Five subtypes of muscarinic receptors are expressed in gastric smooth muscles of guinea pig

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Abstract

Muscarinic receptors play key roles in the control of gastrointestinal smooth muscle activity. However, specific physiological functions of each subtype remain to be determined. In this study, the nonselective cation channel activated by carbachol (I_{CCh}) was examined in circular smooth muscle cells of the guinea pig gastric antrum using patch-clamp technique. 4-DAMP inhibited I_{CCh} dose-dependently with IC_{50} of 1.1 ± 0.1 nM ($n = 6$). GTPγS-induced current, however, was not inhibited by 10 nM 4-DAMP. I_{CCh} was not recorded in pertussis-toxin (PTX)-pretreated smooth muscle cells of gastric antrum. I_{CCh} values in response to 10 μM CCh at a holding potential of 60 mV were -330 ± 32 pA ($n=4$) and -15 ± 3 pA ($n = 6$) in the control and PTX-treated cells, respectively ($P < 0.01$). Sensitivities to nanomolar 4-DAMP and PTX suggest the possible involvement of m4 subtype. Using sequence information obtained from cloned guinea

pig muscarinic receptor genes, it is possible to amplify the cDNAs encoding m1-m5 from guinea pig brain tissue. Single cell RT-PCR experiments showed that all five subtypes of muscarinic receptor were present in circular smooth muscle cells of the guinea pig gastric antrum. Together with our previous results showing that G_o protein is important for activation of ACh-activated NSC channels, our results suggest that I_{CCh} might be activated by acetylcholine through m4 subtype as well as m2 and m3 subtypes in guinea-pig stomach.

Introduction

Muscarinic acetylcholine receptors (mAChRs) are known to be involved in the regulation of smooth muscle contraction in various organs including gastrointestinal tract (Eglen et al., 2001). However, it is difficult to identify the specific receptor subtype(s) that mediate muscarinic action of acetylcholine (ACh) in smooth muscle due to a lack of subtype specific ligands. To date, five different but related mAChRs, designated as m1-m5, have been identified and cloned. The amino acid sequences of mAChRs showed that each subtypes share the common structural motifs, 4 extracellular domains, 7 hydrophobic transmembrane domains and 4 intracellular domains (Bonner et al., 1987; Bonner et al., 1988), which are prototypical members of G-protein-coupled receptor superfamily. The m1, m3 and m5 subtypes are coupled to guanine nucleotide binding regulatory proteins ($G_{q/11}$ proteins) and stimulate phosphatidylinositol turnover, whereas m2 and m4 are linked to signalling pathway via pertussis-toxin sensitive G proteins ($G_{i/o}$ family)(Hulme et al., 1990).

In gastrointestinal smooth muscle, ACh or carbachol (CCh) activates a nonselective cation (NSC) channel as well as elicits intracellular calcium release from sarcoplasmic reticulum (Inoue and Isenberg, 1990; Komori and Bolton, 1990; Kim et al., 1997). However, the pretreatment with pertussis-toxin (PTX) inhibited NSC channel activation but not inositol triphosphate-dependent calcium release (Komori and Bolton, 1990), suggesting the different signalling pathways for these responses. The m2 and m3 have been

shown to be involved in ACh-activated NSC channels in guinea-pig ileum, stomach (Zholos and Bolton, 1997; Rhee *et al.*, 2000) and canine colonic circular smooth muscle (Zhang *et al.*, 1991). In guinea pig ileum (Zholos and Bolton, 1997), m2 antagonists competitively inhibited I_{CCh} with changes in the EC_{50}, but without any effect on maximal cation current. On the other hand, a selective block of the m3 receptor strongly reduced the maximum cation current without change in the EC_{50}, suggesting that m3 receptor activation exert a potent modulatory effect on channel opening in an entirely unexpected way. In contrast, a low concentration of 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), which has a selective antagonistic action on m4 and m5 subtypes in mammalian cells (Braeuner-Osborne and Brann, 1996) as well as m1 and m3 (Elgen *et al.*, 2001), increases the EC_{50}, while it does not reduce the maximal current in guinea pig stomach (Rhee *et al.*, 2000).

Recently, we have shown that G_o protein is important for activation of ACh-activated NSC channels (Kim *et al.*, 1998). Moreover, G_o protein has been shown to couple m4 in neuroendocrine cells to voltage-gated calcium channels (Kleuss *et al.*, 1991, 1992, 1993; Liu *et al.*, 1994), and sympathetic neurons to N-type calcium channels (Pinkas-Kramarski *et al.*, 1990; Bernheim *et al.*, 1992; Delmas *et al.*, 1998). These results indicate the possible involvement of m4 receptor in ACh-activated NSC channel, which is not known to be expressed in stomach smooth muscle. In this study, we analyze the effect of 4-DAMP on the isolated guinea pig stomach smooth muscle cells and investigated whether the m4 subtype is expressed by single cell RT-PCR analysis.

Materials and Methods

Materials

4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) was purchased from Research Biochemical International (Natick, MA). Carbamylcholine chloride (CCh), pertussis toxin (PTX) and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The GeneAmp RNA PCR Kit was purchased from Perkin Elmer (Norwalk, CT). The restriction enzymes were obtained from New England BioLab (Bevely, MA).

Whole-cell patch clamp

Fresh, single smooth muscle cell was isolated enzymatically from the antral circular layer of guinea pig stomach as previously described (Kim *et al.*, 1995). Isolated smooth muscle cells were stored at 4°C until use, and all experiments were performed within 10

h after cell dispersion. An aliquot of single smooth muscle cells in suspension was transferred to the recording chamber (0.3 ml) mounted on an inverted microscope (Diaphot 300; Nikon, Tokyo, Japan). Solutions were perfused through the chamber by gravity at a rate of approximately 2 ml/min and the experiments were performed at room temperature (approximately 25°C). Whole-cell currents were measured using the standard patch clamp technique. Patch pipettes (free-tip resistance of 2-4 $M\Omega$) were connected to the head stage of a patch-clamp amplifier (Axopatch-1D; Axon Instrument). In each experiment, liquid junction potentials were corrected with an offset circuit. Whole-cell currents were filtered at 10 kHz (-3 dB frequency), and the data were displayed on a digital oscilloscope (PM 3350A; Philips, Netherlands), a computer monitor, and a pen recorder (RS 3200; Gould). For later analysis, data were saved in a digital tape recorder (DTR-1204; Biologic, France). Data analysis was performed with pCLAMP software 6.0.2 (Axon Instruments) and Origin 5.0 (Microcal Software Inc., USA). The cells had an average capacitance of 50 ± 3 pF. The series resistance (10-15 $M\Omega$) compensation was not introduced.

Nonselective cation currents in whole-cell voltage-clamp mode were measured in CsCl-rich external solution containing 135 mM CsCl, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.35 with CsOH). The internal pipette solution contained 135 mM CsCl, 3 mM Mg-ATP, 5 mM di-tris-creatine phosphate, 0.5 mM tris-GTP, 2 mM EGTA and 10 mM HEPES (pH 7.2 with CsOH). The use of Cs-rich external and bath solutions result in a larger cation current than normal that improved the signal to noise ratio of the recording (Kim *et al.*, 1995). Cells were voltage-clamped at -60 mV, unless otherwise stated.

Determination of nucleotide sequences of guinea pig muscarinic receptors

The nucleotide sequences of muscarinic receptor were determined by sequencing of cloned RT-PCR products. Total RNA was extracted from guinea-pig tissues by the guanidinium thiocyanate method of Chomczynski and Sacchi (1987). RNA was reverse transcribed using 1 μg of total RNA in reaction buffer containing 50 mM Tris-HCl pH 8.0, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM each of dATP, dTTP, dGTP and dCTP, 5 units of ribonuclease inhibitor, 2.5 μM random hexamers, and 10 units of AMV reverse transcriptase at 42°C. This mixture was then used for PCR amplification of muscarinic receptor cDNA fragments. Degenerated oligonucleotide primers were designed to yield about 500 bp in size based on the alignment of human, mouse, rat mAChR sequences. The buffer for PCR contained 17 mM Tris-HCl, pH 8.0, 48 mM

KCl, 1.6 mM MgCl₂, 0.2 mM each of dATP, dTTP, dGTP and dCTP, 25 pM each of PCR primers and 2.5 units of Taq DNA polymerase. Samples were incubated at 94°C for 10 min and followed by 35 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 2 min. Then a final extension step was performed at 72°C for 8 min, before cooling to 4°C. PCR products were sized-fractionated on 1.5% agarose gels, and purified for cloning to pGEM T easy vector (Promega). All cloned PCR products were subjected to sequencing using an ABI PRISM 310 automated sequencer (Perkin-Elmer). The nucleotide sequences of 5' UTR and 3' UTR were amplified by RACE method according to the manufacturer's instruction (Roche, Germany).

Single cell RT-PCR

Single smooth muscle cell was isolated by the procedure described previously and collected individually for molecular studies (Kim *et al.*, 1995). Micropippettes were constructed from borosilicate glass with resistance of around 1 MΩ. Cells were transferred to the stage of phase contrast microscope and allowed to stick lightly to the bottom of glass coverslip in small chamber for 10-15 min. The cells were then perfused with sterile phosphate-buffered saline to remove cellular debris. Single smooth muscle cell was selected by positioning the tip of the micropipette near the cell with light suction. After aspiration, the cell extract was expelled from the pipette into PCR tube, which contained 2 units of RNase-free DNase, 5 units of ribonuclease inhibitor, 10 mM Tris-HCl pH 8.0, 50 mM KCl, 5 mM MgCl₂, 1 mM each of dATP, dTTP, dGTP and dCTP, 2.5 µM random hexamers, and 12.5 units of AMV reverse transcriptase. First strand cDNA was synthesized at 42°C. This mixture was then used for PCR amplification of guinea pig muscarinic receptor cDNA. The following guinea pig mAChRs-specific primers were used: m1; (sense, 5' CTGGCCTGTG-ACCTCTGG 3'; antisense, 5' TGAGCTGCTGCTGCT-GCC 3'), m2; (sense, 5' AGCAATGCC-TCAGTCATG 3'; antisense, 5' TTTGATGCATGTTTGCTT 3'), m3; (sense, 5' AGAATCTATAAGGAAACT 3'; antisense, 5' TTTTTGAAAACTGCCGCC 3'), m4; (sense, 5' CTCT-GGGCGCCTGCTATC 3'; antisense, 5' GTCTCTGT-GGTGGACAG 3'), m5 (sense, 5' ACAGAGAAGCGA-ACCAAA 3'; antisense, 5' GAGTGTGTGAGC-AGC-AGC3'). To confirm the specific amplification of the guinea pig mAChRs, each PCR product for m1-m5 was subjected to digestion with *Nco* I, *Sac* I, *Hind* III, *Ava* I and *Eco*0190 I enzymes for 12 h, respectively. Five restriction enzymes have only one restriction site in the PCR target region of guinea pig mAChR sequences. Fragments were size fractionated by 1.5% agarose gels and documented by Polaroid photography under ultraviolet illumination.

Results and Discussion

Inhibition characteristics of carbachol activated nonselective cation current by 4-DAMP

Muscarinic agonists release intracellular Ca^{2+} via the phospholipase C/IP₃ system. In addition, muscarinic agonists depolarize the membrane potential, which triggers action potential generation (Bolton, 1977). This membrane depolarization results from the activation of nonselective cation channel by stimulating muscarinic receptor (Benham *et al.*, 1985). Nonselective cation currents activated by carbachol (I$_{CCh}$) were recorded in the cells voltage-clamped at -60 mV. Carbachol (CCh, 2 M) induced an inward current at holding potential of -60 mV. Ramp pulses from -140 mV to +40 mV for 1 second were applied during the
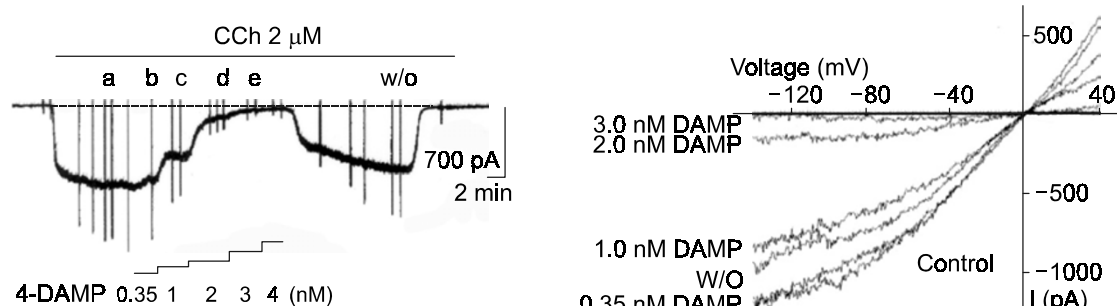

Figure 1. Competitive inhibition of CCh-induced nonselective cation current by the muscarinic receptor antagonist, 4-DAMP. Carbachol (CCh, 2 µM) induced an inward current at holding potential of -60 mV (left panel). Ramp pulses from -140 mV to +40 mV for 1 second were applied during the activation of the current to obtain the current-voltage relationship of I$_{CCh}$ (right panel). Each ramp pulse (a, b, c, d and e) corresponds with each current-voltage relationship at various concentrations of 4-DAMP in right panel. a: control, b: 0.35 nM 4-DAMP, c: 1.0 nM 4-DAMP, d: 2.0 nM 4-DAMP, e: 3.0 nM 4-DAMP. I$_{CCh}$ was activated by carbachol (2 µM), and 4-DAMP inhibited the current dose-dependently.

activation of the current to obtain the current-voltage relationship of I_{CCh}. The current-voltage relationships were similar to those previously reported (Inoue and Isenberg, 1990; Komori and Bolton, 1990; Kim *et al.*, 1995; Kim *et al.*, 1998). 4-DAMP inhibited the current dose-dependently at the range of nM (Figure 1, left panel). The IC_{50} was 1.1 ± 0.1 nM ($n = 6$). 4-DAMP inhibited the current at the whole voltage range without any change of reversal potential (Figure 1, right panel). The IC_{50} was similar to values of m4 (0.8 nM) and m5 (1 nM) muscarinic acetylcholine receptor subtypes (Braeuner-Osborne and Brann, 1996). m1 (3 nM), m2 (25 nM) and m3 (2 nM) has larger IC_{50} values (Braeuner-Osborne and Brann, 1996). How-

ever, Elgen *et al.* (2001) showed different values for m1 (0.4 nM), m2 (4 nM), m3 (0.8 nM), m4 (1 nM) and m5 (5 nM). I_{CCh} was also activated by 0.2 mM GTPγS (Figure 2A, see also Inoue and Isenberg, 1990; Komori and Bolton, 1990; Kim *et al.*, 1995). Similar current was activated after the rupture of the membrane, but was not inhibited by 4-DAMP. This result suggests that 4-DAMP act at an earlier step in the transduction process but does not block the channel directly. When smooth muscle cells were incubated with PTX (5 μg/ml, 3 h at 37°C), no discernible cation current was activated by CCh (Figure 2B). I_{CCh} values in response to 10 μM CCh at a holding potential of -60 mV were -330 ± 32 pA ($n = 4$) and -15 ± 3 pA ($n = 6$) in the control and PTX-treated cells, respectively ($P < 0.01$). PTX treatment has no effect on the voltage-gated calcium current. This result suggests that PTX-sensitive G-protein ($G_{o/i}$) is involved in the opening of nonselective cation channel activated by CCh.

Cloning and sequencing of guinea pig muscarinic receptor cDNAs

Previously, we showed that anti-$G_{\alpha,o}$ antibody blocked NSC channel activation but anti-$G_{\alpha,i}$ did not. We also confirmed the expression of $G_{\alpha,o}$ in guinea-pig gastric smooth muscle by western blot analysis (Kim *et al.*, 1998). G_o protein coupled m4 mAChR to voltage-gated calcium channels in neuroendocrine cells (Kleuss *et al.*, 1991, 1992, 1993; Liu *et al.*, 1994), and sympathetic neurons to N-type calcium channels (Pinkas-Kramarski *et al.*, 1990; Bernheim *et al.*, 1992; Delmas *et al.*, 1998). These observations, together with the inhibitory effect of 4-DAMP and G_o antibody on I_{CCh}, led us to investigate whether m4 and m5 are expressed in guinea-pig stomach in addition to m2 and m3. However, sequence information for guinea-pig mAChRs is not currently available. Therefore, we cloned guinea-pig mAChRs cDNA with RT-PCR using degenerated primers based on sequence homology and determined their sequences. Guinea-pig m1 was cloned partially (458 amino acids) and the other four muscarinic receptors were fully cloned. The m2 predicts a protein of 466 amino acids, m3 587 amino acids, m4 478 amino acids, and m5 530 amino acids. Detailed sequence information is available at the following Genbank accession numbers: m1; AY072058, m2; AY072059, m3; AY072060, m4; AY072061, m5; AY072062. Alignment of predicted protein sequences of guinea-pig mAChRs with the available sequence information from different species showed that m1 is the most conserved among five subtypes and among species, and m5 is the least conserved subtype (Table 1). In all subtypes, inner third loop was the most variable domain among species (m5 > m3 > m2
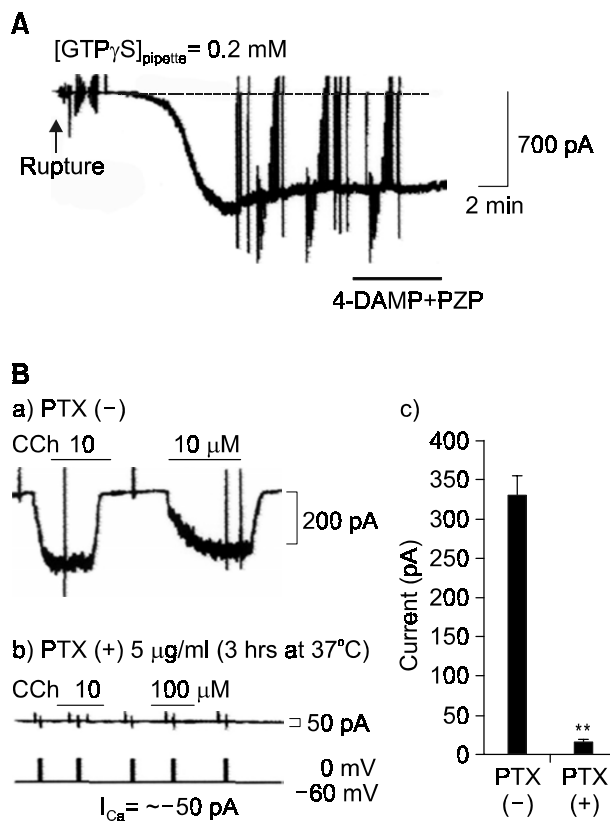
Figure 2. Nonselective cation current induced by the direct activation of PTX-sensitive GTP-binding protein. A, The current was activated by direct activation of G-protein by the intracellular application of GTPγS. GTPγS (0.2 mM) was included in the pipette solution and was dialyzed after the cell membrane rupture. GTPγS-induced current was not inhibited by the treatment with high concentration of 4-DAMP (10 nM) and pirenzepine (50 μM). Upward and downward reflections in trace indicate the currents activated by ramp pulses or step pulses. B. CCh did not activate the currents in cells preincubated with PTX (b,c), while it activated the control cells (a). Upward and downward reflections in trace indicate the currents activated by ramp pulses or step pulses. Voltage-gated calcium current, however, could be activated with membrane depolarization from -60 mV to 0 mV in PTX-treated cells indicating the cells were viable.

Table 1. Comparison of predicted protein sequences of guinea pig mAChRs with the available mAChRs sequences from various species.

Species	m1	m2	m3	m4	m5
Human	NM_00738[a]	NM_00739	NM_00740	NM_00741	AF026263
Rat	M16406	NM_031016	NM_012527	Z49748	M22926
Mouse	J04192				NM_007699
Pig	X04413	M16331	X12712		
Guinea pig	AY072058	AY072059	AY072060	AY072061	AY072062
Bovine	L27103				

Domain	m1	m2	m3	m4	m5
Transmembrane domain	5/154[b] (3.2%)	3/155 (1.9%)	3/156 (1.9%)	2/154 (1.3%)	10/53 (6.5%)
Inner loop (i)	10/230 (4.3%)	32/231 (13.9%)	56/316 (17.7%)	19/242 (7.9%)	76/297 (25.6%)
Outer loop (except N-terminus)	2/52 (3.8%)	3/58 (5.2%)	1/52 (1.9%)	5/54 (9.2%)	4/53 (7.5%)
G-protein binding region	0/20 (0%)	1/20 (5%)	0/20 (0%)	0/20 (0%)	1/20 (5%)
Third inner loop (i3)	7/157 (17.6%)	31/176 (20.4%)	49/240 (9.1%)	17/186 (31.9%)	73/229 (4.5%)
N-terminus	2/24 (8.3%)	3/22 (13.6%)	22/66 (33.3%)	8/30 (26.7%)	12/29 (41.4%)

[a]The numbers indicate the GenBank accession number.
[b]Numbers of non-homologous amino acid residue/amino acid residue of the domain.

> m4 > m1), and G-protein binding domain (N-terminal 20 amino acids of third inner loop, Burstein *et al.*, 1996) was the most conserved (m2 = m5 > m1 = m3 = m4) (Table 1).

Five subtypes of muscarinic receptor are expressed in gastric smooth muscle

In an effort to examine the mAChR subtypes expressed in smooth muscle from guinea-pig stomach, we performed RT-PCR on total RNA from myocytes isolated from guinea-pig stomach. We used the primer sets based on the sequence information of the guinea-pig mAChR genes. The effectiveness of the m1 to m5 mAChR subtypes-specific primers was tested by amplifying mAChR from guinea-pig brain which is known to express all five mAChRs (Drescher *et al.*, 1992; Sharma *et al.*, 1996). When RNA from guinea-pig brain was used, PCR products corresponding to target regions of m1, m2, m3, m4 and m5 were amplified (Figure 3A). The identity of these products was validated by restriction endonuclease analysis which showed that the size of all restriction fragments are corresponding to the correct mAChR based on guinea-pig cDNA sequences (Figure 3C), indicating that primer set was specific to each mAChR. When RNA from a preparation of pure myocytes isolated

from guinea-pig antrum was used, amplified fragments corresponding to PCR target regions of all five mAChR were observed. In order to rule out the contamination with other cell types in a myocyte preparation, we repeated RT-PCR experiments using RNA that was isolated from single myocytes. Even these conditions, all five mAChR mRNAs were amplified (Figure 3B). Moreover, to confirm that these RT-PCR products were amplified from cDNA, we performed control RT reactions in the absence of reverse transcriptase, and found that no DNA was amplified (data not shown).

In this study, we demonstrated that five subtypes of mAChR are present in guinea-pig gastric smooth muscle as well as brain. The radioligand-binding studies showed that the m2 mAChR subtype accounted for 70 to 80% of the receptor population, and the m3 mAChR subtype accounted for 20 to 30% of the receptor population in most smooth muscle preparations (Eglen *et al.*, 1996). From a pharmacological study using competitive antagonists of muscarinic receptors, major role of m2 and m3 subtypes was suggested for NSC channel activation in guinea-pig gastric smooth muscle (Zholos and Bolton, 1977; Rhee *et al.*, 2000). That is, m2 subtype was suggested to play a crucial role in the activation of the NSC channel and m3 subtype to modulate sensitivity
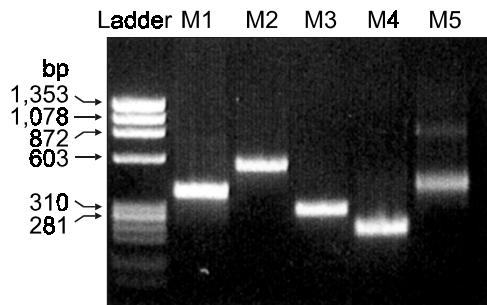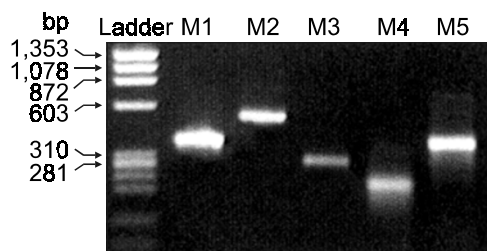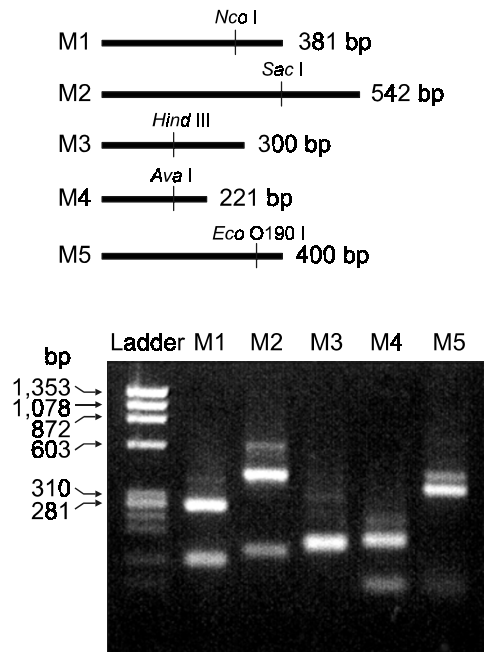
A. Guinea-pig brain

B. Guinea-pig stomach

C. Digestion

Figure 3. Gel electrophoresis of mAChR RT-PCR products from total RNA of guinea pig brain and isolated single antral myocytes. RT-PCR products were resolved on 1.5% agarose gel and visualized by ethidium bromide staining. A, An agarose gel showing amplification of all mAChR subtypes RNA from guinea pig brain. Size markers (φX174 RF DNA/*Hae* III) are indicated on the left. B, Single cell RT-PCR shows amplification of mAChR subtypes RNA from the isolated single gastric myocytes. C, Restriction mapping of mAChR RT-PCR products from RNA of single gastric myocytes.

of the m2-mediated response with no significant change of maximal response. The existence of m4 mRNA and the sensitivities to nanomolar 4-DAMP, PTX and anti-$G_{\alpha,o}$ antibody suggest that NSC channels might be activated by ACh through m4 subtypes as well as m2 subtype in guinea-pig stomach.

In previous study, when subtype-specific antibodies were used to determine the expression pattern of mAChRs in smooth muscle cells from different tissues, all five subtypes were detected in vas deferens, uterus and ileum (Doreje *et al.*, 1991). However, Zhang *et al.* (1991) showed hybridization of a rat cDNA probe only to m2 and m3 mRNA in canine colonic circular smooth muscle. This suggests that the hybridization technique is not sensitive enough to detect relatively low levels of mRNA. Interestingly, in this RT-PCR study, we amplified mRNA for m1 and m5 as well as m2, m3 and m4. The reason for the presence of these additional mAChR subtypes in smooth muscle of guinea-pig stomach is not clear yet. However, the lack of m2 or m3 subtype in knockout mice did not lead to compensatory changes in the expression levels of the remaining mAChR subtypes (Gomeza *et al.*, 1999; Yamada *et al.*, 2001), suggesting the specific functional roles of the individual mAChR subtypes. Therefore, our result of multiple sub-

types in single myocyte may also suggest that various subtypes of mAChRs might interact each other to activate and modulate NSC channel in gastric smooth muscle.

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