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The secretion-stimulated 80K phosphoprotein of parietal cells is ezrin, and has properties of a membrane cytoskeletal linker in the induced apical microvilli

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Stimulation of gastric acid secretion in parietal cells involves the translocation of the proton pump (H,K-ATPase) from cytoplasmic tubulovesicles to the apical membrane to form long, F-actin-containing, microvilli. Following secretion, the pump is endocytosed back into tubulovesicles. The parietal cell therefore offers a system for the study of regulated membrane recycling, with temporally separated endocytic and exocytic steps. During cAMP-mediated stimulation, an 80 kDa peripheral membrane protein becomes phosphorylated on serine residues. This protein is a major component, together with actin and the pump, of the isolated apical membrane from stimulated cells, but not the resting tubulovesicular membrane. Here we show that the gastric 80 kDa phosphoprotein is closely related or identical to ezrin, a protein whose phosphorylation on serine and tyrosine residues was recently implicated in the induction by growth factors of cell surface structures on cultured cells [Bretscher,A. (1989) *J. Cell Biol.*, 108, 921-930]. Light and electron microscopy reveal that ezrin is associated with the actin filaments of the microvilli of stimulated cells, but not with the filaments in the terminal web. In addition, a significant amount of ezrin is present in the basolateral membrane infoldings of both resting and stimulated cells. Extraction studies show that ezrin is a cytoskeletal protein in unstimulated and stimulated cells, and its association with the cytoskeleton is more stable in stimulated cells. These studies indicate that ezrin is a membrane cytoskeletal linker that may play a key role in the control of the assembly of secretory apical microvilli in parietal cells and ultimately in the regulation of acid secretion. Taken together with the earlier studies, we suggest that ezrin might be a general substrate for kinases involved in the regulation of actin-containing cell surface structures.

Key words: cytovillin/cytoskeleton/gastric acid/microvilli/parietal cells

Introduction

The mechanism by which membrane organelles fuse with and deliver their membrane proteins to the plasma membrane is poorly understood. This stems in part from the fact that in many cells exocytosis is constitutive and associated with constant endocytosis as part of the membrane recycling process. Acid secretion by the parietal cell of the stomach is a particularly amenable system for studying this problem because the exocytic process is regulated and separated temporally from the endocytic process.

In a resting or non-secreting stomach the proton pumping ATPase (H,K-ATPase) is kept from working by being sequestered in a cytoplasmic membrane compartment known as tubulovesicles (reviewed in Forte *et al.*, 1989). Tubulovesicles lack a K^+ conductance pathway, thus they are deprived of the supply of K^+ that is necessary for the H^+ for K^+ exchange function of the H,K-ATPase. Stimulation of gastric acid secretion requires the fusion of H,K-ATPase-containing tubulovesicles with the apical membrane of the parietal cell, placing the pump enzyme in parallel with a K^+ conductance in the apical membrane (Forte *et al.*, 1981). This structural reorganization of the apical membrane greatly expands the secretory surface which is reorganized into an elaborate system of deeply invaginated canaliculi (Forte *et al.*, 1977; Helander and Hirschowitz, 1972) consisting of elongated microvilli of variable size and shape. This translocation involves the rearrangement of membrane cytoskeletal proteins such as actin and spectrin (Mercier *et al.*, 1989b). The mediation of this process by cAMP is correlated with the phosphorylation of an 80 kDa protein, referred to as 80K (Urushidani *et al.*, 1987). This protein is not found associated with tubulovesicles prior to stimulation, but is a major component, together with the H,K-ATPase and actin, of the apical membrane isolated from stimulated cells (Urushidani *et al.*, 1989). Our initial studies of the 80K protein indicated that it is a peripheral membrane protein (Urushidani *et al.*, 1989) and undergoes redistribution following stimulation (Hanzel *et al.*, 1989), but provided no information as to its function or identity.

A protein of similar molecular weight, named ezrin (Bretscher, 1983), has previously been identified during the biochemical dissection of the proteins that make up the cytoskeleton of the intestinal brush border microvilli. In addition to the major proteins, actin, brush border myosin I, villin and fimbrin, ezrin is present, stoichiometrically, as a minor component associated with the isolated microvillar cytoskeleton. Moreover, the observation that activation of specific growth factor receptors resulted in the tyrosine phosphorylation of proteins led to the identification of a series of EGF-stimulated phosphoproteins known primarily by their molecular weight. Among them an 81 kDa protein in A431 cells was characterized and referred as to p81 (Hunter and Cooper, 1981), then identified as ezrin (Gould *et al.*, 1986). EGF stimulation in A431 cells caused the formation of microspikes and microvilli leading to membrane ruffling. Ezrin was localized to these surface structures and phosphorylated concurrent with their formation (Bretscher, 1989). Similarly, a protein of ~75 kDa was identified in

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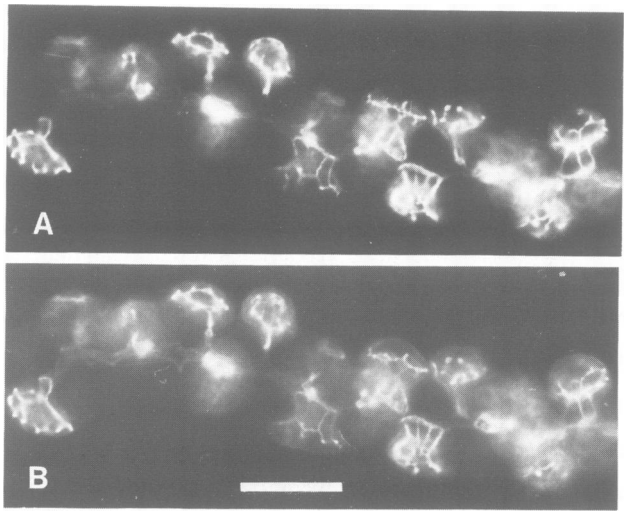

Fig. 1. Simultaneous localization of 80K (**A**) and ezrin (**B**) in resting gastric glands. Fixed glands were probed with anti-80K, visualized with Texas Red (A), and anti-chicken ezrin visualized with fluorescein (B). It is clear that the two probes label the same structures and that they are found exclusively in parietal cells (the black unstained regions between parietal cells are mucous neck cells and chief cells that are also present in gastric glands). For both probes staining was localized to a filamentous network that appeared to have a focus or anchoring point at the apical pole of the parietal cell. The general appearance of this network of staining by these two probes resembles the system of secretory canaliculi that extends from the lumenal surface throughout the parietal cell. Scale bar = 25 μm.

a wide range of cultured human cells, using antibodies to a retrovirus-related peptide. This protein was characterized as a membrane protein highly specific for microvilli and named cytovillin (Pakkanen *et al.*, 1987). Immunological studies (Gould *et al.*, 1986; Pakkanen and Vaheri, 1989) and recent DNA sequencing data (Gould *et al.*, 1989; Turunen *et al.*, 1989) have now established that ezrin, p81 and cytovillin are the same protein.

In this study we show that gastric 80K is homologous to intestinal and placental ezrin. Further ultrastructural and biochemical characterization specifies the properties of the association of gastric ezrin with the secretory microvilli and the membrane skeleton of parietal cells, and suggests a key role for the phosphorylation of 80K/ezrin during the stimulation-induced apical membrane transformations which facilitates gastric acid secretion. Some of this work has been presented in a preliminary form (Mangeat *et al.*, 1990).

Results

80K and ezrin are homologous proteins

Rabbit gastric glands are tubular structures composed predominantly of acid secreting parietal cells, pepsinogen secreting chief cells and mucous cells. The lumen running down the center of the gland is formed by the apical membranes of these cells. In addition to its luminal aspect the apical membrane of the parietal cell invaginates to form a highly anastomosing network, more functionally called the secretory canaliculus, which extends throughout the cells. When gastric glands were simultaneously probed with mouse monoclonal anti-80K (Figure 1A) and rabbit anti-chicken ezrin (Figure 1B), both antibodies stained the same subcellular structures, only present in parietal cells, mainly the secretory canaliculi (see below).

To test for a direct immunological relationship between

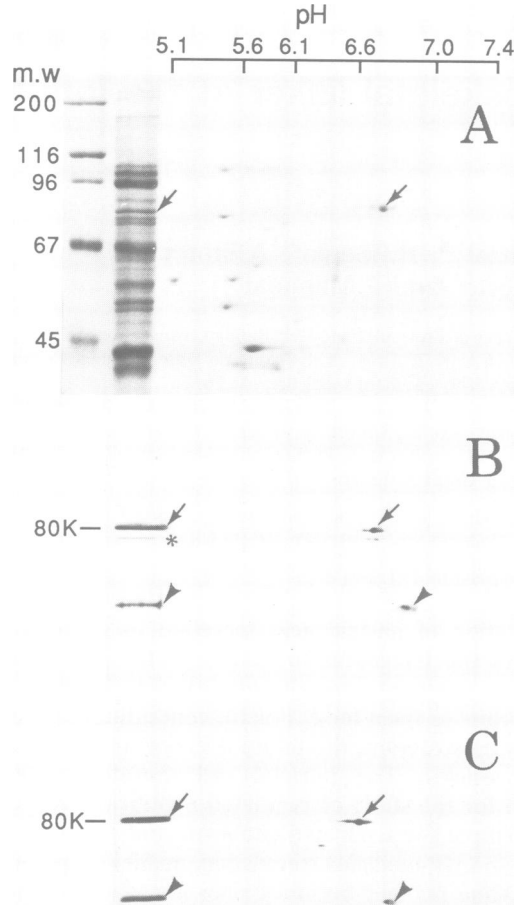

Fig. 2. Gastric 80K is recognized by anti-ezrin antisera. Enriched apical membrane fractions were separated on triplicate 2D gels and: (**A**) stained with Coomassie blue, or transferred to nitrocellulose and probed with rabbit anti-chicken ezrin (**B**) or anti-80K antibodies (**C**). An equivalent sample was run in the second dimension only, to the left of the 2D sample. Arrows indicate 80K; arrowheads indicate a breakdown product, recognized by both antibodies. * indicates an additional peptide recognized only by the polyclonal anti-chicken ezrin.

80K and ezrin, apical membrane-rich fractions of rabbit gastric glands were separated on three parallel two-dimensional C2D gels; one was stained with Coomassie blue (Figure 2A), and the others were transferred to nitrocellulose and probed with either anti-chicken ezrin (Figure 2B), or anti-80K (Figure 2C). The anti-chicken ezrin polyclonal serum recognized the same 80 kDa peptide (arrow) and breakdown products (arrowhead) in the parietal cell membrane sample as did the anti-80K. In addition, anti-chicken ezrin recognized an additional peptide at 77 kDa just below 80K (*), which could be an additional breakdown product, or some additional antibody produced in response to immunization with chicken ezrin. Anti-human ezrin serum gave a similar result except that, like the anti-80K monoclonal, it did not recognize the 77 kDa peptide (data not shown). Western blots of human placental p81 ezrin produced the same pattern as observed for the parietal cell membrane fraction. anti-chicken ezrin recognized both an 81 kDa and a 77 kDa protein; anti-human ezrin and three different clones of anti-80K recognized only the 81 kDa protein (Bretscher, 1989; A.Bretscher, unpublished results).

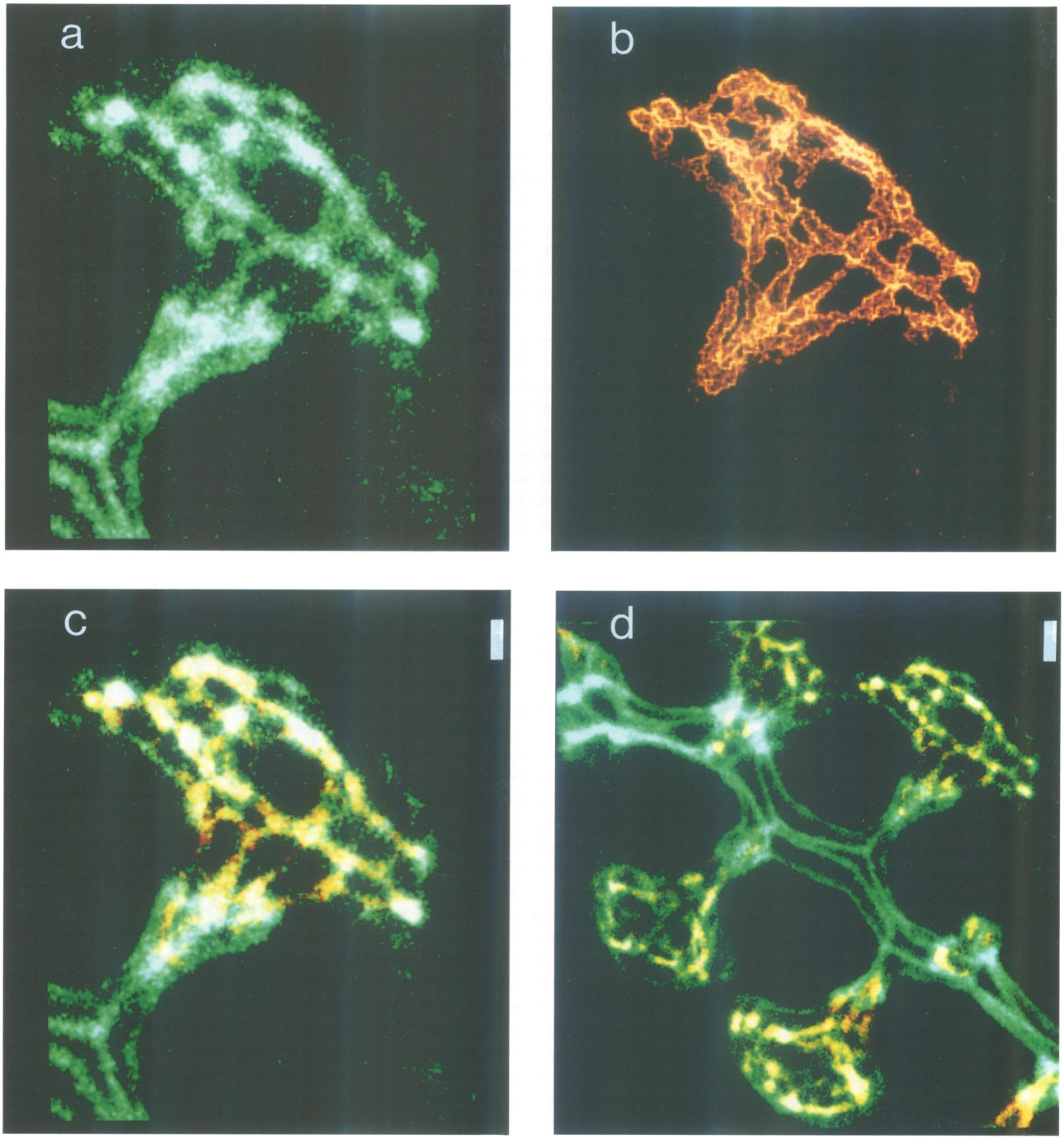

Fig. 3. Simultaneous localization of 80K and F-actin to the parietal cell apical membrane in resting gastric glands. Fixed gastric glands were probed with anti-80K and visualized in Texas Red; FITC−phalloidin was used to localize F-actin. Twenty four serial sections were collected on a confocal microscope and used to generate projections for either F-actin (**a**, green) or 80K (**b**, red). Staining of F-actin (green) is localized to a filamentous network throughout the parietal cell, analogous to the location of the microvilli within the secretory canaliculi, as well as to outlining the entire gland lumen. Anti-80K staining (red) is localized only to the network of secretory canaliculi within parietal cells. When the two projections are merged (**c**) it can be seen that 80K stains a subset of the structures containing F-actin. The image of 80K alone (b) was generated to show only surfaces that allow more detail of the canaliculi to be seen. A more expansive view of the gastric gland is shown in **d**; the single cell represented in a, b and c is present in the upper right corner of d. Scale bar = 3 μm in a, b and c; 6 μm in d.

Thus, we conclude the 80K and ezrin are homologous proteins.

80K/ezrin is mainly concentrated in the secretory microvilli associated with actin and apical H,K-ATPase

Earlier localization of 80K in parietal cells combined with the biochemical enrichment of 80K in an apical membrane fraction had led us to conclude that 80K was an apical membrane protein (Hanzel *et al.*, 1989). The subcellular localization of 80K/ezrin was further investigated in comparison with other cell markers, such as F-actin and the parietal cell H,K-ATPase, by confocal and electron microscopy.

Gastric glands are tubular structures several cells thick,

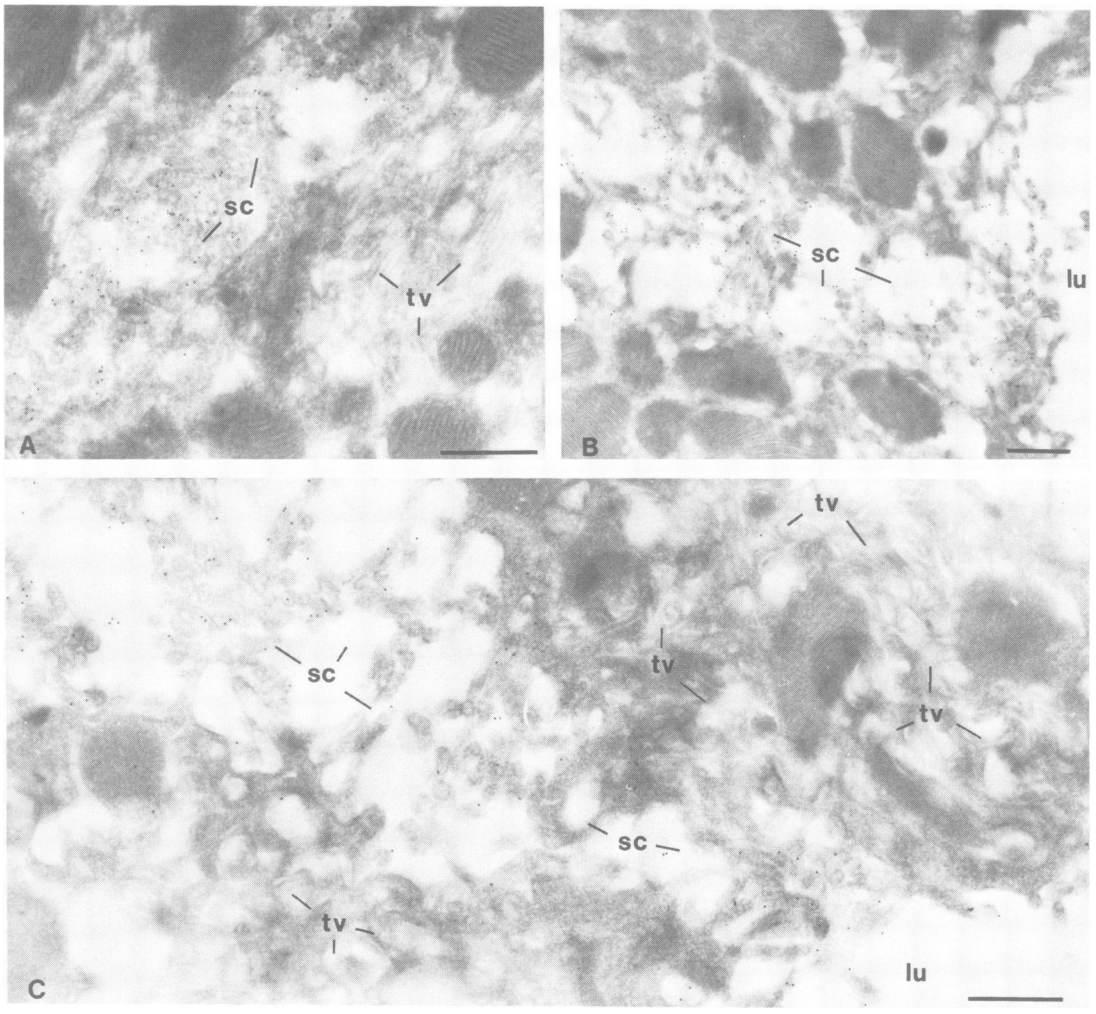

Fig. 4. Ultrastructural localization of ezrin and H,K-ATPase in resting rat parietal cells. (**A**) Section through a parietal cell showing the system of intracellular tubulovesicular membranes (tv), as well as microvilli lining the lumen of the secretory canaliculus (sc). Ezrin (5 nm gold particles) can be localized in the microvillar membrane of the secretory canaliculi and is absent from the tubulovesicles. The majority of H,K-ATPase, labeled with 15 nm gold particles, is sequestered in the tubulovesicular membrane system. H,K-ATPase also colocalized with ezrin in the microvilli membrane of the secretory canaliculus. (**B**) Higher power view of partietal cell showing ezrin and H,K-ATPase colocalized within microvilli of the secretory canaliculi, whereas only H,K-ATPase is present in tubulovesicles. (**C**) Ezrin is also found localized, although in lesser amount, in basolateral membrane infoldings (arrowheads), where it is not associated with H,K-ATPase. In this field, the basolateral membrane separates a parietal cell from an endocrine cell (ec). Scale bars = 500 nm.

and thus epifluorescence is often obscured by 'out of focus' signal. Confocal microscopy was used to minimize this interference. F-actin, identified with FITC−phalloidin in Figure 3, is concentrated in three distinct regions: the apical membranes of both chief and parietal cells lining the entire gastric gland lumen, the anastomosing secretory canalicular membrane of the parietal cell, and a faint ring just inside of the basolateral membrane of the parietal cell. Simultaneous probing for 80K by double labeling of the same glands showed that 80K is localized to the apical membrane of the parietal cell exactly like actin (Figure 3), and is present in diminished quantities at the more basal sites in the parietal cell. However, unlike actin, 80K is not found at non-parietal cell (mainly chief cells) apices lining the gland lumen. Thus, 80K co-localizes with F-actin within the parietal cell. The labeled structures resulting from three-dimensional reconstruction of serial confocal sections demonstrated that the secretory canaliculus is contiguous with the lumen of the gastric gland. These images are remarkably similar to those drawn by Golgi (1893) a century ago.

We used the anti-ezrin antibody to determine the distribution of ezrin in the parietal cell at the electron microscope level, and to examine possible relationships with the acid producing H,K-ATPase by double labeling with immunogold. In resting tissue (Figure 4), ezrin colocalized with H,K-ATPase in apical microvillar membranes of the secretory canaliculus (Figure 4A and B). However, these two proteins do not colocalize in other membranous structures. The majority of H,K-ATPase was associated with the tubulovesicular membrane system, whereas tubulovesicles were devoid of ezrin (Figure 4A and B). It was also possible to establish that ezrin was frequently present along the basolateral membrane (Figure 4C, arrowheads), although in smaller amounts than its apical distribution. Stimulation of acid secretion results in a 5- to 10-fold increase in the apical surface area of a parietal cell (Helander and Hirschowitz, 1972). In stimulated rat parietal cells (Figure 5) we found that all H,K-ATPase colocalized with ezrin in the expanded apical secretory microvilli. In this state, basolateral membrane infoldings were still labeled with anti-ezrin. The double labeling used here allowed a clear distinction between basolateral membranes (single ezrin labeling,

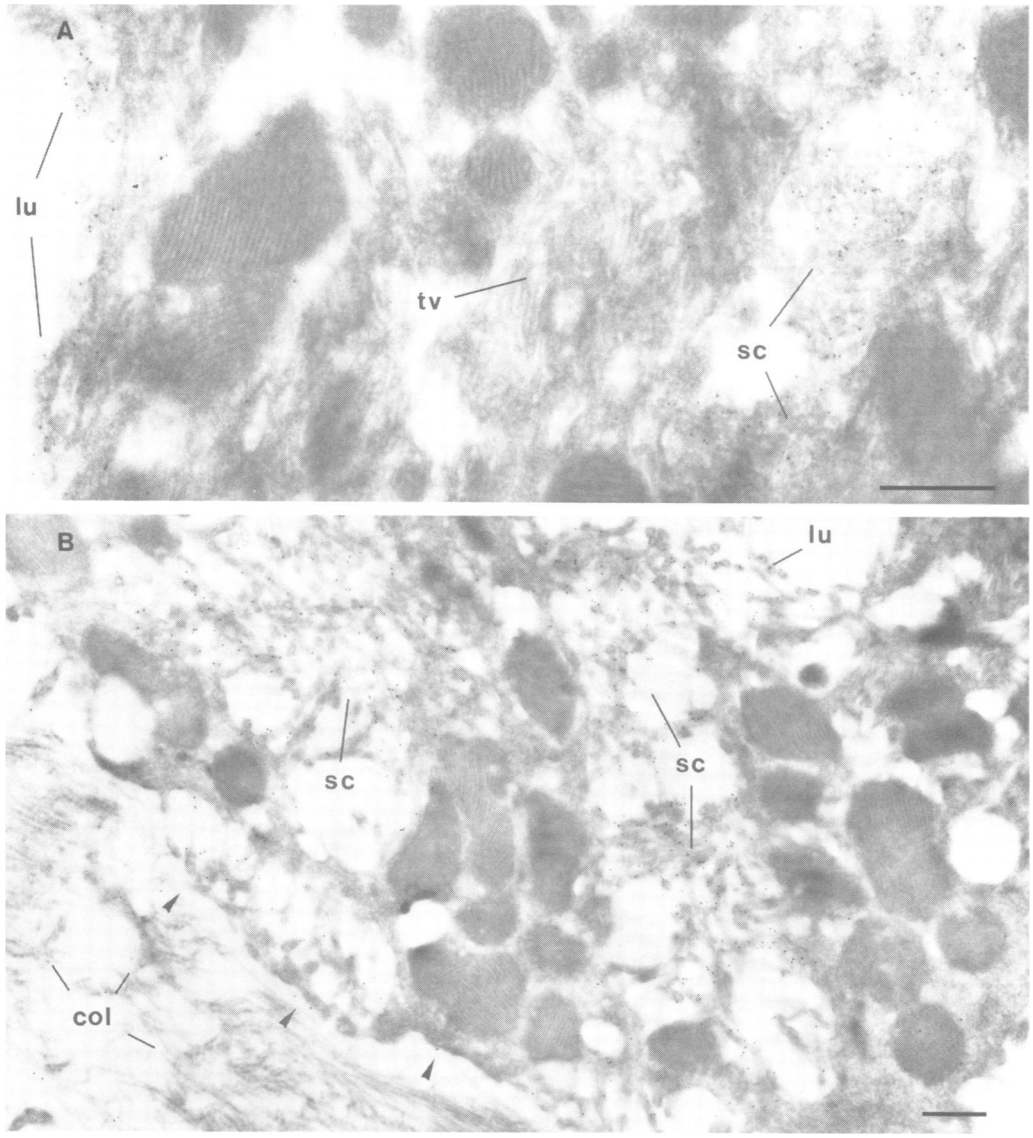

Fig. 5. Ultrastructural characterization of ezrin and H,K-ATPase in stimulated rat parietal cells. (**A** and **B**) Upon stimulation of parietal cells, the H,K-ATPase (15 nm gold particles) containing tubulovesicles fuses with the ezrin-rich (5 nm gold particles) apical membrane, and thus both are colocalized to microvilli of the greatly expanded apical membrane within the secretory canaliculi (sc). Note that some ezrin-specific labeling is still associated with the basolateral membrane infoldings (arrowheads) adjacent to the striated collagen fibers (col). Scale bars = 500 nm.

arrowheads) and the deep invaginations of apical secretory membranes (double ezrin and H,K-ATPase labeling).

The monoclonal antibodies against 80K were very sensitive to fixation, thus to preserve antigenicity, mild conditions were used for tissue fixation at the expense of better morphology. However, immunogold labeling on thin, frozen sections demonstrated that for both resting and stimulated parietal cells anti-80K was distributed like anti-ezrin. As shown in Figure 6, 80K was definitively associated with the microvilli-studded apical membrane, including its extensions into the secretory canaliculi, whereas 80K was absent from the cytoplasmic tubulovesicle membrane network. As for ezrin some sparse anti-80K labeling was identified along the basolateral membrane (data not shown).

80K/ezrin is associated with the detergent-insoluble cytoskeleton fraction

Parietal cell microvilli serve an analogous purpose to intestinal brush border microvilli, increasing the functional surface area of a transporting membrane, but microvilli of stimulated parietal cells are much less uniform in size and shape. The cytoskeletal proteins of brush border microvilli have been extensively characterized by selective detergent extractions which leave the cytoskeleton insoluble and much of the morphology intact (Bretscher, 1983, 1986). We applied the strategy of selective detergent extraction to characterize the association between 80K and cytoskeletal proteins.

Gastric glands were extracted in PIPES-buffered, low salt media, containing 0.1% NP-40 in the absence of Ca^{2+} to preserve the cytoskeleton, including actin and tubulin and their associated proteins. Samples of the detergent-insoluble pellet (25 μg) and total gland protein (50 μg) were subjected to SDS−PAGE and either stained with Coomassie blue (Figure 7, left) or probed with anti-80K (Figure 7, right). The detergent-insoluble pellet is rich in actin (42 kd) and tubulin (55 kd). The heavy 80K immunostaining in the insoluble pellet from extracted glands demonstrates that this cytoskeletal fraction is also enriched in 80K relative to total

gland protein. Immunostaining of the extracted gland remnants revealed that F-actin, 80K and tubulin were all present with respective staining patterns similar to control, unextracted, glands. On the other hand, H,K-ATPase staining was completely lost, confirming that the procedure effectively solubilized integral membrane proteins. When the extraction buffer included 2 mM Ca^{2+}, tubulin was markedly reduced in the insoluble pellet, with little effect on either F-actin or 80K (data not shown).

Salt-sensitive release of 80K/ezrin in digitonin-permeabilized glands is dependent on stimulation

The interaction of digitonin with cholesterol in plasma membranes renders them permeable to solutes as large as proteins, but does not alter integral membrane proteins such as the H,K-ATPase, as evidenced by the ability of digitonin-treated gastric glands to accumulate acid (Hersey and Steiner, 1985; Malinowska *et al.*, 1981). We decided to use a digitonin permeabilization protocol in order to manipulate

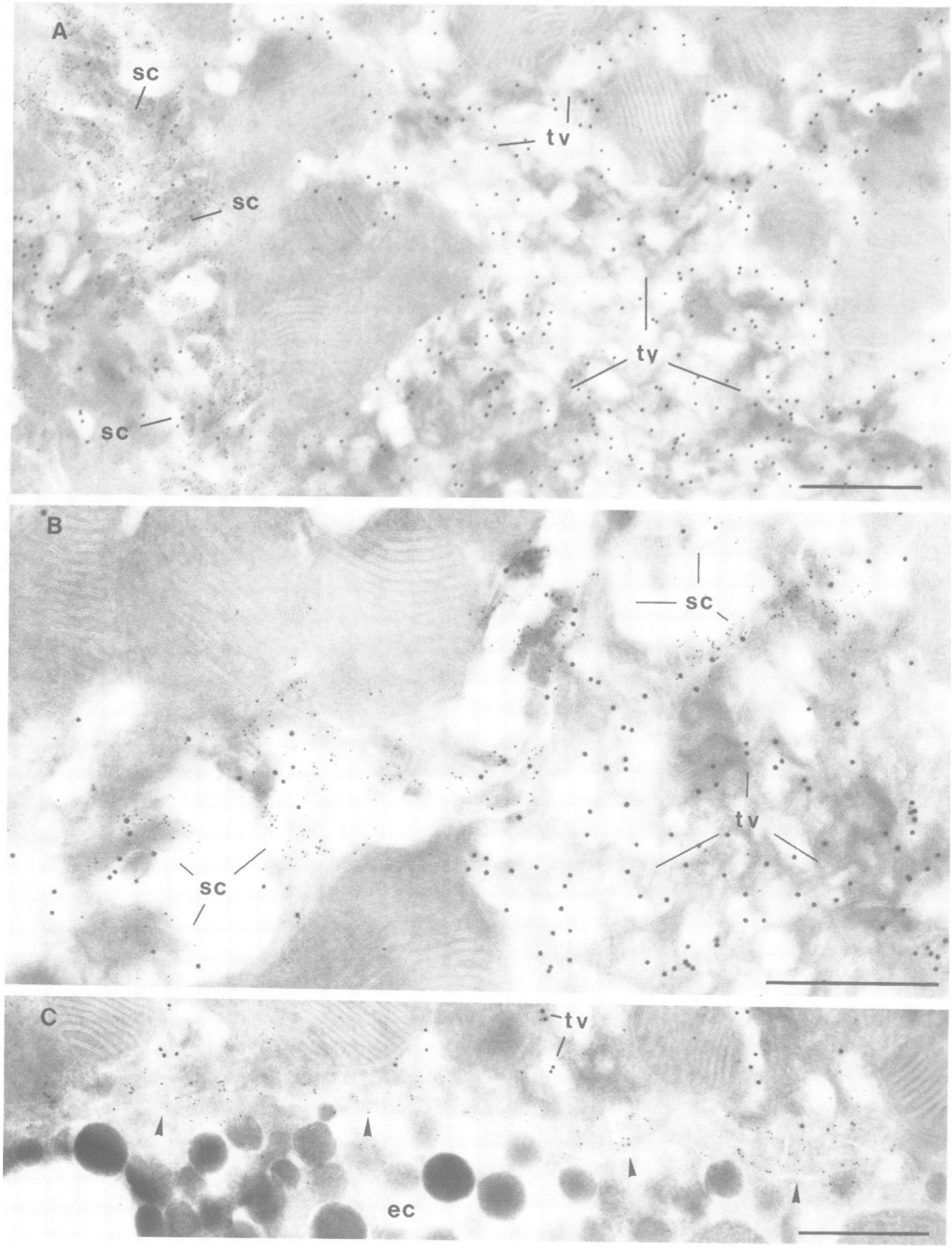

Fig. 6. Ultrastructural characterization of 80K in rat parietal cells. Although a relatively poor preservation of the morphology was achieved with the very mild fixation conditions used to preserve antigenicity, a clear subcellular localization of 80K was observed. In resting tissue (**A** and **C**) 80K is restricted to microvilli-rich apical membrane of the secretory canaliculi (sc), shown mainly in cross section, and the gland lumen (lu). Note that 80K is excluded from the intracellular, tubulovesicular membrane network (tv) which is rich in H,K-ATPase. In stimulated tissue (**B**) 80K is also distributed along the elongated apical microvilli lining both the expanded secretory canaliculi and the gland lumen (lu). Scale bars = 500 nm.

the salt concentration in the cytoplasmic space (i.e. via the bathing medium), to examine the salt sensitivity of 80K association within the parietal cell.

Isolated gastric glands were permeabilized with digitonin in either phosphate-buffered saline (PBS) or buffered isotonic sucrose. Samples were taken before the addition of digitonin and at 5, 10 and 20 min after adding digitonin. The glands were immediately separated into a supernatant, containing released cytoplasmic contents, and a pellet. Approximately 20% of the total protein (17.6% ± 0.9, $n = 9$) was released by the digitonin treatment, most of it within the first 5 min. Supernatant and pellet proteins were fractionated on parallel SDS gels which were either stained with Coomassie blue or probed with anti-80K (Figure 8). When the cytoplasm was equilibrated with physiological salt concentration, in PBS, all of the 80K was released into the soluble fraction in <5 min. When the cytoplasm was equilibrated with isotonic sucrose containing only 10 mM buffer, the bulk of the 80K remained with the permeabilized glands even after 20 min of extraction. Gastric glands that were digitonin-treated in PBS were fixed and stained with antibodies that recognize either 80K or H,K-ATPase. These glands showed a complete loss of 80K, but no reduction in H,K-ATPase staining (data not shown). Altogether, these results are consistent with a salt-sensitive association between actin and 80K, and a weak ionic association between 80K and the apical membrane.

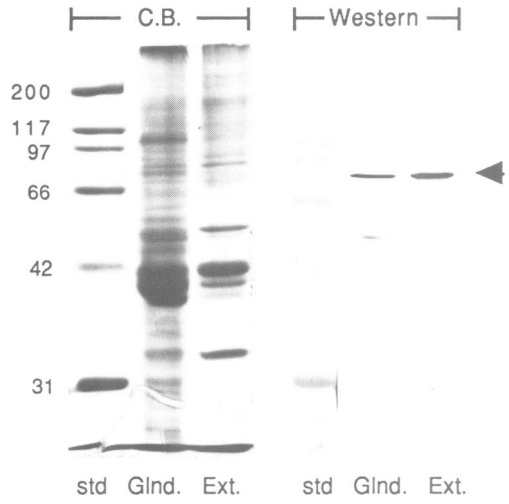

Fig. 7. 80K is retained in the detergent resistant cytoskeleton of rabbit gastric glands. Gastric glands were extracted three times in a low salt, weakly buffered solution containing 0.1% NP-40 and the detergent resistant pellet (Ext.) was solubilized in SDS. Molecular weight standards, total glands (Glnd, 50 μg), and the extracted glands (Ext. 25 μg) were run in duplicate; one gel was stained with Coomassie blue, and the other transferred to nitrocellulose and probed for 80K.

To characterize further the salt sensitivity of the association of 80K with the gastric gland, and to explore the possible functional significance of changes associated with stimulation, we monitored the release of 80K from digitonin-permeabilized glands titrated with salt. Isolated gastric glands were either maintained resting with cimetidine, or maximally stimulated with histamine and IBMX for 30 min. The treated glands were washed in buffered isotonic sucrose and then suspended in the same isotonic media with various concentrations of KCl. Digitonin was added at zero time and each tube was gently shaken at 37°C. After 5 min the glands were quickly spun in a microfuge and the supernatant and pellet were separated. Equal amounts (10 μg protein) of each pellet and supernatant sample were separated on SDS−PAGE, transferred to nitrocellulose, and probed for 80K. 80K was quantified by densitometry of the Western

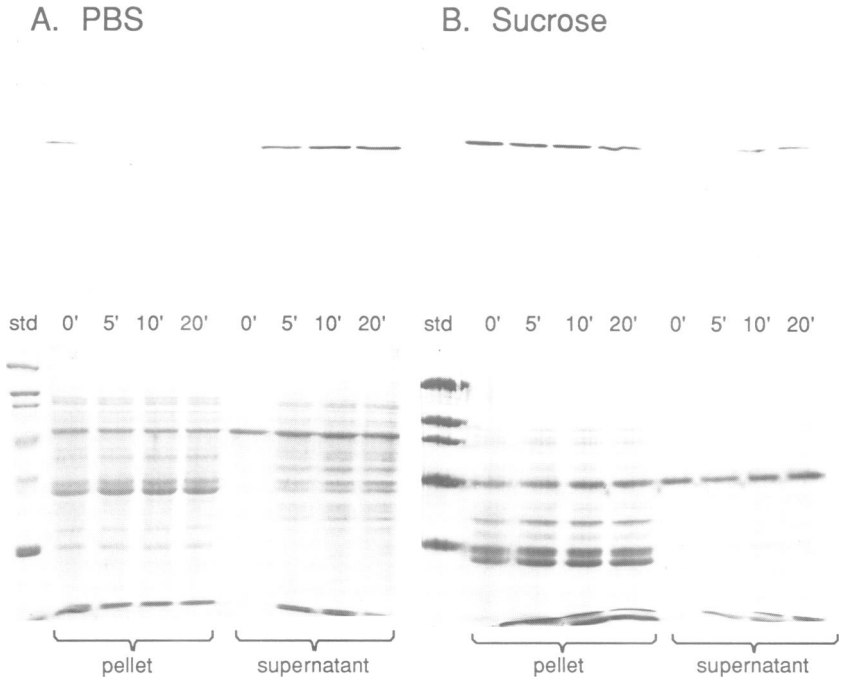

Fig. 8. Release of 80K from permeabilized glands is sensitive to salt concentration. Gastric glands were permeabilized with 20 μg/ml digitonin in either PBS, or isotonic sucrose containing 10 mM buffer (sucrose). The glands were centrifuged at 0, 5, 10 and 20 min after the addition of digitonin, and separated into pellet and supernatant fractions. Samples were run on duplicate SDS−PAGE, one stained for protein (lower panels) and the other transferred to nitrocellulose and probed with anti-80K antibodies (upper panels).

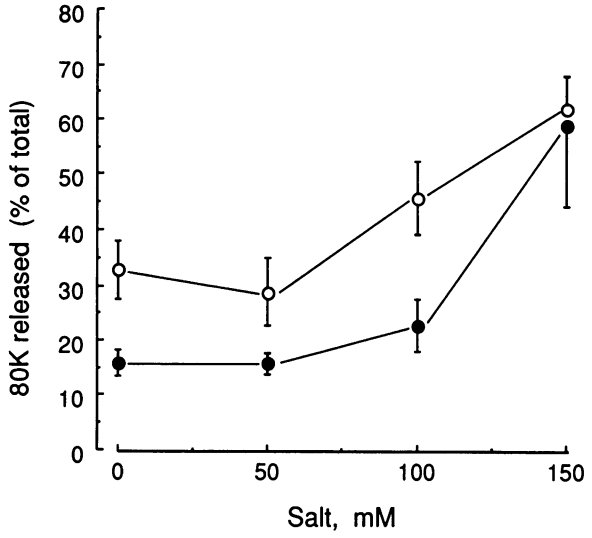

Fig. 9. The salt-sensitive release of 80K from permeabilized gastric glands is influenced by the secretory state. Either resting (○) or maximally stimulated (●) gastric glands were permeabilized with digitonin, as described in Figure 8, in solutions containing various salt (KCl) concentrations. After 5 min the soluble and insoluble fractions were collected, run on gels and 80K was quantitated as described in Materials and methods. Values are the mean ± SEM of three separate gland preparations.

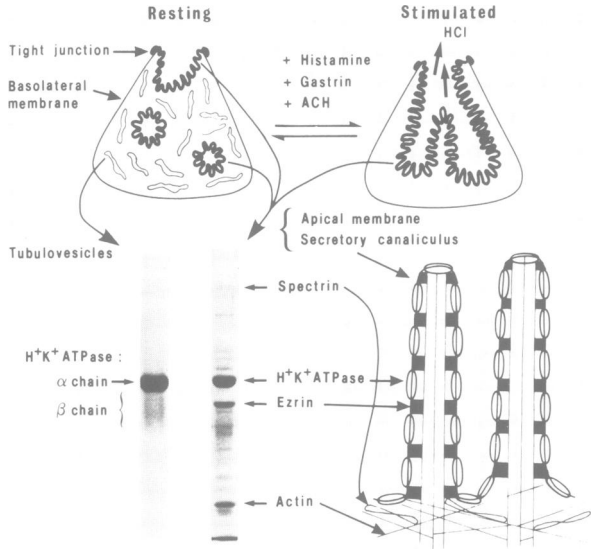

Fig. 10. Schematic organization of resting and stimulated gastric parietal cells. The upper part of the figure represents the reversible morphological changes that occur upon stimulation of gastric secretion. An intracellular pool of resting membranes, tubulovesicles, are inserted into the apical membrane enlarging the surface of the secretory canaliculi up to 10-fold. During this process cortical proteins assemble and elongate the microvilli. In the lower part are shown SDS−PAGE gels (Urushidani *et al.*, 1989) from H,K-ATPase-rich membranes isolated from resting tubulovesicles (left) and stimulated apical microvillar membranes (right), and a tentative proposal for parietal cell microvillar architecture.

blot, and when adjusted for the amount of protein in the corresponding sample, we could determine the amount and percent of total 80K released at each condition.

Figure 9 shows the sensitivity to salt for release of 80K from digitonin-permeabilized parietal cells. For both the resting and stimulated preparations more 80K was released as the concentration of salt was increased; however, consistent differences in salt sensitivity were also associated with stimulation. When the cytoplasm was equilibrated with lower salt concentrations (0, 50 and 100 mM KCl) twice as much 80K was released from resting glands as from stimulated glands. Similar titration curves, showing increased 80K release with increased salt concentration, were obtained from resting glands that were either homogenized or disrupted by sonication rather than permeabilized by digitonin (data not shown).

Discussion

In this paper we have demonstrated the homology of a previously identified gastric 80K phosphoprotein (Urushidani *et al.*, 1989) with intestinal and placental ezrin, and provided additional evidence that gastric 80K/ezrin is a major cytoskeletal component of gastric secretory microvilli. This result was supported by the biochemical, physical, and immunological properties of the gastric protein, identified on 2D gels with different antibodies against gastric 80K and against various sources of ezrin. The subcellular localization to 80K/ezrin in apical secretory microvilli of parietal cells was further substantiated using light confocal and electron microscopy.

Recently ezrin has been demonstrated to be homologous to cytovillin by both immunological cross-reactivity (Pakkanen and Vaheri, 1989) and by cDNA sequence (Gould *et al.*, 1989; Turunen *et al.*, 1989). Both ezrin and 80K were isolated from microvilli-studded apical membranes: ezrin from small intestine and 80K from the parietal cell. Cytovillin has been localized to the apical microvilli of human choriocarcinoma cells. Thus, based on a combination of criteria, including immunological cross-reactivity, cytolocalization and shared physical characteristics, we conclude the homology between 80K and ezrin/p81/cytovillin.

Gastric ezrin was first identified as a parietal cell phosphoprotein (Urushidani *et al.*, 1989) and its phosphorylation concomitant with stimulation of HCl secretion suggested a possible involvement of the phosphoprotein in the requisite apical membrane transformations. As yet, intestinal ezrin has no known function, and has not been shown to be modified during functional activity of the intestine. On the other hand, ezrin in A431 cells, which overexpress EGF receptors (Fabricant *et al.*, 1977), was initially characterized as a substrate for an EGF-stimulated tyrosine kinase (Hunter and Cooper, 1981). In these cells EGF stimulates the formation of microvillar structures (microspikes) within 30 s, membrane ruffling between 2 and 5 min, and rounding up after 10−20 min (Bretscher, 1989). Ezrin is localized to these microvillar structures and is phosphorylated on both tyrosine and serine residues in a time course which parallels the EGF-stimulated morphological changes. Ezrin is rapidly (within 2 min), and transiently (down at 10 min), phosphorylated on tyrosine (Bretscher, 1989). On the other hand, in parietal cells, 80K/ezrin is stably phosphorylated on serine over a time course which parallels the fusion of tubulovesicles with the apical membrane (Urushidani *et al.*, 1987,1989).

The possibility that EGF could influence the phosphorylation of 80K in the parietal cell is quite provocative. Hatt and Hanson (1988) showed that EGF inhibited acid secretion by histamine-stimulated rabbit gastric glands, and this inhibi-

tion was overcome by dibutyryl-cAMP or phosphodiesterase inhibitors. Moreover, EGF reduced the histamine-stimulated increase in cellular cAMP levels. Hatt and Hanson (1988) have proposed that EGF might operate by either of two ways: by activation of a G_i protein which then inhibits the histamine-stimulated adenylate cyclase, or by stimulating the cAMP-specific phosphodiesterase. The observation that EGF stimulates tyrosine phosphorylation of A431 ezrin (Bretscher, 1989) suggests a third possibility, that is, EGF-stimulated tyrosine phosphorylation of gastric 80K/ezrin might itself inhibit stimulation. This would be consistent with a previous *in vivo* study, suggesting that the inhibitory effect of EGF in parietal cells was essentially due to a defect of cytoskeleton-induced morphological transition from resting to stimulated state (Gonzalez *et al.*, 1981). If the site-directed phosphorylations induced by histamine and EGF had opposite effects, 80K would be an important model for dual control converging at a single protein. Preliminary experiments have demonstrated that phosphorylation of 80K in gastric glands was stimulated by EGF, and there were distinct differences in EGF-stimulated versus histamine-stimulated phosphopeptides (Whitney *et al.*, 1990). A minor but significant pool of ezrin is observed at the basolateral membrane of parietal cells. This pool might provide a privileged substrate for EGF receptor tyrosine kinase which has been specifically localized in high amounts in basolateral membranes of parietal cells (Mori *et al.*, 1987).

The homology between 80K and ezrin confirms an earlier proposal that 80K is associated with the actin cytoskeleton of microvilli (Hanzel *et al.*, 1989). Our ultrastuctural results, combined with biochemical fractionation of cytoskeleton-enriched fraction, demonstrate this point, although the molecular nature of this association remains to be determined. Thin, frozen sections, double labeled for ezrin and H,K-ATPase, show that ezrin colocalizes with the proton pump along the microvillar membrane. By comparison with previous double labeling between actin and H,K-ATPase (Mercier *et al.*, 1989a) a close colocalization exists between ezrin and H,K-ATPase, and actin in the microvilli, whereas actin was also found deeper at the base of the secretory microvilli (devoid of H,K-ATPase) in the terminal web-like structure (see Figure 8 in Mercier *et al.*, 1989a). Thus, this suggests that ezrin is more specifically localized along the microvillar axis associated with apical membrane, and is absent from this actin-rich structure in the terminal web. In addition, ezrin is present, although in a much smaller quantity, in the basolateral membrane infoldings, where it might participate in the organization of the actin/spectrin membrane skeleton (Mercier *et al.*, 1989a).

The architecture of parietal cell microvilli is far less documented than that of intestinal brush border. Parietal cell microvilli contain an actin cytoskeleton stabilized by associated proteins, but specific location of microfilaments and the nature and stoichiometry of actin-associated proteins are quite different from that of intestinal brush border. In brush borders microfilaments are localized within the central core of the microvilli, whereas in parietal cells microfilaments are radially arranged subadjacent to the plasma membrane (Forte *et al.*, 1977; Black *et al.*, 1982). Moreover, at the present time only actin, spectrin and ezrin have been identified as gastric cytoskeletal microvillar proteins in association with H,K-ATPase, the major integral microvillar membrane protein of stimulated parietal cells. No cross-reactive froms of villin and fimbrin have been found (P.Mangeat, unpublished results). The lack of these latter proteins is certainly related to the comparative lack of rigidity of parietal cell microvilli, which is a prerequisite for the high dynamic turnover of the apical membrane in these cells associated with resting/stimulated transitions. It is noteworthy to point out that ezrin appears as a major cytoskeletal component of parietal cell microvilli (Urushidani *et al.*, 1989), and that ezrin is present in much larger stoichiometric amounts than in brush borders. A simplified schematic view of parietal cell morphology in resting and stimulated states is shown in Figure 10, along with a proposed relationship of cytoskeletal proteins to apical microvilli. Ezrin has been shown to be a major component of apical microvillar membranes isolated from stimulated parietal cells (Urushidani *et al.*, 1989), and supported by the electron microscopy localizations reported here, in contrast to the absence of ezrin from tubulovesicles or from the region of the terminal web. Thus we propose that ezrin is associated only with actin microfilaments within the microvilli, possibly serving as a microfilament–membrane linker as suggested for the structurally related homologues of ezrin, band 4.1 and talin (see below).

Considering the huge addition of apical membrane that results from the fusion of intracellular tubulovesicles upon parietal cell stimulation, it is of interest how ezrin is mobilized on the apical membrane during new membrane insertion. Is ezrin recruited from the existing basolateral pool, or alternatively from a soluble pool which might exist in resting cells, or is ezrin diluted during this process? Although essentially qualitative, our ultrastructural data do not bring experimental support to a translocation of ezrin from the basolateral side, since the basolateral pool is minor in resting cells and does not appear to change in stimulated cells. No obvious evidence is found either for a large dilution of ezrin in apical microvilli of stimulated cells as compared with those of resting cells. The significant difference, found in salt-sensitive release of 80K from resting and stimulated digitonin-permeabilized cells, suggests that differences in cytoplasmic and membrane-associated pools of ezrin might actually exist *in vivo*, considering the actual intracytoplasmic salt concentration.

It is clear from work presented here that release of 80K from gastric homogenates or detergent-derived cytoskeletal preparations is sensitive to ionic strength. This property is consistent with the methods originally used by Bretscher (1986) to purify intestinal ezrin, and with the more efficient release of ezrin from A431 cells at 100 mM than 10 mM salt (Gould *et al.*, 1986). Permeabilization of gastric glands with digitonin allowed us systematically to manipulate salt concentration with minimum perturbation to hydrophobic protein–membrane interactions. 80K was converted from insoluble to soluble forms with increasing salt (Figure 9). Stimulation of gastric glands, using protocols which typically have produced a 3- to 4-fold increase in phosphorylation of 80K, reduced the fraction of phosphoezrin rendered soluble. These data may be interpreted as a stimulation-induced recruitment of ezrin (possibly by phosphorylation) from a more soluble pool, stabilizing the expanding apical membrane along microfilaments to form the microvilli characteristic of the secretory canaliculi.

Recent cDNA sequencing data has promoted the suggestion that ezrin may be functionally related to two other cytoskeletal proteins involved in connections to the plasma membrane, band 4.1 found in erythrocyte ghosts and talin

found highly concentrated in regions of cell contacts (Gould *et al.*, 1989; Rees *et al.*, 1990). There is a high degree of homology among these three proteins within a 200 amino acid span of the N-terminal domain, e.g. 35% identity between ezrin and band 4.1 and 23% identity between ezrin and talin (Rees *et al.*, 1990). Band 4.1 has been most extensively characterized. The site at which band 4.1 binds with the integral membrane protein glycophorin is located within this N-terminal domain. In addition to its membrane binding, band 4.1 has been shown to bind to several erythrocyte cytoskeletal proteins, as well as promoting the binding of spectrin to F-actin, and is an important regulatory component of membrane–cytoskeleton interactions (reviewed in Mangeat, 1988). Based on structural similarities to band 4.1 it was suggested that ezrin may modulate the association between plasma membrane and microvillar cytoskeleton (Gould *et al.*, 1989). In JEG-3 cells cytovillin/ezrin was shown to be within a few nanometers of the lipid bilayer between the microvillar membrane and the microfilaments (Pakkanen *et al.*, 1987), a position very similar to 4.1. If phosphorylation of ezrin can influence the stability of association between the plasma membrane and microfilaments, or other linking proteins, in a polarized fashion, this may be the key to the stimulation-related volatile surface reorganization in both A431 cells and parietal cells.

Materials and methods

Materials

FITC–phalloidin, Texas Red (Tx-R)–streptavidin were purchased from Molecular Probes; Hanker/Yates from PolySciences. All other chemicals were analytical grade.

Isolation and stimulation of glands

Rabbit gastric glands were isolated as described by Berglindh and Obrink (1976). Glands were maintained in the resting with 10^{-4} M cimetidine, an H2 receptor antagonist. Maximally stimulated glands were treated with 10^{-4} M histamine plus 5×10^{-5} M isobutylmethylxanthine (IBMX).

Detergent extraction of glands

Detergent-insoluble cytoskeletal fractions were prepared with several protocols. The most common was designed to retain both microtubules and microfilaments and was a modification of Duerr *et al.* (1981). All steps were performed at room temperature, including centrifugation. Typically 1 ml of settled glands was washed (1 *g* × 10 min) in (in mM) 100 PIPES pH 6.9, 1 $MgSO_4$, 2 EGTA; extracted three times (15 min shaking, 400 *g* × 5 min) in (in mM) 100 PIPES pH 6.9, 1 $MgSO_4$, 2 EGTA, 0.1% NP-40, 2000 glycerol (0.184 g/ml), 1 PMSF, 0.01 pepstatin, 0.024 TIU/ml aprotinin. In some experiments the EGTA was replaced with 2 mM $CaCl_2$. Detergent-soluble fractions were precipitated overnight in 80% MeOH at −5°C before determining protein concentration and running on SDS–PAGE, the detergent-insoluble pellets were either dissolved in 1 N NaOH or SDS sample buffer.

Permeabilization of glands

Digitonin permeabilization of gastric glands followed the protocol of Hersey and Steiner (1985). Glands were suspended in the test solution (e.g. PBS) and brought to a concentration of 10 mg wet weight/ml. Digitonin was added to a final concentration of 20 μg/ml, and the glands were gently shaken for 5 min at 37°C. One ml samples were quickly separated in a microfuge, and the soluble and insoluble fractions were processed as described above. Proteins >100 kDa were released by this treatment.

Antibodies

Anti-80K monoclonals were as described earlier (Hanzel *et al.*, 1989). Monoclonal antibody 146.14 has been described in Mercier *et al.* (1989a,b) and characterized as anti-H,K-ATPase by Lupo *et al.* (unpublished results). Polyclonal anti-chicken and anti-human ezrin were described by Bretscher (1983 and 1989, respectively).

One and two dimensional gel electrophoresis, Western blot analysis

Procedures were essentially as described earlier (Hanzel *et al.*, 1989; Urushidani *et al.*, 1989). Quantification of Western blots was done by 2D densitometric scanning and interpolation of areas (Bio-Rad). These procedures remained linear over the range quantitated, from 2 to 25 μg protein.

Immunocytochemistry

Essentially, all conditions were as described earlier (Hanzel *et al.*, 1989). Briefly gastric glands were fixed in 4% formaldehyde, permeabilized with 0.5% Triton X-100, and settled on poly-lysine-coated coverslips. The coverslips were processed with antibodies and then mounted on slides in either glycerol:PBS (9:1, v/v), or, to prevent photobleaching, 1.5% Hanker/Yates was added to PBS before mixing with glycerol, and coverslips sealed with nail polish. FITC–phalloidin was used to localize F-actin. Examination and photography was done on a Zeiss Axiophot microscope. Appropriate filters were used to detect FITC (excitation 450–490 nm, barrier 520 nm), and Texas Red (excitation 510–560 nm, barrier 590 nm). Control experiments demonstrated that the red filter excluded any signal from FITC, and the green filters excluded the Texas Red signal.

Ultrastructural immunocytochemistry

Adult rats were fasted overnight. Before being euthanized, rats were treated for 1.5 h with either the H2 antagonist cimetidine (100 mg/kg) (resting conditions), or given standard food (stimulated conditions). For 80K localization, rat gastric tissues were fixed 30 min in PLP fixative according to McLean and Nakane (1974). For double labeling studies with anti-ezrin and anti-proton pump antibodies, tissues were fixed 1 h in 2% paraformaldehyde and 0.03% glutaraldehyde in 100 mM sodium phosphate buffer pH 7.4. Thin, frozen sections were performed according to Tokuyasu (1973, 1989). Tissue were infused with a mixture of 20% polyvinyl-pyrrolydone dissolved in 2.3 M sucrose. These conditions increased the preservation of the specimen, especially in stimulated tissues with mild fixation.

For 80K labeling, monoclonal antibody 4A5 (Hanzel *et al.*, 1989) was used 30 times more concentrated than for light microscopy, followed by affinity-purified rabbit anti-mouse IgG (10 μg/ml), and by colloidal gold-labeled protein A (10 nm). For double labeling experiments sections were first incubated with affinity-purified anti-human placenta ezrin antibody (10 μg/ml) and purified monoclonal antibody 146.14 (Mercier *et al.*, 1989a,b) (20 μg/ml) followed by dilutions (1/20) of colloidal gold-conjugated goat anti-rabbit IgG (5 nm) and goat anti-mouse IgG (15 nm) antibodies. After standard processing, the sections were stained according to the positive–negative method (Griffiths *et al.*, 1983) and observed at 75 keV in a Hitachi H-600 electron microscope.

Confocal microscopy

Gastric glands, fixed and stained as described above, were examined in a PHOIBOS 1000 Laser scanning confocal microscope (Sarastro, Sweden). Single labeled samples were visualized with FITC, excited with 488 nm, and emitted light >515 nm was collected. Doubly labeled specimens were visualized with FITC–phalloidin and Tx-R–strepavidin. Previous confocal studies of doubly labeled samples have scanned the sample twice with settings optimized for each fluorophore (Mossberg and Ericsson, 1990). In order to maintain registration in the *z* (vertical) axis we chose to collect both images simultaneously.

To exclude adequately FITC from the Tx-R channel, we accept only light longer than 630 nm, which is above the 620 nm emission maxima for Tx-R. Empirically, we have found that Tx-R can be adequately excited with the 514 nm line of the argon laser, and it is beneficial to amplify the signal by employing a biotinylated second antibody and Tx-R–strepavidin. For doubly labeled samples, we excited with either the entire emission spectra of the argon laser (no excitation filter), or, if the FITC signal was too strong, only with the 514 nm line. The excitation and emission paths were separated with a 530 nm dichroic longpass filter further reducing the FITC signal. The FITC and Tx-R emission signals were separated with a 565-DCLP, an RG 630 was used for the Tx-R channel, and a 540 DF 30 was used for the FITC channel. This configuration allowed leakage of <2% of the FITC signal into the Tx-R channel and <3% of the Tx-R into the FITC channel, thus no correction was required or made for cross-talk. Three-dimensional reconstruction of serial confocal sections was performed with the program Vanis (Sarastro).

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