Cell Tissue Res (1997) 287:325–333

Cell & Tissue Research

Endocytosis of gastrin in cancer cells expressing gastrin/CCK-B receptor

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Received: 5 January 1996 / Accepted: 13 June 1996

Abstract. Endocytosis of gastrin was studied in a number of gastrin-receptor-expressing cell lines by confocal laser scanning microscopy (CLSM) with the aid of a biologically active fluorescent derivative, rhodamine green heptagastrin. Rapid clustering (within 4–7 min) and internalization of fluorescent ligand upon binding at room temperature and 37° C were observed in the rat pancreatic acinar carcinoma cell line AR42J, human gastric carcinomas AGS-P and SIIA, human colon carcinomas HCT116 and HT29, and in NIH/3T3 cells transfected with human and rat gastrin/cholecystokinin-B receptor cDNA. Internalization was inhibited by hypertonic medium. Fluorescent heptagastrin and transferrin colocalized in the same endocytic vesicles at different stages of internalization suggesting that endocytosis occurred predominantly through a clathrin-dependent mechanism. At 37° C partial colocalization with the lysosomal marker neutral red was detected by CLSM, implying that internalized gastrin accumulated in the lysosomes. Immunoelectron microscopy studies with antibodies against gastrin revealed the presence of the internalized hormone in multivesicular vesicles and endosomes. Almost no hormone was detected in lysosomes with the antibodies to gastrin, suggesting that the degradation of the peptide is rapid in those vesicles. Continuous accumulation of fluorescent label was observed by CLSM in the presence of the protein synthesis inhibitor cycloheximide, suggesting that the gastrin receptor is recycled back to the cell membrane after hormone delivery to intracellular compartments. An estimated average recycling time for the receptor molecules was 1 h in NIH/3T3 cells.

Key words: Cholecystokinin receptor – Fluorescent microscopy – Confocal microscopy – Gastrointestinal hormones – Gastrin – Drug delivery – Cancer cells

This research was sponsored in part by the National Cancer Institute, DHHS under contract with ABL. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government

Abbreviations: CCK, cholecystokinin; CCK-8, CCK octapeptide; RG-7G, rhodamine green heptagastrin; DMEM, Dulbecco's modified Eagle's medium; CLSM, confocal laser scanning microscopy; GPCR, G-protein-coupled receptor: FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline

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Introduction

Gastrin, a gastrointestinal (GI) hormone, stimulates growth of certain gastrointestinal cancers (Lamote and Willems 1988; Smith and Solomon 1988; Townsend et al. 1988) and has a trophic effect on a number of established GI cancer cells (Kusyk et al. 1986; Watson et al. 1989; Hoosein et al. 1990; Ishizuka et al. 1992; Mauss et al. 1994). Studies of binding of ^{125}I-labeled gastrin have demonstrated the presence of specific gastrin receptors on many cell lines derived from different types of cancers (Frucht et al. 1992). We have been using the gastrin receptor as a model for studies of receptor-mediated delivery of cytotoxic agents into cancer cells. Triazene derivative of pentagastrin has been shown to have a specific toxic effect on the cells expressing gastrin receptors (Schmidt et al. 1994). However, the toxicity of the compound was relatively low, most likely due to insufficient activity of the toxic moiety. To design better receptor-targeted drugs a better understanding of endocytic pathways of the ligand is essential. The receptors with high affinity for gastrin from different species have been cloned and their pharmacological properties have been characterized (reviewed by Wank et al. 1994). Novel receptors for gastrin, termed gastrin-preferring receptors, were found to mediate the biological effects of gastrin on fibroblasts, intestinal cells, and colon cancer cells (Bold et al. 1994; Singh et al. 1995). They have significantly lower affinity to shorter forms of gastrin (Singh et al. 1995) and their structure is not yet known. The cloned cholecystokinin (CCK)-A and CCK-B receptors belong to a superfamily of G-protein-coupled receptors (GPCR). The mechanisms of endocytosis of GRCP were studied for a small

number of receptors (Ghinea et al. 1992; Hoxie et al. 1993; Ashworth et al. 1995; Grady et al. 1995; Roettger et al. 1995) but the data are incomplete and controversial. We have previously described the synthesis and characterization of three fluorescent derivatives of heptagastrin (Czerwinski et al. 1995). One of the derivatives, rhodamine green heptagastrin (RG-7G), was found to interact specifically with the gastrin/CCK-B receptor while retaining the biological properties of the parent peptide. The K_d of RG-7G binding to CCK-B receptor is 2 nM, which is very close to that of gastrin; thus it can be considered a specific ligand for CCK-B receptor. Moreover, it was found to have appropriate optical properties which allow it to be used as a model compound for studies of gastrin trafficking by cell imaging techniques. We have also demonstrated that confocal laser scanning microscopy (CLSM) can be used successfully for quantitation of ligand binding to a receptor obviating the need of radioactive derivatives of the hormone (Czerwinski et al. 1995). The present study examines the internalization of a biologically active fluorescent derivative of gastrin in real time in a number of CCK-B receptor expressing cells by means of CLSM and also the intracellular localization of internalized hormone with the aid of immunoelectron microscopy. The main goal of this work is to explore the feasibility of gastrin/CCK-B receptor internalization as a means of delivery of cytotoxic agents to receptor-expressing tumor cells. For the study we have chosen cancer cell lines that were shown to express gastrin/CCK-B receptors. Rat pancreatic acinar cells AR42J express both CCK-A and CCK-B (Zhou et al. 1992) as well as a receptor for glycine extended gastrin (Seva et al. 1994). Human colon cancer cell lines HCT-116 and HT-29 were shown to contain CCK-B receptor mRNA (Lebovitz et al. 1994). Human gastric cancer cell line AGS binds selective CCK-B receptor antagonist and expresses CCK-B receptor mRNA (Shih et al. 1996). NIH/3T3 cells were stably transfected with CCK-B receptor cDNA to produce a model cell line for the drug delivery studies.

Materials and methods

Fluorescent probes

RG-7G was synthesized as previously described (Czerwinski et al. 1995). Rhodamine red concanavalin A, tetramethylrhodamine-transferrin, neutral red and Hoechst 33342 dye were purchased from Molecular Probes (Eugene, OR, USA). Human gastrin II (2–17) was purchased from Research Plus (Bayonne, NJ, USA). Polyclonal antibodies to human gastrin were from DAKO (Carpinteria, CA, USA).

Cells

NIH/3T3 cells, rat pancreatic acinar carcinoma cells, AR42J, human colon carcinomas HCT116 and HT29 were obtained from the American Type Culture Collection (Rockville, Md., USA). Human gastric carcinomas AGS-P and SIIA cells were a kind gift from Dr. Richard Bold (Galveston, TX, USA). Recombinant human and rat gastrin/CCK-B receptor cDNA (Wank et al. 1994) in the mammalian expression vector pCDL-Neo was stably transfected into NIH/3T3 cells using electroporation, and clones were selected in the presence of 250 µg/ml G418 (Life Technologies, Bethesda, Md., USA). Clones derived from single cells were selected for receptor expression using ^{125}I-Bolton-Hunter CCK octapeptide (CCK-8; Amersham, Newford, MA, USA) binding. AR42J cells, NIH/3T3 cells, and HCT116 were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD, USA), containing 10% fetal bovine serum (FBS). AGS-P and SIIA cells were grown in F12 Ham medicum (Gibco, Gaithersburg, MD, USA), and HT29 in RPMI-1640.

Laser scanning confocal microscopy

Cells were grown in Nunc cover-glass chamber slides in medium without phenol red. After incubation with fluorescent heptagastrin the cells were observed on a Zeiss inverted LSM 410 confocal laser scanning microscope. Fluorescence of RG-7G was excited using a 488-nm argon/krypton laser and emitted fluorescence was detected with 515–540-nm band pass filter. For neutral red, rhodamine red, and tetramethylrhodamine, a 543-nm helium/neon laser was used for excitation and fluorescence was detected with a 590-nm band pass filter. The Hoechst 33342 dye was excited with a 364-nm ion laser, and emission was detected through a 420-nm band pass filter.

Demonstration of RG-7G internalization

The cells were incubated for 30 min with 50 nM RG-7G in phenol-red-free medium containing 10% FBS at 20 or 37° C, rinsed with cold phosphate-buffered saline (PBS) and treated with rhodamine red concanavalin A (10 µg/ml in PBS) for 3 min at 4° C. After a final rinse with cold PBS, the cells were observed with a confocal microscope using ×63 oil immersion lens, a pinhole of 35, and an electronic zoom of 3.2 to yield a final magnification of ×2016 of the stored images.

Time-course of RG-7G internalization

The cells were preincubated for 30 min with 100 nM RG-7G at 4° C, rinsed with cold medium and placed under the microscope. Images of a selected group of cells were taken at 1-min intervals at 20° C.

Colocalization of RG-7G with transferrin

The cells were incubated with 100 nM RG-7G and tetramethylrhodamine transferrin (20 µg/ml) in serum free DMEM without phenol red at 20 or 37° C in a CO_2 incubator for various time intervals, rinsed with cold PBS, fixed with 4% formaldehyde in PBS at 4° C for 3 min and immediately observed with the inverted confocal microscope.

Colocalization of RG-7G with the lysosomal marker, neutral red

Cells previously treated for 30 min with 100 nM RG-7G in a CO_2 incubator were incubated with PBS containing 10 µg/ml of neutral red and 10 µg/ml Hoechst 33342 dye for 5 min at 37° C, rinsed and observed with the confocal microscope.

Time-course of RG-7G accumulation within the cells

Cells which were grown almost to confluency were pretreated with 15 µg/ml cycloheximide. After 30 min of incubation 100 nM

RG-7G was added in the medium without phenol red. The cells were incubated in a CO_2 incubator for various time intervals, rinsed with medium and observed with the inverted confocal microscope. A minimum eight images for each incubation time point were recorded and the fluorescence intensity was quantitated by processing the data using Zeiss LSM software.

Immunoelectron microscopy

AR42J cells were incubated for 15 min with 100 nM human gastrin II (2–17) in a CO_2 incubator, rinsed with cold PBS, fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS, pH 7.2, for 1 h at 4° C, and postfixed in 1% OsO_4 in PBS for 1 h at 4° C. To reduce the number of free aldehyde groups, samples were treated with 0.1 M $NaBH_4$ in PBS for 30 min. Dehydration and embedding were carried out at room temperature. Embedding in LR White was performed according to the procedure of Newman (Newman 1989). Polymerization of the blocks was carried out at 50° C for 48 h. Thin sections were prepared on an LKB Nova ultramicrotome using a diamond knife and were then picked up on nickel grids coated with Formvar film. Grids with sections were incubated over a drop of blocking solution [1% bovine serum albumin (BSA) in PBS for 30 min], then were treated with diluted antibodies (rabbit anti-gastrin, 2-17, 1:300), followed by protein A conjugated with 10 nm colloidal gold particles (for 45 min) and washed three times after all labeling steps with 0.05% Tween-20 in PBS. Controls for specificity included incubation with non-immune serum, omission of first antibodies, and substitution with other antibody (anti-spectrin, DAKO, Carpinteria, CA, USA).

Results

The binding and internalization of a fluorescent derivative of heptagastrin, RG-7G, was observed in NIH/3T3 cells stably transfected with human and rat gastrin/CCK-B receptor as well as in rat pancreatic acinar carcinoma cells AR42J, human colonic carcinoma cells HT29 and

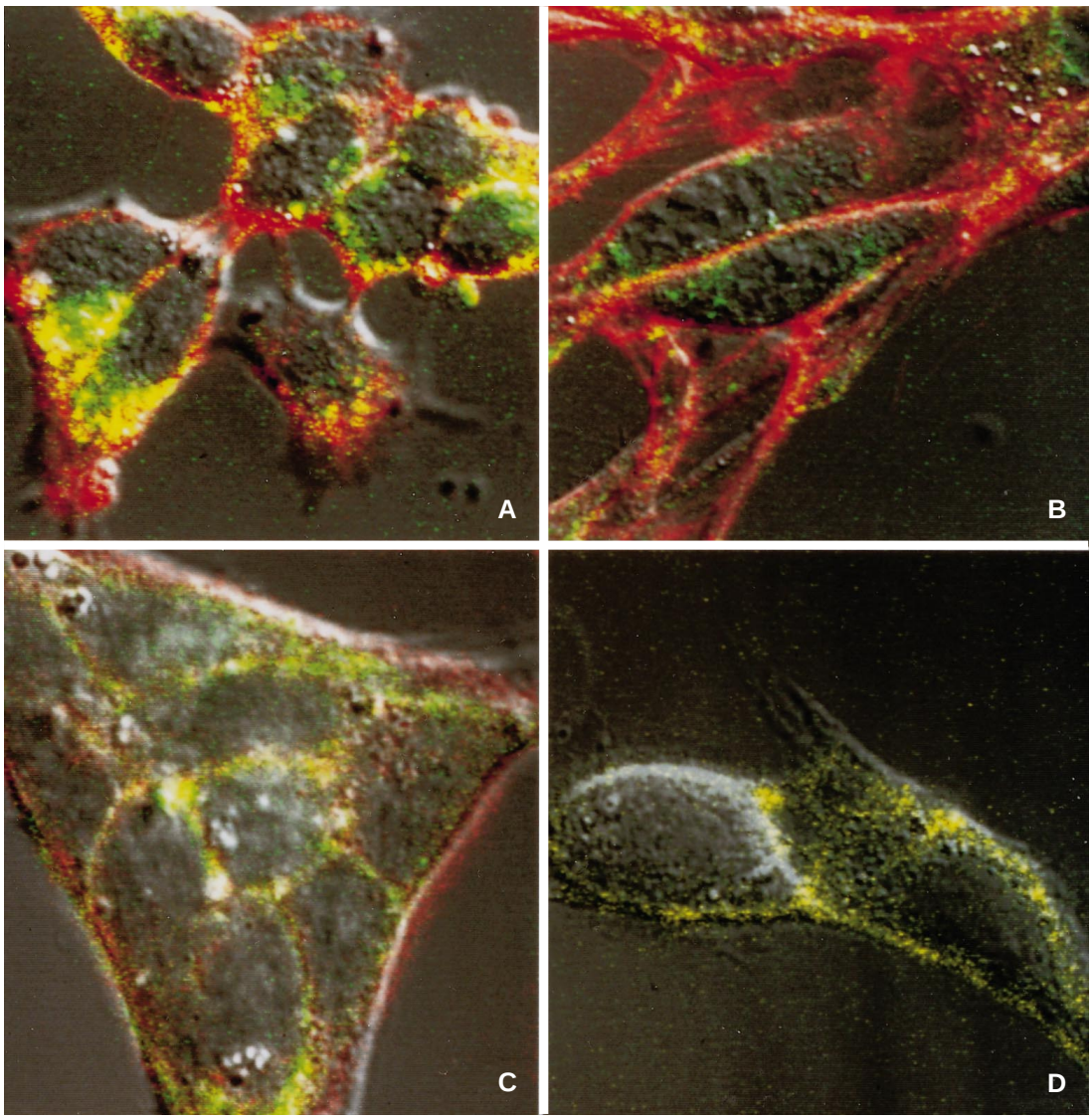

Fig. 1. NIH/3T3 cells stably transfected with human gastrin/cholecystokinin (CCK)-B receptor cDNA (**A**) and AR42J cells (**B**) after incubation with rhodamine green heptagastrin (RG-7G; in *green*) and rhodamine red concanavalin A (in *red*), which marks the surface of the cells. Colocalization of the two, or surface-located fluorescent gastrin appears in *yellow*. Also shown are AR42J cells (**C**) and NIH/3T3 cells stably transfected with human gastrin/CCK-B receptor cDNA (**D**), treated with RG-7G in the presence of 0.3 M sucrose. ×2016

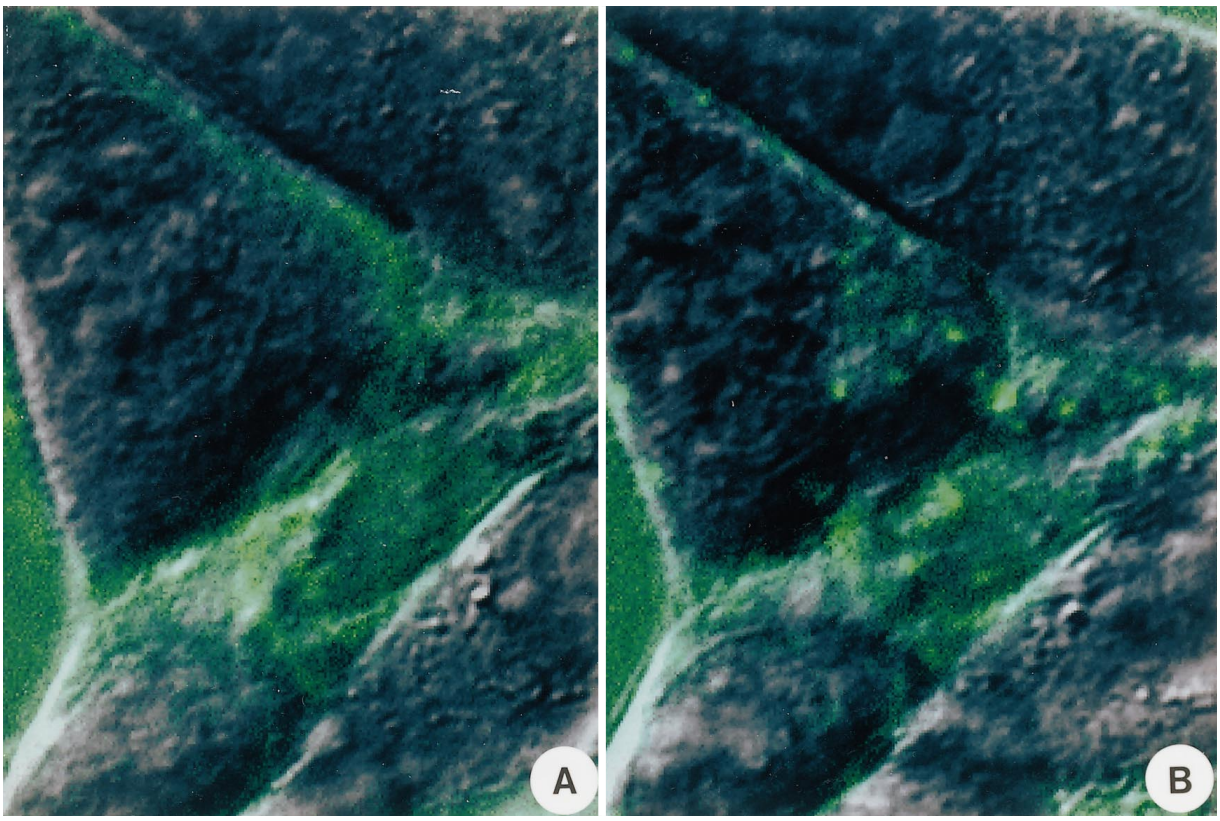

Fig. 2A. NIH/3T3 cells expressing gastrin/CCK-B receptor, saturated with fluorescent gastrin at 4° C. All the compound is on the surface. **B** The same group of cells, after five min at room temperature. Internalized particles containing the compound can be seen clearly. ×2016

HCT116, and in human gastric carcinoma cells SIIA and AGS-P. Preincubation of all cell lines with 10 µM human gastrin for 30 min reduced fluorescence of bound RG-7G to background levels.

Two approaches were used for demonstration of internalization. To distinguish between internalized and surface-bound hormone, the surface of RG-7G-treated cells was labeled with concanavalin A conjugated with rhodamine red (Fig. 1A, B). The major portion of RG-7G did not show colocalization with the red fluorescence of the cell surface marker after 30 min of incubation either at 20 or 37° C in all cell lines tested, but was detected inside the cells. The cells were also washed with a pH 3.5 Hanks' salt solution after incubation with the fluorescent gastrin derivative. The surface-bound ligand is known to be removed from the cell surface under these conditions (Gammeltoft 1990). Even after 40 min of acid treatment the cells retained most of the fluorescence.

To estimate the time needed for internalization, the cells were saturated with RG-7G at 4° C for 30 min, rinsed with medium and observed by confocal microscopy at 20° C at 1-min intervals. At 4° C there was no internalization (Fig. 2A), while at 20° C the bound ligand was clustered into aggregates on the cell surface and there was evidence of internalization as early as after 4–7 min of incubation (Fig. 2B).

Two techniques were used to determine whether gastrin is internalized via clathrin-coated vesicles. First, the cells were incubated simultaneously with RG-7G and tetramethylrhodamine-transferrin, which is a marker for endocytosis via coated pits (Trowbridge et al. 1993). After incubation, the cells were fixed briefly with formaldehyde to prevent movement of intracellular vesicles during the recording of the images which were obtained with the inverted confocal microscope. At various time intervals, ranging from 1 to 20 min, there was significant colocalization of green fluorescence of RG-7G with red fluorescence of tetramethyrhodamine-transferrin (Figs. 3, 4). Second, it was established that hypertonic medium selectively inhibits clathrin-mediated endocytosis (Heuser and Anderson 1989). When gastrin-receptor-expressing cells were incubated in medium containing 0.3 M sucrose prior to addition of the ligand endocytosis of RG-7G was markedly inhibited, and almost all green fluorescence was confined to the surface of the cells (Fig. 1C, D). Endocytosis of transferrin was also inhibited in the same conditions.

Labeling the cells with neutral red, a marker for lysosomes, revealed partial colocalization of the green RG-7G fluorescence and the red fluorescence of the marker (Fig. 5). Immunoelectron microscopy was performed on AR42J cells. The cells incubated with 100 nM human gastrin 2–17 for 15 min at 37° C were fixed and processed for microscopy. The ligand was detected with polyclonal antibodies to human gastrin and protein A conjugated with colloidal gold particles. The label (Fig.

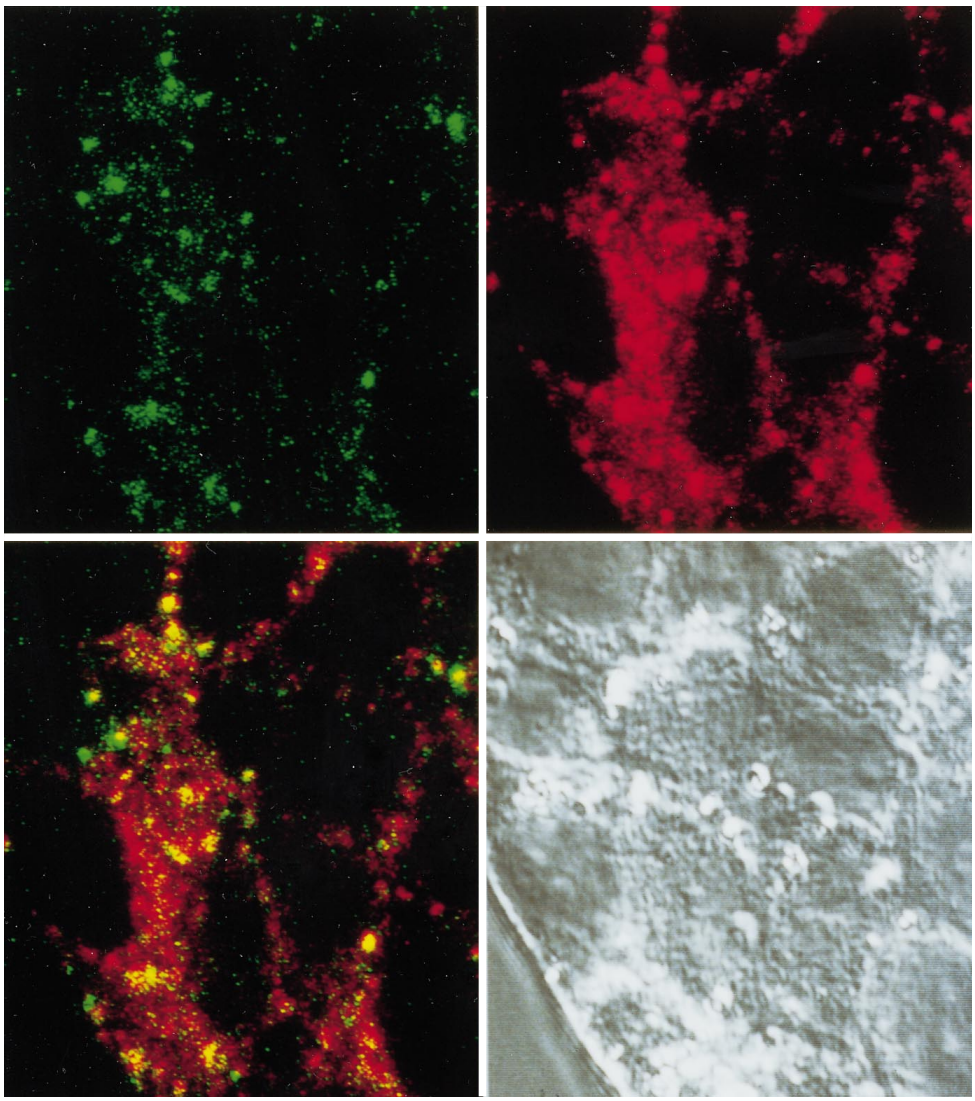

Fig. 3. AR42J cells treated with RG-7G (in *green*) and tetramethylrhodamine-transferrin (in *red*) for 10 min at 37° C. Colocalization of the two appears as *yellow* or *orange*. ×2016

6) was localized on the cell membrane and in multivesicular vesicles. No ligand was detected in lysosomes, probably due to rapid degradation of the peptide within the lysosomes.

Accumulation of RG-7G inside the cells was studied as a function of time. Several chamber slides with attached AR42J cells were preincubated with cycloheximide, an inhibitor of protein synthesis. After addition, of 100 nM RG-7G to the medium, containing cycloheximide, the slides were placed in a CO_2 incubator. The incubation was stopped at various time intervals; the cells were rinsed with medium and observed with an inverted confocal microscope using an identical set of parameters for all observations. At least eight images for each incubation time were stored on an optical disk and the fluorescence was quantitated with the help of Zeiss LSM software. The experiment was repeated four times with essentially the same results. The curve for the accumulation of fluorescence as a function of time (Fig. 7) revealed a pulse pattern, which is consistent with a hypothesis of periodic receptor recycling and subsequent uptake of RG-7G. The time between accumulation intervals indicated that the average recycling time for the receptor molecules was approximately 1 h.

Discussion

Confocal microscopy confirmed that the internalization of gastrin/CCK-B receptor-expressing cancer cells occurred rapidly in all of these cells. The degree of internalization varied in the different cell lines, but it was more than 50% in all studied cells after 30 min of incubation at 37° C. AR42J cells express significantly more receptor molecules than all other cell lines studied, which is the reason why they were used for the majority of illustrations in this paper. The results with the other cell lines were qualitatively the same, although the intensity of fluorescence was somewhat lower.

The pathways of internalization of GPCR are poorly understood and can also be different for different receptors (or for the same receptor in different cells). For example, the thyrotropin-releasing hormone (Ashworth et al. 1995), thrombin (Hoxie et al. 1993), luteinizing hor-

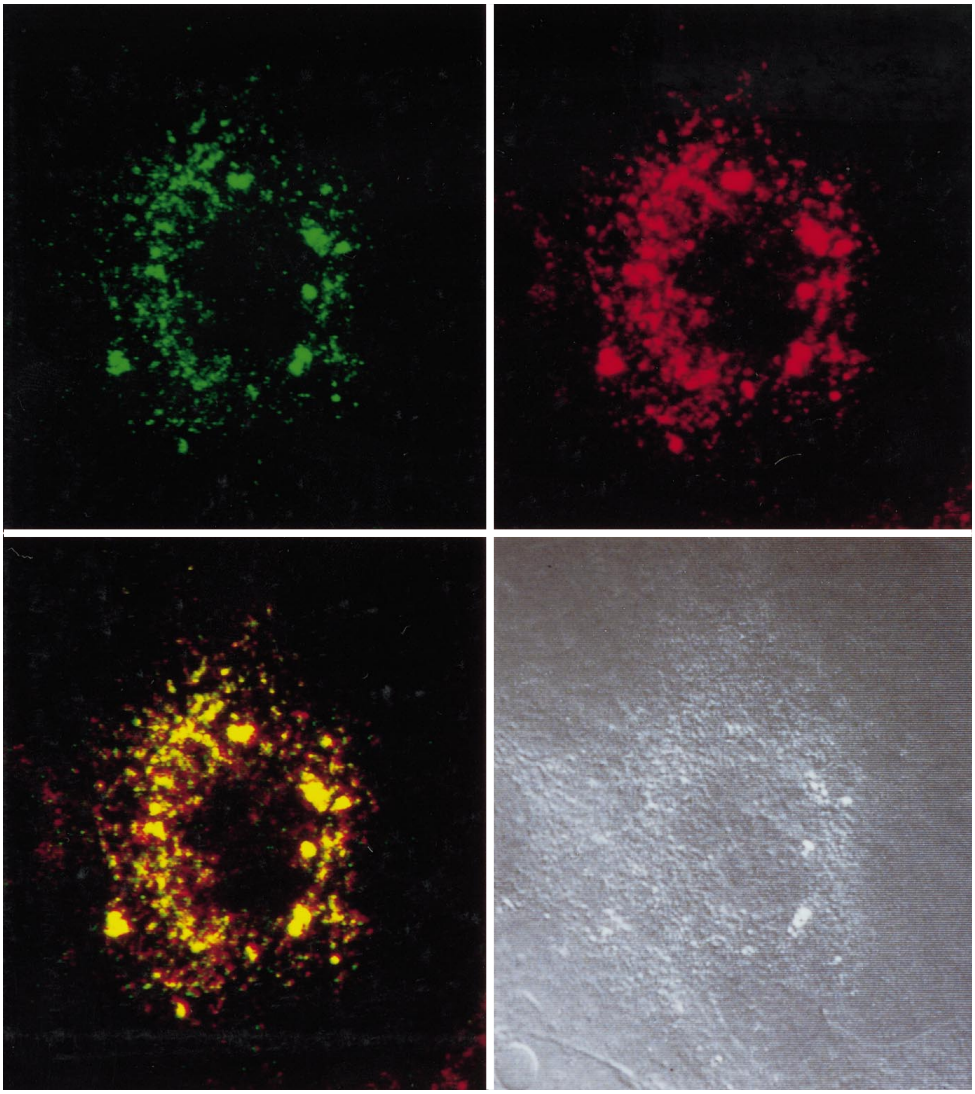

Fig. 4. NIH/3T3 cells transfected with human gastrin/CCK-B receptor cDNA, treated with RG-7G (in *green*) and tetramethylrhodamine-transferrin (in *red*) for 10 min at 37° C. Colocalization of the two appears as *yellow* or *orange*. ×2016

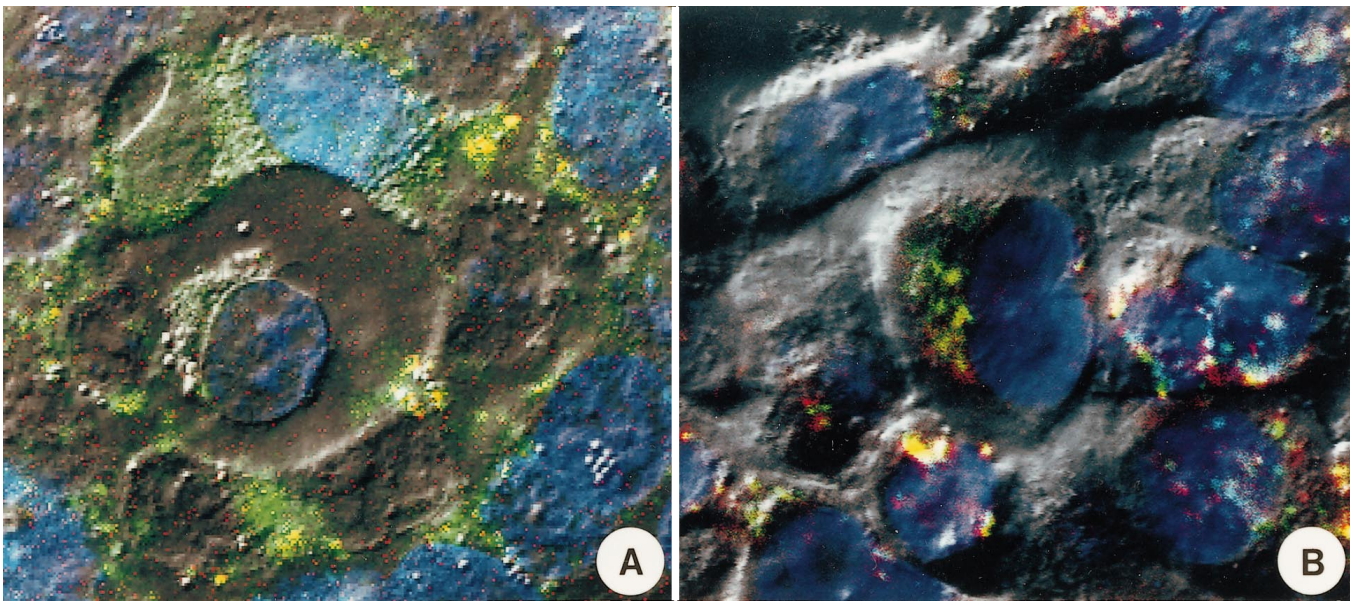

Fig. 5. AR42J cells (**A**) and human gastric adenocarcinoma AGS-P cells (**B**) after incubation with RG-7G, which is in *green*, and labeled with Hoechst 33342 dye (*blue*), which marks the nucleus, and neutral red (in *red*), which is a marker for lysosomes. Partial colocalization of gastrin and neutral red can be observed (*orange*). ×2016

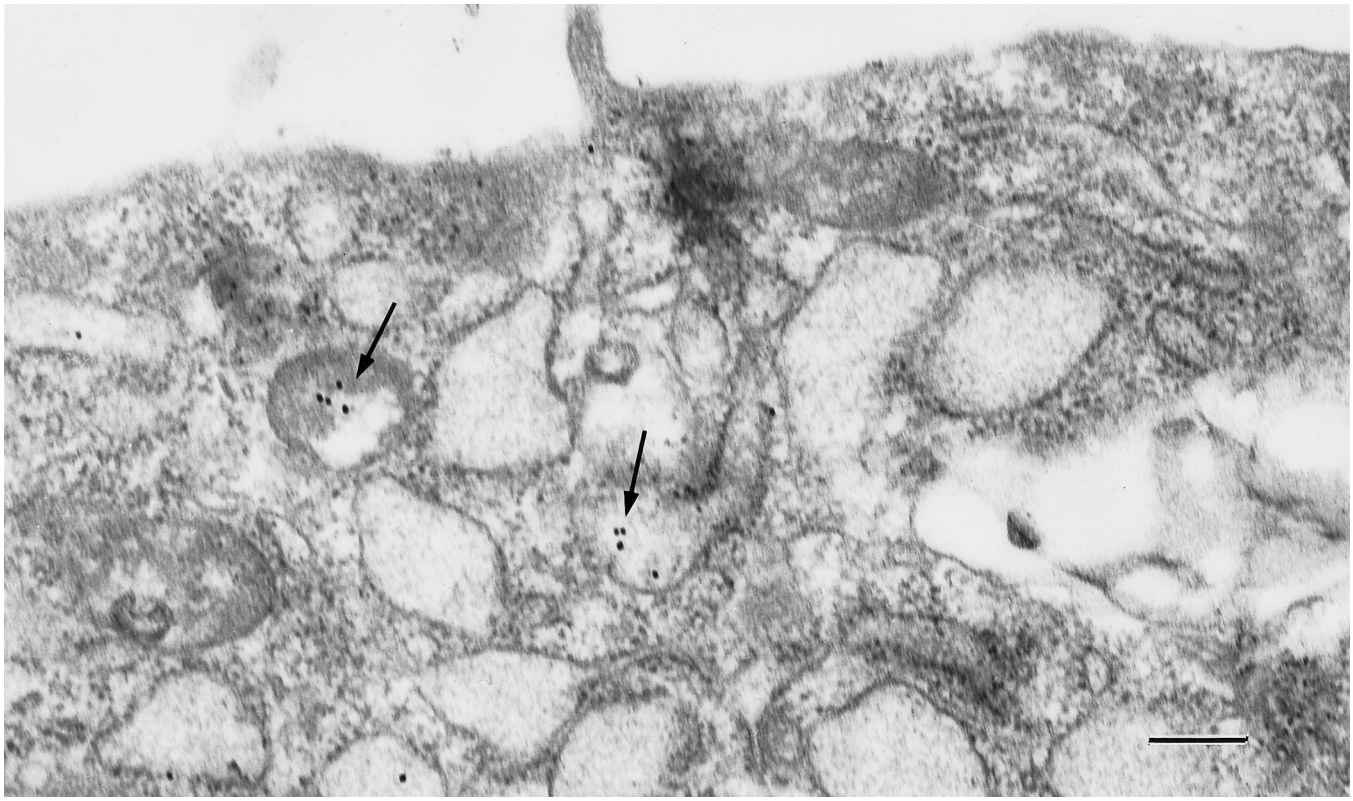

Fig. 6. Immunoelectron microscopy of AR42J cells with the antibodies against gastrin, revealing the presence of the internalized hormone (*arrows*) in multivesicular vesicles and late endosomes. *Bar:* 0.2 μm

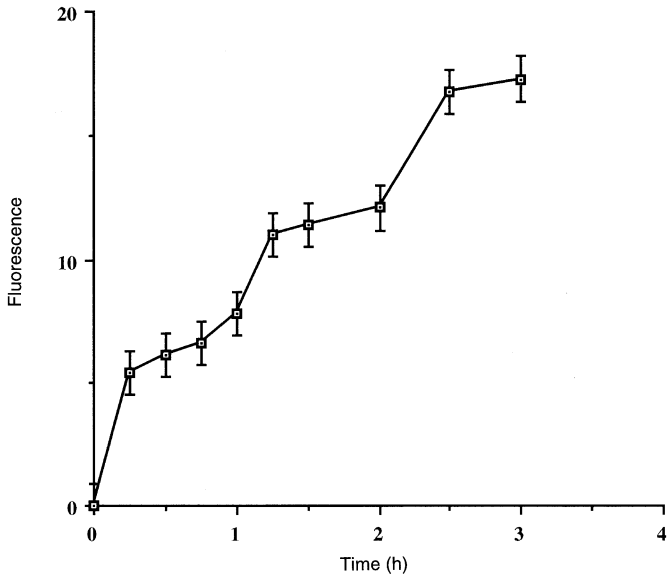

Fig. 7. Time-dependence of RG-7G accumulation in AR42J cells in the presence of cycloheximide. Cells were exposed to 100 nM RG-7G for various time intervals and bound fluorescence was measured by confocal laser scanning microscopy. Each point is the mean+SE of eight measurements

mone (Ghinea et al. 1992), and gastrin-releasing peptide (Grady et al. 1995) receptors were shown to internalize via clathrin-coated pits, while the β-adrenergic receptor was found to undergo internalization both in a clathrin-dependent (von Zastow et al. 1992) and clathrin-independent (Raposo et al. 1989) manner. Existence of dual pathways of internalization was suggested for CCK in CCK-A receptor-bearing CHO cells (Roettger et al. 1995). In all cell lines tested in the present study, the internalization of gastrin was inhibited by hypertonic medium, which is known to inhibit formation of coated pits (Heuser et al. 1989). Fluorescent heptagastrin also showed significant colocalization at various stages of internalization with fluorescent transferrin. Because transferrin is known to be internalized through coated pits (Trowbridge et al. 1993), our data suggest that endocytosis of gastrin in cancer cells also occurs predominantly through clathrin-coated pits. Although we were not able to detect endocytosis through a clathrin-independent mechanism, it should be noted that both methods that were used for demonstration of endocytosis through coated pits are indirect, and thus we cannot exclude the possibility that in some cells another pathway exists. It was shown (Koleske et al. 1995) that caveolae, or non-clathrin-coated invaginations are reduced or absent in oncogenically transformed cancer cells. The reduction in number of these cell membrane structures in cancer cells can be attributed to the dominance of endocytosis through clathrin-coated pits.

Immunoelectron microscopy with antibodies against gastrin was used for ultrastructural characterization of the internalization organelles. We have chosen this approach because it gives more reliable results than those obtained from localization of colloidal gold derivatives of short peptides, although immunolocalization is inherently less sensitive than the latter method. Attachment of

the massive complex of BSA and colloidal gold, as described in an earlier study (Roettger et al. 1995), is likely to modify the properties of the small peptide significantly. This can give rise to equivocal results. On the other hand, detection of small peptides with the antibodies is complicated by the low yields of the fixation procedures. Native gastrin dose not have any amino groups which are necessary for fixation with formaldehyde and glutaraldehyde and thus it cannot be used for immunoelectron microscopy studies. We have used gastrin 2–17, an equally active form of gastrin that lacks N-terminal pyroglutamic acid residue, and therefore can be fixed for immunolocalization. Gastrin was not detected in coated pits by immunoelectron microscopy probably because of the relatively short lifetime of a coated pit [less than 1 min (Gruenberg and Howell 1989)], compared to the fixation time. However, another reason may be that our method is not sufficiently sensitive.

According to the most commonly accepted scheme of receptor-mediated endocytosis (Trowbridge et al. 1993), clathrin-coated pits loaded with receptor-ligand complexes deliver their cargo to sorting endosomes, also called multivesicular endosomes, or carrier vesicles. The present data on immunoelectron-microscopy localization of gastrin is in agreement with this general scheme of endocytosis, because the hormone was found predominantly in multivesicular vesicles of receptor-expressing cells. Colocalization of fluorescent gastrin with the lysosomal marker, neutral red, indicates that the peptide probably ends up in lysosomes. We were not able to detect internalized gastrin in lysosomes with the antibodies to the hormone, probably because rapid proteolysis destroys the peptide. However, the fluorescent tag delivered together with the peptide remains unaffected by the proteolytic enzymes and the acidic environment of the vesicle.

The intracellular stability of the fluorescent moiety allowed for the study of the time-dependence of ligand accumulation in receptor-expressing cells with the help of the method that we developed previously (Czerwinski et al. 1995). The technique has an important advantage over the pulse-chase experiments with radiolabeled ligands that were used by others for studying receptors recycling (Trowbridge et al. 1993), since it allows experiments on intact cells under near physiological conditions. This prevents the disruption of the systems that may be important in endocytosis. The fluorescent label, unlike amino acids labeled with radioactive isotopes, does not get released from the cell after digestion of the peptide. This property of the probe permits the study of kinetics of the ligand uptake. We assume that the plateaus on the fluorescence versus the time curve (Fig. 7) correspond to the periods when almost all of the receptor molecules had been sequestered. When the molecules recycle back to the surface, the next accumulation stage starts. An average recycling time for the gastrin/CCK-B receptor in NIH/3T3 cells deduced from that curve is about 1 h. It was shown that the processing time for receptors such as transferrin, low density β-lipoprotein, and asialoglycoprotein can be as short as 5 min, but linear uptakes of ligands by these receptors for periods up to 45 min were also observed (Trowbridge et al. 1993). It was suggested that the various recycling receptors that enter the cells via coated pits are all processed within the same set of endocytic elements (Trowbridge et al. 1993). However, recent data suggest that the sorting signals and molecular mechanisms regulating internalization of GPCRs can vary among receptors of the different classes (Slice et al. 1994). Thus the timing of GPCR processing may also be different. The recycling time for another GPCR – the luteinizing hormone receptor – was estimated to be between 20 min and 1 h (Ghinea et al. 1992), similar to the recycling time for the gastrin/CCK-B receptor obtained in the present study. The percentage of the gastrin/CCK-B receptor molecules that recycle is high since the amount of fluorescence uptake during the second and the third cycle (Fig. 7) is not much different from that of the first cycle. Thus the receptor could work as an efficient drug delivery system. However, large amounts of internalized ligand remain compartmentalized inside the cells. That means that for toxic moieties to be effective they must be chemically stable and capable of penetrating vesicular membranes in order to reach vitally important cell targets. The search for optimal toxic moieties and the mode of their attachment to the carrier peptide are ongoing in our laboratory.

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