Functionally distinct pools of actin in secretory cells

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Ammar, David A., Phuong N. B. Nguyen, and John G. Forte. Functionally distinct pools of actin in secretory cells. Am J Physiol Cell Physiol 281: C407–C417, 2001.—Acid secretion by the gastric parietal cell is thought to be regulated by the recruitment of vesicles containing the primary proton pump, the H⁺-K⁺-ATPase (HK). We have used latrunculin B (Lat B), which binds to monomeric actin, to investigate actin turnover in the stimulated parietal cell. In isolated gastric glands, relatively high concentrations of Lat B were required to inhibit acid accumulation (ED₅₀ ~ 70 μM). Cultured parietal cells stimulated in the presence of low Lat B (0.1–1 μM) have reduced lamellipodia formation and some aberrant punctate phalloidin-stained structures, but translocation of HK and vacuolar swelling appeared unaffected. High Lat B (10–50 μM) resulted in gross changes in actin organization (punctate phalloidin-stained structures throughout the cell and nucleus) and reduced translocation of HK and vacuolar swelling. Resting parietal cells treated with high Lat B showed minor effects on morphology and F-actin staining. If resting cells treated with high Lat B were washed immediately before stimulation, they exhibited a normal stimulated morphology. These data suggest distinct pools of parietal cell actin: a pool highly susceptible to Lat B primarily involved in motile function of cultured cells; and a Lat B-resistant pool, most likely microvillar filaments, that is essential for secretion. Furthermore, the stimulation process appears to accentuate the effects of Lat B, most likely through Lat B binding to monomer actin liberated by the turnover of the motile actin filament pool.

parietal cell; latrunculin B; secretion; cytoskeleton

Acid secretion by the gastric parietal cell is thought to be regulated by the recruitment of vesicles containing the primary proton pump, the H⁺-K⁺-ATPase (HK), from a broadly distributed compartment of cytoplasmic vesicles to the apical plasma membrane (13, 16). Although the actual mechanism for the movement and fusion of these vesicular structures is not completely understood, there is evidence to suggest the involvement of the actin cytoskeleton (12). Ordinarily, ~90% of parietal cell actin is present in the filamentous (F-actin) form and much of this is highly structured at the elaborate apical canalicular membrane (14). In stimulated gastric mucosa, cytochalasin disrupts apical microvilli and inhibits acid secretion, although relatively high doses and a long incubation time are required (5). Cytochalasin D was also found to inhibit acid secretion and increase the proportion of G-actin in isolated gastric glands (14). Interestingly, the steady-state ratio of F-actin to G-actin (F:G ratio) was not altered when resting parietal cells were stimulated to secrete acid (14), although the stimulation-associated recruitment of HK-rich membranes is known to result in a large increase in apical canalicular surface area. Thus secretion of HCl by the parietal cell appears to require an intact actin cytoskeleton, but it is not certain whether, or how, it changes as the structural and functional activities of the cell are transformed by stimulation.

The present studies were undertaken to reexamine the role of the cytoskeleton in gastric HCl secretion. The principal approach was to determine the structural and functional effects of latrunculin B (Lat B) on isolated gastric glands and cultured parietal cells. The latrunculins (A and B), originally isolated as lethal toxins from the Red Sea sponge Latrunculia magnifica, are membrane-permeable macrodides that disrupt the actin cytoskeleton (23). Steady-state assays have revealed that latrunculin A (Lat A) binds G-actin in a 1:1 ratio with an equilibrium dissociation constant of 2 μM and prevents filament formation; furthermore, kinetic assays indicate that Lat A causes a lag in polymerization (8). These data suggest that the Lat A:G-actin complex is incompetent in both nucleation and polymerization reactions. On the other hand, Lat A does not promote depolymerization of F-actin filaments (2), indicating that its direct interaction is primarily with the free actin monomer. Latrunculin, therefore, appears to act as a thymosin-like substance, sequestering the actin monomer away from sites of actin polymerization, but not influencing the rate of actin turnover. Because the steady state of parietal cell actin is known to exist in the predominant F-actin form, we thought it would be of interest to test the structural and functional effects of agents, like latrunculin, whose action appears to be directed toward the sequestration of cellular actin from the F-actin form.

Methods

Isolation of gastric glands. New Zealand White rabbits were sedated with a subcutaneous cocktail of 100 mg/ml ketamine, 20 mg/ml xylazine, and 10 mg/ml acepromazine

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male. Pentobarbital sodium (Nembutal; Abbott) was administered intravenously to achieve surgical anesthesia, and a midline abdominal incision was made. The aorta was then exposed, clamped, and a cannula was inserted therein for perfusion with PBS (150 mM NaCl, 3.64 mM phosphate, 1 mM CaCl2, and 1 mM MgSO4, pH 7.4) at high pressure. After being cleared of blood, the stomach was removed from the animal, opened, and washed several times with PBS. The animal was killed by a lethal dose of Nembutal. The gastric mucosa was scraped from the smooth muscle layer and finely minced. The minced mucosa was washed three times with PBS and twice with Eagle's minimum essential medium (GIBCO) buffered with 20 mM HEPES, pH 7.4 (HEPES-MEM). The minced mucosa was digested at 37°C for ~30 min in a minimal amount (~20 ml) of HEPES-MEM that contained 25 mg each of collagenase (Sigma) and bovine serum albumin (BSA). The reaction was stopped by diluting the collagenase solution into 200 ml of HEPES-MEM, and large pieces of undigested mucosa were removed by filtering the solution through a nylon mesh. Because of their large size, relatively few gastric glands settled out in 10–15 min, leaving individual cells and debris suspended in the medium. The cell suspension was removed to harvest cells, and the glands were washed by settling three times in HEPES-MEM.

Isolation and primary culture of gastric parietal cells. Cultures enriched in parietal cells were obtained from the above cell suspension. Large debris were removed by strain- ing the cell suspension through a 40-μm mesh. Intact cells were recovered by three repetitions of centrifugation at 200 g for 5 min, followed by resuspension in fresh HEPES-MEM. To destroy contaminating yeast and bacteria, the cells were incubated for 30 min in medium B (DMEM/F-12 (GIBCO) supplemented with 20 mM HEPES, 0.2% BSA, 10 mM glucose, 8 mM epidermal growth factor, 1× SITE medium (Sigma), 1 mM glutamine, 100 U/ml penicillin/streptomycin, 400 μg/ml gentamicin sulfate, 25 μg/ml amphotericin B, and 15 μg/ml genitin or 20 μg/ml novobionicin, pH 7.4). Cells were plated onto Matrigel (Collaborative Biomedical)-coated coverslips in 12-well plates and incubated at 37°C in culture medium A (medium B less amphotericin B).

14C-aminopyrine-uptake assays. Stimulation of gastric glands or parietal cells was quantified using the aminopyrine (AP)-uptake assay. The performance of and calculations for the AP-uptake assay in glands were essentially as described by Berglindh (4) with modifications described by Yao et al. (26). This assay measures the accumulation of AP in acidic spaces caused by the action of the proton-pumping enzyme, HK. In its neutral state, AP freely equilibrates across biological membranes, but protonation of this weak base in acidic spaces gives it a positive charge and traps it. Aliquots (0.5 ml) of the gland suspension were distributed into preweighed 1.5-ml tubes containing 22 nCi/ml of [14C]AP (Amersham; specific activity ~109 mCi/mmol). Glands were stimulated by the addition of histamine (100 μM) and 3-isobutyl-1-methylxanthine (IBMX; 30 μM), or kept in the resting state with cimetidine (100 μM). Where designated, Lat B (Calbiochem), which is known to have similar effects to Lat A (22), was also included. Preparations were incubated for 20 min at 37°C with gentle shaking (~160 oscillation/min) and then centrifuged briefly to pellet the glands. Aliquots of the supernatants were removed and counted in a Beckman liquid scintillation counter. The remaining supernatant was discarded, the pellet was desiccated, and the tube was weighed again. The net weight of the pellet allowed us to estimate the total cell volume of the glands (4). Finally, the pellet was solubi- lized and counted in the scintillation counter. These data were used to calculate the AP accumulation ratio (ratio of

\[ \text{[AP]}_{\text{intracellular}} : \text{[AP]}_{\text{extracellular}} \]. To normalize AP-uptake values among the various preparations, the data are expressed as a fraction of the stimulated control (i.e., with 0 Lat B).

AP assays were also performed on 1- to 2-day-old cultured parietal cells in 12-well plates. Culture media was removed and replaced with 0.5 ml of HEPES-MEM containing 11 nCi/ml of [14C]AP. The resting and stimulated states were achieved with addition of cimetidine or histamine plus IBMX, as above, and the indicated amounts of Lat B were also included. Cultures were gently shacked for 30–40 min at 37°C. At the end of the incubation, the coverslips were removed from the medium, quickly dipped in PBS to remove external radioactivity, and incubated in solubilization buffer (125 mM Tris-Cl, 2% SDS, and 10% 2-mercaptoethanol, pH 6.8) for at least 1 h at room temperature. Cells were then scraped from the coverslip, and the protein content of the cell scrapings was assayed using the filter paper blot method (20). The protein data allowed us to estimate the total cell volume of each culture (4). Aliquots of both the reaction media and cell scrapings were assayed for [14C]AP by liquid scintillation counting. These data were used to calculate AP ratios, as described above.

Detergent extraction of gastric glands and assay of actin content. To assess the relative proportion of free and poly- meric actin, detergent-soluble and -insoluble fractions were prepared. Assays were performed in parallel with AP-uptake experiments, minus the AP. After incubation at 37°C, the glands were centrifuged (400 g) for 1 min and the superna- tant discarded. All subsequent steps were performed at room temperature. Typically, a gland pellet was treated with a 20-fold volume of extraction buffer containing 0.1% Nonidet P-40 (NP-40), 5 mM KH2PO4, 27 mM Na2HPO4 (pH 7.2), 2 mM MgSO4, 2 mM EGTA, 0.2 mM ATP, 0.5 mM diethio- ritol, 2 mM glycerol, and 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor) for 15 min at room temperature with agitation (14). 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).

Actin content was measured in the NP-40 supernatant and pellet by running aliquots on SDS-PAGE (10% running gel), blotting the protein to nitrocellulose or polyvinylidene diflu- roide, and probing for actin using a commercial actin anti- body (C4; ICN). The actin signal was detected by enhanced chemiluminescence with a second antibody coupled to horse- radish peroxidase, the Renaissance kit (NEN), and X-Omat film (Kodak). The density of the signal on the film was quantified by digital imaging using NIH Image software.

Immunofluorescence and confocal microscopy. Cells were held in the basal resting state (resting cells) with 100 μM cimetidine or stimulated with 100 μM histamine plus 30 μM IBMX (stimulated cells) for 30 min. In some cases, a proton pump inhibitor, 10 μM Sch-28080, was included along with the stimulants to inhibit acid secretion (Sch cells). This treatment has been shown to block the osmotic vascular swelling that accompanies HCl secretion but sustains the translocation of HK to the apical membranes. Therefore, it can be used to assess the effects of agents on recruitment of HK, upstream of the proton transport step (24). Cells were fixed by treatment with 3.7% formaldehyde in PBS for 20 min, followed by permeabilization in 0.5% Triton X-100 in PBS for 15 min and blocking in 2% BSA in PBS for 15 min. HK was detected by 30 min of incubation with a mouse monoclonal antibody, 2G11, against its β-subunit (Affinity Bioreagents). The primary antibody was detected by FITC- labeled goat anti-mouse IgG (Jackson Laboratories) in a 30-min subsequent incubation. Actin was detected by coinci-
dent incubation with 80 nM rhodamine-labeled phalloidin (Molecular Probes). All antibody dilutions were made in PBS containing 2% BSA. Coverslips were supported on slides by grease pencil markings and mounted in Gel/Mount (Biomeda). Cells were visualized by conventional fluorescence microscopy with a Nikon Microphot FX-2 using Inovision software to collect images. Confocal microscopy was performed with a Bio-Rad/Leica system.

RESULTS

Lat B inhibits acid accumulation in gastric glands. The AP accumulation assay was carried out in resting (cimetidine) and maximally stimulated (histamine plus IBMX) glands incubated with various concentrations of Lat B for 20 min. For the purpose of normalization, we set the AP-uptake ratio for the maximally stimulated control glands at 100% for each experiment, and the results for treated preparations were scaled accordingly. Figure 1A shows the relative AP-uptake data for resting and stimulated glands in the presence and absence of Lat B. AP accumulation for glands stimulated by histamine plus IBMX was increased 10-fold over resting controls, consistent with results of many others for this widely used assay. In the stimulated preparations, there was no significant effect on AP uptake up to concentrations of 25 μM Lat B, above which a dose-dependent inhibition of AP accumulation occurred. Significant decreases in AP uptakes were seen at 50 μM (64 ± 10% of control), reaching 31 ± 6% of the stimulated control at the highest concentration assayed (100 μM).

Lat B increases steady-state levels of soluble actin. Experiments were designed to measure the effects of Lat B on the release of actin into a detergent-soluble pool. Resting or stimulated gastric glands were incubated with or without Lat B as in the AP experiments above, treated with extraction buffer, and quickly separated into a supernatant and pellet for assay of actin as described in METHODS. In accordance with previous experiments, a relatively small amount of total actin can be extracted by this procedure in control glands, and this is consistent with the small percentage of actin monomer (G-actin) in parietal cells (14). The data of Table 1 show that there was an increase in the detergent-soluble actin (and corresponding decrease in

Table 1. Lat B increases the extractable actin from gastric glands

<table>
<thead>
<tr>
<th>[Lat B], μM</th>
<th>%Total actin</th>
<th>n</th>
<th>%Total actin</th>
<th>n</th>
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<tbody>
<tr>
<td>0</td>
<td>7.1 ± 1.3</td>
<td>13</td>
<td>5.3 ± 1.1</td>
<td>14</td>
</tr>
<tr>
<td>1</td>
<td>7.6 ± 1.5</td>
<td>4</td>
<td>8.3 ± 3.5</td>
<td>4</td>
</tr>
<tr>
<td>2.5</td>
<td>8.8</td>
<td>2</td>
<td>2.3 ± 0.7</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>15.7 ± 3.2*</td>
<td>6</td>
<td>16.9 ± 5.1*</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>25.8 ± 5.3*</td>
<td>4</td>
<td>24.6 ± 3.0*</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
<td>28.6 ± 4.1*</td>
<td>8</td>
<td>21.2 ± 2.5*</td>
<td>11</td>
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<tr>
<td>75</td>
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<td>1</td>
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<td>3</td>
</tr>
<tr>
<td>100</td>
<td>31.4 ± 3.3*</td>
<td>5</td>
<td>32.0 ± 6.7*</td>
<td>5</td>
</tr>
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</table>

Values are means ± SE. Freshly isolated gastric glands were treated with the indicated amounts of latrunculin B (Lat B) in resting or stimulated states. After incubation for 20 min at 37°C, glands were treated with Nonidet P-40 extraction buffer and separated into a soluble and insoluble fraction, as described in METHODS. Samples were run on SDS-PAGE, blotted to nitrocellulose, and immunoprobed for actin. Immunoblots were quantified by densitometry with values expressed as percentage of total actin (supernatant + pellet). *Significant difference with respect to 0 Lat B (P < 0.05).
the cytoskeletal actin) in both resting and stimulated gland preparations as the concentration of Lat B was increased. The extractable actin in stimulated glands increased from \(5.3 \pm 1.1\%\) to \(16.9 \pm 5.1\%\) at 10 \(\mu M\) Lat B and to \(32.0 \pm 6.7\%\) at 100 \(\mu M\). There was no significant difference in the amount of actin extracted from resting or stimulated glands at any given concentration of Lat B.

**Cultured parietal cells are more sensitive than gastric glands to Lat B.** The experiments above on gastric glands are consistent with the expected action of Lat B as a G-actin-sequestering agent, that is, a dose-dependent increase in the detergent-soluble pool of actin from gastric glands. However, it was unexpected that Lat B would display relatively little effect on AP uptake at concentrations \(<50\ \mu M\) where there were clearly documented increases in extractable actin. To explore the effects of Lat B at the cellular level, we used primary parietal cell cultures that have been usefully applied to study stimulation-associated responses of membrane trafficking and cytoskeleton (1, 10). The effect of Lat B on AP uptake by cultured parietal cells is shown in Fig. 1B. As with the intact gastric glands, Lat B produced a dose-dependent inhibition, but the threshold occurred at lower doses. A significant inhibition of AP uptake occurred at 1 \(\mu M\) Lat B. From these limited data, the ED50 for Lat B was considerably higher for intact glands (\(\approx 70\ \mu M\)) compared with the cells in culture (\(\approx 8\ \mu M\)); thus the isolated, cultured cells appeared more sensitive.

**Use of cultured cells to assay morphological changes that occur upon stimulation.** To determine the morphological effects of Lat B on actin, and to ascertain whether Lat B interferes with the translocation of the HK, cultured parietal cells were prepared for immunofluorescence and then examined by conventional light microscopy and by confocal microscopy. Images of “control cells,” not treated with Lat B but in differing states of functional activity, are shown in Figs. 2, 3, A and B, and 4A. In resting parietal cells (Figs. 2A and 3A), there is typically an intense phalloidin staining of the large membrane vacuoles that have been sequestered from the apical membrane to the cell interior in culture (apical membrane vacuoles) and a somewhat weaker phalloidin staining of the membrane surrounding the cell (formerly the basolateral membrane). These locations of phalloidin are consistent with F-actin microfilaments in the apical microvilli and cortical region subadjacent to the basolateral membrane, respectively. The staining of HK is mostly cytoplasmic in resting cells (Figs. 2A and 3A') and generally does not overlap with the phalloidin staining. When parietal cells are stimulated, a dramatic morphological change ensues. One no longer sees discrete phalloidin-stained vacuoles, rather, they have expanded to fill almost the entire cell (Figs. 2B and 3B and B'). Furthermore, there appears to be a greater colocalization of HK staining with the enlarged phalloidin-stained vacuolar structures.
As pointed out in other works (1, 7, 10), these immunofluorescence data are consistent with stimulation-associated recruitment of HK to the apical membrane, but because of the extreme vacuolar swelling in stimulated cells, it is difficult to distinguish a hypothesis of HK/F-actin colocalization from one in which the two probes appear to overlap because of spatial constraints. Accordingly, another assay was used in which the cells

Fig. 3. The morphological effects of Lat B on cultured parietal cells. Cultured parietal cells were maintained in the resting state (rest ctrl), stimulated (stim ctrl), or stimulated in the presence of the indicated concentrations of Lat B (stim + 0.1, 1, 10, or 50 μM) for 30 min at 37°C. Cells were fixed, permeabilized, stained for F-actin and HK, and examined by confocal microscopy. Optical sections were consistent with immunofluorescence data of Fig. 2, with F-actin being localized to the apical membrane vacuoles and the surrounding plasma membrane and HK throughout the cytoplasm in resting control cells (A and A') and a high degree of F-actin and HK colocalization on swollen apical vacuoles in control-stimulated cells (B and B'). For cells stimulated in the presence of Lat B, there was a concentration-dependent progression of morphological alterations. At 0.1 μM Lat B (C and C'), cells were nicely swollen with no consistent differences noted from control-stimulated cells. At 1 μM Lat B (D and D'), cells also looked well stimulated; staining pattern was similar to control cells except for the appearance of some more punctate F-actin staining. By 10 μM Lat B (E and E'), the morphology was distinctly different from control, although much of the HK was still localized to the partially swollen vacuoles; F-actin staining tended to be nonuniform and punctate. Frequently, phalloidin-stained rodlike structures accumulated in the nucleus (E, arrows). At 50 μM Lat B (F and F'), the cells showed little characteristic of the stimulated morphology except for some accumulation of HK on the nonswollen vacuoles. Bar marker, 20 μm.
were stimulated in the presence of a proton pump inhibitor so that the membrane translocation processes would remain intact, but the large ionic and osmotic flow into the vacuoles was blocked. This type of treatment is especially useful because pump inhibitors can determine whether any test agent blocks translocation of the pump or inhibits acid accumulation (1, 10). Immunolocalization of the HK in stimulated cells after treatment with the proton pump inhibitor Sch-28080 (Sch cells) showed that most of the proton pump was

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Fig. 4. Effects of Lat B on cultured parietal cells stimulated in the presence of a proton pump inhibitor. All cell cultures were stimulated for 30 min at 37°C in the presence of 10 μM Sch-28080 as a proton pump inhibitor; Lat B was included at the concentrations indicated. Cells were fixed, permeabilized, stained for F-actin and HK, and optical sections were taken by confocal microscopy at the midcell level to visualize vacuolar inclusions and at the coverslip level to visualize cytoplasmic extensions. When control parietal cells were stimulated in the presence of a pump inhibitor (SCH ctrl), HK was largely cleared from the cytoplasm and colocalized with F-actin at the thickened apical membrane vacuoles that do not swell (because the pump inhibitor prevents osmotic flow; A and A'); large F-actin-rich lamellipodial extensions are also very characteristic of these conditions (A'). Lat B at 0.1 (B and B') and 1 μM (C and C') has virtually no effect on the translocation of HK to the apical vacuoles; however, there was major disruption of the lamellipodial F-actin (B' and C') as well as discontinuities of F-actin staining at the surrounding plasma membrane (B and C). At 10 μM Lat B, translocation of HK to vacuoles is still seen (D'), although F-actin staining appears to be more spotty and punctate with frequent accumulations of rodlike structures in the nucleus (D, arrow), and lamellipodial F-actin is completely disrupted (D'). Bar marker, 20 μm.
cleared from the cytoplasm and colocalized with the vacuoles, which appeared to be slightly larger and thicker walled than resting controls (Figs. 2C and 4A'). The lack of vacuolar expansion is also clear in differential interference contrast images of Sch cells (Fig. 2C), whereas they are greatly expanded in maximally stimulated cells (Fig. 2B). In addition, cells stimulated in the presence of a pump inhibitor usually produced elaborate actin-rich lamellipodia, emanating from the base of the cell (Fig. 2C), which are shown to good advantage in a focal plane near the level of cell attachment to the coverslip (Fig. 4A').

Lat B affects actin structures in stimulated parietal cells at concentrations that do not affect acid accumulation. Cells stimulated in the presence of 0.1 μM Lat B had the same morphological characteristics as control-secreting cells; that is, vacuoles were swollen and there was reasonably good overlap in the probes for F-actin and HK (Fig. 3, C and C'). When Sch cells were treated with 0.1 μM Lat B, the HK translocated from the cytoplasm to the F-actin-rich vacuoles (Fig. 4, B and B'). However, even at these low concentrations, Lat B had major morphological effects on lamellipodial formation (Fig. 4B'), the lamellipodia were very much condensed or absent altogether. Where present, the leading edge of remnant lamellipodia stained brightly with phalloidin, almost as if the rest of the structure had been dragged back toward the cell. Long, actin-rich thin extensions were sometimes seen. Further perturbations of the cytoskeleton were seen in stimulated parietal cells treated with 1 μM Lat B. At this concentration, stimulated cells had intense punctate phalloidin-stained structures that were especially apparent in the plane of the retracted lamellipodia (Fig. 4C'), and the staining of the surrounding plasma membrane was often less intense and discontinuous (Figs. 3D and 4C). However, in most other respects, the morphological response to stimulation in the presence of 1 μM Lat B appeared normal, with large swollen vacuoles (Fig. 3, D and D') and Sch cells showing effective translocation of HK from the cytoplasm to the vacuolar membranes (Fig. 4C').

Lat B causes gross changes in actin at 10–50 μM. At 10 μM Lat B, the number of swollen parietal cells and the magnitude of swelling was much reduced, compared with lower Lat B treatments. Moreover, those cells with a stimulated morphology did not appear normal. Although the vacuoles of the stimulated cells were somewhat expanded, they did not appear to have many large vacuolar spaces. The intense punctate actin structures noted at 1 μM were more common at 10 μM Lat B, and the cells frequently appeared to have rodlike phalloidin staining in their nuclei (Figs. 3E and 4D, arrows). In Sch cells (Fig. 4D'), the cytoplasmic HK appeared to clear in some of the cells; however, the intensity of the staining was not uniform. Often, intense punctate HK staining colocalized with the punctate actin staining. Furthermore, the HK staining frequently appeared heavier on the outer surfaces of vacuolar membranes facing the plasma membrane and lighter on the vacuolar membranes facing toward the center of the cell. All of these characteristics worsened when Lat B was increased to 50 μM (Fig. 3F). Few cells on the stimulated coverslips showed translocation of the HK. Cells that did have some vacuolar accumulation of HK were rarely swollen (Fig. 3F') and looked like stimulated cells treated with a pump inhibitor.

Lat B has a potent effect on F-actin structures in fibroblasts. We performed experiments with fibroblast-enriched cell cultures to assess the effects of Lat B on these actin-rich cells. The results of Fig. 5 indicate that the fine F-actin stress fibers characteristic of control fibroblasts are much diminished with as little as 30 nM Lat B. As Lat B is further increased, the F-actin fibers disappear and are replaced by punctate phalloidin-stained structures. These results demonstrate that in cells of appropriate sensitivity, Lat B has its well-known effect of sequestering actin at very low doses (22, 23).

Pretreatment of resting cells with Lat B has minimal effects on subsequent response to stimulation. In contrast to what was seen for stimulated parietal cells, treatment of cultures that were maintained in the resting condition (cimetidine) with Lat B for 30 min had relatively minimal effects on F-actin staining. With 1 μM Lat B, the pattern of F-actin staining on vacuolar surfaces and surrounding plasma membrane was similar to that in control cells (Fig. 6A). When ≥50 μM Lat B was applied to resting cells for 30 min, the vacuolar F-actin staining appeared normal, but staining at the plasma membrane tended to become spotty, and we often saw the pattern of nuclear rodlike phalloidin staining (Fig. 6A). Because of the striking differences in the morphological effects of Lat B on resting and stimulated cells, we tested the responses of cells pretreated for 30 min in the resting state with Lat B, rinsed briefly, and then stimulated with Sch in the absence of the drug. Stimulation of cells, after being pretreated with 1–50 μM Lat B, produced morphological secretory responses that were indistinguishable from control-stimulated cells (Fig. 6B). The vacuoles appeared swollen, and there was good colocalization of HK and F-actin. The cells were entirely lacking of the punctate phalloidin staining that was so prevalent in cells stimulated in the presence of ≥1 μM Lat B (cf. Figs. 3 and 4). Consistent with the washout results from stimulated cells, the Sch cells pretreated with up to 50 μM Lat B showed good translocation of the HK from the cytoplasm to vacuolar membranes. Thus this aspect of stimulation-associated membrane recruitment was not apparently altered by the pretreatment of resting cells (Fig. 6C). In fact, Sch cells pretreated with 1 μM Lat B displayed excellent lamellipodial formation and appeared similar to control cells in all respects. However, lamellipodial formation in the Sch cells became diminished when the preincubation was 10 μM Lat B and was eliminated at 50 μM Lat B (Fig. 6C), even though all other morphological characteristics of these cells appeared quite normal.
DISCUSSION

The diminution of acid secretion by Lat B, coupled with morphological assessments that show inhibition of secretagogue-dependent translocation of HK, support the hypothesis that actin plays a role in acid secretion. However, the mechanism of Lat B action allows further dissection of this actin-dependent process not previously characterized (15). That increasing Lat B increases the detergent-soluble actin in parietal cell preparations was not an unexpected result. The latrunculins (A and B) appear to act as actin-binding agents, similar to the thymosins, sequestering the actin monomer away from sites of actin polymerization without altering the rate constants for actin turnover. Several lines of evidence support this fact. The ubiquitous thymosin β₄, with a dissociation constant of 2 μM and a cellular concentration of 300–500 μM, is responsible for most of the 150 μM of “unassembled actin” (6). Both latrunculin and thymosin β₄ inhibit nucleotide exchange (3, 27), resulting in a pool of actin in a nonpolymerizable, ADP-bound form. Thymosin β₄ and latrunculin appear to have overlapping binding sites on G-actin, on the basis of competitive binding kinetics (2, 27) as well as mutational studies (3). Furthermore, latrunculin appears to act very unlike profilin, another G-actin-binding protein. Profilin displaces ADF/cofilin from ADP-G-actin and promotes nucleotide exchange (6); in a cell with uncapped actin filaments, profilin acts less like an actin-sequestering agent and more like an agent that increases actin “treadmilling,” promoting barbed end growth. In contrast, latrunculin is not competitive with profilin for G-actin (27), latrunculin does not promote nucleotide exchange (2, 27), and the latrunculin:G-actin complex does not participate in actin polymerization (2, 8, 27). Therefore, when used in our living/dynamic parietal cells, Lat B should decrease the steady-state F-actin:G-actin ratio and alter the functional stability of dynamic actin filaments but leave relatively unchanged more stable structures (22).

We had pointed out earlier that the parietal cell maintains actin predominantly in the filamentous form (~90% of total actin is in the F-actin form), and the F-actin:G-actin ratio did not significantly change between resting and maximally stimulated states (14). Phalloidin staining reveals that parietal cell F-actin is abundant at or near the plasma membranes, especially within the microvillar filaments lining the tortuous secretory canaliculi that constitute the apical plasma membrane (16). Relatively high concentrations of Lat B are required to alter the histamine/IBMX secretory responses of parietal cells in freshly isolated glands. In the 20-min time course of our experiments, the ED₅₀ for inhibition of AP uptake by gastric glands was ~70 μM Lat B, and a significant increase in NP-40-soluble...
Actin did not occur at <10 μM Lat B. The resistance of glandular F-actin to Lat B was validated by the pattern of phalloidin staining, which was fairly normal with Lat B concentrations up to 50 μM, independent of whether the glands were resting or maximally stimulated (data not shown). This lack of gross morphological staining is in contrast to the changes in phalloidin staining seen when gastric glands are treated with cytochalasin D (21). This may be explained by the differing actions of the two drugs. Cytochalasins catalytically sever actin filaments. In contrast, Lat B affects actin filaments uniformly because it depletes the G-actin pool as F-actin turns over.

After they are placed in culture, parietal cells retain many functional characteristics of cells within the gastric gland, but some morphological rearrangement occurs (1). The apical canalicular membranes have been incorporated as vacuolar structures, but they retain their prominent F-actin staining and the ability to recruit HK and secrete acid, demonstrating that an oxyntic secretory phenotype is preserved. However, several novel characteristics are manifest in the cultured parietal cells. They lose the propensity to form contacted epithelial layers, and they take on a highly interactive relationship with the substratum, including lamellar cytoplasmic extensions. These latter structures are clearly stimulated by the cAMP pathway, actively forming long leading edges of motility shortly after activation of histaminergic receptors. A rise in cAMP also produces the oxyntic phenotype of HK recruitment, which promotes HCl secretion into the apical membrane vacuoles, and the consequent

Fig. 6. Effects of Lat B on resting parietal cells and on parietal cells that are stimulated after a preincubation and washout of Lat B. All incubations were at 37°C, after which the cells were fixed, permeabilized, stained for F-actin and HK as described in METHODS, and visualized with a fluorescence microscope. A: resting parietal cells are relatively refractory to Lat B. No significant changes in F-actin staining were observed up to 1 μM Lat B. Even at higher concentrations (e.g., up to 50 μM), the vacuolar staining of F-actin was comparable to controls with some diminution of F-actin staining at the surrounding plasma membrane. B: resting parietal cells were preincubated in the indicated concentration of Lat B for 30 min, the reagent was then washed free, and the cells were stimulated for 30 min. The morphological responses to stimulation were normal, even up to 50 μM Lat B. C: resting parietal cells were preincubated in the indicated concentration of Lat B for 30 min, the reagent was then washed free, and the cells were stimulated for 30 min in the presence of Sch-28080. For all concentrations of Lat B, the recruitment of HK to the secretory vacuoles and colocalization with F-actin was normal. Lamellipodial formation at 1 μM Lat B was also normal (in contrast to Fig. 4), although these extensions were reduced with 10 μM and eliminated with the 50-μM Lat B preincubation. Bar marker, 20 μM.
swelling response of the cells leads to the predominant morphology. In the presence of a pump inhibitor or appropriate protonophores, the HK is recruited to the apical vacuoles, but swelling is minimal and the F-actin-rich lamellipodial extensions become a dominant morphological characteristic.

Parietal cells maintained in culture were more sensitive than intact glands to Lat B, yet inhibition of functional responses, such as AP accumulation and secretagogue-dependent morphological rearrangement, required much higher levels of Lat B than were needed for disruption of more rudimentary cytoskeletal structures. Doses of \( \geq 1 \) \( \mu \)M Lat B were required to block the morphological responses of cultured parietal cells to stimulation by secretagogues, including the stimulation-associated recruitment of HK. It is of interest to note that treatment of Chinese hamster ovary cells with 10 \( \mu \)M Lat B causes disruption of both the actin cytoskeleton and subcellular localization of the epithelial isoform of the Na\(^{+}/H^+\) exchanger (NHE3), which is linked to the actin cytoskeleton through ezrin interactions (19). Notwithstanding the relative refractoriness of the acid secretory functions, there were obvious cytoskeletal effects of Lat B on parietal cells and other cell types in culture at much lower concentrations of the drug.

The most obvious effects of low-dose Lat B on parietal cells were observed on the lamellipodial formations that accompany secretagogue stimulation in the cultured system. These flattened, F-actin-rich, highly mobile protrusions were significantly altered at submicromolar concentrations of Lat B, and we frequently observed accumulations of punctate foci of phalloidin staining at these low drug levels. Some structural parallels can be drawn from studies on cultured fibroblasts. Lat B as low as 0.03 \( \mu \)M effected a destabilization of F-actin stress filaments and the accumulation of punctate phalloidin-stained deposits throughout the fibroblasts. Thus it is clear that Lat B is exerting its putative biochemical action of sequestering actin monomer. However, there also appear to be some relatively resistant F-actin structures in the parietal cell, and it seems likely that the secretory response is more closely tied to the more stable pool of actin filaments.

Precedent exists for pools of cellular actin with differing stabilities and sensitivities to actin-destabilizing drugs. Dendritic spines are micrometer-sized, actin-rich, glutamate receptor-containing protrusions formed at postsynaptic contact sites in the central nervous system. Direct visualization of neuronal cultures expressing green fluorescent protein-actin reveals that these spines show fast (\(~1\) s) changes in shape but not size (11). These changes are actin dependent because cytochalasin D or Lat B abolished motility without significantly depolymerizing the spine actin. These data would suggest the presence of two pools of actin within the spine: a stable, possibly capped, actin spine; and dynamic F-actin structures, possibly bound to membrane-cytoskeletal linkers such as spectrin or to the glutamate receptors themselves (18). Another report concerns the formation of distinct pools of F-actin on ADP-ribosylation factor-stimulated Golgi membranes (17). While Lat A completely blocks formation of all types of actin filaments, there appears to be two distinct pools of actin based on sensitivity to cytochalasin D, salt extraction, and association with the actin-binding protein drebin. One pool is sensitive to cytochalasin D, drebin associated, and salt extractable, forming on both coatamer-coated vesicles and the non-budded Golgi remnants. A second pool forms only on the Golgi remnants, does not associate with drebin, and is resistant to both cytochalasin D and salt extraction.

We propose that the resistance of acid secretory function of parietal cells to Lat B is the result of very stable actin filament turnover pathways that minimize the accessibility of the actin monomer to Lat B. In experiments using standard tissue culture cell lines, maximal morphological effects (i.e., rounding up and loss of F-actin staining) occur at submicromolar concentrations of latrunculin (22, 23). In contrast, we have demonstrated that high doses of Lat B and/or long incubation times are required to disrupt the parietal cell cytoskeleton. We do not believe that the insensitivity of parietal cells to Lat B is due to the inability of the drug to enter the cell, because our present experiments show that the highly mobile lamellipodia are very sensitive to Lat B. For the same reason, we think it is unlikely that the refractory nature of resting parietal cells is due to the exclusion of Lat B by the action of an organic transporter, such as a multidrug resistance transporter. This transporter would have to have prominent activity in resting parietal cells, but stimulation by histamine/IBMX would have to greatly reduce this activity. Instead, we propose that when parietal cells are placed into culture, a more primitive cytoplasmic motile activity becomes manifest that is more sensitive to Lat B. This may be due to the loss of tissue-organizing structures that rigidly maintain actin in the filamentous form, or possibly due to the interaction of the parietal cell with the culturing substrate, such as seen with focal adhesion points (9). If a high F-actin:G-actin turnover in this pool was easily accessible to Lat B, this could represent a significant “drain” on the total cellular actin when the lamellipodial activity was stimulated by cAMP. Such a hypothesis would be consistent with 1) the high sensitivity of lamellipodia, 2) the resistance of F-actin structure in presumed microvillar loci, and 3) the refractory nature of the cytoskeleton to Lat B in cultured parietal cells at rest. For the latter case where there is no activation/turnover of a lamellipodial pool, there is little stress on the flow of actin to the Lat B-sensitive state. Accordingly, we did not see any notable morphological effects of Lat B on resting cells, and, more importantly, the functional responses of cultured parietal cells to histamine were totally unabated by pretreatment with Lat B while in the resting state. The one minor caveat to this statement was that pretreatment with 50 \( \mu \)M Lat B eliminated the subsequent lamellipodial response to histamine, but all other indices of a robust secretory response were in full evidence. The loss of lamellipodia...
with 50 μM Lat B pretreatment may simply be the result of some residual drug because of an inefficient washout.

It is of interest to speculate whether the pools of actin with distinct properties exist within the parietal cells. These potential pools may reflect differences in specific actin isoforms (e.g., β vs. γ), differences in the phenotypic expression of actin-binding proteins (e.g., freshly isolated glands vs. cultured cells), or simply differences in physical location within the cell (e.g., apical vs. basolateral). Furthermore, these pools could be responsible for the distinct polarized distribution of actin isoforms (25), which in turn would likely require specific interactions with cytoskeletal anchoring, bundling, and motor proteins.

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