Quantitative and Functional Characterization of Muscarinic Receptor Subtypes in Insulin-Secreting Cell Lines and Rat Pancreatic Islets

Tiina P. Iismaa, Elizabeth A. Kerr, Justine R. Wilson, Lee Carpenter, Natalie Sims, and Trevor J. Biden

Expression of muscarinic receptors in rat islets, RINm5F cells, and INS-1 cells was established by reverse transcriptase–polymerase chain reaction (RT-PCR) and quantified by RNase protection. Both methods indicated that m3 and m1 receptors were expressed approximately equally in the various cellular preparations and to a much greater extent than the m5 subtype. However, the cell lines, especially RINm5F cells, expressed less of a given receptor subtype than did islets. Immunohistochemistry indicated that m3 receptors were expressed throughout the islet core. Binding studies using the radiolabeled muscarinic receptor antagonist QNB demonstrated a maximal binding capacity of INS-1 cells of 23.0 ± 2.9 fmol/mg protein. Functional analyses were undertaken using INS-1 cells stably transfected with either m1 or m3 receptor cDNAs. Overexpression of either receptor did not affect basal responses but markedly enhanced maximal responses to the muscarinic receptor agonist carbachol. Although maximal hydrolysis of phosphatidylinositol 4,5-bisphosphate (Ptd InsP2) was twofold greater in m1-transfectants as compared with m3-transfectants, cell lines overexpressing either receptor gave essentially equivalent secretory responses to a full range of carbachol doses. The results demonstrate that both m1 and m3 muscarinic receptors are well expressed in pancreatic β-cells, functionally linked to signaling pathways, and capable of initiating insulin secretion with equal potencies. *Diabetes* 49:392–398, 2000

Release of acetylcholine from vagal efferents on pancreatic islet cells plays an important role in the cephalic phase of insulin secretion. Thus, the neurotransmitter directly evokes a rise in plasma insulin in the immediate postprandial period in rats and humans, before any glycemic elevation (1,2). Acetylcholine also potentiates nutrient-stimulated insulin secretion, an effect best demonstrated in vitro, where confounding postabsorptive influences occurring in vivo are absent (1,2). Thus, potentiation has been demonstrated using perfused pancreas (3) and isolated islets (4–6), and the direct stimulation has been shown in pancreas perfusions (3), insulin-secreting cell lines (7), and cultured (6,8) but not freshly isolated (9,10) islets. The latter preparation might be less responsive because of damage to acetylcholine receptors on the cell surface sustained during collagenase digestion, which is necessary for islet isolation. Acetylcholine has also been shown to prime the β-cell in vitro such that a subsequent glucose challenge elicits a larger secretory response than in an unprimed cell (5).

The effects of acetylcholine on the β-cell are mediated by muscarinic cholinergic, rather than nicotinic, receptors (1,2,5,10). Five subtypes of muscarinic receptors have been defined at the molecular level (m1–m5), all of which are members of the G-protein coupled receptor family (11). The m2 and m4 receptor subtypes couple mainly via pertussis toxin-sensitive G-proteins to inhibition of adenylate cyclase. The functional responses of the m1, m3, and m5 subtypes are generally insensitive to pertussis toxin, are mediated via Gq/11, and are associated with activation of phospholipase C (PLC) and hydrolysis of phosphatidylinositol 4,5-bisphosphate (Ptd InsP2) (11). The signaling pathways activated by muscarinic receptor agonists in β-cells are insensitive to pertussis toxin (12,13) and are not accompanied by inhibition of adenylate cyclase (6,9), but they do involve Ptd InsP2 hydrolysis (7,8,10,13). Thus, functional islet muscarinic receptors fall into the m1/m3/m5 category, rather than m2/m4. This is broadly consistent with some limited pharmacological analyses suggesting that m3 is the major receptor subtype present in pancreatic β-cells (14–17). However, mRNA for other receptor subtypes has also been detected by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of extracts of rat islets (17) and insulin-secreting RINm5F cells (18). The relative amounts of these receptor subtypes have never been quantified, nor has the capacity of the minor subtypes to contribute to insulin secretion been assessed.

This is important because muscarinic receptor agonists couple to multiple signaling pathways in pancreatic β-cells, including hydrolysis of both Ptd InsP2 and phosphatidylinositol (Ptd Ins) (7,8,19); mobilization of intracellular Ca^{2+} stores (7,10); activation of protein kinase C (20,21); stimulation of Na^+ influx and resultant depolarization of the plasma membrane potential (22,23); activation of Ca^{2+} influx through both volt-

From the Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, Australia.

Address correspondence and reprint requests to Dr. Trevor Biden, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst NSW 2010 Australia. E-mail: t.biden@garvan.unsw.edu.au.

Received for publication 8 February 1999 and accepted in revised form 3 November 1999.

[^{3}H]QNB, L-[benzilic-4,4′-^{3}H]quinuclidinyl benzilate; CMV, cytomegalovirus; EC_{50}, molar concentration of carbachol that produced 50% of maximal response; HPLC, high-performance liquid chromatography; IC_{50}, concentration of competing ligand that displaced 50% of the specific binding of QNG; KRB, Krebs-Ringer bicarbonate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLC, phospholipase C; Ptd Ins, phosphatidylinositol; Ptd InsP2, phosphatidylinositol 4,5-bisphosphate; RT, reverse transcriptase; TEM buffer, 50 mmol/l Tris-HCl (pH 7.4), 1 mmol/l EDTA, and 5 mmol/l $MgCl_2$.

age-dependent and -independent means (6,22,24–26); and finally, a paradoxical inhibition of Ca^{2+} influx manifest under some conditions (26). The extent to which these various pathways operate independently, or are hierarchical, is not well understood, nor is their potential established for selective coupling to one or other of the different muscarinic receptor subtypes. Agents capable of selectively reproducing the priming and/or potentiatory effects of acetylcholine would be of potential therapeutic benefit in the treatment of type 2 diabetes because they would augment insulin secretion in a fashion tightly coupled to the prevailing plasma glucose concentration, an advantage not seen with therapies such as sulfonylureas. The aim of the current study was therefore to determine the relative abundance of the muscarinic receptors mediating secretion in pancreatic islets and a variety of insulin-secreting cell lines, and to modulate that relative abundance and hence determine the potential of the individual receptor subtypes to activate secretion.

RESEARCH DESIGN AND METHODS

Materials. Tissue culture media were from Gibco BRL (Gaithersburg, MD). All radionucleotides were from Amersham Australia (Sydney), and scintillation fluid was from Canberra Packard (Sydney, Australia). High-performance liquid chromatography (HPLC) columns and glass microfiber filters (GF/B and GF/C) were purchased from Whatman International (Maidstone, U.K.), and Coomassie protein assay kits were from Bio-Rad Australia (Sydney). RNase Protection Kits and Expand RT were supplied by Boehringer Mannheim Australia (Sydney). DNaseI, T4 polynucleotide kinase, SP6 and T7 RNA polymerase, pGEM-3Z vector Riboprobe in vitro transcription systems, and RNase protection assay kits were obtained from Promega (Madison, WI). The sources of other molecular biological reagents were as follows: plasmids pREP8 and pRc/CMV from Invitrogen (San Diego, CA); DNA polymerase from Perkin-Elmer Cetus (Norfolk, CT); Superfect from Qiagen (Melbourne, Australia); RNA extraction kits from 5 Prime 3 Prime (Boulder, CO); and Nick columns from Pharmacia (Uppsala, Sweden). The Vectastain ABC system was purchased from Vector Laboratories (Burlingame, CA), and antibodies were from Research & Diagnostic Antibodies (Berkeley, CA) (m1 and m3 receptors), ICN Pharmaceuticals (Costa Mesa, CA) (glucagon and somatostatin), and Zymed Laboratories (South San Francisco, CA) (neuron-specific enolase). Kits for radioimmunoassay of rat insulin were obtained from Linco Research (St. Louis, MO). All other biochemicals and specialized reagents were from Sigma (St. Louis, MO). Various cDNAs were generous gifts of the following investigators: human m1 receptor, R.G. Graham; rat m3 receptor, P.R. Schofield; rat m1 and m5 receptors, J. Codina; and rat cytoplasmic actin, P. Gunning.

Cell and islet culture. Islets were isolated from adult male Wistar rats (220–270 g) by collagenase digestion, purified on a Histopaque 1077 gradient, and hand-picked under a binocular microscope (19). Islets were maintained for 48–72 h in medium 199 supplemented with 10% (vol/vol) newborn calf serum, 14 mmol/l $NaHCO_3$, 11.1 mmol/l glucose, 500 IU/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin. RINm5F cells were routinely cultured in 75 cm^2 flasks with 20 ml of RPMI 1640 medium containing 24 mmol/l $NaHCO_3$, 10 mmol/l HEPES, 10% (vol/vol) fetal calf serum, 50 IU/ml penicillin, and 50 µg/ml streptomycin. INS-1 cells were maintained under identical conditions except for additional inclusion in the medium of 1 mmol/l sodium pyruvate and 50 µmol/l β-mercaptoethanol.

RT-PCR analysis of mRNA expression. RNA was isolated using a commercial kit. Total RNA (2.5 µg) was treated with RNase-free DNaseI, and random-primed cDNA was synthesized from half of each sample using Expand RT as recommended by the manufacturer. PCR was performed using AmpliTaq Gold DNA polymerase and rat gene-specific sense/antisense primer pairs (20 µmol/l in 100 µl reaction volume) as follows: m1F, 5′-TCTGAGACACCAGGCAAAGG-3′ and m1R, 5′-CTTGACTGTATTTGGGGAGC-3′, corresponding respectively to nucleotides 682–701 (sense) and 981–962 (antisense) of the m1 coding sequence (GenBank Accession M16406); m3F, 5′-CACAGGCAGTTCTCGAAGCT-3′ and m3R, 5′-AGGACGGTAGCTTGGTAGAG-3′, corresponding to nucleotides 846–865 (sense) and 1153–1134 (antisense), respectively, of the m3 coding sequence (M16407); and m5F, 5′-GAAACAGTTGTGAACACCCG-3′ and m5R, 5′-CTCTTTGACCAGAACCATTC-3′, corresponding to nucleotides 1027–1046 (sense) and 1309–1289 (antisense), respectively, of the m5 coding sequence (M22926, J04706). A Perkin-Elmer Cetus DNA thermal cycler was used with the following temperature parameters: incubation at 94°C for 12 min, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min (m1) or 55°C for 1 min (m3 and m5), and 72°C for 1 min, then incubation at 72°C for 10 min at the end of the final cycle. One-twentieth to one-third of each reaction was examined by Southern blot analysis (27) using ^{32}P end-labeled (T4 polynucleotide kinase) antisense oligonucleotide probes corresponding to the coding sequence within the amplified regions as follows: m1, 5′-CTTCCAGCTGTAGGCCTGCAG-3′ (nucleotides 825–805); m3, 5′-CCTGGTCTCTGAGCCAATGTC-3′ (nucleotides 1083–1063); and m5, 5′-CTGACTCTTGAGTCTGTG-3′ (nucleotides 1119–1102).

Ribonuclease protection assay of mRNA expression. Fragments corresponding to the coding sequences of rat m1 (nucleotides 428–790; 363 bp); m3 (nucleotides 98–446; 349 bp); m5 (nucleotides 748–983; 236 bp); and rat cytoplasmic β-actin (nucleotides 416–597; 182 bp, EMBL Accession V01217, J00691) were cloned into *Hin*cII-cleaved pGEM-3Z vector. Probe (antisense) and reference (sense) RNA were generated from linearized DNA template using bacteriophage SP6 or T7 RNA polymerase. Either [α-^{32}P]dCTP or [α-^{32}P]dGTP (both 3,000 Ci/mmol) was used to generate ^{32}P-labeled antisense RNA. Unincorporated nucleotides were removed by ethanol precipitation of transcripts in the presence of 2.5 mol/l ammonium acetate. Reference RNA was quantified by measuring A_{260}; radiolabeled probe RNA was further purified over a Nick column, and specific activity was determined by Cerenkov counting. Total RNA was prepared as above. Ribonuclease protection assays were carried out using commercial kits essentially as recommended. Briefly, probe RNAs (~1 × 10^5 cpm) were co-precipitated either with 10 µg total RNA or with known amounts of reference RNAs in the presence of 10 µg yeast tRNA; resuspended in hybridization buffer (40 mmol/l PIPES, 400 mmol/l NaCl, 1 mmol/l EDTA, 80% [vol/vol] deionized formamide, pH 6.4); denatured (95°C, 5 min); and hybridized overnight at 60°C. After RNase A/RNase T1 (37°C, 30 min) and proteinase K (37°C, 15 min) digestion, samples were deproteinized, concentrated, resolved by electrophoresis in a 6% polyacrylamide/8 mol/l urea gel, and visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Bands were quantified using IPLabGel software.

Immunohistochemistry. Pancreases of anesthetized Wistar rats (220–270 g) were perfused with ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) as for collagenase digestion. Pancreatic tissue was dissected, post-fixed overnight at 4°C in fresh 4% paraformaldehyde, and embedded in paraffin using standard procedures. Immunohistochemistry was carried out as described (28) on serial 5 micron sections using the Vectastain ABC system. Antibodies were used at the following dilutions: m3 (1:1,000); m1(1:2,000); somatostatin (1:2,000); glucagon (1:2,000); and neuron-specific enolase (1:200).

Transfection of mammalian cell lines. The mammalian expression vector pHis/CMV was constructed by ligating the 1102–4577 bp *Bgl*II single digest fragment of pREP8 containing the gene (*hisD*) that confers histidinol resistance to mammalian cells, to the 4.235 kb *Nsp*V and *Swa*I restriction endonuclease fragment of pRc/CMV that contains the cytomegalovirus (CMV) promoter, the ColE1 replication origin, and the bacterial gene encoding β-lactamase. The full-length coding sequences of human m1 (1,522 bp) (29) or rat m3 receptors (1,770 bp) (30) were subcloned into the resultant pHis/CMV expression construct. INS-1 cells were transfected in six well plates (80% confluent) using 2.5 µl Superfect reagent/µg plasmid DNA per well and passaged in the presence of 10 mmol/l L-histidinol to select for stable transfectants. Stably tranfected clonal cell lines were obtained by dilution cloning of the pooled transfected cells in 96-well plates and selecting for colonies in the presence of 10 mmol/l histidinol.

Radioligand binding assays. Pools of cells, transfected with either m1 or m3 receptor cDNA, were removed by scraping from 150 cm^2 flasks, washed once in PBS, and then resuspended in 5 mmol/l Tris-HCl (pH 7.4) plus protease inhibitors (2 mmol/l phenylmethylsulfonyl fluoride, 2 µmol/l pepstatin A, 2 mmol/l benzamidine, 20 µg/ml leupeptin, 10 U/ml aprotinin A). After 30 min at 4°C, cells were freeze-thawed once and then centrifuged (10 min × 900*g* at 4°C) to remove cellular debris. Membranes were collected by centrifugation (40 min × 100,000*g* at 4°C), resuspended at 5–10 mg/ml in 20 mmol/l TrisHCl (pH 7.4) plus protease inhibitors, and stored at –20°C. Aliquots of membranes (2–100 µg protein) were incubated with L-[benzilic-4,4′-^3H]quinuclidinyl benzilate ([^3H]QNB) (0.1–10 nmol/l; 43.5 Ci/mmol) for 45 min at 37°C in 1 ml of 50 mmol/l Tris-HCl (pH 7.4), 1 mmol/l EDTA, 5 mmol/l $MgCl_2$ (TEM buffer) plus protease inhibitors. Nonspecific binding was determined as residual radioactivity in the presence of 5 µmol/l atropine; it was never >15% for transfected cells or >40% for untransfected cells. A manifold filtration unit (Millipore, Bedford, MA) was used to retain membranes plus bound radioactivity on GF/B filters that had been pretreated for 1 h in 0.3% polyethylenimine and rinsed in PBS. The membranes on the filters were then washed with 2 × 5 ml ice-cold TEM buffer and then air-dried. Radioactivity was determined by liquid scintillation spectroscopy using 5 ml Econofluor scintillant. Values for B_{max} and K_m were determined by Scatchard analysis (31).

Functional assays. For secretory studies, INS-1 cells were grown in 24-well plates. After aspiration of culture medium, the wells were washed twice in modified Krebs-Ringer bicarbonate (KRB) buffer containing 5 mmol/l $NaHCO_3$, 1 mmol/l $CaCl_2$, 0.5% bovine serum albumin, and 10 mmol/l HEPES (pH 7.4). After a 30-min preincubation in 0.5 ml KRB at 37°C, a further 0.5 ml of prewarmed KRB containing 2.8 mmol/l glucose and final concentrations of carbachol as indicated were added, and the cells were incubated for a further 15 min. An aliquot of the

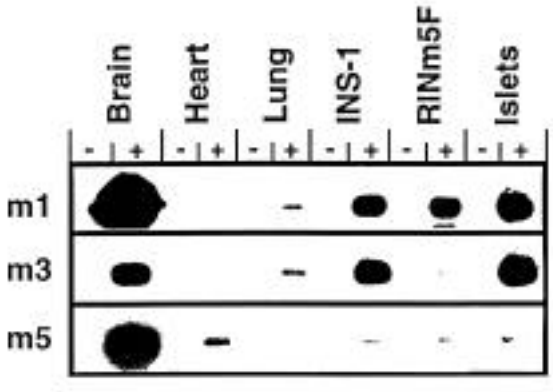

FIG. 1. RT-PCR amplification of muscarinic receptor RNA extracted from insulin-secreting cell lines, rat islets, and other tissues. Samples of total RNA (1.25 µg) were reverse transcribed (+) or not (–), and amplified by PCR using primers specific for m1, m3, or m5 muscarinic receptors. Aliquots of each reaction (5 µl for brain, 35 µl for others) were then analyzed by Southern blotting using radiolabeled oligonucleotide probes specific for each receptor subtype.

medium was then removed for analysis of insulin content by radioimmunoassay. For inositol phosphate studies, cells were grown in 12-well plates in the presence of inositol-free RPMI 1640 supplemented with [^{3}H]inositol (10 µCi /ml) for the last 48 h of culture. Clonal cell lines were preincubated for 10 min in KRB containing 2.8 mmol/l glucose and then stimulated or not with 0.1 mmol/l carbachol for 5 min. Incubations were terminated with 0.1 ml of 100% trichloracetic acid, and inositol trisphosphate (InsP3) was extracted and separated from Dowex 1 × 8 formate columns by elution with ammonium formate, as reported previously (8). For analysis of Ptd Ins and Ptd InsP2 hydrolysis, inositol phosphate isomers were separated by HPLC from pooled transfected cells stimulated for 1 min with 0.5 mmol/l carbachol (8). HPLC analysis was performed using a Whatman partisphere PAC column (12.5 × 0.4 cm) eluted with ammonium phosphate (pH 3.8). The exact gradient comprised an initial 5-min wash with water, followed by 0–0.1 mmol/l ammonium phosphate over 35 min and 0.1–1.0 mmol/l over 55 min. Radioactive peaks were quantified with a Radiomatic Flo-one beta (Series A-100) on-line detector, using Ultima Flo AP scintillant. Counts were internally corrected for loading and recovery by expressing results as a percentage of total free inositol. Hydrolyses of Ptd Ins and Ptd InsP2 were quantified as the accumulation of the inositol monophosphates Ins(1)P1 and Ins(4)P1, respectively, as previously verified (19). Expression of results and statistical and data analysis. Unless otherwise indicated, results are expressed as means ± SE with the number of observations in brackets. Statistical significance was determined with Student's *t* test. The concentration of competing ligand that displaced 50% of the specific binding of QNB (IC_{50}) and the molar concentration of carbachol that produced 50% of maximal response (EC_{50}) were calculated from sigmoidal plots fitted using the Prism Version 2 program (GraphPad Software, San Diego, CA).

RESULTS

As an initial step in determining the muscarinic receptor subtypes present in pancreatic β-cells, we used primers specific for the m1, m3, and m5 receptors for RT-PCR amplification of RNA derived from clonal β-cell lines, rat pancreatic islets, and various rat tissues. The results are shown in Fig. 1. In each instance, controls lacking RT were run in parallel for detection of contaminating genomic DNA, but no signal was detected in these control lanes. Both RINm5F and INS-1 cell lines, as well as pancreatic islets, gave hybridization bands indicating the presence of m1 receptor RNA, but to a much lesser extent than that derived from rat brain. As expected (11), the m1 receptor was only poorly detected in heart and lung. In contrast, the signals corresponding to m3 receptor RNA were equivalent in islets, INS-1 cells, and brain under these loading conditions. The m3 receptor signal was lower in RINm5F cells and lung. A band corresponding to m5 RNA was detected in brain and to a very much lesser degree in heart, lung, islets, and the two insulin-secreting cell lines.

Additional experiments were undertaken using RNase protection to quantify the muscarinic receptor expression in pancreatic β-cells. A representative gel is shown in Fig. 2, and the collated data is summarized in Table 1. Protected bands ran at the appropriate sizes as predicted from the length of the respective probes (Fig. 2, *right*). Brain RNA was also quantified as a positive control and demonstrated expression of m3 and m1 receptors in an approximate ratio of 2:1. Islets, whether freshly isolated or cultured for 48 h, gave comparable results and indicated that m1 and m3 receptors are expressed in approximately equal amounts (Table 1). There was a clear tendency for both cell lines to express lower levels of either receptor RNA, but this only attained statistical significance for m1 expression in RINm5F cells ($P < 0.02$ vs. either islet preparation). Precise quantification of m5 receptor RNA was precluded by the very low levels found in brain and the various β-cell preparations (<0.05 pg/µg total RNA).

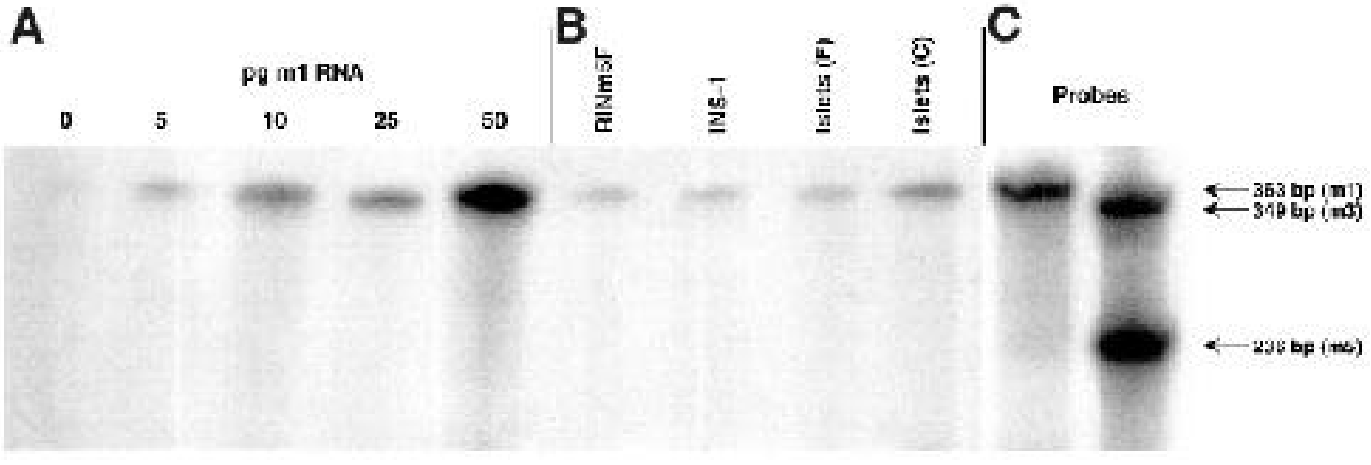

FIG. 2. Analysis of m1 muscarinic receptor expression in pancreatic β-cells by RNase protection. The 0–50 pg of reference RNA (*A*) or 10 µg total RNA isolated from islets or cell lines as indicated (*B*) were protected from RNase digestion by hybridization with radiolabeled 363 bp m1 muscarinic receptor RNA probe. Hybridized fragments were resolved by electrophoresis on 6% polyacrylamide/8 mol/l urea gels and visualized using a phosphorimager. Also shown are the electrophoretic mobilities of each of the muscarinic receptor RNA probes used in the study (*C*).

TABLE 1
Expression of muscarinic receptor mRNA in insulin-secreting cell lines, rat brain, and freshly isolated or cultured rat islets.

Cell type	Receptor mRNA (pg/µg total)	
	m1	m3
Brain	2.5 ± 0.5	1.2 ± 0.4
Islets		
Fresh	1.8 ± 0.3	1.6 ± 0.5
Cultured	1.7 ± 0.4	1.8 ± 0.6
RINm5F	0.4 ± 0.1	0.6 ± 0.1
INS-1	1.2 ± 0.4	0.7 ± 0.3

Data are means ± SE of four or five individual determinations. RNA was quantified by RNAse protection.

To determine whether m3 receptors were expressed on β-cells, as opposed to other cell types present in islets, immunohistochemistry was undertaken on serial pancreatic sections using antibodies to m3 receptors and various cellular markers. Representative results are shown in Fig. 3. Against the background of pancreatic acinar tissue, islets were plainly visible when incubated with an antibody for neuron-specific enolase, a marker for cells of neuroendocrine lineage (Fig. 3*B*). The great majority of these cells are well documented to be β-cells, whereas α-cells, staining for glucagon content, are confined to the periphery of the islet (Fig. 3*A*). Somatostatin-containing δ-cells showed a similar peripheral distribution but were much less abundant than the α-cells (Fig. 3*D*). Staining for m3 receptors was more intense in islet cells than in the surrounding acinar tissue (Fig. 3*C*). Both central (β) and peripheral (α) cells appeared to express the m3 receptor.

To assess the functional role of the different receptor subtypes in regulating insulin secretion, a transfection strategy was employed using INS-1 cells. In preliminary experiments, it was found that transfection with full-length m1 and m3 receptor cDNAs in the antisense orientation resulted in secretory responses to muscarinic receptor agonists that were essentially identical to those in control transfectants. This suggested that the antisense approach had not decreased muscarinic receptor expression and so was not pursued. On the other hand, transfection with muscarinic receptor cDNA in the sense orientation did increase receptor expression as assessed by specific binding of the hydrophilic muscarinic receptor antagonist QNB. A representative Scatchard plot for untransfected INS-1 cells is shown in Fig. 4. From this and other experiments it was calculated that INS-1 cells expressed maximal muscarinic receptor binding

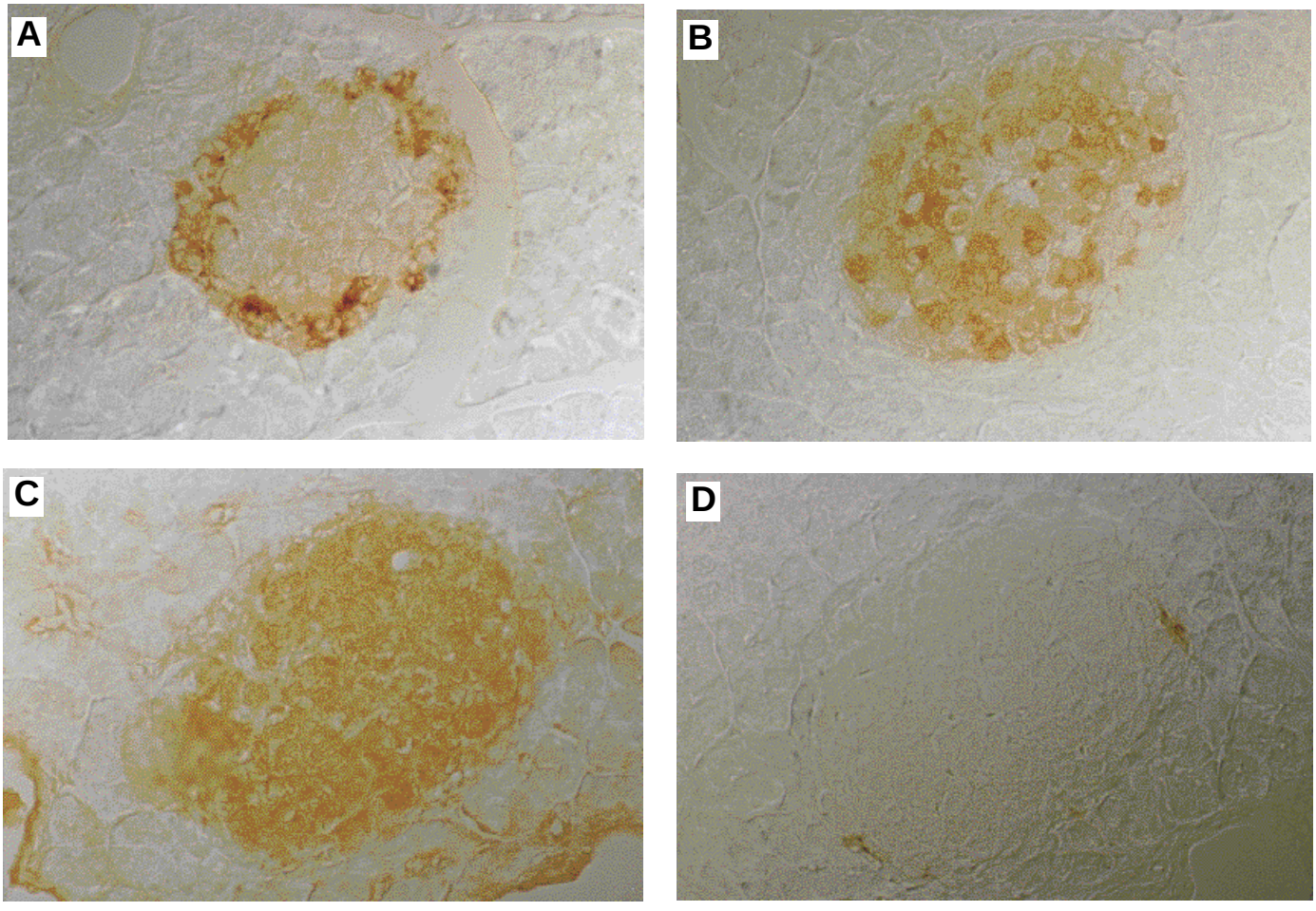

FIG. 3. Immunohistochemistry of serial sections of rat pancreas stained with antibodies against glucagon (*A*), neuron specific enolase (*B*), muscarinic m3 receptor (*C*), or somatostatin (*D*). Pancreases were fixed with 4% paraformaldehyde and embedded in paraffin. Serial 0.5 micron sections were taken. Immunohistochemistry was carried out using antibody concentrations as described in RESEARCH DESIGN AND METHODS.

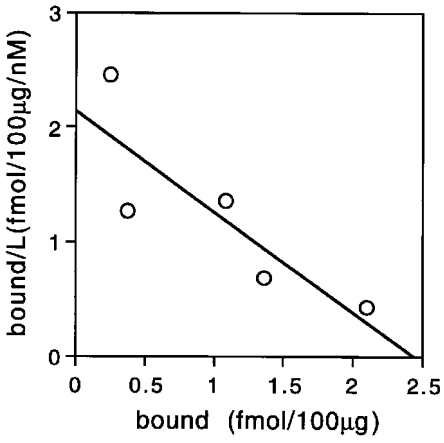

FIG. 4. Scatchard plot of QNB binding to membranes from untransfected INS-1 cells. Membranes (100 µg protein) were incubated with 0.1–5.0 nmol/l [^{3}H]QNB. Specific binding was determined by subtraction of that seen in the presence of 5 µmol/l atropine and plotted against the QNB concentration. Points are means of duplicate determinations from a representative experiment from a total of eight.

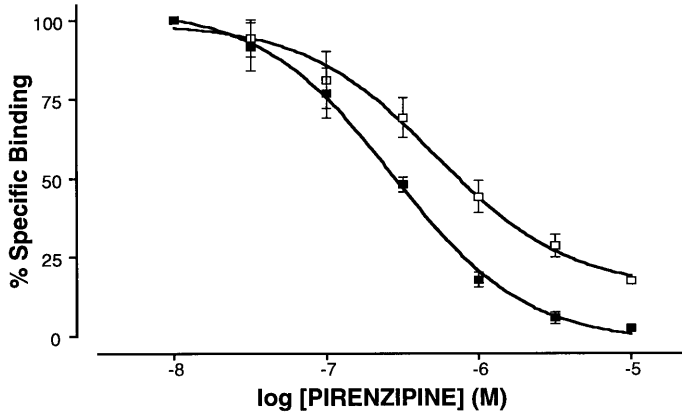

FIG. 5. Pirenzipine displacement curves of QNB binding to membranes from INS-1 cells overexpressing m1 (■) or m3 (□) muscarinic receptors. Membranes (2.5 µg protein for m1; 10 µg for m3) of stably transfected cell pools were incubated with 0.5 nmol/l [^{3}H]QNB and 0.01–10.0 µmol/l pirenzipine. Results are expressed as a percentage of the maximal binding seen in the absence of pirenzipine, after subtraction of nonspecific counts as determined in the presence of 5 µmol/l atropine. Points are means of two separate experiments each assayed in duplicate.

of 23.0 ± 2.9 fmol/mg protein (n = 8). This was increased to 14.5 ± 4.2 and 0.42 ± 0.07 pmol/mg protein, respectively, in m1- and m3-transfected cells (n = 3). To confirm that the transfected receptor cDNA did indeed result in overexpression of the appropriate functional receptor, antagonist binding experiments were undertaken using the m1 receptor antagonist pirenzipine. The displacement curves (Fig. 5) demonstrate that m1-transfected cells have a higher affinity for pirenzipine than those transfected with m3 receptor cDNA. From two independent experiments, the IC_{50} for pirenzipine displacement of [^{3}H]QNB was $2.3 \pm 1.1 \times 10^{-7}$ and $5.9 \pm 2.7 \times 10^{-7}$ mol/l for m1- and m3-expressing cells, respectively. Using the Cheng-Prusoff equation (31), these values were converted to K_i values of 68 and 330 nmol/l for m1 and m3 receptors, respectively.

The ability of the transfected cell pools to couple to PLC was next investigated (Fig. 6*A*). Hydrolysis of Ptd InsP2 in response to a supramaximal concentration of the muscarinic agonist carbachol was markedly increased by receptor overexpression. This was more apparent for m1 (~30-fold over control) than for m3 receptor transfectants (~15 times control), a finding that is consistent with the greater overexpression of m1 versus m3 receptors as assessed by binding analysis. Hydrolysis of Ptd Ins (as opposed to Ptd InsP2) was not detectable in any of the INS-1 cells whether transfected or not (results not shown). Insulin secretion was also assessed (Fig. 6*B*). Overexpression of either m1 or m3 receptors did not affect basal secretion as compared with vector-transfected controls. However, whereas control cells responded to carbachol with a doubling of the secretory response, this effect was much more pronounced in the cells overexpressing either receptor subtype. Despite the fact that the m1 cells exhibited a larger stimulation of PLC, they had a slightly, though significantly ($P < 0.05$), lower capacity for carbachol-stimulated insulin secretion relative to their m3-transfected counterparts.

Stably transfected, clonal cell lines were established from the transfected cell pools by dilution cloning. Two representative cell lines were chosen in which muscarinic receptor expression was less divergent than in the cell pools. The lev-

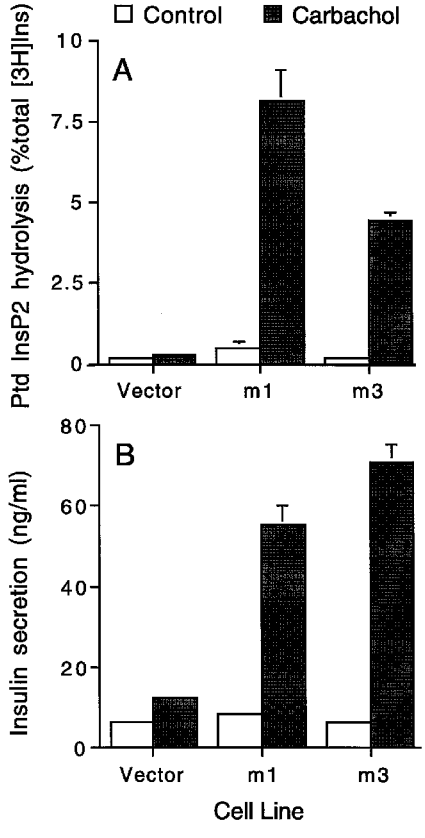

FIG. 6. Functional responses to carbachol in pools of INS-1 cells stably transfected with vector alone, or m1 or m3 muscarinic receptor cDNA. *A*: Ptd InsP2 hydrolysis. Transfected cells, pre-labeled with [^{3}H]inositol, were stimulated for 1 min in KRB medium plus or minus 0.5 mmol/l carbachol. Inositol phosphates were extracted and separated by anion exchange HPLC, and radioactivity in Ins(4)P1 was determined. Results are expressed as a percentage of total counts in free inositol and represent means ± SE of four independent determinations. *B*: Insulin secretion. Cells were stimulated for 15 min in KRB medium plus or minus 0.5 mmol/l carbachol, and insulin secreted into the medium was quantified by radioimmunoassay. Results represent means ± SE of 12–24 independent determinations.

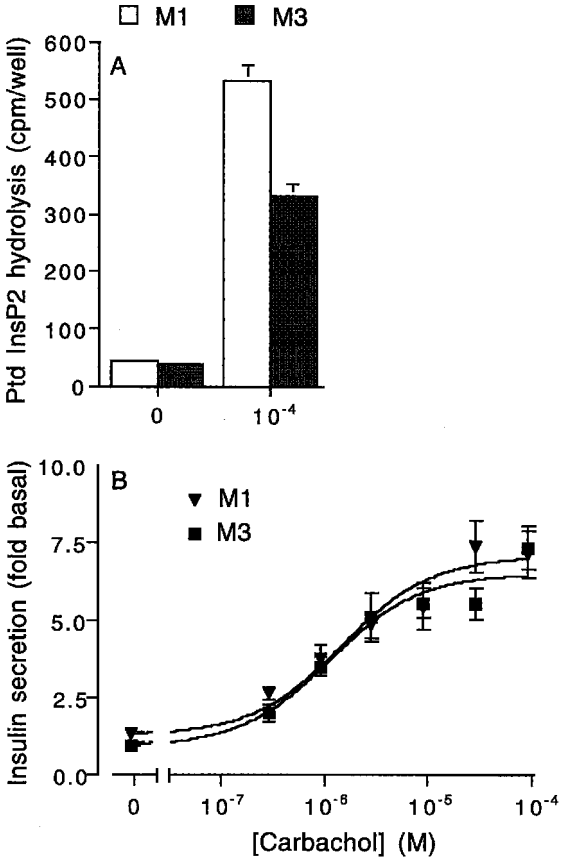

FIG. 7. Functional responses to carbachol in clonal cell lines stably overexpressing m1 or m3 muscarinic receptors. Cell lines were established from transfected pools by dilution cloning. *A*: Ptd InsP2 hydrolysis. Transfected cells, prelabeled with [^{3}H]inositol, were stimulated for 5 min in KRB medium plus or minus 0.1 mmol/l carbachol. Inositol phosphates were extracted and separated by anion exchange chromatography, and radioactivity in InsP3 was determined. Results represent means ± SE of eight independent determinations. *B*: Insulin secretion. Cells were stimulated for 15 min in KRB medium with 0–0.1 mmol/l carbachol, and the insulin secreted was determined by radioimmunoassay. Results represent means ± SE of 6–12 independent determinations.

els of expression were 3.0 ± 0.2 and 0.3 ± 0.1 pmol/mg protein in INS m1.2 and INS m3.4, respectively. These cell lines were then used for further functional analyses. Again, the m1 transfectants exhibited a maximal Ptd InsP2 hydrolysis that was approximately double that of the cells overexpressing m3 muscarinic receptors (Fig. 7*A*). In contrast, the two cell lines exhibited very similar secretory responses (Fig. 7*B*), confirming the lack of correlation between maximal secretion and Ptd InsP2 hydrolysis observed in the cell pools. EC_{50} for carbachol-stimulated insulin secretion was calculated as 1.4 ± 0.7 µmol/l (n = 3) and 1.7 ± 0.7 µmol/l (n = 2) for Ins m1.2 and Ins 3.4 cell lines, respectively. This demonstrates that both receptors couple to secretion with equal potencies, which are independent of maximal binding.

DISCUSSION

Analysis of muscarinic receptor mRNA content, by either RT-PCR amplification or RNase protection, gave essentially similar results when comparing the various tissues and cell lines for a given receptor subtype. Thus, for m1 receptor content, the ranking was brain > islets > INS-1 > RINm5F. For m3, the ranking was islets > brain > INS-1 > RINm5F. Consistent with these results, two previous studies using RT-PCR have documented the existence of m3 receptor RNA in rat islets and RINm5F cells (17,18), and m1 receptor RNA in islets (17). No expression of m1 or m5 receptor RNA, as visible PCR bands on agarose gels, was detected in the earlier study of the cell line (18), whereas these subtypes were clearly documented here using the more sensitive technique of hybridizing PCR bands with specific radiolabeled probes. However, our results are consistent with a relatively low expression of m1 RNA in RINm5F cells (certainly lower than in islets) and of very low expression of m5 receptor RNA in brain and the various β-cell extracts. The results for m5 are consistent with its previously documented very low expression in all tissues except for isolated areas of the brain (11).

RNase protection has been used previously to quantify m1 muscarinic receptor message in adult rat brain at a level of 3.6 ± 0.8 pg/µg total RNA (32), which is comparable to the results presented here. The current study is the first to quantify muscarinic receptor RNA in β-cells and indicates that m1 and m3 are expressed in approximately equal amounts in isolated islets and at levels broadly comparable with those found in brain. The results also suggest, in keeping with the data obtained using RT-PCR analysis, that both receptor subtypes are expressed in somewhat lower amounts in the two studied β-cell lines, especially RINm5F cells.

Immunohistochemical analysis of pancreatic tissue has previously documented muscarinic receptor expression in islet cells, but the antiserum used was unable to distinguish between different receptor subtypes (33). Our results indicate the existence of m3 muscarinic receptor protein in the islet core (β-cells) as well as in the peripheral α-cells. However, because the earlier study showed particularly intense staining co-localizing with α-cells, we cannot exclude the possibility that m1 receptors (not detected by our m3 antiserum) are the predominant subtype in α-cells.

Most of the several published studies addressing the control of insulin secretion by muscarinic receptors have emphasized the role of the m3 receptor. However, given the relatively poor discrimination between m1 and m3 receptors by muscarinic receptor antagonists, a minor contribution by m1 receptors would have been difficult to demonstrate. We have taken an alternative approach of selectively increasing muscarinic receptor expression by transfection of INS-1 cells with m1 and m3 receptor cDNA. Overexpression of the receptors was achieved, as evidenced by both radioligand binding and functional criteria. Most importantly, overexpression of either receptor resulted in marked enhancement, relative to untransfected cells, of both insulin secretion and Ptd InsP2 hydrolysis in response to carbachol. Basal responses were unaffected. It was also apparent that the m3-transfected cell pools responded slightly better in terms of secretion, but only half as optimally on Ptd InsP2 hydrolysis, as compared to the m1 transfectants. Broadly consistent results were found in the two cell lines selected by dilution cloning. The INS m1.2 and INS m3.4 cell lines displayed essentially similar secretory potential, despite a larger Ptd InsP2 response in the m1-transfected cells. These results suggest that signaling pathways in addition to activation of PLC might be important for insulin release or that submaximal Ptd InsP2 hydrolysis suffices for maximal secretion.

The two selected cell lines displayed maximal QNB binding of ~3 and 0.3 pmol/mg membrane protein for the m1 and m3 transfectants, respectively. These values are within the range of many cell lines expressing heterologous muscarinic receptors that have been previously used for functional analysis (34–36). Therefore, these cell lines should prove valuable tools in the further analysis of the mechanisms by which muscarinic receptor agonists stimulate insulin secretion. Indeed, the current study has already documented that either receptor subtype couples to the secretory pathway with equal potency. These results, as well as the expression of m1 receptors in the β-cell quantified here for the first time, suggest that the m1 receptor does have the potential to activate pathways leading to insulin release and hence might play at least a limited role in mediating the secretory response to acetylcholine in vivo. The poor correlation between Ptd InsP2 hydrolysis and insulin secretion also suggests that other signaling pathways, such as Ca^{2+} influx (6,22,24), might be more important for secretory regulation. Delineation of those pathways might be aided by future studies to determine which mechanisms are activated downstream of each receptor subtype.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council of Australia and a research grant from Aza Research.

We thank Brooke Carter and Dr. Chris Mitchell for help with the inositol phosphate assays and Dr. Karen McCrea for advice on the binding studies. Critical comments on the manuscript from Drs. Chris Mitchell, Amanda Sainsbury, and Karen McCrea are also gratefully acknowledged.

REFERENCES

1. Ahren B, Karlsson S, Lindskog S: Cholinergic regulation of the endocrine pancreas. *Prog Brain Res* 84:209–218, 1990
2. Stubbe JH, Steffens AB: Neural control of insulin secretion. *Horm Metab Res* 25:507–512, 1993
3. Conaway HM, Griffey MA, Whitney JE: Characterization of acetylcholine-induced insulin secretion in the isolated perfused dog pancreas. *Proc Soc Exp Biol Med* 150:308–312, 1975
4. Malaisse WJ, Malaisse-Lagae F, Wright PH, Ashmore J: Effects of adrenergic and cholinergic agents upon insulin secretion in vitro. *Endocrinology* 80:975–978, 1967
5. Zawalich WS, Zawalich KC, Rasmussen H: Cholinergic agonists prime the β-cell to glucose stimulation. *Endocrinology* 125:2400–2406, 1989
6. Wollheim CB, Siegel EG, Sharp GWG: Dependency of acetylcholine-induced insulin release on Ca^{++} uptake by rat pancreatic islets. *Endocrinology* 107:924–929, 1980
7. Wollheim CB, Biden TJ: Second messenger function of inositol 1,4,5-trisphosphate: early changes in inositol phosphates, cytosolic Ca2+, and insulin release in carbamylcholine-stimulated RINm5F cells. *J Biol Chem* 261:8314–8319, 1986
8. Biden TJ, Davison AGM, Prugue M: Regulation by membrane potential of phosphatidylinositol hydrolysis in pancreatic islets. *J Biol Chem* 268:11065–11072, 1993
9. Gagerman E, Idahl L-A, Meissner HP, Taljedal I-B: Insulin release, cGMP, cAMP, and membrane potential in acetylcholine-stimulated islets. *Am J Physiol* 235:E493–E500, 1978
10. Morgan NG, Rumford GM, Montague W: Studies on the role of inositol triphosphate in the regulation of insulin secretion from isolated islets of Langerhans. *Biochem J* 228:713–718, 1985
11. Bonner TI: New subtypes of muscarinic acetylcholine receptors. *Trends Pharmacol Sci* 10:11–15, 1989
12. Dunlop ME, Larkins RG: Muscarinic-agonist and guanine nucleotide activation of polyphosphoinositide phosphodiesterase in isolated islet-cell membranes. *Biochem J* 240:731–737, 1986
13. Wollheim CB, Biden TJ: Signal transduction in insulin secretion: comparison between fuel stimuli and receptor agonists. *Ann N Y Acad Sci* 488:317–333, 1986
14. Henquin JC, Nenquin M: The muscarinic receptor subtype in mouse pancreatic B-cells. *FEBS Lett* 236:89–92, 1988
15. Santos RM, Rojas E: Muscarinic receptor modulation of glucose-induced electrical activity in mouse pancreatic B-cells. *FEBS Lett* 249:411–417, 1989
16. Verspohl EJ, Tacke R, Mutschler E, Lambrecht G: Muscarinic receptor subtypes in rat pancreatic islets: binding and functional studies. *Eur J Pharmacol* 178:303–311, 1990
17. Boschero AC, Szpak-Glasman M, Carneiro EM, Bordin S, Paul I, Rojas E, Atwater I: Oxotremorine-m potentiation of glucose-induced insulin release from rat islets involves M3 muscarinic receptors. *Am J Physiol* 268:E336–E342, 1995
18. Tang SH, Sharp GWG: Identification of muscarinic receptor subtypes in RINm5F cells by means of polymerase chain reaction, subcloning, and DNA sequencing. *Diabetes* 46:1419–1423, 1997
19. Biden TJ, Prugue ML, Davison AGM: Evidence for phosphatidyl-inositol hydrolysis in islets stimulated with carbamoylcholine: kinetic analysis of inositol polyphosphate metabolism. *Biochem J* 285:541–549, 1992
20. Easom RA, Landt M, Colca JR, Hughes JH, Turk J, McDaniel M: Effects of secretagogues on protein kinase C-catalysed phosphorylation of an endogenous substrate in isolated pancreatic islets. *J Biol Chem* 265:14938–14946, 1990
21. Jones PM, Persaud SJ, Howell SL: Protein kinase C and the regulation of insulin secretion from pancreatic B cells. *J Mol Endocrinol* 6:121–127, 1990
22. Henquin JC, Garcia MC, Bozem M, Hermans MP, Nenquin M: Muscarinic control of pancreatic B cell function involves sodium-dependent depolarization and calcium influx. *Endocrinology* 122:2134-2142, 1988
23. Gilon P, Henquin JC: Activation of muscarinic receptors increases the concentration of free Na+ in mouse pancreatic B-cells. *FEBS Lett* 315:353–356, 1993
24. Sanchez-Andres JV, Ripoll C, Soria B: Evidence that muscarinic potentiation of insulin release is initiated by an early transient calcium entry. *FEBS Lett* 231:143–147
25. Gilon P, Nenquin M, Henquin JC: Muscarinic stimulation exerts both stimulatory and inhibitory effects on the concentration of cytoplasmic Ca^{2+} in the electrically excitable pancreatic B-cell. *Biochem J* 311:259–267, 1995
26. Yada T, Hamkawa N, Yaekura K: Two distinct modes of Ca^{2+} signalling by ACh in rat pancreatic β-cells: concentration, glucose dependence and Ca^{2+} origin. *J Physiol* 488:13–24, 1995
27. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989
28. Sims NA, White CP, Sunn KL, Thomas GP, Drummond ML, Morrison NA, Eisman JA, Gardiner EM: Human and murine osteocalcin gene expression: conserved tissue restricted expression and divergent responses to 1,25-dihydroxyvitamin D3 in vivo. *Mol Endocrinol* 11:1695–1708, 1997
29. Peralta EG, Ashkenazi A, Winslow JW, Smith DH, Ramachandran J, Capon DJ: Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic receptors. *EMBO J* 6:3923–3929, 1987
30. Braun T, Schofield PR, Shivers BD, Pritchett DB, Seeburg PH: A novel subtype of muscarinic receptor identified by homology screening. *Biochem Biophys Res Commun* 149:125–132, 1987
31. Haylett DG: Direct measurement of drug binding to receptors. In *Textbook of Receptor Pharmacology*. Foreman JC, Johansen T, Eds. Boca Raton, FL, CRC Press, 1996, p. 121–149
32. Ludlam WH, Zang Z, McCarson KE, Krause JE, Spray DC, Kessler JA: mRNAs encoding muscarinic and substance P receptors in cultured sympathetic neurons are differentially regulated by LIF or CNTF. *Dev Biol* 164:528–539, 1994
33. Van der Zee EA, Buwalda B, Strubbe JH, Strosberg AD, Luiten PGM: Immunocytochemical localization of muscarinic acetylcholine receptors in the rat endocrine pancreas. *Cell Tissue Res* 269:99–106, 1992
34. Buckley NJ, Bonner TI, Buckley CM, Brann MR: Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* 35:469–476, 1989
35. Wang SZ, el-Fakahany EE: Application of transfected cell lines in studies of functional receptor subtype selectivity of muscarinic agonists. *J Pharmocol Exp Ther* 266:237–243, 1993
36. Richards MH, van Giersbergen PLM: Human muscarinic receptors expressed in A9L and CHO cells: activation by full and partial agonists. *Br J Pharmacol* 114:1241–1249, 1995