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Immunocytochemical localization of muscarinic acetylcholine receptors in the rat endocrine pancreas

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Summary. Immunocytochemical application of the anti-muscarinic acetylcholine receptor antibody M35 to pancreas tissue revealed the target areas for the parasympathetic nervous system. Immunoreactivity in the endocrine pancreas was much higher than that in the exocrine part. Moreover, the endocrine cells at the periphery of the islets of Langerhans displayed the highest level of immunoreactivity. Based on these findings in the mantle of the islets, two types of islets have been distinguished: type-I islets with intensely stained mantle cells, and type-II islets with a much lower concentration of these cells. On average, type-I islets were larger (244.8 μm ± 6.1 SEM) than type-II islets (121.5 μm ± 3.8 SEM). M35-immunoreactivity was present on the majority of D cells, which were characterized by their immunoreactivity to somatostatin [of 446 D cells 356 (79.8%) were M35-immunopositive]. However, only a small proportion of the intensely stained mantle cells belonged to the D cell population. Therefore, it is concluded that the majority of the intensely stained mantle cells represent glucagon-secreting A and/or pancreatic polypeptide-secreting F cells. The intensity of M35-immunoreactivity at the periphery and central core of the islets paralleled the density of cholinergic innervation, suggesting a positive correlation between the intensity of cholinergic transmission and the number of muscarinic acetylcholine receptors at the target structures. The present study further revealed some striking parallels for the muscarinic acetylcholine receptor characteristics between the (endocrine) pancreas and the central nervous system.

Key words: Pancreas, endocrine – Immunocytochemistry – Muscarinic acetylcholine receptor – Acetylcholine – Somatostatin – Rat (Wistar)

The endocrine pancreas plays a key role in the regulation of blood glucose levels through insulin release (Fischer et al. 1972; Strubbe and Steffens 1975; Strubbe and Bouman 1978). In the rat endocrine islets of Langerhans, the central portion contains insulin-producing cells (B cells), whereas the mantle consists of glucagon-, somatostatin- and pancreatic polypeptide-producing cells (A, D and F cells, respectively) (Bonner-Weir 1991; Miller 1981; Munger 1981; Orci and Unger 1975). Acetylcholine (ACh), released by parasympathetic nerve fibers, stimulates both insulin release from B cells and somatostatin release from D cells (Honey and Weir 1980; Miller 1981). The released somatostatin inhibits the secretory activity of the A and B cells (Arimura and Fishback 1981; for review, see Wood et al. 1983). The stimulatory action of ACh on the endocrine pancreas is blocked by atropine (Frohman et al. 1967; Grill and Östenson 1983; Honey and Weir 1980), indicating that ACh exerts its influence through muscarinic acetylcholine receptors (mAChRs). The close interaction between the parasympathetic innervation, mAChRs, and the inhibitory processes of somatostatin within the endocrine pancreas are essential for the maintenance of normoglycemia during food intake (Schusdziarra et al. 1979).

In previous studies, binding of receptor ligands was performed to shed more light on the autonomic innervation and their postsynaptic receptor distribution. Binding of tritiated mAChR-ligands demonstrated a predominant homogeneous receptor population of the M3 receptor subtype in the endocrine and exocrine pancreas (Henquin and Nenquin 1988; Korc et al. 1987; Peralta et al. 1987; Verspohl et al. 1990). However, more detailed cellular analysis of the endocrine cells endowed with mAChRs has been hampered by the large distances that the energy quanta travel in autoradiographic emulsions (Hamel and Beaudet 1987; Kuhar et al. 1981). Recently, receptor protein immunocytochemistry enabled us to visualize mAChRs at the (sub)cellular level in relation to the cholinergic innervation in the central nervous system (CNS) (Van der Zee et al. 1989). The mAChRs were visualized by employing the extensively characterized monoclonal antibody M35 raised against purified mAChR-protein from bovine forebrain homogenates

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(André et al. 1983, 1984, 1987; Leiber et al. 1984). Since mAChRs play a preeminent role in the regulation of the pancreatic secretory processes, we aimed to substantiate further the mAChR-characteristics of the endocrine pancreas. Therefore, we examined the cellular localization of mAChRs in the endocrine pancreas in relation to the inhibitory peptide somatostatin and the cholinergic innervation.

Materials and methods

Nine young adult male Wistar rats (±300 g body weight) were used. Fixation of the pancreas was carried out by transcardial perfusion of 400 ml fixative consisting of 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) at pH 7.4. The pancreas was dissected and stored overnight at 4° C in 30% buffered sucrose for cryoprotection. Serial sections from the tail of the pancreas (that part of the pancreas part adjacent to the spleen and containing the highest concentration of islets) were cut on a cryostat microtome at a thickness of 20 µm. The sections were thaw-mounted on gelatin-coated slides.

Immunocytochemical procedure

Tissue sections were processed for different immunocytochemical staining procedures. Prior to the first antibody incubation, sections were rinsed in phosphate-buffered saline (PBS), immersed in 0.01% H_2O_2 in PBS, rinsed again, and incubated for 1 h at room temperature (RT) in various normal sera (dependent on the source of the secondary antibodies) to suppress nonspecific antibody binding. Thereafter, the sections were incubated overnight at 4° C, followed by one of three immunocytochemical procedures: (1) mouse anti-mAChR IgM (M35), diluted 1:1000 in 1% normal rabbit serum (NRS); (2) goat anti-choline acetyltransferase (ChAT) IgG [kindly donated by Dr. L.B. Hersh (Bruce et al. 1985)], diluted 1:1000 in 1% NRS and 0.5% Triton X-100; (3) rabbit anti-somatostatin (SOM) IgG (S309, kindly donated by Dr. R. Benoit), diluted 1:6000 in 1% normal goat serum (NGS) and 0.5% Triton X-100. After incubation, the sections were thoroughly rinsed in PBS, preincubated for 1 h with the appropriate normal sera followed by the second antibody incubation for 2 h at RT. Second antibodies were (1) biotinylated rabbit IgG anti-mouse-IgM [mu-chain directed; 1:200; Zymed (San Francisco, USA)] in 1% NRS; (2) rabbit anti-goat IgG [1:100; Sigma (St. Louis, USA)] in 1% NRS; (3) goat anti-rabbit IgG (1:100; Zymed). Sections were again rinsed in PBS (1 h) before the third incubation step (2 h at RT) in (1) HRP-conjugated streptavidin (1:200; Zymed); (2) goat peroxidase-anti-peroxidase (PAP) [1:500; Dakopatts (Glostrup, Denmark)]; (3) rabbit PAP (1:500; Dakopatts). Finally, the sections were processed by the diaminobenzidine(DAB)-H_2O_2 reaction (30 mg DAB and 0.01% H_2O_2/100 ml TRIS buffer).

Double-labeling experiments for the study of the coexpression of mAChRs and somatostatin in single endocrine cells were carried out with fluorescent techniques. The sections were exposed to one of the primary antibodies as for single labeling described above. S309-incubation was followed by phycoerythrin-conjugated goat anti-rabbit IgG [1:200, 2 h at RT; Tago (San Mateo, USA)]. After completion of the S309 staining, the sections were incubated with M35, followed by biotinylated rabbit anti-mouse IgM (1:50, 2 h at RT, Zymed) and fluorescein isothiocyanate(FITC)-conjugated streptavidin (1:200, 2 h at RT, Zymed). Following immunolabeling, the sections were rinsed in PBS, mounted in distilled water and coverslipped in a 1:1 mixture of PBS and glycerin. The sections were studied and photographed with a Ploemopak Leitz fluorescent microscope with the appropriate filter blocks for FITC and phycoerythrin labels, yielding a green and red fluorescence, respectively. Standard control experiments for both single and double labeling were performed by (1) omission of primary antibodies in the incubation cycle; (2) primary antibodies incubated with the non-matching secondary antibodies (in the case of doublelabeling in the sequence of S309 – biotinylated rabbit anti-mouse-IgM – FITC-streptavidin and M35 – phycoerythrin-conjugated goat anti-mouse IgG); and (3) replacing the primary antibody by normal sera. In all cases, the controls yielded negative results, i.e., the absence of any detectable labeling. In the case of fluorescence double labeling, the control experiments excluded the appearance of possible cross-reactivity of secondary antisera during the incubation cycle.

AChE histochemistry

Tissue selected for acetylcholine-esterase (AChE) histochemistry was fixed by immersion in 2.5% glutaraldehyde in PBS overnight at 4° C before processing. The thaw-mounted sections were stained for AChE using silver-nitrate intensification after Hedreen et al. (1985). In short, the sections were incubated for 2 h at RT in a sodium-acetate-buffered solution (0.1 M, pH 6.0), containing acetylthiocholine iodide (25 mg/50 ml medium), sodium citrate (0.1 M), copper sulphate (0.03 M) and potassium ferricyanide (5 mM). The sections were subsequently reacted in sodium sulphide (1%, pH adjusted to 7.8) and silver nitrate (1%) for 90 s each, to reveal black staining of AChE-rich neurons and fibers.

Analysis

After immunocytochemical staining, sections were dehydrated, cleared in xylene, coverslipped and examined by use of a standard light microscope. The interrelation between AChE-positive and ChAT-positive fibers innervating the islets was studied on adjacent sections. The size of the endocrine islets was measured (the longest diameter of the islet in the plane of section) and the number of D cells and intensely stained mantle cells (ism cells, see Results) established. Islets were arbitrarily classified as type I or type II, depending on whether they contained high or low numbers of ism cells, respectively.

Results

Immunolocalization of muscarinic acetylcholine receptors

Both the exocrine and endocrine pancreas reacted specifically with the mAChR-antibody M35. The basic distribution pattern of M35-immunoreactivity became apparent throughout the entire tail of the pancreas. The islets of Langerhans revealed a high level of immunoreactivity, with a variable intensity of M35-immunoreactivity among the endocrine cells. Based on the characteristics of the M35-immunoreactive elements, two types of islets could be distinguished. Ism cells predominated at the periphery of the first type of islet (type I) (Fig. 1 A). These cells were not randomly arranged, but formed a chain of immunopositive cells at the periphery of the islets. The ism cells often surrounded the peripheral blood vessels of the islets (large arrows in Fig. 1 A). The number of ism cells varied considerably between islets. Nevertheless, islets with a nearly complete string of ism cells and islets endowed with only fragments of this rim were considered to be type-I islets. The type-I islets with abundant ism cells forming an almost complete cord in the peripheral area tended to be grouped together

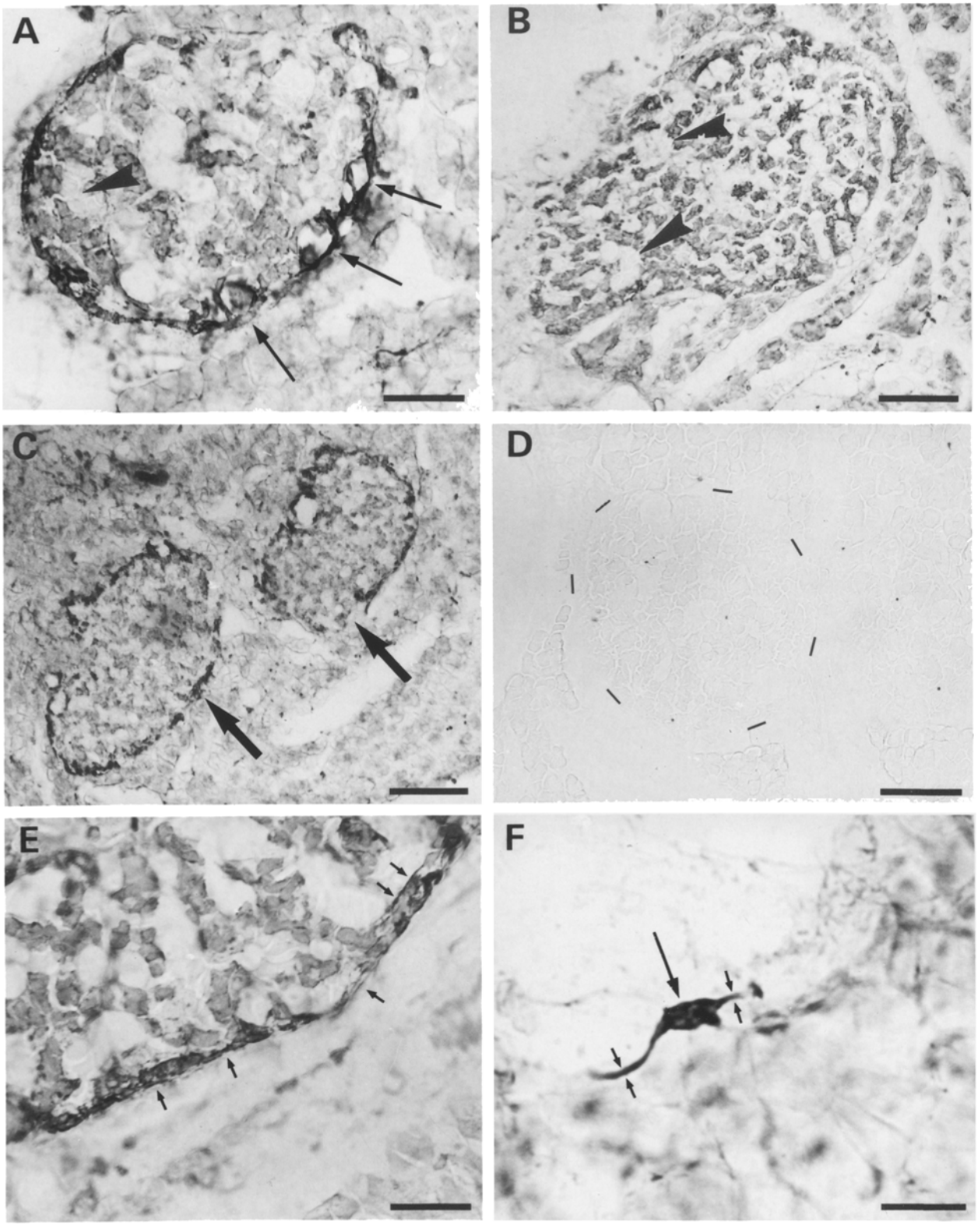

Fig. 1A–F. Photomicrographs of M35-immunoreactivity in the rat pancreas. Two types of islets can be distinguished. At the periphery of type-I islets (**A**), intensely stained endocrine cells are present. Often, these cells form a cord around blood vessels (*arrows* in **A**) and encircle the islet. Type-II islets (**B**) lack this type of cell. Type-I islets are frequently found within close vicinity of each other (**C**; *arrows* indicate type-I islets). Faintly stained or even immunonegative clusters of cells are shown by *large arrowheads* in **A** and **B**. After omission of the primary antibody, the sections reveal no immunoreactivity (**D**; *small lines* delineate an immunonegative islet). In **E**, M35-immunoreactive fiber-like elements (*small arrows*) are present at the mantle of the islets. Small M35-positive nerve cells (**F**) are observed scattered throughout the pancreas, displaying strong labeling in the cell soma (*large arrow*) and the proximal processes (*small arrows*). *Scale bars*: **A** 50 μm, **B** 100 μm, **C** 125 μm, **D** 80 μm, **E** 40 μm, **F** 20 μm

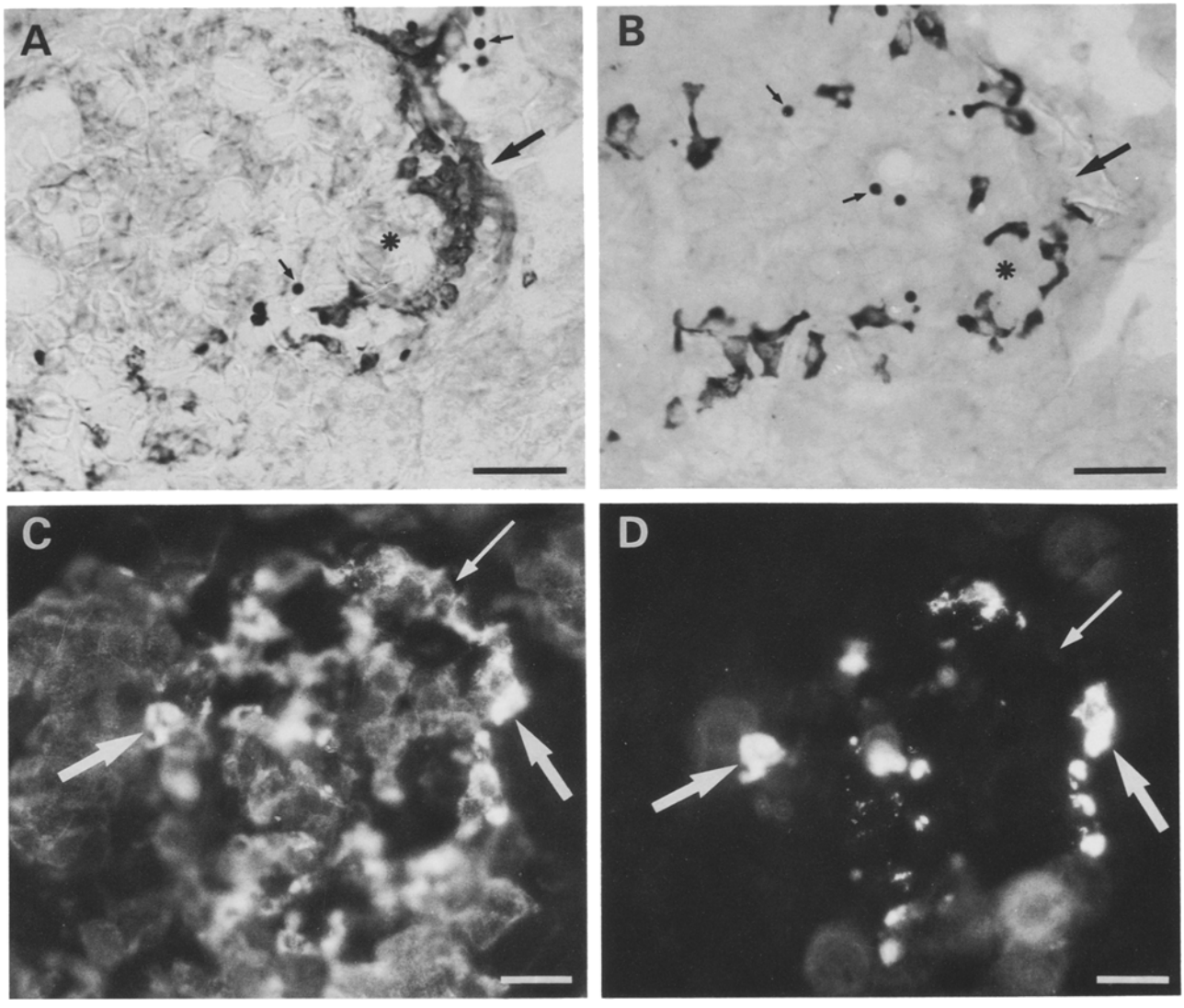

Fig. 2. Photomicrographs of adjacent sections (**A**, **B**) immunoprocessed for mAChRs (**A**) and somatostatin (**B**). Although intensely M35-positive endocrine cells and D cells are found in the periphery of the islets, the adjacent sections reveal only a partial overlap of the both markers. The *large arrows* in **A**, **B** indicate a region that displays large numbers of mAChRs, but that does not contain D cells (*small arrows* indicate erythrocytes). Fluorescent photomicrographs (**C**, **D**) depict the localization of mAChRs (**C**) and somatostatin (**D**) within a single section, clearly indicating the colocalization of these markers (*large arrows*). However, the majority of intensely M35-labeled cells are somatostatin-negative (*small arrow*). The *asterisks* in **A**, **B** serve as a landmark. *Scale bars*: **A**, **B** 40 μm; **C**, **D** 35 μm

in small clusters of 2–5 islets (Fig. 1 C, two islets). The second type of islet (type II) was characterized by the lack of ism cells at the periphery of the islet (Fig. 1 B). Serial sectioning revealed that type-II islets were not merely type-I islets transected in such a way that the ism cells were out of the plane of section. Of 602 islets examined (in 6 animals), 313 (52%) belonged to type I. On average, the latter type of islet was larger than type II [longest diameter: 224.8 μm (±6.1 SEM) vs 121.5 μm (±3.8 SEM) for type I and II, respectively].

In addition to heavily M35-immunoreactive endocrine cells, the mantle of the islet consisted of strongly immunopositive, delicate fiber-like elements (small arrows in Fig. 1 E). The core of the islets contained predominantly less intensely stained endocrine cells. Clusters of weakly immunopositive cells or immunonegative cells were found in both types of islets (arrowheads in Fig. 1 A, B). Omission of the primary antibody during the incubation procedure yielded negative results (Fig. 1 D), as in all other immunocytochemical control experiments.

In the exocrine pancreas, the acini were characterized by a faint to moderate staining intensity for M35. In addition to the labeled acinar cells, strong immunoreactive nerve cells were found, scattered heterogeneously throughout the exocrine tissue (Fig. 1 F). Apart from their clearly stained cell bodies (large arrow in Fig. 1 F), dendritic and possibly axonal processes were observed invading the surrounding tissue (small arrows in Fig. 1 F).

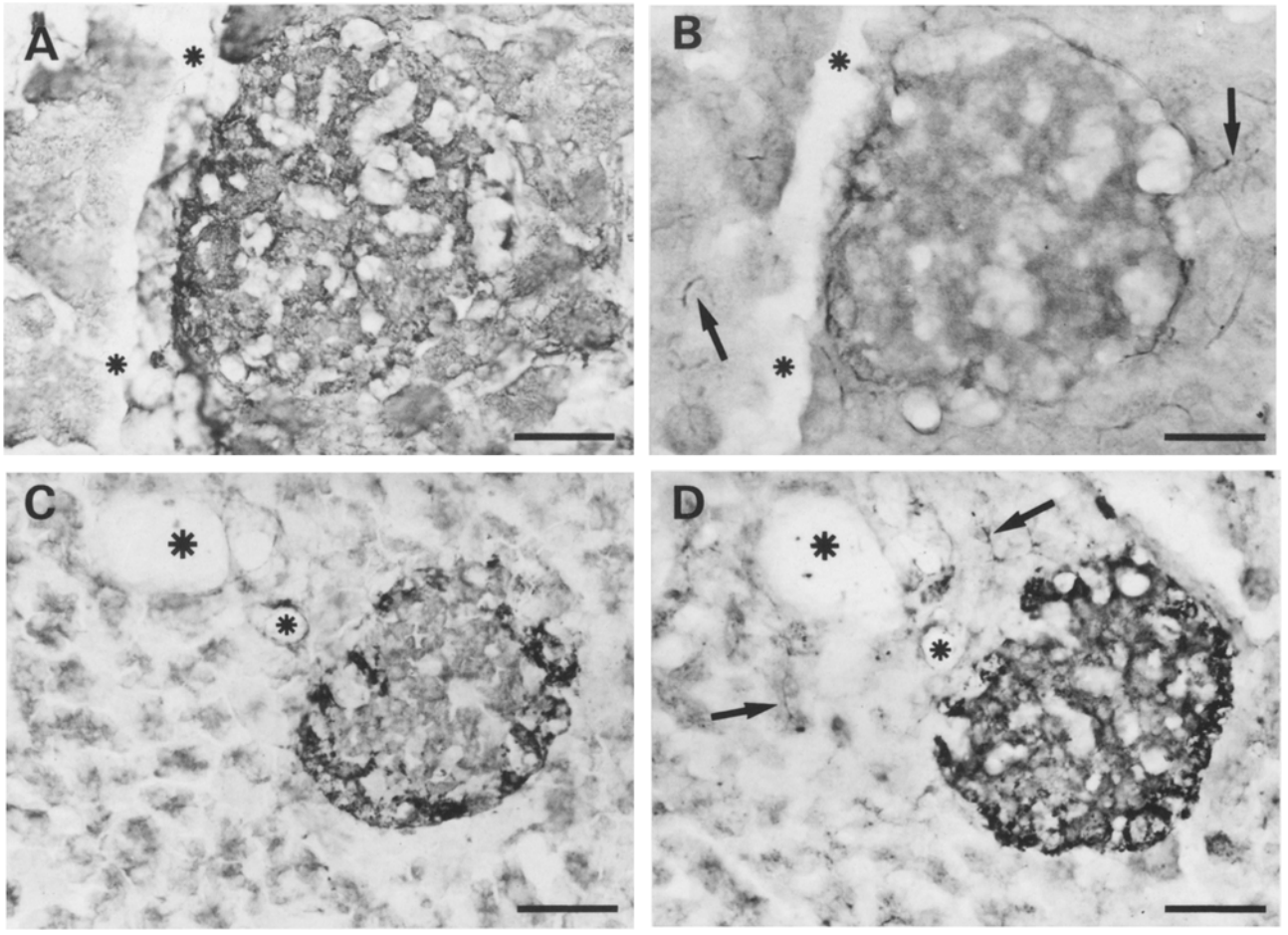

Fig. 3. Adjacent sections (**A**, **B** and **C**, **D**) immunostained for M35 (**A**, **C**) and ChAT (**B**, **D**). ChAT-immunoreactivity dominates at the periphery of the islets and some delicate fibers are present in the surrounding tissue (*small arrows* in **B**, **D**). Islets devoid of intensely stained mantle cells (ism cells) (type-II islets) (**A**) reveal only low to moderate levels of ChAT-immunoreactivity (**B**), whereas in islets with ism cells (type-I islets, **C**), strong ChAT-positive elements are found at the rim of the islets. The *asterisks* indicate landmarks for orientation. *Scale bars*: **A**, **B** 100 μm; **C**, **D** 125 μm

Type of islet cells immunoreactive for mAChRs

The staining pattern for M35 observed in the islets of Langerhans suggested that various endocrine cell types expressed mAChRs. B cells formed the majority of endocrine cells and were predominantly located in the central part of the islet. In this area, numerous cells were immunopositive for M35, albeit with considerable variation in staining intensity between clusters of B cells. However, they were always less intensely stained compared with the ism cells at the periphery of the islet. Adjacent sections showed that the number of ism cells exceeded the number of somatostatinergic D cells (Fig. 2A, B). In 20 sectioned islets, 1981 ism cells were counted versus 818 D cells. Furthermore, immunofluorescence double labeling for somatostatin and M35 also revealed that only a small part of the ism cells expressed somatostatin (Fig. 3C, D). Conversely, 90 (20.2%) of the 446 D cells studied appeared to be single labeled, demonstrating that 79.2% of the D cells were endowed with mAChRs. However, most D cells displayed only a faint immunoreactivity for M35, indicating expression of low levels of mAChRs.

Cholinergic innervation of the islets of Langerhans

The observation of a cord of ism cells expressing abundant amounts of mAChRs in type-I islets prompted us to determine whether the difference in mAChR-expression between the two types of islets was related to the density of cholinergic innervation. In the current study, the cholinergic innervation of the postsynaptic muscarinic cholinoceptive elements of the pancreas was visualized by means of ChAT-immunocytochemistry and AChE-histochemistry.

Adjacent sections revealed that all islets displayed both ChAT-immunoreactivity and AChE-deposits. With regard to ChAT, the ACh synthesizing enzyme, the immunopositive fibers were found most densely at the periphery of the islets, and less dominantly in the central core (Fig. 3B, D). Centrally, the cholinergic innervation revealed considerable variation between (groups of) cells (e.g., Fig. 3D). Compared with type-II islets, the mantle of type-I islets clearly revealed a stronger cholinergic innervation (Fig. 3B, D). The amount of mAChRs thus parallelled the density of cholinergic innervation.

With regard to AChE, the ACh degrading enzyme,

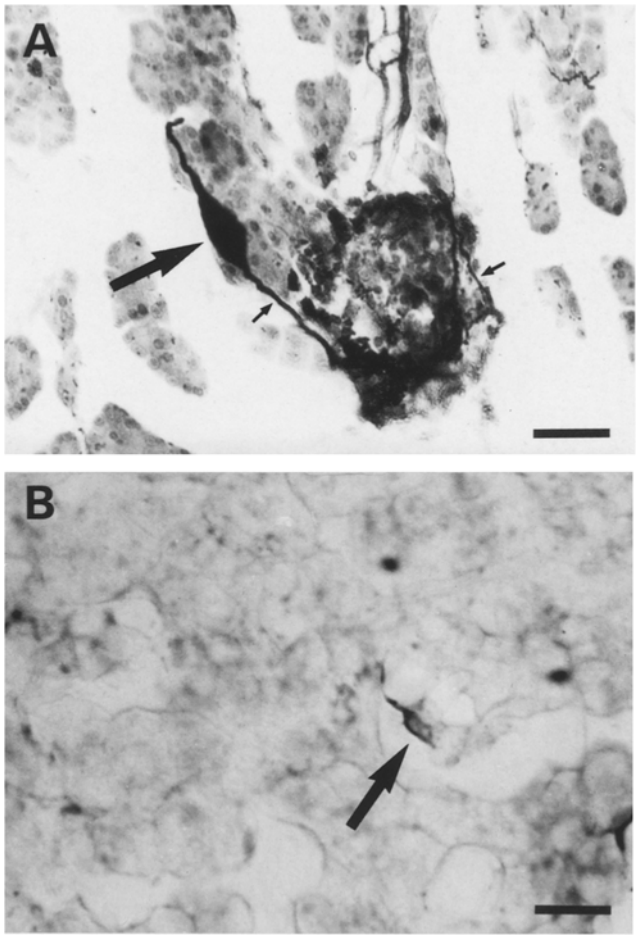

Fig. 4. Photomicrographs of a typical example of an AChE-positive ganglion (**A**, *large arrow*) innervating a nearby islet (*small arrows* in **A**) and a ChAT-immunoreactive nerve cell in the acinar tissue (*large arrow* in **B**). Ganglion cells depicted in (**A**) were systematically ChAT-negative, whereas the nerve cells in (**B**) were always AChE-negative, illustrating the conflicting findings for the two generally used cholinergic markers. *Scale bars*: **A** 150 μm; **B** 20 μm

the reaction product in the islets of Langerhans was found at the periphery and in the central core. Several heavily stained ganglion cells were observed, sometimes densely innervating nearby islets (small arrows in Fig. 4A). A comparison of ChAT and AChE labeling showed that the markers only partly overlapped. For example, only some of the AChE-positive ganglion cells showed ChAT-immunoreactivity, whereas AChE-positive ganglion cells densely innervating islets located nearby were consistently ChAT-negative. A further discrepancy between AChE-labeling and ChAT-labeling was found in the type of nerve cells described above as possessing mAChRs (Fig. 1F). Morphologically identical small nerve cells were frequently ChAT-immunoreactive (Fig. 4B), but always AChE-negative.

Discussion

The currently presented data revealed the visualization of mAChRs in the endocrine and exocrine tissue of the rat pancreas by way of receptor immunocytochemistry. The distribution of mAChRs was in agreement with binding studies employing tritiated cholinergic agents, showing the presence of mAChRs in the islets of Langerhans and in acinar tissue (Appert et al. 1981; Grill and Östenson 1983; Ng et al. 1979; Verspohl et al. 1990).

The differential distribution of five subtypes of mAChRs has been shown in the CNS (Bonner et al. 1987; Buckley et al. 1988). A predominantly homogeneous mAChR-population has been found in the rat pancreas (Waelbroeck et al. 1987); this population is of the M3 mAChR-subtype in mammalian pancreatic islets and in the exocrine tissue (Henquin and Nenquin 1988; Korc et al. 1987; Verspohl et al. 1990). Furthermore, rat pancreas contains mRNA of the M3 mAChR-type (Peralta et al. 1987). The present results thus show that M35 recognizes the M3 subtype of mAChRs. Recently, Vasudevan and coworkers (1991) have demonstrated that M35 binds to the M3-receptor subtype in a cell line transfected with the M3 DNA-code. These results are in agreement with earlier findings in the CNS that indicate that M35 does not discriminate between mAChR-subtypes (Van der Zee et al. 1989).

The endocrine tissue displays larger amounts of M35-immunoreactivity than the surrounding exocrine tissue. A heterogeneous distribution of mAChRs is found in the islets of Langerhans. Some cells at the periphery of the islets express large numbers of mAChRs and have been designated as ism cells. The endocrine cells in the central portion, most probably B cells, exhibit moderate levels of M35-immunoreactivity. However, not all B cells appear to be clearly M35-positive. Certain clusters of B cells, most probably representing units of B cells around central capillaries (Bonner-Weir 1988), are weakly immunopositive or even immunonegative. This may correspond with the differential density of cholinergic innervation observed within the endocrine islets (see below) and may be related to different signal-transduction thresholds that are observed, for example, for glucose responses of B cells (Schuit et al. 1988; Pipeleers 1987). The glucose sensitivity of B cells may be sensitized by vagal input through increasing protein kinase C activity and calcium flux (Zawalich et al. 1989).

Concerning the type of endocrine cells endowed with mAChRs, the endocrine activity of A, B, D and F cells is influenced by cholinergic stimulation in an atropine-sensitive manner, implying the presence of cholinergic receptors on all these types of cells (Östenson and Grill 1985; Hermansen 1980; Honey and Weir 1980; Miller 1981; Schwartz et al. 1978). The current study has revealed that M35-immunoreactivity is present throughout the islets, indicating that B cells in the central core and A, D and F cells in the mantle of the islets express mAChRs. Immunofluorescence double labeling for SOM and mAChRs has shown that the majority of D cells (79.8%) contains mAChRs. The finding that mAChRs are expressed in some but not all SOMergic cells is in accord with previously published data from the CNS (half of all SOMergic cells in the rat dorsal hippocampus possess mAChRs; Van der Zee et al. 1991a). However, only some of the ism cells belong to

the D cell population; thus, the majority of ism cells most probably represents A and/or F cells. Pancreatic polypeptide release from F cells may be a suitable parameter for the vagal tonus (Ahren et al. 1986), suggesting a dense cholinergic innervation of the F cells. Furthermore, ism cells are most frequently found in close proximity to capillary walls as clearly delineated cell cords, a finding characteristic for A cells (Orci and Unger 1975).

The cholinergic innervation of islets was studied by means of ChAT-immunocytochemistry and AChE-histochemistry. The results obtained by AChE-histochemistry resembled earlier findings (Coupland 1958; Luiten et al. 1986). Both the labeling intensity for AChE and ChAT in the pancreatic tissue was highest at the periphery of the islets. Assays measuring the activity of both enzymes revealed a 10-fold higher concentration of ChAT in the islets versus the surrounding tissue (Godfrey and Matschinsky 1975), which is in agreement with our findings. However, a striking discrepancy became apparent between the two cholinergic markers. Only some of the AChE-positive somata appeared to be ChAT-positive, whereas the small ChAT-immunoreactive nerve cells were clearly devoid of AChE-staining. These results resemble the partial colocalization of both enzymes in the CNS, where intensely AChE-positive neurons do not always contain ChAT (Eckenstein and Sofroniew 1983). In addition, small ChAT-immunoreactive interneurons in, for example, rodent cortex are consistently AChE-negative (Levey et al. 1984). Furthermore, the appearance of M35-immunoreactive nerve cells and ChAT-positive somata provide additional evidence for an intramural ganglionic organization. The present results further demonstrate that AChE is not as specific a cholinergic marker as ChAT. Therefore, in discussing the relationship between mAChR-expression and cholinergic innervation, we will concentrate on ChAT-immunoreactivity.

The differentiation of type-I and -II islets may be caused by a difference in (1) the degree of cholinergic neurotransmission, and/or (2) the architectural organization of the vascularization of the islets. (1) Type-I islets have a stronger cholinergic innervation than type-II islets, suggesting a positive correlation between the numbers of mAChRs and the intensity of cholinergic innervation. This correlation may even be found within islets for individual or clustered B cells. Similarly, the strongest mAChR-expression visualized by M35 in the rat cortex has been observed in areas provided with the most dense cholinergic innervation (Van der Zee et al. 1989). In agreement, isolated and subsequently microencapsulated islets (Fritschy et al. 1991), which are chronically lacking cholinergic innervation, still display the type-II M35-immunoreactivity pattern but lack the strongly stained ism cells (own unpublished data). Endocrine cells provided with a large amount of mAChRs probably represent cells with a high rate of receptor-turnover caused by high activity of (cholinergic) transmission. Similarly, a class of highly active hippocampal interneurons has revealed the highest density of M35-immunoreactivity throughout the rat CNS (Van der Zee et al. 1991b). (2) The type of vascularization of the islets depends on their size (Bonner-Weir 1991). In small islets, the efferent capillaries pass through exocrine tissue before coalescing, whereas in large islets the efferent capillaries coalesce at the edge of the islet. Since ism-cells are frequently found in close proximity to the capillary walls as mentioned above, vascular architectural characteristics of the islets in combination with differences in cholinergic innervation most likely cause the differentiation between type I and II as visualized by M35-immunocytochemistry.

Finally, it is of interest that the present study has revealed parallel findings between the (endocrine) pancreas and the CNS, concerning the relationship of (1) the level of M35-immunoreactivity and the density of cholinergic innervation, (2) the colocalization of mAChRs and somatostatin, and (3) the differentiation between the two cholinergic markers ChAT and AChE. Pearse (1977) has observed that neurons and peptide-producing endocrine cells share similar ultrastructural characteristics and cytochemical features, such as amine precursor uptake and decarboxylation activity (the APUD concept). He suggests that neurons and the APUD cells are derived from a common ancestral cell of a "neural origin, perhaps coming from the neural crest" (Pearse and Takor Takor 1976). Our current findings indicate a similar relationship but now this relationship is based on the resemblance of the anatomical features of the muscarinic cholinoceptive system of the CNS and the (endocrine) pancreas. Therefore, the present study provides a new approach for investigating the functional relationships between the CNS and the endocrine pancreas in the regulation of blood glucose homeostasis.

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