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Analysis of the Distribution of Na^+/H^+ Exchanger Isoforms among the Plasma Membrane Subfractions of Bovine Kidney Cortex: Reevaluation of Methods for Fractionating the Brush-Border and the Basolateral Membranes[1]

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Distribution of the NHE1 and the NHE3 isoforms of Na^+/H^+ exchanger in the plasma membranes of bovine kidney cortex was analyzed. Fractionation of the plasma membranes by centrifugation on a Percoll density gradient resulted in clear separation of the basolateral membranes (BLM) from the brush-border membranes (BBM), with Na^+,K^+-ATPase and aminopeptidase M as their respective marker enzymes. Under these conditions, a 110 kDa protein cross-reactive with an anti-NHE1 antibody was detected exclusively in the BLM fractions, while a 90 kDa protein cross-reactive with an anti-NHE3 antibody was detected in the BBM fractions. A conventional Mg^{2+}-precipitation method for obtaining the BBM, which is adequate with rabbit kidney as a starting material, turned out to be inadequate with bovine kidney cortex, since a considerable amount of the 110-kDa NHE1 protein was detected in the bovine kidney BBM fraction prepared by this procedure, together with the 90-kDa NHE3 protein. Percoll density gradient centrifugation is thus strongly recommended for the fractionation of BBM and BLM of bovine kidney cortex. The bovine NHE1 isoform was shown to be unique in that it is far less sensitive to the inhibition by ethylisopropylamiloride than that of other species.

Key words: basolateral membrane, bovine kidney cortex, brush-border membrane, ethylisopropylamiloride, Na^+/H^+-exchanger.

Na^+/H^+ exchanger (NHE) of mammalian cells is an intrinsic protein of the plasma membrane that mediates 1:1 exchange of Na^+ and H^+ ions in various tissues (for recent reviews see Refs. *1* and *2*). Occurrence of NHE isoforms designated as NHE1 to NHE5 has been reported based on the information obtained through cDNA cloning and chromosomal mapping in recent years (*3-7*). The NHE1 isoform is expressed ubiquitously in every tissue, while the NHE3 isoform is most abundantly expressed in the kidney cortex (*6*). The NHE1 isoform was expressed in the basolateral surfaces of polarized cells in culture, while on introduction of the NHE3 cDNA into these cells the NHE3 isoform was targeted to the apical surfaces (*8*). By using a NHE1-specific antibody, the NHE1 protein was in fact detected in the basolateral membranes (BLM) of intestinal and renal epithelia of rabbit (*9, 10*). These findings together with several lines of biochemical information, mainly on the inhibitor-sensitivity of the NHE activity of isolated membrane vesicles, led to the view that in renal and intestinal epithelial cells NHE1 is localized to the BLM, while NHE3 is expressed in the brush-border membranes (BBM) (*11*). More recently, distribution of the NHE3 isoform in animal tissues was investigated directly in rabbit and canine kidneys using antibodies specific to rat NHE3, and the results of these studies indicated that NHE3 is mainly localized to the BBM (*12, 13*).

In one of our previous studies we showed that a 110 kDa protein cross-reactive with an antibody against a C-terminal peptide of human NHE1 is present in the BBM fraction from bovine kidney cortex prepared according to the commonly used Mg^{2+}-precipitation procedure (*14*). Subsequent immunoaffinity purification demonstrated that the 110 kDa protein was in fact a reconstitutively active bovine Na^+/H^+ exchanger, but that the amiloride sensitivity of the exchanger was much lower than that of typical NHE1 transporters found in other species. It was uncertain whether this entity in fact represented the bovine NHE1. Bovine NHE isoforms, on the whole, thus have not been well characterized so far. In particular, an immunologically NHE1-related exchanger with low amiloride-sensitivity is an unusual entity, and its characteristics as well as its precise localization would merit further studies. The occurrence and distribution of NHE3 and its possible relatedness to the 110 kDa NHE with low amiloride-sensi-

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Abbreviations: BBM, brush-border membrane; BLM, basolateral membrane; EIPA, ethylisopropylamiloride; KLH, keyhole limpet hemocyanin; NHE, Na^+/H^+ exchanger.

tivity are also intriguing. For this reason we prepared antibodies raised against C-terminal peptides of human NHE1 and rabbit NHE3, respectively, and cautiously examined the distribution of proteins cross-reactive with these antibodies among the membrane fractions obtained from bovine kidney cortex.

MATERIALS AND METHODS

BBM from rabbit and bovine kidney cortex was prepared by a magnesium-precipitation method essentially according to Aronson (*15*) with slight modification in that homogenization was carried out in a homogenization buffer consisting of 0.25 M sucrose and 10 mM Tris-HEPES buffer (pH 7.4) containing 0.5 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg antipain, twice for 1 min each, using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) at the speed of 5,000 rpm.

Plasma membranes of rabbit and bovine kidney cortex containing both BBM and BLM were first prepared as described previously (*16*), and then further fractionated by either sucrose or Percoll density gradient centrifugation. Sucrose density gradient centrifugation was carried out as described in Ref. *16*. For Percoll density gradient centrifugation, the crude plasma membranes were suspended in the homogenization buffer containing 11 or 13% Percoll (Pharmacia Biotech, Tokyo) for bovine or rabbit membranes, respectively, at a protein concentration of 1 mg/ml, and then centrifuged at 27,000 rpm for 25 min in a Beckman 60Ti rotor. After the centrifugation fractions of appropriate size were collected from the top of the tube using ISCO Model 640 density gradient fractionator. Membranes were recovered by centrifugation for 90 min at 50,000 rpm, resuspended in a solution consisting of 0.25 M mannitol, 10 mM $MgSO_4$, and 1 mM Tris-HEPES buffer (pH 7.4), and stored frozen at −80°C.

An NHE1-specific polyclonal antibody raised against a C-terminal peptide (794Cys----815Gln) of human NHE1 conjugated to keyhole limpet hemocyanin (KLH) as an antigen (*14*) was used. An NHE3-specific polyclonal antibody was raised against a C-terminal peptide (Cys-816Ser ----832Met) of rabbit NHE3, conjugated *via* *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Pierce, Rockford, IL, USA) to KLH (Sigma, St. Louis, MO, USA), as an antigen, and purified in a similar manner as described previously (*14*). Immunoblotting using these isoform-specific antibodies was carried out as previously described (*14*). Immunoreactive proteins were detected by using horseradish peroxidase-conjugated Protein A and the ECL detection reagents (Amersham, Buckinghamshire, UK).

Activity of aminopeptidase M was measured according to the procedure of Haase *et al.* (*17*) in a reaction medium consisting of 20 mM HEPES/Tris (pH 7.4), 100 mM mannitol, and 0.3 mg/ml L-leucine-4-nitroanilide. Membrane vesicles containing 5–10 μg of protein in 8 μl were added to 92 μl of the reaction medium, and the appearance of 4-nitroaniline was monitored at 405 nm at 25°C. Activities of Na^+/H^+ exchange and Na^+/K^+ ATPase were measured as described in Ref. *16*. Ethylisopropylamiloride (EIPA) was purchased from Research Biochemicals, Natick, MA, USA. Protein was determined by the method of Lowry *et al.* (*18*) with bovine serum albumin as a standard.

RESULTS

Preparation of Anti-NHE3 Antibody and the Specificity of Its Reaction—Anti-NHE3 antibody was raised in rabbit utilizing a KLH-coupled peptide with a sequence corresponding to the C-terminal portion of rabbit NHE3 as an antigen (Fig. 1), and then affinity-purified as described under "MATERIALS AND METHODS." The C-terminal amino acid sequence of rabbit NHE3 was used because the amino acid sequence of bovine NHE3 remained unavailable. No significant similarity in amino acid sequence is noted between this peptide and any portion of other NHE isoforms so far reported. Moreover, there seemed to be a sound reason to assume that the anti-NHE3 antibody would cross-react with bovine NHE3, since 11 out of 17 amino acid residues of this rabbit NHE3-C-terminal peptide were conserved among rabbit, human, and rat NHE3 isoforms.

The purified antibody detected a 90 kDa protein on immunoblotting analysis in the rabbit kidney cortex membrane prepared by the Mg^{2+}-precipitation procedure as shown in Fig. 2. The binding of the anti-NHE3 antibody was specific in that it was abolished by preincubating the antibody with an excess amount of the NHE3-peptide.

Cross-Reaction of Anti-Human NHE1 and Anti-Rabbit NHE3 Antibodies with Bovine NHE Isoforms—Distribution of proteins that cross-react with anti-human NHE1 and anti-rabbit NHE3 antibodies was examined by im-

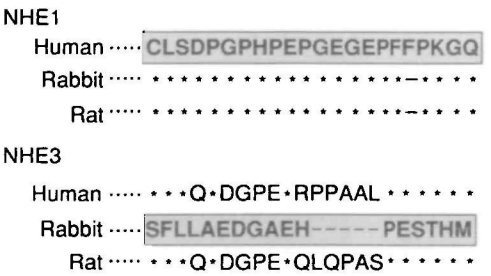

Fig. 1. **Sequences of peptides representing the C-terminal portions of human NHE1 and rabbit NHE3 isoforms against which anti-NHE1 and anti-NHE3 antibodies were raised.** Amino acid sequences of peptides used as antigens are shaded. Sequences representing the corresponding portions of rabbit (*9*) and rat (*6*) NHE1 and human (*26*) and rat (*6*) NHE3 are shown for reference, with coincidence among the three species indicated by asterisks.

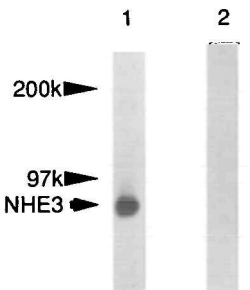

Fig. 2. **Anti-NHE3 antibody specifically detects a 90 kDa protein in the BBM from rabbit kidney cortex.** Immunoblots of rabbit kidney BBM proteins which were analyzed by SDS-PAGE and probed by anti-NHE3 antibody are shown. For lane 2, immunoblotting was performed using the anti-NHE3 antibody preincubated with 0.2 mg/ml of the NHE3-C-terminal peptide.

munoblotting using bovine kidney BBM vesicles prepared by the Mg^{2+}-precipitation procedure. With the anti-NHE1 antibody, we detected a 110 kDa protein as previously reported. In addition, anti-NHE3 detected a 90 kDa protein (Fig. 3). The proteins detected by two antibodies were clearly distinct from each other, and most likely represented bovine NHE1 and NHE3, respectively.

The occurrence of NHE1 in the BBM seemed unusual, since the NHE1 isoform is currently assumed to occur in the BLM in polarized epithelial cells. Although previous biochemical studies including ours indicated that NHE was mostly localized to the BBM in the kidney epithelia (*16, 19-21*), the previous conclusion should be reexamined in the light of our current knowledge on NHE isoforms. In this context we may not dismiss the possibility that the NHE1 protein was actually present in the contaminating BLM, since the BBM vesicles as prepared by the Mg^{2+}-precipitation were not completely free from contamination by the BLM (*16*). This prompted us to undertake a rigorous study on the distribution of NHE isoforms among membrane fractions derived from bovine kidney cortex.

Distribution of the NHE1 and the NHE3 Proteins among Fractions Recovered after Fractionation of the Plasma Membrane from Bovine Kidney Cortex by Sucrose Density Gradient Centrifugation—The plasma membrane fraction obtained from bovine kidney cortex was further fractionated by centrifugation on a 35-48% sucrose density gradient (Fig. 4). Major peaks of the Na^+,K^+-ATPase and the aminopeptidase M activities that indicate the positions of the BLM and the BBM, respectively, are fairly well separated on the gradient (Fig. 4A).

The distribution of the NHE activity (Fig. 4B) roughly coincided with that of aminopeptidase M except for a small satellite peak of the NHE activity, which was not clearly discernible in previous studies, in the BLM-containing fractions. Immunoblotting analysis of these fractions indicated that fraction 2, a representative of the BLM and the satellite peak of the NHE activity, in fact contained the NHE1 protein (Fig. 4C). The NHE3 protein was not detected in this fraction. On the other hand, immunoblotting analysis of the heavier membrane fractions consisting the major peak of the NHE activity indicated that they contained both the NHE1 and the NHE3 proteins.

Occurrence of the NHE1 isoform in the BBM fractions appeared to be consistent with the results in the preceding section as well as those of our previous works. However, on further examination of the data in Fig. 4, we noticed that a small but significant amount of the Na^+,K^+-ATPase activity was recovered in fractions 5 to 8, in which the aminopeptidase M activity was also concentrated. This implied that we could not exclude the possibility that fractions 5 to 8, which largely represented the BBM, were still contaminated by the BLM and that the NHE1 protein found in these fractions actually originated from the BLM. To test whether this is indeed the case we tried to find other methods of membrane fractionation that would afford better resolution of the BLM and the BBM.

Fractionation of the Plasma Membrane from Bovine Kidney Cortex by Percoll Density Gradient Centrifugation and the Analysis of Distribution of NHE Isoforms among the Membrane Fractions—After several trials we found that an 11% Percoll density gradient served very well for the purpose of separating the BLM of bovine kidney cortex from the BBM as judged by cross-contamination between Na^+,K^+-ATPase and aminopeptidase M. As shown in Fig.

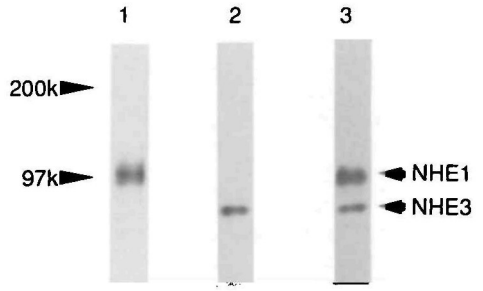

Fig. 3. **Immunoblotting analysis for the proteins of bovine kidney BBM with anti-NHE1 and anti-NHE3 antibodies.** Proteins in the BBM from bovine kidney cortex prepared by the Mg^{2+}-precipitation procedure (40 μg) were analyzed by SDS-PAGE and probed by anti-NHE1 antibody (lane 1), anti-NHE3 antibody (lane 2), or a mixture of anti-NHE1 and anti-NHE3 antibodies (lane 3).

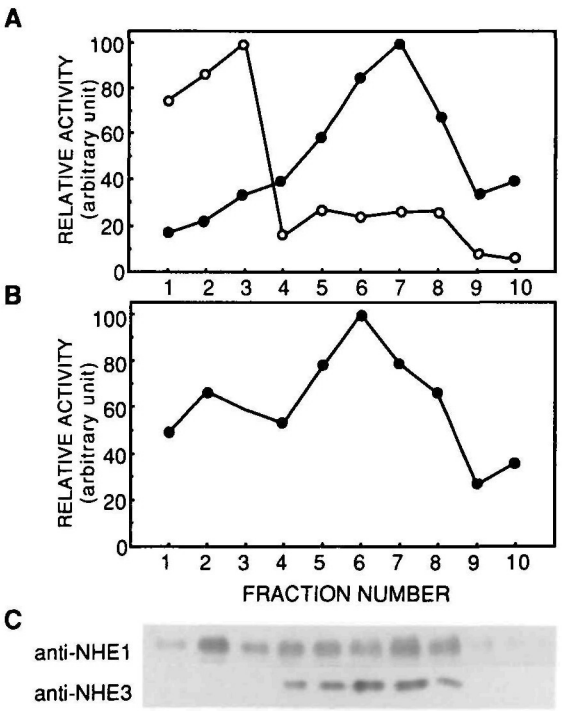

Fig. 4. **Fractionation of the plasma membranes of bovine kidney cortex by sucrose density gradient centrifugation.** Thirteen milligrams of the crude plasma membranes of bovine kidney cortex was fractionated by 35-48% sucrose density gradient centrifugation at 197,000 × *g* for 2.5 h in a Beckman SW-41Ti rotor, and 1-ml fractions were collected from the top of the tube. A: Aliquots were taken from each fraction and assayed for the aminopeptidase M (●) and the Na^+,K^+-ATPase (○) activities. The aminopeptidase M activity of fr. 7 and the Na^+,K^+-ATPase activity of fr. 3 were arbitrarily taken as 100. B: Membrane vesicles were recovered from each fraction, and the $^{22}Na^+$-uptake activity was measured as previously described using 50 μg of protein (*14*). The activity was expressed in terms of the amount of $^{22}Na^+$ transported by the total protein in each fraction, with the value of fr. 6 being arbitrarily taken as 100. C: Aliquots of the membranes recovered in B, which were equivalent to 4% of each fraction in amount, were analyzed by SDS-PAGE, and the proteins cross-reactive with the anti-NHE1 and the anti-NHE3 antibodies were analyzed by immunoblotting.

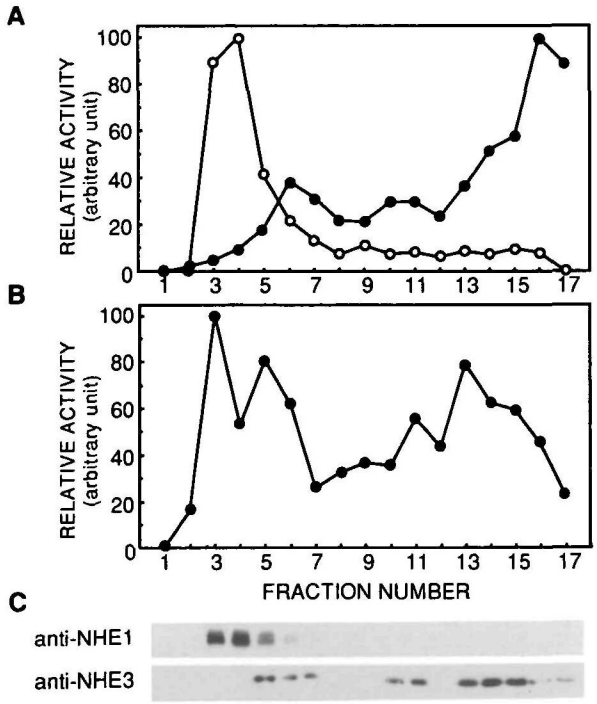

Fig. 5. **Fractionation of the plasma membranes of bovine kidney cortex by Percoll density gradient centrifugation.** Thirty-five milligrams of the crude plasma membranes of bovine kidney cortex were fractionated by 11% Percoll density gradient centrifugation as described under "MATERIALS AND METHODS," and 2-ml fractions were collected. A: The aminopeptidase M (●) and the Na^+,K^+-ATPase (○) activities were assayed in aliquots of each fraction as in Fig. 4. The aminopeptidase M activity of fr. 16 and the Na^+,K^+-ATPase activity of fr. 4 were arbitrarily taken as 100. B: Membrane vesicles were recovered from each fraction, then the $^{22}Na^+$-uptake activity was measured as previously described using 50 μg of protein (*14*). The activity was expressed in terms of the amount of $^{22}Na^+$ transported by the total protein in each fraction, with the value of fr. 3 being arbitrarily taken as 100. C: Aliquots of the membranes recovered in B, which were equivalent to 6% of each fraction in amount, were analyzed by SDS-PAGE, and the proteins cross-reactive with the anti-NHE1 and the anti-NHE3 antibodies were analyzed by immunoblotting.

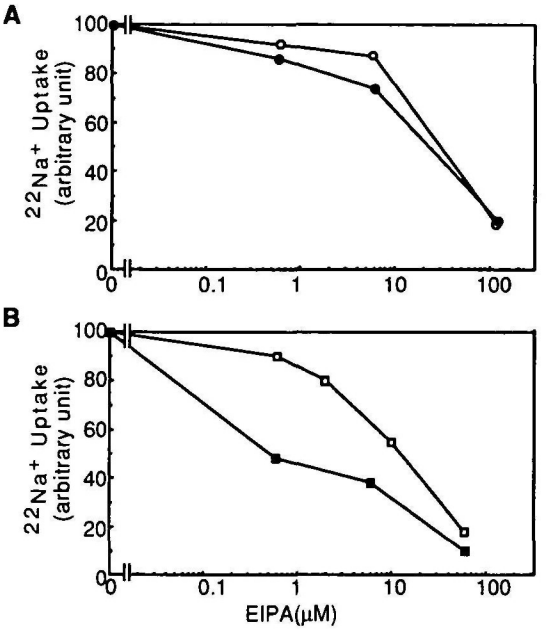

Fig. 6. **EIPA sensitivity of the $^{22}Na^+$-uptake by the BBM and the BLM of bovine and rabbit kidney cortex.** A: The $^{22}Na^+$-uptake by the BBM (fr. 11 of Fig. 5) (○) and the BLM (fr. 3 of Fig. 5) (●) of bovine kidney cortex was measured in the presence of various concentrations of EIPA, as indicated. B: The $^{22}Na^+$-uptake by the BBM (○) and the BLM (●) of rabbit kidney cortex fractionated by Percoll density gradient centrifugation was measured in the presence of various concentrations of EIPA, as indicated.

5A, Na^+,K^+-ATPase, which represents the distribution of the BLM, was recovered exclusively in fractions with lower densities with a prominent peak in fractions 3 and 4. Tailing of the enzyme toward higher density fractions and its comigration with aminopeptidase M noted in the sucrose density gradient fractionation was practically abolished under these conditions, and most importantly, this was accompanied by disappearance of the NHE1 protein from higher density fractions on immunoblotting analysis (Fig. 5C). The NHE3 protein was completely absent from the BLM fraction in accordance with the results described in the preceding section.

The distribution of aminopeptidase M on the Percoll density gradient was rather complicated, and the enzyme activity was found scattered over a number of fractions, from 5 to 17, making up the bottom 3/4 of the gradient. This is due to the fact that the density gradient was almost flat in this part. The distribution of the enzyme was not further improved when the sample was centrifuged for a longer time. The activity appeared in a few peaks of various heights, suggesting some heterogeneity in the membranes accommodating the enzyme. This was also the case with the NHE3 protein. Moreover, the distributions of aminopeptidase M and NHE3 over the gradient were roughly coincident, except for fractions 16 and 17, in which the aminopeptidase M activity was the highest, while the NHE3 protein (Fig. 5C) as well as the NHE activity (Fig. 5B) was definitely less in quantity than each other fraction. The NHE3 protein is localized to the renal BBM, but may be absent from a portion of it.

Thus, the results shown in Fig. 5 collectively indicate that the NHE1 protein is colocalized with Na^+,K^+-ATPase and limited to the BLM, while the NHE3 protein is exclusively localized to the BBM, where aminopeptidase M is located.

Sensitivity of Bovine NHE Isoforms to Inhibition by EIPA—Percoll density gradient centrifugation provided us with samples of NHE1 and NHE3 that were essentially free from cross-contamination. Using these samples, we examined their sensitivity to inhibition by EIPA. This is particularly interesting because our previous results using a Mg^{2+}-precipitated "BBM" preparation, which was shown above to consist of both NHE1- and NHE3-containing vesicles, showed that amiloride-sensitivity of the NHE reaction was rather low in spite of the presence of considerable amount of the NHE1 protein in the membranes (*16*).

The results of experiments using the BLM (fraction 3 of Fig. 5) and the BBM (fraction 11 of Fig. 5) vesicles were consistent with the previous results (Fig. 6). The EIPA-sensitivity of the NHE reaction by both NHE1 and NHE3 was practically indistinguishable and rather low. Parallel experiments with the BLM and the BBM vesicles similarly

prepared from rabbit kidney cortex, containing the NHE1 and the NHE3 proteins, respectively, without cross-contamination (data not shown), indicated that rabbit NHE1 showed much higher sensitivity to EIPA in accordance with previous observations in the literature. The EIPA sensitivity of rabbit NHE3 was roughly the same as that of bovine NHE1 and NHE3. Thus NHE1 of bovine kidney is unique in that its amiloride-sensitivity is definitely low as compared to that of other species.

DISCUSSION

Using antibodies specific to NHE1 and NHE3 proteins, we detected a 110 kDa protein and a 90 kDa protein related to NHE1 and NHE3, respectively, in the bovine kidney brush-border membranes that had been prepared according to the Mg^{2+}-precipitation procedure. This is consistent with our previous finding that the Mg^{2+}-precipitated BBM preparation from bovine kidney cortex contained a 110 kDa protein that is cross-reactive with the anti-NHE1 antibody, and that the protein could be purified to give a reconstitutively active NHE, using an immunoaffinity sorbent with the immobilized anti-NHE1 antibody (*16*).

The observed association of the NHE1 protein, which is distinct from the NHE3 protein, with the Mg^{2+}-precipitated BBM preparation from bovine kidney raised two questions: (1) does the NHE1 isoform of bovine kidney cortex in fact occur in the BBM, even though this would be exceptional in view of what has been recorded to date in the literature (*22*)?, and (2) does bovine NHE1 in fact show an unusually low amiloride-sensitivity as compared to the NHE1 isoform from other species (*23-25*)?

As to the first question, a series of experiments to analyze the distribution of the NHE1 protein led us to the conclusion that the conventional Mg^{2+}-precipitation procedure, which is appropriate for obtaining rabbit kidney BBM, does not work satisfactorily to separate bovine kidney BBM from BLM, and that the NHE1 protein found in the BBM preparation actually originated from the basolateral membrane contaminating the BBM preparation. This was indicated by analyzing the membrane fractions obtained after Percoll density gradient centrifugation. In 11% Percoll Na^+,K^+-ATPase was recovered in a single peak after centrifugation in association with the NHE1 protein, but distinctly separated from aminopeptidase M and the NHE3 protein, indicating that NHE1 and NHE3 were confined to the BLM and the BBM, respectively. The observed localization is in agreement with the current view on the distribution of NHE isoforms in polarized epithelia (*22*).

The BBM and the BLM from bovine kidney appeared difficult to separate, since even sucrose density gradient centrifugation resulted in cosedimentation of a part of the BLM with the BBM. The present results indicate that this difficulty may be obviated by using Percoll density gradient centrifugation. Clear-cut separation of distinct membrane segments is a prerequisite for the biochemical analysis of the properties of polarized epithelia. The present protocol for preparing pure BLM and BBM may extend the usefulness of bovine kidney, which is easily obtainable in quantity, as a material for such biochemical analysis.

As to the second question, the development of a procedure to obtain the BBM and the BLM preparations free from cross-contamination enabled us to definitely show that EIPA-sensitivity of bovine NHE1 was much lower than that of rabbit NHE1, being practically indistinguishable from that of bovine and rabbit NHE3. This is in agreement with our previous result that the NHE molecules purified on an anti-NHE1 antibody affinity sorbent showed a low amiloride sensitivity (*16*). Bovine NHE1 is thus unique among the NHE1 transporters from various species, but the molecular basis for this low EIPA/amiloride-sensitivity as well as its possible physiological implication remains obscure. The structure of bovine NHE1, especially at the amiloride/Na^+-binding site, is highly intriguing.

Sensitivity to amiloride and its derivatives is currently used as a convenient characteristic to distinguish the NHE isoforms occurring in given tissues from each other (*1, 23*). The present result may warn us against using this convention too naively.

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