A New Method for Cell Permeabilization Reveals a Cytosolic Protein Requirement for Ca\textsuperscript{2+}-activated Secretion in GH\textsubscript{3} Pituitary Cells*

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(Received for publication, December 12, 1988)

Ca\textsuperscript{2+} is a major regulator of exocytosis in secretory cells, however, the biochemical mechanisms underlying regulation remain to be identified. To render the secretory apparatus accessible for biochemical studies, we have developed a cell permeabilization method (cell cracking) which utilizes mechanical shear. GH\textsubscript{3} pituitary cells subjected to cracking were permeable to secretory apparatus accessible for biochemical studies, indicating regulation remain to be identified. To render the permeable cells at the secretory proteins, prolactin (PRL) and a proteoglycan, but not lysosomal enzymes.

Extensively washed permeable cells were incapable of releasing PRL in response to Ca\textsuperscript{2+} and MgATP addition. However, addition of cytosol was found to restore Ca\textsuperscript{2+}-activated, MgATP-dependent PRL release. The cytosolic factor responsible for activity was thermolabile and protease sensitive. The protein was partially purified, and its molecular mass was estimated to be equivalent to that of a globular protein of 200-350 kDa by molecular sieve chromatography. Inhibitors of calmodulin or protein kinase C (trifluoroperazine, calmidazolium, H-7) failed to inhibit Ca\textsuperscript{2+}-activated PRL release, and the required cytosolic protein could not be replaced by purified calmodulin, calmodulin-dependent protein kinase II, protein kinase C, or calpactin I. Further purification and characterization of the cytosolic protein should reveal the nature of biochemical events involved in regulated secretory exocytosis.

Cytoplasmic Ca\textsuperscript{2+} is a major intracellular regulator of secretory exocytosis in endocrine, exocrine, and neural cells. Although the mechanisms responsible for stimulus-evoked cytoplasmic [Ca\textsuperscript{2+}] increases are relatively well defined (1), the pathway(s) by which elevations in [Ca\textsuperscript{2+}] stimulate secretion remains to be identified. Direct Ca\textsuperscript{2+}-lipid bilayer interactions are inadequate for promoting membrane fusion over the range of [Ca\textsuperscript{2+}] found in activated secretory cells (2). It is likely that Ca\textsuperscript{2+}-dependent effectors mediate the actions of Ca\textsuperscript{2+} on secretion. Numerous Ca\textsuperscript{2+}-dependent enzymes and binding proteins have been suggested to be involved in stimulus-secretion coupling (3-6), however, there is little direct experimental evidence available to evaluate their role.

The major obstacles to biochemical studies of regulated secretion are the inaccessibility of the secretory apparatus in intact cells and the failure of cell disruption techniques to adequately preserve the structural integrity required for function. Several techniques which have been employed previously for accessing the intracellular environment of secretory cells have limitations for biochemical studies. Patch clamp electrode recording of capacitance changes provides access only in individual secretory cells (7, 8). High voltage fields stably permeabilize cells only to small molecules (9). Permeabilization with detergents (10-12) or hydrophobic peptides (13) provides macromolecular access but is limited by potential effects on membrane-associated processes. Homogenization techniques are generally too disruptive except for highly specialized cell types (14, 15).

The motivation for the present work was to develop a permeabilization method for GH\textsubscript{3} pituitary cells which would allow biochemical studies of regulated exocytosis. The regulation of prolactin (PRL) secretion by Ca\textsuperscript{2+}-activated or protein kinase C-mediated pathways in GH\textsubscript{3} cells has been extensively studied in intact (16, 17) and in electropermeabilized (18-20) cells. In this report, we describe a new method for cell permeabilization which provides macromolecular access and which preserves cellular structure sufficiently to enable the study of Ca\textsuperscript{2+}-activated, MgATP-dependent PRL release. This system has allowed identification of a cytosolic protein required for Ca\textsuperscript{2+}-activated PRL release which has been partially purified and characterized.

**EXPERIMENTAL PROCEDURES**

Cell Culture—GH\textsubscript{3} cells (American Type Culture Collection) were grown as monolayer cultures in F-10 medium (GIBCO) supplemented with 15% horse serum plus 2.5% fetal bovine serum (HyClone). The decreasing PRL content of GH\textsubscript{3} cells upon continuous passage prompted the initiation of new cultures at 3- to 6-month intervals. In order to enhance PRL secretory granule content, cultures were incubated for 48-120 h in F-10/Dulbecco's modified Eagle's medium (1:1) medium supplemented with 15% horse serum, 300 nM insulin, 1 nM 17β-estradiol, and 10 nM epidermal growth factor (21). Hormones were purchased from Sigma.

Cell Permeabilization—In order to minimize nonincubation control values for PRL secretion experiments, monolayers were washed 3 times in F-10 medium (37 °C) containing 0.1% BSA, incubated in the same for 30-60 min and washed an additional 3 times (16). Cells were detached using Hank's Mg\textsuperscript{2+},Ca\textsuperscript{2+}-free buffer with 0.001 M EDTA.

* This work was supported by National Science Foundation Grant DCB 8512441 and United States Public Health Service Grant DK 40428. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PRL, prolactin; BSA, bovine serum albumin; EGTA, (ethyleneglycol-bis(oxyethylenenitrito))tetrasacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; SEM, scanning electron microscope; TEM, transmission electron microscope; HVE, high voltage electron microscope; H-7, 1-(5-isouquinolinesulfonyl)-2-methylpiperazine; W-7, N-(6-aminoxyethyl)-5-chloro-1-naphthalene sulfonamide; AMPPCP, adenylyl (5′-y-methylene)diphosphonate; ATP[S], adenosine-5′-O-(3-thiotriphosphate); AMPPNP, adenylylimidodiphosphate.
After washing by centrifugation (800 X g) in a cold isotonic buffer containing 0.1 mM EGTA, cells were resuspended in cold K-glucose buffer (0.02 M Heps, pH 7.2, 0.12 M potassium glutamate, 0.02 M NaCl, 0.005 M glucose, 0.002 M EGTA, 0.1% BSA), a buffer previously found to be optimal for Ca2+-dependent PRL release from electrically permeabilized (20) GH3 cells. Permeabilization was achieved by single passage of a suspension (10^7-10^8 cells/ml) of chilled cells through a stainless steel ball homogenizer. The ball homogenizer has been previously described (22) and consists of a bored (0.3750 inch) chamber into which was fitted a 0.3749-inch tungsten carbide ball, establishing an overall clearance of 0.0001 inch. Cell permeabilization, monitored with 0.4% (w/v) trypan blue and phase contrast optics, was routinely 95-99%. Alternatively, permeable cells were viewed under fluorescence optics in the presence of 20 μg/ml fluorescein-wheat germ agglutinin (Vector Laboratories) as shown in Fig. 1A.

**PRL Secretion Assays.—**Permeabilized cells were used in either of two formats for PRL release experiments. Either cracked cells with residual cytoplasm were added directly to reactions or the cells were washed 3 times by centrifugation (800 X g) using 20 volumes of K-glucose buffer for each wash. In the latter protocol, the washed permeable cells (termed cell ghosts) were resuspended in K-glucose buffer and added to reactions after release incubations were conducted at 30 °C for 5 min or 37 °C for 5 min. Activity was assayed by filtration onto Whatman GFA filters (19). Zero time values corresponding to 30% of the incubation values have been subtracted.

Sulfated proteoglycan release was measured as previously described (19) except that cells were labeled with 10 μCi/ml ^35S
d-glucosamine (Du Pont-New England Nuclear). This assay was conducted with extensively washed GH3 cells ghosts due to the necessity of removing unincorporated ^35S radioactivity. Following incubation of labeled ghosts and centrifugation, supernatants were analyzed by determining phosphotungstic acid (0.5%/trichloroacetic acid 6%)-insoluble material by filtration onto Whatman GFA filters (19). Zero time values corresponding to 30% of the incubation values have been subtracted.

For microscopy, intact cells or washed ghosts were suspened in 0.02 M Heps, pH 7.2, 0.12 M potassium succinate, 0.02 M NaCl, 0.005 M glucose, 0.002 M EGTA, 0.1% BSA, and 0.2 M sucrose. For TEM and HVEM analysis, chilled suspensions were fixed for 5 min in 2% glutaraldehyde (Ladd Research Industries, Inc.), sedimented in a microcentrifuge and allowed to fix at 4 °C for an additional 16 h. For SEM analysis, suspensions were fixed for 2 h by mixing with 4 volumes of 1% paraformaldehyde, 2.5% glutaraldehyde in 0.075 M sodium phosphate, pH 7.5.

For TEM analysis, cell pellets were washed with buffer, post-fixed in 2% OsO4, dehydrated with ethanol, post-stained with 1% aqueous uranyl acetate and lead citrate using standard techniques (24). Samples were examined using a Hitachi H-600 microscope. For HVEM analysis, washed cell pellets were post-fixed in 1% OsO4, stained with 1% uranyl acetate, dehydrated in ethanol, and critical point dried as described (26). Samples were examined with an AEI EM-7 high voltage electron microscope, operated at 1 MeV. For SEM analysis, fixed cell pellets were washed, dehydrated in ethanol, critical point dried, and sputter-coated with a thin film of platinum as described (26). Samples were examined at low voltage (1-2 kV) with a Hitachi S-900 microscope.

**Other Materials—**H-7 and W-7 were obtained from Seikagaku America, Inc., trifluoperazine from Smith, Kline and French, calmodiazolium from Behring Diagnostics, and ATP analogs from Boehringer Mannheim GmbH, B. W. Porter (University of Wisconsin) provided purified calmodulin-dependent protein kinase II (Cam II), purified calcitonin I (46) was kindly provided by D. S. Drust and C. E. Creutz (University of Virginia). Calmodulin was purchased from Sigma, and B. W. Porter (University of Wisconsin) provided purified rat brain protein kinase C (approximately 40% pure).

**RESULTS**

**Permeabilization by Cell Cracking.—**The permeabilization method described under "Experimental Procedures" allowed the preparation of highly permeable GH3 cells which were structurally well preserved. When GH3 cells were passed a single time through the ball homogenizer, 95-98% of the cells were rendered trypan blue stainable ("cracked" cells). To further assess the extent of permeabilization, cracked cells were incubated with fluorescein-wheat germ agglutinin (33 kDa). As shown in Fig. 1A, this fluorescent lectin bound to the surface of intact cells (left panel) whereas both plasma membrane and nuclear membrane of the cracked cells were labeled (right panel). Hence, cell cracking results in the permeabilization of cells to a 33 kDa probe.

Scanning electron microscopy showed that a large tear in the surface membrane was present in cracked cells (Fig. 1B).
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FIG. 1. Microscopic examination of intact and permeable GH\textsubscript{3} cells. Suspended GH\textsubscript{3} cells were prepared and permeabilized as described under "Experimental Procedures." A, fluorescence microscopy of intact cells (left) and cracked cells (right) stained with wheat germ agglutinin-fluorescein. For electron microscopy, intact cells (left) and cell ghosts (right) were prepared and fixed as described under "Experimental Procedures." B, SEM images at X 5,000. C, HVEM images at X 3,000. D, TEM images at X 3,000. In each case, images shown for cracked cells (right panels) were representative for the proportion of permeable cells (95-98%) estimated by trypan blue staining.

In spite of the high degree of permeability achieved, a normal cytoplasmic ultrastructure was evident in cracked cells examined with either HVEM (Fig. 1C) or TEM (Fig. 1D) techniques. TEM images (Fig. 1D) showed that dense granules of approximately 100 nm were preserved upon cell cracking. These were similar to the PRL secretory granules previously identified in GH\textsubscript{3} cells (21).

Biochemical studies of cracked cells also indicated that there was substantial structural preservation. Markers for the Golgi (UDP-galactosyltransferase), lysosomes (acid phosphatase, N-acetylglucosaminidase), and PRL vesicles/granules (immunoreactive PRL) were each present at a level at least 80% of the total detected in the same number of intact cells (not shown).

Ca\textsuperscript{2+}-activated PRL Release Is Preserved in Cracked Cells—Incubation of the cracked cells at 30–37°C resulted in an increase of PRL measured in the high speed supernatant of the reaction mix. The rate and extent of PRL release were enhanced by inclusion of Ca\textsuperscript{2+} and MgATP in the reaction mixtures (Fig. 2). The maximal extent of PRL release observed under optimal incubation conditions (see below) represented 25 ± 6% (mean ± S.D. of six determinations) of the total intracellular PRL pool.

With MgATP present, Ca\textsuperscript{2+} stimulated the release of PRL 2–10-fold. Free ionic Ca\textsuperscript{2+} was stimulatory over the range of 0.1–1 μM (Fig. 3A) with half-maximal activation observed at 0.6 μM. A Hill plot of data pooled from several experiments indicated cooperativity of Ca\textsuperscript{2+} activation with a Hill coefficient equal to 2 (not shown). At Ca\textsuperscript{2+} concentrations which exceed those of an activated cell (>10 μM), PRL release was suboptimal (Fig. 3B). In the absence of MgATP, Ca\textsuperscript{2+} had little influence on PRL release by cracked cells (Table II). These results demonstrate that cracked cells retain a PRL release mechanism which is similar in its Ca\textsuperscript{2+} sensitivity to that of intact or electropermeabilized GH\textsubscript{3} cells (18–20).

Requirement of a Cytosolic Factor for Ca\textsuperscript{2+}-activated PRL Release—Cracked GH\textsubscript{3} cells were stable to manipulation and could be washed extensively by centrifugation. Washed cracked cells are membranous "ghosts" which are devoid of soluble cytoplasmic factors but which retain secretory granules and a normal cytoplasmic ultrastructure (Fig. 1, C and D). Incubation of cell ghosts with Ca\textsuperscript{2+} plus MgATP did not result in PRL release (Figs. 3A and 4A). However, addition of crude GH\textsubscript{3} cell cytosol (high speed supernatant) to the ghost cell incubation completely restored Ca\textsuperscript{2+}-activated, MgATP-dependent PRL release (Figs. 3A and 4A).

A limited tissue survey showed that cytosol fractions prepared from GH\textsubscript{3} cells, rat liver, rat brain (Fig. 4B), bovine pituitary, and PC12 cells (not shown) reconstituted PRL release in GH\textsubscript{3} cell ghosts. Cytosol from GH\textsubscript{3} cells contained contaminating rat PRL which increased nonincubation control values in the assay. Because of its greater activity, abun-
The cytosol used either with the presence of 2 mM MgATP. Incubations contained 2 pg cells and GH3 cell ghosts. Approximately 15-30 pg of cytosolic protein/0.2 ml of incubation. Concentrated GH3 cell cytosol was prepared from a high speed supernatant of cracked cells by concentration with a Centricon-30 device. Concentrated GH3 cell cytosol was tested for activity in a PRL release assay utilizing cell ghosts, 2 mM MgATP and either 1 mM or 1 nM Ca\(^{2+}\). PRL release observed at 1 nM Ca\(^{2+}\) was subtracted from that at 1 mM Ca\(^{2+}\) and compared with that of untreated cytosol (100%).

The activity of cytosol in supporting Ca\(^{2+}\)-dependent PRL release was found to be nondialyzable (not shown), thermolabile (Table I), and susceptible to inactivation by treatment with proteases (Table I) but not with RNase or DNase (not shown), indicating that a protein(s) in the cytosol was responsible for activity. The general presence of protein was not possible for activity. The general presence of protein was not adequate since a variety of purified proteins failed to exhibit activity in a PRL release assay utilizing cell ghosts, 2 mM MgATP and either 1 mM or 1 nM Ca\(^{2+}\). PRL release observed at 1 nM Ca\(^{2+}\) was subtracted from that at 1 mM Ca\(^{2+}\) and compared with that of untreated cytosol (100%).

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**FIG. 2.** Time course of PRL release from cracked GH3 cells. Cell suspensions were permeabilized as described under "Experimental Procedures" and incubated at either 30 or 37 °C for the indicated times either with (●) or without (○) 1 mM free Ca\(^{2+}\) plus 2 mM MgATP. The total PRL present at zero time was 19.6 ng.

**FIG. 3.** Ca\(^{2+}\)-dependence of PRL release from cracked GH3 cells and GH3 cell ghosts. Cells were prepared and permeabilized as described under "Experimental Procedures." A, PRL release incubations were conducted at indicated free Ca\(^{2+}\) concentrations in the presence of 2 mM MgATP. Incubations contained 2 × 10^5 cracked cells (●) or cell ghosts (○, △) and were conducted at 30 °C for 15 min. Cell ghosts were incubated without cytosol (○) or in the presence of concentrated cytosol (△). Cracked cell incubations (●) contain approximately 15-30 μg of cytosolic protein/0.2 ml of incubation. Concentrated GH3 cell cytosol was prepared from a high speed supernatant of cracked cells by concentration with a Centricon-30 device. The cytosol used (△) was 15-fold concentrated corresponding to 300 μg cytosol protein/0.2 ml of incubation. B, PRL release incubations were conducted at indicated free Ca\(^{2+}\) concentrations in the presence of 2 mM MgATP. Incubations contained cell ghosts with rat brain cytosol (2.8, 5.6, 8.4, 14, or 42 μg protein/0.2 ml) as indicated. Incubations were at 30 °C for 15 min. PRL release in the absence of cytosol was <1 ng at all Ca\(^{2+}\) concentrations. PRL values at zero time have been subtracted.

**FIG. 4.** Ca\(^{2+}\)-activated PRL release from GH3 cell ghosts requires a cytosolic factor. A, ghosts were prepared and incubated at 30 °C for 15 min with 2 mM MgATP, with (●) or without (○) 1 μM Ca\(^{2+}\) plus varying amounts of crude cytosol prepared from the same cells. Cytosol was added back intact (1.0X) or diluted (0.5X and 0.1X). The 1.0X corresponds to the concentration of cytosol that would be present in cracked cell incubations which is approximately 15 μg/0.2 ml of incubation. B, ghosts were incubated at 30 °C for 15 min with 2 mM MgATP with (closed symbols) or without (open symbols) 1 μM Ca\(^{2+}\). Indicated amounts of cytosol protein (per 0.2 ml of incubation) prepared from GH3 cells (○), rat liver (●, ■), or rat brain (△, ▲) were included.

**TABLE I**

| Treatment          | % Activity remaining |
|--------------------|----------------------|
| None               | 100                  |
| Heat/10 min        |                      |
| 40 °C              | 108.8                |
| 45 °C              | 97                   |
| 50 °C              | 14.7                 |
| 55 °C              | 0                    |
| 60 °C              | 0                    |
| Proteolysis/15 min |                      |
| Papain             | 3.5                  |
| Control            | 79.5                 |

- Following indicated treatments, rat brain cytosol was tested for activity in a PRL release assay utilizing cell ghosts, 2 mM MgATP and either 1 μM or 1 nM Ca\(^{2+}\). PRL release observed at 1 nM Ca\(^{2+}\) was subtracted from that at 1 mM Ca\(^{2+}\) and compared with that of untreated cytosol (100%).
- Cytosol was incubated with papain-agarose (Sigma, activated for 1 h at 37 °C with 0.05 mM cysteine) for 15 min at 35 °C at a ratio of 1 unit of papain/50 mg of protein. Reactions were terminated by chilling and addition of antipain (2 μg/ml) and leupeptin (0.5 μg/ml) followed by centrifugation. An incubation of cytosol without papain addition was conducted in parallel (control).
Requirement for Nucleotides in Ca\textsuperscript{2+}-activated PRL Release—Ca\textsuperscript{2+}-activated PRL release by both cracked cells and cell ghosts (supplemented with cytosol) was found to be dependent upon inclusion of MgATP in the reactions (Fig. 5A), with maximal release observed by 1–2 mM MgATP. In contrast, MgGTP, MgUTP, and MgCTP were much less effective (Fig. 5A). A requirement for the γ-phosphate of ATP was evident from the findings that MgADP, MgAMP, Mg-AMPPCP (not shown), MgATP[S], and MgAMPPNP (Fig. 5B) failed to substitute for MgATP. Nonhydrolyzable ATP analogs were found to inhibit ATP-dependent PRL release (Fig. 5B) and the inhibition by ATP[S] was not readily reversed (Fig. 5C), similar to results reported for catecholamine release from saponin-permeabilized chromaffin cells (27).

A representative experiment in Table II illustrates the interdependence of Ca\textsuperscript{2+}, MgATP, and cytosol factor. Optimal PRL release by GH\textsubscript{3} cell ghosts was Ca\textsuperscript{2+}, MgATP, and cytosol dependent.

Evidence for PRL Release by Exocytosis—Several criteria have been utilized to assess whether PRL release by cracked cells occurred by exocytotic discharge. We have found that Ca\textsuperscript{2+}-activated PRL release required structural integrity of the cracked cells, was accompanied by the Ca\textsuperscript{2+}-, MgATP-, and cytosol-dependent release of other secretory proteins, and occurred under conditions which did not result in the release of lysosomal proteins. As shown in Fig. 6, passage of GH\textsubscript{3} cells a second or third time through the ball homogenizer resulted in a substantial reduction of Ca\textsuperscript{2+}-activated PRL release in spite of the fact that PRL was retained in sedimentable structures (see Fig. 6, legend). Light microscopic examination indicated that, by a second passage through the ball homogenizer, there was a marked disruption of cellular integrity (not shown). Multiple passes of the cells through the ball homogenizer resulted in the preparation of PRL vesicles/granules and membranes, however, these did not participate in a Ca\textsuperscript{2+}-activated process which releases PRL.

Intact and electropermeable GH\textsubscript{3} cells secrete a sulfated proteoglycan by a Ca\textsuperscript{2+}-regulated mechanism (19). As shown in Table III, Ca\textsuperscript{2+}-activated, MgATP- and cytosol-dependent release of the proteoglycan by cracked cells was observed.

Acid phosphatase and N-acetylgalactosaminidase are not secreted by intact GH\textsubscript{3} cells. Less than 2.7 and 4.1%, respectively, of the cellular content of these enzymes was released by cracked cells in incubations in which release of 30% of the PRL content was observed (Fig. 7). These data suggest that PRL release by permeable GH\textsubscript{3} cells occurred by Ca\textsuperscript{2+}-regulated exocytic discharge rather than by a nonspecific lytic mechanism.

Partial Purification of the Cytosolic Factor—Reconstitution of Ca\textsuperscript{2+}-activated PRL release by GH\textsubscript{3} cell ghosts served as a quantitative bioassay (Fig. 4B) to monitor the purification of the cytosolic protein. Partial purification has been achieved as summarized in Table IV using phenyl-Sepharose, protamine-agarose and Mono Q chromatography. The elution characteristics from Mono Q chromatography are shown in Fig. 8 where Ca\textsuperscript{2+}-dependent PRL releasing activity was found to be eluted between 0.1 and 0.3 M KCl. Overall purification achieved by sequential chromatography with three columns was approximately 40-fold (Table IV). Although activity was very stable in crude cytosol, purified fractions lost activity at 0 or −20 °C. It is likely that the degree of purification has been underestimated due to inactivation. Analysis of the partially purified material by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that multiple proteins were present, precluding a conclusion about the molecular characteristics of the factor at this time.

Molecular sieve chromatography of the rat brain cytosolic factor indicated that the activity displayed molecular weight heterogeneity, eluting more broadly than calibrating proteins. The major peak of activity was observed to elute at the position of a globular protein of about 350 kDa on a calibrated TSK G3000-SW column (Fig. 9). Gel filtration on S300 Sephacryl indicated an apparent molecular mass similar to catalase (210 kDa) with the activity eluting broadly on this column as well (not shown). The basis for the size heterogeneity of the cytosolic factor is not known, and clarification of this property must await full purification.

Attempts to Identify the Cytosolic Factor—A number of Ca\textsuperscript{2+}-dependent enzymes and binding proteins, many of them cytosolic, have been suggested as potential Ca\textsuperscript{2+} targets involved in the regulation of secretion (see “Discussion”).

![Fig. 5](image-url)
Cytosolic Protein Required for Ca\(^{2+}\)-activated Secretion

### TABLE II

| Incubation conditions | PRL release (ng/incubation) |
|-----------------------|-----------------------------|
| Ca\(^{2+}\) | ATP | cytosol |
| - | - | - | 0.8 ± 0.4 |
| + | - | - | 1.0 ± 0.2 |
| - | + | - | 0.6 ± 0.4 |
| + | + | - | 1.1 ± 0.5 |
| - | + | + | 1.6 ± 0.1 |
| + | + | + | 2.6 ± 0.3 |
| + | + | + | 10.6 ± 0.4 |

*PRL release incubations at 30 °C for 15 min were conducted with GH\(_3\) cell ghosts and 2 mM MgATP, 1 nM or 1 µM Ca\(^{2+}\) (+ or −) and 50 µg of cytosol as indicated. Values shown are the mean ± range of duplicate determinations.

### FIG. 6

**Effect of cell disruption on Ca\(^{2+}\)-activated PRL release by GH\(_3\) cell ghosts.** Cell suspensions were passed through the ball homogenizer 1, 2, or 3 times as indicated. Cellular material was pelleted at 10,000 × g for 5 min and resuspended in Kglu buffer for PRL release incubations. Incubations were conducted at 30 °C for 15 min with 2 mM MgATP in the presence or absence of 1 µM Ca\(^{2+}\) or 30 µg of rat brain cytosol as indicated. The PRL content of 10,000 × g pellets was 34, 27, and 21 ng for 1X, 2X and 3X cells, respectively.

### FIG. 7

**GH\(_3\) cell ghosts release PRL but not lysosomal enzymes.** GH\(_3\) cell ghosts were prepared and incubated at 37 °C for 5 min with 1 µM Ca\(^{2+}\), 2 mM MgATP, and indicated amounts of partially purified cytosolic factor (phenyl-Sepharose fraction). Supernatants and pellets from the incubations were analyzed for PRL (○), acid phosphatase (△), and N-acetylglucosaminidase (□) content as described under “Experimental Procedures.” Ordinate values represent the content of supernatants normalized to the content of pellets from unincubated controls.

### TABLE III

| Release of sulfated proteoglycan by GH\(_3\) cell ghosts |
|----------------------------------------------------------|
| ³⁵S\(^{35}\)O-proteoglycan released* | % total |
| Complete | 27.1 |
| − Cytosol | 4.5 |
| − ATP | 10.1 |
| − Ca\(^{2+}\) | 9.7 |
| − Incubation | 4.7 |

*³⁵S\(^{35}\)O labeling of cells and analysis of proteoglycan release was as described under “Experimental Procedures.” The complete incubation was at 30 °C for 15 min and contained ³⁵S\(^{35}\)O-labeled GH\(_3\) cell ghosts, 30 µg of rat brain cytosol, 2 mM MgATP, and 1 µM Ca\(^{2+}\). Data shown are the means of duplicate determinations expressed as a percentage of the total secretory proteoglycan pool determined by extraction of cells with 0.1% Triton X-100.

summarized in Table V, we have examined the effects of several inhibitors on Ca\(^{2+}\)-dependent PRL release and the ability of several purified proteins to substitute for the cytosolic factor. Inhibitors which are known to interfere with hydrophobic interactions (28) required by calmodulin and protein kinase C (trifluoroperazine, calmidazolium) had no effect of Ca\(^{2+}\)-activated PRL release, even when tested at high concentrations. W-7 and pimozide were also without effect (not shown). H-7, an inhibitor of protein kinase C (28), also had no influence. In contrast, neomycin, a drug used to inhibit polyphosphoinositide metabolism (29), fully inhibited Ca\(^{2+}\)-activated PRL release at low concentrations (K\(_i\) = 20 µM).

The purified proteins which were tested (calmodulin, Ca\(^{2+}\)-calmodulin-dependent protein kinase II, protein kinase C, calpain I) failed to substitute for the cytosolic factor.

### DISCUSSION

**Regulated Exocytosis in Cracked GH\(_3\) Cells**—A variety of methods have been employed to permeabilize secretory cells. Electropermeabilization is widely employed since it produces plasma membrane pores without damaging intracellular organelles. However, the permeability achieved allows only the exchange of small molecules (9, 18–20). Methods used for inducing macromolecular lesions have employed agents which either lack a well-defined permeabilization mechanism or which have the potential for affecting intracellular mem-
branes. Digitonin and saponin have been successfully used to permeabilize secretory cells by several workers (10-12, 27) whereas others have found that such detergents are inhibitory to Ca\(^{2+}\)-activated secretion (9). In order to avoid the use of detergents, which promote PRL release in the absence of Ca\(^{2+}\) and MgATP in GH\(_3\) cells,\(^5\) we sought a nonchemical method for cell permeabilization. Balch and Rothman (30) described the use of a metal ball homogenizer to disrupt cells in isotonic buffers for the isolation of intact organelles. We have modified the homogenizer (22) and found that a single passage of cells through the device produces a large tear in the plasma membrane (Fig. 1B) without damaging intracellular organelles (Fig. 1, C and D). Cells cracked open by this method preserve their cytoplasmic ultrastructure and retain functional Ca\(^{2+}\)-activated exocytotic mechanisms.

The conclusion that Ca\(^{2+}\)-activated, MgATP-dependent PRL release from cracked cells faithfully reflects the physiological secretory process of intact cells is based on biochemical evidence. The available evidence strongly mitigates against a nonspecific lytic mechanism for PRL release. PRL release by cracked cells was found to be activated by 0.1-1.0 \(\mu\)M Ca\(^{2+}\), precisely the [Ca\(^{2+}\)] effective with electropermeable GH\(_3\) cells and measured to be the range of [Ca\(^{2+}\)] effective in intact cells (18-20). Ca\(^{2+}\)-activated PRL release by cracked cells was also MgATP-dependent. This is in accord with the expectation provided for numerous (10, 31, 32) but not all (14, 15, 33) secretory cells, although the underlying role for MgATP remains to be established. Cracked cells exhibit a specificity for ATP since other nucleotides were much less effective and hydrolysis of the \(\gamma\)-phosphate appeared to be obligatory.

Our evidence suggests that ultrastructural integrity is crit-
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The amount of PRL released by cracked cells has been found to correlate with the size of the releasable PRL pool in intact cells. Maximally, 30% of the PRL content was released by permeable cells during incubation under optimal conditions. Secretagogue treatment of intact cells prior to permeabilization resulted in a corresponding reduction in Ca\textsuperscript{2+}-activated PRL release by permeable cells.\textsuperscript{2} Growth of GH\textsubscript{3} cells has been observed. The observation that PRL and proteoglycans were released by cracked cells whereas lysosomal enzymes were not is consistent with the idea that Ca\textsuperscript{2+} activation in this system involves an unmasked non-specific lytic mechanism. A requirement for structural integrity is expected for vectorial exocytosis.

Additional studies indicated that a second secretory protein, a proteoglycan, was coreleased by cracked cells by a Ca\textsuperscript{2+}-activated, MgATP-dependent PRL release by cracked cells. Disrupting cell structure by passing cells through the bulb homogenizer markedly reduced Ca\textsuperscript{2+}-activated PRL release in spite of the fact that PRL granules/vesicles remained intact. This observation indicates that isolated membrane elements, in the absence of adequate structural organization, fail to engage in Ca\textsuperscript{2+}-activated PRL release. Hence, it is very unlikely that Ca\textsuperscript{2+} activation in this system involves an unmasked non-specific lytic mechanism. A requirement for structural integrity is expected for vectorial exocytosis.

Further indirect studies have generated a large number of hypotheses concerning the Ca\textsuperscript{2+} regulation of exocytosis (2–6). Studies have suggested the participation of calmodulin (38, 39), Ca\textsuperscript{2+}-dependent protein kinase II (40), calcineurin (15), protein kinase C (31), phospholipase C (41, 42), phospholipase A (43), Ca\textsuperscript{2+}-dependent membrane-binding proteins (44–47), Ca\textsuperscript{2+}-dependent calmodulin-binding proteins (48), or Ca\textsuperscript{2+}-dependent cytoskeleton-associated proteins (49). Although there is no direct evidence available, the similarity in the Ca\textsuperscript{2+} activation characteristics suggests that common mechanisms underlie Ca\textsuperscript{2+}-activated secretory exocytosis.
cytosis in diverse systems. It is possible that several Ca\textsuperscript{2+}-dependent mechanisms act either sequentially or in concert. Different requirements in various systems might reflect different rate-limiting steps involving vesicle/granule transport to the plasma membrane, docking of granules at exocytotic sites, or the membrane fusion reactions involved in exocytotic discharge.

Our results with permeable GH\textsubscript{3} cells have a direct bearing on several of the proposed mechanisms for Ca\textsuperscript{2+}-regulated exocytosis. We have been unable to demonstrate inhibition of Ca\textsuperscript{2+}-activated PRL release using high concentrations of calmodulin inhibitors. Direct addition of purified calmodulin or calmodulin-dependent protein kinase II was unable to substitute for the cytotoxic factor in our system. These results argue against a major role for calmodulin in Ca\textsuperscript{2+}-activated PRL release and indicate that the cytotoxic factor is not likely to be a calmodulin-regulated enzyme.

Several results also argue against a role for protein kinase C as the Ca\textsuperscript{2+} effector system involved in Ca\textsuperscript{2+}-activated PRL release. H-7, a protein kinase C inhibitor (28), had no effect on PRL release supported by the cytotoxic factor, and purified protein kinase C was unable to substitute for the cytotoxic factor. In spite of the conclusion that protein kinase C may not itself constitute the Ca\textsuperscript{2+}-dependent effector, several studies document that protein kinase C activation can stimulate PRL secretion in GH\textsubscript{3} cells (16, 20). Possibly, as suggested by Baker and Knight (50) for chromaffin cells, protein kinase C activation can sensitize the Ca\textsuperscript{2+}-dependent effector system to low Ca\textsuperscript{2+} concentrations. Our preliminary results indicate that PRL release from cracked cells can also be stimulated by protein kinase C activators at low Ca\textsuperscript{2+} concentrations.

Neomycin was found to be a very potent inhibitor of Ca\textsuperscript{2+}-activated PRL release. Neomycin inhibition has previously been reported for a large number of secretory systems (41, 51, 52). It is known that neomycin binds phosphatidylinositol 4,5-bisphosphate and inhibits the metabolism of this lipid by several enzymes, including phospholipase C (29). Hence, neomycin inhibition might indicate the involvement of phospholipase C in Ca\textsuperscript{2+}-activated PRL release (42). The cytotoxic factor described in this study exhibits chromatographic properties similar to at least one of the brain cytotoxic isoenzymes (53). However, the cationic nature of neomycin and the possibility of inhibitory actions not involving phospholipase C preclude definitive interpretation at this time.

One of the steps of cytotox factor purification involves Ca\textsuperscript{2+}-dependent hydrophobic interaction chromatography on phenyl-Sepharose. As detailed elsewhere,\textsuperscript{3} interaction of the factor with this hydrophobic matrix at low ionic strength was Ca\textsuperscript{2+}-dependent, a property which implies that the cytotoxic factor is itself a Ca\textsuperscript{2+}-dependent enzyme or binding protein. In addition, the cytotoxic factor was shown to interact with liposomes in a Ca\textsuperscript{2+}-dependent manner.\textsuperscript{4} Hence, the cytotoxic factor may be similar to one of the recently characterized Ca\textsuperscript{2+}-dependent membrane-binding proteins (annexins, 44–47). However, direct tests have shown that calpactin I is incapable of substituting for the cytotoxic factor. In addition, the native molecular mass of the cytotoxic activity (200–350 kDa) is substantially greater than those reported for many of these Ca\textsuperscript{2+}-binding proteins (3, 5, 6, 44–47).

At present, neither the subunit molecular weight nor the identity of the cytotoxic factor with a previously identified protein are known. Future efforts to fully purify this Ca\textsuperscript{2+}-dependent cytotoxic factor should indicate the nature of Ca\textsuperscript{2+}- dependent events associated with regulated secretion.

Acknowledgments—We greatly appreciate the help provided by H. Ris and P. Cooke for HVEM studies and by V. W. Welshons and J. Pawley for SEM studies which were conducted at the Integrated Microscopy Resource (IMR) in Madison. The IMR is funded as a National Institutes of Health Biomedical Research Technology Resource (RR570). TEM studies were conducted with the help of P. J. Lewandoski at the Centralized Electron Optics Facility, Anatomy Department, University of Wisconsin.

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