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Identification of Apical Membranes from Tight Epithelia Using Spin-Labeled Amiloride and Electron Paramagnetic Resonance Spectroscopy

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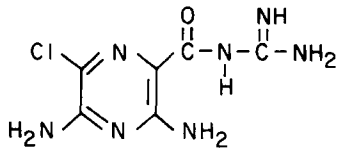
Summary. Apical cell membranes from Na^+-transporting epithelia were identified in centrifugal fractions prepared from homogenates of rainbow trout kidney, gill and frog skin using a spin-labeled, nitroxide derivative of amiloride and electron paramagnetic resonance spectroscopy. Spin-labeled amiloride (ASp) is a potent inhibitor of Na^+ transport. Frog skin short-circuit current was inhibited by 50% in the presence of 7×10^{-8} M ASp, whereas 4×10^{-7} M amiloride was required to obtain the same effect. ASp is a suitable probe for the amiloride binding site based on analytical criteria: Unbound ASp produces an EPR signal linear with concentration and detectable at micromolar concentrations. Estimates of ASp binding can usually be made on less than 100 μg of membrane protein. While ASp binds non-specifically to many materials, amiloride- or benzamil-displaceable binding occurred only in trout gill and kidney, and in frog skin, but not in trout skeletal muscle. ASp binds to membrane fractions produced by differential centrifugation of trout gill, kidney and frog skin. In trout gill and kidney, 81% and 91%, respectively, of the amiloride-displaceable ASp binding is found in the $10{,}000 \times g$ fraction. All of the ASp binding in frog skin is found in the $10{,}000 \times g$ fraction. These data indicate that spin-labeled amiloride is a useful probe for the identification of the amiloride binding site, and electron paramagnetic resonance spectroscopy will allow the amiloride binding site to be used as a molecular marker for apical membranes.

Key Words amiloride · electron paramagnetic resonance · membrane isolation · Na^+ transport · trout gill · trout kidney · frog skin

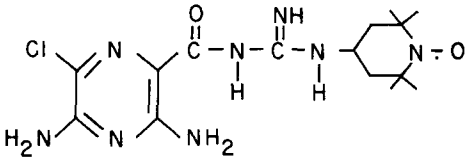
Introduction

Transepithelial Na^+ transport across absorptive epithelia entails two discrete steps, one transferring the ion from the bathing medium across the apical membrane, the other extruding it from the cell into the extracellular fluid compartment. Most of our information on the two transfer steps has been gathered from combined electrical and tracer experiments on intact epithelia, an approach that has been used for more than thirty years. More recently, considerable attention has been directed at the study of transport in membrane vesicles. The latter may form spontaneously from isolated membrane fragments (reviewed by Sachs, Jackson & Rabon, 1980; Murer & Kinne, 1980) or may require the incorporation of a purified transport system, such as the Na,K-ATPase or sarcoplasmic reticulum Ca^{2+}-ATPase, into artificial phospholipid vesicles (reviewed by Hokin, 1981). The simplification afforded by vesicles as compared with epithelial cells *in situ* makes them useful for studying some aspects of transfer by one or the other membrane. In such experiments, unequivocal results may depend on obtaining a population of vesicles as homogeneous as possible; i.e. with insignificant contamination by other cell components, especially other membranes. In practice, this involves a scheme for isolating the desired membrane fraction from a homogenate of the epithelium. Obviously, a molecular or morphological marker capable of distinguishing the target membrane from others is a critical requirement. For a number of epithelial membrane systems unique enzymes provide the necessary markers. Thus, the Na,K-ATPase, found in the plasma membrane of most animal cells (Neville, 1976), is located only on the basolateral aspect of most Na^+-transporting epithelial cells. Brush-border membranes can be identified morphologically with the electron microscope (Hopfer et al., 1973; Kessler et al., 1978) as well as by the presence of alkaline phosphatase (Kinne et al., 1975), glutamyl transpeptidase (Kinsella & Aronson, 1980; Sacktor et al., 1981), and other enzymes.

In this regard, isolation of apical membranes from tight epithelia (without brush-border membranes) has been hampered by the lack of a suitable enzyme marker. Thus far, the only useful procedure has been to label the apical membrane by covalent



Amiloride



Spin Label

Fig. 1. Structural formulae of amiloride and spin-labeled amiloride. The nitroxide reporting group, with its unpaired electron, replaces one of the protons on the terminal nitrogen atom of the guanidino moiety

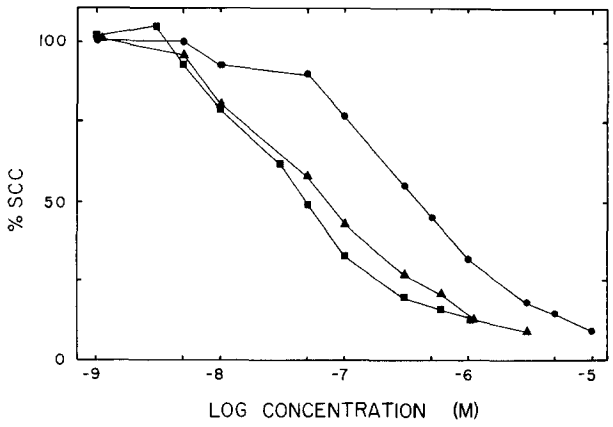


Fig. 2. Relative SCC from frog skins exposed to three inhibitors of apical Na^+ transport. Points are the mean relative SCC of three frog skins. Circles indicate amiloride, triangles ASp, and squares represent benzamil at the concentrations given in the figure

binding of ^{125}I (Rodriguez & Edelman, 1979; Chase & Al-Awqati, 1981). While this method has been effective, its use is restricted to epithelial sheets which permit the iodination reagents access only to one side. Thus, it has not been useful for kidney tissue, and its effectiveness in epithelia with a heavy mucous coat (e.g. gills) is questionable.

Inhibition of apical sodium transport by the diuretic, amiloride, is known to involve binding to specific sites on the apical membrane. In fact, [^{14}C]- and [^3H]-amiloride have been used to estimate both binding constants and number of such binding sites in frog skin (Cuthbert & Shum, 1974). Amiloride binding is very reversible; its inhibition of frog skin short-circuit current (SCC) is completely reversed within minutes of removal from the bath. This characteristic makes isotopically labeled amiloride unsuitable as a marker for identifying apical membranes through a membrane isolation procedure. An indirect approach, the assay of amiloride-sensitive sodium transport into vesiculated membranes, has been used by other workers (LaBelle & Valentine, 1980; LaBelle & Lee, 1982). In conjunction with the assay of mitochondrial and basolateral marker enzymes, this technique is useful, but only in those fractions containing a large proportion of the membrane material as sealed vesicles. Any apical membrane that is present in sheets or unsealed vesicles cannot be identified.

Recently, an electron spin-labeled derivative of amiloride (ASp) was synthesized in the Merck Sharp & Dohme Research Laboratories. This compound (4-([amino{(3,5-diamino-6-chloro-2-pyrazyl) carbonyl}methyl]amino)-2,2,6,6-tetramethylpiperidinyloxy free radical hemihydrate) has a nitroxide group substituted for one of the protons on the terminal nitrogen atom of the guanidino moiety of amiloride (Fig. 1). ASp was reported to be an effective transport inhibitor in frog skin and, when bound, produced a reduced EPR signal (Briggman et al., 1982), although the information has appeared only in abstract form. In our hands, the electron paramagnetic resonance (EPR) of ASp is detectable at micromolar concentrations and the EPR signal is proportional to the amount of free, i.e. mobile or unbound, ASp in solution. This study describes ASp inhibition of Na^+ transport across isolated frog skin and its use as an apical membrane marker through the initial stages of a cell fractionation procedure.

Materials and Methods

Inhibition of Frog Skin SCC by ASp, Amiloride and Benzamil

A 3.14 cm^2 piece of abdominal skin from *Rana pipiens* (Wm. A. Lemberger Co., Oshkosh, Wis.) was mounted in a Ussing-type chamber bathed with aerated Ringer's solution (composition in mM: 116 NaCl, 4 KCl, 2 $CaCl_2$, 5 $NaHCO_3$ – pH 7.2) on both mucosal and serosal sides. After the SCC stabilized, the test compound was introduced on the mucosal side of the skin (*see* Fig. 2 for concentrations) and the SCC recorded when it reached a new constant value. Following the test period (10 min or less) the skin was washed twice with Ringer's solution and the SCC allowed to recover to its pretreatment level. This procedure was chosen over a cumulative dosage method because longer exposures to ASp and benzamil required unacceptably long wash-out times to regain even near-normal SCC. Each of the three com-

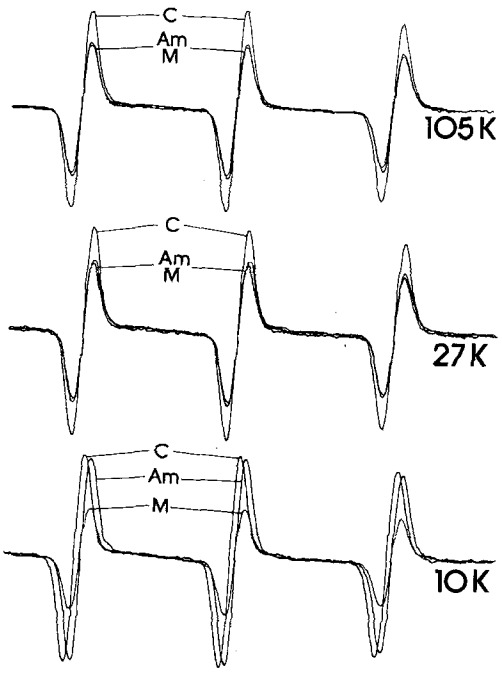


Fig. 3. The effect of trout gill membrane fractions and amiloride on the EPR signal of ASp. The control signal (*C*) (ASp alone) was reduced by all three membrane fractions (*M*). Addition of 0.8 mM amiloride (*Am*) reversed the binding by the 10K fraction but had no effect on ASp binding to the other two fractions

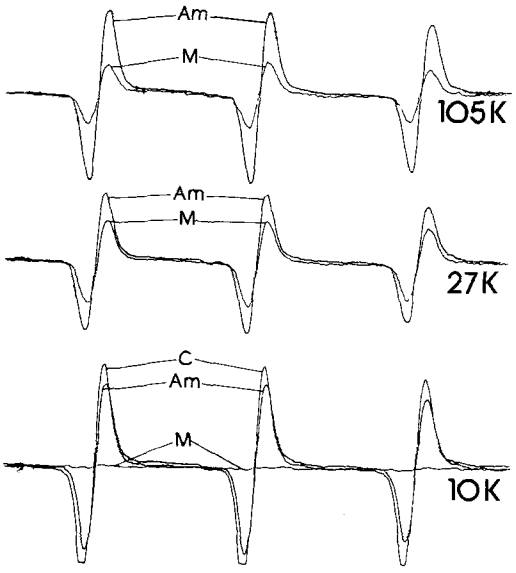


Fig. 4. The effect of trout kidney membrane fractions and amiloride on the EPR signal of ASp. The control signal (*C*) (ASp alone) was reduced by all three membrane fractions (*M*). Addition of 0.8 mM amiloride (*Am*) incompletely reversed the binding in all three fractions. The difference between the signals with and without excess amiloride is interpreted as ASp bound to the amiloride binding site

pounds was tested on the same skin; the experiment was replicated on three separate skins. The results were normalized to a percentage of the pretreatment SCC and expressed as the mean of the three experiments.

Quantitation of Spin-Labeled Amiloride

The signal measured by the EPR spectrometer (Varian model E-9) is proportional to the quantity of free ASp in the instrument's resonant cavity. In order to relate this signal to the absolute amount of ASp present, precision pipettes (Van-Lab 100-μl Micropipettes, VWR Scientific) were filled with 0, 3.0, 6.0, 9.0, 15.0, 22.5 and 30.0 μM ASp dissolved in 20 mM MOPS – pH 7.3 and 30 mM KCl and the EPR spectrum recorded. Each concentration was run in duplicate, one set of the tubes containing 0.8 mM amiloride. Inclusion of amiloride in this experiment was necessary because a small enhancement of the spin signal occurred whenever amiloride and ASp were present together, although amiloride alone produced no signal. While the exact volume of solution in the sensitive area of the instrument's resonant cavity was unknown, it remained constant throughout all of our measurements. The area under the EPR signal is proportional to the amount of "reporting" spin-label present in the sample. However, we found it to be adequate and simpler to relate the sum of the three peak heights to spin-label concentration (*see* Figs. 3–5 for examples of typical nitroxide EPR signals). Equations (linear) were fit to the data using standard regression techniques and the correlation coefficient r was calculated (Steel & Torrie, 1960).

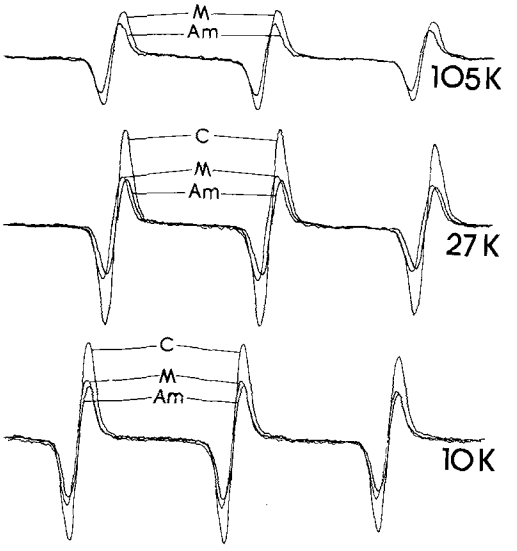


Fig. 5. The effect of trout skeletal muscle membrane fractions and amiloride on the EPR signal of ASp. The control signal (*C*) (ASp alone) was reduced by all three membrane fractions (*M*). Addition of 0.8 mM amiloride (*Am*) did not reverse spin-label binding in any of the three fractions

Preparation of Membrane Fractions

The ability of ASp and EPR spectroscopy to detect the presence of amiloride binding sites in various cell fractions was tested by preparing membranes from gill, kidney and skeletal muscle of

rainbow trout and from frog skin. Sodium transport in rainbow trout gill is amiloride-sensitive (Kirschner, Greenwald & Kerstetter, 1973), while amiloride sensitivity in skeletal muscle and kidney has not been reported. Rainbow trout (*Salmo gairdneri*) were obtained from the Garden Springs hatchery, Spokane Wash., and maintained in large tanks at 12°C until sacrificed. Crude particulate fractions were prepared by homogenizing the tissue, using a glass and Teflon homogenizer, in 10 to 15 volumes of homogenizing medium (250 mM sucrose, 20 mM Tris-acetate – pH 7.6, 5 mM EDTA, 1 mM dithioerythritol (DTE), and 0.1% Na-deoxycholate (DOC)). Early in this work, a number of preparations were made without using detergent. The DOC treatment had no effect on ASp binding, but enzyme activities were substantially increased. The homogenate was then fractionated by differential centrifugation into pellets obtained at $1000 \times g$ – 15 min, $10{,}000 \times g$ – 20 min, $27{,}000 \times g$ – 30 min, and $105{,}000 \times g$ – 60 min. Following centrifugation, the 10K, 27K and 105K $\times g$ pellets were resuspended in approx. 100 volumes of homogenization medium without DOC and recentrifuged. The 1K $\times g$ pellet was discarded. Finally, the washed pellets were resuspended in buffered sucrose (150 mM sucrose, 20 mM MOPS – pH 7.5) at a protein concentration of 1 to 3 mg/ml. These suspensions were used for both ASp-EPR spectroscopy and enzyme assays. Note that DTE is not present in the resuspension medium. In the presence of DTE, the EPR signal of ASp decreased in a time-dependent manner, probably because the paramagnetic nitroxide group was reduced to hydroxylamine.

Particulate fractions were obtained from frog skin using the methods described above with the following modifications: The ventral skin was removed from three frogs and the outer layers scraped from the fibrous dermis with a scalpel blade. The scrapings were then homogenized and the homogenate fractionated as described above. The resulting pellets were prepared for EPR spectroscopy as above except that the $1{,}000 \times g$ pellet was tested, not discarded.

Marker Enzyme Assays

The distribution of mitochondrial and basolateral membranes was determined by assaying for the "marker" enzymes, succinate-cytochrome *c* reductase (SCR) and Na,K-ATPase. A SCN^- inhibitable, anion-stimulated ATPase was also assayed, not as a marker, but to determine whether or not its distribution is correlated with that of the amiloride binding site.

Na,K-ATPase activity was determined by a modification of the method of Hokin et al. (1973). Briefly, 20 to 40 μg of membrane protein was assayed in 0.5 ml of reaction buffer (composition in mM: 250 NaCl, 60 KCl, 8 $MgCl_2$, 5 Na_2-ATP, 50 imidazole – pH 7.2). One tube of an assay pair also contained 0.5 mM ouabain; each assay pair was run in duplicate. The reaction mixture was incubated at 30°C for 30 min and the reaction stopped by plunging the tubes into an ice-water mixture, followed immediately by the addition of 1 ml of 5% sodium dodecylsulfate (SDS). Inorganic phosphate was determined by a modified Fiske-SubbaRow method (Peterson, 1978). Na,K-ATPase activity was the ouabain-inhibitable component of the total ATPase activity.

Succinate-cytochrome *c* reductase was assayed according to Fishbein and Stowell (1968). Membrane protein (10 to 40 μg) was added to a reaction mixture (50 μM cytochrome *c*, Sigma, grade III, 28 mM $NaHPO_4$ – pH 7.4, and 2 mM NaCN) and the reaction started by the addition of Na-succinate (final concentration 25 mM) to the reaction cuvette and an equal volume of distilled water to the cuvette serving as the reagent blank. The reaction was monitored over a 15-min period at 550 nm in a double-beam spectrophotometer (Beckman, model 24).

An anion-stimulated ATPase was assayed in trout gill and kidney membranes using the method of Bornancin, de Renzis and Naon (1980). Enzyme activity was measured by incubating 20 to 40 μg membrane protein in 0.5 ml of reaction mixture (composition in mM: 20 Tris-acetate – pH 7.8, 5 $MgCl_2$, 20 NaCl, 2.5 Na_2-ATP, 0.5 ouabain) at 30°C for 30 min. One tube of an assay pair also contained 10 mM NaSCN, a potent inhibitor of the enzyme; each assay pair was run in duplicate. The reaction was stopped by plunging the tubes into an ice-water mixture, followed immediately by 1 ml of 5% SDS. Anion-stimulated ATPase activity was calculated as the SCN^- inhibitable component of the total ATPase activity. Inorganic phosphate was determined as stated above.

Membrane protein was estimated by the Lowry method as modified by Peterson (1977) using bovine serum albumin standards. The protein was precipitated prior to the Lowry assay to remove interfering substances known to be present in the membrane suspensions.

ASp Binding to Frog Skin, Trout Gill, Kidney and Skeletal Muscle Membrane Fractions

The determination of ASp binding to each of the three particulate fractions obtained from trout gill, kidney and skeletal muscle homogenates and four frog skin fractions required the EPR spectrum from four samples with the following composition: Two control tubes both containing ASp at 6×10^{-6} M, one of which also contained 8×10^{-4} M amiloride, provided a reference (standard) signal. Another pair of samples, identical to the two described above, also contained the membrane material to be tested at a protein concentration of approx. 1 mg/ml. The unbound ASp content of each sample was then calculated by comparing the EPR signals from each of the two protein-containing samples to their respective standards. The amiloride-displaceable binding was calculated by difference. The results are expressed as nanomoles ASp bound per mg protein. This experiment was repeated on five membrane preparations from trout gill and kidney, on two preparations from skeletal muscle, and on one frog skin preparation.

Results

Inhibition of Frog Skin SCC by ASp, Amiloride and Benzamil

Because subsequent experiments depended on the ability of amiloride or of an amiloride derivative to displace ASp from the amiloride binding site, it was important to determine the relative affinities of ASp, amiloride and benzamil for the site. This was accomplished by determining the dose-response relationship of these compounds on the SCC of frog skin.

The nitroxide derivative of amiloride is a potent inhibitor of SCC in isolated frog skin (Fig. 2). ASp produced 50% inhibition of SCC at 7.2×10^{-8} M (SE = 1.3×10^{-8}) whereas 3.9×10^{-7} M (SE = 0.5 $\times$

Table 1. Distribution of marker enzymes, ASp binding and protein in trout gill membrane fractions

Fraction	Na,K-ATPase	Succinate cytochrome *c* reductase	Anion ATPase	ASp binding	Protein
10,000 × *g*	27%[a]	65%	73%	81%	34%[d]
	(81.9 ± 12.7)[b]	(43.2 ± 15.5)	(537 ± 21.1)	(1.5 ± 0.3)[c]	(5.6 ± 0.8)[e]
27,000 × *g*	30%	24%	11%	19%	15%
	(208.4 ± 31.5)	(30.4 ± 7.2)	(186.8 ± 18.1)	(0.8 ± 0.3)	(2.4 ± 0.3)
105,000 × *g*	43%	12%	16%	0	51%
	(89.3 ± 17.5)	(4.2 ± 0.9)	(72.5 ± 13.8)		(8.6 ± 1.4)

[a] %total activity (post 1,000 × *g* supernatant) present in each fraction.
[b] Specific activity: nmol (mg protein)$^{-1}$ min^{-1}. Values are mean ± SEM, $n = 5$.
[c] Specific activity: nmol bound (mg protein)$^{-1}$. Values are mean ± SEM, $n = 6$.
[d] %total protein (post 1,000 × *g* supernatant) present in each fraction.
[e] mg protein.

10^{-7}) amiloride was required for 50% inhibition in the same skins. The value for amiloride agrees well with that determined by Benos, Mandel and Balaban (1979), 3×10^{-7} M. Benzamil was slightly more potent than ASp, half inhibiting the SCC at 4.8×10^{-8} M (SE = 0.5×10^{-8}).

Inhibition of all three compounds was reversible, but recovery from both ASp and benzamil required longer washout times than from amiloride. At the higher concentrations used (>3×10^{-7} M) control values for the SCC were reached in 10 to 15 min following the removal of either ASp or benzamil, while recovery from amiloride exposure took less than 10 min even at the highest concentration tested.

Quantitation of Spin-labeled Amiloride

The relationship between the magnitude of the EPR signal and the amount of unbound ASp is linear over the entire range of concentrations used in this study. The addition of amiloride or benzamil to the ASp solution at a constant concentration of 0.8 mM increased the slope of the standard curve by approximately 5 to 7%, necessitating the use of separate standards for the binding-displacement measurements. The relationship between ASp concentration and signal magnitude (SM) is given by the following equations: with amiloride SM = 24.3[ASp] − 3.4, without amiloride SM = 23.2[ASp] − 5.25. (Signal magnitude is in arbitrary units, ASp concentration in μM. The correlation coefficient $r > 0.999$ for both curves.) The basis of this phenomenon is unknown. Attempts at eliminating it by changing vehicle salt type (to tetraethylammonium chloride or NaCl) and concentration as well as substituting polyethylene tubing for the glass pipettes in this experiment were unsuccessful. The presence of excess amiloride or benzamil also reduced the variability in the magnitude of the spin signal. The coefficient of variation was 0.8% in the presence of 0.8 mM amiloride or benzamil and 3.0% without the unlabeled compound.

ASp Binding to Frog Skin, Trout Gill, Kidney and Skeletal Muscle Membrane Fractions

Spin-labeled amiloride binds to membranes prepared from trout gill (Fig. 3), kidney (Fig. 4) and skeletal muscle (Fig. 5) but the binding is reversed by excess amiloride only in gill and kidney membranes. Binding to skeletal muscle membranes was not reversed by amiloride in any of the three centrifugal fractions or in unfractionated homogenate.

It is clear, even without using quantitative methods, that trout kidney membranes contain far more of the amiloride binding site than does the gill (*compare* Figs. 3 and 4). This is borne out by Tables 1 and 2 which show that the total amiloride-displaceable binding was about 83 nmol in kidney and 10 nmol in gill. Furthermore, the binding sites from both tissues are predominantly in the 10K centrifugal fraction. In fact, enough ASp was bound in the 10K kidney fraction shown in Fig. 4 to obliterate the EPR signal. Further evidence that the ASp is bound to specific membranes is that changes in the homogenization techniques used to produce the membranes resulted in a different pattern of ASp binding in the particulate fractions. The data in Fig. 4 are from kidney membranes prepared in homogenization medium without DOC while the tissue used for Table 2 was homogenized in 0.1% DOC. The specific activity of amiloride-displaceable binding sites changed from 10K > 105K > 27K in Fig. 4 to 10K > 27K > 105K in Table 2.

Table 2. Distribution of marker enzymes, ASp binding and protein in trout kidney membrane fractions

Fraction	Na,K-ATPase	Succinate cytochrome *c* reductase	Anion ATPase	ASp binding	Protein
10,000 × *g*	5.7%[a] (73.1 ± 21.8)[b]	82% (179.2 ± 22.9)	62% (493 ± 76.4)	91% (11.3 ± 2.0)[c]	41%[d] (6.7 ± 1.5)[e]
27,000 × *g*	31% (585.6 ± 104.3)	16% (76.4 ± 6.1)	21% (368.2 ± 19.4)	4% (1.2 ± 0.7)	17% (2.7 ± 0.7)
105,000 × *g*	61% (468.4 ± 51.0)	2.5% (4.9 ± 0.4)	17% (122.1 ± 18.9)	5% (0.6 ± 0.6)	42% (6.8 ± 1.5)

[a] %total activity (post 1,000 × *g* supernatant) present in each fraction.
[b] Specific activity: nmol (mg protein)$^{-1}$ min^{-1}. Values are mean ± SEM, $n = 5$.
[c] Specific activity: nmol bound (mg protein)$^{-1}$. Values are mean ± SEM, $n = 4$.
[d] %total protein (post 1,000 × *g* supernatant) present in each fraction.
[e] mg protein.

In frog skin, all of the detectable, amiloride-displaceable binding was found in the 10K fraction. These membranes bound approximately 1.1 nmol (mg protein)$^{-1}$.

Distribution of Marker Enzymes and ASp Binding in Crude Particulate Fractions

Fractionation of trout gill tissue (Table 1) resulted in incomplete separation of membranes from mitochondria as well as the apical and basolateral region of the cell membrane. Na,K-ATPase, characteristic of the basolateral membrane (DePierre & Karnovsky, 1973), is found in highest specific activity, 208 nmol P_i (mg protein)$^{-1}$ min^{-1}, in the 27K fraction. However, as a consequence of protein distribution, this marker is distributed in approximately equal quantities among the three fractions. Succinate-cytochrome *c* reductase, the marker of inner mitochondrial membrane (Trumpower & Katki, 1978), is localized mainly in the 10K and 27K fractions and is only a minor contaminant of the 105K pellet. Anion-stimulated, SCN^- inhibitable ATPase is known to be associated with mitochondrial membranes (Van Amelsvoort et al., 1977) and possibly with apical cell membrane in chloride-transporting epithelia (Bornancin et al., 1980). If this enzyme is present only in mitochondrial membranes, the ratio of A^--ATPase activity to the activity of the mitochondrial marker should be reasonably constant. The ratio of A^--ATPase : SCR specific activities is approximately 12 in the 10K fraction and 17 in the 105K membranes, not a significant difference. Finally, approximately 80% of the membranes binding ASp are localized in the 10K fraction.

The fractionation of trout kidney tissue also resulted in a significant, albeit incomplete, segregation of membrane markers (Table 2). In kidney the majority of the Na,K-ATPase activity is present in the 27K and 105K pellets, the 10K pellet containing less than 6% of the basolateral membranes isolated by this procedure. Membranes containing SCR were especially concentrated in the 10K pellet, specific activity 179 nmol cytochrome *c* reduced (mg protein)$^{-1}$ min^{-1}, and were only a contaminant in the 105K pellet, specific activity 5 nmol cytochrome *c* reduced (mg protein)$^{-1}$ min^{-1}. The ratio of A^--ATPase : SCR activity increased significantly from 2.8 in the 10K fraction to 24.9 in the 105K membranes. Kidney 10K membranes are by far the most concentrated source of ASp binding sites to be found in either tissue. This fraction contained over 90% of the ASp binding sites with a specific activity of 11.3 nmol ASp bound per mg protein. In contrast to gill, detectable ASp binding was always present in the 27K membranes and usually present in the 105K fraction.

Discussion

The amiloride binding site is a useful, natural marker for apical (luminal) cell membranes of tight, Na^+-transporting epithelia. Unfortunately, Na^+-transporting epithelia are found in organs as morphologically diverse as vertebrate kidney, frog skin, fish gills and toad urinary bladder. Such heterogeneity of origin makes uniform methods for the isolation of apical membranes impossible. Unambiguous identification of membranes prepared from diverse sources is a problem because characteristic enzymatic and morphological markers are not necessarily conserved between preparations. The difficulties of using the marker enzymes useful in common mammalian systems are presented by Rodriguez and Edelman (1979) for a single organ, toad bladder.

Their solution, the $^{131}I/^{125}I$-iodination of the apical and basolateral membranes, is probably impractical and surely difficult in all but sheet epithelia. Clearly, the identification of a naturally occurring marker of apical membrane, common to all tight Na^+-transporting epithelia, would be very useful.

The data on frog skin (Fig. 2) show that the nitroxide derivative of amiloride, like the parent compound, is a potent, reversible inhibitor of sodium transport across frog skin. This result was expected as structure-activity trials conducted during the development of amiloride indicated substitutions similar to that shown in Fig. 1 had relatively little effect on drug potency (Cragoe, 1979). In fact, we found the affinity of the binding site is considerably higher for the spin-labeled compound. Since there is overwhelming evidence that the amiloride site is present in epithelia at the body surface of freshwater vertebrates, crustaceans, molluscs and annelids (Kirschner et al., 1973; Kirschner, 1979, 1983), the derivative should bind to the site in all of them. It is usually assumed that amiloride's naturetic action on the kidney has the same basis, and the data presented above suggest that the spin-labeled compound binds to the tubular sites as well.

Substantial reduction in amplitude of the EPR signal was noted in all tissues tested, including kidney, gill and skeletal muscle from trout (Figs. 3, 4 and 5). A fraction of the total reduction is not reversed when excess amiloride or benzamil are added. Such nonspecific "binding" might be due to any event that immobilizes or modifies the spin-label; e.g. solution in the lipid phase of the membranes or reduction of the nitroxide to a hydroxylamine which is not paramagnetic (Jost & Griffith, 1978). In any event, this fraction is not of interest in our context.

It is precisely the bound fraction that is released when amiloride or benzamil is added that reports the presence of the amiloride binding site. There is substantial evidence for this. First, the fact that amiloride and benzamil, both inhibitors of sodium transport, compete for the site, is a necessary, if not sufficient, condition. Second, the label binds in the submicromolar range, although instrumental limitations have not permitted measurements of binding near the K_i for frog skin SCC inhibition. The average concentration of free ASp in equilibrium with the bound ligand in the experiments in Tables 1 and 2 was 2.88 ± 0.53 μM and 2.02 ± 0.56 μM (mean $\pm$ SEM), respectively. It is therefore unlikely to involve such alternative sites of amiloride inhibition of epithelial Na^+ transport as the Na,K-ATPase (Soltoff & Mandel, 1983), modification of basolateral membrane permeability to K^+ (Davis & Finn, 1982), or by acting as a permeant weak base which dissipates the pH gradient necessary for Na^+-H^+ exchange (Dubinsky & Frizzell, 1983) since these require concentrations approaching millimolar. Further, the number of binding sites reflected the transport capacity of the tissue. For example, sodium reabsorption in trout kidney amounts to 950 μeq $(100\ g)^{-1}\ hr^{-1}$ (Holmes & Stainer, 1966), while in gill it is about 30 μeq $(100\ g)^{-1}\ hr^{-1}$. The specific activity of binding was 11.3 nmol $(mg\ protein)^{-1}$ for kidney membranes and 1.3 nmol $(mg\ protein)^{-1}$ for gill. Muscle, which has no Na^+ transport system comparable to the apical membrane of epithelia, showed no reversible binding.

It is clear from the distribution of marker enzymes and ASp binding that we have not successfully isolated apical membranes. Indeed, the objective of the simple cell fractionation scheme used in this study was not to purify any membrane type, but rather to observe how enzyme activity associated with inner mitochondrial membrane and basolateral plasma membrane becomes distributed as a consequence of their sedimentation properties, and how the distribution of the amiloride site correlates with that for the marker enzymes. Based on ASp binding, the $10{,}000 \times g$ pellet contains the largest fraction of apical membranes from either tissue. This fraction is often discarded and higher speed pellets used as a source of vesicles. In fact, it is difficult to predict how the apical membrane will distribute in centrifugal fractions; this may vary among tissues and with the homogenization procedures employed. Membrane vesicles demonstrating amiloride-sensitive Na^+ transport have been isolated from toad bladder in a post $8{,}000 \times g$ fraction ($210{,}000 \times g$ – 30 min; LaBelle & Valentine, 1980). Also from toad bladder, ^{125}I-labeled apical membranes were isolated by sucrose gradient fractionation of a $100{,}000 \times g$ – 60 min pellet (Rodriguez & Edelman, 1979). Finally, from the same tissue, Chase and Al-Awqati (1981; 1983) produced vesicles capable of amiloride-sensitive Na^+ transport using membranes from a $22{,}000 \times g$ – 30 min pellet. The preparation of surface membranes from diverse tissues can be even more variable (for a review *see* Neville, 1976). This finding underscores the need for a reliable and universal marker for apical membrane.

From an analytical standpoint, the detection of ASp by standard EPR techniques is adequate for performing binding studies as well as following the amiloride site through a cell fractionation procedure. In particular, the reversibility of binding, which has precluded the use of radioactive amiloride for this purpose, presents no problem and, in fact, is the basis for distinguishing between specific binding of ASp to the inhibitory site and nonspecific binding. The relationship between EPR signal and

unbound ASp is linear over at least a tenfold change in concentration beginning at 3 μM in our experiments. A satisfactory measurement can be made on 100 μg of gill membrane protein, less if the material is particularly rich in amiloride binding sites (e.g. 10 to 20 μg of kidney membrane protein). Since we have used neither maximum power of the microwave beam nor maximum signal amplification, it is clear that the sensitivity of the method can be extended.

Finally, ASp might aid in studying an outstanding question related to Cl^- transport in epithelia like gills and skins. There is no question that Cl^- is absorbed actively by a mechanism independent of Na^+ transport, electrical neutrality being maintained by counter transfer of HCO_3^- (reviewed by Kirschner, 1983). The apical membrane step is active, and it has been suggested that a SCN^--sensitive, anion-stimulated ATPase may be involved (Liang & Sacktor, 1976; de Renzis & Bornancin, 1977; Bornancin et al., 1980). However, this has been disputed. Mitochondrial specific activity of this enzyme is quite high and these organelles have been described as the sole source (Van Amelsvoort et al., 1977). Other data, including the enzyme distribution shown for kidney tissue in Table 2, indicate that a fraction of the ATPase does not distribute with mitochondrial markers (Bornancin et al., 1980). If Cl^- and Na^+ transfer occur in the same cells, co-purification of ASp binding and a transport-related, anion-stimulated ATPase should occur and would be easy to follow. Such an observation would place a fraction of the ATPase on the apical membrane and would provide support for a role in Cl^-/HCO_3^- exchange. Independent distribution of ASp and the anion-stimulated ATPase in a purification scheme would indicate either that the enzyme is not involved in anion transport or that Na^+ and Cl^- are transported by different cells in the epithelia. Thus, use of spin-labeled amiloride should resolve one question or raise another one.

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