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Expression cloning and characterization of the canine parietal cell gastrin receptor

(G protein-coupled receptor/cholecystokinin/gastric mucosa)

ALAN S. KOPIN*†, YOUNG-MEE LEE*, EDWARD W. MCBRIDE*, LAURENCE J. MILLER‡, MING LU§, HERBERT Y. LIN¶, LEE F. KOLAKOWSKI, JR.‖, AND MARTIN BEINBORN**

*Division of Gastroenterology and GRASP Digestive Disease Center, New England Medical Center, Tufts University School of Medicine, Boston, MA 02111; ‡Gastroenterology Research Unit, Mayo Graduate School of Medicine, Rochester, MN 55905; §Department of Cell Biology, Baylor College of Medicine, Houston, TX 77035; ¶Whitehead Institute for Biomedical Research, Cambridge, MA 02142; ‖Endocrine Unit, Massachusetts General Hospital, Boston, MA 02114; and **Department of General Pharmacology, Medical School, Hannover, Federal Republic of Germany

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ABSTRACT **Gastrin is an important stimulant of acid secretion by gastric parietal cells and is structurally related to the peptide hormone cholecystokinin (CCK). The pharmacologic properties of the parietal cell gastrin receptor are very similar to the predominant CCK receptor in the brain, CCK-B. Neither the gastrin nor the CCK-B receptor have been cloned thus far, making it difficult to resolve whether these two receptors are distinct. We have isolated a clone encoding the canine gastrin receptor by screening a parietal cell cDNA expression library using a radioligand-binding strategy. Nucleotide sequence analysis revealed an open reading frame encoding a 453-amino acid protein with seven putative hydrophobic transmembrane domains and significant homology with members of the β-adrenergic family of G protein-coupled receptors. The expressed recombinant receptor shows the same binding specificity for gastrin/CCK agonists and antagonists as the canine parietal cell receptor. Gastrin-stimulated phosphatidylinositol hydrolysis and intracellular Ca^{2+} mobilization in COS-7 cells expressing the cloned receptor suggest second-messenger signaling through phospholipase C. Affinity labeling of the expressed receptor in COS-7 cells revealed a protein identical in size to the native parietal cell receptor. Gastrin receptor transcripts were identified by high-stringency RNA blot analysis in both parietal cells and cerebral cortex, suggesting that the gastrin and CCK-B receptors are either highly homologous or identical.**

Gastrin is a peptide hormone produced by gastric antral G cells. The principal physiologic effect of this hormone is to stimulate secretion of hydrochloric acid by gastric parietal cells. Gastrin also modulates growth and differentiation of the gastric mucosa during normal development (1). Physiologic effects of this peptide are triggered when gastrin binds to its plasmalemmal receptor, tentatively identified by affinity labeling as a protein with apparent M_r of 74,000 (2, 3). Agonist stimulation of gastrin receptors on parietal cells results in phosphatidylinositol hydrolysis and elevation of free cytosolic calcium concentration ($[Ca^{2+}]_i$) (4, 5), mediated through guanine nucleotide-binding proteins (G proteins) (6). Gastrin, cholecystokinin (CCK), and CCK-related peptides comprise a hormone family, characterized by the identical carboxyl-terminal pentapeptide amide structure, a domain critical for receptor binding.

The corresponding target receptors for this hormone family can be divided into two main classes, CCK-A, and CCK-B/gastrin receptors, based on their agonist and antagonist-binding specificities (7, 8). The peripheral or "alimentary" receptor (CCK-A), found on pancreas, gallbladder, and certain brain nuclei, has a 1000-fold higher affinity for CCK than for gastrin. CCK-B/gastrin receptors are found in the brain (CCK-B), on smooth muscle cells, and on parietal cells (gastrin receptors). Agonist-binding studies on brain membranes and parietal cells show a 6- to 10-fold and a 1- to 2-fold higher affinity for CCK than for gastrin, respectively (9). These small differences in agonist binding have created controversy regarding the existence of subtypes within this receptor class. Pharmacologic antagonists are unable to differentiate between the central CCK-B and parietal cell gastrin receptors. Whether the CCK-B/gastrin receptor class is heterogeneous awaits isolation and direct comparison of putative subtypes.

Up to the present, none of the cDNAs encoding CCK/gastrin receptors have been cloned. In an effort to begin to understand the molecular basis for the pharmacologic divergence within this receptor family, we have isolated and characterized a cDNA encoding the canine parietal cell gastrin receptor.††

METHODS

Isolation of Canine Parietal Cells. Canine parietal cells were enzymatically dispersed as described by Soll *et al.* (10). Isolated cells were enriched in a Beckman XJ-10 elutriation system and collected at 900 rpm between flow rates of 50 and 80 ml/min. Further enrichment to 95% purity, as estimated by Papanicolaou staining, was achieved by isopycnic density centrifugation over a discontinuous Percoll gradient.

Construction of a Parietal Cell cDNA Expression Library. RNA was prepared from $\approx 1.5 \times 10^8$ parietal cells by acid phenol extraction (11) followed by oligo(dT)-cellulose chromatography. Six micrograms of poly(A)$^+$ RNA were converted to double-stranded cDNA (12). After the addition of *Bst*XI adaptors (Invitrogen, San Diego), the cDNA was size-selected over a 5–20% potassium acetate gradient, and fractions >1.5 kb were ligated into the expression vector pcDNA-1 (Invitrogen, San Diego).††

Isolation of a Gastrin Receptor cDNA. Two million primary recombinants in pools of 3,000–10,000 were produced by transforming *Escherichia coli* MC1061/p3 by electroporation (13). Bacterial stocks representing each pool were stored in glycerol. DNA representing each pool was prepared by the alkaline lysis procedure and then transfected into COS-7 cells adherent to glass flaskettes (Nunc) by using DEAE-dextran (14). Forty-

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Abbreviations: CCK, cholecystokinin; CCK-A, peripheral CCK receptor; CCK-B, predominant CCK receptor in brain; $[Ca^{2+}]_i$, free cytosolic calcium concentration.

†To whom reprint requests should be addressed.

††The sequence of the cDNA encoding the protein reported in this paper has been deposited in the GenBank data base (accession no. M87834)

eight hours after transfection, cells were incubated for 60 min at 37°C in Hanks' buffer/25 mM Hepes, pH 7.4/0.1% bovine serum albumin (solution A)/50 pM ^{125}I-labeled CCK-8 (the CCK octapeptide labeled at Asp[1]; New England Nuclear, 2200 Ci/mmol; 1 Ci = 37 GBq) and 50 pM ^{125}I-labeled D-Tyr-Gly-[(Ahx^{28,31})-CCK-26–33] (where Ahx is 2-aminohexanoic acid), a CCK analog that includes a free amino group available for fixation (15). After four washes in ice-cold solution A, the cells were fixed in phosphate-buffered saline, pH 7.4/2.5% glutaraldehyde, dipped in 0.5% gelatin, and dried at 37°C. Slides were exposed to Kodak NTB2 photoemulsion for 3 days (16) and examined by dark-field microscopy. The positive pool was sequentially divided until a single positive clone (GR-1) was obtained.

Nucleotide Sequence. The gastrin receptor cDNA was subcloned into M13mp18/19, and single-stranded templates from both strands were sequenced by the dideoxynucleotide chain-termination method (17) using modified T7 DNA polymerase (United States Biochemical). Sequence was analyzed by the Genetics Computer Group, Inc. program GAP (18) and the Klein, Kanehisa, DeLisi analysis of hydropathy (19).

Northern (RNA) Blot Hybridization Assays. Poly(A)$^+$ RNA from adult canine tissues was separated on a 1.2% agarose/0.66 M formaldehyde gel and transferred to a Nytran membrane by capillary blotting. A 1400-base-pair *Pst* I–*Xba* I fragment of GR-1, radiolabeled with [α-^{32}P]dCTP by priming with random hexamers (20), was hybridized to the membrane overnight at 42°C in 50% (vol/vol) formamide/5× standard saline citrate/20 mM sodium phosphate, pH 6.6/1× Denhardt's solution/0.5% SDS/10% (wt/vol) dextran sulfate/salmon sperm DNA at 100 μg/ml. The blot was washed for 40 min in 0.2× standard saline citrate/0.1% SDS at 70°C. The autoradiogram was exposed for 45 hr with two intensifying screens at −80°C.

Binding Experiments. COS-7 cells (1.5 × 10^6) were plated in 10-cm culture dishes (Nunc) and grown in Dulbecco's modified Eagle's medium/10% fetal calf serum in 5% CO_2 at 37°C. After an overnight incubation, cells were transfected with 5 μg of GR-1 DNA (14). Twenty-four hours after transfection, cells were split into 24-well dishes (Costar), 5000 cells per well. After an additional 24 hr, competition binding experiments were done in solution A/0.15 mM phenylmethylsulfonyl fluoride/40 pM ^{125}I-labeled CCK-8 as radioligand. Equilibrium binding occurred after incubation for 80 min at 37°C. Cell monolayers were subsequently washed three times, and bound radioactivity was quantified after cell hydrolysis in 1 M NaOH. Radioligand saturation experiments were done in an analogous manner over a range from 2.5 to 1000 pM ^{125}I-labeled CCK-8 (NEN) with nondisplaceable binding in the presence of excess unlabeled competitor assessed in parallel wells.

Binding parameters were also measured in isolated plasma membranes from COS-7 cells transfected with clone GR-1 DNA. Binding was performed for 60 min at 22°C. Separation of bound and free radioligand was achieved by receptor-binding filter-mat filtration, as described (21).

Competition and saturation binding data were analyzed by using computerized nonlinear curve fitting (22).

Affinity Cross-linking. For affinity labeling the recombinant receptor, COS-7 cell membranes from GR-1-transfected cells were allowed to bind ^{125}I-labeled D-Tyr-Gly-[(Ahx^{28,31})-CCK-26–33] for 60 min at 22°C, followed by separation of bound from free radioligand by centrifugation (15). The membrane pellet was cross-linked by using 100 μM disuccinimidyl suberate at 4°C for 5 min, with excess cross-linker quenched with Tris buffer. Labeled membranes were separated on a 10% SDS/PAGE gel and visualized by autoradiography.

Measurement of $[Ca^{2+}]_i$. Forty-eight hours after transfection, COS-7 cells were loaded with the Ca^{2+} fluorophore fura-2 in modified Krebs–Ringer bicarbonate buffer. Fluorescence changes after stimulation of cells with 10^{-6} M gastrin were measured as described (23).

Measurement of Inositol Phospholipids. COS-7 cells transfected with GR-1 were cultured for 24 hr in inositol-free Dulbecco's modified essential medium (GIBCO)/*myo*-[^{3}H]inositol at 10 μCi/ml (American Radioligand) before analysis. After 1-hr equilibration in modified KRB buffer (see above), the cells were stimulated with 10^{-6} M gastrin for 10 sec and harvested in methanol/HCl. The aqueous phase was extracted with chloroform, lyophilized, and analyzed by strong anion-exchange HPLC (24).

RESULTS AND DISCUSSION

We have isolated a gastrin receptor clone from a canine parietal cell cDNA expression library. The cDNA has an open reading frame encoding a 453-amino acid protein, with a predicted M_r of 48,518 (Fig. 1). Hydropathy analysis (19) reveals seven hydrophobic segments, corresponding to the transmembrane domains characteristic of the G protein-coupled receptor family. Examination of other regions of the deduced amino acid sequence reveals a large number of the amino acid "signatures" present in the vast majority of known G protein-coupled receptors (L.F.K., unpublished data). The amino terminus of the cloned receptor lacks a signal sequence; however, it includes three potential asparagine-linked glycosylation sites (N-X-S/T) at amino acid positions 7, 30, and 36. There are two cysteine residues (C-127 and C-206), which may be involved in an intrachain disulfide bond similar to that found in rhodopsin (26). The third cytoplasmic loop and the carboxyl terminus are rich in threonine and serine residues, which may serve as potential sites of phosphorylation analogous to those found in rhodopsin and in the β_2-adrenergic receptor (27).

The gastrin receptor shares significant amino acid identity with the β-adrenergic G protein-coupled receptor family (Fig. 2). Translated clone GR-1 protein has the highest degree of amino acid identity (35%) with the neuropeptide Y-Y1 receptor FC-5 (28) (Claes Wahlestedt, personal communication). Additional receptors that have high amino acid identity with the gastrin receptor include the human dopamine D_4

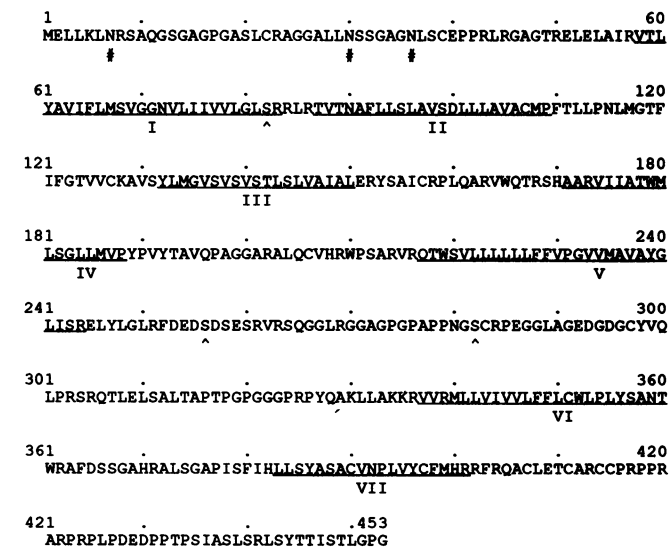

FIG. 1. Deduced amino acid sequence of the canine parietal cell gastrin receptor. Putative transmembrane domains of the gastrin receptor are underlined and indicated by Roman numerals. Consensus N-linked glycosylation sites are indicated by #. Potential protein kinase C or casein kinase II phosphorylation sites, as predicted by patterns found in the Prosite data base (25), are indicated by ^.

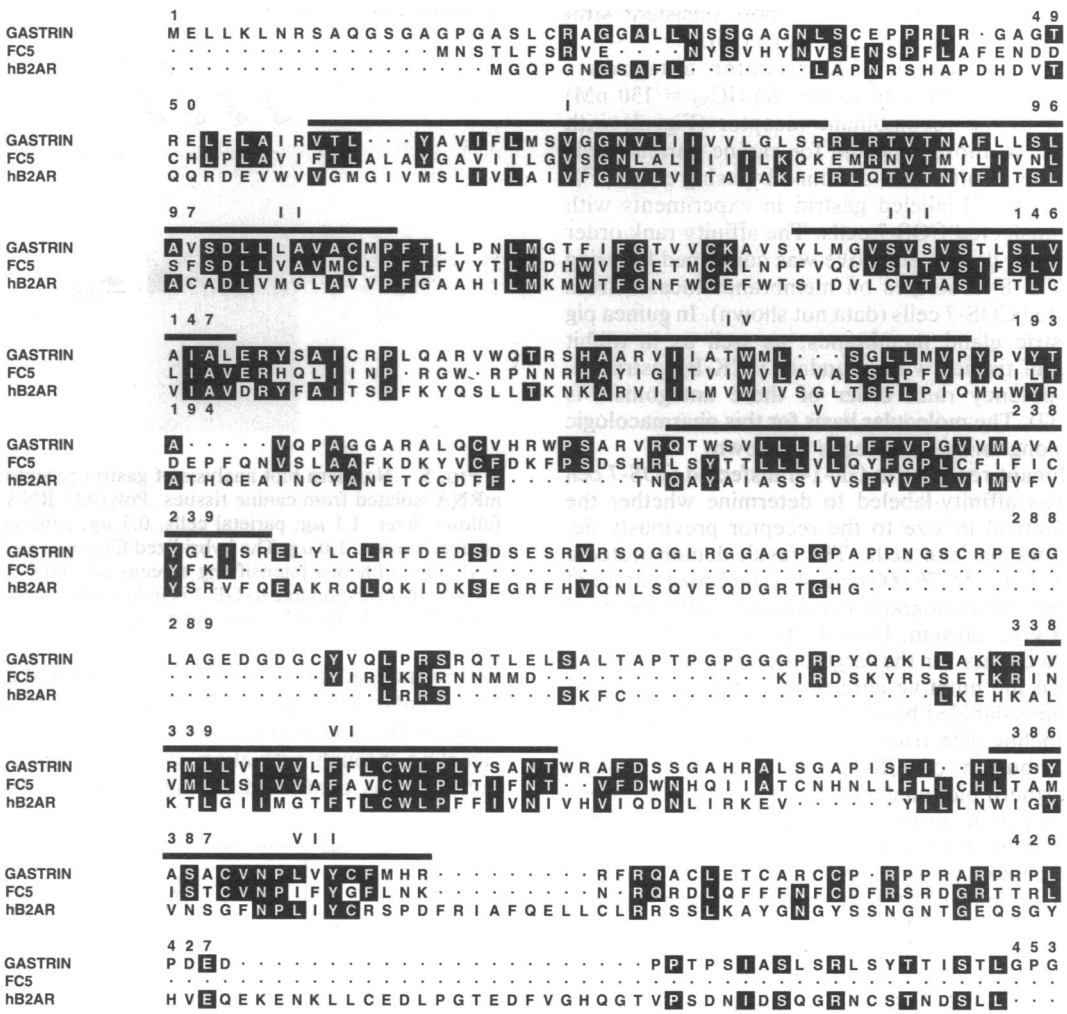

FIG. 2. Primary structure of the canine parietal cell gastrin receptor and alignment with known G protein-coupled receptors. Shaded amino acids are identical in at least two of the three receptors. Bars over sequences represent transmembrane segments predicted by the Klein, Kanehisa, DeLisi algorithm. Numbering corresponds to amino acids in the gastrin receptor. FC5, neuropeptide Y-Y1 receptor; hB2AR, human β_2-adrenergic receptor.

receptor (28%), *Drosophila melanogaster* 5-hydroxytryptamine receptor (27%), and the human β_2-adrenergic receptor (26%). Phylogenetic analysis reveals that GR-1 protein defines another branch of the neuropeptide ligand class of G protein-coupled receptors (L.F.K., data not shown). The significant amino acid identity of the gastrin receptor with both peptide hormone and biogenic amine receptors is noteworthy.

Pharmacologic characterization of receptors expressed on COS-7 cells transfected with GR-1 DNA demonstrated binding specificity typical of CCK-B/gastrin receptors (Fig. 3). ^{125}I-labeled CCK-8 binding revealed a single homogeneous class of receptors, as confirmed by a Hill coefficient near unity (0.93 ± 0.07) for homologous competition with unlabeled CCK. Other structurally related members of this hormone family competed for binding of ^{125}I-labeled CCK-8 in a concentration-dependent manner. The calculated IC_{50} values for CCK-8, gastrin, and CCK-8-desulfate were 0.09 nM, 0.26 nM, and 1.4 nM, respectively. This result is consistent with the radioligand affinity, as derived from saturation binding experiments with GR-1-transfected COS-7 cells (K_d = 0.08 nM) and canine parietal cells (K_d = 0.27 nM). Comparable results were obtained by using isolated membranes prepared from transiently transfected COS-7 cells (IC_{50} values for CCK-8, gastrin, and CCK-8-desulfate were 0.08 nM, 0.4 nM, and 1.5 nM, respectively). The same affinity rank order for all tested agonists was confirmed by using ^{125}I-labeled gastrin (Amersham) as the radioligand (data not shown). All of the

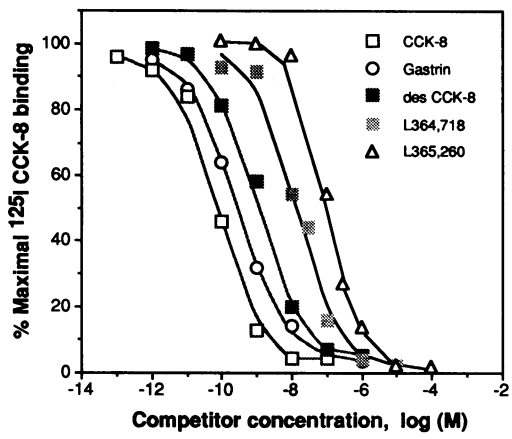

FIG. 3. ^{125}I-labeled CCK-8 receptor binding. Binding of ^{125}I-labeled CCK-8 to COS-7 cells transiently transfected with GR-1 is shown in the presence of increased concentrations of CCK-8, gastrin I, CCK-8-desulfate (des CCK-8), L364,718, and L365,260. Each point represents the mean of three experiments. Maximum binding in the absence of competitor (100%) was 1.3 fmol per well. Untransfected cells showed no saturable binding.

agonist-binding data presented above are consistent with results obtained with canine parietal cells (10).

The nonpeptide gastrin/CCK receptor antagonists L-364,718 (IC_{50} = 19 nM) and L-365,260 (IC_{50} = 130 nM) (Glaxo) bound to the recombinant receptor (Fig. 3) with affinities similar to those reported for canine parietal cells (29). Comparable results were obtained by using either ^{125}I-labeled CCK-8 or ^{125}I-labeled gastrin in experiments with intact GR-1-transfected COS-7 cells. The affinity rank order for the antagonists described above was confirmed by using ^{125}I-labeled CCK-8 in studies on membranes located from GR-1-transfected COS-7 cells (data not shown). In guinea pig brain and gastric gland membranes, as well as in rabbit parietal cells, all tissues with abundant CCK-B/gastrin receptors, the potency rank order of these antagonists is reversed (30–32). The molecular basis for this pharmacologic divergence among animal species is unknown.

The recombinant receptor in GR-1-transfected COS-7 cell membranes was affinity-labeled to determine whether the receptor is identical in size to the receptor previously described on canine parietal cells. The result demonstrated a protein centered at $\approx M_r$ 76,000 (Fig. 4). The broad nature of this band in the autoradiograph is consistent with the band representing a glycoprotein. Indeed, the carbohydrate moiety probably accounts for the large difference in molecular weight between the putative core protein and the apparent size of the affinity-labeled band. The size agrees with previous affinity-labeling data from canine parietal cells (3).

Tissue distribution of GR-1 was assessed by high-stringency Northern blot analysis (Fig. 5). Receptor mRNA was detected in gastric parietal cells, pancreas, and cerebral cortex. All of these tissues are reported to have gastrin/CCK-B type receptors (9, 33). The canine pancreas is notable for substantial amounts of CCK-B/gastrin receptors relative to other species.

Gastrin binding to the recombinant receptor elicits a typical biological response in GR-1-transfected COS-7 cells, providing further confirmation that GR-1 encodes a functional receptor protein. Gastrin (10^{-6} M) triggered a marked increase in $[Ca^{2+}]_i$ from 46.5 ± 6.9 nM to 142.4 ± 16.2 nM (n = 3, $P < 0.05$), whereas untransfected cells did not respond (Fig. 6*A*). After chelation of extracellular calcium by EGTA (2.5 mM), gastrin transiently increased $[Ca^{2+}]_i$ from 17.6 ± 1.0 to 88.5 ± 11.6 nM (n = 3, $P < 0.05$), suggesting that the

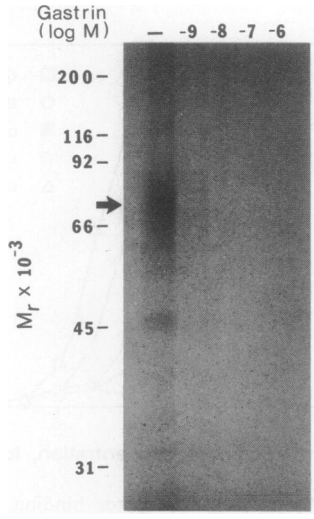

FIG. 4. Affinity labeling of the canine parietal cell gastrin receptor on transfected COS-7 cells by using bifunctional chemical cross-linking. A broad band, centered at M_r 76,000, is indicated by the arrow; labeling was fully abolished by gastrin in a concentration-dependent manner. Results are typical of four similar experiments.

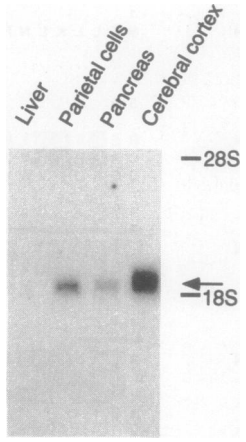

FIG. 5. Northern blot analysis of gastrin receptor transcripts in mRNA isolated from canine tissues. Poly(A)$^+$ RNA was loaded as follows: liver, 1.1 μg; parietal cells, 0.3 μg; pancreas, 0.5 μg; and cerebral cortex, 1.0 μg. The hybridized filter was exposed to Kodak XAR film with two intensifying screens at −80°C for 2 days. The transcript corresponding to GR-1 is indicated by an arrow. Positions of the 28S and 18S rRNA are indicated.

gastrin-induced increase in $[Ca^{2+}]_i$ originated primarily from intracellular Ca^{2+} pools. The pattern of $[Ca^{2+}]_i$ response suggests that the recombinant receptor triggers intracellular signaling through activation of phospholipase C. This hypothesis was confirmed by measurement of inositol phos-

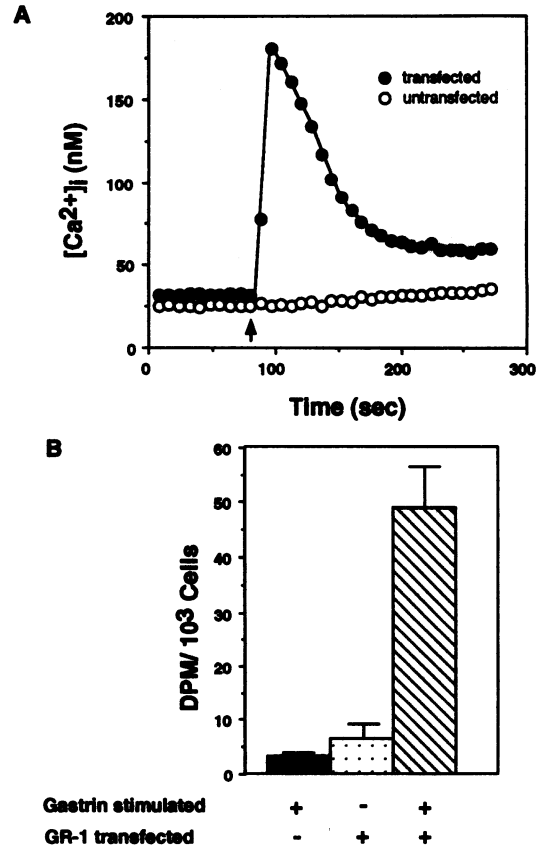

FIG. 6. Second-messenger signaling in transfected COS-7 cells after stimulation by gastrin I (1 μM). (*A*) In fura-2-loaded COS-7 cells, $[Ca^{2+}]_i$ was determined from fluorescence emission ratios at 340/380 nm. The arrow indicates the addition of gastrin I. Data shown are representative of three experiments. (*B*) Inositol 1,4,5-trisphosphate measurements (mean ± SEM of n = 3) in untransfected and GR-1-transfected COS-7 cells. DPM, dpm.

pholipid metabolites in GR-1-transfected COS-7 cells 10 sec after gastrin stimulation (Fig. 6*B*). The time point was chosen to just precede the gastrin-induced $[Ca^{2+}]_i$ peak that occurred at 16 sec. Gastrin (10^{-6} M) increased the level of inositol 1,4,5-trisphosphate by 741 ± 115% over control, unstimulated GR-1-transfected COS-7 cells ($n = 3$, $P < 0.01$). Inositol 1,3,4,5-tetrakisphosphate which may, together with inositol 1,4,5-trisphosphate, modulate intracellular calcium levels, also increased by 272 ± 15% over control ($n = 3$, $P < 0.01$). Our results agree with previous reports of the second-messenger pathways linked to the native parietal cell gastrin receptor (4, 5, 34).

Several lines of evidence suggest that the cloned cDNA encodes the parietal cell gastrin receptor. The recombinant receptor was isolated from a library produced from a highly enriched preparation of parietal cells. RNA blot analysis of mRNA from isolated parietal cells shows that the GR-1 transcript is highly expressed in these cells. The binding of agonists and antagonists, the affinity labeling, and the signal-transduction data are all indistinguishable from previous observations with canine parietal cells, which have been shown to have a single homogeneous class of receptors (3–5, 10, 34).

The abundance of the GR-1 transcripts, in both canine cortex and parietal cells (Fig. 5), supports the view that CCK-B and gastrin receptors are highly homologous, if not identical. The question of CCK-B/gastrin receptor identity will be answered conclusively only after the nucleotide sequences encoding both the parietal cell and brain receptors are available in the same species.

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