Fast freeze-fixation/freeze-substitution reveals the secretory membranes of the gastric parietal cell as a network of helically coiled tubule

A new model for parietal cell transformation

John M. Pettitt*, Danielle C. Humphris, Simon P. Barrett, Ban-Hock Toh, Ian R. van Driel and Paul A. Gleeson

Department of Pathology and Immunology, Monash University Medical School, Commercial Road, Prahran, Victoria 3181, Australia

*Author for correspondence

SUMMARY

The parietal cell of the gastric mucosa undergoes rapid morphological transformation when it is stimulated to produce hydrochloric acid. In chemically fixed cells, this process is seen as a reduction in number of cytoplasmic ‘tubulovesicles’ as the apical surface of the cell progressively invaginates to increase the secretory surface area. It is widely believed that the tubulovesicles represent stored secretory membrane in the cytoplasm of the unstimulated cell, which is incorporated into the apical membrane upon stimulation, because they share H⁺,K⁺-ATPase activity with the apical membrane. However, fusion of tubulovesicles with the apical membrane concomitant with parietal cell activation has never been convincingly demonstrated. We have used fast freeze-fixation and freeze-substitution to study stages of morphological transformation in these cells. Tubulovesicles were not seen in the cytoplasm of any of our cryoprepared cells. Instead, the cytoplasm of the unstimulated cell contained numerous and densely packed helical coils of tubule, each having an axial core of cytoplasm. The helical coils were linked together by connecting tubules, lengths of relatively straight tubule. Lengths of straight connecting tubule also extended from coils lying adjacent to the apical and canalicular surfaces and ended at the apical and canalicular membranes. Immunogold labelling with α- and β-subunit-specific antibodies showed that the gastric H⁺,K⁺-ATPase was localized to the membranes of this tubular system, which therefore represented the configuration of the secretory membrane in the cytoplasm of the unstimulated parietal cell. Stimulation of the cells with histamine and isobutylmethylxanthine lead to modification of the tubular membrane system, correlated with progressive invagination of the apical membrane. The volume of the tubule lumen increased and, as this occurred, the tight spiral twist of the helical coils was lost, indicating that tubule distension was accounted for by partial unwinding. This exposed the cores of cytoplasm in the axes of the coils as rod-shaped elements of a three-dimensional reticulum, resembling a series of microvilli in random thin sections. Conversely, treatment with the H₂ antagonist cimetidine caused severe contraction of the tubular membrane system and intracellular canaliculi. Our results indicate that tubulovesicles are an artifact of chemical fixation; consequently, they cannot have a role in parietal cell transformation. From our findings we propose an alternative model for morphological transformation in the parietal cell. This model predicts cytoskeleton-mediated control over expansion and contraction of the tubular membrane network revealed by cryopreparation. The model is compatible with the localization of cytoskeletal components in these cells.

Key words: gastric parietal cell, morphological transformation, freeze-fixation, freeze-substitution, H⁺,K⁺-ATPase, proton pump

INTRODUCTION

The parietal cells in the gastric epithelial glands are specialized for hydrochloric acid secretion. The secretory surface in these cells is not permanent but rapidly develops when the cell is stimulated to acid production and disappears when the stimulus is withdrawn. In chemically fixed glands, the unstimulated or ‘resting’ cell has a series of interconnected cytoplasmic compartments, the intracellular canaliculi, which are open to the apical surface. Microvilli are present on both the apical and canalicular membranes. The subapical and pericanalicular cytoplasm contains a large number of vesicular and/or short tubular structures called ‘tubulovesicles’ which, together with the secretory canaliculi, occupy much of the cytoplasmic volume in the apical part of the cell. The change to the secretory state is accompanied by extensive membrane rearrangement. In chemically fixed cells, this is seen as an increase in the apical and canalicular surface area, depletion of the cytoplasmic tubulovesicles and the development of numerous microvilli on the extending secretory surface. When
acid secretion ceases and the cell reverts to the resting state, the membrane configuration is evidently reversed, since tubulovesicles are again seen in the cytoplasm and the secretory surface is visibly diminished (Forte et al., 1981, 1990; Mercier et al., 1989a).

Careful ultrastructural examination of the transition between the unstimulated and stimulated states has not provided a solution to the enigma of how morphological transformation in the parietal cell occurs. Two hypotheses have been advanced to account for the process. One of these predicts membrane interconversion. According to this hypothesis, the tubulovesicles are a membrane reservoir which is utilized to extend the secretory membrane surface area in response to a physiological stimulus for acid secretion. The hypothesis requires that the excess membrane is subsequently retrieved from the secretory surface and restored to the cytoplasm as discrete tubulovesicles when the stimulus is withdrawn (Forte et al., 1977). The evidence adduced in support of this view includes the following findings: (1) immunocytochemical results show that in the unstimulated cell the gastric H^+,K^-ATPase (proton pump) is localized to both the tubulovesicular and canalicular membranes (Smolka et al., 1983; Mercier et al., 1989a; Pettitt et al., 1993); (2) morphometric data reveal a positive correlation between the increase in secretory surface area and the decrease in tubulovesicle membrane surface area during the conversion to the secretory state (Ito and Schofield, 1974); (3) freeze-fracture images demonstrate that the membranes of the secretory surface and the tubulovesicles are structurally similar (Ito and Schofield, 1974; Black et al., 1980); (4) inhibition experiments show that blocking protein synthesis does not prevent membrane rearrangement in the stimulated cell, indicating that membrane biogenesis is not necessary for secretory membrane extension (Orrego et al., 1966). Given the rapidity at which the cell responds to the presence and withdrawal of the stimulus, and the size of the tubulovesicle population in the unstimulated cell, it is clear that membrane recycling to take place it must occur over a very brief period and involve numerous fusion or fission events as tubulovesicle membrane is inserted or removed from the secretory surface. However, there is no convincing evidence from electron microscope studies for membrane recycling on such a scale (Berglindh et al., 1980; Forte and Soll, 1989).

In opposition to the concept of membrane recycling, Berglindh et al. (1980) have proposed that parietal cell transformation involves a process of osmotic expansion. On this view, the tubulovesicle component in the unstimulated cell, rather than being composed of individual vesicles, represents a vesicular continuum which has relatively few contact points with the apical and canalicular membranes. This hypothesis requires structural continuity between the apical surface and the tubulovesicular system, and predicts that volume flow associated with KCl flux is the diluting force in the system of tubulovesicles which produces morphological transformation. The major concern with this explanation is whether the appearance of the cell after experimentally induced osmotic swelling corresponds to normal morphological transformation which follows stimulation, or merely imitates the condition (Gibert and Hersey, 1982).

These alternative hypotheses differ fundamentally in the mechanisms they propose to account for the morphological transformation that accompanies the initiation and cessation of acid secretion in the parietal cell. Moreover, central to the membrane interconversion hypothesis is the supposition that transformation precedes secretion (Forte and Soll, 1989), while the concept of osmotically induced morphological transformation includes no such constraint (Berglindh et al., 1980). Any hypothesis for transformation must be able to account for the observation that the proton pump is inactive in the unstimulated state and active in the stimulated state (Forte and Soll, 1989; Rabon and Ruben, 1990).

We have investigated the organization of the secretory membrane system in the stimulated and unstimulated gastric parietal cell using fast freeze-fixation and freeze-substitution to minimize fixation artifacts (Kellenberger, 1991). To assist in the interpretation of the features revealed by these procedures we have used computer graphics to align images of consecutive sections and generate mosaic displays. These displays were used to construct models. With these methods we show that the principal locus of the proton pump in the unstimulated parietal cell is a system of interconnected helically coiled tubule. This membrane arrangement contrasts with the appearance in aldehyde-fixed cells where the gastric proton pump is seen to be localized to discrete cytoplasmic vesicles - the so-called ‘tubulovesicles’ (Smolka et al., 1983; Mercier et al., 1989a,b; Jones et al., 1991; Pettitt et al., 1993). Our results suggest that the tubulovesicles visualized in such preparations are an artifact of chemical fixation. Accordingly, an hypothesis which implicates these structures in parietal cell transformation cannot now be sustained.

MATERIALS AND METHODS

Animals

Adult BALB/c mice were obtained from the Monash University Central Animal Facility and housed under standard conditions at the Monash University Medical School Animal Facility. Animals were killed by CO_2 asphyxiation, the abdomen was immediately opened and the stomach removed.

Isolation of gastric glands

Gastric glands were isolated from the mucosa using a modification of the method of Gespach et al. (1980). Stomachs were opened by cutting from the oesophagus to the pylorus and rinsed in warm phosphate buffered saline (PBS). Tissue specimens of the gastric wall in the region of the corpus and fundus were removed, rinsed in warm PBS and incubated at 37°C in a solution containing 3 mM EDTA, 0.5 mM dithiothreitol (DTT) in PBS in a centrifuge tube. After 15 minutes, this solution was renewed and the treatment was continued for a further 10 minutes. The tube containing the samples was then shaken vigorously for 1 minute to release the glands from the submucosal tissues. The tissue remnants were removed from the tube and the isolated glands pelleted by centrifugation at 340 g for 3 minutes.

In vitro histamine and cimetidine treatment of mucosal tissues and isolated glands

Stomachs obtained from animals that had been fasted overnight were opened by longitudinal incision and rinsed in warm PBS. The intact stomach was rinsed in incubation buffer containing: 132.4 mM NaCl, 5.4 mM KCl, 5.0 mM Na_2PO_4, 1.0 mM NaH_2PO_4, 1.2 mM MgSO_4, 1.0 mM CaCl_2, 2 mg/ml glucose and 2 mg/ml BSA, adjusted to pH 7.6. The rinse buffer was discarded and replaced. Parietal cells were stimulated by the addition of 1x10^-4 M histamine (Sigma Chemical Co., St Louis, MI, USA) plus 5x10^-5 M IBMX (3-isobutyl-1-methyl...
xanthine: Sigma Chemical Co.) to the incubation buffer maintained at 37°C (Hanzel et al., 1991). Tissue specimens were excised from the corpus and fundus regions of the stomachs at intervals from 5 to 60 minutes post-incubation and freeze-fixed as described below. Gastric glands isolated from the stomachs of fasted animals were treated similarly. It has been shown that the individual response of parietal cells to the presence of histamine varies considerably - in a given time, morphological change is more extensive in some cells than in others (Mangeat et al., 1990). Therefore, to assist the interpretation of the ultrastructural features in cells exposed to histamine by excluding differences possibly attributable to starvation, these experiments were duplicated with tissues and glands from non-fasted animals. The effect of histamine treatment on parietal cells in the glands was followed in complementary experiments using the fluoroaphore 9-aminocacidine (Sigma Chemical Co.) as described by Mangeat et al. (1990).

Parietal cells in stomach tissue and isolated gastric glands were maintained in the resting state by the addition of the H₂ receptor antagonist cimetidine (Sigma Chemical Co.) to the incubation buffer at a final concentration of 1×10⁻⁵ M (Hanzel et al., 1991). Samples were removed at intervals for freeze-fixation, as during histamine treatment. Duplicate preparations of untreated tissues and glands were also prepared for comparison.

**Chemical fixation of tissue specimens and isolated gastric glands**

The methods used for chemical fixation and post-fixation processing of specimens were as previously described (Pettitt et al., 1993).

**Freeze-fixation of tissue specimens and isolated glands**

Tissue specimens were placed on a piece of nylon transfer membrane (Hybond-N+, Amersham International, UK) that was attached to the specimen carrier of a Reichert KF80/MM80 freeze-fixation unit (Reichert, Vienna, Austria) with double-sided Scotch tape, and the excess liquid was removed from the surface of the specimen with filter paper. The specimen was frozen by controlled impact against a metal mirror cooled to −196°C with liquid nitrogen (LN₂). The nylon membrane with the frozen specimen attached was prised from the specimen carrier under LN₂ and transferred under LN₂ to the chamber of a freeze-substitution apparatus. For isolated gastric glands, pellets were resuspended in 1% low gel temperature agarose (Bio-Rad Laboratories, Hercules, CA, USA) plus 15% sucrose in PBS. A drop of the suspension was placed on a Formvar-coated copper wire loop (Lancelle et al., 1986), the loop was attached to the specimen injector assembly of the KF80/MM80 unit and plunged into liquid propane maintained at −185°C and −190°C with LN₂. Alternatively, a small Teflon ring was attached to the KF80/MM80 specimen carrier with double-sided Scotch tape and sufficient of the gland/agarose suspension to form a prominent meniscus was pipetted into the centre of the ring. The agarose was allowed to gel and the suspension frozen by controlled impact against an LN₂-cooled metal mirror. The Teflon ring containing the frozen suspension was freed from the specimen carrier and transferred to a freeze-substitution chamber under LN₂.

**Freeze-substitution**

Freeze-substitution was performed in a Reichert CS-Auto freeze-substitution apparatus (Reichert, Vienna, Austria). Tissue specimens and glands for morphological study were transferred to a pre-cooled medium containing 1% osmium tetroxide, 3% glutaraldehyde, 1% uranyl acetate in methanol (Müller et al., 1980). The optimal substitution time, 55 hours at −90°C followed by a temperature rise of 5 deg. C/hour terminating at −20°C, was determined by assessing the results from a series of trials. At the end of the substitution process, the substitution medium was removed and the material rinsed twice in pre-cooled methanol. The temperature in the chamber was raised to +10°C, the material removed, rinsed in acetone and embedded in Spurr’s resin at room temperature. Tissue specimens and glands for immunocytochemical study were freeze-substituted in 0.5% glutaraldehyde in medium in the presence of a Linde 3A molecular sieve (BDH Chemicals, Poole, UK) using the same substitution program. After the second methanol rinse, the temperature in the chamber was raised to −4°C and the material was infiltrated at this temperature with LR Gold resin (London Resin Co., Woking, UK). The chamber was cooled to −20°C and the resin polymerized by ultraviolet light.

**Section cutting**

Sections (90 nm and 300 nm thick) of epoxy-embedded specimens were mounted on uncoated nickel grids and stained with 2% aqueous uranyl acetate and lead citrate. Serial sections, 90 nm in thickness, were collected on Formvar/carbon-coated slot grids and double-stained. Sections (90 nm) of LR gold-embedded material were mounted on Formvar/carbon-coated copper grids.

**Immunoelectron microscopy**

Immunolabelling was performed on LR Gold sections with monoclonal antibodies 1H9 and 2B6, specific for the α- and β-subunits of the H⁺,K⁺-ATPase, respectively, and with monoclonal antibody ET-1 in controls, following the procedure previously described (Callaghan et al., 1990; Pettitt et al., 1993). The immunolabelled sections were contrasted with 1% aqueous potassium permanganate.

**Electron microscopy and computer imaging**

Sections were examined in a JEOL 100C transmission electron microscope operating at 60 kV for the thin (90 nm) sections and 100 kV for the thick (300 nm) sections. Micrographs of serial sections were taken at instrumental magnifications of 10,000, 20,000 and 30,000 and the negatives photographically enlarged.×3. Fiducial points were marked and the images digitized over the range of magnifications to span the difference in scale, using IBM-compatible reconstruction software described by Young et al. (1987: distributed by the High Voltage Electron Microscope Laboratory, University of Colorado, Boulder, CO, USA). Areas showing cytoplasmic membrane concentration adjacent to the apical membrane and canaliculi in the generated displays were selected and identified on the original negatives. These areas were then photographically enlarged to 150,000-180,000, profiles of the selected structures were traced and the tracings further enlarged (300,000-360,000). New fiducial points were marked, the interfiducial distances chosen with regard to the dimensions in the final enlargement of the object under study, and the tracings digitized. The generated displays were rotated and the reconstructions were captured at different orientations and printed. The printed displays were translated into drawings and used to prepare models.

**RESULTS**

Conventional aldehyde primary fixation, and osmium post-fixation of unstained murine parietal cells, produced images in which the apical region of the cell contained profiles of intra-cellular canaliculi with evident microvilli. Numerous profiles of tubulovesicles were present in the subapical cytoplasm and in the cytoplasm surrounding the canaliculi (Fig. 1A).

The general gross morphology of parietal cells in fast freeze-fixed, freeze-substituted tissue specimens and isolated gastric glands corresponded to that seen in cells after chemical fixation. Unstained cells showed no inward extension of the apical membrane and the cytoplasm contained profiles of intra-cellular canaliculi. Microvilli were present on the apical and canalicular membranes (Fig. 2A). However, no tubulovesicles were detected in the cytoplasm of fast freeze-fixed, freeze-substituted unstained cells. In stimulated parietal cells the apical membrane was deeply invaginated and had incorporated

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the intracellular canaliculi. Numerous microvillus-like extensions had developed on the invaginated membrane (Fig. 3B).

**Apical and canalicular microvilli**

The variation seen in the morphology of the microvilli on the apical membrane of the fast freeze-fixed unstimulated cell is illustrated by the thick section in Fig. 4A. The microvilli varied in length and in diameter and had irregularly spaced constrictions and prominent distal dilations. They also varied in shape. Many of the longer microvilli were more or less perpendicular to the membrane surface, but others were recurved, and occasionally both forms branched at varying distances beyond their origin. Some of the shorter microvilli had a loose helical twist. Tilting thick tangential sections of the apical membrane revealed no change in the appearance of these structures. Typically, the canalicular compartments in the unstimulated cell were small and the morphology of the canalculus microvilli did not correspond to that of the microvilli on the apical membrane. Random thin sections showed that the lumen of the canalculus was frequently bridged by rods of cytoplasm. These cytoplasmic bridges were more or less circular in cross-section and appeared to be connected to each other laterally and vertically throughout the luminal space by microvillus-like projections of uniform diameter (Fig. 5B).

**Membranes of the subapical and pericanalicular cytoplasm**

Fast freeze-fixation revealed a complex system of membranes in the cytoplasm beneath the apical surface and intracellular canalici of the unstimulated parietal cell. The range of profiles seen in random thin sections showed that the membrane in this system consisted of tubule of constant diameter organized in regular helical configurations, each helical unit being composed of from 5-7 gyres of variable
pitch. These helical coils of tubule (HC) were numerous and densely packed in the cytoplasm. They showed no regular arrangement and in random sections they were seen in different orientations (Fig. 5A). Serial sections verified that there was no preferential alignment of HCs in respect of the apical and canalicular membranes. The various profiles showed that the gyres of tubule in the majority of HCs were closely wound, successive gyres of the helix usually in contact but not fused and occasionally slightly skewed to the central axis of the helix (Figs 5A, 6A,B). Some HCs were less symmetrical and the gyres of tubule less tightly bound. Each HC enclosed an axial core of cytoplasm ~120-150 nm in diameter (Fig. 5A,D).

Random thin sections and serial sections of well-preserved cells showed continuity between the HCs and lengths of relatively straight tubule of the same diameter, which were frequently seen distributed between the profiles of the HCs (Fig. 5D). Thick sections of these preparations revealed that the tubules formed interconnections between the HCs (Fig. 1B). Reconstructions of images from serial sections confirmed this relationship and demonstrated that the connecting tubule (CT)
Fig. 3. Thin sections of fast freeze-fixed/freeze-substituted stimulated and unstimulated parietal cells. (A) Cell after 30 minutes of histamine treatment. The helical coils close to an intracellular canaliculus (c) are noticeably enlarged and their gyres have partially unwound (arrows), so that the structures appear as lengths of loosely coiled, inflated tubule. Bar, 0.25 µm. (B) Cells in gastric gland after 30 minutes histamine treatment. The apical membranes of the parietal cells (p) in this gland are fully invaginated and the cells are completely transformed. Bar, 5 µm. (C) Unstimulated cell after 30 minutes cimetidine treatment. The canalicular compartment is reduced and the helical coils in the pericanalicular cytoplasm are seen as stacks of flat cisternae and concentric membranes (arrows). Bar, 0.5 µm.

Fig. 4. Fast freeze-fixed/freeze-substituted unstimulated and stimulated parietal cells. (A) Thick section of unstimulated cell. The microvilli on the apical membrane vary in length and shape and have distal dilations. Some microvilli are branched (arrowhead) and others have a helical form (arrow). Bar, 1 µm. (B) Thick section of cell after 30 minutes of histamine stimulation. The structures exposed at the extending secretory surface are rods of cytoplasm interconnected in three dimensions to form a reticulum. This is clearly seen left of centre at the base of the invagination. The arrow indicates a helical coil in the subapical cytoplasm. The coil has not yet become distended. Bar, 0.5 µm. (C) Thin section of cell after 30 minutes of histamine stimulation. The apical surface is invaginating (asterisk). In thin sections the individual elements of the reticulum seen in Fig. 3B resemble microvilli arising from the apical membrane. The arrow indicates a helical coil which is distending. Bar, 1.0 µm. (D) Thin section of unstimulated cell after 30 minutes of cimetidine treatment. The canalicular compartment is reduced and has only a few short microvilli. In axial view, the helical coils appear as series of concentric membranes enclosing cytoplasmic cores (arrow). Bar, 0.2 µm.
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Fig. 5. Fast freeze-fixed/freeze-substituted unstimulated parietal cells. (A) Thin section. Helical coils (arrows) are densely packed and occur in different orientations in the cytoplasm beneath an intracellular canaliculus (c). Bar, 0.5 µm. (B) Thin section of an intracellular canaliculus. The lumen is bridged by rods of cytoplasm which appear to be connected together by microvillus-like projections. Bar, 0.5 µm. (C) Thick section. Three connecting tubules (arrows) pass from helical coils (seen in near axial view) towards an intracellular canaliculus (c). The structures immediately below and to the left of the connecting tubules are canalicular microvilli. Bar, 0.25 µm. (D) Thin section. A length of relatively straight connecting tubule (small arrows) departs from a helical coil (large arrow) and traverses the cytoplasm. The core of axial cytoplasm is clearly seen in the coil. Bar, 0.25 µm.

Fig. 6. Fast freeze-fixed/freeze-substituted stimulated parietal cell after 30 minutes of histamine treatment. (A, B) Representative areas of two consecutive thin (90 nm) sections from a series of serial sections. The helical coils in A (indicated by the arrows) are continuous with the tubule coils indicated in B. Sections from this series were used for digitizing and preparing reconstructions. Bar, 0.5 µm.
could extend for some distance into the cytoplasm. These longer lengths of CT formed links between widely separated HCs lying on the same and different planes. Our electron micrographs showed that CTs of variable length extended from HCs lying close to the apical and canalicular membranes and terminated at these membranes. Points of CT-membrane association were evident in both tangential thick sections (Fig. 5C) and in thin sections normal to the membrane surface. In Fig. 7A a pair of parallel CTs ends at the apical membrane, with one tubule on either side of a microvillus. The separation between the two CTs decreases as they pass from the apical membrane into the subapical cytoplasm and disappear beneath an HC. The termination of a third CT at the apical membrane lies immediately out of the plane of the section and is therefore indistinct. However, the alignment of the profile in relation to the apical surface suggests that the CT ends at the membrane between two neighbouring microvilli. Below the apical membrane this CT curves sharply, and passes over the pair of parallel CTs to join an HC.

Acutely tangential sections that glanced the surface of the canalicular or apical membrane exposed arrays of CTs. This appearance is illustrated in Fig. 7B, where there are profiles of CTs on two sides of a canalicular compartment. The CTs on either side of the compartment have a nearly parallel arrangement and they end at the canalicular membrane on the same level. The CTs which follow the contour of the canalculus before leaving the plane of section are presumably associated with the canalicular membrane on another level.

**Effect of histamine treatment**

Our cryopreparations showed that not all parietal cells responded to histamine stimulation and that the degree of response varied considerably, even between cells within a gland. These observations agree with the findings from parietal cell culture (Mangeat et al., 1990). We could detect no obvious morphological or ultrastructural differences between responsive cells in fasted and non-fasted animals. Because the response of the cells to the presence of histamine was not consistent, we adopted invagination of the apical membrane as the criterion for stimulation and the extent of invagination as an indicator of the degree of transformation. Therefore, all the cells in the gland in Fig. 3B, which was exposed to histamine for 30 minutes before freeze-fixation, are regarded as fully stimulated and completely transformed.

Distinctive changes in the form of the HC system were evident in cells stimulated with histamine for 10-30 minutes. These changes were correlated with the progress of apical membrane invagination and the development of numerous microvillus-like structures on the extending secretory surface (Fig. 4C). The HCs close to the invaginating apical membrane in activated parietal cells exhibited varying amounts of tubule distension from moderate to pronounced. The process appeared progressive and resulted in the loss of the helical organization (Figs 3A, 7D). Gradual modification of HC structure due to expansion made interpretation of random profiles problematical, especially that of advanced stages. However, collectively these profiles suggested that while the volume of the tubule lumen was increased, distension did not affect membrane thickness nor did it change the association between the HC and its axial core of cytoplasm (Fig. 7C). With expansion of the tubular system, the axial cytoplasmic cores of the HCs were exposed as rods of cytoplasm, circular in cross-section and uniform in diameter, that were connected to each other in three dimensions to form a reticulum; this organization is evident in the thick section in Fig. 4B. In random thin sections of activated cells the structural elements of the developing cytoplasmic reticulum resembled microvilli arising from the invaginating apical membrane (Fig. 4C). HCs were present in the cytoplasm of fully transformed cells but notably at a much lower frequency than in the cytoplasm of cells exhibiting an intermediate stage in transformation.

**Effect of cimetidine treatment**

Our cryopreparations showed that, as with histamine, the response of parietal cells to cimetidine differed from cell to cell. After 30 minutes incubation in the antagonist some parietal cells still possessed a relatively extensive secretory surface, in contrast to others where the surface was reduced and intracellular canaliculi had re-formed. The canalicular compartments which developed in the responsive cells were contracted structures with little luminal volume and relatively few stubby microvilli (Fig. 4D; compare with Fig. 5A). Profiles of HCs and CTs were present in the cytoplasm beneath the apical and canalicular membranes. The helical twist of many of these HCs was compressed and in near axial sections the gyres of tubule appeared as a stack of flat membranous cisternae (Fig. 3C). The diameter of the tubule lumen was invariably reduced and in some HCs the lumen was completely closed, in which case the HC took the form of concentric membranes enclosing a cytoplasmic core (Figs 3C, 4D).

**The proton pump is localized to HC and CT membrane**

Indirect immunogold post-embedding labelling experiments on thin sections of fast freeze-fixed unstimulated parietal cells with monoclonal antibodies specific for either the α- or β-subunits of the gastric proton pump (H+,K+-ATPase) (Pettitt et al., 1993) showed colloidal gold particles in each case associated with the apical membrane, the canalicular membranes, and the membranes of the HCs and CTs (Fig. 2C,D). Fig. 2E illustrates a cross-section of an HC; the axial core of cytoplasm is clearly seen. Binding of colloidal gold particles is restricted to the membrane of the tubule, no particles are attached to the core. Sections treated with isotype-matched antibody of irrel-
relevant specificity showed negligible non-specific attachment of colloidal gold label to the membranes of the HCs and CTs (Fig. 2B). These results clearly show that the gastric H⁺,K⁺-ATPase is localized to the membranes of the HCs and CTs.

**Computer-assisted three-dimensional reconstruction of HCs and associated CTs**

The model shown in Fig. 8A-D was constructed from a sequence of profiles in a series of consecutive sections of a parietal cell exposed to histamine for 30 minutes prior to freeze-fixation. The structure of HCs in a preparation of a partially activated cell was used for this analysis in order to facilitate digitization. Slight expansion of the HCs reduced the number of overlapping profiles between sections, which made the limits of separate profiles easier to discern. Possible errors in the reconstruction were therefore avoided. The images selected for processing were close to the invaginating apical membrane and the degree of tubule dilation is illustrated by the HCs in Fig. 6A,B. Digitized images from seven serial sections aligned as a mosaic (data not shown) were used for the reconstruction. The depth portrayed by the model in the orientation shown in Fig. 8A is ~0.9 µm.

**DISCUSSION**

We have used impact freezing or plunge freezing and freeze-substitution to preserve the structure of the gastric parietal cell for a detailed study of its secretory membrane system. Freeze-fixation improves morphological preservation (Gilkey and Staehlin, 1986), but it also improves the time resolution of structural stabilization. With fast freeze-fixation structural stabilization takes place in milliseconds (Ryan and Purse, 1985; Baatsen, 1993) rather than the seconds or minutes required for chemical crosslinking at room temperature (Kellenberger et al., 1992). Both of these virtues are of significance in our study. During parietal cell transformation the apical membrane is reorganized and it is generally accepted that this process involves rapid translocation of membrane contained in structures termed tubulovesicles (Ito and Schofield, 1974; Forte et al., 1977; Black et al., 1981; Mercier et al., 1989a,b; Hanzel et al., 1991; Smith et al., 1993). While tubulovesicle profiles have sometimes been seen in close apposition to the invaginating surface membrane in chemically fixed stimulated parietal cells (see Fig. 5 of Forte et al., 1977), conclusive proof of membrane recycling is still lacking.

Our work extends published information by: (1) presenting a more accurate description of the organization of the H⁺,K⁺-ATPase-containing membrane system in the unstimulated parietal cell; (2) revealing that the vesicular organization of this membrane system in chemically fixed parietal cells is an artifact of specimen preparation; and (3) demonstrating that parietal cell transformation does not take place through the recycling of tubulovesicles.

**Organization of the apical microvilli and gastric H⁺,K⁺-ATPase-containing cytoplasmic membrane system in the unstimulated cell**

Earlier transmission EM studies on thin sections of chemically fixed material have revealed that the microvilli which decorate the apical membrane in the unstimulated parietal cell are not regular in shape (Ito and Schofield, 1974). Our thick sections of fast-frozen cells provide information additional to this description by showing that these structures interdigitate, vary in length and take a variety of forms, all of which have bulbous tips. Moreover, some of the shorter apical microvilli exhibit a marked helical twist about their long axis. Our results also indicate that these apical microvilli are different structures from the canalicular ‘microvilli’ which develop in the activated cell undergoing transformation.

We have interpreted the basic unit of organization in the parietal cell secretory membrane system of the unstimulated cell as consisting of a helically wound coil of tubule - an HC.
This interpretation is based upon the appearance of these structures in random thick and thin sections, visual analysis of their organization in serial sections, and computer-assisted reconstruction of individual units. HCs lying on the same plane are connected together by lengths of non-coiled tubule of the same diameter (CTs), which also connect HCs lying on different planes. These elements were recognized in all our sections and revealed in the reconstructions. Similar tubule was seen to arise from at least some HCs located close to the apical and canalicular surfaces and to terminate at the apical and canalicular membranes. Non-coiled tubular elements of the same dimensions but present in greater abundance have been recognized in the pericanalicular cytoplasm of a fast-frozen, acetone/osmium-substituted parietal cell and equated with tubulovesicles (see Fig. 15b of Ito, 1981). They have also been detected in an ultra-high resolution scanning EM study of aldehyde-fixed stimulated parietal cells where they were seen to connect a tubulovesicular network with the intracellular canaliculi at infrequent points along the canalicular membrane (Ogata and Yamasaki, 1993). These authors describe the tubules in their preparations as being structurally continuous with the canalicular membrane at the points of contact. It is tempting to speculate that the CTs arising from HCs and ending at the apical surface or a canalicular compartment in our freeze-fixed cells are permanently joined with the apical or canalicular membranes (see Fig. 7A,B); however, as they stand, our observations do not directly demonstrate membrane continuity. A more precise picture of the relationship of the internal tubular network to the cell surface should emerge with higher resolution data combined with in vivo tracer studies. Our results from in vitro experiments with heavy metal tracers, designed to determine whether the lumen of the network in the unstimulated parietal cell is open to the lumen of the gland, were inconclusive (unpublished results). Two factors were responsible for this. First, incubation of mucosal tissues and isolated gastric glands with heavy metal colloids prior to freeze-fixation resulted in post-mortem changes to parietal cell ultrastructure. Secondly, heavy metal colloids suspended in methanolic substitution media for post-fixation tracing had a tendency to precipitate at the temperature required for freeze-substitution.

Changes in organization associated with stimulation

Even with rapid freeze-fixation the sequence of histamine-induced changes in the parietal cells was difficult to follow; nevertheless, features of the transformation process could be identified. First, modification and the eventual disappearance of HC/CT organization is clearly correlated with progressive invagination of the apical membrane. Our immunogold labelling experiments show that the proton pump is localized to the membranes of the HCs and CTs in unstimulated cells, whereas in the stimulated cell the pump is confined to the invaginated apical membrane (Smolka et al., 1983; Mercier et al., 1989a,b; Mangeat et al., 1990; Smith et al., 1993).

Obviously, the membrane of the HCs and CTs is utilized to increase the apical membrane surface area during transformation. Secondly, histamine causes expansion of the HCs and CTs, which is brought about by dilation of the tubule lumen. Tubule dilation is apparently accommodated by the tight spiral twist of the HC, since this is lost when dilation is pronounced. Evidently, the helices partially uncoil to allow the tubule lumen to enlarge. Thirdly, the process of dilation and uncoiling does not alter the relationship of the tubule to the axial cytoplasmic core, which becomes exposed as an element of a reticulum.

Exposure of parietal cells to cimetidine caused a reduction in the luminal volume in responsive cells and severe contraction or total collapse of the HC-CT system. Interestingly, the canalicular membranes in these cells had few ‘microvilli’, which were short, peg-like protrusions.

Helical coils, connecting tubules and tubulovesicles

Tubulovesicles are universally encountered in large numbers in chemically fixed gastric parietal cells and, indeed, their presence in the cytoplasm is considered characteristic of the unstimulated state (Forte and Soll, 1989). However, not one of our cryopreparations showed these structures, whereas they were always present in the cytoplasm of unstimulated cells after chemical fixation (see Fig. 1A). We have previously demonstrated that antibodies against the proton pump strongly label the tubulovesicle membranes in chemically fixed unstimulated parietal cells (Callaghan et al., 1990; Jones et al., 1991; Pettitt et al., 1993). In the present study we have shown that the proton pump is localized to the membranes of the HCs and CTs in freeze-fixed unstimulated cells. This shared feature clearly suggests that the HCs/CTs and tubulovesicles are interrelated structures.

One possibility is that tubulovesicles are a structural and temporal intermediate between the HC/CT system and the apical membrane in the cell’s transformation cycle. However, this interpretation is not supported by our finding that tubulovesicles are generally absent from fast freeze-fixed parietal cells: neither has the HC/CT system been detected in chemically fixed cells. The different morphology of freeze-fixed and chemically fixed parietal cells most likely reflects the different fixation procedures. On this interpretation, tubulovesicles are the remains of HCs and CTs which have broken down and become vesiculated. In other cell types aldehyde fixation has been shown to cause cytoplasmic tubules and membranes to fragment and vesiculate (Mersey and McCully, 1978; Heath et al., 1985; Wilson et al., 1990; Hyde et al., 1991; Morgenstern, 1991). Chandler (1984) has suggested that this is attributable to the inability of aldehydes to arrest lipid mobility within membranes undergoing rapid change in structure, even though membrane-associated proteins are cross-linked. It is interesting, therefore, that aldehyde fixation prior to freeze-fracture reveals numerous tubulovesicles in the cytoplasm of unstimulated parietal cells (Ito and Schofield, 1974; Black et al., 1980).

We conclude from the present study that the tubulovesicular elements found in gastric parietal cells result from artificial vesiculation during chemical fixation of the tubule comprising the HCs and CTs, which retains its in vivo morphology when the cell is rapidly frozen.

How does transformation occur?

Our findings are not consistent with the membrane recycling hypothesis for parietal cell transformation (Forte et al., 1977). Our results show that the membrane containing the gastric H⁺,K⁺-ATPase in the cytoplasm of the unstimulated parietal cell is a continuous system of interconnected helically coiled tubules that is probably always in contact with, if not a
permanent extension of the apical plasma membrane of the cell. Upon stimulation of the cell to acid secretion, these coils simply distend and progressively unwind to extend the secretory surface. Fig. 9 shows the arrangement of the membrane system envisaged for the unstimulated cell. The stimulation of the parietal cell results in the conversion of an inactive H⁺,K⁺-ATPase to an active enzyme of the unstimulated parietal cell. Electron micrographs show that the coils and tubules are densely packed in the cytoplasm. The number depicted in the diagram has been reduced for clarity.

How does tubule distension occur when the cell is stimulated? There is considerable evidence for the involvement of the cytoskeletal proteins actin, spectrin, ezrin and ankyrin in the process of parietal cell transformation (Mercier et al., 1989b; Hanzel et al., 1991; Soroka et al., 1993; Smith et al., 1993). The biochemical and immunocytochemical localization studies of these investigators have indicated that cytoskeletal components are associated with the secretory membrane in the stimulated parietal cell but not with the membranes of the unstimulated cell. This suggests that cytoskeletal proteins play a central role in the morphological transformation of the cell. Actin-associated proteins are known targets for modulation by second messengers. Experimental histamine stimulation of parietal cells via H₂-receptors is known to result in an increase in cytosolic calcium levels and the activation of adenylate cyclase with an associated elevation of cellular cAMP levels (Helander and Keeling, 1993). The increased level of cAMP correlates with phosphorylation of the 80 kDa ezrin molecule in parietal cells (Urushidani et al., 1987; Hanzel et al., 1991), and an increase in cytosolic calcium may stimulate nucleotide exchange of actin monomers, possibly mediated by profilin, and induce actin polymerization (Machensky and Pollard, 1993). Thus, we suggest that receptor-mediated signalling results in the attachment of the actin-spectrin complex to specific points of the tubule membrane, possibly via ezrin or ankyrin (Berryman et al., 1993). If the HC/CT network is not continuous with the cell membrane in the unstimulated cell, this event is presumably preceded or soon followed by fusion of those CTs in contact with the apical and canaliculus membranes to points on these membranes, thereby bringing the network into open communication with the gland lumen. Actin polymerization could then bring about distension of the tubule and, as a consequence, the helical structure would partially unwind. Significantly, phallacidin fluorescence (Soroka et al., 1993) and immunofluorescence (Mercier et al., 1989b) show that actin and spectrin are specifically localized to the secretory membranes in the stimulated parietal cell. The uncoiling would reveal the cores of the HCs as a series of anastomosing cytoplasmic rods, which in random thin sections would resemble microvilli. Our observations support the view of Vial et al. (1985) and Ogata and Yamasaki (1993) that these structures are not the same as the ‘classical’ microvilli of the intestinal brush border, which have a central core of actin filaments, or the microvilli with peripherally arranged microfilaments that occur on the apical membrane of the unstimulated parietal cell (Forte et al., 1977; Black et al., 1982). Indeed, cytoplasmic extensions in the canalicular lumen, similar to the organization that fast freeze-fixation has revealed, have been illustrated in chemically fixed stimulated parietal cells and described as ‘elaborate in shape’ (see Fig. 5 of Forte et al., 1977) and ‘pleomorphic microvilli’ (see Fig. 7 of Ito et al., 1977), implying that parietal cell microvilli have more than one form. Finally, a necessary requirement for our model is that parietal cell volume increases substantially with stimulation, and this has been seen to occur (Berglindh et al., 1980; Mangeat et al., 1990).

To date the dynamics of morphological transformation in the gastric parietal cell and the status of the cytoskeletal proteins in membrane rearrangement have been studied in chemically fixed cells which contain the gastric proton pump sequestered in a class of vesicles that cannot be identified in fast freeze-fixed, freeze-substituted cells. It is clear, therefore, that more reliable methods of preparation must be used for further analysis of transformation and, in particular, to understand the precise role of the cytoskeleton in the process.

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