

## Subcellular distribution of tissue kallikrein and Na,K-ATPase $\alpha$ -subunit in rat parotid striated duct cells

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**Abstract.** Intracellular protein distribution and sorting were examined in rat parotid striated duct cells, in which tissue kallikrein is apical, and Na,K-ATPase is basolateral. Electron-microscopic immunogold cytochemistry, with both polyclonal and monoclonal antibodies, demonstrated these enzymes at opposite poles of the cells and in distinct intracellular sites. Kallikrein was found within apical secretory granules, whereas Na,K-ATPase was present on basolateral cell membranes. In addition, kallikrein was localized throughout cisternae of all Golgi profiles, whereas Na,K-ATPase ( $\alpha$ -subunit) was found only in small peripheral vesicles and/or lateral cisternal extensions of a basal subset of Golgi profiles. These differences in the subcellular distribution of the two marker antigens were most clearly seen with double immunogold labelling. Our results suggest that kallikrein, an apical, regulated secretory protein, and Na,K-ATPase, a basolateral, constitutively transported membrane protein, are segregated at (or prior to) the level of the Golgi apparatus rather than in the trans-Golgi network (TGN), as was expected.

**Key words:** Kallikrein – Adenosine triphosphatase, sodium, potassium – Secretion – Intracellular membranes – Golgi apparatus – Parotid – Transport – Rat (Sprague-Dawley, Wistar)

### Introduction

Most proteins destined for export from eukaryotic cells are segregated from cytosolic proteins early in processing, by sequestration in an internal membrane system

*Abbreviations:* ATP adenosine tri-phosphate; HBSS Hanks' balanced salt solution; GaM goat anti-mouse; GaR goat anti-rabbit; PBS phosphate-buffered saline; RaM rabbit anti-mouse; RER rough endoplasmic reticulum; TGN trans-Golgi network

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consisting of rough endoplasmic reticulum (RER), Golgi membranes, and a variety of vesicles, vacuoles and granules, traversed sequentially (Palade 1975). At least two pathways exist for the transit of proteins to the cell periphery: 1) a regulated pathway, for secretory material stored in granules and discharged in response to specific stimuli, and 2) a constitutive pathway, for macromolecules which leave the system without selective retardation (Burgess and Kelly 1987; Farquhar 1991). Most components of the plasma membrane apparently follow the constitutive pathway (Caplan and Matlin 1989). Currently under intense investigation are the mechanisms underlying this sorting and traffic among the various intracellular membrane compartments (Bartles and Hubbard 1988; Mostov et al. 1992).

Polarized epithelial cells exhibit much less cell surface membrane fluidity than nonpolarized cells, and most membrane proteins studied so far are non-randomly distributed on either the apical or the basolateral epithelial cell surface (Rodriguez-Boulant and Sabatini 1978; Farquhar 1991; Mostov et al. 1992). The trans-Golgi network (TGN) of membranes has emerged as a likely site of sorting for most proteins that enter the internal membrane system of the cell (Griffiths and Simons 1986).

We have investigated this protein sorting pathway in parotid striated duct cells using electron microscopic immunogold labelling of two proteins that sort to opposite poles of the cells: tissue kallikrein and Na,K-ATPase. Tissue kallikrein is one member of a family of serine proteases found in salivary glands, kidneys, and several other tissues (Clements 1989). Among other actions, kallikrein has been implicated in ion transport processes, perhaps by means of local kinin production (Bhoola et al. 1992). Na,K-ATPase is an evolutionarily conserved ion transport enzyme, found in most animal cell membranes as a complex consisting of separately translated and processed  $\alpha$ - and  $\beta$ -subunits. Although the  $\alpha$ -subunit appears to carry out all enzyme functions and to be the target of inhibitors, functional cell surface expression of the enzyme appears to require association of the  $\alpha$ - and  $\beta$ -subunits (Geering 1990).

In the present study, we have confirmed our previous observations on the localization of tissue kallikrein in small, apical secretory granules of parotid striated ducts (Simson et al. 1983) and of Na,K-ATPase along invaginated basolateral cell membranes (Graves et al. 1989). Using double-label immunogold cytochemistry, we have found that kallikrein (an apical regulated secretory protein) and Na,K-ATPase (a basolateral integral membrane protein) are segregated at the level of the Golgi apparatus within these cells. These findings have been presented in abstract (Simson 1989).

## Materials and methods

### Tissue preparation

Parotid glands of rats (Sprague-Dawley and Wistar strains) were fixed by cardiac perfusion. The fixative consisted of 2% paraformaldehyde, 1% glutaraldehyde in phosphate-buffered saline (PBS) at room temperature or 3% paraformaldehyde combined with either 0.1% or 0.5% glutaraldehyde in prewarmed (37°C), oxygenated Hanks' buffered salt solution (HBSS). The latter fixative was used for most of the samples illustrated. A brief (1–2 min) rinse with buffer (PBS or HBSS) preceded administration of the fixative. Some tissues were fixed by drip fixation for purposes of comparison with perfusion-fixed material. Following the fixative perfusion (5–10 min) parotid glands were excised, minced into fine slivers (< 0.5 mm), and placed in additional fixative for 30 min to 1 h. Tissues were rinsed in buffer, then transferred to 50 mM NH<sub>4</sub>Cl in buffer for 30 min, dehydrated in a graded series of alcohols at decreasing temperatures, and embedded in Lowicryl at –40°C (Roth et al. 1981). Tissues fixed with 2% paraformaldehyde, 1% glutaraldehyde were dehydrated in dimethyl formamide and embedded in Lowicryl K4 M at –20°C.

### Immunostaining

For light-microscopic immunocytochemistry, semi-thin sections (1 µm) were cut on a Reichert-Jung Ultracut ultramicrotome and transferred to polylysine-coated glass slides. These were screened by an immunostaining procedure by use of protein A-gold with silver amplification (Taatjes et al. 1987). The advantage of initial screening with this method at the light-microscopic level is that the same concentrations of primary antibody could then be used on thin sections for electron microscopy. Following the initial screening, thin sections (80–100 nm) were cut from the same block face for electron-microscopic immunocytochemistry and picked up on nickel grids that had been previously coated with parlodion and carbon. Thin sections were allowed to dry overnight before immunostaining.

### Antibodies

The primary antibodies used in the present study were: (1) rabbit polyclonal anti-rat tissue kallikrein, affinity-purified (Chao et al. 1983), (2) an immunoglobulin fraction obtained from rabbit antiserum against toad (*Bufo marinus*) Na,K-ATPase  $\alpha$ -subunit (Girardet et al. 1981), and (3) a mouse monoclonal anti-rat tissue kallikrein antibody (V4D11), characterized previously (Chao and Chao 1987), kallikrein affinity-purified from an Ig fraction of ascitic fluid.

The primary antibodies used in this study have been well characterized (see references cited above), and have been used in previous studies from our laboratories (Chao et al. 1983; Graves et al.

1989; Simson et al. 1992). The polyclonal anti-kallikrein antiserum was kallikrein-affinity purified; it does, however, cross-react with other kallikrein gene family members in submandibular glands. We have shown previously (Simson et al. 1992) that, of the kallikrein gene family members so far identified, parotid striated ducts contain only authentic tissue kallikrein in small, apical granules. The monoclonal anti-kallikrein antibody has been shown to bind only to tissue kallikrein (and not to other members of the kallikrein gene family) in Western blots of submandibular gland homogenates. The polyclonal antibody to the  $\alpha$ -subunit of *Bufo marinus* Na,K-ATPase reacts with the  $\alpha$ - but not the  $\beta$ -subunit of the toad enzyme (Girardet et al. 1981), and binds to conserved sequences of the mammalian enzyme, particularly in distal tubules of the kidney and in salivary gland striated ducts (see Graves et al. 1989).

Secondary antibodies (affinity-purified, Cappel-Organon Technika, West Chester, Pa) were: (1) rabbit anti-mouse immunoglobulin (RaM), used to detect the monoclonal primary antibody; (2) goat anti-mouse immunoglobulin (GaM), also used to detect the monoclonal primary antibody; and (3) goat anti-rabbit immunoglobulin (GaR), used to detect polyclonal primary antibodies.

These secondary antibodies were used to coat colloidal gold particles of different sizes (8 or 14 nm). Ig-gold complexes were employed as second step reagents for localizing primary antibodies and, in the absence of primary antibodies, as controls.

### Colloidal gold

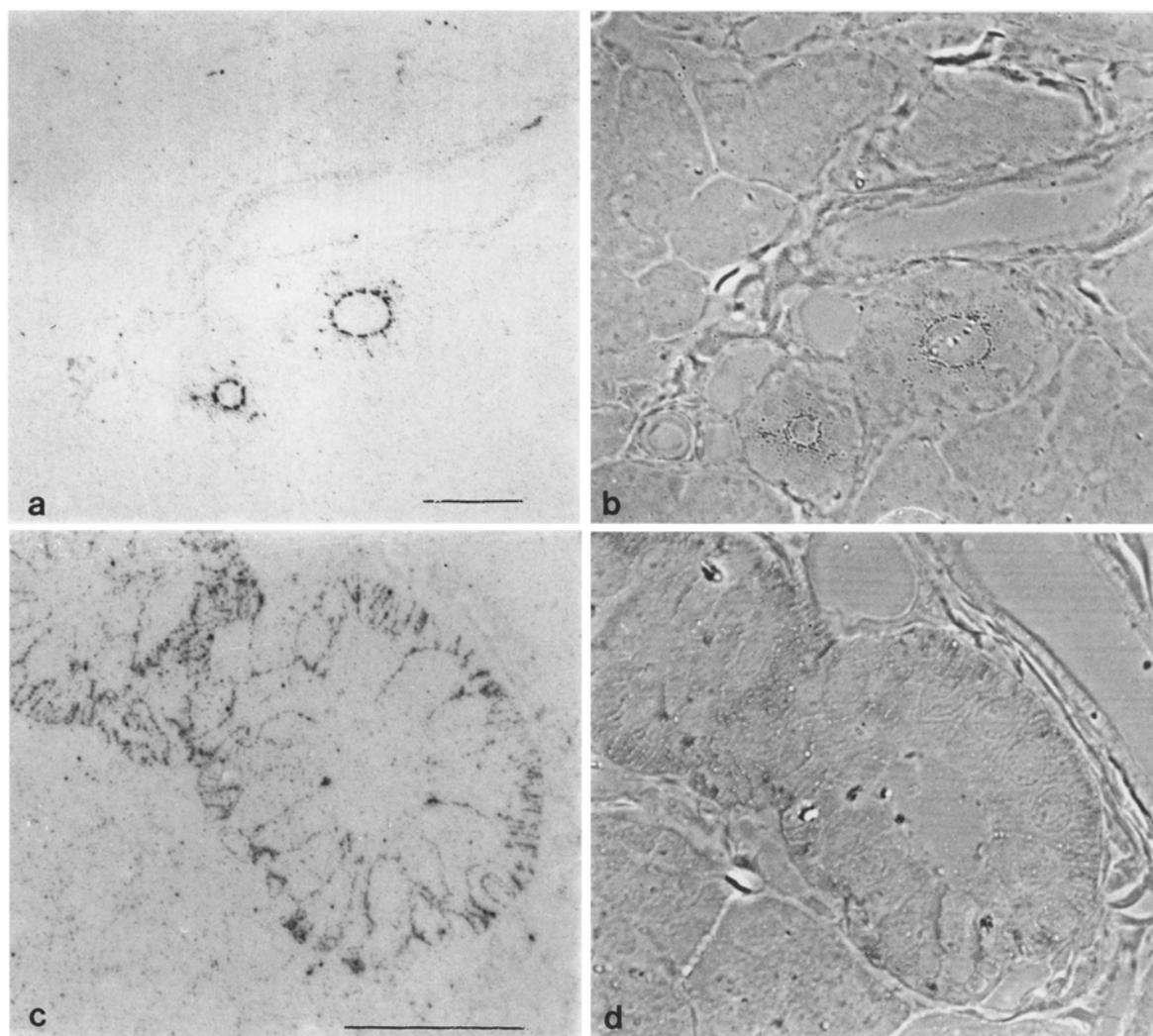
Colloidal gold particles of 14 nm were prepared with the citrate-reduction method (Frens 1973). Gold particles of 8 nm were prepared by the tannic acid/citrate reduction method (Slot and Geuze 1985). The colloidal gold particles were used within a day of preparation for coating with either protein A (Roth et al. 1978) or immunoglobulins (Lucoq and Baschong 1986). Protein A was purchased from Pharmacia (Uppsala, Sweden).

### Electron-microscopic procedures

The protein A-gold technique was basically that of Roth and co-workers (1978), with an initial blocking step with either 0.5% bovine serum albumin (BSA) or 0.5% ovalbumin. Either 14 nm or 8 nm gold, coated with protein A, was used in the second step of the procedure. Grids were rinsed thoroughly with PBS between incubation steps by a combination of jet washing and passing through PBS-containing wells. In single-label studies only a single primary antibody was used as the first step of the procedure. The ranges of antibody dilutions used were: rabbit anti-kallikrein, 1:100–1:500; rabbit anti-Na,K-ATPase, 1:50–1:200; V4D11, 1:50–1:100. The primary antibody was followed (after thorough rinsing in PBS) either with protein A-gold or with colloidal gold coated with an appropriate secondary antibody (RaM, GaM, or GaR). In incubations with the monoclonal antibody (V4D11) as the primary reagent followed by protein A-gold, RaM was usually used as an intermediate step to provide better and more consistent binding of the protein A-gold. Double-label incubations were performed with primary antibodies originating in different species: e.g., polyclonal (rabbit) anti-Na,K-ATPase with monoclonal (mouse) V4D11 (anti-kallikrein), mixed at appropriate dilutions in the first incubation step, followed by a mixture of GaR-gold of one size and GaM-gold of another size, as described previously (Simson et al. 1989). The protein A-gold or antibody-gold suspensions were used at OD<sub>525</sub> of 0.1–0.5 for 14 nm gold and 0.02–0.1 for 8 nm gold.

### Controls

Several control procedures were included. Some sections were incubated with RaM alone followed by protein A-gold or with a combi-



**Fig. 1a-d.** Semi-thin Lowicryl K4 M sections of rat parotid gland stained with antibody + protein A-gold followed by silver enhancement. In **a, b**: V4D11, 1:200 dilution. **a** Bright-field illumination: immunostained apical rim of striated duct cells and absence of staining in surrounding acinar tissue. **b** Phase-contrast illumination: acinar tissue and blood vessels above striated ducts. In **c, d**:

rabbit anti-Na,K-ATPase, 1:100. **c** Bright-field illumination showing immunostaining primarily along basolateral invaginated membranes of striated duct cells. Apical membranes not stained. Note fine meshwork of staining along acinar cell membranes (*lower left*). **d** Phase-contrast image. Bars: 20  $\mu$ m. **a, b**  $\times$  1300; **c, d**  $\times$  2600

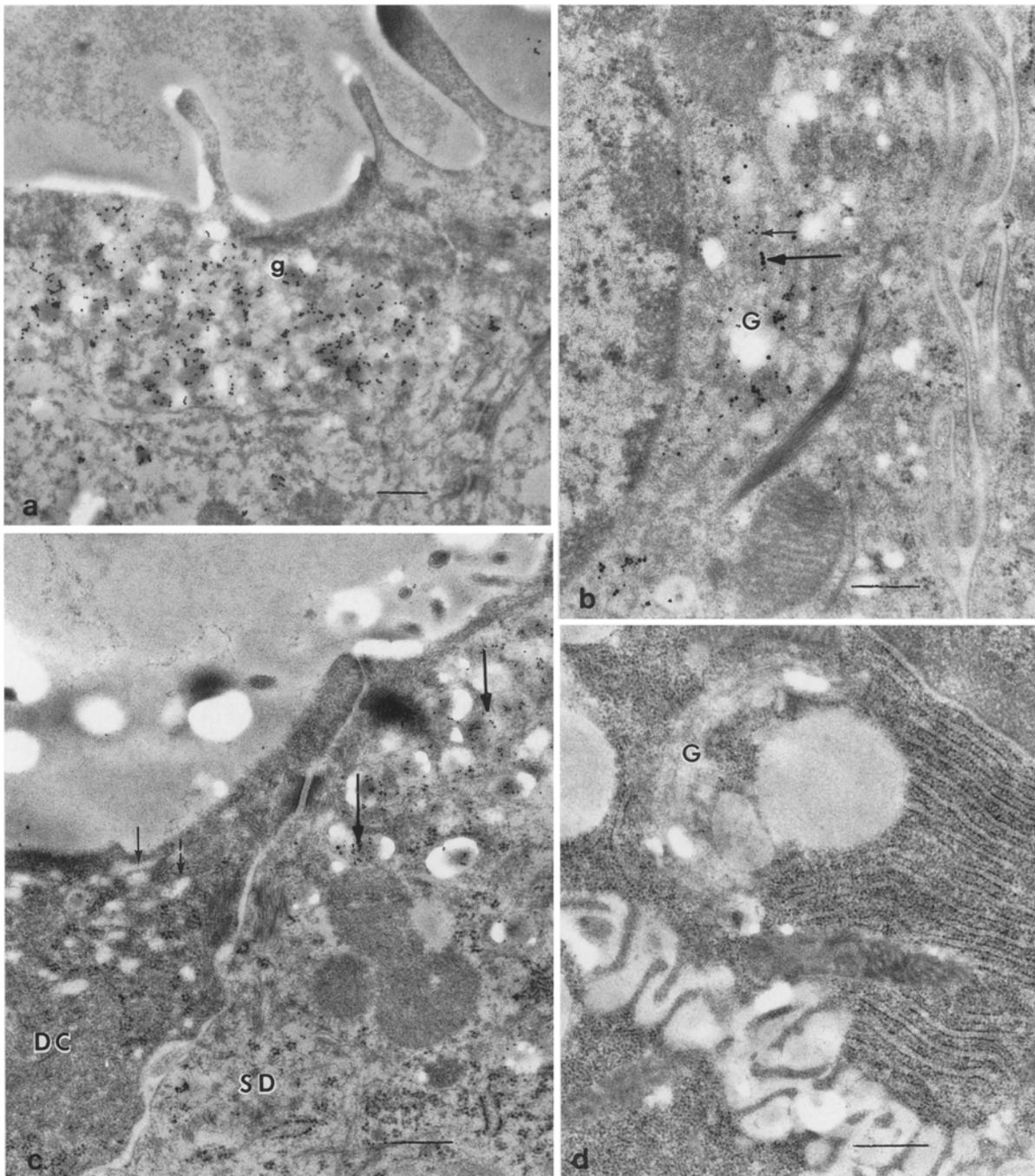
nation of GaR-gold and GaM-gold. Some sections were incubated with protein A-gold or antibody-gold alone after the initial BSA or ovalbumin blocking step. In some experiments only a single antibody was used in the initial incubation step, but both antibody-gold complexes (GaR-gold + GaM-gold) were used in the second step of the procedure. This provided a control for possible cross-reactivity of the secondary antibody-gold with an inappropriate primary antibody. After immunostaining, sections were rinsed twice each with PBS and double distilled water and allowed to dry overnight. They were then stained with uranyl acetate and lead acetate prior to viewing with a Zeiss EM 10 electron microscope.

Micrographs of all observed Golgi profiles (total = 85) were analyzed; simple percentages were calculated in terms of position in the cell and immunostaining with antibodies against kallikrein and/or Na,K-ATPase.

## Results

### *Cellular localization: light microscopy*

Staining of semi-thin sections of parotid glands with antibodies to either kallikrein or Na,K-ATPase, followed by protein A-gold and silver amplification, confirmed the segregation of these enzymes in striated duct cells to the apical and basal poles, respectively (Fig. 1). Kallikrein immunoreactivity was present as a rim at the apex of striated duct cells in the zone known to contain small secretory vesicles. In addition, perinuclear staining was detected with anti-kallikrein antibodies, suggesting localization in the Golgi apparatus. Antibody to Na,K-ATPase stained basal striations and the lateral cell surface to the cell junctions, but did not stain the apical surface or any obvious apical structures.



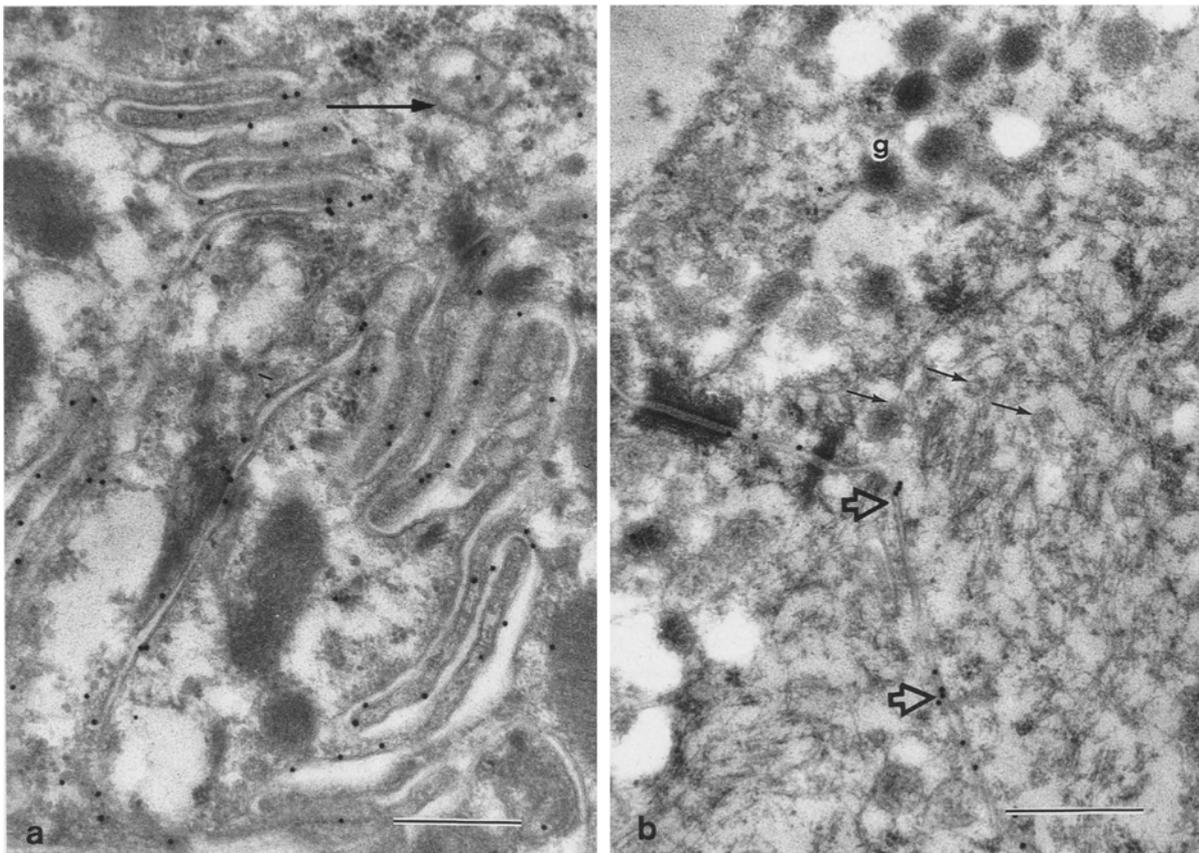
**Fig. 2a-d.** Immuno-gold staining for kallikrein. **a** V4D11, 1:200; GaM-gold, 14 nm. Apical region of striated duct, showing specific staining of small apical granules (g). **b** Lateral Golgi stack (G), double stained with monoclonal (V4D11, 1:50) and polyclonal (rabbit, 1:200) antibodies to kallikrein; secondary antibodies: GaM-gold, 14 nm + GaR-gold, 8 nm. No difference in distribution of large and small gold particles (*long and short arrows*). **c** Polyclonal anti-

kallikrein, 1:200, followed by GaR-gold, 8 nm + GaM-gold, 14 nm, secondary antibodies. With this sequence, smaller gold particles label granules (*large arrows*) in typical striated duct cell (SD). Nearby cell with darker cytoplasm (DC) contains small, unstained apical vesicles (*small arrows*). **d** Acinar cell, same sequence as **c**; no staining over Golgi apparatus (G). Bars: 0.5  $\mu$ m. **a**  $\times$  18 000; **b-d**  $\times$  23 000

#### *Intracellular localization: electron microscopy*

**Kallikrein.** Immunogold staining for kallikrein at the ultrastructural level extended the light-microscopic observations and was consistent from experiment to experiment, regardless of the primary antibody used (rabbit anti-kallikrein or V4D11) or of the secondary colloidal

gold marker (protein A-gold, GaR-gold or GaM-gold). The major sites of kallikrein localization in striated duct cells were apical granules (Fig. 2a) and cisternae of Golgi stacks (Fig. 2b). Occasional cells, exhibiting no staining over apical vesicles or Golgi stacks, were found adjacent to cells with stained granules. (Fig. 2c, cell on left). These probably represented "dark cells", or "pillar cells," a cell



**Fig. 3a,b.** Immuno-gold staining in striated ducts with rabbit antibody to  $\alpha$ -subunit of Na,K-ATPase, 1:50, followed by protein A-gold, 14 nm. **a** Gold particles distributed along invaginated basolateral plasma membranes. Multivesicular body (*arrow*) gold labelled.

**b** Lateral border between striated duct cells; labelling of lateral membranes (*large, open arrows*). Apical granules (*g*) and coated vesicles (*small arrows*) unlabelled. Bars: 0.5  $\mu$ m.  $\times$  37 000

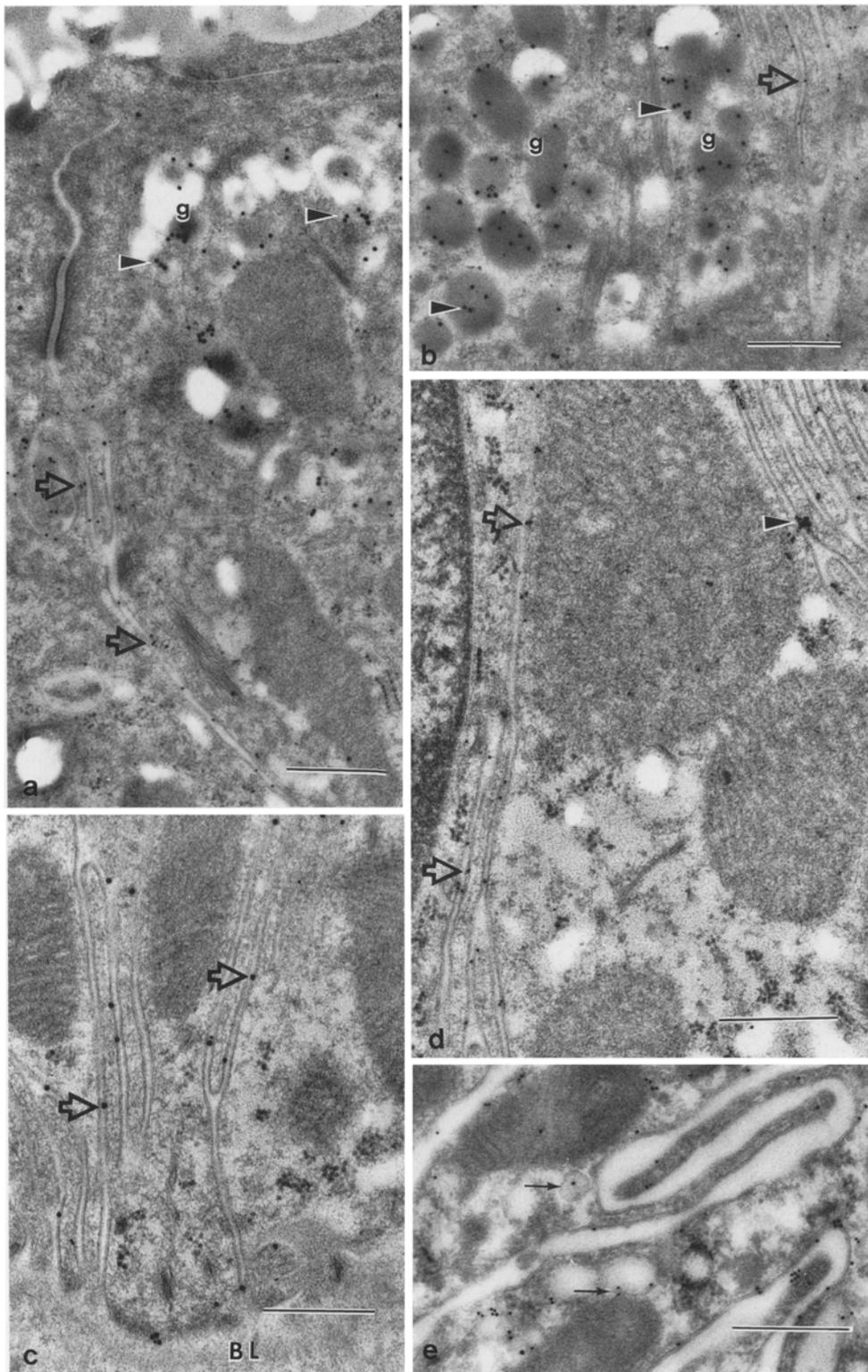
type found in many transporting epithelia, whose existence in salivary gland ducts has been questioned (see Pinkstaff 1980; Hazen-Martin and Simson 1986).

In typical striated duct cells, Golgi stacks were perinuclear, usually between the nucleus and lateral plasma membrane, but sometimes apical or basal to the nucleus. All such Golgi profiles in striated duct cells stained for kallikrein, but no staining was seen in acinar cell Golgi profiles (Fig. 2d). When both polyclonal and monoclonal antibodies against kallikrein were mixed in the first immunostaining step, followed by a mixture of appropriate secondary antibodies coated onto different sizes of colloidal gold, the two particle sizes were identically distributed (Fig. 2b), indicating that the epitopes recognized by the two antibodies to kallikrein were present in the same subcellular compartments. Gold particles were occasionally seen over short profiles of RER; this labelling was low compared with the labelling of secretory granules and Golgi cisternae. Occasional gold particles were present along the outer surface of the apical plasma membrane and in the lumen; this staining probably indicated secreted kallikrein. Fixation with high concentration glutaraldehyde (1%) resulted in markedly reduced immunostaining for kallikrein with both antibodies.

**Na,K-ATPase  $\alpha$ -subunit.** The rabbit polyclonal antibodies against the  $\alpha$ -subunit of *Bufo marinus* Na,K-ATPase

provided excellent immunostaining of rat striated ducts at the electron microscopic level. Gold particles were localized along the highly invaginated basolateral plasma membranes of striated duct cells (Fig. 3a). The straight (non-invaginated) lateral plasma membranes between the upper poles of these cells were also well labelled (Fig. 3b), but no staining was seen apical to the junctional complex (Figs. 3b,4a). Less intense staining was also present along the basal and lateral plasma membranes of acinar cells (not shown), but not on membranes lining either the lumen or intercellular canaliculus. Immunostaining for Na,K-ATPase was not reduced in high concentration glutaraldehyde-fixed tissues.

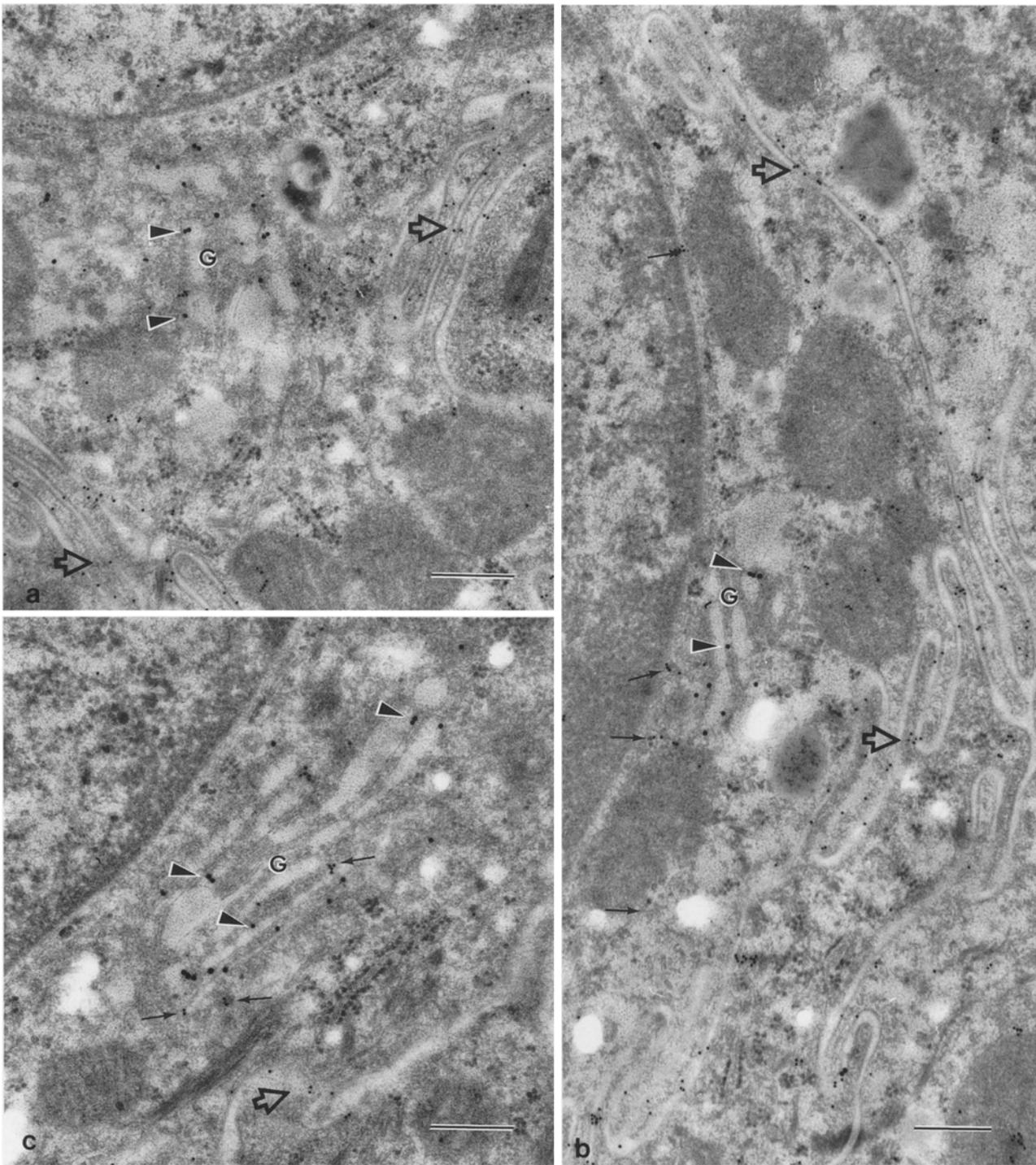
**Double immunogold labelling.** When a mixture of mouse monoclonal anti-kallikrein (V4D11) and rabbit polyclonal anti-Na,K-ATPase antibodies was used as the first step in the immunostaining procedure, followed by a mixture of the respective secondary antibodies (GaM and GaR) coated onto different sizes of colloidal gold particles (8 nm and 14 nm), the separate subcellular localizations of the two antigens were obvious (Figs. 4,5). Antibodies to kallikrein stained most granules and vesicles in the apical zone of striated duct cells, whereas nearby lateral plasma membranes were clearly stained for Na,K-ATPase, basal to the junctional complex (Fig. 4a,b). The basal region of the cells was stained primarily with antibody to



**Fig. 4a-e.** Double immunogold labelled striated duct cells with monoclonal antibodies to kallikrein (V4D11) and polyclonal antibodies to  $\alpha$ -subunit of Na,K-ATPase. Primary antibody dilutions: 1:50. **a,b** Secondary antibodies: GaM-gold, 14 nm + GaR-gold, 8 nm. In **a**, apical granules (*g*) are labelled with large gold particles (*arrowheads*), indicating kallikrein; lateral plasma membranes (*open arrows*) labelled with small gold particles, indicating Na,K-ATPase. Granule contents extracted because of low (0.1%) concentration of glutaraldehyde in fixative. No staining on apical plasma membrane above junctional complex. In **b**, granule (*g*) preservation improved with higher (0.5%) concentration glutaraldehyde. Immunostaining for kallikrein (*arrowheads*) still seen (unlike after 1% glutaraldehyde). Lateral membranes labelled with small gold particles (*open arrows*) showing Na,K-ATPase. **c** Secondary antibodies: GaM-gold, 8 nm and GaR-gold, 14 nm. In this case, large gold particles indicate Na,K-ATPase. Basolateral field shows labelling for Na,K-ATPase (*open arrows*) along invaginated membranes; membrane in contact with basal lamina (*BL*) poorly labelled. **d,e** Secondary antibodies: GaM-gold (14 nm) + GaR-gold (8 nm). In **d**, plasma membrane well labelled with small gold particles (*open arrows*), showing Na,K-ATPase. One cluster of large particles (*arrowhead*), presumably represents non-specific binding. In **e**, vesicles labelled for Na,K-ATPase with small gold particles (*small arrows*) near basolateral membranes (1% glutaraldehyde-containing fixative). *Bars*: 0.5  $\mu$ m. **a,b**  $\times$  28 000; **c-e**  $\times$  34 400

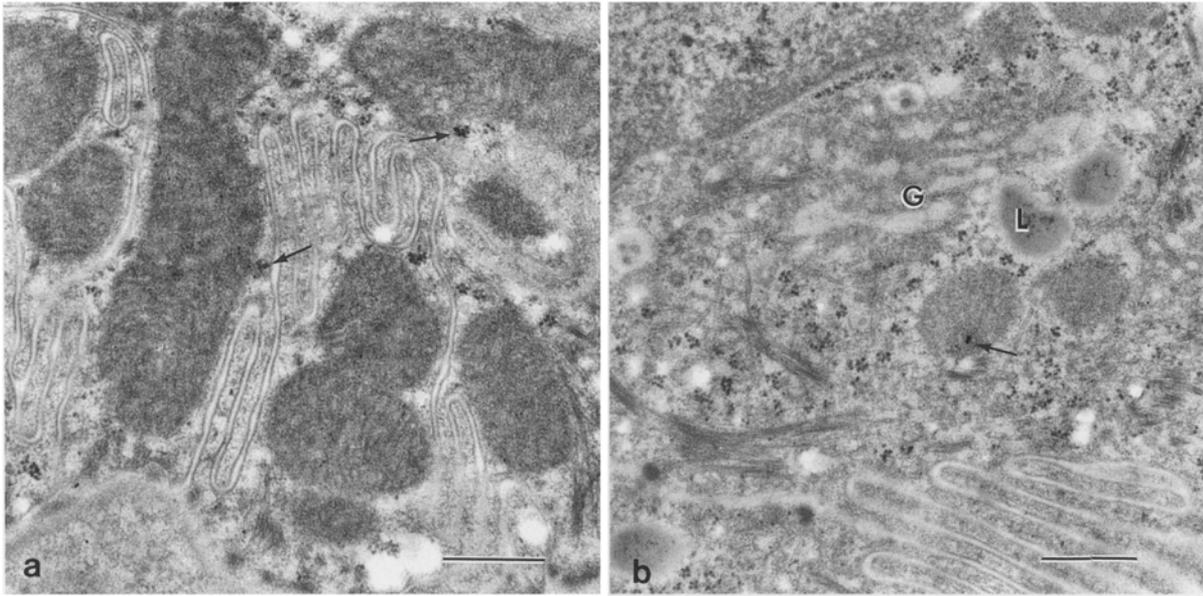
Na,K-ATPase (Fig. 4c-e) along invaginated basolateral plasma membranes; the membranes in contact with the basal lamina itself exhibited little staining (Fig. 4c). Vesicles associated with basolateral membranes were usually stained for Na,K-ATPase (Fig. 4e). Golgi stacks in the vicinity of these membranes, by contrast, stained primarily for kallikrein (Fig. 5a).

The distribution of staining in the Golgi apparatus of striated duct cells was examined in detail, since this organelle, particularly the TGN, was the anticipated site of sorting of the two antigens of interest. In these cells, the Golgi apparatus consisted of several small perinuclear stacks of cisternae (2-5 cisternae per stack), most of which (60%) were found lateral to the nucleus, in the



**Fig. 5a-c.** Golgi stacks in double immuno-gold labelled striated duct cells. Primary antibodies: monoclonal anti-kallikrein (V4D11) and polyclonal anti- $\alpha$ -subunit of Na,K-ATPase, dilutions: 1:50. Secondary antibodies: GaM-gold, 14 nm + GaR-gold, 8 nm. **a** Basal Golgi stack (G) labelled primarily with large gold particles (*arrowheads*) for kallikrein; nearby basolateral membranes (*open arrow*) labelled with small gold particles for Na,K-ATPase. **b** Lateral Golgi stack (G): Golgi cisternal membranes labelled throughout

with large gold particles (*arrowheads*) indicating kallikrein; plasma membrane labelled with small gold particles for Na,K-ATPase (*open arrows*). Few small gold particles associated with nuclear envelope (*small arrows*). **c** Lateral Golgi stack (G); majority of cisternae labelled for kallikrein (*arrowheads*), but some labelling for Na,K-ATPase seen on lateral cisternal extensions and in vesicles of peripheral Golgi field (*small arrows*). Lateral cell membrane (*clear arrow*) cut tangentially (lower right). Bars: 0.5  $\mu$ m.  $\times$  27 000



**Fig. 6a,b.** Control sections. RaM (1:50) used in place of specific primary antibody. **a** Secondary antibodies: GaM-gold, 14 nm + GaR-gold, 8 nm. Almost no staining of cellular structures. Gold particles near mitochondria (*small arrows*) may indicate non-specific

binding. **b** Secondary antibodies: GaM-gold, 8 nm and GaR-gold, 14 nm. Basal Golgi apparatus (G), shows no staining of cisternae or associated structures, including nearby lysosomes (L). One gold particle over mitochondrion (*arrow*). Bars: 0.5  $\mu$ m.  $\times$  27 000

narrow corridor of cytoplasm connecting the basal and apical regions of the cell (Fig. 5b). Some Golgi stacks (25%) were basal to the nucleus and a few (15%) were apical. In double immunostained sections, all Golgi stacks, regardless of their position, exhibited staining for kallikrein. Some (about 1/3 of the profiles seen) stained with the antibody to Na,K-ATPase in the peripheral Golgi field, either in small, smooth, Golgi-associated vesicles or in lateral cisternal extensions. Coated vesicles were frequently found in the vicinity of the Golgi apparatus but did not stain for either kallikrein or Na,K-ATPase. Multivesicular bodies usually stained for Na,K-ATPase (Fig. 3a), and sometimes for kallikrein as well (not shown). Some staining with antibody to Na,K-ATPase was associated with the nuclear envelope (Fig. 5b).

**Controls.** When either normal rabbit serum or RaM was substituted for the primary antibody, essentially no staining was seen in striated duct cells (Fig. 6a,b). A few particles were present over mitochondria, in apparently non-specific, antibody binding. This nonspecific mitochondrial staining was rarely seen in acinar cells, however, which exhibited very low background staining (not shown, but see Fig. 2d). When a single primary antibody was followed by a mixture of two secondary antibodies coated onto different sizes of colloidal gold (as in Fig. 2c), only one size gold particle was observed, reflecting the distribution of the primary antibody.

## Discussion

The two proteins, whose subcellular distributions have been analyzed in the present study, are normal endogenous constituents of parotid striated duct cells. In this cell

type, tissue kallikrein has been shown previously to be localized apically (Simson et al. 1979, 1983), whereas Na,K-ATPase is basal (Graves et al. 1989). Mechanisms for establishing the polarity of these proteins must depend upon differences in molecular signals that interact with the subcellular sorting machinery. The two molecules examined in this study differ in several important respects. Kallikrein is a secreted protein (Simson et al. 1979), whereas Na,K-ATPase is an integral membrane protein. Kallikrein is hydrophilic and contains carbohydrate side groups (Fiedler 1979), whereas the  $\alpha$ -subunit of Na,K-ATPase has several hydrophobic domains but no carbohydrates (Shull et al. 1985; Tamkun and Fambrough 1986). Nonetheless, secretory and membrane proteins are considered to follow a common intracellular pathway through the Golgi apparatus, to be sorted in the TGN (Griffiths and Simons 1986; Caplan and Matlin 1989; Farquhar 1991).

In the present study, the pattern of immunostaining for kallikrein indicates that it follows the classical pathway for secretory proteins established by Palade and co-workers for pancreatic zymogens (see Palade 1975). This exocrine pathway, well characterized for a wide variety of secretory proteins (Farquhar 1991), involves synthesis in RER, transfer (via transition vesicles) to the Golgi apparatus, and storage in secretion granules that await an appropriate discharge signal. Transit from RER to storage granule normally involves modification of carbohydrates in the Golgi apparatus (Farquhar 1991) and further processing, such as proteolytic trimming, in a post-Golgi compartment, e.g., condensing vacuole or immature secretory granule (Orci et al. 1987). In the present study, although immunostaining for kallikrein in endoplasmic reticulum was weak, that in the Golgi apparatus was marked and consistent throughout the cisternae.

Hence, although the early, unprocessed form of the enzyme may be poorly recognized by the antibodies used, the form present in the Golgi apparatus exists in a conformation comparable to the form present in secretory granules.

In contrast to kallikrein, Na,K-ATPase is a basolateral, integral membrane protein and is expected to follow the constitutive secretory pathway in these cells. The constitutive pathway is generally considered to be the same as that followed by regulated secretory proteins up to the final Golgi station or TGN (Burgess and Kelly 1987; Caplan and Matlin 1989). At the TGN, the pathways are believed to diverge, with small, constitutive vesicles transported quickly (within 10–30 min) to the cell surface and discharged without awaiting a further secretory signal (Burgess and Kelly 1987). The evidence accumulated to date suggests that many normal plasma membrane proteins, as well as viral membrane and/or coat proteins, follow this latter pathway, intercalated into the membranes of small vesicles (Rodriguez-Boulan and Sabatini 1978; Strous et al. 1983; Orci et al. 1987).

Results of the present study suggest that the  $\alpha$ -subunit of Na,K-ATPase (expected to follow the constitutive pathway) is, in fact, already segregated from tissue kallikrein (which follows the regulated secretory pathway) at the level of the main Golgi stack. The Na,K-ATPase  $\alpha$ -subunit, destined for basolateral membrane insertion, appears to skirt the main Golgi cisternae and to form a peripheral (and probably transient) connection with this organelle. Differences in the intensity of labelling for the two marker proteins in the Golgi apparatus might result from differences in transit times through this organelle; however, differences in distribution would not. Several reasons can be postulated for a differential distribution of the two proteins within Golgi stacks. Kallikrein is a soluble secretory protein which could diffuse into the cisternae from transition vesicles upon fusion of the two compartments. Na,K-ATPase, by contrast, is integrated into the membrane via several transmembrane loops (Shull et al. 1985), and would diffuse less readily in the cisternal membrane plane. Moreover, Na,K-ATPase is largely bound to ankyrin, which would further limit its diffusibility in the membrane (Nelson and Veshnock 1987; Koob et al. 1988; Morrow et al. 1989). Kallikrein is a sialylated protein (Fiedler 1979) and thus must contain signals directing it to sialylation sites in Golgi cisternae. The  $\alpha$ -subunit of Na,K-ATPase, by contrast, has little or no carbohydrate (Tamkun and Fambrough 1986) and may not be directed to Golgi cisternae. If the Na,K-ATPase  $\alpha$ -subunit forms a complex with the glycosylated  $\beta$ -subunit prior to insertion into the plasma membrane, an association (perhaps transient) of the  $\alpha$ -subunit with the Golgi apparatus would allow complex formation to occur before transport to the plasma membrane.

A lack of consensus currently exists concerning subcellular sites of synthesis of the two subunits of Na,K-ATPase (cytosolic vs membrane-associated), the time course of cell surface appearance of newly synthesized enzyme (45 min vs > 4 h), and the site(s) of targeted delivery of the enzyme (exclusively basal vs random deliv-

ery with high apical turnover) (Hiatt et al. 1984; Fisher et al. 1984; Geering et al. 1985, 1987; Caplan et al. 1986, 1990; Boll et al. 1991; Hammerton et al. 1991; Mays and Nelson 1992). These studies have been performed on animals of several species, in different developmental stages, and in different tissues. While both subunits are relatively conserved in terms of amino acid composition (see Geering 1990), interpretation of the data is complicated by the fact that both subunits have tissue-specific isoforms (Fambrough and Bayne 1983; Felsenfeld and Sweadner 1988). The time course for processing of the enzyme, and perhaps even its intracellular route of traffic and targeting to the cell surface, may be quite different, for example, in *Xenopus* oocytes (Ackerman and Geering 1990) and chick sensory neurons (Tamkun and Fambrough 1986). Very little is known about the actual intracellular routes of transport of Na,K-ATPase from sites of synthesis to the plasma membrane. Differences in data and interpretation in these studies may also reflect species or tissue differences in sorting and membrane targeting of the subunits. The lack of agreement in these studies suggests that the  $\alpha$ -subunit, at least, may not follow the canonical model for membrane protein synthesis and targeting (also suggested by Caplan et al. 1986). This subunit differs from most proteins examined in cell membrane trafficking studies, in that it lacks a hydrophobic N-terminal signal peptide but possesses multiple hydrophobic domains in the primary translation product (Shull et al. 1985).

Nonetheless, the present study, in conjunction with data from other studies, may provide some insight and offer plausible interpretations of apparently contradictory data. Since our immunocytochemistry localized only the  $\alpha$ -subunit, the subcellular localization of the  $\beta$ -subunit remains conjectural; additional studies need to be performed. However, the  $\beta$ -subunit is highly glycosylated in its mature form, and we postulate that it traverses the main Golgi stacks during processing. Complete glycosylation of the  $\beta$ -subunit requires 45–90 min (or longer) to complete (Tamkun and Fambrough 1986; Ackermann and Geering 1990), whereas the  $\alpha$ -subunit apparently binds stably to the  $\beta$ -subunit within 15 min of synthesis (Tamkun and Fambrough 1986). Other evidence suggests early postsynthetic (10–20 min) stabilization and activation of the  $\alpha$ -subunit (Caplan et al. 1990; Geering 1990). This may result, at least in part, from association with the  $\beta$ -subunit. It is possible that newly synthesized  $\alpha$ -subunit binds to previously synthesized and glycosylated  $\beta$ -subunit in lateral Golgi cisternal extensions and shuttle vesicles. If this hypothesis is true, newly synthesized  $\alpha$ - and  $\beta$ -subunits might appear at the cell surface with different time courses. Complete processing of core sugars of the  $\beta$ -subunit is apparently not necessary for Na,K-ATPase activity of the enzyme complex (Geering 1990). Shuttling of vesicles between cell membrane and basolateral Golgi stacks could result in a prolonged time course of glycosylation of the  $\beta$ -subunit, and a sizeable intracellular vesicle-bound pool of the enzyme.

Another unexpected finding in the present study was that only about a third of the Golgi profiles were associated with vesicles stained for Na,K-ATPase. These were

either basal or lateral to the nucleus of striated duct cells, near membranes into which the enzyme would be inserted. In contrast, kallikrein was demonstrable in all Golgi profiles in these cells, whether apical (in the expected direction of secretory transport) or basal. This suggests that Golgi stacks of a given cell may be "regionally specialized" to subserve processing and/or traffic of a subset of molecules for export. Examples already exist of regional subcellular localization of contents in endoplasmic reticulum (Deschuyteneer et al. 1988; Ellinger and Pavelka 1992). The controversy concerning membrane delivery of Na,K-ATPase in MDCK cells, that is, whether the enzyme is delivered exclusively to the basolateral cell surface (Caplan et al. 1986, 1990; Boll et al. 1991), or randomly delivered to both apical and basal surfaces, with selective removal of apical enzyme (Hammerton et al. 1991; Mays and Nelson 1992) may reflect the fact that in one system (Hammerton et al. 1991) the MDCK cells had recently been induced to form tight junctions and achieve polarity, whereas in the other system (Caplan et al. 1986), MDCK cells had exhibited stable polarity for the duration of culture. If "regionally specialized" Golgi stacks can contribute to polarized delivery of cell-surface constituents, as suggested by observations in the present studies, then in "newly polarized" cells, the Golgi stacks may not have achieved the regional differentiation required for non-random delivery of Na,K-ATPase to the cell surface membrane.

Much of the work on cell traffic in polarized epithelial cells has taken advantage of either virally infected or genetically engineered tissue culture cells (Rodriguez-Boulan and Sabatini 1978; Strous et al. 1983; Orci et al. 1987; Tooze et al. 1987). These models have provided numerous insights into the mechanisms of intracellular traffic. It is clear, however, that tissue culture and genetic engineering are artificial systems that may result in anomalous information for unexpected reasons. In the system investigated in the present study, two endogenous cell products, targeted to separate cellular domains by means of normal subcellular sorting machinery, were localized simultaneously with double-label immunogold cytochemistry. Our results suggest that kallikrein and Na,K-ATPase are sorted prior to or during transit through the Golgi compartment, rather than in the TGN, where most secretory and membrane proteins appear to be sorted.

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