Cell. Mol. Life Sci. (2009) 66:2525–2537
DOI 10.1007/s00018-009-0052-0

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

The role of kinesin, dynein and microtubules in pancreatic secretion

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Received: 31 March 2009 / Revised: 8 May 2009 / Accepted: 13 May 2009 / Published online: 2 June 2009

Abstract The regulated secretion of pancreatic zymogens depends on a functional cytoskeleton and intracellular vesicle transport. To study the dynamics of tubulin and its motor proteins dynein and kinesin during secretion in pancreatic acinar cells, we infused rats with 0.1 μg/kg/h caerulein. Electron and fluorescence microscopy detected neither dynein nor kinesin at the apical secretory pole, nor on the surface of mature zymogen granules. After 30 min of secretagogue stimulation, kinesin and the Golgi marker protein 58 K were reallocated towards the apical plasma membrane and association of kinesin with tubulin was enhanced. Disruption of acinar cell microtubules had no effect on initial caerulein-induced amylase release but completely blocked secretion during a second stimulus. Our results suggest that mature zymogen granule exocytosis is independent of intact microtubules, kinesin and dynein. However, microtubule-dependent mechanisms seem to be important for the replenishment of secretory vesicles by redistribution of Golgi elements towards the apical cell pole.

Keywords Microtubules · Motor proteins · Pancreatic acinar cells · Zymogen granule maturation · Zymogen granule secretion

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Introduction

Pancreatic acinar cells are highly specialized for the synthesis, storage, and secretion of digestive enzyme precursors. In these cells, exocytosis of zymogen granules is tightly regulated to allow release only in response to physiological stimulation. Pancreatic proenzymes are thought to be sorted from constitutively secreted proteins in the Golgi complex and packed into secretory vesicles that bud off the *trans*-Golgi-network to undergo further maturation to zymogen granules. Mature zymogen granules accumulate in the apical region of the highly polarized acinar cells where they occupy as much as 30% of the cell volume [1]. Upon stimulation by secretagogues such as cholecystokinin (CCK) or its analogue caerulein, a Ca^{2+} release from intracellular stores triggers fusion of zymogen granules with the apical plasma membrane and the release of their content into the apical lumen [2–4].

Although regulated exocytosis has been studied for many years in the exocrine pancreas, this multistep process is only incompletely understood at the molecular level. It is, however, of fundamental pathophysiological interest to understand the mechanisms involved in the maturation and

secretion of zymogen granules, because acute pancreatitis, a life-threatening disease, is characterized by a break-down of cellular polarization and regulated exocytosis [5].

It is well established that actin and the microtubule cytoskeleton are critically involved in major trafficking pathways of secretory vesicles in eukaryotic cells. In pancreatic acinar cells, a disruption of the actin cytoskeleton by cytochalasin or actin monomer binding proteins inhibits stimulated secretion [6–9]. Disassembly of the actin cytoskeleton in rat pancreatic acinar cells during secretagogue-induced pancreatitis results in a complete inhibition of digestive enzyme release [10]. Recent studies demonstrated zymogen granules to be coated with actin and associated with myosin V, and further suggest an involvement of actin and its motor proteins in exocytosis [11–13]. The role of microtubules in zymogen granule transport, however, remains elusive since the antimicrotubular agent colchicine in most studies failed to suppress the secretory response when applied to pancreatic acini [14–17]. Moreover, past studies showed conflicting results regarding the localization and role of microtubule motor proteins, kinesin and dynein, in pancreatic acinar cells. An association of kinesin with apically clustered zymogen granules that increased in response to a secretory stimulus has been reported [18]. In contrast, Kraemer et al. [19] demonstrated that kinesin was absent from zymogen granule membrane fractions and localized exclusively to the basolateral region, whereas dynein and dynactin were found to be associated with zymogen granule membranes. More recently, a comprehensive proteomic study of zymogen granule membrane proteins identified 101 proteins, but neither dynein nor kinesin could be identified as zymogen granule binding motor protein [12]. It therefore remains to be established to what extent microtubules and their motor proteins participate in the secretory process. To study this in detail, we determined the subcellular distribution of β-tubulin and its motor proteins, kinesin and dynein, in the exocrine pancreas in resting cells as well as in response to a secretory stimulus. Additionally, the functional involvement of microtubules was addressed by treatment of freshly isolated pancreatic acini with colchicine. Our results suggest that secretion from mature zymogen granules in pancreatic acinar cells is independent of microtubules and motor protein involvement. Zymogen granule formation and supply, on the other hand, seem to require intact microtubules that presumably mediate transport of Golgi elements towards the apical cellular region.

Materials and methods

Reagents

If not otherwise stated, all chemicals were obtained from Sigma (Taufkirchen, Germany) or Merck Eurolab (Darmstadt, Germany), Amersham Pharmacia (Freiburg, Germany), or Bio-Rad (Hercules, CA, USA). Biotin *N*-hydroxy-succinimide ester was from Pierce (Rockford, IL, USA), BSA-c basic blocking solution was from Aurion (Wageningen, The Netherlands), collagenase from *Clostridium histolyticum* (EC. 3.4.24.3) was from SERVA (Heidelberg, Germany, lot no. 14007, collagenase activity 1.50 PZ-U/mg), Cy3 marked streptavidin was from Dianova (Hamburg, Germany), digoxigenin *N*-hydroxy-succinimide ester was from Boehringer (Mannheim, Germany), LR White was from London Resin (Basingstoke, Hants, UK), polylysine slides were from Menzel Gläser (Braunschweig, Germany), Vectastain Elite ABC-Kit (avidin-biotin-peroxidase complex) and Vectashield were from Vector Laboratories (Burlingame, CA, USA).

Animals

All experiments involving animals were performed in accordance with the Helsinki Declaration and The Guiding Principles in the Care and Use of Animals.

Antibodies

The antibodies (Ab) used were as follows: goat anti-mouse IgG Cy^{TM}3-labeled Ab (Jackson Immuno Research, West Grove, PA, USA), goat anti-mouse Biotin-conjugated IgG (Vector Laboratories), goat anti-mouse IgG horseradish peroxidase conjugated (Sigma), goat anti-mouse biotin-SP-conjugated IgG (Jackson Immuno Research), goat anti-mouse IgG labeled with 12 nm colloidal gold (Dianova), goat anti-rabbit IgG labeled with 6 nm colloidal gold (Dianova), goat anti-rabbit Alexa-594 labeled IgG (Molecular Probes, Eugene, OR, USA), mouse monoclonal antibody (mAb) raised against Golgi 58 K protein/formiminotransferase cyclodeaminase (FTCD) (Sigma), anti-dynein mAb cytoplasmic 74 kD intermediate chains (Chemicon International, Temecula, CA, USA), mAb raised against kinesin heavy chain (Chemicon International), anti-β-tubulin mAb (Sigma), mouse IgG (Dianova), rabbit polyclonal antibody (Ab) raised against tubulin (Sigma), sheep FITC-conjugated anti-digoxigenin Ab (Boehringer Mannheim, Germany).

Preparation of tissue samples

Male Wistar rats (Charles River, Sulzfeld, Germany) were fasted for 12–18 h with free access to water, infused with 0.9% NaCl or with the CCK analog caerulein (0.1 μg/kg/h), and sacrificed at intervals between 0 and 4 h (see Figure legends). Pancreas tissue samples were excised, snap-frozen in liquid nitrogen for protein analysis or flushed with PBS (13.7 mM NaCl, 2.7 mM KCl, 80.9 mM Na_2HPO_4,

and 1.5 mM KH_2PO_4, pH 7.4) to remove trapped blood, fixed overnight at +4°C in 4% formaldehyde in PBS and routinely processed for paraffin embedding and sectioning. 5-µm-thick paraffin sections were mounted on polylysine micro slides, thoroughly air-dried and stored in a cool place until use.

Immunohistochemistry

Tissue samples were fixed in buffered 4% formaldehyde and routinely embedded in paraffin. 4-µm sections of the paraffin blocks were dewaxed in xylene, rehydrated in graded alcohols, pre-treated for antigen retrieval in 10 mmol/L citric acid, pH 6.0, in a pressure cooker as described by us earlier [20–22] and immunoreacted overnight at 4°C with primary mAbs against dynein, kinesin, and Golgi 58 K, diluted to a final concentration of 2–5 µg/mL. After immunoreacting with primary mAbs and following washing in PBS, the sections were treated for 10 min with methanol containing 0.6% H_2O_2 to quench endogenous peroxidase. For bright-field microscopy, bound primary mAbs were detected using HRP-avidin-biotin complex (Vectastain 'Elite' ABC kit, Vector Laboratories) and NovaRed substrate kit (Vector Laboratories), counterstained with Ehrlich hematoxylin for 30 s and mounted with an aqueous mounting medium GelTol (Immunotech, Marseille, France).

Single antibody immunofluorescent labeling

After blocking non-specific binding sites (Fc-receptors) with BSA-c basic blocking solution, sections were incubated overnight at 4°C with mAbs raised against dynein, kinesin, and Golgi 58 K, and washed with PBS. Detection of bound primary mAbs was performed with goat anti-mouse IgG Cy^{TM}3-labeled secondary Abs. After counterstaining with DAPI (Sigma) for 15 s, samples were mounted with Vectashield (Vector Laboratories). In the controls, incubation with primary Abs was omitted or mouse IgG at the same final concentration replaced primary Abs. No specific immunolabeling was observed in the controls.

Dual antibody immunofluorescent labeling

For dual antibody immunolabeling, mAbs were haptenylated with biotin (for dynein) or digoxigenin (for kinesin, and for the 58 K Golgi marker), using biotin and digoxigenin *N*-hydroxy-succinimide esters according to the manufacturer's instructions. Bound digoxigenin-haptenylated anti-kinesin or anti-58 K Golgi mAbs were visualized with sheep FITC-conjugated anti-digoxigenin Abs. Bound biotinylated anti-dynein Abs were detected with Cy3-labeled streptavidin. Double immunolabeling of dynein and 58 K Golgi was performed using secondary fluorophore-conjugated Abs (BD PharMingen, Hamburg, Germany) raised against the corresponding IgG isotypes (FITC-anti-IgG1 for anti-58 K Golgi and Cy3-anti-IgG2b for anti-dynein), as described by us elsewhere [23].

Microscopy and image processing

Immunostained sections were examined on motorized Zeiss Axiophot2 microscope (Carl Zeiss Vision, Jena, Germany) equipped with appropriate filters. Separate images for DAPI staining and for fluorophore (FITC or Cy3) immunolabeling were captured digitally into color-separated components using an AxioCam digital microscope camera and AxioVision multi channel image processing (Carl Zeiss Vision). Images shown in Figs. 1, 2 and 4 are representative of six independent experiments with similar results.

Immunoelectron microscopy

Sections of pancreatic tissue (1–2 µm) were fixed with 2% formaldehyde in PBS overnight at +4°C, washed three times in PBS for 15 min, subsequently dehydrated in ethanol and embedded in LR White. Ultrathin sections (50–100 nm) were incubated overnight at +4°C with anti-dynein or anti-kinesin mAbs and with a rabbit polyclonal Ab to tubulin. Bound primary mAbs were labeled for 2 h at room temperature with anti-rabbit IgG and anti-mouse IgG goat secondary Abs conjugated with colloidal gold particles (6 and 12 nm, respectively), washed three times for 10 min in PBS and distilled water, counterstained with uranyl acetate and finally examined with a CM 10 transmission electron microscope (Philips, Hamburg, Germany).

Western blotting

Pancreatic tissue was homogenized with a Dounce S glass homogenizer (Braun, Melsungen, Germany) in iced Triton-X-100 lysis buffer containing protease inhibitors (1 mL/mg tissue, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 0.01 M sodiumpyrophosphate, 0.1 M sodium fluoride, 1 mmol/L dihydrogenperoxide, 1 mmol/L L-phenyl–methyl–sulfonyl–fluoride (PMSF) and 0.02% soybean-trypsin-inhibitor). Protein concentration was determined by a modified Bradford-assay (Bio-Rad) and equal amounts of protein were used in subsequent experiments. SDS polyacrylamide gel electrophoresis was performed in a discontinuous buffer system and gels were blotted on nitrocellulose

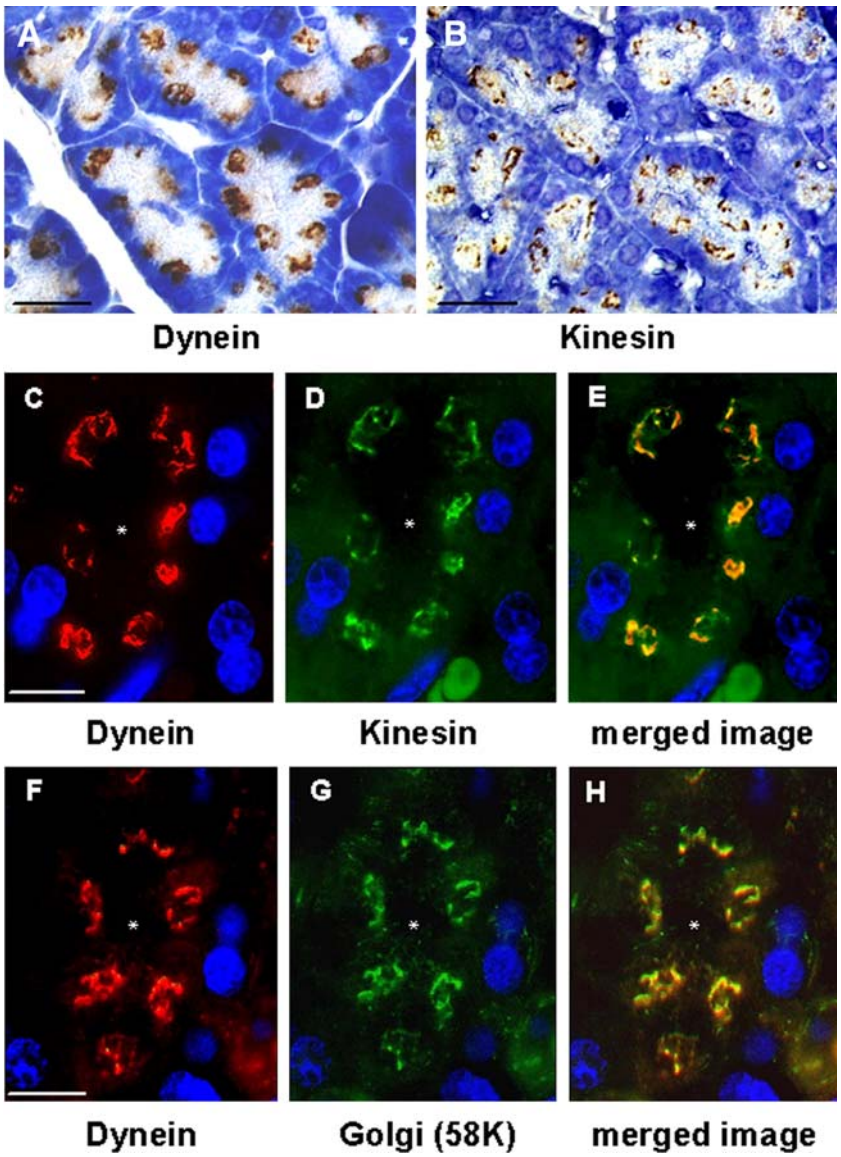

Fig. 1 **a** and **b** Perinuclear localization of dynein and kinesin in resting pancreatic acini. Pancreatic paraffin sections from rats were labeled with monoclonal antibodies raised against dynein intermediate chain (**a**) or kinesin heavy chain (**b**), and detected using the HRP-ABC kit and the NovaRed substrate kit resulting in a brownish stain. Nuclei were counterstained with hematoxylin. Images shown are representative for at least five experiments. *Bars* 20 μm. **c–e** Colocalization of dynein and kinesin in resting pancreatic acini. Dynein was visualized with Cy3 label (**c** *red*) and kinesin with FITC-label (**d** *green*). Nuclei were counterstained with DAPI and appear in *blue*. **e** Merged image of the dynein and kinesin labels, with *orange* color representing the colocalization of both proteins. *Asterisks* denote acinar lumens. All experiments were repeated at least five times. *Bar* 10 μm. **f–h** Colocalization of dynein and the Golgi marker 58 K in resting pancreatic acini. Dynein was labeled with Cy3 (**f** *red*), and Golgi with FITC (**g** *green*). Nuclei were counterstained with DAPI and appear in *blue*. In **h**, the *red* and *green* signals were merged, resulting in an *orange* color indicating the colocalization of dynein and 58 K Golgi. *Asterisks* denote acinar lumens. All experiments were repeated at least five times. *Bar* 10 μm

membranes (Hybond C; Amersham Pharmacia). After overnight blocking in NET-gelatine (10 mmol/L Tris/HCl pH 8.0, 0.15 mmol/L NaCl, 0.05% TWEEN 20, 0.2% gelatine) immunoblot analysis was performed using monoclonal antibodies followed by enhanced chemoluminescence detection (ECL Kit; Amersham Pharmacia) using horseradish peroxidase conjugated goat anti-mouse IgG.

Purification of tubulin via temperature-dependent disassembly/reassembly

Cytoplasmic microtubules rapidly disassemble at low temperatures. Incubation and centrifugation at 4°C allow the recovery of tubulin and motor proteins in the supernatant, while cellular debris and insoluble proteins are

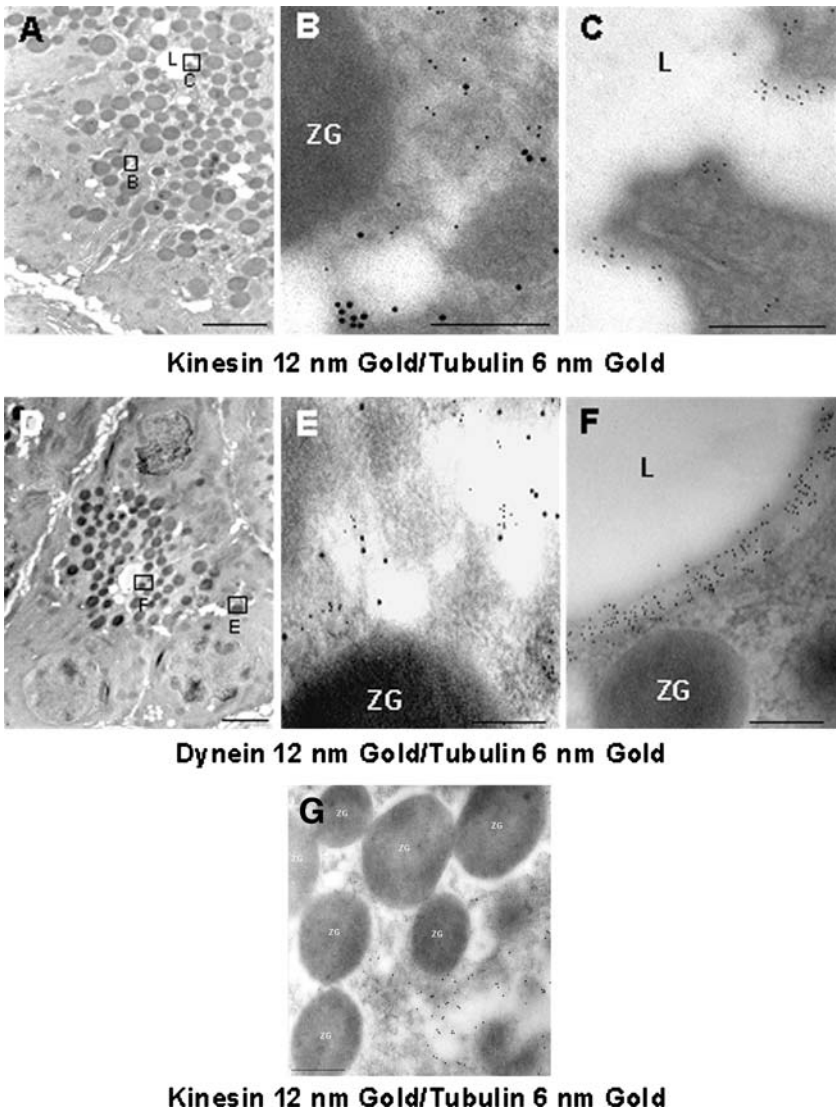

Fig. 2 Immunoelectron microscopic localization of kinesin, dynein, and tubulin in resting acinar cells. Ultrathin sections of pancreatic tissue were incubated either with anti-kinesin and anti-tubulin antibodies (**a**–**c**, **g**), or with anti-dynein and anti-tubulin antibodies (**d**–**f**) followed by 6 and 12 nm gold-conjugated secondary antibodies (see "Materials and methods"). Lumen of acinar cells (*L*) and zymogen granules (*ZG*) are marked. *Boxes* denote regions of higher magnification shown in **b**, **c**, **e**, **f**. Neither dynein nor kinesin can be detected in the apical cellular region (**c**, **f**) or at the surface of zymogen granules (**b**, **e**, **g**). Tubulin, detected with 6 nm gold particles colocalizes with kinesin and dynein in the perinuclear region (**b**, **e**), and can additionally be found near the apical lumen of acinar cells (**c**, **f**). Images shown are representative for at least five experiments. *Bars* 5 μm (**a**, **d**), 1 μm (**g**) or 0.25 μm (**b**, **c**, **e**, **f**)

removed with the pellet. Reassembly occurs at 37°C and polymerized tubulin together with its associated motor proteins can subsequently be collected by centrifugation at 25°C. Tubulin was purified from pancreatic tissue after homogenization in H buffer (0.5 mM $MgCl_2$, 20 mM MES, 80 mM NaCl, 1 mM EGTA, 10 μG/mL aprotinin, 10 μg/mL leupeptin, 2 mM PMSF, 10 mM sodium tetrapyrophosphate, 100 mM NaF, 0.02% soybean-trypsin-inhibitor, 1 mL/mg protease-inhibitor (Sigma), 0.36 mM GTP) by two cycles of temperature-dependent disassembly/reassembly [24–27]. Homogenates were centrifuged at 100,000*g* for 1 h at 4°C, and disassembled tubulin together with its motor proteins was recovered in the supernatant. Reassembly occurred at 37°C for 20 min in H buffer supplemented with taxol (20 μM), inorganic tripolyphoshate (PPPi, 3 mM) and glycerol (4 M) followed by centrifugation at 100,000*g* (25°C), the pellet was resuspended in H buffer, and incubated at 4°C for 15 min to disassemble microtubules. Remaining microtubules were separated by centrifugation at 4°C (100,000*g*) and the supernatant was used to repeat the reassembly/disassembly cycle. The microtubules containing pellet of the second cycle was resuspended in RIPA lysis buffer (100 mM Tris pH 8.0, 300 mM NaCl, 100 mM EDTA, 0.2% $NaDodSO_4$, 2% Triton-X-100, 2% sodium deoxycholate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mM PMSF, 10 mM sodium tetrapyrophosphate, 100 mM NaF, 0.02% soybean-trypsin-inhibitor, 1 mL/mg protease–inhibitor) (Sigma), and protein concentration was determined by a modified Bradford-assay (Bio-Rad). 1 μg protein was subjected to SDS-PAGE, transferred to nitrocellulose membranes and Western blot was performed with anti-tubulin, anti-dynein and anti-kinesin antibodies.

Preparation of rat acinar cells

Acini were freshly prepared from pancreases of male Wistar rats by collagenase (SERVA) digestion, suspended in incubation medium (IM) containing NaCl (96 mmol/L), KCl (6 mmol/L), HEPES (24.5 mmol/L), NaH_2PO_4 (2.5 mmol/L), $MgCl_2$ (1 mmol/L), glucose (11.5 mmol/L), $CaCl_2$ (0.5 mmol/L), Na-pyruvate (5 mmol/L), Na-glutamate (5 mmol/L), Na-fumarate (5 mmol/L), minimum essential medium (1% v/v), bovine serum albumin fraction V (1% w/v) at pH 7.5, and adjusted to a biovolume concentration of 2 mm^3/mL. After equilibration for 30 min, acini were cultivated at a temperature of 37°C.

Measurement of amylase release in isolated pancreatic acini

Freshly isolated acini in IM medium (see above) were stimulated with caerulein concentrations of 0.1 nM and constantly agitated at 37°C. At this concentration, caerulein evokes a secretory response similar to physiological concentrations of CCK. Aliquots were centrifuged at 50*g* for 5 min, and amylase activity was measured photometrically in the supernatant with ethylidene-pNP-G7 as a substrate using the Infinity Amylase Test Kit (Thermo Electron, Melbourne, Australia). Values were calibrated against an α-amylase activity standard. The data reported represent means ± SEM obtained from multiple determinations in three or more separate experiments for each group. Data points in the graphs indicate means ± error bars (SEM).

Colchicine treatment of isolated pancreatic acini

Pancreatic acini were transferred to 5 mL vials with aliquots of IM medium and incubated in the presence or absence of colchicine (100 μM) obtained from Sigma for 60 min. Caerulein was added at concentrations of 0.1 μg/kg/h and constantly agitated for 5 min at 37°C. As a control, non-colchicine-treated, unstimulated acinar cells were used. After centrifugation at 50*g* for 5 min, amylase activity was measured as described above. For the measurement of amylase secretion upon repeated caerulein stimulations, aliquots of agonist stimulated, non-colchicine-treated acinar cells were washed in IM medium to remove caerulein, incubated for 5 h in the presence or absence of 100 μM colchicine and subsequently stimulated again using 0.1 nM caerulein. Amylase release was measured as described above. Secretion was expressed as a percentage of initial acinar amylase total content.

To study the acinar cell cytoskeleton after colchicine treatment, isolated acini were incubated for 1 h in the presence or absence of colchicine, fixed with 4% formaldehyde in PBS and incubated with an anti-tubulin antibody. After washing, bound primary antibodies were visualized with goat anti-rabbit Alexa-594 labeled IgG. Immunostaining was analyzed using an IX70 microscope (Olympus) including a TILL Photonics imaging system (Gräfelfing, Germany).

Results

Microtubule motor proteins dynein and kinesin colocalize with the Golgi complex

To investigate the intracellular distribution of the microtubule motor proteins dynein and kinesin in resting pancreatic acini, paraffin sections of rat pancreas were labeled with anti-kinesin and anti-dynein monoclonal antibodies and detected with goat anti-mouse biotin-labeled secondary antibodies and avidin-biotin-peroxidase. In addition, the tissue was stained with hematoxylin. As shown in Fig. 1a and b, brown stainings representing dynein (a) and kinesin (b) appeared in the perinuclear region and not in the apical nor in the basolateral cellular region. We further investigated the colocalization of both molecular motors in double immunolabeling experiments using biotinylated anti-dynein and digoxygenated anti-kinesin antibodies. Bound primary antibodies were visualized with sheep FITC-conjugated anti-digoxigenin and Cy3-labeled streptavidin resulting in red dynein and green kinesin signals, respectively (Fig. 1c, d). The overlap of dynein and kinesin signals indicates that both motor proteins colocalize in resting pancreatic acinar cells (Fig. 1e). The anti-kinesin and anti-dynein antibodies labeled a perinuclear region in resting pancreatic acinar cells where the Golgi complex would be expected. To test whether the cellular distribution of kinesin and dynein is restricted to the Golgi apparatus, we extended our experiments by using an antibody detecting the 58 K formiminotransferase cyclodeaminase that associates with the cytoplasmic surface of the Golgi complex [28]. Staining with antibodies raised against dynein (red channel) and 58 K (green channel) indeed displayed a similar signal distribution. The resulting pseudo-orange color of the composite image confirmed the colocalization of both proteins (Fig. 1f–h). Comparable results were obtained in double-labeling experiments using antibodies raised against kinesin and 58 K (data not shown).

These observations indicate that both, kinesin and dynein colocalize with the Golgi complex in resting acinar cells suggesting a role of these molecular motors in Golgi function.

Tubulin, kinesin, and dynein are absent from mature zymogen granules

To gain a better insight into the subcellular events, immunoelectron microscopy of acini in fixed pancreatic tissue samples was performed. In addition to the anti-dynein and anti-kinesin heavy chain antibodies, we used a monoclonal antibody raised against β-tubulin. Differentially sized gold particles representing kinesin (12 nm, Fig. 2a–c, g), dynein (12 nm, Fig. 2d–f) and β-tubulin (6 nm, Fig. 2a–g) were visible within the perinuclear region, whereas neither kinesin nor dynein was detectable in the apical cellular region, further confirming the immunofluorescence microscopy results (Fig. 2c, f). In contrast, anti-β-tubulin antibodies were also targeted close to the apical cell membrane (Fig. 2c, f). Tubulin, dynein and kinesin were clearly absent from the surface of mature zymogen granules (Fig. 2b, e, g), and may thus not be directly involved in zymogen granule secretion.

Kinesin and the Golgi complex extend towards the apical cellular region after exocytosis

Intravenous infusion with CCK or caerulein has been shown to result in significant changes in zymogen granule formation and exocytosis in pancreatic acini [5, 29].

To analyze the intracellular distribution of motor proteins during hormonal stimulation, we have infused male Wistar rats with 0.1 µg/kg/h caerulein. Similar to physiological concentrations of CCK, this caerulein dose stimulates pancreatic secretion but does not cause a blockade in secretion or disruption of the acinar cytoskeleton [5]. Acini were prepared from male rats after different time points of infusion ranging from 15 min to 4 h (Fig. 3). Due to technical limitations, 15 min was the shortest in vivo stimulation period that could be monitored. Immunofluorescence microscopy of fixed pancreatic acini was performed using anti-dynein (Fig. 3a–e), anti-kinesin (Fig. 3f–j), and anti-58 K (Fig. 3k–o) monoclonal antibodies. As shown in Fig. 3a–e, fluorescence signals representing dynein exhibited almost identical distribution after different time points of caerulein stimulation. In contrast, kinesin redistributed during stimulation and appeared to extend its localization towards the apical cell pole after 30 and 60 min in the presence of caerulein (Fig. 3h, i). After a prolonged agonist stimulation time of 4 h, however, kinesin had returned to the perinuclear localization found in resting acini (Fig. 3j).

Immunofluorescence visualization of the Golgi marker 58 K in stimulated acini revealed a staining pattern similar to that of kinesin. Agonist stimulation for 15 min did not

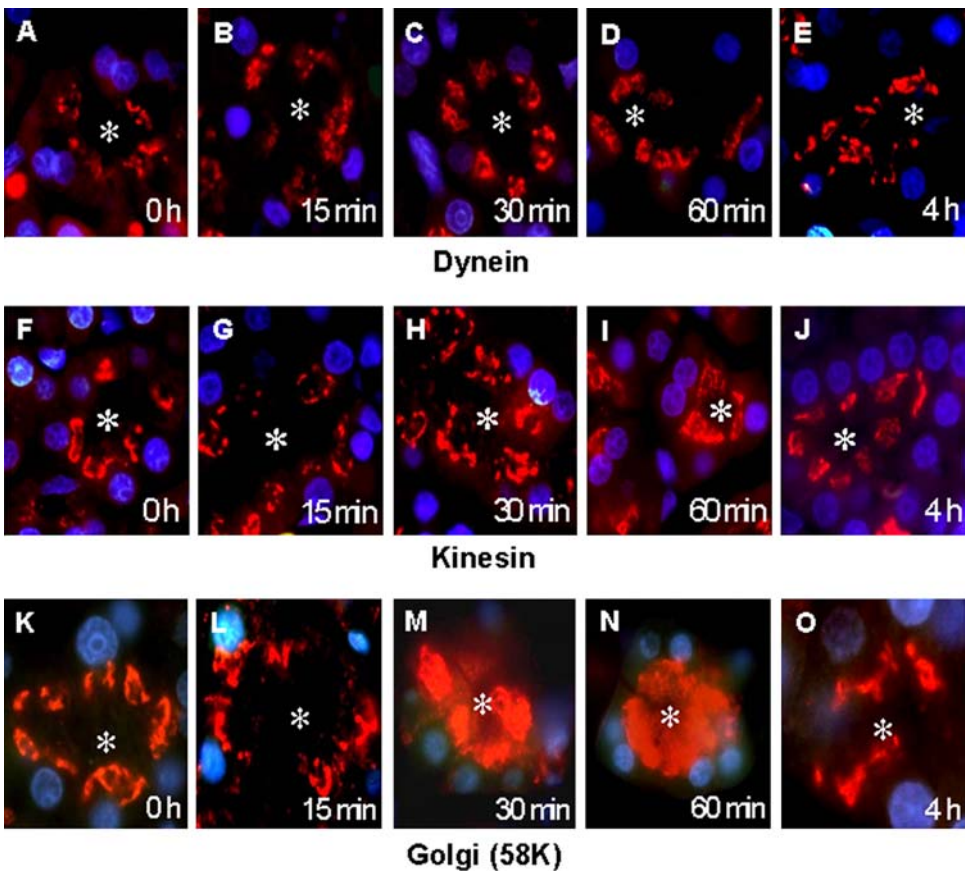

Fig. 3 Localization of dynein, kinesin and the Golgi marker 58 K in stimulated acini. Pancreatic paraffin sections from rats infused with 0.1 µg/kg/h caerulein for 15, 30, 60 min, or 4 h, were labeled for dynein (**a**–**e**), kinesin (**f**–**j**), and 58 K (**k**–**o**) using mAbs and Cy3-marked secondary Abs. Nuclei were counterstained with DAPI and appear in *blue*. *Asterisks* denote acinar lumens. During secretion (15 min, **b**, **g**, **l**) microtubule motor proteins (dynein and kinesin) and 58 K are still localized in the perinuclear region, whereas after 30 and 60 min of hormonal stimulation, the 58 K Golgi marker and kinesin were reallocated towards the apical lumen (**h**, **i**, **m**, **n**), significantly reducing their distance to the apical plasma membrane. All experiments were repeated at least five times and recorded at ×60

cause changes in the perinuclear Golgi localization (Fig. 3k–l) whereas after 30 and 60 min a dramatic extension towards the apical cellular region could be detected (Fig. 3m–n). After 4 h of stimulation, the Golgi signal was redistributed to the perinuclear region and no obvious difference between resting and stimulated acini was observed (Fig. 3o). Since the Golgi marker and kinesin undergo a comparable spatial and temporal reallocation upon agonist stimulation, it is likely that kinesin is involved in the caerulein-induced Golgi dynamics.

Association of kinesin with tubulin is enhanced in stimulated acinar cells

To determine whether the translocation of kinesin and the 58 K Golgi marker during caerulein stimulation is reflected by biochemical alterations, we examined pancreatic protein levels of kinesin and dynein and their association with tubulin. Pancreatic homogenates prepared after infusion of male rats with 0.1 µg/kg/h caerulein were assessed for kinesin, dynein, and tubulin content by Western blot analysis using monoclonal antibodies (see "Materials and methods"). No significant differences in signal intensity between resting and stimulated acini could be detected in three independent experiments, suggesting that caerulein infusions for up to 48 h neither resulted in different expression levels of tubulin and its motor proteins nor in their degradation (Fig. 4a).

To examine the association of kinesin and dynein with tubulin in stimulated acini, we purified tubulin using a method based on temperature-dependent microtubule disassembly/reassembly [24–27]. Pancreatic tissue obtained from rats after different periods of agonist stimulation was homogenized and subjected to two cycles of temperature-dependent polymerization as described in "Materials and methods". Purified microtubule fractions displayed similar signal intensities of dynein and tubulin, respectively (Fig. 4b). Hence, equal amounts of dynein associated with tubulin were isolated from resting and stimulated cells indicating that the association of dynein with tubulin is not affected during hormonal stimulation for up to 4 h. In contrast, association of kinesin with tubulin altered significantly during agonist treatment. Signal intensities of kinesin were low in resting cells and after 15 min of stimulation, whereas after 30 min, an increased kinesin signal was detectable and persisted at 60 and 120 min in the presence of caerulein. After 4 h of hormonal treatment, kinesin signal was reduced to the level found in resting acini. Since total kinesin concentration in pancreatic extracts did not change significantly during stimulation (Fig. 4a), it is likely that the increase in microtubule-bound kinesin is due to an enhanced binding of kinesin to tubulin during secretory stimulation. Strikingly, this temporal enhancement of kinesin associated with tubulin coincides with the onset of Golgi translocations (Fig. 3m–o), further supporting an involvement of kinesin in Golgi dynamics.

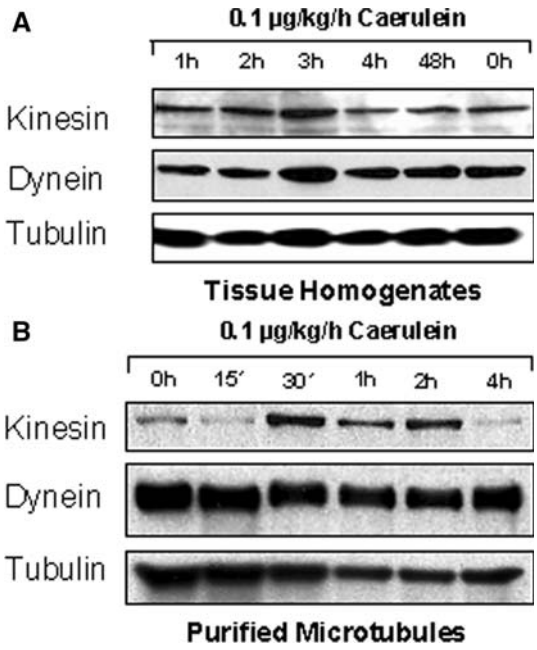

Fig. 4 In vivo expression and association of tubulin, dynein, and kinesin in stimulated acinar cells. Pancreatic tissue from rats infused with 0.1 µg/kg/h caerulein for the time intervals indicated was homogenized. **a** Equal protein loads from each lysate were subjected to standard SDS PAGE and Western blotting with mAbs. No significant differences in expression levels of tubulin and its motor proteins (kinesin heavy chain, dynein intermediate chain) can be detected during stimulation for up to 48 h. **b** Tubulin together with its associated motor proteins was purified by two cycles of temperature-dependent disassembly/reassembly as described in "Materials and methods". Precipitates were resuspended in lysis buffer and equal amounts of protein were used for Western blots. No significant differences in association of dynein with tubulin between resting and stimulated acini are detectable whereas affinity of kinesin to tubulin was significantly enhanced after 30 min of caerulein stimulation. All experiments were repeated at least three times

Amylase release occurs immediately after caerulein stimulation

To determine the time course of amylase release in pancreatic acinar cells, we stimulated pancreatic acini with 0.1 nM caerulein and monitored amylase activity. As shown in Fig. 5, amylase is released immediately after the onset of caerulein treatment. About 66% of secreted amylase was measured within the first 20 min, whereas only 34% was detected between 20 and 40 min (Fig. 5), suggesting that most of the zymogen granules are secreted within the first 20 min.

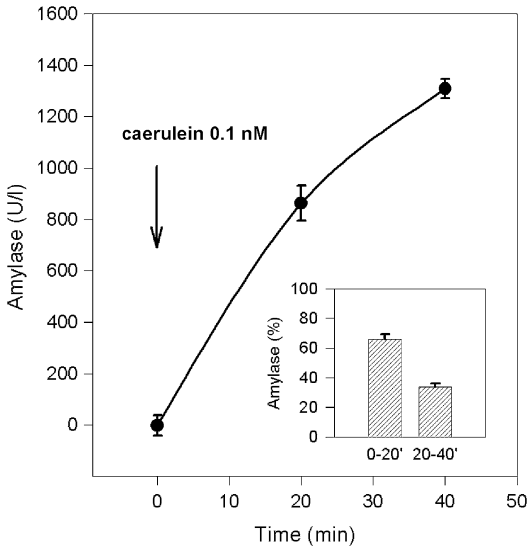

Fig. 5 Time course of amylase release in pancreatic acini. Freshly isolated pancreatic acini were stimulated with caerulein concentrations of 0.1 nM and amylase activity was monitored in the cell-free medium up to 40 min after secretagogue treatment. Values were calibrated against an α-amylase activity standard. All values represent means ± SEM for three or more separate experiments. In the *histogram*, secretion within the two time intervals is expressed as a percentage of total amylase secretion. In all cases, background secretion values of unstimulated cells have been subtracted from secretion values of stimulated cells

Regeneration of zymogen granules after a secretory stimulus depends on intact microtubules

To investigate the role of microtubules in agonist-induced secretion and zymogen granule supply, isolated acinar cells from rat pancreas were incubated with colchicine to depolymerize microtubules prior to caerulein stimulation. As shown in Fig. 6a and b, preincubation with colchicine for 1 h resulted in a complete disruption of acinar cell microtubules. Amylase release from cells stimulated with 0.1 nM caerulein, however, was not impaired by colchicine pretreatment suggesting that intact microtubules are not required for the instant secretion of mature zymogen granules (Fig. 6c). We therefore tested whether colchicine treatment had an effect on amylase secretion following granule depletion. Acini stimulated with caerulein for 40 min were washed and subsequently incubated for 5 h in the presence or absence of colchicine. After 5 h, zymogen granules were fully regenerated in the non-colchicine-treated control cells, because at this time point an additional caerulein stimulus resulted in a second release of amylase to a nearly identical extent as the first stimulus. However, pretreatment of acini with colchicine for 5 h after the first stimulus almost completely abolished the second amylase release (Fig. 6d). These results support the conclusion that microtubule-dependent mechanisms are not involved in the initial fusion events following a secretory stimulus that lead to an immediate amylase secretion from preformed, mature zymogen granules but play a role in the recovery of the secretory vesicle pool.

Discussion

The present study was undertaken to elucidate the role of microtubules and their motor proteins in the rat exocrine pancreas. Using immunofluorescence and electron microscopy of rat pancreatic tissues, we first examined the intracellular localization of kinesin and dynein in resting pancreatic acini. Remarkably, neither kinesin nor dynein was detected at the apical pole of acinar cells where exocytosis occurs. At the electron microscopic level, kinesin and dynein were absent from zymogen granules and also from the apical terminal microfilament web and the plasma membrane. Since both kinesin and dynein colocalize with the 58 K Golgi marker in a perinuclear region, it seems reasonable to conclude that neither dynein nor kinesin is involved in zymogen granule secretion. Previous studies on pancreatic acini and acinar cells from other groups are highly controversial. Marlowe et al. [18] observed an association of kinesin with apically clustered zymogen granules in the rat pancreas and cultured pancreatic acini that increased during regulated secretion in cultured acinar cells. Similarly, Ueda et al. [15] demonstrated that kinesin localized to zymogen granules in rat streptolysin-O-permeabilized isolated acini and anti-kinesin antibodies inhibited intracellular movement of zymogen granules in non-stimulated pancreatic acini. Immunoblot analyses of purified zymogen granules from isolated rat pancreatic acinar cells demonstrated the presence of actin, myosin V, and kinesin in the granule fraction [13]. In contrast, Kraemer et al. [19] found dynein but not kinesin to be associated with zymogen granules purified from the rat pancreas. In complete accordance with our results, neither dynein nor kinesin could be identified on zymogen granule membranes from rat pancreas in a recent proteomic study performed by Chen et al. [12]. Furthermore, kinesin has been localized to the Golgi complex in other polarized epithelial cells such as rat hepatocytes [30] and Madin–Darby canine kidney (MDCK) cells [31].

These contradictory observations may result from the type and actual state of pancreatic acinar cells examined, from the specificity of antibodies used, and from the methods by which zymogen granules were isolated. For instance, various studies were performed using cultured and dissociated pancreatic acinar cells, which tend to dedifferentiate rapidly and in which the secretory function has been reported to be severely impaired [32]. Moreover, the purification methods used to isolate zymogen granules

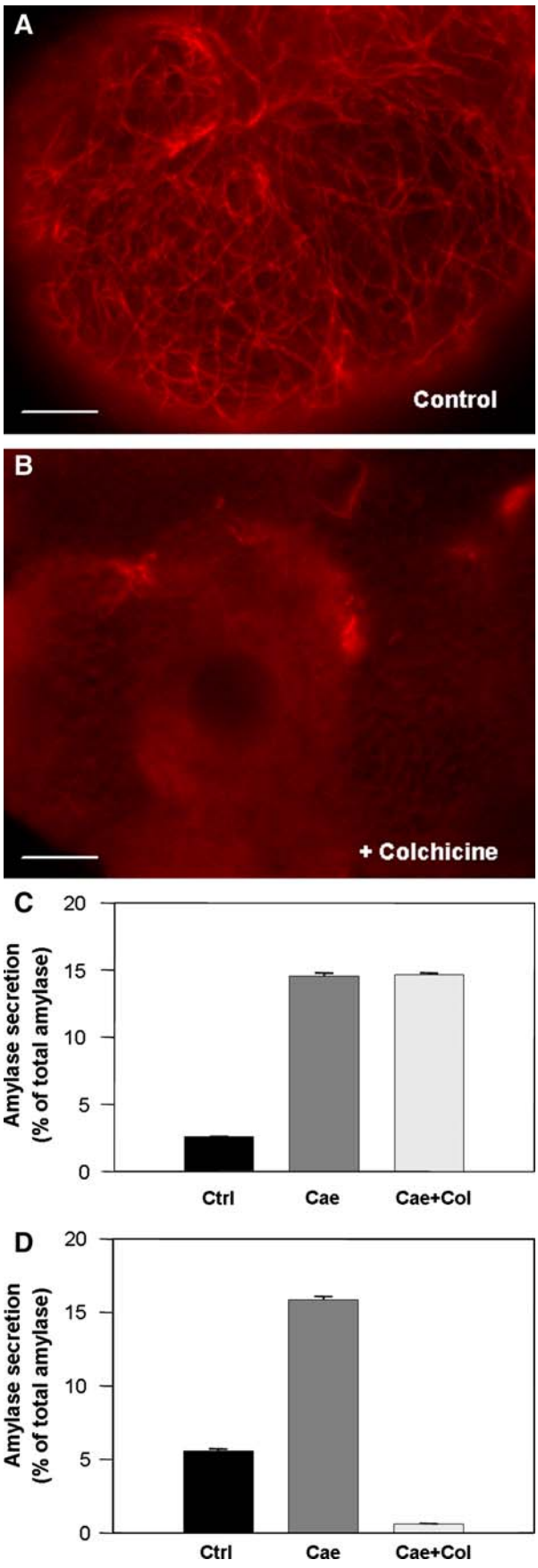

Fig. 6 Effect of colchicine on the microtubule cytoskeleton and caerulein-stimulated amylase secretion in isolated acinar cells. **a**,**b** Immunostaining of acinar cell cytoskeleton in the presence or absence of colchicine. Isolated acini were incubated for 1 h with or without colchicine, fixed and immunostained with anti-tubulin primary mAbs in addition to anti-mouse-IgG-Alexa-594 secondary Abs. Immunostaining was analyzed using an IX70 microscope including a TILL Photonics imaging system. Pretreatment of colchicine for 1 h completely disrupted microtubules in pancreatic acinar cells. *Bars* 5 µm. **c** Caerulein induced amylase secretion. Acinar cells from rat pancreas were isolated, preincubated for 1 h in the presence (*Cae* + *Col*) or absence of colchicine (100 µmol/L, *Cae*) and subsequently stimulated with caerulein (0.1 nM). Unstimulated and non-colchicine-treated cells were used as control (*Ctrl*). Amylase secretion was measured during the first 20 min after stimulation and expressed as percentage of total amylase content in resting acinar cells. Data presented are the mean ± SEM of five experiments. No difference in secretion can be observed with or without preincubation with colchicine. **d** Effect of colchicine on repeated caerulein induced amylase secretion. Pre-stimulated non-colchicine-treated cells were washed to remove caerulein, incubated for 5 h in the presence (*Cae* + *Col*) or absence of 100 µmol/L colchicine (*Cae*) and subsequently stimulated again with 0.1 nM caerulein. Unstimulated and non-colchicine-treated cells were used as control (*Ctrl*). Amylase secretion was measured during the first 20 min after caerulein treatment as described above. Preincubation of colchicine prior to the second stimulation led to substantial inhibition of amylase release. The results shown are representative for at least five experiments

from cells and tissues may differ in purity, thus accounting for the reported variability of microtubule motor protein distribution in acinar cells.

In vivo stimulation of the exocrine pancreas resulted in a significant redistribution of the Golgi complex towards the acinar cell apex as visualized by fluorescence microscopy. In parallel, we observed an extension of kinesin from the perinuclear region towards the apical cellular region concurrent with the spatial and morphological alterations of the Golgi apparatus. Moreover, a significant enhancement of kinesin association with tubulin was observed after 30 min of secretagogue administration. Because morphological and biochemical alterations both occur simultaneously, it is attractive to predict that the Golgi complex expands towards the apical plasma membrane along microtubules and that this event is mediated by kinesin. Indeed, it has been reported previously that large tubulovesicular structures of the *trans*-Golgi network can extend outward from the centrosome along microtubules [33–37]. Furthermore, the Golgi complex has been shown to relocate during the development of MDCK cell surface polarity, and this process seems to be directly related to microtubule organization [38]. Finally, kinesin and dynein have both been reported to play a role in Golgi localization and transport [39–42]. In polarized cells, however, microtubules are supposed to be organized with their plus ends pointing to the basolateral surface and their minus ends towards the apical cell pole [43–47]. Therefore, the plus-end directed or anterograde family of motors like kinesin

should mediate movements towards the basolateral region whereas transport to the apical pole would be expected to be driven by dynein. In cell lines derived from columnar epithelium such as polarized MDCK and Caco-2 cells, however, an additional meshwork of microtubules of mixed polarity underlying the apical region has been identified, [43, 44, 48] and apical transport in these cells involves kinesin [37, 49].

We have used isolated pancreatic acini to investigate the time course of amylase release and the role of microtubules in zymogen granule secretion. Our results provide evidence that most zymogen granules are released within the first 20 min after caerulein treatment and a clear reduction of release activity can already be observed between 20 and 40 min of stimulation. Such a rapid amylase release from pancreatic acinar cells has also been reported by Torgerson and McNiven [50]. Moreover, multiphoton excitation imaging revealed that exocytosis of zymogen granules starts within seconds after secretagogue stimulation [51]. In contrast, the redistribution of kinesin and the 58 K Golgi marker was observed 30 min after the onset of caerulein administration. Well in line with our findings, Dahan et al. [52] have previously reported that hormonal stimulation for 30 min leads to an increase in the fragmentation and vesiculation of Golgi subregions in pancreatic acinar cells. Thus, it is likely that the apical extension and subsequent fragmentation of Golgi compartments involved in regulated secretory cargo exit plays a role in the regeneration of the pool of secretory vesicles.

The microtubule disrupting compound colchicine had no effect on the initial caerulein-induced amylase release but prevented amylase release in response to a delayed second stimulus almost completely. Since the microtubule cytoskeleton has been demonstrated to be totally disrupted after 60 min of colchicine treatment, the initial caerulein-induced amylase release appears to be completely independent of microtubule function. Similarly, as Ueda et al. [15] reported a pretreatment of streptolysin-O-permeabilized acini with the anti-microtubular agent nocodazole for 90 min did not affect amylase secretion. Moreover, Ishihara et al. [14] demonstrated that colchicine failed to inhibit exocytosis in the initial 1-min period but partially suppressed the later phase of the secretory response. In earlier studies, Jamieson [17] found that treatment with colchicine inhibited amylase release only by 25–30% and a 40% inhibition of amylase secretion could only be produced when high concentrations of colchicine were applied for at least 210 min [16].

These findings and our data provide evidence that the microtubule cytoskeleton is not directly involved in zymogen granule fusion events and exocytosis. However, since perturbations of the microtubule cytoskeleton lead to a complete disruption of the Golgi complex [40, 42], zymogen granule formation and replenishment is likely to be affected by colchicine treatment. This would explain the substantial inhibition of enzyme secretion following a second secretory stimulus in colchicine-treated acini. Our observation that exocytosis of mature zymogen granules in

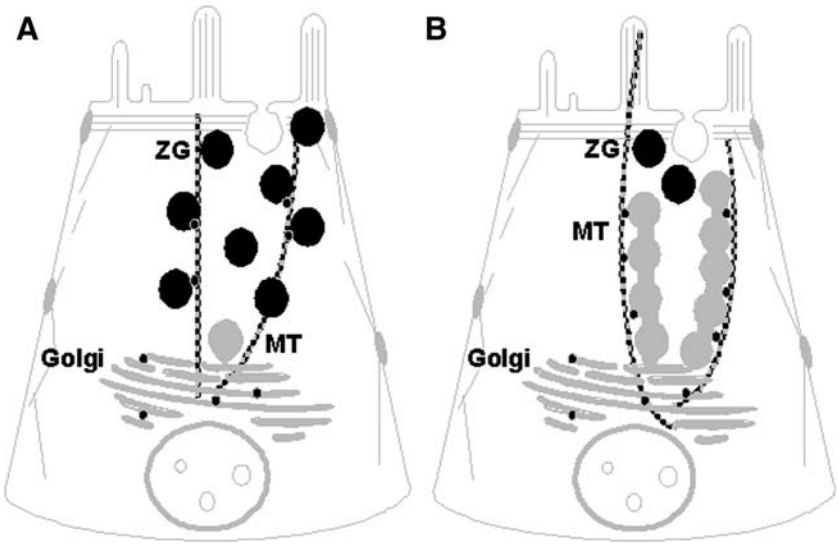

Fig. 7 Model of zymogen granule replenishment in the pancreatic acinar cell after exocytosis. **a** The conventional view would predict that secretory vesicles bud off the *trans*-Golgi network as condensing vacuoles and are subsequently transported along microtubules (*MT*) and motor proteins (*black dots*) towards the apical plasma membrane. During this process, these vacuoles mature to zymogen granules (*ZG*). **b** Our data suggest that a redistribution of Golgi compartments is involved in the recovery of a depleted zymogen granule pool. Following stimulated secretion of stored zymogen granules Golgi cisternae extend towards the apical pole to replenish releasable zymogen granules. This process is supposed to be microtubule-dependent and driven by motor protein kinesin

pancreatic acini is independent of the microtubule cytoskeleton is supported by comparable data from polarized MDCK cells. In these cells, kinesin is essential for the formation and translocation of Golgi tubules but not for the exocytosis of protein containing vesicles [37].

Increasing evidence supports a crucial role for the actin cytoskeleton rather than for the microtubule network in zymogen granule fusion events [8, 12, 53, 54]. Finally, multiphoton excitation imaging in intact pancreatic acini has demonstrated that zymogen granules located more distantly from the plasma membrane become fusion competent as soon as apical granules have fused with the adjacent plasma membrane [51]. This sequential fusion mechanism of compound exocytosis allows the cells to rapidly release their granule content located within deeper layers of the secretory vesicle pool. Based on our data, we cannot clearly exclude that an intracellular traffic of granules to the apical plasma membrane is the microtubule-dependent step needed to restore the available secretory pool of acinar cells after granule depletion. However, the compound fusion mechanism described does not require transport of granules more distant from the plasma membrane to become available for enzyme release [51]. Based on the kinetics of Golgi and kinesin redistribution shown here, we would like to suggest a model in which a microtubule-dependent and kinesin-driven extension of the Golgi towards the apical pole is necessary for the recovery of a depleted zymogen granule vesicle pool (Fig. 7).

To summarize, our results provide morphological and biochemical evidence that kinesin and dynein are absent from zymogen granules but associate with the Golgi apparatus in the rat exocrine pancreas. Release of mature and fusion competent zymogen granules does not require intact microtubules. Hormonal stimulation not only regulates exocytosis but also drives the extension of the Golgi complex towards the apical cell surface presumably mediated by microtubules and kinesin recruitment. This mechanism suggests a fundamental role of kinesin and the microtubule cytoskeleton in restoring the pool of secretory vesicles in pancreatic acinar cells.

Acknowledgments This work was supported by grants from the Deutsche Forschungsgemeinschaft (Le 625/8-1 and 9-1 to M.M.L., SFB 293 B7 to J.S. and M.M.L.), the Alfred Krupp Foundation and BMBF (KF0409001MD6) as well as the IZKF Münster (IZKF H3 to J.S. and M.M.L.). We wish to thank U. Breite, H. Heitmann, V. Samoilova, U. Naumann, and S. Rackow for expert technical assistance and R. Ossig for helpful discussions.

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