Biochemical Evidence for the Presence of an Amiloride Binding Protein in Adult Alveolar Type II Pneumocytes*

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An amiloride binding protein in adult rat and rabbit alveolar type II (ATII) cells was characterized using three different antibodies against epithelial Na⁺ channel proteins. We found that 1) polyclonal antibodies raised against epithelial Na⁺ channel proteins from bovine kidney cross-react with a 135-kDa protein in ATII membrane vesicles on Western blots; 2) using the photoreactive amiloride analog, 2'-methoxy-5'-nitrobenzamil (NMBA), in combinations with anti-amiloride antibodies, we found that NMBA specifically labeled the same M, protein; and 3) monoclonal anti-idiotypic antibodies directed against anti-amiloride antibodies also recognized this same M, protein on Western blots. We also demonstrated a low benzamil affinity binding site (apparent $K_D = 370 \text{ nM}$) in rabbit ATII cell membranes and both high and low benzamil affinity binding sites (apparent $K_D = 6 \text{ nM}$ and $230 \text{ nM}$) in bovine kidney membranes using $[^3H]Br$-benzamil as a ligand. Pharmacological inhibitory profiles for displacing bound $[^3H]Br$-benzamil were also different between ATII cells and bovine kidneys. These observations indicate that adult ATII pneumocytes express a population of epithelial Na⁺ channels having a low affinity to benzamid and amiloride and a pharmacological inhibitory profile different from that in bovine kidney.

The alveolar epithelium is composed of two morphologically and functionally distinct cell types: type I and type II cells (Crapo et al., 1978; Haies et al., 1981). Alveolar type I cells are elongated and cover more than 90% of the alveolar surface area in the normal lung and have a primary physiological role in alveolar gas exchange. Alveolar type II (ATII) cells are cuboidal in shape and occupy less than 10% of the alveolar surface area. Nonetheless, ATII cells are critical for normal alveolar function. ATII cells synthesize pulmonary surfactant which stabilizes alveoli during respiration by lowering surface tension (Tierney, 1989) and are also involved in alveolar repair subsequent to lung injury by nitrogen dioxide or oxygen (Adamson and Bowden, 1974; Evans et al., 1975). In addition, ATII cells are thought to play an important role in the maintenance of normal lung fluid balance by transporting actively fluid from the alveolar space into plasma (Matalon, 1991). Studies from isolated adult lungs (Basset et al., 1987; Goodman et al., 1987) and lungs in intact animals (Basset et al., 1987b; Nielson and Lewis, 1990) suggest that the lung fluid transport is coupled to active transport of Na⁺ across the alveolar epithelial cells involving both apical Na⁺ conductive pathways and a basolateral Na⁺/K⁺-ATPase. The evidence for amiloride-inhibitable Na⁺ absorption in adult ATII cells comes from studies in cultured ATII cells (Mason et al., 1982; Cheek et al., 1989), $^{22}$Na⁺ influx studies in membrane vesicles (Matalon et al., 1991), and more recently in whole-cell patch clamp and $^{22}$Na⁺ uptake studies into freshly isolated ATII cells (Matalon et al., 1992). These studies suggest that adult ATII cells have a Na⁺-selective ion channel on their apical membranes with a low affinity to amiloride.

Recently, two classes of epithelial Na⁺ channels have been described with distinct pharmacological characteristics: high amiloride affinity (H-type) and low amiloride affinity (L-type) Na⁺ channels (Smith and Benos, 1991). A high amiloride affinity Na⁺ channel protein purified from bovine kidney and toad renal A6 cells has a molecular mass averaging 730 kDa and is comprised of at least six nonidentical subunits held together by disulfide bonds with apparent molecular masses of 315, 150, 95, 70, 55, and 40 kDa (Benos et al., 1987). Similar results were obtained in A6 cells by Kleyman et al. (1991) using RA 6.3, a monoclonal anti-idiotypic antibody that recognizes presumptive amiloride binding sites. Although some of the functional properties of low amiloride affinity Na⁺ channels have been characterized (Smith and Benos, 1991; Benos et al., 1992), little is known about the biochemistry of these Na⁺ channels. Therefore, the main purpose of this study is to provide evidence for the existence of a low amiloride affinity binding site in mammalian ATII cells. We further showed that this amiloride binding protein has a molecular mass of 135 kDa and may represent a component of a low amiloride affinity Na⁺ channel.

MATERIALS AND METHODS

ATII Cell Isolation—ATII cells were isolated from the lungs by an enzymatic dissociation method according to the procedures reported previously (Matalon et al., 1991). Rat ATII cells were isolated and used for Western blot analyses, because the anti-Na⁺ channel antibodies were raised in rabbit. Rabbit ATII cells were also isolated and used for equilibrium binding assays, because relatively large amounts of protein were required to perform the assays. However, as addressed...
under "Results," it seems that there is no species difference in alveolar epithelial Na⁺ transport between rat and rabbit lungs. Adult New Zealand White rabbits (weighing 1.9-2.2 kg) or Sprague-Dawley rats (weighing 200-220 g) were sacrificed with an intravenous or peritoneal injection of sodium pentobarbital (65 mg/kg animal), respectively. Following removal of alveolar macrophages by extensive lavage, the lung already inflated with 37 °C solution, Jokilik's modified minimum essential medium containing 10% fetal bovine serum and DNase (50 μg/ml). The crude cell suspension was filtered through nylon gauze of decreasing pore size (150, 41, and 15 μm).

Following removal of alveolar macrophages by extensive lavage, the lungs were digested with warmed (37 °C) Jokilik's modified minimum essential medium containing elastase (1.3 units/ml) and DNase (10 μg/ml). The resulting pellet was resuspended with PBS and processed for Western blot analysis, photoaffinity labeling, and binding assay.

Membrane Vesicle Preparation—Membrane vesicles from ATII cells, alveolar macrophages, and Chinese hamster ovary cells were prepared by a differential centrifugation method as described previously (Nair and Ho, 1986; Sariban-Sohraby and Benos, 1986). Alveolar macrophages and non-epithelial Chinese hamster ovary cells were used as a negative control as they do not have amiloride-sensitive Na⁺ channels (Tousson et al., 1989; Matalon et al., 1991, 1992). After the cells were homogenized in homogenization buffer (sucrose 250 mM and triethanolamine 10 mM, pH 7.4) supplemented with protease inhibitors, the homogenate was spun at 500 x g for 10 min to remove nuclei and unbroken cells. The supernatant was then spun at 6,000 x g for 10 min to sediment mitochondria. The supernatant was recovered and spun at 95,000 x g for 1 h (Beckman Ultracentrifuge, LB-60M). The resulting pellet was resuspended in PBS, and protein concentration was measured using the Comassie Blue G-250 binding assay kit from Bio-Rad. Fresh bovine kidneys were collected from a local abattoir. After papillary tips were excised from the kidney and transferred to homogenization buffer, papillary membrane vesicles were prepared as above. The membrane vesicles were stored at -70 °C until used for Western blot analysis, photoaffinity labeling, and binding assays.

Photoaffinity Labeling—The procedure employed was modified from Benos et al. (1987) and Kleyman et al. (1989). ATII cell membrane vesicles (0.5 ml, 0.15 mg/ml in PBS) were placed into a 13 x 100-mm glass test tube. After preincubation with the photoreactive amiloride analog, NMBA (2'-methoxy-5'-nitrobenzamid) for 20 min in the dark to equilibrate NMBA binding to its binding site, photolysis was carried out with constant stirring. The synthesis of NMBA and its mechanism of photoincorporation have been described previously (Kleyman et al., 1989). The sample was placed 30 cm away from the light source inside a water-jacketed glass chamber and 12 cm from the ultraviolet light (passing through a 313-nm narrow band-pass filter that is directly attached to a heat absorbing filter, Oril) emitted from a 150-watt Xenon lamp. After photolysis, membrane vesicles were washed with PBS and collected by repeated centrifugation using an Eppendorf centrifuge (16,000 x g) for 30 min. The resulting pellet was resuspended with PBS and processed for SDS-PAGE and Western blotting. The photolabeled proteins were detected by anti-amiloride antibodies that have been previously raised and characterized (Kleyman et al., 1986).

Electrophoresis and Western Blotting—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Bio-Rad Mini-Protein II slab gel apparatus using the discontinuous buffer system of Laemmli (1970). Procedures for SDS-PAGE and Western blotting were described in detail previously (Sorscher et al., 1988). In all experiments, SDS-PAGE was carried out on 7.5% acrylamide gels, except for the experimental condition of Fig. (1). In order to exclude possible interactions between the Fe portion of intact IgG and the samples, the Fe portion of the purified IgG of anti-Na⁺ channel antibodies was removed using the immunoPure F(ab')² preparation kit from Pierce Chemical Co. and the resultant F(β)γ fragment used for Western blotting. The results obtained using the intact antibodies and F(β)γ fragments were identical.

Binding Assay—[3H]Br-benzamil was prepared (14.4 Ci/mmole) and used as a ligand to label putative amiloride-sensitive epithelial Na⁺ channels; it is known that benzamil binds to H-type epithelial Na⁺ channels with a higher affinity than amiloride (Benos, 1988; Kleyman and Cracoe, 1988). Either freshly isolated rabbit ATII cell membranes or bovine renal papillary membranes (routinely 50 μg) were incubated at 4 °C in 0.1 ml of a solution containing PBS, pH 7.4, and the required concentrations of [3H]Br-benzamil. After 60 min of incubation, the amount of specific binding was separated from non-specific binding by successive 30-min incubations of incubation continually terminated by rapid filtration at 0 °C through Whatman GF/B glass fiber filters that have been pretreated with 0.3% polyethyleneimine for 1 h. It was reported previously that this pretreatment increased the specific binding of most radiolabeled ligands to their membrane receptors (Bruns et al., 1983). Competition experiments of specific [3H]Br-benzamil binding to membranes were performed by adding unlabeled amiloride analogs, such as amiloride (1 nM to 1 μM), benzamil (1 nM to 1 mM), or EIIPA (1 nM to 1 mM), into the incubating solution. Non-specific binding was determined in parallel incubations in the presence of an excess amount of unlabeled amiloride (1 mM). Hill plots of the specifically bound [3H]Br-benzamil were fitted according to the following equation: log f/(1-f) = n log (L/Kd). In this model, n is the theoretical number of ligand binding sites per receptor molecule, Kd = dissociation constant, L = free ligand concentration, and f = B/Bmax is fractional receptor site occupancy (Bennett and Yamamura, 1985). All data in binding assays are presented as the mean ± S.E.

RESULTS

In order to demonstrate the presence of an amiloride binding protein in adult ATII cells, three different antibodies related to epithelial Na⁺ channel protein epitopes were used. We used membrane vesicular membranes derived from freshly isolated rat and rabbit ATII cells. First, polyclonal antibodies raised against epithelial Na⁺ channel proteins purified from bovine kidney were used. The antibodies cross-reacted with a polypeptide with an apparent molecular mass of 135 kDa from rat ATII cell membranes on Western blots under both reduced (10 mM dithiothreitol) and nonreduced conditions (Fig. 1, lanes 2 and 3). The ineffectiveness of a sulfhydryl group reducing agent (10 mM dithiothreitol) on the apparent molecular mass of the 135-kDa protein was also observed in photoaffinity labeling studies (Fig. 3B). Besides the 135-kDa polypeptide, two other faint bands with molecular mass about 150 and 125 kDa were also detected by anti-Na⁺ channel antibodies (Fig. 1, lane 3). In the membrane vesicles from bovine renal papilla, the antibodies recognized four polypeptides with apparent molecular masses of 300, 165, 150, and 95 kDa, respectively.

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Fig. 1. Western blot analysis of membrane vesicular proteins from bovine kidney papilla (lane 1) and adult rat ATII cells (lanes 2 and 3) incubated with F(ab')² fragments of anti-Na⁺ channel antibodies raised against purified Na⁺ channel proteins from bovine renal papilla for 2 h except lane 3 (for 4 h). The membrane vesicles from rat ATII cells were also incubated with the preimmune serum for 2 h (lane 4). All experiments were performed under reducing conditions (10 mM dithiothreitol) except lane 2. 15 μg of protein were loaded on lane 1, 30 μg of protein on lane 2, and 50 μg of protein on lanes 3 and 4. Note triplet polypeptides (150, 135, and 125 kDa) labeled with the antibodies in lane 3.

1 2 3 4

- 135 kDa

1234
respectively (Fig. 1, lane 1). Occasionally, a 55-kDa polypeptide was also detected. The observation of multiple polypeptides (150, 135, and 125 kDa) around the 135-kDa in ATII cell membranes and duplicate polypeptides (165 and 150 kDa) around 150 kDa in bovine kidney membranes was reproducible independent of different membrane preparations. Preimmune serum showed no reactivity against ATII cell membrane proteins (Fig. 1, lane 4).

In a second approach, a recently synthesized photoreactive amiloride analog, NMBA, was used in combination with polyclonal anti-amiloride antibodies. This procedure was used previously to identify Na⁺ channels in bovine kidney membrane vesicles (Kleyman et al., 1989). The photolabeled ATII cell membrane vesicles were first separated by SDS-PAGE, then NMBA bound protein, presumably an amiloride binding protein, was resolved by anti-amiloride antibodies. Figs. 2 and 3 show the results from photoaffinity labeling experiments in membrane vesicles from rat and rabbit ATII cells, respectively. Rat ATII cell membrane vesicles were photolabeled with 0.2 μM NMBA for varying periods of time (Fig. 2A) or for 10 min with varying concentrations of NMBA (Fig. 2B). In these experiments, NMBA specifically labeled a triplet of polypeptides (150, 135, and 125 kDa) with the strongest binding to the 135-kDa polypeptide. NMBA also periodically labeled 230-, 70-, 55-, 45-, and 20-kDa polypeptides. However, these polypeptides, except the 70-kDa polypeptide, seem to be nonspecific, because (a) the photoincorporation of NMBA into these polypeptides was not blocked by excess addition of benzamil and (b) similar molecular mass polypeptides were detected in alveolar macrophage membrane preparations (Fig. 2A). A 230-kDa polypeptide was also detected regardless of photoactivation (Fig. 2A). On the other hand, the photoincorporation of NMBA into the 135-kDa polypeptide was competitively inhibited by high concentrations (300 μM) of unlabeled benzamil (Fig. 2A). There was also an apparent competitive inhibition by benzamil on the NMBA photoincorporation into the 70-kDa protein. Similar observations were made about time course, dose response, and NMBA labeling pattern were obtained in rabbit ATII cell membrane vesicles (Figs. 3, A and B). These findings are consistent with the idea of no species difference in alveolar epithelial Na⁺ transport between rat and rabbit lungs. This idea is also supported by previous demonstrations: (a) the presence of an amiloride-blockable active Na⁺ transport system in intact rabbit (Nielson and Lewis, 1990) and rat (Basset et al., 1987b)
lungs; (b) the presence of low amiloride affinity Na⁺ conductive pathways in freshly isolated rat and rabbit ATII cells (Matalon et al., 1991, 1992).

Monoclonal anti-idiotypic antibodies (RA 6.3) directed against anti-amiloride antibodies were used as a third independent method to identify the presence of an amiloride binding protein in adult ATII cells. This antibody has been shown to inhibit Na⁺ transport across A6 cell monolayers and used to identify an amiloride binding protein as part of an H-type Na⁺ channel in A6 cells (Kleyman et al., 1991). RA 6.3 recognizes a polypeptide in rat ATII cell membrane vesicles with an apparent molecular mass of 135 kDa on Western blots, implying that this polypeptide is an amiloride binding protein (Fig. 4). Fig. 5 shows competition experiments between NMBA and RA 6.3, showing that NMBA and RA 6.3 compete for the same binding site. Compared with the control, a condition without RA 6.3 addition (Fig. 5, lane 1), preincubation of rat ATII cell membrane vesicles with RA 6.3 (1:100 dilution) for 3 h before adding 2 μM NMBA (Fig. 5, lane 2) attenuated the intensity of photolabeled (10 min) 135-kDa protein, that is, the amount of NMBA that had been photoincorporated. Non-specific mouse ascites (1:100 dilution) did not interfere with NMBA photoincorporation (Fig. 5, lane 3). The NMBA-labeled polypeptides with molecular mass values around 50–70 kDa in the control (Fig. 5, lane 1) were covered by excess amounts of reduced (10 mM dithiothreitol) IgGs in Fig. 5, lanes 2 and 3.

In order to characterize further an amiloride binding site in adult ATII cells, [³H]Br-benzamil was used as a ligand to titrate an amiloride binding site in equilibrium binding studies. Fig. 6 shows the results of such equilibrium binding studies, in which increasing concentrations of [³H]Br-benzamil were added to a fixed amount of rabbit ATII cell membrane proteins. Fig. 6A shows the concentration dependence of the specific [³H]Br-benzamil binding to rabbit ATII cell membranes. Scatchard analysis of specific [³H]Br-benzamil binding to rabbit ATII cell membranes (Fig. 6B) showed a single population of binding site with an apparent dissociation constant (Kₐ) of 370 nM. The total number of binding sites was 12.5 pmol/mg of protein. A Hill plot of specifically bound [³H]Br-benzamil to rabbit ATII cell membranes revealed that the Hill coefficient was close to 1.0 (Fig. 6C), suggesting that there is no cooperative binding of [³H]Br-benzamil to its binding site. The same experiment was performed using bo-

![Image of Western blot analysis of membrane vesicle proteins from freshly isolated rat ATII cells (lane 1) and rat alveolar macrophages (lane 2) incubated with monoclonal anti-idiotypic antibody, RA 6.3, raised against anti-amiloride antibodies. 45 μg of protein were loaded on each lane. Anti-idiotypic antibody specifically recognized a 135-kDa polypeptide.](image1)

![Image of competition experiments between NMBA and anti-idiotypic antibody RA 6.3. Membrane vesicles from rat ATII cells were photolabeled for 10 min with 2 μM NMBA in the absence (lane 1) or presence (lane 2) of RA 6.3. Preincubation of membrane vesicles with RA 6.3 for 3 h (lane 2), before adding NMBA, attenuated the intensity of photolabeled 135-kDa protein, suggesting that NMBA and anti-idiotypic antibody compete for the same binding site. Preincubation with non-specific mouse ascites for 3 h did not interfere with NMBA photoincorporation (lane 3). The photolabeled polypeptides with molecular masses around 50–70 kDa in lane 1 were covered by excess amount of reduced (10 mM dithiothreitol) IgGs in lanes 2 and 3.](image2)

![Image of specific binding of [³H]Br-benzamil to rabbit ATII cell membranes as a function of [³H]Br-benzamil concentration. The incubation time was 90 min on ice. Each datum point represents the mean ± S.E. of three measurements. The data from this plot were also used for Scatchard plot (B) and Hill plot (C). B. Scatchard plot of specific [³H]Br-benzamil binding to rabbit ATII cell membranes. ATII cell membranes were incubated with 0.5–800 nM of [³H]Br-benzamil in the absence or presence of 1 mM amiloride. The Kₐ given by the inverse of the slope, was 370 nM, and the total number of binding sites (the x axis intercept) was 12.5 pmol/mg of protein. C. Hill plot of the specifically bound [³H]Br-benzamil to ATII cell membranes were fitted according to the following equation; log [f/(1 - f)] = n log ([L]/Kₐ). In this model, n = the theoretical number of ligand binding sites per receptor molecule, Kₐ = dissociation constant, [L] = free ligand concentration, and f = B/Bₜₐ₇ = fractional receptor site occupancy.](image3)
vire renal papillary membranes as a control to titrate a high amiloride affinity binding site (Fig. 7). The Scatchard plot for the specific [3H]Br-benzamil binding to kidney papillary membranes is curvilinear (Fig. 7B), suggesting (a) negative cooperativity of ligand binding, (b) multiple independent receptor sites, or (c) interconverting affinity states (Limbird, 1986). If we assume that the ligand binds to two populations of binding sites, the curvilinear shape would be resolved into two linear components with the best fitting linear regression curve: $K_{d1} = 6$ nM, $B_{max1} = 0.5$ pmol/mg protein, $K_{d2} = 230$ nM, and $B_{max2} = 11$ pmol/mg protein. In order to determine whether a single binding site or two binding site model was a better representation of the data, we performed an F statistic, whether a single binding site or two binding site model was a better representation of the data, we performed an F statistic, and found that it was highly significant ($p < 0.001$) to reject the single binding site hypothesis. The ratio of the respective amounts of high affinity to low affinity binding sites was 1:22.

The results of competition experiments between [3H]Br-benzamil and unlabeled amiloride analogs are shown in Fig. 8A for bovine kidneys and Fig. 8B for rabbit ATII cells. In bovine kidneys, the concentration of unlabeled amiloride that reduces the specific [3H]Br-benzamil binding by 50% (apparent $K_i$) is 0.5 μM (Fig. 8A). Benzamil and EIPA displaced [3H] Br-benzamil binding with apparent $K_i$ of 50 nM and 4 μM, respectively, showing the structure-inhibitory profile as benzamil > amiloride > EIPA. In contrast, the structure-inhibitory profile for ATII cells is very much different from bovine kidney in that EIPA > amiloride > benzamil. Apparent $K_i$ values for benzamil, amiloride, and EIPA in ATII cells are 100, 45, and 5 μM, respectively (Fig. 8B).

Finally, in ATII cells low [3H]Br-benzamil concentrations (50 nM) were used in another competition experiment to favor the occupancy of any possibly existing high amiloride affinity binding site, which might not be detected in Scatchard analysis. However, as shown in Fig. 9, the apparent $K_i$ of amiloride is 11 μM, which is close to the $K_i$ (30 μM) obtained by using high concentrations (300 nM) of [3H]Br-benzamil, indicating further that ATII cell membranes contain a single population of a low amiloride affinity binding site. But, it is important to note that these $K_i$ values of amiloride in ATII cells are...
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more than 50 times less potent than the \(K_i\) of amiloride (0.5 \(\mu M\)) in bovine kidney.

**DISCUSSION**

Apically located amiloride-sensitive Na\(^+\) channels can be divided into at least two main groups based upon their sensitivity to amiloride and its analogs (Smith and Benos, 1991; Oh and Benos, 1992). The first class includes classic epithelial Na\(^+\) channels present in the apical membranes of high electrical resistance epithelia, such as frog skin, toad urinary bladder, and toad renal A6 cells. These channels have a high affinity to amiloride with a \(K_i\) less than 1 \(\mu M\) at physiological external Na\(^+\) concentrations. We will refer to these channels as the H-type Na\(^+\) channel ("H" denotes a high amiloride affinity). The other class has a low affinity to amiloride with \(K_i > 1 \mu M\) and are called L-type Na\(^+\) channels ("L" denoting a low amiloride affinity). These L-type Na\(^+\) conductive pathways have been found in various epithelial cells, including adult ATII cells (Matalon et al., 1991, 1992).

Several observations from the previous and present studies suggest that a polypeptide with a molecular mass of 135 kDa in adult ATII cells could be a component of an epithelial Na\(^+\) channel, probably an L-type Na\(^+\) channel, and not other amiloride-blockable transporters. First, the specificity of the anti-Na\(^+\) channel antibody and the anti-idiotypic antibody (RA 8.3) has been well characterized through immunocytochemical studies (Brown et al., 1989; Tousson et al., 1989; Kleyman et al., 1991), demonstrating that these antibodies do not cross-react with other amiloride-sensitive transporters (such as Na\(^+\)/H\(^+\) or Na\(^+\)/Ca\(^++\) exchangers or the Na\(^+\)/K\(^+\)/ATPase). Recently, anti-Na\(^+\) channel antibody was also used to immunolocalize putative L-type Na\(^+\) channels in rat ATII cells (Matalon et al., 1992), canine lingual epithelial cells (Simon et al., 1992), and guinea pig cochar hair cells (Hackett et al., 1991). Matalon et al. (1992) also demonstrated that the antibodies did not recognize any specific proteins in membrane preparations from rat alveolar macrophages and non-epithelial Chinese hamster ovary cells on Western blots. Second, as shown in Fig. 2A, high concentrations of NMBA (0.2 \(\mu M\)) were needed to photolabel an amiloride binding site in rat ATII cells, and high concentrations of benzamil (300 \(\mu M\)) were required to block the photoincorporation of NMBA. In contrast, it was reported previously that 5-25 nm concentrations of NMBA were used to photolabel an amiloride binding protein of a H-type Na\(^+\) channel in bovine kidney and A6 cells, and their NMBA photoincorporation was blocked by 2.5-5 \(\mu M\) benzamil (Kleyman et al., 1989). Therefore, it can be inferred that ATII cell membranes contain a low affinity benzamil (amiloride) binding site. Third, and perhaps most importantly, it is well documented that the structure-inhibitory profile expected for H-type Na\(^+\) channels is benzamil > amiloride > EIPA (Benos, 1985; Kleyman and Cragoe, 1988). Similar results were obtained for the H-type Na\(^+\) channel found in bovine renal papillary membranes (Fig. 8A). In contrast, the structure-inhibitory profile for ATII cells is very much different, showing EIPA > amiloride > benzamil (Fig 8B). Matalon et al. (1991, 1992) also demonstrated similar pharmacological inhibitory profiles for amiloride and its analogs in \(^{22}\)Na\(^+\) uptake studies into ATII cells and their membrane vesicles. This particular structure-inhibitory pattern, that is, the higher or comparable sensitivity to EIPA as compared with amiloride, benzamil, or phenamil, is a distinct characteristic of L-type Na\(^+\) channels (Asher et al., 1987; Moran et al., 1988; Benos et al., 1992).

The \(K_i\) values of all amiloride analogs deduced from competition experiments in ATII cells seems to be overestimated because of the large difference observed between the calculated \(K_i\) of benzamil (370 nM) from Scatchard analysis and the apparent \(K_i\) of benzamil (100 \(\mu M\)) from competition experiments. This large overestimation of \(K_i\) of benzamil in competition experiments can be explained in part by nonspecific binding of high concentrations of [\(^{3}H\)]Br-benzamil (300 nM) to lipid membranes, thus requiring excess amounts of unlabeled benzamil to displace nonspecifically bound [\(^{3}H\)]Br-benzamil. This explanation is reasonable, because benzamil is a derivatized amiloride having a benzyl ring structure on its guanidinium moiety. Thus, benzamil is much more hydrophobic than amiloride. Another possibility could be the overestimation of free concentrations of unlabeled benzamil because of its high nonspecific binding to lipid membranes or poor dissolution in the incubation medium. Alternatively, inadequate separation of free from bound [\(^{3}H\)]Br-benzamil to membranes may also contribute to an inaccurate estimation of specifically bound [\(^{3}H\)]Br-benzamil. Nevertheless, even though the apparent \(K_i\) values of amiloride analogs obtained from competition experiments are not exact, the dramatically different structure-inhibitory profiles in ATII cells indicate that the amiloride binding sites on the Na\(^+\) channels in bovine kidneys and ATII cells are pharmacologically distinct.

The ATII cells appear to contain a single low benzamil affinity binding site with an apparent \(K_i\) of 370 nM (Fig. 6). In contrast, bovine kidney membranes may possess two distinct populations of benzamil binding sites, namely one with a \(K_i\) of 9 nM and a second with a \(K_i\) of 230 nM (Fig. 7). It is interesting to compare the \(K_i\) values of high and low benzamil affinity binding sites in bovine kidneys from this study and those in pig kidneys (Barbry et al., 1989). Barbry et al. (1989) reported that they observed two binding sites from pig kidneys both in crude membrane preparations and in purified Na\(^+\) channels. Their experimentally determined \(K_i\) values of benzamil (50 and 400 nM) are in good agreement with those from the present study (6 and 230 nM). The ratio of the respective amounts of high affinity to low affinity binding sites was also comparable in bovine kidneys (1:22) and in pig kidneys (1:14) (Barbry et al., 1989). In fact, the presence of both high and low affinity benzamil or amiloride-sensitive Na\(^+\) transport systems has been demonstrated in rat colonic enterocytes (Bridges et al., 1988, 1989), in toad urinary bladders (Garty, 1984; Asher et al., 1987), and in membrane vesicles made from bovine renal papilla (Sariban-Sohraby and Benos, 1986).

The anti-Na\(^+\) channel antibodies recognized tripeptide poly-peptides (150, 135, and 125 kDa) around 135 kDa in ATII cell membranes and duplicate poly-peptides (165 and 150 kDa) around 150 kDa in bovine kidney membranes. NMBA was also photoincorporated into the tripeptide poly-peptides in rat and rabbit ATII cells with similar molecular masses. This observation of multiple poly-peptides around the major amiloride binding protein in ATII cells and bovine kidneys may indicate differentially processed forms of an amiloride binding protein. It is known that one of the H-type Na\(^+\) channel subunits, namely, the 150-kDa poly-peptide, binds amiloride (Smith and Benos, 1991; Benos et al., 1992). Considering (a) the similar molecular mass of an amiloride binding protein in ATII cells and bovine kidney and (b) cross-reactivity by anti-Na\(^+\) channel antibodies, it is possible that a conservation of an amiloride binding subunit occurred throughout epithelial Na\(^+\) channel evolution.

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