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ARTICLE

Beta cell chromogranin B is partially segregated in distinct granules and can be released separately from insulin in response to stimulation

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Abstract
Aims/hypothesis We investigated, in three beta cell lines (INS-1E, RIN-5AH, betaTC3) and in human and rodent primary beta cells, the storage and release of chromogranin B, a secretory protein expressed in beta cells and postulated to play an autocrine role. We asked whether chromogranin B is stored together with and discharged in constant ratio to insulin upon various stimuli.
Methods The intracellular distribution of insulin and chromogranin B was revealed by immunofluorescence followed by three-dimensional image reconstruction and by immunoelectron microscopy; their stimulated discharge was measured by ELISA and immunoblot analysis of homogenates and incubation media.
Results Insulin and chromogranin B, co-localised in the Golgi complex/trans-Golgi network, appeared largely segregated from each other in the secretory granule compartment. In INS-1E cells, the percentage of granules positive only for insulin or chromogranin B and of those positive for both was 66, 7 and 27%, respectively. In resting cells, both insulin and chromogranin B were concentrated in the granule cores; upon stimulation, chromogranin B (but not insulin) was largely redistributed to the core periphery and the surrounding halo. Strong stimulation with a secretagogue mixture induced parallel release of insulin and chromogranin B, whereas with 3-isobutyl-1-methylxantine and forskolin ± high glucose release of chromogranin B predominated. Weak, Ca^{2+}-dependent stimulation with ionomycin or carbachol induced exclusive release of chromogranin B, suggesting a higher Ca^{2+} sensitivity of the specific granules.
Conclusions/interpretation The unexpected complexity of the beta cell granule population in terms of heterogeneity,

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molecular plasticity and the differential discharge, could play an important role in physiological control of insulin release and possibly also in beta cell pathology.

Keywords Beta cells · Beta cell lines · Carbachol · Chromogranin B · INS-1E · Insulin · Ionomycin · Islets, dissociation of storage and secretion · Regulated secretion

Abbreviations

BAPTA	1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid)
$[Ca^{2+}]$	Ca^{2+} concentration
$[Ca^{2+}]_i$	Ca^{2+} concentration in the cell cytosol
CgB	chromogranin B
3D	three-dimensional
IBMX	3-isobutyl-1-methylxantine
ICA512/IA-2	protein tyrosine phosphatase-like protein-2
LAMP1	lysosomal-associated membrane protein 1
mAb	monoclonal antibody
pAb	polyclonal antibody
PMA	phorbol 12-myristate 13-acetate
SG	secretory granule
SM	secretagogue mixture
TGN	trans-Golgi network

Introduction

Most endocrine cells are known to secrete not one, but two or more hormones accompanied by additional proteins and peptides that are believed to be co-stored within the same secretory granules (SGs) and co-discharged by the same exocytic process [1, 2]. In various cell types, however, storage of secretory proteins has been shown to occur, completely or in large part, within distinct SGs. Examples are: (1) three peptides from the egg-laying hormone precursor processing of *Aplysia californica*, stored in three types of SGs [3, 4]; (2) growth hormone, prolactin and the granin secretogranin II of the acidophilic cow pituitary cells, each contained in a distinct type of SG [5]; and (3) galanin and vasopressin of neurohypophysial neurons, stored in two distinct and one mixed SG population [6]. It remains largely unknown whether the exocytosis of distinct SGs in single cells occurs under the same or different controls.

Pancreatic beta cells and beta cell lines are also known to synthesise, in addition to insulin, a number of other secretory proteins, including members of the granin family, chromogranin A and chromogranin B (CgB) [7–10]. Based on intracellular distribution studies [7, 8] and on data obtained in other cell lines (AtT20 and PC12), these granins are believed to play important roles both in the sorting, packaging and processing of secretion products [11–14], and in the storage of Ca^{2+} within SG lumina [15]. Moreover chromogranin A and/or peptides derived from its processing have been reported to play autocrine, paracrine and endocrine functions upon discharge [16–19], while CgB has been shown to induce an autocrine inhibition of insulin release from beta cells [20]. However, the functional significance of the latter process in the intact animal remains unclear. Despite the long-standing interest in granins, previous studies have not focused on heterogeneity of the SG compartment in insulin-producing cells.

In this paper, therefore, we have addressed several questions concerning the biology of CgB in beta cells. In view of the possible differences between the various beta cell models, our work was carried out in rat, mouse and human primary islet cell cultures and in three lines, INS-1E, RIN-5AH and betaTC3, with results that, overall, were consistent. Using a combination of immunofluorescence (followed by image deconvolution and three-dimensional [3D]-reconstruction) with immunoelectron microscopy and secretion assays, we investigated: (1) whether and to what extent CgB is co-stored with insulin within the same SGs; (2) the distribution of the granin in the architecture of the SG lumen; and (3) whether its release can occur independently of insulin. Our results demonstrate that co-localisation of insulin and CgB occurs in only a fraction of the SGs; that the intragranular distribution of CgB in the core or the halo of SGs can change in part upon cell stimulation; and that, depending on the secretagogue, release of the granin can occur concomitantly with or independently of insulin. The peculiar properties of CgB secretion could open a new line of functional research in beta cells, which remains to be developed.

Methods

Cells and antibodies INS-1E [21], RIN-5AH [22] and betaTC3 [23] cells were grown at 37°C, under a 5% CO_2 humidified atmosphere, in RPMI 1640 (INS-1E and betaTC3) or 11 mmol/l glucose-containing DMEM (RIN-5AH) media supplemented with 10% (vol./vol.) fetal clone serum III (Hyclone, Logan, UT, USA), 100 U/ml penicillin per streptomycin and 2 mmol/l ultra-glutamine (Cambrex, Verviers, Belgium). The medium for INS-1E cells also contained 1 mmol/l pyruvate (Cambrex), 10 mmol/l HEPES pH 7.4 and 50 μmol/l β-mercaptoethanol; that for RIN-5AH cells also contained 1 mmol/l pyruvate. Human islets were obtained from post-mortem donors through the North Italian Transplant Organization, with the approval of the local Ethics Committee. Mouse and rat islets were isolated by the collagenase digestion method [24, 25] and partially dissociated with Accutase (Innovative Cell Technology, San Diego, CA, USA) at 37°C for 10 min. Mouse/rat and human islets were cultured for up to 2 days in RPMI 1640

containing 10% (vol./vol.) serum and final wash culture media (Mediatech, Manassas, VA, USA), respectively.

The polyclonal antibodies (pAb) were a guinea pig anti-insulin (DAKO, Milan, Italy) and two rabbit anti-CgBs, gifts of P. Rosa (Consiglio Nazionale delle Ricerche, Milan, Italy) and A. Laslop (PE-11; University of Innsbruck, Austria) [26, 27]. The mouse monoclonal antibodies (mAb) were an IgG2a anti-rat CgB (CIRO) from our laboratory [28]; an anti-insulin (Sigma-Aldrich, Milan, Italy) and an anti-human CgB (67-C7-2), gift of W. B. Huttner (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany); an anti-trans-Golgi network (TGN) marker (TGN38) and an anti-GM130 (Golgi complex marker; BD Biosciences, San Jose, CA, USA); an anti-lysosomal-associated membrane protein 1 (LAMP1) (LYC16; Calbiochem, Darmstadt, Germany); two anti-protein tyrosine phosphatase-like protein-2 (ICA512/IA-2), mICA512cyto, gift of M. Solimena (Technical University of Dresden, Germany) [29] and 76F from our laboratory [30]. INS-1E lysates and incubation media were immunoblotted to assess antibody specificity (Electronic supplementary material [ESM] Fig. 1) for the anti-CgB mAb. Secondary antibodies were purchased from Jackson ImmunoResearch Europe (Soham, UK). The other reagents were from Sigma-Aldrich.

Immunocytochemistry, image analysis and immunoelectron microscopy Immunofluorescence was performed as described [31]. Image deconvolutions and 3D cell reconstructions were performed on 150 nm optical Z-stack sections acquired by Deltavision wide-field microscopy system and processed with softWoRx3.4.5 software (Applied Precision, Issaquah, WA, USA). Figures were assembled from exported tiff files with Adobe Photoshop CS version 8 for MacOS X, according to the code of image manipulation ethics [32].

Conventional electron microscopy was performed in Epon-embedded ultra-thin sections of cells and rat islets fixed with 2% (vol./vol.) glutaraldehyde and 2% (vol./vol.) OsO_4 [33]. For immunoelectron microscopy, INS-1E cell pellets were fixed at room temperature in a mixture of 4% (wt/vol.) formaldehyde/0.2% (vol./vol.) glutaraldehyde, washed in 100 mmol/l phosphate buffer pH 7.4, dehydrated in ethanol and infiltrated in LR White Resin (Polyscience, Eppelheim, Germany), which was polymerised for 24 h at 50°C. Ultra-thin sections were incubated with an anti-insulin mAb and an anti-CgB pAb in phosphate buffer, then washed and reacted with goat anti-mouse and anti-rabbit Ig-coated colloidal gold particles (6, 12 or 20 nm, respectively). After extensive washes, sections were stained with uranyl acetate and lead citrate and examined in a LEO 912AB (LEO Electron Microscopy Ltd. Cambridge, UK) or 100 kV Tecnai 12 Biotwin (FEI, Hillsboro, OR, USA) electron microscopes. Quantification of labelling was performed on printed micrographs (at least 20 for each condition). Labelled SGs were counted manually by point counting. Results were expressed as per cent of total SGs labelled in the depth of the core, its periphery/halo or both.

Secretion assays INS-1E cells were used at sub-confluence, human islets (30 islets per condition) were cultured overnight at 37°C before use. Cells and human islets were preincubated for 1 h in low (2.8 mmol/l) glucose KRB (4.8 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 10 mmol/l HEPES pH 7.4, 2 mmol/l KH_2PO_4, 8.3 mmol/l $NaHCO_3$, 134 mmol/l NaCl, 2 mmol/l $CaCl_2$). The medium was then discarded and replaced with fresh low-glucose KRB, alone (basal secretion) or supplemented with various secretagogues (stimulated secretion). A secretagogue mixture (SM), containing 16.7 mmol/l glucose, 1 mmol/l 3-isobutyl-1-methylxantine (IBMX), 0.1 μmol/l phorbol 12-myristate 13-acetate (PMA) and 10 μmol/l forskolin [34]) was used to induce maximal secretion; IBMX, PMA or forskolin (above concentrations) were also given alone or in combination with high glucose (20 mmol/l); ionomycin was used at 0.1 to 10 μmol/l, carbachol at 10 to 100 μmol/l. Incubation media, collected at the indicated time-points, were centrifuged at 400×*g* for 2 min at 4°C to remove any detached cells and gross fragments, and stored at −80°C until use. Cell monolayers and islets were suspended on ice, for 30 min at 4°C, in a lysis buffer (50 mmol/l HEPES pH 7.5, 150 mmol/l NaCl, 15 mmol/l $MgCl_2$, 1 mmol/l EGTA, 10% [wt/vol.] glycerol, 1% [wt/vol.] Triton X-100, 0.5 mmol/l phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors), then thoroughly homogenised by freeze-thawing and centrifugation at 14,000×*g* for 15 min at 4°C before storage at −80°C. The stimulated release data shown in the results were subtracted from the basal release (in all cases very low, see ESM Fig. 1 for CgB).

Insulin and CgB assays Insulin content of media and lysates was measured by a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacturer's instructions. The release values were multiplied by the total volume and expressed as per cent of the insulin content of the cells, defined by the formula: 100 × insulin (medium)/ insulin (medium) + insulin (lysate). Mean values were obtained from triplicate wells.

CgB was assayed by a modified dot–blot procedure [35]. Briefly, 30 μg of cell lysate protein (BCA assay; Pierce Biotech, Rockford, IL, USA), and 600 μl media from the release experiments, diluted in 10% (vol./vol.) methanol, 0.5% (wt/vol.) sodium deoxycholate, 50 mmol/l Tris pH 7.4 and 150 mmol/l NaCl added with protease inhibitors, were loaded on to a 0.22 μm pore size nitrocellulose membrane (Biorad, Milan, Italy) in a dot–blot apparatus. Protein-loaded membranes were air-dried and then processed as for

western blotting [31] using a mixture of two anti-CgB antibodies: the pAb PE-11 [27] (1:400) and the mAb CIRO (1:1,000). Chemiluminescence of bound antibodies, revealed by the Western Femto Signal (Pierce Biotech, Rockford, IL, USA), was acquired every 30 s in a UVP Bioimaging System (UVP, Upland, CA, USA). Dot intensities were integrated with LabWorks Image Acquisition and Analysis software (UVP). The percentage of released CgB using optical densities instead of absolute values was calculated as for insulin. Values given are averages of three to four experiments±SEM.

Fura-2 assay of Ca^{2+} concentrations in the cytosol Suspensions of INS-1E cells (3×10^6/ml) in RPMI 1640, prepared from sub-confluent cultures, were loaded with 2 μmol/l Fura-2/AM (Merck Biosciences, Darmstadt, Germany) for 20 min at room temperature, then washed and transferred to KRB supplemented with 200 μmol/l sulfinpyrazone. Basal fluorescence ratios at 340 and 380 nm excitation wavelengths, and the changes of fluorescence induced by 0.5 to 10 μmol/l ionomycin, were acquired at 37°C, under continuous stirring, in a fluorometer (LS50; Perkin Elmer, Waltham, MA, USA) [36].

Statistical analyses Experiments were performed in triplicate and repeated up to four times. Replicates of independent experiments were used to calculate averages±SEM. Differences were analysed by unpaired two-tailed Student's *t* tests with $p\leq0.05$ taken to be significant.

Results

Dissociation of insulin and CgB in various beta cell types: immunofluorescence To establish whether insulin and CgB are stored in the same or in separate SGs, we investigated a number of well-known insulin-secreting cell lines as well as primary human, rat and mouse islet cell cultures, using antibodies against the two proteins and various intracellular organelle markers. The results were analysed by deconvolution of the Z-stack images followed by 3D-reconstruction.

Figure 1 illustrates the results obtained with the rat beta cell line INS-1E. More than 75±6% (mean±SEM; $n=298$) of the cells were double-positive for insulin and CgB. A large amount of insulin/CgB co-localisation was observed in the perinuclear area corresponding to both the TGN (revealed by the marker TGN38; Fig. 1b,c,e,f,h,i) and the Golgi complex (revealed by the marker GM130, not shown). In contrast, only a fraction of SGs at the cell periphery were positive for both insulin and CgB, while the others were immunolabelled only for either one (Fig. 1a,d,g,b,e,f). To rule out the possibility that some of the insulin staining was due to lysosomes, dual labelling for insulin and the lysosomal marker LAMP1 was performed, showing little if any co-localisation at the cell periphery, while in a central region, corresponding to the Golgi complex–TGN, some co-localisation was observed (ESM Fig. 2). A similar pattern was observed in RIN-5AH (ESM Fig. 3a–c), a cell line not used for further functional studies because of its

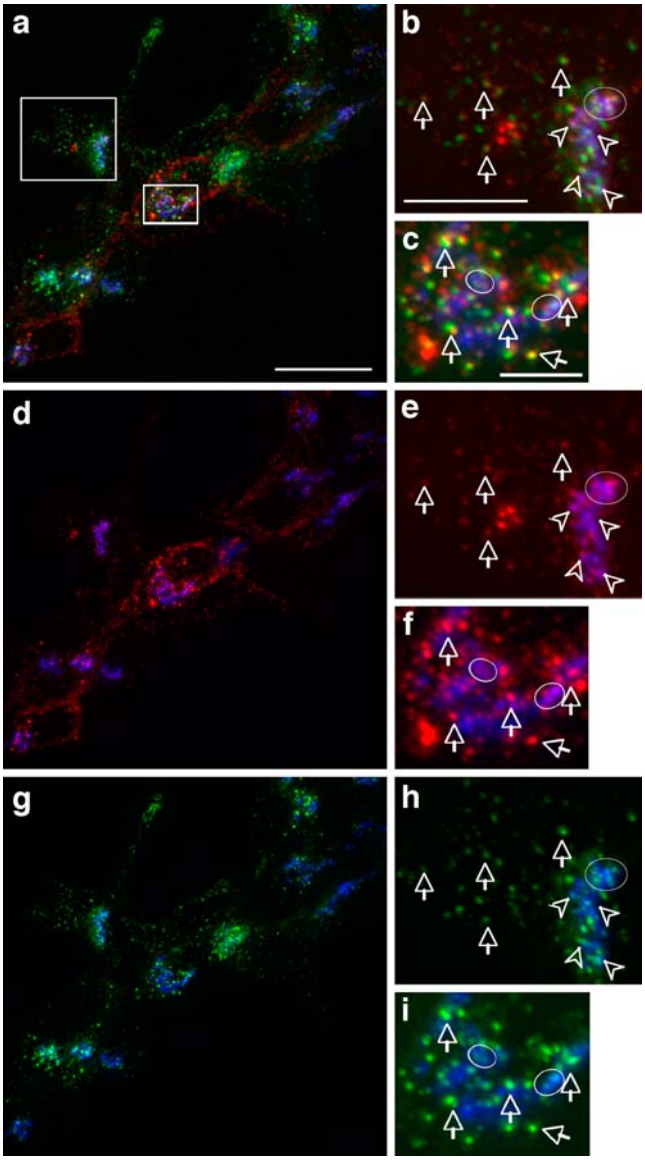

Fig. 1 Deconvolution and 3D reconstruction of triple-labelled, merged immunofluorescence images acquired by the Deltavision system. Red, insulin; green, CgB; blue, TGN38. **a**–**c** Triple merged fluorescence images of INS-1E cells; double fluorescence images for insulin and TGN38 (**d**–**f**) and for CgB and TGN38 (**g**–**i**). Insets (**a**) are shown in close-up (**b**, **c**, **e**, **f**, **h**, **i**). Scale bars (valid for all same-sized panels): 13 μm (**a**), 3 μm (**b**) and 5 μm (**c**). Circles (**b**, **c**), triple merged signals (white); arrowheads (**b**), insulin/CgB-positive structures in the TGN area; arrows, punctate co-localisations of insulin and CgB at the periphery of the cytoplasm (**b**), most likely SGs, and in the TGN area (**c**) (yellow in both cases). Circles, arrowheads, arrows (**e**, **f**, **h**, **i**) indicate the same structures labelled by only one of the two colours

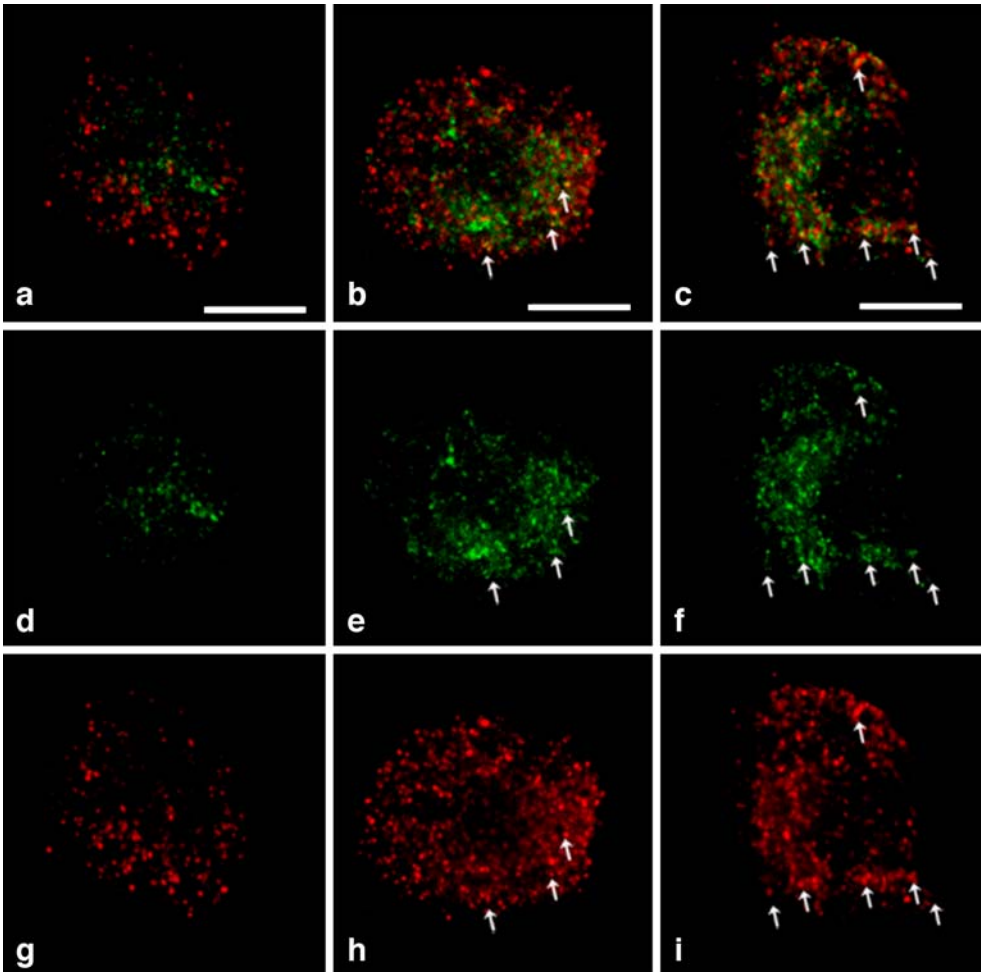

Fig. 2 Deconvolution and 3D reconstruction of immunofluorescence images showing primary rat beta cells stained with anti-insulin (red) and anti-CgB (green). **a**–**c** Merged fluorescence images; (**d**–**f**) the corresponding individual fluorescence images for CgB; and (**g**–**i**) for insulin. Arrows, dually immunolabelled SGs (yellow in the merged images). Scale bars: 3 μm (valid for all same-sized panels)

heterogeneity in insulin and CgB expression (T. Giordano, C. Brigatti, M. L. Malosio, unpublished data). In preparations of primary rat (Fig. 2), mouse (ESM Fig. 4) and human (ESM Fig. 3d,e) islet cells, and in another insulin-secreting line, the mouse betaTC3 (ESM Fig. 3f), mixed SGs were rare (Fig. 2; ESM Figs 3e,f and 4b,c). Most SGs positive for either insulin or CgB exhibited a distinct distribution, at the periphery of the cell (insulin) or more spread throughout the cytoplasm (CgB). In human primary islet cells, CgB was present not only in beta cells but also in other, insulin-negative cells (ESM Fig. 3d) not yet investigated for endocrine function.

Both in betaTC3 and INS-1E cells, the co-localisation of insulin with two other SG proteins, the membrane protein IA-2/ICA-512 and the other major granin, chromogranin A, was much higher than that with CgB (ESM Fig. 3g–i).

Dissociation of insulin and CgB in the SGs: ultrastructural immunocytochemistry SGs were further investigated at the ultrastructural level. In view of the focus of this paper on mature organelles, the immature SGs distributed in the proximity of the TGN were not taken into consideration. In conventional Epon-embedded sections of rat primary beta cells and INS-1E, the SGs, distributed both at the periphery and more deeply in the cytoplasm, exhibited their well-known structure, with a dense core surrounded by a clear halo of variable thickness (Fig. 3a,b). Sections of INS-1E cells embedded in LR White were double immunogold-labelled for insulin and CgB. The labelling of structures other than SGs, including nuclei, remained at the background level (fewer than ten gold particles/μm^2), both in resting and in stimulated cells. In contrast the SGs appeared much more strongly, however differentially labelled (Fig. 3c–h). In resting cells, 66±1.93% (mean±SEM) of the immunogold-labelled SGs were positive for insulin alone, 27±2.38% were mixed and 7±3.7% were positive for CgB alone.

We also investigated the effects of six different conditions on intragranular distribution of insulin and CgB in INS-1E cells: (1) incubation in low glucose KRB (resting cells); (2) 5 min stimulation with 5 μmol/l ionomycin; (3–6) 20 min stimulation with high glucose, forskolin, PMA or SM. At least 20 immunogold-labelled ultra-thin sections per group were chosen at random and their SGs analysed

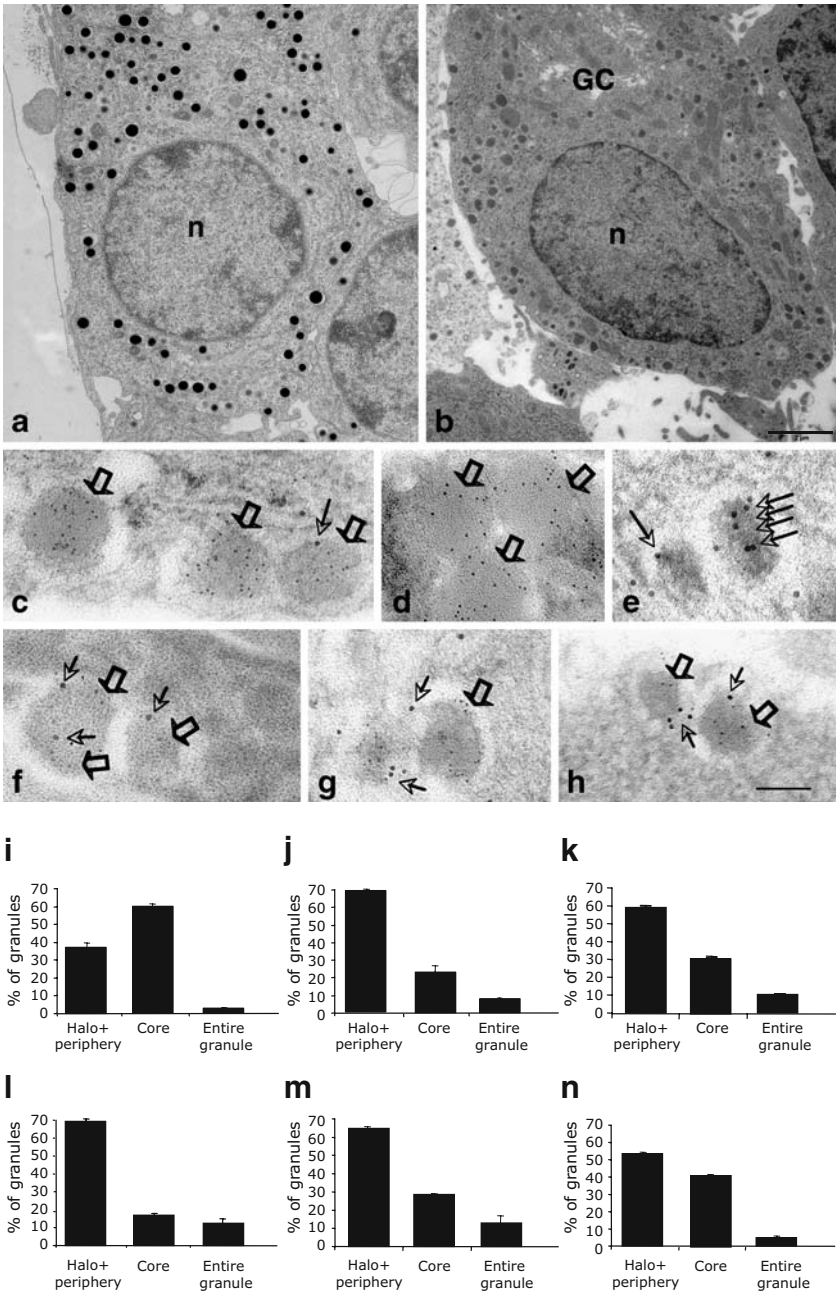

Fig. 3 Conventional ultrastructure of rat primary beta cells and INS-1E cells, with immunogold labelling for insulin (6 nm particles) and CgB (12 nm particles). **a** Typical ultrastructure of primary rat beta cells and (**b**) INS-1E cells with SGs characterised by a dense core surrounded by a clear halo, as revealed by conventional electron microscopy. GC, Golgi complex; n, nucleus. **c**–**h** Different patterns of SG immunogold labelling in resting INS-1E cells: positive for insulin only (**c**, **d**), for CgB only (**e**) and for both (**c**, **f**–**h**). The intra-SGs distribution of the two proteins is variable, both in the core and at its periphery/submembrane halo. Thin arrows, CgB-positive gold particles (12 nm); open arrows, insulin-positive gold particles (6 nm). Scale bars (valid for all same-sized panels): (**b**) 2 µm; (**h**) 0.1 µm. **i**–**n** Quantitation of distribution of CgB-specific immunogold particles within the SGs of INS-1E cells at rest (2.8 mmol/l glucose) (**i**), after 5 min treatment with 5 µmol/l ionomycin (**j**) and after 20 min with 20 mmol/l glucose (**k**), 10 µmol/l forskolin (**l**), 0.1 µmol/l SM (**m**) and 0.1 µmol/l PMA (**n**). Populations of 89 (**i**), 90 (**j**), 111 (**k**), 261 (**l**), 211 (**m**) and 227 (**n**) SGs were scored for labelling in the halo/periphery only (halo+periphery), in the core only (core) and in both areas (entire granule)

for insulin and CgB distribution. In resting cells, insulin gold particles appeared almost always localised in the SG core (Fig. 3c–h), whereas the distribution of CgB particles varied, with ~60±1.6% (mean±SEM) of the SGs (n=89) exhibiting a higher labelling in the core rather than in the periphery and in the clear halo (Fig. 3i). Stimulation with ionomycin failed to induce any changes in insulin (not shown), but induced a visible redistribution of CgB. Only 23±3.1% (mean±SEM) of the SGs (n=90) exhibited labelling of the core and 69±0.75% labelling of the periphery/halo (Fig. 3j). Interestingly, an intra-SG distribution of CgB similar to that of the ionomycin-stimulated cells was also found upon stimulation with high glucose (n=111; Fig. 3k), forskolin (n=261; Figs 3l and 4a–g) and SM (n=211; Figs 3m and 4o–s). In contrast, in the cells stimulated with PMA, the distribution of CgB was approximately the same in the halo/periphery (53±0.53% [mean±SEM]) and in the core (42±0.37%) (n=227) (Figs 3n and 4h–n).

Release of insulin and CgB Stimulated release of the two proteins was investigated in INS-1E cells. Maximal responses were induced by SM including 16.7 mmol/l glucose, 1 mmol/l IBMX, 0.1 µmol/l PMA and 10 µmol/l forskolin, administered for 3, 5 and 20 min. Release of insulin and CgB, moderate in the initial 5 min, increased

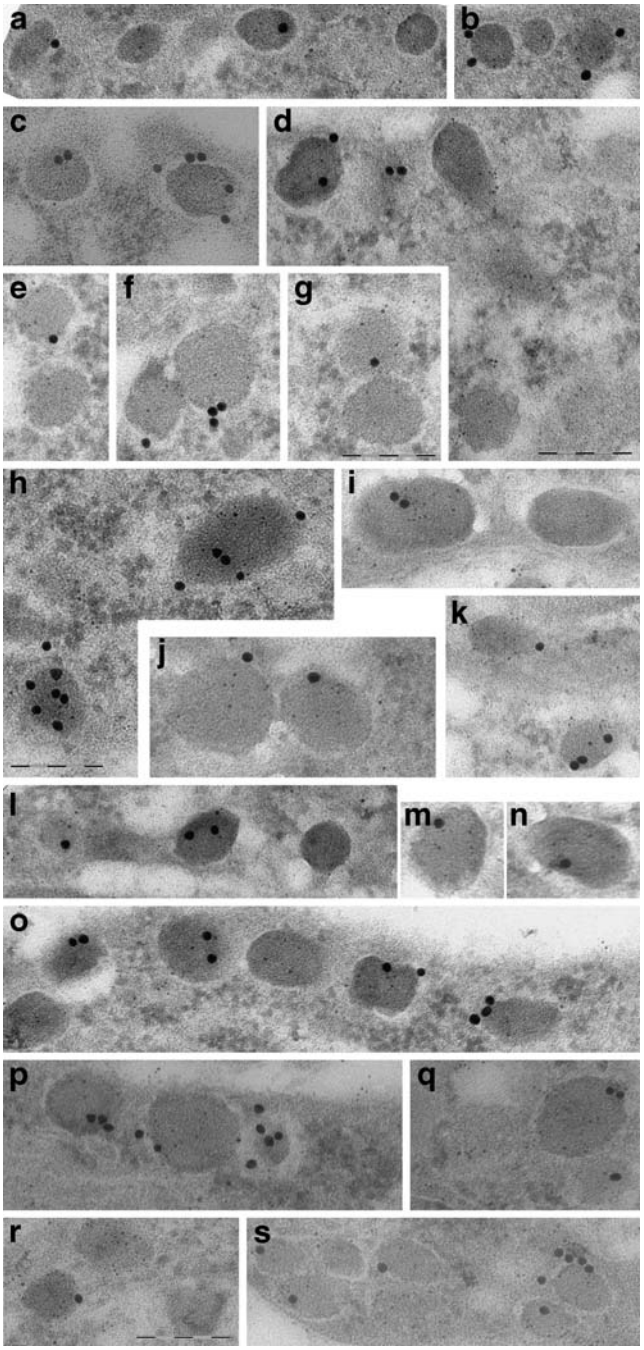

Fig. 4 Immunogold labelling of INS-1E cells upon secretagogue application. Different patterns of SGs immunogold labelling with insulin (6 nm particles) and CgB (20 nm particles) are visible in INS-1E cells exposed to 20 min treatments with forskolin (10 μmol/l) (**a**–**g**), PMA (0.1 μmol/l) (**h**–**n**) and SM (**o**–**s**). Scale bars (**d**, **g**, **h**, **r**): 0.2 μm (valid for other panels of corresponding groups)

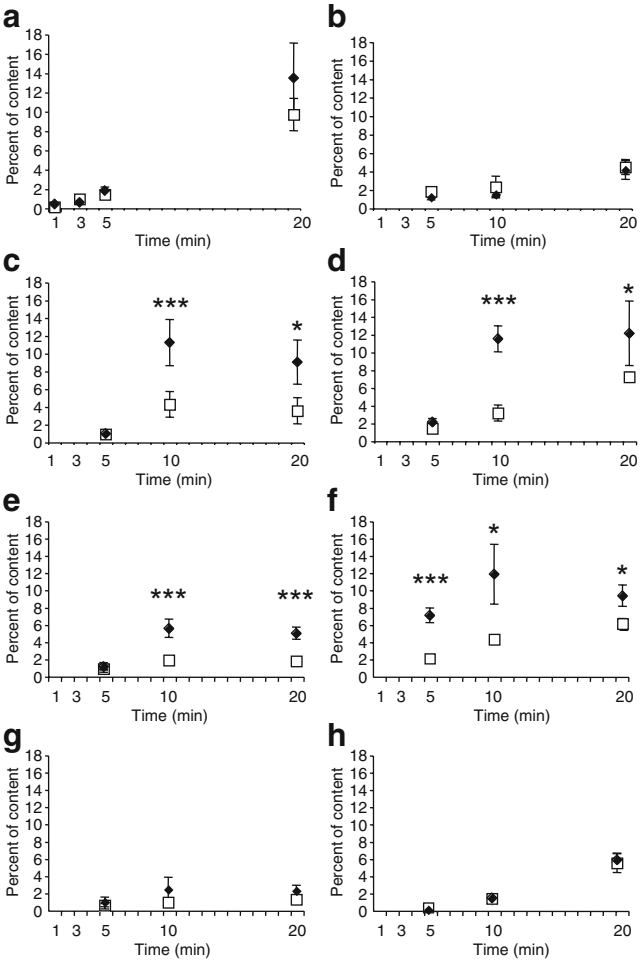

Fig. 5 Time course of insulin (squares) and CgB (diamonds) release from INS-1E cells induced by various secretagogues. Stimulation was with SM (**a**), high glucose (**b**), IBMX (1 mmol/l) (**c**), IBMX+glucose (**d**), forskolin (10 μmol/l) (**e**), forskolin+glucose (**f**), PMA (0.1 μmol/l) (**g**) and PMA+glucose (**h**). Data shown are averages of two to three experiments run in triplicate (±SEM). *$p \leq 0.05$; ***$p \leq 0.001$ (Student's two-tailed unpaired *t* test)

thereafter reaching after 20 min 13.4±3.6% (mean±SEM) and 9±1.75% of the total cell content, respectively (Fig. 5a). The various components of SM were also administered separately from each other or in combination with high glucose. With high glucose alone, the release of insulin and CgB occurred in parallel, reaching 4.5±0.8% (mean±SEM) and 4.2±1.0%, respectively, after 20 min (Fig. 5b). Parallel, but lower release was observed with PMA (Fig. 5g) and with PMA+glucose (Fig. 5h). IBMX and forskolin triggered a per cent release of CgB that was significantly higher than that of insulin at both 10 and 20 min (Fig. 5c,e). The combination with high glucose increased insulin release, without, however, reaching the levels of CgB (Fig. 5d,f). In addition, CgB release upon forskolin + glucose appeared earlier, being significant already at 5 min and maximal at 10 min of stimulation (Fig. 5d). An even more evident dissociation between insulin and CgB release was induced by ionomycin, an ionophore drug that triggers concentration-dependent increases of Ca^{2+} concentrations ($[Ca^{2+}]$) in the cytosol ($[Ca^{2+}]_i$) (ESM Fig. 5). The CgB release responses, visible already after 5 min at 0.1 μmol/l ionomycin, reached a plateau at 1 μmol/l, although at levels much lower (~1/10) than those induced by SM (Figs 5a and 6a). In contrast, no

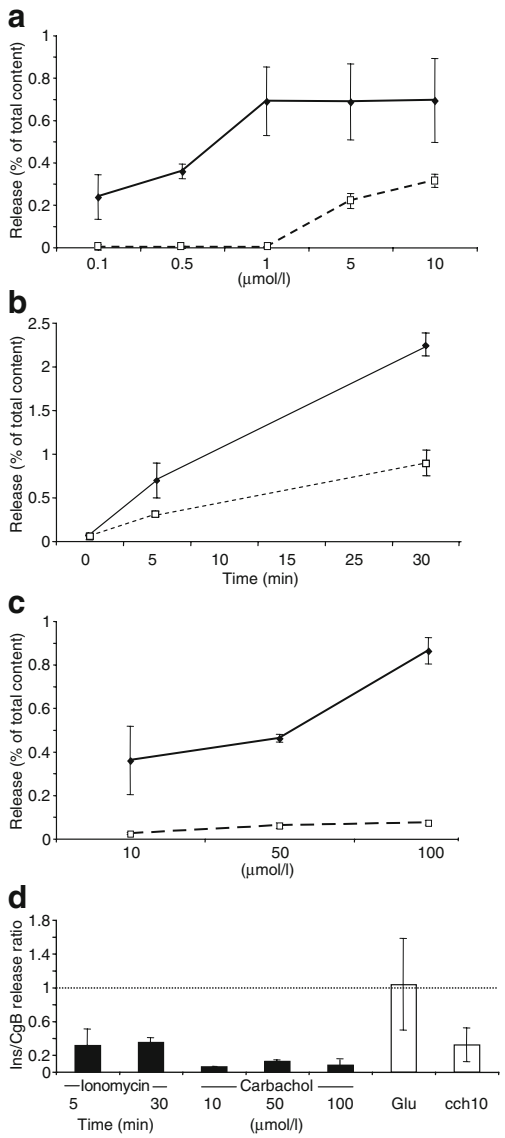

Fig. 6 Dissociation between insulin and CgB release induced in INS-1E cells by stimulation with ionomycin and carbachol. **a** Concentration-dependence of insulin (dashed line) and CgB (solid line) release responses induced by 5 min application of ionomycin. **b** The same responses induced by application of 10 μmol/l of the ionophore for 5 and 30 min. **c** The concentration-dependent release responses induced by 20 min application of carbachol (10–100 μmol/l). **d** Ratios between the per cent insulin and CgB release responses induced in INS-1E cells (black bars) by ionomycin (as in **b**), carabachol (10–100 μmol/l) (as in **c**) and human islets (white bars) incubated either with high glucose (Glu) or 10 μmol/l carbachol (cch10). Dotted line (**d**), represents the ratio of insulin and CgB equal to 1, meaning identical. The data shown are averages of three experiments run in triplicate±SEM

release of insulin was induced by ionomycin up to 1 μmol/l, the maximal concentration for CgB. At 5 and 10 μmol/l, some insulin release appeared, remaining however proportionally much lower than that of CgB (Fig. 6a). The greater stimulation of CgB vs insulin release by 10 μmol/l ionomycin, visible at 5 min stimulation, was maintained at 30 min (Fig. 6b). In a few control experiments, in which cells were pre-loaded with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; 30 min pre-incubation with 50 μmol/l BAPTA-AM) before stimulation, both insulin and CgB release induced by 10 μmol/l ionomycin were reduced to ~50% (not shown). These results confirm the Ca^{2+}-dependence of the ionomycin-induced release stimulation. A complete dissociation of the release of CgB from that of insulin, similar in extent to that observed with 0.1 to 1 μmol/l ionomycin, was also induced by carbachol, an agonist of muscarinic receptors, administered for 20 min at 10 to 100 μmol/l. (Fig. 6c). Figure 6d summarises some of the release results in terms of insulin:CgB release ratios observed both from INS-1E cells, ranging from 0.35±0.05 (mean± SEM) with 10 μmol/l ionomycin to <0.1±0.004 with 10 μmol/l carbachol, and from human islets stimulated for 20 min, ranging from 1±0.55 with high glucose to 0.32±0.2 with 10 μmol/l carbachol.

Discussion

Transport of proteins by endocrine cells is often envisaged as the flow of a mixture, transported from the endoplasmic reticulum to the Golgi complex and then to the SGs [37], which are finally exocytosed upon appropriate stimulation [38]. Previous studies had shown, however, that in various cell types the secretory proteins flowing out of the Golgi do not remain intermixed, but are sorted into different types of SGs [4–6, 39, 40]. In insulin-secreting cells, such separate sorting has never been reported. Rather, the various secretory proteins, including the granins, were believed to be co-stored and co-released with insulin [7, 8, 41]. Our data in six different insulin-secreting cell types, including rat, mouse and human primary beta cells, show that intermixing of CgB with insulin takes place only up to the Golgi–TGN area. In the SGs such a co-storage is not the rule, but is restricted to only a fraction of the organelles.

The appreciation of the dissociation between insulin and CgB was made possible by the use of an appropriate image analysis system. SGs positive only for insulin or CgB, hardly distinguishable by conventional confocal microscopy, became obvious when the cells were analysed by deconvolution and 3D-reconstruction. This result can be explained by the improvement of the resolution and also by the fact that imaging of the SGs in 3D is more likely to reveal their real structure. Moreover, the multiplicity of the SGs was confirmed by immunoelectron microscopy. The heterogeneity of beta cell SGs, moreover, may not be limited to insulin and CgB, since additional secretory proteins are also expressed by beta cells [42–44]. During their transport along the secretory pathway, these proteins might either remain intermixed with insulin, as chromogranin A [7], or segregate away from the hormone and

possibly also from CgB, contributing to further increase the complexity of the SG population.

An unexpected result of our ultrastructural immunocytochemistry studies was the intragranular redistribution of CgB taking place upon stimulation of INS-1E cells. In a previous study, the granin had been reported to be localised mostly in the core [41], whereas in our resting cells such a localisation was observed in only 60% of the SGs. This discrepancy, however, might be only apparent, due to differences both in the reagents employed (fixatives, hydrophilic resin, antibodies), and in the procedures used for data analysis. Our novel finding is the large redistribution of CgB, from the depth of the core to its halo/periphery, which we observed when cells were exposed to distinct types of stimulation with ionomycin, high glucose, forskolin, IBMX and SM, while upon stimulation with PMA, CgB remained almost evenly distributed between core and halo/periphery of SGs. The change of CgB distribution looks specific since it was accompanied neither by a redistribution of insulin, which remained confined to the core of SGs, nor by any changes of cell ultrastructure. Other organelles, from the endoplasmic reticulum to the Golgi complex, are known to modify their internal environment, with consequences on their protein structure and distribution, in response to changes of the cytosolic milieu [45]. Changes of $[Ca^{2+}]$ within the SGs, reported also in beta cells following stimulation [46, 47] could also trigger similar effects and sustain the redistribution of CgB. The significance of this redistribution is not clear. However, in a different system (PC12 cells), the catecholamines localised preferentially in the halo are known to be released more readily through small exocytic fusion pores [48, 49], and a similar possibility has also been envisaged for the release of a pool of insulin [50].

The most important findings of this work concern the release of CgB, in particular the demonstration that the granin is not always released in parallel to insulin (as occurs in response to strong stimulation by four secretagogues administered together in the SM, and also after application of PMA±high glucose) but can predominate over insulin (stimulation with IBMX and forskolin±high glucose) or even take place independently (stimulation with ionomycin at concentrations ≤1 μmol/l, and with carbachol). Even at higher ionomycin concentrations, when some insulin release did take place, its percentage remained about threefold lower than that of CgB. These results strongly suggest that discharge of the SGs containing only CgB is regulated by intracellular signals that are distinct from those regulating the SGs containing insulin, both alone and possibly also in combination with CgB. This conclusion appears consistent with that of another report, which appeared during revision of this manuscript and suggested that non-specified chromogranin-containing SGs could represent a store for K_{ATP} channels destined to be inserted into the plasma membrane upon secretagogue application [51]. Since the response to ionomycin and in considerable part also that to carbachol are mediated by the increase of $[Ca^{2+}]_i$, it may be hypothesised that the SGs containing only CgB are more sensitive to Ca^{2+} than those containing insulin.

In conclusion, our results demonstrate the co-existence in various insulin-secreting beta cells of various types of SGs containing, separately, insulin or CgB, or the two proteins together. These various types of SGs appear to be functionally different, since they could be discharged, preferentially or even exclusively, in response to different stimuli. Co-existence in single cells of different SG populations, containing single secretory proteins or protein mixtures, had already been reported in other cell systems; however, their differential release had never been observed. It will be interesting to establish whether the levels and the release of CgB are altered in specific pathological conditions. Changes of CgB release may in fact result in alterations of insulin secretion and could therefore contribute to various types of metabolic diseases.

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