

Functional Properties of Human Muscarinic Receptors Hm1, Hm2 and Hm3 Expressed in a Baculovirus/Sf9 Cell System

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Abstract – The human muscarinic acetylcholine receptor (mAChR) subtypes Hm1, Hm2 and Hm3 have been expressed in insect cells (*Spodoptera frugiperda*, Sf9) using the baculovirus expression system. Expression of relevant DNA, transcript and receptor proteins was identified by PCR, Northern blotting and [³H]QNB binding, respectively. As assessed by [³H]QNB binding sites, yields of muscarinic receptors in membrane preparations in this study were as about 5-20 times high as those in mammalian cells reported in previous studies. The [³H]QNB competition binding studies with well-known subtype-selective mAChR antagonists showed that the receptors expressed in Sf9 cells retain the pharmacological characteristics expected for the m1, m2 and m3 muscarinic receptors. The m1-selective antagonist, pirenzepine, displayed a considerably higher affinity for Hm1 by 110-fold and 35-fold than for Hm2 and Hm3, respectively. The m2-selective methoctramine displayed a significantly higher affinity for Hm2 than for Hm1 and Hm3 (10- and 26-fold, respectively). p-F-HHSiD exhibited high affinity for Hm3 that is not significantly different from those for Hm1, but 66-fold higher than its affinity for Hm2. The functional coupling of the recombinant receptors to second messenger systems was also examined. While both Hm1 and Hm3 stimulated phosphoinositide hydrolysis upon activation by carbachol, Hm2 produced no response. On the other hand, activation of mAChRs induced the inhibition of forskolin-stimulated cyclic AMP formation in Hm2-expressing cells, whereas the significant dose-dependent increase in or poor response on cyclic AMP formation were produced in Hm1 or Hm3-expressing cells, respectively. These results indicate the differential coupling of recombinant Hm1, Hm2 and Hm3 receptors expressed in Sf9 cells to intracellular signalling system.

Key words □ Muscarinic receptor subtypes, Antagonist binding, Sf9 cells, Baculovirus expression system, Second messenger system

Muscarinic acetylcholine receptors (mAChR) are members of a large family of G protein coupled receptors. Five mammalian muscarinic receptor genes (m1-m5) have been cloned, and expression studies have shown that the individual receptor subtypes differ in their functional and ligand-binding properties. They classified into two distinct groups with respect to their second messenger coupling: m1, m3, and m5 receptor couple to G-proteins that stimulate phosphatidylinositol hydrolysis whereas m2 and m4 receptors couple preferentially to G-proteins that inhibit adenylyl cyclase (Peralta *et al.*, 1988; Bonner, 1989). However, m1 or m3 muscarinic receptor-mediated stimulation of adenylyl cyclase activity has also been observed in some cell lines (Peralta *et al.*, 1988; Felder *et al.*, 1989; Gurwits *et al.*, 1994; Burford and Nahorski, 1995; Suh and Kim, 1995) The muscarinic recep-

tors have also been classified pharmacologically with muscarinic antagonists such as pirenzepine, methoctramine and hexahydrostiladifenidol (p-F-HHSiD), which display subtype-selective binding and inhibition of muscarinic responses. The baculovirus expression system using the *Spodoptera frugiperda* (Sf9) insect cells is now widely used for production of high levels of recombinant proteins and many G-protein-coupled receptors have been successfully expressed in insect cells (Parker *et al.*, 1991; Rincken *et al.*, 1994; Kukkonen *et al.*, 1996; Heitz *et al.*, 1997). Although mAChR subtypes also have been reported to be expressed in insect cells using the baculovirus expression system, functional properties for their intracellular signaling responses to activation by muscarinic agonist have not been clearly characterized.

In the present study, we have expressed the recombinant human m1, m2 and m3 mAChR subtypes (Hm1, Hm2 and Hm3) in Sf9 cells, and evaluated the ligand binding and func-

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tional properties of the receptors. The 1-quinuclidyl [phenyl-4-³H]benzilate ([³H]QNB) competition binding assays with some subtype-selective muscarinic antagonists and the measurement of coupling to second messenger systems such as inositol 1,4,5-triphosphate [(1,4,5)P₃] and cyclic AMP were performed in those Hm1-, Hm2- or Hm3-expressing cells.

MATERIALS AND METHODS

Materials

[³H]QNB (48.0 Ci/mmol), [³H]inositol 1,4,5-triphosphate (0.1 μCi/ml), [³H]adenine (1 mCi/ml) and α-[³²P]dCTP (3000 Ci/mmol) were obtained from Amersham (Buckinghamshire, UK). DNA purification kit, Prime-a-Gene DNA labeling kit and X-gal were from Promega (Madison, USA). Restriction enzymes and Taq polymerase were obtained from TaKaRa (Otsu, Japan), Posco (Sungnam, Korea), and Boehringer Mannheim (Mannheim, Germany), and T4 ligase was from New England Biolab (Beverly, USA). Fetal bovine serum, a 0.24-9.5 kb RNA Ladder and vanadyl ribonucleoside complex were purchased from Gibco BRL (Gaithersburg, USA). Pirenzepine, methocramine, *p*-F-HHSiD, 4-DAMP and U73122 were from Research Biochemical International (Natick, USA), and U73343 was from Biomol (Plymouth Meeting, USA). Carbachol, atropine, forskolin and 3-isobutyl-1-methyl xanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, USA) (GeneScreen nylon membrane was obtained from New England Nuclear (Boston, USA). Jetsorb[®] gel extraction kit was from Genomed (Research Triangle Park, USA). Scintillation cocktail (ScintiSafe[™] Econo2) was from Fisher Scientific (New Jersey, USA). All other chemicals were reagent grades and purchased from Sigma (St. Louis, USA).

Cell culture and virus infection

Sf9 cells (ATCC CRL 1711) were subcultured as monolayers or in suspension at 27°C in TNM-FH medium (Sigma) supplemented with Pluronic F-68 (10%), amphotericin-B (0.25 μg/ml), strep-to-mycin (5 mg/ml), and penicillin-G (5000 units/ml) and 10% fetal bovine serum. For virus infection, monolayers were inoculated with appropriate virus at an indicated multiplicity of infection (MOI). For the mock-infected control, tissue-culture medium alone was added. The residual inoculum was replaced with medium after one-hour adsorption period, and infected cells at 27°C.

Construction of baculovirus transplacement vector and recombinant baculovirus

The Hm1, Hm2 and Hm3 human muscarinic receptor genes which have been cloned into pCMV plasmid vector were obtained from Dr. Lee (Medical School of Hanyang University). To construct transplacement vectors containing Hm1, Hm2 and Hm3 cDNAs, the 1.5-kb *Bam*HI fragment of Hm1pCMV, the 1.57-kb *Pst*I-*Kpn*I fragment of Hm2pCMV and the 1.91-kb *Bam*HI fragment of Hm3pCMV were subcloned into the *Bam*HI or *Pst*I-*Kpn*I sites of pBlueBack (pBB, Invitrogen) baculovirus transfer vector, downstream of the polyhedrin promoter (Fig. 1).

To generate recombinant baculoviruses, 4 μg of each transplacement vector DNA (containing each wild-type and chimeric receptor cDNA) and 1 μg of linearized parental viral DNA (Back-N-Blue, Invitrogen) were cotransfected into Sf9 cells by using insectinPlus (Invitrogen). The recombinant

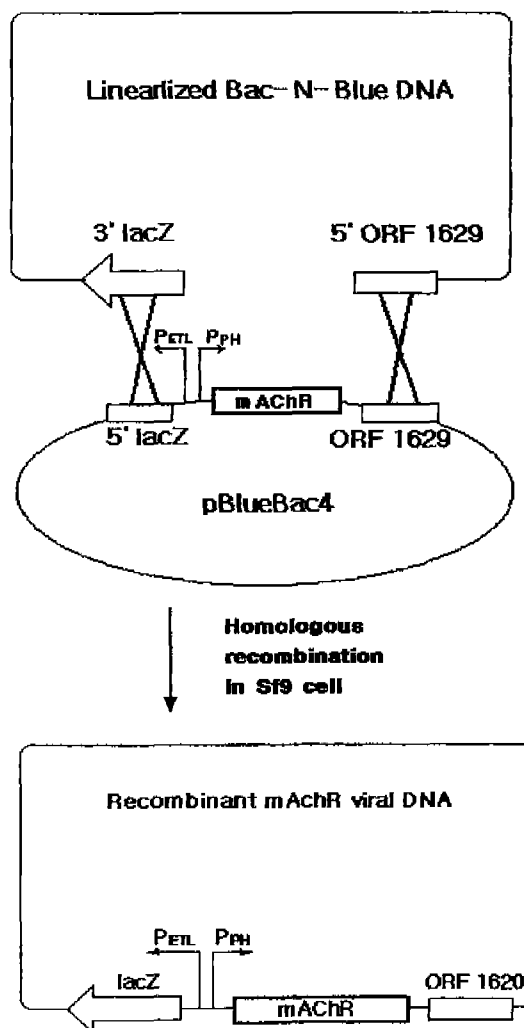


Fig. 1. Schematic representation of the construction of recombinant baculovirus vector (see text for description).

viral clones were isolated by plaque assay, the recombinant viruses were amplified in Sf9 cell monolayers, and their titers were measured according to the standard baculovirus expression manual (O'reilly *et al.* 1994). Expression of relevant DNA, transcript and receptor proteins was confirmed by polymerase chain reaction (PCR), Northern blotting and [³H]QNB binding, respectively.

Purification of viral DNA and PCR analysis

Total DNA containing substantial amounts of viral DNA was extracted late in infection according to O'reilly *et al.* (1994), and used as a template for PCR identification of recombinant baculovirus DNA. Sf9 cells (5×10^6 cells/100 mm dish) infected with 1 ml primary passage stock of recombinant viruses or mock were collected and lysed in lysis buffer containing 30 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate and 1% NP-40 on ice for 5 min, and centrifuged at $1,000 \times g$ for 5 min at 4°C. The pellet was washed in cold phosphate buffered saline (PBS; pH 6.2) and was resuspended in extraction buffer containing 10 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 0.5% sodium dodecyl sulfate (SDS). Proteinase K was added to a final concentration of 1 mg/ml and the sample incubated at 37°C for overnight. The sample DNAs were extracted sequentially with phenol, phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol (24:1), and precipitated with 2.5 volume of 100% ethanol. To identify recombinant muscarinic receptor viral DNA, the sequences of the 377-bp from Hm1, the 402-bp from Hm2 and the 519-bp from Hm3, comprising entire i3 loop of each receptor subtype, were amplified from template viral DNA (about 10 ng) by 30 cycles, consisting of 1 min at 95°C, and 1 min at 50°C, and 2 min at 72°C. The PCR products were identified by 1.2% agarose gel electrophoresis.

Extraction of cytoplasmic RNA and Northern analysis

Sf9 cells (5×10^6 cells/100 mm dish) were infected with mock, wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus; AcMNPV), or three recombinant muscarinic receptor viruses at a MOI of 10. At 48 h after infection, cells were scraped and collected into conical tubes, and centrifuged at $1,000 \times g$ for 5 min. Cell pellets were washed three times with 10 ml ice-cold PBS (pH 6.2) and lysed in 0.8 ml lysis buffer containing 0.14 M NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.0), 0.5% NP40 and 10 mM vanadyl ribonucleoside complex on ice for 5 min, and centrifuged at $2,000 \times g$ for 5 min. The supernatant was mixed with an equal volume of extraction buffer (0.2 M Tris-HCl, pH 7.5; 25 mM EDTA; 1.3 M NaCl; 2% SDS). Proteinase K was added to a

final concentration of 0.2 mg/ml, and the sample incubated at 37°C for 30 min. Samples of cytoplasmic RNA were applied to denaturing (formaldehyde)-agarose (1%) gels, separated by electrophoresis and transferred to nylon membranes (GeneScreen, NEN). RNA was cross-linked to the nylon membrane by UV irradiation. Blots were prehybridized for 3 hours at 42°C in 5×SSPE (1×SSPE=150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA), 5×Denhardt's, 50% formamide, 0.5% SDS, 10% dextran sulfate, and 200 µg/ml of denatured salmon sperm DNA. Hybridization was carried out at 42°C in the same buffer with addition of ³²P-labeled probe. The probe, a 1.57-kb *Pst*I-*Kpn*I fragment of Hm2 cDNA in pBB4, was labeled with [α -³²P]dCTP using a Prime-a-Gene DNA labeling system (Promega). The nylon membrane was washed and exposed to an X-ray film with an intensifying screen at -80°C.

Sf9 cell membrane preparation and radioligand binding assays

Two dishes of Sf9 cells (10^7 cells/100 mm dish) were infected with each recombinant virus at a MOI of 20. At 60 h after infection, mock and virus-infected cells were homogenized in a ice-cold buffer containing 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml pepstatin A, and 10 µg/ml leupeptin. The homogenate was centrifuged at $1,000 \times g$ for 5 min to remove nuclei and cell debris. Crude membranes were collected by centrifugation at $15,000 \times g$ for 20 min and used in binding assays. In saturation assays, aliquots of membrane homogenates (50-100 mg protein) were incubated in assay buffer (20 mM Tris-HCl, pH 7.4/12.5 mM MgCl₂/1 mM EDTA), containing various concentrations (0.01-10 nM) of [³H]QNB for 2 h at 30°C in a total volume of 1 ml. In the [³H]QNB displacement studies, 8-10 different concentrations of unlabeled muscarinic antagonists were incubated with 0.3 nM [³H]QNB and cell membranes. Nonspecific binding was determined in the presence of 2 µM atropine, and specific binding was expressed as the difference in bound radioactivity in the presence and absence of atropine. Binding was terminated by rapid filtration with a 24-well Cell Harvester (Brandel) through GF/B filters presoaked in 0.1% polyethylenimine. The filters washed three times with 4-ml of ice-cold assay buffer and the bound radioactivity was determined in a liquid scintillation counter (Beckman LS6000TA). Binding data from the saturation experiments were analyzed using the computer program LIGAND to obtain the dissociation constant K_D and the binding capacity B_{max}. Data from displacement experiments were fitted to the following equation with a nonlinear least squares

curve-fitting program (SigmaPlot); $[\text{Bound } [^3\text{H}]\text{QNB}] = \frac{B_{\text{max}} \text{IC}_{50}}{([\text{X}] + \text{IC}_{50}) + B_0}$; B_{max} and B_0 are bound $[^3\text{H}]\text{QNB}$ in the absence of muscarinic ligands and presence of 2 mM atropine, respectively. $[\text{X}]$ is the concentration of a given unlabeled antagonist. IC_{50} values were converted to K_i values according to the Methods of Cheng and Prusoff (1973).

Measurement of inositol 1,4,5-trisphosphate $[\text{I}(1,4,5)\text{P}_3]$

The mass of intracellular $\text{I}(1,4,5)\text{P}_3$ level was determined by competition assay with $[^3\text{H}]\text{I}(1,4,5)\text{P}_3$ in binding to $\text{I}(1,4,5)\text{P}_3$ binding protein. Sf9 cells were infected with indicated recombinant viruses, and at 48-72 h after infection, cells were collected by centrifugation at $1,000\times g$ for 5 min, washed twice and resuspended in PBS (pH 6.2). Aliquots of cells were incubated in 0.5 ml of buffer at room temperature in the absence and the presence of appropriated concentrations of carbachol for 2 min. Reactions were terminated by addition of 0.1 ml of ice-cold 20% perchloric acid and the perchloric acid extracts were neutralized with 20% KOH. Aliquots of neutralized extracts (water-soluble inositol phosphate) were incubated in assay buffer containing 0.1 M Tris buffer (pH 9.0), 4 mM EDTA and 4 mg/ml bovine serum albumin for 15 min at 4°C with binding protein (400 μg protein) in the presence of 0.01 μCi of $[^3\text{H}]\text{I}(1,4,5)\text{P}_3$. Standards [0-40 pmole of $\text{I}(1,4,5)\text{P}_3$] were assayed concurrently. Bound $[^3\text{H}]\text{I}(1,4,5)\text{P}_3$ was separated from free radioligand by centrifugation at 4°C and measured by liquid scintillation counting. The $\text{I}(1,4,5)\text{P}_3$ binding protein was prepared from bovine adrenal cortex according to a method of Challis *et al.* (1990).

Measurement of cyclic $[^3\text{H}]\text{AMP}$

Intracellular cAMP was determined by measuring the formation of cyclic $[^3\text{H}]\text{AMP}$ from $[^3\text{H}]\text{adenine}$ nucleotide pools as described by Suh and Kim (1995), using a method modified from that of Salomon (1991). Sf9 cells (2×10^6 cells/well) in 6 well-dishes were infected with indicated recombinant viruses. At 48 hr after infection, cells were preloaded for 24 h with 3 μCi $[^3\text{H}]\text{adenine}$ in 1.5 ml of complete medium, and labelled cells were washed twice with PBS (pH 6.2). After pretreatment with 1 mM IBMX with or without atropine for 15 min, cyclic AMP formation was stimulated with 10 μM forskolin in the presence of IBMX in PBS (pH 6.2) for 10 min at room temperature, with or without carbachol. The reaction was stopped by aspirating medium off and adding 1 ml of ice-cold 5% (v/v) perchloric acid containing 1 μM cold cyclic AMP. The plates were left on ice for 30 min to extract water-soluble cyclic AMP. Then, the extracts were transferred to Eppendorf tubes and centrifuged at $5,000\times g$ for 5 min to pre-

cipitate the cell debris. Cyclic $[^3\text{H}]\text{AMP}$ and $[^3\text{H}]\text{ATP}$ were separated by sequential chromatography on dowex AG50W-X4 (200-400 mesh) cation exchanger and neutral alumina column. $[^3\text{H}]\text{ATP}$ fraction was obtained by elution with 2 ml of distilled water from Dowex column and then sequential elution with 3.5 ml of distilled water was loaded into the alumina column. The alumina column was washed by adding 4 ml imidazole solution (0.1 M, pH 7.2) and the eluants were collected into scintillation vials containing 15 ml of scintillation fluid. The radioactivity for cyclic $[^3\text{H}]\text{AMP}$ and $[^3\text{H}]\text{ATP}$ was measured by liquid scintillation counting. Increase of

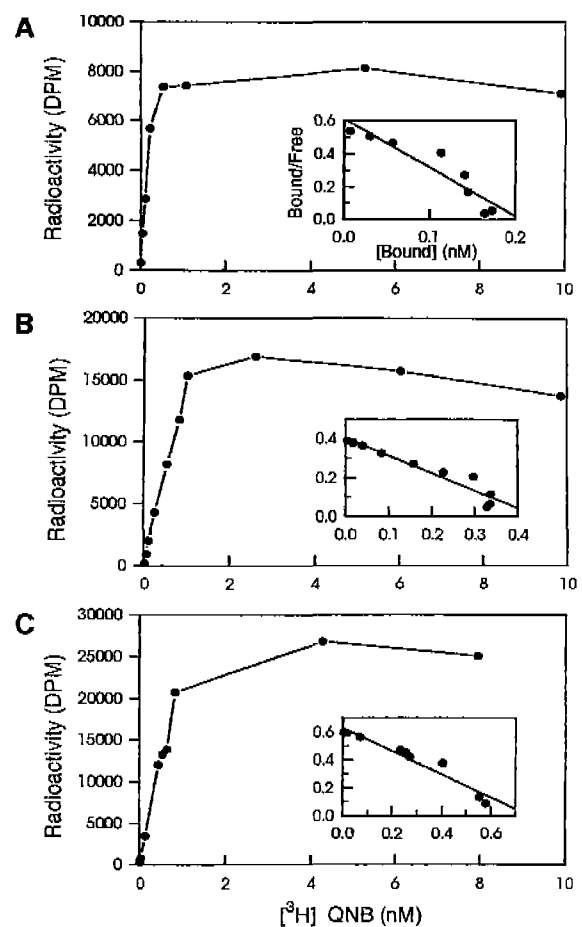


Fig. 2. PCR analysis of recombinant viral DNA. DNA sequences were amplified from DNA template extracted from mock-infected Sf9 cells for negative controls (-), or from each of recombinant Hm1pBB4, Hm2pBB4 and Hm3pBB4 vector DNA templates for positive controls (+), or from viral DNA templates extracted from cells infected with recombinant Hm1, Hm2 or Hm3 viruses (S), using primers flanking entire i3 loop of each receptor subtype. Major bands for amplified recombinant DNA products of the 377-bp from Hm1, the 402-bp from Hm2 and the 519-bp from Hm3 were detected in agarose gel analysis.

intracellular cAMP concentration was calculated as cyclic $[^3\text{H}]\text{AMP}/[^3\text{H}]\text{ATP} + \text{cyclic } [^3\text{H}]\text{AMP} \times 10^3$.

RESULTS

Identification of recombinant muscarinic receptors expressed in insect cell using a baculovirus system

Recombinant baculoviruses for three human muscarinic receptor subtypes (Hm1, Hm2 and Hm3) were produced, and the receptors were expressed at high levels in the Sf9 insect cells. Production of the relevant recombinant baculoviral

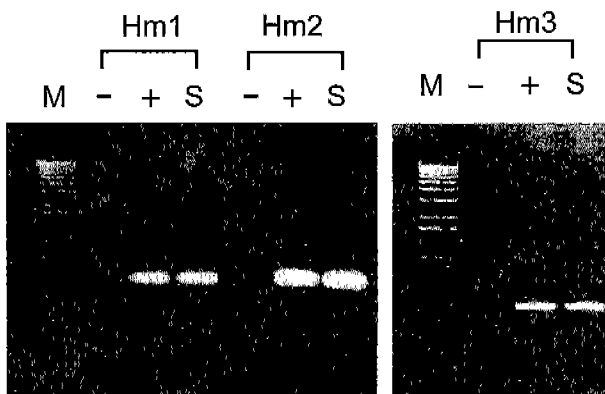


Fig. 3. Northern blot analysis of cytoplasmic RNA isolated from Sf9 cells 48 h after infection with mock or wild-type baculovirus. AcMNPV and infection with recombinant viruses containing Hm1 cDNA, Hm2 cDNA or Hm3 cDNA. The Hm1, Hm2 and Hm3 messages with correct size were abundantly produced in Sf9 cells infected with recombinant viruses whereas the muscarinic receptor mRNAs were not present in cells infected with mock and AcMNPV.

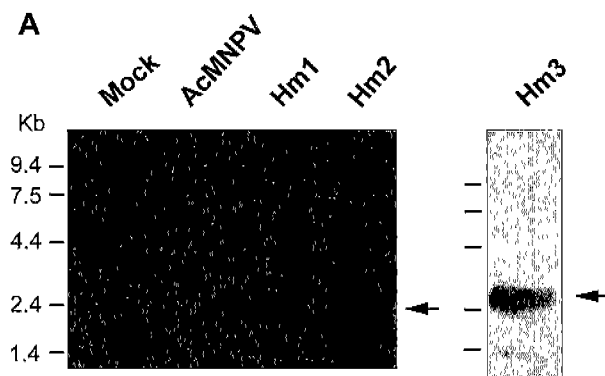


Fig. 4. Specific $[^3\text{H}]\text{QNB}$ binding to mAChR subtypes expressed in insect Sf9 cells. Saturation isotherms for $[^3\text{H}]\text{QNB}$ was obtained from membranes prepared from insect cells infected with recombinant Hm1 (A), Hm2 (B) or Hm3 (C) baculovirus. The result shown is a representative data of three or more independent experiments. Binding assay was performed in triplicate on membranes. The inset shows Scatchard plot of the data.

DNA was confirmed by PCR analysis (Fig. 2). Abundant messages of correct size were observed in Sf9 cells infected with each recombinant receptor virus by Northern blotting of cell RNA (Fig. 3). No RNA band was detected in mock or wild-type virus (AcMNPV) infected cells, indicating no endogenous expression of mAChRs in Sf9 cells. The binding activity for the recombinant muscarinic receptor proteins was determined by direct radioligand binding assay using a non-selective muscarinic antagonist, $[^3\text{H}]\text{QNB}$. $[^3\text{H}]\text{QNB}$ binding isotherms of recombinant Hm1, Hm2, Hm3 subtypes in crude membrane preparations from Sf9 cells were consistent with the presence of a single binding site with homogenous affinity (Fig. 4). The numbers of receptors expressed (B_{max}) and their affinities (K_D) are summarized in Table I. The specific $[^3\text{H}]\text{QNB}$ binding was demonstrated in Sf9 cells infected with each recombinant muscarinic receptor viruses, whereas no detectable binding could be found in mock and AcMNPV infected cells. Yields of muscarinic receptors assessed as $[^3\text{H}]\text{QNB}$ binding sites in membrane preparations in this study were as about 5-20 times high as those in mammalian cells (Wess *et al.*, 1990; Lei *et al.*, 1988), and similar to those in Sf9 cells (Vasudevan *et al.*, 1995) reported in previous other studies.

Binding affinities of muscarinic antagonists to the Hm1, Hm2 and Hm3 receptors expressed in Sf9 cells

mAChRs expressed in Sf9 cell membranes were further characterized pharmacologically by examining subtype specificity of muscarinic antagonists. Table I shows the K_i values of the muscarinic receptor antagonists pirenzepine, methoctramine, HHSiD and 4-DAMP, which were obtained from the displacement curves of the $[^3\text{H}]\text{QNB}$ binding to the Hm1,

Table I. Ligand binding properties of mAChR subtypes expressed in Sf9 cells

		Hm1	Hm2	Hm3
$[^3\text{H}]\text{QNB}$	K_D	0.29±0.07	0.54±0.06	0.30±0.04
	B_{max}	1,418±529.6	3,347±892.0	2,952±367.6
Pirenzepine	K_i	0.52±0.11	57.03±8.08	15.60±2.03
Methoctramine	K_i	24.58±3.75	3.56±0.68	65.71±7.18
<i>p</i> -F-HHSiD	K_i	0.90±0.18	21.68±4.70	0.40±0.05
4-DAMP	K_i	0.59±0.03	4.59±0.76	0.75±0.11

B_{max} values derived from Scatchard plot indicate maximum number of $[^3\text{H}]\text{QNB}$ binding sites (fmol)/mg of membrane protein. K_D (nM) represents the dissociation constant. The inhibition constant K_i (10^2 nM) for each muscarinic antagonist was derived from its IC_{50} values in $[^3\text{H}]\text{QNB}$ /antagonist competition curve. Data are presented as mean± standard errors of at least three independent experiments, each performed in triplicate.

Hm2 and, Hm3 receptors produced in Sf9 cells. The relative order of affinities of muscarinic antagonists for each subtype were as follows: Hm1, pirenzepine \geq 4-DAMP \geq p-F-HHSiD > methoctramine; Hm2, Methoctramin \geq 4-DAMP > p-F-HHSiD > pirenzepine; Hm3, p-F-HHSiD \geq 4-DAMP > pirenzepine > methoctramine. Pirenzepine that has been known to have the highest affinity for the m1 receptors also displayed a considerably higher affinity for Hm1 by 110-fold and 35-fold than for Hm2 and Hm3, respectively, in our study. The m2-selective methoctramine displayed a significantly higher affinity for Hm2 than for Hm1 and Hm3 (10- and 26-fold, respectively). p-F-HHSiD and 4-DAMP exhibited high affinities for Hm3 that are similar to those for Hm1, but 66- and 6-fold higher, respectively, than those for Hm2.

Functional coupling of the Hm1, Hm2 and Hm3 receptors expressed in Sf9 cells

Stimulation of mock-infected Sf9 cells with carbachol induced no effect on I(1,4,5)P₃ generation and cyclic AMP formation indicating no existence of mAChR in the insect cells (Fig. 4), which coincides with the result from the binding assay. The carbachol-induced increases in I(1,4,5)P₃ levels were observed in cells expressing Hm1 and Hm3 receptors, but not in Hm2-expressing cells (Fig. 4). On the other hand, a muscarinic agonist carbachol produced increase, decrease and no change in forskolin-stimulated cyclic AMP formation in Hm1-, Hm2- and Hm3-expressing cells, respectively (Fig. 5). These results indicate the differential coupling of recombinant Hm1, Hm2 and Hm3 receptors expressed in Sf9 cells to intracellular signalling system.

DISCUSSION

The present study examines the binding and signaling properties of three human mAChR subtypes, Hm1, Hm2 and Hm3, when expressed in Sf9 insect cells using the baculovirus expression system. The level of expression did not significantly differ from one subtype to the other, ranging from 1.4 to 3.4 pmole/mg membrane protein ($1^{\circ}3 \times 10^6$ cells/mg protein) under our experimental conditions. As a single displacement curve needs only about 4 to 10 pmole of mAChR, only a few dishes (1×10^7 cells/100 mm dish) of culture are sufficient to obtain membrane receptors for ligand binding assays. The subtype specificity of all muscarinic antagonists tested in this study was in general agreement with the results for mAChR subtypes expressed in mammalian systems (Dörje *et al.*, 1991; Bolden *et al.*, 1992; Barbier, *et al.*, 1998) or in Sf9 cells

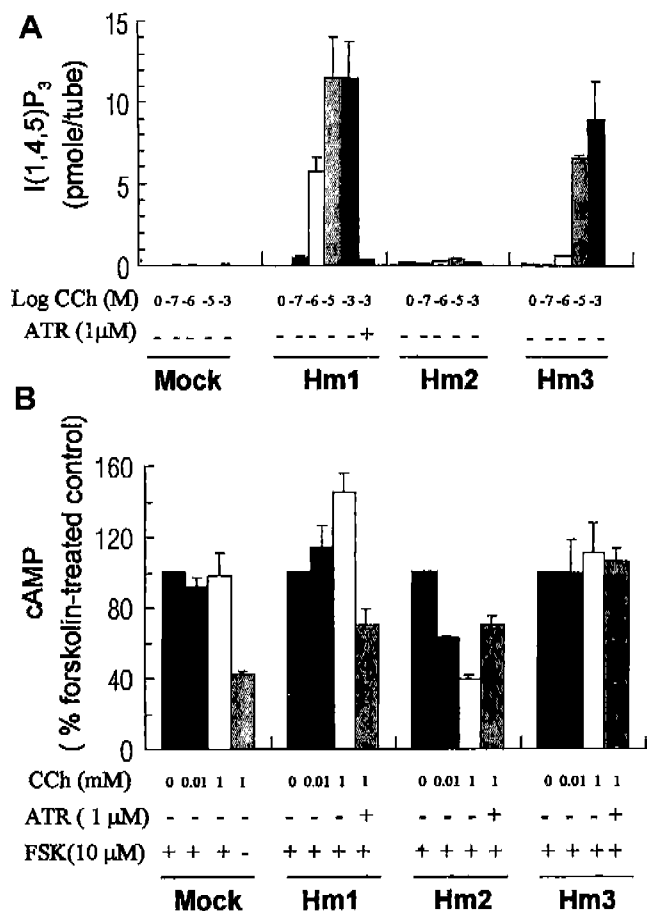


Fig. 5. Carbachol-induced I(1,4,5)P₃ formation and cAMP responses in Sf9 cells infected with mock, recombinant Hm1, Hm2 or Hm3 receptors. **A.** Infected cells were pretreated for 10 min with or without atropine (ATR, 1 μM) and incubated for 1 min with increasing concentrations of carbachol (CCh). The formation of I(1,4,5)P₃ was determined by competition assay with [³H]I(1,4,5)P₃ in binding to I(1,4,5)P₃ binding protein. **B.** Infected cells were loaded with [³H]adenine for 24 h and pretreated with IBMX (1 mM) for 15 min with or without ATR (1 μM), and then treated with forskolin (FSK, 10 μM) for 10 min with or without CCh in the presence of IBMX. The cAMP level was assayed as described in MATERIALS AND METHODS.

(Dong *et al.*, 1995). These results confirm that this system could be useful for screening of subtype-specific ligands, although molecular size of mAChR expressed in Sf9 cells is much less than those estimated for mAChR in mammalian tissues. Apparent molecular sizes of m1 or m2 subtype expressed in Sf9 cells were estimated to be 52 kDa (Heitz *et al.*, 1997) or 60 kDa (Nakata *et al.*, 1994) whereas those estimated for cerebral (mostly m1) or atrial (m2) mAChR were 70–80 kDa (Peterson *et al.*, 1984; Haga and Haga, 1985).

Specific receptor-mediated cell signalling can be investigated as long as the cell line used has the necessary compo-

nents to transduce the receptor signal into a cellular response. Agonist-stimulated mAChRs expressed in Sf9 cells produced intracellular signalling responses, suggesting that Sf9 cells endogenously express intracellular signaling proteins that couple mAChRs that expressed heterologously in these cells. The m1 and m3 receptors are known to activate primarily pertussis toxin-insensitive G protein $G_{q/11}$ that couples to phospholipase C while m2 receptors couple preferentially to pertussis toxin-sensitive $G_{i/o}$ protein that inhibit adenylyl cyclase. In present study, carbachol-stimulated recombinant Hm1 and Hm3 receptors showed increased PI responses, and Hm2 stimulation induced the inhibition of cyclic AMP formation in Sf9 cells. The receptors we have expressed, therefore, appear to couple to endogenous Sf9 G-proteins, $G_{q/11}$, and $G_{i/o}$. This is partially supported by results from the immunoblotting and ADP-ribosylation studies using Sf9 cells (Heitz *et al.*, 1995; 1997) that indicates the presence of $G\alpha_{q/11}$ and a pertussis-toxin substrate which has the relative molecular mass of mammalian $G\alpha_o$. The identities of $G\alpha_i$ and $G\alpha_s$ in Sf9 cells have not been clearly shown. The mAChR-mediated stimulation of adenylyl cyclase activity has been observed in Chinese hamster ovary cells transfected with m1 or m3 receptors (Peralta *et al.*, 1988; Burford and Nahorski, 1995), m1-transfected A9L cells (Felder *et al.*, 1989) and neuronal SK-N-BE(2)C cells which endogenously have m3 receptors (Suh and Kim, 1995). In our study, agonist-activation of Hm1 expressed in insect Sf9 cells also produced the enhancement of cyclic AMP formation. It is not clear whether this Hm1-stimulated cyclic AMP formation in Sf9 cells occurs as a consequence of elevated Ca^{2+} levels following phospholipase C activation as suggested in A9L and neuronal cells (Felder *et al.*, 1989; Suh and Kim, 1995) or via activation of $G\alpha_s$, as suggested in Chinese hamster ovary cells (Burford and Nahorski, 1995). Hm3 receptors in Sf9 cells appear to differently couple to signalling system because this receptor did not mediate any change in cyclic AMP response to agonist stimulation, and the cellular mechanism for this result should be further studied.

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