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CONTROL OF Na^+ AND WATER ABSORPTION ACROSS VERTEBRATE 'TIGHT' EPITHELIA BY ADH AND ALDOSTERONE

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SUMMARY

Salt and water balance in vertebrates is controlled by the release of two blood borne hormones: aldosterone and antidiuretic hormone (ADH). It is the purpose of this chapter to review the mechanisms (at the plasma membrane level) by which these hormones cause an increase in salt (sodium) and water movement in the target tissues. The primary effect of aldosterone is to increase the Na^+ permeability of the lumen-facing (apical) membrane by activation of pre-existing quiescent channels at short times, and by the incorporation of newly synthesized channels after prolonged exposure. Other effects might involve an increase in energy supply and synthesis of Na^+-K^+ ATPase which is responsible for Na^+ extrusion from cell cytoplasm to blood. Similarly, ADH stimulates pre-existing quiescent apical membrane Na^+ channels. The second effect of ADH is to increase epithelial water permeability. Evidence strongly suggests that water channels exist in cytoplasmic vesicles which, upon ADH challenge, fuse into the apical membrane causing a rapid increase in apical membrane hydraulic conductivity. The movements of vesicles are dependent on an intact cytoskeleton. Regulation of electrolyte and non-electrolyte transport will be discussed in the light of the above two mechanisms.

INTRODUCTION

One of the greatest challenges that vertebrates (as well as invertebrates) face is the necessity to maintain and regulate plasma volume and ionic composition. This demand for plasma homeostasis is met by a group of organ systems that share a common property of being able selectively to absorb from, or secrete into, the external environment the necessary quantity of electrolytes and non-electrolytes. Close inspection of these organ systems shows that the fundamental building block is a layer of closely packed cells that are tightly joined together by continuous loops of protein strands called the tight junctions. On the microscopic level there are two parallel pathways that electrolytes and non-electrolytes can follow to transverse this planar array of cells called an epithelium. First, they can move between the cells only after permeation across the tight junctions and, secondly, they can enter the cell across one membrane and exit across the other. An epithelium, then, acts as a barrier to movement of substances

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between two compartments. In all instances one of these compartments is the plasma while in many other instances (but not all) the opposing compartment is linked to the outside world.

Since epithelia are known selectively to absorb (movement into the plasma) or secrete (movement away from the plasma) electrolytes and non-electrolytes, this implies that the transport properties of the two membranes must be quite different, and in addition that the transport properties of the individual cell membranes differ among organ systems and within an organ system.

Not all epithelia are created equal in their ability to restrict or enhance the movement of electrolytes and non-electrolytes. Some epithelia possess the ability to absorb copious quantities of ions and water in an isosmotic manner and as a consequence cannot support large osmotic or ionic gradients. Other epithelia are very efficient salt absorbers, but have very low water permeabilities, indicating that they can support large osmotic and ion gradients. Indeed Frömter & Diamond (1972) separated epithelia into two general categories: leaky and tight. Epithelia which fit into the leaky class absorb an isosmotic solution, cannot support large osmotic or ionic gradients, have low electrical resistances, do not generate a spontaneous transepithelial potential and have a high permeability to water. In contrast, tight epithelia have a hyperosmotic absorbate, support large osmotic and ionic gradients, have a high transepithelial electrical resistance, can generate large spontaneous transepithelial potentials and have a low permeability to water. The difference in the electrical resistance between tight and leaky epithelia is a consequence of the resistance of the 'tight' junctions. Thus, leaky epithelia tend to have low resistance junctions ($= 100\,\Omega cm^2$) while tight epithelia have high junctional resistance (usually $>500\,\Omega cm^2$). This variability of junctional resistance helps to account for the ability of tight epithelia to support large ionic gradients and develop transepithelial spontaneous electrical potential differences. However, whether it can also account for the difference in water permeability is presently disputed, although the general trend indicates that there is minimal water flow through the junctions.

Although certainly not a strict rule, the rate of ion transport by tight epithelia seems to be under more rigid control by plasma hormones and neurotransmitters than the rate of ion transport by leaky epithelia.

It is in part this hormonal control of salt and water transport that allows an animal to rapidly re-establish plasma salt and water balance after perturbation. Two of the most well known hormones for regulating salt and water balance in many vertebrates are aldosterone and antidiuretic hormone (vasopressin). In the remainder of this paper I will attempt to review the mechanism at the membrane level by which these two hormones stimulate salt and water transport in tight epithelia.

BACKGROUND

Even though the main emphasis of this chapter is to discuss the alteration of membrane transport properties induced by either aldosterone or antidiuretic hormone (ADH) a brief overview of the steps (from the signals required for hormone release to the biochemical manifestations at the target cells) will indicate the multiplicity of events involved for the simple end product of salt and water transport.

Aldosterone

The single end result of aldosterone release is an increase in Na^+ absorption in a number of target tissues, such as sweat and salivary ducts, large intestine, kidney and urinary bladder. The ability of aldosterone to stimulate K^+ transport (i.e. secretion), in addition to Na^+ absorption, is restricted to the kidney.

Aldosterone is released from the zona glomerulosa of the adrenal glands. There are a multiplicity of signals that result in an increased plasma aldosterone level, among these signals are: angiotinoin II, potassium and sodium ions and adrenocorticotropic hormone. These stimuli do *not* act by a mechanism of increasing the release of prepackaged aldosterone, but rather by stimulating the rate of aldosterone synthesis. Once released into the bloodstream approximately one-half binds to plasma albumin and transcortin while the remaining half is free.

On reaching the target cells, there is a lag period before there is any visible sign of increased Na^+ transport. This lag phase (which is usually 60–90 min in duration and independent of concentration) suggested that an intermediate step might be represented by the synthesis of proteins.

Edelman and co-workers (see Feldman, Funder & Edelman, 1972) demonstrated the following sequence of events after diffusion of aldosterone into the cell cytoplasm. First, aldosterone rapidly binds to a cytoplasmic receptor. This steroid-receptor complex is then translocated to the nucleus. The interaction of steroid-receptor complex with nuclear chromatin causes enhanced transcription of mRNA. Finally, the mRNA is used to synthesize new cytoplasmic proteins (aldosterone-induced proteins, AIP). The exact role of each of these induced proteins has not been elucidated, with the exception of citrate synthase which is a regulatory enzyme of the TCA cycle in mitochondria.

Although it is tempting to speculate that only proteins are important for ion transport, recent evidence also suggests that the lipid composition of the cellular membranes might play a role in the aldosterone response (Goodman, 1981). The following evidence supports a role of lipid metabolism. First, aldosterone stimulates the endogenous activity of phospholipase A. Secondly, there is an increase in both fatty acid synthesis and desaturation. Finally, there is an increase in membrane phospholipid polyunsaturated fatty acid content. Inhibition of acetylcoA carboxylase (one of the enzymes responsible for fatty acid biosynthesis) reversibly blocks the aldosterone-stimulated Na^+ transport.

Antidiuretic hormone

The effects of ADH on the target tissue are complex and the most important are listed below.

(i) A rapid increase in Na^+ transport.
(ii) An increase in water permeability of distal convoluted tubule and collecting duct of the kidney.
(iii) An increased permeability to urea (at least in amphibian urinary bladder).

Biosynthesis of ADH takes place in the perinuclear region of cells in the hypothalamus. After biosynthesis, ADH migrates in secretory granules to the posterior

pituitary gland. Release of pre-packed ADH (vasopressin) from the posterior pituitary occurs by exocytosis into the plasma.

The two primary stimuli for ADH release are plasma volume and plasma osmolarity. Thus, increased plasma osmolarity (by as little as 2 %) is sensed by osmoreceptors (perhaps located in the region of the anterior hypothalamus) and causes an increased release of ADH and a concomitant decrease in urine flow (i.e. an increase in water absorption). The second signal is a decrease in plasma volume, which, if reduced by as little as 6 %, also results in an increase in the circulating levels of ADH. The exact location of the volume receptors is unknown but they are certainly not strictly localized to the region of the CNS as are the osmoreceptors. Decreased plasma volume and a hyperosmotic plasma both stimulate ADH release. However, in severe Na^+ depletion there is conflicting information. If osmolarity is to be maintained volume must decrease. Conversely if volume is to be maintained the plasma will become hypo-osmotic. It is the latter which occurs.

The primary target organ of ADH is the kidney and, specifically, the distal tubule and cortical collecting duct. The loop of Henle causes the interstitial fluid to be hyperosmotic (by as much as 800 mosmol l^{-1}) due to solute concentration by a countercurrent multiplier mechanism and then by making the distal tubule and cortical collecting duct water permeable (with ADH), causing a flow of water from tubular lumen to the interstitium; the flow of water continuing until lumen and interstitium become isosmotic.

One can summarize the current concept of the action of ADH once it reaches the target cell by the following scheme. First, ADH binds to a membrane-bound receptor. This hormone-receptor complex then stimulates adenylate cyclase, resulting in an increase in cell cAMP levels, and a possible phosphorylation or dephosphorylation of a protein kinase.

The relationship between the protein kinase and/or cAMP and subsequent hydro-osmotic and Na^+ transport response is at present not clear.

MECHANISM OF ALDOSTERONE ACTION

Since aldosterone stimulates protein synthesis and its antinatriuretic action is dependent on this process, one can assess the role of the synthesized proteins (AIPs) in relation to a model for Na^+ transport.

The model currently favoured (and the first one developed for ion transport by any epithelium) was described by Koefoed-Johnsen & Ussing (1958). The model visualized Na^+ entering the cell down its net electrochemical gradient. Once inside the cell it is extruded across the basolateral membrane with a 3:2 exchange with K^+. The potassium then diffuses back into the plasma down its net electrochemical gradient through K^+-selective pathways. There are at least three obvious mechanisms for aldosterone action which can be postulated on the basis of this simple model (Fig. 1). All of these involve an increase in the rate of entry of Na^+ across the apical membrane.

(1) An increase in basolateral pump density or kinetics. Thus, an increase in entry could be facilitated by increasing the net electrochemical driving force across the apical membrane by decreasing cell Na^+ *via* the basolateral pump.

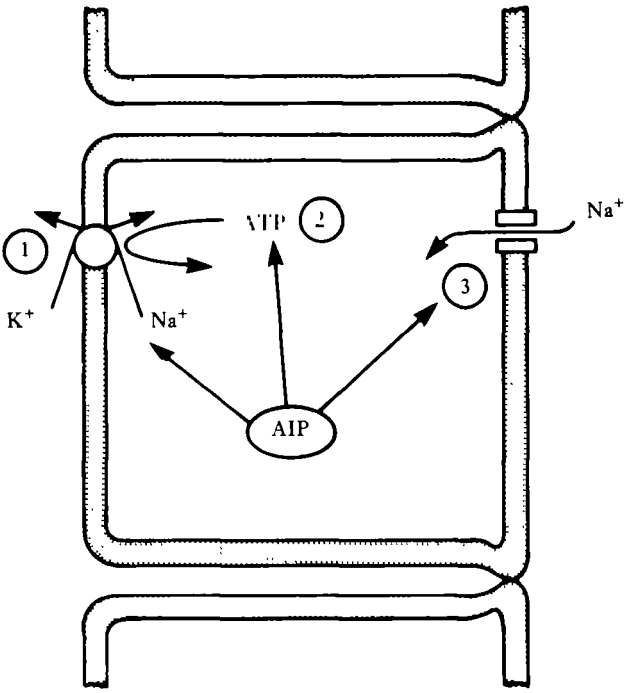

Fig. 1. Line drawing depicting the three most obvious points of action of aldosterone-induced proteins (AIP). All three actions must increase the rate of Na^+ entry across the apical membrane. Mechanisms (1) and (2) act by increasing the net driving force for Na^+ entry by decreasing intracellular Na^+ activity ($a_i Na^+$). More specifically mechanism (1) might decrease $a_i Na^+$ by increasing the Na^+-K^+ pump density, turnover rate, number of ions translocated per cycle and/or the apparent binding affinity of the sites (i.e. decrease the K_m). Mechanism (2) would increase the energy available to the Na^+-K^+ pump by increasing cytoplasmic ATP concentrations. This increase in 'phosphate' potential might result in a decrease in $a_i Na^+$ if the pump is working near equilibrium (see Chapman & Johnson, 1978). The last mechanism (3) is a direct increase in the permeability of the apical membrane by activating pre-existing channels, inserting new channels or modifying pre-existing conducting channels.

(2) Increase in the energy available to the pump. This would result in a stimulation of transport similar to that above.
(3) Increase in the Na^+ permeability of the apical membrane. This would result in an accelerated Na^+ entry rate, which would express itself as a net increase in Na^+ transport.

In this section of the paper I will assess these three hypotheses and demonstrate that the third is well established while the first two are still controversial.

Pump, energy or permeability

It is obvious that to achieve an increase in Na^+ transport, the rate of Na^+ entry must also increase. At first glance one might immediately speculate that the easiest mechanism is to increase the number of parallel pathways in the apical membrane either by inserting new pathways or by activating pre-existing membrane pathways. A less likely, but still possible, mechanism would be to increase the net driving force for Na^+ entry through a fixed number of channels by decreasing the cell Na^+ activity and/or

making the intracellular electrical potential more negative. This can be achieved either by increasing the energy supply to or by altering the kinetics or density of Na^+ pumps. The last possibility is that aldosterone could indirectly increase apical Na^+ permeability if high intracellular Na^+ activity blocks the apical Na^+ pathways (Lewis & Diamond, 1976). Thus, stimulation or alteration of pump kinetics could decrease cell Na^+ and thereby increase apical Na^+ permeability.

To distinguish between these possibilities required a wide spectrum of experimental techniques. The first question to ask is whether there is an increase in apical Na^+ permeability following aldosterone challenge to a target epithelium? Using microelectrode techniques, in conjunction with more conventional transepithelial measurements, investigations on a series of epithelia demonstrate a clear increase in apical membrane conductance. However, such conductance increases could result from an increase in intracellular Na^+ activity. To determine whether there is a real permeability increase requires the direct measurement of intracellular Na^+ activity ($a_i Na^+$). Such a measurement has been performed on the rabbit urinary bladder by Wills & Lewis (1980). These authors found that $a_i Na^+$ (7 mM) did not measurably change as a function of net Na^+ transport. From the measured $a_i Na^+$ and apical Na^+ conductance these investigators were able to demonstrate that the apical membrane permeability did indeed increase proportionately with the net rate of Na^+ transport. Since $a_i Na^+$ did not decrease, these measurements provide evidence that aldosterone directly increases apical Na^+ permeability.

The above experiments do not preclude an effect of aldosterone on the basolateral Na^+ pump. To study this possibility (on the rabbit urinary bladder) Lewis & Wills (1981) utilized the antibiotics nystatin or gramicidin D to eliminate the apical membrane as a resistive barrier, which then allows a selective and controlled loading of Na^+ into the cells. Since the pump is electrogenic, one can measure the pump current at increasing values of $a_i Na^+$ in the presence and absence of aldosterone. Similarly at a constant and elevated [Na^+], the dose response curve for serosal K^+ activation can also be generated. Both sets of experiments indicated that aldosterone did *not* alter either pump kinetics nor density. Garg, Knepper & Burg (1981) observed an increase in Na^+-K^+ ATPase activity in the cortical collecting duct after rabbits were placed on low Na^+ diets for 8–11 days. However, Petty, Kokko & Marver (1981) reported that the increase in Na^+-K^+ ATPase activity induced by aldosterone in cortical collecting tubules was inhibited if the rabbits were simultaneously infused with amiloride. These authors concluded that stimulation of Na^+-K^+ ATPase activity resulted from increased cell [Na^+] and was not primarily an effect of aldosterone. The difference between the bladder and collecting duct might then represent the magnitude of increase in Na^+ transport. The role of metabolism has not been determined in the mammalian urinary bladder. In the toad bladder, however, aldosterone did not cause an increase in transport in substrate-depleted preparations until substrate (e.g. pyruvate) was added to the bathing solutions. The results of this experiment were difficult to assess, since it is well established that high intracellular Na^+ activity is known to inhibit the apical Na^+ pathways (Wills & Lewis, 1980). To determine whether this metabolic effect on apical permeability was a result of decreasing cell [Na^+], due to an increased energy supply to the pump, Palmer & Edelman (1981) calculated the intracellular Na^+ activity (using current-voltage relationships) before

[illegible]d during the stimulation of transport by exogenous pyruvate. These authors found a small decrease in $a_i Na^+$ which was not highly correlated with the increase in Na^+ transport rate. In many tight epithelia the trigger for decreasing apical membrane permeability is not directly mediated by $a_i Na^+$, but rather by intracellular Ca^{2+} accumulation. Upon return of a metabolite (e.g. pyruvate) Ca^{2+} is both extruded from the cell (either by a Na^+-Ca^{2+} exchanger or Ca^{2+} pump) and re-accumulated in the mitochondria. The time course for the cell to reach Ca^{2+} homeostasis might be quite different from the decrease in $a_i Na^+$ and more compatible with the measured increase in Na^+ transport. This question can only be answered using Ca^{2+}-selective microelectrodes. Finally the apical permeability might be directly mediated by the phosphorylation or dephosphorylation of membrane proteins. Which of the above hypotheses will turn out to be correct for the metabolic regulation of transport will require further research.

Mode of enhanced apical permeability

As outlined above, one of the primary effects of aldosterone is to increase apical Na^+ permeability, and (at least in the mammalian urinary bladder) not to alter pump density nor kinetics.

The following questions now can be asked:

(i) Is the Na^+ pathway a carrier or a pore?

(ii) Are the number of pathways increased or are the properties of an individual pathway altered to allow increased transport?

(iii) If the number of pathways are increased, do they lie dormant in the membrane to be activated or are they inserted into the membrane as new protein?

The technique required to answer these questions was first successfully implemented by Lindemann & Van Driessche in 1977. These authors determined the opening and closing rates (whether spontaneous or induced by a blocker) of a single channel by measuring the microscopic current fluctuations of many channels and then decomposing these current fluctuations into their individual amplitude-frequency components (using Fourier transformations). The resulting plot (called here a power spectral density) of the square of the current (A^2s) *versus* frequency will have a characteristic shape which is determined by the type (carrier or pore) and kinetics (rate of shuttling or open and closed probabilities) of the transport process. As an example carrier 'noise' will be small and flat at low frequencies and then increase as the frequency increases (Kolb & Läuger, 1978). Spontaneously fluctuating (open→ closed→ open) pores, will have a high and flat power content at low frequencies, and the power will then start decreasing as a function of frequency with a slope of −2 (Fig. 2A, B). This latter spectrum is called a single time constant relaxation or Lorentzian spectrum and was the type measured by Lindemann & Van Driessche (1977) to describe amiloride blockage of the Na^+ pathway in the apical membrane of frog skin. From this data these authors concluded that there were indeed Na^+ pores or channels, as opposed to a carrier mechanism, in the apical membrane. More recently similar conclusions have been arrived at, using the same technique, for the hen coprodaeum (Christensen & Bindslev, 1982), toad urinary bladder (Palmer, Li, Lindemann & Edelman, 1982) and rabbit urinary bladder (Loo, Lewis & Diamond, 1982). None of [illegible]ese investigators was able to measure a spontaneous open–close fluctuation of the

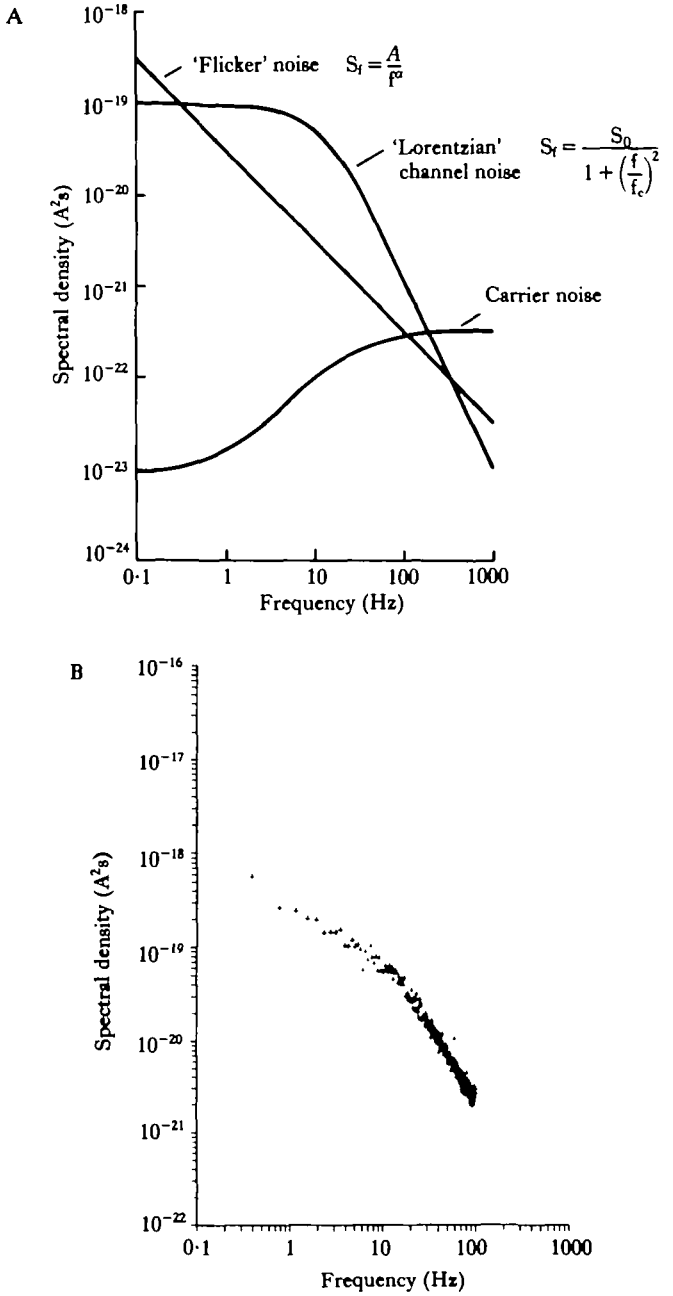

Fig. 2. (A) Graphical representation of the single-sided power spectral density (S_f *versus* frequency) for channel noise, carrier noise (see Kolb & Läuger, 1978) and 'flicker' noise. From a simple model of a spontaneously opening and closing channel (or a pharmacological block) one will measure a Lorentzian or single time constant spectrum. However a Lorentzian spectrum does not mean that the process under study is a spontaneously or induced channel opening and closing phenomenon. (B) An example of an amiloride (1·4 μM) induced Lorentzian spectrum. The single channel current (i) and channel density (M) estimated from this and other measurements are $i = 0{\cdot}53 \times 10^{-12}$ A, $M = 37{\cdot}5 \times 10^6$ (channels for 2 cm^2 of tissue). Of interest is that this spectrum had to be fitted by the sum of a linear component ('flicker' type noise) and a Lorentzian component, where $A = 17{\cdot}4 \times 10^{-20}$ A^{2}s, $\alpha = 1{\cdot}1$, $S_0 = 8{\cdot}5 \times 10^{-20}$ A^{2}s and $f_c = 12{\cdot}4$ Hz.

channel, rather all had to induce fluctuations using either amiloride or triamterene. In two of these preparations (toad and rabbit urinary bladder) the single channel currents (and permeabilities) were independent of aldosterone action and this indicates that the number of channels are increased as a function of aldosterone action, as opposed to an increase in permeability of a single channel.

The last question posed (activation of pre-existing channels or insertion of new channels by aldosterone) is more difficult to answer and might indeed involve both mechanisms, perhaps temporarily dissociated. It is even conceivable that the two mechanisms occur in different tissues.

In the toad urinary bladder, Palmer & Edelman (1981) performed the following experiment to differentiate between channel activation or insertion. First, they treated one-half of the bladder for 1 h with DSA (diazosulphanilic acid – a protein-modifying agent reported to irreversibly inhibit 70 % of the amiloride-sensitive current), they next exposed both halves of the bladder to aldosterone and then measured the Na^+ current 5 h later. The results of this experiment indicated that aldosterone caused a proportional increase in Na^+ current, and not an equal change in Na^+ transport as would be predicted from insertion of new channels from a cytoplasmic store. This sort of data can, perhaps, explain the difference in channel density measured using amiloride binding and fluctuation analysis, where amiloride binding revealed many more channels than predicted and measured using fluctuation analysis. In the simplest model, amiloride can bind to both active and inactive channels, where fluctuation analysis can measure only active conducting pathways. For toad bladder in the first 5 h after aldosterone challenge, new channels do not seem to be inserted but rather pre-existing channels are 'mysteriously' activated. Because of the 'short' duration of aldosterone incubation these experiments do not preclude synthesis and insertion of channels after extended (e.g. 48-h) periods of aldosterone incubation.

The other end of the spectrum is represented by experiments on the hen coprodaeum (Cuthbert, Edwardson, Bindslev & Skadhauge, 1982). This epithelium has no amiloride-sensitive Na^+ transport if the animal is maintained on a normal sodium diet. Animals fed for 9 days or longer on a low Na^+ diet had coprodaeums with large amiloride-sensitive currents. Using radiolabelled benzamil these investigators found an enhanced benzamil binding to the apical membrane of low Na^+ diet tissues and no binding to tissues from animals on a normal Na^+ diet (the authors stress that this may not be significant). Measurements of cytoplasm benzamil binding in these two tissues revealed a very high level of specific binding, indicating a large reservoir of possibly cytoplasmic Na^+ channels. We might interpret this to mean that the cytoplasm has a large store of channels and that aldosterone might act by stimulating the movement of channels into the apical membrane. Whether pre-existing inhibited channels are activated at short times has not been assessed in this tissue.

The third tissue is the rabbit urinary bladder. As previously discussed, this tissue reacts *in vitro* to aldosterone by increasing its rate of Na^+ transport. Alternately, placing rabbits on low Na^+ diets for 1–2 weeks also results in an enhancement of the rate of amiloride-sensitive Na^+ transport (Lewis & Diamond, 1976). The mammalian urinary bladder is a highly distensible organ system. Upon filling the apical cells change from a goblet shape to an oblate spheroid. To accommodate this geometric change, cytoplasmic vesicles are moved toward and fused with the apical membrane.

This directed movement is facilitated by a dense microfilament network (Lewis & de Moura, 1982). Upon bladder collapse these vesicles are retrieved from the apical membrane and stored in the cytoplasm awaiting the next expansion-contraction cycle. These authors were able to fuse the cytoplasmic vesicles into the apical membrane by applying a rapid series of hydrostatic pressure pulses (called punching). This series of pressure pulses not only increased vesicle fusion but also apical membrane removal. It is important to note that the cytoplasmic vesicles are structurally identical to the apical membrane, which is composed of polygonal plaques (about 1 μm in diameter and 12 nm in thickness) that occupy about 73 % of the apical area, the remaining 27 % is normal lipid-type bilayer (Staehelin, Chlapowski & Bonneville, 1972). These apical plaques are interconnected to the vesicles and desmasomes in the lateral and basal membrane by a dense network of microfilaments (Minsky & Chlapowski, 1978). The apical membrane is thus a planar array of fused cytoplasmic vesicles. The transport ability of the vesicles is easily assessed by measuring the amiloride-sensitive Na^+ transport before and after 'punching' the preparation (Fig. 3). Lewis & de Moura (1982) found that the rate of transport was increased by a factor of 10 after a series of punches. Thus, like the coprodaeum, the mammalian urinary bladder possesses an intracellular store of Na^+ channels. Placing rabbits on low Na^+ diets (for 2 weeks) results in an increase in apical Na^+ transport rate and a greater increase after punching (when compared to bladder from control animals). Here one might conclude that aldosterone increases the channel density in a vesicle, perhaps indicating *de novo* synthesis of channels or activation of pre-existing channels. At present we cannot differentiate between these possibilities. The difference in density of channels between the apical membrane and cytoplasmic vesicles might result from some property of the urine which degrades amiloride-sensitive channels into non-selective amiloride-insensitive channels.

In summary, one must conclude that aldosterone increases the density of Na^+ channels in the apical membrane, perhaps by first activating quiescent channels and next by channel insertion, the latter step occurring after extended aldosterone exposure. The basolateral membrane response is not as clear and seems to depend on the tissue under investigation, as some investigators report increases in the density of Na^+ pumps, while others find no density increase. Availability of energy by increases in citrate synthase certainly is an important factor and might itself mediate activation of apical Na^+ channels and/or basolateral pumps.

It is obvious that much more work is required to differentiate between rapid and long term alterations in Na^+ transport by aldosterone.

MECHANISM OF ADH ACTION

Even though the chain of intracellular events following ADH treatment have not been clearly defined (some links are missing) the physiological response is obvious. First, ADH after a very brief lag phase (1–2 min) causes an increase in the water permeability of the target epithelium and also an increase in the Na^+ transport ability (although this is only a transient increase in some instances). Most of the work that has been performed on ADH action has centred on the toad urinary bladder epithelium, and in general one can state that the prime site of transport modificati

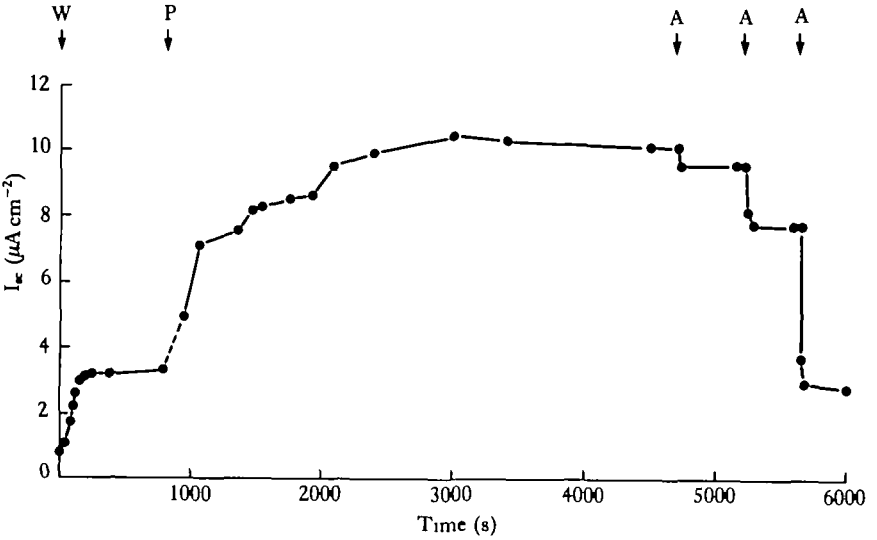

Fig. 3. An example of the effect of 'punching' the rabbit urinary bladder on the net rate of Na^+ transport. A punch consists of removing both serosal and mucosal solutions, and then rapidly raising and lowering the mucosal solution 12 times. At $t = 0$ a saturating dose of amiloride was washed (isovolumetrically) from the mucosal solution (W). Thus the control tissue had an amiloride-insensitive I_{sc} of 0·8 μA cm^{-2}, and an amiloride current of 2·4 μA cm^{-2}. At P the tissue was 'punched' and allowed to recover. The total current reached a value of 10·1 μA cm^{-2}. Submaximal doses of amiloride were added at each label, A, to final concentrations of 0·033, 0·13 and 1·4 μM respectively. Note that the amiloride-sensitive current post-punching is much greater than the pre-punch value.

is at the apical membrane. Since the response of the system to hormone challenge is quite rapid (compared to the aldosterone response), it is assumed that protein synthesis plays a negligible role in the rapid phase of the response, although no-one will deny that the response is mediated by protein structures. To clarify this problem I will consider in turn the response of Na^+ transport and then water permeability to ADH challenge. The basic questions to be asked are similar to those posed for the aldosterone response. Does this hormone stimulate pre-existing channels or does it cause a mobilization of a cytoplasmic store of channels (Na^+ and water) whose ultimate destination is the apical membrane?

Na^+ transport

After addition of ADH (arginine vasopressin 20 units ml^{-1}) Na^+ transport rapidly increases by up to 120 % in the first 10 min. In toad urinary bladder it briefly plateaus and then decreases with a slower time course. A similar rate of activation occurs in frog skin. However, the Na^+ transport reaches and stabilizes at a plateau value until ADH is removed from the serosal bathing solution. At this point, I_{sc} (i.e. Na^+ transport) decays monotonically toward the baseline value. Similar changes in transport are also induced by addition of cAMP and prostaglandin E_2 (Helman, Els, Cox & Van Driessche, 1981).

As in the case of aldosterone, ADH might stimulate Na^+ transport by increasing single channel Na^+ conductance, or increase channel density either by activation of [redacted]escent channels or by insertion of cytoplasmic stores. Using fluctuation analysis

Lindemann and his co-workers (Li, Palmer, Edelman & Lindemann, 1982), working on toad urinary bladder, and Helman *et al.* (1981), working on frog skin, found that ADH caused an increase in channel density and not in single channel conductances. This data indicates (as also found for aldosterone) that channels are either activated or inserted. In an attempt to differentiate between these possibilities Palmer & Edelman (1981), using DSA, eliminated 60% of the Na^+ transport for a 1-h period. Stimulation with ADH resulted in a proportional, not additive, increase in Na^+ transport. This experiment, they claim, is evidence that ADH activates a pre-existing quiescent channel, which is as susceptible to pharmacological inactivation as is a conducting pathway. Other evidence that indicates activation and not insertion is the amiloride binding studies of Cuthbert & Shum (1975), in which the amount of labelled amiloride bound to the apical surface before and during ADH challenge did not significantly change. Lastly, Stetson, Lewis, Alles & Wade (1982) (after inhibiting the water permeability increase that is normally elicited by ADH) were not able to measure a surface area increase (using capacitance as a measure) during ADH stimulation of Na^+ transport. This data indicates that Na^+ channels are not inserted as an integral protein of a lipid vesicle, but cannot rule out the insertion of single proteins.

In summary, ADH stimulates an increase in the density of apical Na^+ channels by activation of pre-existing pathways.

Water response

There is considerable evidence that the increase in water permeability of the apical membrane is not correlated with the stimulation of dormant water 'channels' in the apical membrane, but rather by the insertion of vesicles into this membrane which contain proteins capable of permitting copious movements of water but not ions.

Masur, Holtzman & Walter (1972) and Taylor, Mamelak, Reaven & Maffly (1973) offered the first piece of evidence that vesicle translocation might mediate the water response. Gronowicz, Masur & Holtzman (1980) reported from morphometric analysis that ADH caused a decrease in the number of granules which were immediately opposed to the apical membrane. Taylor *et al.* (1973) demonstrated that microtubule as well as microfilament blocking agents could reduce the water permeability.

Using freeze-fracture techniques, Kachadorian, Wade & DiScala (1975) showed that particle aggregates were present in the apical membrane only during ADH challenge, and the density of aggregates was directly proportional to the rate of osmotic water flow or the hydraulic conductivity of the membrane (Wade, Kachadorian & DiScala, 1977). In the resting state aggregates were found in the cytoplasm only in association with tubular vacuoles of approximately 0·1 μm diameter and up to 1 μm in length (Wade, Stetson & Lewis, 1981). Upon ADH stimulation these aggregates disappear from the cytoplasm and after ADH removal they reappear in the cytoplasm again associated with tubular vacuoles.

Pharmacological inhibition of water permeability without inhibition of Na^+ transport is possible using the drug (local anaesthetic) methohexital (Levine, Levine, Worthington & Hays, 1976). Consistent with the hypothesis of translocation of these aggregates, pre-treatment with methohexital and subsequent ADH addition inhibi

appearance of aggregates in the apical membrane. Partial inhibition of the hydro-osmotic response is also attained by blockade of the microfilament/microtubule system using either colchicine or cytochalasin B (Taylor *et al.* 1973).

Since the available evidence points towards membrane translocation and fusion as a mechanism for the induction of the hydro-osmotic response, one should be able to measure, using electrical techniques, a change in the membrane area *via* a determination of the membrane capacitance.

Two groups have recently been successful in measuring a capacitance increase following ADH addition (Warncke & Lindemann, 1981; Stetson *et al.* 1982).

In the absence of an osmotic gradient Stetson *et al.* (1982) measured a 36% increase in apical capacitance and a 110% increase in Na^+ transport after a 25 min exposure to ADH. There are a number of alternate hypotheses that one can develop to explain a capacitance increase in the absence of any real increase in membrane area. First, cell swelling might alter membrane thickness, dielectric constant and/or the dimension of the lateral spaces. Secondly, there might be an increase or decrease in the resistance of a single cell type in an epithelium composed of more than one cell population. These artifacts were tested by swelling or shrinking the cells and monitoring capacitance, and by increasing apical resistance of one cell type (granule cells) using amiloride. None of these manoeuvres resulted in a significant increase in apical capacitance. Since the capacitance increase seems to reflect a real apical membrane area increase, one can ask the following questions: (i) does the capacitance increase occur under more physiological conditions (e.g. with an osmotic gradient)? (ii) As opposed to being a primary action of ADH, is it a secondary reflection of an increase in Na^+ transport rate? (iii) Is the apical area increase directly correlated with the increase in the hydro-osmotic response?

To answer these questions capacitance changes were measured (i) with an osmotic gradient favouring water movement from mucosa to serosa, (ii) with Na^+ transport inhibited by amiloride and, (iii) after pre-incubation of the tissue with methohexital (a known blocker of the hydro-osmotic response).

In the presence of an osmotic gradient (2×) the apical capacitance increased by approximately 18%, a value less than that observed in the absence of a gradient. This data is in agreement with the finding that particle aggregate density in the apical membrane is also less in the presence of a gradient than in iso-osmotic conditions. Pre-treatment of the bladder with a saturating dose of amiloride and subsequent ADH challenge caused a 48% increase in capacitance, and in addition a small, but highly significant, increase in I_{sc} (which might represent an ionic conductance of the water channels) indicating a primary response to ADH and not a secondary response to Na^+ transport. Lastly, pre-treatment with methohexital followed by ADH completely inhibited the capacitance change but not the increase in Na^+ transport. A similar experiment with methohexital pre-treatment plus a saturating dose of amiloride demonstrated no increase in capacitance *nor* the small increase in I_{sc} measured previously in the presence of amiloride but absence of methohexital.

It is interesting to note that the percentage increase in capacitance during ADH stimulation is correlated with the physiological condition under which the experiment was performed. The maximum change in capacitance was elicited in the absence of Na^+ transport and osmotic gradient; the next largest change was isosmotic conditions

but with normal Na^+ transport and the smallest change was in the presence of an osmotic gradient with Na^+ transport intact. The common denominator of these experiments is that for minimal cell swelling one gets the greatest capacitance change while in the presence of swelling the change in capacitance is blunted. Since ADH stimulates an increase in cell cAMP concentration, the simplest interpretation is that cell swelling causes a dilution in cell cAMP. This dilution has been measured in isolated cells (Eggena, Christakis & Deppisch, 1975). Addition of exogenous cAMP (10 mM-dibutyryl-cAMP) to ADH stimulated bladders in the presence of an osmotic gradient results in an enhancement of the hydraulic conductivity to within 80% of the response in the absence of a gradient. Thus cell cAMP levels certainly modulate the increase in water permeability through a negative feedback loop. The inability of high levels of exogenous cAMP to completely over-ride the inhibitory influence of reduced osmolarity might reflect an impairment of the cytoskeletal system in swollen cells.

Taken together, the membrane effects of ADH can be summarized (Fig. 4) as a stimulation of pre-existing Na^+ channels on the apical membrane, since pharmacological agents inhibit proportionately the ADH-stimulated current and the baseline current. Inhibition of microtubules and microfilaments does not alter the ADH-induced current increase, and the amiloride-sensitive current increase is not associated with a measurable capacitance increase. Lastly, amiloride binding to the apical membrane does not change following ADH challenge. In contrast, the hydro-osmotic responses strongly suggests a movement of cytoplasmic vesicles into the apical membrane. The evidence comes from the following findings. First, the appearance of particle aggregates in the luminal membrane and the disappearance of similar aggregates from the cytoplasm. Next, interference with the cytoskeleton (both microtubules and microfilaments) blocks the increase in water permeability. Lastly, apical membrane area increases only during the hydro-osmotic response.

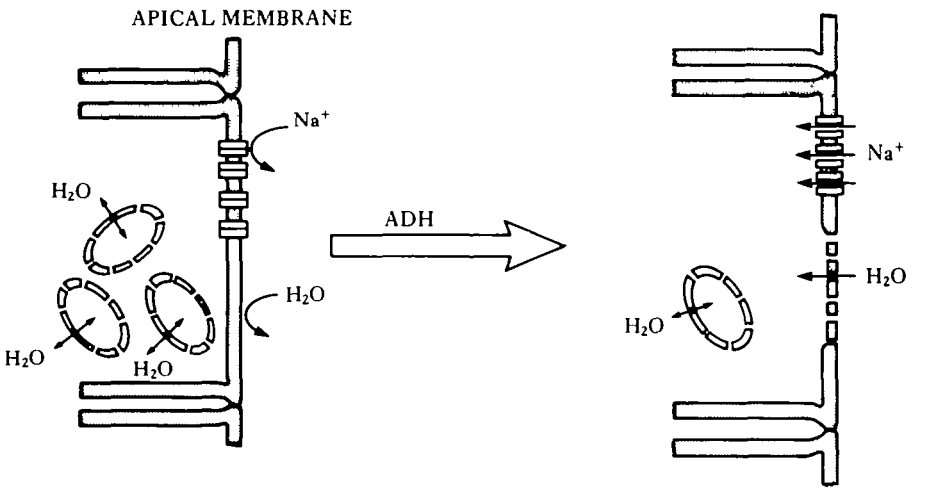

Fig. 4. A possibly fanciful drawing of the effects of antidiuretic hormone on epithelial Na^+ and water movement. ADH activates pre-existing quiescent apical Na^+ channels and mobilizes cytoplasmic vesicles which fuse into the apical membrane, thus dramatically increasing the epithelial water permeability and Na^+ transport.

CONCLUSIONS

I have attempted to demonstrate that the control of membrane electrolyte and non-electrolyte permeability is a complex system involving many cytoplasmic events which ultimately result in transport regulation. Two major strategies that the cells use to adjust membrane permeability are either activation of pre-existing quiescent pathways or the insertion of proteins or vesicles containing transport proteins into the appropriate membrane. The latter system relying heavily on an intact cytoskeleton, a structure which will soon be subjected to more widespread research by transport physiologists.

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