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Glucagon-Like Peptide-1 Does Not Mediate Amylase Release From AR42J Cells

JIE ZHOU,[1] CHAHRZAD MONTROSE-RAFIZADEH,[1] ANDRZEJ M. JANCZEWSKI,[2] MARCO A. PINEYRO,[1] STEVEN J. SOLLOTT,[2] YIHONG WANG,[1] AND JOSEPHINE M. EGAN[1]*

[1]*Diabetes Section, Gerontology Research Center, National Institute on Aging, National Instituites of Health, Baltimore, Maryland*

[2]*Laboratory of Cardiovascular Sciences, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, Maryland*

In this study, AR42J pancreatic acinar cells were used to investigate if glucagon-like peptide-1 (GLP-1) or glucagon might influence amylase release and acinar cell function. We first confirmed the presence of GLP-1 receptors on AR42J cells by reverse trasncriptase-polymerase chain reaction (RT-PCR), Western blotting, and partial sequencing analysis. While cholecystokinin (CCK) increased amylase release from AR42J cells, GLP-1, alone or in the presence of CCK, had no effect on amylase release but both CCK and GLP-1 increased intracellular calcium. Similar to GLP-1, glucagon increased both cyclic adenosine monophosphate (cAMP) and intracellular calcium in AR42J cells but it actually decreased CCK-mediated amylase release ($n = 20$, $P < 0.01$). CCK stimulation resulted in an increase in tyrosine phosphorylation of several cellular proteins, unlike GLP-1 treatment, where no such increased phosphorylation was seen. Instead, GLP-1 decreased such protein phosphorylations. Genestein blocked CCK-induced phosphorylation events and amylase secretion while vanadate increased amylase secretion. These results provide evidence that tyrosine phosphorylation is necessary for amylase release and that signaling through GLP-1 receptors does not mediate amylase release in AR42J cells. J. Cell. Physiol. 181:470–478, 1999.

Recent work suggests that pancreatic exocrine and endocrine cells are derived from common endodermal precursor cells (Teitelman, 1996). It therefore seems possible that both acinar and islet cells may share common characteristics such as regulation of hormone secretion. It is also possible, as has been suggested, that islet hormones influence acinar function because venous drainage from islets flows directly through the acini and they are therefore subjected to higher levels of islet hormones than what is present in blood (Park et al., 1993). Glucagon-like peptide-1 (GLP-1) is a peptide that is released from the gut in response to food and that augments insulin release. Exendin-4, a peptide produced in saliva by the Gila monster lizard, shares a 52% amino acid homology with GLP-1 and is an insulinotropic agent that acts through the GLP-1 receptor (Goke et al., 1993). It has been reported that exendin-4 potentiates cholecystokinin (CCK)-induced amylase release from rat pancreatic acini (Malhotra et al., 1992). Because GLP-1 receptors are present in islets and in the gut, both of which are of endodermal origin, it raises the possibility that GLP-1 receptors may be present in acinar tissue and would be subjected to the same concentrations of GLP-1 as islets. GLP-1 is released from the gut in response to food and carbohydrate ingestion and CCK is released when fat and protein enter the duodenum resulting in both hormones being elevated within the same time interval after eating. Because CCK is the major stimulus to amylase release from the exocrine pancreas, we used it here to compare the effect of GLP-1 on acinar function. We used the AR42J cells (Christophe, 1994), which are derived from a rat pancreatic exocrine tumor, as a model of acinar tissue. We then looked closely at some aspects of the signal transduction system through which GLP-1 is already known to work in beta cells (Goke et al., 1993; Holz et al., 1995; Yada et al., 1993).

We show that GLP-1 receptors, which are G-protein coupled, are present in AR42J cells. When the cells were stimulated by GLP-1 there was a rise in intracellular cyclic adenosine monophosphate (cAMP) and calcium, which in beta cells augment insulin secretion. However, basal or CCK-stimulated amylase secretion was not increased by GLP-1. GLP-1 did not appear to increase intracellular tyrosine phosphorylations, which play a major role in CCK-stimulated amylase secretion. Glucagon, which also increased intracellular cAMP and calcium, did not increase amylase release or

*Correspondence to: J.M. Egan, Diabetes Section, Box #23, GRC/NIA, 5600 Nathan Shock Drive, Baltimore, MD 21224.

E-mail: egan@vax.grc.nia.nih.gov

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tyrosine phosphorylations. Therefore, amylase and insulin secretion are not similarly regulated.

MATERIALS AND METHODS

Materials and cell culture

GLP-1, glucagon, exendin-4, and exendin 9–39 (a GLP-1 receptor antagonist) were obtained from Bachem (Torrance CA). CCK, insulin, Tween-20, Triton X-100 phenylmethylsulfonyl fluoride (PMSF), genistein, Na-orthovanadate and isobutyl methylxanthine (IBMX) were obtained from Sigma Chemical Co. (St. Louis, MO). The rat pancreatic cell line, AR42J, was from American Type Culture Collection (Rockville, MD). Monoclonal and polyclonal antityrosine antibodies were purchased from Upstate Biotechnology, Inc (Lake Placid, NY). Horseradish peroxidase-linked donkey anti-rabbit second antibody, cAMP [^{3}H] assay kits and enhanced chemiluminescent detection system (ECL) were from Amersham Life Science (Arlinton Heights, IL). The polyvinylidene difluoride (PVDF) membrane and precast sodium dodecyl sulfate (SDS)-polyacrylamide gels were from NOVEX (San Diego, CA). Benzamidine, leupeptin, and aprotinin were obtained from ICN (Costa Mesa, CA). Protein assay reagent was from Bio-Rad Laboratories (Hercules, CA). Protein G Plus/protein A agarose beads from Oncogene Science, Inc. (Uniondale, NY). Maloney murine leukemia virus reverse transcriptase was from Bethesda Research Laboratories (Gaithersburg, MD). Random hexanucleotide primer was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Sequenase 2.0 kit was from United States Biochemicals (Cleveland, OH).

AR42J cells were maintained in Dulbecco's modified Eagle medium (DMEM: Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Cells from passage 23–36 were used throughout this study. Cells were routinely plated at about 10^5 cells/ml onto 12-well cluster dishes and incubated in a humidified incubator at 37°C with 95% air and 5% CO_2. Because AR42J cells responded poorly to CCK, we routinely incubated the cells with 10 nM dexamethasone for 48 h before use as this was known to induce CCK responsivity in a concentration-dependent manner (Logsdon et al., 1987).

Reverse transcript-polymerase chain reaction and partially sequence analysis of the GLP-1 receptor. cDNA was synthesised from total cellular RNA using Maloney murine leukemia virus reverse transcriptase (RT) and random hexanucleotide primer. Polymerase chain reaction (PCR) amplification (30 cycles) was performed (Saiki et al., 1988) from first-strand cDNA using recombinant *Taq* DNA polymerase (Amplitaq, Perkin-Elmer, Cetus). Oligonucleotide primers were on 5′- and 3′-end of the pancreatic GLP-1 receptor sequence (Thorens, 1992), $^{5'}$ACAGGTCTCTTCTGCAACC$^{3'}$ and $^{5'}$AAGATGACTTCATGCGTGCC$^{3'}$, respectively. PCR products were then resolved on a 1% agarose gel and visualized using ethidium bromide. The PCR products were subcloned in pBluescript vector and sequenced using the chain termination technique and Sequenase 2.0 kit. The specificity of the PCR product was also determined by the *Bst*X1 restriction enzyme.

Western blotting of the GLP-1 receptor

AR42J cells were grown in 60-mm^2 dishes as described above. When the cells reached 80% confluency, they were washed twice with Krebs-Ringer balanced buffer (KRBB) containing 115 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, and 25 mM HEPES and frozen in liquid nitrogen. The frozen cells were scraped and solubilized in SDS-polyacrylamide gel sample buffer at 70°C for 10 min, eluted with mini-resin column, and subjected to 4%–12% SDS-polyacrylamide gel. After the gel was electrotransfered to PVDF membrane, the blot was blocked with 5% nonfat milk in TBST buffer (20 mM Tris-HCl [pH 7.5], 137 mM NaCl and 0.1% Tween 20) for 1 h at room temperature, and then incubated with antibody to the N-terminus of the GLP-1 receptor (gift from Dr. Joel Habener, Massuchusetts General Hospital, MA) (Heller et al., 1997) at 1:1500 for 1 h at room temperature. PVDF membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antisera for 1 h at room temperature. After a series of washes in TBST, the blots were developed using the ECL detection system. Autoradiographs were quantified using ImageQuantTM software (version 3.3) on a Molecular Dynamics laser densitometer. In this experiment, the stably transfected pancreatic GLP-1 receptor cDNA cell line, CHO/GLPR, was used as a positive control for the presence of the GLP-1 receptor, and CHO/Neo cell line was used as negative control (Montrose-Rafizadeh et al., 1997). We also used preimmune serum to blot under the same conditions and no specific GLP-1 receptor bands can be found. Aliquots (10 μl) of solubilized cell lysates were used to determine protein concentration, which was estimated by the Bradford method (Bradford, 1976).

Measurement of cAMP

Cells were grown in 12-well dishes and treated with hormones and reagents ± IBMX for 1 h. They were then washed three times in ice-cold PBS and lysed with 1 ml ice-cold 0.6 M perchloric acid. The lysates (950 μl) were transfered to microcentrifuge tubes and the pH adjusted to 7.0 using 5 M K_2CO_3. After centrifugation for 5 min at 10^4 rpm, the supernatant was vacuum dried, and then recovered in 200 μl Tris/EDTA buffer. After addition of 0.15 mM Na_2CO_3 (50 μl) and 0.15 mM $ZnSO_4$ (50 μl), followed by incubation for 15 min on ice, the salt precipitate was removed by centrifugation for 15 min at 3.5×10^3 rpm and 50 μl of supernatant was assayed using a cAMP [^{3}H] assay kit, (Steiner et al., 1972). At the same time we used GLP-1– (10 nM) or exendin-4– (0.1 nM) treated AR42J cells from 0 min to 30 min and collected the samples for total intracellular cAMP assay. Cellular protein was measured by the Bradford method using bovine γ-globulin as standard (Bradford, 1976).

Measurement of intracellular calcium, $[Ca^{2+}]_i$, in single AR42J cells

AR42J cells were loaded with the fluorescent Ca^{2+} probe, indo-1 acetoxymethyl ester (indo-1/AM). The loading solution consisted of 50 μg of indo-1/AM (Molecular Probes Inc.), 30 μl of dimethyl sulphoxide

(DMSO) and 5 μl of 25% (w/w in DMSO) Pluronic F-127 (BASF Wyandott Corp.) This mixture was added to 2.0 ml of cells in Hank's balance salt solution (final indo-1 concentration of 25 μM) and gently mixed on a shaking plate for 1h. The cells were then centrifuged at 400*g* for 60 sec, resuspended in standard bathing solution, consisting of 137 mM NaCl; 5 mM KCl; 1.3 mM $MgSO_4$; 5 mM $CaCl_2$; 20 mM HEPES; pH adjusted with NaOH to 7.4. Both loading with indo-1/AM and the experiments were carried out at room temperature (22°–24°C). The cell suspension was placed in a chamber on the stage of an inverted fluorescence microscope (Spurgeon et al., 1990). The emission field was restricted to a single cell. Indo-1 was excited at 350 ± 5 nm every 5 ms, and the fluorescence emission was split into wavelength bands of 410 ± 5 and 490 ± 5 nm, respectively. The 410:490 fluorescence ratio (ratio F410/F490), corrected for autofluorescence, was used as an index of $[Ca^{2+}]_i$, using the methodology described in detail previously (Spurgeon et al., 1990). The cell autofluorescence was assessed in a large number of indo-1 nonloaded cells from the same batch. In a typical experiment, the standard bathing solution was exchanged rapidly (< 200 ms) with one of the test solutions injected from a micropipette placed in close vicinity to the cell (Janczewski and Lakatta, 1993,). Routinely, the cells were exposed to the test solution for 240–300 sec. Thereafter, the test solution was washed out, while $[Ca^{2+}]_i$ was monitored for an additional 120–180 sec. The test solutions were prepared just prior to the experiment by adding the hormones in standard bathing solution.

Tyrosine phosphorylation studies

AR42J cells were preincubated in KRBB for 2 h at 37°C. Then the solution was removed and fresh KRBB was added, followed by placing the cells on a 37°C hot plate for 5 min. After addition of various reagents (see Fig. 7) for 5 min, the reaction was terminated by submersion of the dishes in liquid nitrogen. The frozen cells were scraped and lysed in buffer containing 20 mM Tris-HCl: pH 8.0, 137 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.2 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM Na-orthovanadate, 1 mM benzamidine. Insoluble material was removed by centrifugation at 15,000*g* for 15 min and the supernatant was collected for immunoprecipitation with monoclonal antityrosine antibody. The immunoprecipitation pellet was collected with protein G Plus/protein A agarose beads and separated by electrophoresis in 4%–20% SDS-polyacrylamide gels under reducing conditions followed by electrotransfer to PVDF membrane and immunoblotting with a polyclonal antiphosphotyrosine antibody. The blots were developed using the ECL detection system. Total protein content in the clarified cell lysates was assayed using the Bradford method (Bradford, 1976).

Amylase assay

For amylase secretion, cells were washed free of medium with phosphate buffered saline. Incubation was then carried out in no-serum DMEM containing 15 mM HEPES, 0.2% bovine serum albumin, and 0.01% soybean trypsin inhibitor. The hormones and reagents of interest were added for 50 min at 37°C. The incubation medium was then immediately removed for amylase determination and the cells were again washed with ice-cold PBS. Lysate buffer containing 130 mM Tris-HCl, 10 mM $CaCl_2$, 75 mM NaCl, and 0.2% Triton X-100 (pH 8.0) was added to the cells and the lysates were then collected for total amylase activity (Ceska et al., 1969). The released amylase was expressed as the percentage of the total amylase activity of the cells.

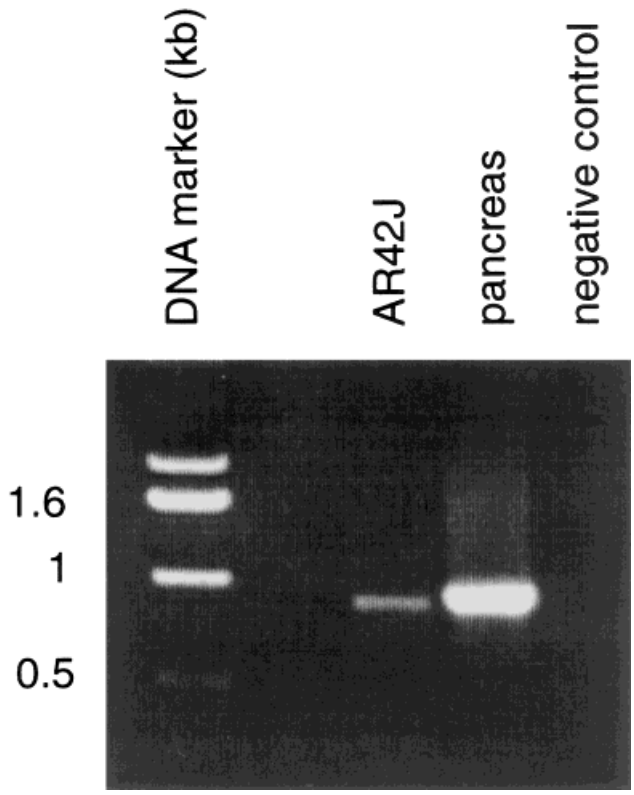

Fig. 1. Reverse transcript-polymerase chain reaction (RT-PCR) of glucagon-like peptide-1 (GLP-1) receptors in AR42J cells and rat pancreas. cDNA was amplified for 30 cycles using primers in the 5′ and 3′ end of the rat pancreatic GLP-1 receptor. PCR products were resolved on a 1% agarose gel and visualized using ethidium bromide. From left to right: lane 1, DNA marker; lane 2, blank; lane 3, AR42J cells; lane 4, rat pancreas; lane 5, water control. In lanes 3 and 4 we see the expected 928 bp band, corresponding to the GLP-1 receptor.

Statistical analysis

Where applicable, results were expressed as the mean ± SEM and subjected to unpaired Student's *t* test. Within group comparisons were analyzed using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

GLP-1 receptor by RT-PCR and partial sequence analysis

The presence of GLP-1 receptor mRNA was detected in AR42J cells by using RT-PCR. Figure 1 shows that by using primers identical to the known pancreatic GLP-1 receptor sequence (Thorens, 1992), we can detect a PCR product of predicted size 928 bp (Egan et al., 1994) in AR42J cells and rat pancreas, but not in PCR of water control. The absence of any genomic DNA

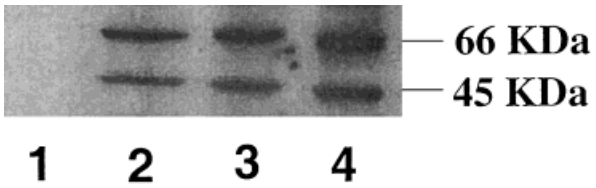

Fig. 2. Western blot analysis of glucagon-like peptide-1 (GLP-1) receptor expression in CHO/Neo cell line (lane 1), AR42J (lane 2, 3) and CHO/GLPR (CHO cells overexpressing the GLP-1 receptor) cell line (lane 4). Cells were solubilized and GLP-1 receptors were detected after Western blotting with antibody to the amino-terminus of the GLP-1 receptor. The positions of the molecular markers, in kilodalton (KDa), are on the right. The 65- and 45-KDa bands have been shown to correspond to the mature and core-glycosylated GLP-1 receptors, respectively (Montrose-Rafizadeh et al., 1997a).

contamination is established as our primers span intronic sequences that would yield PCR bands of 1.8 kb (data not shown). We did not observe any additional bands corresponding to contaminating genomic DNA PCR in our PCR reactions. The PCR reactions were cloned, partially sequenced, and identified to be the beta cell GLP-1 receptor (Egan et al., 1994).

Immunoblot of GLP-1 receptor

Using an antibody against the N-terminal region of the GLP-1 receptor, we obtained specific bands at 65 and 45 KDa in the positive control cells, the stable transfected pancreatic GLP-1 receptor cDNA cell line, CHO/GLPR cells (Montrose-Rafizadeh et al., 1997), and in the AR42J cells, but not in the negative control CHO/Neo cell line. These have been shown to correspond to the mature and core-glycosylated GLP-1 receptors, respectively (Fig. 2). Preimmune serum was used in our experiment and no specific GLP-1 receptor bands were found.

cAMP levels

Ten minutes exposure of AR42J cells to GLP-1(10 nM) caused a 1.5-fold increase in intracellular cAMP levels while exendin-4 (0.1 nM) increased 3-fold at the same time point. By 20 min, with both peptides, cAMP was again back to basal levels (data not shown). Glucagon (10 nM), on the other hand, caused a 2-fold increase in cAMP levels in the presence and absence of CCK (Fig. 3), and levels remained elevated even after 1 h of treatment.

$[Ca^{2+}]_i$ responses to CCK in AR42J cells

Under the present experimental conditions, AR42J cells responded to 1 nM CCK with a transient increase $[Ca^{2+}]_i$ in most AR42J cells (85%, n = 35). Figure 4A shows a representative example of the CCK-induced $[Ca^{2+}]_i$ transients, which commenced after 5–25 sec after exposure to CCK and peaked within the next 5–15 sec. The peak $[Ca^{2+}]_i$, assessed from the peak indo-1 fluorescence ratio (IFR), exceeded the resting IFR by 2.5–3.5-fold. Relaxation of the $[Ca^{2+}]_i$ transients commenced immediately after the peak and usually consisted of an initial rapid phase, followed by a plateau, and a slower final phase. After the $[Ca^{2+}]_i$ transient, baseline $[Ca^{2+}]_i$ decreased below the level of resting $[Ca^{2+}]_i$, measured prior to the exposure to CCK (Fig. 4A). During the subsequent rest, baseline[$Ca^{2+}]_i$ showed a gradual increase but usually did not fully recover to the control levels within 10 min. $[Ca^{2+}]_i$ transients elicited by a repeated exposure to CCK prior to a full recovery of resting $[Ca^{2+}]_i$ were reduced by 30%–40% versus the preceeding $[Ca^{2+}]_i$ (data not shown for repeated exposures to CCK under control conditions, but cf. Fig. 5B,C).

The CCK-induced $[Ca^{2+}]_i$ transients were almost completely abolished in cells pretreated with 10 μM

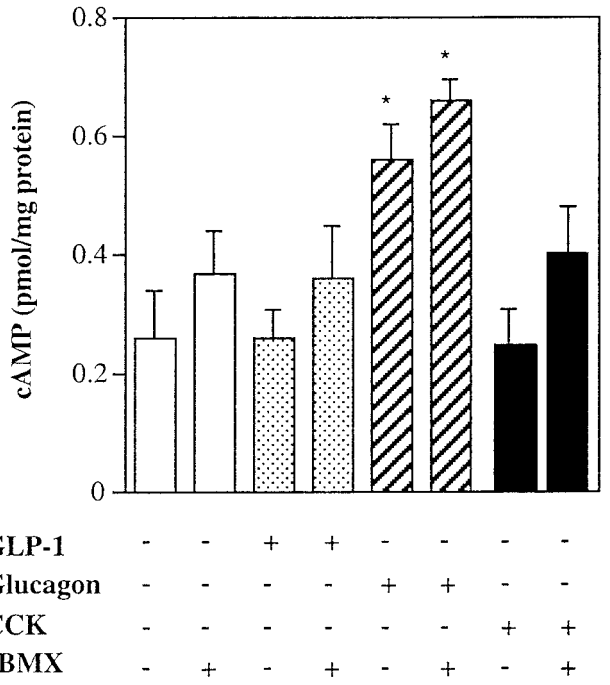

Fig. 3. Effects of glucagon-like peptide-1 (GLP-1) (10 nM), glucagon (10 nM), and cholecystokinin (CCK) (1 nM) treatment ± and isobutyl methylxanthine (IBMX; 100 nM) for 1 h on intracellular cyclic andenosine monophosphage (cAMP) levels in AR42J cells. Results are mean ± SEM of three experiments, * $P < 0.05$.

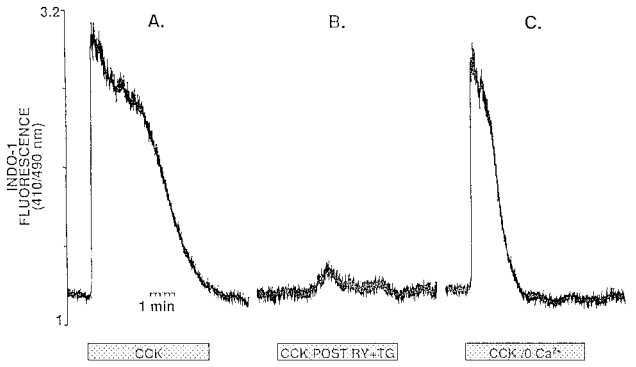

Fig. 4. Effects of cholecystokinin (CCK) on intracellular free $[Ca^{2+}]_i$ in single AR42J cells. The bar indicates the time of exposure to 10 nM CCK in three different cells. **A:** Typical $[Ca^{2+}]_i$ response observed in at least 85% of the cells. **B:** the $[Ca^{2+}]_i$ response to CCK is almost completely abolished following 60 min exposure to 10 μM ryanodine (RY) and 500 nM thapsigargin (TG). **C:** the $[Ca^{2+}]_i$ transient is abbreviated by reduction of extracellular Ca^{2+} during exposure to CCK.

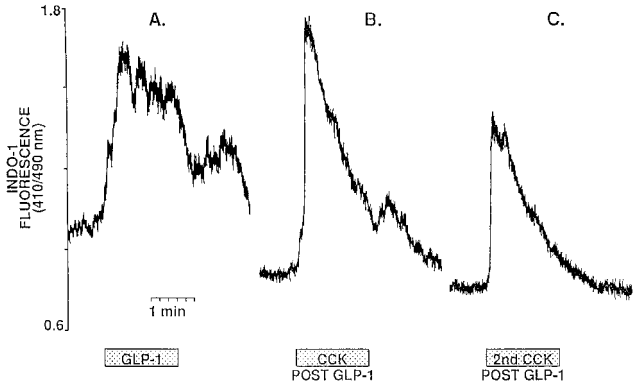

Fig. 5. Effects of glucagon-like peptide-1 (GLP-1) on $[Ca^{2+}]_i$ and cholecystokinin (CCK)-induced $[Ca^{2+}]_i$ transients in single AR42J cells. The same cell was studied in **A-C. A:** exposure to 1 nM GLP-1 induced small, slow, prolonged $[Ca^{2+}]_i$ transients in approximately 50% of AR42J cells and reduced the amplitude of the subsequent exposure to 10 nM CCK. **B:** (3.2 vs. 1.7. ratio 410/490 nm. **C:** amplitude of the $[Ca^{2+}]_i$ transient is further reduced in response to a second exposure to CCK applied in < 10 min.

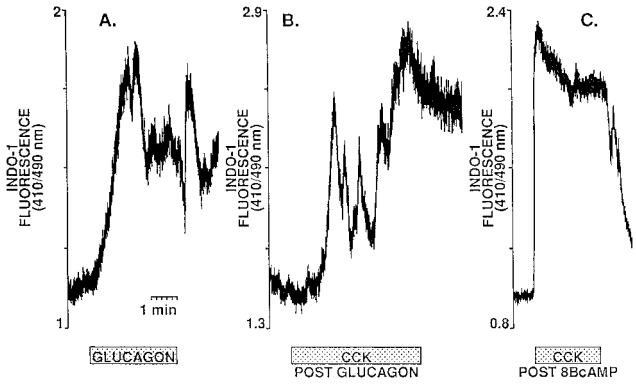

Fig. 6. Effects of glucagon and 8-bromo-cAMP (8-Br-cAMP) on $[Ca^{2+}]_i$ in single AR42J cells. **A:** glucagon (10 nM) induced slow, small, prolonged $[Ca^{2+}]_i$ transients in approximately 70% of cells. **B:** In cells treated with 10 nM glucagon for 3–10 min, the subsequent $[Ca^{2+}]_i$ transients induced by 10 nM CCK showed a slow rate of rise as well as a prolonged relaxation phase. **C:** Brief (1–5 min) exposures to 100 nM 8-Br-cAMP attenuated the relaxation of CCK-induced $[Ca^{2+}]_i$ transients.

ryanodine and 500 μM thapsigargin (Fig. 4B; n = 7). These results support the concept that in acinar cells, the endoplasmic reticulum (ER) is the major source of changes in $[Ca^{2+}]_i$ induced by CCK (Muallem et al., 1988; Ochs et al., 1983). Consistent with this idea, exposures to CCK added to a nominally Ca^{2+}-free superfusing solution (Fig. 4C) did not appreciably affect the rate of rise or the magnitude of the $[Ca^{2+}]_i$ transients (n = 5). However, as shown in Figure 5C, a reduction in the extracellular Ca^{2+} shortened the duration of the $[Ca^{2+}]_i$ transients, suggesting, as shown before (Muallem et al., 1988; Ochs et al., 1983), that extracellular Ca^{2+} may play a role in sustaining the delayed component of the $[Ca^{2+}]_i$ transient initiated by CCK-induced ER Ca^{2+} release. NaF did not mimic CCK's effects on intracellular calcium. Done numerous times, we could not appreciate any alterations in $[Ca^{2+}]_i$ in AR42J cells in response to NaF using the same cell preparations that consistently gave clear CCK responses (data not shown).

$[Ca^{2+}]_i$ responses to GLP-1 in AR42J cells

Exposure to GLP-1, of which 1 nM achieved maximum amplitudes, elicited $[Ca^{2+}]_i$ responses in approximately 50% (n = 27) of AR42J cells. The GLP-1-induced transients (Fig. 5A) displayed considerable variability, but usually developed at a slower rate and attained smaller amplitudes (1.5–2.5-fold increase over resting IFR) than the $[Ca^{2+}]_i$ responses to CCK. Moreover, the GLP-1-induced [$Ca^{2+}]_i$ transients relaxed at a slower rate than those induced by CCK (Figs. 5A vs. 4A and 5B, C). Figure 5B shows the effects on $[Ca^{2+}]_i$ of CCK applied < 10 min after an exposure to GLP-1 in the same cell. In experiments of this type, the CCK-induced transients retained their characteristic configuration (as in Fig. 4A) but reached smaller amplitudes (3.2 vs. 1.7 IFR). The latter effect can be attributed, at least in part, to a reduction of the [$Ca^{2+}]_i$ content, indicated by a reduction in the baseline IFR, and/or partial depletion of the ER Ca^{2+} content (see Fig. 4). On exposure to CCK for a second time the amplitudes were even smaller (Fig. 5C). Pretreatment with ryanodine (100 μM) and thapsigargin (500 μM) virtually abolished $[Ca^{2+}]_i$ responses to GLP-1 (data not shown). Taken together, these results indicate that CCK and GLP-1 have access to the same intracellular pools of Ca^{2+}, presumably the ER, but perhaps release Ca^{2+} by differing mechanisms. Exendin-4 (0.1 nM) had similar effects to GLP-1 (1 nM) on $[Ca^{2+}]_i$. The GLP-1 antagonist, exendin 9–39 (Goke et al., 1993), inhibited GLP-1-induced calcium transients when used at a 10-fold higher concentration than GLP-1.

Effects of glucagon and 8-bromo-cAMP on $[Ca^{2+}]_i$ AR42J cells

Exposures to glucagon (10 nM) induced $[Ca^{2+}]_i$ responses in 70% (n = 12) of AR42J cells. The $[Ca^{2+}]_i$ transients commenced briefly after exposure to glucagon, developed at a relatively slow rate, peaked at 200%–250% of the resting IFR level and showed a prolonged, slow relaxation (Fig. 6A). The $[Ca^{2+}]_i$ transients induced by CCK shortly after treatment with glucagon (or with both treatments added simultaneously) showed an attenuated rate of rise and a very slow rate of relaxation (Fig. 6B, compare with Fig. 4A, 3.2 vs. 1.9 IFR). Similarly, brief (60–300 sec) exposures to 0.1 μM 8-bromo-cAMP, a membrane-permeable form of cAMP, usually did not markedly affect the rate of rise of the CCK-induced $[Ca^{2+}]_i$ transients but markedly slowed their rate of relaxation (Fig. 6C).

Tyrosine phosphorylation studies

In the absence of any stimulation, some proteins exhibited a basal level of phosphorylation which was increased in the presence of CCK and NaF. Five proteins (30, 46, 66, 120, and 190 KDa) were the most obviously influenced in the presence of CCK with at least a 2-fold increase in the phosphorylation levels of those proteins. Note that insulin induced phosphorylation of a protein at 97 KDa, corresponding to the insulin receptor beta subunit. Instead, GLP-1 decreased such proteins phosphorylation compared with the basal

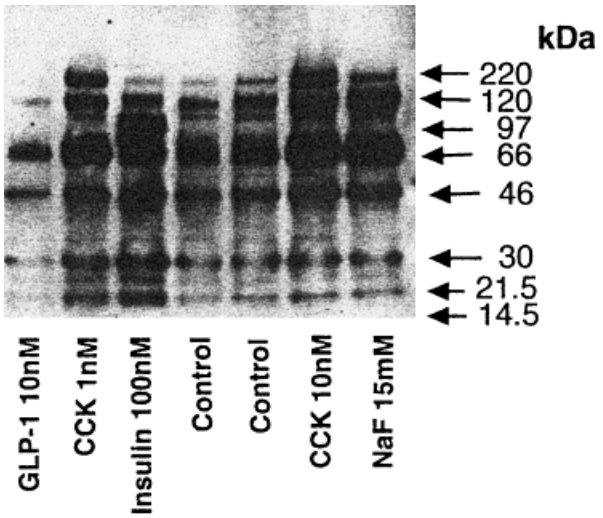

Fig. 7. Protein tyrosine phosphorylation in AR42J cells in response to various stimuli. A representative antiphosphotyrosine immunoblot of total cellular proteins from untreated (control) cells and 5 min-treated cells as indicated (n = 3). Note the increase in tyrosine phosphorylation with cholecystokinin (CCK) and sodium fluoride (NaF) of 30-, 46-, 66-, 120-, and 190-KDa bands. Glucagon-like peptide-1 (GLP-1) did not have any effect on those proteins. Insulin caused increased phosphorylation of 97-KDa band, corresponding to the insulin receptor β-subunit.

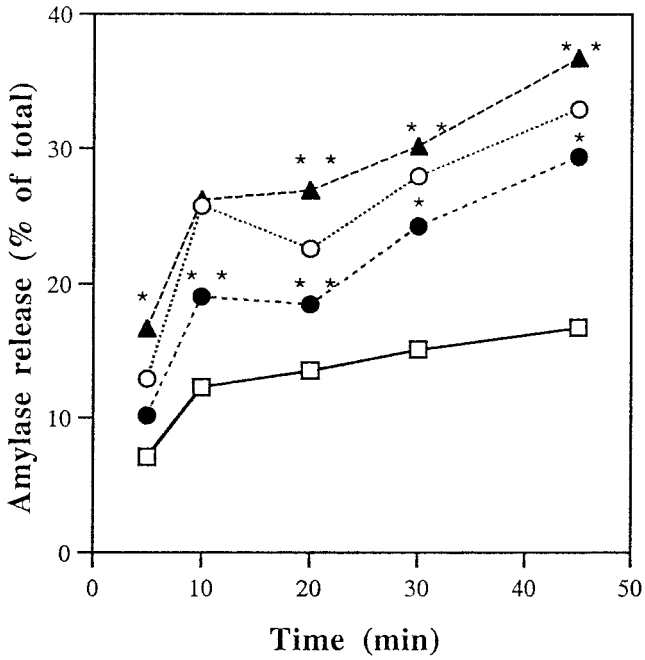

Fig. 8. Time course of the actions of vanadate (1 mM) (▲) and genestein (300 μM) (●) on cholecystokinin (CCK)-mediated (1 nM) amylase release from AR42J cells. Amylase release from CCK-treated (1 nM) (○) cells or control (no treatment) (□) cells is also shown. Amylase values are expressed as a percentage of the released amylase into the medium over the total amylase activity of the cells. Results are the mean ± SEM of four experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, vanadate or genestein plus CCK treatment vs. CCK treatment alone.

level (Fig. 7). Genistein decreased tyrosine phosphorylations induced by CCK (data not shown) and diminished CCK-mediated amylase release, as already shown in Figure 8.

Amylase release

CCK was a potent stimulus of amylase release. Maximum stimulation was seen at 10 nM in our experiments (Fig. 9). Although glucagon (10 or 100 nM) by itself had no effect on amylase release, when combined with CCK it inhibited but did not fully abolish CCK-induced amylase release (Fig. 10; n = 20, $P < 0.01$). GLP-1 and insulin, either alone or combined with CCK, did not influence amylase release (Fig. 10). We also examined exendin-4 (concentrations ranging from 10 pM to 10 nM) for potential effects on amylase release, and, similar to GLP-1, it did not appear to influence amylase release (data not shown). As GLP-1 and glucagon raise cAMP levels in AR42J cells we looked at the effect of 8-bromo-cAMP (8-Br-cAMP), a cAMP analogue, on amylase release. While 8-Br-cAMP appeared to have no effect on amylase release when given alone, it reduced CCK-induced amylase release from 28.79% to 23.55% of total amylase (n = 9, $P < 0.05$). We also used thapsigargin and ryanodine, specific inhibitors of ryanodine receptors/ER Ca^{2+} release channels and of the ER Ca^{2+} pumps, respectively, alone and in combination with CCK, to investigate the role of a rise of intracellular calcium on amylase release. The combination of thapsigargin and ryanodine decreased, but did not fully inhibit, CCK-induced amylase release (Fig. 11; n = 3, $P < 0.01$). NaF, which mimics CCK's effects on amylase release in acinar tissue (Vajanaphanich et al., 1995), did likewise in the AR42J cells (data not shown), with 15 mM NaF being equipotent to 1 nM CCK. The addition of GLP-1 together with NaF to the cells did not increase amylase release above that of NaF alone. Genestein (300 μM), the tyrosine

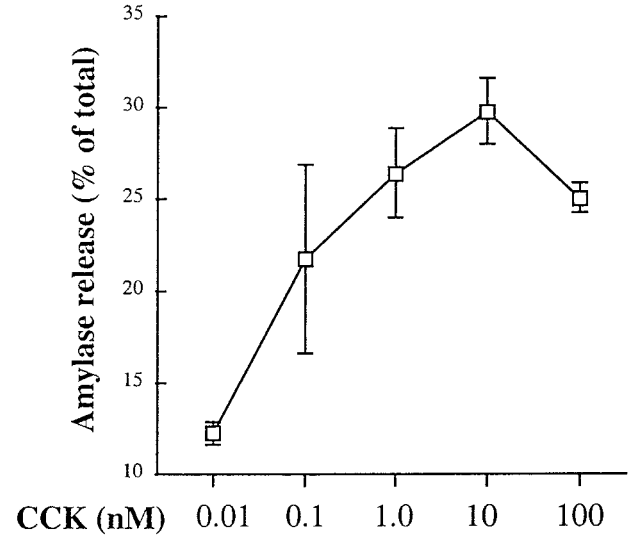

Fig. 9. Cholecystokinin (CCK) concentration-response curve of amylase release from AR42J cells. Cells were treated with CCK at the concentrations shown for 50 min. Amylase values are expressed as a percentage of the released amylase into the medium over the total amylase activity of the cells. Results are the mean ± SEM of 15 experiments.

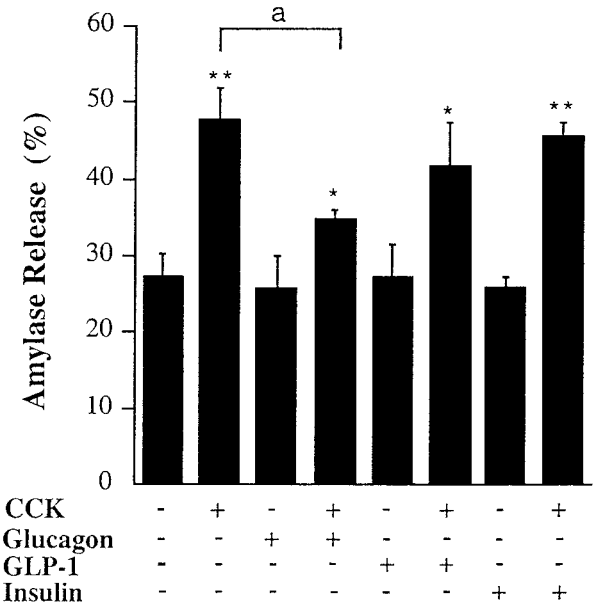

Fig. 10. Effects of glucagon (10 nM), glucagon-like peptide-1 (GLP-1) (10 nM), and insulin (100 nM) ± cholecystokinin (CCK) (1 nM), on amylase release from AR42J cells. Dexamethasone-induced AR42J cells were incubated for 50 min in presence of the hormones. Amylase values are expressed as a percentage of the released amylase into the medium over the total amylase activity of the cells. Results are mean ± SEM of 20 experiments, * $P < 0.05$, ** $P < 0.01$, treatment versus no treatment. a = $P < 0.01$.

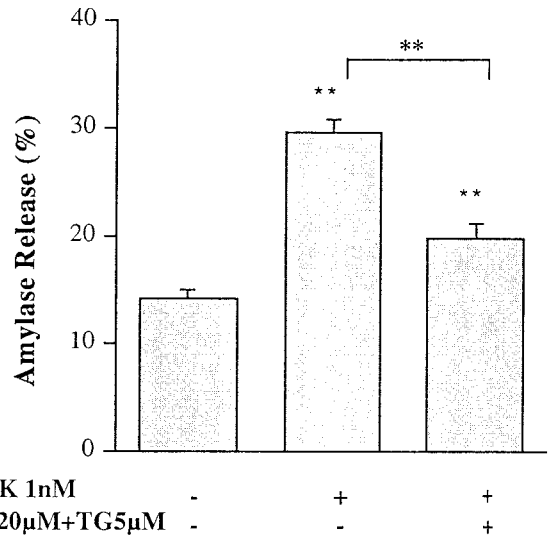

Fig. 11. Effects of ryanodine (RY) and thapsigargin (TG) in the presence or absence of cholecystokinin (CCK) on amylase release from AR42J cells. Amylase values are expressed as a percentage of the released amylase into the medium over the total amylase activity of the cells. RY and TG were added 30 min prior to addition of CCK, which was then added for 50 min. Results are mean ± SEM of three experiments, ** $P < 0.01$.

kinase inhibitor, decreased CCK-mediated amylase release, especially at the early time points of the CCK treatment, while vanadate (1 mM), a tyrosine phosphatase inhibitor, increased significantly basal and CCK-mediated amylase release (Fig. 8). We have shown that when beta cells of the pancreas are treated with GLP-1 for 24 h there is an increase in glucose- and GLP–1-mediated insulin release (Wang et al., 1995). We therefore looked for any long-term effects that GLP-1 might have on amylase release. Preincubation of AR42J cells for 8, 24, 48, or 72 h with GLP-1 (10 nM) and insulin (100 nM) did not increase basal or CCK-induced (1 nM) amylase release (data not shown).

DISCUSSION

The GLP-1 receptor is present on AR42J cells and it is identical to that of beta cells. GLP-1 and exendin-4 clearly increased cAMP and intracellular calcium, similar to its effects in beta cells (Holz et al., 1995), in these cells but did not appear to increase amylase release, alone or with CCK, in AR42J cells. We also demonstrated an increase in cAMP and intracellular calcium with glucagon though the pattern of the increase was different from GLP-1. Tyrosine phosphorylations may be involved in downstream signaling by CCK (Ferris et al., 1999, Rosado et al., 1998). The peptides we used here did not cause any increase in tyrosine phosphorylation in AR42J cells. Malhotra et al. (1992), using rat acinar cells, stated that exendin-4, which is present in the saliva of the Gila monster lizard and that like GLP-1 is insulinotropic by acting through the GLP-1 receptor, potentiated CCK-induced amylase release and increased intracellular cAMP. They did not, however, discuss the effects of GLP-1 in acinar cells. The effects they describe with exendin-4 occured at just one time point with a very high concentration (10^{-8} M) and may have been due to the small number of experiments they carried out.

AR42J cells respond in a physiological manner to CCK as evidenced by induction of amylase release in a concentration-dependent manner and increased intracellular calcium. CCK also induced protein tyrosine phosphorylation as had previously been shown (Lutz et al., 1993). CCK induced substantial increases of 190, 120, 66, 46, and 30 KDa in tyrosine phosphosubstrates on the basis of apparent molecular masses when separated on SDS-polyacrylamide gels. Two of those phosphorylations, 120 and 66 KDa, have already been described (Lutz et al., 1993). Inhibition of tyrosine phosphorylation by genistein inhibited amylase release and also decreased tyrosine phosphorylation events, in agreement with what Rosado et al., (1998) have already shown. This suggests that in AR42J cells, as in acinar cells, that tyrosine phosphorylation is involved in regulated amylase secretion. Insulin induced phosphorylation of most probably its own receptor beta subunit at 97 kd. NaF, a well-known activator of G proteins (Rivard et al., 1995), has previously been shown to mimic the effects of CCK in acinar cells in that it increases amylase release and increases tyrosine kinase activity in acinar cells (Rivard et al., 1995). We also show that NaF mimics the effects of CCK on tyrosine phosphorylation events in AR42J cells and therefore lends credence to the hypothesis that a fluoride-sensitive G protein exists that functions as a transducer between the CCK receptor and tyrosine phosphorylation (Rivard et al., 1995).

GLP-1 receptors belong to a family of G-protein-

linked receptors that includes glucagon-secretin-vasoactive intestinal peptide receptors. After binding of GLP-1 to its receptor there is a rise in cAMP in AR42J cells and in beta cells of the islets of Langerhans (Widman et al., 1996), indicating that the receptor is coupled to the adenylyl cyclase system by a stimulatory G-protein. The CCK receptor has been shown to be coupled to G_i subtypes as well as G_s subtypes in acinar cells (Schnefel et al., 1990).

There are conflicting reports in the literature on the effects of glucagon on amylase release. At least some of the conflicting data may be due to the use of natural glucagon and synthetic glucagon. Natural glucagon increases CCK-induced amylase release but this is probably due to the presence of secretagogues in the preparations (Pandol et al., 1983). Synthetic glucagon, on the other hand, increased cAMP, presumably through activation of adenylyl cyclase by a G_s subunit of a membrane associated G protein, in acinar preparations but no increase in amylase release was observed (Pandol et al. 1983). In vivo, glucagon inhibits exocrine pancreatic function stimulated by CCK (Schonfeld and Muller, 1994), in keeping with our data, and 8-Br-cAMP does likewise. More recent experimental data (Wettergren et al., 1998) have provided evidence that GLP-1 acts through neural pathways to inhibit gastropancreatic function in vivo via the vagus nerve. It is also possible that glucagon does likewise and this could explain the disparate results often found by investigators between the different experimental methods.

Acinar cells exhibit functional changes in diabetes mellitus. The severity of the alterations become more pronounced with worsening of diabetes and increasing duration (Semakula et al., 1996). As glucagon levels are increased in diabetes this may be a factor in the reduction seen in acinar function.

Similar to CCK, the rise in intracellular calcium induced by GLP-1 was from the endoplasmic reticulum. However, the pattern of the calcium gradients was not the same as with CCK, implying that the signaling to the release of calcium by CCK was possibly different from that by glucagon and GLP-1. GLP-1 did not increase tyrosine phosphorylation events. This demonstrates once again the importance of tyrosine phosphorylation for regulated amylase release. It also demonstrates that pathways independent of an elevation of intracellular calcium are important for the secretion of amylase. NaF did not increase intracellular calcium but did increase amylase release. This is further underscored by the results obtained in the presence of thapsigargin and ryanodine. While they prevented any rise in intracellular calcium they reduced but did not prevent, CCK-induced amylase release. Therefore, a rise of intracellular calcium may be necessary for the full expression of CCK-induced amylase release but of itself it is clearly not sufficient to induce amylase release in AR42J cells. GLP-1 cause $[Ca^{2+}]_i$ increase, but lack the protein tyrosine phosphorylation effects on AR42J cells and failed to cause amylase release.

Any cell type may contain diverse beta subunits of the GTP-binding proteins (von Weizsacker et al. 1992). This could mean that depending on the subtype activated, i.e., G_i, G_s by CCK, G_s by glucagon or GLP-1, a different $G_{\beta y}$ subunit may be released. A specific $G_{\beta y}$ might then be required for the tyrosine phosphorylation events we see in AR42J cells as already described for mitogen-activated protein kinase activation (Hawes et al. 1995). It also raises the possibility that if two different $G_{\beta y}$ subunits are released by the action of one hormone they might have additive or antagonistic effects on various downstream events.

In summary, we demonstrate that GLP-1 receptors are present on AR42J cells. Their activation by GLP-1 and exendin-4 leads to increased cAMP and intracellular calcium but did not, however, lead to an increase in amylase release nor did it alter the CCK-mediated amylase release.

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