Insulin and secretagogues differentially regulate fluid-phase pinocytosis in insulin-secreting β -cells

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The physiological role of the β -cell insulin receptor is unknown. To evaluate a candidate function, the insulin regulation of fluid-phase pinocytosis was investigated in a clonal insulinoma cell line (β TC6-F7) and, for comparison, also in Chinese hamster ovary cells transfected with the human insulin receptor (CHO-T cells). In CHO-T cells, the net rate of fluid-phase pinocytosis was rapidly increased 3–4-fold over the basal rate by 100 nM insulin, with half-maximal stimulation at 2 nM insulin, as assayed by cellular uptake of horseradish peroxidase from the medium. Wortmannin, an inhibitor of phosphatidylinositol (PI)-3-kinase, blocked insulin-stimulated pinocytosis with an IC_{50} of 7.5 nM without affecting the basal rate of pinocytosis. In insulin-secreting β TC6-F7 cells, the secretagogues glucose and carbachol (at maximally effective concentrations of 15 mM and 0.5 mM respectively) augmented fluid-phase pinocytosis 1.65-fold over the basal rate. Wortmannin also inhibited secretagogue-stimulated pinocytosis in these β -cells with an IC_{50} of 7 nM but did not

affect the basal rate of pinocytosis measured in the absence of secretagogues. Wortmannin did not influence either basal or secretagogue-induced insulin secretion. Although these β TC6-F7 cells have cell-surface insulin receptors, adding exogenous insulin or insulin-like growth factor 1 did not affect their rate of fluid-phase pinocytosis, either in the absence or presence of secretagogues. From these observations, we conclude that: (1) in both insulin-secreting β -cells and in conventional, insulin-responsive CHO-T cells, a common, wortmannin-sensitive reaction, which probably involves PI-3-kinase, regulates fluid-phase pinocytosis; (2) the insulin-receptor signal transduction pathway is dissociated from the regulation of fluid-phase pinocytosis in the insulin-secreting β -cell line we studied; and (3) the enhancement of fluid-phase pinocytosis associated with secretagogue-induced insulin release in β TC6-F7 cells is not attributable to autocrine activation of β -cell surface insulin receptors.

INTRODUCTION

Insulin-secreting β -cells, such as the β TC-3 insulinoma cell line, express insulin receptors on their cell surface [1,2]. When glucose or other secretagogues trigger insulin release, the exocytosed insulin can bind and activate the β -cell's own surface insulin receptor [1]. Some earlier studies have suggested that this autocrine loop could provide a feedback mechanism for the regulation of β -cells by their own secretory product. For example, exogenous insulin inhibits β -cell insulin secretion in isolated pancreatic islets [3–8], as well as *in vivo* [9–11]. It is uncertain whether this inhibitory effect on secretion results from a direct interaction of insulin with the β -cell insulin receptor. The β -cell insulin receptor might influence other β -cell processes besides secretion, such as gene expression and cell growth. However, the physiological role of the β -cell insulin receptor has not been clearly defined.

Insulin stimulates fluid-phase pinocytosis, the uptake of extracellular medium into cells by vesicular endocytosis, in several insulin-responsive cell types [12–14]. Recent studies show that endocytosis of the plasma membrane is obligatorily coupled to exocytosis in specialized secretory endocrine and neuronal cell types [15,16]. Because β -cells have neuroendocrine characteristics [17,18], and because electrophysiological measurements indicate that endocytosis of the β -cell plasma membrane can be rate-limiting for insulin exocytosis [19], it is possible that endocytosis is coupled to exocytosis in β -cells as well. In the present study we examined whether activation of the β -cell insulin receptor modulates vesicular endocytosis in the β -cell. In principle, such regulation could provide a mechanistic link between the β -cell

surface insulin receptor and the control of β -cell secretory vesicle trafficking.

Phosphatidylinositol (PI)-3-kinase activity is implicated in controlling vesicle traffic in many cell types [20–23]. Insulin stimulation of the insulin receptor kinase leads to the marked augmentation of PI-3-kinase catalytic activity *in vivo* [24–27] and the PI-3-kinase is apparently important in mediating the ability of insulin to cause the redistribution of intracellular vesicles between the cell interior and the plasma membrane in several insulin-responsive cell types [28–31]. Isolated rat pancreatic islets also contain PI-3-kinase activity [32] and we recently found, in a cultured β -cell line, that insulin promotes the activating association of the 85 kDa PI-3-kinase regulatory subunit with insulin receptor substrate 1 (IRS-1), a major substrate and intracellular effector of the insulin receptor tyrosine kinase [1]. Therefore, through PI-3-kinase, the insulin receptor signal transduction pathway might influence vesicle trafficking in β -cells. Potentially, autocrine insulin receptor activation in the β -cell could participate in the initiation of re-endocytosis of the β -cell plasma membrane after insulin secretion. Alternatively, coupling of β -cell exocytosis with endocytosis might be entirely independent of any such autocrine feedback, as is apparently the case for other endocrine cell types. In the present study we distinguished between these possibilities.

MATERIALS AND METHODS

Materials

Reagents obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) included: aprotinin, BSA (immunoglobulin-free), *o*-

Abbreviations used: CHO-T cells, Chinese hamster ovary cells transfected with the human insulin receptor; IGF-1, insulin-like growth factor 1; IRS-1, insulin receptor substrate 1; HRP, horseradish peroxidase; KRBB, Krebs–Ringer bicarbonate buffer; PI, phosphatidylinositol.

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dianisidine, bacitracin, EDTA, horseradish peroxidase (HRP; P-8250) and pig insulin. 1,4-Dithio-D,L-threitol and Hepes were from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Wortmannin obtained from either Sigma or Boehringer Mannheim was of equal potency. ^{125}I -labelled Protein A was from ICN (Costa Mesa, CA, U.S.A.) and enhanced chemiluminescence reagents were from Amersham (Arlington Heights, IL, U.S.A.). Protein A-Trisacryl beads were from Pierce Chemical Co. (Rockford, IL, U.S.A.). Lysine-fixable fluorescein dextran was from Molecular Probes (Eugene, OR, U.S.A.). Tissue culture media were from Gibco-BRL (Grand Island, NY, U.S.A.), and sera were from Hyclone (Logan, UT, U.S.A.). All other reagents were of reagent grade and were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Cell culture

Insulinoma cells ($\beta\text{TC6-F7}$ clone [33]) were kindly provided by Dr. Shimon Efrat (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) and were propagated in Dulbecco's modified Eagle's medium containing 25 mM glucose and supplemented with 100 i.u./ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin sulphate and 7.5% (v/v) heat-inactivated horse serum and 2.5% (v/v) fetal bovine serum, at 37 °C in a water-saturated 10% CO_2 in air atmosphere. Cells were passaged weekly and used for experiments between passages 28 and 58. For use in experiments, the $\beta\text{TC6-F7}$ cultures (40–60% confluent) were preincubated for 16 h in modified Dulbecco's modified Eagle's medium containing 8 mM glucose and 0.1% (v/v) fetal bovine serum. Chinese hamster ovary cells transfected with the human insulin receptor (CHO-T) were a gift from Dr. Richard Roth (Stanford University, Palo Alto, CA, U.S.A.) and were propagated in Ham's F-12 medium containing 100 i.u./ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin sulphate and 5% (v/v) fetal calf serum at 37 °C in a water-saturated 1.5% CO_2 in air atmosphere. Cultures were passaged weekly and used for experiments when 50% confluent and after a 16 h preincubation in Ham's F-12 medium containing 0.1% fetal calf serum.

Insulin release

Cells were grown in 6-well (3.5 cm diam.) Falcon plates to densities of $(1-2) \times 10^5$ cells per well. After each well had been washed three times with 4 ml of glucose-free, Hepes-buffered, Krebs-Ringer bicarbonate buffer (KRBB) (120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 25 mM NaHCO_3 , 20 mM Hepes) pre-equilibrated with 5% CO_2 in air at 37 °C, pH 7.40 ± 0.05 , the cells in each well were pre-equilibrated in 2 ml of KRBB also containing 0.1% BSA for 30 min, followed by a timed incubation in a fresh aliquot of the same buffer also containing the secretagogues indicated in the text. The medium was collected and after centrifugation at 10000 *g* for 5 min at 4 °C the insulin concentration in the supernatant was determined in triplicate by radioimmunoassay with rat insulin standards (a service of the Radioimmunoassay Core Facility of the University of Pennsylvania Diabetes Center).

Fluid-phase pinocytosis

The cellular uptake of HRP, a marker of the extracellular space, was assayed essentially as described by Steinman et al. [34]. Briefly, cell cultures in 6-well plates were preincubated in Hepes-buffered KRBB for 15 min at 37 °C in a 5% CO_2 in air atmosphere. After the addition of HRP to a final concentration of 1 mg/ml, the plates were further incubated under the same conditions for the timed periods indicated in the text. To

terminate uptake, the medium was removed, the well was washed quickly with 2 ml of KRBB containing 0.05% BSA at 37 °C, followed by five consecutive washes at 0–4 °C with 3 ml of PBS (5 min per wash). After removal of the last wash solution, the cells were solubilized at 0 °C in 0.25 ml of 0.1% (v/v) Triton X-100, 20 mM Hepes, pH 7.4. The cells were scraped from the dish and incubated in the lysis buffer for 10 min at 0 °C before centrifugation at 10000 *g* for 10 min at 4 °C. The supernatant was collected and the HRP concentration determined spectrophotometrically with *o*-dianisidine at 460 nm or with equivalent results at 405 nm in a microplate reader after quenching of the colour reaction with 100 mM HCl [35]. Assays were routinely performed in triplicate for each condition. Preliminary control experiments demonstrated that HRP accumulation was linearly proportional to cell number and to HRP concentration, and did not occur when uptake studies were performed at 0–4 °C. Non-specific adsorption of HRP on plastic tissue-culture surfaces was negligible, as assessed in control experiments performed under identical conditions but without cells. For cell enumeration, replicate dishes were trypsinized for 5 min, diluted in PBS and counted either in a standard Neubauer haematocytometer or in a Coulter counter.

Data analysis

All data represent means $\pm$ S.E.M. obtained for at least three independent experiments. The statistical significance between means was analysed by using Student's *t* test; $P \leq 0.05$ was considered significant.

Immunoprecipitations

Standard cell lysis and immunoprecipitation procedures were used as previously described [1]. Briefly, $\beta\text{TC6-F7}$ or CHO-T cultures grown in 15 cm diam. plates were washed and preincubated at 37 °C in Hepes-buffered KRBB with the protocols described above. After incubation with hormones or secretagogues as indicated in the text, the cultures were quickly washed three times with PBS at 0 °C, then the cells were solubilized at 0 °C in 3 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM NaVO_3 , 0.7 mM bacitracin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin and 0.1 mM PMSF). After clarification by centrifugation at 10000 *g* at 4 °C for 30 min, the lysate was incubated for 16 h at 4 °C with specific antibodies as indicated in the text. The immunoglobulins were adsorbed on immobilized Protein A beads (15 μl of a 50% slurry per ml of lysate) for 2–4 h at 4 °C with gentle agitation. The immunocomplexes were washed twice at 4 °C by brief centrifugation and resuspension in 1 ml of wash buffer (1% Triton X-100, 0.1% SDS, 100 mM NaCl and 50 mM Tris/HCl, pH 7.3) and washed once more in a similar buffer lacking NaCl. After removal of excess wash buffer, the immunoprecipitated proteins were solubilized in 30 μl of SDS/PAGE sample buffer with 50 mM dithiothreitol at 100 °C for 3 min.

Electrophoresis and immunoblotting

Immunoprecipitated proteins were separated on a one-dimensional SDS/PAGE (7.5% T acrylamide) gel 0.75 mm thick, as described [1], in a Bio-Rad miniature slab gel apparatus, run at 175 V (constant). Standard molecular mass markers were myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), BSA (66.2 kDa) and ovalbumin (42.7 kDa). Electrotransfer of proteins from the gel to nitrocellulose (Schleicher and Schuell, BA85) was performed for 2 h at 125 V (constant) at 5–15 °C in a Bio-Rad miniature transfer apparatus, as described

by Towbin et al. [36], but with 0.05 % SDS added to the transfer buffer to enhance the elution of high-molecular-mass proteins. Nitrocellulose blots were incubated overnight at 4 °C in blocking buffer [5 % BSA, 1 % ovalbumin in TNA (10 mM Tris/HCl, pH 7.2, 100 mM NaCl, 0.05 % NaN₃)]. The nitrocellulose blots were incubated for 2 h at 22 °C with anti-phosphotyrosine antibodies (2 μ g/ml in blocking buffer), or with other blotting antibodies as indicated in the Figure legends. The blots were then washed twice for 10 min in TNA, once for 5 min in TNA containing 0.05 % Nonidet P40, and then twice more for 10 min each in TNA. The blots were then incubated with 5 μ Ci/ml of ¹²⁵I-labelled Protein A (6–30 μ Ci/ μ g) in blocking buffer for 1 h at 22 °C, and then washed again as described above. Bound antibodies were quantified on a Phosphorimager (Molecular Dynamics) or detected by autoradiography using Kodak XAR film with Cronex Lightning Plus intensifying screens at –80 °C for 12–72 h. For some experiments, immunoblots were processed with an enhanced chemiluminescence protocol (Amersham) following the manufacturer's instructions.

RESULTS

Fluid-phase pinocytosis in CHO-T cells

Fluid-phase pinocytosis was first examined in the CHO-T cell line, which expresses the human insulin receptor at a high level and has been widely used for investigating insulin signalling [37]. In these assays, HRP was used as a marker for the extracellular fluid [34]. As shown in Figure 1, these CHO-T cells demonstrate significant uptake of the extracellular medium. In basal, hormone-free conditions, pinocytic HRP uptake into CHO-T cells occurred at a constant rate, with progressive intracellular accumulation over 1 h at 37 °C. On the basis of the extracellular concentration of HRP used in these experiments, and the assayed cellular HRP content after a 1 h incubation period, these cells pinocytose extracellular medium at a basal rate of approx. 40 nl/h per 10⁶ cells, in agreement with pinocytosis rates previously reported for a variety of cell types [34]. In separate experiments with fluoresceinated dextran as an independent marker of the extracellular medium, CHO-T cells were observed by fluorescence microscopy to internalize the extracellular medium within numerous distinct vesicles distributed throughout

Figure 1 Insulin stimulates fluid-phase pinocytosis in CHO-T cells

CHO-T cells grown to a density of 2×10^5 cells per well in 6-well plates were incubated in KRBB containing HRP (1 mg/ml) at 37 °C in the absence (■) or presence (▲) of 100 nM insulin. At the indicated times, pinocytic cellular uptake of HRP was assayed spectrophotometrically as described in the Materials and methods section. Each point represents a single determination and the experiment shown is representative of five independent experiments.

Figure 2 Effect of insulin concentration on fluid-phase pinocytosis in CHO-T cells

CHO-T cells were grown to 2×10^5 cells per well in 6-well plates and incubated in KRBB containing HRP (1 mg/ml) at 37 °C for 1 h in the presence of the indicated insulin concentrations, before assay of pinocytic HRP uptake. Each point represents the mean $\pm$ S.E.M. for triplicate wells, and is representative of two independent experiments.

the cytoplasm (results not shown), consistent with fluid uptake through internalized pinosomes.

Insulin markedly enhanced fluid-phase pinocytosis in these CHO-T cells, as is evident in Figure 1. During the first 10 min after the addition of 100 nM insulin, the initial rate of pinocytosis increased approx. 3-fold compared with controls, but thereafter the rate of pinocytosis returned to approximately the basal rate. In the experiment shown in Figure 1, the net accumulation of HRP nearly doubled after 1 h in the presence of insulin. Averaging five independent experiments, 100 nM insulin increased HRP accumulation over 1 h by 3.44 ± 1.56 -fold. This augmentation of pinocytosis occurred at physiological insulin concentrations, i.e. detectable at 100 pM insulin, maximal at 100 nM insulin, and with a concentration yielding half-maximal effect of 2 nM insulin (Figure 2). This insulin concentration dependency is consistent with the insulin-stimulated pinocytosis resulting from insulin binding to its own receptor.

On activation of the insulin receptor, the IRS-1 protein undergoes tyrosine phosphorylation, enabling IRS-1 to bind and activate the 85 kDa regulatory α -subunit of PI-3-kinase [24,38]. Previous studies implicate PI-3-kinase activity in insulin's regulation of vesicle trafficking [20–22], as well as receptor-mediated endocytosis [23]. Wortmannin, a fungal metabolite, inhibits PI-3-kinase at nanomolar concentrations [39]. As shown in Figure 3, wortmannin potently inhibited insulin-stimulated fluid-phase pinocytosis in CHO-T cells by approx. 50 % at a concentration of 7.5 nM, with complete inhibition at 50 nM. Wortmannin only marginally affected the basal rate of pinocytosis, even at 50 nM, the highest concentration tested. Other studies have shown that insulin-stimulated PI-3-kinase activity in CHO-T cells is comparably inhibited by wortmannin over the same concentration range [40]. Thus these observations suggest that in CHO-T cells, insulin-stimulated PI-3-kinase activity is involved in the regulation of fluid-phase pinocytosis.

Fluid-phase pinocytosis in β -cells

Next we examined fluid-phase pinocytosis in cultured β TC6-F7 cells, an insulin-secreting, clonal β -cell line. This β -cell line originated in mice expressing a transgene encoding the SV40 T antigen under the control of the insulin promoter. During

Figure 3 Effect of wortmannin on basal and insulin-stimulated fluid-phase pinocytosis in CHO-T cells

CHO-T cells (2×10^5 cells per well) were incubated as described in the legends to Figures 1 and 2 at the indicated wortmannin concentrations, in the absence (○) or in the presence (●) of 100 nM insulin. Wortmannin, prepared as a 10 mM stock solution in DMSO, was added to achieve the indicated final concentrations. DMSO vehicle alone was added to controls to achieve the same final concentration (0.001 %, v/v). Insulin was prepared as a stock solution in 10 mM HCl; equivalent amounts of HCl were added to controls. Each point represents the mean $\pm$ S.E.M. for triplicate wells, and the experiment shown is representative of three independent experiments.

prolonged propagation in culture, β TC6-F7 cells maintain a highly differentiated phenotype, with high insulin production and correct glucose responsiveness [33]. Glucose, in the presence of the cholinergic agonist carbachol, markedly stimulated insulin release, inducing an approx. 5-fold increase in insulin secretion over basal levels during incubation for 100 min (Figure 4). Neither glucose nor carbachol alone were effective secretagogues, consistent with previous reports ([33], and results not shown).

Increased fluid-phase pinocytosis accompanies secretagogue-induced insulin release from β TC6-F7 cells, as shown in Figure 5. When these β -cells are exposed to glucose in the presence of carbachol, the net rate of HRP internalization increased signifi-

Figure 4 Time course of stimulated β -cell insulin secretion

β TC6-F7 cells were plated in 12-well dishes and grown to 10^5 cells per well. Incubation in KRBB containing 0.1% BSA at 37 °C was for the indicated times, in the absence (■) or in the presence (□) of 15 mM glucose and 0.5 mM carbachol. Insulin released into the supernatant was determined by radioimmunoassay. Each point represents the mean $\pm$ S.E.M. for triplicate wells, and the experiment shown is representative of three independent experiments.

Figure 5 Time course of stimulated fluid-phase pinocytosis in β -cells

β TC6-F7 cells were plated in 6-well dishes and grown to 10^5 cells per well. Incubations were at 37 °C in KRBB containing 0.1% BSA in the absence (■) or in the presence (▲) of 15 mM glucose and 0.5 mM carbachol, and pinocytic HRP uptake was assayed at the indicated times. Each point represents the mean $\pm$ S.E.M. for triplicate wells, and the experiment shown is representative of five independent experiments.

Figure 6 Glucose concentration dependence of stimulated fluid-phase pinocytosis in β -cells

β TC6-F7 cells were grown to 10^5 cells per well in 6-well dishes. Incubations and pinocytic HRP uptake assays were performed as described in the legend to Figure 5, at the indicated glucose concentrations, at a constant carbachol concentration (0.5 mM). Each point represents the mean $\pm$ S.E.M. for triplicates and the experiment shown is representative of two independent experiments.

cantly. In ten independent experiments, 25 mM glucose and 0.5 mM carbachol increased pinocytosis on average by 1.65 ± 0.21 -fold during a 2 h incubation. Neither glucose nor carbachol alone stimulated fluid-phase pinocytosis (results not shown), suggesting that secretory activity of these cells is required for augmented pinocytosis. In separate experiments with cells preloaded with HRP, secretagogues did not affect the rate of HRP exocytosis from β -cells or from CHO-T cells (results not shown). Therefore the net increase of HRP accumulation within these β -cells during stimulated secretion is attributable to an increased rate of uptake, not to a decreased rate of HRP efflux from the cells. Figure 6 shows the glucose concentration dependence for the stimulation of fluid-phase pinocytosis in β TC6-F7 cells, assayed at a constant carbachol concentration. Half-maximal stimulation occurred at a glucose concentration between 5 and 10 mM. The same concentration range is half-maximally effective in promoting insulin secretion in these cells [33]. This result

Figure 7 Effect of wortmannin on basal and secretagogue-stimulated fluid-phase pinocytosis in β -cells

β TC6-F7 cells (10^5 cells per well) were incubated as described in the legend to Figure 5 for 2 h at 37 °C in KRBB containing 0.1% BSA and the indicated wortmannin concentrations, in the absence ($\square$) or in the presence ($\blacksquare$) of 15 mM glucose and 0.5 mM carbachol. Pinocytic cellular HRP uptake was measured as described for Figure 5, and is expressed on the ordinate as the percentage of the maximal level of pinocytosis (secretagogue-stimulated in the absence of wortmannin). Each point represents the mean $\pm$ S.E.M. for triplicate wells, and the experiment shown is representative of three independent experiments.

Table 1 Effect of wortmannin on β -cell insulin secretion

Insulin released from β TC6-F7 cells (10^5 cells per well) incubated for 2 h at 37 °C in KRBB with 0.1% BSA, also containing the indicated additions. Results are insulin concentrations (nM) and are expressed as means $\pm$ S.E.M. for triplicate wells. Similar results were obtained in two additional independent experiments.

Addition	Insulin secreted (nM)	
	Without wortmannin	With 20 nM wortmannin
None (control)	2.13 $\pm$ 0.25	2.21 $\pm$ 0.27
0.5 mM Carbachol	3.44 $\pm$ 0.21	3.21 $\pm$ 0.10
15 mM Glucose	3.00 $\pm$ 0.75	2.90 $\pm$ 0.32
15 mM Glucose/0.5 mM carbachol	12.46 $\pm$ 2.40	10.98 $\pm$ 0.54

indicates that enhanced insulin release from these β -cells correlates with increased fluid-phase pinocytosis.

The involvement of PI-3-kinase in β -cell fluid-phase pinocytosis was also investigated. Wortmannin inhibited secretagogue-stimulated pinocytosis approx. 50% at a concentration of 7 nM, with 90% inhibition at 10 nM, as shown in Figure 7. The basal rate of pinocytosis measured in the absence of secretagogues was only minimally influenced by wortmannin. Thus, as in insulin-stimulated CHO-T cells, the augmentation of pinocytosis in β -cells by secretagogues might also involve PI-3-kinase activity. Wortmannin does not inhibit β -cell insulin secretion, as is evident in Table 1, where even a high wortmannin concentration (20 nM) failed to impair significantly the insulin release induced by glucose and carbachol. Therefore the ability of wortmannin to inhibit secretagogue-induced pinocytosis in β -cells is not due to wortmannin inhibition of insulin secretion.

Because the insulin that is secreted into the extracellular medium by the β -cells can bind to the cells' own surface insulin receptors [1], and because insulin binding to its receptor stimulates fluid-phase pinocytosis in other cell types (such as CHO-T; see Figure 1), we tested whether adding exogenous insulin in the

Table 2 Effect of hormones on basal and secretagogue-stimulated fluid-phase pinocytosis in β -cells

Pinocytic HRP uptake in β TC6-F7 cells (10^5 cells per well) was assayed after a 2 h incubation at 37 °C in KRBB with 0.1% BSA, also containing the indicated additions. Results are expressed as the means $\pm$ S.E.M. of triplicates, and the results are representative of four independent experiments. Statistical significance compared with controls: * $P \leq 0.05$.

Addition	HRP uptake (A_{405})
None (control)	0.366 $\pm$ 0.023
15 mM Glucose/0.5 mM carbachol	0.539 $\pm$ 0.022*
100 nM Insulin	0.400 $\pm$ 0.019
100 nM IGF-1	0.381 $\pm$ 0.043
15 mM Glucose/0.5 mM carbachol/100 nM insulin	0.552 $\pm$ 0.018*
15 mM Glucose/0.5 mM carbachol/100 nM IGF-1	0.501 $\pm$ 0.029*

absence of secretagogues would stimulate fluid-phase pinocytosis in β TC6-F7 cells. We observed that added insulin did not influence the rate of fluid-phase pinocytosis in these β -cells. At all insulin concentrations tested and even at the maximal hormone levels shown in Table 2, insulin was ineffective in stimulating fluid-phase pinocytosis in these β -cells. Preincubating the β -cells in 100 nM insulin for 2 h did not affect subsequent stimulation of pinocytosis induced by secretagogues. Identical results were obtained with insulin-like growth factor 1 (IGF-1) (Table 2). Although insulin did not stimulate pinocytosis in β TC6-F7 cells, we demonstrated under the same experimental conditions that exogenous insulin was indeed activating the insulin receptor tyrosine kinase of these β -cells. Insulin receptor immunoprecipitation and anti-phosphotyrosine immunoblotting experiments showed that exogenous insulin induced the tyrosine phosphorylation of cellular proteins, including the 95 kDa insulin receptor β -subunit, in a manner essentially identical with that already demonstrated in the parental β TC-3 cell line ([1], and results not shown). Collectively these results indicate that insulin receptor activation is not necessarily coupled to the regulation of fluid-phase pinocytosis. Moreover, the results indicate that the effect of secretagogues to augment pinocytosis in insulin-secreting β -cells is not mediated via autocrine activation of the β -cell surface insulin receptor.

DISCUSSION

Previous studies have shown that cultured insulin-secreting β -cell lines express authentic insulin receptors [1,2]. Moreover we recently detected expression of the mRNA encoding the insulin receptor in freshly isolated rat islet β -cells [41]. Therefore it is probable that normal pancreatic islet β -cells possess cell-surface insulin receptors. When cultured β -cells release insulin, the extracellular insulin can bind back to the β -cells' own surface insulin receptors [1]. Insulin binding triggers the receptor's tyrosine kinase activity, promoting receptor autophosphorylation and the tyrosine phosphorylation of intracellular effector proteins such as IRS-1. In turn, IRS-1 interacts with other effector proteins including PI-3-kinase, thereby propagating the insulin signal downstream. This signalling pathway mediates certain of insulin's biological effects in conventional insulin target tissues such as liver and skeletal muscle, where the diverse actions of insulin on cellular metabolism and function have been characterized in great detail (reviewed in [42]). However, in β -cells, which like other cells express IRS-1 [1] and probably PI-3-kinase [32], the potential regulatory effects of β -cell insulin receptor activation on β -cell structure and function have not

been investigated systematically. The goal of the present investigations was to evaluate a candidate physiological function for the β -cell insulin receptor: the regulation of β -cell fluid-phase pinocytosis. For comparison we analysed the effect of insulin on fluid-phase pinocytosis in CHO-T cells, a cell line known to be insulin-responsive.

In endocrine and neuronal cells, the exocytotic fusion of secretory vesicles with the plasma membrane is followed by enhanced vesicular endocytosis of the cell surface [15,43]. Interrupting the endocytic component of secretory vesicle trafficking can also disrupt vesicular exocytosis [44]. Exocytosis and endocytosis are apparently balanced, perhaps to keep the cell surface area constant. However, the molecular mechanism that couples these two processes is uncertain. In intact islet β -cells, morphometric measurements show that insulin secretion is accompanied by increased vesicular internalization of the extracellular medium [45]. Endocytosis of the β -cell plasma membrane might also be rate-limiting for insulin secretion, as evidenced by membrane capacitance measurements on isolated β -cells [19]. On the basis of these considerations, and because insulin is known to stimulate vesicular endocytosis in several non-secretory cell types [12–14], in the present study we determined whether insulin receptor activation could influence endocytic vesicular trafficking in the β -cell. By regulating endocytosis and thereby indirectly affecting exocytosis, the β -cell insulin receptor might enable β -cell secretion to be influenced by the circulating insulin level.

In our experiments we observed that exogenous insulin did not influence the rate of fluid-phase pinocytosis in insulin-secreting β -cells, even though exogenous insulin was activating the β -cell-surface insulin receptor tyrosine kinase under the same assay conditions. And although fluid-phase pinocytosis did increase significantly when the β -cells were exposed to secretagogues (glucose and carbachol), this stimulation was not even partly mimicked in the absence of secretagogues by the direct addition of exogenous insulin. Although low-level preactivation of the β -cell-surface insulin receptor probably occurs before insulin addition (owing to a low basal rate of constitutive insulin secretion from the β -cells), had there been any significant effect of exogenous insulin on pinocytosis then the addition of hormone at saturating concentrations would have activated the receptors fully, yielding a measurable augmentation of HRP uptake. Moreover, it is important to note that secretagogues stimulated pinocytosis equally well whether or not the β -cells had been pretreated and then continuously exposed to high, saturating exogenous insulin levels during the uptake assay period. This latter observation demonstrates that the initiation of secretagogue-induced fluid-phase pinocytosis is not dependent on autocrine insulin receptor activation. Autocrine activation of β -cell IGF-1 receptors by insulin, which might result from the very high insulin concentrations that are likely to exist near the β -cell surface during secretion, is also not involved, because saturating doses of exogenous IGF-1 also had no effect on β -cell pinocytosis. Evidently activation of the cell-surface insulin receptor (or IGF-1 receptor) is not obligatorily linked to the stimulation of fluid-phase pinocytosis in the β -cells we studied. A similar dissociation between insulin receptor activation and fluid-phase pinocytosis has been described previously in rat adipocytes [13], a cell type with well-characterized metabolic responses to insulin. Hence the inability of exogenous insulin to induce fluid-phase pinocytosis in the β -cell does not preclude the possibility that insulin might influence yet other aspects of β -cell physiology via the insulin receptor. Further investigations will be required to define the physiological consequences of insulin receptor activation in the insulin-secreting β -cell. Moreover it will be important (although difficult) to extend such studies to islet β -cells, because cultured

β -cell lines, such as β TC6-F7, do not necessarily retain all the normal characteristics of islet β -cells *in vivo*.

A second issue addressed by our study pertains to the role of PI-3-kinase in regulating fluid-phase pinocytosis. We discovered that in CHO-T fibroblasts, nanomolar concentrations of wortmannin, an irreversible and non-competitive inhibitor of PI-3-kinase (reviewed in [39]), effectively blocked the stimulatory effect of insulin on fluid-phase pinocytosis. This observation implicates PI-3-kinase activity in the hormonal regulation of fluid-phase pinocytosis and is consistent with the reduced membrane ruffling and pinocytosis noted in cells expressing a mutant p85 α subunit of the PI-3-kinase [46]. Additionally we found that secretagogue-induced fluid-phase pinocytosis in β TC6-F7 β -cells was inhibited by wortmannin at nanomolar concentrations. Because wortmannin might not be an entirely specific inhibitor of PI-3-kinase activity, even at nanomolar concentrations [47], it remains to be demonstrated directly that PI-3-kinase activity is indeed required for vesicular pinocytosis in β -cells. Nevertheless these observations provide new evidence that in insulin-responsive CHO-T cells and also in insulin-secreting β -cells, a common, wortmannin-sensitive reaction, most probably involving PI-3-kinase, participates in both insulin-dependent and insulin-independent fluid-phase pinocytosis.

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