State of actin in gastric parietal cells

JOHN G. FORTE, BERNICE LY, QINFEN RONG, SHOJI OGIHARA, MARLON RAMILO, BRIAN AGNEW, AND XUEBIAO YAO

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Forte, John G., Bernice Ly, Qinfen Rong, Shoji Ogihara, Marlon Ramilo, Brian Agnew, and Xuebiao Yao. State of actin in gastric parietal cells. Am. J. Physiol. 274 (Cell Physiol. 43): C97–C104, 1998.—Remodeling of the apical membrane-cytoskeleton has been suggested to occur when gastric parietal cells are stimulated to secrete HCl. The present experiments assayed the relative amounts of F-actin and G-actin in gastric glands and parietal cells, as well as the changes in the state of actin on stimulation. Glands and cells were treated with a Nonidet P-40 extraction buffer for separation into detergent-soluble (supernatant) and detergent-insoluble (pellet) pools. Two actin assays were used to quantitate actin: the deoxyribonuclease I binding assay to measure G-actin and F-actin content in the two pools and a simple Western blot assay to quantitate the relative amounts of actin in the pools. Functional secretory responsiveness was assayed by aminopyrine accumulation. About 5% of the total parietal cell protein is actin, with about 90% of the actin present as F-actin. Stimulation of acid secretion resulted in no measurable change in the relative amounts of G-actin and cytoskeletal F-actin. Treatment of gastric glands with cytochalasin D inhibited acid secretion and resulted in a decrease in F-actin and an increase in G-actin. No inhibition of parietal cell secretion was observed when phalloidin was used to stabilize actin filaments. These data are consistent with the hypothesis that microfilamentous actin is essential for membrane recruitment underlying parietal cell secretion. Although the experiments do not eliminate the importance of rapid exchange between G- and F-actin for the secretory process, the parietal cell maintains actin in a highly polymerized state, and no measurable changes in the steady-state ratio of G-actin to F-actin are associated with stimulation to secrete acid.

THE CYTOSKELETAL SYSTEMS, either actin-based microfilaments or tubulin-based microtubules, maintain fundamental cell structure and have been suggested to play a key role in the trafficking of membranes, especially in the regulated recruitment and turnover of membranes associated with a wide variety of transport processes, e.g., pancreatic insulin release (29), antidiuretic hormone (ADH)-regulated water secretion (30), chromaffin granule secretion (31), and gastric HCl secretion (6, 21). For several of these regulated vesicular transport processes, a meshwork of filamentous actin (F-actin) in the cell cortex just beneath the plasma membrane apparently represents a barrier for vesicles that are to be recruited to the surface, and access of vesicles to the target plasma membrane is proposed to occur through regulated depolymerization of F-actin filaments to the monomeric, G-actin, form (4, 18, 31, 36). Indeed, the continuous, rapid interconversion of F-actin and G-actin, carefully controlled by a host of actin-binding proteins, is a hallmark of cytoskeletal remodeling associated with mobility of cells. On the other hand, stable actin filaments are essential to vesicular traffic in a number of systems. Microfilamentous cores of intestinal microvilli have been proposed to represent “rails” on which membrane vesicles might be conveyed to the surface by molecular motor proteins, such as myosin I (13). Moreover, myosin I and the unconventional myosins have been implicated as the protein motors to facilitate the cytoplasmic streaming of organelles along actin bundles in algal cells (1, 2). It is not unreasonable to predict that a volatile F-actin pool (to afford membrane-membrane interaction) and a stable F-actin pool (to provide conveyance and microvillar support) may cooperate in regulated vesicular trafficking.

Regulated secretion of HCl by the gastric parietal cell involves an elaborate surface remodeling as cytoplasmic vesicles containing the proton pump, H+ -K+ -ATPase, are recruited to the apical surface resulting in the transition from short to long microvillar extensions (14, 15). The observed inhibition of secretion by microfilament and microtubule inhibitors has been taken as suggestive evidence that the cytoskeleton plays a central role in the apical membrane remodeling associated with stimulation of the parietal cell (6, 21). Because of the close relationship of actin microfilaments to the extended secretory microvilli and the implied importance of the cytoskeleton to parietal cell secretion, we sought a more direct test of the state of actin associated with secretory activity. The purpose of the present experiments was to assay the relative amounts of F- and G-actin in gastric glands and parietal cells and to determine the changes in the state of actin when parietal cells were stimulated to secrete HCl. Accordingly, we used the deoxyribonuclease (DNase I) binding assay to measure G-actin and total actin content before and after isolated gastric glands or parietal cells were stimulated by histamine. We also used a simple Western blot assay to quantitate the relative amounts of actin in the detergent-soluble and detergent-insoluble pools taken from the glands and cells in resting and secreting states. For all experiments, the functional secretory responsiveness was assayed by the ability to accumulate weak base within an acidic space (aminopyrine accumulation assay). Changes in the secretory response and state of gastric glandular actin were also measured after applying agents that specifically alter the state of the actin cytoskeleton. The experiments show that parietal cells are rich in actin, especially in the F-actin form (~90% of total actin), and that there are no significant changes in the steady-state levels of F-actin and G-actin when the the cells are stimulated to secrete acid. Cytochalasin D promoted a depolymerization of F-actin to G-actin and inhibited acid secretion, whereas stabilization of actin filaments using phalloidin did not inhibit acid secretion.
METHODS

Isolation of gastric glands. Gastric glands were isolated from New Zealand White rabbits essentially as described by Berglindh (5) and more recently described by Yao et al. (39). Gastric mucosa from perfused stomach was digested for 15 min at 37°C with pronase (0.5 mg/ml) in MEM containing 1 mg/ml bovine serum albumin (BSA; fraction V) and 2 mg/ml glucose. The partially digested mucosa was then subjected to three washes of MEM and incubated for an additional 30–40 min in the same incubation medium with 0.8 mg/ml collagenase (Sigma; −350 U/mg). After digestion with collagenase, cells were washed three times in a medium containing (in mM) 114.4 NaCl, 5.4 KCl, 5.0 Na₂HPO₄, 1 NaH₂PO₄, 1.2 MgSO₄, 1 CaCl₂, 0.5 dithiothreitol (DTT), 10 glucose, 1 pyruvate, and 10 N₂-ethanesulfonic acid (HEPES; pH 7.4), as well as 2 mg/ml BSA and 10 mg/ml phenol red. Cells were then resuspended in the same medium containing 10 mg/ml BSA, placed on top of nycodenz gradients (see below) and centrifuged at room temperature in a swinging bucket rotor at 1,000 g for 15 min. The uppermost band at the first gradient interface was collected as white blood cells.

Isolation and separation of glandular epithelial cells. Cells were prepared by a modified method described by Chew and Brown (11). Gastric mucosa from perfused stomach was digested for 15 min at 37°C with pronase (0.5 mg/ml) in MEM containing 1 mg/ml bovine serum albumin (BSA; fraction V) and 2 mg/ml glucose. The partially digested mucosa was then subjected to three washes of MEM and incubated for an additional 30–40 min in the same incubation medium with 0.8 mg/ml collagenase (Sigma; −350 U/mg). After digestion with collagenase, cells were washed three times in a medium containing (in mM) 114.4 NaCl, 5.4 KCl, 5.0 Na₂HPO₄, 1 NaH₂PO₄, 1.2 MgSO₄, 1 CaCl₂, 0.5 dithiothreitol (DTT), 10 glucose, 1 pyruvate, and 10 N₂-ethanesulfonic acid (HEPES; pH 7.4), as well as 2 mg/ml BSA and 10 mg/ml phenol red. Cells were then resuspended in the same medium containing 10 mg/ml BSA, placed on top of nycodenz gradients (see below) and centrifuged at room temperature in a swinging bucket rotor at 1,000 g for 8 min. The top interface from the gradient was collected as the parietal cell-rich fraction, which was characterized at a purity of 85–90% by mitochondrial staining with nitrotetrazolium blue (5). A fraction, which was characterized at a purity of 85–90% by mitochondrial staining with nitrotetrazolium blue (5), aK was drawn from a rabbit into a heparinized syringe, and the cells were harvested and washed three times with phosphate-buffered saline (PBS; in mM: 149.6 NaCl, 3 K₂HPO₄, 0.6 NaH₂PO₄, 1 MgSO₄, and 1CaCl₂) by centrifugation at 2,500 × g for 15 min. Cells were resuspended in PBS and placed on top of a second nycodenz gradient (see below) and centrifuged for 15 min. The uppermost band at the second gradient interface was collected as glandular epithelial cells.

Isolation of white blood cells. Blood (~20 ml) was withdrawn from a rabbit into a heparinized syringe, and the cells were harvested and washed three times with phosphate-buffered saline (PBS; in mM: 149.6 NaCl, 3 K₂HPO₄, 0.6 NaH₂PO₄, 1 MgSO₄, and 1CaCl₂) by centrifugation at 2,500 × g for 15 min. Cells were resuspended in PBS and placed on top of the second nycodenz gradient described for parietal cell isolation, followed by centrifugation at 2,500 × g for 10 min in a swinging bucket rotor (see above). The uppermost band at the first gradient interface was collected as white blood cells.

Permeabilization with α-toxin. In cases in which phalloydin was added to stabilize F-actin filaments, cells were permeabilized with α-toxin, which renders the cells permeable to molecules in the range of 1 kDa while maintaining 60–80% of the functional secretory responsiveness (35, 38). Before treatment with α-toxin, intact glands or cells were washed once in a K⁺-rich permeabilization medium (K medium) containing (in mM) 20 HEPES, 10 Tris (pH 7.4), 100 KCl, 1,2 MgSO₄, 1 NaH₂PO₄, and 40 mannitol. Glands or parietal cells were resuspended in K medium at a 10% cytocr and incubated with α-toxin at 37°C for 45 min in the case of glands and 20 min in the case of isolated cells. Cimetidine (100 µM) was also included in this and all subsequent steps to antagonize endogenous histamine that might be released. After permeabilization, the suspensions were diluted to a 5% cytocr in K medium to which 10 mM succinate and 1 mM pyruvate were included as oxidative substrates. The extent of parietal cell permeabilization was routinely estimated by trypan blue uptake as described by Thibodeau et al. (35).

Stimulation of parietal cells and measurement of aminopyrine accumulation. [¹⁴C]Aminopyrine was added to the cellular suspension in either MEM (intact glands and cells) or K medium (permeabized cells) to a final concentration of 4 × 10⁻⁴ mM. Aliquots (0.5 ml) of the suspension were distributed into preweighed 1.5-ml tubes already containing secretagogues or activators as specified in each individual experiment. The performance of and calculations for the aminopyrine uptake assay were essentially as described by Yao et al. (39). Intact preparations were stimulated with histamine (100 µM) and 3-isobutyl-1-methylxanthine (IBMX; 50 µM). α-Toxin-permeabilized cells were stimulated by adding 0.1 mM adenosine 3',5'-cyclic monophosphate (cAMP) and 1 mM ATP to the K medium (35).

Detergent extraction of glands and cells. Detergent-soluble and detergent-insoluble fractions were prepared as follows. All steps were performed at room temperature. Typically, a gland or cell pellet was treated with a 20-fold volume of extraction buffer (usually ~500 µl) containing 0.1% Nonidet P-40 (NP-40), 5 mM K₂HPO₄, 27 mM NaH₂PO₄ (pH 7.2), 2 mM MgSO₄, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.2 mM ATP, 0.5 mM DTT, 2 M glycerol, and 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor) for 15 min with agitation. The preparations were then centrifuged (400 g) for 1 min and separated into a soluble fraction (NP-40 supernatant) and an insoluble cytoskeletal fraction (NP-40 pellet). Preliminary experiments were carried out to optimize the time of extraction. To minimize interconversion between actin forms, the NP-40 supernatant extracts were assayed for G-actin within 1–5 min of initial extraction. The NP-40 cytoskeletal pellets were resuspended in ~500 µl of extraction buffer and subsequently assayed for G-actin and total actin. Because our preliminary tests showed virtually no G-actin remaining in the NP-40 pellets, assays on this fraction were delayed until the NP-40 supernatants were completed.

DNase I assay of G-actin and total actin. The assay of G-actin and total actin was essentially according to the DNase I inhibition assay (8) with modifications described by Heacock and Bamburg (19). The DNA solution consisted of 80 µg/ml DNA (type XIV from herring testes, Sigma Chemical, D6898) in 0.1 M Tris·HCl, 4 mM MgSO₄, and 1.8 mM CaCl₂ (pH 7.5). The DNase I stock solution was made up as 10 mg/ml DNase I (bovine pancreas, Sigma Chemical, DN-25) in 0.125 M Tris·HCl, 5 mM MgCl₂, 2 mM CaCl₂, and 1 mM Na₃ EDTA (pH 7.5) and stored at −20°C. For the DNase working solution, the stock DNase was diluted 1:100 in 20 mM imidazole, 30 mM NaCl, and 15% glycerol (pH 7.0) and maintained on ice until use.

Assays for G-actin were carried out at room temperature by adding 20 µl of DNase I working solution and the desired amount of unknown sample, or standard G-actin, to 3 ml of DNA solution in a quartz cuvette and immediately measuring the rate of DNA hydrolysis at 260 nm in a Varian spectrophotometer. The resulting curve for (optical density)dt has three portions: a slow initial rate and a major linear portion, followed by a saturating portion. The slope of the linear portion of the curve was inversely proportional to the concentration of G-actin as determined by standard curves constructed with purified G-actin (the higher the concentration of G-actin, the greater the inhibition of DNA hydrolysis rate). The volume of unknown samples added to the cuvette was adjusted so that the slopes fell within the linear range, and assays were always carried out in duplicate or triplicate.
To determine total actin, standards or unknown samples were treated on ice for 15 min with an equal volume of guanidine hydrochloride solution to depolymerize F-actin. The depolymerizing solution contained 1.5 M guanidine hydrochloride, 1 M sodium acetate, 1 mM CaCl₂, 1 mM ATP, and 20 mM Tris·HCl (pH 7.5). After a brief centrifugation to remove remnant nuclear material, aliquots were combined in a cuvette with DNase I and DNA solutions for actin assay as described above. F-actin was calculated as the difference between total actin and initially measured G-actin.

Actin measurements by Western blotting. In some cases total actin content in the NP-40 supernatant and pellet was measured by running aliquots on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% running gel), blotting the protein to nitrocellulose, and probing for actin using a commercial actin antibody (Amersham Life Science). The G-actin was immediately measured in supernatant fraction (means ± SE); for 5, 10, and 15 min, n = 3; for all other time points, n = 2. B: values for actin in 1 set of samples. G-actin (G) and total actin were measured by deoxyribonuclease I assay; F-actin (F) is reported as total actin – G-actin. Sup, supernatant; Pel, pellet.

RESULTS

Time course of protein extraction. To establish an appropriate time of extraction, we evaluated the time course of treatment with NP-40 extraction buffer at room temperature, followed by separation into supernatant and pellet fractions. Figure 1 shows the time course of protein and actin extraction from isolated parietal cells. Protein was rapidly released into the supernatant (~45% of total protein was released within 1 min) and reached a steady state by 10–15 min, at which time ~80% of total cell protein was in the supernatant and 20% in the residual pellet (Fig. 1A). The relative amounts of G- and F-actin in the supernatant and pellet fractions, as measured by the DNase 1 assay, are shown in Fig. 1B. The majority of parietal cell actin remained with the pellet throughout the extraction, and most of the pellet actin was in the F-actin form. The supernatant contained both G- and F-actin. G-actin increased initially and leveled off by 10 min. A small amount of G-actin was initially present in the pellet, and this decreased so that by 15 min there was no measurable G-actin in the pellet. The combined G-actin, supernatant plus pellet, remained constant at ~10% of total parietal cell actin throughout the time of extraction. The F-actin in the supernatant showed a slight decline over the first 5–10 min of extraction. Because the total G-actin-to-F-actin ratio remained constant over the time course of extraction, it appeared that the extraction conditions were stable. Also, on the basis of these experiments we decided to treat cells and glands with extraction buffer for 15 min as a standard protocol of extraction.
Steady-state levels of G-actin and F-actin do not vary with secretory state of gastric glands

Table 2. Steady-state levels of G-actin and F-actin do not vary with secretory state of gastric glands

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<thead>
<tr>
<th></th>
<th>Resting Glands</th>
<th>Stimulated Glands</th>
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<tr>
<td></td>
<td>G-actin</td>
<td>F-actin</td>
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<tr>
<td>Supernatant, µg/mg protein</td>
<td>5.2 ± 0.9</td>
<td>15.1 ± 4.6</td>
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<tr>
<td>Supernatant, % of total</td>
<td>9.9 ± 2.9</td>
<td>25.6 ± 3.2</td>
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<tr>
<td>Pellet, µg/mg protein</td>
<td>0.7 ± 0.6</td>
<td>35.5 ± 5.2</td>
</tr>
<tr>
<td>Pellet, % of total</td>
<td>1.0 ± 0.7</td>
<td>63.3 ± 3.6</td>
</tr>
<tr>
<td>Total, µg/mg protein</td>
<td>5.9 ± 1.0</td>
<td>50.6 ± 9.5</td>
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<tr>
<td>Total, % of total</td>
<td>10.9 ± 2.7</td>
<td>89.1 ± 2.7</td>
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Values are means ± SE for 3 separate gastric gland preparations. Actin in each fraction is expressed both as µg actin (G-, F-, or total actin) per mg of total cellular protein (supernatant + pellet) and as % of total actin. Protein is expressed as % of total protein. Before extraction, glands were treated for 20 min with either 10^{-4} M cimetidine (resting) or 10^{-4} M histamine + 5 × 10^{-5} M 3-isobutyl-1-methylxanthine (IBMX; stimulated). For each experiment, parallel sets of gastric glands were taken for [14C]aminopyrine uptake assay as an evaluation of degree of stimulation; aminopyrine uptake ratios were 24.4 ± 1.4 for resting and 132 ± 34.3 for stimulated glands. There were no significant differences in steady-state levels of G- or F-actin between resting and stimulated preparations (P > 0.1, t-tests).

Insoluble cytoskeletal fraction contained the largest pool of actin (~64% of total), recovered almost exclusively as F-actin. About 15% of the total gastric glandular actin was measurable as G-actin; the remaining 85% was measured only after guanidine treatment and therefore taken to be F-actin. The data for isolated cells, also depicted in Table 1, indicate that the more highly purified parietal cell populations contained a higher proportion of F-actin than either the parietal cell-poor fractions or the intact gastric glands. In isolated cell fractions, judged to be 85–91% parietal cells, F-actin represented ~90% of the total actin, and most of this was found in the NP-40-insoluble cytoskeletal fraction. On the other hand, cell fractions relatively poor in parietal cells contained less total actin, which in turn was somewhat more skewed toward the G-actin form (i.e., ~80% of total actin occurred as F-actin).

Concerns have been raised regarding methodology that may lead to inaccurate measurements of G-actin and F-actin in cells (19). The high percentage of F-actin in gastric glands, parietal cells, and even gastric nonparietal cells could be the result of polymerization of G-actin induced by the NP-40 extraction buffer. To test this hypothesis, white blood cells, which contain relatively high proportions of G-actin (24, 25, 33) were isolated and extracted with the same NP-40 extraction buffer and conditions used for the gastric samples. The last column in Table 1 illustrates the distribution of actin in white blood cells. About one-half of the total actin in the white blood cells was measured as G-actin in the NP-40 supernatant; relatively small amounts of F-actin (~7% of total) were extracted into the NP-40 supernatant (Table 1). These results support the notion that the high F-actin percentage is not an artificial state induced by the NP-40 extraction buffer, and they demonstrate that parietal cells belong to the class of cells in which the distribution of actin is very heavily skewed toward the filamentous cytoskeletal form.

Measurements of actin in resting and stimulated gastric glands. We next sought to evaluate the steady-state levels of actin forms in gastric glands and parietal cells during various states of secretory activity. Table 2 shows actin and protein measured in freshly isolated gastric glands maintained in a resting state compared with glands stimulated with secretagogues to secrete acid. Stimulated glands demonstrated good secretory responsiveness, showing five- to sixfold increases in [14C]aminopyrine uptake ratios over the resting counterparts. However, there were no significant differences between the resting and stimulated glands for the steady-state distribution of actin in the NP-40 supernatant and pellet or in the distribution of G- and F-actin in those fractions. For both resting and stimulated preparations, actin represents ~5% of the total glandular protein, and this was predominantly in the F-actin form. As with previous results, G-actin comprised only ~10% of the total actin.

An experimental time course of stimulation and inhibition is shown in Fig. 2. Gastric glands were treated with histamine for 20 min and then exposed to a powerful histamine-H2 blocker, SKF-9347 (7), to restore the glands to a resting state. Aliquots of glands were taken at various times over the treatment regimen and assayed for G- and F-actin in the NP-40 supernatant and total actin in the pellet. Because there was typically negligible G-actin in the pellet, total pellet actin was assumed to be equivalent to pellet F-actin. Again there were no significant differences in the steady-state distribution of actin forms between resting glands and those stimulated to secrete acid. Over the 60 min after acid secretion was inhibited by SKF-9347, there was a slight decrease in the amount of F-actin that sedimented with the NP-40 pellet, but this reached the 5% level of significance only for the preparations at 30 min postinhibition.

A summary of experiments for resting and stimulated parietal cells is shown in Table 3. As was the case for the gastric glands, there were no significant differences between resting and stimulated cells in the steady-state levels and distribution of actin forms.

Cytochalasin D produces secretory changes and changes in the state of gastric glandular actin. Figure 3 shows that treatment of gastric glands with cytochalasin D disrupted actin filaments, as judged by a significant decrease in the NP-40-insoluble cytoskeletal F-actin pool and an increase in G-actin content. In addition, there was a slight increase in the F-actin in the NP-40 supernatant. These changes in the relative distribution of G- and F-actin occurred at both 1 and 10 µM cytochalasin. Aminopyrine accumulation in response to histamine plus IBMX was also significantly...
depressed in these same cytochalasin-treated glands, consistent with earlier studies showing inhibition of acid secretion in functionally intact gastric mucosa (6).

Stabilization of actin filaments by phalloidin does not alter the secretory response. To ensure ready access of gastric cells to phalloidin, glands or parietal cells were permeabilized with α-toxin, a model that we previously showed to be functionally responsive to cAMP. We used rhodamine-labeled phalloidin to visualize the ready permeation of phalloidin into parietal cells (not shown). Treatment of α-toxin-permeabilized gastric glands with 1 or 10 μM phalloidin did not interfere with their ability to respond to cAMP as a stimulant (Fig. 4).

| Table 3. Steady-state levels of G-actin and F-actin do not vary with secretory state of parietal cells |
|-------------------------------------------------|-------------------------------------------------|
| Resting Glands                                   | Stimulated Glands                                |
| G-actin, µg/mg protein                          | G-actin, µg/mg protein                          |
| 4.9 ± 2.0                                       | 5.4 ± 2.1                                       |
| F-actin, µg/mg protein                          | F-actin, µg/mg protein                          |
| 11.0 ± 2.2                                      | 9.3 ± 1.7                                       |
| Protein                                         | Protein                                         |
| 82.4 ± 1.3                                      | 79.6 ± 2.4                                      |
| Supernatant, % of total                         | Supernatant, % of total                         |
| 9.8 ± 2.2                                       | 10.3 ± 2.3                                      |
| Pellet, µg/mg protein                           | Pellet, µg/mg protein                           |
| 0.2 ± 0.2                                       | 0.2 ± 0.1                                       |
| Pellet, % of total                              | Pellet, % of total                              |
| 0.4 ± 0.2                                       | 0.3 ± 0.2                                       |
| Total, µg/mg protein                            | Total, µg/mg protein                            |
| 5.0 ± 2.0                                       | 5.5 ± 2.1                                       |
| Total, % of total                               | Total, % of total                               |
| 10.1 ± 2.2                                      | 10.6 ± 2.3                                      |

Values are means ± SE for 5 separate parietal cell preparations. Actin in each fraction is expressed both as µg actin (G-actin, F-actin, or total actin) per mg of total cellular protein (supernatant + pellet) and as % of total actin. Protein is expressed as % of total protein. Before extraction, cells were treated for 20 min with either 10^-4 M cimetidine (resting) or 10^-4 M histamine + 5 × 10^-5 M IBMX (stimulated). For each experiment, parallel sets of parietal cells were taken for [3H]aminopyrine uptake assay as an evaluation of degree of stimulation. Aminopyrine index of stimulation (ratio of aminopyrine accumulation of stimulated cells/aminopyrine accumulation of resting cells) for 5 preparations was 7.84 ± 1.0-fold increase. There were no significant differences in the steady-state levels of G- or F-actin between resting and stimulated preparations (P > 0.1, t-tests).
Fig. 4. Phalloidin does not inhibit stimulation of acid secretion by α-toxin-permeabilized gastric glands. Glands were permeabilized with α-toxin as described in METHODS and incubated for 20 min with 10⁻⁴ M cimetidine (resting) or with 10⁻⁴ M cAMP + 10⁻³ M ATP (stimulated) containing 0, 1, or 10 μM phalloidin as indicated. After 20 min, glands were taken for [¹⁴C]aminopyrine uptake assay as an index of acid secretion. Values are means ± SE for 3 separate preparations.

guanidine were necessary to disrupt the phalloidin-F-actin association and depolymerize actin for assay. To avoid this difficulty, we used the alternative Western blot assay of actin in the NP-40 supernatant and pellet fractions from gastric glands and parietal cells. A major disadvantage is that the blotting method does not distinguish between G- and F-actin but simply assays actin association and depolymerize actin for assay. To overcome this limitation, both the blotting method and the DNase I assay were used. Table 4 shows the results of aminopyrine accumulation and actin measurements for α-toxin-permeabilized parietal cells treated with 5 μM phalloidin in the resting and stimulated states. For control cells, with no phalloidin added, the results were similar to those obtained with the DNase I assay (cf. Tables 2 and 3), with the exception that the blotting method indicated proportionally less actin in the NP-40 supernatant (~15% of total) than the DNase I assay (~33% of total). This difference between the two methods was consis-

tent over many experimental measurements, and we have no explanation for the discrepancy. In any event, there were no changes in the distribution of actin in the NP-40-soluble and -insoluble fractions associated with stimulation of the parietal cells. Treatment of parietal cells with 5 μM phalloidin produced a small decrement in the NP-40 supernatant actin and a slight increase in cytoskeletal actin, consistent with a stabilization of F-actin (Table 4). Similar to what was observed for gastric glands, phalloidin did not inhibit the transition from rest to stimulation in α-toxin-permeabilized cells treated with cAMP.

DISCUSSION

From results presented here we draw the following conclusions. Parietal cells are rich in actin, representing ~5% of the total protein, with ~90% of the actin present in the filamentous form (F-actin). Treatment of gastric glands with cytochalasin D resulted in the expected inhibition of acid secretion as well as a decrease in F-actin and an increase in G-actin. On the other hand, hyperstabilization of actin filaments with phalloidin produced no inhibition of parietal cell secretion. Stimulation of acid secretion via the cAMP pathway resulted in no measurable change in the relative amounts of G-actin, short filamentous actin, and cytoskeletal F-actin. These data are consistent with the hypothesis that microfilamentous actin is essential for membrane recruitment underlying parietal cell secretion. However, activation of secretion involves little or no change in steady-state F-actin-to-G-actin transformation. This last conclusion was most surprising in view of the rather profound apical membrane transformations associated with stimulation and the evidence that comes from several other secretory systems.

In stimulated adrenal chromaffin cells, disassembly of cortical actin was found to precede catecholamine secretion and was correlated with increased levels of cytosolic Ca²⁺ (10). Disassembly of actin was also observed in digitonin-permeabilized chromaffin cell actin treated with micromolar levels of Ca²⁺ (9). Using immunogold electron microscopy to double-label chromaffin granules and F-actin, Nakata and Hirokawa (27) found that the cytoskeletal reorganization in response to stimulation of cultured chromaffin cells was not massive but appeared to involve discrete depolymerization of the cortical cytoskeleton proximal to each exocytic site. In the case of ADH-regulated water transport, rapid depolymerization of F-actin was observed in toad bladder epithelial cells stimulated with ADH or 8-bromoadenosine 3’5’-cyclic monophosphate (12). Concomitant with exocytosis and increased water transport, monomeric G-actin increased from 37 to 54% of the total actin (18). In most cells the concentration of G-actin greatly exceeds its critical concentration for polymerization, indicating that G-actin-binding proteins are required to sustain the steady state and suggesting that regulation of the G-actin sequestration system may be fundamental to the exocytic event. Confocal microscopy (20) and quantitative immunogold techniques (15) were used to demonstrate that actin

<table>
<thead>
<tr>
<th>Aminopyrine uptake ratio</th>
<th>Control Cells</th>
<th>Phalloidin-Treated Cells</th>
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<tr>
<td></td>
<td>Resting</td>
<td>Stimulated</td>
</tr>
<tr>
<td>Supernatant actin, % of total</td>
<td>7.5±0.8</td>
<td>67.4±4.6</td>
</tr>
<tr>
<td>Pellet actin, % of total</td>
<td>13.9±0.1</td>
<td>14.9±2.6</td>
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<tr>
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<td>86.1±0.1</td>
<td>85.1±2.6</td>
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</table>

Values are means ± SE for 3 separate parietal cell preparations. After isolation, cells were permeabilized with α-toxin and separated into 2 batches, 1 treated as control and 1 treated with 5 μM phalloidin. Aliquots from each batch were incubated for 20 min with either 10⁻⁴ M cimetidine (resting) or 10⁻⁴ M cAMP + 10⁻³ M ATP (stimulated), after which all cells were treated with NP-40 extraction buffer and assayed for actin. Actin was measured by Western blot of aliquots of NP-40 supernatant and pellet material and is expressed as % of total actin measured for the cell preparation. Parallel sets of parietal cells were taken for [¹⁴C]aminopyrine uptake assay to evaluate the degree of stimulation.
depolymerization in ADH-treated toad bladder cells occurred only at the apical pole, and that this cortical layer of actin selectively depolymerized between microvilli but not within the microvilli per se. On the other hand, net depolymerization of F-actin by low levels of cytochalasin B produced no increase in agruphore fusion or water flow (37). To test whether the cortical actin cytoskeleton provides a barrier for cholecystokinin (CCK)-regulated exocytosis in pancreatic zymogen secretion, Muallem et al. (26) measured the release of amylase from acinar cells permeabilized with streptolysin O so that they could introduce β-thymosins as specific monomeric actin-binding proteins. Low concentrations of the actin monomer-sequestering peptides triggered a rapid and robust exocytosis with a profile similar to the initial phase of CCK stimulation, but high concentrations of the polypeptides inhibited all phases of exocytosis, suggesting that some basic cytoskeletal structure was essential for the secretory event.

O’Konski and Pandol (28) offered alternative evidence for a functionally stable pool of F-actin, showing that diminished secretory activity caused by hyperstimulating acinar cells was associated with marked degradation of the apical cytoskeleton. Collectively, these studies are consistent with the hypothesis that cortical F-actin impedes access for membrane recruitment and that some transient and selective depolymerization must occur for secretion; however, they also indicate that a minimal actin cytoarchitecture is necessary for exocytosis, possibly acting as a structural support for apical surface activity and/or a pathway for vesicular transport.

Remodeling of the apical membrane-cytoskeleton has been implicated in acid secretion by parietal cells. Although there is some disagreement on the specific source of the expanded secretory surface (15, 32), morphological evidence clearly demonstrates the conversion of short to long microvilli when cells are stimulated. A major question is, How do the microvilli grow: do they extend from the tips, or do they “grow” into the cell? Related to this are the nature and dynamics of the microfilament support system for the microvilli. Because of the morphological arrangement within the restricted space of parietal cell canaliculi, secretion-dependent microvillus growth would have to occur into the cytoplasmic space and could not occur by tip elongation as usually happens in the case of filapodia and microspikes or developing intestinal microvilli. This view is consistent with the observation that the bases of the microvilli extend deep into the cytoplasm of maximally stimulated cells. Thus it seems reasonable to conclude that microvillus elongation occurs by adding membrane at the microvillar base, with consequent extension or “growth” into the cell. In resting parietal cells, organized bundles of microfilaments have been shown to extend into the cytoplasm 1 μm or more beneath the short microvilli (6), potentially representing the structural framework for elongation. However, it is not clear whether filament length is fixed or whether they undergo regulated growth and extension to accommodate elongated microvilli. Moreover, the polarity of the microfilamentous bundles within parietal cell microvilli has not been determined. The presumed action of membrane-cytoskeletal attachment proteins such as ezrin or nonconventional myosins, both of which are strongly expressed in parietal cells (3, 17), would provide the bonding sites for shaping the expanded membrane surface into elongated microvilli.

The present data indicate that parietal cell microvillus elongation occurs without a major shift in the steady-state G-actin-to-F-actin ratio. This apparent intransigence of the steady-state actin forms does not preclude the importance of actin turnover associated with secretory activation. It is possible that the turnover between G- and F-actin may be rapid and continuous, so that depolymerization from one pool is compensated by polymerization into another pool within the time resolution of our measurements. Alternatively, there could be a large pool of stable microfilamentous actin, for example, associated with microvilli, and a smaller pool of cortical dynamic actin between microvilli, whose depolymerization would be difficult to detect in the large background of stable F-actin filaments. Both of these general possibilities would allow for activation-dependent cytoskeletal reorganization within an apparently constant G-actin-to-F-actin ratio. On the other hand, the addition of phalloidin to α-toxin-permeabilized parietal cells did not inhibit or prevent the cAMP-mediated accumulation of aminopyrine by glands or parietal cells, suggesting that stabilization of parietal cell F-actin did not alter the response to stimulation. In this regard, phalloidin stabilization of actin filaments in the T84 intestinal cell line did not alter the activation and insertion of Cl− channels at the apical membrane, although cAMP-induced activation of the Na+/K⁺-2Cl⁻ cotransporter at the basolateral membrane appeared to be microfilament dependent and responsible for inhibitory effects in the phalloidin-loaded state (22, 23). These observations suggest that disorganization of the apical actin network may not be required for regulated exocytic recruitment of transport proteins in all cases. In fact, secretion of insulin by permeabilized pancreatic islet cells was reportedly enhanced by stabilizing F-actin with phalloidin (34).

In summary, the results of this study support the conclusion that filamentous actin is important for initiating or maintaining the regulated secretion of HCl by the parietal cell. The parietal cell maintains actin in a highly polymerized state, with 90% of total actin as F-actin, and there were no measurable changes in the steady-state ratio of G-actin to F-actin associated with stimulation of parietal cells to secrete acid. In addition, treatment of α-toxin-permeabilized parietal cells with the F-actin stabilizing agent phalloidin did not inhibit secretory activity. Although these latter data are consistent with a functional role for a cytoskeletal framework in parietal cell activation, they do not eliminate the importance of the rapid recycling of actin between the F- and G-actin forms as secretion and recovery from secretory activity occur. More dynamic measurements of actin turnover and cytolocalization of the dynamic
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