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EDITORIAL REVIEW

Regulation of Na^+ channels in the cortical collecting duct by AVP and mineralocorticoids

Although aldosterone is considered to be the primary hormone responsible for regulating the reabsorption of Na^+ and secretion of K^+ in the collecting duct, recent studies in the rat cortical collecting duct (CCD) have shown that arginine vasopressin (AVP) also stimulates Na^+ reabsorption and K^+ secretion. In fact, AVP acts synergistically with mineralocorticoids to augment these transport processes in this epithelium [1–4]. Although the physiologic role of this response to AVP has not been fully explored, it seems likely that it may relate more to maximizing urinary concentrating ability than to the regulation of Na^+ and K^+ balance [2, 3, 5]. Because of the high water permeability of the rat CCD in the presence of AVP [2, 3], the enhancement of Na^+ reabsorption by AVP also causes an increase in net volume reabsorption in the absence of a transepithelial osmolality difference [1]. The resulting reduction of flow to the medullary collecting duct in vivo would promote a maximal negative free water clearance [6]. Finally, stimulation of K^+ secretion by AVP may be necessary to maintain normal K^+ excretion when salt and water flow in the CCD is reduced during antidiuresis [7].

In the rat CCD both mineralocorticoids and AVP (via its intracellular second messenger, cAMP) increase the amiloride-sensitive Na^+ conductance of the luminal membrane [8–10]. Similar synergism between AVP and mineralocorticoids in regulating Na^+ transport has been observed in the toad bladder and in cultured A6 cells, which are derived from *Xenopus* distal tubule [11–14]. These model systems have provided the foundation of our understanding of how both mineralocorticoids and AVP act either to increase the number or to alter the functional state of amiloride-sensitive Na^+ channels in the apical membrane. This Na^+ channel and its regulation are the subjects of this review.

Fluctuation analysis and patch-clamp techniques have shown the Na^+ channel to be highly selective to Na^+ in comparison to K^+, and to have a relatively low conductance of 4 to 5 pS. However, amiloride-blockable channels with a lower Na^+ selectivity and higher conductances have been observed in some tissues or under special experimental conditions [15–17]. Electrophysiological studies have also shown that changes in the Na^+ permeability of the apical membrane produced by hormone action are the result of changes in the number of channels in the membrane or in the fraction of the time they spent in the conducting state, rather than being the consequence of changes in the unit conductance of the channel [15, 18, 19].

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The Na^+ channel protein has now been isolated, purified and reconstituted into artificial membrane systems [20]. Biochemical studies have shown that the channel is a heteromeric assembly of 5 to 7 subunits, one of which is phosphorylated as a consequence of AVP action, and another of which may be methylated in the presence of aldosterone. Other of these subunits may be G-proteins or the targets of protein kinases and/or lipoxygenase products. Thus there is good reason to suspect that this important ion channel is also subject to regulation by mechanisms other than those involved in the actions of AVP and mineralocorticoids.

Effects of aldosterone on Na^+ transport

Aldosterone has long been regarded to be the primary hormone regulating cation transport in the collecting duct and colon, and in anuran epithelial models of the collecting duct including the toad bladder and frog skin [reviewed in 14, 21–24]. In the mammalian nephron, aldosterone acts primarily on the cortical collecting duct where it increases Na^+ reabsorption and K^+ secretion [25–27]. Figure 1 is a schematic representation of the primary events involved in mediating the effects of aldosterone on Na^+ transport. Aldosterone combines initially with cytoplasmic receptors. The aldosterone-receptor complex then migrates to the nucleus where it binds to DNA and promotes the synthesis of mRNA and specific proteins [28]. The subsequent transport response is completely dependent on the synthesis of these proteins because it can be prevented by inhibitors of mRNA or protein synthesis [21, 28]. The initial event in the transport response is an increase in the amiloride-sensitive Na^+ conductance of the apical membrane, which allows increased Na^+ entry into the cell down its electrochemical potential gradient. There is a subsequent increase in Na^+,K^+-ATPase synthesis resulting in increased pump activity in the basolateral membrane [25, 29–31]. The synthesis of citrate synthase and other Krebs cycle enzymes is also increased [32–34].

The rate-limiting step in Na^+ transport is the apical membrane Na^+ permeability. Increasing the Na^+ permeability of the apical membrane with the ionophore amphotericin B causes an immediate increase in Na^+ transport that is equivalent to that produced by aldosterone [35]. Crabbé [36] showed that the application of exogenous aldosterone produced a consistent increase in Na^+ transport in toad bladders which had been previously depleted of endogenous hormone. This stimulation was later found to involve an increase in apical membrane Na^+ permeability [14], resulting in a small increase in intracellular Na^+ concentration which might directly increase Na^+,K^+-ATPase pump activity [37–39].

The time course of the transport events in the response to aldosterone has been best described in the toad bladder [30, 40].

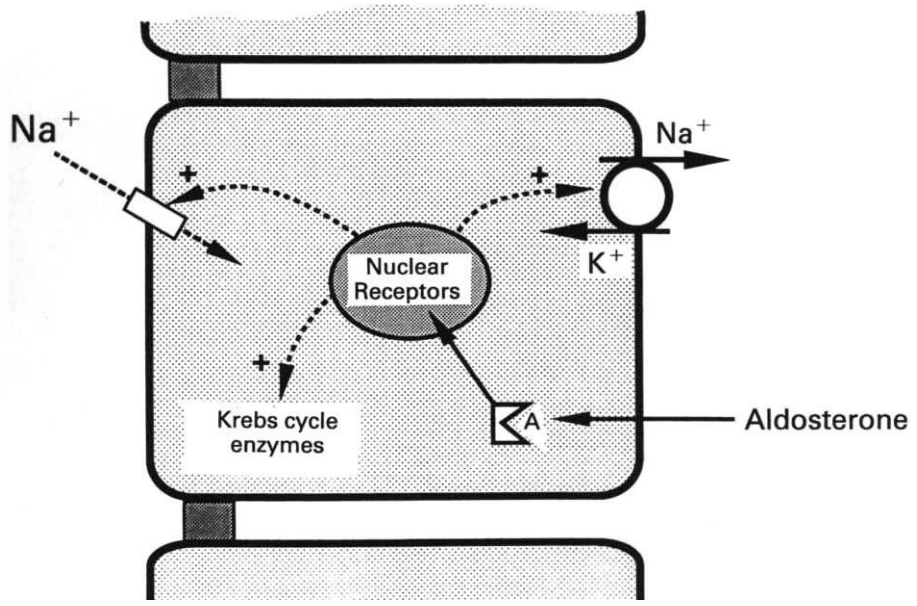

Fig. 1. *Effects of aldosterone in responsive epithelia.* Aldosterone enters epithelial cells by diffusion and combines with a cytoplasmic receptor. The receptor-aldosterone complex migrates to the nucleus where it combines with nuclear receptors on the genomic DNA. Initially there is an increase in apical membrane Na^+ conductance (left side of cell). This is followed by an increase in the synthesis of citrate synthase (and presumably other Krebs cycle enzymes), and an increase in the synthesis of the α and β Na^+,K^+-ATPase subunits, leading to an increase in basolateral membrane pump activity (right side of cell). Although the increase in apical membrane Na^+ conductance is dependent on protein synthesis, most evidence indicates that an activator protein is synthesized and not new channels.

In several studies it has been shown that the increase in apical membrane Na^+ permeability is the earliest event in the action of aldosterone [21, 30, 40]. In the toad bladder, there is a latent period of ~45 minutes after aldosterone addition before the short-circuit current (used to measure Na^+ transport) begins to increase. The increase is accompanied by a decrease in transepithelial resistance—the result of an increased amiloride-sensitive Na^+ permeability of the apical membrane. The Na^+ permeability and the short-circuit current reach a maximum ~three hours after aldosterone is added [30, 40]. Although the Na^+ permeability then remains constant, there is a further increase in the short-circuit current during the period 2.5 to 8.0 hours after hormone treatment. The increased Na^+ transport during this time is coincident with a two- to fourfold increase in Na^+,K^+-ATPase activity resulting from increased synthesis of both the α and β subunits of Na^+,K^+-ATPase [29–31, 40].

Aldosterone initiates the same sequence of events in the cortical collecting duct (CCD), which is the primary target of specific aldosterone binding in the nephron [26, 41]. Acute application of aldosterone to the isolated perfused rabbit CCD, increases Na^+ reabsorption within two to five hours [42], although more than three to six hours is required for an effect in the rat CCD [2]. As shown in Figure 2, electrophysiological studies from the laboratory of O'Neil and his associates [43–45] demonstrated that less than one day after mineralocorticoid treatment there was a doubling of the apical membrane amiloride-sensitive Na^+ conductance in CCD's from normal (adrenal-intact) rabbits with no measurable change in Na^+,K^+-ATPase activity. Only after two to three days of treatment was there any significant increase in apical membrane K^+ conductance and Na^+,K^+-ATPase activity. After long-term (two days to two weeks) aldosterone treatment of adrenalectomized rabbits, Na^+,K^+-ATPase activity in the CCD has been shown to increase by up to fivefold [25, 46]. The latent period between the administration of aldosterone and its effect on Na^+,K^+-ATPase activity has been found to increase in proportion to the basal Na^+,K^+-ATPase activity present before aldosterone

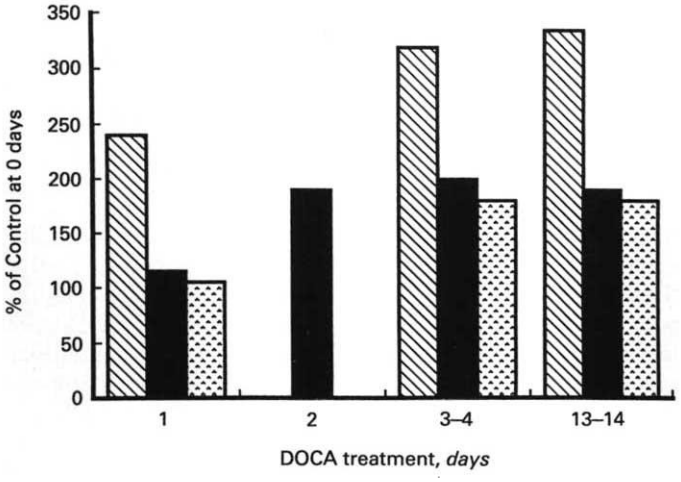

Fig. 2. *Time of the effects of deoxycorticosterone acetate (DOCA) on cation transport processes in the rabbit CCD.* Symbols are: (▨) G_{Na}; (■) Na^+,K^+-ATPase, (⊡) AVP + amiloride. Note that although the Na^+ conductance of the apical membrane (G_{Na}) is more than doubled within one day of DOCA treatment, the Na,K-ATPase activity and the K^+ conductance of the apical membrane are significantly increased only after 2 to 4 days of treatment. Adapted from the plot of O'Neil [43], and based on the data of O'Neil and Hayhurst [44] and Sansom and O'Neil [45].

treatment. Thus the latent period was shortest in CCD's from adrenalectomized rabbits and highest in those from normal rabbits [43, 46].

More recent studies have shown that there is an increase in functional Na^+,K^+-ATPase pumps in the CCD basolateral membrane (determined by ^{3}H-ouabain binding and $^{86}Rb^+$ uptake) within 15 to 30 minutes after aldosterone administration and before any increase in the enzymatically-measured Na^+,K^+-ATPase activity [47, 48]. This increase may be due to a latent pool of the enzyme which is activated or inserted into

the basolateral membrane in response to an acute rise in intracellular Na^+ [47, 48].

Increased Na^+ transport by the pump would also be expected to occur simply as a consequence of increased Na^+ entry across the luminal membrane, even with no increase in functional Na^+,K^+-ATPase. Barlet-Bas et al [49] have recently shown that the intracellular Na^+ concentration in the CCD is nearly the same as the $k_{0.5}$ for Na^+ on the pump, so that a small increase in the Na^+ concentration would lead to a large increase in pump rate. It is presently debated whether or not the rise in intracellular Na^+ concentration is required for the aldosterone-induced increase in Na^+,K^+-ATPase activity [43, 50, 51].

Changes in Na^+,K^+-ATPase activity associated with changes in plasma aldosterone levels have been shown to be paralleled by an equivalent increase in the basolateral membrane surface area of principal cells in the rat and rabbit CCD. Treatment of rabbits for two weeks with high doses of either DOC or dexamethasone more than doubled this membrane surface area [52], but more recent experiments show that the effect is due to the mineralocorticoid actions of both hormones. Stanton et al [53] observed that adrenalectomy decreased the membrane surface area of principal cell basolateral membranes by 35%, and this was reversed by low level infusion of aldosterone but not dexamethasone. A higher but still physiologic rate of aldosterone infusion increased the surface area by more than 110% compared to nonadrenalectomized controls [53]. It might be speculated that the additional membrane area is associated with the newly inserted Na^+,K^+-ATPase pumps.

The mechanism by which aldosterone causes the early rise in the Na^+ conductance of the apical membrane of the CCD or other aldosterone-responsive epithelia remains unknown, although it appears to involve activation of Na^+ channels already present in the apical membrane rather than the insertion of newly synthesized channels [54]. This activation may be associated with methylation of inactive channels in the membrane. Sariban-Sohraby et al [55] have shown that incubation of apical membrane vesicles from A6 cells with a methyl donor results in an increase in Na^+ transport associated with an increased incorporation of methyl groups into membrane proteins. Wiesmann et al [56] have shown that blockers of methyltransferase also block the increase in short-circuit current produced by aldosterone. Although the evidence for methylation has been criticized as nonspecific with respect to the proteins involved [23], new evidence discussed below continues to implicate methylation in the action of aldosterone.

Effects of AVP and its analogs on Na^+ transport

Since the earliest studies of Leaf, Anderson and Page [57] vasopressin and its analogs, such as vasotocin and oxytocin, have been known to increase Na^+ transport in the toad urinary bladder; however, a similar effect in the nephron has taken longer to demonstrate and it may be species specific. In the rat and mouse AVP is found to increase NaCl reabsorption by the thick ascending limb, whereas in the rabbit it does not [58]. The species difference in the response to AVP has been associated with the ability of AVP to stimulate cAMP generation in the thick ascending limb of the mouse and rat but not the rabbit [59]. In the first studies with the isolated perfused rabbit CCD, Frindt and Burg [60] showed that AVP produced only a transient increase in the lumen-to-bath ($J_{l\to b}$) flux of Na^+ with no change in the bath-to-lumen backflux ($J_{b\to l}$). After 15 to 30 minutes of AVP treatment $J_{l\to b}$ reached a peak only 20% over control and then returned to or below control levels within 30 to 60 minutes after AVP addition. The Na^+ transport response to vasopressin or its analogs (such as oxytocin and arginine vasotocin) also exhibits tachyphylaxis in the toad bladder and frog skin. There is a latent period of three to five minutes after hormone addition, then the short-circuit current rises by two- to fourfold within 10 to 20 minutes. The short-circuit current declines to a lower level over 30 to 60 minutes but, in contrast to the rabbit CCD, Na^+ transport is still substantially elevated over control at 60 minutes in most cases [11, 12, 22, 23, 57, 61]. More recently it has been shown that AVP produces a more impressive and stable increase in Na^+ reabsorption in the rat distal tubule and collecting duct. Costanzo and Windhager [62] demonstrated that AVP or cAMP increased Na^+ reabsorption by 40 to 80% in the rat CCD microperfused in vivo, and subsequently, Elalouf, Roinel and DeRouffignac [63], using microperfusion of the distal tubule of the Brattleboro rat, demonstrated almost a doubling of Na^+ reabsorption.

The observation that AVP stimulates Na^+ reabsorption contrasts with the widely held belief that AVP is a "natriuretic hormone." However, Valtin [64] believes that the natriuretic effect seen with vasopressin in earlier studies might be attributable both to hemodynamic changes, as well as indirect effects related to prostaglandin production or oxytocin action via the V_1 receptor. In support of this position, Gellai et al [65] found that infusion of physiologic levels of synthetic AVP in conscious, euvolemic Brattleboro rats depressed Na^+ excretion. The physiologic role of AVP in the regulation of Na^+ transport by the CCD remains to be clarified, but it seems likely that it may be required to maximize negative free water clearance by reabsorbing maximal amounts of isotonic fluid in the cortex [2, 3, 6].

The actions of AVP on cation transport have been studied in more detail in the rat CCD using in vitro tubule perfusion. Tomita, Pisano and Knepper [1] observed no net Na^+ reabsorption in untreated control tubules; however, after the rats were pretreated with high doses of DOC 7 to 12 days before experimentation there was significant net Na^+ reabsorption. Acute addition of AVP to these DOC-treated CCD's produced a stable fourfold stimulation of Na^+ transport. Isotopic flux studies in our laboratory established that AVP significantly stimulates $J_{l\to b}$ (using $^{22}Na^+$) with no effect on $J_{b\to l}$, and showed that AVP and DOC acted synergistically to increase Na^+ reabsorption [2, 3, 66]. As shown in Figure 3, the addition of AVP to CCD's from DOC-treated rats produced a far greater increase in $J_{l\to b}$ and in net flux than in CCD's from untreated rats. In both untreated and DOC-treated groups, $J_{l\to b}$ rose to a maximum within 15 to 20 minutes after adding AVP, remained stable for at least 100 to 150 minutes, and decreased to control levels after removal of the hormone [2, 3]. We have observed the same synergism of AVP and DOC in stimulating Na^+ reabsorption in the prehypertensive Dahl salt-sensitive rat CCD [66].

Vasopressin (or vasotocin or oxytocin) and aldosterone also exhibit synergy in stimulating short-circuit current in the toad bladder [11, 13, 14], and in cultured A6 cells [12]. In contrast, CCD's from DOC-treated rabbits do not even exhibit the transient stimulation of Na^+ transport by AVP that is observed in tubules from non DOC-treated rabbits [3]. However, it is

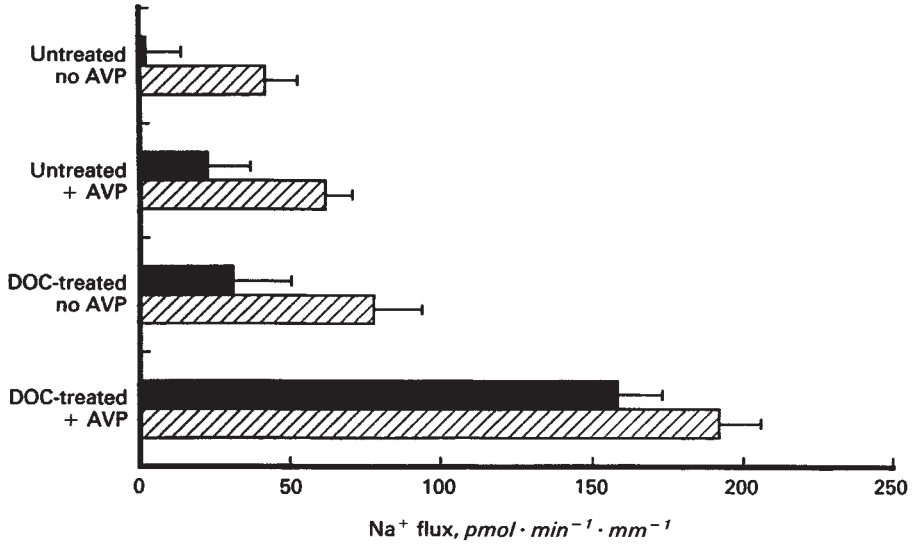

Fig. 3. *Synergism between DOC and AVP in stimulating Na^+ reabsorption in the rat CCD.* Symbols are: (■) net; (▨) lumen-to-bath. The unidirectional flux from lumen-to-bath (as measured by $^{22}Na^+$) and the net flux (calculated as the difference between unidirectional lumen-to-bath and bath-to-lumen $^{22}Na^+$ fluxes in different experiments) are shown for four groups of experiments: 1) CCD's from rats that received no DOC pretreatment and no in vitro AVP; 2) The same CCD's as in group 1 after the addition of 220 pM AVP to the bathing solution; 3) CCD's from rats that had received 5 mg DOC pivalate 4 to 10 days before experimentation with no in vitro AVP; 4) The same CCD's as in group 3 after the addition of 220 pM AVP to the bathing solution. The bath-to-lumen flux was not significantly different among groups, averaging 34 to 47 pmol · min^{-1} · mm^{-1}. Data are from Reif et al [2] and Chen et al [3].

quite interesting that primary cultures of immunodissected rabbit CCD cells acquire a stable stimulatory effect of AVP on Na^+ transport, as measured by the amiloride-sensitive short-circuit current, which is additive to that produced by aldosterone [67].

As is the case with aldosterone, the initial effects of vasopressin and its analogs in the toad bladder and CCD involves an increase in the amiloride-sensitive Na^+ permeability of the apical membrane. In the toad bladder AVP causes an increase in Na^+ uptake that is associated with a fall in the resistance of the apical membrane [22, 23, 61, 68]. The increased Na^+ uptake results in an increase in the intracellular Na^+ activity, indicating that apical entry rather than basolateral pumping is rate-limiting to transport and is increased by the hormone [14, 22, 23]. Using electron probe ultramicroanalysis of toad bladder tissue sections, Rick, Spancken and Dörge [39] have shown that the increase in intracellular Na^+ is greater after AVP treatment than after aldosterone treatment, and they noted that these results were consistent with a primary effect of both hormones on Na^+ entry but with a greater stimulation of Na^+,K^+-ATPase activity by aldosterone.

Na^+ transport in the toad bladder is blocked by amiloride at low concentrations in the presence or absence of vasopressin analogs and/or aldosterone, indicating that the apical entry stimulated by both types of hormone is mediated by the same Na^+ channel [11, 23, 69]. In the rat CCD as well, both AVP and DOC increase the amiloride-sensitive Na^+ conductance of the apical membrane [8–10]. Using intracellular microelectrodes in principal cells of the rat CCD, we have established that AVP and DOC decrease the fractional resistance of the apical membrane in an additive manner, as shown in Figure 4. The effects of either or both hormones together is completely reversed by their removal or by the application of 1 μM luminal amiloride, which increases the fractional resistance of the apical membrane to the same high level in all cases [8–10].

The effects of AVP analogs on Na^+ transport in the toad bladder are mediated by cAMP as is the increased water permeability [70]. We have recently demonstrated that this is also the case in the rat CCD. Dibutyryl-cAMP, isobutylmethylxanthine (a phosphodiesterase inhibitor), and forskolin (a direct stimulator of adenylate cyclase) produced the same changes in apical membrane fractional resistance and/or transepithelial voltage and resistance as did AVP [9].

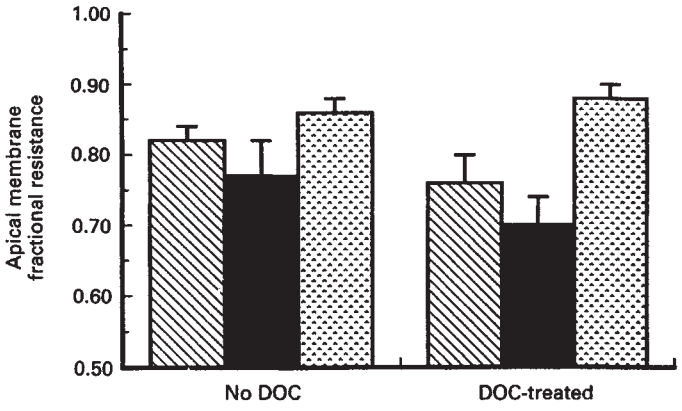

Fig. 4. *Effect of AVP and DOC on the fractional resistance of the apical membrane in the isolated perfused rat CCD.* Symbols are: (▧) control; (■) AVP; (⊟) AVP + amiloride. The fractional resistance of the apical membrane (the fraction of the total transcellular resistance that is due to the apical membrane) was determined by passing transepithelial current pulses while measuring transepithelial and basolateral membrane voltage deflections [8, 10]. The fractional resistance was significantly decreased by 220 pM AVP in CCD's from both untreated and DOC-treated rats and the effect was reversed by the application of 0.1 μM amiloride to the lumen. Note that the average fractional resistance was significantly lower during the control period in CCD's from DOC-treated rats, but in both groups the final fractional resistance with amiloride was the same at 0.87.

AVP and its analogs, in a manner similar to aldosterone, also cause an increase in Na^+,K^+-ATPase activity in the toad bladder and in the rat CCD subsequent to the increase in apical membrane Na^+ permeability. After 18 hours of oxytocin treatment in the toad bladder, Girardet et al [11] observed a 1.9- and 1.6-fold increase in the synthesis of α and β Na^+,K^+-ATPase subunits, respectively. Tomita et al [71] have reported that AVP produced a 50 to 75% stimulation of Na^+,K^+-ATPase activity

in microdissected CCD segments but not in other nephron segments. This effect required at least three days of in vivo AVP treatment, and no change in Na^+,K^+-ATPase activity was observed after treatment of CCD's with AVP for one to three hours in vitro. Thus, although the long-term effects of both AVP and aldosterone involve an increase in Na^+,K^+-ATPase synthesis, the initial effect of both is to increase the amiloride-sensitive Na^+ conductance of the apical membrane. The only difference in their effects is the time course. Aldosterone action requires de novo protein synthesis and thus has a latency of at least 45 minutes. AVP on the other hand begins to exert its action, mediated by cAMP, in three to five minutes and this effect is maximal in 20 to 30 minutes.

Characteristics of the Na^+ channel from fluctuation analysis and patch clamp

The operational characteristics of the amiloride-sensitive Na^+ channel in the apical membrane have been best characterized by the electrophysiological techniques of fluctuation ("noise") analysis and patch clamping. Although the degree of selectivity to Na^+ and the unit conductance of this channel vary among epithelia and even within epithelia, the defining characteristic of the channel (or class of channels) is its sensitivity to blockage by the diuretic amiloride. Amiloride is a weak inhibitor of several Na^+-dependent transporters including the Na^+,K^+-ATPase and the Na^+/Ca^{+2} antiporter, as well as various Na^+-organic solute symporters; however, only the conductive Na^+ channel of tight epithelia is inhibited with an IC_{50} less than 1 μM at a physiologic Na^+ concentration [69]. Even more potent as Na^+ channel inhibitors are those amiloride analogs with hydrophobic group substitutions on the terminal nitrogen of the guanidino moiety, which include benzamil, phenamil and 6-bromomethylamiloride [69].

Noise analysis has been used successfully to study the Na^+ channel in the toad bladder and frog skin. To remove the contribution of the basolateral membrane to the noise spectrum, it is necessary to depolarize it with a high extracellular K^+ concentration. Thus it should be recognized that the channel is being studied under rather nonphysiologic conditions. Nevertheless, these studies have clearly indicated that both AVP and aldosterone increase the number of open channels in the apical membrane, without altering the unit conductance per channel [15, 18, 19].

The special contribution of noise analysis has been the characterization of multiple states of the channel and the kinetics of transitions between these states. In the frog skin the amiloride-sensitive current increased linearly with an increase in the extracellular Na^+ concentration, indicating the absence of saturation by substrate [72], and that each channel had a current in excess of 10^6 ions/sec (>10 pA) in the open state. The noise analysis data were best fitted by a kinetic model of a three-state channel fluctuating between open, closed (nonconducting) and open-occluded (amiloride-blocked) states according to the scheme: closed $\leftrightarrow$ open $\leftrightarrow$ open-occluded [15, 73, 74]. According to this analysis, the total number of channels in the membrane (N) is the sum of the number of open, conducting channels (N_0), the number of closed channels (N_1), and the number of open-occluded channels (N_2). However, Eaton and Marunaka [75] have recently provided evidence that there may be an additional closed-occluded state of the channel, with the possibility of transitions between the closed-occluded and open-occluded states. They suggest that it may be possible in the future to resolve whether the three or four state model applies by using a combination of fluctuation and single-channel patch clamp analysis.

The earliest attempts at studying single amiloride-sensitive Na^+ channel kinetics incorporated isolated membrane vesicles from A6 cells into lipid bilayer membranes; however, the channels described in these studies had lower Na^+ selectivity and higher unit conductances than expected [76, 77]. Helman et al [78] subsequently used patch-clamp technology in the rabbit CCD, but these investigators saw Na^+ channel activity only in inside-out patches and could not resolve single channel events. Palmer and Frindt [79] were subsequently able to examine single Na^+ channel characteristics in patches of the rat CCD luminal membrane. The channels they described had a unit conductance of 4 to 5 pS, which was quite comparable to the estimates obtained by noise analysis in the toad bladder and frog skin [15, 74]. The channels were non-rectifying, and had a selectivity of Na^+ to K^+ of 10:1 to 40:1, although they were less selective for Na^+ in comparison to Li^+. The channels were found to be relatively unstable in excised patches, and also had a slight voltage dependence such that depolarization decreased their open probability (P_0) [79, 80]. The channels were also found to have long mean open and closed times of 3 to 4 seconds, with an open probability (P_0) on the order of 0.5, and amiloride produced more frequent opening and closing [79, 80].

Given a total number of channels N (open and closed) in the apical membrane of a given epithelium, with an open probability P_0, and a unit conductance γ, the total conductance of that membrane would be equal to γNP_0. Because noise analysis data indicates that AVP and aldosterone both increase the apical membrane conductance by increasing the number of active channels without any change in the unit conductance [15, 18, 19], the effects of both hormones must involve an increase in the product NP_0.

Using the whole cell method of patch-clamping in the rat CCD, Frindt, Sackin and Palmer [81] have found that the total amiloride-sensitive Na^+ conductance of principal cells is increased when the rats are maintained on a low Na^+ diet. They analyzed their data to indicate that the low sodium diet increased the number of active Na^+ channels from ~100 per cell on a normal Na^+ diet to ~3,000 per cell on the low Na^+ diet. Furthermore, there was a twofold increase in cell membrane capacitance [81], which was consistent with the known increase in the basolateral membrane surface area as a consequence of elevated aldosterone levels [53]. However, what is actually measured by this technique is the number of channels open at any given time or NP_0, and not the total number of channels N. As discussed in more detail below, preliminary evidence in A6 cells indicates that aldosterone increases P_0 and N [82] while AVP increases N [83], and thus both hormones increase the total membrane Na^+ conductance but by different mechanisms.

Is there more than one type of amiloride-sensitive Na^+ channel?

Although channels with the above characteristics have been found in a variety of tight epithelia, less Na^+-selective but

amiloride-inhibitable channels have also been observed in some studies. These observations have led investigators to question whether these channels are different manifestations of the same basic channel protein in different cells and under different conditions, or whether there may be a family of different amiloride-blockable channel proteins. Hamilton and Eaton [84] observed the presence of two different Na^+ channels in patch clamp studies of A6 cell apical membrane. One channel had about the same conductance (3 to 5 pS) and Na^+:K^+ selectivity (20:1) as the channels observed in the rat CCD and as expected from noise analysis, while the other channel had a high conductance of 7 to 10 pS and a low Na^+:K^+ selectivity of 3 or 4:1 [16, 84]. In spite of these differences both channels were blocked by a low concentration of amiloride (0.5 μM). Hamilton and Eaton [16] were able to establish that the presence of a higher proportion of low-conductance, high-selectivity channels was positively correlated with a high Na^+ transport rate in cell cultures. Sariban-Sohraby, Burg and Turner [85] had previously established that A6 cells had relatively low rates of amiloride-sensitive $^{22}Na^+$ uptake when they had been grown on plastic petri dishes, but the rate was markedly higher in cells that had been grown on permeable membrane supports. Hamilton and Eaton [16] observed that only 15% of the patches from A6 cells grown on plastic contained Na^+ channels and only 8% of that 15% had more than one channel. In contrast, 44% of patches from cells grown on permeable supports contained Na^+ channels and more than half of these had multiple channels. Although patches from either cell culture regimen contained both high and low selectivity channels, in patches from cells grown on the permeable supports the mean open and closed times were two orders of magnitude higher than from cells grown on plastic [16].

A low selectivity channel has also been identified by Light et al [17] in the apical membrane of inner medullary collecting duct cells (IMCD) which had been freshly isolated or grown in primary culture. These channels did not discriminate between Na^+ and K^+, but had a Na^+:Cl^- selectivity ratio of 13:1. The unit conductance of the channels was ~28 pS, and they were blocked by 0.5 μM amiloride. No high Na^+-selectivity, low-conductance channels were observed in any of the patches [17]. In spite of these unique characteristics, the low selectivity conductance channels appear to mediate amiloride-sensitive electrogenic Na^+ reabsorption in the IMCD.

There is some basis for speculating that the high and low selectivity channels could have the same precursor. Lewis and his coworkers [86–89], using the rabbit urinary bladder, which is another epithelium in which Na^+ reabsorption is mediated by amiloride-sensitive channels but at quite low rates, proposed that the Na^+ channels which have been recently inserted in the apical membrane of the bladder have characteristics different from those which have been present for longer. The "older" channels are less Na^+-selective (2 or 3:1 Na^+:K^+) and less susceptible to inhibition by amiloride than the "younger" channels. These investigators have hypothesized that once the channels are inserted into the membrane they are progressively degraded by urokinase [88], kallikrein [89], or other urine proteases [90] to produce a less selective and less amiloride-sensitive channel that is eventually released into the urine.

Similarity of the high and low selectivity channels is also indicated by the fact an anti-idiotypic antibody to the Na^+ channel was able to immunoprecipitate channel proteins from A6 cells grown on either plastic or on permeable supports, suggesting that it recognizes both high and low conductance channels [91]. However, in pulse-chase experiments with ^{35}S-methionine, Kleyman, Kraehenbuhl and Ernst [91] found that a 60 minute chase was required before the antibody recognized newly synthesized channel proteins. They interpreted their experiments to indicate that the channel requires post-translational processing or assembly in order to demonstrate binding with the anti-idiotypic antibody, and possibly before the protein can function as a channel. A final resolution of this possibility and the possibility that high and low selectivity channels are products of the same channel precursor will most likely depend upon isolation and characterization of the two channel proteins, and cloning and sequencing of the channel message or messages.

Isolation, reconstitution and expression of the Na^+ channel protein

Several laboratories have been successful in isolating proteins with the amiloride-binding characteristics of the functional Na^+ channel; however, there has been variable success in reconstituting normal Na^+ transport function of the isolated protein into artificial membrane systems. Using purified apical membranes vesicles from A6 cells which were incorporated into artificial lipid bilayers, Sariban-Sohraby et al [76] and Olans et al [77] were able to demonstrate Na^+ channel activity. However, in spite of the fact that the channels derived from A6 cells were grown on permeable supports, they had a relatively low Na^+/K^+ selectivity (2:1) and a variable unit conductance of 4 to 80 pS.

Benos and Sariban-Sohraby and their coworkers [20, 92, 93] subsequently purified the channel protein from homogenates of A6 cells and bovine renal medulla using a combination of detergent-solubilization and wheat-germ agglutinin-affinity chromatography, followed by either amiloride-affinity chromatography or molecular weight exclusion HPLC to obtain a single peptide with a molecular weight of ~700 kDa. Each step of the purification procedure was monitored by enrichment of ^3H-methylbromoamiloride binding, with a final enrichment of ~5,000-fold compared to the homogenate. Furthermore, the purified protein when reconstituted in lipid bilayer membranes or phosphotidyl choline vesicles exhibited highly-selective Na^+ transport activity that was inhibited 30% by 10 nM bromoamiloride [20, 92]. The isolated protein was also used to produce a polyclonal antibody which could immunoprecipitate the protein, and which has proved useful on immunoaffinity columns for easier preparation of purified protein [94].

When the channel protein was subjected to treatment with the reducing agents dithiothreitol or β-mercaptoethanol, five distinct polypeptides were consistently separated by SDS-polyacrylamide gel electrophoresis [20]. The subunits were apparently integrated into the original 700 kDa protein by disulfide links, because subunits were not observed after denaturing treatments. As summarized in Table 1, the polypeptides, referred to as α, β, γ, δ, and ϵ, had molecular weights of approximately 315, 150, 95, 70, and 55 kDa, respectively. Smaller molecular weight polypeptides of 30 to 45 kDa were also isolated in some preparations, making it possible that

Table 1. Characteristics of subunits from the Na^+ channel protein isolated from A6 cells and bovine renal medulla by Benos et al[a]

Subunit	Average molecular wt (range) *kDa*	Characteristics
α	315 (300–330)	Phosphorylated by cAMP-dependent protein kinase[b]; recognized by polyclonal antibody to the native channel protein[c]; glycosylated[d]
β	150 (130–180)	Amiloride binding site[a]; ankyrin and fodrin binding sites[e]; glycosylated[d]
γ	95 (90–110)	Methylated by carboxymethyltransferase[f]; ADP-ribosylated by pertussis toxin[g]; recognized by polyclonal antibody to the native channel protein[c]; glycosylated[d]
δ	70 (65–85)	No present information
ε	55 (50–65)	Sometimes observed to bind methylbromoamiloride[a]; cDNA cloned and expressed[h]
	40 (30–45)	Variably present and variable size[a]; ADP-ribosylated by pertussis toxin[g]

[a] Benos et al [20]
[b] Sariban-Sohraby et al [95]
[c] Sorscher et al [94]
[d] Smith and Benos [96]
[e] Smith et al [97]
[f] D. J. Benos, personal communication
[g] Ausiello et al [98]
[h] Cunningham et al [99]

additional subunits were present, although the sum of the molecular weights of the five major polypeptides was quite close to that of the intact peptide [20]. Benos et al [20] demonstrated that the β subunit, and occasionally the ϵ subunit selectively bound ^{3}H-methylbromoamiloride. As shown in Table 1, and as will be discussed below (*Subunits as regulators of channel activity*), other characteristics of the various subunits have since been identified. However, given the very limited amounts of the individual subunits that can be produced by biochemical isolation, it has not been possible to test them individually for amiloride-sensitive Na^+ channel activity by incorporation into artificial membrane systems.

Kleyman et al [100, 101] have also used high-affinity amiloride analogs and anti-amiloride antibodies to identify and purify polypeptides from A6 cells and bovine renal cortex. In their most recent work, Kleyman, Kraehenbul and Ernst [91] developed an anti-idiotypic monoclonal antibody that mimics the inhibitory effect of amiloride on Na^+ transport in A6 cells after they have been given a mild treatment with trypsin, which apparently makes the amiloride-binding site accessible to the antibody. This monoclonal antibody also immunoprecipitated a 700 to 750 kDa protein from A6 cells, which appeared to be the same as identified by Benos et al [20] and Sorscher et al [94]. Under reducing conditions, the protein of Kleyman et al [91] was observed to have four or five subunits with molecular weights in the ranges: 230 to 260, 180, 110 to 140, 70 and 50 kDa, with the latter subunit being variably present. The monoclonal antibody recognized the 140 kDa subunit, which corresponds to the 150 kDa β subunit of Benos et al [20] and Sorscher et al [94].

Other investigators have also isolated amiloride-binding proteins from A6 cells and kidney, but none of these proteins have been shown to give a convincing increase in amiloride-sensitive Na^+ transport when reconstituted into artificial membrane systems. Barbry et al [102, 103] used ^{3}H-phenamil to purify an amiloride-binding homodimer with subunits of ~88 kDa from pig kidneys; however, when the peptide was reconstituted into liposomes only modest amiloride-sensitive $^{22}Na^+$ uptake was observed. Barbry et al [104] subsequently used oligonucleotide probes, synthesized on the basis of partial sequencing of the 88 kDa polypeptide, to obtain a cDNA for the polypeptide from a human kidney cDNA library. This cDNA was of the appropriate size and coded for a protein of 713 amino acids. The corresponding mRNA, when introduced into three different cells including *Xenopus* oocytes, was found to express a glycoprotein with the appropriate binding characteristics for amiloride analogs, but no Na^+ channel activity was expressed. Furthermore, the hydropathy plot of the predicted protein was not consistent with a transmembrane protein [104]

Preliminary reports of cloning and sequencing other putative subunits of the channel have come from two other laboratories. Cunningham et al [99] have cloned and expressed the 55 kDa ϵ subunit from a 6.5 kb cDNA isolated from a bovine renal papilla library. Expression of this protein in *Xenopus* oocytes was associated with enhanced Na^+-selective current, but this current was not inhibited by amiloride. Thus, while it may be possible that this protein is part of the transmembrane channel or is the channel itself, it apparently lacks the amiloride binding site which is associated with the β subunit. Staub et al [105] have used an antibody reactive with both the Na^+ channel and the α-subunit of Na^+,K^+-ATPase [40] to isolate a partial cDNA clone from an A6 expression library, but the clone has no homology with the Na^+,K^+-ATPase α-subunit and probably reflects a portion of one of the channel subunits [105].

The similarity of the sizes of the amiloride-binding polypeptides isolated by Benos et al [20] (130 to 180 kDa), Kleyman et al [91, 101] (130 to 140 kDa), and Barbry et al [103] (176 kDa dimer), and the fact that none of them has been convincingly demonstrated to increase Na^+ transport activity when reconstituted or expressed, suggests that all three laboratories may have identified the same protein which is a regulatory subunit of the Na^+ channel or otherwise associated with the channel. The differences in molecular weight of this subunit could be attributable to any one or a combination of factors including: species differences, differing degrees of glycosylation, artifactual or physiologic proteolysis, post-translational modification or assembly, or alternative mRNA splicing [91, 101].

Several groups have shown that poly A(+) RNA extracted from A6 cells and cultured IMCD cells expresses amiloride-sensitive Na^+ channel activity when injected into *Xenopus* oocytes [106–109]. In all cases the RNA channel expressed was inhibited by low concentrations of amiloride; however, while the channel expressed by A6 mRNA had a very high Na^+:K^+ selectivity ratio, the ratio was only 1:4 for channels expressed by mRNA from the IMCD cells [109], supporting the notion that there may be more than one amiloride-sensitive Na^+ channel specified by different genes. Size selection of the mRNA has shown that the expression of Na^+ channel activity is associated with mRNA's coding for polypeptides with a molecular weight range of 50 to 160 kDa [106], with the 17 S RNA fraction (70 to 80 kDa) being the most likely [108].

Subunits as regulators of channel activity

As summarized in Table 1, considerable evidence is now emerging that at least some of the Na^+ channel subunits identified by Benos et al [20] may regulate channel activity by their interaction with protein kinases, methylation, or that they may be GTP-binding (G) proteins. Sariban-Sohraby et al [95] have shown that the 315 kDa α-subunit was selectively phosphorylated when isolated from A6 cells that had been loaded with $^{32}P_i$ and then exposed to AVP. Furthermore, when the isolated Na^+ channel protein was incubated in vitro with γ-^{32}P-ATP, cAMP and the catalytic subunit of cAMP-dependent protein kinase, the same subunit was phosphorylated. Thus, although the α subunit is unlikely to be the channel itself, its phosphorylation appears to be at least part of the mechanism by which AVP increases the number of active Na^+ channels.

Methylation of some component of the channel has been implicated in the mechanism by which aldosterone activates Na^+ channels. In the studies of Sariban-Sohraby et al [55], when A6 cells were incubated with the methyl donor S-adenosylmethionine the amiloride-sensitive Na^+ uptake was more than doubled, and this effect was prevented by methylation inhibitors. Furthermore, Kemendy and Eaton [82] have shown that methylation inhibitors prevent the increase in Na^+ channel P_0 in excised patches from aldosterone-treated A6 cells. There is preliminary evidence that the γ subunit is methylated in the presence of carboxymethyltransferase (D. J. Benos, personal communication), and thus this subunit may be involved in the activation of quiescent Na^+ channels by aldosterone.

The above observations indicate that two of the Na^+ channel subunits may mediate the stimulatory action of AVP and mineralocorticoids on Na^+ transport. Many other autacoids, including prostaglandins and bradykinin, have been implicated in the regulation of Na^+ transport in the CCD. These agents appear to be primarily inhibitory, and have been linked to the phosphoinositide signalling system both by changes in intracellular Ca^{+2} and activation of protein kinase C (PKC) primarily in the rabbit CCD [110]. Some of the Na^+ subunits in Table 1 have characteristics suggesting they may be involved in the response to PKC.

Activation of PKC has been associated with inhibition of Na^+ channel activity in a variety of tight epithelia. In particular it has been shown that phorbol esters and diacylglycerol analogs, which activate PKC, inhibit Na^+ transport in A6 cells [111], LLC-PK_1 cells [112] and the isolated rabbit CCD [113]. It is thought that either PKC or a G-kinase may be a part of the pathway by which atrial natriuretic peptide inhibits Na^+ transport in LLC-PK_1 and IMCD cells via its intracellular messenger cGMP [114–116]. Light, Corbin and Stanton [116] have shown that cGMP inhibits P_0 in inside-out patches from IMCD cells both directly and indirectly via a cGMP-dependent protein kinase which in turn appears to act via regulation of a G-protein. Preliminary evidence (D. J. Benos, personal communication) suggests that β and/or ϵ subunits may mediate inhibition of Na^+ channel activity at least by PKC because they are selectively phosphorylated in the presence of this kinase.

G-proteins are now strongly linked to the regulation of the amiloride-sensitive Na^+ channel. In LLC-PK_1 cells, Mohrmann, Cantiello and Ausiello [112] demonstrated that an elevation of cGMP produced by nitroprusside was associated with both inhibition of amiloride-sensitive Na^+ transport and ADP-ribosylation of a G-protein localized to the apical membrane. Pertussis toxin and GDP-β-S has been found to inhibit completely amiloride-sensitive Na^+ channels in LLC-PK_1, A6 and IMCD cells, whereas addition of GTP or GTP-γ-S to inside-out membrane patches from these cells restored channel activity by increasing P_0 in a dose-dependent manner [112, 117, 118]. The G-protein involved is of the G_i type, because addition of the purified α_i-3 subunit to inside-out patches produces the same increase in P_0 as GTP or GTP-γ-S [117, 118]. It seems likely that this activity is mediated by the γ subunit or the variable 40 kDa subunit of the Na^+ channel (Table 1), both of which have been shown to be ADP-ribosylated by pertussis toxin [98].

The mechanism by which the G-protein activates the Na^+ channel may be indirect in spite of the fact that it appears to be one of the subunits of the channel protein. Kim et al [119] have suggested that the $\beta\gamma$ subunit complex from the G-protein may activate phospholipases in the apical membrane as they do in other systems. Mepacrine, an inhibitor of phospholipase A_2, has been found to decrease both basal and aldosterone-stimulated Na^+ transport in toad bladder [120]. Cantiello et al [121] have shown that this effect is due to the decreased production of a lipoxygenase product, and also demonstrated that arachidonic acid was able to restore channel activity in A6 cells even after pertussis toxin or mepacrine treatment. Even more specifically, the 5-lipoxygenase inhibitor nordihydroguaiaretic acid inhibited P_0 and N in inside-out patches from A6 cells, while leukotriene D_4 restored both parameters [121].

Thus it has been proposed that the Na^+ channel is tonically activated by a G-protein so that pertussis toxin inhibits basal Na^+ transport and activation of the G-protein stimulates it, and that this action is mediated by a leukotriene [117, 121]. However, this mode of G-protein regulation is quite unusual in that no receptor has been identified that would activate the G-protein, and one must question to what autacoid such a receptor would normally respond. Would it be activated by intracellular second messengers rather than by extracellular autacoids? Is it even possible that the α subunit of the G-protein might migrate from the basolateral membrane to become associated with the channel in the apical membrane. At this point the channel-associated G-protein or proteins are potentially very potent and important regulators of Na^+ transport, but the signals to which they respond in the intact tissue remain unknown.

Intracellular calcium as a regulator of channel activity

Increases in intracellular Ca^{2+} have long been known to be associated with a decrease in apical membrane Na^+ permeability in toad bladder [122, 123], frog skin [124], and the rabbit cortical collecting duct [110, 125, 126]. However, it does not appear that Ca^{2+} interacts directly with the channel protein. Using excised patches of the luminal membrane of rabbit CCD, Palmer and Frindt [125] showed that changes in the Ca^{2+} concentration of the medium had no effect on either the number (N) or open probability (P_0) of the channels; however, in cell attached patches, 1 to 10 μM ionomycin decreased NP_0 to less than 10% of control levels in 10 minutes. These investigators proposed that intracellular Ca^{2+} was indirectly regulating the channel, possibly via calmodulin, PKC, or phospholipase-dependent products. However, there is as yet no information

about which of the channel subunits might be involved in the final effect of intracellular Ca^{2+}.

Is the effect of AVP on Na^+ transport mediated by insertion of channels or activation of channels already present in the apical membrane?

Various investigators have argued in favor of and against the hypothesis that AVP increases the number of Na^+ channels in the apical membrane of target epithelia by insertion of channels from an intracellular pool. Garty and Edelman [54] provided convincing evidence for an insertion mechanism by using trypsin to inactivate channels that were accessible to the extracellularly applied enzyme. Using the toad bladder they discovered that trypsin irreversibly inhibited Na^+ transport and that the effect was prevented when the channel was protected by prior addition of amiloride. They found that treatment with trypsin prevented the stimulation of Na^+ transport by aldosterone even though the trypsin was removed before addition of aldosterone [54]. This effect would be compatible with the view that aldosterone activates channels already present in the apical membrane, perhaps by methylation [55], and that it does not cause the insertion of channels that are either newly synthesized or present in an intracellular pool. On the other hand, prior trypsin treatment does not reduce the stimulatory response to AVP, indicating that the channels mediating the AVP-dependent stimulation were not previously exposed to trypsin on the extracellular surface [54]. This result could indicate that AVP causes insertion of Na^+ channels in much the same way that it causes insertion of water channels, that is, by fusion of subapical membrane vesicles containing the channels with the apical membrane [127]. Alternatively, AVP might activate Na^+ channels already present in the membrane by a steric rearrangement that exposes a site that was previously unavailable to the action of trypsin, or else the channels activated by AVP might be different from those acted on by aldosterone.

The idea that Na^+ channels can be inserted from a cytoplasmic pool is also supported by the observations of Lewis and his associates [86, 89] that abrupt pulses of hydrostatic pressure increased amiloride-sensitive Na^+ transport and luminal membrane area in the rabbit urinary bladder by incorporation of subapical membrane vesicles.

Although Sariban-Sohraby et al [95] have demonstrated in vitro phosphorylation of the Na^+ channel α subunit in the presence of ATP and cAMP-dependent kinase, it is possible that this phosphorylation alone is insufficient to increase Na^+ transport. Lester, Asher and Garty [128] have shown that membrane vesicles isolated from toad bladders treated with AVP have an increased rate of amiloride-sensitive $^{22}Na^+$ uptake compared to controls. However, when the vesicles isolated from control bladders were loaded with cAMP, ATP and cAMP-dependent protein kinase, there was no stimulation of transport. On the other hand, in studies using patch-clamping of toad bladder luminal membrane, Frings, Purves and Macknight [129] observed that the addition of protein kinase A, cAMP and ATP to an excised patch which had initially shown a single Na^+ channel that had become quiescent, resulted in a resumption of channel activity. However, this effect was observed in only three of nine attempts.

Lester et al [128] interpreted their observations to indicate that although the phosphorylation process occurs in vitro, another element or elements of the normal cell are needed in order to increase channel activity. For example, an intact cytoskeleton might be required to insert new channels from subapical membrane vesicles, or to rearrange channel subunits within the membrane, after phosphorylation of the α subunit. In fact, Smith et al [97] have have shown that the β subunit of the Na^+ channel protein has binding sites for both ankrin and fodrin (Table 1).

Immunohistochemical approaches have provided evidence both for and against the presence of a pool of Na^+ channels in the cytoplasm of other AVP responsive epithelia. A polyclonal antibody against the purified Na^+ channel protein developed by Sorscher et al [94] was found to bind only to the apical membrane and not to any cytoplasmic elements in A6 cells or rat or bovine renal medullary collecting ducts [130, 131]. However, it should be recognized that it would be difficult with this technology to resolve the presence of specific binding to small membrane vesicles directly under the plasma membrane. It is also possible that the channel subunits reacting with the antibody are continuously present only in the plasma membrane, and that they combine with another subunit or subunits which are introduced into the membrane by vesicle fusion and are not recognized by the antibody. In contrast to these results, the anti-idiotypic monoclonal antibody of Kleyman et al [91], which is directed against the amiloride-binding subunit, does exhibit intracellular binding. Using surface labeling techniques, this antibody was shown to react only with the apical membrane, as expected. However, using immunostaining of sections of A6 cell monolayers, Kleyman et al [91] observed antibody binding in the subapical cytoplasm, apparently to vesicles, as well as apical membrane binding. Lesser but significant immunoreactivity was also observed in other regions of the cytoplasm, including the basolateral region.

Some results of patch clamp studies also support the insertion mechanism of AVP action. Although AVP increased amiloride-sensitive Na^+ transport in intact IMCD cells, addition of AVP or 8-Br-cAMP caused no increase in NP_0 in cell-attached patches, and there was no change in P_0 when excised patches were exposed to ATP and the catalytic subunit of cAMP-dependent protein kinase [17]. Perhaps the most convincing support for the insertion mechanism comes from patch-clamp studies in the laboratory of Eaton and his collaborators [82, 83]. When A6 cells were treated with aldosterone, excised patches showed an increase in P_0 for the Na^+ channel that could be prevented by methylation inhibitors, which also decreased the average number of channels (N) observed in the patches from 2.1 to 0.4 [82]. In contrast, in excised patches from A6 cells treated with AVP there was no change in P_0, but there was a change in the number of channels observed in the patches [83]. Statistical analysis of more than 200 excised patches showed that there was an average of 2.0 ± 1.5 ($\pm$ SD) channels per patch from control cells, but in patches from AVP-treated cells there was a bimodal distribution: approximately one-half of the patches had 1.8 ± 1.2 ($\pm$ SD) channels but the other half had an average of 9.2 ± 1.5 ($\pm$ SD) channels [83]. This result would be compatible with an action of AVP to cause the insertion of new channels into the membrane resulting in "hot spots" of increased channel density that are variably present in the small

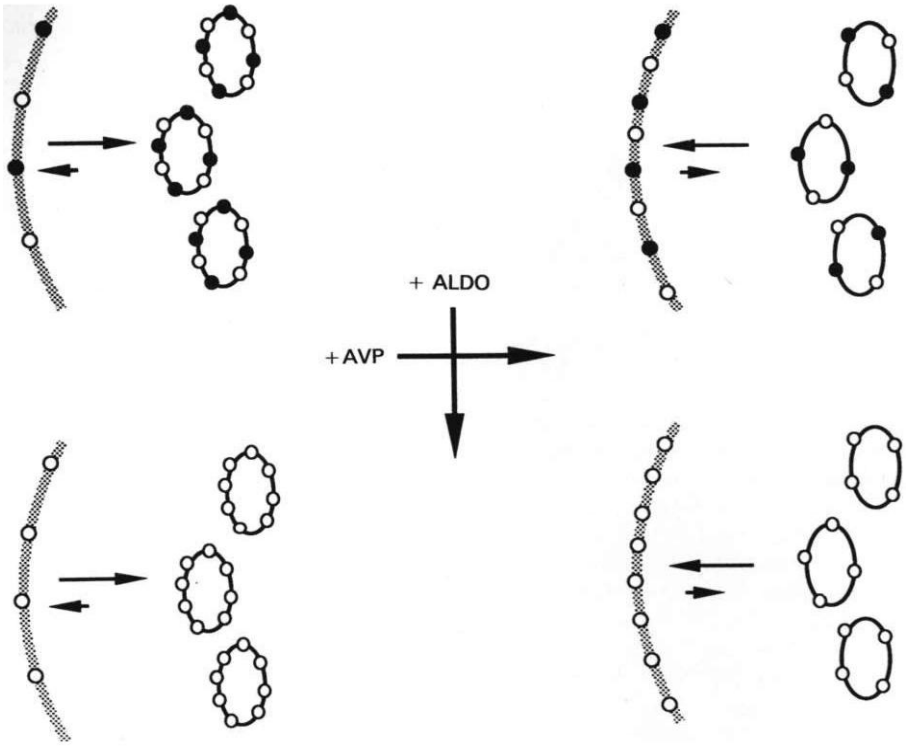

Fig. 5. *A model to explain synergism between AVP and mineralocorticoids in stimulating Na^+ reabsorption by increasing the number of active Na^+ channels.* Each of the four panels depicts the apical plasma membrane on the left with underlying subapical membrane vesicles to the right. Both the plasma and vesicle membranes are assumed to contain two types of Na^+ channels. Inactive channels, shown by closed circles, which are incapable of Na^+ transport, and active channels, shown by open circles. The active channels fluctuate between an open (conducting) state and a closed state, and may be blocked by amiloride, whereas the inactive channels are nonconducting at all times unless converted to open channels. The model assumes that, as in the case of the water channel, the open and closed channels in the plasma membrane are in equilibrium with the vesicular pool. In the absence of vasopressin, the equilibrium is biased toward retrieval, while in the presence of the hormone channels are shifted from the vesicles into the plasma membrane. It is assumed that aldosterone operates primarily by converting inactive channels to active ones, both in the plasma membrane and in the subapical membrane vesicles. Therefore, AVP produces a greater increase in the number of active Na^+ channels in the apical membrane in mineralocorticoid treated cells because those channels inserted from the subapical pool are predominantly in the active form. The same synergism would result if the pool of channels resided in the plasma membrane rather than in subapical membrane vesicles, and if this reserve pool were normally inaccessible to degradation by trypsin until the channels or subunits of the channels were reoriented in the membrane through the action of AVP.

patched membrane area. Marunaka and Eaton [83] also found that when AVP or dibutyryl-cAMP was added to the medium, there was no increase in the number of channels in previously-formed cell-attached patches. These and the similar observations on cell attached patches of IMCD cells by Light et al [17] may indicate that when the membrane is dissociated from the underlying cytoskeleton by formation of the patch, Na^+ channels can no longer be inserted.

Insertion and activation: A possible mechanism for AVP and aldosterone synergism?

The distinct actions of aldosterone and AVP in stimulating the Na^+ channel discussed in the preceding section may provide an explanation for the synergism in their actions as observed in both the rat CCD [2, 3] and in the toad bladder [11, 13]. We offer the scheme shown in Figure 5 as a working hypothesis. It would appear that Na^+ channels exist in both an active and inactive state and that aldosterone increases Na^+ transport by increasing the number of active channels (bottom panels in Fig. 5), perhaps by methylation of inactive channels [55, 82]. In the model we presume that there is an additional pool of Na^+ channels in subapical membrane vesicles, which is also converted from the inactive to the active form through the action of aldosterone. We presume also that this cytoplasmic pool of channels is in equilibrium with the channels in the apical plasma membrane. If AVP were to act to shift this equilibrium from retrieval to insertion (from left to right in Fig. 5) in a way similar to its effect on water channels [127], this would account for the increase in Na^+ transport and in the relative number of channels observed after AVP treatment. However, the increase in the number of active Na^+ channels produced by insertion in the aldosterone-treated epithelium would be much greater than that in the untreated epithelium because the additional channels inserted would all be in the active form.

Obviously, the support for this model is presently at best only circumstantial and is complicated by the lack of direct evidence for a cytoplasmic pool of channels. However, the "inaccessible" pool of channels do not necessarily have to be in subapical membrane vesicles for the model in Figure 5 to apply. This pool of channels could also reside in direct association with the apical membrane and be reoriented within the membrane through the action of AVP via the cytoskeleton as discussed above. This mechanism would produce the same synergism of AVP and aldosterone actions as the vesicle shuttle mechanism presented in Figure 5.

Summary

A variety of experimental approaches have shown that AVP and mineralocorticoids stimulate Na^+ transport through their effects on the number and kinetic properties of amiloride-sensitive Na^+ channels in the apical membrane. The different mechanisms by which AVP and mineralocorticoid act on the Na^+ channel provide a basis for synergism in their actions, perhaps by a scheme such as that proposed in Figure 5. However, the details of this interaction will require a better understanding of the molecular details involved in activating quiescent channels, increasing their open probability, and reorientating or inserting channels to an operational position in the apical membrane.

Electrophysiological and biochemical approaches have gone a long way toward elucidating some of these molecular details. But the latter approach in particular has indicated that the Na^+ channel may have multiple regulatory subunits and thus be a target for several intracellular second messengers and autacoids other than those involved in the actions of AVP and aldosterone. The challenges for future research in this area are multiple. It seems likely that the primary amino acid sequence of the channel subunits will soon become available from cloning and sequencing approaches, but the application of this knowledge to understanding how the subunits are integrated into the complete protein and mediate regulatory signals will be a formidable task. It will be important to determine the normal extracellular signals (other than aldosterone and AVP) and the associated intracellular second messengers that alter channel activity. It will also be important to understand how some species such as the rabbit may "turn off" the stimulatory effect of AVP on Na^+ reabsorption in the CCD, and how this regulatory process is altered when these cells are cultured. At the whole animal level, it will also be important to investigate whether changes in one or more of the normal regulatory pathways that impinge on the Na^+ channel might be involved in a diminished ability to excrete a salt load, as is observed in some models of hypertension. All of these issues need to be understood at the molecular level, and it seems likely they will provide exciting physiological insights at all levels.

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Note added in proof

Kleyman et al (1991) have recently provided evidence supporting the model of aldosterone and AVP interaction presented in Fig. 5. In their experiments, these investigators used apical membrane surface labeling by radioiodination followed by immunoprecipitation of labeled Na^+ channels with a specific antibody in order to assess the relative density of channels in the apical membrane of A6 cells. They also used pulse-chase labeling to measure the rates of channel biosynthesis. They found that aldosterone, which increased short-circuit current by 6.3-fold in the cultured monolayers, had no effect on the density of apical membrane channels or the rate of channel biosynthesis. On the other hand, AVP produced a more than two-fold increase in the apical pool of Na^+ channels. Kleyman et al (1991) interpreted their results to show that aldosterone increases apical membrane Na^+ conductance by activating channels already present in the membrane whereas AVP acts by increasing the number of channels in the apical membrane.

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