which a staircase-generator was triggered by the programming unit of the fast-flow machine and a staircase-shaped voltage signal was applied to the command voltage input of the clamp circuit. We used between six and thirty negative going steps of 20 msec duration. Gain was adjusted such that $V$ changed by a constant increment of 4–20 mV per step.

Two 'transient recorders' (Biomation 802) were used to record the time course of the short-circuit current during each P-T-P cycle and, with higher time resolution, the current response to the voltage staircase.

In a later stage of this investigation it became possible to use a mini-computer (Nova 1220, Data General) for generation of the command voltage staircase and for sampling of the current signal. In these on-line voltage clamp experiments data handling was considerably more rapid. Results of the mathematical analysis became available a few minutes after a set of P-T-P cycles was completed.
Current and voltage sign convention. A current going from the solution bathing the outside of the skin towards that bathing inside (inward current) is defined as positive. The voltage difference between these two solutions is denoted $V$ and defined as $V = \Psi_o - \Psi_i$ where $\Psi_o$ and $\Psi_i$ are the potentials of the outer and inner solution respectively. $V$ is considered to be made up of at least two voltage steps in the epithelium (Ussing & Windhager, 1964; Rawlins et al. 1970), for instance at the apical and basal membrane of cells in the stratum granulosum. These steps will be called $V_o$ and $V_i$, viz. $V_o = \Psi_o - \Psi_e$ and $V_i = \Psi_o - \Psi_i$, where $\Psi_e$ is the potential within the granular cells.
Analysis of current–voltage data. A computer program was written to fit the constant field equation (eqn. (3), page 147) to each set of current–voltage points obtained with one staircase of command voltages. The program varied the parameters $I_{Na}$ and $(Na)_o$ of eqn. (3) until the sum of all squared deviations of data points from the theoretical curve became minimal. The ‘first guess’ value for $P_{Na}$ was computed from

$$P_{Na} = \frac{I_{Na}'(V') - I_{Na} \cdot \exp \left[ -F(V' - V)/RT \right] A(V)}{(Na)_o \cdot [1 - \exp \left( -F(V' - V)/RT \right)]},$$

(1)

where $I_{Na}$ and $I_{Na}'$ are Na currents at voltage $V$ and $V'$, respectively, and

$$A(V) = F \cdot \left( \frac{FV}{RT} \right) / \left(1 - \exp \left( \frac{-FV}{RT} \right) \right).$$

$F$, $R$ and $T$ have their usual meaning. This computation was performed for all pairs of subsequent voltage steps of the staircase and the arithmetic mean of $P_{Na}$ values thus found was used as the ‘first guess’. Using this value of $P_{Na}$, $(Na)_c$ was then computed for each voltage of the staircase:

$$(Na)_c = [(Na)_o - P_{Na} \cdot A(V)] \cdot \exp (FV/RT).$$

(2)

The arithmetic mean of these values of $(Na)_c$ was used as the ‘first guess’ of $(Na)_c$ in the subsequent fitting routine. Activities rather than concentrations were used in all computations. It is noteworthy that our determination of the reversal potential $V_r$ and of $(Na)_c$ does not rely on the measurement of small current signals obtained in the vicinity of $V_r$, but on all current measurements between $V_r$ and zero mV.

It soon became apparent that a fine adjustment of the parameters $P_{Na}$ and $(Na)_c$ with the least square fitting procedure did not significantly alter the values introduced as ‘first guesses’. Therefore, the somewhat time consuming least square fit could be omitted once we had convinced ourselves that the current–voltage data were adequately described by the constant field equation. In all cases the quality of the fit was checked by inspection of a scope display (see Figs. 6 and 8). Fitting was not possible in cases where the inner membrane was not depolarized with high (K)$_o$.

This analysis can also be applied to situations where $(Na)_o$ is larger than $(Na)_c$. It fails, however, when $(Na)_o = 0$ mm. Only the numerical value of the product $P_{Na} \cdot (Na)_c$ can then be found. Direct application of eqns. (1) and (2) to our data neglects residual electrical properties of the K-depolarized inward facing membranes. Possible errors of estimation of $P_{Na}$ and $(Na)_c$ due to this will be discussed on pp. 153–158. Computations were performed on a Nova 1220 or PDP-8/E.

Drugs. Four drugs were used to increase or decrease $P_{Na}$. Benz-thiazolyl-ghanidinium (BTG) is known to increase $P_{Na}$ of the Na-selective membrane just like benz-imidazolyl-ghanidinium (BIG) (Zeiske & Lindemann, 1974) by lowering the apparent Na affinity of the binding site responsible for the self-inhibition phenomenon of the Na channels. A similar, although larger effect can be obtained by adding para-chloromercury-phenyl-sulphonate (PCMB) or para-chloromercury-benzoate (PCMB) to the outer solution (Dick & Lindemann, 1975). The acetyl-guanidine diuretic N-amidino-3,5 diamino-6-chloropyrazine-carboxamid (amiloride) is known to block the Na-channels in competition with Na (Ehrlich & Crabbé, 1968; Nagel & Dörge, 1970; Cuthbert & Shum, 1974).

BTG and BIG were obtained from Riedel-de-Haën, PCMB and PCMBS from Merck and amiloride was a present from Sharp and Dohme, München.

Solutions. Sulphate solutions were used throughout since the membranes of the epithelial cells are almost impermeable to this anion (Koefoed-Johnsen & Ussing, 1958; MacRobbie & Ussing, 1961). Accordingly, the cell volume remains constant when Na of the inside bathing solution is replaced by K (Ussing, Biber & Bricker,
Compositions of the solutions are listed in Table 1. All changes of \([Na]\) were done by replacement with K at a constant anion concentration in order to keep the ionic strength as constant as possible. Thus, for all practical purposes, the Na- (and K) activity coefficient of outside solutions, \(\gamma_o\), is the same for all solutions listed in Table 1. Measurement of the Na activity in the S-100 solution with a Na-electrode (model 94-11, Orion Research, Cambridge, Mass.) gave \(\gamma_o = 0.56\). The K activities were measured in most of the solutions by means of a micropipette K-electrode; I. Astrup and G. R. I. Christoffersen, unpublished). The voltage signal of this electrode was shown to be unaffected by the presence of Na even in solutions where \((Na)\) and \((K)\) were equal. These measurements gave \(\gamma_k = 0.54\). The value 0.55 was used in calculating the Na activities of the Ringer solutions. Activities will be denoted with round brackets.

**Table 1. Electrolyte composition of the Ringer solutions**

<table>
<thead>
<tr>
<th>Solution</th>
<th>[Na] (m-mole/l.)</th>
<th>[K] (m-mole/l.)</th>
<th>[SO₄] (m-mole/l.)</th>
<th>Activity (Na) (m-mole/l.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-5</td>
<td>5</td>
<td>95</td>
<td>50</td>
<td>2.75</td>
</tr>
<tr>
<td>S-10</td>
<td>10</td>
<td>90</td>
<td>50</td>
<td>5.50</td>
</tr>
<tr>
<td>S-15</td>
<td>15</td>
<td>85</td>
<td>50</td>
<td>8.25</td>
</tr>
<tr>
<td>S-20</td>
<td>20</td>
<td>80</td>
<td>50</td>
<td>11.0</td>
</tr>
<tr>
<td>S-30</td>
<td>30</td>
<td>70</td>
<td>50</td>
<td>16.5</td>
</tr>
<tr>
<td>S-50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>27.5</td>
</tr>
<tr>
<td>S-80</td>
<td>80</td>
<td>20</td>
<td>50</td>
<td>44.0</td>
</tr>
<tr>
<td>S-100</td>
<td>100</td>
<td>0</td>
<td>50</td>
<td>55.0</td>
</tr>
</tbody>
</table>

* In addition all solutions contained 1 mm Ca Gluconate, 5 mm Tris and were titrated to pH = 7.4.

**RESULTS**

**Time course of current response to a step change of \((Na)₀**

Fig. 2A shows the response of the short-circuit current to a stepwise increase of \((Na)₀). Initially, a rapid increase of current is observed. Instead of increasing further (dashed line), however, the current soon reaches a peak value and then reclines to a much smaller plateau value. This 'recline' behaviour has previously been reported (Lindemann & Gebhardt, 1973) and was attributed to a delayed decrease of \(P_{Na}\) in response to the increase in \((Na)₀). Prolonged exposure to high \((Na)₀\) (not shown) results in a slow further decrease of current due to an increase of \((Na)c\) (Morel & Leblanc, 1973, 1975).

After the membrane had been exposed to the test solution for 8–10 sec the current became constant. Apparently \(P_{Na}\) had now reached its steady-state value. At this time the clamping voltage was changed in a staircase fashion (arrow labelled S) and the resulting current deflexion recorded. The exposure cycle was repeated after 2 min while blocking the Na channels with 30 μM amiloride contained in the \(T\)-solution. After almost 6 sec of
exposure with amiloride, the initially large current had dropped to a small steady-state value (see Fig. 2 B, Ami). This remaining amiloride-insensitive current will be called $I_{\text{leak}}$. Neither a larger amiloride concentration nor

![Graph](https://via.placeholder.com/150)

**Fig. 2.** A, response of short-circuit current to an increasing step of $(\text{Na})_o$ (substituting $(\text{K})_o$). The dashed line shows the expected time course at constant Na permeability. At $S$ the command voltage was changed in fifteen steps of 4-2 mV each to obtain a current–voltage curve after completion of the permeability change (the ‘recline’ of short-circuit current). B, response to the same $(\text{Na})_o$ step in the presence of parachloro-mercurybenzoate (PCMB, 1 mM), benz-thiazolyl-guanidinium (BTG, 1 mM) and amiloride (Ami, 30 μM) in the outside solution. PCMB was present in the $P$- and $T$-solution, BTG and amiloride only in $T$-solution.
a prolonged exposure time brought a further decrease in this current. It is clear, therefore, that all amiloride-blockable Na channels were blocked at the time the $I_{\text{leak}}$–staircase was recorded. In this way current–voltage curves were obtained with and without the amiloride sensitive Na current ($I_{\text{Na}}$). $I_{\text{Na}}$ could then be computed by subtraction.

Fig. 3. Current–voltage curves obtained 10 sec after a step of $(\text{Na})_o$ from 2.8 to 11 mM, substituting $(\text{K})_o$: ○, total current ($I_{\text{total}}$) of control, no drugs added; △, $I_{\text{total}}$ with 1 mM BTG in $T$-solution; ○, $I_{\text{leak}}$, 30 μM amiloride in $T$-solution. The $I_{\text{Na}}$–$V$ curves of the following figures were constructed by subtracting the leak current from the total current.

In all our experiments with high $(\text{K})_i$ the conductance of the leak pathway was relatively large, i.e. comparable to that of the cellular Na pathway. This was also observed by other groups working with frog skin preparations which included the corium and therefore the subepithelial glands (Ussing et al. 1965). Following these authors we suppose that the large shunt conductance is due to depolarization and stimulation of glands and subsequent partial opening of gland ducts.

The current response in the presence of BTG and PCMB is also shown
Fig. 4. For legend see facing page.
in Fig. 2B. These drugs increase the current over that of the control (Fig. 2A) in a fashion expected from a 'recline inhibitor'. The increase in current can be explained by a prevention of the decrease of $P_{Na}$ which otherwise occurs when ($Na$)$_0$ increases (Zeiske & Lindemann, 1974; Dick & Lindemann, 1975).

**Shape of current–voltage curves. Rectification of the amiloride-sensitive current**

A current–voltage curve of the total current obtained with 11 mM-($Na$)$_0$ is shown in Fig. 3 (filled circles). At this Na activity the curve was found to be almost linear in the range 0 to $-100 \text{ mV}$. It is composed, however, of two non-linear additive components. When $V$ becomes increasingly more negative, the outward directed leak current (open circles) increases more than proportionally while the amiloride blockable current, which is computed as the difference of the two curves, shows the opposite tendency (compare Fig. 4). The presence of 1 mM BTG increased both the inward and outward directed total current (triangles), but not the leak current.

The data points of Fig. 4A, B and C were computed as $I_{total} - I_{\text{leak}}$ at each corresponding voltage. This current difference, $I_{Na}$, is assumed to be identical with the Na current through the Na-specific channels. We shall show below that this assumption is correct (pp. 150 and 151). The direction of rectification of $I_{Na}$ in Fig. 4 is as expected from a membrane where the Na activity at the outer surface is larger than that at the inner surface (Goldman rectification). Inward going rectification was also found by Helman & Miller (1971): under conditions where a low shunt conductance was assured by inside solutions of small K concentration and by a mounting technique of minimal edge damage, the total current increased over-proportionally with depolarizing voltage.

For a voltage range between the reversal potential $V_r$ (at which $I_{Na} = 0$) and 0 mV the $I_{Na} - V$ data points obtained with the same ($Na$)$_o$ could be fitted with the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949), here written with Na activities for the outer and cellular compartment:

$$I_{Na} = \frac{F^2V|RT}{1 - \exp \left(-\frac{FV}{RT}\right)}\left[(Na)_o - (Na)_c \exp \left(-\frac{FV}{RT}\right)\right].$$

(3)

It is noteworthy that in a more exact treatment ($Na$)$_o$ and ($Na$)$_c$ must be multiplied with the partition coefficients $\beta_o$ and $\beta_c$. These coefficients

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Fig. 4. Current–voltage curves of Na channels obtained at six different ($Na$)$_o$. The curves were recorded 10 sec after changing ($Na$)$_o$ from 2.8 mM to the values indicated. The continuous lines are constant field curves fitted to positive current readings, using the $P_{Na}$ and ($Na$)$_c$ values indicated.
relate Na activities at the surfaces of the Na-selective membrane to Na activities in the outer bulk-solution and in the cytosol. Like Hodgkin & Katz (1949) we are using the assumption $\beta_0 = \beta_e$ and consider $\beta$ to be part of $P_{Na}$. Examples of the fit are seen in Figs. 4, 5, 6 and 8. Note that the agreement is good between $V_r$ and 0 mV.

![Graph](image)

**Fig. 5.** Current–voltage curves of amiloride blockable Na channels. The constant field equation was fitted to current readings between 0 and -40 mV (continuous lines), yielding the indicated $(Na)_s$ and Na permeabilities. 

A, note the large deviation of outward currents from the constant field curve both at low and B, high outer Ca concentration.

A deviation from the theoretical curve towards smaller outward currents often occurred at large negative voltages (Fig. 5). This rectification in excess of the ‘Goldman rectification’ is also observed when the Na current
is increased by BTG in the T-solution and also when [Ca]₀ is raised from 1 to 10 mM (Fig. 5B). If amiloride were to block outward Na current less effectively than inward Na current, the resulting over-estimation of the leak current would explain the observed deviation. Unfortunately, there is no information on amiloride blocking at large hyperpolarizing voltages, although it was shown by Morel & Leblanc (1973, 1975) that outward Na current is blocked by amiloride at zero membrane voltage.

Fig. 6. Constancy of (Na)₀ and V₀ as computed from the voltage dependence of the amiloride blockable current at different total membrane conductances: ○, control (no drugs in T-solution); □, BTG, 1 mM in T-solution; ×, amiloride 0·35 μM in T-solution; ●, amiloride, 0·71 μM in T-solution. The $P_{Na}$ (10⁻⁵ cm/sec) values, respectively, 3·31, 1·98, 1·37 and 0·94 were obtained by fitting the constant field equation to the current readings. The four (Na)₀ values thus obtained were: 2·41, 2·39, 2·39 and 2·30 mM.

The rectification in excess of that predicted by the constant field function led us to use only current–voltage data points in the range from zero mV to $V_r$ for the determination of $P_{Na}$ and (Na)₀, as described on p. 142. This restriction has the further advantage that mainly large current values enter into the analysis.
At positive membrane voltages, another type of deviation from the constant field equation was found (not shown). Here $I_{Na}$ is smaller than predicted and at large voltages it even decreases with increasing voltage. The resulting negative slope of the $I_{Na}$--$V$ curve reflects a voltage dependent closure of the Na channels which has been described before for amphibian skin and urinary bladder epithelium (for a review of the literature, see Lindemann, 1971, Gebhardt & Lindemann, 1974).

**Specificity of amiloride**

In the toad skin exposed to Na$_2$SO$_4$-Ringer on both sides amiloride inhibits the active transcellular Na current without affecting the shunt conductance (Hviid Larsen, 1973). This suggests that in frog skin too, the amiloride sensitive current can be treated as a Na current passing the outer membrane through only one type of channel. If this is true, the reversal potential, $V_r$, of $I_{Na}$ should be the Na equilibrium potential and therefore: 

(a) be independent of changes in membrane conductance and 
(b) change with Na activities at the two sides of the membrane according to the Nernst equation. Both implications could be verified experimentally.

(a) If two or more ionic species were to carry the amiloride-blockable current through separate channels, for instance Na through the outer membrane and K through the paracellular shunt, $V_r$ would be affected by a change in the conductance of just one type of channel. It is unlikely that so different structures as Na-specific membrane channels and paracellular pathways have the same concentration dependence for amiloride blockage as well as for BTG stimulation. We therefore produced conductance changes with different concentrations of amiloride as well as with BTG and analysed for $V_r$ in each case (see Fig. 6). In spite of a threefold change of the slope of the current--voltage curve, $V_r$ was found in each case to be −38 mV, indicating that amiloride affects just one type of channel which is also affected by BTG. This result was also obtained when BIG was employed instead of BTG.

(b) In Fig. 4 $V_r$ was shown to increase with (Na)$_o$. In a more detailed analysis of this dependence a constant intracellular Na activity was maintained by equilibrating the outer surface of the skin with the same $P$-solution (S-5 was chosen) before stepping (Na)$_o$ up in the $T$-period. In subsequent $P$--$T$--$P$ cycles (Na)$_o$ was varied from 2·75 to 27·5 mm. $V_r$ varied linearly with the log. of (Na)$_o$ from −11 to −70 mV (Fig. 7). The slope of the regression line is not significantly different from 58 mV per tenfold change in (Na)$_o$. This indicates that $V_r$ is the Na-equilibrium potential and thus the amiloride blockable channels pass Na.

It seems justified, therefore: (a) to treat the amiloride blockable current as
a current carried by Na; and (b) to interpret the 'permeability coefficient' obtained by curve fitting with eqn. (3) as $P_{Na}$ of the Na channels for the voltage range investigated.

Fig. 7. Dependence of the reversal potential ($V_r$) of the amiloride blockable current on $(Na)_o$. Pooled data from five epithelia. The regression line extrapolates to $(Na)_o = (Na)_c = 1.7$ mM at zero $V_r$.

Fig. 8. A, $I_{Na} - V$ curves at five different $(Na)_o$. Continuous lines: constant field curves of best fit, all for $(Na)_c = 2.2$ mM. Inset, $P_{Na}$ values obtained from the fit. B, inverse of $P_{Na}$ plotted versus $(Na)_o$ to obtain the maximum $P_{Na}$ ($P_{max}$) and $K_m$.

**Dependence of $P_{Na}$ on $(Na)_o$**

Once the possibility of estimating the permeability of the Na channels from fits with the constant field equation was established, the dependence of $P_{Na}$ on $(Na)_o$ could be analysed in more detail. In these experiments $(Na)_c$ was kept constant by pre-equilibration with $S-5$ solution. $I_{Na} - V$
curves of a series obtained from the same skin preparation during exposure to $T$-solutions of different $(Na)O$ are depicted in Fig. 8. Changing $(Na)O$ from 5·5 to 28 mm caused the steady-state $P_{Na}$ to decrease from $2·7 \times 10^{-5}$ to $1·45 \times 10^{-5}$ cm/sec (Fig. 8, inset).

As illustrated in the right part of Fig. 8, the dependence of $P_{Na}$ on $(Na)O$ can be linearized by plotting the inverse of $P_{Na}$ against $(Na)O$ and computing a regression line. The linearized plot allows us to find the maximum $P_{Na}$ ($P_{max}$), corresponding to zero mm$(Na)O$, from the intercept of the regression line with the ordinate. The value of $P_{max}$ was quite different from skin to skin, ranging from $1·2 \times 10^{-5}$ to $4 \times 10^{-5}$ cm/sec. The second parameter obtained from the linearized plot, denoted $K_m$, is the negative of the intercept of the regression line with the Na-activity axis (abscissa). The numerical value of this apparent dissociation constant ranged from 5 to 33 mm. There was no correlation between $P_{max}$ and $K_m$ values of individual skins.

**DISCUSSION**

*Voltage clamping of the Na-selective membrane*

We have attempted to reduce membrane potential and resistance of the inward facing membrane of the transporting cells by exposing the inside of the skin to a solution of high K activity. This approach relies on a high passive K permeability of the inward facing membrane, which will make it behave like a K electrode when exposed to sulphate Ringer solution. Substantial, albeit indirect, support for this hypothesis comes: (a) from the relationship between inside K concentration, $(K)_i$, and transepithelial potential difference (Koefoed-Johnsen & Ussing, 1958); as well as (b) from the volume changes of the epithelial cells due to changes in ionic composition and osmolarity of the solution bathing the inside of the skin (MacRobbie & Ussing, 1961; Ussing et al. 1965).

We found that with increased $(K)_i$ the voltage dependence of an inward going amiloride blockable Na current obeys the equation for simple electrodiffusion through homogeneous membrane channels. This relationship could not be observed in skins exposed to low $(K)_i$. One remarkable effect of the K treatment, therefore, is that now the skin behaves as expected from a one membrane preparation with respect to the voltage dependence of the transcellular amiloride blockable current. However affirmative this result may be with respect to the assumed depolarization of the inner membrane, the final interpretation of our quantitative analysis has to take possible non-zero values of the inner membrane potential and resistance into account. To begin with, therefore, we shall discuss to what extent possible membrane potentials and resistances of the inner membrane may have influenced our data.
The pathway of the amiloride blockable current can be represented by the equivalent circuit shown in Fig. 9. In this diagram the outer membrane appears as a Na conductor and the inner membrane as a K conductor in parallel to a constant current source, i.e. a rheogenic Na/K pump. In the steady state, which in our experiments prevails at short-circuit conditions in $P$-solution, the following relationship will hold:

$$I_K = I_{Na}^{sc}/r,$$

$$I_{pump} = (1-1/r) I_{Na}^{sc},$$

$$V_{1}^{sc} = R_K I_{Na}^{sc}/r + E_K.$$  

Here $I_{Na}^{sc}$ is the amiloride blockable current at short-circuit conditions and $r$ the negative coupling ratio of the Na/K pump, i.e. the active Na flux divided by the (negative) active K flux. $I_{pump}$ is the current generated by the Na/K pump. $V_1^{sc}$ is the steady-state inner membrane potential at short-circuit conditions. $R_K$ is the resistance to K ion movement across the inner membranes and $E_K$ the K equilibrium potential. If next we take into account that the current–voltage curves in our experiments were always obtained within such a short time interval that $(Na)_c$ changes very little, it can be assumed that $I_{pump}$ is constant for the entire range of Na currents displayed during the step voltage changes. The membrane potential across the inner membrane is then given by:

$$V_1 = I_{Na} R_K + E_1 = I_{Na} R_K - \left(1 - \frac{1}{r}\right) R_K I_{Na}^{sc} + E_K,$$
where $E_1$ is the instantaneous open-circuit voltage of the inner membrane. The transepithelial potential difference, $V (= V_o + V_1)$, is given by:

$$V = I_{Na} R_K - \left(1 - \frac{1}{r}\right) R_K I_{Na}^{ec} + E_K + I_{Na} R_{Na} + E_{Na},$$

where $R_{Na}$ is the voltage dependent resistance to Na ion movement across the outer membrane. Solution with respect to $I_{Na}$ yields an expression for the non-steady-state current–voltage curve:

$$I_{Na} = \frac{-E_{Na} - E_K}{R_{Na} + R_K/r} + \frac{V}{R_{Na} + R_K}.$$

(4)

Since the K activity in the interspace compartment is high and close to that of the cellular compartment, $R_K$ will be assumed constant, i.e. independent of $I_{Na}$. The ‘Goldman rectification’ observed may then be ascribed to the Na channels of the outer membrane. From this assumption we obtain for arbitrary values of $V_o$ the integral resistance (e.g. Finkelstein & Mauro, 1963):

$$R_{Na} = \frac{(RT)^2}{P_{Na} F^3 V_o} \frac{(1 - \xi) \ln [(Na)_c \xi/(Na)_o]}{(Na)_c \xi - (Na)_o},$$

(5)

where $\xi = \exp (-FV_o/RT)$. By combination of eqns. (4) and (5) and for known values of the circuit components (including $P_{Na}$) non-steady-state current–voltage curves of the circuit shown in Fig. 9 can be constructed. As will be demonstrated below, the current–voltage curves of the circuit, even for the most unfavourable choice of $R_K$ and $E_K$, are still non-linear when $(K)_i$ is large, and within the range $0 \text{mV} > V > V_r$ practically indistinguishable from the current–voltage curve of a simple homogeneous pathway with constant field properties. This means that fitting of the constant field equation (by the method described on p. 142) to current–voltage data pairs obtained from eqn. (4) is possible. The apparent $P_{Na}$ and $(Na)_c$ values estimated by this fitting procedure, however, are not exact but depend on $R_K$ and $E_1$.

Choice of values of the circuit components. Rawlins et al. 1970 measured the resistance profile in the isolated epithelium of toad skin with micro-electrodes advanced through the inside bathing solution. The resistance recorded between the tip of a micro-electrode located within the outermost living cells (the cells of Stratum granulosum) and the inner bath was unmeasurably small with 100 mM KCl-Ringer as inner solution. Unfortunately, in this situation considerable swelling of epithelial cells must be expected (Ussing et al. 1965), which increases the Na permeability of the Na-selective membrane (Share & Ussing, 1965). Because of this effect of swelling on the outer membrane we preferred using sulphate solutions in the present study. In sulphate solutions of high K concentration swelling does not occur (MacRobbie & Ussing, 1961).
I/V-CURVE OF Na⁺ CHANNELS IN FROG SKIN

According to Rawlins et al. (1970) the resistance of the inner membranes does not decrease as much in SO₄ solutions as in Cl solutions. The resistance recorded between granular cells and the inner bathing solution was found to drop from 780 Ω cm² to no less than 400 Ω cm² when K₂SO₄-Ringer was used instead of Na₂SO₄-Ringer. The voltage between granular cells and outer solution was only 2–3 mV with Na₂SO₄-Ringer on both sides of the epithelium. For the present purpose we shall use the 400 Ω cm² value as an upper limit and calculate the error introduced when the resistance of inward facing membranes is that large. Unfortunately, the authors do not indicate the membrane potentials obtained in epithelia bathed in K₂SO₄-Ringer, and no one else has as yet published micro-electrode recordings from cells of the stratum granulosum in skins exposed to high (K)_i. However, Cereijido & Curran (1965), who recorded from cells of unknown intra-epithelial location, reported that with an inside bathing solution containing 58 mM-K₂SO₄ the cells remain 28 mV negative to the inner solution under open-circuit conditions. Unfortunately, as in many other micro-electrode studies, it is not clear to which extent impalement shunt artifacts, a Donnan potential of the cytosol and KCl leakage from the micro-electrode have contributed to this value. The choice of E_K, therefore, cannot yet be based on direct electrical measurements.

In this situation it would be helpful if E_K could be calculated from measurements of intracellular K activities. However, values for granular cells in SO₄-solutions are presently not available. In our calculations we shall therefore include the unfavourable case (K)_c = 75 mM at (K)_i = 55 mM. This would result in E_K = -8 mV.

In several tissues an electrogenic pump with r equal to about 3/2 has been demonstrated (for review see Thomas, 1972). For lack of precise information on r for amphibian skin, r = 3/2 will be used in our calculations. In skins pre-equilibrated with (Na)_o = 2.7 mM the short-circuit current was at most 6 μA/cm² which now gives

\[
\left(1 - \frac{1}{r}\right) R_K I_{Na}^{e} = +0.8 \text{ mV.}
\]

E₁, therefore, should not exceed −10 mV.

**Error analysis.** The error analysis of estimation of P_Na and (Na)_c will now be carried out numerically for the following three sets of conditions: (1) R_K = 400 Ω cm², E₁ = −10 mV; (2) R_K = 400 Ω cm², E₁ = 0 mV; and (3) R_K = 0 Ω cm², E₁ = −10 mV. For selected values of P_Na, (Na)_o and (Na)_c, current–voltage data pairs have been calculated from eqns. (4)
Fig. 10. Theoretical current–voltage curves of the amiloride blockable pathway of Fig. 9 computed from eqns. (4) and (5). Results of three sets of inner membrane conditions are shown: (1) $R_K = 400 \, \Omega \, \text{cm}^2$, $E_I = -10 \, \text{mV}$; (2) $R_K = 400 \, \Omega \, \text{cm}^2$, $E_I = 0 \, \text{mV}$; (3) $R_K = 0 \, \Omega \, \text{cm}^2$, $E_I = -10 \, \text{mV}$. A, D and G, ($Na)_e = 3.0 \, \text{mM}, (Na)_o$ is varied (mM): $\bigcirc$, 30; $\bullet$, 20; $\square$, 15; $\blacksquare$, 10; $\times$, 5; while $P_{Na}$ remains constant at $4 \times 10^{-5}\, \text{cm/sec}$. The continuous lines are constant field curves fitted to the calculated current values. B, E and $H$, plot of reversal potential ($V_r$) versus log $(Na)_o$. Slope of regression line is for: $B$, 63; $E$, 63; and $H$, 59 mV/dec. C, E and I, ($Na)_e = 3.0 \, \text{mM}, (Na)_o = 10 \, \text{mM}, P_{Na}$ is varied ($\times 10^{-5}\, \text{cm/sec}$): $\square$, 4.0; $\bigcirc$, 3.0; $\times$, 2.0; $\bullet$, 1.0. The continuous lines are constant field curves fitted to the calculated current values.
and (5) and were subsequently fitted with the constant field equation as described on p. 142. The apparent permeability and intracellular activity obtained by this fitting are denoted $P_{Na}^*$ and $(Na)_o^*$, respectively. The ratios $P_{Na}^*/P_{Na}$ and $(Na)_o^*/(Na)_c$ are the error factors of the estimated $P_{Na}$ and $(Na)_c$ values due to the influence of the inner membrane. The results are presented in Fig. 10 and Table 2 and can be summarized as follows.

Table 2. Error factors of estimation of $P_{Na}$ and $(Na)_o$ in worst case calculations. $P_{Na}^*$ and $(Na)_o^*$ are the apparent values of permeability and intracellular activity for the following inner membrane conditions: (1) $R_K = 400 \, \Omega \, \text{cm}^2$, $E_i = -10 \, \text{mV}$; (2) $R_K = 400 \, \Omega \, \text{cm}^2$, $E_i = 0 \, \text{mV}$; (3) $R_K = 0 \, \Omega \, \text{cm}^2$, $E_i = -10 \, \text{mV}$. The true permeability and activity values are: $P_{Na} = 4 \times 10^{-5} \, \text{cm/sec}$ and $(Na)_o = 3.0 \, \text{mm}$.

<table>
<thead>
<tr>
<th>$(Na)_o$ (mm)</th>
<th>$P_{Na}^*/P_{Na}$</th>
<th>$(Na)_o^*/(Na)_o$</th>
<th>$P_{Na}^*/P_{Na}$</th>
<th>$(Na)_o^*/(Na)_o$</th>
<th>$P_{Na}^*/P_{Na}$</th>
<th>$(Na)_o^*/(Na)_o$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.98</td>
<td>0.67</td>
<td>0.83</td>
<td>1.0</td>
<td>1.23</td>
<td>0.67</td>
</tr>
<tr>
<td>10</td>
<td>0.90</td>
<td>0.67</td>
<td>0.73</td>
<td>1.0</td>
<td>1.23</td>
<td>0.67</td>
</tr>
<tr>
<td>15</td>
<td>0.80</td>
<td>0.63</td>
<td>0.68</td>
<td>0.96</td>
<td>1.23</td>
<td>0.66</td>
</tr>
<tr>
<td>20</td>
<td>0.73</td>
<td>0.59</td>
<td>0.63</td>
<td>0.93</td>
<td>1.23</td>
<td>0.66</td>
</tr>
<tr>
<td>30</td>
<td>0.65</td>
<td>0.56</td>
<td>0.55</td>
<td>0.87</td>
<td>1.23</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Fitting of the constant field equation to the sets of current–voltage data pairs calculated from eqns. (4) and (5) is possible. Only for the combination of large $R_K$ and large $(Na)_o$ do the points deviate systematically from the constant field curve, in a manner not observed in our experiments (compare Figs. 10A and D with 4, 5 and 8). For $R_K$ larger than zero the apparent $(Na)_c$ decreases with increasing $(Na)_o$ (Table 2), tending to increase the slope of the regression line relating $V_r$ to log $(Na)_o$ (Fig. 10B, $E$ and $H$). The apparent $(Na)_c$ values, however, deviate far more from each other than found experimentally, where the variation of $(Na)_c$ with $(Na)_o$ was small and not systematic. For variation of $P_{Na}$ (the other circuit components being constant) the reversal potential remains constant (Fig. 10C, $F$ and $I$). The conclusion derived from the results shown in Fig. 6, therefore, is not affected by assumed reasonable values of $R_K$ and $E_i$.

It is interesting to note that for $R_K$ values larger than zero the apparent value of $P_{Na}$ decreases with increasing $(Na)_o$ (Table 2). In other words the presence of $R_K$ causes $P_{Na}^*$ to vary with $(Na)_o$ while $P_{Na}$ is actually constant and this variation goes in the direction observed in our experiments. However, we can advance several arguments which show that the dependence of $P_{Na}$ on $(Na)_o$ present in our data is not a computational artifact based on neglect of $R_K$.

(a) The artifact should appear undelayed while the observed decrease of $P_{Na}$ develops with a time constant of seconds.
(b) The transepithelial voltage, recorded in the fast-flow chamber under open-circuit conditions, responds to a step increase of \((Na)_o\) with a 'recline' just like the short-circuit current. This voltage recline is readily explained by a delayed decrease of \(P_{Na}\) (Lindemann & Gebhardt, 1973). Its observation under conditions of reduced or zero transcellular current flow excludes the inner membrane as the origin.

(c) The artifactual absolute decrease of \(P^*_Na\) should become larger when the true value of \(P_{Na}\) is increased with drugs, because the relative contribution of \(R_K\) to the total resistance increases (compare eqns. (4) and (5)). In contrast to this expectation, when \(P_{Na}\) is increased with BIG, the dependence of \(P_{Na}\) on \((Na)_o\) disappears (Zeiske & Lindemann, 1974).

We conclude, therefore, that the decrease of \(P_{Na}\) with increasing \((Na)_o\) is real. However, due to the uncertainty in the numerical values of \(R_K\) and \(E_1\) our analysis may wrongly estimate the true values of \(P_{Na}\) and \((Na)_c\). Both \(R_K\) and \(E_1\) tend to cause an underestimation of \((Na)_c\). In the most unfavourable case \((Na)_c\) is underestimated about 40%, in the likely case 5–10%. The effects of \(R_K\) and \(E_1\) on estimation of \(P_{Na}\) go in opposite directions. Large \(R_K\) values bring about a significant underestimation of \(P_{Na}\) (at most 40%), whereas unfavourable values of \(E_1\) cause an over-estimation of \(P_{Na}\) of at most 23%. Therefore, with respect to absolute values of \(P_{Na}\) and \((Na)_c\) our method is only approximate.

**Na current rectification**

Our analysis has shown that at small negative voltages the often almost linear current–voltage curve of the Na-selective membrane and its paracellular shunt is composed of two non-linear additive components of opposite curvature. Of these, the amiloride-sensitive component is called \(I_{Na}^{\text{INa}}\) and the remaining one \(I_{leak}\). Obviously, all conclusions regarding the shape of the \(I_{Na}^-V\) curve depend heavily on the fact that amiloride present in the outer solution will close the Na channels reliably. This appears to be the case for inward Na current at small negative voltages (Fig. 2B). At zero membrane voltage the outward Na current from Na-loaded epithelia is also blocked by amiloride (Morel & Leblanc 1973, 1975). The relationship

\[
I_{Na} = I_{\text{total}} - I_{\text{leak}}
\]

used in our computation appears to be justified, therefore, for small negative membrane potentials. The case is less clear for large negative membrane potentials. Here the strong rectification of \(I_{Na}\) in excess of 'Goldman rectification' may either be due to a membrane process or be explained by an incomplete blockage of \(I_{Na}\) which results in an over-estimation of the leak current and therefore an underestimation of \(I_{Na}\).

At small negative voltages the \(I_{Na}^-V\) curve of the Na channels is
non-linear at large (Na)\textsubscript{o} but linearity is approximated when the Na activity at the outer side of the membrane is suddenly lowered (Fig. 4). This disappearance of rectification as (Na)\textsubscript{c} and (Na)\textsubscript{o} approach each other is as expected from homogeneous (or symmetrical) transport channels (Goldman rectification). A more detailed analysis of this rectification showed that in the range between zero mV and the reversal potential of \(I_{Na}\) the voltage dependence of the Na current can indeed be described by the constant field equation (eqn. (3)). Fitting of the \(I_{Na-V}\) data with this equation permitted an approximate determination of the parameters \(P_{Na}\)

Fig. 11. Plot of \(P_{\text{max}}/P_{Na}\) versus (Na)\textsubscript{o}/\(K_m\). Data points (192) from fourteen epithelia were pooled. \(P_{Na}\) was obtained by fitting \(I_{Na-V}\) data points with the constant field equation. \(P_{\text{max}}\) and \(K_m\) were determined from regression lines as shown in Fig. 8. \(P_{\text{max}}\) was 2.07 (±0.75) \(\times\) 10\textsuperscript{-5} cm/sec and \(K_m\) was 14.2 (±8.99) mm (means ± s.d. of observation). The straight line is the regression line and is identical to the line predicted by eqn. (7) in the text.
and \((\text{Na})_0\). It should be noted that the \(P_{Na}\) values obtained apply only to the voltage range for which this analysis has been made. In the range mentioned, the constant field equation could still be used when the steady-state \(P_{Na}\) was increased with BIG, BTG or PCMB, or when it was partially decreased with amiloride (Figs. 5 and 6) or with solutions of large \((\text{Na})_0\) (Figs. 4 and 8).

At positive membrane potentials a voltage dependent closure of Na channels occurs which gives rise to considerable deviations of the \(I_{Na} - V\) curve from predictions based on eqn. (1) (see p. 150). The phenomenon has been tentatively attributed to an occupation of Nachannels by Ca-ions (Heckmann, Lindemann & Schnakenberg, 1972).

**Dependence of \(P_{Na}\) on \((\text{Na})_0\)**

Perhaps the most useful result of the quantitative analysis of the \(I_{Na} - V\) data is the approximate determination of the steady-state permeability coefficient for Na and its dependence on \((\text{Na})_0\). \(P_{Na}\) could be determined repetitively in a single skin preparation, using different outer Na concentrations, while the cellular ionic composition was shown by analysis to vary little. In this way the following empirical relationship between the steady-state \(P_{Na}\) and \((\text{Na})_0\) was found (Fig. 8):

\[
P_{Na} = \frac{P_{\text{max}} K_m}{K_m + (\text{Na})_0}.
\]  
(6)

The values of \(P_{\text{max}}\) and \(K_m\) varied considerably from skin to skin. Therefore, if results from many preparations are to be pooled, \(P_{Na}\) and \((\text{Na})_0\) must be normalized with respect to \(P_{\text{max}}\) and \(K_m\):

\[
P_{\text{max}}/P_{Na} = 1 + \frac{(\text{Na})_0}{K_m}.
\]  
(7)

Results from a series of fourteen experiments are shown in Fig. 11. Clearly, the deviation of data points from the expected line of unit slope is small. We conclude that eqn. (6) adequately describes the relationship between \(P_{Na}\) and \((\text{Na})_0\) for the investigated range of Na concentrations.

By substituting eqn. (6) into eqn. (3) we find that the steady-state Na current can be predicted for constant \((\text{Na})_c\) by:

\[
I_{Na} = \frac{P_{\text{max}} K_m}{K_m + (\text{Na})_0} \cdot \frac{F \alpha V/RT}{1 - \exp \left(-\frac{F \alpha V/RT}{RT}\right)} [(\text{Na})_0 - \text{c}. \exp \left(-\frac{F \alpha V/RT}{RT}\right)]
\]  
(8)

for variation of \(V\) between \(E_{Na}\) and 0 mV and for variation of \((\text{Na})_0\) between 0 and 27.5 mm. A possible dependence of \(P_{Na}\) on \((\text{Na})_c\) is not included in this formula.

In this context it is useful to recall that after a step increase of \((\text{Na})_0\) the Na current is transiently larger than its steady-state value (Fig. 2 A). Lindemann & Gebhardt (1973) concluded that the 'recline' of \(I_{Na}\) is not mediated by an increase of \((\text{Na})_c\) because under open-circuit conditions,
**I/V-CURVE OF Na⁺ CHANNELS IN FROG SKIN**

I.e. when Na influx is very small or zero, the potential shows the same recline behaviour. The independence from an increase of \((\text{Na})_c\) is confirmed in the present study.

It is obvious that the current cannot initially exceed its steady-state value in cases where the saturation behaviour is caused by saturation of the transporting channels with Na. We must conclude, therefore, that the relationship given by eqn. (6) is not caused by direct channel saturation.

An alternative explanation would be that Na acts as a negative effector which decreases diffusional rate constants of the transport process. As previously suggested, Na could bind to a modifying site in the vicinity of the Na channel (Dick & Lindemann, 1975; Fuchs et al. 1975; Zeiske & Lindemann, 1975). The binding would have to cause a conformational change which closes the adjacent channel either partly or completely. If the binding of Na to the modifying site were a reversible and fast (frequent) process, the change of channel structure from the open to the closed configuration would have to be a rare event to explain the remarkably slow relaxation (seconds) of \(P_{\text{Na}}\) towards its steady-state value. The change from the closed to the open state would have to be even less frequent, however, to permit equilibrium states of low \(P_{\text{Na}}\). Transport through open channels would occur by simple, non-saturating electrodiffusion.

It is interesting to note that a number of compounds can partially or completely prevent the apparent saturation of the Na current with increasing \((\text{Na})_o\). This was reported for BIG and BTG (Zeiske & Lindemann, 1974) as well as for PCMBS and PCMB (Dick & Lindemann, 1975). In the framework of the model outlined above, this effect can be explained by interference with the binding of Na to the modifying site.

**Dependence of Na influx on \((\text{Na})_o\)**

Direct measurements of Na uptake at the outer surface of frog skin have been made by a number of investigators (e.g. Biber & Curran, 1970; Erlij & Smith, 1973). The unanimous finding was that the Na influx saturated with increasing \((\text{Na})_o\) and could be described by an empirical equation of the type:

\[
J_{\text{Na}}^{12} = J_{\text{Na, max}}^{12} \cdot \frac{(\text{Na})_o}{K_m + (\text{Na})_o} + \alpha \cdot (\text{Na})_o
\]

in which \(J_{\text{Na}}^{12}\) is the unidirectional flux of sodium from the outer solution into the epithelium, \(J_{\text{Na, max}}^{12}\) the maximal influx via a saturating system, \(K_m\) an apparent Michaelis constant and \(\alpha\) an apparent permeability coefficient. The coefficient \(\alpha\) was strongly dependent on the choice of the extracellular marker: the linear term was completely (Erlij & Smith, 1971; 1973) or almost completely (Biber et al. 1972) eliminated when the smaller mannitol molecule was used instead of inulin.
Are the results obtained from such tracer uptake experiments consistent with the conclusions of the present study? From our results it is possible to predict the saturating component of the Na influx through the apical membrane at varying \((Na)_o\) and constant \((Na)_c\). The right part of eqn. (3) can be split up into two terms representing the influx and the outflux component, respectively. With \(J^{\text{out}}_\text{Na} = I_{Na}/F\), and after replacement of \(P_{Na}\) with the right part of eqn. (6), the expected influx is given by:

\[
J^{\text{in}}_\text{Na} = \frac{P_{\text{max}}K_m}{K_m + (Na)_o} \cdot \frac{FV/RT}{1 - \exp \left(-\frac{FV}{RT}\right)}
\]  

(10)

It is useful to re-write this in order to facilitate comparison with eqn. (9):

\[
J^{\text{in}}_\text{Na} = J^* (Na)_o / (K_m + (Na)_o),
\]  

(11)

where

\[
J^* = P_{\text{max}} K_m (FV/RT) / (1 - \exp\left(-\frac{FV}{RT}\right)).
\]  

(12)

It can be seen that eqn. (11) represents a saturation curve of the Michaelis–Menten type only if \(V\), the voltage at the outer membrane, is independent of \((Na)_o\), i.e. clamped to a constant value. Cereijido & Curran (1965) as well as Biber & Curran (1970) maintain that this condition is met when the epithelium is short-circuited (in Cl solutions). Indeed, calculations based on a two-membrane model show that the expected change of \(V\) with \((Na)_o\) is considerably diminished when \(P_{Na}\) decreases with \((Na)_o\) according to eqn. (6). Only when \(V\) is constant does the parameter \(J^*\) of eqn. (11) become independent of \((Na)_o\). It then indicates the maximum Na influx for this voltage. Even then, however, \(J^*\) is not an independent constant but will change proportionally if \(K_m\) is altered (eqn. (12)).

Constancy of \(V\) will hardly be expected when the epithelium is in the open-circuit state. This condition was used by Erlij & Smith (1973). To facilitate comparison with their results we have made calculations which, based on representative samples of our data, show which apparent values of \(K_m\) and \(J^{\text{in}}_{Na, \text{max}}\) would have been obtained if \(V\) had been allowed to vary as under open-circuit conditions. It turned out that the saturation curve, because of the experimental error of data points, can still have the appearance of a pure ‘Michaelis–Menten type’, although \(V\) becomes more negative with increasing \([Na]_o\) and the factor \(J^*\) of eqn. (11) varies from 2 to 5 n-mole. sec\(^{-1}\).cm\(^{-2}\). However, both \(K_m\) and \(J^{\text{in}}_{Na, \text{max}}\) are underestimated by a factor of about 0.5 when \(V\) decreases with increasing \((Na)_o\), compared to the case where \(V\) is clamped to zero.

In conclusion, the \((Na)_o\) dependence of \(J^{\text{in}}_{Na}\), as revealed by direct measurements of Na uptake through the outer border of the epithelium at constant \(V\), seems satisfactorily consistent with the behaviour predicted by eqn. (8) derived in the present study. Apparently, the kinetics of Na influx from the outer solution into the granular cells can be described by
a model consisting of a single type of Na channel, the permeability of which is \((\text{Na})_0\) dependent. In the steady state, this dependence can be studied by tracer uptake experiments or by recording the current–voltage curve. The latter technique is just as Na specific as a tracerflux analysis if the amiloride-sensitive current is used. It has the advantage of good time resolution, however, which permits the investigation of non-steady-state phenomena.

**Mechanism of Na-transport through the outer membrane**

In the physiological voltage range (between \(V_r\) and zero mV) the Na current through the Na-selective membrane of frog skin varies with voltage as predicted by the constant field equation. As mentioned above, this equation was derived for the case of steady-state electrodiffusion through homogeneous channels of uniform voltage drop. It may be stated, therefore, that we did not find indications for the existence of more complicated Na-transport mechanisms than simple electrodiffusion in this voltage range. The diffusion could occur through pores or be mediated by carriers.

Arguments for the involvement of charge translocating carriers in Na uptake have been advanced. They are based on the observation that the Na flux through the outer membrane saturates with increasing \((\text{Na})_0\) and is affected by the membrane potential (Biber & Sanders, 1973). In our results this saturation is expressed as the linear increase of the inverse steady-state permeability with \((\text{Na})_0\) (Fig. 11). We do not think, however, that the saturation argument for carrier transport is as such valid, since transport through narrow pores can also saturate (Heckmann et al. 1972). Furthermore, the transient Na currents observed after a step increase of \((\text{Na})_0\) considerably exceed the steady-state ‘saturating’ currents. This observation excludes direct saturation of the Na-translocating pore or carrier with Na, and has led us to suggest a ‘negative effector’ mechanism instead. It may be added that a recent analysis of Na-current fluctuations (Van Driessche & Lindemann, 1976; Lindemann & Van Driessche, 1977) favours Na diffusion through pores.

It is a pleasure to thank Dr J. Brombach for writing assembler programs which made on-line voltage clamping of frog skin a convenient routine technique in our laboratory. We are indebted to Dr B. Cirne for help with some of the experiments, to Mr W. Zeiske for discussions about the mechanism of action of benz-imidazolylguanidinium, to Mr J. Astrup for help with the K-activity analysis, to Mr G. Ganster for construction and servicing of electronic apparatus and to Mr H. Jung for construction of mechanical devices used in the study. This work was supported as project C 1 within Sonderforschungsbereich 38, ‘Membranforschung’, by the Deutsche Forschungsgemeinschaft and by the Danish Natural Science Research Council.
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