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Receptors controlling sodium ion translocation in biological membranes

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In recent years much attention has focussed on drug-receptors in cell membranes. In the majority of these studies the receptors under investigation have been for neurotransmitters or hormones. In this article attention will be given to receptors for chemical agents which block sodium ion translocation across biological membranes.

Two distinct types of sodium channel are found in living membranes. These are the voltage dependent sodium channels of electrically excitable tissues and 'channels' in epithelial membranes through which sodium ions pass without prior triggering. In the former the fully activated I/V relations of the sodium channels are either linear, that is ohmic as in squid axon, or are non-linear and fit the Goldman equation, as for example in Xenopus nodes. Hence ionic movements in these sodium channels have much in common with free diffusion down electrochemical gradients. The voltage dependence of the sodium ion conductance or permeability is explained in terms of a 'gating' process in which the electric field across the membrane governs the orientations of charged membrane molecules controlling ion flow. Recently techniques have been developed to record 'gating currents' in squid axon membranes, that is capacitative currents representing the movement of charged gating particles (Armstrong & Bezanilla, 1974; Keynes & Rojas, 1974) and there is much evidence, even though it is incomplete, to suggest that these displacement currents are formally equivalent to the Hodgkin-Huxley m gating process controlling the activation of the inward sodium current.

In epithelia the sodium entry process is less well understood. Measurement of sodium influx through the mucosal membranes of amphibian skins and bladders, membranes considered reasonably equivalent to parts of the mammalian nephron, have shown that there is a non-linear relation between sodium influx and sodium concentration of the mucosal bathing solution, even when the potential across the membrane is held constant by a voltage clamp, probably indicating that entry is not by simple free diffusion down an electrochemical gradient.

Using frog skin, Biber & Curran (1970) found that sodium uptake could be resolved into a linear and a hyperbolic component, only the latter being associated with uptake into the transporting compartment. The saturating component was half saturated at a concentration of 14·3 mM sodium. Using a different extracellular marker (mannitol instead of inulin) to allow for contaminating extracellular sodium Erlij & Smith (1973) found only a hyperbolic component of uptake. However, their skins were open circuited and it is not possible to calculate exactly how the potential change associated with changing the sodium concentration may have affected uptake.

What influx measurements in epithelia indicate is that sodium ions probably interact with the membrane during transit. The saturating influx suggests a carrier facilitated diffusion mechanism, or even a saturating active step. Careful studies by groups working with frog skin (Smith, Martin & Huf, 1973) and toad bladder (Finn & Rockoff, 1971) suggest that sodium moves down an electrochemical gradient into the transporting compartment. However, other work (Hvid Hansen & Zerahn, 1964) with lithium, an ion which can also be handled by epithelial transport, shows it accumulates in the tissue in a way which suggests it moves up an electrochemical gradient. Difficulties of interpretation of the nature of the entry step in epithelia relate directly to problems of locating and identifying the sodium transport pool, that is the sodium which has entered the epithelium but has yet to be transported into the serosal compartment. A downhill active entry step remains a further, if not very sensible, possibility.

The foregoing paragraphs have emphasized the difference between sodium entry mechanisms in excitable membranes and in sodium transporting epithelia. There are, however, some similarities if one looks for them. According to Hille (1968a, b) entry to the sodium channels in excitable membranes is controlled by an ion selectivity filter, which can be blocked by tetrodotoxin or saxitoxin. It appears that the interaction of these drugs is not with the gating particles, as gating currents are usually

measured in the presence of tetrodotoxin to prevent any sodium current due to trace amounts of sodium in the bathing solutions. Interestingly enough the gating currents are blocked by the local anaesthetic, procaine (1%) (Keynes & Rojas, 1974).

The nature of the ion selectivity filter in excitable membranes has been investigated principally by Hille (1968a, b; 1975a, b) who concluded that an ionized acidic grouping with a pKa of around 5·0 was an essential part of the sodium channel, as g_{Na} fell to low values in acidic solutions. Later, Woodhull (1973), in electrophysiological experiments with frog nodes of Ranvier, showed that the pH effect was voltage dependent in that the inhibitory effect of protons on P_{Na} diminished at more positive voltages. The interpretation was made that the ionizable group, which had a pKa of 5·4 at zero membrane potential, lies one quarter of the way across the membrane from the outside. In addition protons modified the gating properties of the channels by an effect on surface charge.

Apparently Na ions pass through the channels without crossing high energy barriers. By the use of organic cation probes acting as sodium substitutes, Hille (1971) concluded that the selectivity filter was an oxygen lined channel with a bore of 3–5 Å, and in which the ionized grouping, possibly a carboxylic acid, served to lower the free energy required to dehydrate cations during passage through the selectivity filter.

Both toxins (tetrodotoxin and saxitoxin) have high affinities (around 10^9 M^{-1}) for the channel and interact with a probable stoichiometry of 1:1. Blocking activity of tetrodotoxin (pKa 8·8) is reduced at alkaline pH indicating that the cationic form is the active moiety (Camougis, Takman & Tasse, 1967). Radiolabelled toxins can be used to estimate the density of sodium channels in excitable membranes. When the specific binding of saxitoxin to desheathed rabbit vagus nerves was measured as a function of pH it appeared that protons competed with the toxin for a binding site with an apparent pKa of 5·9 (Henderson, Ritchie & Strichartz, 1973).

In electrophysiological experiments on frog nodes of Ranvier, Ulbricht & Wagner (1975) concluded that tetrodotoxin cations compete with protons for the same binding site in the channels but, unlike protons, the blocking effect of tetrodotoxin was not potential dependent.

The effects of tetrodotoxin and saxitoxin cited above have been taken as evidence for the Kao-Nishiyama hypothesis (1965) that the charged guanidinium grouping in these toxins plugs the ion selectivity filter, the rest of the molecule preventing the toxin from proceeding further and also contributing to the total binding interaction between toxin and the channel.

Fig. 1 shows drawings of CPK-space filling models of tetrodotoxin and saxitoxin and it is self-evident that the guanidinium grouping in these substances is prominently exposed and might well be able to interact with the channel in the way described. Tetrodotoxin and saxitoxin have no inhibitory effect on sodium transport in amphibian epithelia, however two other substances do so. These are amiloride and triamterene and it is clear from Fig. 1 that these too have either an exposed guanidinium (amiloride) or a similar isosteric grouping (triamterene) making it tempting to extend the Kao-Nishiyama hypothesis even further to the blocking effect of these substances in epithelia. The effects of amiloride are exclusive in that no effects upon voltage dependent sodium channel conductance have been observed. Presumably both the toxins and diuretic compounds owe their exclusivity to the non-guanidinium part of the molecule.

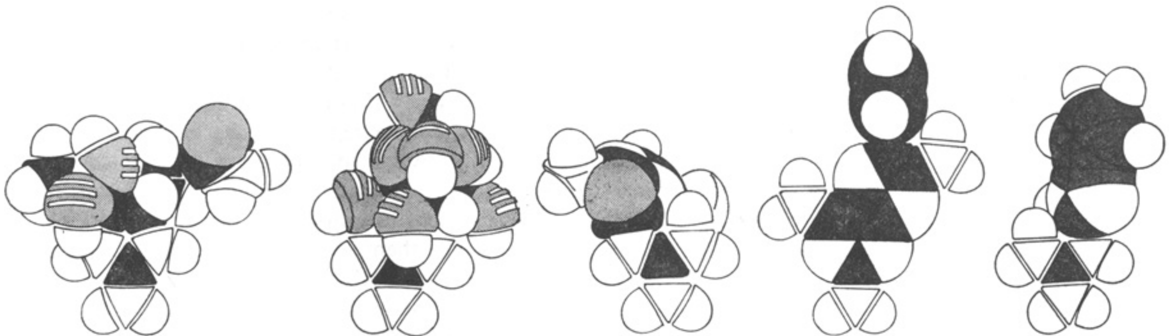

FIG. 1. CPK-space filling models of, from left to right, saxitoxin, tetrodotoxin, amiloride, triamterene and benzimidazoleguanidine.

Recently experiments have been performed in my laboratory to examine the pH dependence of the inhibitory action of amiloride. When the pH of the solution bathing the mucosal surface of frog skin (*Rana temporaria*) was changed between pH 7–11 there was little change in the short circuit current. At all pH values between these limits the SCC was reduced to zero by amiloride, 10^{-4}M. In these experiments the pH of the serosal solution was buffered with tris at pH 7·6, while the pH of the unbuffered mucosal solution was controlled with a pH-stat. At pH values between 7 and 3 the short circuit current declined to a minimum at pH 4 and then increased again at pH 3. At pH 3 none of the short circuit current could be inhibited by amiloride, and the current was probably due to the mucosal to serosal movement of protons. At pH 4 most of the current was amiloride sensitive, and at pH 5–7 the current could be totally inhibited by amiloride.

A plot of the amiloride sensitive short circuit current against pH had the form of a simple titration curve for a monobasic acid with a pKa around 5·0, very similar to the behaviour with pH of the sodium conductance in excitable membranes. Further experiments to determine the apparent affinity of amiloride as an inhibitor of the amiloride sensitive short circuit current showed that affinity was maximal at around pH 8. At more alkaline pH values the fall off in affinity corresponded to a reduction in the amount of ionized (i.e. guanidinium) form of amiloride (pKa = 8·7). At pH values less than 8 the reduction in value of the apparent affinity of amiloride corresponds very closely to the predicted values assuming that protons and the charged form of amiloride compete for binding with a negatively charged grouping with a pKa of around 5.

Thus, at least in a superficial way, the similarity between the sodium entry mechanism in excitable membranes and epithelia is striking. Hille's proposed ionic selectivity filter controls entry to a channel through which sodium ions pass by free diffusion, while in epithelia this is not so, although the entry mechanisms have similar characteristics in both instances. Whether the amiloride binding site occupies a sentinel position in respect of a channel through which ion flow saturates or guards access to a saturable carrier, or is part of a carrier system is not in the least clear.

In 1973, I developed a method for determining the density of specific amiloride binding sites, and hence the density of the sodium entry sites, in the mucosal surface of frog skin. Although all binding studies are bedevilled by non-specific binding, I was helped considerably by the realization that sodium and amiloride compete with each other for the entry site. By performing experiments with solutions in which the sodium concentration was reduced to low values (1–2·5 mM), the apparent affinity of amiloride was increased by at least one order of magnitude with consequent reduction in the proportion of the non-specific binding. By measuring both specific (displaceable) binding simultaneously with the short circuit current in the same tissue, it was possible to know both the amount bound and the reduction in sodium ion transport. The amount bound at 100% occupancy was then derived by simple calculation. As the slope of inhibition curves for amiloride had indicated a stoichiometry of 1 : 1 with the receptor the number of sodium entry sites was deduced (Cuthbert, 1973).

Around 400 sites μm^{-2} were found in frog skin when the mucosal bathing solution contained 2·5 mM NaCl. This density is of the same order of magnitude as the density of sodium channels found in various excitable membranes (for references see Keynes, 1975).

Together with Dr Wing Shum, I examined the effect of ADH on amiloride binding in frog skin (Cuthbert & Shum, 1974). ADH increases sodium transport through this tissue. The sequence of changes is not completely understood although it is known that ADH interacts with receptors in the serosal surface of the epithelium. As a consequence of this the following effects have been described: (i) activation of adenyl cyclase and generation of cAMP, (ii) dephosphorylation of specific membrane proteins and (iii) calcium release from the mucosal surface. Following ADH, sodium influx through the mucosal surface is increased, possibly as a result of the actions given above or other unknown effects.

ADH had no effect on the density of sodium channels in frog skin. However, the nominal current flowing through each channel was approximately doubled by hormone treatment, from 1·29 to $2{\cdot}55 \times 10^{-16}$ A (Cuthbert & Shum, 1974). The value of the current was obtained simply by dividing the total short circuit current by the total number of channels. Further, after ADH the apparent affinity of amiloride was reduced by a factor of 2–3. Consideration of these facts has led to the suggestion that ADH, or more properly its second messenger(s), acts as an allosteric activator of the mucosal entry sites (Cuthbert, 1974). This interpretation means that ADH increases the proportion of channels which are operative at any instant, explaining the

apparent increase in current in each channel and the change in affinity. The macroscopic affinity of amiloride will be an average affinity of the ligand for the operative and non-operative channels. As an inhibitor amiloride must have a higher microscopic affinity (and perhaps exclusive affinity) for the non-operative channels and the proportion of these will be reduced by ADH, thus reducing the apparent overall affinity. We have shown in separate experiments in isolated epithelial cells that ADH shifts the binding curve for amiloride to the right as would be expected if the proposed allosteric mechanism is correct (Cuthbert & Shum, 1975).

Another hormone which increases sodium transport in amphibian epithelia is aldosterone. Again one of the final effects of this hormone is an increase in the sodium permeability of the mucosal face of the cells. The stimulating effect of aldosterone is blocked by inhibitors of transcription and translation, and it seems certain that the steroid stimulates DNA dependent RNA synthesis (Rossier, Wilce & Edelman, 1974).

Most of the experiments with aldosterone have been performed with cells isolated from the mucosal epithelium of toad bladders (*Bufo marinus*). It was shown that a specific saturable binding component for amiloride could be detected with properties expected for sodium entry sites. The evidence was that the concentration of amiloride which half-saturated the channels corresponded to the concentration which caused a 50% inhibition of sodium transport (approximately 10^{-8}M at a sodium concentration of 1·1 mM). Furthermore, the inhibition of amiloride binding by triamterene was quantitatively predictable from the relative affinities of the two ligands determined from inhibition studies (Cuthbert & Shum, 1975). These were the criteria we had used previously to detect the specific binding component for amiloride in intact frog skin (Cuthbert & Shum, 1974).

In a series of experiments it was shown that aldosterone increases the density of amiloride binding sites, and presumably of sodium entry sites, in cells isolated from toad bladder epithelium. We conclude that the aldosterone-induced protein represents the *de novo* synthesis of new channels, or alternatively is an agent which uncovers latent channels (Cuthbert & Shum, 1975). The extent of the increase in channel density depends on the concentration of aldosterone and the conditions of the experiments.

Thus the two hormones can complement one another in a very practical way. ADH acts rapidly and transiently to increase the proportion of functioning sodium entry sites, making possible quick and frequent adjustments of the extent of sodium reabsorption. Aldosterone acts more slowly and increases the number of sites available for sodium reabsorption, and provides a way of sustaining an increase in sodium reabsorption during sodium deprivation, without impairing the ability of the system to adjust to transient needs with ADH.

We have estimated that the half-life of sodium channels in toad bladder epithelial cells is approximately 60 h, and we were unable to detect any difference in the rate of disappearance of the channel population in control and aldosterone treated tissues (Cuthbert & Shum, 1976a).

It might be supposed from what has been written that the question of sodium entry into epithelia and its modification by hormones is becoming reasonably clear. However there are some confusing features which suggest that there are other more subtle control mechanisms at work. For example we found in frog skin that the density of sodium channels was dependent upon sodium concentration. At zero sodium in the mucosal solution we found 86 ± 4 binding sites μm^{-2} in 45 determinations, while at 1·1 mM Na the corresponding value was 201 ± 14 binding sites μm^{-2} in 15 determinations. In a few measurements at 2·5 mM sodium the binding site density appeared to be even larger (378 ± 98 binding sites μm^{-2} in 4 determinations) (Cuthbert & Shum, 1974). Of course there is a considerable scatter between individual skins, but both the values for 1·1 and 2·5 mM Na are significantly different from the value at zero sodium. Using a suspension of toad bladder epithelial cells and an amiloride concentration of $2{\cdot}2 \times 10^{-8}$M no specific (i.e. displaceable binding) was detected at zero sodium, while at 1·1 mM there were 822 ± 15 sites μm^{-2} occupied by the ligand. This effect of sodium was not mimicked by potassium.

One is immediately reminded of other situations in which the levels of receptor macromolecules in membranes are determined by existing concentrations of specific ligands. For example, the density of insulin receptors in fat cells is controlled by insulin concentrations (Soll, Kahn & others, 1975), the density of β-receptors in pineal tissue is controlled by β-receptor agonists (Kebabian, Zatz & others, 1975); in both instances the control is a negative feedback. The response of pineal tissue to β-agonists is particularly interesting as it does not involve the *de novo* synthesis of new protein, rather the uncovering of latent receptors. Perhaps in transporting tissues there are dormant sodium translocating sites

which can be exposed by Na or alternatively by aldosterone-induced protein.

We have found, for example, at low sodium concentrations that when frog skin is voltage clamped at −50 mV (serosa -ve) there is an 80% increase in the number of amiloride binding sites compared to the density at 0 mV. Clamping at +50 mV reduces the density seen at 0 mV by 30% (Cuthbert & Shum, 1976b). We were very careful when we did these experiments to make sure that the potential dependent effects were not due to change in concentration of amiloride at the membrane surface. However, no change in apparent affinity was seen which would be anticipated if the local concentration did not reflect the bulk concentration.

It has not been possible to examine amiloride binding at high sodium concentrations as the effective affinity for it is reduced because of competition with sodium ions. There is evidence however to suggest that mucosal permeability is reduced at high sodium concentrations. Lindemann devised a method by which the mucosal sodium concentration could be changed very rapidly (25 m s). On changing from a low to a high sodium concentration the level of sodium transport rose abruptly to a peak value and then declined, with a time constant of a few seconds, to a plateau, as if some fraction of the sodium entry sites has shut off. More interestingly, the decline could be prevented by benzimidazoleguanidinium (BIG, Fig. 1) (Zeiske & Lindemann, 1974). Furthermore, BIG causes an increase in sodium transport in high sodium solutions when applied in the mucosal bathing solution.

If these interpretations are correct then at low sodium concentrations there may be subtle autoregulatory events designed to increase sodium uptake, while the converse is true at high sodium concentrations. It is difficult to reconcile the stimulatory actions of BIG with the Kao-Nishiyama hypothesis, that is if the guanidinium group is the active moiety of all the compounds shown in Fig. 1, it is difficult to see how occupancy of an ion selective pore by this group can both inhibit and increase sodium influx. Alternatively, if the two state model suggested earlier is correct one might expect that BIG has a higher affinity for the operative forms of the channel while amiloride and triamterene have selective affinities for the non-operative forms.

It is important not to ignore morphological considerations in considering sodium entry into epithelia. There are two major cell types in toad bladder epithelium; mitochondria rich (M-R) cells, constituting around 15% of the total number, and a majority of granular cells. The mitochondria rich cells are flask shaped with only a small proportion of their surface exposed to the mucosal face. Each M-R cell is surrounded by a rosette of granular cells (Ferguson & Heap, 1970). Scott, Sapirstein & Yonder (1974) have managed to separate the two cell types and find that ADH sensitive adenyl cyclase and nuclear aldosterone-receptors are present only in the M-R cells. On the other hand, Goodman, Bloom & others (1975) have found, using an immunofluorescent method, that all cells stain equally well for cAMP. These results suggest many possibilities for the transepithelial route taken by sodium. For example, the mucosal surface area of the M-R cells is so small that sodium influx exclusively through these sites is perhaps unlikely. Perhaps cAMP generated in the M-R cells passes to the granular cells through tight junctions to modify the properties of mucosal surfaces of the granular cells. If sodium enters through the granular cell membranes is it pumped out of the granular cells, or alternatively does it enter the M-R cells before it is extruded into the serosal compartment? It is vitally important to examine amiloride binding in the separated cell types as this may indicate the site of sodium entry.

Finally, to return to the comparison between sodium channels in excitable tissues and epithelia, it is instructive to make some simple calculations with the data that are available. Hille (1970) has compared the properties of sodium channels in amphibian nodes of Ranvier with the physical characteristics of a pore with a radius of 3 Å. In Ringer solution (110 mM sodium) the convergence resistance of a 3 Å pore is 1 nmho, and from diffusion theory such a pore would have maximally $1{\cdot}3 \times 10^8$ new encounters with sodium ions each second. Unit conductances of 0·1–0·2 nmho have been seen at nodes with sub-threshold depolarizations, although in squid axon channel conductance is estimated as 2·5 pmho.

In frog skin, with 1·1 mM Na bathing the mucosal surface, the convergence resistance of a 3 Å pore would be 0·02 nmho with $1{\cdot}3 \times 10^6$ ions entering the pore each second. The maximal recorded current through channels in frog skin is 2×10^{-16} A. Even if the channels open only intermittently (see Cuthbert, 1974), the maximal current is probably not greater than 5×10^{-16} A, which corresponds to 3000 ions s^{-1}. The potential across the mucosal face of epithelial cells with low Na bathing the mucosal surface is about 10 mV indicating a conductance of only 0·05 pmho. Thus, although there may be

similarities between the initial entry stages for sodium with both types of channel, the subsequent translocation steps may be very different.

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