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# Structure and Function of Amiloride-sensitive $Na^+$ Channels

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## Introduction

Sodium-reabsorbing epithelia such as the renal distal tubule and collecting duct, distal colon, and sweat duct play a major role not only in the function of the specific organ system in which they are found, but also in whole body sodium and fluid homeostasis. Further, net transepithelial $Na^+$ transport across these epithelia requires localization of the $Na^+$, $K^+$-ATPase to the basolateral plasma membrane [39, 70, 102, 147], and $Na^+$-selective ion channels to the apical or luminal plasma membrane [13, 43, 128, 140]. These $Na^+$-entry channels are rate limiting for overall transepithelial transport and are regulated hormonally, for example, by the peptide hormones vasopressin and oxytocin and the mineralocorticoid hormone aldosterone. These ion channels are inhibited by the diuretic drug amiloride and many of its congeners [13, 15, 71]. Current wisdom has held that amiloride-sensitive $Na^+$ channels are restricted to epithelial tissues, but recent work by Van Renterghem and Lazdunski [157], Kupitz and Atlas [79], and Bubien and Warnock [22] clearly demonstrate the presence of regulated, amiloride-sensitive $Na^+$ channels in nonepithelial cells, namely, vascular smooth muscle, oocytes, and human and rat lymphocytes, respectively. Experimental evidence from several laboratories has indicated that amiloride-sensitive $Na^+$ channels are also present in distal regions of the mammalian lung (*see refs.* 96, 100, 110, and 163 for discussion). Single $Na^+$ channel activity has been measured in two ways: either by the patch clamp technique [26, 32, 40, 48, 53, 54, 68, 88, 90, 92, 113, 114, 116, 117, 125, 126, 160, 161], or reconstitution studies in planar lipid bilayers into which amiloride-sensitive $Na^+$ channels have been incorporated [18, 31, 64, 108, 109, 111, 112, 132].

Electrophysiological and pharmacological evidence accumulated over the past five years indicates that there may not be a unique class of amiloride-sensitive $Na^+$ channel, but rather a family of these channels [42, 114, 140]. Epithelial $Na^+$ channels can be differentiated as having either a high (H) or low (L) sensitivity to amiloride (i.e., an apparent equilibrium inhibitory dissociation constant ($K_i$) of less than or greater than 1 μM at high extracellular [$Na^+$], respectively, *see* Fig. 1). Further, within each group, the channels display either high or low $Na^+$ *vs.* $K^+$ selectivity. Extracellular conditions as well as whether the channels are observed in native *vs.* cultured cells can affect the relative distribution of the various channel types [55, 90]. Eaton and collaborators [90, 92] and Palmer and Frindt [116, 117] have provided evidence that highly sodium-selective channels exist in the cortical regions of renal collecting tubules, whereas relatively nondiscriminatory amiloride-sensitive $Na^+$ channels are present in the medullary collecting ducts [77]. While the definition of H- and L-type channels has arbitrarily arisen based on sensitivity of the channel to amiloride, it is likely that this criterion will not stand the test of time (*cf.*, ref. 172). Perhaps a better way to differentiate easily between H- and L-type channels is the relative inhibitory efficacy of EIPA compared to

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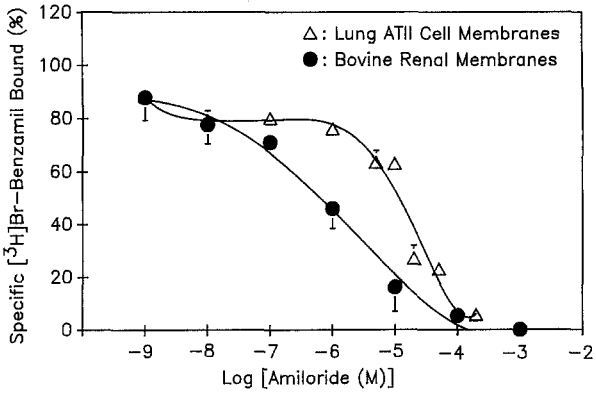


**Fig. 1.** Bromobenzamil binding displacement by amiloride in apical membrane vesicles prepared from rabbit lung alveolar type II pneumocytes and bovine renal papillary collecting tubules.

amiloride. H-type channels are relatively insensitive to EIPA, whereas for L-type channels EIPA inhibits better or at least as well as amiloride.

Palmer [114] has proposed another classification system for epithelial $Na^+$ channels, based primarily upon single channel conductance followed by $Na^+/K^+$ selectivity. He divides epithelial $Na^+$ channels into three groups: types Na(5), Na(9), and Na(28). The type Na(5) channels have a very low single channel conductance (4–5 pS), a very high selectivity for $Na^+$ over $K^+$ (>10:1), and a high amiloride-sensitivity ($K_i$ ~ 0.1–0.5 μM). These channels are observed in native tissues, and in cultured cells. The type Na(9) channels have a moderate conductance (7–15 pS), and a lower Na/K selectivity (3:1) and are the dominant channel observed in cells cultured on nonpermeable supports. In contrast to the Na(5) channels, the mean open-closed transition states of Na(9) channels are much faster (10–100 msec *vs.* sec), and their *I-V* curves do not display Goldman-type rectification. According to Palmer [114], current transitions of both Na(5) and Na(9) channels are weakly voltage dependent: Na(5) channels are opened by hyperpolarizations, while the Na(9) channels are opened by depolarizations. The third type of epithelial $Na^+$ channel, Na(28), displays a relatively large single channel conductance (>20 pS) shows little or no discrimination between $Na^+$ and $K^+$, and has been observed in primary cultures of rat inner medullary collecting duct cells [88] and cultured mouse cortical collecting ducts [2]. This channel is primarily regulated by cGMP [86–89]. Perhaps the most distinguishing and useful criterion for differentiating amiloride-sensitive $Na^+$ channels is their mode of regulation. Regardless of the classification system used, the point to be made is that there exists a variety of amiloride-sensitive $Na^+$ channels with different biophysical characteristics, different amiloride analogue pharmacologies, and different tissue distributions. How these channels are related to one another on a biochemical and molecular level is at present obscure. There is as yet no direct evidence all of these channels simply represent post-translational variants of the same gene product, or are derived from different gene families. This issue is perhaps one of the most intriguing of the field, and many different laboratories are, in one form or another, working hard towards its resolution.

The regulation of amiloride-sensitive $Na^+$ channels differs markedly in various tissues both between and within species. For example, although the basic transport characteristics of amiloride-sensitive $Na^+$ channels are similar in the principal cells of cortical collecting ducts (CCD) from the rat and rabbit, this transport system responds very differently to arginine vasopressin (AVP) and mineralocorticoids [134]. In the rat CCD, AVP produces a dramatic and sustained stimulation of $Na^+$ transport that is synergistic with the stimulatory effects of mineralocorticoids like aldosterone. In contrast, in the rabbit CCD, AVP produces only a transient stimulatory effect on $Na^+$ transport. Moreover, AVP-stimulated $Na^+$ transport is inhibited by prostaglandin $E_2$ ($PGE_2$), increased $[Ca^{2+}]_i$ and PKC activation in the rabbit CCD; however, these agents have no effect in the rat CCD. If, on the other hand, rabbit CCD cells are grown in primary tissue culture, AVP produces a stable stimulation of amiloride-sensitive $Na^+$ transport that is additive to that induced by aldosterone. Further, in these cultured cells, $Na^+$ transport is stimulated rather than inhibited by $PGE_2$.

In cultured epithelial cells, the expression of amiloride-sensitive $Na^+$ channels may depend upon the culture conditions and degree of differentiation of the cells. Yue et al. [171] observed a decreased $Na^+$ conductance concomitant with $Na^+$ channel internalization and degradation in adult rat alveolar type II cells with increasing length of time in culture. A6 cells express few $Na^+$ channels when grown on petri dishes or impermeable supports [129], and when patch clamp experiments are performed on cells grown under these conditions, the predominant channel is an H-type, low $Na^+$ selectivity channel [26]. When the same cells are grown on permeable supports they express more channels which are H-type with high Na selectivity [54, 55]. There are also some differences in the responses of these channels to regulatory influences such as pertussis toxin (*see below*). However, mRNA from cells grown under either condition will result in expression of similar H-type, high $Na^+$ selectivity channels when injected in *Xenopus* oocytes [115]. It would appear that there are simply more channels expressed when message is taken from cells grown on permeable supports. It may be that higher conductance, lower selectivity amiloride-sensitive channels are just different manifestations of the more prototypical low conductance, highly $Na^+$-selective channel seen in native tissue (*see,* for example, ref. 83). As another example of this, Chinet et al. [32] reported that low $Na^+/K^+$ selective

(1.33), relatively high conductance (20 pS) amiloride-sensitive channels were present in excised patches of human nasal epithelium. The channels' selectivity for $Na^+$ over $K^+$ was much higher in cell-attached patches (i.e., >6), although the single channel conductance was the same. The development of improved and better defined culture conditions should maximize and standardize the expression of $Na^+$ channels, thereby allowing more useful inter-laboratory comparisons to be made [67, 142].

During the past decade, a number of ligand and voltage-gated ion channels have been purified in a functionally competent state, and the genes for many of these channels have now been cloned [28, 30, 80, 121, 150, 152, 154, 155]. This work has proceeded because of the availability of high affinity probes for these channels, and the existence of rich biological sources containing high densities of these channels. In contrast, an elucidation of the molecular details of epithelial $Na^+$ channels has lagged behind those of nerve and muscle membranes, primarily because of the lack of tissues with high channel density, and by the lack of suitable molecular probes. The existence of amiloride-sensitive $Na^+$ channels in lymphocytes may change this situation. In spite of these drawbacks, several significant contributions have been made: for example, (i) the successful synthesis of radioactive, high affinity analogues of amiloride [11, 74, 82]; (ii) the use of these analogues as molecular probes for the isolation and purification of amiloride-sensitive channel protein from A6 cells and bovine renal papillary collecting ducts [8–11, 14, 108, 110]; (iii) the generation and use of polyclonal and monoclonal antibodies against this channel protein for immuno-detection both in vivo and in vitro [21, 73, 75, 144, 151]; and, most notably, (iv) the recent expression cloning of a rat distal colon amiloride-sensitive $Na^+$ channel [24, 25, 93].

This work has now enabled researchers in this area to embark upon studies concerning the cellular and molecular biology of epithelial $Na^+$ channels previously not possible. For example, antibodies raised against polypeptides comprising epithelial sodium channels have been used to examine channel biosynthesis and interaction with the cytoskeleton [141], for building immunoaffinity matrices for rapid channel isolation [108], and to screen cDNA expression libraries [146]. The availability of functionally competent purified channel protein as well as expressible channel cDNA will enable the development of appropriate experiments to elucidate the nature of channel block by amiloride and structurally related compounds, channel regulatory mechanisms, and to determine which subunit (or the minimal number of subunits) is (are) required for channel activity [24, 25, 93, 145, 146].

In this review, we have three main objectives. First, we will summarize what is known about the biochemistry of amiloride-sensitive $Na^+$ channels. Second, we will discuss the hormonal and second messenger regulation of these channels, particularly in light of certain controversies surrounding channel activation. Third, we will discuss the exciting new molecular biological findings concerning these channels. Throughout, we will highlight areas where we feel major research efforts need to be concentrated.

## Biochemistry of Amiloride-sensitive $Na^+$ Channels

### $Na^+$ Channels Can Be Purified and Functionally Reconstituted

As indicated earlier, electrophysiological and pharmacological evidence intimates the existence of several classes of amiloride-sensitive ion channel. Preliminary evidence suggests that these classes of epithelial sodium channels differ in their biochemical composition as well. The amiloride analogue structure/inhibitory patterns displayed by L-type $Na^+$ channels are dissimilar to those of the H-type $Na^+$ channel. Both phenamil and benzamil can inhibit L-type channels with the same or slightly greater affinity than amiloride, and EIPA has the same inhibitory potency as the parent amiloride molecule [140]. For H-type channels, phenamil and benzamil are better inhibitors than amiloride, but EIPA is virtually ineffective as an inhibitor. As stated earlier, it is becoming increasingly clear that the amiloride analogue pharmacological profile rather than the amiloride inhibitory constant is a more meaningful distinguishing characteristic between H- and L-type channels.

At present, six different laboratories have succeeded in purifying and reconstituting putative amiloride-sensitive, epithelial $Na^+$ channels. Cherskey [31] reported the isolation and reconstitution of an amiloride-sensitive ion channel from bovine trachea. This channel, isolated by amiloride affinity chromatography, was reconstituted into planar lipid bilayers, and displayed the following characteristics: linear current-voltage relation, when bathed with symmetrical salt solutions, 100:1 $Na^+$ *vs.* $K^+$ selectivity, single channel conductance of 6 pS, and a $K_i$ of amiloride of 0.1 μM. However, no biochemical characteristics of this affinity-purified protein were or have been reported. Branco and Varanda [18] succeeded in incorporating native toad urinary bladder apical membrane vesicles into solvent-free planar bilayers. They observed a voltage-dependent block by amiloride, with a $K_i$ of 0.98 μM, and a channel $P_{Na}/P_K$ of 2. The single channel conductance was 160 pS. Unfortunately, no biochemical information concerning this channel protein has been forthcoming. Work from our laboratory [13–15] has described the biochemical characteristics of H-type $Na^+$ channels isolated from bovine kidney papilla and amphibian A6 cells. This putative channel protein

has, in the native state, a molecular mass averaging 730 kD, and is comprised of at least six nonidentical associated proteins held together by disulfide bonds. These polypeptides have apparent molecular masses of 300, 150, 95, 70, 55, and 40 kD. Reconstitution of the purified native channel protein into planar lipid bilayers showed high amiloride sensitivity ($K_i$ = 10–50 nM; ref. 101), a $Na^+/K^+$ selectivity ratio of 3–7, and a wide range of single channel conductances (from 4 to 80 pS). EIPA had no effect on channel activity up to 10 μM. Moreover, as discussed below, reconstituted channel activity could be modulated by several post-translational modifications known to affect channel function in vivo, and could be experimentally attributed to one or more of the associated proteins in the isolated complex. Only the 150 kD polypeptide binds amiloride, a finding later substantiated by Kleyman et al. [74]. This 150 kD polypeptide also binds ankyrin, and is linked to the actin cytoskeleton via ankyrin's interaction with fodrin [141]. Interestingly, the 150 kD polypeptide was purified independently of the other associated proteins (except possibly the 41 kD G protein) by Sariban-Sohraby et al. [127], reconstituted into liposomes, and shown by patch clamp to produce 5–10 pS, amiloride-blockable channels. These results indicate that this polypeptide itself may be sufficient to form a conductive pore. These observations also suggest that the channel complex isolated consists of polypeptides constituting the conductive pathway and other regulatory proteins that are not themselves pores.

Barbry and collaborators [8–11] have purified and characterized an amiloride-binding protein from the pig kidney outer medulla and cortex. They found in native membrane vesicles that both amiloride and EIPA inhibited $^{22}Na^+$ influx with equal potency ($K_i$ = 6 μM). The protein purified from this preparation was reconstituted into lipid vesicles, and showed electrogenic amiloride-sensitive $Na^+$ transport ($K_i$ for amiloride, phenamil, EIPA = 10 μM, suggestive of an L-type channel). This purified protein had a total molecular mass of 185 kD, and consisted of two identical 105 kD polypeptides cross-linked by disulfide bonds [10]. Recently, this group cloned an amiloride-binding protein from a human kidney cDNA library by using an oligonucleotide probe designed from amino acid analysis of tryptic fragments of the purified 105 kD subunit from pig kidney [7]. Transfection into eukaryotic cell lines showed that the cloned cDNA encoded an amiloride-binding protein. Surprisingly, the amiloride analogue pharmacological binding profile was indicative of an H-type channel, i.e., benzamil > amiloride > EIPA. This protein was glycosylated, but did not contain any consensus phosphorylation sites, and also had a very hydrophilic hydropathy plot. Further, these investigators failed to detect amiloride-sensitive $Na^+$ channel activity in transfected mammalian cells or in a *Xenopus* oocyte expression system. Verity and Fuller [158] have made essentially similar findings, and further demonstrated that the tissue distribution of this cloned amiloride-binding protein was not as would be expected for a $Na^+$ channel, and that its expression was not regulated by corticosteroids. Novotny et al. [105] have now reported that this amiloride-binding protein is a diamine oxidase, an enzyme that regulates cell growth through the metabolism of putrescine and histamine. These observations are also consistent with those of Goldstein et al. [49] who showed that epithelia contain a high affinity amiloride receptor other than the $Na^+$ channel. These findings underscore the difficulty with protein reconstitution approaches. Because the human amiloride-binding protein clone can express a protein capable of binding amiloride (with high not low affinity) but not of inducing channel activity, it is likely that the channel activity observed in the protein reconstitution experiments was due to a minor component not detected biochemically. Moreover, it is apparent that caution must be exercised when interpreting protein purification data using amiloride binding as a sole criterion.

Blazer-Yost and Recio-Pinto [16] have immunoaffinity purified to homogeneity a 700–800 kD protein from toad urinary bladder. They used a monoclonal antibody raised against a 65–70 kD aldosterone-induced glycoprotein from toad bladders as their affinity matrix. After reduction, this large 700–800 kD polypeptide dissociated into five different molecular mass proteins, each showing a microheterogeneity with regard to isoelectric mobility. Upon incorporation of the holo-protein complex into planar bilayers, a range of channel types were observed. Low conductance channels (<20 pS) had fast dwell kinetics and were sensitive to amiloride. Channels with higher conductances (up to 100 pS) were slower, but were amiloride insensitive. All of the channels were cation selective, with a $P_{Na}/P_K$ of about 3. Because of the sensitivity of single channel recording techniques, interpretation of biochemical purification-reconstitution experiments must be tempered by the fact that a small amount of "contaminant" protein in the preparation, undetected by standard biochemical evaluation methods, may be mediating the observed conductance changes. However, the use of specific purification protocols, coupled with specific antibodies and independent verification methods (i.e., amiloride-binding enrichment, concomitant biochemical modification of associated polypeptides within the purified channel complex), support the concept that the biochemical structures identified do in fact constitute an amiloride-sensitive channel complex. Although protein purification and reconstitution are useful and essential procedures, the ultimate identity of any candidate protein as an ion channel necessitates molecular biological approaches.

In bilayers, purified and reconstituted epithelial $Na^+$ channels have conductances ranging from 4–80 pS, yet these different conductance channels display comparable $Na^+/K^+$ selectivity (5:1), amiloride sensitivity ($K_i$ = 100–700 nM), and kinetics. Because the prototypical amil-

oride-sensitive $Na^+$ channel has a very low conductance and high $Na^+/K^+$ permselectivity, can this or any other candidate channel protein complex exhibiting aberrant conductance and/or selectivity properties be the "*correct*" one? The literature is replete with examples of a given class of specific ion channel type that has widely varying biophysical characteristics [57]. For example, inward-rectifying $K^+$ channels have single channel conductances ranging from 3 to 45 pS, and glutamate-receptor cation channels show conductances of 2 to more than 100 pS. For the latter channels, different though homologous cDNAs have been cloned [136], suggesting different structural isoforms of the same class of channel. On the other hand, mutations in the cDNA encoding a specific channel type, biochemically produced post-translational modifications, or toxin treatment, can dramatically alter single channel conductance and/or ion selectivity [58, 63, 124, 137]. Further, membrane lipid composition can dramatically influence channel open times [23]. In fact, event histogram analysis of bovine renal bilayer channel records indicate a major subconductance state of 10–12 pS. Thus, the observed biophysical diversity among amiloride-sensitive $Na^+$ channels can, in part, result from any of the above factors.

Clearly, a major cautionary warning must be issued when interpreting bilayer reconstitution studies, for two reasons: first, the extreme sensitivity of the assay enables the detection of only a single protein molecule, and second, because a purified protein has been extracted from its native environment and inserted into a totally artificial one, it may not behave as expected. Under such conditions, it is not surprising that different channel conductance states will be observed [120]. But because the proteins we have purified from bovine kidney and A6 cells are at first glance remarkably similar in size and general composition, and because they reconstitute regulatable, high amiloride-sensitive $Na^+$ channels, features seen in mammalian papillary collecting ducts in vivo [143, 156], and because native epithelial $Na^+$ channel regulators (e.g., PKA, PKC phosphorylation) can be experimentally assigned to one or more of these purified polypeptide components, this purification-reconstitution approach appears as a viable and reasonable way to elucidate channel regulatory characteristics. What is not yet known is whether there are unique and distinct channel proteins, or if a single channel prototype with slight biochemical differences, or differences in intracellular environment (or a combination of any of these factors), underlie the expanding biological repertoire of amiloride-sensitive channels.

## Antibodies Aid in Elucidating Immunological Similarity Between Different $Na^+$ Channels

Polyclonal antibodies generated against purified H-type $Na^+$ channels from bovine kidney have been used to immunolocalize the channel to the apical membranes of numerous $Na^+$ transporting epithelia, and can cross-react against functional L-type epithelial channels, but not against any other transport or cytoplasmic proteins [51, 108, 139]. The specificity of these antibodies against amiloride-sensitive $Na^+$ channels has been well characterized in rabbit and bovine alveolar type II cells (99, 100, 110], and have been used to immunolocalize presumptive L-type $Na^+$ channels in fixed [100] or frozen rat lung alveolar type II cells [99], canine lingual epithelial cells [139], and guinea pig cochlear hair cells [51]. However, these antibodies do not cross-react with the fusion protein of Barbry et al. [7], nor do antibodies raised against Barbry's purified protein or their fusion protein cross-react with the purified bovine H-type $Na^+$ channel protein (*unpublished observations*), observations consistent with the idea that the 700 kD complex isolated from bovine kidney and A6 cells does not contain diamine oxidase. These H-type channel antibodies cross-react with a 135 kD polypeptide of the alveolar ATII cell membranes. Anti-idiotypic antibodies and NMBA photolabeling studies consistently recognize the same molecular mass protein in renal or alveolar tissue [72]. Because the anti-$Na^+$ channel antibodies reacted with at least four different subunits of the H-type $Na^+$ channels from bovine kidney and A6 cells, but with only one polypeptide of the presumptive L-type $Na^+$ channels in ATII cells, the idea of a single type of $Na^+$ channel with two different amiloride affinity sites may not be true. However, this cannot be rigorously excluded until a biochemical isolation and purification scheme for L-type channels has been worked out. Our laboratory has nonetheless proposed the hypothesis that different amiloride-sensitive $Na^+$ channels with distinct biochemical structures exist. Further, these channels have highly interactive components that modulate the activity of the conduction pathway(s). This hypothesis is consistent with the findings of widely variable kinetic and pharmacological characteristics of amiloride-sensitive $Na^+$ channels [13, 37, 43, 140].

It should be emphasized that the biochemical structure of L-type epithelial $Na^+$ channels has not yet been elucidated, but strides in this direction are being made. We have recently been successful in purifying and reconstituting an L-type cation channel from bovine distal lung and rabbit alveolar type II cells [137]. Preliminary biochemical analysis of this immunopurified protein reveals only a single polypeptide of Mr ~ 130 kD. These immunopurified proteins exhibited single $Na^+$ channel activity when reconstituted into planar lipid bilayers. Amiloride, from only one side of the bilayer, inhibited the mean current and single channel open probability ($P_o$) in a dose-dependent fashion, with an apparent $K_i$ of 8 μM. Furthermore, 1 μM EIPA reduced $P_o$ from its control value of 0.50 to 0.25, and 4 μM EIPA completely inhibited channel activity. Very similar results were obtained using either anti-$Na^+$ channel antibodies or anti-

αrENaC antibodies (137; *see below*) in the immunoaffinity column purification protocol. Interestingly, these channels could be activated by phosphorylation mediated by PKA + ATP (only on the site opposite to which amiloride is effective) even in the apparent absence of a 300 kD subunit. Thus, these new results support the hypothesis that L- and H-type channels are functionally and biochemically distinct, and that immunopurified proteins isolated from mammalian distal lung epithelial cells can function as an ion channel. While certainly not yet comprehensively understood, especially because the subunit or subunits mediating ion conduction have not been conclusively identified, it is becoming increasingly clear that amiloride-inhibitable ion channels are equipped with a multiplicity of inputs that modulate channel activity.

## Regulation of Amiloride-sensitive $Na^+$ Channels

Perhaps the most unique physiological feature of amiloride-sensitive $Na^+$ channels is the wide variety of regulatory signals to which they respond, reflecting the channels' function as the limiting barrier for $Na^+$ reabsorption in many epithelia. Channel activity is regulated by the hormones vasopressin [13, 43], aldosterone [41], insulin [65], and atrial natriuretic factor [101]. These hormonal effects have been shown, in some cases, to be not only additive, but synergistic [65], suggesting multiple regulatory sites. In addition, channel activity is altered by a number of physiological conditions including stretch [84], basolateral tonicity [166], alterations in apical $Na^+$ concentration [91], and intracellular $Ca^{2+}$ and pH [117, 138]. Proposed intracellular mediators include protein kinase A [64, 111, 122], protein kinase C [91, 169], tyrosine kinase [95], heterotrimeric G proteins [5], leukotrienes [26, 27], and transmethylation reactions [130].

In all instances of channel regulation reported, alterations occur either in channels kinetics (open probability or open time), channel number, or both. Channels continue to behave as a single population without significant alteration in single channel conductances within a given system. Several hypotheses have been proposed to account for these types of regulatory response: channel kinetics could be altered by a direct action on the channel or some regulatory component [36, 43, 64, 111], channel number could be affected either by activation of quiescent channels already resident in the membrane [43, 44], by insertion of preformed channels from some subapical domain [74, 84, 94], or by *de novo* synthesis of new $Na^+$ channel protein and/or activating subunits [42, 43]. Given the extraordinary versatility of channel response to such a wide variety of regulatory inputs, it is also evident that multiple mechanisms may interact. Herein lies, perhaps, the key to the biochemical complexity of the channel described above.

Although the molecular events mediating hormone regulation of channel activity have not been completely elucidated, significant progress has been made in recent years through the combination of biochemical studies with electrophysiological techniques which permit direct assessment of channel function. In this section, we will emphasize recent studies of channel regulation by vasopressin, G proteins, and aldosterone.

### Vasopressin Regulates $Na^+$ Channel Activity

Vasopressin (antidiuretic hormone, ADH) stimulates a 2–4-fold increase in $Na^+$ transport across high transepithelial electrical resistance epithelia. The maximal increase in $Na^+$ transport occurs within 10–20 min and is due to an increased apical membrane density of open $Na^+$ channels. Accompanying the ADH-stimulated increase in $Na^+$ transport is an increase in adenylate cyclase activity and a rise in intracellular cAMP levels. Typically, the ADH-induced increase in $Na^+$ transport is transient. After reaching a maximal value, $Na^+$ transport falls to baseline and the epithelium is desensitized to a second application of ADH. However, in the rat cortical collecting tubule, the effects of antidiuretic hormone are sustained [134]. The molecular events underlying the actions of vasopressin on $Na^+$ transport as well as those differentially producing a transient or sustained response are not known. Because cAMP levels are increased, cAMP-dependent protein kinase (PKA) may activate quiescent $Na^+$ channels within the membrane via phosphorylation of a channel subunit. Alternatively, PKA may modify other proteins that cause the insertion of new $Na^+$ channels or regulatory channel subunit into the apical membrane from an intracellular pool.

In an attempt to elucidate whether there is recruitment of channels from an intracellular pool, the following experiment was performed [111]. Three individual groups of confluent A6 cells grown on filters were metabolically labeled with [$^{35}$S]-methionine. One group acted as a control. The apical surface of the cells of the other two groups were trypsinized because trypsin inhibits most of the baseline $Na^+$ transport via proteolysis of the channels [44]. After this trypsinization step, the apical membrane surface was biotinylated in the absence of or following ADH stimulation. Biotinylated $Na^+$ channels were first immunoprecipitated with anti-$Na^+$ channel antibodies followed by a second precipitation using streptavidin. This double immunoprecipitation procedure should yield only surface exposed (and not intracellularly located) $Na^+$ channels. The rationale of this experiment is that because apical trypsinization de-

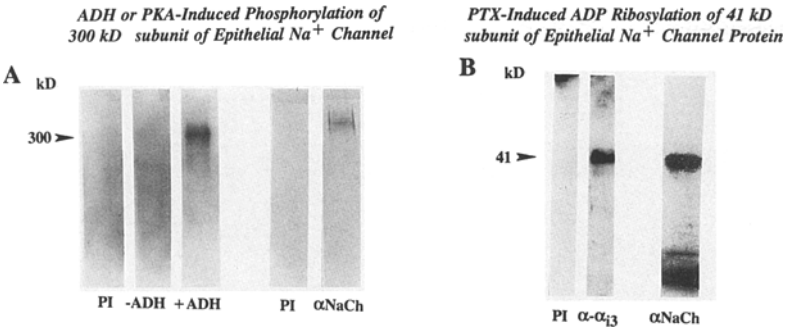


**Fig. 2.** PKA-mediated phosphorylation and PTX-induced ADP-ribosylation of purified epithelial $Na^+$ channel protein. (*A*) Autoradiogram of immunoprecipitated $Na^+$ channel protein from confluent A6 cell monolayers either treated with 100 U/ml of arginine vasotocin (*+ADH*) or nontreated controls (*–ADH*). Lane labeled *PI* refers to $Na^+$ channel immunoprecipitated protein from A6 cells treated with ADH for 30 min. In this case, only preimmune immunoglobulin G was used in place of primary anti-$Na^+$ channel antibody. (Data taken from ref. 111) The fourth and fifth lanes represent partially purified bovine $Na^+$ channel protein immunoprecipitated with anti-$Na^+$ channel antibody (αNaCh) following in vitro phosphorylation with catalytic subunit of protein kinase A plus $^{32}$[P]-ATP. (*B*) Western blot analysis of purified A6 $Na^+$ channel protein probed with an anti-$\alpha_{i-3}$ G-protein antibody ($\alpha$-$\alpha_{i-3}$) and visualized with $^{125}$[$I^-$]-labeled protein A. There was no specific binding using preimmune IgG in place of the antibody. (Data taken from ref. 5) The third lane represents an autoradiograph of immunopurified bovine $Na^+$ channel protein following in vitro pertussis-toxin-induced ADP-ribosylation.

creases net $Na^+$ transport across these epithelia due to the closure of apical $Na^+$ channels as a result of proteolytic cleavage (also with the resultant loss of anti-$Na^+$ channel antibody recognition [151]), then if new $Na^+$ channels were recruited from a subapical membrane pool by ADH treatment, they would be subsequently biotinylated and would be detected in the immunoprecipitated samples. However, no new channel protein was seen after ADH treatment. Thus, vasopressin treatment did not alter apical membrane surface expression of channel protein, providing biochemical support that total channel protein density in the apical membrane is not altered by vasopressin treatment. However, it is important to point out that following a slightly different, but essentially similar procedure, Kleyman and co-workers reach an entirely different conclusion [29, 74]. They found that the surface iodination of apical $Na^+$ channels increased after ADH treatment. The reasons for this disparity in conclusions and results are not known but they may relate to the culture conditions used or to the method of labeling. Also, noise analysis of apical membrane current fluctuations and capacitance measurements in frog skin before and after vasopressin or forskolin treatment indicates that elevation of intracellular cAMP levels simultaneously increase the apical membrane surface area and $Na^+$ channel density [1, 6, 38]. However, the correlation between increased channel density and surface area may be fortuitous because vasopressin is also known to promote fusion of subapical membrane water channel containing vesicles [159]. Early studies have unequivocally demonstrated that the ADH effects on $Na^+$ transport and water permeability can be separated (*see* ref. 43 for discussion). In any case, the ultimate resolution of this issue has not yet been achieved.

An alternative hypothesis to the vesicular fusion one is that a phosphorylation reaction induced by vasopressin may be the effector gating an apical membrane $Na^+$ channel. As shown in Fig. 2, one subunit of a purified epithelial $Na^+$ channel protein can be specifically phosphorylated both in vivo and in vitro by either antidiuretic hormone or catalytic subunit of protein kinase A + ATP, respectively. If a PKA-phosphorylation of a regulatory subunit of the channel is important, then it can be hypothesized that PKA-mediated phosphorylation should increase the open probability of channels reconstituted into planar lipid bilayer membranes. This is, in effect, what we observed (Fig. 3; 64, 111, 133).

## G Proteins Regulate $Na^+$ Channel Activity

The observation that amiloride-sensitive $Na^+$ channels are associated with the $\alpha_{i-3}$ subunit of the heterotrimeric G proteins immediately suggested that this subunit might have a regulatory role. G proteins are known to modulate a number of ion channels in nonepithelial tissues and potentially act through a number of signaling pathways (for review, *see* 57). Cantiello and his colleagues [26, 27] have provided strong evidence that modulation of apical membrane $Na^+$ channels by G proteins is mediated through phospholipase A2 activation and the generation of lipoxygenase metabolites. Channel activity in excised patches is inhibited by the PLA2 inhibitor mepacrine, and activity could be restored by addition of arachidonate or leukotriene D4 [27]. Moreover, channel activation induced by GTP $\gamma$S or addition of exogenous $\alpha_{i-3}$ to patches could be blocked by lipoxygenase inhibitors.

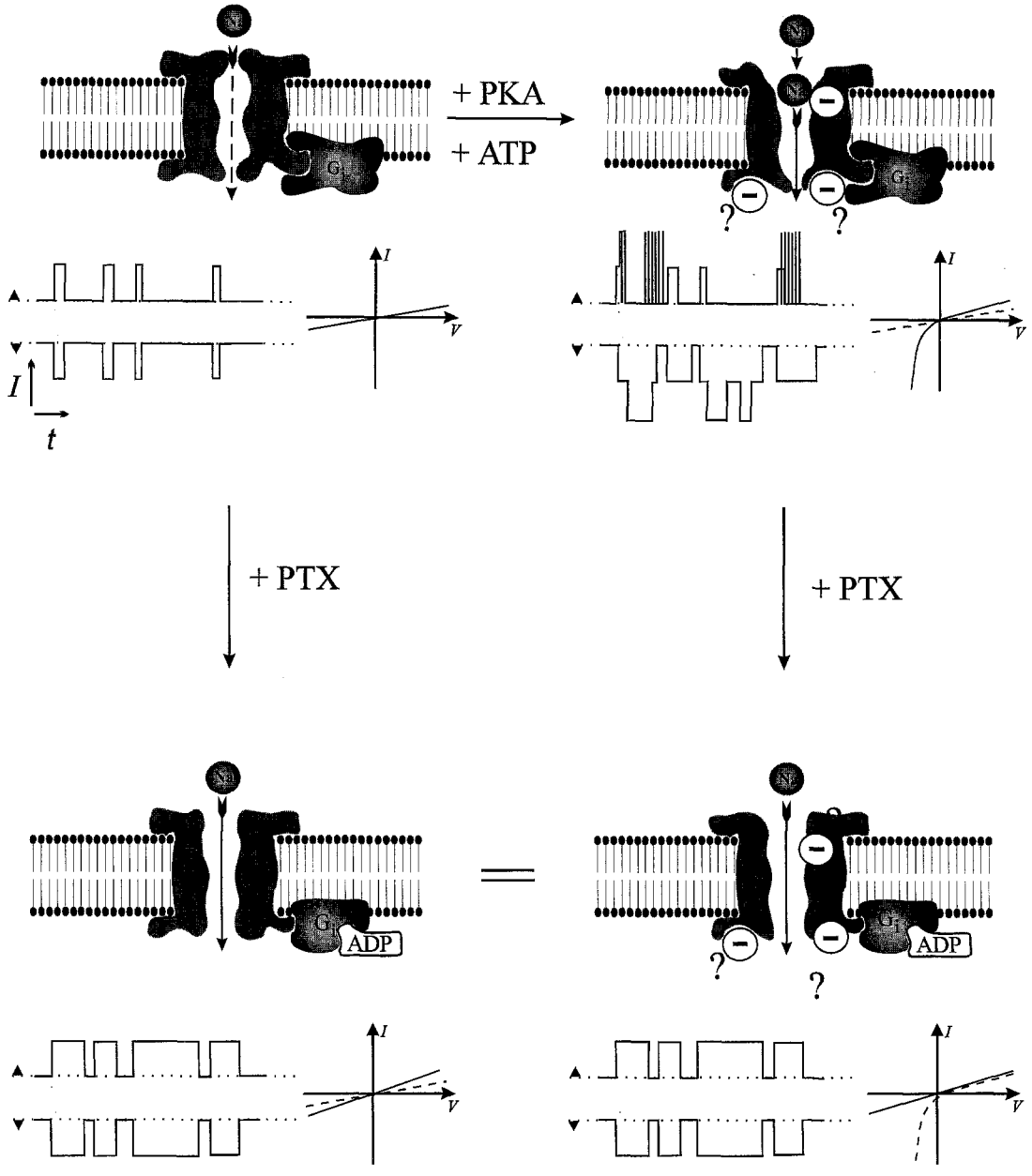


**Fig. 3.** Schematized examples of the effect of PKA-mediated phosphorylation and ADP-ribosylation induced by pertussis toxin (PTX) on single bovine renal $Na^+$ channel activity. For each condition a cartoon of an epithelial $Na^+$ channel, idealized single channel records, and associated current (*I*)-voltage (*V*) curves are depicted. For the *I-V* curves, the dotted trace indicates the *I-V* relationship prior to final treatment. Control records shown are in the upper left hand corner. Channel activity is low, and the *I-V* curve is linear. Upon phosphorylation with PKA + ATP (upper right-hand corner), channel activity dramatically increases, and the *I-V* curve becomes inwardly rectified. ADP-ribosylation with PTX either increases or decreases channel activity, depending upon the previous phosphorylation state of the channel protein. If the channels are not phosphorylated, PTX increases activity (lower left). PTX treatment of PKA-phosphorylated channels results in a significant diminution in channel activity (lower right). Interestingly, the *I-V* curve reverts to being linear.

Some controversy, however, related to the exact role of G proteins in modulating $Na^+$ channel activity does exist. Cantiello, Ausiello, and their colleagues (*personal communication*), studying an H-type channel with low $Na^+/K$ selectivity (i.e., Na(9)) from A6 cells grown on nonporous supports, have repeatedly demonstrated that exogenous $\alpha_{i\text{-}3}$ or GTP $\gamma$S activate the channel, while GDP and pertussis toxin (PTX) inhibit channel activity [5, 26]. They concluded from their experiments that the activated G protein was stimulatory in nature. Eaton and colleagues, studying an H-type channel with high $Na^+/K^+$ selectivity from A6 cells grown on permeable supports and pretreated with aldosterone, found virtually the opposite: GTP inhibits, and pertussis toxin and GDP ac-

tivate the channel [92, 107]. They concluded that the activated G protein was inhibitory in nature.

The G-protein modulation of apical membrane $Na^+$ channels appears also to be mediated through phospholipase A2 activity and the generation of leukotrienes [27]. These channels are inhibited by the phospholipase A2 inhibitor mepacrine in excised patches. As indicated earlier, the 41 kD protein identified as the $\alpha_{i\text{-}3}$ subunit of the $G_{i\text{-}3}$ copurifies with this $Na^+$ channel complex from A6 cells and bovine renal papilla, and has been localized adjacent to the channel by confocal microscopy. Whether this polypeptide is subjected to hormonal regulation by agents known to modulate apical $Na^+$ permeability is not at present known. Hawk and Schafer [56], speculate that this $G\alpha_{i\text{-}3}$ may migrate from the basolateral membrane to become associated with the channel in the apical membrane. Recently, J.K. Bubien, R.S. Jope and D.G. Warnock (*submitted*) proposed a model reconciling these disparate observations based on experiments conducted on human lymphocyte amiloride-sensitive $Na^+$ channels using the whole-cell configuration of the patch clamp technique. In their scheme, PTX would open nonphosphorylated $Na^+$ channels but inactivate previously phosphorylated ones, and cAMP treatment would open $Na^+$ channels in the absence of PTX treatment and would close them in cells pretreated with PTX. The predictions of this hypothesis have been validated in experiments using purified bovine renal $Na^+$ channel protein reconstituted into planar lipid bilayers. As schematized in Fig. 3, we found that the catalytic subunit of protein kinase A + ATP stimulates channel activity by increasing channel open probability [64, 111]. There are two other important features of this PKA-mediated phosphorylation activation. This phosphorylation reaction works only from one side of the bilayer (the side opposite on which amiloride inhibits), and it converts the channel from one whose probability of being open ($P_o$) is independent of voltage to one whose $P_o$ is dependent on applied potential with no effect on single channel conductance, i.e., the mean *I-V* curve becomes inwardly rectified. Moreover, the number of active channels in the membrane is increased, because of a shift in the probability of each individual channel to a more open configuration. In addition, the frequency of and time spent in channel subconductance states increases. Because of the methodology we use in incorporating channels, it is likely that there are silent channels already resident in the bilayer which, upon phosphorylation, become active, as opposed to new channel-containing vesicles fusing with the bilayer. We found that PTX can activate nonphosphorylated $Na^+$ channels, but can inhibit them if the channel is first activated by exposing the cytoplasmic face to protein kinase A and ATP (Fig. 3). Thus, there are several possibilities to consider. First, the amiloride-sensitive $Na^+$ channel studied by Prat et al. [122, 123] may be different than those examined by O'Hara et al. [107]. Each of these channels has different $Na^+$ to $K^+$ selectivities as well as a different single channel conductance. If this is the case, these channels may simply respond to PTX differently. On the other hand, the results shown in Fig. 3 indicate that the effects of PTX may be stimulatory or inhibitory depending upon the previous phosphorylation state of the protein. Regardless, the physiological regulation of these channels will be complex because, as indicated earlier, in addition to these phosphorylation pathways, epithelial $Na^+$ channel activity is also modulated by methylation, arachidonic acid metabolites, interactions with the cytoskeleton, as well as by intracellular calcium and pH. Although it is not currently known which subunit or subunits are involved in the actual conduction through this channel, it is clear that multiple subunits of the channel protein complex are involved in the regulation of this activity.

## Aldosterone Regulates $Na^+$ Channel Activity

The $Na^+$ transport response to aldosterone occurs in three phases. During the initial latent period (30–90 min) receptor binding, translocation and gene activation occur with subsequent transcription and translation of new proteins. During the early phase (30 min–4 hr) there is an increase in transport and a parallel increase in tissue conductance. This appears to be entirely due to activation of amiloride-sensitive $Na^+$ channels. The late phase (4–12 hr) shows a continued rise in transport rate with little further increase in conductance and is associated with increased $Na^+/K^+$ ATPase message and protein (*reviewed in* refs. 41, 43, 60).

Increased channel activity induced by aldosterone is entirely dependent on new protein synthesis, but multiple lines of evidence suggest that these proteins induce activation of previously quiescent channels rather than *de novo* synthesis of new channels. Experiments using amiloride-induced fluctuation analysis show a definite increase in channel number in response to aldosterone [113, 118], but studies using either covalent modification or trypsin inactivation of pre-existing channels [44, 119] suggest that new channels per se are not inserted into the membrane. Wills et al. [167] demonstrated no change in apical membrane surface area of A6 cells in response to aldosterone. These findings are consistent with the observations of Kleyman et al. [72] who used NMBA photolabeling in conjunction with antiamiloride antibodies. These investigators showed that aldosterone does not alter either the cellular pool or the apical expression of $Na^+$ channels in A6 cells. Immunoprecipitation of the surface radiolabeled $Na^+$ channels with antiamiloride, anti-idiotypic antibodies revealed that there was no difference between control and aldosterone-treated A6 monolayers, further supporting the hypothesis that activation of quiescent channels occurs in response to aldosterone. Sim-

ilar conclusions were drawn by Garvin et al. [45] using [$^3$H]-phenamil binding as a marker for apical membrane channels. Our own laboratory has also shown by immunocytochemical means that aldosterone treatment does not promote the fusion of any $Na^+$ channel-containing vesicles in the apical membrane [151]. Finally, Duchatelle and colleagues [36], using the patch clamp technique, reported that exposure of A6 cells to aldosterone resulted in increased density of functional channels with a time course similar to that of the increase in short-circuit current observed in the intact epithelia. They also noted that aldosterone increased the mean open time and the open probability of the channels. These interesting results suggest that aldosterone may have more than one action at the channel, both activating previously quiescent channels and altering their kinetics. Asher and Garty [4] also proposed a dual action of aldosterone on the $Na^+$ channel in vesicle studies [3]. They found that apical membrane vesicles prepared from toad urinary bladder cells during the early phase of aldosterone action had rates of $Na^+$ uptake similar to control vesicles, while those prepared after more prolonged exposure to the hormone had an increased rate of $Na^+$ uptake compared to control, as initially reported by Sariban-Sohraby et al. [129]. They concluded that aldosterone induced an early effect on channel activity which was labile from vesicle-to-vesicle preparation, and a more sustained effect which was stable throughout the vesicle preparation.

The mechanism of aldosterone activation of channels remains the subject of intense study, though several hints emerged from older studies. First, Yorio and Bentley showed that aldosterone action in toad urinary bladder could be inhibited by the PLA2 inhibitor mepacrine [170]. Second, aldosterone action could be inhibited by blockers of acylation reactions which interrupt membrane localization of induced proteins [50, 135]. Third, aldosterone action is dependent on carboxyl methylation reactions [165], and vesicle studies suggest that this carboxyl methylation serves to activate the channel. Moreover, inhibition of methylation reactions with 3-deazaadenosine blocks the increase in open probability induced in patches by aldosterone [37, 69, 92]. Since G-protein localization in a number of tissues is dependent on acylation and carboxyl methylation reactions [33], a unifying hypothesis would propose that aldosterone acts through regulation of the channel-associated G protein as suggested by Hawk and Schafer [56]. In order to test this possibility, our laboratories examined the regulation of $G\alpha_{I\text{-}3}$ by aldosterone in A6 cells [66].

Poly $(A)^+$ mRNA from A6 cells grown overnight in the presence or absence of aldosterone was subjected to Northern blot analysis using a specific $\alpha_{i\text{-}3}$ oligonucleotide probe. A second probe for actin was used as a control. Aldosterone induced a nearly twofold increase in $\alpha_{i\text{-}3}$ message. A6 cells metabolically labeled with [$^{35}$S]-methionine were immunoprecipitated with antibodies to the consensus GTP binding site of G proteins. Western blot analysis was also carried out with this antibody as well as an antibody to the $\alpha_{i\text{-}3}$. These studies indicated that aldosterone stimulated metabolic labeling and quantitatively increased the amount of the 41 kD peptide, again by nearly twofold. Immunoprecipitation of metabolically labeled cells with the polyclonal anti-$Na^+$ renal channel antibody demonstrated increased association of the newly synthesized subunit with the channel.

Because G proteins are thought to be targeted to membranes by post-translational modifications including carboxyl methylation and acylation [33], we also examined the effect of aldosterone on palmitoylation, myristoylation, and prenylation of this 41 kD protein. Using isotopically tagged lipids, it was found that aldosterone stimulated the prenylation and palmitoylation, but not the myristoylation, of this G protein. Moreover, inhibitors of palmitoylation and prenylation significantly inhibit the early stimulation of short-circuit current by aldosterone in intact A6 epithelia. These results suggest that aldosterone may stimulate channel activity by regulating expression and targeting of the channel-associated regulatory G protein (*e.g., see* refs. 164 and 168).

Although the G protein may be the site of the regulatory carboxyl methylation induced by aldosterone, it has not been demonstrated that the 41 kD G protein is actually methylated. In contrast, Sariban-Sohraby et al. [131] found that aldosterone induced the GTP-dependent methylation of a 90 kD protein in the apical membrane of A6 cells. This protein may be a polypeptide comprising part of the epithelial $Na^+$ channel. These results imply that direct carboxyl methylation of a distinct subunit might have an effect on channel activity. We have examined this possibility using a purified renal $Na^+$ channel reconstituted in lipid bilayers. We found that carboxyl methyl transferase isolated from A6 cells results in methylation of the 90 kD subunit, but not the 41 kD subunit, of the purified $Na^+$ channel. Furthermore, methylation of the channel reconstituted into bilayers produced a marked increase in open probability in a GTP-dependent manner. Taken together, these results are consistent with two effects of aldosterone on the channel: a direct effect on channel kinetics induced by carboxyl methylation of the 90 kD subunit, and, possibly, activation of channels induced by increased association of the regulatory G protein with quiescent channels.

In addition to the $Na^+/K^+$ ATPase pump, other proteins are induced by aldosterone [34, 41, 46]. Interestingly, one of the other aldosterone-induced proteins has a molecular mass of 70 kD and is a glycoprotein. This protein shows cross-reactivity with antibodies raised against purified bovine renal $Na^+$ channel, and antibodies raised against it, cross-reacts with a 70 kD subunit of a purified bovine $Na^+$ channel protein in immunoblots [34, 148, 149]. Duchatelle et al. [36] suggest that at least one

of the aldosterone-induced proteins must act as a regulatory protein. In any case, they do suggest that the major action of aldosterone is the activation of channels by a post-translational modification. Likewise, Kleyman et al. [72, 73] suggest that post-translational modification of this same subunit may be a mechanism by which aldosterone regulates these $Na^+$ channels.

### Cytoskeletal Interactions with $Na^+$ Channels Are Important Determinants of Channel Activity

We have previously demonstrated that apical epithelial $Na^+$ channels are associated with the cytoskeleton and that their lateral mobility (as determined by fluorescence photobleach recovery) is severely restricted by this association [141; P.R. Smith, K.J. Angelides and D.J. Benos, *in preparation*]. Recent patch clamp data of Prat et al. [122, 123] have shown that actin disruption activates $Na^+$ channels, whereas cross-linking of actin inactivates channels, thereby demonstrating the cytoskeletal $Na^+$ channel association is involved in regulating $Na^+$ channel activity. Upregulation of $Na^+$ channel activity by aldosterone and/or ADH may be mediated through the cytoskeleton with dissociation of the channels from the cytoskeleton resulting in their activation. P.R. Smith, K.J. Angelides and D.J. Benos (*in preparation*) have used fluorescence photobleach recovery experiments to test this hypothesis by measuring lateral mobility and mobile fraction of $Na^+$ channels in filter-grown A6 cell monolayers before and after 16 hr of aldosterone treatment, and at various times after antidiuretic hormone treatment. Monolayers were labeled with rhodamine conjugated anti-$Na^+$ channel Fab′ fragments, and lateral diffusion coefficient and mobile fraction of the labeled $Na^+$ channels were measured by the spot photobleaching method. Either parameter, namely, lateral diffusion coefficient or mobile fraction of the $Na^+$ channels, were unaltered by antidiuretic hormone treatment. However, in aldosterone-treated monolayers, there was a threefold increase in mobile fraction when compared to controls, again with no change in lateral diffusion coefficient. This threefold increase in mobile fraction suggests that aldosterone-induced upregulation of $Na^+$ transport may be mediated through the cytoskeleton. Preliminary experiments also suggest that the aldosterone-induced increase in mobile fraction of $Na^+$ channels can be inhibited by the carboxymethylation inhibitor 3-deazaadenosine. Based upon these observations, we propose that aldosterone upregulation of $Na^+$ transport in intact epithelia may involve a methylation event that may in turn alter the association of $Na^+$ channels with the cytoskeleton. Interestingly, preliminary results show that aldosterone also increases the phosphorylation of a high molecular weight protein, possibly spectrin, and that the combination of methylation and phosphorylation may detach $Na^+$ channels from their linkage to the cytoskeleton. While highly speculative, this hypothesis nonetheless provides a way to reconcile these seemingly disparate observations. Bear in mind, however, that methylation may have a dual role—one involving the channel's interaction with the cytoskeleton, and the other impinging directly upon channel open probability. However, as usual, the validity of these hypotheses must await experimental tests.

## Molecular Biology of Epithelial $Na^+$ Channels

With the great improvement in modern day electronics and computers, the electrical activity of single epithelial $Na^+$ channels has been extensively investigated. On the other hand, epithelial $Na^+$ channels have been refractory to molecular biological analysis for several reasons. First and foremost, owing to the enormous complexity of the eukaryotic genome, any starting genetic material having only a minute amount of the channel coding gene mixed within a large background of undesired "contaminants" renders the isolation of the particular gene of interest a very difficult and inefficient task. Second, because multicomponent, heterogeneously constructed ion channels, such as epithelial $Na^+$ channels, may require more than one polypeptide for activity, identification of more than one gene encoding a functional channel drastically increases the experimental complexity of cloning.

Epithelial ion channels are relatively rare proteins that exist at a low membrane density in the range of a few hundred to several thousand channels per cell. Purification of epithelial $Na^+$ channels from bovine kidney or A6 epithelial results in minute yields in the range of less than 0.001% of total cellular protein, concomitant with a few thousand-fold enrichment of $Na^+$ channel activity [14]. The absence of biological tissues with a high density of these channels has been somewhat of a deterrent to investigators wishing to undertake a full frontal assault on the biochemical and molecular characterization of these channels at the protein level. This is especially evident when examining the literature comparing the volume of papers relating to epithelial $Na^+$ channel biochemistry and molecular biology to those pertaining to other channels, such as the neuronal voltage-activated $Na^+$ channel that was cloned by Noda and colleagues in 1984 [104].

Preliminary efforts attempting to clone epithelia $Na^+$ channel(s) have focused on the expression of amiloride-sensitive $Na^+$ fluxes or currents in *Xenopus laevis* oocytes injected with total or size fractionated mRNA. Hinton and Eaton [59], using total mRNA from A6 epithelia, and Asher et al. [3], using total mRNA from toad urinary bladders, were able to express functionally competent amiloride-sensitive $Na^+$ channels. In both cases, pretreatment of the epithelium with aldosterone, for 20–24 hr, resulted in a larger expression of $Na^+$ currents, implying that aldosterone upregulates $Na^+$ channel mRNA levels in these mineralocorticoid-responsive ep-

ithelia. Kroll et al. [78] were also able to express amiloride-sensitive $Na^+$ channels in oocytes injected with total mRNA isolated from human nasal polyp and bovine tracheal epithelia. In all cases, the expressed $Na^+$ channels retained similar pharmacological and electrophysiological characteristics as the native channel.

Injections of size fractionated mRNA from different epithelial preparations were subsequently utilized to yield more specific information about epithelial $Na^+$ channels of interest. George et al. [47] injected size fractionated mRNA from A6 epithelia into *Xenopus* oocytes and observed that $Na^+$ uptake was largest in oocytes injected with mRNA in the 1.4 to 4.4 kb size range. These findings were later confirmed by Palmer et al. [115] who observed that mRNA isolated from A6 epithelia with a sedimentation coefficient of 17 (near the 2.0 to 2.2 kb size range of the mRNA, and a predicted 70 kD for translated protein) reconstituted the largest amiloride-sensitive current in *Xenopus* oocytes.

It is of interest that in these experiments, equal channel expression was obtained with mRNA isolated from cells which had not been pretreated with aldosterone as from aldosterone-treated cells. These findings are in contrast to those of Hinton and Eaton [59] and Asher et al. [2] reported above, but are consistent with the data of Kleyman et al. [72] who found that aldosterone did not increase the total cellular pool of $Na^+$ channels.

Although extremely important and necessary as a first step in studying the molecular biology of $Na^+$ channels, these types of studies are limited in scope, and do not allow for the characterization of the structure-function relationship of these channels. Taking this technique of injecting a mixture of cellular mRNA in *Xenopus* oocytes to the next level, Canessa and colleagues [24] and Lingueglia and colleagues [93] have independently isolated a $Na^+$ channel clone from the corticoid hormone-stimulated rat distal colon by expression cloning. This approach involves the expression of smaller and smaller pools of cDNA until a single clone which induces amiloride-sensitive $Na^+$ currents is identified. For example, Canessa et al. [24] screened 60,000 clones that were systematically subdivided into 10 pools until eventually one clone was identified. This first $Na^+$ channel clone, termed αrENaC [25] or RCNaCH [93], expressed currents that displayed a high affinity for amiloride ($K_i$ near 100 nM), and a high $Na^+$ *vs.* $K^+$ selectivity. The nucleotide sequences of this clone predicted a protein of approximately 79 kD molecular mass, two transmembrane spanning regions, and a large 400 residue extracellular loop containing four potential N-glycosylation sites. Depending upon the extent of glycosylation, the αrENaC translation product could give rise to a higher mass polypeptide, and indeed in vitro translation of RCNaCH [93] results in a glycoprotein which migrates at a mass of 92 kD. The relationship between this rat clone and the $Na^+$ channel(s) studied by Cherskey [31], Branco and Veranda [18], Barbry and collaborators [8–11], Blazer-Yost and Recio-Pinto [16], Sariban-Sohraby et al. [127], and Benos and collaborators [13–15] is not known. Based on amiloride sensitivity alone, it would appear that this clone is related to the $Na^+$ channel(s) purified from bovine trachea [31] and kidney [13–15]. However, such a criterion for determining relationship between these $Na^+$ channels is weak at best, and at present we cannot exclude or include a relationship between this rat clone and other purified $Na^+$ channels. It is noteworthy to mention that in preliminary Western blot experiments using highly purified bovine renal $Na^+$ channel protein, rabbit polyclonal antibodies made against an αrENaC fusion protein weakly recognized both the 70 and 90 kD polypeptides, providing the best available evidence for some relationship between the rat clone and the bovine renal $Na^+$ channel. Interestingly, the cloned rat colon epithelial $Na^+$ channel shares a high degree of homology with a family of stretch-activated channels from *Caenorhabditis elegans* [35], namely, the degenerins (*see below*).

The membrane currents observed in oocytes following injection of αrENaC cRNA were smaller than those observed with injection of total rat colon mRNA, suggesting the lack of expression of other $Na^+$ channel subunits that are required for full channel conductance. Subsequently, the Canessa group has confirmed the presence of other $Na^+$ channel subunits in the rat colon [25]. Two additional subunits were identified, and termed β and γ rENaC. Expression of all three rENaC subunits together results in the largest expression of amiloride-sensitive $Na^+$ current, at levels much higher than that which could be achieved by injection of total size fractionated mRNA, or by injection of each clone individually or in pairs. All three subunits were similar in size (79, 72.1, and 74.9 kD, respectively) and share a high degree of identity with each other and with the stretch-activated channels from *C. elegans*. Single channel recordings of oocytes expressing this channel revealed that the channel had a single channel conductance of 4.6 pS at 100 mM $Na^+$. Interestingly, a recent report by Huang and Chalfie [61] also indicate that the *C. elegans* mechanosensitive channel is a trimer structure of similarity-sized subunits. These observations indicate the possibility that both the rat colon $Na^+$ channel and *C. elegans* mechanosensitive channels share a common ancestral gene coding for both channels.

Northern blot analysis of the distribution of the rat colon $Na^+$ channel subunits in other tissues have indicated that all three rENaC subunits are present in the rat kidney and lung [24, 25, 106]. However, the distribution of mRNA levels varied between different subunits, indicating a possible variable stoichiometry of these subunits in different tissues. Voilley et al. [162] have extended these observations and obtained a sequence of a human lung homologue of RCNaCH. However, the precise lo-

cation in the lung where the RNA was initially isolated was not specified. This information is critical for reconciling the extant electrophysiological observations of $Na^+$ channel type (H- or L-) along the pulmonary tree [96, 99, 162]. In any case, the predicted protein products of RCNaCH and its human lung homologue were nearly identical (>80% identity). Labeled probes to the RCNaCH cDNA were also found to hybridize total mRNA in the pancreas and the thyroid. Using a short nucleic acid sequence from αrENaC as a primer for reverse transcription PCR, Li et al. [85] have also cloned this $Na^+$ channel subunit from the rat lingual circumvillate papillae and adjacent tongue sections which lack taste buds, in addition to the olfactory epithelium. This group also detected alternatively spliced forms of this $Na^+$ channel subunit in which 24 base pairs were deleted toward the middle of the translated region. O'Brodovich et al. [106] reported that the mRNA levels for αrENaC were very low in 17–18 day fetal lungs, but that these levels increased dramatically at days 20–22, coinciding nicely with the conversion of lung epithelia from a secretory to a reabsorptive structure.

It is clear that these $Na^+$ channel clones described by Canessa et al. [24, 25] and Lingueglia et al. [93], or other alternatively spliced forms of these clones, are distributed in many epithelial tissues. It is also evident that the single channel characteristics of the rat clones coexpressed in *Xenopus* oocytes resemble that of the small conductance, high selectivity, highly amiloride-sensitive $Na^+$ channel observed in tight epithelia [114]. However, it is well documented that $Na^+$ channels from renal epithelia are cAMP sensitive, while those in rat colonic epithelia are not responsive to cAMP [19, 20]. Even though the colon $Na^+$ channel carries potential cAMP phosphorylation sites, no reports yet exist demonstrating activation of this clone with cAMP. Thus, it is uncertain at present whether these clones transcribe subunits of cAMP-activated epithelial $Na^+$ channels found in renal epithelia. It is interesting to note that stretch-activated $Na^+$ channels endogenous to *Xenopus* oocytes are also amiloride sensitive, albeit at much higher concentrations than this cloned epithelia $Na^+$ channel [52]. The similarity between the degenerins in *C. elegans* and the colon $Na^+$ channel clone may argue in favor of the colon clone being a mechanosensitive $Na^+$ channel, similar to channels endogenous to *Xenopus* oocytes and hair cells [51, 52, 81]. It is possible that when expressed in oocytes these colon clones complement the endogenous stretch-activated $Na^+$ channels to induce large $Na^+$ currents with higher amiloride sensitivity. Alternatively, it is also possible that endogenous oocyte-associated components may interact with the expressed colon clones to alter their electrical, regulatory, and/or pharmacological characteristics. However, it is equally plausible that the rat colon clones constitute the basic building block for a tight-epithelium $Na^+$ channel, which may become susceptible to the actions of cAMP in other epithelia by interaction with additional subunits which confer PKA phosphorylation, or by interaction with other tissue-specific cytoplasmic factors. Indeed, such a hypothesis may be favored since, as indicated earlier, antibodies against an αrENaC fusion protein recognize the 70 and 95 kD subunits from the purified bovine kidney $Na^+$ channel preparation. These ideas remain to be tested and await the cloning of individual $Na^+$ channel-associated proteins or $Na^+$ channels from cAMP-sensitive tissues. In any case, these landmark papers open the door for many exciting and critical studies.

Stanton et al. [145] in primary cultures of rat inner medullary collecting duct cells, and Ahmad et al. [2] in M1 mouse cortical collecting duct cells have partially cloned a cGMP-activated, nonselective cation channel using primers derived from the cGMP-gated rod photoreceptor channel. In patch clamp studies, these channels exhibit fast kinetics and display single channel conductances of 28–34 pS. These channels are inhibited by submicromolar concentrations of amiloride and are thus thought to be important in $Na^+$ reabsorption in the nephron. From the deduced amino acid sequences, these Na(28) channels are highly homologous to the cGMP-gated rod channels, and thus are unique in comparison to ENaC.

Given the multimeric nature of the epithelial $Na^+$ channel [15] it is not obligatory that all subunits conduct $Na^+$ or bind amiloride or its analogues. Utilizing this rationale, one other potential $Na^+$ channel subunit or associated regulatory protein has been cloned. Staub et al. [146] made the observation that an antibody raised against the α-subunit of amphibian $Na^+/K^+$ ATPase stains both the apical and basolateral membranes of A6 epithelia. They used this antibody to screen an A6 cDNA expression library. From the non-α-$Na^+/K^+$ ATPase positive cDNAs, they were able to clone a 130 kD protein termed Apx. This protein, when expressed in *Xenopus* oocytes, did not reconstitute amiloride-sensitive $Na^+$ currents. However, in coinjection experiments, Apx antisense mRNA was found to inhibit amiloride-sensitive $Na^+$ current induced by injection of total A6 mRNA, thus implicating a possible regulatory role for Apx. Further, antibodies raised against Apx fusion protein or against peptides synthesized from the primary sequence of Apx recognized only a single band of 130–150 kD on Western blots of purified A6 channel protein (*unpublished observations*). As with other $Na^+$ channel related clones, the relationship between this associated protein to purified or biochemically characterized $Na^+$ channel(s) is not known and awaits further studies.

## Summary

A new molecular biological epoch in amiloride-sensitive $Na^+$ channel physiology has begun. With the application

of these new techniques, undoubtedly a plethora of new information and new questions will be forthcoming. First and foremost, however, is the question of how many discrete amiloride-sensitive $Na^+$ channels exist. This question is important not only for elucidating structure-function relationships, but also for developing strategies for pharmacological or, ultimately, genetic intervention in such diseases as obstructive nephropathy, Liddle's syndrome, or salt-sensitive hypertension where amiloride-sensitive $Na^+$ channel dysfunction has been implicated [17, 62].

Epithelia $Na^+$ channels purified from kidney are multimeric. However, it is not yet clear which subunits are regulatory and which participate directly as a part of the $Na^+$ conducting core and what is the nature of the gate. The combination of electrophysiologic techniques such as patch clamp and the ability to study reconstituted channels in planar lipid bilayers along with molecular biology techniques to potentially manipulate the individual subunits should provide the answers to questions that have puzzled physiologists for decades. It seems clear that the robust versatility of the channel in responding to a wide range of differing and potentially synergistic regulatory inputs must be a function of its multimeric structure and relation to the cytoskeleton. Multiple mechanisms of regulation imply multiple regulatory sites. This hypothesis has been validated by the demonstration that enzymatic carboxyl methylation and phosphorylation have both individual and synergistic effects on the purified channel in planar lipid bilayers.

Of the multiple mechanisms proposed for channel regulation, evidence is now available to support the ideas that channels may be activated (or inactivated) by direct modifications including phosphorylation and carboxyl methylation, by activation or association of regulatory proteins such as G proteins, and by recruitment from subapical membrane domains. The observation that channel gating is achieved primarily through regulation of open probability without alterations in conductance may simplify future understanding of the molecular events involved in gating once the regulatory sites have been identified. As more $Na^+$ channels or $Na^+$ channel subunits are cloned from different epithelia, it will become possible to piece together the puzzle of epithelial $Na^+$ channels. It is interesting to observe that renal $Na^+$ channel proteins contain a subunit which falls into the 70 kD range. This size protein is in the range reported for the aldosterone-induced proteins [12, 46, 153]. Recent reports indicate that polyclonal antibodies directed against the bovine renal $Na^+$ channel cross-react with GP70, an aldosterone-induced protein [149], especially in light of the recent cloning of an epithelial $Na^+$ channel whose subunit sizes are 70–80 kD [24, 25]. It is tempting to speculate that this size polypeptide forms the basic building block of amiloride-sensitive $Na^+$ channels, which can then be subsequently modified and custom-tailored in different epithelia by the addition of various other associated regulatory proteins.

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