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Muscarinic receptor subtypes in rat pancreatic islets: binding and functional studies

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Cholinergic agents are potent modulators of insulin release that act via muscarinic receptors. We now investigated the muscarinic receptor subtype present in rat pancreatic islets in binding and functional studies. Binding of 5 nM [^{3}H]N-methylscopolamine ([^{3}H]NMS) was half maximal at 30 min. At 60 min, the maximal total binding was 1.29% and the non-specific binding (presence of 100 μM atropine) was 0.18% of the total radioactivity per 10 μg islet protein. Unlabelled atropine inhibited [^{3}H]NMS binding with an IC_{50} of ca. 30 nM. The rank order of antagonist high-affinity binding was atropine > sila-hexocyclium methyl sulfate (SiHC; $M_1 > M_3 > M_2$) > pirenzepine ($M_1 > M_2 \approx M_3$) = methoctramine ($M_2 > M_1 > M_3$). The high-affinity K_ds were 8.5, 56, 1300 and 1300 nM, respectively. The high affinity K_d of the muscarinic receptor agonist, arecaidine propargyl ester (APE), was 8.1 nM. The EC_{50} for the biological effects of APE on insulin and glucagon secretion was 3.2 and 2.3 nM. The rank order for the high-affinity biological effects of antagonists (inhibition of APE-mediated insulin/glucagon release) was almost the same as for binding. The data indicate that rat pancreatic islets contain neither an M_1 subtype (high-affinity for pirenzepine) nor an M_2 subtype (high-affinity for methoctramine) receptor. However, the data evidence an M_3 receptor subtype, since SiHC in the absence of the M_1 receptor subtype shows a relatively high affinity to the receptors in rat pancreatic islets.

Muscarinic receptor subtypes; Islets of Langerhans (rat); Insulin; Glucagon

1. Introduction

The pancreatic islets of Langerhans are innervated by parasympathetic nervous fibres (Coupland, 1958) which are organized in a periinsular plexus with direct innervation to the cells (Miller, 1981). Cholinergic stimulation of insulin release is thought to participate in the secretory response of the pancreatic B-cell to food intake, e.g. in the cephalic phase of insulin secretion (Malaisse, 1972). The importance of cholinergic innervation for islet function is suggested by the 10-fold higher concentration of choline acetyltransferase in the islets versus that in the adjacent exocrine tissue (Godfrey and Matschinsky, 1975). In vivo and in vitro administration of muscarinic agonists stimulates insulin secretion (Malaisse, 1972). The stimulatory action of acetylcholine on the endocrine pancreas is abolished by atropine (Grill and Östenson, 1983; Kaneto and Kosaka, 1974; Holst et al., 1981; Honey and Weir, 1980); the IC_{50} for inhibition of acetylcholine-induced insulin release by the antagonist methylscopolamine is less than 0.4 nM (Grill and Östenson,

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1983). Binding studies with [^{3}H]N-methylscopolamine ([^{3}H]NMS) (Malaisse et al., 1985) or [^{3}H]quinuclidinylbenzilate (Palafox et al., 1986) demonstrated the presence of muscarinic receptors in islets. The IC_{50} of inhibition of [^{3}H]NMS binding in rat pancreatic islets was approximately 5 nM when unlabelled N-methylscopolamine was used (Östenson and Grill, 1985).

At least three different muscarinic receptor subtypes have been proposed on the basis of different affinities for antagonists such as pirenzepine (Hammer and Giachetti, 1982), methoctramine (Melchiorre, 1988; Melchiorre et al., 1987), AF-DX 116 (Micheletti et al., 1987) and hexahydro-sila-difenidol as well as sila-hexocyclium (SiHC; Lambrecht et al., 1988; Eltze et al., 1988; Waelbroeck et al., in press). The subtypes have been termed M_1 (neuronal type), M_2 ($M_{2\alpha}$; cardiac type) and M_3 ($M_{2\beta}$ smooth muscle/glandular type) receptors (for recent reviews see Mitchelson, 1988; Melchiorre, 1988; Mutschler et al., 1988). Five receptor subtypes were identified by molecular cloning studies and were found to be the products of distinct genes. These subtypes were termed m1 to m5 (Bonner et al., 1987; Buckley et al., in press). The antagonist binding properties of the individual cloned m1, m2 and m3 receptors and their patterns of expression in various tissues correspond closely to those of the pharmacologically defined M_1, M_2 and M_3 receptors (Peralta et al., 1987; Akiba et al., 1988; Maeda et al., 1988; Buckley et al., in press).

The limited selectivity of available ligands has long hampered receptor subtype characterization in pancreatic islets. The results of functional studies ruled out the presence of M_2 cardiac type receptors in mouse islets (Henquin and Nenquin, 1988) as well as that of the M_1 subtype in rat endocrine pancreas (Otsuki et al., 1985). In the present study an attempt was made to characterize the muscarinic receptor subtype in both functional and binding studies with rat islets of Langerhans. Pirenzepine ($M_1 > M_2 \approx M_3$) (Hammer et al., 1980), methoctramine ($M_2 > M_1 > M_3$) (Melchiorre et al., 1987; Melchiorre, 1988), and SiHC ($M_1 > M_3 > M_2$) (Lambrecht et al., 1987; Waelbroeck et al., 1989) were used for the purpose.

2. Materials and methods

2.1. Animals

Wistar rats of either sex, weighing between 180 and 250 g, were used. They were kept on a standard pellet diet (Altromin, Lage/Lippe, W. Germany) and tap water ad libitum at 22°C with a 12-h light/dark rhythm.

2.2. Drugs and chemicals

The following compounds were purchased or synthesized: atropine nitrate from Merck (Darmstadt, W. Germany), arecaidine propargyl ester (APE) (Mutschler and Hultzsch, 1973) and sila-hexocyclium (SiHC) (Tacke et al., 1989) synthesized in one of our laboratories. Pirenzepine dihydrochloride was from Boehringer Ingelheim (Ingelheim, FRG) and methoctramine (N,N′-bis[6-(2-methoxybenzyl)aminohexyl]-1,8-octanediamine) tetrahydrochloride was kindly provided by Dr. Carlo Melchiorre, University of Bologna (Italy). [^{3}H]N-methylscopolamine [^{3}H]NMS; 72 Ci/mmol = 2664 GBq/mmol) was from Amersham Buchler (Braunschweig, W. Germany); pilocarpine hydrochloride, soybean trypsin inhibitor (SBTI), bacitracin and HEPES from Sigma Chemical Co. (St. Louis, MO); bovine serum albumin (BSA) fraction V from Miles Laboratories (Elkart, IN); collagenase (CLS grade) from Worthington Biochemicals Corp. (Freehold, NJ). Insulin radioimmunoassay kits were supplied by Isotopendienst West, GmbH (Dreieich, W. Germany). Rat insulin was purchased from the Novo Research Institute (Copenhagen, Denmark). The glucagon kit including glucagon standards was from Serono (Freiburg, W. Germany).

2.3. Isolation of rat pancreatic islets

The procedure for isolation of pancreatic islets was the one described by Lacy and Kostianovsky (1967) with slight modifications as described earlier (Verspohl and Ammon, 1980). Three rats were pretreated with 0.3 ml of 4% pilocarpine hydrochloride i.p. The animals were killed with ether after 3 h and the pancreases were isolated, minced,

and washed twice with 20 ml ice-cold Hanks solution containing 3.7 mM glucose, 1 mg/ml bacitracin, 0.2 mg/ml SBTI and 0.2% albumin. Pancreas pieces were soaked and shaken in a water bath at 37°C in the presence of 650 U collagenase/g tissue suspension. After 15-18 min of incubation the tissue suspension was passed into 10 ml of ice-cold Hanks solution. The islets were separated by sedimentation and collected as described elsewhere (Lacy and Kostianovsky, 1967). This method yields 300-500 islets/rat pancreas. The islets were carefully selected in order to avoid contamination with exocrine tissue since the presence of muscarinic receptors on acini is well known.

2.4. Binding experiments

After the islets were isolated, they were washed in ice-cold Hanks solution using centrifugation. Fifty islets were incubated in 0.3 ml Krebs-Ringer bicarbonate buffer plus 20 mM HEPES (KRBH) buffer, pH 7.4, containing 5 mg/ml bovine albumin, 1 mg/ml bacitracin and 0.1 mg/ml SBTI; 5 nM [^{3}H]NMS, with or without 100 μM unlabelled atropine, was then added. Binding experiments were performed in the presence of 16.7 mM glucose since elevated binding has been shown clearly with long-term high glucose concentrations or hyperglycemia (Östenson and Grill, 1985; 1987). The incubation was terminated by cooling the samples (4°C), and the incubation mixtures were then filtered through glass microfibre filters (Whatman GF/C, U.K.) for 2 s under reduced pressure. The filters were then washed twice with 0.2 ml ice-cold KRBH-buffer, which took less than 15 s. The dissociation experiment at 4°C illustrated in fig. 1 (dotted line) showed that no major dissociation was to be expected during the washing procedure. The filters were suspended in the scintillation cocktail (toluene, ethylene glycol monomethylether and 2,5-diphenyloxazole), shaken vigorously and counted in a β-scintillation spectrometer after luminescence had disappeared.

To establish the apparent receptor affinity (dissociation constant, K_d) the competitive inhibition of specific [^{3}H]NMS binding by various concentrations of unlabelled antagonists was analyzed. This was done by fitting plots for bound drugs versus free drugs by means of a non-linear least-squares computer program that analyzed the data in terms of one non-saturable and one or two saturable components (Dixon, 1974).

2.5. Insulin and glucagon secretion

To measure insulin secretion, five islets were incubated for 60 min at 37°C in the aforementioned KRBH buffer after having been primed for 60 min with 16.7 mM glucose. Insulin and glucagon released into the medium by the islets was assayed with radioimmunoassay kits using rat insulin or glucagon as a standard. Each compound had been checked for non-interference with the insulin and glucagon radioimmunoassays. For statistical evaluation of whole curves, multiple comparisons of means were carried out by two-way analysis of variance (F-test). Dunnett's test was used thereafter if several conditions were to be compared to the control experiments. EC_{50}s of biologic effects were determined by means of the RS/1 statistics pack (BBN Software Products Corp.).

2.6. Protein determination

The protein content was measured in extra batches within each binding experiment. The protein content of the solubilized pancreatic islets (solubilized with 0.1 N NaOH) was measured using bovine serum albumin as a standard (Bradford, 1976).

3. Results

3.1. Time course of [^{3}H]NMS binding

The binding of 5 nM [^{3}H]NMS to isolated rat islets at 37°C was half maximal after 30 min and maximal binding occurred at 60 min (fig. 1). The velocity of binding was much lower than that shown earlier by Grill and Östenson (1983) and by Malaisse et al. (1985). At 60 min, maximal binding was 1.29% of added radioactivity per 10 μg islet protein. Non-specific binding (determined in the

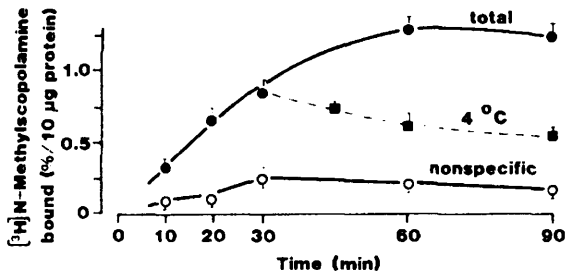

Fig. 1. Time course of [^{3}H]NMS binding to isolated rat islets of Langerhans. Fifty islets were incubated in 0.3 ml KRH-albumin buffer for 90 min at 37 °C with 5 nM [^{3}H]NMS in the absence (●) and presence (○) of 0.1 mM atropine. After washing at 30 min some batch-incubated islets were re-incubated in radioactivity-free medium at 4 °C in order to determine the dissociation velocity in the absence of ligands (■). The results are expressed as % bound per 10 µg islet protein. Each value represents the mean ± S.E. of three to four separate experiments.

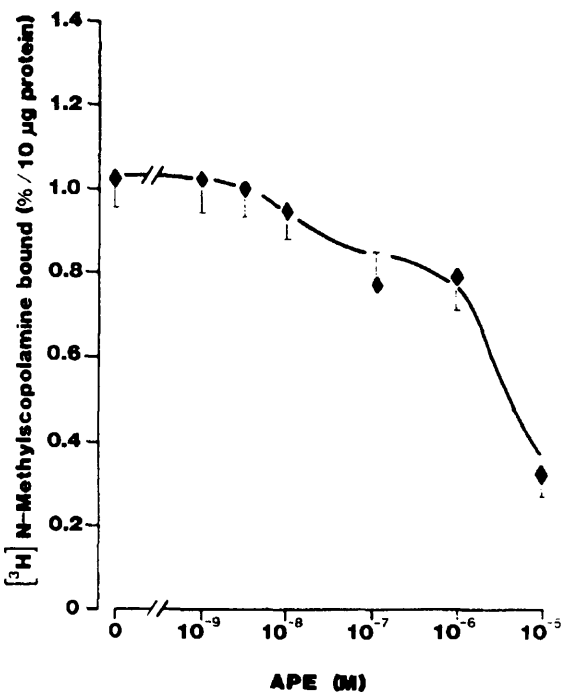

Fig. 2. Inhibition by the agonist, APE, of [^{3}H]NMS binding to isolated rat islets of Langerhans. Fifty islets were incubated in 0.3 ml KRH-albumin buffer for 60 min at 37 °C with 5 nM [^{3}H]NMS and increasing concentrations of agonist. The results are expressed as % bound per 10 µg islet protein. Each value represents the mean ± S.E. of four separate experiments.

presence of 100 µM atropine) ranged from 0.10 to 0.26% of added radioactivity per 10 µg islet protein. All subsequent binding studies were therefore carried out for 60 min. At 30 min, dissociation of labeled ligand at 4 °C was slow, i.e. the half life was more than 60 min.

TABLE 1

Effects of muscarinic receptor agonists and various antagonists on receptor binding, insulin release and glucagon release. Activities were calculated as dissociation constants of high- and low-affinity binding (K_{d1} and K_{d2}) and as half maximally regulated insulin or glucagon release (EC_{50}) of high-affinity effect. The values were calculated from data shown in figs. 2-7. [a] NS vs. pirenzepine ($P < 0.05$); [b] NS vs. atropine ($P > 0.05$). Number of experiments in parentheses.

Compound	K_{d1} (nM)	K_{d2} (nM)	EC_{50} (nM)	
			Insulin secretion	Glucagon secretion
Agonist				
APE	8.06 (4) ±1.04	5840 (4) ±631	3.23 (12) ±0.25	2.34 (12) ±0.31
Antagonists				
Atropine	8.46 (4) ±1.13	463 (4) ±54	9.18 (3) ±0.94	11.3 (3) ±0.99
SiHC	55.6 (4) ±3.89	1940 (4) ±128	6.32 (3) [b] ±1.03	364 (3) ±27.3
Pirenzepine	1340 (4) ±211	9100 (4) ±641	432 (4) ±22.6	1510 (4) ±213
Methoctramine	1280 (4) [a] ±154	55000 (4) ±6100	2130 (3) ±198	2380 (3) [a] ±468

3.2. *Muscarinic receptor agonist, APE*

APE inhibited [^{3}H]NMS binding in a concentration-related manner (fig. 2). Inhibition was essentially complete over four orders of magnitude (1-10000 nM). Binding analysis of the competition-inhibition curve revealed more than a single binding site with a K_{d1} of 8.06 nM and K_{d2} of 5840 nM (table 1) and a capacity of $B_{max1} = 36.8$ and $B_{max2} = 91.9$ fmol/10 µg islet protein.

In rat pancreatic islets, glucose alone (absence of muscarinic receptor agonist) increased insulin release (fig. 3). APE increased insulin release in a dose-dependent biphasic manner that was independent of the glucose concentration (3.0 mM = substimulatory and 16.7 mM = stimulatory). Glu-

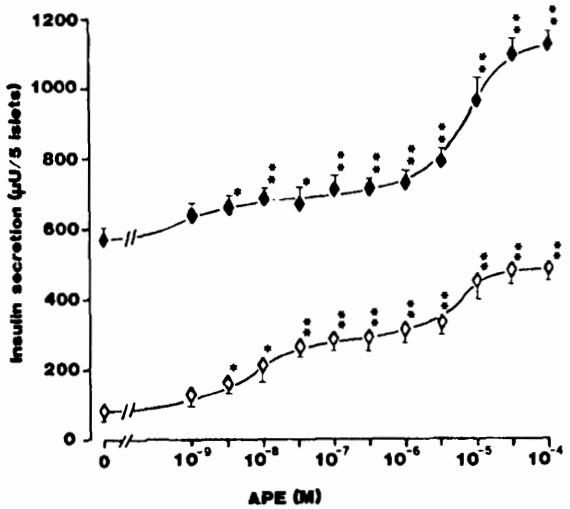

Fig. 3. Stimulation of insulin release by the muscarinic receptor agonist, APE. Five islets were incubated in the same buffer with various APE concentrations for 60 min at 37 °C in the presence of non-stimulatory glucose (3.0 mM (◇)) or stimulatory glucose (16.7 mM (◆)). The results are expressed as μU insulin secreted per five islets over 60 min. Each value represents the mean ± S.E. of 12 separate experiments ($F = 5.12$, $P < 0.01$ at 16.7 mM glucose; $F = 4.46$, $P < 0.01$ at 3 mM glucose; * $P < 0.05$ and ** $P < 0.01$ both vs. absence of APE).

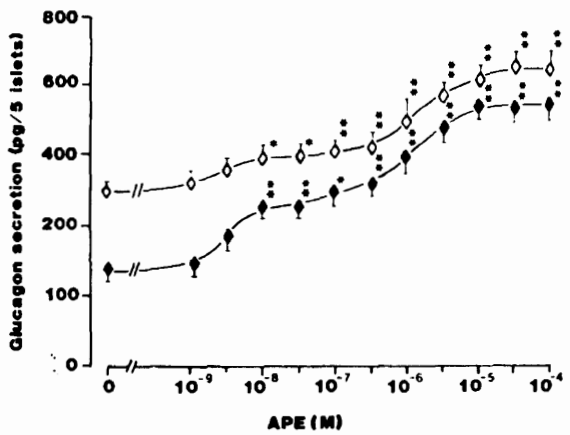

Fig. 4. Stimulation of glucagon release by the muscarinic receptor agonist, APE. Five islets were incubated in the same buffer with various APE concentrations for 60 min at 37 °C in the presence of non-stimulatory glucose (3.0 mM, ◇) or stimulatory glucose (16.7 mM, ◆). The results are expressed as pg glucagon secreted per five islets over 60 min. Each value represents the mean ± S.E. of 12 separate experiments ($F = 4.93$, $P < 0.02$ at 3 mM glucose; $F = 4.31$, $P < 0.01$ at 16.7 mM glucose; * $P < 0.05$ and ** $P < 0.02$ both vs. absence of APE).

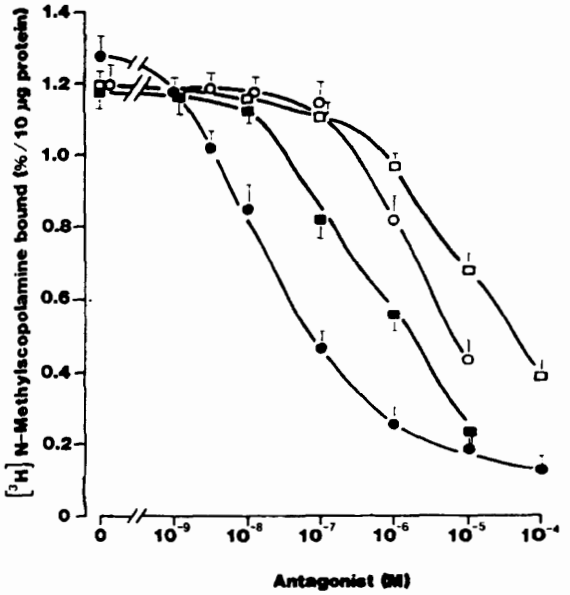

Fig. 5. Inhibition of [^{3}H]NMS binding to isolated rat islets of Langerhans by antagonists: 50 islets were incubated in 0.3 ml KRH-albumin buffer for 90 min at 37 °C with 5 nM [^{3}H]NMS and increasing concentrations of antagonists. The antagonists were: (●) atropine, (■) SiHC, (○) pirenzepine, (□) methoctramine. The results are expressed as % bound per 10 μg islet protein. Each value represents the mean ± S.E. of four separate experiments.

cose alone (absence of muscarinic receptor agonist) decreased glucagon release (fig. 4). APE increased glucagon release in a dose-dependent biphasic manner at both 3.0 and 16.7 mM glucose.

3.3. Muscarinic receptor antagonists

The concentration of unlabelled atropine causing half maximal inhibition of [^{3}H]NMS binding was ca. 30 nM (IC_{50}) (fig. 5). Binding analysis of the competition-inhibition curves generated with unlabelled antagonists revealed more than a single binding site with Hill coefficients of less than 0.6 in all cases. For atropine, e.g. K_{d1} was 8.46 ± 1.13 and K_{d2} was 463 ± 54 nM, respectively. The rank order for inhibition of antagonist high-affinity binding was atropine > SiHC > pirenzepine = methoctramine (table 1).

When islets were stimulated by 10 μM APE in the presence of 16.7 mM glucose, muscarinic re-

ceptor antagonists inhibited insulin release (fig. 6). The rank order of potency was atropine = SiHC > pirenzepine > methoctramine (table 1). No antagonist lowered insulin release below the values obtained with glucose alone (fig. 6), and no antagonist antagonized the effect of glucose alone (data not shown).

In the presence of 3 mM glucose plus 10 μM APE, muscarinic receptor antagonists inhibited glucagon release (fig. 7). The rank order of potency was atropine > SiHC > pirenzepine = methoctramine (table 1). Atropine, but not other antagonists lowered glucagon release below values obtained with glucose alone (fig. 7).

4. Discussion

4.1. Muscarinic receptor agonist

Our data clearly show that muscarinic binding sites are present on rat pancreatic islets. APE

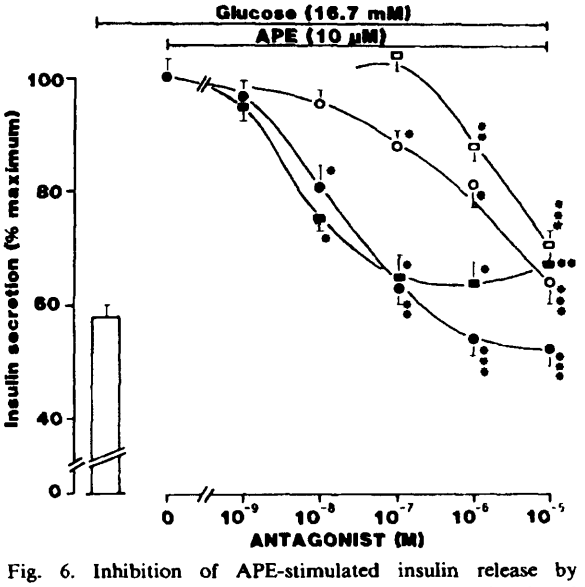

Fig. 6. Inhibition of APE-stimulated insulin release by muscarinic receptor antagonists. Five islets were incubated with increasing concentrations of antagonists in the presence of 16.7 mM glucose plus 10 μM APE for 60 min at 37 °C. The antagonists were: (●) atropine, (■) SiHC, (○) pirenzepine, (□) methoctramine. The results are expressed as maximal % secreted insulin. Each value represents the mean ± S.E. of three separate experiments (*P < 0.05, **P < 0.02, ***P < 0.001 vs. absence of antagonists).

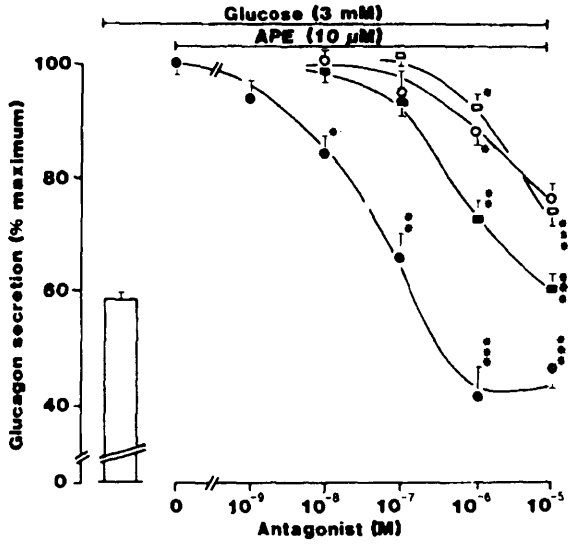

Fig. 7. Inhibition of APE-stimulated glucagon release by muscarinic receptor antagonists. Five islets were incubated with increasing concentrations of antagonists in the presence of 3.0 mM glucose plus 10 μM APE for 60 min at 37 °C. The antagonists were: (●) atropine, (■) SiHC, (○) pirenzepine, (□) methoctramine. The results are expressed as maximal % secreted glucagon. Each value represents the mean ± S.E. of three separate experiments (*P < 0.05, **P < 0.01, ***P < 0.001 vs. absence of antagonists).

which is selective for muscarinic over nicotinic receptors inhibits [^{3}H]NMS binding and stimulates both insulin and glucagon release at similar concentrations in a biphasic, dose-dependent manner.

4.2. Muscarinic receptor antagonists

Since no agonists selective for muscarinic receptor subtypes are available, antagonist were now used to characterize the muscarinic receptor subtype in rat pancreatic islets. There was a clear rank order of affinity: the highest affinity was found for the compound, SiHC, which exhibits a similar affinity for M_1 and for M_3 receptors (Lambrecht et al., 1987; Waelbroeck et al., in press). The low affinity of pirenzepine indicates that the M_1 subtype is not important and the low affinity of methoctramine indicates that M_2 receptor subtypes are not predominant in rat pancreatic islets. The M_3 receptor subtype also appears to be

that most involved in A-cells. This receptor subtype had originally been named M_4. Functional data reported by Henquin and Nenquin (1988) make it likely that this subtype is involved in islets effects.

4.3. Initiating or modulating effect of a muscarinic receptor agonist?

Ambient glucose stimulates insulin secretion and inhibits glucagon secretion (Verspohl and Ammon, 1987). The muscarinic receptor agonist, APE, both initiates and modulates insulin release. The fact that, in contrast to Hermans et al. (1986), we were able to show an initiating (absence of stimulatory glucose) insulinotropic effect of a muscarinic receptor agonist may have been due to species differences (mouse) or to the fact that our islets were primed by an 1-h preincubation in the presence of 16.7 mM glucose or been the result of using a highly specific agonist in contrast to other groups.

The muscarinic receptor agonist, APE, is able to modulate the glucose-mediated inhibition of glucagon release. Our data confirm those of Östensen and Grill (1985) by showing that muscarinic receptor antagonists inhibit the glucagon release stimulated by cholinergic substances.

The receptor subtype population cannot be decided from binding studies alone since rat pancreatic islets are a heterogenous cell population which may obscure a receptor subtype on a cell type which is only present in low amounts. The functional data nevertheless indicate, first that the measured binding sites are related to the B and A cells of rat pancreatic islets and, second, that the muscarinic receptor agonist had an insulinotropic and glucagonotropic effect independent of the glucose concentration used.

4.4. Extrapolation of the in vitro situation

These in vitro data will need further evaluation from in vivo experiments since atropine and possibly other antagonists have been claimed based on the in vivo experiments, to behave differently by not being selective. Atropine and pirenzepine not only do antagonize muscarinic effects but they also antagonize secretin and cholecystokinin-mediated effects (Otsuki et al., 1987; You and Chey, 1988).

Taken together, our data are consistent with the opinion that rat pancreatic islets contain M_3 muscarinic subtypes as also does the exocrine pancreas (Louie and Owyang, 1985; Dehaye et al., 1983). Identifying selective M_3 agonist interacting with the endocrine pancreas but hopefully not with other glands would provide a valuable tool for the development of new insulinotropic drugs. In conclusion, the binding and functional data indicate the predominant presence of an M_3 receptor subtype in the rat pancreatic islets.

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