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Monoclonal antibody to Na,K-ATPase: Immunocytochemical localization along nephron segments

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Monoclonal antibody to Na,K-ATPase: Immunocytochemical localization along nephron segments. To obtain a highly specific reagent that could be utilized for ultrastructural localization of Na,K-ATPase, monoclonal antibodies were produced using microsomal preparations of outer renal medulla of dog and rat enriched for Na,K-ATPase. The monoclonal antibody (C62.4) raised against dog antigen, immunoprecipitated a 96,000 Dalton protein from membranes labeled either with ^{35}S methionine or ^{3}H NAB ouabain. Na,K-ATPase, Na-ATPase, and KpNPPase activity were 25, 60, and 100% maximal after reaction with C62.4. Na,K-ATPase activated with SDS was inhibited, but Na,K-ATPase in tight right-side-out membrane vesicles was not. C62.4 inhibited ouabain binding in the presence of Na,K, and Mg, but did not inhibit ouabain binding in the presence of Mg and Pi. Labeling of broken membranes was readily seen using C62.4 labeled with colloidal gold. Intact right-side-out vesicles showed no evidence of labeling, demonstrating that the antibody is directed to an epitope of the cytoplasmic domain of the enzyme. Differential localization of C62.4 along the nephron was identified. Glomeruli showed no significant antibody binding except by occasional cells in the mesangial regions. Only basal lateral membranes of cells from all tubule segments labeled with C62.4. There was no evidence of specific apical labeling. The thick ascending limb of Henle's loop demonstrated the greatest concentration of antibody binding. In the cortical and outer medullary collecting duct, only principal cells showed abundant antibody binding. Intercalated cells showed no detectable evidence of antibody binding on any surface. These studies demonstrate that Na,K-ATPase is localized exclusively to the basal lateral membrane of renal tubular epithelial cells and varies in density and distribution in different nephron segments.

Localisation immunocytochimique de la Na,K-ATPase le long des segments du néphron à l'aide d'un anticorps monoclonal. Pour obtenir un réactif hautement spécifique pouvant être utilisé pour la localisation ultrastructural de la Na,K-ATPase, des anticorps monoclonaux ont été produits, en utilisant des préparations microsomales de la médullaire rénale externe du chien et de rat enrichies de Na,K-ATPase. L'anticorps monoclonal (C62.4) dirigé contre l'antigène de chien, a provoqué l'immunoprécipitation d'une protéine de 96000 dalton à partir des membranes marquées soit avec la 35S méthionine soit avec la 3H NAB ouabaïne. Les activités de la Na,K-ATPase, de la Na-ATPase et de la KpNPPase étaient égales à 25,60 et 100% de la réaction maximale après emploi du C62.4. C62.4 a inhibé la fixation d'ouabaïne en présence de Na, K et Mg, mais pas en présence de Mg et Pi. Le marquage des membranes brisées fut rapidement obtenu avec du C62.4 marqué à l'or colloïdal. Les vésicules intactes n'ont montré aucune évidence de fixation, ce qui démontre que l'anticorps est dirigé contre un épitope du domaine cytoplasmique de l'enzyme. Une localisation particulière de C62.4 le long du néphron a été établie. Les glomérules n'ont montré aucune fixation significative de l'anticorps sauf pour quelques cellules de la région mésangiale. Seules les membranes cellulaires basdales latérales de tous les ségments du tubule ont été marquées par C62.4. Il n'y a eu aucune évidence de fixation apicale spécifique. La portion large de la branche ascendante de l'anse de Henle a montré la plus grande concentration de fixation de l'anticorps. Dans le tube collecteur cortical et de la médullaire externe, seules les cellules principales ont montré une fixation importante de l'anticorps. Les cellules intercalaires n'ont montré aucune évidence détectable de fixation de l'anticorps à leur surface. Ces études montrent que la Na,K-ATPase est localisée exclusivement aux membranes basales latérales des cellules épithéliales tubulaires rénales et qu'elles varient en intensité et en distribution dans les différentes portions du néphron.

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The renal tubule is a highly specialized organ responsible for transepithelial transport of ions and water. It is rich in sodium potassium ATPase (Na,K-ATPase), an important transport protein capable of coupling the hydrolysis of ATP to the active translocation of sodium and potassium across the cell membrane and "secondary active" transport of other solutes [1]. The anatomic segmentation of the renal tubule correlates with a physiologic segmentation of function. Thus, the localization of Na,K-ATPase at the cellular level is important to the understanding of the role of the sodium potassium pump to the varied tubular transport functions. Previous attempts at ultrastructural localization of Na,K-ATPase in the nephron have used potassium-dependent nitrophenylphosphatase cytochemistry [2, 3], tritiated ouabain radioautography [4], and immunoferritin [5, 6] and immunogold [7] localization using a polyvalent antisera [5]. There are numerous problems associated with each of these methods, ranging from inactivation of the enzyme, loss of adequate fine structural morphology, and cross reactivity of polyvalent antisera [8]. Several investigators [9–11] have demonstrated the feasibility of generating monoclonal antibodies to Na,K-ATPase. Since such reagents are highly specific, a monoclonal antibody to Na,K-ATPase was generated as a probe for use in conjunction with improved immunocytochemical techniques for the ultrastructural localization of the enzyme. The distribution of Na,K-ATPase was examined using this antibody in various nephron segments, along different cell membrane domains and in intracellular compartments.

Methods

Preparation of the monoclonal antibodies

Na,K-ATPase was purified from the outer medulla of dog and rat kidneys by a modification [12] of the method described by

Jorgensen [13]. Na,K-ATPase activity was assayed as described previously [14]. Antigen preparations used had activities of 13 to 20 μM Pi per mg of protein per minute.

BALB/C mice were primed for immunization by injecting 2×10^9 Bordatella pertussis organisms intraperitoneally prior to injection of the prepared antigen. One-hundred to 150 μg of antigen was aggregated by adsorption with potassium alum and was then injected intraperitoneally 2 to 6 hr after the priming procedure. After 3 weeks the mice were bled and their serum tested against the original antigen by an enzyme-linked immunosorbent assay (ELISA). Mice with a high antibody titer were boosted with an intravenous injection of 100 μg of antigen. Three days following the booster dose of antigen, the spleen was removed and viable spleen cells were fused [15] in a ratio of 3:1 with the myeloma cell line SP2/0 in the monoclonal antibody facility of the Department of Pathology. Hybrid cells were grown in microtiter plates for 10 to 14 days and the supernates from each hybridoma was tested for antibody production by ELISA against the original antigen. Hybridomas that exhibited specific antibodies to the antigen by ELISA were cloned and subcloned by limited dilution and tested by ELISA to assure continued antibody production. After cloning, tumor ascites were produced by the intraperitoneal injection of 1 to 5×10^6 hybrid cells into the peritoneum of pristane primed AKD2/F1 mice (Jackson Laboratories, Bar Harbor, Maine, USA).

After removal from the peritoneum, immunoglobulin was precipitated from ascites fluid with 50% ammonium sulfate. The immunoglobulins were re-dissolved in buffer and IgG was affinity purified by using a rabbit anti-mouse IgG coupled to CN bromide activated Sepharose 4B agarose gel (Pharmacia, Uppsala, Sweden). The mouse IgG was eluted with 1 M acetic acid in .15 M sodium chloride neutralized with 1 M Tris base. The antibody was dialyzed overnight with phosphate buffered saline and concentrated with Aquacide II-A (Calbiochem, San Diego, California, USA). Immunodiffusion demonstrated the antibodies to be of immunoglobulin class IgG 1.

Screening by immunoprecipitation

Isolated rat kidneys were perfused with ^{35}S-methionine and ^{35}S-labeled cortical extracts were prepared by Dr. D. Kerjaschki (Department of Cell Biology, Yale University) as described [16]. Radiolabeled cortical extracts (~ 100,000 cpm) were incubated with an excess of antibody (~ 20 μg) and immune complexes were precipitated with agarose beads linked to rabbit anti-mouse Ig antibody. The immune complexes were solubilized by boiling for 3 min in sample buffer (3.65% SDS, 18 mM dithiothreitol, 4.5 mM EDTA, 6M urea, 10% glycerol) and subjected to SDS polyacrylamide gel electrophoresis (3.6 to 8% gradient). The gels were stained with Coomassie blue, dried, and a contact radioautogram was prepared by exposing the dried gel to Kodak X-Omat R film in the presence of a Dupont Cronex Lightening plus screen. The band with radioactivity was compared to the original gel for identification of the specific protein.

For immunoprecipitation of the dog antigen, Maden-Darby canine kidney (MDCK) cells were metabolically labeled with ^{35}S methionine. MDCK cells were grown in 75 cm^2 flasks (Falcon, Oxnard, California, USA) at 37° in a 5% CO_2 atmosphere. Eagle's minimal essential medium (EMEM) with Earle's salts was supplemented with 5% fetal calf serum (Gibco, Madison, Wisconsin, USA), 10 mM Hepes, and 100 U/ml each of penicillin and streptomycin. Cells were harvested for experiments and routinely passaged with trypsin-EDTA (Gibco). For metabolic labeling, MDCK cells were trypsinized and washed twice in methionine free EMEM. Cells were resuspended in EMEM (5×10^6/ml) and ^{35}S-methionine (Amersham, 1000 Ci/mm, Arlington Heights, Illinois, USA) was added (25 m Ci/ml final concentration). Incubation at 37°C proceeded for 2 hr.

In separate experiments, MDCK cells were labeled with the photoaffinity derivative NAB-ouabain [12]. MDCK cells were suspended in 150 μl binding buffer (10 mM Na phosphate, 3 mM mg Cl_2, 150 mM NaCl, 2 mM EDTA, pH 7.3). At 2×10^7 cells/ml and incubated for 10 min in the dark at 37°C with 1 μM ^{3}H 2 nitro 5-azidobenzoyl(NAB)-ouabain (18 Ci/mM) prepared as described previously [12]. This mixture was then diluted to one ml with binding buffer and exposed to UV light from a high-voltage mercury vapor lamp (ACE-Hanovia, Vineland, New Jersey, USA) for 5 min at 25°C to complete photolysis of the NAB-ouabain.

Following metabolic or NAB-ouabain labeling, MDCK cells were washed once and resuspended in precipitation buffer (150 mM NaCl, 10 mm Tris EDTA, pH 7.5, 2 mm EDTA). At a concentration of 5×10^6 cells/ml, this mixture was probe sonicated (sonifier cell disrupter, Heat Systems-Ultrasonics, Inc., Plainview, Long Island, New York, USA) for 30 sec on ice, 40% full output. Sonicates were spun for 10 min at full speed in a Beckman airfuge (160,000g) and the resultant pellets resuspended in precipitation buffer with 1% NP-40 (Sigma, St. Louis, Missouri, USA) (1 ml per 5×10^6 lysed cells). Immunoprecipitation proceeded as according to Dobberstein et al [17] with slight modifications. Aliquots (350 μl) for immunoprecipitation of resuspended cell pellets were mixed with 6 μg of "nonimmune" mouse monoclonal IgG and incubated with constant rotation for 30 min at 4°C. One mg of swollen, washed protein-A beads (Sigma), which had been conjugated with 20 mg affinity purified rabbit anti-mouse IgG (Cappel, Cochranville, Pennsylvania, USA), was then added and incubation continued for another 30 min. The beads were pelleted and 2 μl (4 mg) of either affinity purified antibody C62.4 or "nonimmune" monoclonal IgG were added for a 30-min, 4°C incubation (in the experiment depicted in Figure 1, the antibody C62.4 was premixed either with 12 mg of Jorgensen membranes or buffer and allowed to sit once for 30 min prior to its addition to the precipitation mix). Following this incubation, another 1 mg of protein A-rabbit anti-mouse IgG conjugate was added. After 1 hr at 4°C, the beads were pelleted and washed according to the protocol of Dobberstein et al [17].

For use as a control, nonspecific polyclonal and monoclonal IgG was obtained by ammonium sulphate precipitation followed by affinity column purification of mouse serum and nonimmune tumor ascites. IgG obtained in this manner exhibited no specific binding to rat kidney by indirect immunofluorescence and ELISA. Cross reactivity of monoclonal antibodies was assayed by both immunofluorescence and ELISA.

Functional screening

The screening of antibodies for effects on function of the Na,K-ATPase is complicated by the presence in culture media of substances that are known to affect various aspects of

Na,K-ATPase action (for example, Ca^{++}, P_i). Since isolation of immunoglobulins from a large number of dilute hybridoma supernatants is impractical, two procedures were used to immobilize Na,K-ATPase, permitting removal of the antibody containing medium prior to Na,K-ATPase functional assay. In the first of these, Na,K-ATPase was diluted in phosphate buffered saline (3 μg protein/ml) and was aliquoted into wells of a microtiter plate (50 μl/well) and allowed to stand overnight at 4°C. The wells were emptied and rinsed twice with PBS containing 1% BSA and 20 mM EDTA. Supernatant solutions from hybridoma wells (with 2.2 mM added EDTA) were added (100 μl/well), and the plate was shaken for 45 min at room temperature; PBS and Hypoxanthine-Aminopterin-Thymidine medium were used in control wells at this stage. Wells were emptied and rinsed once with 25 mM imidazole, 1% BSA, 1 mM EDTA, followed by the addition of (^{3}H)-ouabain (0.01 μM, 14/Ci/mmoles) in 60 μl of either *(a)* 120 mM NaCl, 3 mM NaATP, 3 mM $MgCl_2$, 0.1% BSA, 25 mM Tris, pH 7.5, or *(b)* 5 mM Mg^{++}, 3 mM P_i, 2 mM EDTA, 0.2% BSA, 30 mM Tris, pH 7.25 with or without additional unlabeled ouabain (10^{-4}M). After 40 min incubation at room temperature, the wells were emptied, rinsed once with 100 mM KCl, 25 mM imidazole, 1 mM EDTA, pH 7.5 and the bound (^{3}H)-ouabain was removed from the wells with 200 μl 2% SDS and transferred to scintillation vials. Alternatively, it was possible to screen for antibody inhibition of Na,K-ATPase by substituting 200 μl Na,K-ATPase assay medium [14] for the ^{3}H ouabain-binding medium, incubating for 30 min at 37°C, and analyzing for P_i. Note that only approximately 10% of the original Na,K-ATPase activity (and ^{3}H ouabain-binding sites) was retained in control wells on the microtiter plates using this method.

A second screening method employed filtration on cellulose ester filters as a means to immobilize Na,K-ATPase; this provided considerably greater reproducibility (standard deviation 13%) than the use of microtiter plates, but was not suited to a large number of samples. Na,K-ATPase (usually as SDS-permeabilized membranes), 0.4 mg protein/ml was preincubated with 1:10 dilutions of ascites fluid in 0.25 ml of 25 mM imidazole 1% BSA, 20 mM EDTA for 45 min at room temperature and 50 μl aliquots were filtered onto Gelman GN-6 filters on a 30 place Millipore filtration manifold. After rinsing briefly with 1 ml 25 mM imidazole, the vacuum was removed and 200 μl of (^{3}H) ouabain-binding medium (as above with 0.3 μM ^{3}H-ouabain, 3 Ci/mmoles) was applied to the surface of the filters. Following a 45-min incubation at room temperature with shaking, vacuum was reapplied to remove the binding medium, and the filters were rinsed with 100 mM/KCl, 25 mM imidazole, 1 mM EDTA, and transferred to scintillation vials. In control experiments it was found that maximal ouabain binding as determined by this method was 70% that obtained when the incubation with (^{3}H)-ouabain was carried out in a test tube. Na,K-ATPase assay could be carried out with a similar protocol: 1 ml Na,K-ATPase assay medium was applied to the filters in place of the (^{3}H)-ouabain medium, and after 30 min shaking at room temperature the medium was collected through the filter along with a 0.3 ml H_2O rinse, and assayed for P_i [14].

For study of the enzymatic behavior of the antibody-Na,K-ATPase complex, SDS washed microsomes (typically 1 mg/ml, 300 μl) in 25 mM imidazole, 0.5% BSA, 5 mM EDTA were incubated with ascites fluid (typically 5 mg/ml IgG, 40 μl) for 40 min at 22°C, pelleted in an airfuge (Beckman, Palo Alto, California, USA) and resuspended in 0.1% BSA, 25 mM imidazole, 1 mM EDTA (typically in 300 μl). Samples were taken from this for various assays. Assays of Na,K-ATPase activity [14], ouabain binding, and E-P formation [18] and ^{86}Rb deocclusion [19, 20] have been described previously. Na-ATPase activity was assayed the same as Na,K-ATPase, but in 100 mM Na, 1.6 mM Mg, 0.1 mM ATP, 0.2 mM EDTA, 30 mM Tris, pH 7.2. K-dependent nitrophenyl phosphatase (KpNP-Pase) activity was measured by incubation of enzyme in 1 ml 10 mM KCl, 5 mM $MgCl_2$, 10 mM para-nitrophenyl phosphate, 25 mM imidazole for 30 min at 37°C, followed by addition of 2 ml 0.2 N NaOH, 20 mM EDTA, 0.2% EDTA, 0.2% SDS, and determination of absorbance at 410 nm.

Morphological localization

Because glutaraldehyde, even at concentrations as low as 0.25%, altered the antigenicity of the Na,K-ATPase, an alternative fixation protocol was necessary. Fixation using the periodate-lysine-paraformaldehyde (PLP) method of McLean and Nakane [21] as modified by Brown and Farquhar [22] provided adequate structural preservation with excellent retention of antigenicity. The fixative, which is prepared just prior to use, is composed of 0.01 M Na I O_4, 0.75 M lysine, 2% paraformaldehyde, in 0.0375 M Na_2 HPO_4 buffer, pH 6.2.

Sprague-Dawley rats were anesthetized with Inactin (10 mg/100 g body wt). The kidneys were perfused retrograde via the aorta as described previously [23], initially with mammalian Ringer's and followed by fixative. After 5 to 10 min, the kidneys were removed and the tissue was either diced into 1 to 2 mm cubes or cut into large pieces that retained cortical, medullary, papillary relationships, and then postfixed for 6 hr in the perfusate. The tissue was washed 3 to 5 times with 0.1 M Na_2 HPO_4 buffer, pH 7.2. Cryoprotection was achieved by incubating the tissue with 10% DMSO in 0.1 M Na_2 HPO_4 for 1 hr at 4°C. Blocks of tissue were frozen by rapidly plunging them into swirling liquid Freon 22 cooled with liquid N_2. Sixteen μm thick cryosections were cut and placed immediately into 1% Bovine Serum Albumin (BSA) in phosphate buffered saline (PBS).

The immunoperoxidase labeling used in this investigation was modified from that described by Brown and Farquhar [22]. Cryosections were preincubated in a goat anti-rabbit IgG, 5 mg/ml in PBS with 2% BSA for 1 to 3 hr as a general blocking step and to block any endogenous Fc receptors that might be present in the rat kidney. After 3 washes with PBS + 1% BSA, the sections were incubated overnight with 50 to 100 μg/ml of the monoclonal IgG to be tested. The sections were again washed 3 to 5 times with PBS, and incubated for 2 to 4 hr with peroxidase conjugated sheep anti-mouse Fab of IgG. After washing 3 to 5 times, the tissue was fixed for 1 hr with 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) with 5% sucrose. This was followed by three washes with 0.1 M Na cacodylate and 7.5% sucrose followed by three washes in 50 mM Tris-HCl (pH 7.4) with 7.5% sucrose. The tissue was placed in 0.2% diaminobenzidine, in the Tris sucrose buffer for 5 min at which time H_2O_2 was added to produce a final concentration of 0.01%. Incubation was carried out for 5 to 20 min at room temperature. Since the amount of Na,K-ATPase varied in different tubule segments, the incubation times were varied to optimize visualization for each nephron segment; that is, 5 min

for the thick ascending limb, which had the greatest amount, and 20 min for the thin limbs, which had the least amount of enzyme. The reaction was stopped by washing the sections in the Tris buffer. The tissue was osmicated with reduced O_SO_4 [24], dehydrated and embedded in Epon 812. One-half μ semithin sections were cut with glass knives and stained with an Azure II-Methylene blue. Thin sections were cut with diamond knives and viewed unstained and stained with 2% aqueous uranyl acetate and lead citrate. All sections were examined with a Zeiss EM 10B electron microscope. To test the sensitivity and selectivity of this technique, Na,K-ATPase was localized in a variety of tissues less rich in enzyme than the kidney including liver and gastric mucosa. It was easily detected in the liver, and, in gastric mucosa, labeling was present only on basal lateral cell surfaces and was distinct from the microvillous distribution of H,K ATPase seen in parietal cells (unpublished observations).

Immunogold labeling

Colloidal gold (5 nm) solutions were prepared using the method of Faulk and Taylor [25] which employs the reduction of $HAuCl_4$ with white phosphorus. Goat anti-rabbit IgG (Cappel) was bound to the Au as described by Slot and Geuze [26].

Tissue that had been fixed with the PLP method described above was frozen and cryostat sectioned at 16 μm. The sections were incubated with the monoclonal antibody and then with a rabbit anti-mouse IgG (Cappel) 50 to 100 μg/ml. The tissue was then washed with PBS and embedded in Lowacryl K4M employing the procedure of Altman, Schneider, and Papermaster [27]. Thin sections were mounted on formvar-coated grids and labeled with the goat anti rabbit-colloidal gold conjugates by incubating the grids with conjugated antibody for 30 min at room temperature followed by multiple washing with PBS.

Immunofluorescence localization

Fresh, unfixed, and PLP-fixed kidneys were sectioned. Coronal sections (2 to 3 mm) of kidney were frozen with liquid N_2. Eight μm cryosections were picked up on acid-cleaned or gelatinized glass slides and air dried in a humid chamber overnight. Additionally, 0.5 μm thick sections were cut with an LKB ultramicrotome. Sections were incubated with the monoclonal antibody, washed in buffer, and then incubated with fluorescein-labeled goat anti-mouse IgG (Cappel). Sections were examined with a Leitz incident light fluorescence microscope (Ploem, E. Leitz, Wetzlar, West Germany).

Results

Functional screening of C62.4

C62.4 was selected from five different monoclonals in a microtiter plate assay of ^{3}H-ouabain binding. It was noted that ^{3}H-ouabain binding was inhibited 80% by C62.4 in the presence of Na, Mg, and ATP, but C62.4 did not inhibit ouabain binding in the presence of Mg and Pi.

In separate experiments it was found that Na,K-ATPase activity was inhibited 75 to 80% by C62.4 and that maximal inhibition occurred with 1 mg immunoglobulin/mg Na,K-ATPase. Na,K-ATPase that was complexed with the antibody exhibited the following activities compared to control: 25% maximal Na,K-ATPase activity, 60% maximal Na-ATPase activity, and 100% K-nPPase activity. The amount of phosphorylated intermediate (E-P) formed in the presence of (γ-^{32}P)-ATP or $^{32}P_i$ [19] was unaffected by the antibody. The amount of ^{86}Rb that could be bound in the "occluded state" of the enzyme [20] was also unchanged. The rates of release of occluded ^{86}Rb [19, 20] in the presence of ATP, Mg, and P_i were approximately one-half the rates in control samples. In the "occluded state", Rb (or K) is unavailable for exchange with Rb (or K) in the medium. Although detailed studies of the rate of (^{3}H)-ouabain binding were not performed, it was noted that in the presence of ATP, (^{3}H)-ouabain binding occurred at high but not low concentrations of (^{3}H)-ouabain. This indicated that the rate of ouabain binding was slowed by C62.4 rather than the site being blocked by the antibody. Together these results are consistent with the hypothesis that C62.4 acts on Na,K-ATPase by slowing the E_1-E_2 transitions.

In one experiment, the sidedness of antibody binding was investigated by incubation of a preparation of predominantly right-side-out membrane vesicles [28] with antibody before or after treatment with SDS to permeabilize the membranes; in each case, the membranes were then washed and incubated with SDS before assay for Na,K-ATPase activity. It was found that although the antibody inhibited 78% of activity when added after SDS, it inhibited only 25% when added before the membranes were permeabilized, indicating that the antibody binds to the intracellular aspect of the enzyme. C62.4 labeled with colloidal gold bound to SDS-activated membrane fragments in abundance as assayed by electron microscopy. Incubation with a preparation of predominantly right-side-out membrane vesicles showed no evidence of specific binding on the intact vesicles. This observation confirmed the results of ATPase assays with the same preparations and confirmed that C62.4 binds to an intracellular domain of the enzyme. In membrane fragments, colloid gold particles appear to be clustered rather than being evenly distributed. This may reflect either the polyvalency of the antibody-gold complex or the aggregates of enzyme particles seen in Na,K transport vesicles reconstituted with purified Na,K-ATPase [29].

Monoclonal antibody immunoprecipitation of dog antigen with C62.4

MDCK cells labeled with ^{35}S-methionine were sonicated and solubilized with 1% NP-40 and subjected to immunoprecipitation with five monoclonal antibodies initially selected by ELISA or with the same quantity of an unrelated monoclonal IgG. One of these, C62.4 specifically precipitated a single radiolabeled band of molecular weight 96 KD (Fig. 1). No specific bands were precipitated by the other monoclonals or control immunoglobulins. Preincubation of C62.4 with purified Na,K-ATPase completely blocked the precipitation of the labeled polypeptide. Forbush, Kaplan, and Hoffman [12] have shown that NAB-ouabain will specifically bind and upon exposure to ultraviolet light become covalently attached to the α-subunit of Na,K-ATPase. Tritiated NAB-ouabain was used to demonstrate that C62.4 does, in fact, recognize the α-subunit of labeled MDCK cells. Figure 1 shows that this antibody precipitates a 96 KD protein labeled with NAB-ouabain, whereas the unrelated monoclonal IgG did not precipitate any radiolabeled polypeptides. C62.4 that had been preincubated with purified Na,K-ATPase was ineffective. These results confirm with a

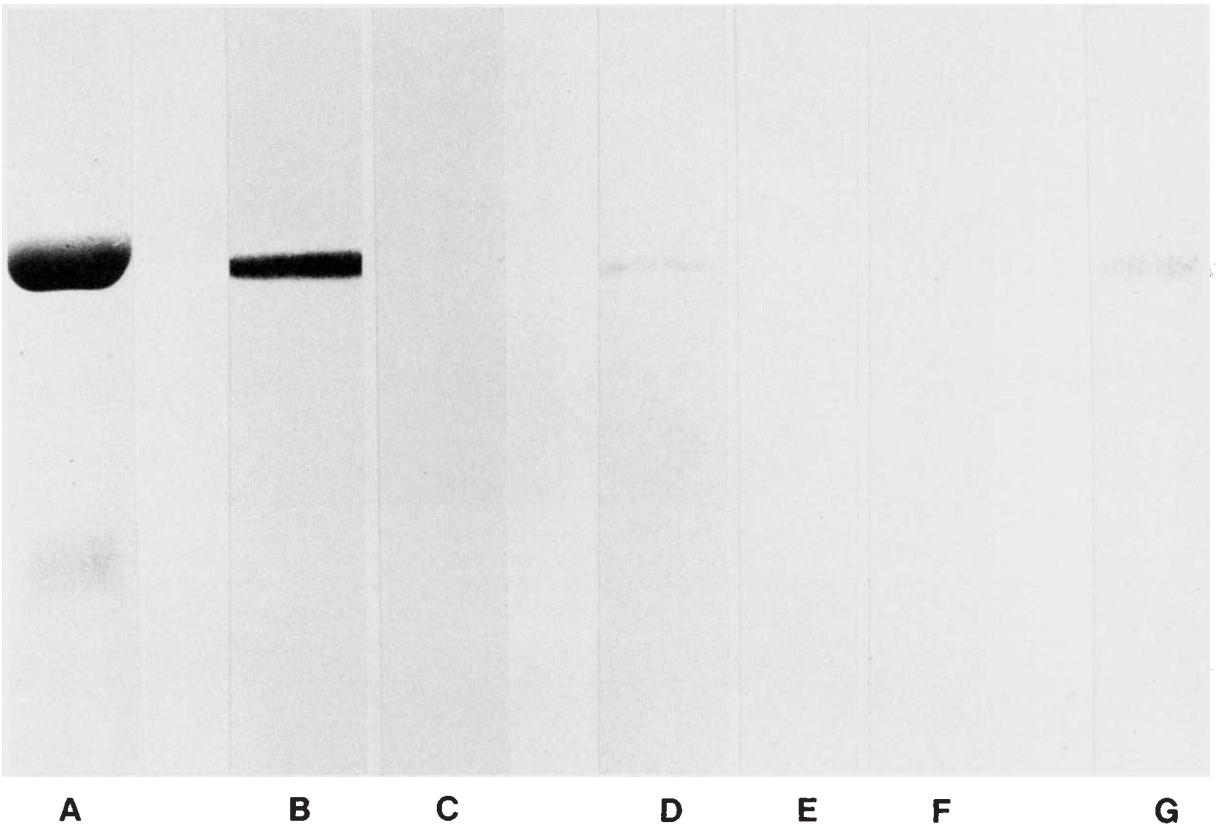

Fig. 1. Lane A *Coomassie blue-stained SDS PAGE of dog kidney membrane preparation enriched in Na^+-K^+-ATPase,* The α-subunit (96 KD) and the β-subunit (~55 KD) are the only bands visible. **Lanes B and C** *Immunoprecipitation of membranes of MDCK cells biosynthetically labeled with ^{35}S methionine.* Incubation with C62.4 precipitates a labeled 96 KD protein **(Lane B).** Preincubation of C62.4 with "cold" purified Na, K ATPase blocks precipitation of any labeled protein. **Lanes D, E, and F** *Immunoprecipitation of membranes of MDCK cells labeled with 3H-NAB ouabain.* Incubation of membranes with C62.4 precipitates a labeled 96 KD protein **(Lane D).** Preincubation of C62.4 with unlabeled purified Na, K-ATPase blocks precipitation of labeled protein **(Lane E).** Incubation of labeled membranes with "nonimmune" mouse monoclonal IgG does not precipitate any labeled protein. **Lane G** *Immunoprecipitation of rat kidney membranes biosynthetically labeled with ^{35}S methionine.* Incubation of membranes with C15.16 precipitates a labeled protein of approximately 94 to 98 KD. **Lanes B** through **G** are visualized by autoradiography.

high degree of confidence that the 96 KD protein precipitated by C62.4 is the α-subunit of Na,K-ATPase.

Immunofluorescent localization in rat kidney with C62.4

Cross reactivity of C62.4 with the Na,K-ATPase prepared from rat outer medulla membranes was demonstrated initially by ELISA. C62.4 was then used as a primary antibody to label fresh-frozen dog and PLP-fixed rat cryosections of kidney. The distribution of the label was identical for both species and methods of preparation. Specific nephron segments were more easily identified with PLP-fixed preparations.

Glomeruli showed no specific binding of C62.4 by indirect immunofluorescence except for occasional cells in the mesangial regions. The proximal tubules labeled along the basolateral aspects with no evidence of luminal binding (Figs. 2**A** and **B**). No detectable fluorescence was present in the thin descending limb of the loop of Henle, whereas definite binding was demonstrated in the most distal portion of the thin ascending limb as it joins the thick ascending limb. The most intense basolateral fluorescence was present in the thick ascending limb of Henle's loop in the outer medullary stripe. This extended into the cortical portion of the ascending limb and the early distal convoluted tubule. No major differences in intensity were seen which differentiated these segments. Almost equally intense was the fluorescent staining of the cortical and outer medullary collecting ducts. In the collecting ducts, occasional cells interpreted as intercalated cells did not significantly bind the antibody and no staining was seen. In the papillary portion, the intensity of basolateral fluorescence of the collecting ducts increased as the collecting duct approached the papillary tip (Figs. 3**A** and **B**).

Ultrastructural immunolocalization

Both colloidal gold and immunoperoxidase localization techniques were utilized in the immunocytochemical localization of Na,K-ATPase to take advantage of each of their respective properties. Amplification of the signal is an advantage of the immunoperoxidase technique, but diffusion of the reaction product reduces the definition of the electron micrograph. Colloidal gold offers the advantage of clear definition of a punctate label, but the polyvalency of the antibody-gold complex results in a lower density of identifiable marker and Lowicryl embedding results in some loss of quality of the electron micrographs. Qualitatively, the nature of the results using both of these methods was essentially identical.

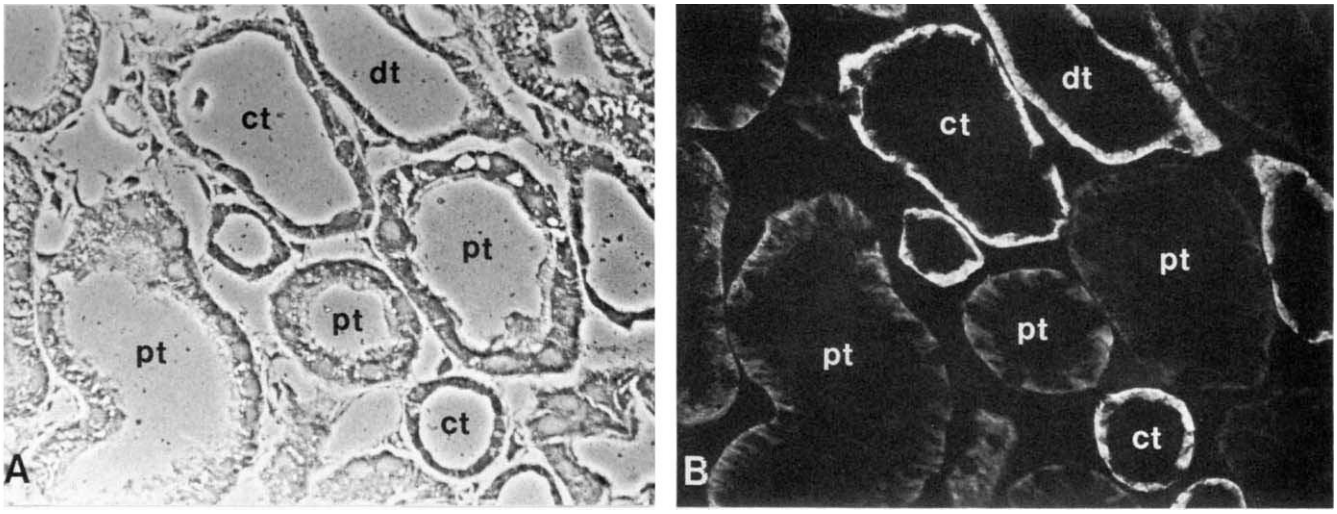

Fig. 2A and **B** *Immunofluorescence photomicrograph and corresponding phase photomicrograph of rat renal cortex.* Sections incubated with C62.4 and fluorescein-labeled goat anti-mouse IgG show a greater intensity of fluorescence in distal tubules (dt) and collecting ducts (ct) as compared to proximal tubules (pt). The intercalated cells of the collecting ducts do not demonstrate any basal fluorescence. ×500

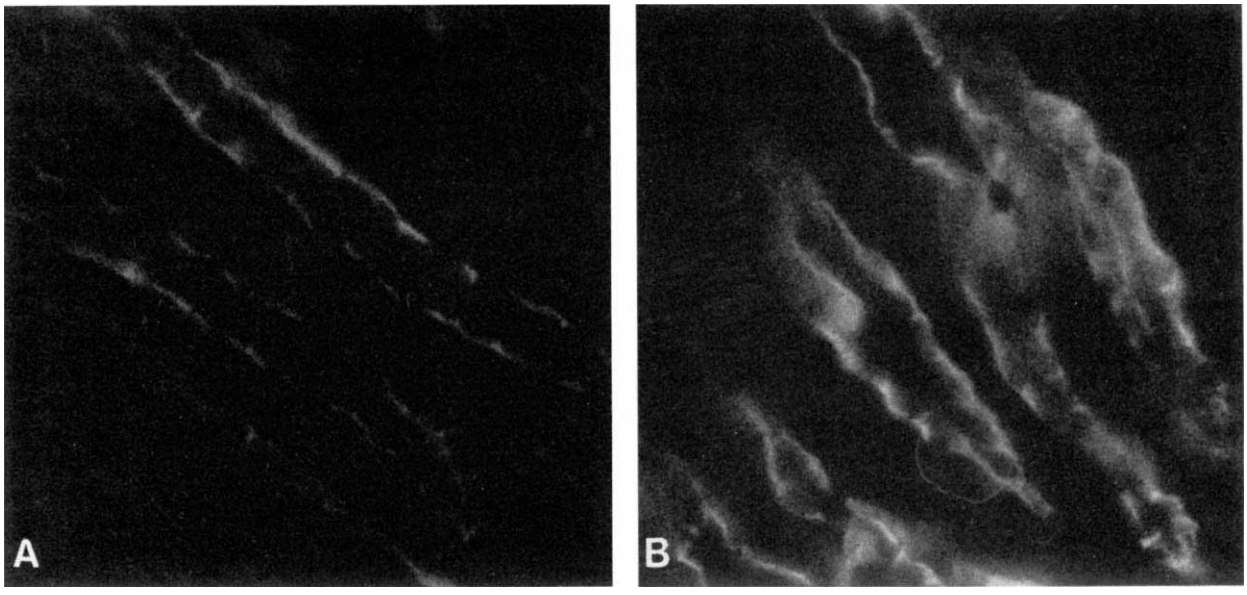

Fig. 3. *Immunofluorescence photomicrographs of papillary collecting ducts in* **A** *the outer region of the papilla and* **B** *at the papillary tip.* Sections were incubated with C62.4 and fluorescein-labeled goat anti-mouse IgG. The intensity of fluorescence in the papillary tip is greater than in the outer region of the papilla. ×500

With both immunoperoxidase and immunogold labeling, the basal and lateral membranes of all segments of the proximal tubule exhibited evidence of binding of C62.4. No evidence of specific binding to the luminal membrane was found. Apical coated pits of proximal tubule cells occasionally demonstrated the presence of reaction product after initial incubation with either C62.4 or control nonspecific mouse IgG, suggesting that apical coated pits may have a nonspecific receptor for immunoglobulin. The binding of the lateral membrane ended sharply at the intracellular junctional complex and distinctly separated the two major cellular membrane domains (Fig. 4A). The findings were similar in all proximal tubule segments and the distribution of reaction product or immunogold was qualitatively the same. The intensity of labeling the basolateral membrane was uneven, with the complex-invaginated regions showing the most intense labeling and the flat subnuclear regions showing the least. This pattern of uneven labeling was seen in all nephron segments (Figs. 5A and B) but was most prominent in the proximal S3 segments and collecting tubules.

Minimal binding of C62.4, which was close to the limits of detection, was present in the thin descending limb of the loop of Henle or in the initial portion of the thin ascending limb of long loop nephrons. That portion of the thin ascending limb just prior to its junction with the thick ascending limb did show more easily detectable evidence of specific binding (Fig. 6), confirming the findings by immunofluorescence localization. The

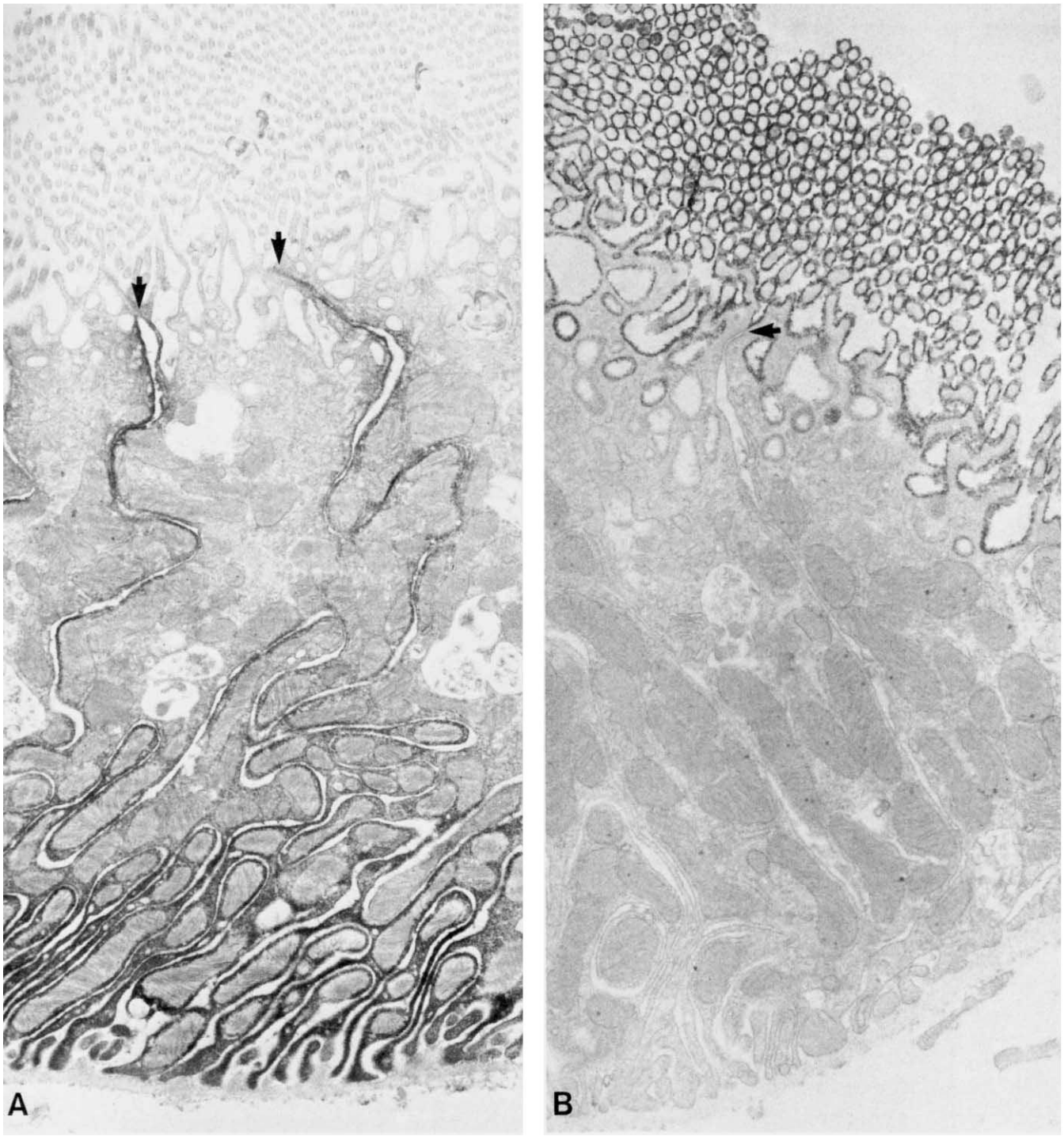

Fig. 4. *Immunoperoxidase labeling of the proximal tubule.* These, and all subsequent micrographs are of the rat kidney. **A** demonstrates the distribution of the α-subunit using antibody C62.4. Note that the reaction product is confined to the cytoplasmic domain of the basolateral membrane. The label stops abruptly at the zonula occludens *(arrows)*. ×10,000 **B** demonstrates the distribution of labeling with a control antibody (C15.16). This antibody immunoprecipitates a 95 KD polypeptide (Fig. 1), which is shown here to be present only in the brush border of the proximal tubule. The label does not extend below the level of the tight junction *(arrow)*. ×18,000

thin limbs of short loop nephrons in the inner stripe of the outer medulla also had minimal binding. Reaction product was most intense in the basal lateral membranes of the thick ascending limb of Henle (Fig. 7). The luminal membrane surface showed no evidence of specific binding. The pattern of binding was similar in medullary and cortical portions of the thick ascending limb as well as in the early distal convolution. Labeling of the basolateral surfaces of macula densa cells was seen (Fig. 7), but

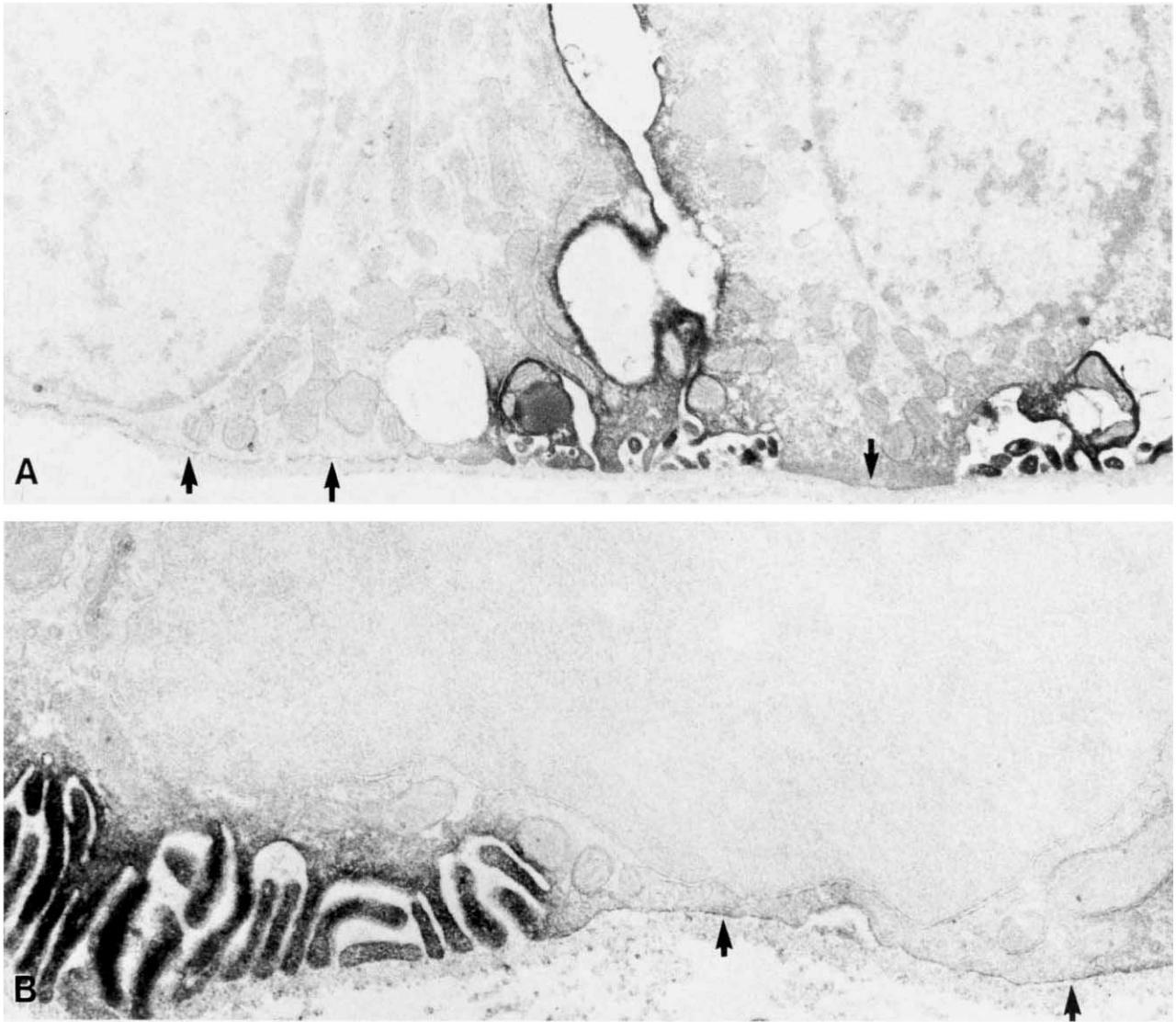

Fig. 5. *In those areas of the basal cell membrane that are not infolded and which directly oppose the basal lamina, reaction product is usually absent* (arrows). **A** Basal portion of two cells of the S3 segment of proximal tubule. **B** Basal portion of a principal cell from a cortical collecting tubule. ×24,000

it was less prominent when compared to cells of the thick ascending limb or distal tubule.

The principal cells of the connecting tubule, cortical collecting tubule, and outer medullary collecting tubule showed evidence of specific binding of C62.4 to the basal lateral membrane (Figs. 8**A** and **B**). The Immunogold technique demonstrated labeling confined to the protoplasmic aspect of the basal lateral plasmalemma particularly well (Fig. 9). This confirmed the intracellular localization of the binding site of C62.4, which had been demonstrated both functionally and morphologically in unfixed membrane fragments and intact right-side-out membrane vesicles. A striking finding was the absence of significant binding of C62.4 to any membrane domains of the intercalated cells (Figs. 8**A** and **B** and 10). The pattern of binding was identical throughout those portions of the collecting tubules which contain intercalated cells. Basal lateral binding of collecting duct epithelium in the inner medulla and papilla was essentially similar to that seen for the principal cells of the cortical and outer medullary collecting tubule (Fig. 11) although reaction product required a somewhat longer period of incubation for visualization. The only intracellular organelle that could be demonstrated to have binding of C62.4 was the medial cisternae of the Golgi apparatus (Fig. 12). Definite binding was not present on any other intracellular membrane profiles or organelles.

Monoclonal antibodies to rat antigen (C15.16)

Several clones selected by ELISA screening produced antibodies to the antigen derived from rat outer medulla. Only one, C15.16, was demonstrated to precipitate a 96 KD protein from ^{35}S-methionine-labeled rat membranes (Fig. 1). Na,K-ATPase activity or ouabain binding of the membranes was unaffected by

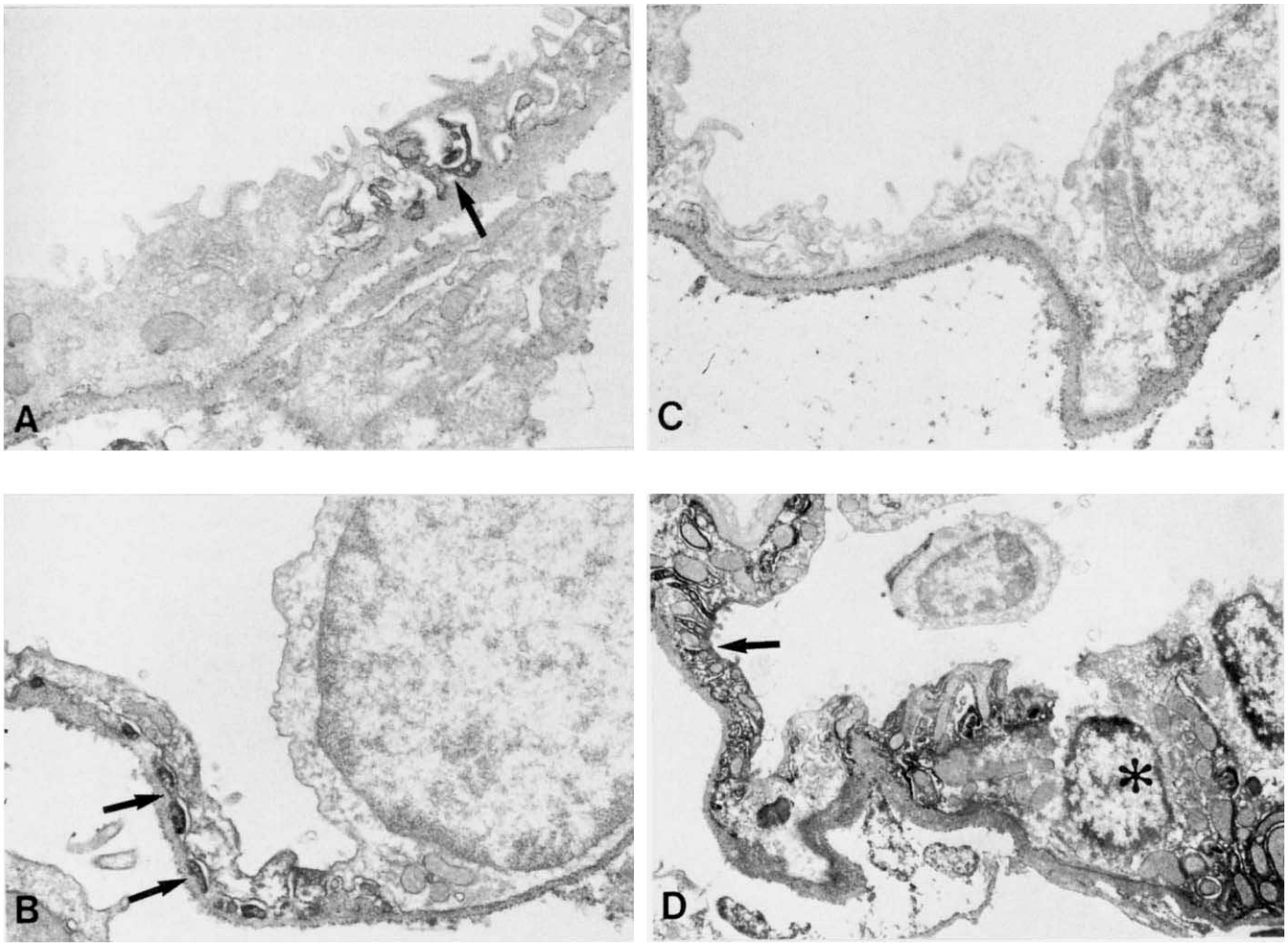

Fig. 6. *Immunoperoxidase labeling of the thin limb of Henle's loop.* Labeling of this segment was weak. **A** is the thin descending limb of the outer medulla. Only incomplete labeling is seen *(arrow)*. **B** and **C** shows the distribution in the inner medullary thin limb. Some profiles exhibit spotty labeling while others are negative. The most intense labeling of this segment occurs in the thin ascending limb just prior to its junction with the thick ascending limb. **D** Label of thin ascending limb *(arrow)*. Thick ascending limb *(star)*. Final magnifications: **A**, ×12,000; **B**, ×9,500; **C**, ×12,000; **D**, ×5,000

incubation with C15.16. In contrast to C62.4, immunofluorescence screening of this antibody revealed binding only to the apical microvilli (brush border) of the proximal tubule. This was confirmed at the ultrastructural level where immunoperoxidase labeling of the apical membrane ended at intercellular junctions with no evidence of basolateral localization (Fig. 4**B**).

Discussion

A monoclonal antibody for the cytochemical localization of Na,K-ATPase was utilized to exploit the inherent advantages of monoclonal antibodies as highly specific reagents. Monoclonal antibodies are preferable to polyclonal antisera for ultrastructural localization of membrane proteins for a variety of reasons. Production of a polyclonal antisera of high specificity requires that the antigen be highly purified, but even with apparently highly purified antigen, production of undesirable antibodies to strongly antigenic minor contaminants can occur [8]. In contrast, relatively impure antigens can be used to generate a variety of monoclonal antibodies and the desired antibody can be selected out with proper screening procedures. With membrane-associated proteins which cannot be easily separated without denaturation or alteration of molecular configuration, the monoclonal antibody technique allows selection of a specific antibody to the desired antigen from the variety of antibodies produced to a relatively crude membrane preparation used as an antigen [30]. Furthermore, since immunoglobulins of polyclonal antisera are heterogeneous, they will recognize multiple antigenic determinants on the same molecule and even the same determinant with differing affinities. In contrast, monoclonal antibodies not only produce a reagent directed to a single antigenic determinant, but also one with a definable and constant affinity, thus providing an ideal probe to examine the distribution of membrane proteins.

The determination of specificity of the monoclonal antibody depends critically on the screening procedures, and definite identification can only be assured when more than one type of screening is carried out. With the monoclonal antibody C62.4, raised against the dog antigen, specificity was initially identified by functional screening and was further characterized by immunoprecipitation of the enzyme labeled by two different methods.

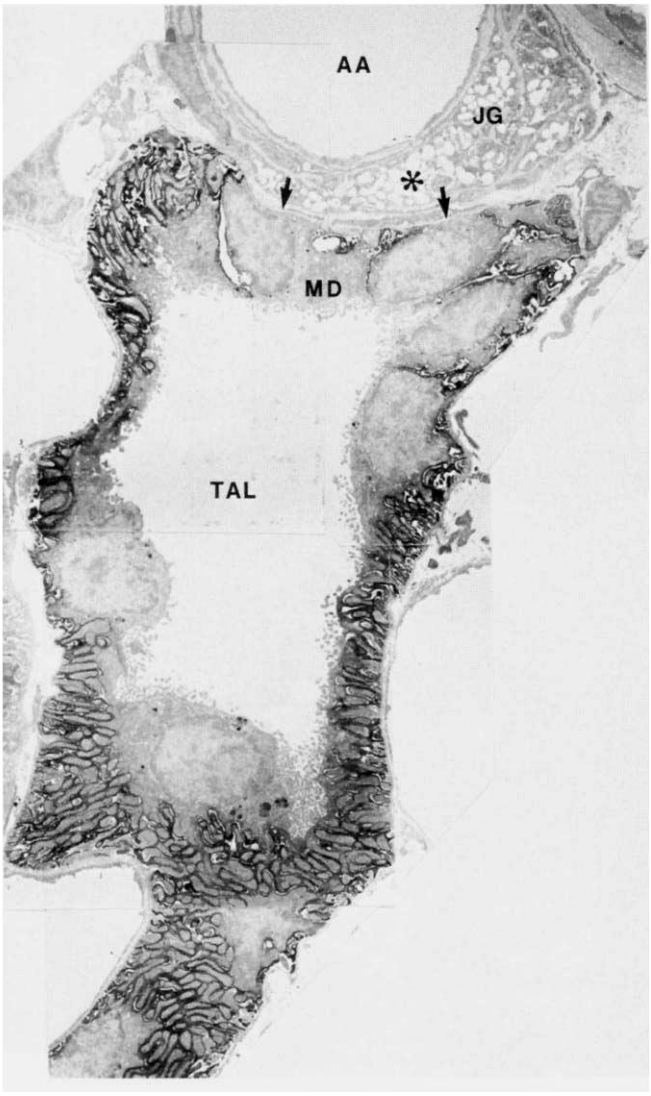

Fig. 7. *Immunoperoxidase labeling of the thick ascending limb (TAL) and macula densa (MD).* The reaction product is very dense in the cells of the TAL. Cells of the macula densa have less prominent basolateral staining as well as fewer infoldings of the basolateral membranes *(arrows).* Renin granules *(star)* of the juxtaglomerular cells (JG) are not well preserved by this method. Abbreviation: AA, afferent arteriole. ×2,900

Since the MDCK tissue culture cell line is derived from dog renal tubular epithelium, it afforded an ideal system for immunochemical screening. Immunoprecipitation of solubilized membrane fragments of cells whose proteins had been labeled with ^{35}S-methionine demonstrated a single 96 KD protein. A more decisive immunoprecipitation was carried out with MDCK cells labeled with the tritiated photoaffinity derivative, NAB-ouabain, which covalently binds specifically to the α-subunit of sodium potassium ATPase. The antibody precipitated a single 96 KD protein labeled with ^{3}H-NAB ouabain. In both instances, addition of excess unlabeled Na,K-ATPase blocked the precipitation of the labeled polypeptide, confirming that the antibody is specific for the α-subunit of Na,K-ATPase.

The need for multiple screening procedures was made evident in examining our results with the monoclonal antibody prepared to rat antigen. Screening by immunochemical methods demonstrated that the antibody precipitated a single 96 KD polypeptide. Since the crude antigen used for immunization had been enriched for Na,K-ATPase, it seemed logical to assume that the peptide was the α-subunit of Na,K-ATPase. Functional screening did not reveal any inhibition of Na,K-ATPase activity and morphologic localization was performed. The antibody localized exclusively to the microvillus brush border of proximal tubular segments, and no evidence of basal lateral localization was found. This result could be interpreted as demonstrating that there may be membrane proteins with antigenic domains shared with Na,K-ATPase that are sorted to different regions of different cell types. Kyte [5, 6] and Papermaster [7] have suggested such a hypothesis based on their findings with polyclonal antisera that labeled brush border as well as the basal lateral domains of renal tubular epithelium. A simpler and more likely interpretation is that there is a highly antigenic contaminating protein (probably a glycoprotein) that co-migrates with the α-subunit of Na,K-ATPase in SDS PAGE.

These results prompted the use of dog kidney as a more abundant source of antigen for functional as well as immunochemical screening. Ball et al [9] has also raised monoclonal antibodies against lamb kidney medulla, one of which demonstrated partial inhibition of enzyme activity. While selection by functional inhibition may give a low yield, it is probably the best initial screening method. Only after having identified a specific antibody in this manner can coprecipitation and cotitration studies be performed to characterize antibodies without functional activities.

Ouabain autoradiography [4], potassium-dependent nitrophenylphosphatase cytochemistry [2], and immunocytochemical localization with polyclonal anti-holoenzyme or anti-alpha subunit [5–7] all have been used to localize the enzyme in kidney tubules. The major site of binding of ^{3}H ouabain was found to be on the basal lateral membrane of the thick ascending limb of Henle's loop with little binding in thin limbs or collecting ducts [4]. Binding to cortical structures was not reported. While ouabain binding is specific for Na,K-ATPase, technical problems associated with long incubation times may make this method insensitive and, with nonspecific ouabain trapping by the tissue, potentially inaccurate. Furthermore, adequate resolution is inhibited by the size of the grains and the thickness of the section.

Potassium-dependent nitrophenylphosphate activity that can be blocked by ouabain has been used more extensively in a variety of tissues, but is complicated by splitting of the substrate by several enzymes, including alkaline phosphatase in the brush border of the proximal tubule. Multiple controls are necessary and techniques used to inhibit non-specific reactions by the addition of cysteine, which inhibits alkaline phosphatase, and dimethyl sulfoxide, which stimulates potassium-dependent phosphatase, are frequently difficult to standardize. Studies using this technique [2, 31, 32] have demonstrated predominantly basolateral labeling of tubular epithelial cells, but distribution along the various nephron segments has varied because of the differences in the activity of the enzyme in the different segments, particularly in those segments with low activity. No definite intracellular structures have been visualized by this technique, although Rostgaard and Miller [32] did find reaction product in a narrow cytoplasmic compartment adjacent to the

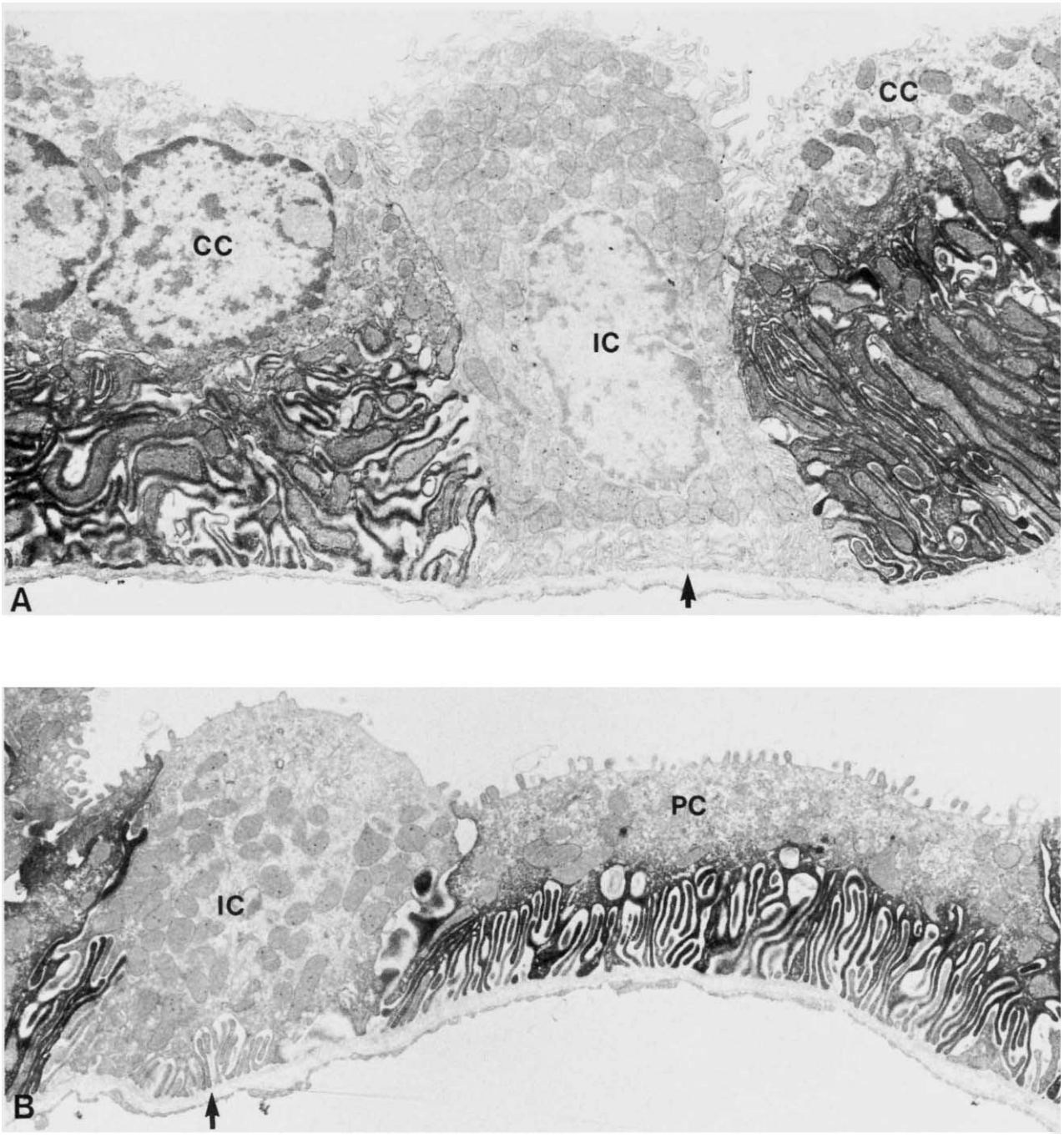

Fig. 8. *Immunoperoxidase labeling of* **A** *the connecting tubule and* **B** *the cortical collecting tubule.* Connecting cells (CC) and principal cells (PC) have dense basolateral labeling. Intercalated cells (IC) from both segments are devoid of reaction product *(arrows).* Final magnification: **A,** ×7,800; **B,** ×9,200

cell membrane. These results are difficult to interpret since the method depends on enzyme activity and thus cannot detect inactive or incomplete forms of the enzyme and diffusion of reaction product in a region of high activity could be misinterpreted as cytoplasmic localization.

Two reports of the use of polyclonal antisera to Na,K-ATPase or to alpha subunits have resulted in findings [5–7] that yield somewhat conflicting results. In the earlier reports with such a technique, anti-holoenzyme bound to basal lateral and luminal membranes of proximal tubules whereas anti-alpha subunit stained only the basal lateral membranes and distal tubules. A complete distribution of labeling along the entire nephron is not described or quantitated [5, 6]. Using a polyclonal antibody generated against a 96 KD protein isolated from partially purified sodium potassium ATPase of toad kidney, Papermaster et al [7] demonstrated binding to the brush border of the proximal tubule as well as the basal lateral membranes of other tubule segments in bovine serum albumin-embedded

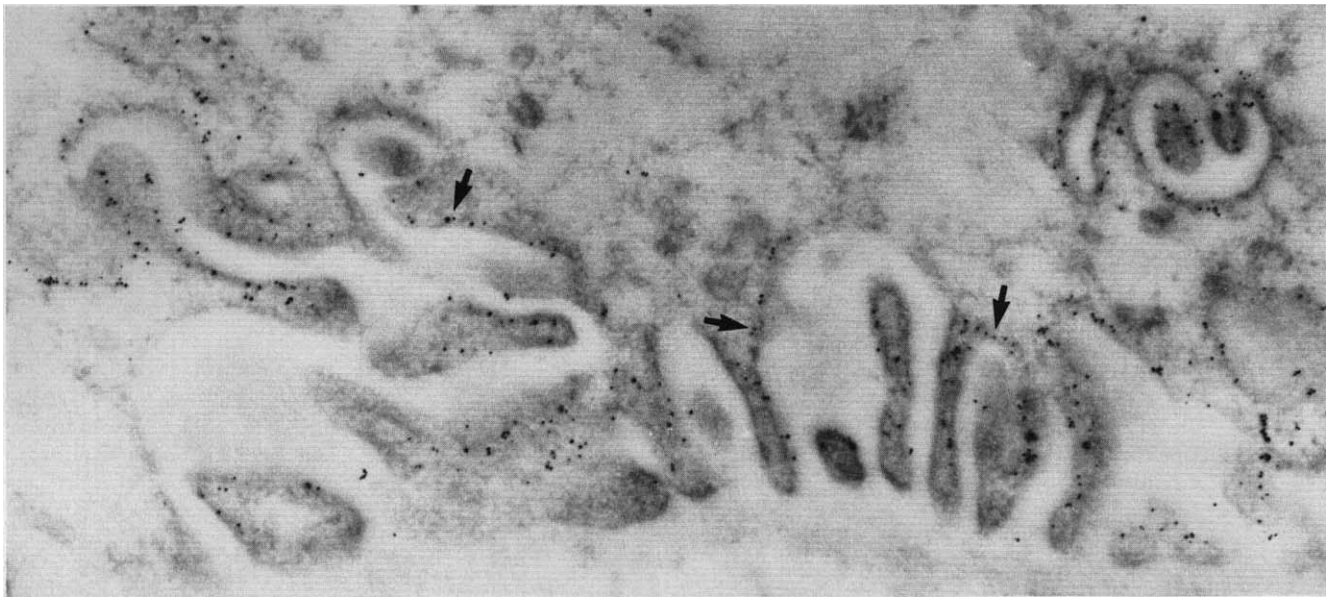

Fig. 9. *Colloidal gold labeling of the basolateral membrane of a principal cell.* The label is localized to the cytoplasmic domain of the membrane *(arrows).* ×37,000

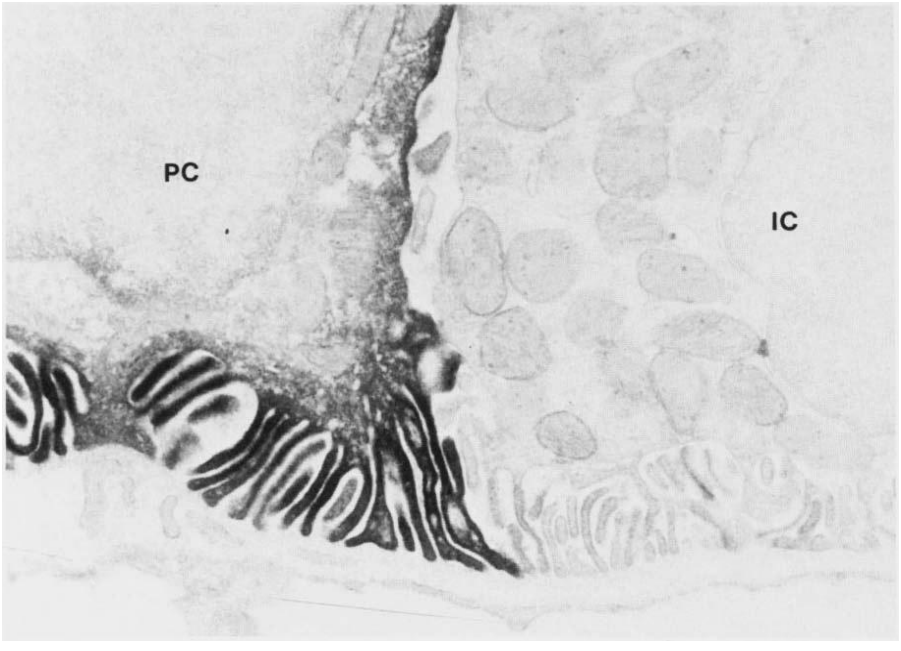

Fig. 10. *Higher magnification micrograph showing the difference in immunoperoxidase labeling between the principal cell (PC) and the intercalated cell (IC) of the cortical collecting tubule.* The cell membranes of the intercalated cell are devoid of label in contrast to the dense labeling of the basal infoldings of the principal cell. 18,600

cryosections. In contrast, in the present study using the monoclonal antibody C62.4, specific binding was limited to the basal lateral membrane surfaces. No luminal staining was seen in any tubular segments. Similarly, exclusively basolateral localization was also observed by Garvel et al [10] with a monoclonal antibody to mouse brain Na,K-ATPase, but this was not described in detail. While the possibility that a luminal membrane protein may share a common domain with Na,K ATPase cannot be absolutely eliminated, it seems likely from our findings that luminal staining seen with polyvalent antisera is the result of the presence of antibodies to a contaminating protein which co-migrates on SDS gels with the alpha subunit.

There was no definite binding of C62.4 to intracellular structures other than the medial cisternae of the golgi complex. Distinct compartmentalization of the golgi has been shown in several studies [33, 34] and has been interpreted as being related to processing of oligosaccharides and terminal glycosylation of proteins. Preliminary results of biochemical experiments (CAPLAN MJ, PALADE GE and JAMIESON JD, manuscript in preparation) provide kinetic evidence for the presence of intracellular pools of Na,K-ATPase, presumably in the cell's post-synthetic processing pathway. The reaction product observed

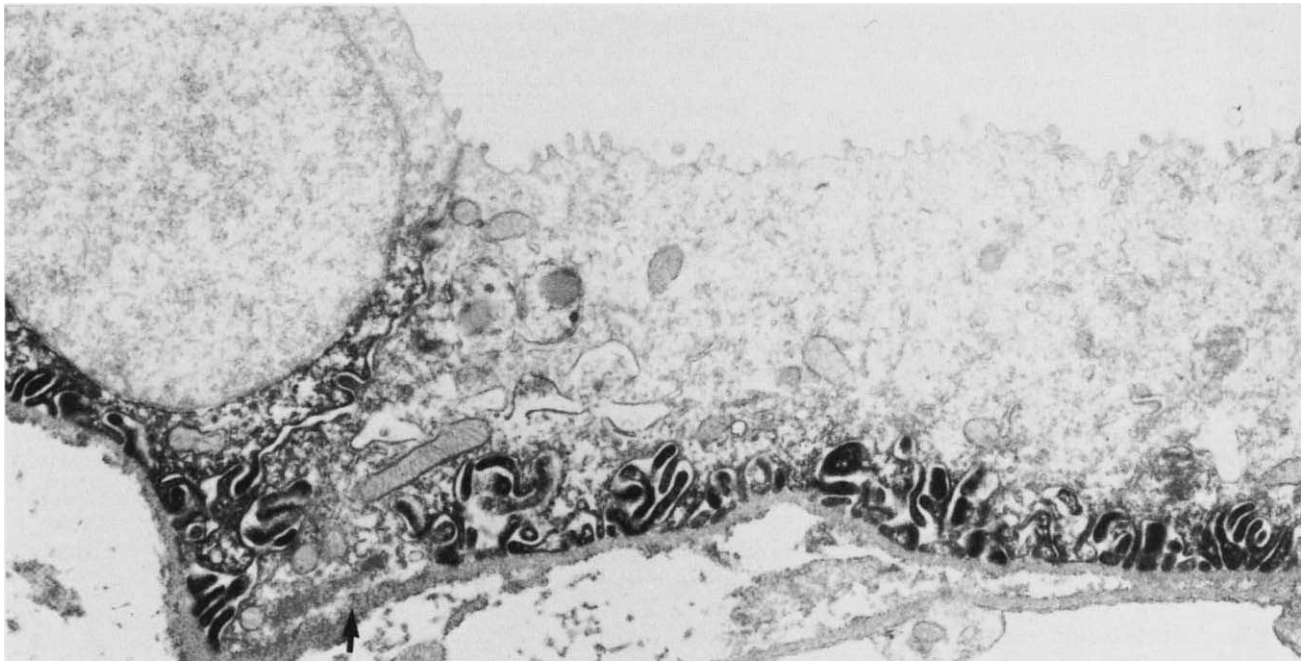

Fig. 11. *Papillary collecting duct.* The reaction product is concentrated in basal infoldings. Note that "flat" areas of basal membrane lack reaction product *(arrow).* Abbreviation: C, capillary ×9,000

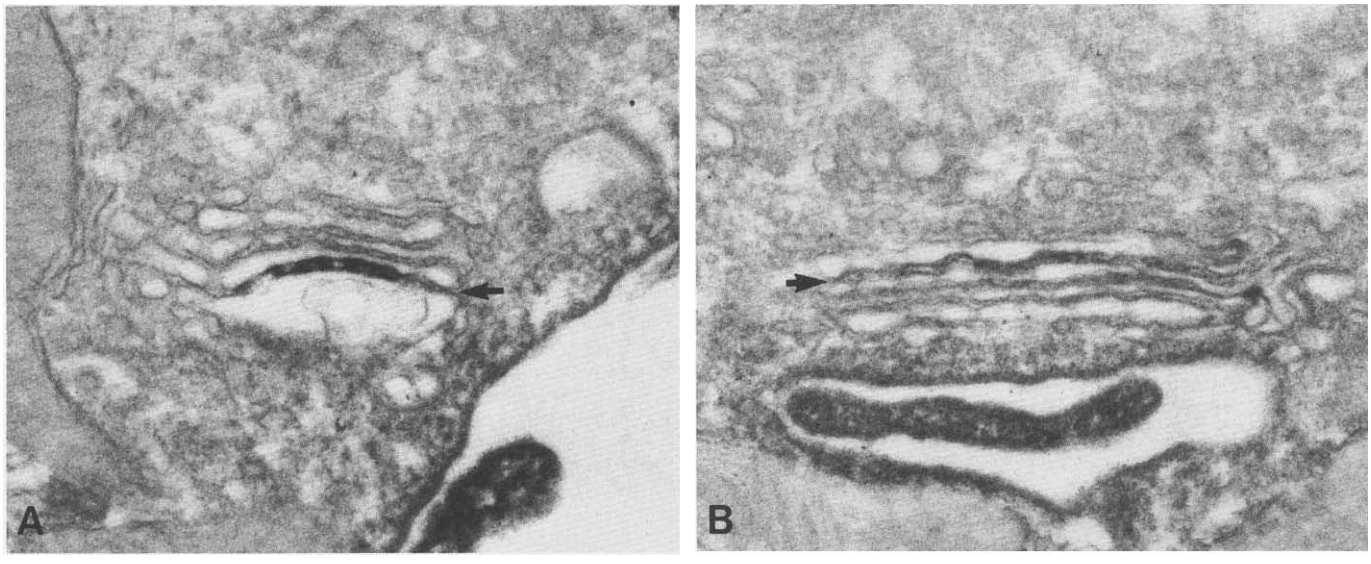

Fig. 12. *Immunoperoxidase labeling of the Golgi apparatus of cells of the thick ascending limb.* Reaction product is limited to the space between the medial cisternae *(arrows).* No other intracellular membranes had demonstrable labeling. ×52,000

in the golgi apparatus may represent the morphological correlate of such pools. Research is under way to quantify and further characterize this apparent concentration of sodium pump in the golgi apparatus and to determine whether it does indeed represent this postulated internal population and how it is transported to the plasmalemma.

The distribution of the enzyme as detected by the monoclonal antibody corresponds well to the functional heterogeneity of the tubular segments as well as to measurements of the activity of Na,K-ATPase in isolated nephron segments of mammalian kidneys [35, 36]. Na,K-ATPase is undoubtedly responsible for generating the driving force for sodium and fluid readsorption and indirectly for the co-transport of amino acids, glucose, and phosphate and the counter transport of protons. Although studies of isolated tubule segments have shown that the activity of Na,K-ATPase declines along the length of the proximal tubule, this was not readily detected by immunofluorescence or ultrastructural localization since the change in activity probably is related to the decrease in the basal lateral membrane surface area rather than to a change in the density of the enzyme per

unit membrane area. The greatest density of labeling by both the immunofluorescence and ultrastructural cytochemical methods used here was in the thick ascending limb of Henle's loop. Both in terms of enzyme activity per gram of tissue and per cm of tubule, the amount of Na,K-ATPase is higher in the medullary thick ascending limb than in the proximal tubule or more distal segments of the nephron [35, 36]. The relative density of immunofluorescence and immunoperoxidase staining observed here corresponds to these differences and is consistent with the rates of transport in this segment.

There has been considerable debate as to whether or not active reabsorption of sodium chloride in the ascending thin limb of Henle's loop contributes to the generation of this medullary hypertonicity. Cytochemical techniques using potassium-dependent nitrophenylphosphatase have demonstrated that descending thin limbs from short loop nephrons in the outer medulla were sites of moderate activity, whereas descending thin limbs and ascending thin limbs of long loop nephrons were unreactive in the rat inner medulla [37]. In our studies, although the enzyme could be detected only in small amounts with labeled antibody, the density was approximately the same in both ascending and descending portions of the thin limb, but was readily detectable in the terminal portion of the ascending thin limb just prior to the transition to the thick limb. Thus, while it is unlikely that the ascending thin limb plays a major role in the generation of the medullary gradient, the presence of detectable activity in the terminal portion suggests that it may make some contribution.

One of the most interesting immunocytochemical findings was the pattern of the immunolabeling in the cortical and outer medullary collecting ducts. The principal cells showed labeling of the basal lateral infoldings, while minimal or no labeling was seen in the intercalated cells. This difference undoubtedly relates to differences in functions of these two cell populations. Important transport functions of the collecting duct include transtubular reabsorption of sodium and potassium across a steep concentration gradient, secretion of potassium, and secretion of hydrogen ion. The principal cells appear to be responsible for sodium reabsorption and potassium secretion and appear to respond to potassium adaptation and mineralocorticoids with an increase in basal lateral membrane surface area and an increase in Na,K-ATPase activity [23]. The intercalated cell, on the other hand, has been demonstrated to respond to changes in acid base balance and potassium depletion by altering luminal membrane surface area [38, 39]. The relative absence of Na,K-ATPase in the intercalated cell suggests that this cell is not significantly involved in the transepithelial movement of sodium and that its principal function may be limited to regulation of proton transport and potassium absorption perhaps via potassium-hydrogen exchange.

While accurate quantitation of intensity of fluorescence is difficult, an apparent increase in immunofluorescence was seen along the length of the papillary collecting duct as it approached the papilla. Since the basal lateral membrane of the papillary collecting duct does not have complex infoldings, the increase in fluorescence intensity may be due to an increase in the density of the enzyme, which correlates with the increasing concentration gradient for sodium along the papillary collecting duct.

Another interesting observation is the uneven labeling of the basal lateral membrane most prominent in the proximal S3 segment, collecting tubule principal cells, and papillary collecting duct cells. The flat basal regions where the plasmalemma was directly opposed to the basal lamina had less binding of C62.4 when compared to the adjacent invaginated plasmalemma. While technical artifacts must be considered, the possibility that Na,K-ATPase may be sorted into microdomains of the cell membrane is an intriguing one. The sorting could be the result of interaction of transmembrane proteins with the extracellular matrix or to the transport activity of that local membrane region.

Monoclonal antibodies provide a highly specific probe for the study of Na,K-ATPase along the nephron. The distribution found with this probe corresponds well with physiologic studies of transepithelial ion movement and biochemical analysis of Na,K-ATPase activity. The use of such a highly specific marker in adapted and adapting states should give better insights into the mechanisms involved in the transepithelial movement of ions as well as possible biosynthetic mechanisms of adaptation.

Acknowledgments

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