Cellular distribution of secretin receptor expression in rat pancreas

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1Division of Digestive Diseases, Department of Internal Medicine, University of Cincinnati, Cincinnati, Ohio 45267-0595; 2Division of Gastroenterology and Hepatology, Department of Internal Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261; and 3Center for Basic Research in Digestive Diseases, Departments of Internal Medicine and Biochemistry/Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905

Ulrich, Charles D. II, Paul Wood, Elizabeth M. Hadac, Elizabeth Kopras, David C. Whitcomb, and Laurence J. Miller. Cellular distribution of secretin receptor expression in rat pancreas. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1437–G1444, 1998.—Secretin is an important regulator of pancreatic function, but the molecular basis of its actions is not well understood. We have, therefore, used in situ autoradiography, photoaffinity labeling, and RNase protection assays with healthy rat pancreas, dispersed acinar cells, and pancreas depleted of acinar cells to explore the cellular distribution and molecular identity of high-affinity secretin receptors in this complex organ. The autoradiographic examination of 125I-labeled [Tyr10]rat secretin-27 binding to normal pancreas demonstrated saturable and specific high-affinity binding sites on both acinar and duct cells, with a uniform lobular distribution, but with no binding above background over islets or vascular structures. Photoaffinity labeling demonstrated that the ductular binding site in acinar cell-depleted copper-deficient rat pancreas represented the same glycoprotein with a molecular weight of 50,000–62,000 that was present on acinar cells. RNase protection assays confirmed the molecular identity of the secretin receptors expressed on these distinct cells. The apparent absence or extreme low density of similar secretin receptors on islets and pancreatic vascular structures suggests that the pharmacological effects of secretin on those cells may either be indirect or mediated by another secretin family receptor that recognizes this hormone with lower affinity.

peptide hormone; hormone receptor; receptor autoradiography; copper-deficient rat

SINCE ITS DISCOVERY AS THE first hormone in 1902 by Bayliss and Starling (3), secretin has been implicated in numerous regulatory events, involving the pancreas (28, 44), biliary tree (1, 2), stomach (25, 46), nerves (37), and even the kidney (5). These span both physiological and pharmacological actions. In the pancreas, secretin has been implicated as a stimulant of ductular bicarbonate and acinar zymogen secretion, islet hormone secretion, and as a regulator of blood flow. The molecular basis of each of these actions is not well understood.

The rat is a particularly useful animal model for the pancreatic effects of this hormone. In this species, analogous to studies in humans, secretin is the principal hormonal stimulant of ductular bicarbonate and water secretion and a contributor to meal-stimulated acinar zymogen secretion (4, 7, 10, 14, 30, 34–36). Islet effects include stimulation of glucagon and somatostatin, although the latter requires high concentrations of secretin and the simultaneous presence of a CCK-like agonist (30, 48). Insulinotropic effects have been described in humans, dogs, and pigs but not in rats. Increases in pancreatic blood flow have been attributed to the effect of secretin to stimulate cardiac output and enhance vasodilation (23), but these require higher concentrations of secretin than those necessary to stimulate pancreatic secretion (32).

A secretin receptor cDNA has been cloned from the mouse/rat neural hybrid NG108-15 cell line and intact rat pancreas (20, 45) and has been found to be prototypic of a new family in the G protein-coupled superfamily of receptors (class II) (44). Other receptors in this family can also respond to high concentrations of secretin, but to date, no structurally unrelated receptor has been shown to respond to this hormone. Affinity labeling of the recombinant secretin receptor and of the predominant high-affinity binding protein naturally expressed in rat pancreas has yielded identical patterns, with a band migrating at a molecular weight (Mw) of 50,000–62,000 (13, 45). This was also the same as that labeled on isolated pancreatic acinar cells (13), which represent almost 90% of pancreatic cells. Because duct cells, islets, and vascular smooth muscle represent relatively minor populations in the pancreas, the molecular identity of secretin receptors on those cells has yet to be determined. Indeed, the broad curves for competition for secretin binding to pancreatic membranes and the precedent for receptor heterogeneity raise the possibility that distinct secretin receptor molecules could be present on different types of cells (4, 44).

The objectives of this study were to determine the cellular distribution of high-affinity secretin receptors within rat pancreas and to establish the molecular identity of secretin receptors expressed in those cells. Cellular distribution in normal rat pancreas was assessed by in situ receptor autoradiography utilizing 125I-labeled [Tyr10]rat secretin-27, an analog that binds specifically and with high affinity to native pancreatic secretin receptors (11, 45). Based on the presence of saturable and specific high-affinity binding to only
pancreatic acinar and duct cells, these were the focus of our molecular receptor characterization. Duct cell receptors were examined in the copper-deficient rat model of acinar cell atrophy (10, 14, 27). Pancreatic plasma membranes prepared from these animals were affinity labeled with \( ^{125}I \)-labeled [Tyr\(^{10}\)-p-NO\(_2\)-Phe\(^{22}\)]rat secretin-27 and found to have the identical molecular characteristics to those prepared from healthy pancreatic membranes. The identity of the ductular and acinar cell high-affinity secretin-binding proteins was further confirmed by RNase protection assays utilizing a \( ^{32}P \)-labeled rat secretin receptor riboprobe. These results provide important insight into the physiological mechanisms through which secretin may or may not control pancreatic exocrine secretion, endocrine secretion, and blood flow in the rat.

**METHODS**

Materials. Synthetic rat secretin and porcine vasoactive intestinal peptide (VIP) were either purchased from Peninsula Laboratories (Belmont, CA) or synthesized at the Mayo Clinic or in the Peptide Facility at the University of Pittsburgh Cancer Institute. Protected amino acids and methylbenzyldryamine resin were from Advanced ChemTech (Louisville, KY) and Peninsula Laboratories (Belmont, CA). All solvents were HPLC grade, and all reagents were either analytic or molecular biology grade.

Animals. Wistar and Sprague-Dawley rats were used as the source of pancreatic tissue in these studies (Harlan Sprague Dawley, Indianapolis, IN). Protocols were reviewed and approved by the Mayo Clinic and University of Pittsburgh Animal Care and Use Committees.

Synthesis of secretin analogs. [Tyr\(^{10}\)]rat secretin-27 and [Tyr\(^{10}\)-p-NO\(_2\)-Phe\(^{22}\)]rat secretin-27 were synthesized, and radiolabeled utilizing either a modified lactoperoxidase method or the solid phase oxidant N-chlorobenzensulfonamide sodium salt (Pierce Chemical, Rockford, IL). Products were purified by reverse-phase HPLC, as we have previously described (45, 47). In situ receptor autoradiography studies utilizing \( ^{125}I \)-labeled [Tyr\(^{10}\)]rat secretin-27 radiolabeled by either method yielded identical results.

In situ receptor autoradiography. Male Wistar rats weighing 300 g were killed after an overnight fast by administration of 100 mg/kg pentobarbital ip. The pancreas and forestomach were then rapidly excised and placed in ice-cold 0.9% sodium chloride. The organs were quickly trimmed, embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), and snap-frozen in an isopentane bath immersed in liquid nitrogen. Blocks containing pancreas and forestomach were serially sectioned (20 µm; utilizing a cryostat with chuck temperature set at \(-18^\circ\)C and with chamber temperature at \(-15^\circ\)C) and thaw mounted on Superfrost/Plus microscope slides (VWR, South Plainfield, N. J.). The tissue-bearing slides were frozen at \(-80^\circ\)C with CaSO\(_4\) desiccant in duct tape-sealed slide boxes until use. Immediately before performing receptor autoradiography, we removed boxes with slides from the freezer and allowed them to reach room temperature to prevent condensation.

The slides were then incubated in a prehybridization buffer \([10 \text{mM} \text{HEPES containing } 0.1 \text{mg/ml soybean trypsin inhibitor (STI)}] \) three times for 8 min at \(22^\circ\)C. The binding buffer was modified from that used by Steiner et al. (39) to include \(10 \text{mM} \text{HEPES, pH } 7.4, 130 \text{mM NaCl, 4.7 mM KCl, 5 mM MnCl}_2, 1 \text{mM EGTA, 1% BSA, 1 mg/ml bactopain, 0.1 mg/ml STI, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 \mu g/ml leupeptin, and 2 \mu g/ml chymostatin. After incubation in binding buffer containing } ^{125}I \)-labeled [Tyr\(^{10}\)-p-NO\(_2\)-Phe\(^{22}\)]rat secretin-27 at 240,000 cpm/ml (300 pM) and between 1 pM and 1 µM noniodinated rat secretin analog for 2 h at room temperature, slides were washed in ice-cold binding buffer without secretin two times for 5 min, rinsed for 5 s in ice-cold distilled water, dried initially in a stream of cold air, and then dried overnight in a duct tape-sealed desiccant-filled box at room temperature.

The slides were then placed in tight apposition to the emulsion side of single-emulsion Hyperfilm (Amersham, Arlington Heights, IL) without use of an intensifying screen. The initial exposure was typically 7 days. Film was developed in D-19 (Eastman Kodak, Rochester, NY), and the slide-mounted tissue sections were subsequently stained with hematoxylin and eosin for comparison to autoradiograms. Autoradiograms were analyzed both visually and using quantitative microdensitometry as we have previously described (47). Because of the nonlinear sensitivity of the film, radioactive standards (Amersham) were exposed to Hyperfilm together with the tissue sections and used to correct the sample densities. Saturable binding was determined by subtracting the density of nonspecific binding (binding in the presence of 1 µM unlabeled peptide) from the density measured in the total binding samples. All results displayed are typical of a minimum of three independent series of experiments.

Membrane preparation from copper-deficient rat pancreas. Rats were fed a diet deficient in copper and supplemented with n-penicillamine (2 g/kg) for 8 wk, with the extent of acinar cell atrophy assessed morphologically (10). After organ removal, the atrophic pancreas was trimmed of fat, and enriched plasma membranes were prepared using a modification of the method previously described (45). In brief, the excised pancreas was brought up to 10% wt/vol in 0.3 M sucrose containing 0.01% STI and 1 mM PMSF and homogenized with a glass Dounce homogenizer, four strokes with a type B pestle followed by four strokes with a type A pestle. The sample was then filtered through cheesecloth, and enough 2.0 M sucrose was added to bring the final concentration to 1.3 M sucrose. This homogenate was then overlaid with 0.3 M sucrose and centrifuged for 3 h at 149,000 g in a swinging bucket rotor. Membranes floating to the 0.3–1.3 M interface were collected, washed, and stored at \(-70^\circ\)C in a modified Krebs-Ringer-HEPES solution with protease inhibitors (KRH) (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 1 mM KH\(_2\)PO\(_4\), 0.2% BSA, 0.01% STI, and 1 mM PMSF).

Photoaffinity labeling. Photoaffinity labeling studies were performed as described previously (45). In brief, initial binding of radiolabeled probe to membranes was performed in the presence of increasing concentrations of secretin and VIP. The membranes were then pelleted by centrifugation, washed, and resuspended in KRH without BSA in 12 × 75 mm borosilicate tubes in preparation for photolysis. This was performed for 30 min at 4°C in a Rayonet model RP-100 apparatus (Southern New England Ultraviolet, Hamden, CT) equipped with 300-nm lamps, with the cooled samples separated from the lamp by 5.7 cm. Photolysis conditions were previously established to assure specific activation of the p-NO\(_2\)-Phe residue (45). Membranes were collected by centrifugation and analyzed by 10% SDS-PAGE followed by autoradiography. The M\(_s\) values for affinity-labeled proteins were calculated from a plot of log M\(_s\) vs. mobility of standard proteins. IC\(_{50}\) values for covalent labeling were determined using densitometric scanning of autoradiograms.

Enzymatic deglycosylation. Affinity-labeled membranes (50 µg) were prepared for deglycosylation by suspension in 50 µl
of 0.1 M sodium phosphate, pH 6.1, containing 50 mM EDTA, 0.1% Nonidet P-40, 0.1% SDS, and 1% 2-mercaptoethanol. Endoglycosidase F (5 U) was incubated with the sample for 12 h at 37°C, and samples were analyzed by SDS-PAGE and autoradiography, as described above.

RNA extraction. Total RNA was prepared from normal rat heart and kidney by the method of Chomczynski and Sacchi (6) and from normal rat pancreas, dispersed pancreatic acini, and pancreatic tissue from copper-deficient rats by the method of Han et al. (15). Dispersed pancreatic acini were prepared by collagenase perfusion and mechanical dissociation, as previously described (12). Pancreatic tissue from copper-deficient rats was trimmed of fat in ice-cold PBS before RNA extraction. In each case, the quality of the purified total RNA was verified through formaldehyde-agarose gel electrophoresis with ethidium bromide staining.

Rat secretin receptor cDNA probe. The sequence corresponding to nt 380-698 of the cDNA encoding the rat secretin receptor was prepared by PCR utilizing a rat pancreatic cDNA library as template (1, 45). This was cloned into the SmaI site of pGEM4Z (Promega, Madison, WI), and its identity was confirmed by double-stranded DNA sequencing.

RNase protection assays. RNase protection assays were performed with total RNA (200 µg), using the RPA II kit (Ambion, Austin, TX) according to the vendor’s instructions (1). In brief, an antisense 32P-labeled riboprobe was transcribed from the rat secretin receptor cDNA fragment in pGEM4Z utilizing T7 RNA polymerase and [32P]UTP (800 Ci/mmol, Amersham). The primary RNA transcript was purified by excision from a 5% acrylamide-8 M urea denaturing gel with subsequent elution at 37°C into a solution of 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% SDS. After hybridization at 45°C for 12 h, the solution was treated with a mixture of RNase A/1 (150–200 U/ml) to digest unhybridized RNA chains, and the protected hybrid (~320 bp) was resolved on a 5% acrylamide-8 M urea gel and detected by autoradiography.

RESULTS

In situ receptor autoradiography. Although radioligand binding to sections of normal and neoplastic rat and human pancreas has been reported (40, 41, 43), determination of the specific cellular distribution of receptors has been impaired by autolysis and poor preservation of tissue architecture. We have been able to minimize these changes using a revised protocol for autoradiography of radioligand binding to unfixed tissue sections. Figure 1 illustrates the results of in situ receptor autoradiography utilizing 100 pM [125I]-labeled [Tyr10]rat secretin-27 and OCT-embedded sections from normal rat pancreas. Figure 1 (left) depicts a representative photomicrograph of a hematoxylin and eosin-stained section of normal rat pancreas after incubation in the prehybridization, binding, and wash buffers. Although some alteration in cellular morphology is evident within this section compared with the paraformaldehyde-fixed section shown in Fig. 2A, higher resolution images of the unfixed tissue (seen in Fig. 2, C, E, and G) clearly demonstrate preservation of islet, vascular, and ductular morphology sufficient to discern cell type-specific radioligand binding.

Figure 1 (middle and right) also shows typical dark-field photomicrographs representing total and nonspecific binding of 100 pM radioligand to the tissue sections. Total binding was determined in the absence of unlabeled competitor, and nonspecific binding was determined in the presence of 1 µM unlabeled secretin analog, with each representing an exposure to the emulsion for 7 days. The specificity of radioligand binding to the circular and longitudinal smooth muscle layers within rat forestomach in the same OCT-embedded frozen section was identical to that reported

![Fig. 1. Autoradiographic localization of secretin receptors in rat pancreas utilizing 125I-labeled [Tyr10]rat secretin-27 and OCT-embedded frozen sections of healthy rat pancreas. Thawed slides were preincubated in 10 mM HEPES, pH 7.4, incubated in binding buffer containing 100 pM radioligand in the presence of increasing concentrations of unlabeled rat secretin analog for 2 h, washed, dried, placed in apposition to Hyperfilm, and exposed for 7 days. Left: low-power photomicrograph of a representative section of hematoxylin and eosin (H&E)-stained normal rat pancreas after incubation with binding buffer containing radioligand in the absence of unlabeled secretin. Examples of individual islets (open arrow), blood vessel traversing an islet (solid arrow), and duct (parallel solid pointers) are identified and enlarged in Fig. 2. Middle dark-field photomicrograph of tritium-sensitive film that overlaid the section at left for 7 days (total binding). Open areas, representing areas of high concentrations of radioligand binding, were present over acinar and duct cells but not over islets or vascular structures. Right: dark-field photomicrograph of control section adjacent to section shown at left that was incubated with the same concentration of radioligand in the presence of an excess of unlabeled secretin (1 µM) [nonspecific binding (NSB)].](http://ajpgi.physiology.org/doi/full/10.1152/ajpgi.00003.2017)
secretin receptors on pancreatic acinar and duct cells, we performed RNase protection assays with a gel-purified 32P-labeled riboprobe. Figure 5 shows recognition of the mRNA encoding this protein with the previously reported rat pancreatic secretin receptor mRNA dimer migrated in the expected position. The intensity of the signal was similar in total pancreas and purified pancreatic acini, with both considerably less intense than the signal from copper-deficient rat pancreas.

Comparison of secretin receptor mRNA expression in whole organ, isolated pancreatic acini, and acinar cell-deficient rat pancreas. To confirm the molecular identity of the mRNA encoding this protein with the previously reported rat pancreatic secretin receptor and to quantify the levels of secretin receptor mRNA relative to total RNA in both pancreatic acinar and duct cells, we performed RNase protection assays with a gel-purified 32P-labeled riboprobe. Figure 5 shows results of a typical assay utilizing equal amounts of total RNA isolated from normal intact pancreas, dispersed pancreatic acini, and pancreas from copper-deficient rats, as well as normal rat heart (positive control) and kidney (negative control) (1). The 32P-labeled riboprobe/mRNA dimer migrated in the expected position. The intensity of the signal was similar in total pancreas and purified pancreatic acini, with both considerably less intense than the signal from copper-deficient rat pancreas.

DISCUSSION

Secretin is a gastrointestinal peptide hormone with multiple effects on the rat pancreas. These include stimulation of bicarbonate and enzyme secretion by low concentrations of hormone (4, 10, 14, 30, 34), and stimulation of certain islet hormones and pancreatic blood flow by higher concentrations of hormone (23, 30,
The molecular basis of these actions is not well understood. The current study focuses on the secretin receptor distribution among the complement of potential hormone-responsive cells in the rat pancreas. We have done this using tissue section autoradiography, photoaffinity labeling, and RNase protection assays in intact tissue, dispersed cells, and acinar cell-depleted pancreas.

The in situ receptor autoradiography utilized a unique protocol that preserved the pancreatic cellular architecture better than other techniques previously applied to this protease-rich organ and employed a low concentration of a selective radioligand (100 pM 125I-labeled [Tyr10]rat secretin-27). Tissue preservation during the incubations was adequate to provide clear morphological identification of each type of cell in the pancreas. The resolution of secretin binding sites was enhanced by the microscopic examination of an autoradiographic emulsion that was present on only a single side of the film that had been held in tight association with the tissue section and used without an intensifying screen. The image of the precipitated silver grains was projected over the morphological section to provide unambiguous cellular localization of receptors. As a further positive control, the secretin analog used as probe in this study was also shown to bind specifically and with high affinity to the muscularis externa of rat forestomach, consistent with the findings of an independent report (39).

In the current series of studies, this technique resulted in saturable, high-affinity radioligand binding only over pancreatic acinar and duct cells, with no signal above background over islets or vascular elements. Of note, there was uniform ligand binding throughout the entire pancreatic lobule, over the relevant cells. The reduced or absent signal over cells that were previously reported to respond to secretin could be explained in several ways. Those cells could have a very
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Fig. 5. RNase protection assays for secretin receptor mRNA utilizing total RNA (200 ng) isolated from intact pancreas, dispersed pancreatic acini, and copper-deficient rat pancreas, as well as normal rat heart (positive control) and kidney (negative control). Total cellular RNA was hybridized to a [32P]-labeled antisense riboprobe transcribed from a rat pancreatic secretin receptor cDNA. The protected hybrid (~320 bp) was resolved in a 5% acrylamide-8 M urea gel and detected by autoradiography. Although the intensity of the signal was similar in both intact pancreas and pancreatic acini, both were considerably less intense than that from copper-deficient rat pancreas.

low density of secretin receptors that is below the threshold for detectability using this technique. The cells could also be responding indirectly through another mediator coming from some cell that is secretin responsive or could be responding by expressing a receptor that might be structurally related to the secretin receptor but that has a lower affinity for this hormone. Neuronal mediation of secretin action would be a good candidate for indirect action (8, 22, 24, 26, 29).

A receptor that may be playing a role in such effects is the VIP-1/pituitary adenylate cyclase-activating polypeptide-2 receptor that binds and is activated by nanomolar concentrations of VIP and micromolar concentrations of secretin (44). Indeed, nerve fibers containing VIP have been localized to pancreatic islets, where VIP can stimulate glucagon secretion (16), and VIP has been reported to stimulate pancreatic blood flow in the rat (21). The higher concentration dependency for islet and vascular responses to secretin in some studies may be consistent with these mechanisms (32, 48).

The molecular identities of the predominant secretin-binding sites present on acinar and duct cells were explored using complementary techniques. Photoaffinity labeling is a method to characterize a radioligand-binding protein biochemically, based on its migration on an SDS-polyacrylamide gel. Unfortunately, G protein-coupled receptors are typically poorly resolved on such gels, and most receptors in a given family are approximately the same size and migrate similarly. Application of this technique to intact pancreas, dispersed acinar cells, and to acinar cell-depleted pancreas (27, 45) resulted in labeled bands migrating similarly, with an apparent M_r of 50,000–62,000. Deglycosylation of this band to yield a protein core of an apparent M_r of 42,000 provided further support for the similar nature of receptors on acinar and duct cells, but this was still not definitive. We, therefore, proceeded to a higher resolution molecular technique. RNase protection assays confirmed the sequence identity of secretin receptor mRNA in each of these tissue and cell preparations.

The demonstration that the same secretin receptor molecule mediates the actions of this hormone on both acinar and duct cells complements and extends previous studies that support direct effects of secretin on both of these cells (4, 14, 30). Secretin binds specifically and with high affinity to membranes from isolated rat pancreatic acinar cells (4) and activates adenylyl cyclase and phospholipase C pathways in these cells (42). In isolated ducts, physiological concentrations of secretin induce intracellular cAMP generation and opening of select chloride channels that drive bicarbonate secretion (14, 30, 33).

Crucial for the current report was the animal model established by Muller in 1970 (27), in which rats fed a diet deficient in copper developed pancreatic acinar cell atrophy in the absence of ductular changes. Subsequently, more consistent atrophy was attained through supplementation of this diet with 0.3%pyrdilamine (10). Morphological analysis of the pancreas from rats fed this diet showed a marked reduction in gland weight due to noninflammatory acinar cell atrophy, with relative enrichment of morphologically intact ductal and islet cells. Biochemical and electrophysiological characterization of ductular elements from these animals has confirmed functional identity with ducts from normal pancreas (10, 14).

Studies establishing the identity of secretin-binding proteins in pancreatic acinar cells and ducts may have considerable impact on future physiological studies and pharmacological strategies. For instance, it will be of importance to define key residues within the ligand-binding domain of the secretin receptor and to delineate specific interactions between residues within secretin and this binding domain, as well as the influence of these interactions on molecular determinants of receptor coupling to specific G proteins (e.g., G_s and G_q) (9, 17–19, 31). With a more detailed understanding of the structures and conformations involved, selective agonists and antagonists might be developed for this receptor. Such agents may help us unravel the physiological roles of specific second messengers activated by secretin in cells bearing these receptors, better assess the contribution of other mechanisms to the physiology of those cells (e.g., vagal stimulation (8, 24, 29)), and determine the therapeutic value of such agents in models reflective of human disease (e.g., cystic fibrosis (38)).

The relative enrichment of secretin receptor-encoding mRNA as a percentage of total RNA isolated from acinar cell-deficient rat pancreas, compared with that in whole organ and isolated acini, suggested the possibility of cell-specific enrichment for this mRNA in ducts. This could reflect enhancement in transcription of secretin receptor mRNA in duct cells and may predict a cell-specific enhancer element. However, these data may also be explained by enhanced stability of secretin
receptor mRNA in duct cells relative to acinar cells, and the abundance of enzyme-encoding mRNA in acinar cells may diminish the relative contribution of similar amounts of secretin receptor mRNA to the total RNA pool in those cells. Similarities in the intensity of the autoradiographic signal over acinar cells and duct cells suggest similar levels of receptor protein expression on each cell.

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