Cell-specific Expression of Epithelial Sodium Channel α, β, and γ Subunits in Aldosterone-responsive Epithelia from the Rat: Localization by In Situ Hybridization and Immunocytochemistry

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Abstract. A highly selective, amiloride-sensitive, epithelial sodium channel from rat colon (rENaC), composed of three homologous subunits termed α, β, and γ rENaC, has been cloned by functional expression and was proposed to mediate electrogenic sodium reabsorption in aldosterone-responsive epithelia. To determine whether rENaC could account for sodium absorption in vivo, we studied the cellular localization of the sodium channel messenger RNA subunits by in situ hybridization and their cellular and subcellular distribution by immunocytochemistry in the kidney, colon, salivary, and sweat glands of the rat. In the kidney, we show that the three subunit mRNAs are specifically co-expressed in the renal distal convoluted tubules (DCT), connecting tubules (CNT), cortical collecting ducts (CCD), and outer medullary collecting ducts (OMCD), but not in the inner medullary collecting ducts (IMCD). We demonstrate co-localization of α, β, and γ subunit proteins in the apical membrane of a majority of cells of CCD and OMCD. Our data indicate that α, β, and γ subunit mRNAs and proteins are co-expressed in the distal nephron (excepting IMCD), a localization that correlates with the previously described physiological expression of amiloride-sensitive electrogenic sodium transport. Our data, however, suggest that another sodium transport protein mediates electrogenic amiloride-sensitive sodium reabsorption in IMCD. We also localized rENaC to the surface epithelial cells of the distal colon and to the secretory ducts of the salivary gland and sweat gland, providing further evidence consistent with the hypothesis that the highly selective, amiloride-sensitive sodium channel is physiologically expressed in aldosterone-responsive cells.

AMILORIDE-sensitive electrogenic transepithelial sodium transport constitutes the rate limiting step for sodium reabsorption in the epithelium of the distal nephron, the distal colon, the ducts of several exocrine glands (for instance the salivary glands and the sweat glands) and the epithelia of the lung (10). In kidney (25) and colon (29), electrogenic sodium transport is upregulated by aldosterone, a mineralocorticoid hormone, allowing the maintenance of sodium balance, blood volume, and blood pressure (30). In the distal nephron of rats submitted to a 1 wk salt restriction, the electrogenic sodium transport is highly efficient at reabsorbing sodium from the urine of collecting ducts, decreasing luminal sodium concentration to levels as low as 1 mM, while the plasma concentration is maintained at 140 mM. To establish such a gradient, the epithelia of the distal nephron expresses an electrogenic sodium transport mediated by an amiloride-sensitive sodium channel located in the apical membrane facing the external compartment (urine), and a ouabain-sensitive sodium pump restricted to the basolateral membrane facing the extracellular compartment (blood).

Current physiological evidence indicates that the amiloride-sensitive epithelial sodium channel is mainly expressed in tissues that fall into the category of tight epithelia in which the paracellular electrical resistance is similar to or larger than that of the plasma membranes. The tight epithelia (distal nephron segments, distal colon) are typically responsive to mineralocorticoid hormones (30). We have recently identified the primary structure of the epithelial sodium channel of the rat distal colon epithelium (rENaC)[1] by expression cloning in *Xenopus laevis* oocytes (3). The channel is composed of three homologous subunits named α, β, and γ rENaC. Coexpression of these subunits

1. *Abbreviations used in this paper:* CCD, cortical collecting ducts; IMCD, inner medullary collecting ducts; OMCD, outer medullary collecting ducts; rENaC, rat epithelial sodium channel.

reconstitutes a channel with ion-selective permeability, single channel conductance and a pharmacological profile similar to that of the native renal channel (4). The α subunit has also been identified from a rat colon cDNA library (21) and a human lung cDNA library (37). The α, β, and γ cDNAs encode proteins of 698, 638, and 650 amino acids, respectively, sharing 35% identity between them. The α, β, γ subunit cDNAs hybridized to transcripts of 3.7, 2.2, and 3.2 kb, respectively, on Northern blots containing poly(A$^+$) RNA from rat renal cortex and medulla and colon suggesting that similar molecules are involved in sodium reabsorption in both organs.

As a first step in understanding the molecular mechanisms of the electrogenic sodium transport in vivo, we have studied the cell specific expression of α, β, and γ subunits in different aldosterone-responsive epithelia by two approaches: (a) localization of subunit mRNAs by in situ hybridization to evaluate the qualitative expression of channel subunit mRNAs; and (b) cell specific expression of α, β, and γ subunit protein by immunocytochemistry.

The main questions we address are the following: (a) are the three subunits coexpressed in cells known to express an amiloride-sensitive electrogenic sodium transport? and (b) are the subunit proteins expressed in the apical membrane exclusively, as expected from electrophysiological data?

Materials and Methods

Immunolocalization of the Three Subunit Proteins of rENaC

Animals. Adult Sprague-Dawley rats (200 g body weight) were kept on a low-salt diet for 1 wk, an experimental manoeuver which raises plasma aldosterone to a high level and induces channel activity in the apical membrane of aldosterone target cells (CCD) (25).

Preparation and Characterization of Antibodies. Isoform specific antibodies were raised against the NH$_2$ terminus or COOH terminus of α, β, and γ subunits. The fusion proteins were generated in the pGX vector (Pharmacia, Uppsala, Sweden), isolated from bacterial lysate (GST-agarose beads), and eluted with reduced glutathion according to the manufacturer's procedure. The GST fusion proteins were injected subcutaneously into rabbits. Rabbit preimmune and immune sera were tested for immunoprecipitation of α, β, and γ rENaC proteins from injected and non-injected oocytes. Oocytes were labeled with [^{35}S]methionine for 12 h and microsomal membranes prepared for immunoprecipitation as described (8). The best immunological responses were observed when the GST fusion proteins from the amino terminus end of α rENaC (amino acids E10 to F77), from the carboxy terminus end of β rENaC (amino acids G559 to E636) and of γ rENaC (amino acids A570 to L650) were used as antigens. These three sera were thus used in the present study.

Preparation of Tissues for Immunocytochemistry. Rats were anesthetized with pentobarbital (40 mg/kg), then perfused through the heart: (a) with a solution of 0.1 M PBS at pH 7.4 containing 5,000 U/l heparin, 0.004 M procaine and 0.075 M L-lysine-monohydrochloride for 3 min; (b) with a fixative mixture (pH 7.4) containing 1 vol of 0.075 M L-lysine-monohydrochloride in PBS and 3 vol of PBS with 4% paraformaldehyde and 0.23% periodate (23) for 15 min. Then the kidneys, distal colon, submandibular glands, and foot pads of fore limbs were dissected and postfixed for 2 h in the same fixative, incubated in PBS containing 30% sucrose for 48 h, frozen and cut into free floating 25-μm-thick sections with a cryostat.

Immunocytochemistry. The rENaC subunits were detected in the tissues by the peroxidase-antiperoxidase technique of Sternberger (33). Briefly, free-floating sections were first treated with 5% H$_2$O$_2$ in PBS for 10 min, in order to inhibit endogenous peroxidases, and with 10% sheep serum in PBS containing 0.4% Triton X-100 for 1 h, to permeabilize the tissues and to lower non-specific background staining. The sections were then incubated: (a) for 48 h with the rabbit antisera (at a dilution of 1:2,000) raised against each subunit of rENaC; (b) for 1 h with 1:40 goat anti-rabbit IgG serum (code No. Z421; DAKOPATTS, Coppenhagen, Denmark); (c) for 1 h

with 1:50 rabbit peroxidase antiperoxidase (code No Z113; DAKOPATTS). These three incubations were carried out in the presence of 0.4% Triton X-100. Peroxidase activity was revealed with 3,3'-diaminobenzidine (0.7 μy/ml) and 0.02% H$_2$O$_2$ diluted in PBS, and the sections were mounted in glycerol. Controls consisted either in omitting the primary antibodies, or by replacing it with rabbit preimmune sera (1:2,000) or rabbit immunoglobulin fraction, or by immunoabsorption using an excess of the corresponding fusion protein. All controls yielded similar results so that only preimmune controls are shown.

We attempted to identify the subunit proteins in colon and kidney tissues by immunoblot (data not shown). Although we could identify proteins with the expected sizes in these tissues, a high background was observed. One possible explanation is that the sodium channel protein is expressed in native tissues at a low copy number (10^2-10^3 molecules per cell) and only in a few percent of the total cell population (5% in the kidney). When we used a kidney cell line (MDCK) transfected and overexpressing α rENaC, it was indeed possible to detect the protein easily by immunoblot with little background (31).
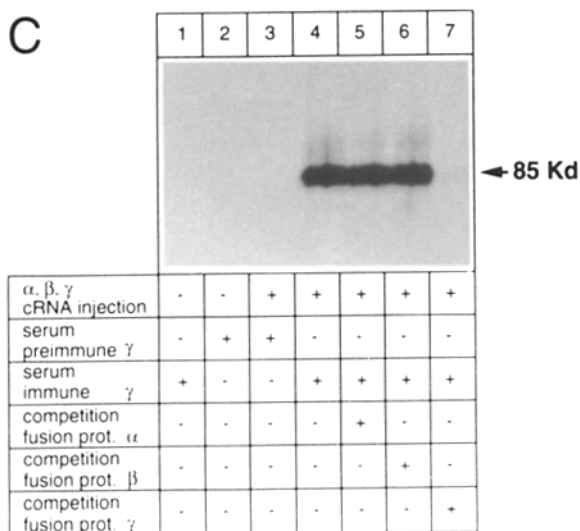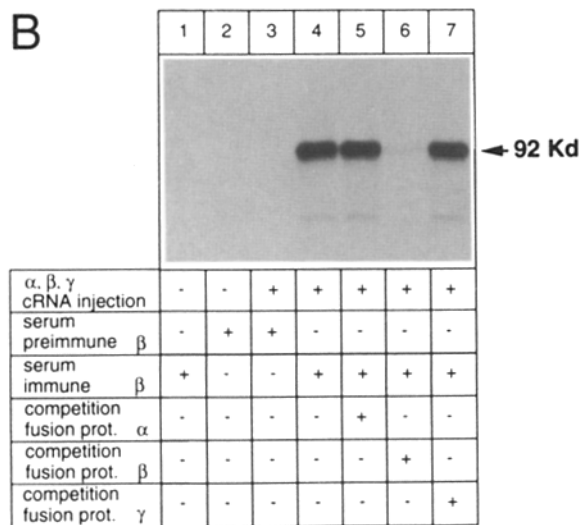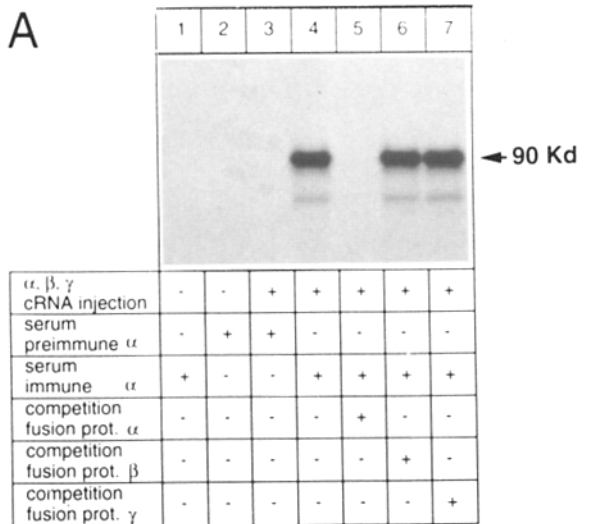
Cell Expression of α, β, and γ Subunit mRNA of the Sodium Channel by In Situ Hybridization

Rat tissues from adult Sprague-Dawley rats were fixed in vivo by intra-aortic perfusion of 4% paraformaldehyde in PBS (130 mM NaCl, 7 mM Na$_2$HPO$_4$, 3 mM NaH$_2$PO$_4$, pH 7.4), as previously described (12). In situ hybridization was performed on 7-μm sections of paraffin-embedded organs. All steps were carried out as previously described (12). After rehydration of sections in graded ethanol solutions, sections were post-fixed in 4% paraformaldehyde (in PBS); proteinase K treatment and acetylation steps were then carried out. Sections were then covered with the hybridization mixture and incubated overnight at 50°C. Post-hybridization treatments consisted of an initial wash in 5× SSC, DTT 10 mM, at 50°C for 30 min, followed by high stringency wash in 50% formamide, 2× SSC, 0.1 M DTT at 65°C for 20 min and several washes in NaCl-Tris-EDTA (0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA) at 37°C. Then, sections were incubated with RNAse A (20 mg/ml) at 37°C for 20 min. After rinsing with 0.1× SSC for 15 min, sections were dehydrated, and dried. Slides were dipped in Kodak NTB2 (melted at 42°C), dried, and exposed at −20°C for 4 wk. To prepare channel subunit riboprobes, part of the 3' untranslated region of α, β, and γ subunit of rat colon epithelial sodium channel were subcloned into the Bluescript vector (Stratagene Corp., La Jolla, CA) and linearized with EcoR1 or XbaI, and antisense or sense ^{35}S-cRNA probes (12) were prepared. After linearization, ^{35}S-labeled RNA probes were synthesized, using either T7 or T3 RNA polymerases. ^{35}S-labeled uridine 5'-triphosphate (1,000 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL) and the other reagents from Promega Biotech (Madison, WI). The hybridization mix was 50% formamide, DTT 1 mM, 2× SSC, 10% dextran sulphate, 1 mg/ml salmon sperm DNA and ^{35}S-labeled cRNA probes. Approximately 1-2 × 10^6 counts/min were applied to each tissue section.

Results

Characterization of Isoform-specific Antibodies

To raise our isoform specific antibodies we selected the NH$_2$ and COOH terminus ends of each subunit. These 9–10-kD fragments are hydrophilic and predicted to be antigenic. The primary sequences are moreover quite divergent between the three subunits (<10% identity) and therefore should allow the generation of isoform specific antibodies. As shown in Fig. 1 (A) immunoprecipitation of [^{35}S]methionine metabolically labeled membranes from water injected oocytes did not reveal any significant signal with preimmune or immune serum (lanes 1 and 2). There is therefore no detectable endogenous background in the oocyte system. When membranes from α, β, and γ subunits cRNA-injected oocytes were submitted to immunoprecipitation by the immune serum, a 90-kD protein was detected (lane 4). The specificity was assessed by the absence of any signal with the preimmune (lane 3) and by the competition with an excess of α rENaC fusion protein (lane 5) and by the lack of compe-

A

	1	2	3	4	5	6	7
α. β. γ cRNA injection	-	-	+	+	+	+	+
serum preimmune α	-	+	+	-	-	-	-
serum immune α	+	-	-	+	+	+	+
competition fusion prot. α	-	-	-	-	+	-	-
competition fusion prot. β	-	-	-	-	-	+	-
competition fusion prot. γ	-	-	-	-	-	-	+

◄ 90 Kd

B

	1	2	3	4	5	6	7
α. β. γ cRNA injection	-	-	+	+	+	+	+
serum preimmune β	-	+	+	-	-	-	-
serum immune β	+	-	-	+	+	+	+
competition fusion prot. α	-	-	-	-	+	-	-
competition fusion prot. β	-	-	-	-	-	+	-
competition fusion prot. γ	-	-	-	-	-	-	+

◄ 92 Kd

C

	1	2	3	4	5	6	7
α. β. γ cRNA injection	-	-	+	+	+	+	+
serum preimmune γ	-	+	+	-	-	-	-
serum immune γ	+	-	-	+	+	+	+
competition fusion prot. α	-	-	-	-	+	-	-
competition fusion prot. β	-	-	-	-	-	+	-
competition fusion prot. γ	-	-	-	-	-	-	+

◄ 85 Kd

tition in the presence of the corresponding fusion proteins of the β (lane 6) or γ subunit (lane 7). Similar results were obtained for the β and γ subunit antibodies (Fig. 1, B, lanes 1-7 and C lanes 1-7).

We attempted to identify the subunit proteins in colon and kidney tissues by immunoblot (data not shown). Although we could identify proteins with the expected sizes in these tissues, a high background was observed. One possible explanation is that the sodium channel protein is expressed in native tissues at a low copy number (10^2-10^3 molecules per cell) and only in a few percent of the total cell population (5% in the kidney). When we used a kidney cell line (MDCK) transfected and overexpressing α rENaC, it was indeed possible to detect the protein easily by immunoblot with little background (31).

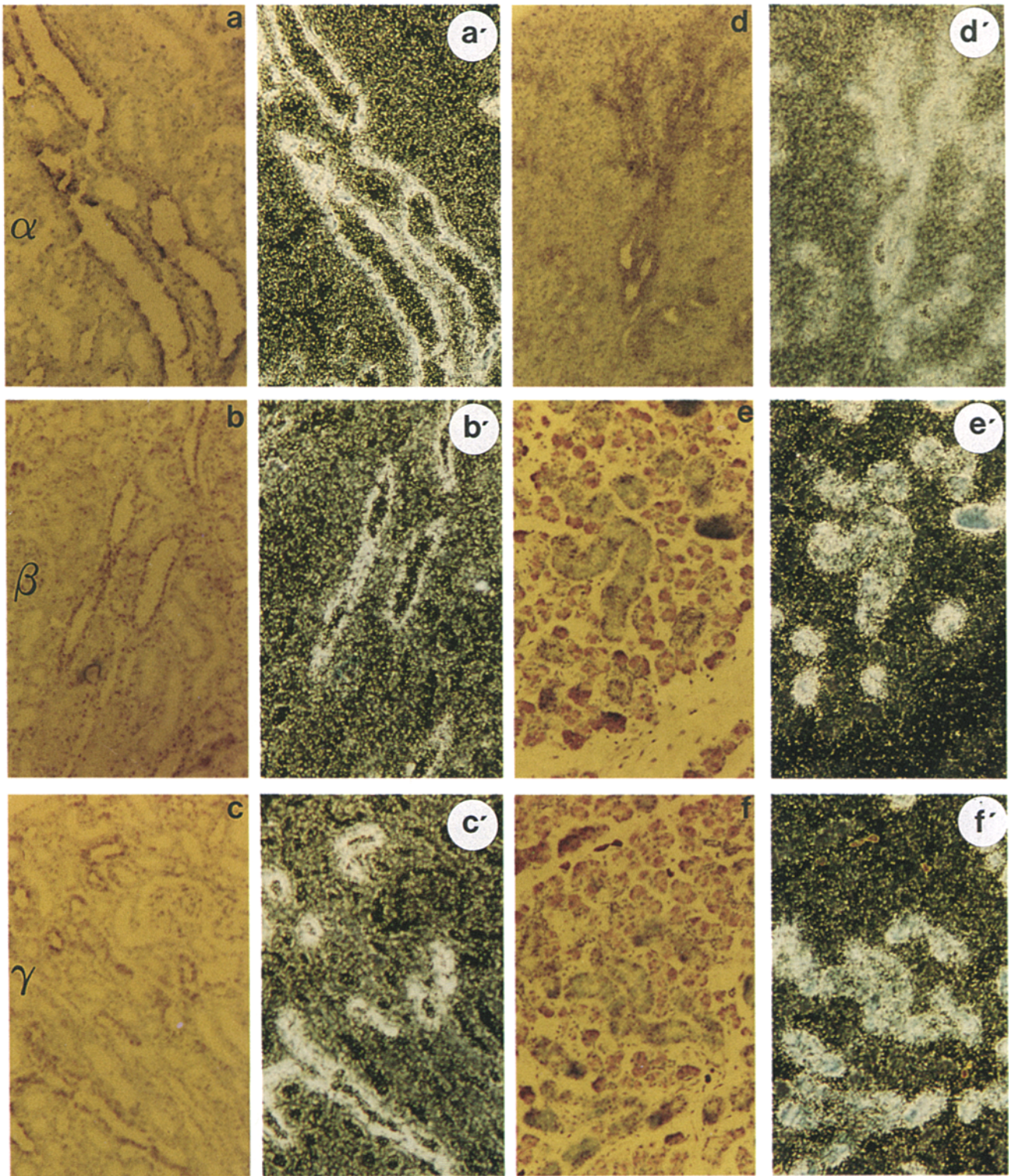### Expression of Sodium Channel Subunits in the Kidney

In the renal cortex (Fig. 2, a and a'/α, b and b'/β, and c and c'/γ), expression of α, β, and γ rENaC mRNAs was found in the distal convoluted tubule and in cortical collecting duct. Control hybridization with sense probes did not show any significant labeling above the background (data not shown).

In the renal outer medulla (Table I), the outer medullary collecting ducts (OMCD) were labeled but the labeling progressively decreased going from the outer stripe (OMCD$_o$) to the inner stripe (OMCD$_i$) of the outer medulla. By contrast, no significant signal was detected in the inner medullary collecting ducts (IMCD). The glomerulus, the proximal tubule and the medullary thick ascending limb of Henle's loop were not labeled (Table I).

In the renal cortex, at low magnification (Fig. 3, A, D, and G), α, β, and γ rENaC immunostaining was observed in the same nephron segments expressing the three mRNA transcripts. The β rENaC immunostaining was more intense, especially in the cortex (Fig. 3 D, β) whereas the γ immunostaining was weak (Fig. 3 G, γ). In the renal cortex, immunostaining was confined to most epithelial cells of DCT, CNT, and CCD, in medullary rays. No significant labeling was observed when the first antibody was replaced by preimmune serum (C, F, and I). At higher magnification, α, β, and γ rENaC immunoreactivities outlined the apical membrane of a majority of cells in these segments (Fig. 3, B/α, E/β, and H/γ), whereas the lateral membrane and the plasmalemma of the basal pole did not display any immunostaining. In contrast, no immunoreactivity was detected in the proximal convoluted or distal straight tubules or in the glomerulus or in vessels (Fig. 3 and Table I).

Figure 1. Characterization of isoform specific antibodies against α, β, and γ subunit rENaC. (*A*) Immunoprecipitation with anti-α serum. Oocytes were either not injected (lanes *1-3*) or co-injected with α, β, γ subunit cRNA of rENaC (lanes *4-7*), subjected to a [^{35}S]methionine pulse protocol, extracted with Triton-X100, and immunoprecipitated, as described, using immune serum α (lanes *4-7*) or preimmune serum (lanes *2* and *3*). Specificity of the immunoprecipitated product (90 kD) is shown in lane *4* (preimmune), lane *5* (competition by fusion protein α), lane *6* (lack of competition by fusion protein β), and lane *7* (lack of competition by fusion protein γ). (*B*) Immunoprecipitation with anti-β serum. (*C*) Immunoprecipitation with anti-γ serum.

Figure 2. Expression of α, β, and γ sodium channel subunit mRNAs in the kidney and salivary gland (sublingual). Sections were hybridized with antisense probes encoding for the α subunit (*a*, *a'*, *d*, and *d'*), the β subunit (*b*, *b'*, *e*, and *e'*), and the γ subunit (*c*, *c'*, *f*, and *f'*). Each section is shown as bright field and as dark field photographs (*a* and *a'*, respectively). A strong hybridization signal, of equivalent magnitude for the three subunit mRNAs, was found over distal tubules and cortical collecting ducts of the kidney cortex. The signal was low and uniform over proximal tubules and glomeruli (*a–c* and *a'–c'*). Similarly, high expression of each of the subunit mRNAs was present over ducts of the salivary glands, whereas signal over acini was very low. (*d–f* and *d'–f''*) Magnification: ×160. Summary of data see Table I. Bar, 125 μm.

Table I. Cell Expression of α, β, γ rENaC mRNA and Protein in Rat Tissues

| | Epithelial sodium channel subunit | | | | | |
| | α | | β | | γ | |
Tissues or organ	mRNA	protein	mRNA	protein	mRNA	protein
Kidney						
Glomerulus (G)	−‡	−	−	−	−	−
Proximal convoluted tubule (PCT)	−	−	−	−	−	−
Proximal straight tubule (PST)	−	−	−	−	−	−
Thin limb (TL)	−	−	−	−	−	−
Thick ascending limb (TAL)	−	−	−	−	−	−
Distal convoluted tubule (DCT)	+*	+	+	+	+	+
Cortical collecting duct (CCD)	+	+	+	+	+	+
Outer medullary collecting duct (OMCD)	+	+	+	+	+	+
Inner medullary collecting duct (IMCD)	−	−	−	−	−	−
Mixed salivary gland (submandibular)						
Serous predominance						
Serous acini	−	−	−	−	−	−
intercalated ducts	−	−	−	−	−	−
mucous tubule	+	+	+	+	+	+
striated ducts	+	+	+	+	+	+
Mucous predominance						
mucous tubule	ND§	−	ND	−	ND	−
striated ducts	+	+	+	+	+	+
Sweat gland						
secretory portion (body)	ND	−	ND	−	ND	−
excretory duct	ND	+	ND	+	ND	+
Digestive tract						
Small intestine						
absorptive cells	−	−	−	−	−	−
goblet cells	−	−	−	−	−	−
Colon						
proximal	−	−	−	−	−	−
distal						
surface cells	+		+		+	
absorptive	ND	+	ND	+	ND	+
goblet cell	ND	−	ND	−	ND	−
crypt cells	−	−	−	−	−	−

Summary of all data collected in the present study by in situ hybridization (mRNA) and by immunocytochemistry (protein).
*, a significant signal above background.
‡, no significant signal above control background.
§ ND, not determined

In the renal outer medulla (outer stripe), at low magnification (Fig. 4, A/α, D/β, and G/γ), labeling was confined to collecting ducts and the labeling decreased going from the outer stripe OMCD$_o$ into the inner stripe (OMCD$_i$). A closer view at high magnification reveals the characteristic labeling of the apical membrane of a majority of epithelial cells in the OMCD$_o$ but 20 to 30% of the cells are negative (Fig. 4, B/α, E/β, and H/γ). Preimmune rabbit sera (Fig. 4, C/preimmune α, F/preimmune β, and I/preimmune γ) did not show any specific immunostaining.

In the inner medulla and the papilla (Table I), the thin segments of the Henley's loop and the IMCD did not display any specific immunoreactivity. The anti–γrENaC antibody gave a strong diffuse signal on IMCD cells that was equally present with the preimmune serum.

Salivary Gland

In the submandibular gland, a mixed salivary gland with serous predominance, the expression of the sodium channel subunit mRNA (Fig. 2, d and d'/α, e and e'/β, f and f'/γ, and Table I) was high in the large interlobular ducts and also in

smaller striated ducts. No significant signal was detected by in situ hybridization in the serous acini of the gland. Control hybridization with sense probes did not reveal any significant labeling above tissue background (data not shown).

At the protein level (Fig. 5 A and Table I), at low magnification, the distribution of α (A), of β (D), and of γ (G) immunoreactivities were similar in the portions of the gland expressing predominantly serous acini (A–C and D–F). At higher magnification, the three subunits were co-localized in the apical membrane of striated ducts and interlobular ducts. Interestingly, in the rat gland, some portions of striated ducts are composed of a majority of typical striated duct cells (with their basal membrane infoldings) and of sparse mucous cells identified by their nuclei located basally and the absence of basal lateral membrane infolding. As illustrated in Fig. 5 (B, *left*), the striated duct cells are positive whereas the mucous cells (*open arrow*) are negative. This pattern was observed for α, β, and γ subunits (Table I). No labeling was observed in serous acini and their intercalated ducts. Preimmune sera gave a weak and diffuse signal (C/α, F/β, and I/γ). In the few lobes of the gland which display a
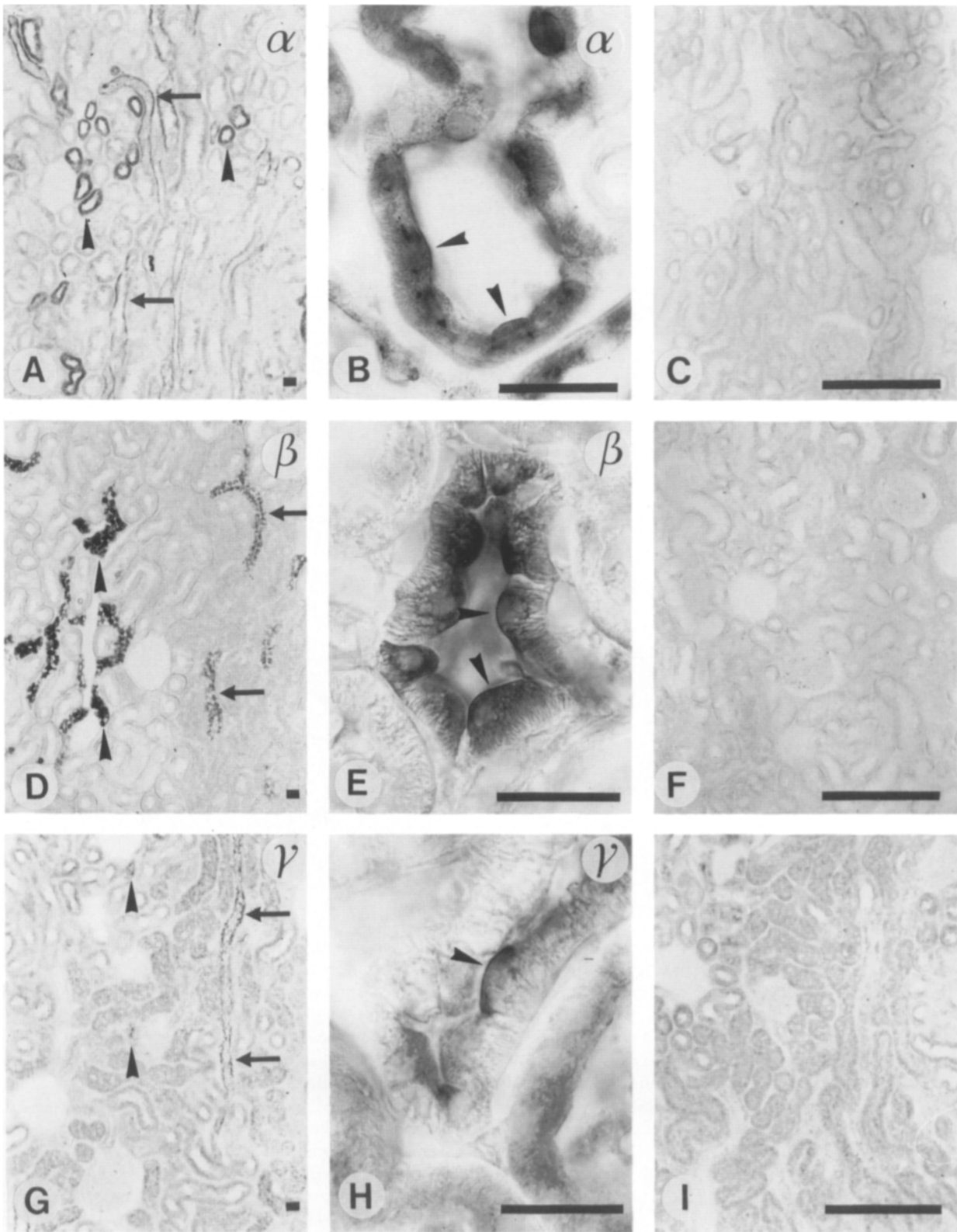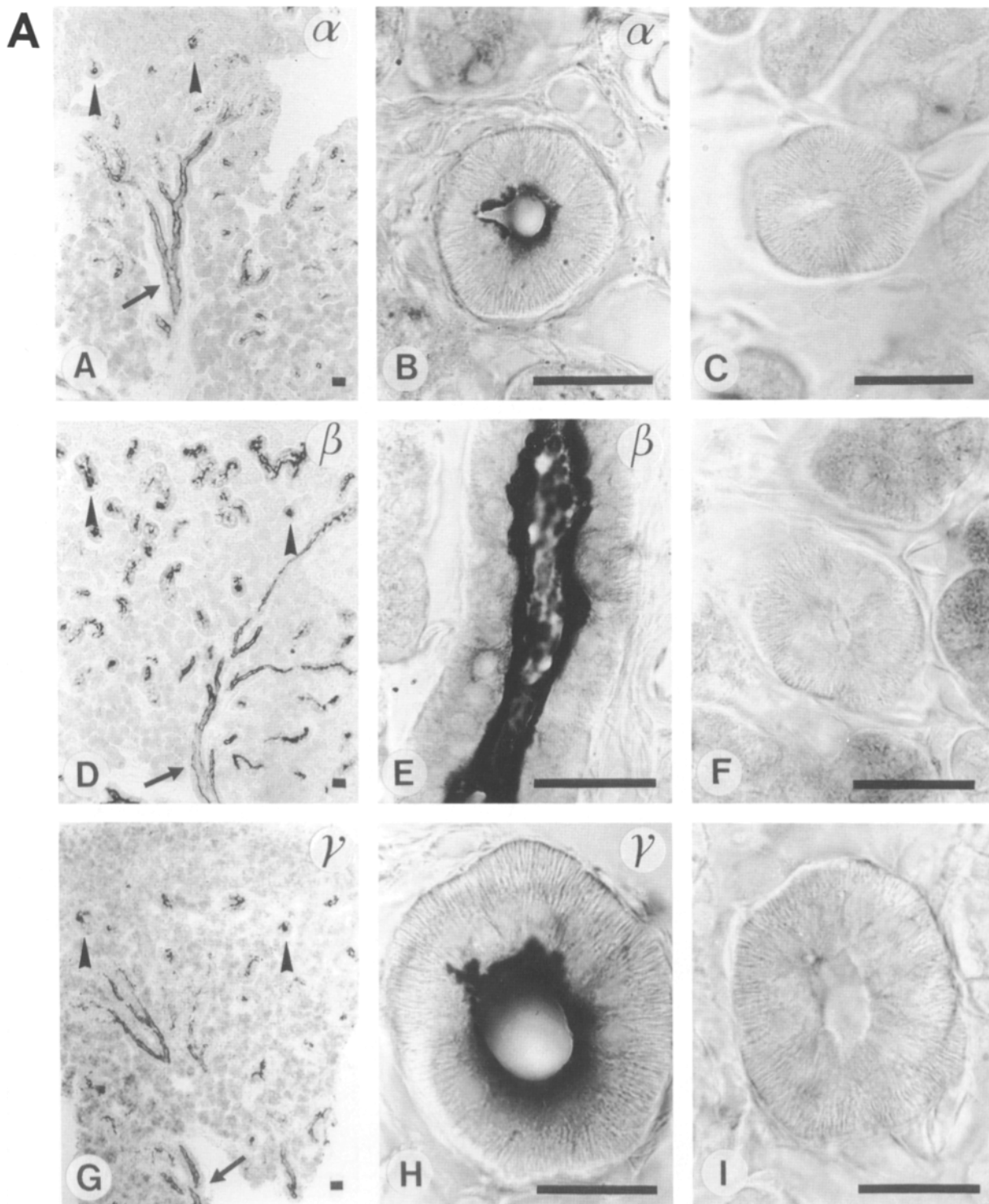
Figure 3. Immunocytochemical localization of rENaC α, β, and γ subunits in the renal cortex. At low magnification, the α (*A*), β (*D*), γ (*G*) subunits of rENaC are detected in most epithelial cells of distal convoluted and connecting tubules (*arrowhead*) in the cortex and in most epithelial cells of cortical collecting ducts (*arrows*) in medullary rays. In contrast, glomeruli, and proximal tubules were not stained. At higher magnification, immunoreactions for α subunit (*B*), β subunit (*E*), and γ subunit (*H*) were observed on the apical membrane (*arrowhead*) of positive cells of distal convoluted tubules and were absent from the lateral membrane and from the plasmalemma of their basal pole. The γ subunit immunostaining was faint, compared to that observed with α and β antisera. Intracellular labeling was observed for all subunits but is more predominant for the β subunit in the distal convoluted tubule. No selective labeling was observed with preimmune sera (*C* [α] , *F* [β], *I* [γ]). Bars, 30 μM.
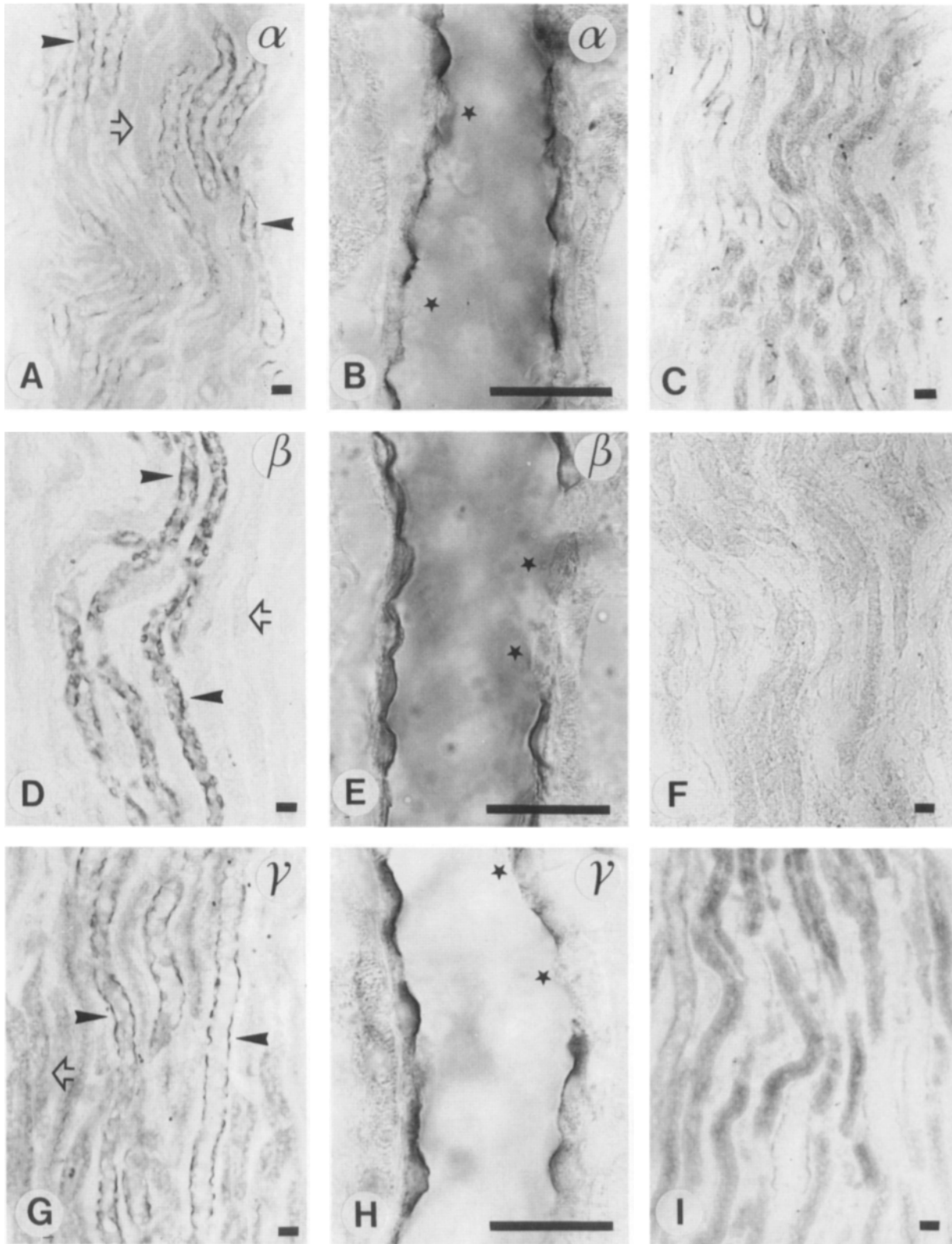
Figure 4. Immunocytolocalization of α, β, and γ rENaC subunits in the outer stripe of the renal outer medulla. At low magnification, immunoreactions for α (A), β (D), and γ (G) rENaC subunits were restricted to collecting ducts (*arrowhead*) and are absent from the proximal tubule and cortical ascending limb (*TAL*) (*open arrows*). At higher magnification, in the majority of epithelial cells of collecting ducts OMCD, the immunoreaction for α, β, and γ subunits outlines the apical membrane. 20–30% of the cells were negative (*asterisks*). No immunoreaction was observed with preimmune sera (C [α]; F [β]; I [γ]). Bars, 30 μM.

Figure 5. (A) Immunocytolocalization of α, β, γ rENaC subunits in the submandibular gland. At low magnification, in the regions of the gland which display serous predominance, the α (A), the β (D), and the γ (G) rENaC subunits were expressed by epithelial cells of striated ducts (*arrowhead*) and small interlobular ducts (*arrows*) whereas serous acini and their intercalated ducts were not labeled. At higher magnification, immunoreaction was confined to the apical membrane of striated duct cells and was absent from their lateral membrane or from their basal labyrinth (*B/α; E/β; H/γ*). No immunoreaction was observed over these cells with preimmune sera (*C/α; F/β; I/γ*). Bars, 30 μM. (B) Immunocytolocalization of β rENaC subunit in striated ducts (*left*) and mucous secreting ducts (*right*). In most regions of the gland, which display a serous predominance (*left*), mucous secreting cells are scattered (*open arrows*) between typical striated duct cells. Mucous cells are identified by their basal nuclei and supranuclear mucous chaplets. As shown here, the mucous cells are negative, whereas the apical membrane of the striated duct cells is positive for β rENaC. In the few lobules with mucous predominance (*right*), mucous cells are negative. The cell limits are seen by overexposure. Bars, 30 μM.
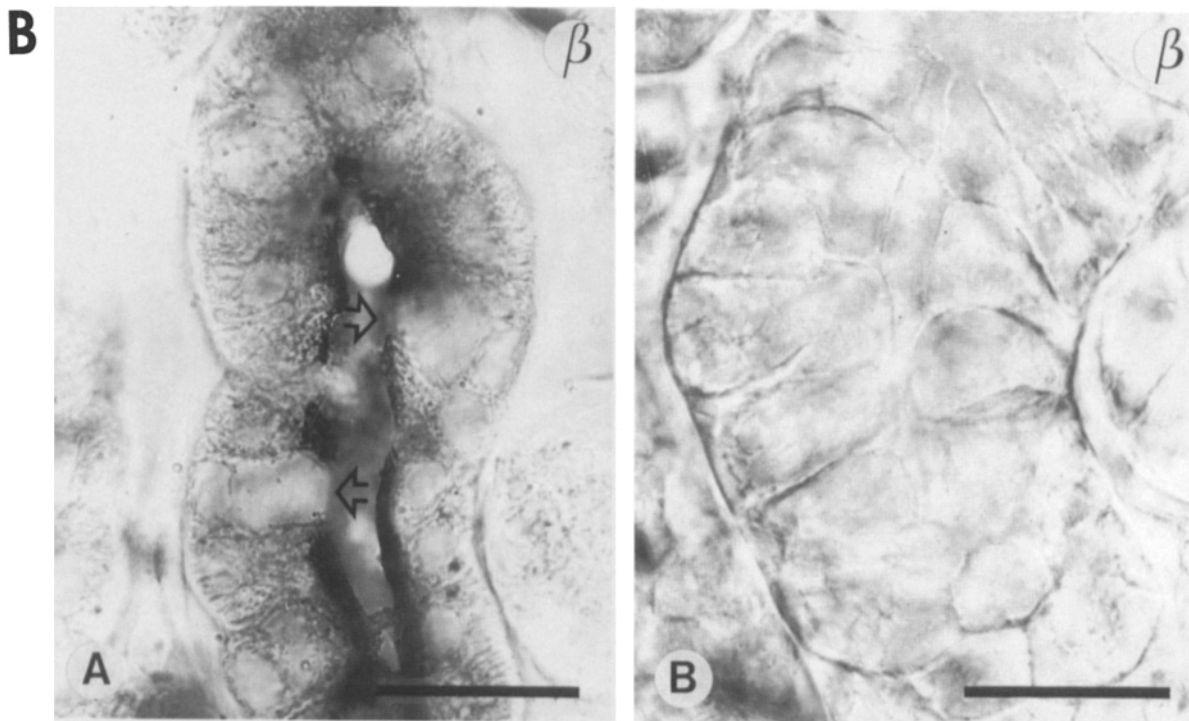
Figure 5.

mucous predominance, the mucous tubules do not express any immunoreactivity for α, for β, or for γ rENaC, whereas the sparse striated ducts do (Fig. 5, *B, right* and Table I).

Sweat Glands

The distribution of mRNA was not studied in this tissue. In the foot pads, at the protein level and at low magnification, α, β, and γ rENaC immunoreactivity was restricted to the sweat glands (Fig. 6 *A/α, D/β,* and *G/γ*). The three subunits were co-localized in most superficial segments of the coiled portion of the gland, which correspond to the excretory duct. At a closer view, the immunostaining outlines the apical membrane of epithelial cells of excretory ducts, whereas the basolateral membrane was devoid of any labeling (Fig. 6, *B/α, E/β, H/γ*). No significant immunoreactivity was observed when the first antibody was replaced by preimmune sera (*C/*preimmune α, *F/*preimmune β, and *I/*preimmune γ).

Colon

As shown in Fig. 7, the distal colon shows expression of α, β, and γ rENaC mRNAs, which is expected since these subunits were cloned from this tissue. Each of the three subunit mRNAs was observed in the superficial cells of distal colon epithelium (Fig. 7, *a* and *a'/*antisense α, *b* and *b'/*antisense β, *c* and *c'/*antisense γ). Control hybridization with sense probes resulted in a weak and uniform signal (*d* and *d'/*sense α, *e* and *e'/*sense β, and *f* and *f'/*sense γ). Crypt cells and the underlying muscular layers were negative. No significant signal was detected in the proximal colon or small intestine for any of the three subunits (Table I for summary of data).

At the protein level (low magnification), in the mucosa of the distal colon (Fig. 8, *A/α, D/β*), α and β rENaC immunoreactivity were restricted to the most superficial epi-

thelial cells facing the lumen of the digestive tube, whereas the cells lining the crypts were unstained. At a higher magnification, the immunostaining was restricted to the tips of microvilli in the striated border of most superficial cells (Fig. 8, *B/α* and *E/β*). In contrast, no labeling could be detected in adjacent goblet cells. A strong intracellular staining was also observed especially for the β subunit. Again, only superficial epithelial cells were labeled but not crypt cells. A few interstitial cells were also labeled by the β antibody but the labeling was non-specific. When preimmune serum was substituted for the primary antiserum, no significant labeling could be observed in the colon mucosa, with the exception of a weak but distinct signal in the sub-apical region of the superficial cells (Fig. 8, *open arrow, C/*preimmune α and *F/*preimmune β). Under the present experimental conditions, we failed to detect a specific labeling with the γ rENaC subunit antibody. It is not clear whether the protein is expressed at very low, undetectable levels or, alternatively, whether our antibody failed to recognize the protein in this specific tissue.

Discussion

Functional Heterogeneity of Epithelial Sodium Channels: Three Types of Channel May Mediate Electrogenic Sodium Transport

Epithelial sodium channels have been divided by Palmer (27) into three types according to four physiological and pharmacological criteria: (*a*) ion selectivity; (*b*) single channel conductance; (*c*) gating kinetics; and (*d*) affinity for amiloride. The type 1 (HS-ENaC[5pS]) of epithelial sodium channel is highly sodium selective (P_{Na}/P_K over 20), is characterized by its low single channel conductance (4–5 pS), its
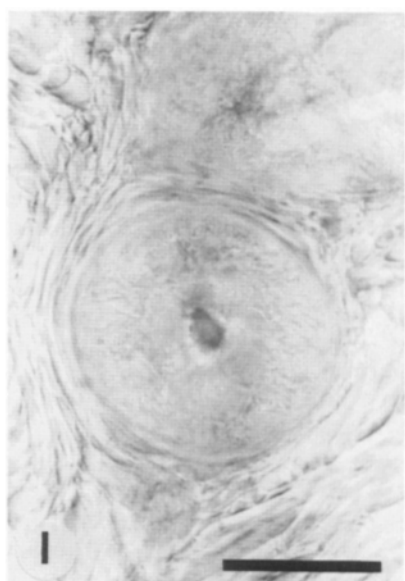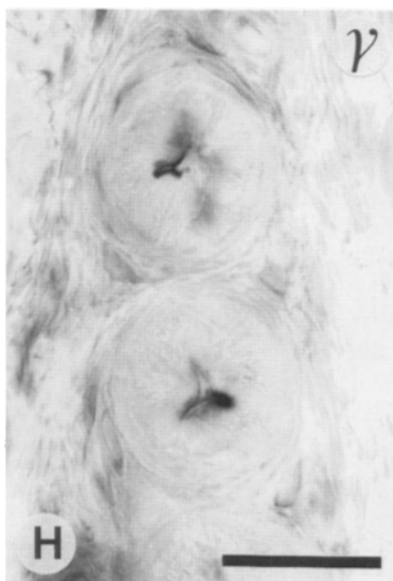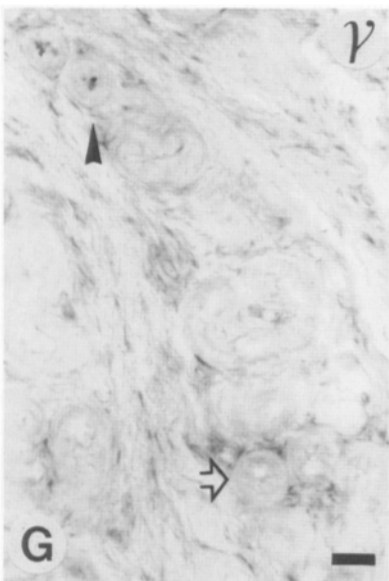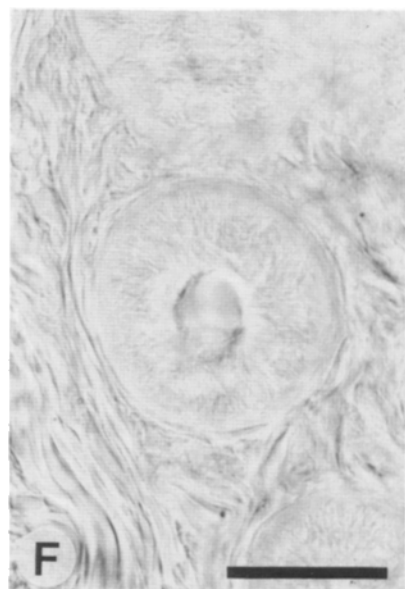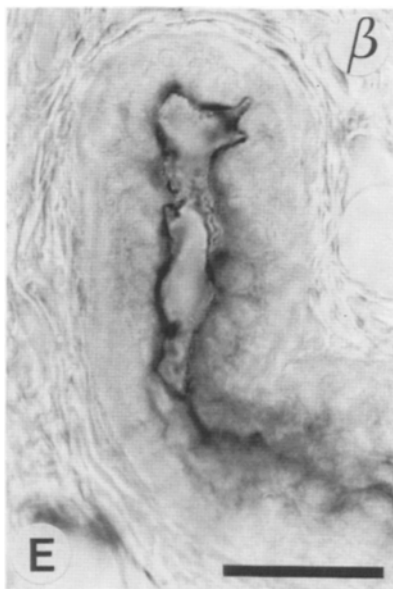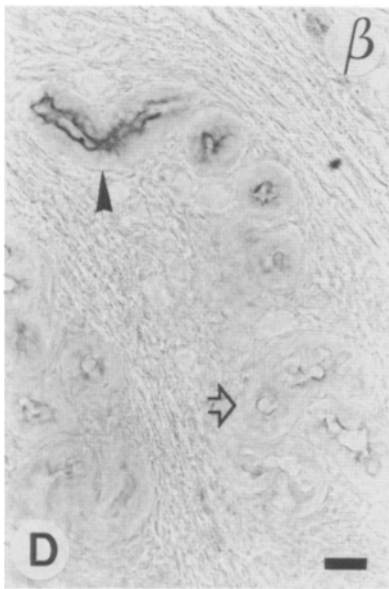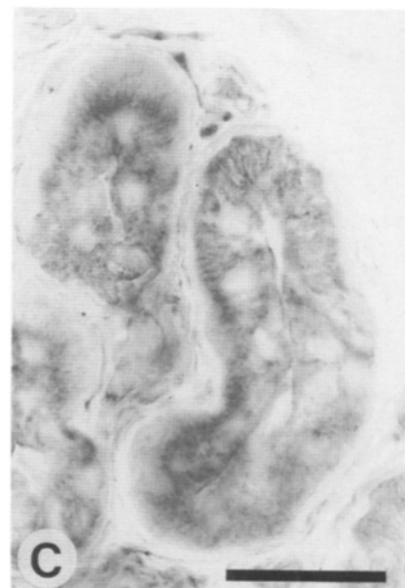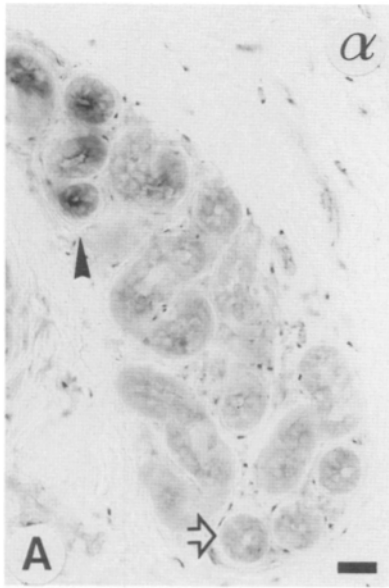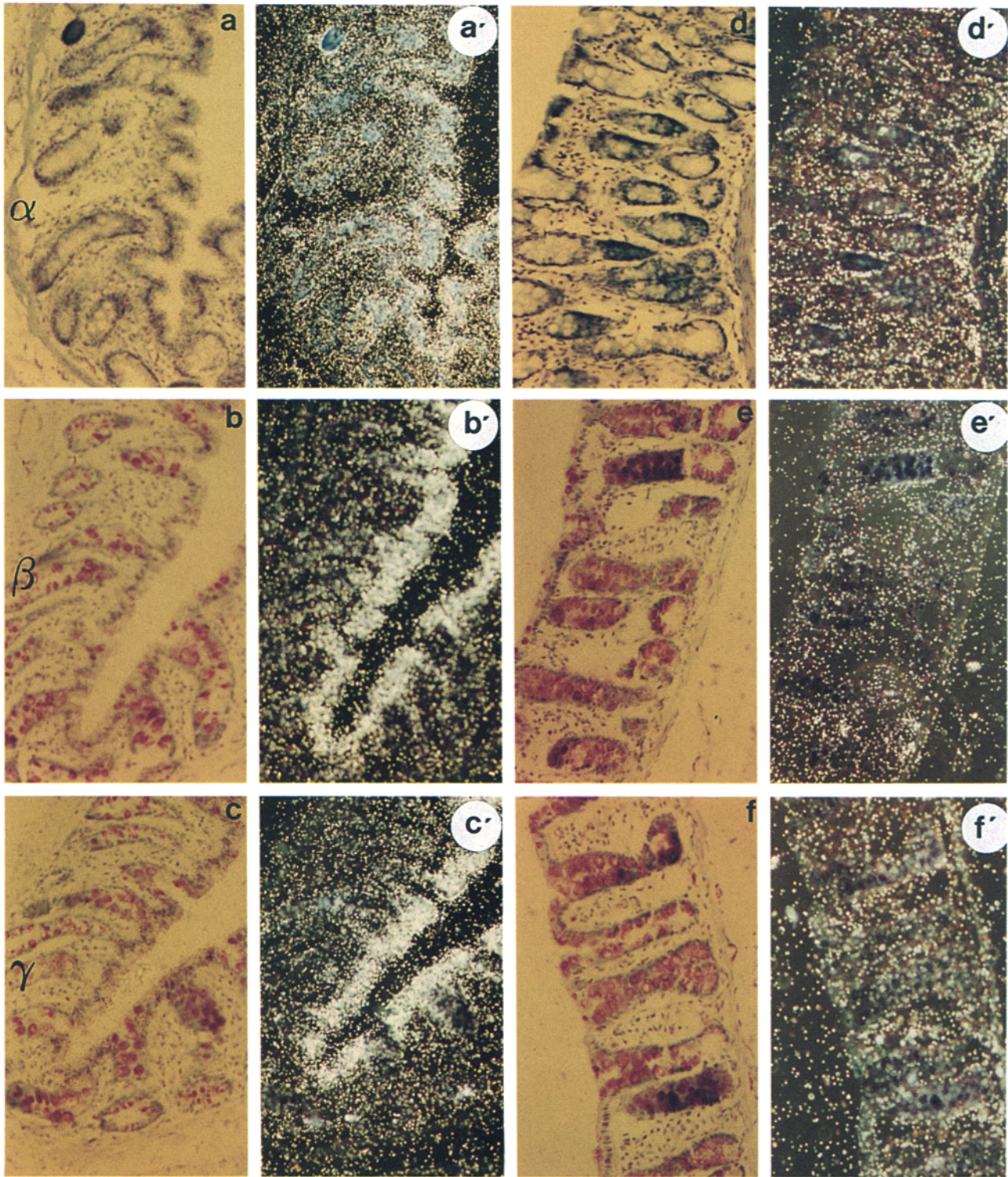
Figure 7. Expression of α, β, and γ rENaC subunit mRNA in the distal colon. Sections were hybridized with antisense probes specific for the α subunit mRNA (*a* and *a'*), the β subunit mRNA (*b* and *b'*), the γ subunit mRNA (*c* and *c'*), or with sense probes for α (*d* and *d'*), β (*e* and *e'*) or γ (*f* and *f'*) subunits. Each section is shown as bright field and as dark field photograph (ex: *a* and *a'*, respectively). All three subunit mRNAs are strongly expressed in the most superficial cells of the distal colon epithelium, while crypt cells are negative. A uniform and low labeling was observed with the sense probes, contrasting with the intense signal observed with the antisense probes. Magnification: ×200. Bar, 100 μm.

Figure 6. Immunocytochemical localization of α, β, and γ rENaC subunits in the sweat glands. At low magnification, in sweat glands of foot pads of fore limbs, immunoreaction for α (*A*), β (*D*), γ (*G*) rENaC subunits was restricted to the most superficial segments of the convoluted tubules which correspond to their excretory ducts (*arrowhead*), whereas the deeper convoluted tubule (secretory coil) (*open arrow*), was not labeled. At higher magnification, the immunostaining outlined the apical membrane of the reabsorptive duct epithelial cells (*B*/α; *E*/β; *H*/γ). No selective labeling was observed in controls (*C*/α; *F*/β; *I*/γ). Bars, 30 μM.
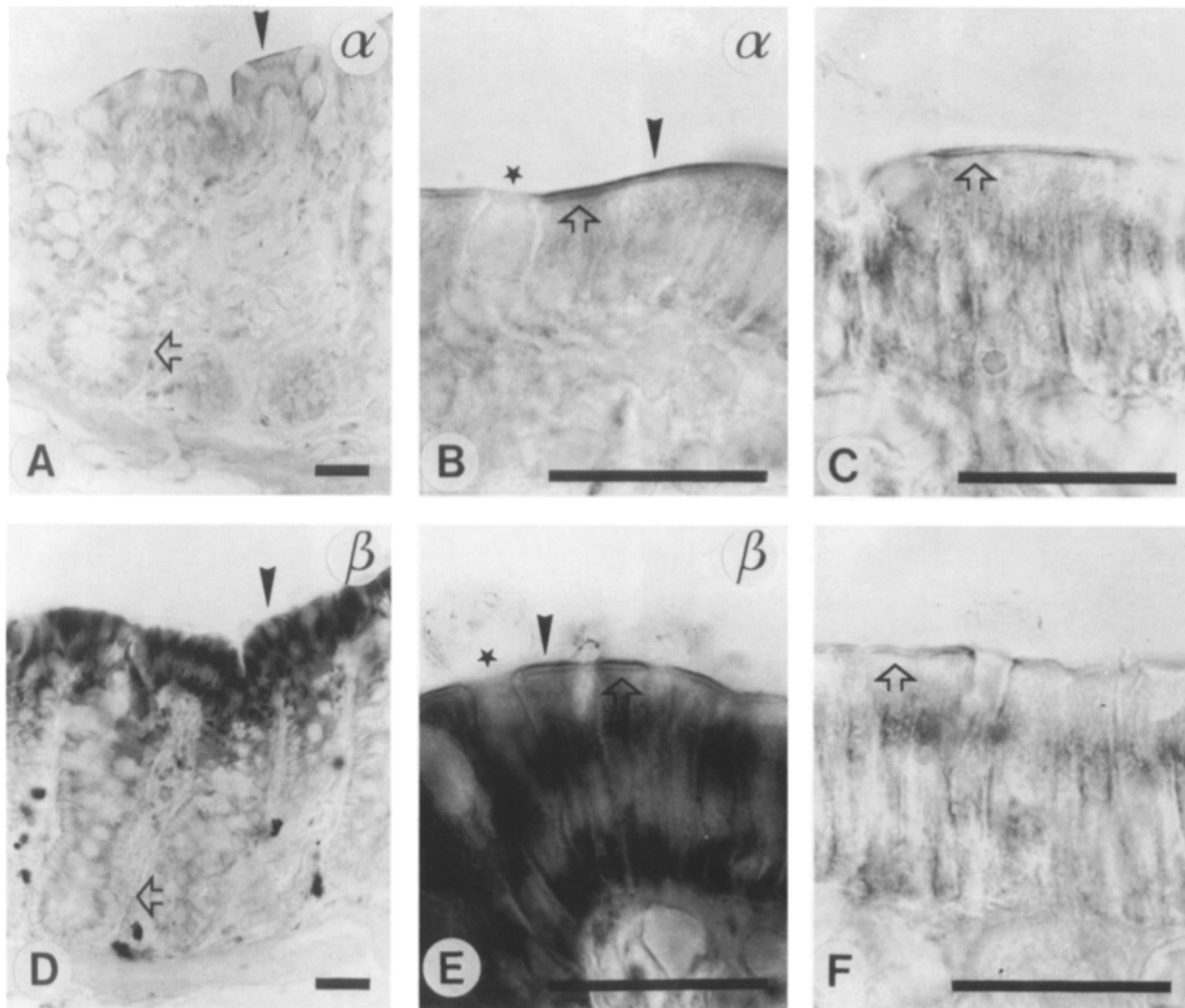
Figure 8. Immunocytolocalization of α, β rENaC subunits in the mucosa of the distal colon. At low magnification, the α (*A*) and β (*D*) rENaC subunits were localized in the superficial cells of the colonic mucosa (*arrowhead*) but are absent from the epithelial cells of the crypts (*open arrow*). At higher magnification, both α (*B*) and β (*E*) antisera, characteristically labeled the tips of microvilli in the brush border of superficial absorptive cells (*arrowhead*), whereas contiguous goblet cells lacked specific staining (*asterisks*). The β antisera also strongly labeled the cytoplasm of superficial cells but not that of the crypt cells. Preimmune sera for α (*C*) and for β (*F*) did not show any specific immunostaining but the background was higher than in other tissues. No specific immunoreaction could be detected, using the γ subunit antisera.

long time of closures and openings (time constants for open and closed time [τ] in the range of a few seconds) and its high sensitivity (K_i in the submicromolar range) to amiloride (26, 27). The type 2 (MS-ENaC[9pS]) is moderately sodium selective (P_{Na}/P_K of 3 to 4), has a higher single channel conductance (9pS), short gating kinetics (τ in the 10 to 100 ms range) and high affinity for amiloride (K_i in the submicromolar range). The type 3 (NS-ENaC[28pS]) is cation nonselective (P_{Na}/P_K of 1), has the highest single channel conductance (28pS), short gating kinetics (τ in the 100 ms range) and high affinity for amiloride (K_i submicromolar range). The amiloride-sensitive electrogenic sodium transport observed in tight epithelia in vivo could be mediated by any of the three types of channels. Indeed, current physiological evidence indicate that type 1 channel is expressed in the apical membrane of CCT and of the distal colon epithelium. Type 2 channel has been described in sweat duct cells and

type 3 in IMCD. When α, β, and γ rENaC are co-expressed in oocytes, a type 1 channel is reconstituted. We will therefore discuss the possible relations between the presently available electrophysiological data and the cellular distribution of α, β, γ rENaC, making the assumption that it would function as type 1 channel in vivo, as predicted from reconstitution experiments in oocyte.

Cellular Expression of α, β, γ rENaC Correlates with the Electrogenic Sodium Transport in Aldosterone-responsive Epithelia

In the kidney (see model of a nephron in Fig. 9 *A*) aldosterone appears to control sodium reabsorption across the entire distal nephron from DCT to IMCD (34). Experimental evidence for a mineralocorticoid-dependent, amiloride-sensitive, electrogenic sodium transport has been provided in
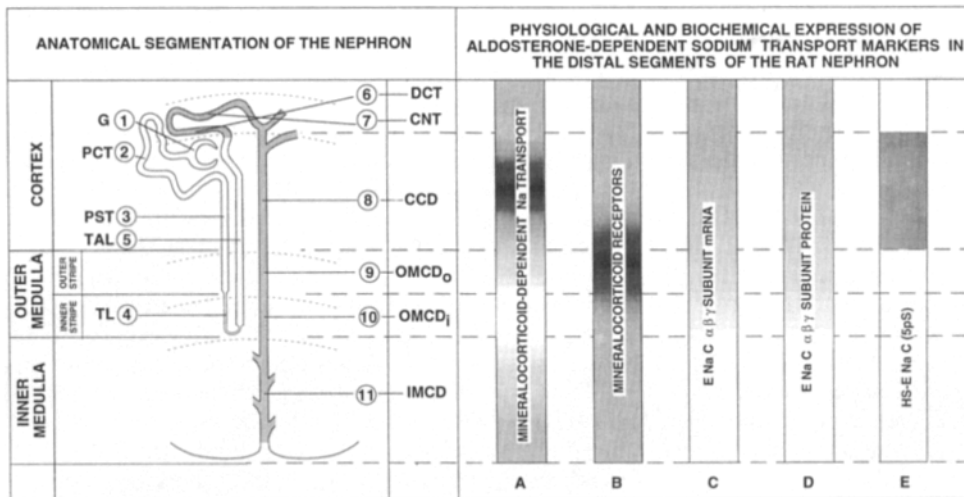
Figure 9. Diagram of the anatomical segmentation of the nephron (*left*) and physiological and biochemical expression of aldosterone-dependent sodium transport markers in the distal segments of the rat nephron (*right*). The anatomical segmentation of the nephron is shown according to the current nomenclature (16). The three main regions are indicated on the left (cortex, outer medulla, inner medulla). *1*, glomerulus (*G*); *2*, proximal convoluted tubule (*PCT*); *3*, proximal straight tubule (*PST*); *4*, thin limb (Henley's loop) (*TL*); *5*, thick ascending loop (*TAL*); *6*, distal convoluted tubule (*DCT*); *7*, connecting tubule (*CNT*); *8*, cortical collecting duct (*CCD*); *9*, outer medullary collecting duct of the outer stripe (*OMCD_o*); *10*, outer medullary collecting duct of the inner stripe (*OMCD_i*); *11*, inner medullary collecting duct (*IMCD*). (*A*) Aldosterone-dependent, amiloride-sensitive electrogenic sodium transport in the distal segment of the rat nephron (5, 24, 34). (*B*) Immunohistolocalization of mineralocorticoid receptors (7). (*C*) Distribution of α, β, γ subunit mRNA (present study). (*D*) Distribution of α, β, γ subunit protein (present study). (*E*) Highly selective low single conductance sodium channel (*HS-E Na C 5pS*) detected by patch clamp in the apical membrane is at present restricted to CCD (25).

vivo (for review see references 30, 34). Consistent with these physiological data, evidence for the presence of mineralocorticoid receptor in the whole distal nephron has been recently demonstrated by immunocytochemistry (7) (Fig. 9 *B*). As shown in Fig. 9 *E*, in the mammalian kidney the highly selective, low conductance type 1 channel (HS-ENaC[5pS]) has been positively characterized by patch clamp, until now, only in the apical membrane of the renal cortical collecting duct of the rat (7).

We have previously characterized in the *X. laevis* oocyte system a HS-ENaC(5pS) cloned from epithelial cells from the distal colon of rat fed for 1 wk on a low-salt diet. The ion selectivity, the single channel conductance, the kinetics and the pharmacological profile of the oocyte-expressed heteromultimeric rat colonic channel were similar to those of the native sodium channel expressed in rat renal CCD. The requirement for at least three subunits in the oocyte expression system suggested that α, β, and γ subunits should be co-expressed in CCD in vivo. However, it is important to note, that the expression of α subunit alone or the combinations of $\alpha\beta$ or $\alpha\gamma$ were sufficient to induce channel activity in oocytes. An important difference was a lower magnitude of the elicited amiloride-sensitive sodium currents; α, $\alpha\beta$, or $\alpha\gamma$ induce 2 to 10% of the maximal activity observed in the presence of $\alpha\beta\gamma$ subunits. It was therefore conceivable that, in vivo, a specific cell could modulate the maximal activity of sodium channels by expressing differentially various combinations of subunits. Our data indicate that this is not the case: α, β, and γ subunits are co-expressed in the apical membrane of the majority of epithelial cells lining the CCD. The CCD and OMCD express two main cell types: the principal (or majority) cells (60 to 80% of the total population) and the intercalated cells or minority cells. According to the cell distribution observed here, it is likely that rENaC is selectively expressed in principal cells, since only a minority of CD cells were unreactive with anti–rENaC antibodies. The principal cells have been shown to be involved in sodium

reabsorption and/or potassium secretion (34) while the intercalated cells are believed to be involved in proton secretion/bicarbonate reabsorption and potassium reabsorption (32, 39).

As summarized in Fig. 9, *C* and *D*, α, β, and γ rENaC are also expressed in DCT where roughly 50% of sodium transport is electrogenic and amiloride-sensitive, and 50% is neutral and mediated by a thiazide-sensitive NaCl co-transporter (24). Similarly, ducts of the outer stripe (OMCD_o) where sodium reabsorption is thought to be 100% electrogenic and amiloride-sensitive (24, 34), express α, β, and γ rENaC. Patch clamp analysis of the apical membrane in these nephron segments may resolve the HS-ENaC (5pS) in the future.

The IMCD is known to reabsorb sodium actively in vivo (5) and in cultured cells (11, 18) but, in contrast to the situation observed in CCD and OMCD, there is no evidence for potassium secretion. In IMCD, there is no electrophysiological characterization of single sodium channel conductances in the apical membrane of intact, native, cells. In cultured cells, however, a non-selective cation channel has been described with low cation selectivity (P_{Na}/P_K about 1), high single channel conductance (28pS), fast gating kinetics (millisecond range) and high affinity for amiloride (0.1 mM) (19, 20). This channel belongs thus to the type 3 (LS-ENaC[28pS]). Because of its non-selectivity, it is uncertain whether this channel is responsible for the electrogenic sodium transport observed in vivo. Since we have no evidence for the expression of α, β, a γ rENaC in IMCD we are left with three possible explanations. First, our detection systems are not sensitive enough to demonstrate the presence of the rENaC channel subunits in IMCD. Second, another type (isoform or spliced variant of rENaC) of sodium channel is expressed in this specific nephron segment and is not recognized by our probes. Our mRNA probes are isoform specific since they have been designed for the 3'UTR of each isoform. The specificity of our current set of antibodies is

also restricted to the NH₂ terminus (α) or COOH terminus (β and γ). Therefore, spliced variants of the same isoform or distinct isoforms of each subunit may escape our detection systems. A third possibility is that a completely different gene product mediates sodium reabsorption in this segment. The channel protein purified from bovine papilla, a tissue containing almost exclusively IMCDs (1) is a good candidate for this function.

In the proximal tubule we have no evidence for expression of α, β, and γ subunits of rENaC. Yet a highly selective sodium channel has been observed by patch clamp of the apical membrane of the proximal straight tubule (PST or pars recta of the proximal tubule) (Fig. 9) a typical leaky epithelium. This channel is not fully characterized but appears to have a somewhat higher single channel conductance (12pS) (9) opening the possibility for the expression of another gene product in leaky epithelia.

In the distal colon, aldosterone induces an electrogenic sodium transport in vivo and in vitro (29). The HS-ENaC (5pS) channel has been characterized by noise analysis in the rabbit (38, 40) but patch clamp data of either rabbit or rat colonic cells are, unfortunately, not yet available due to technical difficulties. The distal colon epithelium consists of two mucosal compartments: the surface epithelium and the crypts. Both compartments are able to transport ions but an amiloride-sensitive sodium transport has recently been assigned to surface epithelium, whereas crypts (15, 22) are thought to express CFTR and to be involved in chloride secretion (35, 36). Our in situ and immunolocalization data are consistent with the physiological data although we could not positively identify the γ subunit at the protein level. The immunolocalization of the three subunit proteins in the colon proved to be more difficult to demonstrate than in other tissues since the preimmune background was high. Experiments using antibodies directed against other domains of the protein will have to be performed to try to solve this problem. Again, the identification of HS-ENaC (5pS) by patch clamp of the apical membrane of native superficial cells, if technically possible, will allow completion of the molecular characterization of this channel in the colon.

In the sweat gland, the proximal tubule or secretory coil secretes an isoosmotic fluid which is then presented to the water impermeable apical membrane of the distal, reabsorptive tubule or excretory duct (28). No evidence for an amiloride-sensitive conductance was obtained in cultured human sweat gland secretory cells (17), whereas there is evidence for such sodium conductance in excretory ducts (2, 13). Patch clamp study of cultured human sweat gland excretory duct provided evidence for an amiloride-sensitive (1 mM) sodium channel that was, however, characterized by a relatively high single channel conductance (15 pS) and was moderately selective (P_{Na}/P_K about 4) (13). These characteristics (type 2) are not predicted from our own immunolocalization data which demonstrate coexpression of the α, β, and γ rENaC subunits in the apical membrane of the excretory ducts suggesting the expression of the type 1 channel (HS-rENaC[5pS]). As discussed by Joris (13), low selectivity epithelial sodium channels have been encountered especially in patching cultured cells; in view of the immunolocalization presented here, the characterisation of the sweat gland channel should probably be re-examined in na-

tive cells. Alternatively, we may be dealing with isoform spliced-variants of rENaC that cannot be distinguished by our probes and that would be functionally distinct.

In the intralobular ducts (6) and the main excretory duct (14) of the mouse mandibular salivary gland, there is evidence for the expression of a highly selective (P_{Na}/P_K over 20), amiloride-sensitive (2 μM) sodium conductance as shown by whole-cell patch of collagenase-treated and dispersed native cells (6). These data are consistent with the distribution of α, β, and γ rENaC in the rat submandibular gland where we find expression in striated and interlobular ducts. Within the striated ducts, cell specific expression was evident since isolated mucous cells were devoid of labeling.

While the cellular distribution of the three subunits of rENaC is now defined in aldosterone-responsive epithelia, this study has raised several new questions. The lack of correlation between the physiological expression of epithelial sodium channels in some tissues and the cellular expression of rENaC in some tight and leaky epithelia suggest that isoform(s) or spliced variant(s) of rENaC may exist to account for distinct biophysical properties observed in these tissues. Alternatively, completely distinct gene products may be involved.

ENaC Is Localized in the Apical Membrane of Tight Epithelia and Excluded from the Basal Lateral Membrane

The model for electrogenic sodium transport in tight epithelia predicts that the expression of the epithelial sodium channel is restricted to the apical membrane and is excluded from the basal and lateral membrane where the sodium pump is located. Our data show that the three subunits of rENaC are coexpressed in the apical membrane of the epithelial cells so far examined in kidney, colon, sweat, and salivary glands. In no instance did we detect immunoreactivity on the lateral or basal membranes, indicating that α, β, and γ rENaC are well polarized and targeted to the apical membrane only. An unexpected observation was that α, β, and γ subunit proteins are not only expressed in the apical membrane of the target cells (as expected from the physiological data) but also intracellularly. In some instances the intracellular pool appears to be large (for instance the β subunit in colon), whereas in other cases it is small (sweat gland). The role of the intracellular pool remains to be investigated. Within the limit of the present immunocytochemical technique, it is not possible to precisely localize the subcellular compartments of this intracellular pool but the tools we have developed should allow us to study this question in detail. The molecular mechanisms by which hormones, such as vasopressin or aldosterone regulate sodium channel activity in the apical membrane of epithelial cells become amenable to experimental studies.

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