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Research Article

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Binding and Internalization of Somatostatin, Insulin, and Glucagon by Cultured Rat Islet Cells

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Abstract

The pathways by which islet B, A, and D cells bind and internalize homologous (self) and heterologous (other) islet hormones were compared. [^{125}I-Tyr]Somatostatin-14 (S-14), ^{125}I-insulin, and ^{125}I-glucagon were incubated with monolayer cultures of neonatal rat islet cells. Tissues were processed for quantitative electron microscopic autoradiography by the probability circle method coupled to morphometry. For all three radioligands and all three cell types surface labeling was rapidly followed by internalization of the radioligands into endocytotic vesicles. The further intracellular movement of the ligand occurred in a time- and temperature-related manner and depended on whether it was homologous or heterologous for the cell in question. Thus [^{125}I-Tyr]S-14 in B and A cells, ^{125}I-insulin in A and D cells, and ^{125}I-glucagon in B and D cells were rapidly transferred from endocytotic vesicles to lysosomal structures. By contrast, [^{125}I-Tyr]S-14 in D cells, ^{125}I-insulin in B cells, and ^{125}I-glucagon in A cells showed poor progression from endocytotic vesicles to downstream vesicular structures. We conclude that (*a*) each of the three radioligands is internalized by islet cells in a time- and temperature-dependent manner; (*b*) after initial internalization the further intracellular progression of the endocytosed radioligand occurs freely in cells heterologous for the radioligand but poorly in cells homologous for the radioligand; and (*c*) binding and endocytosis can be uncoupled from lysosomal degradation of ligand.

Introduction

Receptor-mediated endocytosis is a general process through which polypeptide hormones and other agents that bind to high affinity sites on cell surfaces are internalized (1–3). The intracellular path of the ligand-receptor complex follows a fairly typical route which involves initial binding of the ligand to the plasma membrane, aggregation of the ligand-receptor complexes around coated pits, the internalization of the complexes via endocytotic vesicles, and the transport and fusion of these vesicles with primary lysosomes (1–15). In the resulting secondary lysosomes the complexes undergo dissociation, the ligand is degraded, and the receptor is freed for recycling to the plasma membrane. Internalization occurs rapidly and is a time- and temperature-dependent process (3–10).

Specific, high-affinity binding sites for somatostatin (16–18), insulin (17, 19), and glucagon (17, 20, 21) have been demonstrated on normal whole islets (16, 19), purified islet B cells (21), and neoplastic islet cells (17, 18) by direct binding assays. Using the technique of quantitative electron microscopic autoradiography, we have previously identified specific binding sites for radioiodinated somatostatin-14 (S-14),[1] ^{125}I-insulin, and ^{125}I-glucagon on neonatal islet B, A, and D cells in monolayer culture (22, 23). We have extended these observations and now describe the internalization of each radioligand by the three islet cell types.

Methods

Islet cell cultures and labeling experiments. Monolayer cultures of dispersed islet cells from 3-d-old rats were established in plastic Petri dishes as previously described (22, 23). The medium consisted of MEM with 10% heat-inactivated FCS. The glucose concentration was maintained at 16.5 mM until day 5 of culture when it was reduced to 5.5 mM for 1 d before the labeling experiments which were conducted on day 6. Under these conditions islet cells grew in monolayer clusters rich in A, B, and D cells (24). Petri dishes contained on an average ~ 30–40 clusters, each containing 60–108 islet cells.

Porcine monocomponent insulin (Novo Industrie, Copenhagen, Denmark), porcine glucagon (Novo Industrie), and synthetic tyrosinyl-somatostatin (Tyr-S-14; Bachem Fine Chemicals, Torrance, CA), were radioiodinated by the chloramine-T technique as previously reported (22, 25). The specific activities of the three radioligands were 499 Ci/mmol ^{125}I-insulin, 1,365 Ci/mmol ^{125}I-glucagon, and 1,355 Ci/mmol [^{125}I-Tyr]S-14.

Before commencement of the labeling experiments the maintenance media were discarded and the cells washed with 2 ml MEM-FCS. Paired Petri dishes were then incubated at 15 or 37°C for 5 or 60 min with 1 ml of medium (MEM-FCS, 5.5 mM glucose) containing radioiodinated hormones at the following concentrations: ^{125}I-insulin, 5.05 nM; ^{125}I-glucagon, 0.5 nM; and [^{125}I-Tyr]S-14, 0.83 nM. Control dishes were incubated with the same concentrations of labeled hormones in the presence of excess (0.3–0.6 μM) unlabeled hormones. In parallel incubations the concentration of endogenous islet hormone secretion at 37°C was determined as follows: insulin, 0.46 nM/dish per 60 min; glucagon, 0.05 nM/dish per 60 min; somatostatin, 0.006 nM/dish per 60 min. These secretion rates were calculated to result in hormone concentrations in the medium 10–140 times lower than those of the corresponding ligands for the 37°C, 60-min incubations, and 110–1,600 times lower for the 37°C, 5-min incubations.

At the end of the incubation period the radioactive media were removed, the Petri dishes washed three times with MEM in PBS, and the cultures fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at room temperature for 4 h. The fixed cells were then dehydrated with ethanol, embedded in Epon, and sectioned. High

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1. *Abbreviations used in this paper:* ARG, autoradiographic grains; S-14, somatostatin-14.

resolution autoradiography was performed by coating the sections with a thin emulsion of Ilford L4 according to Caro et al. (26) followed by incubation at 4°C for 4–5 wk. After development with Microdol X, the sections were examined in an electron microscope (model 301; Philips Electronic Instruments, Inc., Switzerland).

Evaluation of grain distribution. For each incubation condition four separate cell clusters were evaluated completely. A total of 1,767 B cells, 786 A cells, and 486 D cells were examined. The total number of grains analyzed was 529 for [^{125}I-Tyr]S-14, 454 for ^{125}I-insulin, and 285 for ^{125}I-glucagon. All determinations were carried out on positive prints enlarged to a final magnification of 39,000. To estimate the binding of the three radioligands to the islet cells, all autoradiographic grains (ARG) present in a given cluster were photographed. The distance between the center of each grain and the surrounding membranes was then measured and expressed as half distances. In the event that an ARG in the intracellular space was exactly equidistant between the different types of cells, we gave half a chance to each cell to be related to the ARG. The half distance was assumed to be 900 Å and we could then apply the universal curve for ^{125}I-radiation. The grain distribution was calculated according to the method of Salpeter and Bachmann (27). We considered grains located within 250 nm of the plasma membrane as noninternalized (10), those located beyond the 250-nm line as internalized. Results were expressed as percent of internalized grains over total grain number.

To estimate the probability that the radiation source lies within a given tissue compartment of the endocrine cells, we applied to the same pictures as the half distances, the 95% probability circle (Whur et al.) (28) coupled to the morphometric analysis of the size of the compartment by the point counting method (29). This method corrects for the overlap of a single developed grain with several different structures. The following compartments were considered: cell membrane, microvilli, endocytotic vesicles, lysosomes, multivesicular bodies, Golgi apparatus, secretory granules, and nucleus. We applied the formula $Rvi^+ = Gi/Pi\ (5 \cdot d^2 \cdot T)$, where Rvi^+ = specific radiation label density, Gi = number of points of the 95% probability circle falling on a compartment i, Pi = number of points of the morphometrical lattice falling on a compartment i, d = the point spacing of the lattice, and T = the section thickness. Data are expressed as Rvi^+, which represents the number of grains per μM^3 of a given compartment (i).

Results

Labeling of islet cells. Autoradiographic grains associated with each radioligand were localized over B, A, and D cells, the three major islet cell types found in this system. The grains were distributed over the plasma membrane as well as intracellularly. The grains were specific and were virtually eliminated when ligands were coincubated with their corresponding unlabeled hormones in excess. The percent of islet cells labeled with each radioligand at 37°C has been previously described (22). In general, there was a time- and temperature-dependent increase in the labeling density of the islet cells by the three radioligands. Labeling with [^{125}I-Tyr]S-14 was maximal over A cells, 58–82% of which contained autoradiographic grains under the different incubation conditions. The percent of B and D cells labeled with [^{125}I-Tyr]S-14 varied between 31 and 40% for B cells and 24 and 35% for D cells under the same conditions. ^{125}I-Insulin-related grains were maximally distributed over B cells (46–74% of cells labeled) followed by A cells (21–48% of cells labeled) and D cells (22–37% of cells labeled). The highest density of ^{125}I-glucagon-related grains occurred over B cells (28–52% of cells labeled) followed by D cells (18–49% of cells labeled) and A cells (10–23% of cells labeled).

Internalization of [^{125}I-Tyr]S-14, ^{125}I-insulin, and ^{125}I-glucagon. At 15°C incubation for 5 min ARG were generally symmetrically distributed around the plasma membranes of B, A, and D cells. Internalization of radioligand occurred in a time- and temperature-dependent manner for each radioligand. Characteristic ligand- and cell-specific differences in internalization were noted, however. For [^{125}I-Tyr]S-14, translocation of label from the region of the plasma membrane to the cell interior occurred efficiently in B and A cells only. The somatostatin-producing D cells exhibited poor internalization even after 60 min at 37°C (Fig. 1). Similar findings were observed in the case of ^{125}I-insulin, which showed 38–40% internalization of all cell-associated ARG after 60 min at 37°C in A and D cells, compared with only 20% in B cells. For ^{125}I-glucagon, extensive penetration of radioligand occurred in B and D cells (~ 60% of all specific ARG); in A cells, however, only ~ 30% of cell-associated ARG were internalized after 60 min at 37°C.

Subcellular distribution of [^{125}I-Tyr]S-14-, ^{125}I-insulin-, and ^{125}I-glucagon-related grains. Morphometric evaluation of the distribution of [^{125}I-Tyr]S-14-related ARG over the plasma membrane and various cytoplasmic compartments is portrayed in Fig. 2. For plasma membrane, the Rvi^+ exhibited a time- and temperature-dependent decrease in B and A but not D cells, confirming the findings in Fig. 1. Labeling of microvilli was demonstrated for each of the three cell types. Only B cells, however, exhibited a time-dependent decrease in Rvi^+; microvilli labeling of A and D cells appeared to be constant. Labeling of the endocytotic vesicles paralleled that of plasma membrane, i.e., high initial labeling followed by significant reduction with time and temperature in B and A but not D cells. By contrast, labeling of the multivesicular bodies and Golgi area followed a reciprocal pattern to that observed for plasma membrane and endocytotic vesicles, i.e., progressive increase in labeling with time and temperature in B and A but not D cells. The lysosomal compartment was significantly labeled in the case of the A and B cells; weak labeling of D cell lysosomes was also observed, but only at the 60-min time points. Labeling of the secretory granules and nucleus was indistinguishable from background for all three cell types.

Significant accumulation of ^{125}I-insulin-related ARG was seen over plasma membranes, microvilli, and endocytotic vesicles of B, A, and D cells (Fig. 3). Labeling of multivesicular bodies, lysosomes, and Golgi was significant only for A and D but not for B cells. Secretory granules and nuclei of all cells exhibited only background labeling. In the case of B cells, labeling was confined to the plasma membrane and endocytotic vesicles and showed no progression with time and increasing temperature to other intracellular compartments. A signifi-

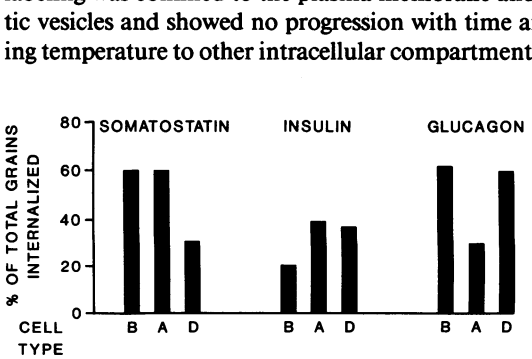

Figure 1. Percent internalization of ^{125}I-labeled hormones within pancreatic endocrine cells (60 min at 37°C). ARG located within 250 nM of the plasma membrane were considered noninternalized; those localized beyond 250 nM were regarded as internalized. For each cell type results are expressed as percent of internalized grains over total grain number.

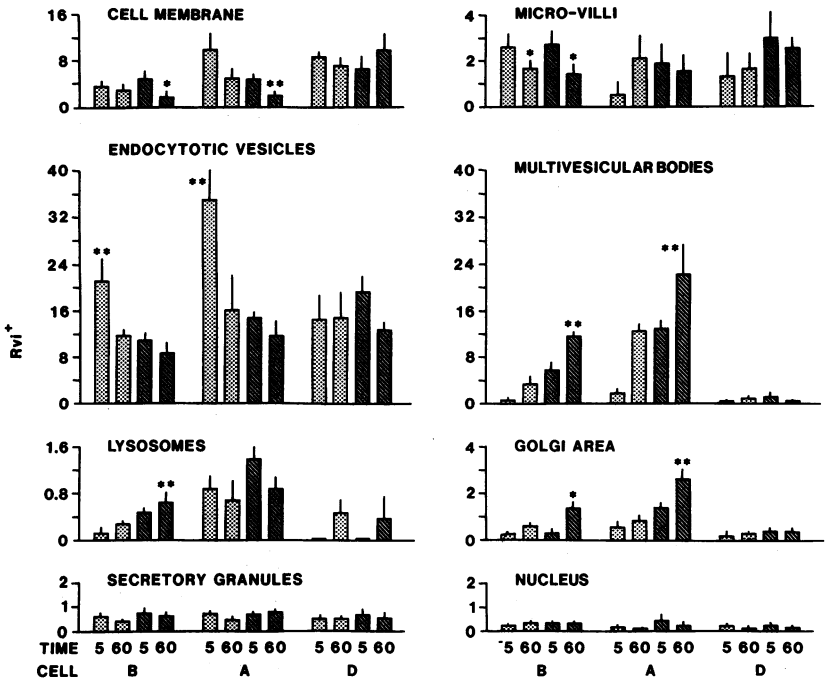

Figure 2. Pattern of autoradiographic labeling of B, A, and D cell compartments after exposure of cultured islet cells to [^{125}I-Tyr^{1}] S-14 for 5′ or 60′ at 15 (*dotted bars*) and 37°C (*hatched bars*). Data are expressed as relative activity Rvi^+ in a given subcellular compartment as evaluated quantitatively with the probability circle method coupled to morphometry. $^*P < 0.01$ vs. the preceding value. $^{**}P < 0.01$ vs. the three other values in the group.

cant time-dependent reduction in ARG accumulation in endocytotic vesicles at 37°C was noted, although this too was not accompanied by increased labeling of distal compartments. By contrast, grains associated with the plasma membrane and endocytotic vesicles of A and D cells decreased with increasing time and temperature of incubation concomitant with the appearance of label in distal compartments such as lysosomes and Golgi.

Labeling of subcellular compartments with ^{125}I-glucagon followed a generally similar pattern to that for [^{125}I-Tyr]S-14 and ^{125}I-insulin (Fig. 4). Thus, after initial labeling of plasma membranes, microvilli, and endocytotic vesicles of all three cell types, the Rvi^+ for multivesicular bodies and Golgi showed an increase only for B and D but not A cells. Lysosomal labeling was observed in all three cell types, being prominent in D cells, weak in B cells, and present only in the 5-min, 15°C incubation condition in A cells.

Discussion

Islet cells influence each other through hormone produced either locally or delivered via the systemic circulation (30–33). There is now good evidence that each of the three islet hormones, insulin, glucagon, and somatostatin, are capable of exerting effects on A, B, and D cells (34–41). Thus, somatostatin is a potent inhibitor of A and B cells (34), insulin is capable of suppressing A and D cells (35, 36), and glucagon is known to stimulate B and D cells (37, 38). Furthermore, each of the three hormones is capable of regulating its own secretion through autofeedback (39–41). All these effects are believed to be mediated through specific receptors located on the plasma membrane. Such receptors have been characterized biochemically for insulin and somatostatin in normal rat islets (16, 19), for glucagon in purified rat B cells (21), and for all three hormones in rat islet cell tumor (17, 18). By autoradiography we

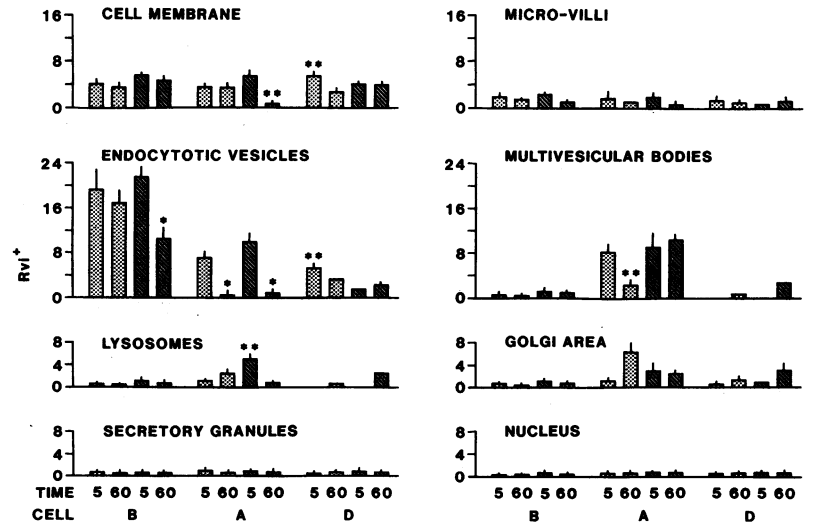

Figure 3. Pattern of autoradiographic labeling of B, A, and D cell compartments after exposure of cultured islet cells to ^{125}I-insulin. Conditions and method of analysis as for Fig. 2. $^*P < 0.01$ vs. the preceding value. $^{**}P < 0.01$ vs. the three other values in the group.

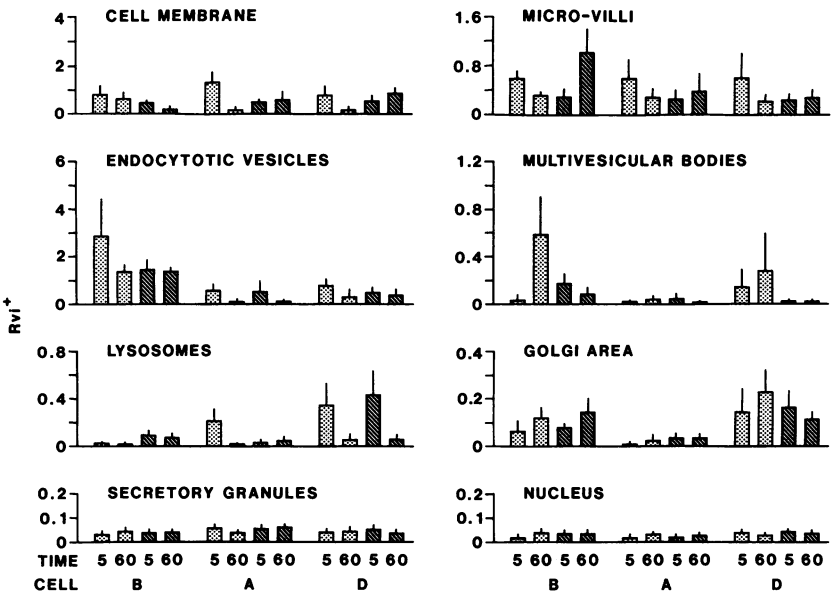

Figure 4. Pattern of autoradiographic labeling of B, A, and D cell compartments after exposure of cultured islet cells to ^{125}I-glucagon. Conditions and method of analysis as for Fig. 2.

have previously demonstrated the existence of specific, saturable binding sites for labeled insulin, glucagon, and somatostatin on B, A, and D cells in monolayer cultures of neonatal rat islets (22, 23). We have now studied the internalization of each radioligand in each of the three islet cell types and traced the fate of the radioligands from their initial site of binding on the plasma membrane to intracellular sites of processing. We have analyzed our results by two quantitative methods. First, we have used the line source analysis of Salpeter to determine that the three radioligands localized to the plasma membrane of the cell at early times of incubation. Second, we used the probability circle method to determine whether internalized grains are preferentially associated with intracellular organelles. These techniques are similar to those applied previously for studying the binding, internalization, and subcellular distribution of ^{125}I-insulin (4, 6–8, 10, 12), ^{125}I-glucagon (9), ^{125}I-human growth hormone (42), ^{125}I-epidermal growth factor (5), and ^{125}I-LDL (43) in a variety of target cells.

Binding of [^{125}I-Tyr]S-14, ^{125}I-insulin, and ^{125}I-glucagon occurred over both plasma membrane and microvilli. Whether grains localized on other specialized regions of the plasma membrane such as coated invaginations, previously described for ^{125}I-insulin (10) but not for ^{125}I-glucagon binding (9), was not investigated in the present study. After initial binding the radioligands were rapidly internalized in a time- and temperature-dependent fashion and became progressively associated with vesicular structures: endocytotic vesicles, multivesicular bodies, lysosomes, and Golgi structures. The intracellular pathway for the three radioligands in the islet cells was thus in general similar to that resulting from receptor-mediated endocytosis reported for LDL and epidermal growth factor in cultured human fibroblasts (5, 43), insulin in lymphocytes, hepatocytes, and adipocytes (4, 6–8, 10, 12), glucagon in hepatocytes (9), human growth hormone in lymphocytes (42), and gonadotropin-releasing hormone (15), corticotrophin-releasing hormone (44), and somatostatin (13, 14) in anterior pituitary cells. There were, however, notable cell- and ligand-specific differences in the rate and extent of accumulation of radiolabel in the various subcellular structures. For all three radioligands and all three cell types, the appearance of label in endocytotic vesicles subjacent to the plasma membrane was virtually simultaneous with binding to plasma membrane and microvilli. In the case of somatostatin, the accumulation of [^{125}I-Tyr]S-14-related ARG in these vesicles was maximal for the 5-min, 15°C incubation and rapidly decreased with increasing time and temperature in B and A but not D cells. This decrease was due to transfer of label to multivesicular bodies, lysosomes, and Golgi vesicles, structures downstream in the endocytotic pathway. Except for a very small accumulation of [^{125}I-Tyr]S-14-related ARG in lysosomes after the two 1-h incubations, such progression of radioactivity beyond endocytotic vesicles to downstream vesicular structures was prominently lacking in D cells. In the case of insulin as well, a similar pattern of labeling of the subcellular compartments was observed. Thus, radioactivity was rapidly translocated from the plasma membrane to endocytotic vesicles in all three cell types and subsequently transferred to the other vesicular structures in only A and D cells. Although the B cells contained the highest overall accumulation of label, this remained concentrated in endocytotic vesicles, with an apparent block in the transfer of radioactivity to other intracellular compartments. In the case of glucagon, despite the poor concentration of ARG in the various intracellular compartments, the pattern of internalization of the surface-bound radioactivity was once again similar to that found with the other two radioligands. Thus, A cells in contrast to B and D cells were conspicuous for their paucity of ARG in multivesicular bodies, lysosomes, and Golgi structures. Overall, then, the data are consistent with the thesis that after initial binding and internalization of all three radioligands in all three islet cell types, the further intracellular progression of the endocytosed ligand to lysosomal and other vesicular structures occurs freely in cells heterologous for the radioligand but poorly in cells homologous for the radioligand. Occasional departures from this general rule, however, were observed, e.g., (*a*) a small accumulation of [^{125}I-Tyr]S-14 ARG in D cell lysosomes (Fig. 2), (*b*) a reduction in the labeling density of ^{125}I-insulin in B cell endocytotic vesicles after 60 min at 37°C (Fig. 3), and (*c*) a small time-dependent reduction in ^{125}I-glucagon labeling of A cell endocytotic vesicles. These inconsistencies are relatively

minor, and in most instances probably reflect internal methodological variations inherent in the morphometric quantitation of weakly labeled subcellular compartments.

The internalization of receptor-bound insulin has been extensively studied in several cell systems (4, 6–8, 10, 12). Likewise, the internalization of glucagon by hepatocytes and peripheral leukocytes has been fully characterized (9, 45). This, however, is the first report of the internalization of either radioligand by islet cells, with findings very similar to those reported for other target systems except for (*a*) the poor intracellular processing of both ligands in islet cells homologous for the ligand, and (*b*) a more rapid rate of internalization of ^{125}I-glucagon in B and D cells compared with hepatocytes (9). The internalization of somatostatin has been studied only in the anterior pituitary using as markers radioiodinated tyrosinated analogue of S-28 or gold-conjugated S-14 (13, 14). The pattern of labeling of the subcellular compartments in the somatotroph with these techniques was comparable to that observed for A and B cells in the present study, with the exception of significant association of ligand with nucleus and secretory granules in one of the two pituitary studies (14). We were unable to demonstrate any significant labeling with [^{125}I-Tyr]S-14 of nuclear structures in any of the three islet cell types. Additionally, despite autoradiographic and biochemical evidence for somatostatin receptors on secretory granules both in the pituitary and in islets (14, 46), no evidence for this was found in the present study. Finally, our demonstration of rapid intracellular translocation of plasma membrane receptor-bound [^{125}I-Tyr]S-14 in normal islet cells differs markedly from the behaviour of [^{125}I-Tyr^{11}]S-14 bound to surface receptors on RIN m5F insulin-producing tumor cells which have been reported to process receptor-bound ligand through degradation in situ by surface-bound enzymes, rather than through internalization by receptor-mediated endocytosis followed by lysosomal degradation (47, 48).

To our knowledge this is the first time that internalization of a hormone by a cell that normally secretes it has been described in detail. Our results show a dissociation between binding and internalization on the one hand, and subsequent lysosomal degradation of the internalized ligand on the other. Thus, the plasma membrane binding of [^{125}I-Tyr]S-14, ^{125}I-insulin, and ^{125}I-glucagon to D, B, and A cells, respectively, was coupled to rapid internalization and the appearance of the ligands in endocytotic vesicles. The subsequent transfer of the radioactivity to lysosomal structures, however, appeared to be inhibited in each of the three cells. These data clearly illustrate that binding and endocytosis can be uncoupled from lysosomal degradation of ligand in islet cells and perhaps all secretory cells that internalize their own (homologous) radioligand. Occasional exceptions to this general rule were observed, probably due to methodological variations, although alternative explanations cannot be excluded. The process through which endocytotic vesicles are directed towards lysosomes is poorly understood but is believed to involve the juxtaposition of endocytotic vesicles and lysosomes during movement of the organelle through the cytoplasm followed by fusion of the membranes of the two structures (49). This step appears to be rate limiting and can be selectively inhibited by perturbations such as low temperature (50). Furthermore, substances within the endocytotic vesicle or the lysosome have been shown to inhibit fusion (49). In the case of insulin, glucagon, and somatostatin cells internalizing their own ligands, such signals for fusion must reside in the ligand-receptor complex. It is conceivable that in cells such as B, A, and D, which release secretory products by exocytosis and internalize some of the released material via autoreceptors, the step requiring fusion of endocytotic vesicles and lysosomes is saturated through continuous lysosomal destruction of a high volume of incoming ligand. Alternatively, the presence of hormone homologous to the ligand in storage vesicles may inhibit competitively the transfer of endocytosed ligand to lysosomal structures.

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