cAMP and Ca^{2+}-mediated Secretion in Parotid Acinar Cells Is Associated with Reversible Changes in the Organization of the Cytoskeleton

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Abstract. The potential involvement of actin and fodrin (brain spectrin) in secretory events has been assessed in primary cultured guinea pig parotid acinar cells, using as a tool affinity purified anti-alpha-fodrin antibody, phalloidin, and immunofluorescence techniques. In resting parotid acinar cells fodrin and actin appeared as a continuous ring under the plasma membrane of most of the cells. Upon stimulation with secretagogues fodrin and actin labeling at the level of the plasma membrane disappeared almost completely. To establish a correlation between secretion and cytoskeletal changes at the individual cell level, anti-alpha-amylase-antibodies were used to label secreted amylase exposed at the surface of secreting cells. The number of cells expressing alpha-amylase on their surface followed bulk secretion of alpha-amylase. A strict correlation between secretion and alteration of the actin-fodrin labeling was observed at the individual cell level. The cytoskeletal changes occurred in parallel with secretion independently of the secretagogue used (carbamoylcholine in the presence of Ca^{2+}, isoproterenol in presence or absence of Ca^{2+}, forskolin, or dibutyryl-cyclic-AMP). The changes were reversible upon removal of the secretagogue.

Since Ca^{2+}, as well as cAMP-mediated secretion, was associated with the same kind of cytoskeletal changes, a reorganization of the cytoskeleton may play an essential part in regulated secretion.

THE cytoskeletal proteins actin and fodrin (brain spectrin) are widely distributed among different cell types. Fodrin is a heterodimer composed of an alpha (240 kD) and a beta (235 kD) subunit. The main characteristics of the proteins of the fodrin-spectrin family are self-assembly and binding to actin filaments, this latter property being inhibited by calcium (for review see references 8 and 9). The existence of F-actin-fodrin networks in vitro and in vivo has been reported. In most cell types, actin filaments and fodrin are colocalized under the plasma membrane, a notable exception being epithelial and endothelial cells where fodrin is mainly detected along the actin cables within the cell (4, 12). The existence of F-actin-fodrin networks under the plasma membrane of secretory cells raises the question of whether a reorganization of this network is required for secretory vesicles to get access to the plasma membrane during regulated secretion.

The first demonstration of such a reorganization during stimulated secretion came from experiments in chromaffin cells of the adrenal medulla. Following stimulation of chromaffin cells, a dramatic redistribution of fodrin (7, 14) and a reduction in the amount of actin associated with the cytoskeleton (3) was observed. Furthermore, the introduction of anti-alpha-fodrin antibodies into permeabilized chromaffin cells induced a 50% inhibition of catecholamine release (15). As nicotine- and potassium-induced secretion in chromaffin cells involves an increase in free calcium, a calcium-based mechanism was at first assumed to control fodrin redistribution under these conditions.

If the reorganization of the cytoskeleton observed in chromaffin cells were not an epiphenomenon, but were directly involved in granule docking or membrane retrieval, one should expect similar changes to occur in other cell types showing regulated secretion. Therefore, we have used in the present study guinea pig parotid acinar cells in primary culture as a model. In contrast to chromaffin cells, the parotid acinar cell is an exocrine cell, and exocytosis can be evoked not only by agonists which involve an increase in free calcium but also by agonists which stimulate secretion via an increase in cAMP (5). If the reorganization of the cytoskeleton were an essential step during regulated secretion, one should observe these changes with both types of agonists. To study the state of the cytoskeleton, we used as a tool affinity purified antibodies directed against alpha-amylase and alpha-fodrin, TRITC-coupled phalloidin, and immunofluorescence techniques.

Our results show clearly at the single cell level that stimulation of secretion is associated with a dramatic reorganization of the F-actin/fodrin web also in exocrine cells and that it occurs irrespective of whether the second messenger involved is free calcium or cAMP. In addition it will be shown that the effect of cAMP cannot be explained by an increase

in cytosolic-free calcium. These findings support the idea that the reorganization of the subplasmalemmal cytoskeleton is not an epiphenomenon but rather directly linked to regulated exocytosis.

Materials and Methods

Materials

Guinea pigs were from Winkelmann (Dernbach, Germany). BSA was from Paesel and Lorei (Frankfort, Germany); MEM from Biochrom KG (Berlin, Germany); FCS from Gibco (Paisley, UK). Dispase, DNAse, hyaluronidase, poly-D-lysine, MEM vitamins, and amino acids were purchased from Boehringer-Mannheim Biochemicals (Mannheim, Germany). Collagenase, soya trypsin inhibitor, carbamoylcholine, isoproterenol, *N*-6,2'-O dibutyryladenosine-3', 5' cyclic monophosphate (dicAMP)[1], 3-isobutyl-1-methylxanthine (IBMX), and phalloidin coupled to rhodamine were obtained from Sigma Chemical Co. (St. Louis, MO). CNBr activated Sepharose 4B was from Pharmacia (Uppsala, Sweden). The various conjugated antibodies were from Dianova (Hamburg, Germany).

Cell Culture

Male guinea pigs of the Pirbright white strain were anesthetized with pentobarbital and killed by heart incision. Parotids were removed and dissected with forceps in KRB (Krebs-Ringer) buffer (12.5 mM Hepes, 135 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 5 mM glucose, 5 mM β-hydroxybutyrate, 40 μg soya trypsin inhibitor/ml, pH 7.4) containing 1.81 mM $CaCl_2$ and 0.81 mM $MgSO_4$ under 100% O_2, incubated for 5 min in EGTA-buffer (0.5 mM EGTA, 116 mM NaCl, 4.8 mM KCL, 15.6 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5 mM glucose, pH 7.4), washed in KRB buffer and subsequently incubated for 2 × 30 min in a shaking water bath in presence of 5 mg/ml dispase and 100 μg DNAse/ml Puck's buffer (120 mM NaCl, 5 mM KCl, 5 mM KCl, 26 mM $NaHCO_3$) under 5% CO_2 and 95% O_2. Cells were washed with KRB buffer plus 0.1% BSA, and incubated for 30 min in presence of 1.5 mg collagenase, 1 mg BSA, 200 μg hyaluronidase, and 0.5 mg soya trypsin inhibitor/ml MEM medium under 5% CO_2 and 95% O_2. Subsequently the cells were passed through an 84-μm nylon mesh and centrifuged at 100 *g* for 2 min. The pellet was resuspended in KRB buffer plus 0.1% BSA. After centrifugation under the same conditions, cells were loaded onto a 4% (wt/vol) BSA gradient in KRB buffer and centrifuged at 100 *g* for 4 min. After two more washes in KRB buffer, cells were resuspended in MEM medium and plated in 24-well plates at a density of 10^6 cells/well and kept at 37°C under 5% CO_2 in MEM medium supplemented with 10% FCS.

Secretion Studies

Parotid acinar cells were used after 12 h in culture. Cells were washed twice in release medium (116 mM NaCl, 5.4 mM KCl, 10 mM Na phosphate, 26 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 2 mM glutamine, 5 mM glucose, pH 7.4) containing either 2 mM $CaCl_2$ or 0.2 mM EGTA. After washing, parotid cells were allowed to stay for 10 min in the release medium. Subsequently an aliquot of the medium was removed to estimate basal release, followed by the addition of the secretagogue. At the end of the stimulation period, medium was withdrawn to evaluate amylase release, and the cells were lysed with cold Lubrol (2 mg/ml) to determine the amylase content of the cells.

In case of pretreatment of the cells with the calcium chelator carboxymethyl-1,2-bis(2-aminophenoxy)ethane N,N,N',N-tetraacetic acid (BAPTA), cells were preincubated for 20 min at room temperature in presence of 20 μm BAPTA (19). Amylase activity was measured according to reference 2.

Production of Antibodies

Fodrin was extracted from bovine brain with a low salt buffer, and purified by ion exchange chromatography and gel filtration (4). Alpha and β subunits were separated on a 5% polyacrylamide gel. After Coomassie blue staining the alpha subunit was excised from the gel, mixed with complete Freund's adjuvant, and injected into a rabbit. Two weeks after the first boost in incomplete Freund's adjuvant, antibodies against the alpha-subunit of fodrin could be detected by immunoblotting. Affinity-purified immunoglobulins (Ig) were obtained by incubation of the antiserum with the alpha subunit of fodrin which had been transferred from a polyacrylamide gel to a nitrocellulose strip (14). Ig were eluted by glycine/HCl, pH 2.8, and immediately neutralized.

Alpha amylase from guinea pig parotid glands was purified according to reference 16. A sheep was immunized by multiple intracutaneous injections of the antigen in Freund's complete adjuvant. Further boosts were performed in Freund's incomplete adjuvant. Amylase was coupled to CNBr-activated Sepharose 4B, and antibodies specific for alpha-amylase were affinity purified on this column. Affinity purified antibodies directed against alpha-amylase were coupled to FITC according to reference 13.

Electrophoretic Methods and Immunoblotting

Electrophoresis was performed according to reference 10, proteins were transferred to nitrocellulose (18), incubated with either affinity purified antibodies against alpha-fodrin followed by incubation with peroxidase-conjugated goat anti-rabbit IgG antibodies diluted 1/1,000, or with anti-alpha-amylase antibodies followed by peroxidase-coupled donkey anti-sheep IgG diluted 1/1,000. The incubations were performed in presence of 3% BSA and 10% FCS in PBS.

Immunocytochemistry

For immunofluorescence studies, 5 × 10^5 parotid acinar cells were seeded in 24-well plates on glass coverslips previously coated for 30 min at room temperature with 10 μg/ml poly-D-lysine. After the various treatments, cells were rapidly rinsed in PBS, fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were subsequently incubated in presence of FITC-coupled anti-alpha-amylase antibodies diluted 1/500 in 10% FCS in PBS for 1 h. After washing in PBS, cells were permeabilized with ice cold acetone (50, 90, 50% vol/vol successively for 3, 5, and 3 min, respectively), and incubated either for 1 h with 3 ng/ml phalloidin coupled to rhodamine or for 2 h with anti-alpha-fodrin antibodies (5.10 μg/ml) in 10% FCS in PBS, followed by goat anti-rabbit IgG antibodies coupled to rhodamine diluted 1/150 in the same buffer for 1 h. Unless otherwise stated, all incubations were performed at room temperature. Coverslips were mounted in a 1:1 mixture of glycerol and PBS. Probes were examined under epi-illumination, using a microscope (IM35; Carl Zeiss, Oberkochen, Germany) and a 100× objective. Micrographs were taken with Kodak 3200 ASA film (Eastman Kodak Co., Rochester, NY).

Results

Characterization of the Anti-Alpha-Fodrin Antibody

Affinity purified anti-alpha-fodrin antibody was characterized by immunoblotting. A bovine brain fraction enriched in fodrin was submitted to SDS-PAGE and proteins were transferred to a nitrocellulose sheet by blotting. The affinity purified anti-alpha-fodrin antibody detected a band of 240 kD (Fig. 1 *a*). When frozen fractions were used, one could detect with the same antibody a lower band (110 kD) which corresponds most likely to a degradation product of fodrin.

The same antibody used after blotting of whole guinea pig parotid cell extracts labeled only a band with 240 kD (Fig. 1 *b*), indicating that these antibodies could be used for immunostaining of alpha-fodrin in parotid acinar cells.

Anti-Alpha Amylase Antibody As a Marker of Secretion at the Level of the Individual Parotid Acinar Cell

The affinity purified anti-alpha-amylase antibodies were used for immunoblotting of a guinea pig protein fraction enriched in amylase and a total parotid gland extract (Fig. 2, *a* and *b*). In both cases, only one band with 52 kD was detected. The same antibody coupled to FITC was used to label parotid acinar cells in culture. Permeabilized cells displayed

1. *Abbreviations used in this paper*: DbcAmp, dibutyryladenosine-3',5' cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine.

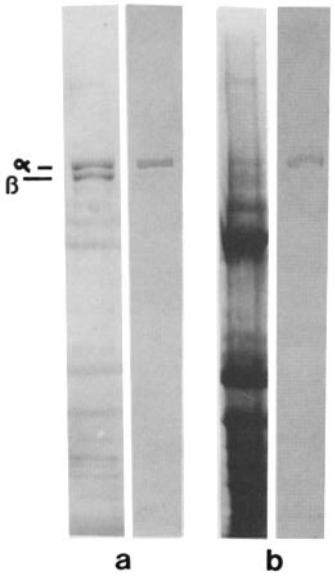

Figure 1. Characterization of anti-alpha-fodrin antibodies. Proteins of a bovine brain preparation enriched in fodrin (*a*) were subjected to SDS-PAGE and subsequently labeled with Coomassie blue (*left*) or transferred to a nitrocellulose sheet and labeled with affinity purified anti-alpha-fodrin antibodies (*right*). The same procedure was repeated for guinea pig parotid acinar proteins (*b*). (*Left*) Coomassie blue labeling of the acrylamide gel. (*Right*) corresponding blot. The localization of the alpha and β subunits of fodrin is indicated.

a strong cytoplasmic labeling, but no labeling at the level of the plasma membrane (Fig. 2 *c*). Commercial FITC-coupled goat-anti-rabbit antibodies were used as a control, and no labeling was detected (data not shown).

When the FITC-coupled anti-alpha-amylase antibody was used in nonpermeabilized parotid acinar cells, no labeling at all was observed (Fig. 2 *d*) in most (90–95%) of the cells. A few cells (typically 5–10% of the cells, depending on the cell preparation) displayed some labeling at the level of the plasma membrane. This staining was present at the cell surface, punctate, or as a bright line at the level of the plasma membrane, either restricted to one pole of the cell or, in a minority of cells, as a continuous ring (Fig. 2, *e* and *f*). Moreover, no cytoplasmic labeling comparable to the one detected in permeabilized cells was observed.

When parotid acinar cells in primary culture were stimulated with various secretagogues one could observe an increase in the number of cells presenting an intense labeling of amylase on the cell surface as well as a rise in the secretory activity of the cells as assessed by measuring the bulk release of amylase (see Tables I–III). Therefore, it seems justified to use the appearance of amylase labeling at the cell surface as a marker for secretion at the single cell level.

Fodrin/Actin Organization in Resting Parotid Acinar Cells

Parotid acinar cells were fixed, labeled with FITC-coupled anti-alpha-amylase antibodies, permeabilized, and incubated in presence of either TRITC-coupled phalloidin or anti-alpha-fodrin antibodies followed by a second antibody coupled to TRITC.

In the resting cells (defined by the absence of alpha-amylase labeling at the plasma membrane level; Fig. 3, *a–d*), both actin filaments and fodrin were detected as a bright continuous ring under the plasma membrane in most of the cells. The correlation between the absence of surface amylase labeling and the presence of continuous subplasmalemmal cytoskeletal ring in the same cell was 100% ($n = 66$) for fodrin and 99% for actin ($n = 102$). Interestingly, one could observe that the few cells presenting an amylase labeling at the surface had a different actin/fodrin labeling. The subplas-

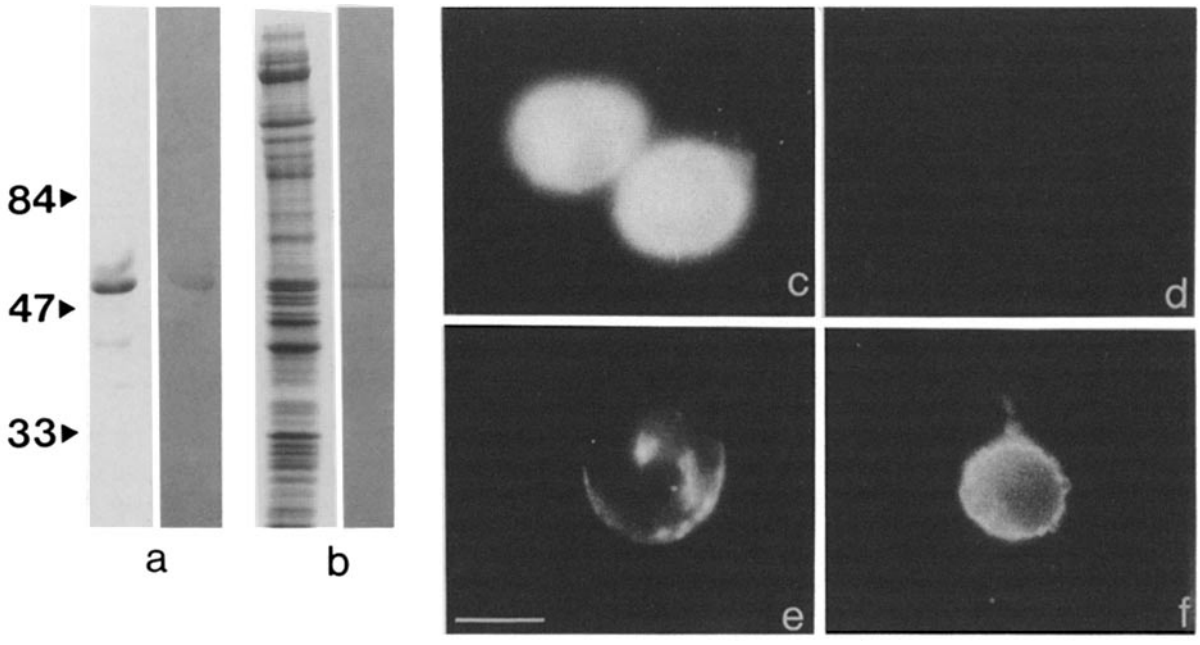

Figure 2. Characterization of anti-alpha-amylase antibodies. Amylase purified from parotid acinar gland (*a*) and a parotid gland whole extract (*b*) were submitted to SDS-PAGE. (*Left*) Coomassie blue staining of the gel. (*Right*) corresponding blot labeled with affinity purified anti-alpha-amylase antibodies. Molecular mass (in kD) of standard proteins are indicated on the left. (*c*) Amylase immunofluorescent labeling in permeabilized parotid acinar cells in culture. (*d–f*) Amylase labeling of non-permeabilized parotid acinar cells in culture. Typically 90–95% of cells present an absence of decoration similar to the one shown in *d*; a minority of the cells present a surface labeling similar to the one shown in *e* and *f*.

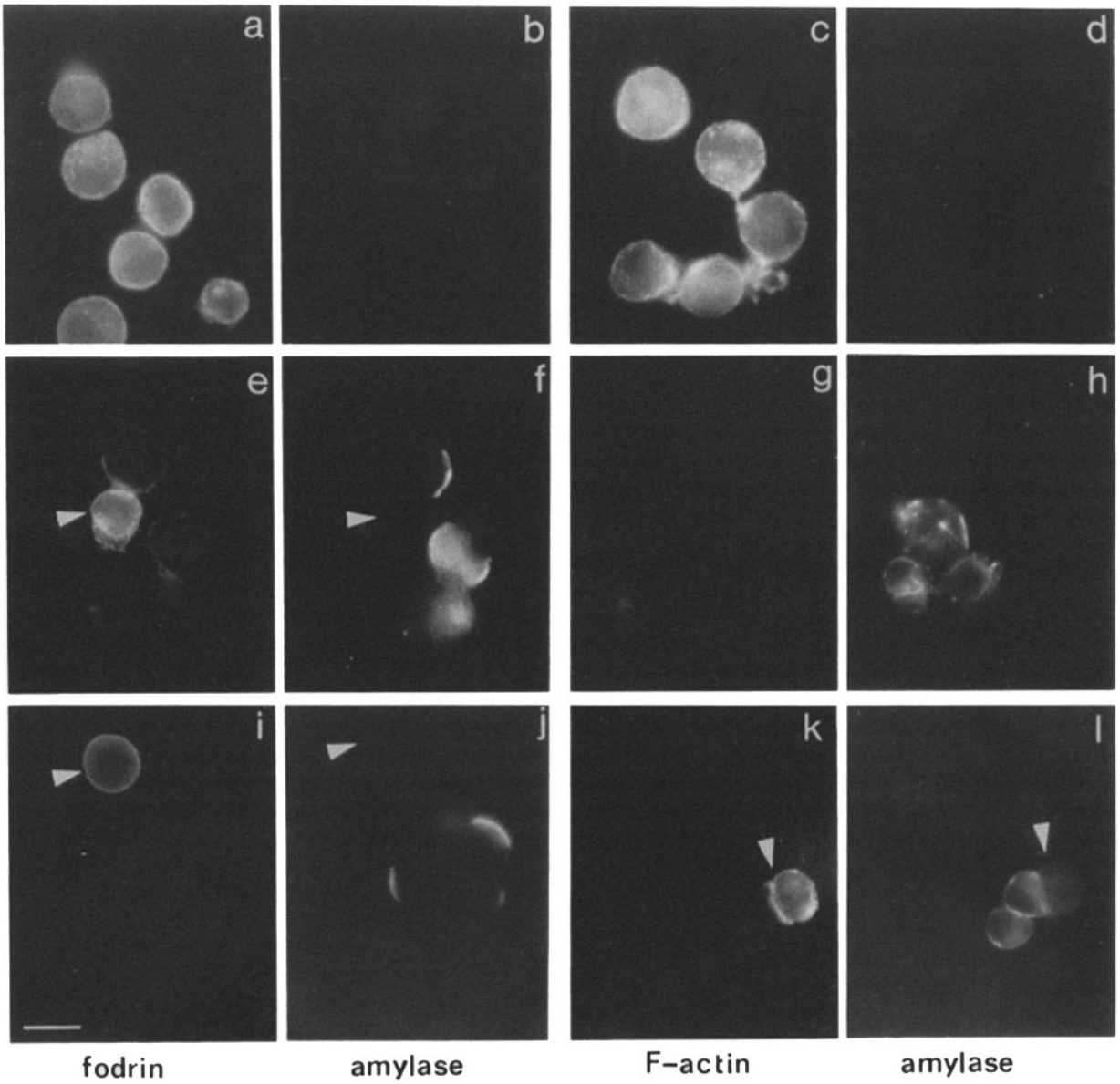

Figure 3. Correlation between secretion and F-actin/fodrin localization at the individual cell level. Fodrin (*a*, *e*, and *i*), F-actin (*c*, *g*, and *k*) labeling, and corresponding (*b*, *f*, and *j*, and *d*, *h*, and *l*, respectively) surface amylase labeling. Parotid acinar cells in culture were incubated in absence (*a–d*), or presence of either 10^{-5} M carbamoylcholine (*e–h*) or 10^{-5} M isoproterenol (*i–l*). After 30 min of incubation, the cells were fixed and labeled with an anti-alpha-amylase antibody coupled to fluorescein. Subsequently, the cells were permeabilized and labeled with either anti-alpha-fodrin antibodies followed by a second antibody coupled to TRITC, or with phalloidin coupled to TRITC. Interestingly, stimulated cells which did not present any amylase surface labeling did not present a weakening or disappearance of fodrin or F-actin fluorescence (*arrowheads*). Bar, 10 μm.

malemmal labeling had disappeared or was at least much bleaker in 90% (n = 37) of these cells (not shown).

Fodrin/Actin Organization in Stimulated Cells: Stimulation with the Muscarinic Agonist Carbamoylcholine

When parotid acinar cells in culture were stimulated with 10^{-5} M carbamoylcholine in presence of 2 mM calcium, one could observe an increase in bulk secretion, accompanied by an increase in the number of cells expressing immunoreactive alpha-amylase on their surface (Table I). Furthermore, at the level of the individual cell, a strict parallelism between amylase detection at the cell surface and disappearance of the actin (100%, n = 86) or fodrin (98%, n = 87) ringlike labeling was observed (Fig. 3, *e–h*). On the other hand, treatment with carbamoylcholine in the absence of added calcium and simultaneous presence of 0.2 mM EGTA did neither stimulate secretion nor induce any rise in the number of cells presenting a surface amylase labeling (Table I). In accordance, a disappearance of the F-actin/fodrin rings under the plasma membrane was no longer visible (data not shown).

Stimulation with the β-Adrenergic Agonist Isoproterenol

Isoproterenol at a concentration of 10^{-5} M was in most of

Table I. Effects of Carbamoylcholine and Isoproterenol on Bulk Secretion of Alpha-Amylase and on the Appearance of Alpha-Amylase on the Cell Surface in the Presence and Absence of Calcium

Experimental condition	Bulk secretion (% of total amylase activity)	Surface labeling (% of examined cells)
Unstimulated	4.2 ± 1.0	4.1
Carbachol	12.1 ± 2.1	14.4
Carbachol − $CaCl_2$ + EGTA	3.7 ± 1.2	3.6
BAPTA alone	3.7 ± 1.7	3.4
Isoproterenol	27.6 ± 2.3	17.0
BAPTA/Isoproterenol −$CaCl_2$ + EGTA	20.5 ± 1.2	16.7

Secretion was measured after stimulation for 30 min. Isoproterenol and carbamoylcholine were both used at a concentration of 10^{-5} M. In the experiments with carbachol + EGTA, calcium in the external medium was replaced by 0.2 mM EGTA, in the experiments performed with isoproterenol + EGTA + BAPTA, the cells were preincubated in the absence of added calcium in the medium with 2.10^{-5} M carboxymethyl-BAPTA for 20 min. Subsequently the cells were stimulated with isoproterenol in presence of 0.2 mM EGTA. The results are from a single experiment where bulk secretion was determined in triplicates ($x \pm$ SEM). In two additional experiments the correlation between bulk secretion and surface labeling was similar although the absolute values differed slightly. In parallel, to assess the degree of surface labeling, parotid acinar cells in culture were fixed after stimulation, labeled with anti-alpha-amylase antibodies coupled to fluorescein, and the percentage of cells presenting a surface labeling was determined from a populaton of 300 cells or more.

Table II. Effects of Agonists Involving cAMP As Messenger on Bulk Secretion of Alpha-Amylase and Appearance of Alpha-Amylase Labeling on the Cell Surface

Experimental conditions	Bulk secretion (% of total amylase activity)	Surface labeling (% of examined cells)
Control	2.3 ± 0.1	10.0
Forskolin	10.7 ± 0.5	20.6
IBMX + DbcAMP	10.7 ± 0.3	24.0
Isoproterenol	18.1 ± 1.0	34.3
Control after removal of isoproterenol	1.6 ± 0.6	14.9

Secretion was measured after stimulation for 30 min. Isoproterenol was used at a concentration of 5×10^{-6} M. IBMX and DbcAMP were both used at a concentration of 5.10^{-4} M. In the experiment "Control after removal of isoproterenol," cells were first challenged with isoproterenol for 30 min, secretagogue was then removed and secretion was measured 30 min later. The results are from a single experiment where bulk secretion was determined in triplicates ($x \pm$ SEM). In two additional experiments the correlation between bulk secretion and surface labeling was similar although the absolute values differed slightly. The degree of surface labeling was measured as given in the legend to Table I.

the cell preparations a more potent agonist than carbamylcholine, and induced a sharp rise in secretion and percentage of cells presenting a surface amylase labeling (Table I). Here too, in most of the cells one could observe a strict correlation between amylase labeling at the cell surface and disappearance or weakening of the fodrin/actin staining (Fig. 3, *i–l*); 94% (n = 100) of the amylase-stained cells presented this altered fodrin labeling. The corresponding value for F-actin was 95% (n = 88). The effect of isoproterenol upon these parameters did not significantly change when external calcium was omitted from the medium and 0.2 mM EGTA was added.

Nevertheless, isoproterenol might have led to a small increase in intracellular calcium despite the removal of external calcium, and this increase could have been responsible for the cytoskeletal rearrangement. We, therefore, did not only replace external calcium by EGTA, but in addition preincubated the parotid acinar cells in culture in presence of 2.10^{-5} M of the calcium chelator BAPTA-carboxymethyl ester for 20 min at 20°C before challenging the cells with 10^{-5} M isoproterenol in presence of 0.2 mM EGTA. Controls were performed in presence of BAPTA. Under these stringent conditions, isoproterenol was still able to induce a significant increase over control in terms of bulk secretion and percentage of cells presenting a surface amylase labeling (Table II). In some cultures, no difference at all was observed in terms of isoproterenol-induced secretion between cells pretreated with BAPTA and non-pretreated cells (data not shown). Moreover, the strong correlation (typically 95%) between surface amylase labeling and fodrin/actin reorganization (Fig. 4, *e–h*) at the level of the individual cell pertained. BAPTA treatment alone did not induce any changes in the F-actin/fodrin labeling. These results indicate that isoproterenol induces not only secretion but also the changes in the F-actin-fodrin web by a mechanism involving cAMP and not by an increase in intracellular free calcium. Furthermore, to reinforce the arguments in favor of a possible involvement of cAMP in the coupling between secretion and reorganization of the cytoskeleton, parotid acinar cells in culture were stimulated with either 10^{-5} M forskolin or 5.10^{-4} M dibutyryl-cAMP in presence of 5.10^{-4} M IBMX. In both cases, one could measure a strong increase of secretion, and a significant rise in the number of cells presenting a surface amylase labeling was observed (Table II). Under both conditions, a strict correlation between secretion and cytoskeleton rearrangement occurred (95%, n = 134; in the case of forskolin, and 100%, n = 82 in the case of dibutyryl-cAMP, respectively) as observed by immunofluorescence at the individual cell level (Fig. 4, *i–p*).

Reversibility of the F-actin-Fodrin Reorganization Process

30 min after removal of isoproterenol, both bulk secretion and the number of cells presenting amylase immunoreactivity on the surface had returned to values close to control (Table II). This was accompanied by a reappearance of F-actin/fodrin labeling indistinguishable from the appearance of unstimulated cells (data not shown). Moreover, 93% (n = 108) of the cells unlabeled with amylase presented a bright continuous fodrin ring, and 100% (n = 100) of these cells presented a continuous subplasmalemmal F-actin labeling. The cells which still presented a surface amylase decoration exhibited an altered cytoskeletal labeling (10 out of 10 counted cells). These data demonstrate the reversibility of the cytoskeletal rearrangement process.

When parotid acinar cells in culture were challenged with increasing concentrations of either carbamoylcholine or isoproterenol before either fixation or measurement of bulk secretion, a dose-dependent increase in overall secretion was accompanied by an increase in the number of cells presenting an immunoreactive alpha-amylase labeling on the surface (Table III). At the individual cell level, one could observe again that the cells characterized by an amylase labeling at the plasma membrane level had also lost their F-actin/fodrin ring, as assessed by immunofluorescence (data not shown).

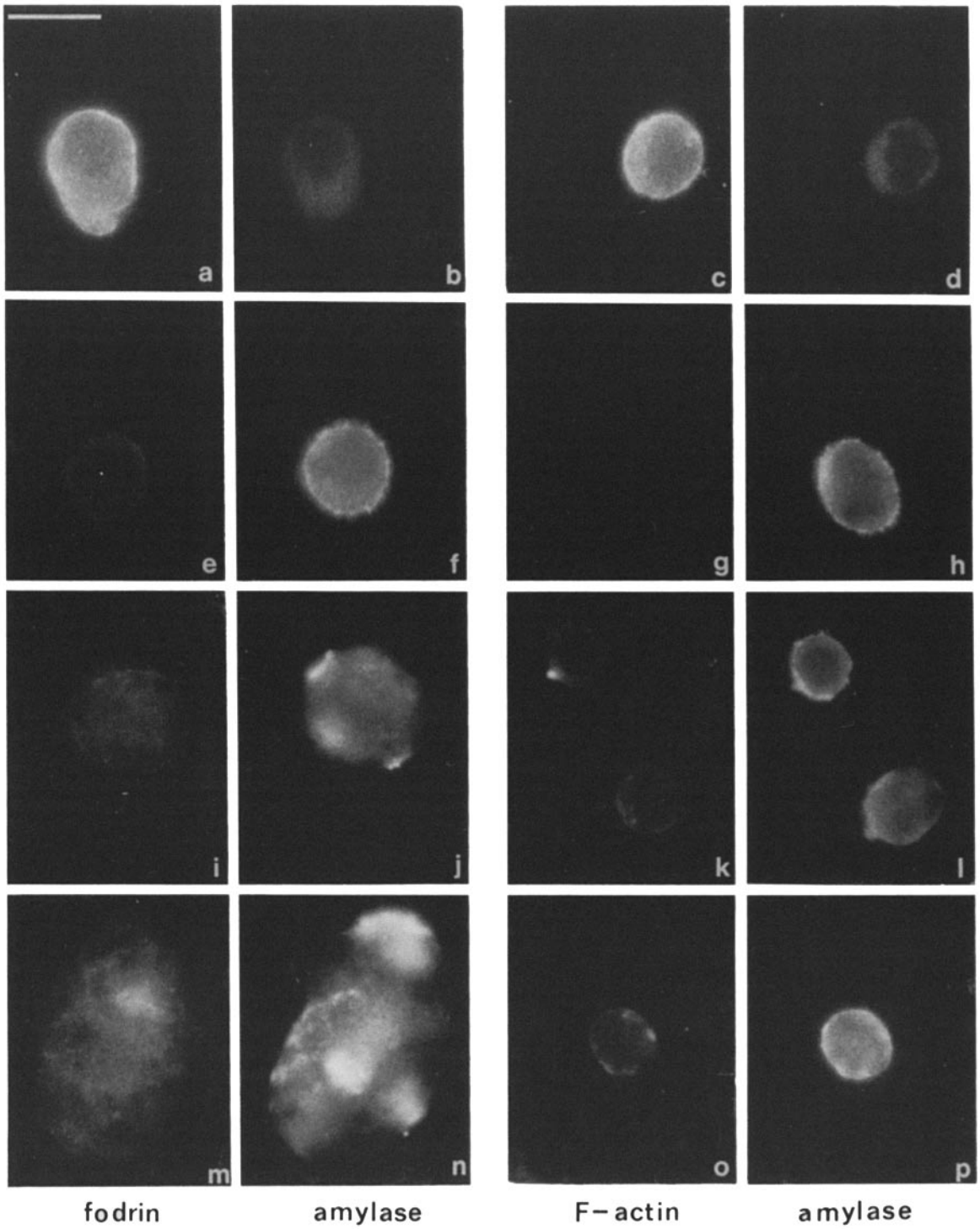

Figure 4. Correlation between secretion and F-actin/fodrin localization at the individual cell level. Fodrin (*a*, *e*, *i*, and *m*), F-actin (*c*, *g*, *k*, and *o*) labeling, and corresponding (*b*, *f*, *j*, and *n* and *d*, *h*, *l*, and *p*, respectively) surface amylase labeling. (*a–d*) Parotid acinar cells were preincubated for 20 min in presence of 2.10^{-5} M BAPTA, and subsequently incubated in presence of 0.2 mM EGTA and absence of secretagogue. (*e–h*) Parotid acinar cells were preincubated for 20 min in presence of 2.10^{-5} M BAPTA, and subsequently incubated in presence of 0.2 mM EGTA and 10^{-5} M isoproterenol. (*i–l*) Stimulation in presence of 10^{-5} M forskolin. (*m–p*) Stimulation in presence of 5.10^{-4} M DbcAMP plus 5.10^{-4} M IBMX. After 30 min of incubation, the cells were fixed and labeled with an anti-alpha-amylase antibody coupled to fluorescein. Subsequently, the cells were permeabilized and labeled with either anti-alpha-fodrin antibodies followed by a second antibody coupled to TRITC, or with phalloidin coupled to TRITC. Bar, 10 μm.

Table III. Effect of Increasing Doses of Carbamoylcholine on Bulk Alpha-Amylase Secretion and Appearance of Immunoreactive Alpha-Amylase on the Cell-Cell Surface

Carbachol, (10^{-5} M)	Bulk secretion (% of total amylase activity)	Surface labeling (% of examined cells)
0	3.8 ± 0.5	7.9
0.01	2.9 ± 0.6	8.5
0.1	4.2 ± 1.2	13.1
1.0	14.0 ± 2.1	15.0
10.0	25.1 ± 1.1	26.7

Parotid acinar cells in culture were stimulated for 30 min in presence of increasing concentrations of carbamoylcholine (carbachol). The results are from a single experiment where bulk secretion was determined in triplicates ($x \pm$ SEM), and are representative for another experiment. The degree of alpha-amylase surface labeling was determined as given in the legend to Table I.

Furthermore, in order to rule out a potential disruptive effect of the permeabilization procedure on the cytoskeleton, 10 μM digitonin were substituted for acetone. The resulting labeling of actin filaments and fodrin in resting cells was indistinguishable from the one obtained with acetone alone as a permeabilizing agent. Moreover, the correlation between F-actin/fodrin rearrangement and positive amylase labeling on the cell surface was again observed upon stimulation with either carbamoylcholine or isoproterenol, and the redistribution observed was indistinguishable from the one observed following acetone treatment (data not shown).

Discussion

In this study we have used anti-alpha-amylase antibodies as a tool to detect secretion at the level of the individual cell. Our assumption was that a fraction of the amylase released following fusion of a secretory granule would remain for some time associated with the plasma membrane. This assumption was based on our observation, that membranes prepared from purified parotid gland secretory granules still bind significant amounts of amylase even after washing with 0.5 M NaCl. In the unstimulated state only few cells exhibited a surface fluorescence following incubation with the FITC-labeled anti-amylase antibody. This most likely reflects the "basal secretion" measured in bulk secretion experiments. However, both the number of unstimulated parotid cells in culture expressing a surface amylase labeling (see Tables I and II) and the intensity of this labeling differed markedly from one culture to the other. Nevertheless, the strict correlation (typically >90%) between the expression of amylase labeling at the cell surface and changes in the cytoskeleton was constant throughout the different experiment conditions used.

Fluorescence labeling of fodrin and F-actin showed in primary cultured guinea pig parotid acinar cells a similar pattern as described previously for chromaffin cells (14), namely a ringlike labeling directly under the plasma membrane. In view of the actin reticulating properties of fodrin in vitro (8) one has to assume the existence of an actin-fodrin network right under the plasma membrane of parotid acinar cells. Our working hypothesis was that, in case this network should inhibit docking and fusion of secretory vesicles in cells with regulated secretion: (*a*) a reorganization of this network should take place; (*b*) this reorganization should occur independently of the signal transmission pathway used to induce secretion; and (*c*) changes of the cytoskeleton should correlate with exocytosis at the level of the individual cell.

The primary cultured guinea pig parotid acinar cell is still able to respond with increased secretion to muscarinic as well as to β-adrenergic agonists. In accordance with the working hypothesis, both types of agonists led to a dramatic reorganization of the cytoskeleton detectable as a disappearance of fodrin and F-actin fluorescence. Moreover, at the level of the individual cell there was an almost perfect correlation between secretion as detected by amylase surface fluorescence and reorganization of the cytoskeleton. A reorganization of the subplasmalemmal fodrin/F-actin web had already been observed in stimulated chromaffin cells (3, 14), and had been related to the increase in free calcium upon stimulation. As it had been claimed that isoproterenol does not only increase cAMP but induces also a moderate rise in free calcium (5), we had to exclude the possibility that the reorganization of the cytoskeleton observed in isoproterenol stimulated parotid acinar cells had resulted from an increase in free calcium and not from the rise of cAMP.

Our results exclude the possibility that isoproterenol induces secretion and reorganization of the cytoskeleton via an increase in free calcium. Whereas carbamoylcholine-induced secretion as well as cytoskeletal changes were completely blocked when external calcium was replaced by EGTA, stimulation of both parameters by isoproterenol was not or very little affected when external calcium had been replaced by EGTA, and the remaining internal calcium chelated by a permeable BAPTA derivative. Therefore, the effect of isoproterenol on secretion as well as on the cytoskeleton must involve an increase of cAMP. Moreover, experiments with forskolin and with DbcAMP show that an increase in cAMP is sufficient to stimulate secretion as well as rearrangement of the cytoskeleton in guinea pig parotid acinar cells.

According to our present knowledge, cAMP can induce regulatory processes either by enhancing the phosphorylation of proteins by cAMP-dependent protein kinases or by releasing RI- or RII-type regulatory subunits. It seems much more likely that the phosphorylation of specific proteins involved in the organization of the fodrin/F-actin network (1) represents the mechanism by which the β-agonist leads to the observed cytoskeletal changes. Interestingly, phosphorylation of the myosin light chain kinase by protein kinase A has been shown to induce a loss of microfilament bundles and morphological changes in fibroblasts (11). It seems possible that muscarinic agonists act also by a protein phosphorylation mechanism as an increase in free calcium can activate calmodulin-dependent protein kinases. We have recently presented evidence that stimulation of guinea pig parotid acinar cells by carbamoylcholine leads indeed to the activation of calcium/calmodulin-dependent protein kinase II (6, 17). Work aimed at analyzing specific protein phosphorylations involved in the cytoskeletal changes described here are under way.

The disappearance of the spectrin/F-actin web is reversible following removal of the agonist. This indicates that fodrin has only undergone rearrangement but not degradation. We do not know whether fodrin remains accessible for the corresponding antibodies or actually redistributes in the cell. In any case, redistribution of fodrin into the cytoplasm would

lead to a drastic dilution compared to the accumulation under the plasma membrane observed in the resting state. This latter interpretation is strengthened by recent EM studies in the rat adrenal medulla, demonstrating that, upon stimulation, fodrin redistributes in the cytoplasm and is even associated with intracellular vesicles (7). Interestingly, however, in chromaffin cells in culture, fodrin has been shown to redistribute as patches (14). On the other hand, in parotid acinar cells in culture one could observe a complete disappearance of the fodrin labeling from the plasma membrane. It is not clear yet whether this reflects only a quantitative difference or whether the steps of reorganization of the cytoskeleton differ also qualitatively.

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