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INSTRUMENTS AND TECHNIQUES

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Adenovirus-mediated gene expression in isolated rat pancreatic acini and individual pancreatic acinar cells

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Abstract In this study we have examined the feasibility of using replication-deficient recombinant adenoviral vectors to transfer and express genes in pancreatic acinar cells in vitro. We infected primary cultures of both isolated pancreatic acini and individual acinar cells with a recombinant adenovirus containing the coding sequence for β-galactosidase. Our data demonstrate that recombinant adenoviruses readily infect pancreatic acinar cells in vitro. Close to 100% infection and maximal β-galactosidase expression were obtained, when acini or acinar cells were infected with 5×10^6 or 10^6 plaque-forming units (pfu) of virus per millitre of acini or acinar cell suspension, respectively. Examination of the time-course of β-galactosidase expression showed that there was a lag of approximately 6 h before β-galactosidase levels increased. Thereafter β-galactosidase expression increased rapidly. By 20 h post-infection β-galactosidase activity had increased from undetectable levels to 2.5–3.0 units/mg of cellular protein. Acini/acinar cells maintained a robust secretory response after adenoviral infection. The cholecystokinin-octapeptide (CCK8) dose/response curves for amylase secretion for acini and acinar cells infected with 5×10^5 and 1×10^5 pfu/ml of virus, respectively, were biphasic, with maximal amylase secretion being stimulated by 1 nM CCK8. In addition, the dose/response curves were identical to those obtained from control, sham-infected, acini/acinar cells. Our findings indicate that replication-deficient recombinant adenoviral vectors will be excellent tools to transfer and express genes in isolated pancreatic acini or acinar cells.

Key words Adenovirus · Exocrine pancreas · Gene expression · Gene transfer

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Introduction

Adenoviruses are double-stranded DNA viruses which infect non-proliferating mammalian cells, producing mild illnesses such as upper respiratory tract infections [7]. When the E1 segment of the adenovirus genome is deleted the virus can still infect cells but cannot replicate [2, 6]. Moreover, the space created by the deletion can be used to insert new genes into the viral genome. These characteristics have prompted a number of research groups to explore the feasibility of using replication-deficient recombinant adenoviruses as vectors for gene therapy. It has been shown that recombinant adenoviruses can efficiently transfer genes to a wide variety of tissues in vivo [1, 2, 4, 8, 15], including exocrine cells [6, 9, 10, 13, 14]. In particular, recent research has demonstrated that recombinant adenoviruses can transfer and express transgenes in all the major cell types present in the exocrine pancreas [6, 10, 13, 14]. These studies were conducted in vivo, by injection of virus into the pancreatic duct [6, 10, 13], and ex-vivo, in isolated perfused gland segments [14].

Pancreatic acinar cells have been intensively studied as models of both regulated protein secretion and stimulus-response coupling. To date, however, it has *not* been possible to transfer and express genes in pancreatic acinar cells in vitro. Acinar cells are terminally differentiated, and no continuous cell line exists which adequately preserves the acinar phenotype. In addition, acinar cells in primary culture lose their acinar characteristics within a few days, and are replaced by cells with the characteristics of duct cells (see [5] for review). This combined

lack of a long-term culture system and a suitable transfection method has severely restricted the type of experimental strategies available to researchers, thereby hampering and undoubtedly slowing research investigating the basic cell biology/physiology of the pancreatic acinar cell.

One way to circumvent these difficulties might be to infect acinar cells in vivo with recombinant adenoviruses encoding the gene of interest, and subsequently isolate cells expressing the transgene for in vitro study. However, this strategy has several drawbacks. On a purely practical level, it necessitates animal surgery, and requires rather large amounts of recombinant virus [10, 13]. In addition, levels of infection and transgene expression are highly variable. Furthermore, in vivo infection efficiencies are typically well below 100% [10, 13, 14], so that, if the cells are subsequently isolated, only a proportion of them will express the transgene. Finally and most seriously, pancreatic infusion of adenovirus in vivo has been reported to cause severe inflammatory pancreatitis [10, 13], which inevitably results in some degree of tissue/cell damage and may thus compromise normal pancreatic cellular physiology.

The aim of the present study was to determine whether replication-deficient recombinant adenoviruses can be used to express transgenes in very short-term (24 h) primary cultures of both pancreatic acini and single acinar cells. Our express aim was to develop a cell preparation and method which would enable us to transiently express proteins in acinar cells and subsequently examine acinar physiology in vitro. Therefore, in contrast to previous, in vivo, studies, we have made detailed quantitative measurements of both infection efficiency and transgene expression. In addition, we have examined whether viral infection and transgene expression per se alter physiological secretory activity. Our data demonstrate that infection of acini or single acinar cells with adenovirus results in a rapid (within 24 h), high, and reproducible level of transgene expression. In addition, adenoviral infection/transgene expression can be obtained with no significant effect on acinar cell secretory function. This is the first demonstration that adenoviral infection per se has no deleterious effect on pancreatic exocrine cell function. Our study establishes that replication-deficient recombinant adenoviruses will be invaluable tools for expressing transgenes in isolated pancreatic acini/acinar cells, and defines conditions which permit optimal transgene expression with minimal or no effects on normal cell function. The ability to express heterologous proteins transiently in isolated pancreatic acini should greatly aid studies examining stimulus-secretion coupling and regulated exocytosis in the pancreatic acinar cell.

Materials and methods

Materials

Collagenase (CLSPA) was purchased from Worthington Biochemicals. EGTA was obtained from Boehringer Mannheim, Indianapolis, Ind, USA. All the other chemicals were purchased from the Sigma, St. Louis, Mo., USA The adenovirus used was a replication-deficient variant of the human adenovirus type 5 (Ad.RSV β Gal) carrying the coding sequence for *Escherichia coli* β-galactosidase, together with the SV40 nuclear localization signal, driven by the Rous Sarcoms virus long terminal repeat promoter. These elements are inserted in a deletion of the E1B region of the viral genome [9]. Viral preparations which had been standardized by limiting dilution plaque assay were kindly supplied by Drs. B.C. O'Connell and B.J. Baum, Gene Therapy and Therapeutics Branch, National Institute of Dental Research, Bethesda, Md., USA

Methods

Isolation of rat pancreatic acini

Rat pancreatic acini were prepared according to a procedure described previously [11, 12]. In brief, the rat was killed by cervical dislocation, the pancreas was removed and cleaned free of fat and connective tissue. It was then diced with a razor blade and placed in a 25-ml Erlenmeyer flask in 5 ml of a modified Kreb's Ringer buffer (KRB) containing: 125 mM NaCl, 4 mM KCl, 12.5 mM HEPES (pH 7.4), 10 mM glucose, 1.5 mM $MgCl_2$, 2.0 mM $CaCl_2$, 0.1% bovine serum albumin (BSA), 0.01% soybean trypsin inhibitor and 400 U CLSPA collagenase (Worthington Biochemical). The flask was then capped with a rubber stopper and shaken at 200 cycles/min in a 37°C waterbath for 15 min. The partially digested tissue was transferred to a 15-ml Corex tube which was capped and vigorously shaken by hand. After 1–2 min the resulting acinar suspension was filtered through surgical gauze and then a 200 µm nylon mesh to remove the undigested clumps of tissue. The filtered acini were pelleted by centrifugation at 200 *g* for 3 min and resuspended into fresh KRB, this centrifugation resuspension step was repeated twice more to remove all traces of the collagenase. The final acinar cell pellet was resuspended in 2–4 ml of the KRB.

Isolation of rat pancreatic acinar cells

Isolation of acinar cells followed the same procedure as for acini except that an additional digestion step with trypsin was included. Thus, after 15 min in collagenase-containing KRB, the partially digested tissue was resuspended in 5 ml of a buffer containing: 125 mM NaCl, 4 mM KCl, 12.5 mM HEPES (pH 7.4), 10 mM glucose, 0.4 mM EGTA and 1 mg/ml trypsin. The resulting suspension was placed in a fresh 25-ml Erlenmeyer flask and shaken at 200 cycles/min at 37°C for a further 3 min. Subsequently the tissue was resuspended in 5 ml of KRB containing 400 U/ml collagenase and placed in a 15-ml Corex tube, shaken, filtered and washed as described above.

Culture of pancreatic acini and acinar cells

The isolated acini/acinar cells were pelleted by centrifugation and resuspended in Waymouth's 751/2 media containing 2% FCS (acini) or M199 containing 10% chick embryo extract and 4% horse serum (acinar cells). This process was repeated three times to remove all traces of the KRB. The final acini/acinar cell pellets were resuspended in 10 ml of the appropriate media (75–100 µg cellular protein/ml of medium) containing 2 nM epidermal growth factor (EGF). The suspensions were then plated on 6-well tissue culture plates or T75 flasks, depending on the type of experiment. Finally a defined concentration of adenovirus made in the appropriate culture media was added to each well/flask. For the control "sham-infected" acini/acinar cells only medium was added to the wells. The acini/acinar cells were then incubated with the virus in a humidified 5%CO_2/95% air atmosphere at 37°C. After 4 h the acini/acinar cells were removed from the culture plates and washed into either fresh M199 containing 10% chick embryo extract, 4% horse serum and 2 nM EGF (acinar cells) or Waymouth's 752/1 media containing 5% FCS and 2 nM EGF (acini). The acini/acinar cell suspensions were then replated on fresh 6-well tissue culture

plates or T75 flasks and cultured at 37°C in a 5%CO_2/95% air atmosphere for 2 to 22 h.

β-Galactosidase assay

Pancreatic acini/acinar cells were harvested at specified times after infection and washed twice with PBS. The acini/cells were then resuspended in 200 µl of lysis buffer (25 mM Tris, pH 8.0, 0.1% Triton X-100) and sonicated for 5 s to lyse the cells. The lysates were then centrifuged at 14,000 *g* for 5 min and 150 µl of the supernatant removed for analysis. For the β-galactosidase assay the supernatants were diluted 1 in 100 with lysis buffer. A 30-µl aliquot of each diluted supernatant was then incubated at 37°C with 270 µl of assay buffer (0.1 M phosphate buffer, pH 7.5, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol and 0.65 mg/ml o-nitrophenyl-β-D-galactopyranoside). After 20 min, the reactions were stopped by addition of 0.5 ml of 1 M Na_2CO_3 and the absorbance of the samples read at 420 nm.

Histochemical staining

At 20 h post-infection the acini/acinar cells were harvested and washed twice with PBS. The acini/acinar cells were then resuspended in 2 ml of PBS containing 2% v/v formaldehyde and incubated for 5 min at room temperature. The fixed acini/acinar cells were then washed twice with PBS to remove all traces of the fixative. Finally, the fixed acini/acinar cells were resuspended in 2 ml of a solution containing: 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) reagent. The suspensions were then incubated at 37°C for 2–4 h to allow the stain to develop. Stained cells were viewed on a Nikon Optiphot microscope and photographed using Kodak slide film. Unstained and stained cells were counted using a haemocytometer.

Secretion experiment

The acini/acinar cell suspensions were removed from the T75 flasks and centrifuged at 300 *g* for 5 min to pellet the cells. The acini/acinar cells were then resuspended in 10 ml of KRB. This centrifugation/resuspension step was repeated twice more to remove all traces of the culture media. The final cell pellets were resuspended in 4–5 ml of KRB, and 150-µl aliquots of the suspensions were then placed into 2-ml Eppendorf tubes, to which 150 µl of KRB containing cholecystokinin-octapeptide (CCK8) was added. The tubes were incubated at 37°C for 30 min before secretion was stopped by placing the experimental tubes in an ice-bath for 5 min. The acini/acinar cells were pelleted by centrifugation in an Eppendorf microfuge at 2000 *g* for 2 min and the supernatant removed. The supernatant and the lysed pellets were then assayed for amylase using the Bernfeld assay [3].

Statistics

Unless otherwise stated the data presented are the means ± SEM from three independent experiments. Statistical significance was calculated by the two-tailed Student's *t*-test for paired or unpaired values where appropriate, with $P<0.05$ representing significance.

Results and discussion

In order to determine if replication-deficient recombinant adenoviruses can be employed to transfer and express genes in pancreatic acinar cells in vitro, we infected isolated rat pancreatic acini and pancreatic acinar cells with increasing concentrations of a recombinant adenovirus that carries the coding sequence for β-galactosidase inserted into the viral genome. Twenty hours post-infection the acini/acinar cells were harvested and the amount of β-galactosidase activity present in the cells quantified (Fig. 1). Uninfected acini/acinar cells had no β-galactosidase activity. However, when acini/acinar cells were infected with the recombinant adenovirus, cellular β-galactosidase activity increased dramatically. With pancreatic acini, maximal β-galactosidase activity (approx. 1.5 units/mg cellular protein) was obtained when acini were infected with 5×10^6 to 1×10^7 pfu/ml of acinar suspension. In comparison, with single acinar cells, maximal β-galactosidase expression (1.9 units/mg cellular protein) was obtained when acinar cells were infected with the lower concentration of 10^6 pfu/ml of cell suspension. Curiously, when acinar cells were infected with higher concentrations of virus (5×10^6 to 10^7 pfu/ml) lower levels of β-galactosidase expression were obtained. This decrease in expression was not due to cell death as no decline in cell viability (as monitored by Trypan blue exclusion) was observed when the cells were infected with 5×10^6 or 10^7 pfu/ml (data not shown). The higher concentrations of virus required to obtain maximal β-galactosidase expression in acini probably reflects the fact that some of the acinar cells present within an acinus are less accessible to the virus. These data demonstrate that recombinant adenoviral vectors can be used to obtain a high level of transgene expression in isolated pancreatic acini or pancreatic acinar cells.

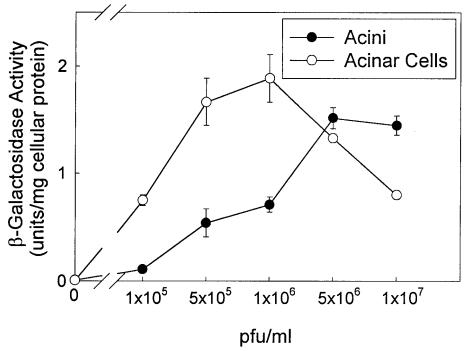

Fig. 1 β-Galactosidase expression in pancreatic acini or acinar cells infected with increasing concentrations of adenovirus. Rat pancreatic acini or pancreatic acinar cells were infected with increasing concentrations of recombinant Ad.RSV β Gal as described in Materials and methods. The acini/acinar cells were harvested 20 h post-infection and the amount of β-galactosidase activity associated with the cells determined. The data presented are the means ± SEM from three separate experiments

We next examined the infection efficiency of the recombinant adenoviruses. For these studies, acini or acinar cells were infected with a maximally effective dose of 5×10^6 or 10^6 pfu/ml, respectively. Twenty hours after infection the acini/acinar cells were harvested and those cells expressing β-galactosidase visualized using the X-Gal histochemical stain (Fig. 2). None of the uninfected acini/acinar cells stained with the X-Gal reagent. However, all the infected acini/acinar cells stained positive with X-Gal, as evidenced by the appearance of blue

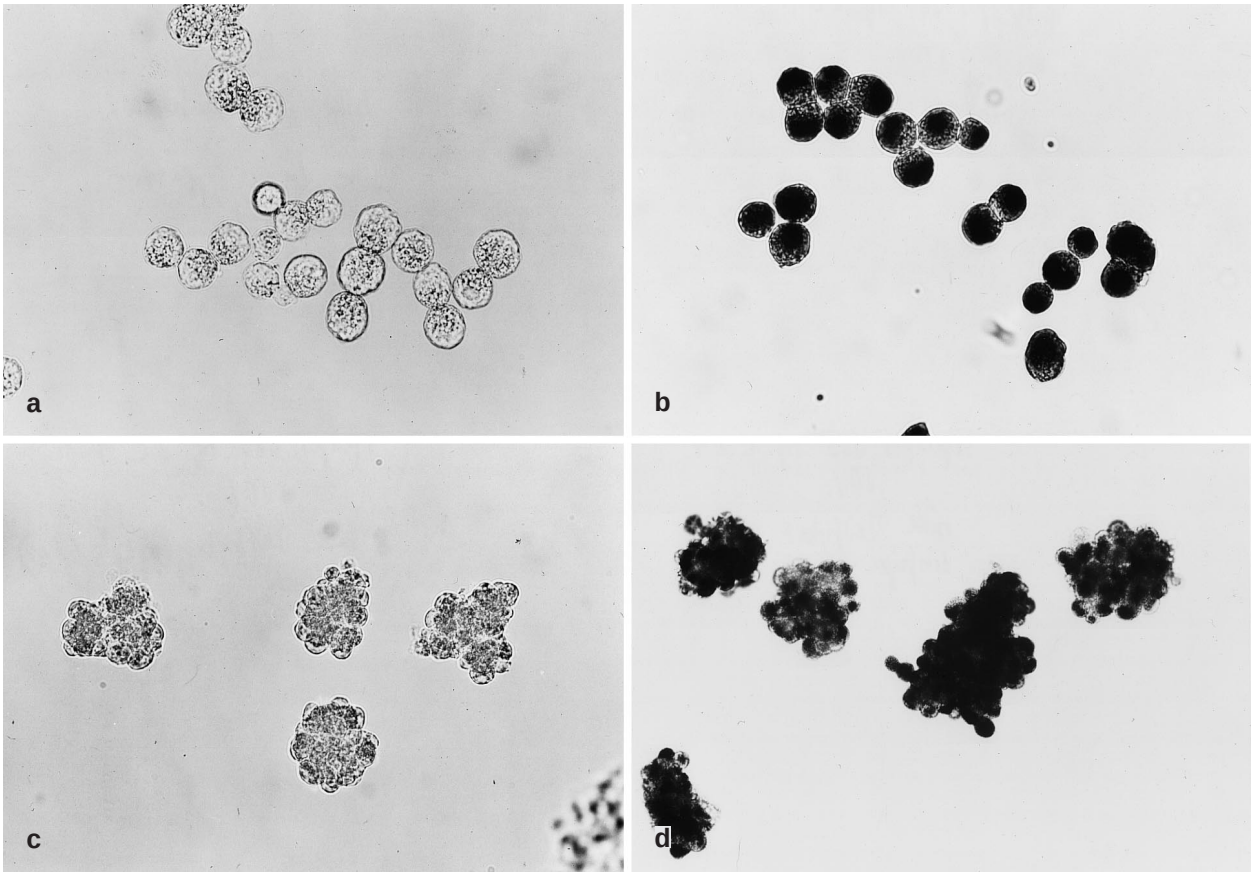

Fig. 2A–D Histochemical evaluation of infection efficiency. All acini and acinar cells were treated with the X-Gal reagent (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). **A** Sham-infected control acinar cells. **B** Acinar cells infected with 1×10^6 pfu/ml of Ad.RSV β Gal. **C** Sham-infected control acini. **D** Acini infected with 5×10^6 pfu/ml of Ad.RSV β Gal. Acini/acinar cells were examined 18–20 h after infection. **A** and **B**: ×1000, **C** and **D**: ×500

staining of nuclear and perinuclear regions, demonstrating that at the concentrations used the adenovirus had an infection efficiency of near 100%.

Next we examined the time-course of β-galactosidase expression. Single acinar cells and acini were infected with 10^6 and 5×10^6 pfu/ml of suspension, respectively. Cells/acini were harvested 6, 12, 20 and 24 h post-infection and the amount of β-galactosidase activity present within the acinar cells determined (Fig. 3). With both the acini and acinar cells only a minimal increase in β-galactosidase expression was observed up to 6 h post-infection. Thereafter, however, the level of expression increased dramatically. With acini, expression increased from 6 to 20 h post-infection but then appeared to plateau. In contrast, with the acinar cells, β-galactosidase expression increased almost linearly from 6 to 24 h post-infection. Why expression of β-galactosidase plateaued in acini but continued to increase in acinar cells is unclear.

To determine if virus-infected cells are still secretory competent, acinar cells/acini were infected with increasing concentrations of virus for 16–18 h, after which the cells were harvested and stimulated with 1 nM CCK8. In

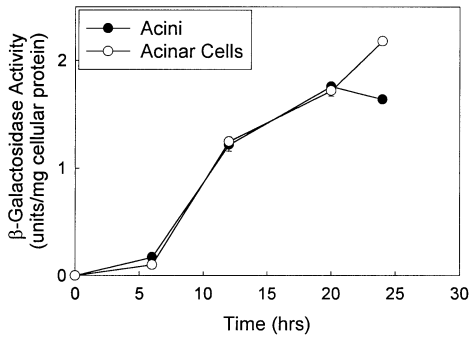

Fig. 3 Time-course of β-galactosidase expression in pancreatic acini or acinar cells infected with adenovirus. Rat pancreatic acini or pancreatic acinar cells were infected with 5×10^6 or 1×10^6 pfu Ad.RSV β Gal/ml of suspension, respectively. Acini/acinar cells were harvested 6, 12, 20 and 24 h after infection and the amount of β-galactosidase activity associated with the cells determined. The data presented are the means ± SEM from three separate experiments

parallel, the acini/acinar cells were stained with X-Gal and the percentage of cells infected determined by light microscopy (Fig. 4). With both acini and acinar cells a significant decrease in the secretory response to CCK8 was observed at very high concentrations of virus. There was not clear correlation between the percentage of cells infected and the decline in the secretory response at very high virus doses. Therefore, adenoviral infection per se does not appear to adversely effect secretory function.

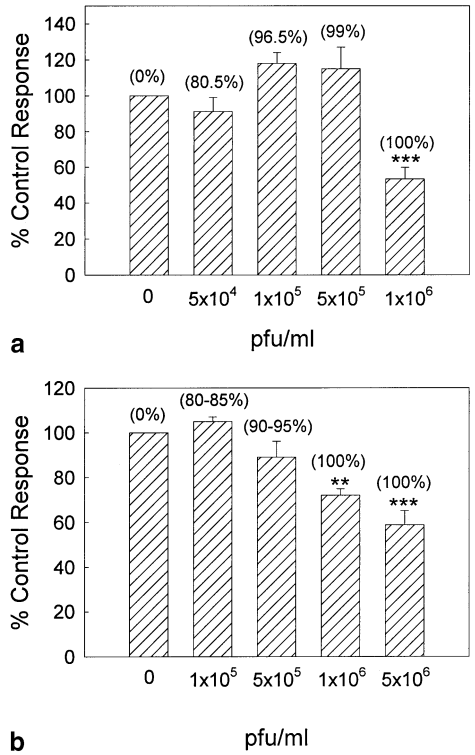

Fig. 4A, B Influence of viral concentration on cholecystokinin-octapeptide- (CCK8-) stimulated amylase secretion. Acinar cells (**A**) and acini (**B**) were infected with increasing concentrations of Ad.RSV β Gal and harvested 16–18 h after infection. The acinar cells/acini were then incubated for 30 min with or without 1 nM CCK8 and the amount of amylase secreted determined. The data presented are the means ± SEM from three separate experiments. The control response represents the increase in the amount of amylase secretion in response to 1 nM CCK8 in sham-infected acini/acinar cells. The numbers in *parentheses* represent the mean value for the % of cells infected as determined by X-Gal staining. A range is given for acini due to the difficulty in obtaining an exact count for the number of individual cells in each acinus. **<*P*=0.01, ***<*P*=0.001 values significantly different from control

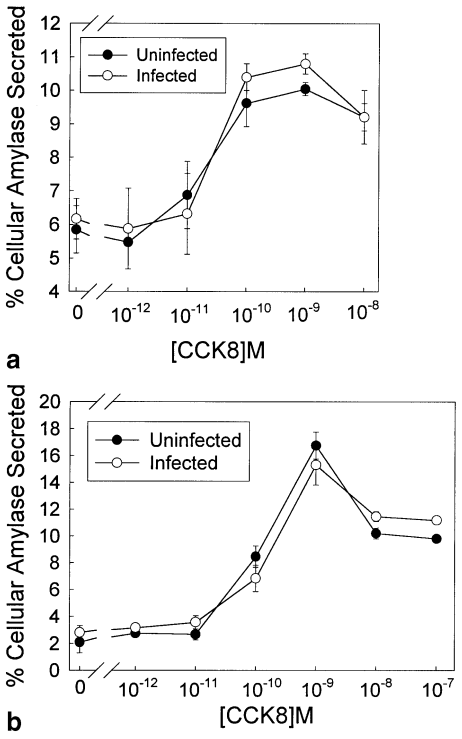

Fig. 5 CCK8 dose/response curve for amylase secretion from control and infected acinar cells (**A**) or acini (**B**). Rat pancreatic acini or pancreatic acinar cells were infected with 5×10^5 or 1×10^5 pfu of Ad.RSV β Gal/ml of cell suspension, respectively. Acini/acinar cells were harvested 16–18 h postinfection and aliquots stimulated with increasing concentrations of CCK8. Amylase secretion is expressed as % of cellular amylase secreted. The data presented are from a single experiment representative of three separate experiments

The decrease in secretion is most likely due to an adverse effect of the gross-overexpression of β-galactosidase observed with the higher viral concentrations. Therefore infecting acini/acinar cells with lower concentrations of virus (1×10^5 to 5×10^5 pfu/ml) allows one to obtain efficient gene transfer and expression (>80% of the cells expressing transgene) without adversely effecting the cells secretory response.

To test this hypothesis, acinar cells and acini were infected with 1×10^5 and 5×10^5 pfu/ml of virus, respectively. After 16–18 h the acini/acinar cells were harvested and used to obtain CCK8 dose/response curves for amylase secretion (Fig. 5). The CCK8 dose/response curves for the uninfected and infected cells/acini were essentially identical. The dose/response curves were biphasic, that is, amylase secretion first increased with increasing concentrations of CCK8 up to a maximum and then declined. Maximal amylase secretion was obtained with 1 nM CCK8 for acini and 0.1–1 nM CCK8 for acinar cells. However, the response obtained from the acinar cells was only approximately a quarter of that seen with the acini. Staining with X-Gal showed that 98% of the acinar cells, and 90–95% of the cells present in the acini, were infected. These data thus show that the acini/acinar cells maintain a robust secretory response after infection with recombinant adenoviruses, and that viral infection and transgene expression does not interfere with stimulus-secretion coupling or the exocytotic process.

In summary, our data demonstrate that replication-deficient recombinant adenoviruses readily infect isolated pancreatic acini and pancreatic acinar cells. Infection is accompanied by a rapid (within 24 h) and high level of transgene expression. The data also show that both acini and acinar cells maintain a robust secretory response to CCK8 after adenoviral infection. The secretory response did decline when acini/acinar cells were infected with very high concentrations of virus. This decline did not appear to be due to viral infection per se but rather due to the gross over-expression of β-galactosidase. However, lowering the concentration of virus allowed us to obtain high levels of infection (90–100%) without influencing the CCK8 dose/response curve for amylase secretion, demonstrating that viral infection and transgene expression can be achieved without interference with the secretory pathway.

The primary culture system employed in the present study offers several advantages over in vivo infection,

currently the only other approach which has been successfully used for gene transfer to acinar cells. Firstly, the in vitro system avoids the need for animal surgery and uses relatively small amounts of recombinant virus. It also allows good control of both infection efficiency and expression level, allowing 90% or more of cells to be transduced without deleterious effects on cell function. Finally, the normal physiology of the cell is not altered by an immune reaction to viral infection, as in the case in vivo [10, 11].

In conclusion, our study indicates that replication-deficient recombinant adenoviral vectors will be excellent tools for transiently expressing genes in isolated pancreatic acini or acinar cells. The ability to rapidly express high levels of heterologous proteins in pancreatic acini should greatly expand the types of experimental strategies available to researchers and, in particular, will have a major impact on the study of stimulus-secretion coupling and regulated exocytosis in the pancreatic acinar cell.

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